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Content

คณะกรรมการจัดการประชุมวิชาการประจำปี ครั้งที่ 32

สมาคมเภสัชวิทยาแห่งประเทศไทย

ร่วมกับโครงการบัณฑิตศึกษา สาขาชีวเวชศาสตร์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ระหว่างวันที่ 22-24 มีนาคม 2553

ณ มหาวิทยาลัยธรรมศาสตร์ วิทยาเขตรังสิต ปทุมธานี

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ก. <u>อนุกรรมการฝ่ายวิชาการ</u>

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ค .อนุกรรมการฝ่ายทะเบียน

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ฉ .อนุกรรมการฝ่ายสถานที่ และนันทนาการ

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ช .อนุกรรมการฝ่ายประชาสัมพันธ์ และประเมินผล

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ซ .อนุกรรมการฝ่ายประเมินการประกวดผลงานวิจัย

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   มหาวิทยาลัยมหิดล

สารจากนายกสมาคมเภสัชวิทยาแห่งประเทศไทย

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เรียน สมาชิกสมาคมเภสัชวิทยาฯ และผู้เข้าร่วมประชุมทุกท่าน

ในนามของสมาคมเภสัชวิทยาฯ ดิฉันขอต้อนรับทุกท่านเข้าร่วมประชุมวิชาการประจำปีครั้งที่ 32 ของสมาคมเภสัชวิทยาแห่งประเทศไทย ซึ่งการประชุมครั้งนี้ได้รับความเอื้อเฟื้อจากสมาชิกชาวเภสัชวิทยาและบุคลากรทุกๆท่านจากโครงการบัณฑิตศึกษา สาขาชีวเวชศาสตร์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ วิทยาเขตรังสิต ปทุมธานี ร่วมเป็นเจ้าภาพในการจัดประชุมครั้งนี้ โดยมี ศ.ดร.เกศรา ณ บางช้าง เป็นประธาน หัวข้อการประชุมครั้งนี้ ประกอบด้วย เทคโนโลยีด้านการพัฒนาและวิจัยยาโรคติดเชื้อและมะเร็ง วิทยาการก้าวหน้าทางเภสัชวิทยาของเคมีบำบัดโรคติดเชื้อ แบคทีเรีย ไวรัส และปรสิตที่สำคัญ รวมถึงเคมีบำบัดโรคมะเร็ง นอกจากนี้ยังมีการนำเสนอผลงานวิจัยของนักเภสัชวิทยา นักศึกษาบัณฑิต และการมอบรางวัลผลงานการนำเสนอผลงานวิจัยดีเด่นประจำปี ในนามของสมาคมเภสัชวิทยาฯ ดิฉันขอขอบคุณวิทยากร คณะกรรมการจัดการประชุม สมาชิก นักศึกษา ผู้ให้การสนับสนุนทั้งจากหน่วยงานภาครัฐและเอกชน และผู้เข้าร่วมประชุมทุกท่านที่ได้มีส่วนทำให้การประชุมครั้งนี้สำเร็จบรรลุวัตถุประสงค์ตามที่หวังไว้ทุกประการ

รองศาสตราจารย์ ดร. จินตนา สัตยาศัย
นายกสมาคมเภสัชวิทยาแห่งประเทศไทย

สารจากประธานจัดงานประชุมวิชาการประจำปีครั้งที่ 32

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เรียน สมาชิกสมาคมเภสัชวิทยาและผู้เข้าร่วมประชุมทุกท่าน

ในนามของคณะกรรมการจัดการประชุม และคณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ดิฉันขอขอบคุณทุกท่านที่ให้ความสนใจเข้าร่วมประชุมวิชาการประจำปีครั้งที่ 32 ของสมาคมเภสัชวิทยาแห่งประเทศไทย ในหัวข้อบรรยายหลักคือ “Chemotherapy 2010: Discovery and Development” โดยมีเนื้อหาการบรรยายในหัวข้อย่อยซึ่งมุ่งเน้นให้ผู้เข้าประชุมซึ่งเป็นนักเภสัชวิทยาและนักวิจัยสาขาทางวิทยาศาสตร์อื่นๆ ที่เกี่ยวข้องได้รับความรู้เกี่ยวกับเทคโนโลยีและวิทยาการก้าวหน้าด้านการพัฒนาและวิจัยยาโรคติดเชื้อและมะเร็งซึ่งเป็นปัญหาสาธารณสุขสำคัญของโลกซึ่งกำลังเป็นที่สนใจ อาทิ เคมีบำบัดโรคติดเชื้อ แบคทีเรีย ไวรัส และปรสิตที่สำคัญ รวมถึงเคมีบำบัดโรคมะเร็ง โดยได้รับเกียรติจากวิทยากรชั้นนำทั้งในและต่างประเทศ มาให้บรรยายเป็นเวลา 2วันเต็ม โดยเฉพาะอย่างยิ่ง Professor Dr. Stephen A Ward จากมหาวิทยาลัย Liverpool ประเทศอังกฤษ ซึ่งเป็นองค์ปาฐกถาในปาฐกถาพิเศษ รองศาสตราจารย์ ดร.จิราวัฒก์ สดาวงศ์วิวัฒน์ ซึ่งมาให้การบรรยายเรื่อง “The Role of Pharmacology in Optimizing Antimalarial Therapy & Approaches Towards Antimalarial Drug Candidates” นอกจากการปาฐกถาและการบรรยายแล้วยังมีการนำเสนอผลงานวิจัยของอาจารย์ นักวิจัย และนักศึกษา ด้านการวิจัยสารจากธรรมชาติ การวิจัยทางเภสัชจลนศาสตร์และเมตาโบลิซึ่ม การวิจัยเคมีบำบัดโรคติดเชื้อและมะเร็ง การวิจัยทางเซลล์วิทยาและชีววิทยาระดับโมเลกุล

ดิฉันและคณะกรรมการฯ หวังเป็นอย่างยิ่งว่าการประชุมครั้งนี้จะเป็นประโยชน์ต่อผู้เข้าร่วมประชุมทุกท่านในการนำไปใช้ในการเรียนการสอนและการวิจัย การจัดงานครั้งนี้เป็นครั้งแรกของมหาวิทยาลัยธรรมศาสตร์ ร่วมกับสมาคมเภสัชวิทยาแห่งประเทศไทย หากมีสิ่งใดขาดตกบกพร่อง ดิฉันขอน้อมรับเพื่อนำไปปรับปรุงแก้ไขต่อไป

ศาสตราจารย์ ดร.เกศรา ณ บางช้าง
ประธานกรรมการประชุมวิชาการ

คำกล่าวเปิดการประชุมวิชาการ ประจำปี ครั้งที่ 32
สมาคมเภสัชวิทยาแห่งประเทศไทยร่วมกับโครงการบัณฑิตศึกษา
สาขาชีวเวชศาสตร์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์
โดย
ศาสตราจารย์ ดร .สุรพล นิติไกรพจน์
อธิการบดีมหาวิทยาลัยธรรมศาสตร์

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ท่านนายกสมาคมเภสัชวิทยาแห่งประเทศไทย ท่านคณะกรรมการจัดการประชุมและท่านผู้เข้าร่วมประชุมทุกท่าน ในนามของมหาวิทยาลัยธรรมศาสตร์ ผมมีความยินดีเป็นอย่างยิ่งที่ได้รับเกียรติมาเป็นประธานในพิธีเปิดการประชุมวิชาการเภสัชวิทยาครั้งที่ 32ของสมาคมเภสัชวิทยาแห่งประเทศไทย ร่วมกับคณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ในวันนี้

ผมขอแสดงความชื่นชมที่ได้ทราบว่าสมาคมเภสัชวิทยาแห่งประเทศไทย ได้จัดให้มีการประชุมวิชาการประจำปีอย่างต่อเนื่องมาจนถึงปีที่ 32การประชุมแต่ละครั้งก็ประสบความสำเร็จอย่างดียิ่ง การจัดการประชุมสัมมนาทางวิชาการของสมาคมฯ นอกจากจะเป็นการส่งเสริมให้นักวิจัยในสาขาวิชาเภสัชวิทยาได้มีโอกาสเผยแพร่ผลงานวิจัยของตัวเองในด้านวิชาการอย่างสม่ำเสมอแล้ว ยังเป็นโอกาส ให้มีการถ่ายทอดเทคโนโลยีและแลกเปลี่ยนความคิดเห็นในหมู่นักวิชาการอันจะนำไปสู่การพัฒนาทางด้านวิชาการของสาขาวิชาเภสัชวิทยาต่อไป นอกเหนือจากการนำเสนอผลงานวิจัยในสาขาเภสัชวิทยาแล้ว ปัจจุบันความก้าวหน้าทางวิทยาศาสตร์และเทคโนโลยีทำให้การที่จะดำเนินการวิจัยให้เป็นผลสำเร็จตามเป้าหมายนั้นจำเป็นต้องมีการเชื่อมโยงกันของสาขาวิชาต่างๆ เป็นสหสาขาวิชา ดังนั้น การประสานงานกันของผู้เชี่ยวชาญของแต่ละสาขาวิชาในการมาทำงานร่วมกัน จึงนับว่าเป็นสิ่งจำเป็นอย่างยิ่ง ดังนั้น การประชุมวิชาการครั้งนี้ นอกเหนือจากนักวิชาการด้านเภสัชวิทยาแล้ว ผมคาดว่าจะมีนักวิชาการจากสาขาวิชาอื่นๆ ที่เกี่ยวข้องมาร่วมด้วย ไม่มากก็น้อย

สิ่งที่น่ายินดีอีกประการหนึ่งคือการที่มหาวิทยาลัยธรรมศาสตร์ได้มีโอกาสในการร่วมจัดการประชุมทางวิชาการกับสมาคมเภสัชวิทยาแห่งประเทศไทยในครั้งนี้ ทุกท่านที่เข้าร่วมประชุมครั้งนี้คงมีโอกาสได้เห็นบรรยากาศของมหาวิทยาลัยธรรมศาสตร์ ศูนย์รังสิตได้ชัดเจนมากขึ้น โดยเฉพาะการขยายตัวด้านการเรียน การสอน และการวิจัยด้านวิทยาศาสตร์สุขภาพที่มหาวิทยาลัยให้การสนับสนุนอย่างเต็มที่ และมีการพัฒนาก้าวหน้าอย่างรวดเร็ว จนเป็นที่ยอมรับในแวดวงวิชาการด้านนี้เพิ่มขึ้นเป็นลำดับ ผมหวังเป็นอย่างยิ่งว่าการประชุมวิชาการครั้งนี้ จะเป็นประโยชน์แก่ทุกท่านที่เข้าร่วมประชุมในการที่จะนำความรู้ที่ได้ไปพัฒนาทั้งด้านการเรียนการสอนและการวิจัยของตนเองต่อไป

บัดนี้ สมควรแก่เวลาผมขอเปิดประชุมวิชาการประจำปี ครั้งที่ 32ของสมาคมเภสัชวิทยาแห่งประเทศไทยเรื่อง “Chemotherapy 2010: Discovery and Development” ขออวยพรให้การประชุมครั้งนี้สัมฤทธิ์ผลทุกประการ และขอให้ทุกท่านที่มาร่วมประชุมจงประสบแต่ความสุข สวัสดี โดยทั่วกัน

ศาสตราจารย์ ดร .สุรพล นิติไกรพจน์
อธิการบดีมหาวิทยาลัยธรรมศาสตร์

บรรณาธิการแถลง

เรียนท่านสมาชิกและผู้เข้าร่วมประชุม

วารสารเภสัชวิทยาแห่งประเทศไทย (Thai Journal of Pharmacology) ฉบับนี้เป็นฉบับแรกของปี 2553และเป็น Proceeding ของงานประชุมวิชาการประจำปีนี้ วารสารฉบับนี้มีความหนาเป็นพิเศษเนื่องจากมีโปสเตอร์นำเสนอผลงานวิชาการมากกว่าทุกปีที่ผ่านมา จึงเชื่อได้ว่างานประชุมวิชาการของสมาคมฯในปีนี้ย่อมคึกคักกว่าทุกปีอย่างแน่นอน

เป็นครั้งแรกที่คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ได้รับเกียรติเป็นเจ้าภาพจัดการประชุมวิชาการครั้งที่ 32 ของสมาคมเภสัชวิทยาแห่งประเทศไทย และมีส่วนให้พวกเราได้มีโอกาสมาเยี่ยมเยือนมหาวิทยาลัยในส่วนวิทยาเขตรังสิต และเชื่อว่าหลายท่านคงได้รับความประทับใจในความสวยงามของสถานที่จัดงานประชุม

วารสารฉบับนี้จักไม่สำเร็จลงได้ด้วยดีถ้าปราศจากความร่วมมืออย่างแข็งขันจากทีมงานฝ่ายเอกสารและวิชาการของคณะกรรมการจัดประชุมครั้งนี้ โดยเฉพาะอย่างยิ่งท่านประธานจัดงาน คณาจารย์ตลอดจนเจ้าหน้าที่ทุกท่านของโครงการบัณฑิตศึกษา คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ดิฉันจึงขอใช้โอกาสนี้แสดงความชื่นชมความสามารถด้านบริหารจัดการและประสานงานอย่างมีประสพการณ์ของมืออาชีพ ดิฉันขอขอบพระคุณทุกท่านที่ได้สละเวลาช่วยงานอย่างเต็มที่

นอกเหนือจากความรู้ที่ทันสมัยสมกับหัวข้อการประชุมในปีนี้แล้ว ดิฉันหวังว่าท่านสมาชิกและผู้เข้าร่วมประชุมจักได้มีโอกาสรู้จักและแลกเปลี่ยนประสพการณ์ด้านงานวิจัยกับเพื่อนสมาชิกจากคณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ และอาจเกิดแนวทางที่จะมีความร่วมมือกันที่จะสร้างผลงานวิจัยด้านเภสัชวิทยากันต่อไปในอนาคต

รศ.ดร.สุพัตรา ศรีไชยรัตน์

บรรณาธิการวารสาร

รายนามวิทยากร

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ศาสตราจารย์ ดร.นพ.ประเสริฐ เอื้อวรากุล	คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล
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ผู้ช่วยศาสตราจารย์ ดร.นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา	ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ศาสตราจารย์ ดร.สุภา หารหนองบัว	คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์
รองศาสตราจารย์ ดร.กอบธัม สถิรกุล	คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล
รองศาสตราจารย์ ดร.อรุณพร อิฐรัตน์	คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์
ผู้ช่วยศาสตราจารย์ ดร .คณิต สุวรรณบริรักษ์	คณะเภสัชวิทยา จุฬาลงกรณ์มหาวิทยาลัย

กำหนดการประชุมวิชาการประจำปี ครั้งที่ 32

สมาคมเภสัชวิทยาแห่งประเทศไทย

ร่วมกับโครงการบัณฑิตศึกษา สาขาชีวเวชศาสตร์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ระหว่างวันที่ 25 – 26 มีนาคม 2553

ห้องประชุมใหญ่ TCC LAND Auditorium

อาคารคณะสถาปัตยกรรมศาสตร์และการผังเมือง

มหาวิทยาลัยธรรมศาสตร์ วิทยาเขตรังสิต ปทุมธานี

Chemotherapy 2010: Discovery and Development

วันที่ 25 มีนาคม 2553

8.00 – 8.45 ลงทะเบียน

8.45 – 9.00 ประธานกล่าวรายงาน

พิธีเปิดการประชุมโดยอธิการบดีมหาวิทยาลัยธรรมศาสตร์

นายกสมาคมฯ กล่าวต้อนรับผู้เข้าร่วมประชุม

9.00 – 10.15 ปาฐกถานำ ดร.จิราวัฒก์ สดาวงศ์วิวัฒน์

เรื่อง The Role of Pharmacology in Optimizing Antimalarial Therapy & Approaches Towards Antimalarial Drug Candidates

Professor Dr. Stephen A. Ward, Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, University of Liverpool, UK

10.15 – 10.30 พักรับประทานอาหารว่าง

10.30 – 12.00 **Session 1: Update on Virology and Pharmacology of Antiviral Agents**

Antiretroviral therapy 2010: From reality to research

ผู้ช่วยศาสตราจารย์พิเศษ แพทย์หญิงจุรีรัตน์ บวรวัฒนุวงศ์

โรงพยาบาลชลบุรี

Influenza H1N1 2009

ศาสตราจารย์ ดร.นพ.ประเสริฐ เอื้อวรากุล

คณะแพทยศาสตร์ ศิริราชพยาบาล มหาวิทยาลัยมหิดล

Anti-influenza Pharmacology

ผู้ช่วยศาสตราจารย์ ดร.วัชรี ลิมปนสิทธิกุล

ภาควิชาเภสัชวิทยา จุฬาลงกรณ์มหาวิทยาลัย

ผู้ดำเนินการอภิปราย: *ผู้ช่วยศาสตราจารย์ ดร.วัชรี ลิมปนสิทธิกุล*

ภาควิชาเภสัชวิทยา จุฬาลงกรณ์มหาวิทยาลัย

12.00 – 13.00 พักรับประทานอาหารกลางวันและชมการแสดงผลงานวิชาการโดยโปสเตอร์ และ Lunch Symposium

13.00 – 14.30 **Session 2: Update on Chemotherapy of Anticancer Agents**

Clinical Application of New Anticancer Agents

ผู้ช่วยศาสตราจารย์ นพ. วิโรจน์ ศรีอุฬารพงศ์

ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

Anticancer Drugs and Food Interactions

รองศาสตราจารย์ ดร.จุฑามาศ สัตยวิวัฒน์

สถาบันวิจัยจุฬาภรณ์

ผู้ดำเนินการอภิปราย: *รองศาสตราจารย์ ดร. วีรพล คู่คงวิริยพันธุ์*

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์

มหาวิทยาลัยขอนแก่น

14.30 – 15.00 พักรับประทานอาหารว่าง

15.00 – 16.30 **Session 3: Stem Cell Therapy**

Induced Pluripotent Stem Cells

ผู้ช่วยศาสตราจารย์ ดร.พญ.กัญญา ศุภปีติพร

ภาควิชากุมารเวชศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ผู้ช่วยศาสตราจารย์ ดร.นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ผู้ดำเนินการอภิปราย: *ผู้ช่วยศาสตราจารย์ ดร.นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา*

ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์

จุฬาลงกรณ์มหาวิทยาลัย

16.30-17.00 ประชุมธุรการของสมาชิกสมาคมเภสัชวิทยาแห่งประเทศไทย

18.00 งานเลี้ยงต้อนรับ

วันที่ 26 มีนาคม 2553

8.45-10.15 น. **Session 4: Chemotherapy and Discovery of Anti-HIV Agents**

Pharmacogenomics and Pharmacokinetics in Anti-HIV Drug Development

Dr. Tim R. Cressey, Harvard School of Public Health, Harvard University Boston, Massachusetts. (Institut de Recherche pour le developpement, Program for HIV Prevention and Treatment, Faculty of Associated Medical Sciences, Chiang Mai University)

ผู้ดำเนินการอภิปราย: *รองศาสตราจารย์ ดร. วิจิตรา ทัศนียกุล*
ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น

10.15 – 10.30 น. พักรับประทานอาหารว่าง

10.30 - 12.00 น. ชมและตัดสินการแสดงผลงานวิชาการโปสเตอร์
โดย คณะกรรมการประกวดผลงาน

12.00 – 13.00 น. พักรับประทานอาหารกลางวัน

13.00 – 14.30 น. **Session 5: Drug Design By Molecular Modeling**

Drug Design Approaches: Molecular Modeling, Computational Chemistry and Combinatorial Chemistry

ศาสตราจารย์ ดร.สุภา หารหนองบัว
คณะวิทยาศาสตร์ มหาวิทยาลัยเกษตรศาสตร์

Drug Discovery: Pharmacokinetic/Pharmacodynamic Fitting and Simulation

รองศาสตราจารย์ ดร.กอบธัม สถิรกุล
คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล

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14.30 – 14.45 พักรับประทานอาหารว่าง

14.45 – 16.15 **Session 6: Chemotherapy and Discovery of Chemotherapeutic Agents: Research on Natural Products**

From Herbal Medicine to Modern Medicine: New Leads for Infectious and Cancer Chemotherapy

รองศาสตราจารย์ ดร.อรุณพร อิฐรัตน์
คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

Marine Compounds as Sources of Chemotherapeutics

ผู้ช่วยศาสตราจารย์ ดร. คณิต สุวรรณบริรักษ์
คณะเภสัชวิทยา จุฬาลงกรณ์มหาวิทยาลัย
ผู้ดำเนินการอภิปราย: *รองศาสตราจารย์ ดร. อรุณพร อิฐรัตน์*
คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

16.15 – 16.30 พิธีมอบรางวัลการเสนอผลงานวิจัยดีเด่น
ปิดการประชุม

Chiravat Sadavongvival Memorial Lecture

The challenge of identifying novel antimalarial drug candidates

Steve Ward

Liverpool School of Tropical Medicine, University of Liverpool, UK

The need for the next generation of novel antimalarials is highlighted by the reports of reduced parasite susceptibility to the artemisinins emerging from the Thai Cambodian borders as early as 2003 and finally confirmed in 2009 (Dorndorp et al 2009, Cui and Su 2009). After the demise of chloroquine and pyrimethamine/sulphadoxine and the loss of efficacy of mefloquine, the use of drug combinations containing an artemisinin was heralded as a very timely solution to the burgeoning malaria problem worldwide. In fact leading scientists of the day when asked about potential resistance to this new drug class were quoted as saying "not in our life-time" Sadly this seems now to have been naïve and over-optimistic. If history tells us anything it is that the malaria parasite is more than well equipped with the evolutionary equipment to thwart our efforts at chemotherapy. Having said that we should not underestimate the huge impact the artemisinin - based combinations are having and will continue to have in the control and treatment of malaria globally over the short to medium term. However it is clear we now need a Plan B.

The Antimalarial drug development path, as with any drug development path, is both time-consuming and expensive. In reality any screening hit identified today would take 10-15 years before it became a therapeutic reality for patients in malaria endemic countries using the accepted drug development paradigm. So it is important that at the outset we are clear on what type of drug we want to develop, often referred to as the Target Product Profile (TPP). The TPP will differ depending on the end use of the drug e.g. treatment of non - severe *falciparum* disease, prophylaxis, radical cure etc. and several organisations including WHO and MMV have TPP's for malaria published on their websites (Wells et al 2009). This has become increasingly important in the so-called "malaria eradication era". The drugs that will be required to eradicate malaria will not necessarily have the same TPP as drugs needed in the control and treatment phases of this programme.

Through the efforts of organizations such as the Medicines for Malaria Venture (MMV), the antimalarial drug development pipeline is as healthy as at any time in recent history. However a cursory glance at the molecules in the portfolio reveal some causes for concern. There are very few drugs that genuinely target novel parasite processes, there is a heavy reliance on variations of existing themes including in the peroxide based drug class and there is a dearth of drugs that are being developed with a view to eliminating liver stage parasites including hyponozoites, gametocytes and other species most notably *P. vivax*. That being said, these specific areas are being specifically targeted for funding as we move forward (Wells et al 2009).

Efforts to rationally develop antimalarial drugs against specific and novel parasite targets has so far proven difficult without a single successful example in the clinic. The malaria genome has been in the public domain for nearly a decade (Gardner et al 2002). Yet despite significant scientific endeavour the number of truly validated is small (Wells et al 2009, Olliaro and Wells 209, Aguero et al 2008). There are many factors that contribute to this but the difficulty in developing a condition knock out strategy for falciparum malaria and a genome where almost half the genes have no know function are significant contributors.

Faced with this poor return on effort, the community has moved towards whole cell *P. falciparum* screening of chemical libraries as a source of new parasite specific antimalarial

"hits". This approach, sponsored in large part by MMV, has proven spectacularly successful. To date some 5 million individual compounds have been screened and up to 20,000 different compounds have been identified that kill *P. falciparum in vitro* with IC50 values in the sub-nanomolar range (Wells et al 2008). The *P.falciparum* genome has approximately five and a half thousand genes and from extrapolation with other cellular systems it might be reasonable to expect that less than 5% of these genes would represent genuinely druggable targets. Based on this argument, many of the 20,000 novel hits must target a common gene product.

This is a fantastic new resource for the community, but there are many hurdles before these hits become drug candidates and then ultimately drugs in the clinic. Even for the biggest of pharmaceutical companies working in one of the "block-buster" therapeutic areas, it would be impossible to rationally triage 20,000 compounds into a hit to lead programme even if substantial singletons can be clustered based on some a priori feature. To do the data justice will require input from the whole malaria community with partnerships between academia and industry. The first challenge will be to ensure that this data reaches the public domain, which unfortunately has not been the case to date.

The development pathway to a drug registration is well established yet attrition rates remain very high. Data presented by Wells and Oliarro (2009) suggest the need for 7 to 10 molecules entering phase I clinical trials in order to ensure one new antimalarial drug combination into the clinic. Consequently we will need all the hits available in order to ensure that the early discovery and development is sufficiently robust to deliver this number of clinical candidates. The fact that this is a significant challenge cannot be overstated. An interesting example of the unforeseen challenges that lie ahead can be exemplified by the isoquine and CDA projects both supported by MMV. In the case of isoquine this was a drug from a known class, the 4-aminoquinolines, that had been rationally redesigned to overcome resistance and idiosyncratic toxicity based on a reactive metabolite (O'Neill et al 2009). Despite over 50 years of successful clinical experience with the drug class, the pre-clinical development programme repeatedly uncovered unexpected toxicities in animal models. When the drug eventually entered phase I trials, a drug associated serious adverse event finally terminated the programme. In the case of CDA, another drug using well-established clinical drugs, it was only at the phase III clinical trial stage, using a very focused protocol design looking at hemotoxicity in detail, that an unexpected hemotoxicity in G6PD deficient patients was uncovered which precluded its development as a treatment for non-severe malaria (Tiono et al 2009). The fact that working with so called well understood drugs and drug classes can still fail to met all the requirements for modern drug registration, stresses the need for greater filtering at the early discovery stage to see if as much risk as possible can be eliminated from the molecules being taken forward. This concern is not restricted to antimalarial development but is a pharma industry wide problem. It is now standard practice to look for toxic and metabolic alerts and dispositional liabilities in lead molecules very early and certainly before they progress to candidates. Specifically for antimalarials, the potential for resistance development is also considered early in development. Although to date it is not clear what would constitute a no-go decision based on resistance acquisition. Finally and with reference to the outputs from whole cell screening we are encouraged to establish a mechanism of action. Although lack of this information isn't a barrier to development and registration, it does pose difficulties and slows the progress of the development process.

Additional challenges for the malaria community are the lack of adequate predictive models to assist in the candidate selection process. In the case of *P. falciparum* there are excellent *in vitro* models and a humanized mouse model. There are no robust *in vitro* assays for *P. vivax* and this is major hinderence to developing drugs against this malaria species. Equally we await a validated *in vitro* hypnozoite assay and a truly validated gametocyte assay (*P.*

falciparum in the first instance) that is predictive of transmission blocking activity. All of these are opportunities for new development and research investment.

In conclusion it is clear that we urgently need new antimalarial drugs with novel mechanisms of action that can effectively treat parasite populations resistant to existing drugs. We are now in a unique position with respect to P. falciparum with several thousand in vitro nanomolar "hits" and the challenge will be to efficiently triage these hits to leads onto candidates and then eventually into clinical drugs. We will also need to look beyond drugs to treat non-severe falciparum malaria and severe malaria as we plan the development programmes that will deliver in 10-15 years. We will need to consider other malaria species and other applications such as radical cure, intermittent presumptive treatments, mass drug administration etc and modify our TPP's accordingly.

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Session 1: Update on Virology and Pharmacology of Antiviral Agents

AIDS 2010 : From Reality to Research

Chureeratana Bowonwatanuwong, M.D.

Internal Medicine Department, Chonburi Hospital, Thailand 2000

Abstract

Since the first reported HIV infected case in Thailand in 1984, the number of accumulated HIV/AIDS case increased to more than 1 million in 2007. With the first Antiretroviral drug (ARV) Zidovudine (AZT) launched in 1987, currently more than 25 effective antiretroviral drugs were approved and available. From the worse prognosis that all AIDS case died within one and a half year returned to good prognosis with same life expectancy and quality as general population by treated with highly active antiretroviral therapy(HAART). These reflected dedicated, effective research work from all related scientists and clinicians and also all contributed institutes.

The milestone of research works that created to save life of HIV infected people were:

1. 1996 – 2001: An achieve of markedly reduce mortality of AIDS patients from various opportunistic infections and boosted immunological status to the treated patients
2. 2001-2006: A successful attempt in suppressing circulated virus to undetectable level and maintaining such level more than 7 years.
3. 1996-2010: A marvelous research in preventing mother to child transmission (PMTCT) from the high rate at 30% in year 1995 to less than 2% in year 2008 or even at o% nowadays.

Current unending research in HIV/AIDS fields are the challenging to improve ARV efficacy and its profile to more adherence and less long term adverse events in which alive treated patients are facing. These 2 decades saga proved that the reality suffers could be definitely solved by the research mind and hands.

Keywords: highly active antiretroviral therapy (HAART), undetectable virus, preventing mother to child transmission (PMTCT), reality, research

Session 1: Update on Virology and Pharmacology of Antiviral Agents

Influenza A 2009 H1N1

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Abstract

Although most cases of influenza A 2009 H1N1 were mild and self-limited, many cases developed severe pneumonia leading to acute respiratory distress syndrome (ARDS) and death. It is not clear what determined the disease severity in these cases. Host factors including pregnancy, obesity, and chronic illnesses are certainly contributing factors, but other unknown host and viral factors may also play pivotal roles. The pandemic influenza A 2009 H1N1 virus has been shown to induce more severe lung pathology in ferrets and monkeys than seasonal influenza viruses. This may be a result of receptor-binding property of the virus, as the pandemic virus binds not only to α2,6-linked sialic acid, expressed mainly in upper airway, but also with a lower affinity to α2,3-linked sialic acid, expressed in alveoli. The ability of this virus to infect alveolar epithelial cells may play a crucial role in pneumonia and ARDS. On the other hand, evidences are pointing to host innate responses as major determinants of disease outcome. Therapeutic approaches aiming at modifying innate inflammatory responses have been proposed but not yet fully explored. Both mechanistic studies and clinical trials addressing these alternative treatments are needed for a better pandemic influenza preparedness.

Keywords: influenza, pandemic, H1N1, pathogenesis, tissue tropism, innate responses.

Session 1: Update on Virology and Pharmacology of Antiviral Agents

Anti-influenza Pharmacology

Wacharee Limpanasithikul

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Abstract

The World Health Organization (WHO) announced the worldwide pandemic infection of the novel H1N1 influenza virus as "2009 H1N1" on 11th June 2009. This virus originally called "swine flu" is a new strain of the influenza A virus that contains mixed segments of genes from pig, bird and human influenza viruses. Although vaccine is the primary need for the prevention of this virus, vaccine production would not be available immediately to prevent the first time of spread of a new strain of influenza virus. Vaccines are being deployed in some well-resourced countries but are not available globally. Antiviral drugs are thus an important strategy for specific protection of this pandemic outbreak. Two groups of antiviral drugs are generally use for the prophylaxis or treatment of influenza infections: the adamantanes (amantadine and rimantadine) and the neuraminidase (NA) inhibitors [oseltamivir (Tamiflu®) and zanamivir (Relenza®)].

Amantadine and rimantadine block viral uncoating in the infected cell. They are effective against influenza A and are associated with several side effects and with rapid emergence of drug-resistant variants. This potential for the development of resistance limits the use of these drugs for the treatment of influenza. They are not recommended for the prophylaxis or treatment of the 2009 H1N1 influenza virus.

The neuraminidase inhibitors oseltamivir and zanamivir interfere with the release of progeny influenza virus from infected host cells, a process that prevents infection of new host cells and thereby inhibits the spread of infection in the respiratory tract. These drugs are effective against all strains of influenza. They are advantage over the adamantanes, which are effective only against sensitive strains of influenza A. The neuraminidase inhibitors should be administered as early as possible, between 24 and 72 hours after the onset of the illness. They are associated with fewer side effects and are less likely to induce drug-resistant influenza than the amantanes.

At present, the 2009 H1N1 virus is susceptible to the NA inhibitors and resistant to the adamantanes. Antiviral treatment is recommended for all hospitalized patients with confirmed, probable, or suspected 2009 H1N1 infection and patients at high risk of complications. Treatment with these drugs is not recommended for low-risk patients with uncomplicated febrile illness. For the greatest benefits, treatment with the neuraminidase inhibitors should be started within 48 hours of the onset of the illness. However, there are evidences that hospitalized patients still benefit from treatment initiation even later. These drugs are use in the same doses and as for seasonal influenza.

Keywords: Anti-influenza, antiviral, H1N1

Session 2: Update on Chemotherapy of Anticancer Agents

Clinical Application of New Anticancer Agents

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Abstract

Recent advance in the development of several novel anticancer agents has led to significant improvement of outcome of cancer treatment. Major achievements in the field of target therapy have been focusing on identification of newer generation of anti-growth factor receptor. Combinatorial therapy with specific monoclonal antibodies like trastuzumab and pertuzumab to target parallel growth factor signaling pathways may lead to improvement of outcome of breast cancer treatment. In addition, a novel toxin conjugated trastuzumab has been shown to possibly break the resistant barrier of HER2 positive advanced breast cancer. Second generation tyrosine kinase inhibitors (TKI) targeting EGFR mutation, for example BIBW2992 and HKI272, have been studied to overcome the secondary resistance occurred following previous exposure to TKI. Several of these agents have now entered into phase III studies in lung cancer. Other non-TKI targeted agents are currently under intensive studies in several indications. Key targeted non-growth factor receptor pathways involve mTOR survival pathway, protein degradation apparatus, and DNA repair mechanism. Everolimus, a mTOR inhibitor is now a proven second line agent following failure to first line multi-target TKI like sunitinib or sorafenib in renal cell carcinoma. Moreover PARP inhibitors have preliminary shown to be effective in the so called triple negative breast cancer which has no a current specific agent of choice. All of these examples strongly illustrate a new era of fighting against cancer.

Keywords: growth factor receptor, anticancer, tyrosine kinase, mTOR, PARP

Session 3: Stem Cell Therapy

Cell therapy by generation of disease-corrected, patient-specific pluripotent stem cells

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Abstract

Several diseases with genetic etiologies have proved to be cured by bone marrow transplantation. However, donor availability constrains its usage. Recent technologies have made human induced pluripotent stem (iPS) cells a foreseeable and realistic source to release this limitation.

iPS cells derived from somatic cells hold promise to develop patient-specific cell therapies and provide experimental platforms to model human diseases. iPS cells are the product of somatic cell reprogramming to an embryonic-like state. Reprogramming mature somatic cells to generate iPS cells occur by the introduction of a defined and limited set of transcription factors. Very recently, iPS-cell technology has been successfully applied to human somatic cells and used for the generation of disease-corrected, patient-specific cells with potential value for stem cell therapy. The generation of patient-specific iPS cells could be used in the treatment of several human inherited diseases especially fatal diseases requiring haematopoietic stem cell transplantation. One of the most suitable candidates is a disorder called Wiskott-Aldrich syndrome (WAS). WAS is an X-linked recessive disorder characterized by immunodeficiency, thrombocytopenia and eczema. Without bone marrow transplantation, most patients die by 10 years old due to recurrent infections, haemorrhage or autoimmune diseases. We have identified a Thai patient with classic WAS with a novel and unique termination codon mutation (p.X503R) in the *WAS* gene. This resulted in an absence of protein with a severe phenotype in this patient. Since bone marrow transplantation cannot be performed due to a lack of a suitably matched donor, disease-corrected, patient-specific iPS cells could be an alternative source for treatment of this fatal disease.

Keywords: human induced pluripotent stem cells, iPS, patient-specific cell therapy.

Session 4: Chemotherapy and Discovery of Anti-HIV Agents

Pharmacogenomics and Pharmacokinetics in Anti-HIV Agent Development

Tim R. Cressey[1,2]

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Abstract

HIV-infected immunocompromised patients require life-long antiretroviral therapy. To date, 26 antiretroviral drugs are approved by the US Food and Drug Administration for the treatment of HIV. Highly Active Antiretroviral Therapy (HAART), normally a combination of three antiretroviral drugs, has dramatically improved the prognosis of HIV/AIDS. However, viral replication under therapy can lead to the selection of drug resistant viruses and subsequent virologic failure. While poor adherence is likely to be the main cause of treatment failure, individual pharmacokinetic variability can also play an important role. Drug-drug interactions, drug-food interactions, sex, age, renal/hepatic function and pregnancy are all sources of pharmacokinetic variability.

In recent years, host genetic polymorphisms have also been shown to explain part of this variability and several pharmacogenetics studies have demonstrated that host genetic polymorphisms can influence antiretroviral drug exposure, toxicity and response to treatment. During antiretroviral drug development, drug hypersensitivity reactions have been reported for several agents. Based on pharmacogenetic research data antiretroviral treatment decisions based on host genetics to prevent the risk of hypersensitivity reactions are now part clinical practice. Specifically, it is now recommended that patients initiating abacavir are screened for the presence of the HLA-B*5701 allele as it is strongly associated with an immunologically mediated hypersensitivity reaction to abacavir, which in rare cases can be fatal.

Antiretroviral drugs within the Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTIs) drug class are commonly used within first-line HAART regimens. Substantial evidence exists that polymorphisms in the *CYP2B6* drug metabolizing enzyme gene are associated with higher NNRTI drug exposure, and in some studies with early drug toxicity (mainly efavirenz related neuropsychological toxicity). The bulk of evidence concerns the *CYP2B6 516G>T* polymorphism, primarily within the variant *CYP2B6*6* allele that also includes the *785A>G* polymorphism, which has been shown to be associated with higher efavirenz plasma exposure but not with time to virologic or toxicity-related failure. To date, in the absence of drug toxicity, it is unclear the benefit of a clinical intervention for patients identified with high NNRTI plasma drug concentrations or who are carriers of a genotype associated with high drug concentrations.

Some antiretroviral drug toxicities do not appear until after months of treatment and clinical and pharmacogenomics data could be combined to individualize antiretroviral treatment. Strong evidence supports the existence of host genetic polymorphisms that predict a higher risk of unconjugated hyperbilirubinemia in patients receiving atazanavir. Perhaps patients with risk alleles for hyperbilirubinemia should not necessarily avoid atazanavir use but may require closer laboratory monitoring. Similarly, the genetics of tenofovir associated nephrotoxicity may become increasingly important as tenofovir slowly replaces zidovudine in HAART regimens throughout the world.

To date, pharmacogenetics analyses of antiretroviral drugs have identified several host genetic polymorphisms associated with antiretroviral drug toxicity and

pharmacokinetics. Understanding the contribution of specific polymorphisms on antiretroviral drug efficacy and/or toxicity may lead to simple yet critical interventions to further optimize these life-saving treatments.

Keywords: Pharmacogenomics, HIV, antiretroviral, HLA-B*5701, *CYP2B6*, efavirenz, hypersensitivity reactions

Session 5: Drug Design By Molecular Modeling

Frontier Research for HIV-1 Reverse Transcriptase Inhibitor Discovery

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Abstract

Three research platforms have been established for innovative research on Anti-AIDS drug discovery. Computational Drug Discovery platform includes structural modification of Natural products and organic syntheses, QSAR, quantum chemical calculations, virtual screening, *de novo* design, combinatorial library design, protein-ligand interaction simulations, large-scale Molecular Dynamics simulations, drug-likeness analysis and ADME/T prediction. In addition, Biological-physicochemical experimental platform has been set up to verify and realize the computational design based on several biophysical technologies, such as biological activity assay, including enzyme assay and cell-based assay, X-ray crystallographic and NMR spectroscopic studies on enzymes, enzyme kinetics study, and isothermal titration calorimetry (ITC), which can be used to determine ligand-receptor interaction and protein-protein/DNA interaction. Another goal is to set up a Development of methodology and nanopolymer platform to develop fluorescence resonance energy transfer materials for biological assay which might lead to high throughput screening technology development. It is hoped that the obtained results will provide drug candidates for drug development, and the platform as to how cooperative and interdisciplinary work can be carried out to further advance this crucial area of research.

Keywords: anti-HIV, Reverse Transcriptase, computer-aided drug design, biological testing.

Session 5: Drug Design By Molecular Modeling

Drug Discovery: Pharmacokinetic/Pharmacodynamic Fitting and Simulation

Korbtham Sathirakul

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Abstract

As widely known, more than 40% of the past failure in drug development is solely due to improper ADME properties of the new chemical entities (NCEs). The NCEs recognized exert highest pharmacological action may not be the optimize NCEs for further drug development. Based on convenience for the patients, the oral or any other extravascular dosage form such as nasal, pulmonary, rectal etc. are the most desired formulations. The extravasular dosing as a means for systemic treatment needs absorption process. Although the NCEs can exert significant pharmacological actions but have very low bioavailabiliy (BA), the pharmaceutical companies may discard these NCEs from their pipe lines. This leads to the concept of "Drug-Likeness" screening. However, the limitation in bioavailability can be the most important for the development of the oral formulations. Recently, the impact of absorptive/secretive processes on a drug's bioavailability has been recognized. Owe to the discovery of significantly vast numbers of the transporters on the surface of the cells which manage the uptake and efflux of NCEs and the possibility of integrate this knowledge with basic mathematical model for fitting the data or simulations, pharmaceutical companies can predict the biopharmaceutics and pharmacokinetics of NCEs more rapidly and precisely. The current available *in vitro* and *in vivo* techniques in combination with new in *silico* will significantly improve the drug discovery and development. Integration of the solid knowledge from chemical structure to pharmacokinetic until the final pharmacodynamics process which is the last objective for the benefits of the patients needs mechanistic approaches. These mechanistic approaches will be illustrated in this article in order to provide an overview and idea on what is available with regards to various experimental model.

Keywords: drug-likeness, pharmacokinetics, pharmacodynamics, Simulation, fittings, mathematical model.

The absorption process can occur in various route of administration. However, the principle of the absorption process via each route of administration is similar. Thus, the author would like to focus entirely on oral drug administration in this article. As widely known, more than 40% of the past failure in drug development is solely due to improper ADME properties of the new chemical entities (NCEs). The NCEs recognized exert highest pharmacological action may not be the optimize NCEs for further drug development. Thus, the focus of NCEs design is not solely on optimizing pharmacodynamic activity but also to ensure adequate pharmacokinetic properties (i.e.absorption, distribution, metabolism,and elimination) to enable suitable dosage forms/regimens to be developed. Oral drug administration is the most common and convenient route for chronic drug therapy [1]. Bioavailability is the term used to express both the extent and the rate at which unchanged drug proceeds from the site of administration to the site of measurement within the body. Oral bioavailability is directly related to the kinetic processes where by drug passes from the gastro intestinal tract (GIT) through the apical membrane of the epithelial cells

(i.e.enterocytes), through the enterocyte cells in to pre-hepatic blood vessels, which collect in the portal vein prior to passage through the liver, before reaching the systemic circulation. The bioavailability of the drug can be described as a fraction of dose escaping from GIT local instability (i.e. degradation, deactivation, insoluble etc.), intestinal mediated metabolism (so-called pre-hepatic first pass metabolism), and hepatic first pass metabolism. Numerous efforts exist to relate the use of physiochemical descriptors of drug molecules to the passive diffusion of drugs through biological membranes to predict the extent of absorption from the GIT. The pH partition hypothesis [2], physical model for passive diffusion [3], absorption potential [4], Lipinski's rule of five [5], quasi equilibrium model [6] and/or rule of unity [7] were used as tools for BA prediction. Although, many drugs have been recognized to penetrate the enterocytes via passive diffusion, recent studies have demonstrated that a number of drug transporters including uptake and efflux systems determine the membrane transport processes. Transporters are membrane proteins that are present in all organisms. These proteins control the influx of essential nutrients and ions and the efflux of cellular waste, environmental toxins, and other xenobiotics. The functions of membrane transporters may be facilitated (equilibrative, not requiring energy) or active (requiring energy). In considering the transport of drugs, pharmacologists generally focus on transporters from two major super-families, ABC (ATP binding cassette) and SLC (solute carrier) transporters. Most ABC proteins are generally active transporters and need ATP hydrolysis. There are 49 known genes for ABC proteins that can be grouped into seven subclasses or families (ABCA to ABCG) [8]. The most famous ABC transporters is P-glycoprotein (P-gp, encoded by ABCB1, also termed MDR1) which firstly discovered in resistant in cancer chemotherapy. The SLC superfamily includes genes that encode facilitated transporters and ion-coupled secondary active transporters that reside in various cell membranes. Transporters are very essential for all living cells. The numbers and types of transporters asymmetrically available in each side of the cell membrane are major contribution to the vectorial transport of any solutes resulted in efficient transfer of solutes across epithelial or endothelial barriers. For example, vectorial transport is important for hepato-biliary and urinary excretion of drugs from the blood to the lumen and in the intestinal absorption of drugs. Transporters work together with drug-metabolizing enzymes to eliminate drugs and their metabolites.

Due to the combination of passive diffusion, presystemic first pass metabolism; especially at the enterocytes and influx/efflux transporter generate the complexity of absorption process. The summary of these processes in enterocytes was illustrated in Figure 1.

Aforementioned above, although the absorption process seems to be complicated, it is not obstacle for a clever human species to understand and make use of combining all the knowledge together to generate the mathematical model in order to be able to fit the experimental results and to perform simulation for any specific scenarios of the interests to predict the outcomes. All the physicochemical, GIT physiological and dosage form/formulation factors as illustrated in Table 1 were included in the many absorption predictive model.

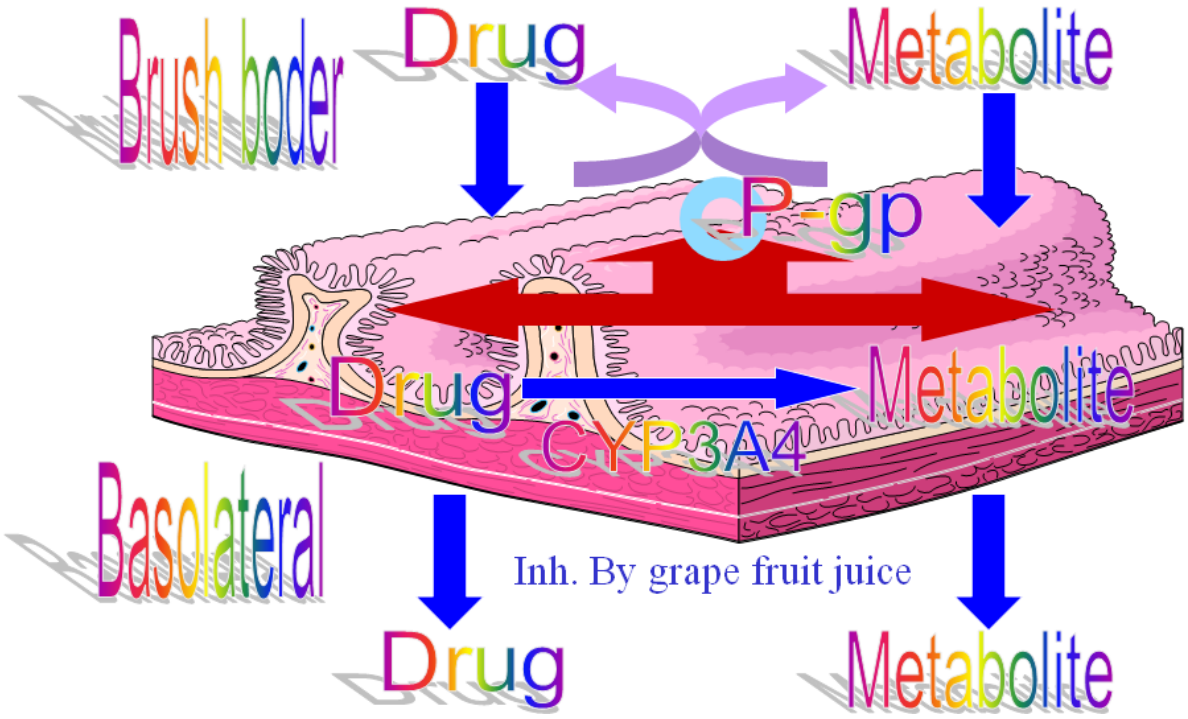


Figure 1: Complexity of absorption process. The P is parent drug and M is metabolite. P and M molecules can get in and out back and forth from the enterocytes before they can reach the systemic circulation.

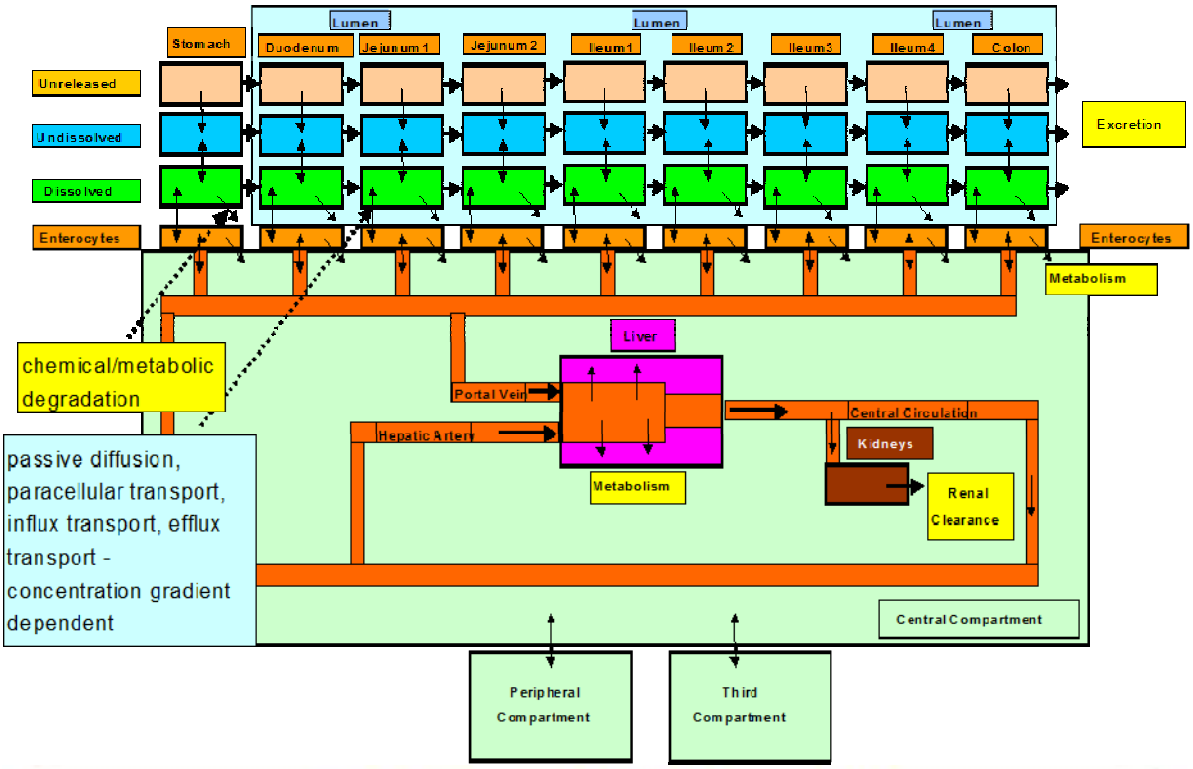
Physiochemical factors of drug substances	Physiological factors of GIT	Dosage form and formulation factors
Solubility	Stomach emptying rate	Dissolution rate
log *P*	Intestinal motility/flow rate	Disintegration rate
pK_a	Membrane surface area	Drug release mechanisms
H-bonding potential	Intestinal metabolism	Excipient effects
Molecular weight/size	Transport mechanisms	
PSA	Native surfactants	
	Intestinal secretions, e.g. mucous, enzymes	
	Intestinal blood/lymph flow	

GIT: gastrointestinal tract; PSA: polar surface area; log *P*: octanol/water partition coefficient.

Table 1 Factors influencing gastrointestinal absorption of drugs.

The mechanistic approaches with the system of equations with a few assumptions were developed to explain and predict BA of the NCEs. However, this approaches need an integration of the data obtained from *in vitro*, *in vivo* and/or in *silico*. Clearly, whole animal studies could not be used as a screening tool in a nearly development stage; therefore, *in vitro* models of intestinal absorption have been developed [9]. This in vitro system includes membrane-based (PAMPA: parallel artificial membrane permeation assay in high throughput fashion) [10], cell culture-based (including Caco-2 (Human colon adenocarcinoma cell, MDCK (Dog kidney epithelial cells) etc.) [11], and *ex vivo* models (Ussing chamber technique) [12, 13]. Each method has its pros and cons. The ability to accurately predict the oral absorption of drugs based solely on *in vitro* data provides an opportunity to assess the

developability, from an absorption point of view, of NCEs before any preclinical or clinical *in vivo* studies are performed. There have been several reports on physiologically based mathematical models that are capable of producing such predictions, and there area few commercially available software packages (e.g.,GastroPlus^{TM}, iDEA^{TM}, Intellipharm^{R} PK, Simp-cyp^{TM} and P K-SimR d etc.) that have been shown to predict the human absorption properties with a fairly high degree of accuracy. These software can support the pharmaceutical scientist to do the pharamcokinetics and/or pharmacodynamics prediction of NCEs in high throughput screening (HTS) fashion. All of the models used in the software are physiologically based. There are several different physiologically based approaches for the prediction of human oral absorption described in the literature. The models can be divided the different approaches into (1)qualitative methods such as the pH-partitioning hypothesis and the absorption potential (AP) concept and (2)quantitative methods including dispersion models, mass balance models, and compartmental absorption and transit models. The qualitative models aim to correlate physicochemical and physiological properties to the oral absorption of drugs in a simple way [14]. The example of the model used in Gastroplus TM using ACAT (Advanced Compartmental Absorption and Transit model) [15] and ADAM (Advanced Dissolution Absorption Metabolism) model [16] are illustrated below in Figure 2. As described earlier, absorption is a complex process that depends on several physiological and physicochemical properties. Thus these valuable models can facilitate the possibility of identification of potential risk for poor absorption based on a limited set of in vitro data in early drug discovery. The concept is used widely within the industry and is often used as a way to confirm that the physicochemical properties of a drug candidate are within an acceptable range [18]. Although several physiologically based mathematical models as mentioned above designed to predict absorption properties have been available and used for several years, good correlations have been shown between predicted and observed human BA for passively transported NCEs/compounds, slightly poorer correlations are normally obtained when drugs with significance levels of transport via transporter(s) [19]. Thus, the need to include the transporters data in the models is very valuable for the development of the more accurate prediction. Owe to the advance in cellular and molecular level study, the pharmacokinetics is moving to the concept of molecular pharmacokinetics. The roles of drug transporters can be assessed using in vitro and invivo, using techniques spanning from cellular expression systems to gene knock out animals. Research outcomes from such studies have been applied to clinical science and drug development. The studies of membrane vesicles and cultured epithelial cell lines have been used in the field since 1980s and this resulted in sustainable advancement in the field. At the end of 1980s, the molecular nature of drug transporters was unveiled by cDNA cloning and the first clinically important drug transporter, the P-glycoprotein(P-gp), was identified. The existence of further carriers, receptors and/or metabolic enzymes with overlapping substrate specificity complicates the generation and interpretation of suitable data.



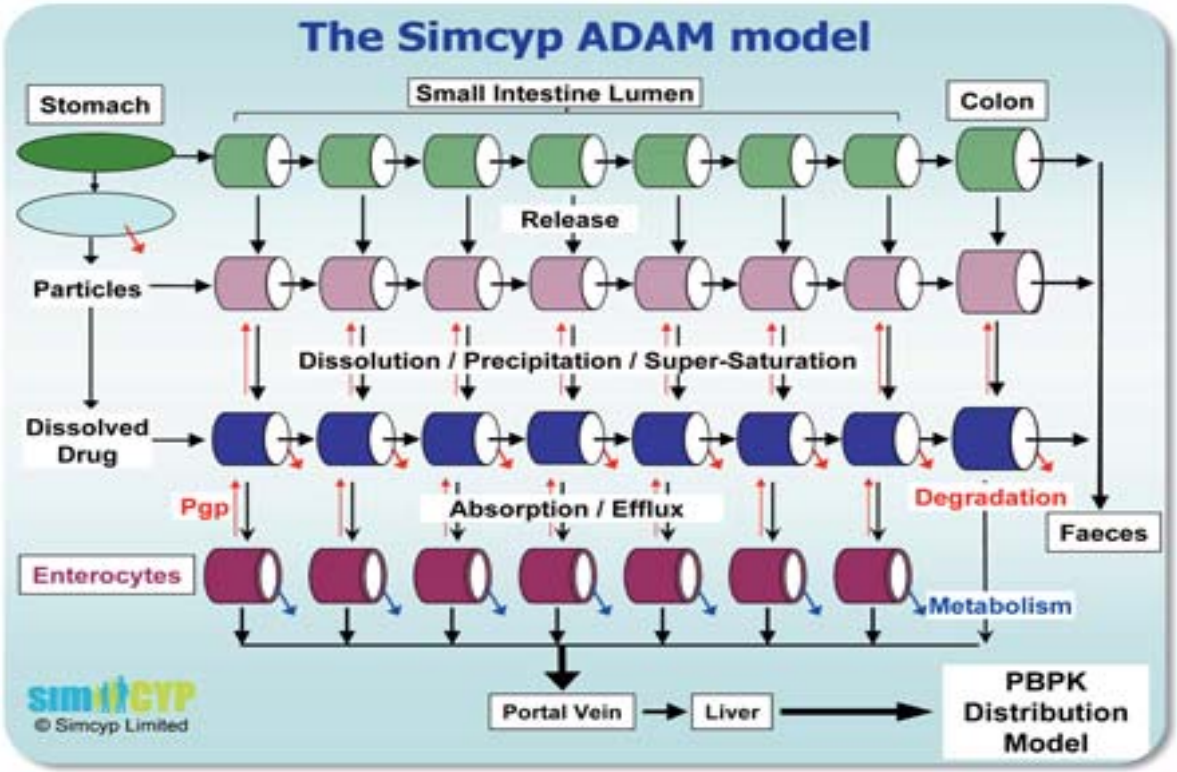


Figure 2 ACAT (Advanced Compartmental Absorption and Transit model) is illustrated in above figure and is illustrated in below figure.

The molecular expression cloning, polymerase chain reaction (PCR) cloning, and ***in silico*** homology screening strategies have been used in the field. It will be very crucial if isolation of transport proteins in sufficient amount and purity can be prepared as required for structural analysis. These data can be summarized and integrated in the mathematical model

for ***in silico*** prediction to improve the accuracy of the model for NCEs which are the substrate of any transporters.

Moreover, we should also consider the differences among the population in term of both inter-subject and intra-subject variability. The model can include a full physiologically-based pharmacokinetic (PBPK) model together with extensive libraries on demographics, developmental physiology and the ontogeny of drug elimination pathways using population pharmacokinetic approaches. Thus, as an example, it is possible to use the data from one age range such as adult to predict those from pediatric and geriatric. The example of the prediction of the clearance of caffeine from birth to adulthood is illustrated in Figure 3 [16].

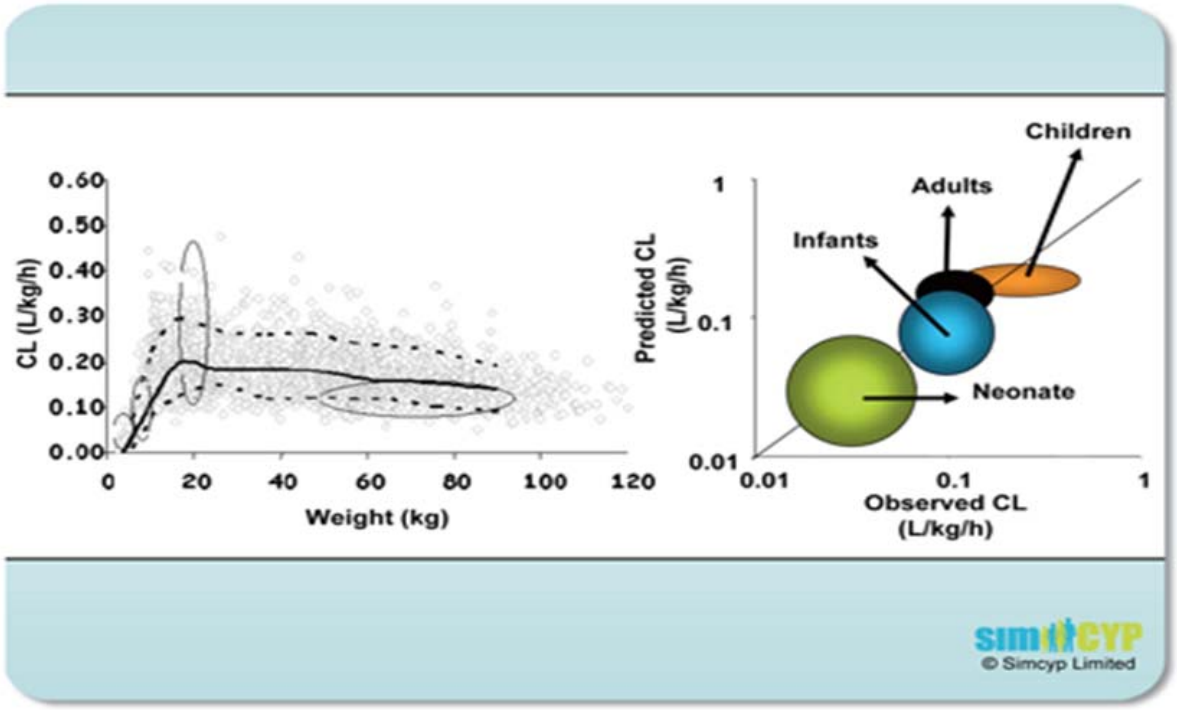


Figure 3 Prediction of the clearance of caffeine from birth to adulthood

In a few decades, it has long been identified that the genetic variability in metabolism process can interfere the pharmacokinetics of medicines and this variability can be observed differently in ethnic groups. There are the bimodal (poor/extensive metabolizers) or multimodal differences (poor/expensive/ultra metabolizers) in clearance among populations. This is recognized as "Genetic Polymorphism". In the midst of the discovery of transporters, it is not surprising that the genetic polymorphism of transport process can be elucidated. Thus, if we need to make more precise perdition, the genetic polymorphism information should be integrated. These aforementioned factors were integrated in few software such as Simcyp™. The population approaches whole body PBPK model with the ADAM™ model were used in this software as depicted in Figure 4.

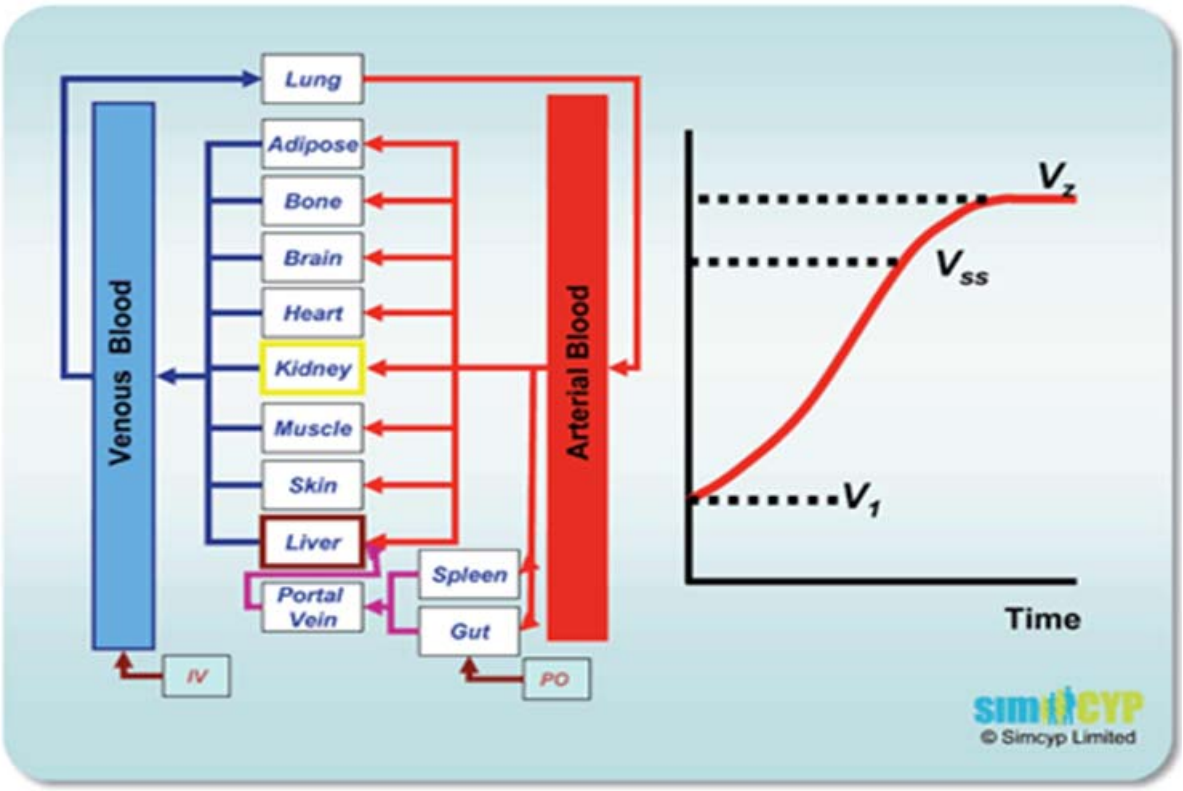


Figure 4 Whole body PBPK

In conclusion, the new era of the new drug discovery and development process is in progress. This new scheme of the process by integrating ***in vitro***, ***in vivo*** and ***in silico*** experiments into mathematical model for the NCEs screening can reduce the cost, attrition rate and time to get the new medicine in the market.

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Session 6: Chemotherapy and Discovery of Chemotherapeutic Agents: Research on Natural Products

Research on Thai medicinal plants for cancer treatment

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Abstract

This topic reviews the research on anticancer effects of medicinal plants originating from Thailand. The selection of the plants for the research was based largely on their ethnomedical use by Thai folk doctors and on the knowledge of Thai traditional medicine. The investigations focused primarily on the plants frequently used in the preparations for cancer treatment. The plants were tested for active components followed by using the National Cancer Institute (NCI) assays for anticancer compounds. The biological assay which correlated with cancer such as, antioxidant, immunology, anti-inflammatory and antimicrobial were also investigated. Plant extracts have been compared with whole plant preparations as well as combinations of many plants for their effectiveness as anticancer medicine. In one preparation composed with many plants which were proved that some plants in the preparation showed cytotoxic activity against cancer cells and plant showed no cytotoxic activity but they exhibited another activity such as antioxidant, anti-inflammation, antimicrobial and enhance immunology. The concept of cancer treatment research in Thailand is moving toward the holistic approach, which requires the knowledge of both body and mind. Most of the current research on the subject in Thailand is on elucidation of biological activity of plant materials to confirm. Informal clinical trials have been conducted on some of the anticancer preparations, but most are unpublished. The present results indicated that using whole herbal preparations has produced gentler effects on the human body because of the synergy of the plants, which make up the preparations. All of these cancer research can supported using cancer preparation of Thai folk doctors

Key Words: Thai medicinal plants, Thai traditional medicine, anticancer herbs

Session 6: Chemotherapy and Discovery of Chemotherapeutic Agents: Research on Natural Products

Marine Compounds as Sources of Chemotherapeutics

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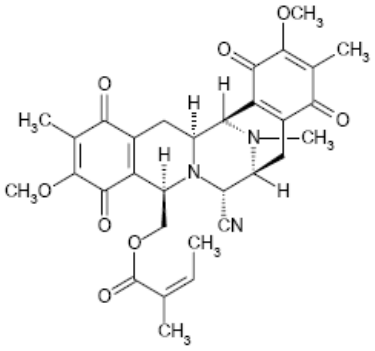
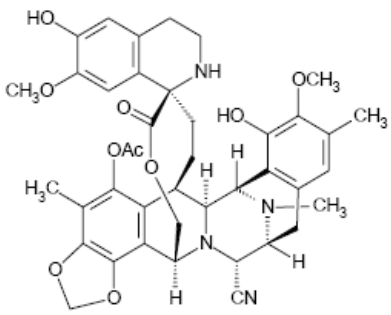
Abstract

Marine organisms have been recognized as a source for new anticancer natural products since the availability of ara-C, a sponge-derived nucleoside, for the treatment of acute myeloid leukemia and non-Hodgkin's lymphoma. Later, searches for new marinederived compounds have been world widely explored to develop potential anticancer agents such as dolastatins, bryostatins, didemnins, aplidins, kalahalides, and ecteinascidins. In this presentation, we also discuss the investigation of two highly cytotoxic tetrahydroisoquinoline alkaloids, including renieramycins and ecteinascidins from the Thai sponge *Xestospongia* sp. and the Thai ascidian *Ecteinascidia thurstoni*, respectively. With large quantities of these natural products in hand, chemical transformations and the structure-cytoxicity relationships of these compounds have been studied for further development of these cytotoxic alkaloids as new anticancer agents from in-the-sea farming Thai marine organisms.



Renieramycin M



Ecteinascidin 770

Keywords: marine natural products, anticancer agents, cytotoxicity, renieramycins, ecteinascidins.

Original article **P01**

ฤทธิ์ต้านมะเร็งลำไส้ใหญ่ของสมุนไพรไทยในเซลล์เพาะเลี้ยง

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บทคัดย่อ

มะเร็งลำไส้ใหญ่และทวารหนัก จัดเป็นมะเร็งที่พบบ่อยในลำดับต้นๆ โดยเฉพาะในประเทศที่พัฒนาแล้ว และเป็นมะเร็งที่คาดว่าสามารถป้องกันได้เนื่องจากสาเหตุหลักเกิดจากลักษณะของอาหารที่รับประทาน จึงมีการศึกษาจำนวนมากที่พยายามค้นหาสารจากธรรมชาติที่สามารถป้องกันการเกิดมะเร็งลำไส้ใหญ่ งานวิจัยนี้เป็นการศึกษาฤทธิ์ต้านเซลล์มะเร็งลำไส้ใหญ่ของสารสกัดจากสมุนไพรไทย 7 ชนิด ได้แก่ เถาวัลย์เปรียง เขยตาย ปัญจขันธ์ พลูคาว อังกาบ ทองพันชั่ง และหญ้าปักกิ่ง โดยทำการทดสอบเบื้องต้นถึงฤทธิ์ในการฆ่าเซลล์มะเร็งลำไส้ใหญ่เพาะเลี้ยง SW480 ด้วย MTT assay ผลการทดลองพบว่า สารสกัดเถาวัลย์เปรียงมีฤทธิ์ในการฆ่าเซลล์ SW480 ได้ดีที่สุด (IC_{50} = 4.86 μg/ml) สารสกัดเถาวัลย์เปรียงจึงถูกเลือกเพื่อนำมาศึกษาในระดับลึกต่อไป โดยทำการค้นหากลไกในการต้านเซลล์มะเร็ง โดยทดสอบฤทธิ์ของสารสกัดต่อการเกิด cell apoptosis ด้วย Flow cytometer และพบว่าสารสกัดเถาวัลย์เปรียงไม่มีผลในการทำให้เซลล์มะเร็งเกิด apoptosis แต่พบว่าเซลล์บางส่วนมีการตายแบบ necrosis และคาดว่าสารสกัดเถาวัลย์เปรียงอาจมีผลต่อกระบวนการแบ่งตัวของเซลล์บางส่วน ซึ่งจะได้ดำเนินการทดสอบผลของสารสกัดนี้ต่อ cell cycle ของเซลล์ SW480 ต่อไป

คำสำคัญ : มะเร็งลำไส้ใหญ่และทวารหนัก, ฤทธิ์ต้านมะเร็ง, สมุนไพรไทย, เซลล์เพาะเลี้ยง

บทนำ

ในปัจจุบัน อัตราการเกิดโรคมะเร็งเพิ่มขึ้นเรื่อยๆ มะเร็งลำไส้ใหญ่และทวารหนัก (colorectal cancer) จัดเป็นมะเร็งที่พบได้มากขึ้นในปัจจุบัน โดยพบว่าสาเหตุหลักเกี่ยวข้องกับอาหารที่รับประทาน ในประเทศที่กำลังพัฒนาอัตราการเกิดมะเร็งลำไส้ใหญ่จะสูงขึ้นในกลุ่มคนเมืองเมื่อเปรียบเทียบกับคนในชนบท โดยที่คนในเมืองจะรับประทานอาหารประเภทเนื้อสัตว์มาก อาหารที่มีปริมาณไขมันหรือน้ำตาลสูง และอาหารที่มีเส้นใยต่ำ (1).

ในปัจจุบัน มีงานวิจัยจำนวนมากที่ศึกษาสารจากธรรมชาติหรือสารสกัดสมุนไพร เพื่อใช้ในการป้องกันการเกิดมะเร็งลำไส้ใหญ่ โดยใช้ colon adenoma cell lines งานวิจัยนี้จึงทำการทดสอบสารสกัดจากสมุนไพรไทย โดยเฉพาะสมุนไพรที่มีข้อมูลจากภูมิปัญญาชาวบ้านที่มีการนำไปใช้ในผู้ป่วยโรคมะเร็ง ซึ่งได้แก่ เถาวัลย์เปรียง เขยตาย ปัญจขันธ์ พลูคาว อังกาบ ทองพันชั่ง และหญ้าปักกิ่ง มีการศึกษาพบว่า หญ้าปักกิ่งมีฤทธิ์ต้านการกลายพันธ์และลดการเกิด aberrant crypts focus ในลำไส้ของสัตว์ทดลอง (2) ส่วนปัญจขันธ์พบว่ามีฤทธิ์ต้านมะเร็งในสัตว์ทดลอง (3) และยังกระตุ้นการตายของเซลล์มะเร็งหลายชนิดได้ รวมทั้งเซลล์มะเร็งลำไส้ (4) นอกจากนี้ มีรายงานถึงฤทธิ์ต้านเซลล์มะเร็งเม็ดเลือดขาวของพลูคาว (5) ส่วนการศึกษาของอังกาบ เถาวัลย์เปรียง เขยตาย และทองพันชั่ง ยังไม่พบรายงานฤทธิ์ในการต้านมะเร็งใดๆ การศึกษาในครั้งนี้ จึงมีวัตถุประสงค์ เพื่อศึกษาฤทธิ์ของสมุนไพรไทยดังกล่าวในการต้านเซลล์มะเร็งลำไส้ใหญ่เพาะเลี้ยง SW480

วิธีการทดลอง

1. การเตรียมสารสกัดจากสมุนไพร นำส่วนของพืชที่ต้องการสกัดมาบด แล้วอบให้แห้งที่อุณหภูมิ 55°C การสกัดจะใช้การหมักสมุนไพรแห้งด้วย methanol เป็นเวลา 3 วัน และผ่านกระบวนการกรอง จากนั้นระเหยให้แห้งภายใต้ความดันต่ำและเก็บไว้ที่อุณหภูมิ -70 °C

2. การเพาะเลี้ยงเซลล์ (Cell culture) SW480 human colon carcinoma cells จะถูกเลี้ยงด้วยอาหารเลี้ยงเซลล์ DMEM/F12 ที่มี 10% FBS และ 1% penicillin-streptomycin ในที่อุณหภูมิ 37 0°C และ 5% CO_2 ใน 75 cm^3 culture flask

3. การวัดการมีชีวิตของเซลล์ (Cell viability assay) ทดสอบโดย MTT [3-(3,5-dimethylthiazol-2,5-diphenyltetra-zolium bromide)] assay โดยเลี้ยงเซลล์ ใน 96 well microplate ด้วยสารสกัดแต่ละชนิดเป็นเวลา 24 ชม. การเกิดสี formazan จะถูกประเมินโดยการค่าการดูดกลืนแสงที่ 570 nm ด้วย ELISA reader

4. การวัด Cell apoptosis ด้วย Flow cytometer cell apoptosis สามารถวัดได้โดยใช้ Annexin V-FITC apoptosis detection kit จากนั้นวิเคราะห์เซลล์ด้วย Flow cytometer โดยเซลล์ที่มีชีวิต (viable cell) จะได้เป็น annexin V-negative&PI-negative ส่วนเซลล์ early apoptosis จะได้เป็น annexin V-positive&PI-negative และเซลล์ late apoptosis จะได้เป็น annexin V-positive&PI-positive

5. การวัด lactate dehydrogenase (LDH) activity โดยวัดการทำงานเอนไซม์ LDH ที่ถูกปล่อยออกจากเซลล์ที่ตายแบบ necrosis สู่อาหารเลี้ยงเซลล์ ด้วยการใช้ pyruvate และ NADH เป็นสารตั้งต้นของปฏิกิริยาและติดตามการลดลงของ NADH ด้วยการวัดค่าการดูดกลืนแสงที่ 340 nm

ผลการทดลอง

การทดสอบฤทธิ์ของสารสกัดสมุนไพรทั้ง 7 ชนิด ในการยับยั้งเซลล์ SW480 โดยเลี้ยงเซลล์ด้วยสารสกัดสมุนไพรแต่ละชนิดที่ความเข้มข้น 0-1,000 µg/ml เป็นเวลา 24 ชม. จากนั้นนำไปวัด cell viability ด้วย MTT assay แล้วคำนวณหาค่า IC_{50} ผลการทดลอง ดังแสดงในตารางที่ 1 พบว่าสารสกัดเถาวัลย์เปรียงมีฤทธิ์ในการยับยั้งการเจริญเติบโตหรือฆ่าเซลล์ SW480 ได้ดีที่สุด ส่วนหญ้าปักกิ่ง ไม่พบว่าสามารถยับยั้งเซลล์ได้ที่ความเข้มข้นสูงสุดที่ใช้ จึงไม่สามารถคำนวณหาค่า IC_{50} ได้

ตารางที่ 1 ค่า IC_{50} ของสารสกัดสมุนไพรในการยับยั้งการเจริญเติบโตของเซลล์ SW480

Plant extract	Scientific name	IC_{50} (µg/ml)
เถาวัลย์เปรียง	*Derris scandens* (Roxb.) Benth.	4.86
ปัญจขันธ์	*Gynostemma pentaphyllum* Makino	118.50
เขยตาย	*Glycosmis pentaphylla* (Retz.) DC.	> 1,000
ทองพันชั่ง	*Rhinacanthus nasutus* (L.) Kurz	> 1,000
พลูคาว	*Houttuynia cordata* Thunb.	> 1,000
หญ้าปักกิ่ง	*Murdannia loriformis* (Hassk.) Rolla Rao & Kammathy	> 1,000
อังกาบ	*Barleria cristata* L.	> 1,000

หมายเหตุ ND = non detectable

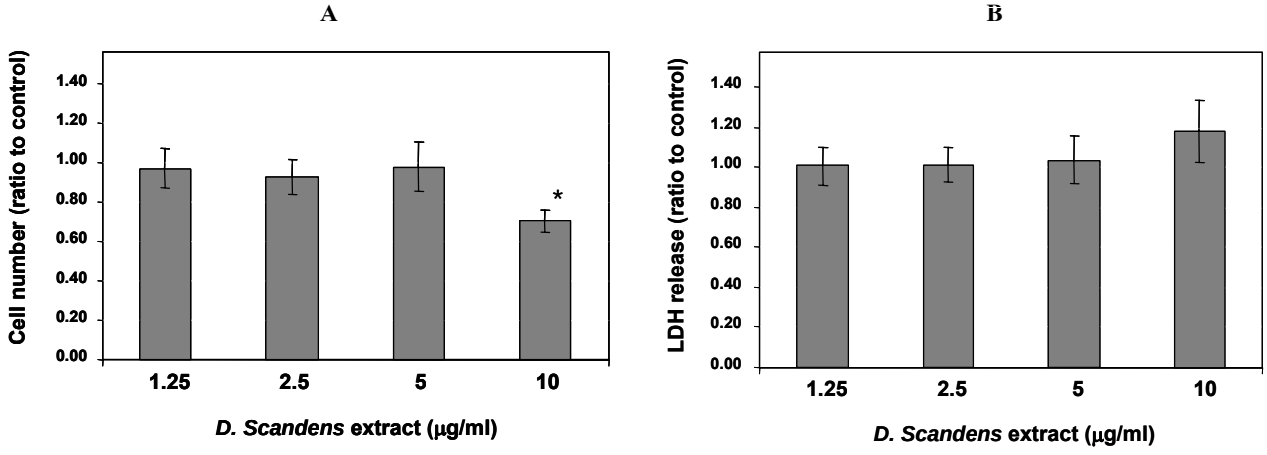
เมื่อเปรียบเทียบระหว่างสมุนไพรทั้ง 7 ชนิด เถาวัลย์เปรียงมีฤทธิ์ต้านเซลล์มะเร็งได้ดีที่สุด จึงถูกเลือกเพื่อนำไปศึกษาหากลไกในระดับลึกต่อไป โดยคาดว่าสารสกัดเถาวัลย์เปรียงอาจเหนี่ยวนำให้เซลล์มะเร็งตายหรือทำให้เกิดการหยุดการแบ่งตัวของเซลล์โดยรบกวนกระบวนการ cell cycle ของเซลล์ จึงได้ทดสอบฤทธิ์ของสารสกัดเถาวัลย์เปรียงในการเหนี่ยวนำให้เซลล์เกิดการตายแบบ apoptosis ด้วย Flow cytometer เนื่องจากการทดสอบฤทธิ์ยับยั้งหรือฆ่าเซลล์มะเร็งด้วย MTT assay ได้ทำการทดลองโดยการเลี้ยงเซลล์ด้วยสารสกัดเป็นเวลา 24 ชม. ซึ่งพบว่าสารสกัดเถาวัลย์เปรียงที่ความเข้มข้นน้อยกว่า 10 μg/ml ทำให้จำนวนเซลล์ลดลงไปจำนวนมาก ทำให้ไม่มีเซลล์มากพอที่จะใช้ในการประเมินการเกิด apoptosis ด้วย Flow cytometer จึงได้ปรับการทดลองเป็นเลี้ยงเซลล์ SW480 ด้วยสารสกัดเถาวัลย์เปรียงที่ความเข้มข้น 1.25-10 μg/ml เป็นเวลา 4 ชม. แล้วประเมินการเกิด apoptosis หลังจากนั้น 48 ชม. ผลการทดลองพบว่า ร้อยละของเซลล์ที่นำไปทดสอบด้วย Flow cytometer พบว่าเซลล์ที่มีการตายแบบ apoptosis และ necrosis ไม่มีความแตกต่างกันทั้งเซลล์กลุ่มควบคุมและเซลล์ที่เลี้ยงด้วยสารสกัดเถาวัลย์เปรียงที่ทุกความเข้มข้น (ตารางที่ 2)

ตารางที่ 2 ผลการวัด cell apoptosis ของสารสกัดเถาวัลย์เปรียงต่อการเจริญเติบโตของเซลล์ SW480

***D. Scandens* Extract (μg/ml)**	**Live cells (%)**	**Early apoptosis (%)**	**Late apoptosis (%)**	**Necrosis (%)**
Untreated cells	85.12 ± 3.21	6.88 ± 2.45	5.95 ± 1.45	2.05 ± 0.58
0.25% DMSO	85.61 ± 1.77	6.72 ± 2.09	6.19 ± 1.20	2.30 ± 0.90
1.25	84.50 ± 2.75	5.99 ± 1.82	6.50 ± 1.10	3.02 ± 0.78
2.5	87.40 ± 4.54	5.11 ± 2.23	6.10 ± 2.35	1.40 ± 0.49
5	83.69 ± 2.78	7.25 ± 1.89	6.63 ± 1.14	2.42 ± 0.37
10	86.61 ± 2.31	6.71 ± 1.39	5.20 ± 1.14	1.48 ± 0.29

หมายเหตุ ค่าที่แสดงเป็น Mean±SEM จาก 3-5 การทดลอง

อย่างไรก็ตาม หลังจากเลี้ยงเซลล์ SW480 ด้วยสารสกัดเถาวัลย์เปรียงเป็นเวลา 4 ชม. ได้ทำการนับจำนวนเซลล์ที่เหลืออยู่และนำอาหารเลี้ยงเซลล์ไปทดสอบการหลั่ง lactate dehydrogenase (LDH) ซึ่งเป็นสัญญาณของการเกิด cell necrosis จากการที่เยื่อหุ้มเซลล์ถูกทำลาย และมีการหลั่ง LDH ออกมาภายนอกเซลล์ ผลการทดลองดังแสดงในรูปที่ 1 พบว่าสารสกัดเถาวัลย์เปรียงที่ความเข้มข้น 1.25-5 μg/ml ทำให้เซลล์ตายไม่มากนัก โดยยังมีจำนวนเซลล์และการหลั่ง LDH ใกล้กับกลุ่มควบคุม (สัดส่วนใกล้เคียง 1) ยกเว้นสารสกัดที่ความเข้มข้น 10 μg/ml ทำให้จำนวนเซลล์ลดลงเล็กน้อย (รูปที่ 1A) และมีการหลั่ง LDH เพิ่มขึ้นเล็กน้อยเช่นกัน (รูปที่ 1B)



รูปที่ 1 ผลของสารสกัดเถาวัลย์เปรียงต่อเซลล์ SW480 หลังจากเลี้ยงเซลล์ SW480 ด้วยสารสกัดเถาวัลย์เปรียงที่ความเข้มข้น 1.25-10 μg/ml เป็นเวลา 4 ชม. แล้วนับจำนวนเซลล์ที่เหลืออยู่ (A) ส่วนอาหารเลี้ยงเซลล์นำไปวัดระดับของ LDH (B) โดยจำนวนเซลล์และการหลั่งของ LDH คำนวณเป็นค่าสัดส่วนกับกลุ่มควบคุมซึ่งเป็นเซลล์ที่เลี้ยงด้วย 0.25% DMSO ผลการทดลองที่แสดงเป็นค่า Mean±SEM จาก 3-5 การทดลอง

วิจารณ์ผลการทดลอง

จากผลการทดลองเปรียบเทียบสมุนไพรทั้ง 7 ชนิด ในการยับยั้งหรือฆ่าเซลล์มะเร็งลำไส้ใหญ่ SW480 พบว่าสารสกัดเถาวัลย์เปรียงมีฤทธิ์ที่ดีที่สุด โดยมีค่า IC_{50} = 4.86 μg/ml จึงได้ถูกเลือกเพื่อทำการศึกษาในระดับลึกต่อไปถึงกลไกของฤทธิ์ฆ่าเซลล์มะเร็ง เถาวัลย์เปรียงเป็นสมุนไพรที่มีการนำมาใช้เพื่อลดการอักเสบ สารสำคัญที่พบเป็นสารกลุ่ม isoflavones (6) และมีรายงานว่าสารสกัดเถาวัลย์เปรียงสามารถกระตุ้นภูมิคุมกันได้ (7) นอกจากข้อมูลจากภูมิปัญญาชาวบ้านที่ใช้รักษามะเร็งแล้ว ยังไม่เคยมีรายงานว่าสารสกัดเถาวัลย์เปรียงมีฤทธิ์ในการต้านมะเร็ง ในการทดสอบฤทธิ์ต้านเซลล์มะเร็งของสารสกัดเถาวัลย์เปรียงนี้ ทำโดยประเมินผลของสารสกัดต่อการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ SW480 ซึ่งการต้านเซลล์มะเร็งอาจเป็นผลมาจากการทำให้เซลล์ตายหรือเซลล์หยุดเจริญเติบโต การประเมินผลของสารสกัดในการทำให้เซลล์มะเร็งตาย ได้ทำการทดสอบการตายทั้งแบบ apoptosis และ necrosis ซึ่งผลการศึกษานี้พบว่าสารสกัดเถาวัลย์เปรียงไม่ทำให้เซลล์เกิด apoptosis และมีแนวโน้มที่จะทำให้เซลล์ตายแบบ necrosis แต่อย่างไรก็ตาม สัดส่วนของเซลล์ที่ตายแบบ necrosis ดูหมือนว่าจะต่ำกว่าสัดส่วนของจำนวนเซลล์ที่ลดลงไปเล็กน้อย แสดงว่าสารสกัดเถาวัลย์เปรียงอาจมีผลอย่างอื่นอีก ซึ่งอาจเป็นฤทธิ์ในการรบกวนกระบวนการแบ่งตัวของเซลล์ จึงทำให้เซลล์หยุดการเพิ่มจำนวน ซึ่งฤทธิ์ดังกล่าวนี้ จะทำการทดสอบผลของสารสกัดเถาวัลย์เปรียงต่อ cell cycle ของเซลล์ SW480 ต่อไป

สรุปผลการทดลอง

สารสกัดจากเถาวัลย์เปรียงมีฤทธิ์ในการต้านเซลล์มะเร็งลำไส้ใหญ่ SW480 ได้ดีที่สุด เมื่อเปรียบเทียบกับ ปัญจขันธ์ เขยตาย พลูคาว อังกาบ ทองพันชั่งและหญ้าปักกิ่ง โดยกลไกในการต้านเซลล์มะเร็งนี้ไม่ได้เป็นผลมาจากการทำให้เซลล์ตายแบบ apoptosis แต่ทำให้เซลล์บางส่วนเกิดการตายแบบ necrosis ส่วนผลต่อ cell cycle ของเซลล์ SW480 จะได้ทำการทดสอบต่อไป

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณกองทุนวิจัยมหาวิทยาลัยนเรศวร (RX-AR-027-2552)

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Original article **P02**

Preliminary study on antimalarial activities of Thai herbal medicines

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Abstract

Malaria remains one of the most serious causes of mortality and morbidity in the tropics. The problems of multi-drug resistance *Plasmodium falciparum* have been aggravating and up to present, there is still no new clinical effective antimalarials to replace artemisinin and derivatives. It is therefore an urgent need to search for new promising antimalarial drug targets. In this study, we assess the *in vitro* antimalarial activity of the ethanolic extracts of the four Thai medicinal plants/regimen against the chloroquine(CQ)-resistant (K1) and CQ-sensitive (3D7) strains of *P.falciparum.* The ethanolic extracts of all medicinal plants/regimen showed promising antimalarial activity against both CQ-resistant and CQ-sensitive strains of *P.falciparum*, of which the extract of *Piper chaba* Hunt exhibiting the most potent antimalarial activity with IC_{50} of less than 10 μg/ml against both CQ-resistant and CQ-sensitive strains. Proteomics study of the extracts of these four plants/recipe are being investigated to identify their molecular targets of antimalarial action.

Keywords : *Plasmodium falciparum*, Thai herbal medicine, antimalarial, proteomics, drug resistance

Introduction

Malaria remains one of the most serious causes of mortality and morbidity in the tropics. The problems of multi-drug resistance *Plasmodium falciparum* have been aggravating and up to present, there is still no new clinical effective antimalarials to replace artemisinin and derivatives (1). It is therefore an urgent need to search for new promising antimalarial drug targets. Thai medicinal plants have been increasingly applied as an alternative treatment for various infectious diseases including malaria. The ultimate goal of the study was to screen a total of 30 medicinal plants/recipes for their *in vitro* antimalarial activity against a total of 30 *P. falciparum* isolates collected from the Thai-Myanmar border. Promising candidates were planned for investigation of their protein targets. Here, were present the preliminary results on the antimalarial activity of the ethanolic extracts of three Thai medicinal plants namely *Piper chaba* Hunt, *Atractylodes lancea*, *Zingiber officinale* Roscoe, and one recipe-- Prasapraoyai, against the laboratory strains—chloroquine (CQ)-resistant K1 and CQ-sensitive 3D7. Standard antimalarial drugs CQ, mefloquine (MQ) and artesunate (ARS) were used as control drugs for activity against K1 and 3D7 strains. The plants/recipe have been used for centuries in Thai folklore medicine to treat various diseases. The *Piper chaba* Hunt is a well-known medicinal plant for the treatment of a variety of symptoms such as stomachache and fetch-up. The *Atractylodes lancea* has been claimed to be an effective diuretics and antipyretics. The *Zingiber officinale* Roscoe is commonly used to treat several symptoms including common cold, anarcartharsis and diarrhea. The Prasapraoyai recipe has been used to treat fever in children. In addition, further study on proteomics was also planned, in order to identify protein targets of these plant extracts.

Methods

Plant Materials*:* The ethanolic extracts of the four medicinal plants/recipe were prepared at the Applied Thai Traditional Medicine Center, Faculty of Medicine, Thammasat

University. Plant materials were collected from various parts of Thailand and some were purchased from the city markets. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand, where the herbarium vouchers have been kept. A duplicate set has been deposited in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand.Stock solutions of the ethanolic extracts were prepared at concentration of 1 mg/ml.

Parasite Culture: CQ-sensitive (3D7) and CQ-resistant (K1) strains of *P. falciparum* were maintained in continuous culture in O^+ human erythrocytes suspended in RPMI 1640 culture medium (at 37°C under a gas mixture of 5% CO_2, 5% O_2, and 90% N_2) according to the standard method described by Trager and Jensen (2). The culture medium was supplemented with 25 mM sodium bicarbonate, 10 mg/ml gentamicin sulfate, 25mM HEPES (pH7.4), 80 ml/l human B or AB serum. To obtain specific life cycle stages of *P. falciparum*, 5% sorbitol treatment was used (3).

Assessment of Antimalarial Activity In Vitro: Antimalarial activity screening of the four medicinal plants/recipe were performed in a 96-well microtiter plate based n SYBR green-I-based assay (4-5). Experiments were repeated three times, and triplicate in each experiment. The concentration range of the extracts used was 0.78- 100 µg/ml. Assays were initiated at 2% parasitemia and 1% hematocrit. Fifty percent inhibitory concentration (IC_{50}) values were calculated for each plant extract based on dose effect analysis (CalcuSyn^{TM} software).

Results

Figure 1A, B, C and D showed dose response curves of the four medicinal plants/recipe in 3D7 and K1 strain *P.falciparum in vitro*. The median (range) IC_{50} values of the ethanolic extract of *Piper chaba* Hunt for 3D7 and K1 strains *P.falciparum* were 4.1 (4.7-3.8) and 5.3 (7.1-3.9) µg/ml, respectively (Figure 1A). The IC_{50} values for the extract of *Atractylodes lancea* in 3D7 and K1 strains were 17.3 (57.8- 17.0) and 19.5 (49.5-7.1) µg/ml, respectively (Figure 1B). The extract of Prasapraoyai recipe showed median (range) IC_{50} values of 12.6 (15.8- 9.1) and 16.3 (32.6- 15.7) µg/ml, respectively (Figure 1C). The corresponding IC_{50} values of the extract of *Zingiber officinale* Roscoe were 36.7 (125.6-27.0) and 16.9 (68.8-15.2) µg/ml, respectively (Figure 1D).

Table1. *In vitro* antimalarial activity of *Piper chaba* Hunt, *Atractylodes lancea*, Prasapraoyai regimen, and *Zingiber officinale* Roscoe against 3D7 and K1 *P. falciparum* strains. CQ, MQ and ARS were used as control drugs

Ethanolic extract or drugs	Median IC_{50} (range) (µg/ml) of parasite strain	
	3D7	K1
1.*Piper chaba* Hunt	4.1 (4.7-3.8)	5.3 (7.1-3.9)
2.*Atractylodes lancea*	17.3 (57.8- 17.0)	19.5 (49.5-7.1)
3.Prasapraoyai regimen	12.6 (15.8- 9.1)	16.3 (32.6- 15.7)
4.*Zingiber officinale* Roscoe	36.7 (125.6- 27.0)	16.9 (68.8-15.2)
5.Chloroquine*	9.4 (11.6-9.3)	128.7 (139.2-109.3)
6.Mefloquine*	20.8 (28.0-19.2)	10.4 (10.7-10.3)
7.Artesunate*	2.1 (2.5-2.0)	1.9 (2.1-1.9)

* Unit of median IC_{50} (range) is nM.

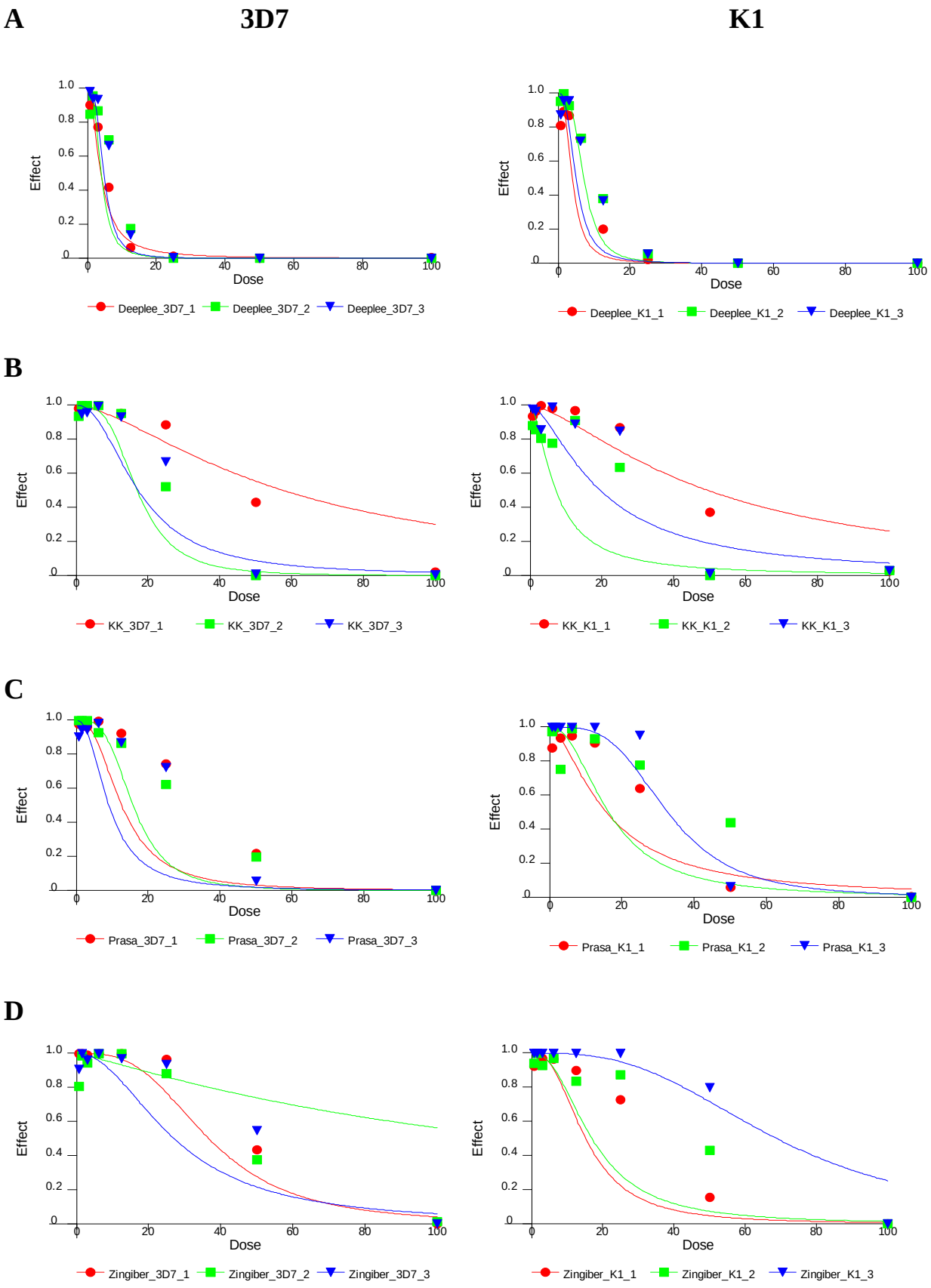

Figure 1 Dose response curves of the four medicinal plants/regimen (A) *Piper chaba* Hunt, (B) *Atractylodes lancea*, (C) Prasapraoyai regimen, and (D) *Zingiber officinale* Roscoe in 3D7 and K1 *P. falciparum* strains.

Discussion

The ethanolic extract of *Piper chaba* Hunt exhibited the most potent antimalarial activity with IC_{50} of less than 10 μg/ml against both CQ-resistant and CQ-sensitive strains. Based on the criteria for categorization of plant extracts with antimalarial activity (6), it could be classified as 'high activity' (6). The antimalarial activity of *Atractylodes lancea*, Prasapraoyai regimen and *Zingiber officinale* Roscoe are classified as 'moderate activity' with IC_{50} varying between 10-100 μg/ml. It is noted however that the antimalarial activity of all extracts except that of *Zingiber officinale* Roscoe, were similar against CQ-sensitive and CQ-resistant falciparum strains. Proteomics study of the extracts of these four plants/recipe are being investigated to identify their molecular targets of antimalarial action.

Conclusion

Ethanolic extracts of the four medicinal plants/recipe namely *Piper chaba* Hunt, *Atractylodes lancea, Zingiber officinale* Roscoe, and Prasapraoyai recipe showed promising antimalarial activity against both CQ-resistant (K1) and CQ-sensitive (3D7) *P. falciparum* strains.

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Original article **P03**

Study on cytotoxic and antioxidant activities of *Pseuderathemum platiferum*

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Abstract:

The aim of this investigation was to determine the total phenolic content, cytotoxic and antioxidant activities of *Pseuderanthemum palatiferum* extracts (Payawanon in Thai). It was used to treat various diseases in Veitnam. Its leaves which were used to treat cancer were extracted by different methods and got 11 crude extracts. These extracts were tested cytotoxic activity against two types of lung cancer cells (COR-L23 and A549) and liver cancer cells (HepG2) by SRB assay. They were also tested antioxidant activity by DPPH assay and determine total phenolic content using the Folin–Ciocalteu method.. They were found that the dry leaves extract by macerated with 95%EtOH showed cytotoxic activity against COR-L23 and A549 (IC_{50} = 31.70 and 40.68 µg/ml respectively). The dry leaves extract by decoction method exhibited the highest antioxidant activity (EC_{50} value of 5.95 µg/ml). The total phenolic content of dry leaves extract by soxhlet extraction with methanol showed the highest content followed by fresh leaves extract by maceration with 95%EtOH and fresh leaves extract by decoction (GAE = 137.17, 127.58, 126.75 mg/g, respectively). In the conclusion, *P*. palatiferum have non specific cytotoxic effect against lung cancer and also showed high total phenolics content and high antioxidant activity. Thus, these results suggest that this plant could be supported ethnomedical used to treat cancer .

Keywords: *Pseuderanthemum palatiferum*, Cytotoxicity, SRB assay, Antioxidant activity, Phenolic content

Introduction

Pseuderanthemum palatiferum (Nees) Radlk is a shrub or small tree occurring in Northern Veitnam.(Cuong and Quynh, 1999) It belong to the family of the Acanthaceae.(HO *et al*., 2000) This plant is a medicinal plant in Vietnam used by some population to treat various diseases such as digestive disorders, stomach trouble, large intestine inflammation, bleeding injury, kidney and liver diseases and cancer. (Dieu *et al*., 2005) Its leaves also is vegetable . The phytochemical investigation of *P. platiferum* leaves found that its compounds were flavonoids, apigenin, triterpenoids saponin, β-sitosterol, stigmasterol, kaempferol, and salicylic acid. (Hung *et a*l., 2004). The objective of this study, we aims to study on cytotoxic activity against lung cancer cells and also investigate antioxidant activity by DPPH assay and determine total phenolic content.

Methodology

Plant material

Pseuderanthemum palatiferum (Nees) Radlk were collected from Amphor Wichenburi, Petchaboon Province, Thailand. Authentication of plant materials were carried out at the herbarium of the Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept to specify plant and species identified.

Preparation of sample

The method of extracts were devided 3 methods. The dry leaves were dried at 50°C, powdered and macerated with 95 % ethanol. It was filtrated and concentrated to dryness

under reduced pressure to be ethanolic extracts (EtOH). The dried leaves were also extracted by soxhlet apparatus using ordering polarity solvent as hexane, chloroform and methanol for 8 hours each. They were concentrated by evaporator. The decoction was also used for the dry leaves extract . They were prepared according to folk medicine. It were boiled in distilled water and the final volume reduced to1/3volume then filtered and dried by the lyophilizer. The fresh leaves were extracted by the method of dry leaves extracts above . Another method for fresh leaves which differ dry leaves extract method, they were blended, filtrate and dry by lyophillizer The percentage of yields were showed in Table 1. The water extracts were dissolved in sterile water and the ethanolic extracts were dissolved in DMSO and all stock solution were filtrated by sterile filter paper (0.2μm) before cytotoxic testing.

In vitro Assay for Cytotoxicity

Human cell lines

Two type of lung cancer cells such as human large cell lung carcinoma (COR-L23) and carcinomic human alveolar basal epithelial cells (A549) were used to test. Human hepatocellular liver carcinoma cell line (HepG2) was also studied. COR-L23, A549 were cultured in RPMI 1640 medium supplement with 10% heated foetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin. HepG2 were cultured in Minimum Essential Media (MEM) with Earle Salt (without glutamine medium supplement) with 10% heated foetal bovine serum, 50 IU/ml penicillin and 50 μg/ml streptomycin and 1% non-essential amino acid and maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity (Keawpradub *et al., 1999*). According to their growth profiles, the optimal plating densities of CORL23, A549 and HepG2 were determined $1x10^3$, $1x10^3$ and 3 x 10^3 cells/well respectively to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay

Cytotoxicity assay

The brief of the assay , the cell pellets were resuspensed and viable cells were counted to give a final concentration of $1x10^3$, $1x10^3$ and $3x10^3$ cells/well for CORL23, A549 and HepG2 .100 μl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24h the cells were treated with the extracts. Each extract was initially dissolved in an amount of DMSO for the ethanolic extracts and sterile distilled water for the water extracts. The extracts were diluted in medium to produce 5 concentrations and 100 μl of each concentration was added to each well of the plates in 6 replicates to obtain final concentrations of 100, 50, 10, 1, 0.1 μg/ml for extract . The final mixture used for treating the cell contained not more than 1% of the solvent , the same as in solvent control wells. The plates were incubated for selected exposure times of 72 hours. as indicated. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 μl of fresh medium were added. The plates were incubated for recovery period of 6 days and cell number were analyzed by SRB assay (Skehan et al 1990). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines [10] extracts with IC_{50} values < 20 μg /ml were considered active.

Evaluation of the antioxidant activity

The ability of the extract to annihilate the DPPH radical was determined by the method of Yamasaki *et al*, (1994). In this assay expressed as EC_{50} (μg/ml) according to the DPPH radical scavenging assay using BHT as a standard. Samples for testing were prepared by dissolution in absolute ethanol (1, 10, 50, and 100 μg/ml in final conc.). A sample solution was mixed with the same volume of 6 x 10^{-5} M DPPH in absolute ethanol. After 30 min incubation in darkness and at ambient temperature, the absorbance was recorded at 520 nm.

(The changes in color from purple to a residual pale yellow color). The percentage inhibition was calculated using Eq.1. Estimated EC_{50} values are presented as the average of quadruplicate analyses (Yamasaki *et al*., 1994). The data are mean ±SD. Determination was done in triplicate.

$$\text{Percentage of inhibition} = [Abs_{control} - Abs_{sample}/Abs_{control}] \times 100 \quad \text{(Eq.1)}$$

The scavenging activity of the *Pseuderanthemum palatiferum* extracts is shown in Table 2.

Determination of total phenolic content

The content of total phenolic content in samples was determined by the Folin–Ciocalteu's (FC) reagent (Miliauskas *et al*., 2004) using gallic acid as standard. This method is based on the reduction of a phosphowolframate–phophomolybdate complex by phenolics to blue reaction products. For the preparation of calibration curve were dilution of 5, 10, 20, 40, 80 and 100 µg/ml. Aqueous gallic acid solutions were mixed with 100µg/ml FC reagent and 20µg/of sodium carbonate. Preparation of the sample were dilution of 500 µg/ml. Sodium carbonate of 20µg/ml and 100mg/ml of Folin–Ciocalteu's reagent were added. The absorbance of the standards and samples was measured at 765 nm after 30 min of reaction at room temperature. The results were expressed as mg of gallic acid equivalents (GAE). All tests were run in triplicate and averaged. The amounts of total phenolics in the studied herbs are shown in Table 2.

Results

Table 1. Percentage yield and IC_{50} of cytotoxicity assay of *Pseuderanthemum palatiferum* extracts.

Part of used	Methods	Extracts	% yeild	IC_{50}(µg/ml)		
				COR-L23	A549	HepG2
Fresh leaves	Blended	Water	2.53	>100	>100	>100
	Decoction		1.93	36.05± 0.1	64.94± 0.6	>100
	Maceration	95%EtOH	1.66	71.45± 0.3	78.23± 1.1	>100
	Soxhlet extraction	Hexane	0.13	48.61± 0.6	>100	52.84± 1.4
		Cholofrom	1.92	>100	>100	>100
		Methanol	0.66	>100	>100	>100
Dry leaves	Decoction	Water	26.42	>100	>100	>100
	Maceration	95%EtOH	9.10	31.70± 0.9	40.68± 1.5	65.63± 2.5
	Soxhlet extraction	Hexane	1.59	>100	>100	>100
		Cholofrom	2.15	>100	>100	>100
		Methanol	7.79	>100	>100	>100

Discussion

Its dry leaves extract by decoction showed the highest percentage of yield (26.42%). Cytotoxic activity of its dry leaves extract by maceration with 95%EtOH showed the best cytotoxic effect against COR-L23 and A549 (IC_{50} = 31.70 and 40.68 µg/ml respectively) but it show less cytotoxic effect against HepG2 (IC_{50} = 65.63 µg/ml). It showed as specific activity for lung cancer treatment better than liver cancer. Dry leaves extract by decoction exhibited the highest antioxidant activity, followed by dry leaves extract by soxhlet extraction with methanol (EC_{50} = 5.95 and 10.95 µg/ml, respectively) while BHT showed less antioxidant activity than two these extracts (EC_{50} = 11.35 µg/ml) . The total phenolic contents were observed in dry leaves extract from soxhlet extraction with methanol, fresh

leaves extract by maceration with 95% EtOH and followed by fresh leaves extract by decoction (GAE = 137.17, 127.58, 126.75 mg/g, respectively)

Table 2. Antioxidant activity by DPPH assay (EC_{50} μg/ml) and the total phenolic content of the *Pseuderanthemum palatiferum* extracts (n=3)

Part of used	Methods	Extracts	DPPH assay EC_{50} (μg/ml)	total phenolic content GAE(mg/g)
Fresh leaves	Blended	Water	>100	29.07±1.47
	Decoction		12.32±0.54	126.75±3.33
	Maceration	95%EtOH	9.14±0.99	127.58±1.20
	Soxhlet extraction	Hexane	12.44±1.90	26.39±7.33
		Chloroform	>100	28.71±2.02
		Methanol	>100	29.80±5.14
Dry leaves	Decoction	Water	5.95±4.59	53.57±4.81
	Maceration	95%EtOH	53.22±2.61	65.18±12.20
	Soxhlet extraction	Hexane	15.57±1.72	31.17±4.96
		Chloroform	34.42±7	64.42±8.36
		Methanol	10.95±0.52	137.171±1.71
BHT			11.35±0.20	-

Conclusion

We have reported the *in vitro* antioxidant and cytotoxic activities of *P. palatiferum* in Thailand. The most of the antioxidant activity and total phenolic content were fresh leaves maceration with 95%EtOH. While dry leaves maceration with 95%EtOH shown inhibition to the growth of a human lung cancer cell line. Thus the result is preliminary screening for supplements seem to protect against cancer and health promotion.

Acknowledgements

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Original article **P04**

Induction of apoptosis in human B-lymphoma cells by citral

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Abstract

This study intended to evaluate anti-tumor activity of citral on human B-lymphoma cell line Ramos. Citral caused Ramos cell death in a concentration-dependent (37.5, 75 and 150 μM) as well as time dependent (3, 6 and 12 h) manner. By staining with annexin V-FITC and propidium iodide (PI) and detecting with flow cytometer, citral induced cell death mostly in apoptotic pattern. Other types of cell death were also detected only when the cells were treated with high concentrations of citral for 6 and 12 h while apoptosis was still dominate. Normal human peripheral blood mononuclear cells (PBMCs) were much more resistant than Ramos cells to apoptotic induction by citral. These results demonstrated the beneficial effect of citral which rich in Thai herbs and spices in oncology.

Keywords**:** citral, apoptosis, Ramos cells

Introduction

Citral, 3,7-dimethyl-2,6-octadienal, is a monoterpenoid containing a mixture of cis-isomer neral and trans-isomer geranial. It is in the volatile oils of many Thai herb and spice such as lemon grass (*Cymbopogon citratus*), lemon balm (*Melissa officinalis*), and *Litsea cubeba*. It is widely used in industries as favoring agent in food, cosmetics and detergent (Opdyke, 1979). Its pharmacological activities have also been studied. It has been reported to have antibacterial (Hayes, 2002), antifungal (Silva, 2008), anti-parasitic (Hierro, 2006) as well as anticancer properties. It inhibited breast cancer cells, MCF-7, growth with IC_{50} 180 μM (48 h) and induced apoptosis in these cell less than 50% at 200 μM (48 h). It also induced apoptosis in many leukemic cell lines at IC_{50} 47 μg/ml (16 h). In our study, the effect of citral on B-lymphoma cell line Ramos was investigated.

Materials and Methods

Chemicals and reagents

Citral was obtained from Sigma-Aldrich (USA). Reagents for cell culture were from Gibco USA. Apoptosis kit for flow cytometer was purchased from Promega, USA.

Cells

Human B-lymphoma cells (Ramos) were obtained from ATCC (USA). Human peripheral blood mononuclear cells (human PBMCs) were prepared from heparinized blood of healthy male blood donors, age 20-35 years old with informed consent at the National Blood Bank, Thai Red Cross Society by ficoll gradient centrifugation. Both cell types were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 0.5% L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin, at 37^0C in 5% CO_2/95% air.

Determination of apoptotic induction by citral

Ramos cells or PBMCs at $1x10^6$ cells/ml were treated with citral at the concentrations of 37.5, 75 and 150 µM in 0.5% ethanol for 3, 6 and 12 h. Apoptotic cells were determined by detecting the exposure of phosphatidylserine (PS) on the outer cell membrane by annexin V-FITC and PI staining assay with fluorescence flow cytometer. Mint oil and 0.5% ethanol were used as the negative controls in the study.

Statistical analysis

Data were presented as mean ± S.E. Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test according to the statistic program, SPSS version 17. Any p-value < 0.01 was considered statistically significant.

Results

The apoptotic effect of citral on Ramos cells

Citral statistically induced Ramos cell death mostly by apoptosis in the time- (3, 6 and 12 h) and concentration- (37.5, 75 and 150 µM) manner (Table 1 and Figure 1). Non-apoptotic cell death was detected only at the high concentrations of citral (75 and 150 µM) for longer time exposure (12 h), but apoptosis was still the main type of cell death. Ten µg/ml etoposide slightly induced cell death at 3-12 h of exposure but it caused 45% cytotoxicity to Ramos cells at 24 h exposure (data not shown).

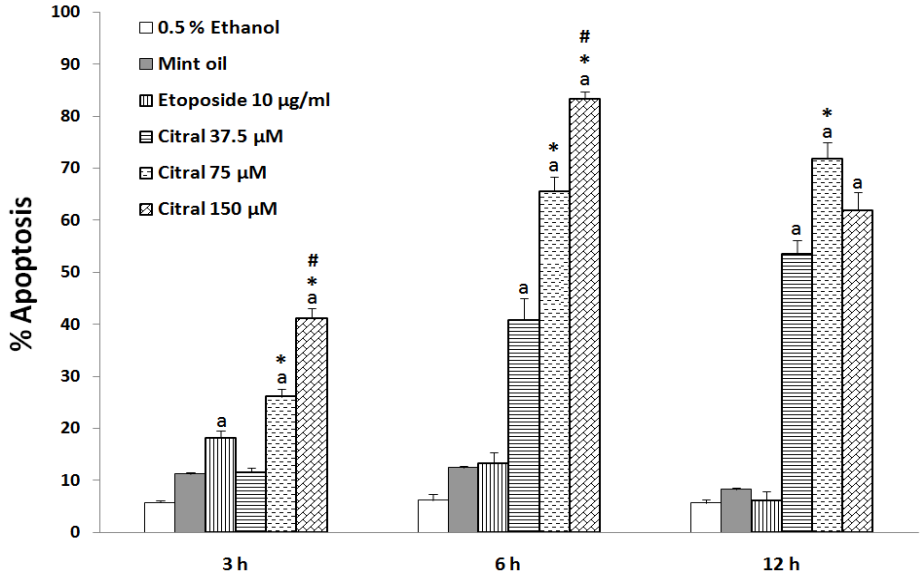

Figure 1. Effect of citral on Ramos cells apoptosis. Ramos cells ($1x10^6$ cells/ml) were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. Mint oil and 0.5% ethanol treated cells were the negative controls while 10 µg/ml etoposide was used as the positive control. The treated cells were stained with annexin V-FITC/PI. The types and the percentage of cell death were detected by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells. The data are expressed as mean ± S.E. of three independent experiments (n=3). a p < 0.01 significantly different from 0.5% ethanol, * p < 0.01 significantly different from 37.5 µM citral, and # p < 0.01 significantly different from 75 µM citral.

Table 1. Effect of citral on Ramos cell death.

	% Cell death					
	3 h		6 h		12 h	
	Total death	**Apoptosis**	**Total death**	**Apoptosis**	**Total death**	**Apoptosis**
0.5% Ethanol	6.17 ± 0.38	5.73 ± 0.37	6.63 ± 1.15	6.22 ± 1.05	5.83 ± 0.59	5.63 ± 0.60
Mint oil	12.83 ± 0.45	11.20 ± 0.35	13.30 ± 0.28	12.42 ± 0.41	12.87 ± 1.77	8.23 ± 0.39
Etoposide 10 µg/ml	19.20 + 1.07	18.23 ± 1.25	15.22 ± 2.47	13.17 ± 2.18	26.03 ± 1.50	6.10 ± 1.85
Citral 37.5 µM	12.33 ± 0.80	11.50 ± 0.85	43.20 ± 4.69	40.72 ± 4.30	68.37 ± 4.37	53.53 ± 2.62
Citral 75 µM	27.43 ± 1.10	26.07 ± 1.44	70.83 ± 2.87	65.47 ± 2.91	93.07 ± 1.47	71.83 ± 3.16
Citral 150 µM	43.03 ± 1.90	41.10 ± 1.93	90.57 ± 2.28	83.30 ± 1.49	98.87 ± 0.03	61.97 ± 3.37

Note: Ramos cells ($1x10^6$ cells/ml) were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. Mint oil and 0.5% ethanol treated cells were the negative controls while 10 µg/ml etoposide was used as the positive control. The treated cells were stained with annexin V-FITC/PI. The types and the percentage of cell death were detected by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells.

The apoptotic effect of citral on PBMCs

Citral had no cytotoxic effect on PBMCs after 3 h of treatment. It induced cell death only in apoptosis pattern, in a concentration- and time-dependent manner, at 6 and 12 h of exposure (Figure 2). Its effect on PBMCs was much less than on Ramos cells after 3 and 6 h of treatment. It had less than 50% cytotoxic to the normal cells at 150 µM for 12 h exposure, but this effect was still less than its effect on Ramos cells which is 98.87 ± 0.03% cytotoxicity.

Discussion and Conclusion

Citral is a volatile oil presents in several Thai herbs and spices such as lemon grass (65-85%). We demonstrated that citral induced cell death mainly by apoptosis in human B-lymphoma cells, Ramos cells, in a concentration- and time-dependent manner. Ramos cell apoptosis was significantly detected after 3 h exposure to 75 and 150 µM citral. Citral at both concentrations caused more than 90% Ramos cell death after 12 h exposure. It also induced almost 70% apoptosis after 12 h exposure at 37.5 µM. Etoposide at 10 µg/ml had little cytotoxicity on the cells at 3-12 h exposure. It has been reported that citral exhibited cytostatic in MCF-7 breast cancer cells. It inhibited these cell proliferation with IC_{50} 180 µM after 48 h exposure (Chaouki, 2009). It induced MCF-7 cell death by 50% after 48 h exposure at 200 µM. Citral also induced apoptosis in other hematopoietic cancer cell lines including BS 24-1, Jurkat and U937 with IC_{50} 47 µg/ml at 16 h exposure (Dudai, 2005). We also evaluated the selectivity and safety of citral. Citral at 37.5 µM had 68% cell death on Ramos cells without any cytotoxicity on normal human PBMCs after 12 h exposure. Apoptotic effect of citral on Ramos cells was much higher than on normal blood cells, especially at the short time of exposure, 3 and 6 h.

The results from our study demonstrated that citral induces human B-lymphoma cell apoptosis within a short time of exposure. Citral may be developed as an agent for the management of lymphoma cancer. However, more in depth studies both *in vitro* as well as *in vivo* in appropriate relevant animal models are needed to strengthen this suggestion.

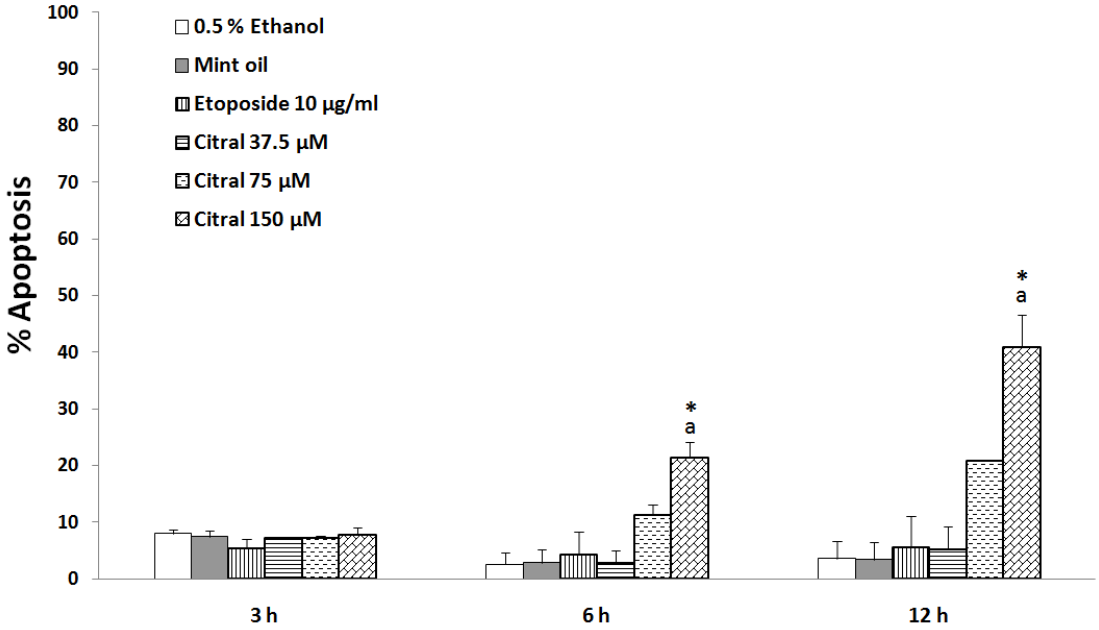

Figure 2. Effect of citral on normal cells (human PBMCs). PBMCs ($1x10^6$ cells/ml) were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. Mint oil and 0.5% ethanol treated cells were the negative controls while 10 µg/ml etoposide was used as the positive control. The treated cells were stained with annexin V-FITC/PI. The types and the percentage of cell death were detected by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells. The data are expressed as mean ± S.E. of three independent experiments (n=3). a $p < 0.01$ significantly different from 0.5% ethanol, * $p < 0.01$ significantly different from 37.5 µM citral.

Acknowledgments

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Original article **P05**

Phytochemistry of Thai plants and their antiprotozoal activities

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Abstract

The *in vitro* antiprotozoal activities of 12 chloroform extracts from 6 species of Thai plants were evaluated by three strains of protozoa including *Crithidia fasciculata*, *Plasmodium falciparum* chloroquine sensitive strain (3D7) and chloroquine resistant strain (K1). Anticrithidial activity of the chloroform extracts was studies by treatment of cultured promastigotes with various concentrations of the chloroform extracts. Growth inhibition by the sample was measured by microculture tetrazolium (MTT) assay. The antiplasmodial activity was determined by using SYBR Green I against the chloroquine-resistance K1 strain and chloroquine-sensitive strain of *Plasmodium falciparum*. In each assay, the 50% inhibitory concentration (IC_{50}) value for each sample was derived by the drug concentration-response curves.

The results were demonstrated that, the leaf extracts from *Aglaia odorata* (Meliaceae) and *Azadirachta indica* (Meliaceae) showed good active results against all of selected protozoa. The highest anticrithidial activity was found in leaf extract of *Azadirachta indica* (IC_{50} < 7.8125 μg/ml). While the highest antiplasmodial activity 3D7 strain was found in leaf extracts of *Aglaia odorata* and *Azadirachta indica* (IC_{50} < 7.8125 μg/ml). It was shown that the leaf extract of *Azadirachta indica* (IC_{50} 3.846 μg/ml) gave the highest antiplasmodial activity in K1 strain. The plant extracts which showed the most and moderate activities have potential to be developed for antiprotozoal drugs in the future.

Keywords**:** Phytochemistry, Thai plants, antiprotozoal, IC_{50}

Introduction

The use of medicinal plants in the treatment of parasitic diseases is an old practice. Human parasitic infections are serious problems in tropical and subtropical developing country, despite the discover of new antiprotozoal (Mesia *et al.*, 2007).

This present study deals with the *in vitro* evaluation of the antiprotozoal activity of Thai plants against *Crithidia fasciculata,* the chloroquine-sensitive 3D7 strain and the chloroquine - resistant K1 strain of *Plasmodium falciparum*. *Crithidia fasciculate* is a kinetoplastid which occurs in nature as a commensal gut parasite in insects (Kariem *et al*., 1995). This organism is harmless to laboratory workers and it is easy to be cultured in large amounts. Therefore, *Crithidia fasciculata* can be used as a model of flagellated parasites such as, *Trypanosoma* spp., *Leishmania* spp. Malaria is the world's most important tropical disease (Kaur *et al.*, 2009). Resistance to chloroquine has steadily increased especially *Plasmodium falciparum*, so the discovery of new antimalarial drugs is necessary.

The aims of this study were to evaluate *in vitro* the antiprotozoal activities of chloroform extracts from Thai plants, and examine the active substances of these extracts.

Materials and Methods

Plant samples from four families i.e., *Harrisonia perforata* (Simaroubaceae), *Aglaia odorata, Azadirachta indica, Swietenia macrophylla* (Meliaceae), the unknown species cf.

Mitracarpus (Rubiaceae) and *Derris trifoliata* (Fabaceae) were collected during June to October 2009. These plant samples and their biological activities are showed in Table 1

Plant samples: leaf, stem bark, aerial parts and root were dried, grinded and extracted with methanol at room temperature for seven days, filtered, and concentrated. The aqueous residues were extracted with chloroform, and then evaporated to dryness. The chloroform extracts were applied to test for antiprotozoal activities by continuous culture in 96-wells plate.

Table 1: Plant samples and their biological activities

Family	Botanical names	Main constituents	Biological activities
Fabaceae	*Derris trifoliata* Lour.	Alkaloids, flavonoids, tannins, triterpenoids (Khan *et al*., 2006)	- anticancer (Chihiro, 2004) - anticarcinogenic agents (Kennedy, 1998)
Meliaceae	*Aglaia odorata* Lour.	rocaglamides, terpenoids, lignans, alkaloids (Tu, 2007)	- antifungal, herbicide (Tu, 2007) - antileukemia (Hayashi, 2001)
Meliaceae	*Azadirachta indica* A. Juss.	Limonoids, alkaloids, flavonoids, coumarins, tannins (Hout *et al*., 2006)	- antimalarial for chloroquine-resistant strain of *Plasmodium falciparum* (Badani, 1987) - antibacterial and anti-inflammatory (Doraboba, 2004) - herbicide (Nagpal *et al*., 1996)
	Swietenia macrophylla King	triterpenoids, limonoids, flavonoids, tannins (Mootoo, 1999)	Seed: antimalarial (Kadota, 1990; Soediro, 1990)
Rubiaceae	cf. *Mitracarpus* sp.	favonoids, tannins (Frabi *et al*., 2009)	- *Mitacarpus frigidus* : antimicrobial, antileishmanial, antioxidant (Frabi *et al*., 2009) - *Mitacarpus villosus*: antifungal (Irobi and Daramola, 1993)
Simaroubaceae	*Harrisonia perforata* (Blanco) Merr.	Limonoids, quassinoids, chromones (Tanaka *et al*., 1995)	Antimalarial (Nguyen-Pouplin *et al*., 2007)

By Thin layer chromatographic technique (TLC) all chloroform extracts were also examined for alkaloids and terpenoids by spraying with Dragendorff's reagent and anisaldehyde-sulphuric acid reagent respectively.

Results and Discussion

The results from *in vitro* antiprotozoal testing of 12 chloroform extracts are presented in Table 2. The extracts which were considered as the most active against *Crithidia fasciculata* are leaf extracts of *Aglaia odorata* (Meliaceae) and *Azadirachta indica* (Meliaceae). For antimalarial, the extracts which were considered as the most active against 3D7 strain are leaf extracts of *Aglaia odorata* (Meliaceae) and *Azadirachta indica* (Meliaceae) and the most active against K1 strain are leaf extract of *Azadirachta indica* (Meliaceae) and aerial parts of cf. *Mitracarpus* sp.(Rubiaceae).

From the active compound examination, it was found that all of the extracts give positive reaction with Dragendoff's reagent and anisaldehyde-sulphuric acid reagent. Therefore, these demonstrate that all extracts have alkaloids and terpenoids. In previous

report, antiprotozoal activity may be due to the presence of alkaloids, terpenes, flavonoids, or saponins according to these phytochemical groups exhibited in vitro an antiprotozoal activity at the different extents (Phillipson and Wright,1991; Schwikkard *et al*., 2002)

Table 2: Results of plants screened for antiprotozoal activities

Botanical names	Local names	Part	Aniprotozoal activity IC_{50} (μg/ml)		
			Cf	**3D7**	**K1**
Aglaia odorata Lour.	ประยงค์	Leaves Stem bark	9.585 19.25	<7.8125 116.7	93.62 >400
Azadirachta indica A. Juss.	สะเดาอินเดีย	Leaves Stem bark	<7.8125 12.83	<7.8125 19.36	3.846 190.6
Derris trifoliata Lour.	ถอบแถบน้ำ	Leaves Stem bark	448.0 165.2	17.75 32.53	22.98 22.19
Harrisonia perforata (Blanco) Merr.	สีฟันคนทา	Leaves Stem bark	57.93 52.72	nd nd	356.2 109.0
Swietenia macrophylla King	มะฮอกกานีใบใหญ่	Leaves Stem bark	>1000 196.5	>1000 154.8	>1000 89.10
cf. *Mitracarpus* sp.	-	Aerial parts Root	>1000 >100	102.0 nd	<7.8125 >100
Chlroquine	-	-	nd	0.004	0.51
Miltefosine	-	-	5.8 μM	nd	nd

Cf: *Crithidia fasciculata*, **3D7**: the chloroquine-sensitive strain of *Plasmodium falciparum*,
K1: the chloroquine-resistance strain of *Plasmodium falciparum*, **nd**: not determined

IC_{50} < 10 μg/ml = most active, 10 < IC_{50} ≤ 50 μg/ml = high considered moderately active, 50 < IC_{50} ≤ 100 μg/ml = moderately active, 100 < IC_{50} ≤ 500 μg/ml = less active, IC_{50} > 500 μg/ml = none active

Conclusion

This investigation has demonstrated the antiprotozoal property of selected plant extracts. Most of plant extracts have antiprotozoal more than one of the selected protozoa. The extracts from leaves of *Aglaia odorata* (Meliaceae) and leaves of *Azadirachta indica* (Meliaceae) were found to be most active against all the selected protozoa. The plant extracts which show most active and moderately active will be separated by chromatographic technique, purification and structural elucidation of active compounds in the extracts towards development of antiprotozoal drugs.

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Original article **P06**

In vitro antimicrobial activities of meropenem, ciprofloxacin, colistin alone and in combinations against meropenem-resistant *Pseudomonas aeruginosa*

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Abstract

Determinations of *in vitro* antimicrobial activities of meropenem, ciprofloxacin, colistin alone and in combinations against 20 clinical isolates of *P. aeruginosa* have shown that 16 isolates (80%) were multidrug-resistant (MDR) isolates. All isolates were resistant to meropenem (MIC_{90}=32 μg/mL) whereas 4 out of 20 isolates (20%) were susceptible to ciprofloxacin (MIC_{90}=64 μg/mL). Furthermore, all isolates were susceptible to colistin (MIC_{90}=2 μg/mL). With checkerboard method, it has been shown that meropenem in combination with ciprofloxacin or colistin and ciprofloxacin in combination with colistin had synergistic effects with drug concentration within the therapeutic serum level against 3, 6 and 4 isolates, respectively. For triple combination of meropenem, ciprofloxacin and colistin, the synergistic effect was observed in 5 isolates. Taken these results into account, meropenem in combination with colistin may be optimal alternative treatment for meropenem-resistant *P. aeruginosa* infections.

Keywords: *Pseudomonas aeruginosa*, Meropenem, Resistant

Introduction

P. aeruginosa is one of the major causes of nosocomial infections. Empirical therapies are used for *P. aeruginosa* infections and combination therapy is preferred (1). However, increasing of MDR *P. aeruginosa* has been a problem worldwide (2). Carbapenems are drugs of choice for the treatment of MDR pseudomonal infection. However, carbapenem-resistant *P. aeruginosa* is also emerging and increasing (2). Therefore, new therapies are needed urgently. Previous studies reported the synergistic effects between carbapenems with aminoglycosides and/or fluoroquinolones against *P. aeruginosa*. Nevertheless, resistances to these agents have also been documented which limit therapeutic options (1-3). Recent study has shown that polymyxins (old class of antibiotics) which were abandoned earlier because of their toxicities may be the only available active agents existing against MDR *P. aeruginosa* (3). Therefore, this study was aimed to investigate *in vitro* activities of meropenem, ciprofloxacin, colistin and the combinations against meropenem-resistant *P. aeruginosa.*

Materials and methods

Bacterial isolates

Twenty isolates of *P. aeruginosa* were clinically isolated from the patients at Siriraj Hospital between years 2006-2008. *Escheriachia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains.

Antimicrobial agents

Antimicrobial disks were amikacin, ciprofloxacin (BBL™: Beckton Dickinson, USA), cefepime, meropenem (BBL™: BENEX Limited, USA) and piperacillin/tazobactam (Oxoid: Oxoid, Basingstoke, Hants, England). Standard laboratory powders were meropenem and sodium

carbonate and ciprofloxacin HCL (Siam Bhesach CO., Ltd., Bangkok, Thailand). Colistin sulfate (Sigma Aldrich, St Louis, MO).

Antimicrobial susceptibility testing

Susceptibility to each 5 board-spectrum antimicrobial agents (amikacin, ciprofloxacin, cefepime, meropenem, piperacillin/tazobactam) against all 20 *P. aeruginosa* isolates were determined by disk diffusion method according to CLSI (4). An isolate was considered to be MDR isolate when it resists to 3 or more of 5 broad-spectrum antimicrobial agents as described previously. Agar dilution method (5) was performed to determine MICs of meropenem, ciprofloxacin and colistin. The susceptibility were interpreted by referring to CLSI (6)

Evaluation of the combination effects

The combination effects of meropenem, ciprofloxacin and colistin were determined by checkerboard microdilution method, which modified from previous study (7). After incubated at 37 ^{o}C for 18 hours, FIC index (ΣFIC) was calculated according to the following formula: FIC index = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone) + (MIC of drug C in combination/MIC of drug C alone). FIC index < 1.0, 1.0 and > 1.0 was used to define synergy, additive and antagonism, respectively according to previous study (7).

Results

Antimicrobials susceptibility

From disk diffusion method, all isolates were resistant to meropenem whereas 80%, 65%, 70% and 30% of the tested isolates were resistant to ciprofloxacin, amikacin, cefepime and piperacillin/tazobactam, respectively. Sixteen isolates (80%) were MDR *P. aeruginosa*. MICs showed that most of the tested isolates were resistant to both meropenem and ciprofloxacin (40%), whereas all tested isolates were susceptible to colistin (Table 1).

Table 1 Antimicrobial susceptibility, MIC_{50} and MIC_{90} of meropenem, ciprofloxacin and colistin against 20 clinical isolates of *P. aeruginosa*

Susceptibility	Meropenem		Ciprofloxacin		Colistin	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Susceptible (S)	0	0	4	20	20	100
Intermediate (I)	8	40	0	0	0	0
Resistant (R)	12	60	16	80	0	0
Range (µg/mL)	8-32		0.5-128		1-2	
MIC_{50} (µg/mL)	16		64		1	
MIC_{90} (µg/mL)	32		64		2	

Susceptibility breakpoint (CLSI 2007) ; Meropenem : (S) ≤ 4 µg/mL, (I) = 8 µg/mL, (R) ≥ 16 µg/mL ;
Ciprofloxacin : (S) ≤ 1 µg/mL, (I) = 2 µg/mL, (R) ≥ 4 µg/mL ; Colistin : (S) ≤ 2 µg/mL, (I) = 4 µg/mL, (R) ≥ 8 µg/mL.

Combination effects

The combination effects between meropenem, ciprofloxacin and colistin against 20 clinical isolates of *P. aeru*ginosa were shown in Table 2

Table 2 Combination effects of meropenem, ciprofloxacin and colistin against 20 clinical isolates of *P. aeruginosa*

Antimicrobial combinations	FICI range	[a]Synergy (ΣFIC < 1)	Additive (ΣFIC = 1)	Antagonism (ΣFIC > 1)	[b]No. of isolates showed synergy within therapeutic serum level
		No. of isolates (%)	No. of isolates (%)	No. of isolates (%)	
MEM + CIP	0.75-1.063	6(30)	10(50)	4(20)	3
MEM + COL	0.563-1.060	6(30)	4(20)	10(50)	6
CIP + COL	0.560-1.060	6(30)	4(20)	10(50)	4
MEM + CIP + COL	0.623-1.060	13(65)	1(5)	6(30)	5

Abbreviations; MEM, meropenem; CIP, ciprofloxacin; COL, colistin; FICI, fraction inhibitory concentration index.
[a]Synergy : 1) MEM + CIP at concentration range from 4-16 and 0.25-64 µg/mL, respectively.
2) MEM + COL at concentration range from 1-8 and 1 µg/mL, respectively. 3) CIP + COL at concentration range from 0.06-16 and 1 µg/mL, respectively. 4) MEM + CIP + COL at concentration range from 1-16, 0.06-64 and 0.06-1 µg/mL, respectively.
[b]Therapeutic serum level : MEM ≈ 25 µg/mL (500 mg IV infusion q 8 hr), CIP ≈ 4 µg/mL (400 mg IV infusion q 8-12 hr)

Discussion and conclusion

The present study observed low level of meropenem resistance (MIC_{90}=32 µg/mL) . This may caused by loss of OprD porin in conjunction with overexpression of multidrug efflux pump (1,8). Moreover, high level of resistance of ciprofloxacin (MIC_{90}=64 µg/mL) were also observed. This may caused by overexpression of multidrug efflux pump together with the target sites mutation (1,2). In combination study, although, antagonism (FIC index > 1) was observed, there was no change in MIC values when tested with drug alone and drug in combinations. Triple combination of meropenem, ciprofloxacin and colistin showed the highest synergistic effect against *P. aeruginosa* (13 out of 20 isolates). When, the therapeutic serum concentration level is taken into account, meropenem in combination with colistin may be the best alternative treatment of meropenem-resistant *P. aeruginosa* infection (Table 2). Colistin may disturb bacterial membrane and increase cell's permeability which resulting in the enhancement of penetration of the antibiotic into cell (7). However, further *in vitro* and *in vivo* studies are needed prior to its application in the clinical setting.

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Original article **P07**

Neurotonic Thai plants reduce reactive oxygen species production in SH-SY5Y neuroblastoma cells

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Abstract

Oxidative stress is considered an important causative factor in several neurodegenerative diseases. This study was aimed to determine the antioxidant properties of seven neurotonic Thai plants with possible neuroprotective effect in humans. Antioxidant power was evaluated by ferric reduction, lipid peroxidation inhibition and intracellular reactive oxygen species (ROS) suppression. The results showed that the extracts from *Terminalia bellirica* (Gaertn.) Roxb. and *Albizia procera* (Roxb.) Benth. could act as ferric reducing agents, whereas those of *Cassia fistula* L. and *Stephania suberosa* Forman seemed to be potent inhibitors of lipid peroxidation. These plant extracts could also effectively suppress the formation of intracellular ROS in differentiated SH-SY5Y neuroblastoma cells. In the conclusion, most of the selected plants demonstrated strong antioxidant activity by acting as metal reducing agents, lipid peroxidation inhibitors, and/or intracellular ROS suppressants. This study provides the potential mechanisms of Thai neurotonic plants as neuroprotective agents which could be beneficial in the prevention or delay of neurodegenerative processes.

Keywords: Antioxidant, reactive oxygen species, neuroprotection, Thai plant, herbal medicine

Introduction

There is substantial evidence showing the relationships of ROS production and the induction of cell death and pathogenesis of neurological disorders including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (1). Thus, there are large numbers of experiments published showing the neuroprotective effect of antioxidants including vitamins and natural substances in *in vitro* and in animal models for neurodegeneration (2). Although the efficacy of these antioxidants for treatment of neurodegenerative disorders is still unclear, their potentials as alternative therapy or nutritional supplement to slow down the progression of those neuronal diseases have received much attention. In Thailand, there exist a number of herbal medicines that are believed to possess rejuvenating and neurotonic effects. For some of these plants, their beneficial effects for Alzheimer's disease by inhibiting acetylcholinesterase (AChE) activity were previously demonstrated (3). This study was aimed to test the ability of these plant extracts to suppress the oxidative stress in test tube and cell culture models.

Methods

Preparation of plant extracts

The specific parts of plants were collected, cut into small pieces and dried in a hot-air oven at 50 ^{o}C. The dried plant materials were coarsely powdered and macerated with 95% ethanol for 3 days. The extracts were filtrated, dried under reduced pressure, and kept at -20 °C until use.

Cell culture preparation

Human neuroblastoma SH-SY5Y cells were grown in DMEM/Ham's F-12 containing 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were maintained at 37 °C in a CO_2 incubator containing 5% CO_2. The medium was then changed to DMEM supplemented 1% FBS and 10 μM retinoic acid and the cells were allowed to differentiate for 6 days

Lipid peroxidation determination

Each plant extract was added to the brain homogenate before induction of lipid peroxidation by 400 μM $FeCl_2$ and 200 μM ascorbic acid. TBAR solution (10% trichloroacetic acid, 7% thiobarbituric acid, and 4% HCl final) was added to the mixtures, which were then heated to 95°C for 1 h. After spinning, the clear supernatant was read out on a plate reader at 532 nm.

Ferric reducing antioxidant power (FRAP assay)

The FRAP reagent was freshly prepared by mixing 3 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$ (10:1:1) together. To be tested, plant extracts were added to FRAP reagent. The absorbance was read out at 593 nm after 1 h of reaction.

Determination of reactive oxygen species (ROS)

A fluorescent DCFH-DA (10 μM) probe were added to the medium and incubated at 37°C for 30 min. The differentiated SH-SY5Y cells were incubated with the extract for 30 min before adding the free radical generator APPH. The fluorescent product 2',7'-dichlorofluorescein (DCF) was monitored spectrofluorometrically (Ex 485 nm and Em 530 nM).

Results

Our result showed the increasing amount of Fe^{2+} ion in the presence of increasing concentrations of all plant extracts in a dose-dependent manner (Figure 1). The extract from *T. bellirica* exhibited the highest reducing activity, followed by *A. procera*, *C. rotundus*, *C. fistula*, *T. divaricata*, *S. suberosa*, and *B. superba*. All selected neurotonic plant extracts also inhibited the lipid peroxidation reaction of brain homogenate in a dose-dependent manner. The IC_{50} values of lipid peroxidation inhibitory activity of all plant extracts were calculated and are shown in table 1. The production of ROS inside SH-SY5Y cells was decreased after incubating with the tested plant extracts in a dose-dependent manner (Figure 2). The decrease in the ROS level was not the result of the decrease of cultured cell numbers because the extracts at all tested concentrations did not have an effect on the SH-SY5Y cell viability (data not shown).

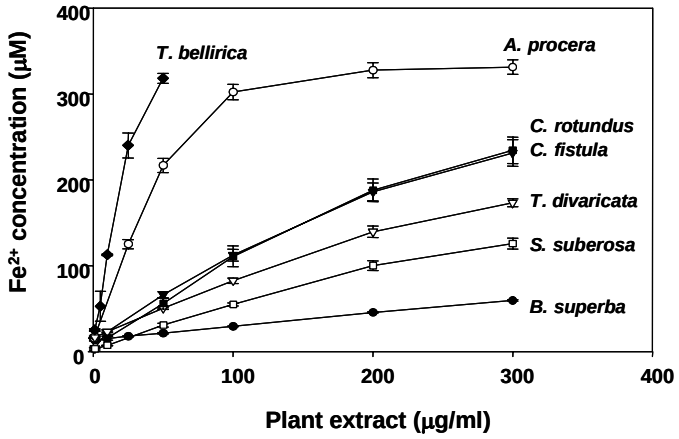

Figure 1 Ferric ion reducing activity of plant extracts. The results are mean±SEM.

Table 1 List of selected neurotonic plants and their lipid peroxidation inhibitory activities

Plant	Part	IC_{50} (μg/ml)	95% confidence interval (CI)
Stephania suberosa Forman	Rhizome	5.93	3.50-8.32
Cassia fistula L.	Root	6.47	4.15-10.09
Albizia procera (Roxb.) Benth.	Root	29.32	16.23-52.98
Tabernaemontana divaricata (L.) R. Br. ex Roem. & Schult.	Tuber	63.86	41.37-98.57
Terminalia bellirica (Gaertn.) Roxb.	Stem bark	161.7	61.27-426.9
Cyperus rotundus L.	Tuber	329.5	84.51-1285
Butea superba Roxb.	Fruit	902.9	173.2-4707

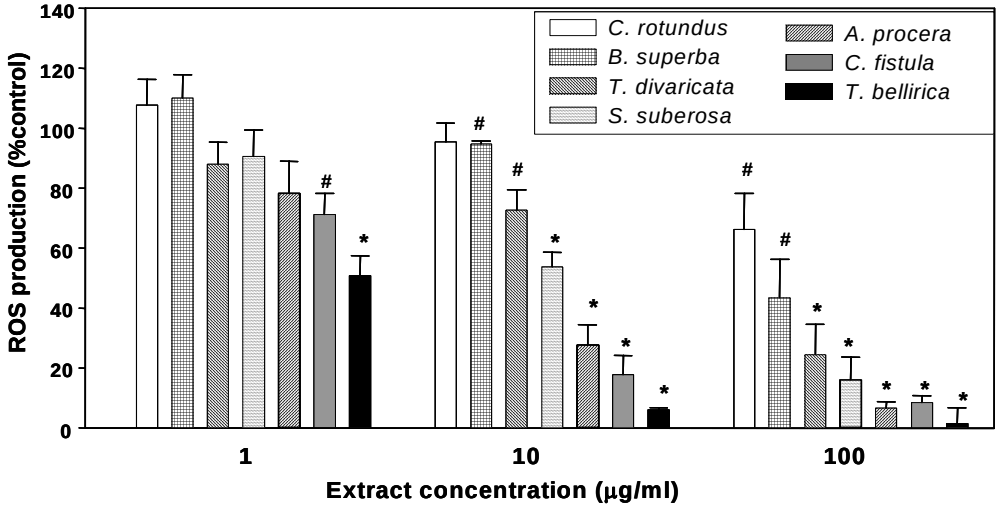

Figure 2 Effect of plant extracts on intracellular ROS production. The results are mean±SEM. (# p-value ≤ 0.05, * p-value ≤ 0.005)

Discussion

The extracellular Fe^{2+} was found to protect the intracellular space from H_2O_2, probably by initiating the Fenton reaction outside the cell (4). The increase in the Fe^{3+}/Fe^{2+} ratio was demonstrated as iron-induced oxidative stress in the blood sample of patients compared to healthy controls (5). These neurotonic plants could potentially prevent neuronal cells from the extracellular oxidative stress by suppressing the Fe^{3+}/Fe^{2+} ratio. The lipid peroxidation inhibitory effect of most extracts was proportional to their metal reducing activity (correlation analysis not shown). Metal reduction occurring in an aqueous compartment might consequently lead to the oxidation suppression of cellular lipid components. The ferric reducing activities of some of these plants seemed to be correlated with their intracellular ROS lowering effects. However, the metal-reduction ability did not seem to be the only mechanism of action for this purpose, the lipid peroxidation inhibitory effect was perhaps involved with the intracellular ROS decrement.

Taken together, some of these selected neurotonic Thai plants exhibit strong antioxidant activities which could be beneficial as neuroprotective agents in patients with certain neurodegenerative disorders or as supplement to prevent naturally degeneration of neuronal cells in risk group people.

Acknowledgements

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Original article **P08**

Potentiative effects of standardized extract of *Centella asiatica* on vinblastine-induced cell death

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Abstract

The purpose of this study was to investigate the influence of a standardized extract from *Centella asiatica* (ECa-233) and asiaticoside (ATS) on vinblastine (VBL)-induced cytotoxicity in porcine renal epithelial cell line (LLC-PK_1) and its *MDR_1*-gene-transfected epithelial cell line (LLC-MDR_1). The cells were co-treated with VBL (at various concentrations) and either ECa-233 or ATS for 72 hr prior to determination of cell viability with the MTT assay. Our results showed that the noncytotoxic ECa-233 and ATS could enhance the cytotoxicity of VBL in both cell types. It was likely that both ECa-233 and ATS were able to inhibit the function of P-glycoprotein, a known membrane efflux pump of VBL and might be useful in cancer chemotherapy.

Keywords: Extract of *Centella asiatica*, asiaticoside, vinblastine, LLC-PK_1, LLC-MDR_1.

Introduction

Centella asiatica is a pan-tropical plant in Thailand. This herbal plant has been used to relieve symptoms in various conditions including mental disorders, inflammation, circulatory problems and immune system deficiencies. In addition, this plant contained the pharmacological activities in wound healing antitumor and cognition enhancement in experimental rats (1). As known, the major components in *Centella asiatica* included triterpene saponins, in particular, asiaticoside and madecassoside, and their aglycone (asiatic acid and madecassic acid, respectively) (2). Asiaticoside at sub-cytotoxic concentration could induce apoptosis, and enhance the cytotoxic effect of vincristine in cancer cells (3).

This study was to examine the potentiative effects of our standardized extract of *Centella asiatica* (ECa-233), in comparison with asiaticoside, on vinblastine-induced cytotoxicity in porcine renal epithelial cell line (LLC-PK_1) and its *MDR_1*-gene-transfected epithelial cell line (LLC-MDR_1).

Methods

Cell culture: LLC-PK_1 cells [ATTC no. CL-101] and LLC-MDR_1 cells [kindly provided by Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands)] were cultured in M199 supplemented with 10% FBS and 100 unit/ml penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

Test materials: Standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) were kindly provided by Assoc. Prof. Dr. Ekarin Saifah and Assist. Prof. Dr. Chamnan Patarapanish, Faculty of the Pharmaceutical Sciences, Chulalongkorn University, respectively. ECa-233 contained triterpenoid glycosides at least 80% with the ratio between madecassoside and asiaticoside of 1.50 ± 0.50.

Cytotoxicity study: Cells were cultured in 96-well plates at a seeding density of $2x10^3$ cells/ well for 24 h prior to experiment. VBL-induced cytotoxicity in presence or absence of our test materials was determined after 72 hr-incubation by the MTT assay (4). Briefly, medium containing MTT (0.4 mg/ml) was added to each well and incubated for 4 hours. The formazan crystal were dissolved in DMSO and the absorbance was read at an excitation wavelength of 570 nm (reference wavelength 620 nm) using a microplate reader (Anthos Labtec HT2 version 1.21E, Australia). Percent cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the control vehicle.

Statistical analysis: All values were presented as mean ± SEM. One-way ANOVA followed by the Dunnett's test were performed for statistical comparisons. *P* value ≤ 0.05 was considered significant.

Results

Our preliminary results indicated that ECa-233 (up to 1000 µg/ml) and ATS (up to 100 µg/ml) had no effect on viability of LLC-PK_1 and LLC-MDR_1 cells, as determined by the MTT assay. In this study, both ECa-233 and ATS were able to significantly shift the concentration response curve of VBL-induced cytotoxicity leftward in both LLC-PK_1 and LLC-MDR_1 cells (Fig.1). The effects of our test materials were comparable with those of verapamil, a known P-gp inhibitor, which was used as positive control in this study. As expected, the degree of VBL resistance in LLC-MDR_1 was 5.1-fold higher than the resistance in LLC-PK_1, suggesting the higher level of *MDR_1* expression in LLC-MDR_1 cell type (Table1). In addition, our test materials and verapamil elicited the greater influence on VBL-induced cytotoxicity in LLC-MDR_1 than in LLC-PK_1.

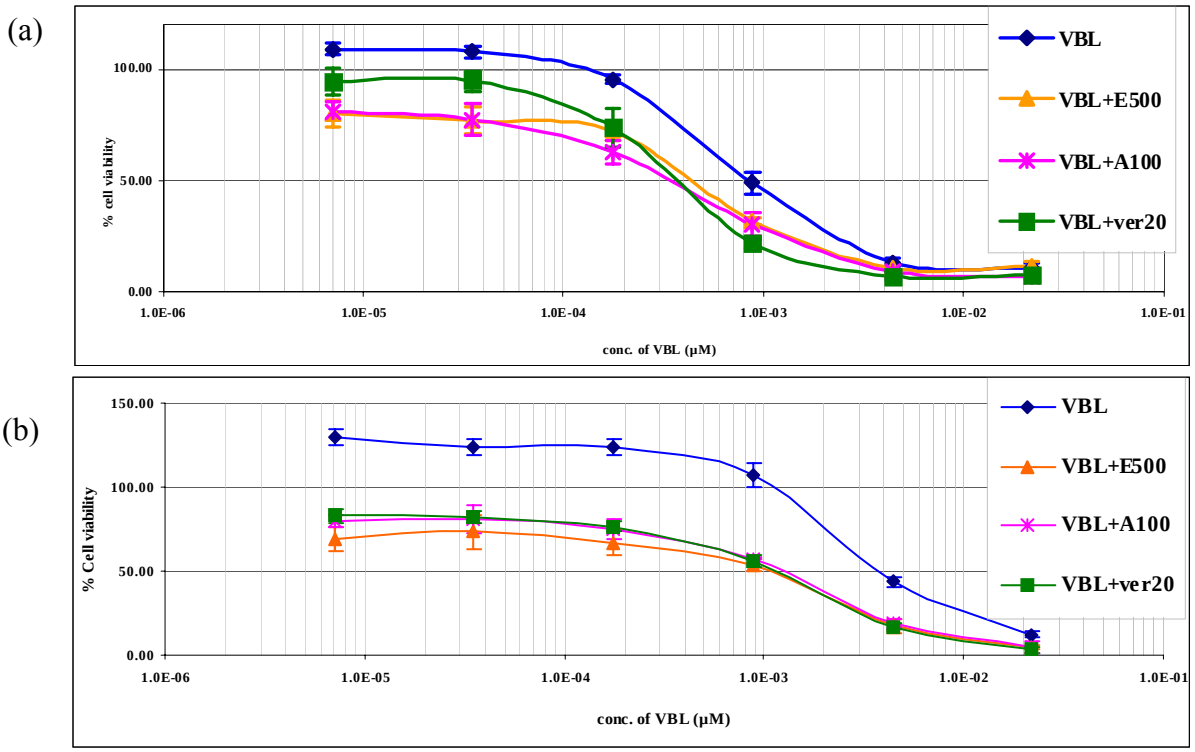

Figure 1 Concentration response curve of VBL-induced cytotoxicity in LLC-PK_1 cells (a) and LLC-MDR_1 cells (b), as determined by the MTT assay. The cells were cultured with a full range of concentrations of VBL in the presence or absence verapamil 20 µM or ECa-233 500 µg/ml or ATS 100 µg/ml for 72 hours. The results were presented as the mean ± S.E of at least triplicate determination.

Table 1 The apparent IC_{50} values of VBL in LLC-PK_1 and LLC- MDR_1 cells. The values were determined in the co-treatment with ECa-233 or ATS or verapamil. Each value represented the mean and S.E of at least three independent experiments.

Groups	LLC-PK_1		LLC-MDR_1	
	IC_{50} of VBL (mean ± S.E, nM)	Fold reversal of MDR[a]	IC_{50} of VBL (mean ± S.E, nM)	Fold reversal of MDR[a]
Vinblastine (VBL)	1.01 ± 0.09		5.19 ± 0.63	
VBL + ECa-233 500 µg/ml	0.48 ± 0.10*	2.45	0.82 ± 0.20*	5.97
VBL + ATS 100 µg/ml	0.44 ± 0.06*	2.10	1.04 ± 0.02*	4.85
VBL + verapamil 20 µM	0.41 ± 0.11*	2.28	0.96 ± 0.15*	5.11

*$P < 0.05$ vs vinblastine group

[a] The fold reversal of MDR was defined as the ratio of the IC_{50} value for VBL to that for VBL with the modulating agent.

Discussion & Conclusion

This study demonstrated that ECa-233 and asiaticoside (ATS) could potentiate the VBL-induced cytotoxicity in LLC-PK_1 and LLC-MDR_1 cells. Cellular accumulation of VBL resulted in cytotoxicity and cell death. Because VBL was a subsurate of P-gp efflux pump, cells with the MDR phenotype over-expression became resistance to VBL treatment . Generally, the presence of P-gp inhibitors such as verapamil could enhance the effectiveness of VBL through increase VBL accumulation in the cells (5). The enhancing effect of P-gp inhibitors on VBL-cytotoxicity was stronger in LLC-MDR_1 cells than in LLC-PK_1 cells. Hence, it was very likely that both ECa-233 and ATS increased VBL toxicity in LLC-PK_1 and LLC-MDR_1 cells through inhibition of P-gp function. These findings suggested that ECa-233 and ATS could be modulators of P-gp functions and might be useful in cancer chemotherapy.

Acknowledgements

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Original article **P09**

Cytotoxic and antioxidant activities of the ethanolic extract from *Dioscorea birmanica* prain & burkill

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Abstract

The objective of this research is to investigating on antioxidant and cytotoxic activities against cancer cells of *Dioscorea birmanica* Prain & Burkill extract which Thai traditional medicine used to treat cancer patients . Cytotoxic activity was tested against two type of lung cancer cell line (CORL23 and A549) and one type of normal lung cell (MRC5) by sulforhodamine B assay The antioxidant activity evaluated by DPPH assay. The results were found that the ethanolic extract of *Dioscorea birmanica* Prain & Burkill showed high cytotoxic activity against lung cancer cells ; A 549 and COR-L23 (IC_{50}= 7.45± 0.31, 8.71 ± 0.29 µg/ ml respectively) but no cytotoxic activity against normal cancer cells MRC5 (IC_{50}= 94.76. ± 1.25 µg/ ml).

The antioxidant activity of the ethanolic extract of this plant was evaluate as EC_{50} as 9.35 ± 0.62µg/ml . Six fractions were isolated by vaccuum liquid chromatography by ordering polarity of solvent (hexane, hexane: chloroform 1:1, chloroform, chloroform:methanol 8:2, chloroform :methanol 1:1 and methanol were code as F1-F6 respectively). It found that F4 showed the highest cytotoxic against A595 and F5 showed the highest activity against COR-L23 (6.07 and 16.44 µg/ml respectively). Two these fraction less active against normal cell. F3 showed the highest antioxidant activity followed by F4 and F5 (11.5, 14.9 and 15.5 respectively). Thus it is concluded that F4 and F5 should be continuous isolated cytotoxic and antioxidant compounds for discovery anticancer drug from this plant in the future

Keywords: cytotoxic activity, antioxidant activity, *Dioscorea birmanica* Prain & Burkill, lung cancer cell line

Introduction

Malignant disease is the leading cause of death in Thailand [1]. There are several types of cancer which are the cause of death in worldwide, lung cancer is the first leading in worldwide. Plant-based systems have a long history of use in traditional health care [2]. World Health Organization estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care and at least 119 chemical substances derived from 90 plant species can be considered as important drugs currently in use in one or more countries [3] (Farnsworth *et al.,* 1985). 74% of these 119 compounds were discovered as a result of chemical studies directed at the isolation of the active substances from plants basic used from traditional medicine [4]. Therefore, the usage of ethnopharmacology, or traditional use, is channeled for discovery of new biologically-active molecules[2]. Thai traditional medicine was used to treat cancer patients for long time ago. From the selective interview southern folk doctor was found that *Dioscorea birmanica* is a one plant to use for treat cancer patients [5]. Thus it should be continue investigation for cytotoxic activity against lung cancer cells which is a biggest problem for people in worldwide.

The objective of this investigation is to test cytotoxic activity against two type of lung cancer and comparison with lung normal cell and also test antioxidant activity. The

bioassay guide fractionation would be used to led to isolate cytotoxic compounds against lung cancer cells and antioxidant activity.

Materials and methods

Plant materials and preparation of extracts

The rhizome of *Dioscorea birmanica* Prain & Burkill were washed and then dried at 50 ◦C, powdered and extracted by methods corresponding to those used by traditional doctors. Dried ground plant material (1kg) was macerated with 95% ethanol for 3 days and the marc was macerated 2 times filter and then concentrate to dryness by evaporator.The extracts of plant were calculated percentage of yield and dissolved in dimethyl sulfoxide (DMSO) before bioassay.

Bioassay guild fractionation from *Dioscorea birmanica* Prain & Burkill extracts

The dried powder rhizomes of *Dioscorea birmanica* Prain & Burkill were macerated with 95% ethanol, and then concentrated under reduced pressure to obtain 11.13% of ethanolic extract. The crude soluble fraction was separated by quick column chromatography over silica gel eluting with a gradient system of increasing polarity as follow, using hexane (1000 ml), hexane:chloroform (1:1) (1000 ml), chloroform (1000 ml), chloroform:methanol (8:2), chloroform:methanol (1:1) and methanol (1000 ml) respectively and drying by rotary evaporation. Each fraction was dried and evaporated to yield 0.36 0.63, 2.85,,9.77,,50.02,and 25.67%, respectively, ,these fractions being denoted as DB1, DB2 ,DB3,DB4,DB5 and DB6. All of these fraction and the ethanolic extract of this plant were tested cytotoxic and antioxidant activities for leading to discovery a cytotoxic compound against lung cancer cells .

In vitro assay for cytotoxic activity

Cell culture

Two different types of human cancerous cell lines as the large cell lung carcinoma (COR-L23) and non small cell lung carcinoma (A549) , normal human lung cell as normal lung fibroblast cell line (MRC-5) were used to test in this study. COR-L23 and A549 are cultured in RPMI 1640 medium supplement with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin [6]. MRC-5 is cultured in DMEM culture medium containing 10% heated fetal bovine serum and 1% of 10,000 U penicillin and 10 mg/ml streptomycin. The cells are maintained at 37^{o}C in an incubator with 5% CO_2 and 95% humidity.

Cytotoxic assay

The sulforhodamine B (SRB) assay was used in this study to estimate cell numbers indirectly by staining total cellular protein with the SRB. The protocol was based on that originally described by Skehan, et al. (1990) [7] In brief, cells at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single cell suspensions. The viable cells were counted by trypan blue exclusion using a haemocytometer and diluted with medium to give a final concentration of $1x10^4$, $1x10^4$and $5x10^4$ cells/ml for COR-L23, A549 and MRC-5 respectively. 100 μl/well of these cell suspensions would be seed in 96-well microtiter plates and incubate to allow for cell attachment. After 24h the cells were treated with various concentrations of the extracts. The extracts were diluted in medium to produce the required concentrations 100 μl/well of each concentration would be added to the plates to obtain final concentrations of 1, 10, 50, 100 μg/ml for the extract and 0.1, 1, 10, 50 μM for pure compound, the final mixture would used for treating the cell contained not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium would be

removed. The wells were washed with medium, And 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40% trichloroacetic acid (TCA) per well, incubated at 4°C for 1 hour in the refrigerator and washed 5 times with tap water to wash non viable cells, so viable cells were fixed as monolayer in each well. 50µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with 1% acetic acid until only dye adhering to the cells are left. The plates dry and 100 µl of 10 mM Tris base (tris (hydroxy methyl) aminomethane, pH 10.5) was added to each well to solubilize the dye. To shake the plates gently for 20 minutes on a gyratory shaker. Read the absorbance (OD) of each well (4 replicate) on Microplate reader at 492 nm as an indication of cell number. To measure cell survival as the percentage absorbance compared with the control (non-treated cells). The IC_{50} values calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine.

***In vitro* assay for Antioxidant activity**

DPPH radical scavenging assay [8] pipetted sample solution in each concentration 100 µl in 96-well plate, added DPPH solution 100 µl in each sample and mixed.(Final concentration of sample 100, 50, 10, 1, 0.5 µg/ml). The absorbance (A) was measured at 520 nm. Calculated by formula %inhibition = [(Acontrol – Asample) / Acontrol] × 100 and EC_{50} value calculated by linear regression analysis by prism program.

Results and discussion

The percentage of yield and antioxidant activities exhibited in table 1. The cytotoxic activity against all cells was showed in table 2 **.**

Table 1. The percentage yields and antioxidant activity by DPPH assay of the extracts (n=3)

Code of Extract	Solvent	%yield	Antioxidant by DPPH assay as EC50 (µg/ml)±SEM
DBE	ethanol	11.13	9.35 ± 0.62
F1	Hexane	0.36	>100
F2	Hexane:Chloroform 1:1	0.63	>100
F3	Chloroform	2.85	11.50 ± 0.59
F4	Chloroform:methanol 8:2	9.77	14.85 ± 1.40
F5	Chloroform:methanol 1:1	50.22	15.51 ± 0.30
F6	Methanol	25.67	45.33 ± 1.7
BHT			10.35±0.40

The ethanolic extract of *Dioscorea birmanica* Prain & Burkill showed high activity against A 549 and CORL23 (IC_{50} = 7.45 and 8.71 respectively) but this extract showed no cytotoxic activity against normal lung cell (IC_{50} = 94.76 µg/ml). These results showed that this extract showed specific cytotoxic activity against only cancer cell but not kill normal cells. In addition, the ethanolic extract also showed high antioxidant activity because it showed higher antioxidant activity than BHT as positive control. This extract is good two points for discovery cancer drug which should kill only cancer cells but not kill normal cells and also good for being antioxidant product in the future. F4 and F5 showed high potentcy against CORL23 and A545 and less active for normal cell MRC5. F4 showed the highest cytotoxic against A595 and F5 5 showed the highest activity against CORL23 (6.07 and 16.44 µg/ml respectively). Two these fraction less active against normal cell. F3 showed the

highest antioxidant activity followed by F4 and F5 (11.5, 14.9 and 15.5 respectively). F4 and F5 should be continuous isolated cytotoxic and antioxidant compounds

Table2 Cytotoxic acrtivity showed as IC_{50} value (µg/ml) and SEM of *Dioscorea birmanica* Prain & Burkill extract and its fraction against lung cancer cell line (COR L-23 and A 549) and normal cell line (MRC -5) at exposure time (72 hrs) (n = 3)

Code of Extract	Solvent	Cytotoxic activity IC_{50} µg/ml ±SEM		
		COR L23	A549	MRC5
DBE	ethanol	8.71 ± 0.29	7.45 ± 0.31	94.76 ± 1.25
F1	Hexane	96.75 ± 1.25	100 ± 0	100 ± 0
F2	Hexane:Chloroform 1:1	75.66 ± 1.48	100 ± 0	100 ± 0
F3	Chloroform	67.52 ± 2.045	23.01 ± 0.43	83.51 ± 1.20
F4	Chloroform:methanol 8:2	20.22 ± 1.67	6.07 ± 0.25	38.77 ± 1.63
F5	Chloroform:methanol 1:1	16.44 ± 1.23	6.14 ± 0.08	85.57 ± 1.70
F6	Methanol	100 ± 0	89.34 ± 1.74	100 ± 0

Conclusion

It was concluded that the ethanolic extract of this plant showed cytotoxic activity against human lung cancer cell but no cytotoxic against normal lung cells and it also showed high antioxidant activity . From bioassay guide fractionation , fraction which isolated by Chloroform and methanol showed potential for find out cytotoxic and antioxidant compounds for discovery anticancer drugs and antioxidant product from this plant in the future

Acknowledgements

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Original article **P10**

PEITC inhibits cholangiocarcinoma via induction of mitochondrial dysfunction

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Abstract

Phenethyl isothiocyanate (PEITC), a natural compound found abundantly in cruciferous and other vegetables, has been shown to possess cancer chemopreventive activity. The purpose of this investigation was to examine the cytotoxic effect of PEITC in cholangiocarcinoma cells (CCA). Cholangiocarcinoma cells, KKU-100 and human liver Chang cells used for comparison in the study. Effects of PEITC on cell growth and induction of apoptosis was determined by fluorescent dye staining using acridine orange and ethidium bromide. Cultured cells were exposed to PEITC for 3, 12, 24 and 48 hours following assessment of cell viability and apoptotic cell death. PEITC can induce a large proportion of cells to undergo apoptosis in a dose-time dependent manners. The PEITC induced depletion of intracellular antioxidant GSH in the cell lines. Moreover, a rapid collapse of the mitochondrial transmembrane potential, as measured by JC-1 staining, was observed concurrently with an apparent apoptosis in both cells. Furthermore, Western blot analysis were used to examine the antioxidant and survival response related proteins. The results revealed that PEITC increased levels of Nrf2 and cyclin D1 in both cell lines, and Bax and Trx protein expression was up-regulated in KKU-100. The effect of PEITC on cell growth and apoptosis may contribute to cancer chemopreventive properties. In conclusion, our data lucidly evidence the chemopreventive merits of dietary phytochemical PEITC in suppression of cholangiocarcinoma.

Keywords: Phenylethyl isothiocyanate; Cholangiocarcinoma; Apoptosis; Cytotoxicity

Introduction

Cholangiocarcinoma (CCA) is a highly malignant adenocarcinoma originating from the cholangiocytes. The highest incidence of this cancer has been reported in Northeastern Thailand (1). Intrahepatic CCA is the most common type of CCA in Thailand and countries in Southeast Asian region. Infestation of *Opisthorchis viverrini* has been classified as a definite risk factor of the disease (2). Drug resistance in several cancers is often characterized by the increased activation of certain transcription factors, which provide the anti-apoptotic and pro-oncogenic signals.

Phenethyl isothiocyanate (PEITC), a hydrolysis product of a group of naturally occurring thioglucoside and glucosinolate compounds, found in cruciferous vegetables. PEITC has been extensively investigated for its chemopreventive activity against several cancers. The ability of PEITC to induce apoptosis has been suggested to be due to the disruption of mitochondrial redox status via the electrophilic isothiocyanate moiety (3). It seems probably that at low concentrations, PEITC causes mild oxidative stress, which consequently stimulates the cell to build up its antioxidant/cytoprotective defense contributing to chemopreventive effect. However, the relatively high concentrations result in

oxidative stress and irreversible changes in redox status, and mitochondrial dysfunction which leads to growth inhibition and apoptotic cell death in cancer cells (4).

The aim of this study was to examine the effects of PEITC on the induction of apoptotic cell death and mechanisms involving with were also investigated, including cellular redox status, mitochondrial energetics and adaptive antioxidant response of CCA cells.

Methods

In these experiments, cell cultures used in this study included KKU-100 and Chang liver cells. Cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 12.5 mM Hepes and 100 mg/ml gentamicin under 5% CO_2 in air at 37^o C. The media was renewed every 3 days, trypsinized with 0.25% trypsin-EDTA, and subcultured in the same media.

The cytotoxicity assay and induction of apoptosis was assessed by acridine orange/ethidium bromide (AO/EB) method. Cells were cultured at appropriate density and treated with PEITC at 1, 3, 10, 30 μM for 3, 12, 24 and 48 hours. Then cells were stained with AO/EB for fluorescence microscopic examination using an inverted microscope. The number of viable and apoptotic cells were counted.

To determine the pro-oxidant effect of PEITC, cultured cells were treated with 10 μM PEITC at 0, 3, 6, 12, 24 and 48 hours. Cells were washed and collected for assay of glutathione (5).

To examine the effect of PEITC on the mitochondrial transmembrane potential, cells were treated with 3, 10 and 30 μM PEITC for 1 or 3 hours, then the assay was performed using 5,5',6,'-tetrachloro- 1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining method (6). Fluorescent signals were examined by using a spectrofluorometer and fluorescence images were captured by fluorescence microscope. The intact mitochondria were stained with red fluorescence, while the depolarized mitochondria were stained with green fluorescence.

In Western blot analysis determined the level of Nrf2, cyclin D1, Bcl-xl, Bax and Trx expression, both cell lines were treated with PEITC at varied concentrations and cultured for 48 hours. Equivalent amounts of whole cell lysate proteins (40μg) were subjected to separation on 10% SDS-polyacrylamide gel electrophoresis and blotted on to a PVDF membrane. After blotting was carried out, the signals were visualized using ECL kit. The bands were determined intensities by Gel pro Analysis.

Results

KKU-100 and Chang cells were treated with varied concentrations (1, 3, 10 and 30 μM) of PEITC for various times (3, 12, 24 and 48 hours). PEITC induced growth suppression and apoptosis rapidly within 3 h and in a dose–dependent manner in both cell lines (Fig.1). The pro-oxidant effect of PEITC was assessed by assay of redox status in both cell lines. The total GSH levels in treated cells were declined rapidly in both cell lines. However, the levels were restored rapidly within 12 h in Chang cells. The cellular redox ratio is considered an indicative of redox balance, there was no change in KKU-100 but clearly suppressed in Chang cells, indicating that PEITC was more potent in induction of oxidative stress in Chang cells (Fig. 2). Depolarization of mitochondrial transmembrane potential often precedes an induction of apoptotic cell death. PEITC induced dissipation of mitochondrial transmembrane potential in both cell types, where JC-1 forms red fluorescent aggregates (JC-1-aggregates) in intact mitochondria and JC-1 monomers with green fluorescence in depolarized mitochondria (Fig.3). PEITC induced a rapid fall in the mitochondrial transmembrane potential at the concentration as low as 3 μM. It is apparent that Chang cells were shown to be more sensitive than KKU-100 cells by rapid change in mitochondrial transmembrane potential. Western blot analysis of antioxidant, proliferative and apoptotic-

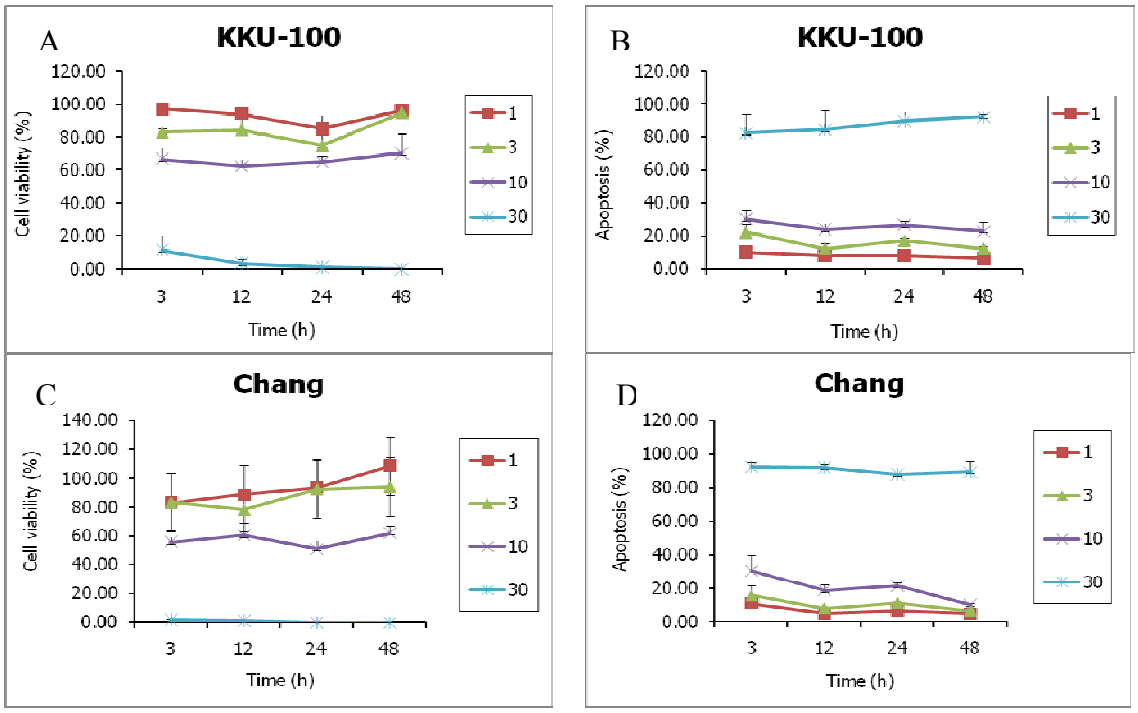

Figure 1 Cytotoxic effects of PEITC on KKU-100 and Chang cells. Cultured cells were incubated with varied concentrations of PEITC and at various time periods, cytotoxicity of the cells were examined by fluorescent staining method. (A, C) Percent viable cells and (B, D) apoptotic cells of KKU-100 and Chang cells, respectively, are showed. Data represent mean ± SE, each from 3 experiments.

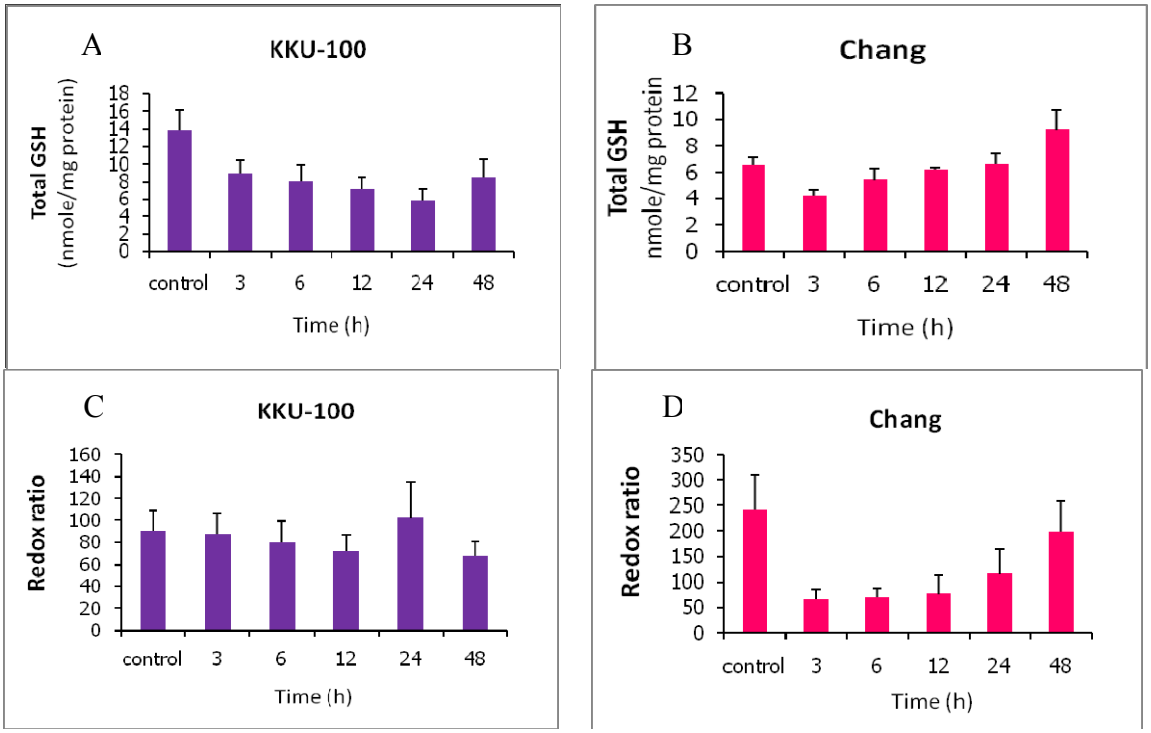

Figure 2 Effect of PEITC on redox status of KKU-100 and Chang cells. Cultured cells were incubated with 10 µM of PEITC and at various time periods redox status of cells were examined by glutathione assay. (A, B) Total intracellular glutathione (GSH) and (C, D) redox ratio (GSH/GSSG) of KKU-100 and Chang cells, respectively, are showed. Data represent mean ± SE, each from 3 experiments.

related proteins in CCA cells and Chang cells were analyzed after cells were treated for 48 hours. The results showed that PEITC increased level of cyclin D1 and Nrf2 in dose-dependent, whereas Trx and Bax were up-regulated in KKU-100 cells. However, there was no change in Bcl-XL in both cell lines (Fig.4).

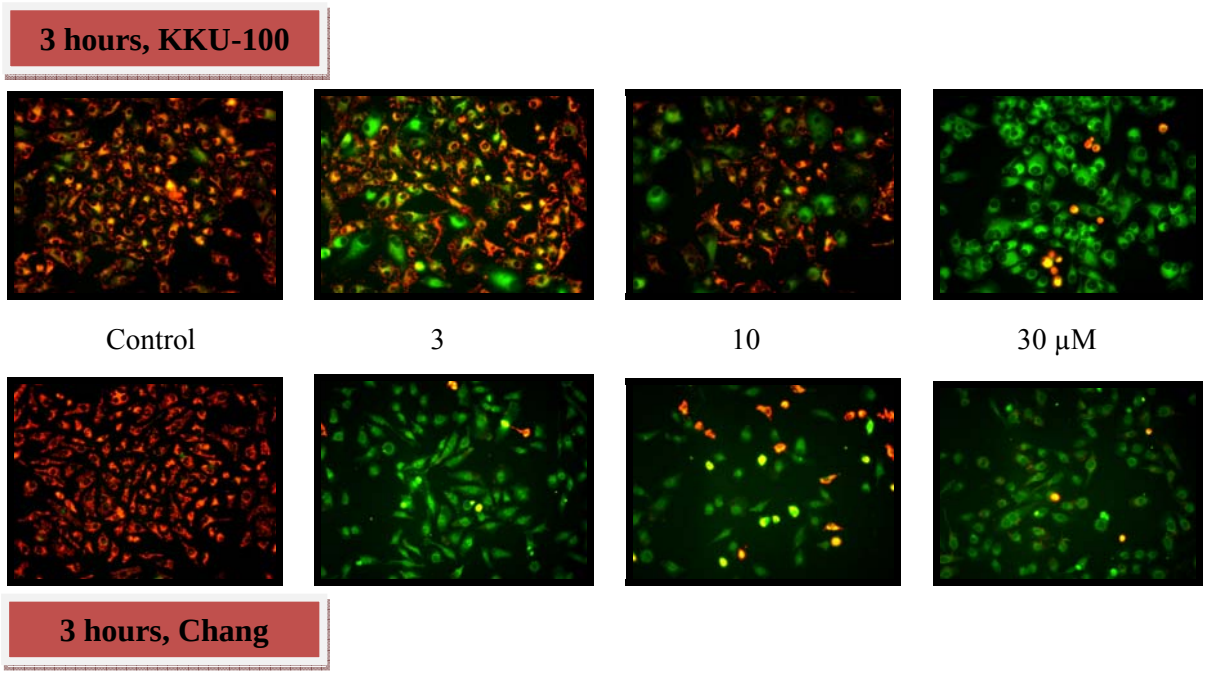

Figure 3 Effect of PEITC on mitochondrial transmembrane potential in KKU-100 and Chang cells. Cultured cells were treated with 3, 10 and 30 μM of PEITC for 3 h, and cells were stained with JC-1.

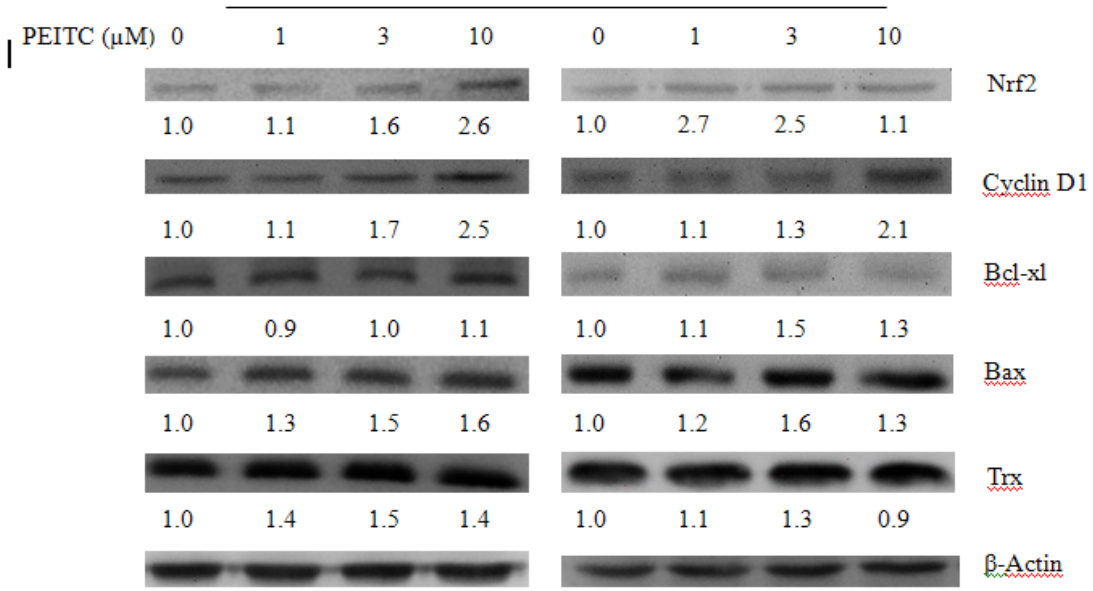

Figure 4 Western blot analysis of antioxidant (Nrf2, Trx), proliferative (cyclinD1) and Bcl-2 family proteins (Bcl-xl, Bax) proteins. KKU-100 and Chang cells were treated with PEITC at various concentrations for 48 hours. Representative pictures are showed and values denoted were mean from 3 experiments.

Discussion

Several dietary isothiocyanates from a variety of cruciferous vegetables such as *Brussica* species, are considered to be promising chemopreventive agents. PEITC is one of the most commonly investigated isothiocyanates (ITCs). In this study we have shown that treatment of CCA cells and normal liver cells with PEITC promotes rapid cell growth arrest and apoptosis in a comparable potency. The cytotoxicity was concurrently induced with rapid dissipation of mitochondrial transmembrane potential and decline of cellular GSH.

The redox capacity of the cells can be altered due to a depletion of reduced GSH, the important antioxidant defense mechanism in the cell. The rapid loss of cellular glutathione may be associated with cell death, where our finding is consistent with recent reports demonstrating association of reactive oxygen formation, loss of GSH and induction of apoptosis by PEITC in leukemic cells (7). Our data suggest that mechanism of PEITC-mediated an early growth inhibition of CCA involves the mitochondria. The loss of mitochondrial transmembrane potential can be detected with JC-1 as early as in the first hour indicating a rapid disruption of the inner membrane of mitochondria. This effect is similar to resveratrol which was shown to inhibit uveal melanoma tumor cells proliferation and stimulate apoptosis (8). Western blot analysis showed that treatment with PEITC increased Bax accumulation in KKU-100 cells. Severe oxidative stress induces translocation of Bax or Bak causing mitochondrial outer membrane permeabilization (9) and leading to cell apoptosis. This suggests PEITC induces cell killing via activation of Bax in KKU-100 cells.

Moreover, increased levels of Nrf2 were observed in both cell lines. Upregulation of Nrf2 mediated antioxidant and pro-survival pathway protecting cells from oxidative injury, however, activation of Nrf2 indeed protects cancer cells from chemotherapeutic agents (10). Trx, a Nrf2 down-stream regulated gene was slightly increased in KKU-100 cells, but unchanged in Chang cells. Trx is thought to have anti-apoptotic effects and identified as an interacting partner of the apoptosis-signaling kinase ASK-1 (11). Thus, up-regulation levels of Trx may be an adaptive responses to resist apoptosis. Cyclin D1 was increased with PEITC treatment. It has been shown that the cancer cells exert its survival through regulation of cell cycle and subsequent events lead to cell survival. In contrast, other investigator has reported that PEITC significantly suppressed the expression of cyclin D1 (12). PEITC did not inhibit Bcl-xl which is anti-apoptotic protein but other investigator has reported different observations showing that PEITC strongly inhibited Bcl-xl expression (13).

Thus, PEITC induced apoptosis in the CCA may be at least mediated via increased cellular stress, increased Bax expression and leading to inner mitochondrial membrane disruption. This is implied that other factors play role in modulating PEITC induced cell killing. Moreover, PEITC is seemed to inhibit KKU-100 and Chang cells in a non-selective manner. Thus, this may limit its utility in use in vivo.

Conclusion

PEITC can inhibit CCA proliferation and stimulate apoptosis. The mechanism of action may involve in activation of the mitochondrial apoptotic pathway via depletion of cellular GSH. In addition, increased level of Nrf2 and Trx may be the adaptive survival responses of cancer cells. An investigation of the key cellular signaling molecules as targets of the PEITC is warranted.

Acknowledgements

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Original article **P11**

Cytotoxic and antioxidant activities of two species of ginger extracts

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Abstract

Ginger or Khing (Thailand), a plant that belongs to the family Zingiberaceae, is one of the herbs commonly used in Thai traditional medical formulas. From selective interviews with Thai folk doctors and review Thai traditional medicine textbooks found that the word ginger was classified in two terms, Khing and Khing-Haeng. Folk doctors described that Khing-Haeng is more pungent than Khing but Khing-Haeng is becoming extinct. Thus, nowadays they used Khing instead Khing- Haeng. Khing is identified as Zingiber *officinale* and Khing-Haeng as *Zingiber ligulatum*. Thus, the objectives of this research are the investigation and comparing biological activities, using *in vitro* cytotoxicity against lung cancer cell lines (COR-L23) and antioxidant activity. Only ethanolic extracts of *Zingiber officinale* and *Zingiber ligulatum* showed similar antioxidant activity with EC_{50} value of 15.10 ± 2.50 and 15.89 ± 2.92 μg/ml, respectively. For cytotoxic activity, only the ethanolic extract of *Zingiber officinale* showed activity against COR-L23 with IC_{50} value of 7.90 ± 1.90 μg/ml but *Zingiber ligulatum* showed less activity (IC_{50} =42.27 ± 2.28 μg/ml). These results revealed that *Zingiber officinale* possessed high cytotoxic activity against lung cancer cells and confirmed using *Zingiber officinale* replace *Zingiber ligulatum* which nearly disappear .

Keywords: *Zingiber officinale* (Khing), *Zingiber ligulatum* (Khing-Haeng), cytotoxicity, antioxidant activity.

Introduction

Ginger or Khing (Thailand), a plant that belongs to the family Zingiberaceae, is one of the herbs commonly used in Thai traditional medical formulas. Generally, the word Khing refers to the edible ginger, *Zingiber officinale.* From selective interviews with Thai folk doctors and review Thai traditional medicine textbooks found that the word ginger was classified in two terms, Khing and Khing-Haeng. Folk doctors described that Khing, refers to the edible ginger, King haeng is more pungent taste than Khing refers to be the medical ginger. Practically, they used Khing more than Khing-Haeng because Khing-Haeng is becoming extinct. Thus, nowadays they also used Khing instead Khing- Haeng as medical ginger. Khing was growing widely in Thailand so it find easily for commercial . Resulting from our investigated and identification , they are 2 species of the family Zingiberaceae ; Khing was identied as Zingiber *officinale* Rosc. and Khing-Haeng as *Zingiber ligulatum* Roxb.

The previous reported about of *Z. officinale* found that its have a variety of biological activities including anticancer (Katiyar *et al.*, 1996; Lee and Surh, 1998; Bode *et al*.,2001; Chung *et al*., 2001; Keum *et al*., 2002; Leal *et al.,* 2003; Miyoshi *et al*., 2003; Wang *et al.*, 2003), antioxidation and anti-inflam**m**ation (Aeschbach *et al*., 1994; Habsah *et al*., 2000; Surh, 2003), anti-platelet aggregation (Tjendraputra *et al*., 2003), anti-fungal (Ficker *et al*., 2003) and neuroprotective (Kim and Kim, 2004). Surprisingly, there have been no reports on biological activities of *Zingiber ligulatum*. The objectives of this research are the investigation and comparing biological activities, using *in vitro* cytotoxicity against lung cancer cell lines (COR-L23) and antioxidant activity.

Methods

Plant materials

Khing (*Z. officinale*) was collected from amphor Khaoko, Phetchabun province. and Khing-Haeng (*Z. ligulatum*) was collected from amphor Saentum, Trad province. Their herbarium were collected for identification by expertee from Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept.

Extraction

The extraction procedures used were similar to those practiced by folk doctors (ethanolic extract and water extract). Dried ground material was marcerated with 95% ethanol, and then filtered and concentrated to dryness under reduced pressure. For water extract, plants were decocted, and then filtered and dried by freeze drying. The percentage yields of extracts were calculated.

In vitro assay for Antioxidant activity

Antioxidant activity determined using DPPH assay, according to modified method of Yamasaki *et al*. (1994). The mixture was incubated at 25^{O}C for 30 min. Then the decrease in absorbance due to DPPH was measured at 540 nm using a micro-plate reader. The antioxidant activity of each extract expressed as IC_{50} (mg/ml).

In vitro assay for cytotoxic activity

Plant extracts were diluted and tested the cytotoxicity against COR-L23 using sulphorhodamine B (SRB) assay (Skehan *et al*., 1990). The monolayered of cell cultures in 96-well plate were treated with sample for 4 replications. The plates were incubated for an exposure time at of 72 hours, and then the medium was removed and washed. The plates were incubated for a recovery period of 3 days. The survival percentage was measured colorimetric using SRB assay and IC_{50} value was calculated by means of GraphPad Prism (version 4.0) program.

Results and Diccussion

Table 1. The percentage yields, cytotoxicity against COR-L23 and Antioxidant activity by DPPH assay of the extracts of Khing and Khing-Haeng (n=3)

Plant or Sample	Part of used	Extract	% Yield	Antioxidant	Cytotoxicity
				EC_{50} ± SEM (µg/ml)	IC_{50} ± SEM (µg/ml)
Zingiber officinale (Khing)	Rhizome	Water (ZO1)	8.15	> 100	> 100
		EtOH (ZO2)	4.29	**15.10 ± 2.50**	**7.90 ± 1.90**
Zingiber ligulatum (Khing-Haeng)	Rhizome	Water (ZL1)	15.84	> 100	> 100
		EtOH (ZL2)	10.70	**15.89 ± 2.92**	42.27 ± 2.28
BHT	-	-	-	11.36 ± 0.21	-

The results of antioxidant activity and cytotoxic activity of the extracts from *Zingiber officinale* and *Zingiber ligulatum* are shown in Table 1. The ethanolic extract of *Zingiber officinale* and *Zingiber ligulatum* showed moderate antioxidant activity with EC_{50} values of 15.10 ± 2.50 and 15.89 ± 2.92 µg/ml, respectively. For cytotoxic activity, only ethanolic extracts of *Zingiber officinale* was exhibited against COR-L23 with IC_{50} values 7.90 ± 1.90 µg/ml.

Conclusion

The results from our testing showed that the ethanolic extracts of *Zingiber officinale* and *Zingiber ligulatum* have similar antioxidant activity while only the ethanolic extracts of *Zingiber officinale* possessed high cytotoxic activity against lung cancer cells. Its confirmed using *Zingiber officinale* instead *Zingiber ligulatum* of Thai folk doctors for treatment cancer.

Acknowledgements

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Original article **P12**

Effect of lipopolysaccharide pre-treatment on the replication of Japanese encephalitis virus in microglia

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Abstract

Microglia are resident cells of the central nervous system (CNS) and become activated to function as brain macrophage in response to infectious pathogens. Our recent findings further demonstrate that microglia support the replication of Japanese encephalitis virus (JEV) and remain productively infected for up to 16 weeks. Thus, microglia may serve as a viral reservoir for JEV infection of neurons in the CNS in addition to provide the first line of defense against invading pathogens. Interestingly, in the same study, no increase in nitric oxide levels in the supernatant of microglial cultures was observed following JEV infection over a 5 day period. This suggests that microglia may not be activated in response to JEV infection. Therefore, using the same experimental paradigm this study aimed to determine whether activation of microglia prior to JEV infection would reduce a subsequent viral production. Briefly, mouse BV-2 microglia were plated at a density of 5×10^5 cells/well in 6-well plates. Then, the cultures were treated with lipopolysaccharide (LPS) prior to the infection with JEV at a MOI of 5 pfu/cell. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay. Results clearly demonstrated that LPS pre-treatment dramatically reduced viral titer in the growth media compared with that of infected cultures without LPS pre-treatment. Thus, cellular activation by LPS prior to the infection appears to reinforce a natural innate immune mechanism of microglia against JEV infection.

Keywords: Japanese encephalitis virus, microglia, LPS

Introduction

Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae*, which includes West Nile, Dengue, Tick-borne encephalitis and Yellow fever viruses. The JEV genome consists of a positive single-stranded RNA approximately 11 kb in length [1]. JEV replicates exclusively in the cytoplasm and mature on intracellular membranes, but not on plasma membrane of infected host cells [2]. JEV is the most common cause of human arthropod-borne encephalitis in Asia with a high mortality rate. The clinical manifestation of JEV infection including fever, headache, vomiting and altered consciousness leading to neurological sequelae in some of those who survive [3]. In infected human cases, JEV antigens were localized mainly in neurons suggesting that neurons are the principal target cells of JEV [4]. However, recent study have demonstrated that microglia support the replication of JEV and remain productively infected for up to 16 weeks [5]. Thus, microglia may serve as a viral reservoir for JEV infection of neurons in the central nervous system (CNS).

Microglia are the resident immune cells of the CNS and have a critical role in host defense against invading pathogens [6]. Activation of microglia *in vitro* can be induced by a wide range of stimuli, including lipopolysaccharide (LPS) which is outer membrane glycolipids of gram-negative bacteria. LPS activates microglia through toll-like receptor 4 (TLR4) resulting in the production of proinflammatory cytokines, chemokines and reactive

oxygen/nitrogen species [7]. Specifically, Nitric oxide (NO) and interferons (IFNs) secreted by activated microglia play a critical role in antimicrobial action and inhibition of viral replication in the CNS [6, 8]. However, our previous results demonstrated that infection of microglia with JEV did not induce NO production over a 5 day period [5]. Thus, we hypothesize that an absence or a delay of microglial activation following JEV infection may be beneficial to the replication of JEV. To test this hypothesis, cultures of microglia were infected with JEV with or without LPS pre-treatment. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay as described in the methods section.

Materials and methods

Cell cultures, C6/36 cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% L-Glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin and used to propagate JEV. LLC-MK2 and BV-2 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 5-10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% L-Glutamine at 37°C in a 5% CO_2 incubator. All cell culture reagents were purchased from Hyclone (USA).

JEV propagation, C6/36 cells were grown in tissue culture flasks until sub-confluence. As previously described [5], the culture medium was replaced with serum-free MEM containing the JEV strain Beijing-1 (accession No.L48961) at multiplicity of infection of 1 pfu/cell. Viral infection was allowed to proceed for 120 min at 28°C with constant agitation. Subsequently, fresh complete MEM was added to the cells. At day 4 post-infection, the medium containing progeny virus was collected for determination of viral titer by standard plaque assay.

JEV infection of microglial cells, BV-2 microglia were plated at a density of 5×10^5 cells/well in 6-well plates and incubated with 0.5 μg/ml of LPS (Sigma, USA) for 6 h at 37°C. Untreated cultures were served as control. Then, the cells were washed with PBS 3 times and subsequently inoculated with JEV at a MOI of 5 pfu/cell for 2 h at 37°C followed by washing with PBS to remove unabsorbed viruses. Uninternalized extracellular viruses were inactivated by washing with acid glycine buffer followed by washing with PBS. Then, fresh serum-containing medium was added into the cells and the cells were incubated at 37°C in a 5% CO_2 incubator. The media containing progeny virus were collected at 24 h post-infection for determination of viral titer by standard plaque assay.

Standard plaque assay, To determine viral titer, LLC-MK2 cells in 6-well plates were inoculated with 200 μl of serially diluted virus-containing medium for 1 h and 30 min at 37°C with agitation. Then, 1% Seakem LE agarose (Cambrex, USA) mixed with nutrient overlay (Earle's Balanced Salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS) was added to each well. The plates were incubated at 37°C in a 5% CO_2 incubator for 7 days. Then, the cultures were fixed with 3.7% formaldehyde and agarose plugs were removed. Plaques were counted after staining with 1% crystal violet solution.

Statistical analysis, All data were presented as mean ± SEM. One way analysis of variance followed by LSD was used to compare the significance between the control and the treatment groups. The p-value of less than 0.05 was set for the significant difference.

Results

BV-2 cells were infected with JEV at a MOI of 5 pfu/cell at 37°C for 2 h. At 24 h post-infection, the growth medium was collected and serially diluted to quantify progeny viruses by standard plaque assay (Fig. 1 A). The calculated viral titer was $5\pm0.5\times10^5$ pfu/ml (Fig. 1 B). No plaques were detected in uninfected control cultures.

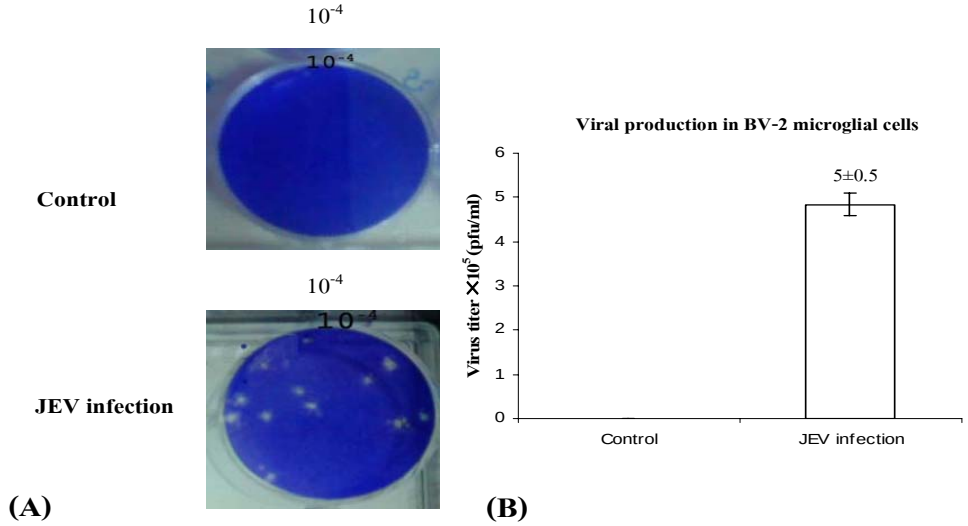

Figure 1. JEV infection of microglia. BV-2 cells were infected with JEV at a MOI of 5 pfu/cell. Virus-containing growth medium was collected at 24 h post-infection, serially diluted and used to inoculate LLC-MK2 cells, according to a standard plaque assay procedure. This experiment was repeated three times independently and each was performed in triplicate. (A) Plaques were stained with 1% solution of crystal violet. (B) The viral titers are displayed graphically.

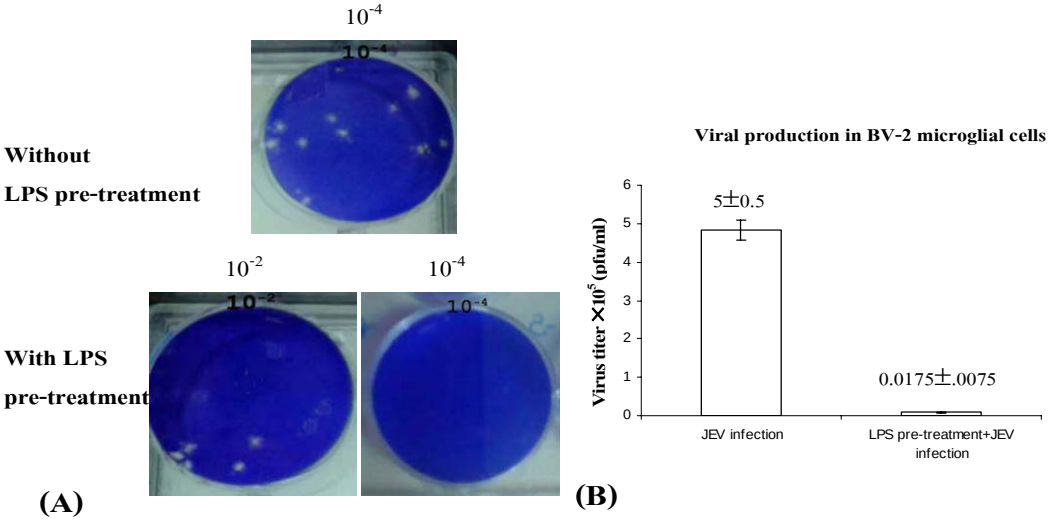

Figure 2. JEV infection of microglia with or without LPS pre-treatment. BV-2 cells were pre-treated with LPS (0.5 μg/ml) before JEV infection (5 pfu/cell). Virus-containing growth medium was collected at 24 h post-infection, serially diluted and used to inoculate LLC-MK2 cells. This experiment was repeated three times independently and each was performed in triplicate. (A) Plaques were stained with 1% solution of crystal violet. (B) The viral titers are displayed graphically.

To test whether activation of microglia prior to JEV infection reduces the amount of progeny viruses in the growth medium, BV-2 cells were pre-treated with LPS for 6 h followed by challenge with JEV at MOI of 5 pfu/cell. At 24 h post-infection, progeny viruses in the growth medium were quantified by standard plaque assay (Fig. 2 A). The calculated viral titer in the infected cultures without LPS pre-treatment was $5\pm0.5 \times 10^5$ pfu/ml, whereas

that of infected cultures with LPS pre-treatment was $1.75\pm0.95 \times 10^3$ pfu/ml (Fig. 2 B). The results clearly demonstrated that LPS pre-treatment dramatically reduced viral titer in the growth media compared with that of infected cultures without LPS pre-treatment.

Discussion

Since it was shown that replication of JEV in microglia did not induce the production of NO, a marker associated with microglial activation, an absence or a delay of microglial activation following JEV infection may be beneficial to the replication of JEV. To test this hypothesis, cultures of microglia were infected with JEV with or without LPS pre-treatment. At 24 h post-infection, the growth media containing progeny virus were collected for determination of viral titer by standard plaque assay as described in the methods section. In this experimental paradigm, microlgial activation by LPS occurs through TLR4 which is linked to a variety of intracellular signaling cascades leading to transcription activation of genes involved in the innate immune response. These genes include inducible nitric oxide synthase (iNOS) and interferons (IFNs) [7]. This information strongly supports the present finding that LPS pre-treatment dramatically reduced the production of infectious progeny of JEV from infected microglia. Additional supportive evidence include a recent report by Boivin et al [8] showing that pre-treatment with TLR antagonists induced early expression of several immune genes in the brain and resulted in a significantly lower viral load in a mouse model of Herpes Simplex Virus Type 1 Encephalitis. In summary, the present study demonstrated that cellular activation through TLR4 prior to the infection appears to reinforce a natural innate immune mechanism of microglia against JEV infection.

Acknowledgements

We would like to thank Dr. Thananya Thongtan for providing C6/36, JEV and LLC-MK2 cells. This work was supported by a grant to PC.

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Original article **P13**

Enhancing cell proliferation and protection hydrogen peroxide-induced cytotoxicity in PC12 cells by rice extracts

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Abstract

Rice is well-known as source of vitamin E, beta-glucan and gamma-oryzanol which may be useful for the treatment of Alzheimer's disease. Three varieties of rice, white rice: white-hom-ma-li (MLW) and glong-hom-ma-li(MLG); red rice, Sung-yod (SY) were extracted by various methods to obtain 14 fractions. Each fraction was studied for cytotoxicity and protection neuronal cell from hydrogen peroxide-induced toxicity. By MTT reduction assay, all fractions at dose of 100 µg/ml had no cytotoxic effect on PC12 cell culture. Moreover, proliferative effect were detected only at dose of 50 µg/ml in lyophilized rinse-water from SY (SYGW), MLG (MLGW), MLW(MLWW), ethanolic extract from ML bran (MLBMEt) as 119.33 , 119.69, 120.14, 112.41 respectively by 4 independence experiments. At dose of 50 and 100 µg/ml, each fraction of MLWW, MLGW, ethanolic extract of ML bran , MLG bran and SY bran (MLBMEt, MLGMEt SYBMEt), supercritical extract from ML bran (MLBSUP), and cold-press of SY bran (SYBEX) could protect PC12 cells from oxidative stress induced by hydrogen peroxide with 83-92% effectiveness against that of vitamin E. These results concluded that various fractions from rice: rinsed water, ethanolic extract, supercritical extract and cold-press extract had protective effect on neuronal cell PC12 from oxidative stress induce by hydrogen peroxide. This neuroprotective effect may be due to the combination of more than one active principle in rice that could be potential candidates for use as nutraceutical for neurodegenerative diseases.

Keywords: neuronal cell, PC12, rice extracts, antioxidant

Introduction

Oxidative neuronal cell damage has been implicated in neurodegenerative disorders such as Alzheimer's disease (AD) (1). Many studies indicate that the brain of an AD patient is subjected to increased oxidative stress resulting from free radical damage (2). These oxidative stress-induced damages disrupt cellular function and membrane integrity, thereby leading to apoptosis (3).

Natural antioxidants have been reported to play a major role in blocking oxidative stress induced by free radicals. Rice is well-known as source of beta-glucan, gamma-oryzanol and vitamin E, especially, wheat germ and rice bran are major sources of these antioxidants (4,5). In particular, strong reduction in oxidative stress within neurone cells was one of the major role of vitamin E in brain function and in the prevention of neurodegeneration (6). A new potential antioxidant agent from rice bran, water-soluble oryzanol enzymatic extract derived from rice bran, was proved to prevent brain protein damage due to lipid peroxidation (7). In addition, there was hydrophilic antioxidants in purple rice bran which was much greater antioxidative effect than that of its lipophilic antioxidants and anthocyanins and gamma-tocols.(8).

The present study is to investigate cytotoxic and protective actions of various fractions of rice extract against hydrogen peroxide-induced rat pheochromocytoma line PC12 injury. Cell viability was measured with blue formazan that had been metabolized from colorless [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide] MTT by mitochondrial dehydrogenases which are only active in live cells. This assay provides a sensitive measurement of the normal metabolic status of cells which reflects early cellular redox changes (9).

Methods

Tested materials. Three varieties of rice, white rice, White-Hom-ma-li (MLW) and Glong-hom-ma-li(MLG); red rice, Sung-yod, (SY) were extracted by various methods to obtain 14 fractions. The water soluble fractions were 3 fractions from rinsed water of 3 kinds of rice: MLWW, MLGW, SYGW and 2 fractions from freeze dried of boiled rice bran: MLBBOIL, SYBBOIL. The water non-soluble fractions obtained from maceration of rice in ethanol: MLWMEt, MLGMEt, SYGMEt and maceration of rice bran : MLBMEt, SYBMEt. Also, cold press method and supercritical extraction were used for extracting bran of ML and SY to obtain MLBEX, SYBMEX, MLBSUP and SYBSUP respectively.

Cell culture Transplantable rat pheochromocytoma, PC12 cells were purchased from American Type Culture Collection (ATCC) and grown on collagen-coated tissue plates in Dulbecco's modified minimal essential media(DMEM, high glucose formula with L-glutamate and pyruvate) supplemented with 5% (v/v) fetal bovine serum ,10% horse serum, 1% penicillin (100 U/ml) / streptomycin (100 µg/ml) under 5% CO_2 in air at 37 ºC and cells were subcultured twice a week. When the cells reached 70% confluence growth, undifferentiated and viable cells were cultured at a density of 5 $x10^4$ cells in 96-well coated plates. Cells were used for experiments 48 h. after seeding.

Intervention for cytotoxicity. Each of rice extracts at concentrations of 50, 100 µg/ml/ well was added, and incubated for 48 h.

Intervention for antioxidative stress study. After treated cells with rice extract for 24 h, hydrogen peroxide 500 µM was added into each well. New media was changed after 2 h of incubation. Vitamin E 10 mM was employed as positive control agent(9).

Cytotoxicity test. The viability measurement was assessed using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide] reduction assay. After exposing the cells to an intervention, MTT (5mg/ml) was added to each culture well. Then incubation at 37 ºC for an additional 2 h, the formazan crystals were dissolved by additional 100 µl of dimethyl sulfoxide (DMSO), and the plate were shaken vigorously to ensure complete solubilization. Formazan absorbance was assessed at 570 nm by a microplate reader (biotek, power wave XS). Values are expressed as percentage of viable cells. Four independence experiments were done for each of rice extract.

Statitic analysis. Statistical analysis was performed by one way analysis of variance, ANOVA, followed by Duncan post hoc comparison. The homogenous groups with vitamin E $p < 0.05$ were demonstrated. All data are presented as mean and standard error of mean.

Results

Cytotoxicity At dose of 100 µg/ml all of 14 rice extracts had no cytotoxic effect on PC12 cell culture growing in proliferative media. Moreover, proliferative effect were detected only at dose of 50 µg/ml in lyophilized rinse-water from SY (SYGW), MLG (MLGW), MLW(MLWW), ethanolic extract from ML bran (MLBMEt) as 119.33 , 119.69, 120.14, 112.41 respectively by 4 independence experiments.(Fig.1) At dose of 100 µg/ml of these 4 mentioned groups had no significant difference in proliferation when cultured in proliferative media, but slightly showed neurite outgrowth when cultured in

differentiative media (DMEM with % 1 FBS % 5 HS; data non showed). All other water-non-soluble fractions had no effect on cell proliferation.

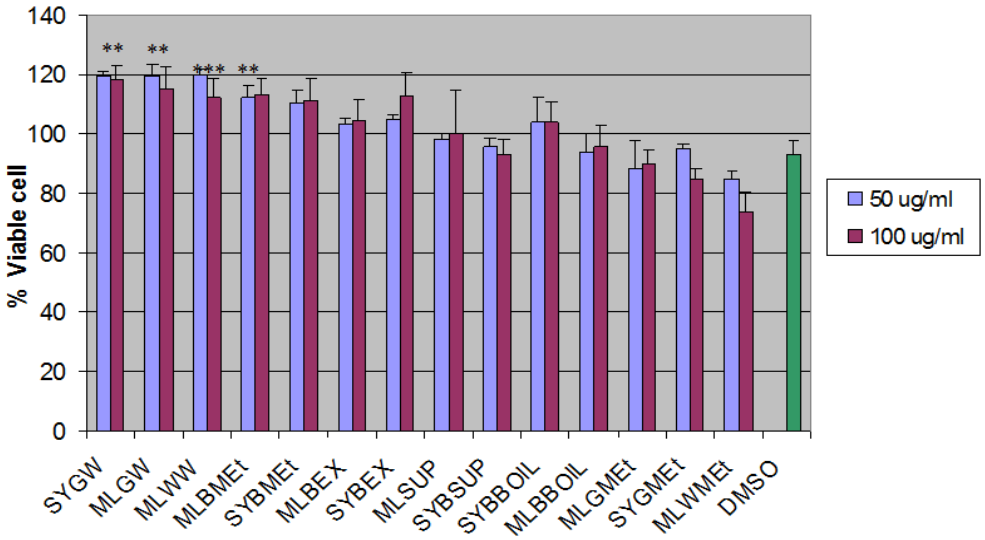

Figure 1. Percentage of PC12 cell growth (mean ± SEM) by MTT assay after treated with rice extracts exposure time 48 hours by independent experiment (N=4). SYGW, MLGW, MLWW, and MLBMEt could proliferate PC12 growth significantly. (***p< 0.001,**p< 0.01) when compared to 2% DMSO(control).

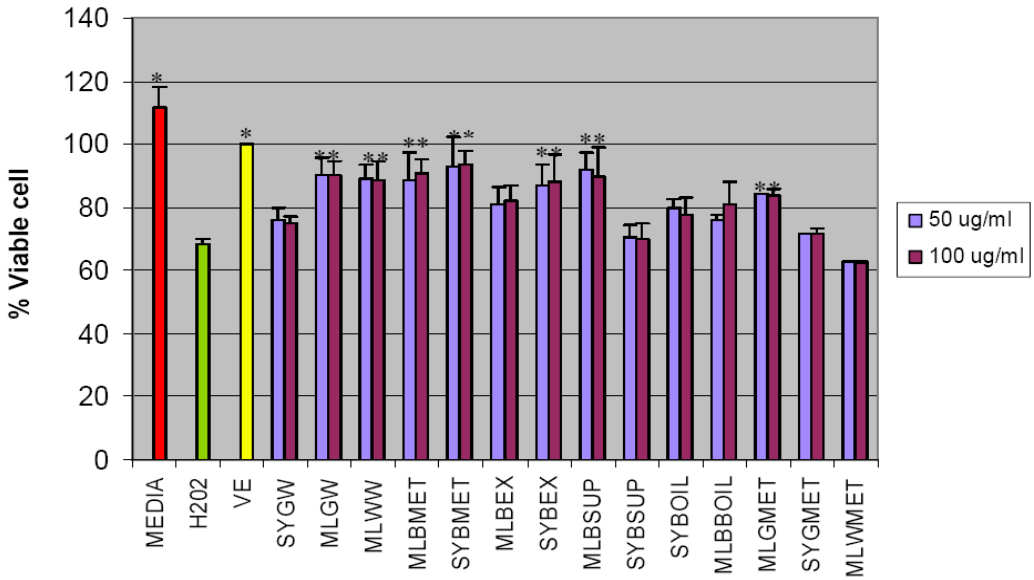

Figure 2. Percentage of viable cells in relative to 100% of vitamin E by MTT assay after treated PC12 cells with each of rice extract for 24 h followed by addition of vitamin E 10 mM for 2 h. The homogenous groups of experiment compared with vitamin E were displayed significantly (* p< 0.05).

Cell protective effect. After exposure of PC12 cells to hydrogen peroxide(H_2O_2) 500 µM for 2 h , survival cells marked decreased from 115 (media or untreated H_2O_2 group) to 68% whereas the group given vitamin E 10 nM 24 h prior to treated with H_2O_2 maintained the number of viable cells as same as the media (untreated H_2O_2) group. At dose of 50 and 100 µg/ml given, rinsed water fraction of ML and MLG (MLWW , MLGW), ethanolic extract of ML, MLGand SY bran (MLBMEt, MLGMEt, SYBMEt), supercritical extract from ML bran (MLBSUP), and cold-press of SY bran (SYBEX) could rescue cells from

oxidative stress induced by hydrogen peroxide. The number of viable cells in these mentioned groups were homogenously in range of 83 to 92 % of vitamin E ($p< 0.05$)

Discussion

After exposure each of 14 fractions of rice extract at dose of 100 µg/ml to PC12 cells in complete media, no cytotoxic effect were found. At dose of 50 µg/ml, cell proliferation was founded in 3 water-soluble fractions from SY, MLG, MLW and ethanolic extract from ML bran which may be due to hydrophilic active principle in rice that could be rinsed off. When cultured PC12 with each of these fractions in differntiative media (only supplemented with % 1 FBS, % 5 HS, and nerve growth factor 2 ng/ml), cells did not proliferate but produced slightly neurite outgrowth, belbing cell and lysis cells (data not shown). These results demonstrated that rice extracts had no cytotoxic on neuronal cell in normal condition.

The 7 fractions of rice extract that provided significant protection to the PC12 cell from H_2O_2–induced injury were 2 water-soluble fractions: MLWW, MLGW and 5 water-non- soluble fractions :MLBMEt, MLGMEt SYBMEt, MLBSUP, and SYBEX. The water-soluble fractions might composed of beta-glucan or hydrophilic antioxidant (8) whereas the water non-soluble fractions might composed of oryzanols and vitamin E in different ratio.

Conclusion

The present findings indicated that 7 rice extract fractions exert neuroprotective effects against H_2O_2 toxicity, which might be potential candidates for use as nutraceutical for neurodegenerative diseases.

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Original article **P14**

The screening of bacteria with antimalarial activity, a novel alternative to drugs

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Abstract

Malaria remains one of the important human diseases in the world with 1-2 million deaths annually. An increasing in drug resistance of malaria parasites particularly, *P. falciparum*, to available drugs has led to a novel drug development. Antimalarial drug development can follow several strategies, ranging from modifications of existing agents to the design of novel agents that act against new targets. Several compounds isolated from natural products including plant and microorganisms such as bacteria and fungi will be a rich source many bioactive substances with diverse structures for optimization to obtain better therapeutic agents. The objective of this work is to find a new compound from bacterial origin to combat against drug resistant malaria parasite, *P. falciparum*. The bacterial extract prepared from two isolated bacteria with anti-MRSA activity (strain SOPB1 and WARY7-4) show inhibitory activity toward chloroquine resistant malaria strain K1 as shown by a decrease up to 50% in the parasite number at every concentration used. Moreover, these two substances also inhibit the parasite development and reinfection as shown by pyknotic formation and died parasite at the end of incubation. To our knowledge, this work is the first report on the antimalarial activity of bacterial extract. These two bacterial extracts are promising target used for treatment of drug resistance malaria *P. falciparum* and might be exploited as a novel drug for mono- or combination therapy for malaria in the future.

Keywords: bacteria, antimalarial activity, screening

Introduction

Malaria remains one of the most important diseases of man with over half the world's population at risk of infection and 1-2 million deaths annually. There are four different species of human malaria parasites namely, *Plasmodium falciparum*, *P. vivax*, *P. malaria*, and *P. ovale*. *P. falciparum* is the most dangerous malaria species as it often leads to the death and can be fatal within 2-3 hours of the first symptom (Hyde *et al.*, 2002). A major contributor to malarial morbidity and mortality is certainly the increasing resistance of malaria parasites to available drugs (Olliaro and Bloland, 2001). Resistance is primarily seen in *Plasmodium falciparum*, the most virulent human malaria parasite. Considering increasing resistance to available agents, there is broad consensus that there is a need to develop new antimalarial drugs (Ridley, 2002). Antimalarial drug development can follow several strategies, ranging from modifications of existing agents to the design of novel agents that act against new targets (Rosenthal 2003). Nature remains a rich source for compounds for medical applications. Several compounds isolated from nature including plant and microorganisms such as bacteria and fungi will be a rich source many bioactive substances with diverse structures for optimization to obtain better therapeutic agents.

The objective of this work is to find a new compound from bacterial origin to combat against drug resistant malaria parasite, *P. falciparum*.

Methods

Sensitivities of *P. falciparum* strain K1 used in this study to chloroquine, quinine, mefloquine and artesunate were investigated by an *in vitro* drug sensitivity assay based on the incorporation of [^{3}H] hypoxanthine into parasite nucleic acids or radioisotopic technique (Desjardins *et al.*, 1979). Two selected isolated bacteria (strain SOPB1 and WARY7-4) with anti-MRSA activity isolated from many areas of Thailand (Aunpad and Na-Bangchang 2007) was used as a source for preparation of bacterial extract to test the activity against chloroquine resistant malaria standard K1 strain. Synthesis of the bacterial extract from isolated bacteria was carried out in Tryptic soy broth (TSB medium, Difco, USA). A 200 ml flask was inoculated with 1% (10^6 CFU/ml) of an overnight culture of each isolate. The cultures were incubated at 37^{o}C for 16-18 hours with shaking at 200 rpm (New Bruchwics, England). The cell culture were collected in a 50-ml falcon tube and stored –20^{o}C for freezing. In order to obtain highest efficiency, the freeze culture cell was concentrated by lyophilization until dryness and this preparation was designated as bacterial extract and tested for antimalarial activity. In order to examine the effect of bacterial extract on malaria, the malaria was cultured with various concentrations of bacterial extracts for 48 hours. The culture were taken at 0, 6, 12, 24 and 48 hour for parasite number counting as a count parasite/10000RBC under microscope and the morphology of malaria was also observed under microscope.

Results

The level of chloroquine and artesunate susceptibility was determined by using the criteria of Pickard and colleague, (Pickard *et al.,* 2003). There was no evidence indicating artesunate resistant so far therefore artesunate susceptibility was not classified. The result shows that strain K1 has mean IC_{50} against chloroquine, quinine, mefloquine and artesunate at 100.50 nM, 314.982 nM, 10.940 nM and 0.424 nM, respectively. The strain K1 was therefore sensitive to quinine, artesunate and mefloquine and resist to chloroquine. The parasite number was decreased from 1 to 0.5 count parasite/10000RBC at 12 hour of incubation at every concentration of bacterial extract from strain SOPB1 (Table 1). The morphology of standard K1 strain grown with bacterial extract strain SOPB1 at 48 hour was different when compared to those of control without bacterial extract as shown in Fig. 1. At high concentration (10 mg/ml and 5mg/ml), the parasite can not develop into trophozoite stage. The parasites developed into early trophozoite stage at lower concentration of bacterial extract (2.5 mg/ml) however they can not reinfect the red blood cell (Fig. 1). At the concentration of 1.25 mg/ml of bacterial extract, the parasite developed into mid trophozoite however they can not reinfect the red blood cell. Interestingly, the parasite grown with bacterial extract strain SOPB1 developed slowly when compare to that of control.

The effect of bacterial extract prepared from strain WARY7-4 toward standard K1 strain was shown in Table 2. At high concentration (10 mg/ml), the parasite developed to ring stage after 6 hour of incubation which was the same as control group however the parasite formed pyknotic at 12 hour of incubation and stopped growing at 24 hour of incubation. At lower concentration (5mg/ml), the parasite developed slowly and died at 24 hour after incubation (Fig. 2).

Discussion

Two isolated bacterial extract strain SOPB1 and WARY7-4 show inhibitory activity toward chloroquine resistant malaria strain K1 as shown by a decreased up to 50% in the parasite number at every concentration used. Moreover, these substances also inhibit the parasite development and reinfection as shown by pyknotic formation and died parasite at the end of incubation.

Table 1 Effect of bacterial extract strain SOPB1 to strain K1

Substance concentration	**Count Parasite/10000RBC**				
	0 hour	**6 hour**	**12 hour**	**24 hour**	**48 hour**
Control	1.0	1.0	1.0	1.0	4.0
10 mg/ml	1.0	1.0	0.5	0.02	not found
5.0 mg/ml	1.0	0.5	0.5	0.03	not found
2.5 mg/ml	1.0	1.0	0.5	0.05	0.02
1.25 mg/ml	1.0	1.0	0.5	0.05	0.02

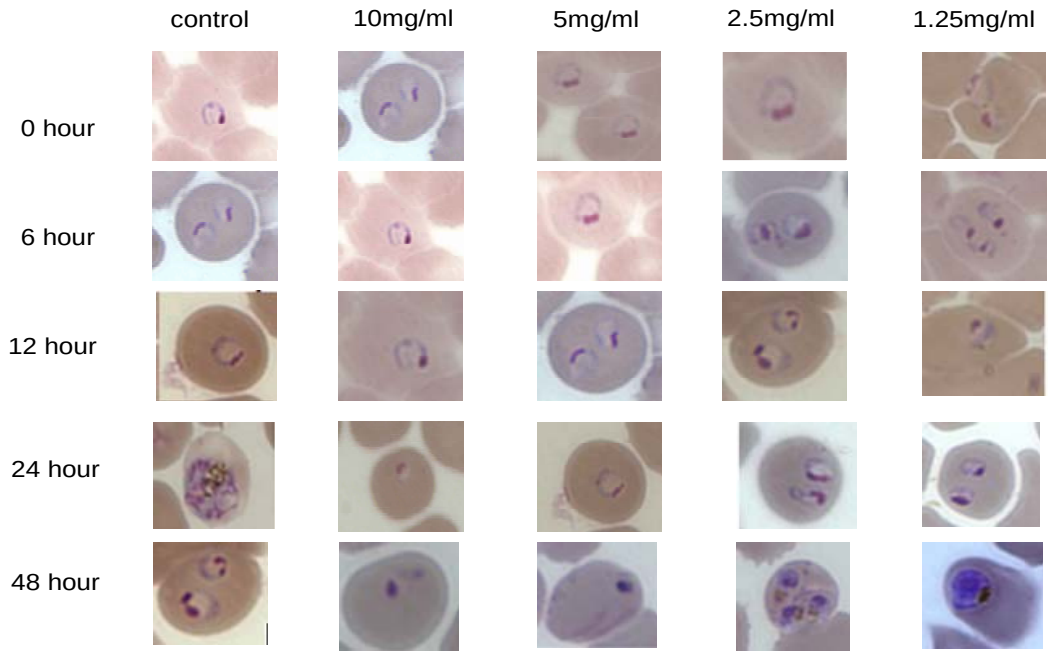

Figure 1 The morphology of standard K1 strain grown with various concentration of bacterial extract strain SOPB1 at different time (0-48 hour).

Table 2 Effect of bacterial extract strain WARY7-4 to strain K1**.**

Substance concentration	**Count Parasite/10000RBC**				
	0 hour	**6 hour**	**12 hour**	**24 hour**	**48 hour**
Control	1.0	1.0	1.0	1.0	4.0
10 mg/ml	1.0	1.0	0.5	0.02	not found
5.0 mg/ml	1.0	0.5	0.5	0.03	not found
2.5 mg/ml	1.0	1.0	0.5	0.7	0.5
1.25 mg/ml	1.0	1.0	0.5	0.6	0.5

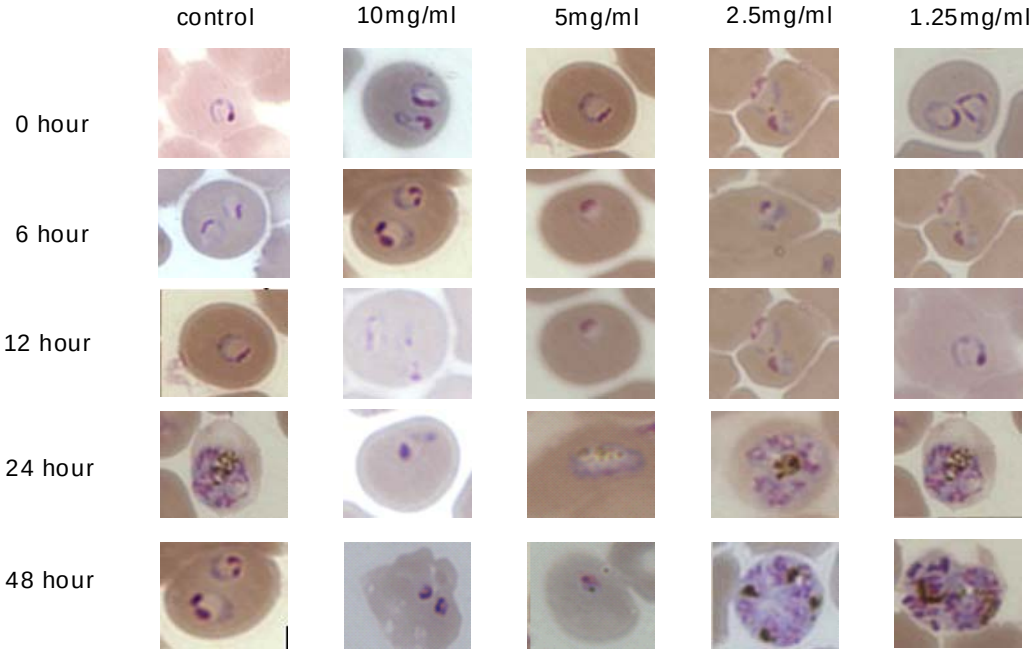

Figure 2 The morphology of standard K1 strain grown with various concentration of bacterial extract strain WARY7-4 at different time (0-48 hour).

Conclusion

It is to our knowledge; this work is the first report on the antimalarial activity of bacterial extract. Those two bacterial extracts are promising target used for treatment of drug resistance malaria *P. falciparum* and might be exploited as a novel drug for mono- or combination therapy for malaria in the future.

Acknowledgements

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Original article **P15**

Cytotoxic effects of the ethanolic extract from Benjakul formula and its compounds on human lung cancer cells

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Abstract

Benjakul have also been used for cancer therapy by folk medicine. The ethanolic extracts, five fractions and three pure compounds from *Benjakul* were investigated for cytotoxic activities against two types of human lung cancer cell lines (COR-L23 and A549) and one normal human lung myofibroblasts (MRC-5). The cytotoxic activities of the *BEN*-3 fraction which fractionated with vacuum liquid chromatography method by chloroform solvent showed stronger cytotoxic activities against COR-L23 and A549 cells than the ethanolic extracts and other fractions (IC_{50} values 28.09 and 34.43 μg/ml, respectively). Its pure compounds, plumbagin exhibited the greatest cytotoxic activity against COR-L23, A549 and MRC5 with IC_{50} values 0.36 , 0.59 and 2.17μg/ml, respectively Piperine and gingerol exhibited cytotoxic activities against CORL23 more than A549. In conclusion, all *Benjakul* extracts shown high cytotoxic activities against COR-L23 but less activities against other cells.

Keywords: cytotoxic activity; Benjakul**;** COR-L23**;** A549**;** MRC-5

Introduction

Benjakul is thai traditional medicine formula that composed of 5 plants, root of *Plumbago indica* Linn. (Plumbaginaceae), root of *Piper sarmentosum* Roxb. (Piperaceae), stem of *Piper interruptum* Opiz. (Piperaceae), fruit of *Piper longum* Linn. (Piperaceae) and rhizome of *Zingiber officinale* Roscoe. (Zingiberaceae). In Southern folk medicine of Thailand, it was used as an adaptogenic drug for cancer patients [1]. Previous work, the ethanolic extract, fractions and three pure compounds that isolated from Benjakul; plumbagin, piperine and 6-gingerol shown cytotoxic activities against human large cell breast carcinoma cell line, MCF-7 [2]. In the present study, we investigated cytotoxic activities of them against human large cell lung carcinoma cell line, COR-L23, human lung adenocarcinoma epithelial cell line, A549 and normal human lung myofibroblasts, MRC-5.

Method

Plant material and preparation of extracts

Five plants of Benjakul formula were purchased from folk doctor (August, 2009). All plants were cleaned immediately of extraneous material and were dried in at 50 °C. The dried powdered plant material of Benjakul (1 kg each) was extracted by maceration with 95% ethanol. The extracts was concentrated by rotary evaporator then was freeze-dried.

Fractionation of ethanol extract of Benjakul

Sixty gram of the ethanolic extract of Benjakul were separated to be fractions using vacuum liquid chomatography by ordering increase polarity of solvents. Five fractions being denoted as BEN-1 (hexane), BEN-2 (hexane:chloroform 1:1), BEN-3 (chloroform), BEN-4 (chloroform:methanol1:1) and BEN-5 (methanol). Plumbagin, gingerol and piperin were isolated from the ethanolic extract of this preparation followed the method of Sakpakdejaroen

thesis [2]. All compounds and extracts were dissolved in DMSO before tesing cytotoxic assay.

Cytotoxicity testing

Two types of lung cancer cells and one normal human lung cell lines were used in the initial screening of the extract, fractions and pure compounds . The lung cancer cell lines were A549 (human small cell lung carcinoma cell line) and COR-L23 (human large cell lung carcinoma cell line), while the normal cell line used was MRC-5 (normal human lung myofibroblasts). The culturing of the cancer cells was as described by Keawpradub *et al*. (1999) [3] while the MRC-5 cells were cultured as described by Itharat *et al*. (2004) [4].

The cells were seeded in 96-well microtiter plates for all the experiments and 100 µl of cell suspension used in each well. The plates were incubated at 37 °C to allow for cell attachment. After 24 h the cells were treated with the extract, fractions or pure compounds. All of them were initially dissolved in either DMSO. 100 µl/well of each concentration was added to the plates in four replicates to obtain final concentrations of 1, 10, 50, 100 µg/ml in the wells for the extract and fractions and 0.05 - 20 µg/ml for pure compounds. 2% of DMSO being used in the solvent control wells. The plates were incubated for 72 h. After that, the medium was removed and the wells were then washed with PBS, and 200 µl of fresh medium were then added. The plates were incubated at 37 °C for a recovery period of 72 h and cell growth was then analysed using the SRB assay [5]. It was carried out as previously described Itharat et al. (2004) [4].

The plates were fixed with 100 µl of ice-cold 40% TCA per well and incubated at 4 °C for 1 h and were washed with cold water (five times). SRB stain (50 µl; 0.4 in 1% acetic acid) was added to each well and left in contact with the cells for 30 min and were washed with 1% acetic acid (five times). The fixed plates were dried at room temperature and 100 µl of 10 mM Tris base pH 10.5 were added to each well to solubilise the dye. The plates were shaken and the absorbance (OD) of each well was read on a Power Wave X plate reader at 492 nm. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations. All experiments were done in three replicates on each plate.

Results and discussion

The results of cytotoxic activity of ethanolic extract, fractions and three pure compounds of Benjakul against three human cell lines (COR-L23, A549 and MRC-5) are shown in table 1.

Table 1. Cytotoxic activity of ethanolic extract, fractions and three pure compounds of Benjakul on three human cell lines.

Cell lines	% yield	IC_{50} (µg/ml)[a]		
		COR-L23	A549	MRC-5
Ethanolic extract	9.11	43.64±4.34	48.96±3.68	60.83±3.59
BEN-1	2.02	38.33±1.66	72.85±2.83	74.67±4.08
BEN-2	2.22	43.69±1.88	76.33±4.37	44.54±2.06
BEN-3	20.73	28.09±0.86	34.43±2.68	32.16±2.43
BEN-4	53.25	43.04±3.10	56.26±5.23	78.26±7.47
BEN-5	16.69	>100	>100	>100

Cell lines	% yield	IC_{50} (µg/ml)[a]		
		COR-L23	**A549**	**MRC-5**
Plumbagin	4.18	0.36±0.02	0.59±0.09	2.17±0.77
Piperine	7.81	17.00±0.14	>20	>20
6-Gingerol	0.54	15.91±0.91	>20	>20

[a] IC_{50} values were expressed as the mean ± S.D., determined from the results of SRB assay in three replicates.

Following the criteria for cytotoxic activity of extracts and pure compounds by the National Cancer Institute guidelines (NCI) [6] with IC_{50} values <30 µg/ml and <4 µg/ml, respectively. The ethanolic extract showed cytotoxic activity against two lung cancer cells (COR-L23 and A549) more than normal lung cells with IC_{50} = 43.64 , 48.96 and 60.83 µg/ml, respectively. The cytotoxic activity of the BEN-3 fraction against two human lung cancer cell exhibited higher activity than the ethanolic extract and the other fractions however it showed active against only COR-L23 but it showed IC_{50} less 30 µg/ml (IC_{50}=28.09 µg/ml). For the pure compounds, plumbagin exhibited the greatest cytotoxic activity against COR-L23 and A549 with IC_{50} = 0.36 µg/ml and 0.59 µg/ml, respectively, whereas piperine and 6-gingerol showed cytotoxic activity against COR-L23 stronger than A549, however the effect of them were less than NCI criteria. All s of samples showed less cytotoxic activity against normal cells (MRC-5).

Conclusion

In conclusion, the present study supports using of Benjakul formula for cancer treatment as to be claimed by folk doctors. Plumbagin as its active cytotoxic showed the most activity, so it may develop for chemotherapeutic drug in the future.

Acknowledgements

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Original article **P16**

Cytotoxic activity of Hommali 105 rice bran extract against human cancer cells

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Abstract

The objectives of this research are the investigation for the cytotoxic activity against cancer cells of *Hommali 105* rice bran (*Oryza sativa*) extracts by seven difference extraction methods. The methods of extraction were maceration by 95% ethanol, supercritical fluid extraction, boiling in water, freeze dry expression method and Soxhlet extraction by using different solvent such as hexane, chloroform and methanol. The highest percentage of yield derived from the water extract was 21.7. Cytotoxic activities screening by the SRB assay were carried out against four human cancer cell lines: lung (CORL23), cervical (Hela), prostate (PC3) and breast (MCF-7) and normal human cells line (MRC5). The results found that the chloroform extract showed the highest inhibition against prostate cancer cells, followed by cervical and breast cancer cells (46.9%, 29.6% and 21.58%, respectively), but it had no cytotxic activities against both lung cancer and normal cells . This extract is also selective cytotoxic activities against only human cancer cells depend on hormone such as prostate (PC3) cervical (Hela) and breast (MCF-7) cells.

Keywords: Hommali 105 rice bran , *Oryza sativa* , the extraction, cytotoxicity, SRB

Introduction

Cancer is the first leading cause of death of Thai people nowadays. It is suffer for treatment cancer patients by chemotherapy which can kill both cancer and normal cells. Thus the research try to find out drug which can kill cancer cells but not show cytotocic activity with normal cells. Plants were used to treat cancer for long time ago and it was isolated acive cytotoxic compounds and derived them to against cancer cell such as pacitaxel from Pacific Yew tree. Two types of hydroxy acids were isolated from water extract from rice bran [(10*E*, 12*Z*)-9-hydroxy-10,12-octadecadienoic acid [A] and (9*Z*, 11*E*)-13-hydroxy-9,11-octadecadienoic acid [B]], exhibited cytotoxic against P388 mouse leukemia cells The cytotoxicity of acid A was stronger than that of acid B . [(1)]. From this research make to continuous research about rice bran extract to use to treat cancer cells because rice bran is popular use for health food in Thailand and rice is a main food of Thai people long time ago. Rice bran is prepared as several product for health tonic. Its powder has previose reports as high nutritive food and used for reducing blood cholesterol, antioxidative properties, decreasing the incidence of atherosclerosis disease and having laxative effect. The components of rice bran composed with sterol , gamma-oryzanol, tocopherols, tocotrienols and phenolic compound [(2)]. In Thailand the best rice and popular is Hommali rice and the type is 105. Thus, the objective of investigation is to test cytotoxic activity against four types of human cancer cells of rice bran extract which there is no previous report for cytotoxic study against cancer cell from rice bran extract. Type of the rice bran is homali 105 as the most popular use in Thailand.

Methods

Plant materials and extraction methods

Hommali 105 rice bran used in this study was collected from Surin province, Thailand. Five exraction methods are maceration by ethanol 95%, supercritical fluid extraction, boiling in water extraction and dry by frreeze dryer , expressed extraction method and soxhlet extraction method by using solvent hexane, chloroform and methanol. We got seven extract for cytotoxic testing The percentage of yield showed in table 1

In vitro cytotoxicity testing and SRB assay

The cytotoxicity assay was carried out using sulphorhodamine B (SRB) assay (2). The target cell lines were four type of human cancer cells i.e. lung (COR-L23), prostate (PC3), breast (MCF-7), and cervical (Hela) cancer cell lines and one type of human normal lung cell (MRC5). The 100μg/ml of seven different extracts were tested against all cell lines by SRB assay. The culturing of the cancer cells was as described by Keawpradub *et al*. (1999) [3] and Itharat *et al*. (2004) [4] while the MRC-5 cells were cultured as described by Itharat *et al*. (2004) [4]. The active plants extracts were diluted and tested for calculating IC_{50}.

The monolayered culture of each cell line in a 96-well microtiter plate was treated with each plant extracts for 6 replications. The plates were incubated for an exposure time of 72 hours, then the medium was removed and washed. The plates were incubated for a recovery period of 6 days. The percentage of toxicity was measured colorimetrically using SRB assay and IC_{50} values was calculated by means of Prism program.

Results and discussions

Boiling in water method and dry by freeze dired showed the highest of percentage of yield as 21.73 % and the second is from Supercritical fluid extraction is 8.57%.

Table 1 percentage of yield of rice bran from Hommali 105 extract on different extraction methods

	Code of extract	Method of extraction	% Yield
1	ME	Maceration with ethanol 95%	3.09
2	SUP	Supercritical extraction	8.57
3	BOIL	Boiling in water , filtrate and dry by freeze dryer	21.73
4	EX	Expression	7.00
5	SOXME	Soxhlet extraction by hexane	6.58
6	SOXCH	Soxhlet extraction by chloroform	1.09
7	SOXHE	Soxhlet extraction by methanol	6.58

The results of cytotoxic activity screening showed in table 2 . It was found that soxhlet extraction by chloroform extract showed the highest percentage of inhibition against prostate cancer cells , followed by cervical and breast cancer cells (46.9, 29.6 and 21.58 respectively) but it had no cytotxic activity against both lung cancer and normal cells . This extraction method also is selective cytotoxic activity against only human cancer cells depend on hormone such as prostate (PC3) cervical (hela) and breast (MCF-7) cells

Table 2 Percentage of cytotoxic activity (% Toxicity ± SD)or inhibition of growth all types of cell lines

Extraction methods	CORL-23	Hela	PC3	MCF7	MRC5
MEt	12.05 ± 0.23	-0.21 ± 0.02	-11.95 ± 0.04	**20.53± 0.03**	-19.84±0.03
SUP	**31.14 ± 0.46**	5.54 ± 0.08	2.33 ± 0.04	15.79±0.01	-18.65±0.02
BOIL	**13.04 ± 0.01**	-7.71 ± 0.07	-2.69 ± 0.02	8.70±0.03	-6.55±0.03
EX	8.84 ± 0.20	-2.77 ± 0.07	5.83 ± 0.04	6.84±0.02	-26.98±0.03
SOXHE	0.31 ± 0.04	3.80 ± 0.05	9.62 ± 0.02	15.26±0.02	-28.17±0.02
SOXCH	-10.22 ± 0.08	**29.57 ± 0.05**	**46.94 ± 0.04**	**21.58±0.01**	-8.73±0.04
SOXME	-0.78± 0.09	-2.77 ± 0.05	-4.96 ± 0.05	10.00±0.00	-7.94±0.05

The supercritical fluid extract of rice bran showed also selective cytotoxic against lung and breast cancer cell , less active for cervical and prostate (31.1, 15.8, 5.5 and 2.3% respectively) but this extraction method made normal cells proliferate growth. It is good thing to used this extract for promoting to used in lung cancer patients because it can kill lung cancer but not kill lung normal cells. In addition to macearation method showed also selective cytotoxic against breast and lung cancer cells but it promote growing or proliferation prostate and cervical cell growth.

The rice bran oil from expression showed proliferation normal cell but less than soxhlet extraction by hexane. Characteristic of two extract are oil it showed cytotoxic activity against cancer cell but proliferate normal cells so they should be promoting using rice bran oil in cancer patients. Free fatty acid in these oil should be continuous studied because the previous report (1) found that free fatty acid exhibited cytotoxic against P388 mouse leukemia cells

However, all of method showed less cytotoxic against all type of cancer cell because IC50 value are more than 100 μg/ml The benefit of this study showed that the extraction method make cytotocic against cancer cell difference. Thus, the methods for preparing rice bran to be product for cancer will be chosen appropriately with type of cancer cells such as the soxhlet extraction by chloroform appropriate with prostate and cervical cancer cells , supercritical fluid extraction is the best for lung cancer cells. Rice bran oil from soxlhlet extraction by hexane and expression are good for promote lung normal cell growth

Conclusion

The highest of percentage of yield is water extract by boiling method .The soxhlet extraction by chloroform extract showed the highest percentage of inhibition against prostate cancer cells , followed by cervical and breast cancer cells but it had no cytotxic activity against both lung cancer and normal cells . This extraction method also is selective cytotoxic activity against only human cancer cells depend on hormone such as prostate (PC3) cervical (hela) and breast (MCF-7) cells

Acknowledgements

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Original article **P17**

Anti-tumor activity of *Micromelum hirsutum* extract

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Abstract

We investigated anti-tumor activity of the dichloromethane extract from branches of *Micromelum hirsutum* on human B lymphoma cells, Ramos. The extract exhibited Ramos cell cytotoxicity in a concentration-dependent manner with IC50 41.88 µg/ml at 24 h exposure. It induced cell death mainly by apoptosis at 12 h exposure. This apoptotic induction activity of the extract was markedly mediated by caspase activation. The extract induced Ramos cell accumulation at S and G2/M phase of the cell cycle. Our result demonstrated that the dichloromethane extract from branches of *M. hirsutum* has anti-tumor potential against B lymphoma.

Keywords: *Micromelum hirsutum*, cytotoxicity, apoptosis, cell cycle

Introduction

Micromelum hirsutum is a member of Rutaceae family. Plants in genus *Micromelum* are known to have carbazole alkaloids as parts of their active compounds. Mahanine is the well known carbazole alkaloid which is a major constituent in *M. minutum*. It exhibits many pharmacological activities including anti-mutagenic, antimicrobial, anti-oxidants, anti-inflammatory and anti-tumor activities (Ramsewak *et al*., 1999; Tachibana *et al*., 2001; Nakahara *et al*., 2002; Roy *et al.,* 2004, 2005). It has been identified that the dichloromethane extract of the stem bark of *M. hirsutum* contains six carbazole alkaloids and one lactone derivative of oleic acid. There are one new carbazoles micromeline and five known carbazoles, lansine, 3-methylcarbazole, methyl carbazole-3-carboxylate, 3-formylcarbazole, and 3-formyl-6-methoxycarbazole. The lactone derivative of oleic acid is micromolide. This extract has been demonstrated to exhibit anti-tuberculosis activity (Ma *et al*., 2000). All of these compounds have no reported on anti-tumor activities.

Methods

Plant extract: The precipitated from methanol extract from branches of *M. hirsutum* was partitioned in hexane. The precipitate from hexane was further partitioned in dichloromethane (CH_2Cl_2). The CH_2Cl_2 extract was dissolved in dimethyl sulfoxide (DMSO) and further diluted to various final concentrations at a constant concentration of DMSO (0.5 %).

Cells: Human B-Lymphoma cells, Ramos cells were from ATCC. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS, 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37^{o}C in a humidified atmosphere of 5% CO_2.

Chemicals: Annexin V apoptosis detection kit was obtained from Santa Cruz Biotechnology, USA. The caspase inhibitor Z-VAD-FMK was from Promega, USA. Etoposide was obtained from Ebewe Pharma, Austria. Other chemicals were obtained from Sigma, Germany.

Cytotoxicity testing

Ramos cell at $1x10^6$ cells/ml were treated with CH_2Cl_2 extract from branches of *M. hirsutum* at the concentrations 6.25-100 µg/ml for 24 h. Viability of the treated cells were detected by staining with 1 mg/ml resazurin. Resorufin product in viable cells was determined by microplate reader at 570 and 600 nm. The percentage of cytotoxicity of the extract was calculated by comparing to the untreated control.

Determination of apoptosis

Ramos cells at $1x10^6$ cells/ml were treated with 25, 50 and 100 µg/ml extract for 12 h. The treated cells were stained annexin V-FITC and propidium iodide (PI) and the patterns of cell death were determined by fluorescence flow cytometer. Apoptotic cells were detected as annexin V-FITC positive cells which expose phosphatidylserine (PS) on the outer cell membrane. 0.5% DMSO solution was used as the negative control.

The caspase dependency of apoptotic induction activity of the extract was also determined. Ramos cells were pre-treated with 50 µM pan-caspase inhibitor (Z-VAD-FMK) for 1 h before adding the extract. The treated cells were performed as the above.

Cell cycle analysis

Ramos cells at $1x10^6$ cells/ml were treated with 25, 50 and 100 µg/ml extract for 1.5 h. The treated cells were washed twice with RPMI 1640 medium and further incubated in fresh complete RPMI 1640 medium for 48 h. The cells were collected, fixed with cold 70% ethanol, treated with 4 mg/ml RNaseA and finally stained with 0.05 µg/ml PI. The DNA content of the fixed cells was determined by fluorescence flow cytometry.

Statistical analysis

All assays were perform in at least three independent experiment (n=3). The data were presented as mean ± S.E. Data analysis was performed on SPSS 17.0. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. The p-value < 0.05 was considered statistically significance.

Results

Cytotoxicity of the extract

The extract had cytotoxicity on Ramos cell in a concentration dependent manner with IC50 at 41.88 µg/ml after 24 h exposure (Fig.1).

Apoptotic induction activity of the extract

The extract at the concentration of 25, 50 and 100 µg/ml caused Ramos cell death mainly by apoptosis (Fig. 2). It induced apoptosis in a concentration-dependent manner. This apoptotic activity of the extract was markedly mediated by caspase activation. The pan caspase inhibitor Z-VAD-FMK inhibited the extract-induced Ramos cell apoptosis at all concentrations of the extract used in the study (Fig. 3).

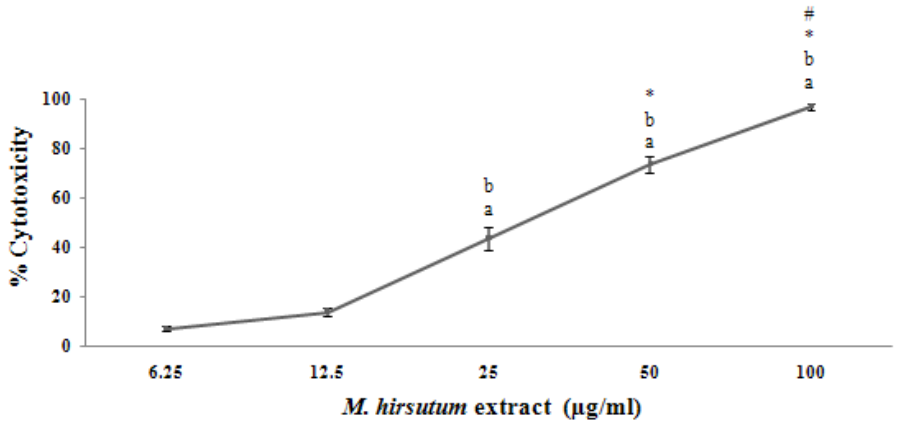

Figure 1: Cytotoxicity of the dichloromethane extract from branches of *M. hirsutum* on Ramos cells. The cells were treated with 6.25, 12.5, 25, 50 and 100 µg/ml for 24 hours. Viable cells were detected by staining with resazurin. The percentage of cytotoxicity was calculated by comparing with the untreated control. The data are expressed as mean ± S.E. of four independent experiments (n=4).
a $p<0.05$ compared to 6.25 µg/ml extract, b $p<0.05$ compared to 12.5 µg/ml extract, * $p<0.05$ compared 50 and 100 µg/ml extract to 25µg/ml extract, # $p<0.05$ compared between 50 and 100 µg/ml extract.

Effect of the extract on the cell cycle

The extract had influence on the cell cycle of Ramos cells. It reduced the proportion of cells in G1 phase but the proportion of the treated cells was increase in S and G2/M phase (Fig. 4). These effects were clearly observed at 25 and 50 µg/ml extract. The 100 µg/ml extract-treated cells were not being able to detect the cell cycle in this study. One µg/ml etoposide induced cell accumulation in G2/M phase.

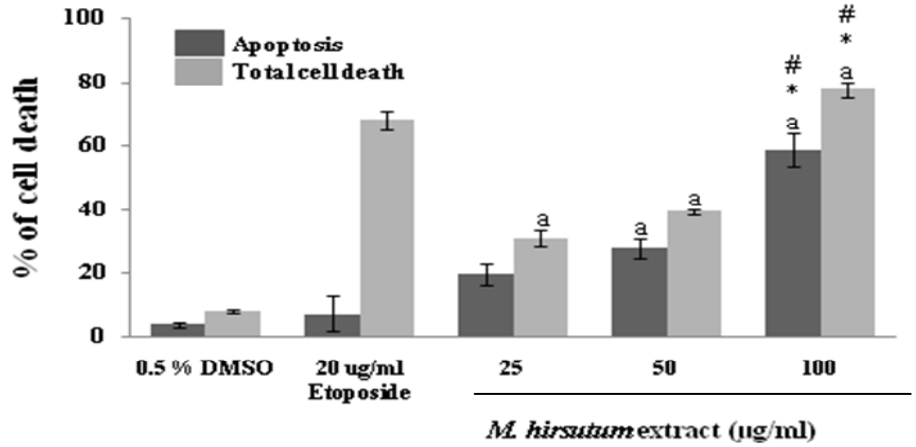

Figure 2: Effect of dichloromethane extract from branches of *M. hirsutum* on Ramos cell apoptosis. The cells were treated with 25, 50 and 100 µg/ml extract for 12 h. The treated cells were stained with annexin V-FITC and PI and the patterns of cell death were determined by fluorescence flow cytometer. Annexin V-FITC positive cells were determined as apoptotic cells. The data are represented as mean ± S.E. of three independent experiments (n=3).
a $p<0.05$ compared to 0.5% DMSO, * $p<0.05$ compared to 25 µg/ml extract, # $p<0.05$ compared between 50 and 100 µg/ml of extract.

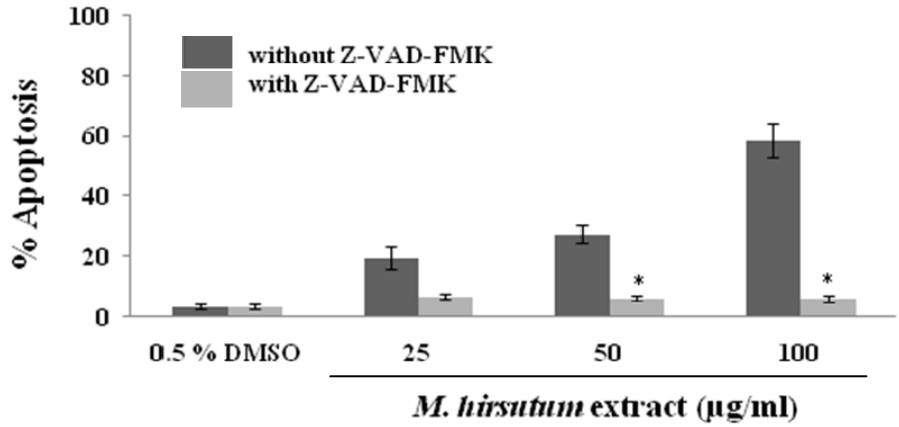

Figure 3: Effect of caspase inhibitor on the extract-induced Ramos cell apoptosis. The cell were pre-treated with or without Z-VAD-FMK for 1 h and then treated with 25, 50 and 100 µg/ml extract for 12 h. The percentage of apoptotic cells were determined by staining with annexin V-FITC/PI and detecting with fluorescence flow cytometer. Annexin V-FITC positive cells were determined as apoptotic cells. The data is expressed as mean ± S.E. of three independent experiments (n=3).

* $p<0.05$ compared between with and without Z-VAD-FMK.

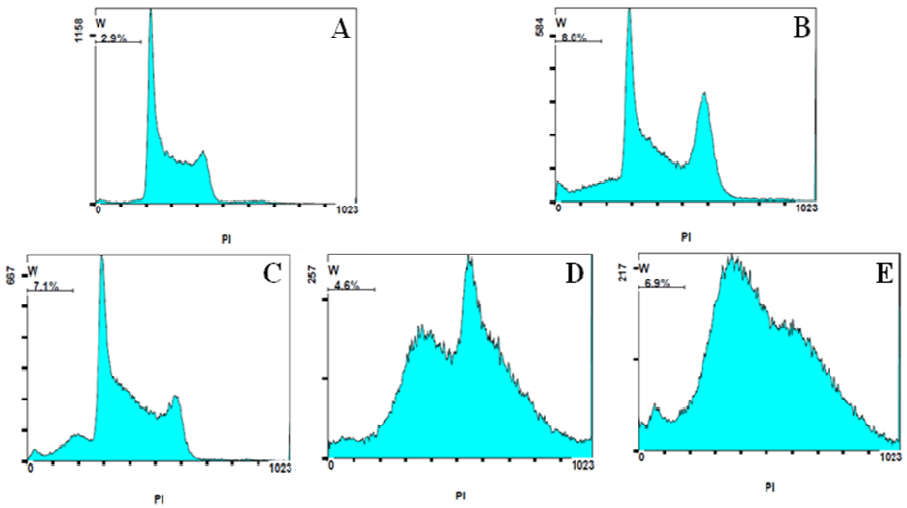

Figure 4: A representative histogram of the effect of dichloromethane extract from branches of *M. hirsutum* on Ramos cell cycle. The cells were treated with 12.5, 25 and 50 µg/ml extract for 1.5 h. The treated cells were washed twice and re-incubated without the extract for 48 h. the cells were fixed, treated with RNase. The cell cycle patterns were determined by fluorescence flow cytometer. (A) 0.5% DMSO; (B) 1 µg/ml etoposide; (C, D, E) 12.5, 25 and 50 µg/ml extract.

Discussion and Conclusion

Plants in genus *Micromelum* contain several carbazole alkaloids which have various pharmacological properties as well as anti-tumor activity. Mahanine is a well known carbazole form *M. minutum* that has been reported to exhibit various pharmacological activitites including anti-oxidant, anti-inflammatory, antimutagenic and anti-tumor activities. Six carbazoles, including new carbazole, micromelin and other five known carbazoles were isolated from the CH_2Cl_2 extract from stem bark of *M. hirsutum*. In this study we investigated anti-tumor effect of the CH_2Cl_2 extract from branches of *M. hirsutum*. The extract had cytotoxic effect on human B lymphoma cells, Ramos, in a concentration-dependent manner with IC50 at 41.88 µg/ml. It induced Ramos cell death mainly by apoptosis (annexin V-FITC positive). The apoptotic effect of the extract was mediated by caspase activation. A pan

caspase inhibitor Z-VAD-FMK, which inhibits caspase 3, 6, 7, 8 and 9 markedly inhibited apoptotic activity of all concentrations of the extract used in the study (25-100 µg/ml). The extract also had effect on the cell cycle of Ramos cells. It induced accumulation of cell in S and G2/M phases. The effects of the extract on Ramos cells cyclins and cyclin-dependent kinase inhibitors (CKIs) are ongoing investigated. These results demonstrated that *M. hirsutum* may contain constituents which are possible candidates for anti-tumor agents.

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Original article **P18**

A High level of nanog-luciferase activity by recombinant Oct-4 protein : toward developmental tool for iPS cell generation

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Abstract

Induced pluripotent stem cells (iPS) technology which enable a generation of cells with human embryonic stem cells (ES) properties from somatic cells provide the possibility for creating various cell types for autologous transplantation in patient. Nevertheless, the problems associated with using retroviruses and oncogenes for reprogramming process need to be resolved before iPS cells can be considered for human therapy. In this study we demonstrate that recombinant protein Oct-4, one of the four key factors for reprogramming, containing protein transduction domain can pass through somatic cell membrane when added to the culture media. Treating mouse embryonic fibroblast with recombinant Oct-4 protein resulted in an activation of nanog-luciferase reporter. These results suggest that the recombinant Oct4 protein is active and may be used to substitute viral vector in iPS cell generation.

Keywords: induced pluripotent stem cells, recombinant Oct-4 protein, protein transduction domain, nanog luciferase activity.

Introduction

Embryonic stem (ES) cells, possess the remarkable property of pluripotency, the ability to give rise to all cells of the organism. With iPS technology it is now possible to generate es-like pluripotent stem cells from somatic cells. Mouse and human iPS cells could be generated from various types of somatic cells by infected with retroviral encoding four transcription factors; Oct4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007; Wernig, Meissner et al. 2007). Human iPS cells could also be generated by a different set of four factors: Oct4, Sox2, Nanog, and LIN28 (Yu, Vodyanik et al. 2007). Potential safety concerns in the present reprogramming protocols still hinder human clinical application, especially the use of retroviral vectors (Yamanaka 2009). To solve this issue, we investigated the possibility of replacing viral vector with recombinant proteins. Previous studies have demonstrated that various proteins such as cre-recombinase, GFP protein can be delivered into cells by conjugating them with a short peptide that mediates protein transduction (PTD). The aim of this project is to develop tool for iPS cells generation based on this technology.

Materials & Methods

The open reading frame of mouse Oct4 was amplified and cloned into the expression vector (Invitrogen). Protein expression plasmid was transfected into host cells using FuGENE HD (Roche). Stably protein-expressed cells were selected, and the recombinant protein was extracted using Protein Extraction Reagent (Pierce, Thermo) Based on 6x-Histidine tagged at C-terminal, recombinant mouse Oct4 protein was purified by affinity chromatography using HisTrap HP (GE Healthcare). For identification of recombinant mouse Oct4 protein, western

blot was used. Briefly, purified protein was separated on 8% SDS-PAGE and blotted onto nitrocellulose membrane (Bio-rad). The blot was blocked with TBST (20 mM Tris-HCl, pH7.6, 136 mM NaCl, and 0.1% Tween-20) containing 5% skim milk and then incubated with Oct3/4 (sc-5279; Santa Cruz Biotechnology) antibody solution at 4°C overnight. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. Signal was detected using Supersignal (Pierce, Thermo). Protein transduction of recombinant protein into cells was examined by immunofluorescence. 10 µg of recombinant mouse Oct-4 protein were added into mouse Neural stem cells (NSC) media and cultured for 6 hours. Then cells were fixed and immunostained using Oct3/4 (sc-5279; Santa Cruz Biotechnology) antibody and Alexa 488-conjugated goat anti-mouse IgG_{2b} (Invitrogen). For luciferase reporter assay, Nanog5P reporter plasmid (addgene) was used in combination with pRL-TR (Promega). All plasmids were transfected into mouse embryonic fibroblast (MEF) using FuGENE HD (Roche). Recombinant Oct-4 protein was added at concentration 10 µg/ml. Dual-Luciferase reporter assay system (Promega) were used according to the manufacturer's protocols. Briefly, Two days after transfection, cells were lysed using 1X passive lysis buffer. Lysates were collected and assayed for luciferase activity by using Centro LB 960 detection system (BERTHOLD).

Results

Purified recombinant mouse Oct-4 protein (rmOct-4) was tested for protein identification using western blot analysis. As a result, single band was detected which correlate to Oct4 protein molecular weight (Figure 1). These data showed purity and specificity of isolated protein. Next, we tested protein transduction efficiency of rmOct-4 by adding 10 µg of rmOct-4 into culture media of mouse neural stem cells (mNSCs). Six hours after transduction, mNSCs were fixed and stained for Oct-4 protein. We found that mNSCs treated rmOct-4 were stained with Oct4 protein in nuclease more than untreated control (Figure 2). This data showed that rmOct-4 protein has ability to pass through nuclear membrane.

Oct-4 protein is essential for maintenance of pluripotentcy in Embryonic stem cells (ESCs). By interaction with SOX2, Oct-4 has been reported to regulate pluripotent specific gene expression, such as Nanog. So, we tested whether rmOct-4 has ability to activate Nanog expression *in vitro* using Luciferase assay. rmOct-4 shown to increase luciferase activity more than 4-folds compare to control. Moreover, combination of SOX2 transfection with rmOct-4 protein enhances luciferase activity to 12 folds. These data demonstrated that rmOct-4 protein can activate Nanog luciferase reporter (Figure3).

Figure 1 Western blot analysis of rmOct-4 protein using Oct3/4 antibody and horseradish peroxidase (HRP)-conjugated secondary antibody.

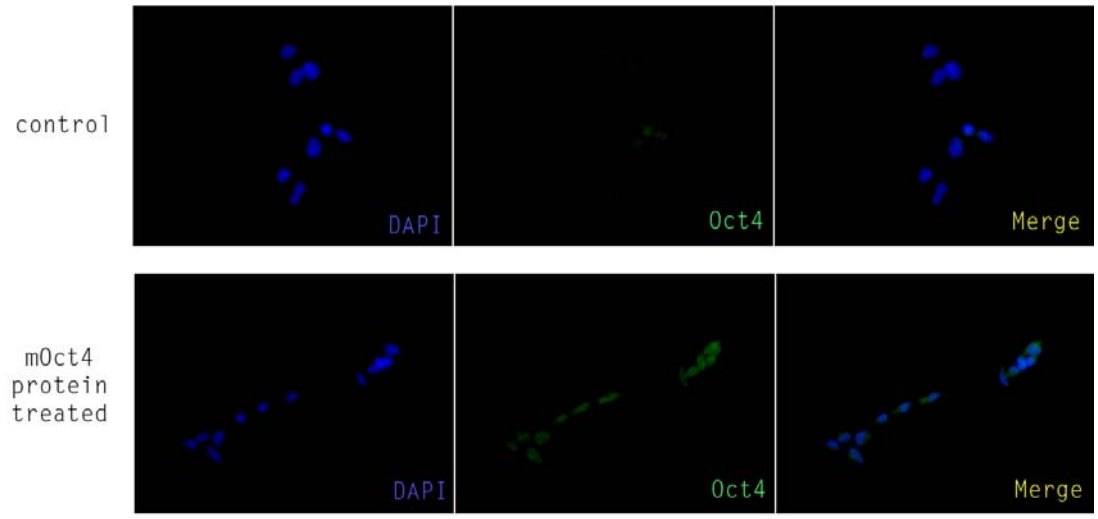

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Figure 2 Protein transduction of rmOct-4 protein into mNSC cells was examined by immunofluorescence. Oct4 (GREEN), DAPI (BLUE) and the images were merged. Magnification: 40X

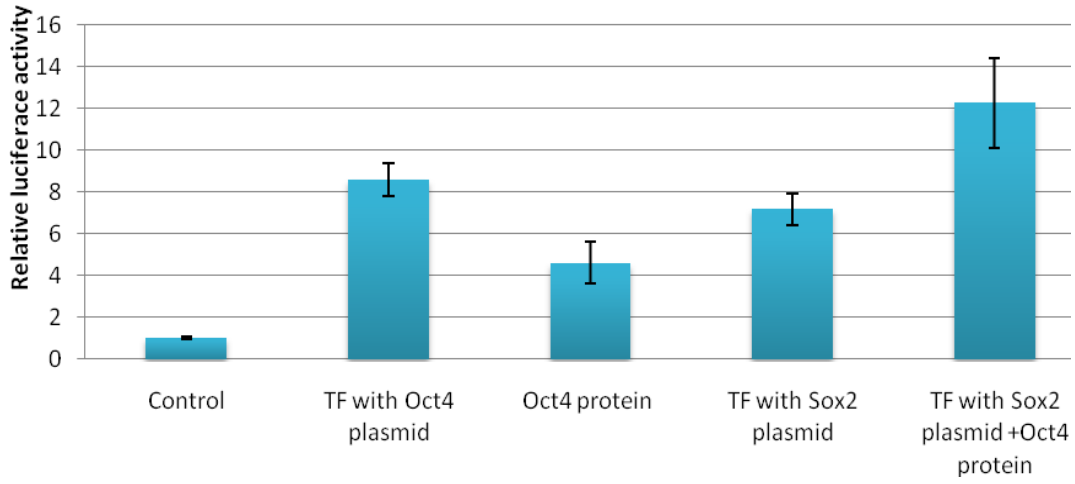

Figure 3 Effect of rmOct-4 protein on luciferase activity in MEF.

Discussion & Conclusion

In this study we show that we have generated a transducible versions of Oct4, a key transcription factor required for pluripotency. Immunofluorescence staining indicated that Oct4-PTD is internalized by cells and can translocate into the nucleus. This recombinant protein, when added to culture media, is not toxic to the cells within the concentration range used in our experiments (10 µg/ml) and can function to activate Oct-4 target genes as shown by nanog reporter assay. Our results suggest that Oct4-PTD may become a useful tool for iPS cells generation. It would provide advantage over those based on gene transfer because it would allow pluripotency genes control without interfering with host cell genome and the protein administration can be stopped when its function is no longer required.

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Original article **P19**

Antioxidant and cytotoxic activities of Thai medicinal plants named Hua-Khao-Yen

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Abstract

Two species of Thai medicinal plants named Hua-Khao-Yen were investigated for their antioxidant and cytotoxic activities against two types of human lung cancer cells (A549 and COR-L23) and human normal lung cells (MRC5). The extract procedures used were similar to those practiced by Thai traditional doctors (water and ethanolic extract). The results found that the ethanolic extract of *Dioscorea membranacea* Pierre (DME) exhibited the highest cytotoxic activities against A549 and COR-L23 cancer cells (IC_{50} values were 15.25±1.36 and 12.63±0.43 μg/ml, respectively), whereas the water extract of *Dioscorea membranacea* (DMW), the water and ethanolic extract of *Smilax corbularia* (SCW and SCE) showed no cytotoxic activity against all cancer cells. SCW, SCE and DME exhibited antioxidant activities by DPPH assay (EC_{50} = 6.40±0.40, 4.20±0.12 and 10.34±1.4 μg/ml, respectively). These results supported using *Dioscorea membranacea* for cancer treatment by Thai traditional doctors.

Keywords: Hua-Khao-Yen, antioxidant, cytotoxic.

Introduction

Thai medicinal plants named Hua-Khao-Yen have mostly been used in Thai traditional medicines preparation for treatment of leprosy, veneral diseases, inflammations, bacterial infections and cancers. Two species of Hua-Khao-Yen including *Dioscorea membranacea* Pierre (Dioscoreaceae) have been possessed potent cytotoxicity against breast cancers with IC_{50} of 11.8±0.75 μg/ml, whereas they were nontoxic to normal cell line (SVK 14) Previous study showed *Dioscorea membranacea* have specific activity against the human large lung carcinoma cell (COR-L23) (Itharat *et al*., 2003 and 2004). *Dioscorea membranacea* also showed high antioxidant activities by DPPH assay (EC_{50} = 12.53 μg/ml). However, no report about antioxidant and cytotoxic activities of *Smilax corbularia* Kunth (Smilacaceae).

The aim of this study was to compare antioxidant and cytotoxic activities of *Dioscorea membranacea* and *Smilax corbularia* extracts against three types of human lung cell i.e. A549, COR-L23 and MRC5.

Methods

Plant material and Preparation of extract

The fresh rhizome of *Dioscorea membranacea* Pierre (Dioscoreaceae) was collected from Chumporn province and the rhizome of *Smilax corbularia* Kunth was collected from Chiangmai province and identified by comparison of authentic herbarium in Department of forestry Bangkok, Thailand where the herbarium vouchers kept.

The plants material were dried at 50°C, powdered and divided into two portions. The first portion (100 g of each plant) was boiled for 30 min in water and the filtrated was freeze dried to obtain water extract of each plant. The second portion (100 g of each plant) was macerated with 95% ethanol and the filtrated was evaporated to dryness under reduced pressure to obtain the ethanolic extract of each plants.

DPPH radical scavenging assay

The following assay procedure was modified from those described by Blois, 1958 and Yamasaki *et al.*, 1994. Samples for testing were dissolved in absolute ethanol or distilled water to obtain the highest concentration of 200 μg/ml. Each sample was further diluted for at least 4 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (100 μl) was mixed with an equal volume of $6x10^{-5}$ M DPPH (in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxytoluene), a well known synthetic antioxidant, was tested in the same system as a positive standard. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The result was expressed as percentage inhibition in the formular below:

$$\%\text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

The EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between %inhibition and concentrations by Prism program.

***In vitro* assay for cytotoxic activity**

The cytotoxicity assay was carried out using sulphorhodamine B (SRB) assay (Skehan *et al*., 1990). Three different types of human cell lines were used i.e. lung adenocarcinoma (A549), large cell lung carcinoma (COR-L23) and normal lung cell (MRC5) (Keawpradub *et al*., 1996). The monolayered culture of each cell line were seeded in 96-well microtiter plate and incubated to allow for cell attachment (18-24 hours). Then treated cell with 4 serial dilution and 6 replications. The plates were incubated for the exposure time at 72 hours, then the medium was removed and added the new medium. The plates were incubated for recovery period of 72 hours. The survival percentage was measured colorimetrically using SRB assay and the IC_{50} values (effective concentration of sample required to inhibit cell growth by 50%) was calculated from dose-response curves plotting between %inhibition and concentrations by Prism program. According to American National Cancer Institute (NCI) guidelines (Suffness and Pezzuto, 1990) crude extract with an IC_{50} values < 30 μg/ml were considered active.

Results and discussion

Water and ethanolic extracts from two species of Thai medicinal plants named Hua-Khao-Yen were investigated antioxidant and cytotoxic activities (Table 1, Fig.1 and Fig. 2). The results indicated that the ethanolic extract of *Smilax corbularia* possessed the highest antioxidant activity by DPPH assay with an EC_{50} value of 4.2 ± 0.12 μg/ml, followed by the water extract of *Smilax corbularia* and the ethanolic extract of *Dioscorea membranacea* (EC_{50} = 6.4 ± 0.4, 10.34 ± 1.4 μg/ml respectively). The water extract of *Dioscorea membranacea* were apparently inactive (EC_{50} > 50μg/ml). Among four crude extracts were tested, only the ethanolic extract of *Dioscorea membranacea* showed appreciable activity against A549 and COR-L23 with IC_{50} = 15.25 ± 1.36 and 12.63 ± 0.43 μg/ml, respectively).

Table 1 Percentage of yield, antioxidant activity (DPPH assay) (μg/ml) and cytotoxic activity (SRB assay) (μg/ml) against two type of lung cancer cells (A549 and COR-L23) and normal lung cell (MRC5).

Plant extracts	**Code**	**%yield**	**Antioxidant activity (n=3)**	**Cytotoxic activity (n=6) IC_{50}(μg/ml) (%inhibiton at concentration 50 μg/ml)**		
				A549	**COR-L23**	**MRC5**
Dioscorea membranacea (EtOH)	DME	4.25	10.34±1.40	15.25±1.36 (84.95)	12.63±0.43 (90.58)	>50 (52.31)
Dioscorea Membranacea (water)	DMW	24.90	>50	>50 (13.44)	>50 (6.09)	>50 (5.74)
Smilax Corbularia (EtOH)	SCE	12.38	4.20±0.12	>50 (20.14)	>50 (23.22)	>50 (7.52)
Smilax Corbularia (water)	SCW	8.25	6.40±0.40	>50 (10.12)	>50 (9.31)	>50 (5.68)
Butylate hydroxytoluene	BHT	-	12.10±1.20	-	-	-

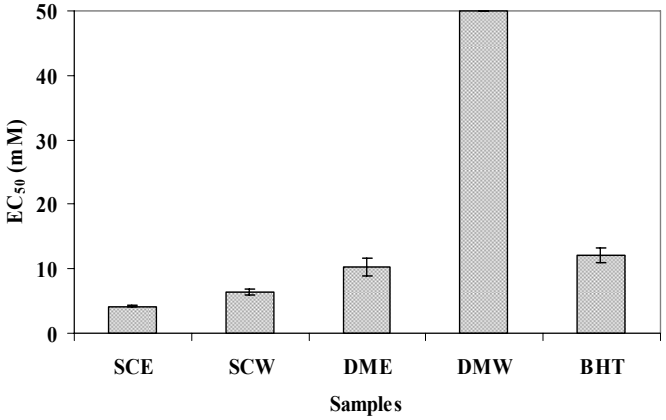

Fig. 1 Antioxidant activity by DPPH assay of Hua-Khao-Yen (EC_{50} (μg/ml)±SEM)**.** SCE = *Smilax corbularia* (EtOH extract), SCW= *Smilax corbularia* (water extract), DME = *Dioscorea membranacea* (EtOH extract), DMW= *Dioscora membranacea* (water extract), BHT = butylated hydroxytoluene.

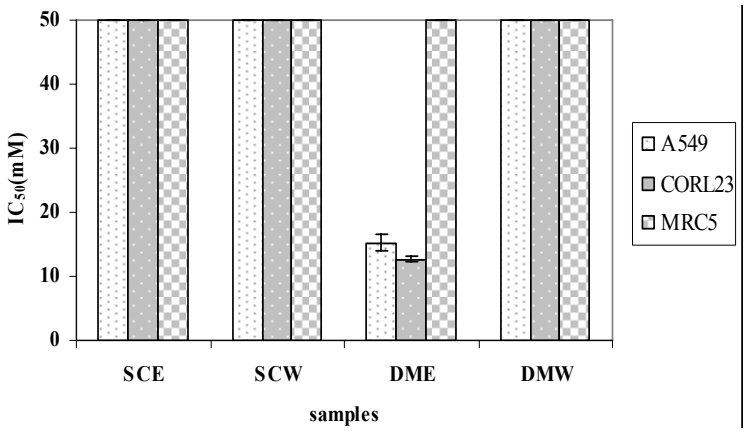

Fig. 2 IC_{50} (μg/ml) of Hua-Khao-Yen against cell lines (n=6) between normal cell (MRC5) and each cancer cell (A549 and COR-L23). SCE = *Smilax corbularia* (EtOH extract), SCW= *Smilax corbularia* (water extract), DME = *Dioscorea membranacea* (EtOH extract), DMW= *Dioscora membranacea* (water extract).

Conclusion

Cytotoxic activity screening of both species extracts by using the SRB assay was carried out against three human cell lines i.e. A549, COR-L23 and MRC5. The results found that the ethanolic extract of *Dioscorea membranacea* exhibited the highest cytotoxic activity against both types of lung cancer cells but no cytotoxic against lung normal cells and also showed high antioxidant activity whereas the water extract of *Dioscorea membranacea* and both extracts of *Smilax corbularia* showed no cytotoxic activity against all cells. However, both extract of *Smilax corbularia* exhibited high antioxidant activity by DPPH assay. This result conclude that *Dioscorea membranacea* showed specific against only cancer and safety for normal cell. *Smilax corbularia* also used in cancer preparation because it showed high antioxidant activity.

Acknowledgements

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Original article **P20**

Antiinflammatory and antimicrobial activities of Thai plant extracts for hemorrhoid treatment

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Abstract

The objective of this research was investigating anti-inflammatory and antimicrobial activities of Thai medicinal plants used to treat hemorrhoid. Inhibitory activity against LPS induced NO production in RAW 264.7 cell lines was used as assay for anti-inflammation. Disc diffusion method and MIC values were evaluated for antimicrobial against gram positive (*Staphylloccus aureus* and *Bacillus subtilis*), gram negative (*Escherichia coli*) and fungi or yeast (*Candida albican*). The extraction method, similar to folk doctors, used maceration in ethanol and boiling in water. Five plants: *Anacyclus pyrethrum* (L.) DC., *Angelica sylvestris* Linn., *Artemisia vulgaris* Linn., *Terminalia chebula* Retz gall and *Picrorrhiza kurroa* Royle. Ex Benth. in hemorrhoid preparation were selected to investigate. The results were found that *Artemisia vulgaris* exhibited the most potent anti-inflammatory activity with an IC_{50} value of 28.09 μg/ml and *Terminalia chebula* Retz gall extract showed the highest antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* with concentration of 0.28 mg/ml, 5 mg/ml and 1 mg/ml respectively.

Keywords: antiinflammatory, nitric oxide, antimicrobial, Thai traditional medicine for hemorrhoid

Introduction

Anacyclus pyrethrum (L.) DC. (AP), *Angelica sylvestris* Linn. (AS), *Artemisia vulgaris* Linn. (AV), *Terminalia chebula* Retz gall (TG) and *Picrorrhiza kurroa* Royle. Ex Benth. (PK) are commonly used for the many formula of Thai traditional medicine and they are ingredients in hemorrhoid preparation. Thus, the investigation of this research focused at antiinflammatory and antimicrobial activities for supporting using these plant in hemorrhoid preparation. The inhibitory against LPS induced Nitric Oxide production in RAW 264.7 cell lines was used as preliminary study of these plant extracts for antiinflammation activity because of nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs and microorganisms cause diverse biological effects. There is no previous report on the antiinflammatory activities of these plants. In addition to antimicrobial was also studied with these plant extracts for hemorrhoid treatment. These results should be used for supporting using these plants as ingredients in hemorrhoid preparation.

Materials and methods

Plant materials

A. sylvestris, *A. pyrethrum*, *A. vulgaris*, *P. kurroa* and *T.chebula* gall were purchased from the Thai herb shop . Plant materials were dried and powdered. The powder was macerated three times with 95% ethanol for 3 days each. The extracts were concentrated under reduced pressure by rotary evaporator.

Antiinflamatory activity by nitric oxide production in RAW 264.7[1]

The murine macrophage cells (Raw 264.7) were cultured in complete medium and incubate at 37 °C in 5% CO_2 unless otherwise stated. The extracts were prepared at a concentration of 10 mg/ml in DMSO as stock solution. After 70–80% confluence, the cells were treated with LPS (10 µg/ml) and extracts were diluted as concentration 3, 10, 30 and 100 µg/ml. The nitric oxide production was determined at 540 nm. Briefly, 100 µl supernatant of samples were pipetted in 96 well plate and put an equal volume of Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide in 5% H_2SO_4)in that 96 well plate and incubated for 10 min at room temperature. The IC_{50} calculated using Prism program. Cytotoxicity testing was also tested to determine that nitric oxide production was not produced by destroy cell membrane. This testing used MTT assay or the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method. The absorbance was determined at 540 nm.

Antimicrobial activity[2,3]

The antimicrobial activities of all plant extracts were determined against *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 90028). The disc diffusion assay (Kirby-Bauer Method) was used to screen for all extracts. Minimal inhibitory concentration was determined as follows: the plant extracts were dissolved in DMSO. The MHB solution was brought for preparing plant extracts to be concentration ranges of 0.156 – 5.0 mg/ml. 100 µl of each concentration was added in a well (96-well plate) containing of 100 µl of MHB and inoculums. All mixtures were incubated at 37°C for 24 hours. MIC values of the plant extracts against bacterial strains were evaluated by resazuin as this minimum dilution after overnight incubation .

Results and Discussion

All ethanolic extracts showed inhibition NO production at concentration 100µg/ml (AP, AS, AV, PK and TC are 49.55, 90.01, 88.71, 73.75 and 90.73 µg/ml respectively), only two extracts such as AV and TG had no cytotoxicity against RAW 256.7 cells and can inhibit NO oxide production more than 50% . They were dilution and evaluated by inhibition nitric oxide production and found that the IC_{50} (n=3) of AV and TG were 28.09±7.02 and 41.94±9.43 µg/ml respectively .It is concluded that AV showed the best inhibitory effect on NO production activity. It can possess that it should be antiinflammatory of hemorrhoid product.

Table 1 Antiinflamatory effect of ethanolic extracts of plants at concentration 100 µg/ml evaluated by % inhibition of Nitric Oxide (NO) production

Extract	Code	%inhibition of NO production (%cytotoxic)				IC_{50} (µg/ml)
		3 µg/ml	10 µg/ml	30 µg/ml	100 µg/ml	
Anacyclus pyrethrum(L.)DC	AP	1.0 (2.0)	3.1 (3.7)	15.5 (3.9)	49.6 (9.1)	-
Angelica sylvestris Linn.	AS	2.2 (13.3)	12.3 (22.6)	35.1 (28.5)	90.0 (46.7)	-
Artemisia vulgaris Linn.	AV	4.8±16.4 (6.7±3.4)	16.4±6.6 (8.9±3.4)	57.2±15.0 (11.6±3.3)	88.7±4.7 (7.7±6.9)	28.09±7.02
Picrorrhiza kurroa Royle. Ex Benth.	PK	0.0 (23.9)	4.5 (42.2)	20.7 (32.9)	73.5 (50.1)	-
Terminalia chebula Retz gall	TG	4.3±1.5 (10.9±6.5)	13.3±3.5 (8.8±2.4)	39.6±6.0 (15.0±6.2)	90.7± 1.8 (16.9±1.4)	41.94±9.43

In the table 2, *Terminalia* gall extract exhibited antimicrobial activity against microorganisms such as *S.aureus, B. subtilis* and *C. albicans.* MIC values were found 281.25 µg/ml 5 mg/ml and 1 mg/ml respectively. It related with the previous report because it contained tannin which can inhibit microbial . *A. sylvestris* can inhibit *S. aureus* and *B. subtilis* (MIC =2.5 , 1 mg/ml respectively). Whereas *P. kurroa* was against *S. aureus* (4.5 mg/ml) and there is no antimicrobial activity from *A. vulgaris* and *A. pyrethrum* extracts.

Table 2 antimicrobial activity of ethanolic extracts ; *A. sylvestris* (AS), *A. pyrethrum* (AP), *A. vulgaris* (AV), *P. kurroa* (PK) and *Terminalia* gall extract (TG) (n=3)

Extract	Inhibition zone(mm.) and MIC (n=3)			
	S.aureus ATCC 25923	*B. subtilis* ATCC 6633	*E.coli* ATCC 25922	*C. albicans* ATCC 90028
AP	-	-	-	-
AS	7.33 ± 0.58 2.5 mg/ml	7.33 ± 0.58 1 mg/ml	-	-
AV	-	-	-	-
PK	11.67 ± 0.58 4.5 mg/ml	8.33 ± 0.58 > 5 mg/ml	-	
TG	16.68 + 0.58 281.25 µg/ml	9.33 + 2.31 5 mg/ml	-	19.33 + 4.04 1 mg/ml

Conclusion

From these results were concluded that the ethanolic extracts *Artemisia vulgaris* was ingredients for antiinflammation of hemorrhoid preparation. *Terminalia chebula* gall was ingredient for antimicrobial activity especially gram positive bacteria cause of wound because of tannin containing in this gall. These results can support using these five plant for relief pain from inflammation and wound from hemorrhoid. However , it should be confirm by another antiinflammation assay such as COXII inhibitor and test all of these plants *in vivo.*

Acknowledgements

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Original article **P21**

Effects of hemin on antimalarial activity of artemisinin and quinoline antimalarial drugs against cultured *Plasmodium falciparum*

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Abstract

Artemisinin and quinoline-containing drugs are important classes of antimalarial drugs. The molecular bases of the mechanisms of action of these drugs have been widely proposed, and one of them involves the interactions of drugs with ferriprotoporphyrin IX (hemin). The influence of hemin on the antimalarial activity of artemisinin and quinoline-containing drugs was studied with TM267R laboratory strain of *Plasmodium falciparum* cultured in human erythrocytes. This study indicates that hemin has a significant synergistic effect only on the activity of artemisinin but has no effect on quinoline-containing drugs.

Keywords: *Plasmodium falciparum*, Hemin, Artemisinin, Quinoline antimalarial drugs

Introduction

Malaria is a vector-borne infectious disease caused by the protozoan *Plasmodium* parasites. Among the five species of human malarias, *Plasmodium falciparum* is the most severe disease and life-threatening disease in the world (1). Quinoline antimalarials are classified into four major subclasses i.e. quinine, 4-aminoquinolines, quinolinemethanols and 8-aminoquinolines. Unfortunately, the efficacy of these drugs has decreased, mainly due to the development and spread of parasite resistance (2). Thus, the development of artemisinins have provided a very important class, which is effective against severe and multiple drug resistant strains of *Plasmodium falciparum.* Nowadays, Artemisinin has also been reported about drugs resistant in *P. falciparum* parasites (3). So, new antimalarial drugs are urgently needed.

Hemin is produced by metabolic breakdown of hemoglobin in the parasite food vacuole. During the red blood cell stage of malaria parasite, it digests host hemoglobin as a food source. Afterward, heme moieties are released and oxidized into hemin. The parasite detoxifies these toxic hemin molecules by polymerizing them into a non toxic complex called hemozoin (4). So, the aim of this study is focused on the effect of hemin on antimalarial potency of artemisinin and quinoline-containing drugs using *in vitro* culture of *Plasmodium falciparum*.

Methods

In these experiments, artemisinin, hemin (ferriprotoporphyrin IX), and four quinoline-containing drugs (quinine, chloroquine, primaquine, and mefloquine) used in this study were purchased from Sigma Chemical CO.

Parasites culture: The *P.falciparum* laboratory strain TM267R which has been maintained in continuous culture was used throughout the study. The parasites with human blood group O erythrocytes were cultured at 37°C in 5% CO_2 + air as previously described (5). Malaria complete medium (MCM) was prepared from stock RPMI 1640 instant medium supplement with 10-15% human heat-inactivated serum.

The effect of hemin on antimalarial drugs activity of standard antimalarial drugs (artemisinin, quinine, chloroquine, primaquine and mefloquine) was assayed in triplicate, in 96-well plates. The parasites at 3-5% parasitemia and 1-3% Hct were used. The concentrations of artemisinin and quinoline-containing drugs were varying from 10^{-4}-10^{-13} M but fixed dose hemin at 10^{-5}-10^{-9} M. After 24 hours incubation, the percentage parasite inhibition in relation to control was calculated to determine 50% inhibitory concentration (IC_{50}) by examining thin smear Giemsa-stained slides. Thin blood film slides from each experimental well were counting the number of parasites in random adjacent microscopic field's equivalent to about 3,000 erythrocytes under oil immersion. Percentage parasitemia was determined.

Statistical analysis: The data were analyzed by GraphPad Prism 5. All data will be presented as mean ± standard error. One-way ANOVA was used to compare the data with the proper match paired. P-value less than 0.05 were considered as significant difference.

Results

The *in vitro* effect of hemin on antimalarial activity of quinoline-containing drugs against *Plasmodium falciparum* (TM267R) was shown in figure 1. IC_{50} of quinoline-containing drugs were not significant different when combined with hemin. This data shown that hemin has no effect on quinine, chloroquine, primaquine, and mefloquine.

We have also designed the *in vitro* study using *P. falciparum* culture to determine the effect of hemin on IC_{50} of artemisinin. The data show that hemin at 10^{-5} and 10^{-6}M decreases IC_{50} of artemisinin significantly (figure 1). The IC_{50} of artemisinin alone is 6.19±0.8 nM. When combined with hemin at concentrations of 10^{-5} and 10^{-6}M, the IC_{50} of artemisinin decreases to 1.07±0.2 and 1.9±0.5 nM, respectively. These indicate that hemin increases artemisinin potency.

Discussion

One hypothesis for the mechanism of action of artemisinin is the reductive activation of its endoperoxide bridge by free heme resulting from the digestion of hemoglobin by *Plasmodium* species (6). This reaction might lead to produced carbon-centered radicals to kill parasite. The previous studies illustrated the interaction of hemin with artemisinin by UV-visible spectroscopy and high performance liquid chromatography/diode array detector/mass Spectrometry (7). It has been shown that artemisinin and its derivatives interacted more strongly with hemin. And our laboratory previous study was done about interactions between hemin and artemisinin using LDL oxidation as a model (8). The result has shown that artemisinin enhances hemin-induced lipid peroxidation. This result indicates that artemisinin is a potent pro-oxidant.

We therefore investigated a possible potentiation of artemisinin activity in infected human erythrocytes by combination with hemin. Our results have shown that combination of artemisinin and hemin produces synergistic effect. Therefore, the activity of artemisinin together with hemin might be involved in free radical reaction that derived from artemisinin molecule. Moreover, hemin had no effect on the activity of quinoline-containing drugs. Indeed, the differences in the activities of these drugs may due to their structural distinctions.

In conclusion, our data suggest an interesting potential about the proposed role of hemin on mechanism of action of artemisinin. These indicate that hemin increases artemisinin potency and may be benefit for coping with the resistance problem. In addition, further studies in an *in vivo* model are essential to generate knowledge on the mechanism of interaction between artemisinin and hemin.

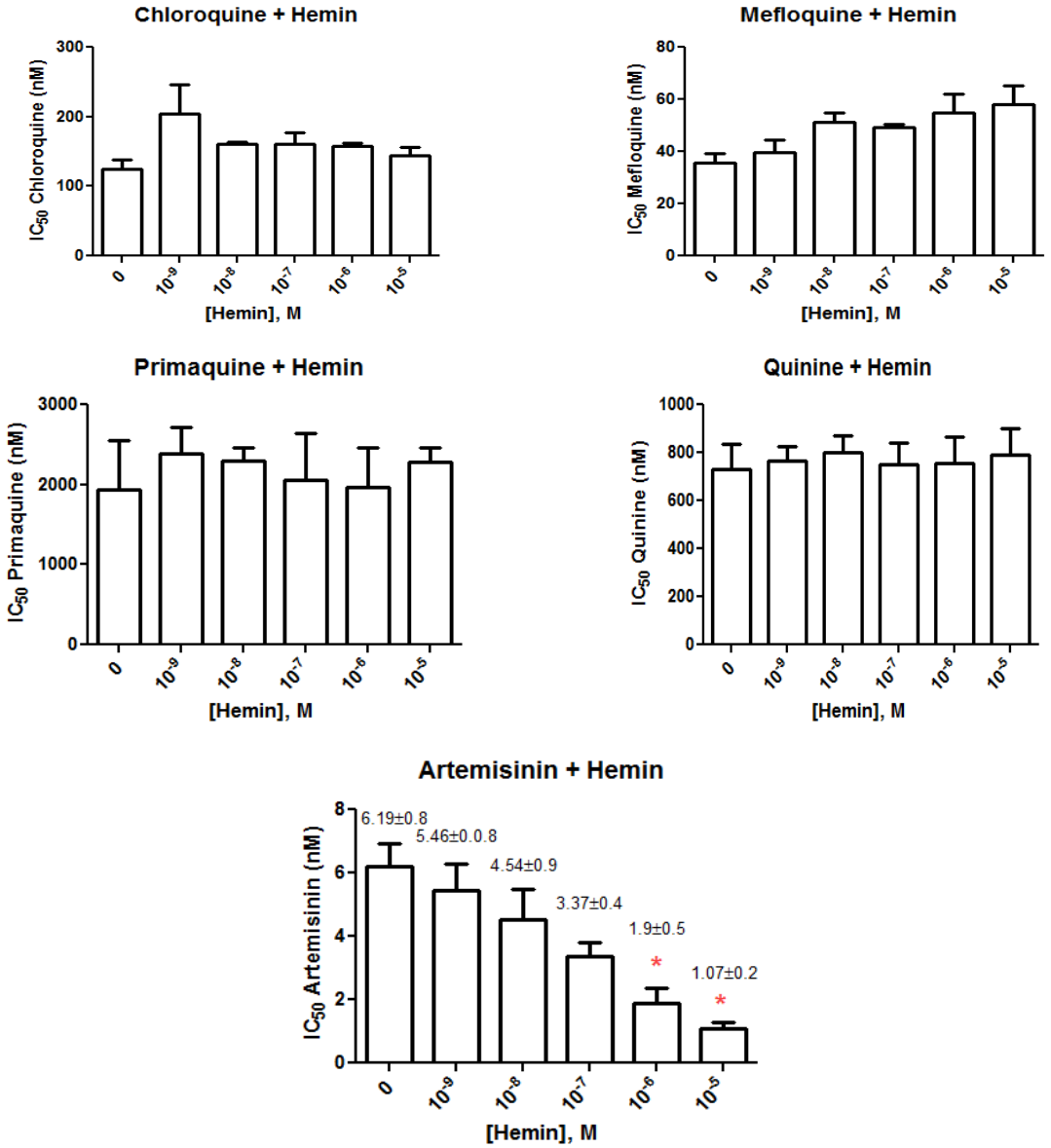

Figure 1 The *in vitro* effect of hemin on Antimalarial activity of artemisinin and quinoline-containing drugs against *Plasmodium falciparum* (TM267R). Values are expressed as mean ± SE (n=3). (* P <0.05).

Conclusion

From this study, hemin enhanced potency of artemisinin but had no effect on antimalarial activity of quinoline-containing drugs on *in vitro* cultured of *Plasmodium falciparum*.

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Original article **P22**

Biological activities of Antidesma thwaitesianum Muell

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Abstract:

Antidesma thwaitesianum Muell is a member of Euphorbiaceae, some parts of it were used to treatment several diseases worldwide but there is no scientific report. The objective of this research is to test biological activity of all parts of this plant extract such as cytotoxic activity against lung cancer cell by SRB assay, antioxidant activity by DPPH assay and antimicrobial activity by disc diffusion method. The results found that the ethanolic extract showed high antioxidant activity because they showed antioxidant activity more than BHT (and wood exhibited the highest antioxidant activity. Antidesma thwaitesianum wood extract also exhibited the highest cytotoxic against lung cancer cells. Its leaves extract showed the highest antimicrobial against *Staphyllococus aureus* and *Candida albican*. In the conclusion, wood of this plant appeared to be the best part used for cancer treatment because it showed both antioxidant and cytotoxic activities and the leaves appeared to be the best part for antimicrobial.

Keywords: *Antidesma thwaitesianum* Muell. cytoxic and antioxidant activities assay.

Introduction

Antidesma thwaitesianum Muell is Thai name Maoluang . Its fruits were use as soft drink and vine in Thaland. Indian and Indonesian like to eat leaves of this plant for food . In Cambodia using its leaves were used as antipyretic, headache and nausea, stem bark used for wound healing in animal and antidiarrhea [1] .The previous report found that root of this plant showed anti HIV-1 integrase [2]. Surprisingly there is no research to investigate cytotoxic activity against cancer cell by comparison all parts of this plant extract. Thus, the objectives of this research were to study on biological activities such as cytotoxic activity against cancer cell, antioxidant and antimicrobial activities. The comparison activities of each part of this plant was also investigated**.**

Methodology

1. Plant Materials

Antidesma thwaitesianum Muell. were collected from Amphor wang-juan, Chonbure Province, Thailand.

2. Preparation of Plant Extracts

The each part of *Antidesma thwaitesianum* Muell. were washed, sliced thinly, dried in an oven at 45°C and made it be powdered. Dried plant material (500 g) were macerated by 1.5 L of 95% ethanol, for 3 days, filtered and dried by using an evaporator. The percentage of yielded were evaluated and showed in table 1.

In vitro assay for Antioxidant activity

DPPH radical scavenging assay [3], pipetted sample solution in each concentration 100 µl in 96-well plate, added DPPH solution 100 µl in each sample and mixed.(Final concentration of sample 100, 50, 10, 1, 0.5 µg/ml). The absorbance (A) was measured at 520

nm. Calculated by formula %inhibition = [(Acontrol – Asample) / Acontrol] × 100 and EC_{50} value calculated by linear regression analysis by prism program.

***In vitro* Assay for Cytotoxic Activity**

The cytotoxicity assay was carried out using sulphorhodamine B (SRB) assay[4]. The target cell lines were human lung cancer cells (COR-L23). the 50 µg/ml of the extracts were tested first against all cell lines by SRB assay and the active plants extracts were diluted and tested for calculating IC_{50}.

The monolayered culture of each cell line in a 96-well microtiter plate was treated with each plant extracts for 6 replications. The plates were incubated for an exposure time of 72 hours, then the medium was removed and washed. The plates were incubated for a recovery period of 6 days. The survival percentage was measured colorimetrically using SRB assay and IC_{50} values was calculated by means of Prism program.

Antimicrobial assay [5,6]

In the preliminary studies, all extracts were evaluated for antibacterial activity by disc diffusion method. All extracts were tested against two types of gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), one type of gram negative bacteria (*Escherichia coli*) and one type of fungi (*Candida albicans*). The active plant extracts were diluted to determine the minimum inhibitory concentration (MIC).

Results:

Table 1 Percentage of yield , cytotoxic activity against lung cancer cells and antioxidant activity of the ethanolic extract of four parts of Antidesma thwaitesianum Muell. (n=3)

Part of plant	% yield	Cytotoxic against CORL23	Antioxidant
		IC_{50} (µg/ml) ±SEM	EC_{50} (µg/ml) ±SEM
leaves	8.40	34.675±2.5	3.88±0.1
Stem bark	8.61	19.320±0.12	3.59±0.5
Wood	7.82	15.411±1.2	2.42±0.12
Root	4.52	22.253±1.9	9.53±0.9
BHT		12.12±1.5	

Table 2 Inhibition zone (mm) ±SD of all part of Antidesma thwaitesianum Muell.extract against four microbial (n=3)

Parts of plant (mg/ml)	Inhibition zone (mm)±SD (n=3)			
	E.coli	*S. aureus*	*B.subtilis*	*C.albican*
leaves	-	17.00± 0.57	-	15.00 ± 0.57
Stem bark	-	10.33 ±0.88	-	-
Wood	-	11.33 ± 0.66	-	-
Root	-	10.00 ±1.15	-	-
Ampicillin (0.003mg/ml)	16±0.0	30.0±0.1	25.1±0.3	*NT*

NT= no test

The results found that the ethanolic extract of all part of this plant extract showed high antioxidant activity and more than BHT and wood exhibited the highest antioxidant activity

(EC_{50}=2.42 µg/ml). Antidesma thwaitesianum wood extract also exhibited the highest cytotoxic against lung cancer cells (IC_{50}=15.4µg/ml). Its leaves extract showed the highest antimicrobial against *Staphyllococus aureus* and *Candida albican*. In the conclusion, wood of this plant was evaluated as the best part for cancer treatment because it showed both antioxidant and cytotoxic activities and leaves is the best for antimicrobial. This result related with using for wound healing (Narod, 2004). Surprisingly, Its root have ever been report that it showed good activity for anti-HIV integrase but it has ever been studied that another part of this plant showed anti HIV. Thus, these studied can conclude that each part should be studied to compare each activity for the highest useful for each activity

Conclusion

In summary from this research can conclude that wood is the best part for using anticancer and antioxidant but leaves is the best part for antimicrobial especially *Staphyllococus aureus*

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Original article **P23**

Study on acute toxicity, anti-oxidant and anticancer activity of ginger in *Opisthorchis viverrini*-carcinogen induced cholangiocarcinoma in hamsters

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Abstract

The study aimed to investigate acute toxicity and anti-cholangiocarcninoma activity of the crude ethanolic extract of ginger (*Zingiber officinale* Roscoe). The extract was resuspended in distilled water before given to hamsters (5 males and 5 females in each group) *via* intragastric gavage. Ethanolic extract of ginger was shown absence of toxicity at the maximum dose of 5,000 mg/kg body weight. The investigation of anti-cholangiocarcinoma activity was carried out in *Opisthorchis viverrini*-dimethylnitrosamine (DMN) induced-cholangiocarcinoma (CCA) hamster model. The crude extract (at the dose of 1,000, 3,000 and 5,000 mg/kg body weight daily or every alternate day for 30 days) was fed to animals at 12 weeks after induction, with confirmation of cholangiocarcinoma by histopathological examination at autopsy. Body weight, food and water consumption were recorded daily. The pathogenesis change was examined by hematoxylin-eosin stained at autopsy. Study is underway to conclude on the anticancer activity of the extract. The anti-oxidant activity was also evaluated using the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay. The ethanolic extract of ginger exhibited moderate anti-oxidant activity with mean (SD) IC_{50} (concentration which produced 50% inhibition of oxidative activity) of 26.68 ± 0.16 μg/ml.

Keywords: cholangiocarcinoma, *Opisthorchis viverrini*, dimethylnitrosamine , anti-cholangiocarcinoma activity, anti-oxidant, acute toxicity, hamsters

Introduction

Cholangiocarcinoma (CCA) is an uncommon adenocarcinoma which arises from the epithelial cells of bile ducts anywhere along intrahepatic and extrahepatic biliary tree excluding the papilla of Vater and the gall bladder (1). *Opisthorchis viverrini* (OV) infection is a high risk factor of cholangiocarcinoma (CCA) (2). Although CCA is a relatively rare cancer worldwide, the highest incidence rate is observed in north-east region of Thailand where the prevalence of infection with OV is also highest (2-3). Thai medicinal plants have been increasingly applied as an alternative treatment for various diseases particularly cancer. The aim of the present study was to study on acute toxicity of the crude ethanolic extract of ginger as well as their anticancer activity in OV-carcinogen induced cholangiocarcinoma in hamsters. In addition, the anti-oxidant activity of the extract was also evaluated.

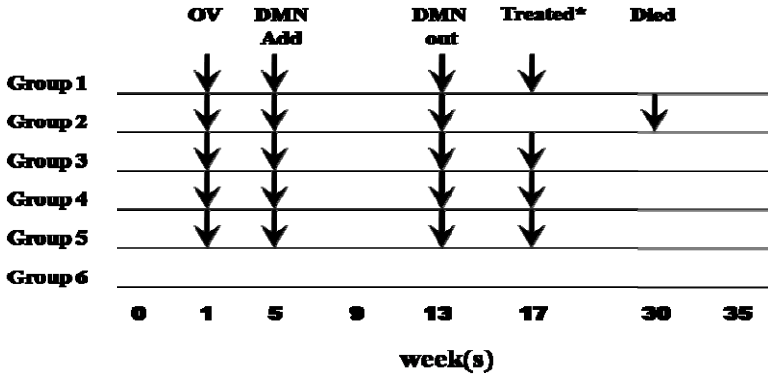

Figure 1. Shematic diagram showing sequences of treatment in each group of hamsters. The arrows (↓) represent the intervention introduced: OV= infection with 50 *Opisthorchis* viv*errini* metacercariae; DMN add = administration of dimethylnitrosamine; DMN out = withdrawal of dimethylnitrosamine; Treated = administration of the ethanolic extract; Died = time of start of death in each hamster

Methods

Acute Toxicity and Anticancer Activity: Syrian golden hamsters (National Laboratory Animal Centre, Thailand), aged 6–8 weeks, weighting 105–120 g were used throughout the experiment. The extract was resuspended in distilled water and given to hamsters *via* intragastric gavage. The acute toxicity (5) of the crude ethanolic extract of ginger was evaluated in a total of 10 (5 males and 5 females) hamsters at the highest dose of 5,000 mg/kg body weight. Distilled water-Tween 80 was given to animals in the control group (5 males and 5 females). Animals were observed individually after dosing at least once during the first 30 minutes, then periodically during the first 24 hours, and thereafter, daily for a total of 14 days (5). At the end of the observational period, the animals were sacrificed under inhalation of ether solution and autopsy was carried out on all the animals. The anticancer activity of the crude ethanolic extract of ginger was evaluated in a total of 80 *Opisthorchis viverrini* (OV)-dimethylnitrosamine (DMN) induced cholangiocarcinoma in hamsters (5 males, 5 females in each group) (Figure 1). The first five groups were OV-infected hamsters, in which 50 metacercariae of OV were fed by intragastric gavage. Four weeks after infection, all were fed with drinking water containing 12.5 *ppm* of DMN daily for 8 weeks (6). Four weeks after DMN withdrawal, hamsters were fed with the ethanolic extract of ginger (prepared in distilled water-Tween 80) at the dose of 1,000, 3,000 and 5,000 mg/kg body weight daily and every alternate day for 30 days (6 groups, 5 males and 5 females each). In the control groups (5 males and 5 females each), animals were treated with distilled water (gastric gavage) and 5-fluorouracil (subcutaneous injection). Body weight, food and water consumption were recorded daily. The progression of CCA was confirmed by pathogenesis changes examined by hematoxylin-eosin stained at autopsy. The study is underway to evaluate the survival time and survival rate (primary endpoint parameters) of the treated compared with control groups.

Anti-oxidant Activity: The anti-oxidant activity was evaluated using the colorimetric free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (7). The decrease in DPPH (scavenging activity) was measured as a reduction in absorbance at the UV wavelength 517 nm.

Results

The ethanolic extract of ginger at the highest dose of 5,000 mg/kg body weight showed no apparent acute toxicity during the observational period. Stomachache and slower movement were however observed during the first administration of the ethanolic extract of

ginger, which lasted until one hour after administration. In the hamsters with OV-carcinogen induced cholangiocarcinoma in all groups, average daily intake of water and food, as well as the average of body weight were similar, but were significantly decreased when compared with the non-induced group (5 males and 5 females). With regards to the anti-oxidant action, the ethanolic extract of ginger exhibited moderate activity with mean (SD) IC_{50} (concentration which produced 50% inhibition of oxidative activity) of 26.68 ± 0.16 μg/ml.

Discussion and Conclusion

The ethanolic extract of ginger is considered non-toxic in the acute toxicity model. The observed disturbance of locomotion may be a result of local irritation of stomach epithelial cells. The progression of chlolangiocarcinoma induced by OV infection in combination with the carcinogen DMN had significant influence on the growth of animals. Study is underway to conclude on the anticancer activity of the extract. The anti-oxidant activity was also evaluated using the free radical DPPH assay and results showed the ethanolic extract of ginger to exhibit moderate anti-oxidant activity. It is noted however that, in a previous study, phenolic extract of ginger was shown to produce more potent activity with IC_{50} of 0.64 μg/ml (8).

Acknowledgement

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Original article **P24**

Anti-pathogenic bacterial activities of fractionated venom of king cobra *(Ophiophagus Hannah*)

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Abstract

This study aimed at measuring the antibacterial activity of the semi-purified fractions isolated from King Cobra venom and investigated the mechanism of action. The protein fractions were isolated from King Cobra venom and designated F1-F7. The F5 showed the highest antibacterial activity. The F5 was further concentrated and separated by Sephadex G-75 column to obtain six fractions (F5.1-F5.6). The antibacterial assay showed that F5.1 could inhibit the growth of gram-positive *S. aureus* and gram-negative *E. coli, S. aeruginosa* and *S.* Typhimurium bacteria. The F5.1 showed antibacterial activity against *S. aureus* with the MIC of 1.8 μg/ml. Its molecular weight was 69 kDa, which was determined by SDS-PAGE. The mechanism of action may be damaging the cytoplasmic membrane, as shown from the scanning electron microscopy. The MIC concentration of F5.1 showed potent cytotoxicity on Human PBMCs cells. The results obtained from this study indicated that the F5.1 had antibacterial activity against the *S. aureus* through membrane damage, it was also cytotoxic to normal human cells. In conclusion, this study provides basic information on antibacterial effect of the semi-purified F5.1 of King cobra venom. The F5.1 should be further purified or modified to obtain the potential antibacterial compound which will be safe to the normal human cells.

Keywords: *Ophiophagus Hannah*, snake venom, fractionated, antibacterial, membrane damage

Introduction

Antibacterial proteins and peptides have been found and isolated from a variety of plants, animals and microorganism. Snake venoms contained a complex mixture of proteins and peptides (90-95%). Antibacterial proteins and peptides were found in the snake venoms, such as L-amino acid oxidase from *Bothrops alternates* (1), PLA_2 myotoxins from *Bothrops asper* snake venoms (2), metalloproteases from *Bothrops jararacussa* (3) and Small peptide from *Naja atra* venom (4). However, King Cobra venom (*Ophiophagus Hannah*) that are rich source of proteins and peptides have a variety of pharmacological activities such as antinociceptive activity, antiplatelet aggregation, anticonvulsant effects and cytotoxicity (5), however the antimicrobial activity has not been investigated.

The aim of this preliminary study was to find the antibacterial activity of fractionated venom of King Cobra against the pathogenic bacteria including both gram-negative and gram-positive bacteria in order to evaluate the potential use of its components as an antibacterial agent.

Materials and Methods

The crude venom of King Cobra was tested for antibacterial properties against the pathogenic bacteria including gram-positive *S. aureus* (ATCC 25923), *S. pyogenes* (ATCC 19615) and gram-negative *E. coli* (ATCC 25922), *S.*Typhimurium (ATCC 25922) and *P. aeruginosa* (ATCC 27853) bacteria by using disc diffusion assay for in vitro susceptibility testing. The F1-F7 obtained from the Snake Venom Research Division, Queen Saovabha Memorial Institute were tested for the antibacterial activity. MIC were determined using microbroth dilution method. The highest active fraction was collected and then purified by Sephadex G-75 column equilibrated and eluted with 20 mM TBS buffer. Protein elution was monitored at uv absorption of 280 nm. Each fraction was collected, dried and resuspended in 10 mM sodium phosphate buffer and tested for antibacterial activity by microbroth dilution method. The molecular weight of the highest potent fraction was determined by 12.5% SDS-PAGE. Tested bacteria was treated with F5.1, the highest potent fraction, at the MIC for 3 and 6 hours. The bacteria was visualized using scanning electron microscopy to determine the bacterial cell structure. Cytotoxicity of the MIC of F5.1 was assessed by using rezasurin assay on the human PBMCs cells. Viability of the cells was measured by microplate reader spectrophotometer.

Statistical analysis

Data was presented as mean ± standard error (mean ± S.E.). Statistical comparisons were made by one-way ANOVA. Any p-value < 0.05 was considered statistically significant.

Results

The crude venom showed concentration-dependent antibacterial activity against the pathogenic gram-positive strain, *S. aureus*, and gram-negative strains such as *E. coli*, *S.*Typhimurium and *P. aeruginosa* (Figure 1). The F5 had the strongest inhibitory activity against *S. aureus* and *P. aeruginosa* with the MIC of 3.90 µg/ml and 62.5 µg/ml respectively (Table 1). The F5 was further separated by Sephadex G-75 column and obtained six fractions (F5.1, F5.2, F5.3, F5.4, F5.5, F5.6). The F5.1 was found to possess the strongest inhibitory activity against all the tested bacteria with the MIC ranging from 1.95 to 7.81 µg/ml. The F5.2 possessed very low antibacterial activity with the MIC of 125-500 µg/ml. The F5.3, F5.4 and F5.6 were inactive against all of the tested bacteria. The F5.5 was active against only *P. aeruginosa* (Table 2). The MIC for *S. aureus* was further determined by measuring the turbidity at 600 nm, the MIC was found to be 1.8 µg/ml (Figure 2). From the SDS-PAGE, the molecular weight of F5.1 was 69 kDa (Figure 3). The mechanism of action of F5.1 was explored, the morphological changes induced by F5.1 on *S. aureus* were examined using SEM. Figure 4 showed the SEM micrographs of the buffer-treated and F5.1-treated cells of *S. aureus* at 3-6 hours of exposure. The buffer-treated *S. aureus* had smooth and normal surface morphology (Fig. 4A, 4C). The F5.1-treated *S. aureus* showed large globular surface protrusions on the bacterial cell surface (Fig. 4B, 4D). The F5.1 had cytotoxic activity on Human PBMCs cells at MIC concentration.

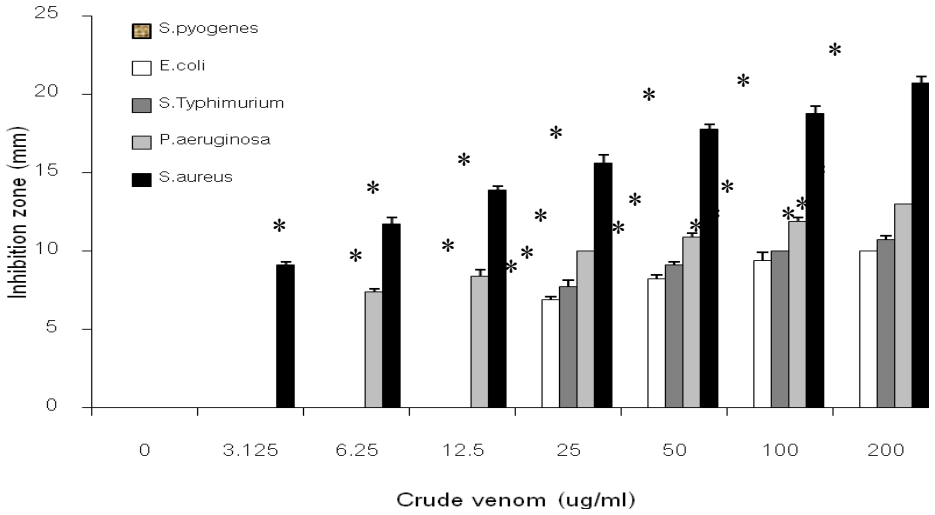


Figure 1 Antibacterial activity of King Cobra crude venom Disc diffusion assay. Antibacterial activity of crude venom of King Cobra. The inhibition zone was shown as mean ± S.E. of five replicate discs. Data was analyzed by one way ANOVA, $p < 0.05$ was considered sigfinicantly different when compared with the control.

Table 1 Antibacterial activity of F1-F7 peak fractions determined by microbroth dilution method

Microorganism	MIC (µg/ml)		MIC (µg/ml) Q-Sepharose						
	Cipro-floxacin	Crude venom	F1	F2	F3	F4	F5	F6	F7
E. coli	0.015	-	-	-	-	500	250	125	250
P. aeruginosa	0.5	-	-	-	-	1,000	62.5	250	250
*S.*Typhimurium	2.0	-	-	-	-	-	1,000	62.5	250
S. aureus	0.5	15.62	-	1,000	-	62.5	3.90	250	500

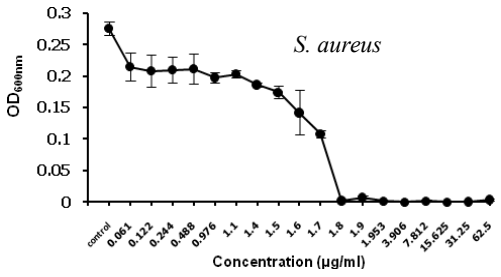
Each concentration was performed a triplication, MIC: Minimum inhibitory concentration, -: no antibacterial activity detected at concentrations up to 1,000 µg/ml

Table 2 Antibacterial activity of Sephadex G-75 peak fractions determined by microbroth dilution method (each concentration was performed a triplication)

Microorganism	Sephadex G-75 fractions of F5 (µg/ml)											
	F5.1		F5.2		F5.3		F5.4		F5.5		F5.6	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	7.81	7.81	500	500	-	-	-	-	-	-	-	-
P. aeruginosa	7.81	7.81	125	250	-	-	-	-	500	500	-	-
*S.*Typhimurium	7.81	62.5	500	500	-	-	-	-	-	-	-	-
S. aureus	1.95	1.95	125	125	-	-	-	-	-	-	-	-

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, -: no antibacterial activity detected at concentrations up to 500 µg/ml

Figure 2 Minimum inhibitory concentration (MIC) of F5.1 peak fractions against *S. aureus* determined by microbroth dilution method (each concentration was performed a triplication)



Microorganism	F5.1 MIC (µg/ml)
E. coli	7.0
P. aeruginosa	6.0
*S.*Typhimurium	6.0
S. aureus	1.8

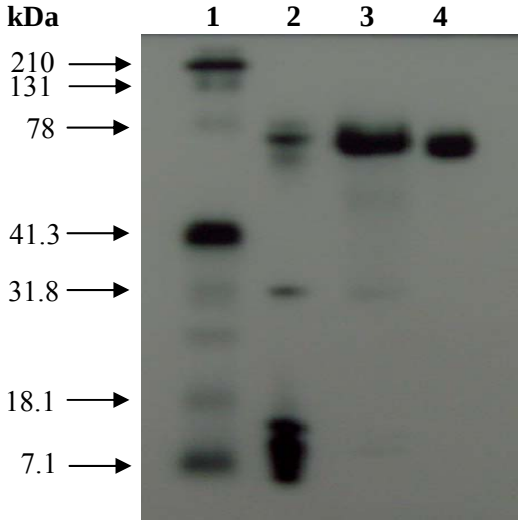


Figure 3 Molecular weight of F5.1 analyzed by 12.5% SDS-PAGE: Lane 1: protein markers including myosin (210 kDa), *β*-galactosidase (131 kDa), bovine serum albumin (78 kDa), carbonic anhydrase (41.3 kDa), soybean trypsininhibitor (31.8 kDa), lysozyme (18.1 kDa) and aprotinin (7.1 kDa); Lane 2: *O.hannah* crude venom; Lane 3: F5; Lane 4: F5.1. The molecular masses (kDa) of the markers are indicated on the left column.

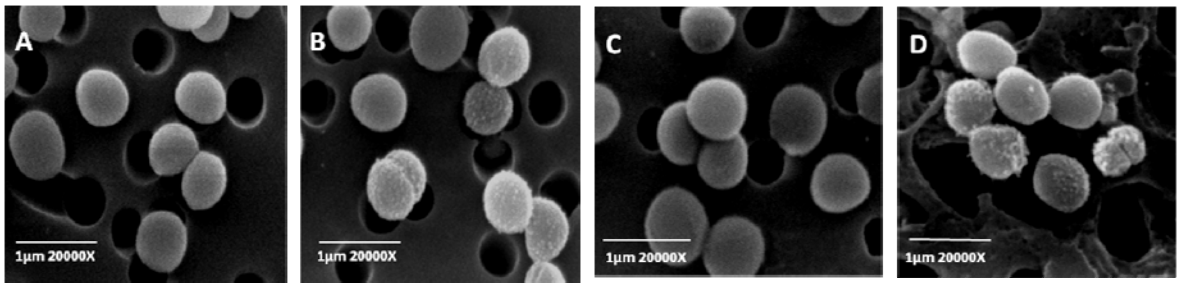


Figure 4 Scanning electron micrographs of *S. aureus* treated with the F5.1. Incubated *S. aureus* at 37 ^{o}C for 3 h, with 10 mM sodium phosphate buffer (pH 7.4) (control, A) and with 1.8 µg/ml F5.1(B) and for 6 h, with10 mM sodium phosphate buffer (pH 7.4) (control, C) and with 1.8 µg/ml F5.1(D).

Discussion and Conclusion

The results obtained from this study indicated that F5.1 with molecular weight of 69 kDa showed inhibitory activity against pathogenic bacteria. *S. aureus* was more susceptible to the F5.1 than *P. aeruginosa, E. coli* and *S.* Typhimurium. The mechanism of antibacterial activity may be through the membrane damage. The MIC of F5.1 was cytotoxic to the human normal cells. From the previous report, the pEM-2, a modified synthetic peptide derived from a snake venom Lys49 phospholipase A_2 showed reduced toxicity towards muscle cells, while retaining high bactericidal potency (3). Therefore, F5.1 should be further studied in terms of

cytotoxic components or modified the amino acid sequence of this protein to decrease toxicity and retain antibacterial activity. It will be useful in the search and developing for new potential antibacterial agents against pathogenic bacteria.

Acknowledgements

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Original article **P25**

Effects of xanthoxylin on melanogenesis

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Abstract

The important role of melanin is skin protection against UV radiation from sunlight which induces DNA damage and skin cancer. The purposes of this study were to determine the effects of xanthoxylin on melanin content by measure melanin content at 405 nm, mRNA expression of regulatory melanogenesis proteins by RT-PCR and dendriticity in mouse B16F10 melanoma cells by photograph the pictures under microscope. This study found that xanthoxylin increased melanin content and mRNA expression of regulatory melanogenesis proteins (tyrosinase and Mitf). In addition, xanthoxylin also increased dendrites of mouse B16F10 melanoma cells and no effect on viability of mouse B16F10 melanoma cells. It could be concluded that xanthoxylin induced melanogenesis, increased dendrites and activated tyrosinase and Mitf expressions in mouse B16F10 melanoma cells.

Keywords: xanthoxylin, melanogenesis, melanin, tyrosinase

Introduction

Melanin from melanocytes in epidermis plays an important role in skin protection against UV radiation from sunlight which induces DNA damage and skin cancer [1]. In melanosomes of melanocytes, tyrosinase is a rate-limiting step enzyme that converts tyrosine to dopachrome, while tyrosinase-related proteins (TRP-2 and TRP-1) convert dopachrome to melanin [2]. Expression of these proteins is regulated by microphthalmia-associated transcription factor (MITF). Melanin-containing melanosomes are transferred from dendrites of melanocytes to keratinocytes. Melanin is an excellent photoprotectant that absorbs the harmful energy of UV radiation (UVR) and helps prevent the UVR from damaging skin cells and penetrating deeper into the tissues. Agents that increase melanin production or melanogenesis have the potential to reduce both photodamage and skin cancer incidence [3].

Xanthoxylin (2'-hydroxy-4', 6' dimethoxyacetophenone) is a common phenolic compound in Rutaceae family. Its several pharmacological activities have been reported including neurotransmitter-mediated contractions in nonvascular smooth muscles [4], antifungal [5], antispasmodic [6], antioedema [7] and inhibitor of prostaglandin synthetase and 5-lipoxygenase [8]. Many phenolic compounds have been investigated for their activities on melanogenesis. This study aimed to elucidate the effects of xanthoxylin purified from *Zanthoxylum piperitum* on melanogenesis and dendricity as well as on proteins involving melanin synthesis in mouse B16F10 melanoma cells.

Methods

Mouse B16F10 melanoma cells (from ATCC), at the density of 1×10^4 cells/well in melanin content assay and mRNA expression or 1×10^3 cells/well in melanocyte dendricity assay, were treated with 6.25-25 μM xanthoxylin for 72 hours. Untreated cells and 10 nM α-

MSH-treated cells were used as the negative and positive control, respectively. The treated cells were used for the following objectives;

1. Determination of melanin content: The treated cells were undergone lysis by 2 M NaOH. Melanin in the cells was dissolved by heating at 60 °C for 5 min and its content was measured at 405 nm.

2. Determination of melanocyte dendricity: The numbers of dendrites of the treated cells were observed under a light microscope at 40X amplification.

3. Determination of mRNA expression of proteins involving in melanogenesis: Total RNA was isolated from the treated cells using TRIzol® reagent and then reversing to cDNA using Improme II™ reverse transcription system reagent. The cDNA was PCR amplified with the specific primers of tyrosinase, MITF, TRP-1 and TRP-2 genes. The PCR products were run on 1.5% agarose gel electrophoresis and semiquantitative determined by a gel documentation.

All assays were performed in 3 independent experiments (n=3). The data were presented in mean ± S.E. One-way ANOVA was used to determine the statistical analysis and p-value < 0.05 was considered statistically significant.

Results

Effect of xanthoxylin on melanogenesis

Xanthoxylin induced melanogenesis in B16F10 cells, in a concentration dependent manner (table 1 and Fig. 1). It significantly increased melanin content at the concentration of 12.5 and 25 µg/ml without increasing in cell proliferation or cell toxicity (data not shown).

Table 1: Effect of xanthoxylin on melanigenesis.

Compounds	% Stimulation of melanin content
Untreated control	100±0.0008
10nM α-MSH(positive control)	307.6±0.0020*
6.25 µg/ml Xanthoxylin	153.3±0.0067
12.5 µg/ml Xanthoxylin	268.8±0.0075*
25 µg/ml Xanthoxylin	650.7±0.0056*#

B16F10 cells were treated with 6.25, 12.5, 25 µg/ml xanthoxylin for 72 h.
Ten nM α-MSH was used as the positive control. Melanin content in the treated cells was determined and compared to the untreated control (n=3). * P<0.01, significantly when compared to untreated control. # P<0.01, significantly when compared to 12.5 µg/ml xanthoxylin.

Effect of xanthoxylin on melanocyte dendricity

Xanthoxylin induced the increase in the number of dendrites in B16F10 cells, in a concentration-dependent manner (Fig. 1)

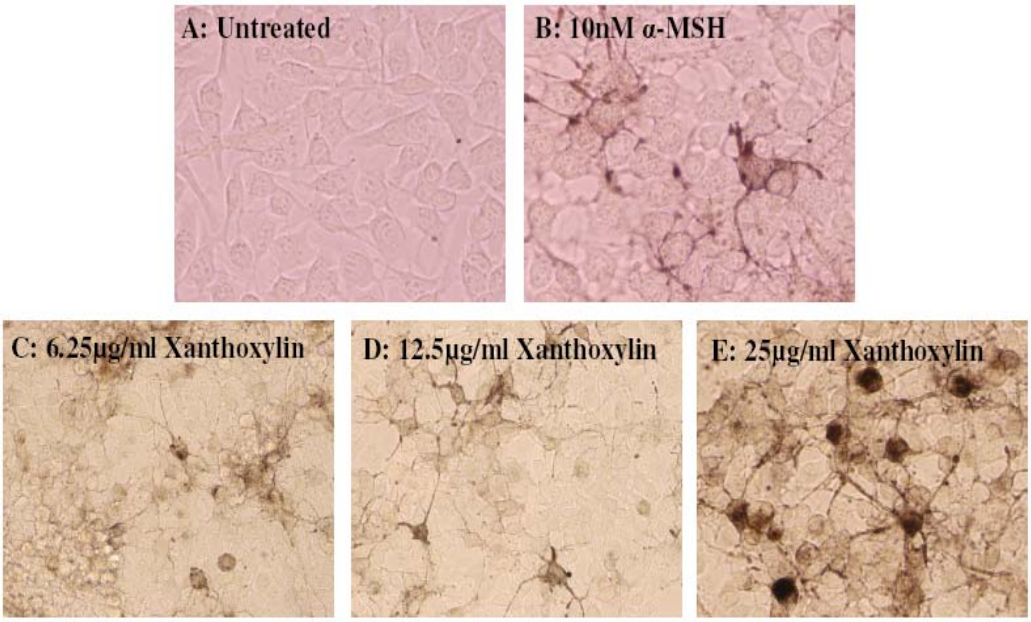

Figure 1: A representative result of the effect of xanthoxylin on melanin content and dendricity. B16F10 cells were treated with 6.25, 12.5, 25 µg/ml xanthoxylin for 72 h. Ten nM α-MSH was used as the positive control. (n=3)

Effect of xanthoxylin on the mRNA expression of proteins involving in melanogenesis

Xanthoxylin, at the concentration of 12.5 and 25 µg/ml, significantly increased the mRNA expression of MITF and tyrosinase when compared to the untreated control (Fig. 2). It didn't induce TRP-1 and TRP-2 expression.

Conclusion

The results from this study suggest that xanthoxylin induced melanogenesis by activating the mRNA expression of the transcription factor, MITF, and tyrosinase which are essential for melanin synthesis in melanocytes. It also increased melanocyte dendricity which is important for transferring melanin-containing melanosomes to keratinocytes. The mechanism of xanthoxylin-induced melanogenesis are ongoing investigated.

Discussion

It is known that darkly pigmented skin has higher protection against skin cancer than fair skin. Enhancer of skin pigmentation is one of main mechanism to reduce both photodamage and skin cancer incidence. However, some enhancers such as ultraviolet radiation (UVR) and psoralen are associated DNA damage and skin cancer development. Any pigmentation enhancers without DNA damage are still needed. In this study, we demonstrated that xanthoxylin induced melanogenesis and dendricity. It didn't have neither proliferative nor cytotoxic activities. This agent may potentially be an echancer of skin pigmentation.

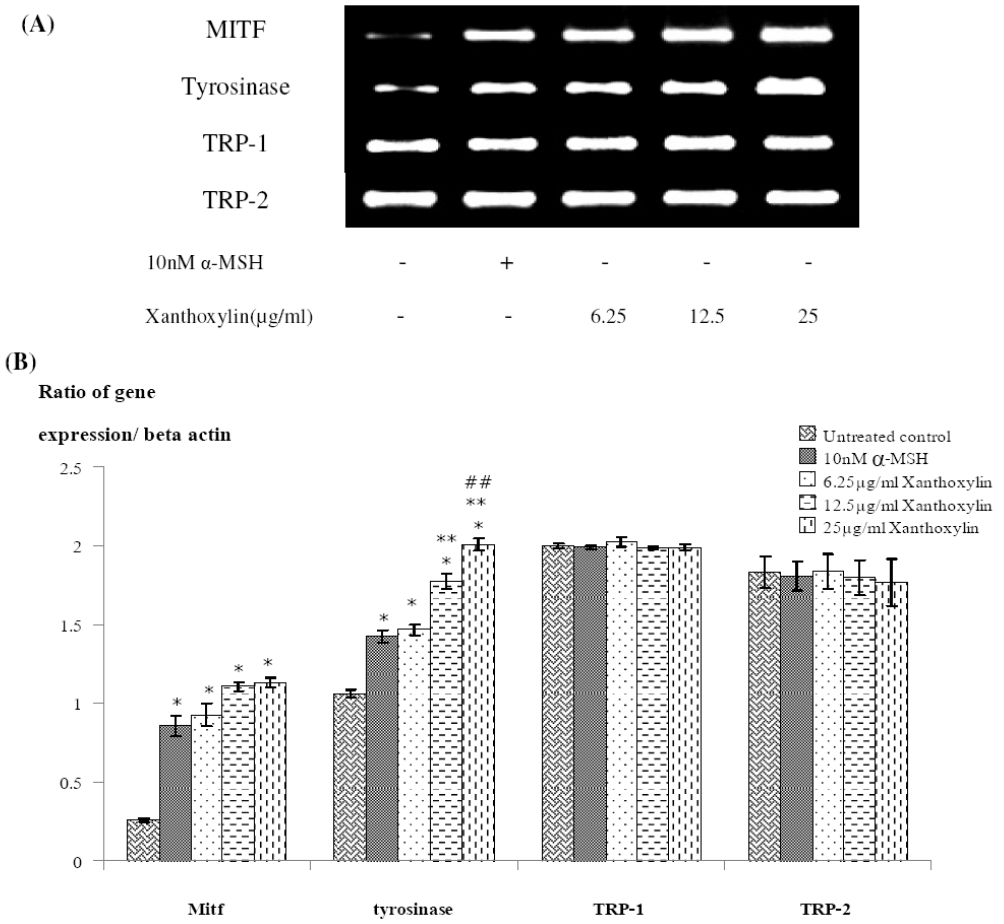

Figure 2: Effect of xanthoxylin on the mRNA expression of proteins in melanogenesis. B16F10 cells were treated with 6.25, 12.5, 25 μg/ml xanthoxylin for 72 h. Ten nM α-MSH was used as the positive control. Total RNA was isolated from the treated cells and reversing to cDNA. cDNA was amplified by PCR using specific primers for MITF, tyrosinase, TRP-1 and TRP-2 genes (n=3). * $P<0.05$, significantly when compared to untreated control. ** $P<0.05$, significantly when compared to 6.25 μg/ml xanthoxylin. ## $P<0.05$, significantly when compared to 12.5 μg/ml xanthoxylin.

Acknowledgements

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Original article **P26**

Screening of antiproliferation activity of Thai medicinal plants against human cholangiocarcinoma cells *in vitro*

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Abstract

Cholangiocarcinoma is a serious public health concern in Thailand with increasing incidence and mortality rates. The present study aimed to investigate antiproliferation activity of crude ethanolic extracts of 28 plants and 5 recipes used in Thai folklore medicine against human cholangiocarcinoma (CL-6) and human hepatocarcinoma (HepG2) cell lines *in vitro*. Antiproliferation activity of the plant extracts against the cancerous cell lines compared with normal cell line (renal epithelial cell) was assessed using MTT assay and the IC_{50} (concentration which inhibits cell growth by 50%) were calculated. The extracts from eight plants and one folklore recipe exhibited promising activity against the cholangiocarcinoa CL-6 cell line with percentage cell survival of less than 50% at the concentration of 50 μg/ml. Among these, the extracts from the five plants and one recipe (AtLEt, KaGEt, ZiOEt, PiCEt, MeFEt and PsPyEt) showed potent cytotoxic activity with mean IC_{50} values of 24.09, 37.36, 34.26, 40.74, 48.23 and 44.12 μg/ml, respectively. In contrast, activities against the hepatoma cell HepG2 varied markedly; mean IC_{50} ranged from 9.67 to 115.47 μg/ml and the only promising extract was from ZiOEt (IC_{50} 9.67 μg/ml). The sensitivity of all the three cells to 5-FU also varied according to cell types, particularly with CL-6 cell (IC_{50} 757 μM). In overall, it can be concluded that ethanolic extracts of AtLEt and ZiOEt contained antiproliferation against CL-6 and Hep-G2 cell line, respectively.

Keywords: Thai medicinal plants, cytotoxic activity, cholangiocarcinoma, CL-6, HepG2

Introduction

Cholangiocarcinoma, malignant epithelial cells that arises within bile duct, is a serious public health in Thailand with increasing incidence and mortality rates. It accounts for approximately 15% of liver cancer worldwide (1). Chemotherapeutic treatment of cholangiocarcinoma is largely ineffective. The standard chemotherapeutic agent, 5-fluorouracil (5-FU) always produces low clinical response rate (2). Numerous cancer research studies have been carried out using medicinal plants in an effort to discover new therapeutic agents that lack toxic effects associated with current therapeutic agents. Traditional medicine is commonly used as an alternative treatment for cancer by Thai people (3). Several Thai traditional folklores have been assessed for their anticancer activities in various human cancerous cell lines with some promising candidates (4). In the present study, the ethanolic extracts of a total of 28 plants and 5 recipres used in Thai folklore medicine were investigated for their antiproliferation activity *in vitro* in two human cancerous cell lines (cholangiocarcinoma: CL-6, hepatocarcinoma: HepG2), and one normal human cell line (renal epithelial cell: HRE).

Methods

Plant materials were collected from various parts of Thailand and some were purchased from the city markets. Ethanolic extracts of all plant materials were prepared. CL-6, HepG2 and HRE used for cytotoxic screening of the medicinal plant extracts were maintained in standard culture medium at 37ºC in a 5% CO_2 atmosphere with 95% humidity. The MTT colorimetric assay (5) was used to screen for cytotoxic activity of all the plant extracts and 5-FU (positive control) in a 96-well plates at a cell density of 10^4 per well and 100 µl culture medium. Each extract was screened initially for its cytotoxicity against all cancerous and normal cell lines at the concentration of 50 µg/ml. The potential candidates which resulted in cell survival of less than 50% were further assessed for their IC_{50} (concentration that inhibits cell growth by 50%) at the concentration range of 250-1.95 µg/ml. Following a 3 h incubation with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (20 µl of 5 mg/ml) at 37ºC, cells were lyzed with DMSO. The yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals. Absorbance (OD) was measured at 570 nm using a microplate reader (Varioscan Flash, Thermo, Finland). The percentage of cytotoxicity compared to the untreated cells was determined. The IC_{50} values were calculated with probit analysis software (CalcySyn™, USA).

Results and Discussion

Results from the initial screening showed that the extracts from eight plant species exhibited promising activity against the cholangiocarcinoa CL-6 cell line with percentage cell survival of less than 50% at the concentration of 50 µg/ml (Figure 1). Crude extracts from AtLEt showed the highest promising activity against CL-6 cell line, whereas it showed no activity against HepG2 cell. CuLEt and MaSEt exhibited high activity against HepG2 (% survival of 13.9 and 29.2%, respectively; Figure 1) but relatively low activity against CL-6 cell line (% survival of 59.9 and 51.4, respectively; Figure 1). Among these, six showed potent cytotoxic activity with IC_{50} of less than 50 µg/ml (AtLEt, KeGEt, ZiOEt, PiCEt, MeFEt and PsPyEt). The extracts of the six plants were further investigated for their spectrum of anticancer activity against HepG-2 cell line. Results showed that the activity against hepatoma cell HepG2 varied markedly; mean IC_{50} ranged from 9.67 to 115.47 µg/ml. The most promising extract was from ZiOEt (IC_{50} 9.67 ± 3.91 µg/ml). The extract from AtLEt exhibited the most potent activity against CL-6 but the activity against HepG2 was only moderate. The results are generally in agreement with that shown in the screening test (Figure 1), confirming that HepG2 was more resistant to the tested ethanolic extracts from Thai traditional folklore than CL-6. The extract from AtLEt appears to be the most potent (IC_{50} 24.09 ± 3.40 µg/ml), whereas that from ZiOEt appears to be the most potent against HepG2 (IC_{50} 9.67 ± 3.91 µg/ml). Further investigation of all the six extracts for their cytotoxic activity against cholangiocarcinoma in hamster model is underway to fully assess the anticancer activity *in vivo*.

Conclusion

Six out of a total of 28 plants and 5 recipes used in Thai folklore medicine exhibited promising cytotoxic activity against CL-6 human cholangiocarcinoma cell line. HepG2 appears to be more resistant to the tested extracts. The extract from AtLEt was more potent against cholangiocarcinoma, whereas that from ZiOEt was more potent against HepG2.

Acknowledgement

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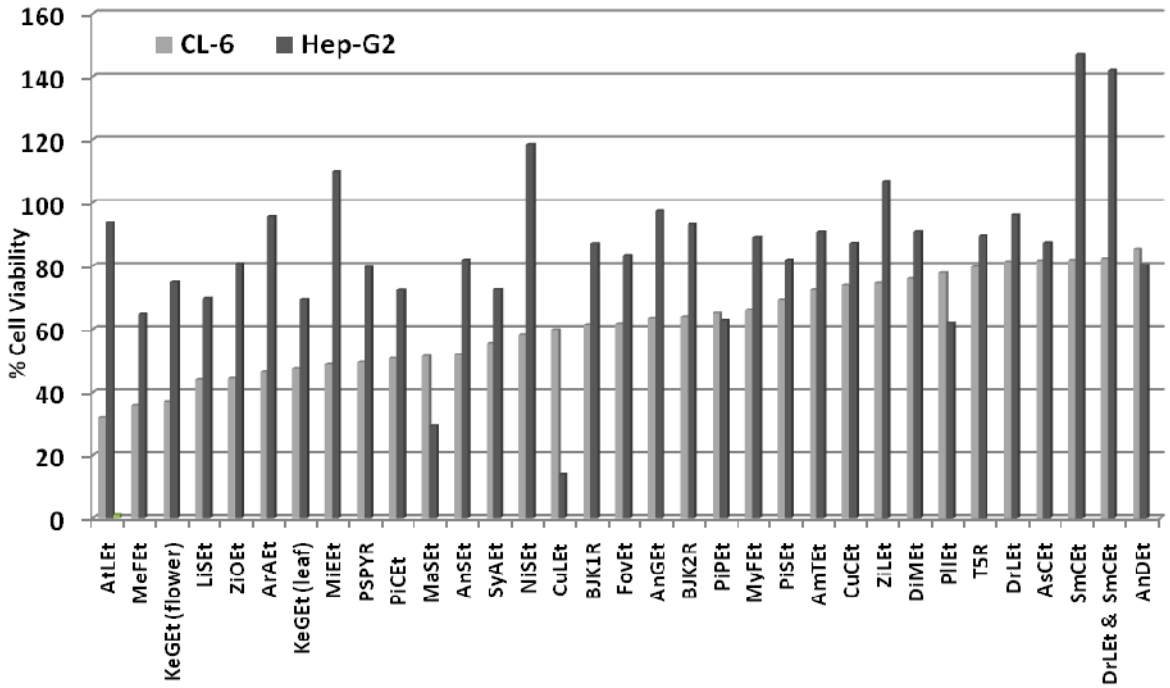

Figure 1. Percentage survival of cancer cell lines (CL-6 and HepG2) treated with ethanolic extract from a total of 28 plants and 5 recipes used in Thai folklore medicine at the concentration of 50 μg/ml.

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Original article **P27**

The anti-apoptosis effect of curcumin I on bax/bcl-2 ratio against 6-OHDA induced SH-SH5Y toxicity

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Abstract

Curcumin is a naturally occurring polyphenolic compound. It has been reported that it exerts anti-oxidative and anti-apoptotic activities. 6-Hydroxydopamine (6-OHDA) is used as a neurotoxin to generate a model of Parkinson's disease. In the present study, we investigate whether curcumin I (diferuloylmethane) could protect SH-SY5Y, dopaminergic cells from 6-OHDA-induced neurotoxicity. The results showed that pretreatment with curcumin I significantly prevented 6-OHDA induced cell viability reduction. Further experiments indicated that curcumin I could protect 6-OHDA-induced apoptosis signaling cascades activation. The results showed that pretreatment with curcumin I could prevent 6-OHDA-induced increasing of Bax/Bcl-2 ratio. This study suggested that curcumin I exerts its protective effects against 6-OHDA induced neurotoxicity. Therefore, curcumin I may be used as a potential compound for preventing oxidative stress-induced neurodegeneration.

Keywords: 6-OHDA, Curcumin I, Bax, Bcl-2, Phospho-P38.

Introduction

The generation of reactive oxygen species (ROS) has been well known to play a pivotal role in the pathogenesis of neurodegenerative diseases including Parkinson's disease (PD). In particular, 6-OHDA, a hydroxylated analogue of dopamine, is known to produce ROS [3] and widely used to mimic a model of PD both *in vivo* and *in vitro* studies[1]. Curcumin has been well known of its anti-oxidative, anti-inflammatory and anti-apoptotic properties. It has been reported that curcumin could prevent MPP^+- induced neurotoxicity in PC-12 cells via its anti-oxidative activity [2]. Also, another study reported that pretreatment with curcumin could protect 6-OHDA-induced cytotoxicity by anti-oxidative modulation in MES23.5 cells[4]. However, little is known about the cytoprotective effects of pure compound isolated from *Curcuma longa* on preventing 6-OHDA-induced dopaminergic cell death. Therefore, this study was taken to investigate whether a pure compound isolated from *Curcuma longa* which is curcumin I has a neuroprotective effect for preventing 6-OHDA-induced neurotoxicity in SH-SY5Y cells.

Methods

Cell cultures and cell viability assay

SH-SY5Y cells were maintained in MEM and Ham F-12 supplemented with 10% fetal bovine serum (FBS), 25 mg/ml of penicillin, 25 U/ml of streptomycin, 1 mM sodium pyruvate and 1 mM non-essential amino acids at 37 °C in an atmosphere of 5 % CO_2. 6-OHDA was dissolved in 0.01 % ascorbic acid in iced cold sterile water to give a stock

solution of 10 mM. Curcumin I was also dissolved in dimethylsulfoxide (DMSO) as a stock solution of 10 mM and the final concentration of DMSO was always less than 0.1 %. MTT (3-(4,5-dimethylthiazol-2,yl)2,5 diphenyltetrazolium bromide) assay was performed in order to determine the protective effect of curcumin I on cell viability in 6-OHDA treated cells. Cells were plated at a density of 3×10^4 cells/well in 96-well plate. After 24 h, cells were pretreated with curcumin I at the concentrations of 1, 5, 10 and 20 μM for 30 min before exposure to 6-OHDA 25 μM for 24 h. Then, 50 μl of 10 mg/ml MTT solution was added to each well and incubated for 4 h at 37 °C.

Western blot analysis

After treatment, cells were lysed in freshly prepared lysis buffer solution. Lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the equal amounts of protein were separated by 12.5 and 10 % SDS-PAGE and transferred to hybond ECL nitrocellulose membranes. Membranes were blocked with 5 % skimmed milk in TBST for 1 h and then incubated with primary anti-Bax, Bcl-2 and actin antibodies in TBST containing 3 % BSA. After incubation with HRP-conjugated anti-IgG antibody, detected proteins were visualized using an enhanced chemiluminescence assay kit. Protein bands were measured using densitometry analysis program.

Data analysis

All data are expressed as mean ± SEM and analyzed using one-way ANOVA. Tukey's multiple comparison post-test was used to calculate the statistical significance. Differences were considered statistically significant when $p < 0.05$.

Results

Pretreatment with curcumin I could significantly prevent 6-OHDA induced cell death in a concentration dependent manner (Figure 1). The present study also found that curcumin I prevented 6-OHDA-induced increase in Bax/Bcl-2 ratio.

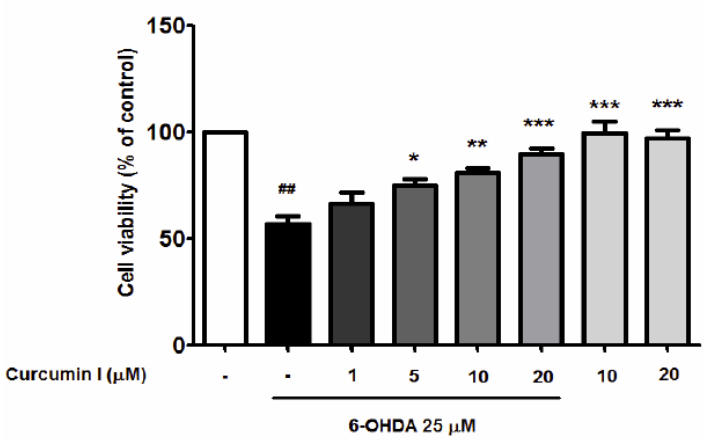

Figure 1 The protective effect of curcumin I on 6-OHDA- induced cell death. Values represent mean ± SEM of at least three separate determinations. * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$ significant difference when compared with only 6-OHDA group, ##$P<0.001$ significant difference when compared with control group.

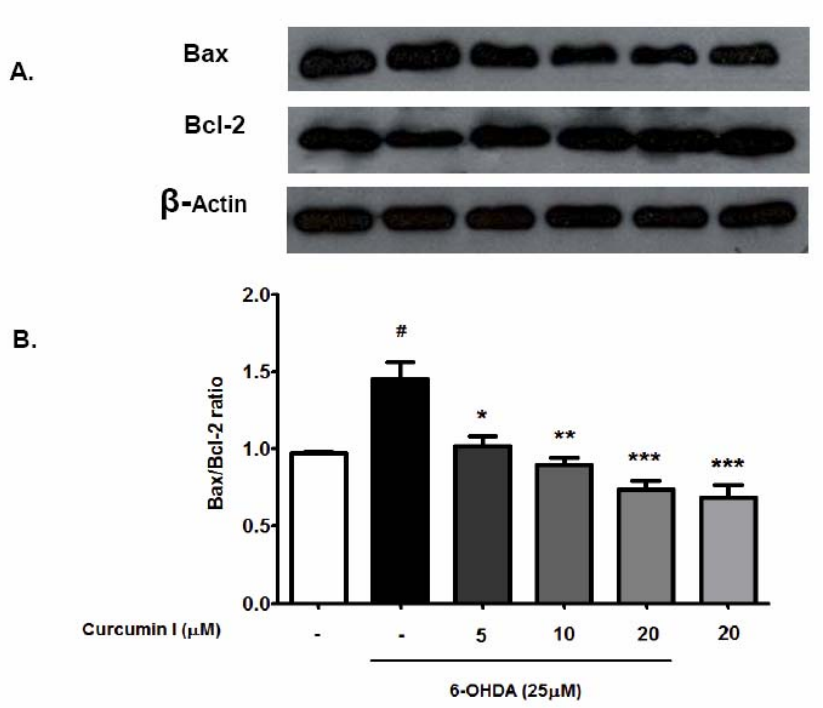

Figure 2. Curcumin I reduces Bax/Bcl-2 ratio in 6-OHDA-treated cells. A. Levels of Bax, Bcl-2 and β-Actin expression. B. Values represent mean ± SEM of Bax/Bcl-2 of at least three separate determinations. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ significant difference when compared with only 6-OHDA group, # = $p<0.001$ significant difference when compared with control group.

Discussion

6-OHDA-treated human neuroblastoma SH-SY5Y cells is a useful *in vitro* model for studying neurodegenerative events that may occur in PD. In this study, we demonstrated that curcumin I protects SH-SY5Y cells against 6-OHDA-induced cytotoxicity in apoptosis signaling pathway. The ratio between Bax and Bcl-2 has been used as an indicator for determining cell undergoing apoptosis. The ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2 increases significantly upon treatment with 6-OHDA whereas curcumin I reduced the expression of Bax and increased the expression of Bcl-2 significantly, thereby ameliorating the 6-OHDA-induced Bax/Bcl-2 ratio elevation in SH-SY5Y cells. The present study showed that curcumin I has significant cytoprotection against 6-OHDA-induced apoptosis SH-SY5Y cells. The cytoprotection of curcumin I may be attributed from its inhibitory effect on the apoptotic signaling as evident from the decrease in Bax/Bcl-2 ratio. The antioxidant property of curcumin I may be one of the major mechanisms participated in its neuroprotection against cell death.

Conclusion

Our results show that curcumin I protects SH-SY5Y cells against 6-OHDA-induced cytotoxicity. Its anti-apoptotic activity of curcumin I may be useful as a potential compound for preventing oxidative stress-induced Parkinson's disease.

Acknowledgements

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Original article **P28**

Protective effects of curcumin against dopamine quinone-induced dopaminergic neurotoxicity in SH-SY5Y cells

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Abstract

Auto-oxidation of 6-hydroxy dopamine (6-OHDA) has been known to generate free radicals and subsequent quinone proteins formation which is toxic to dopaminergic neurons. Curcumin, is a pure compound extracted from *Curcuma longa* which known to possess anti-oxidant, anti-inflammatory and anti-carcinogenic activities. In this study, we investigated the effects of curcumin on 6-OHDA-induced neurotoxicity in SH-SY5Y cells. It was found that curcumin significantly increased the viability of the cells. Along with the increase in cell survival the quinoprotein production was significantly decreased. These results indicate that curcumin has neuroprotective effects and may have a therapeutic potential for treatment of Parkinson disease.

Keywords: 6-OHDA, quinoprotein, *Curcuma longa*.

Introduction

The formation of dopamine quinones has been known to exert toxic effects in dopaminergic cells (Asanuma et al., 2004). These quinones conjugate with the sulfhydryl group of the amino acid cysteine. The dopamine quinone modified proteins were called quinoproteins. Since the sulfhydryl group on cysteine is often found at the active site of functional proteins, quinoproteins inhibit protein function and cause cell death. Curcumin or diferuloylmethane is a purified natural compound extracted from *Curcuma longa* (turmeric). The previous study has reported that curcumin posseses multiple pharmacological properties such as anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities (Joe and Lokesh, 1994).

Therefore, we speculate that curcumin may prevent the degeneration of dopaminergic neurons. In this study, we investigated whether the anti-oxidant property of *C.longa* would prevent the neurotoxic effects of 6-OHDA in SH-SY5Y cells.

Methods

To determine the effects of 6-OHDA and curcumin, on cell viability, SH-SY5Y cells were plated at a density of 2×10^4 cells per well on 96-well culture plates. After treatment with various concentration of 6-OHDA ranging from 10 μM to 200 μM for 24 h, or pretreatment with different concentrations of curcumin for 30 min followed by 24 h of 6-OHDA treatment (25 μM), the medium was removed and the cells were incubated with a solution of 1 mg/ml MTT for 4 h and then measuring absorbance at 570 nm. The protein-bound quinones (quinoprotein) were detected by the nitrobluetetrazolium (NBT)/glycinate colorimetric assay. Cells were plated at a density of $1x10^6$ cells/well in 6-well tissue culture plates and incubated for 24 h. After pretreatment with various concentrations of curcumin for 30 min follow by 24 h of 6-OHDA treatment (25 μM). The cultures were lysated in 10%

trichloroacetic acid (TCA) and NBT reagent was added (0.24 mM NBT in 2 M potassium glycinate, pH 10.0) followed by incubation the mixture in the dark for 2 h under constant shaking. The absorbance of blue-purple color developed in the reaction mixture was measured at 530 nm.

Results

To evaluate the cytotoxic effects of 6-OHDA and curcumin**,** as shown in Fig.1, treatment with various concentration of 6-OHDA ranging from 10 μM to 200 μM for 24 h significantly decreased the cell viability in a dose -dependent manner. 6-OHDA at the concentration of 25 μM, decreased the cell viability to 59 % of control, therefore 6-OHDA at this concentration was used to detect the neuroprotective effect of curcumin. Pretreatment the cells with curcumin at concentration of 1 μM, 5 μM, 10 μM and 20 μM for 30 min followed by treatment with 6-OHDA at the concentration of 25 μM for 24 h significantly increased the cell viability (Fig.2). These results demonstrated the protective effect of curcumin on cell viability in 6-OHDA-induced cell death. To measure the levels of quinoprotein production, as shown in Fig. 3, after treatment the cells with 6-OHDA for 24 hr the level of quinoprotein production was significantly increased to 198.01 ±_7.38 % compared with the control. Pretreatment the cells with curcumin significantly decreased the effect of 6-OHDA-induced quinoprotein production in a concentration-dependent manner. At the concentration of 1, 5, 10, and 20 μM, curcumin reduced quinoprotein production to 184.33 ± 2.14, 153.56 ± 6.87, 100.56 ± 5.29, and 91.27 ± 3.42 %, respectively, compared with the control. Data were represented as mean ± SEM of three separate experiments.

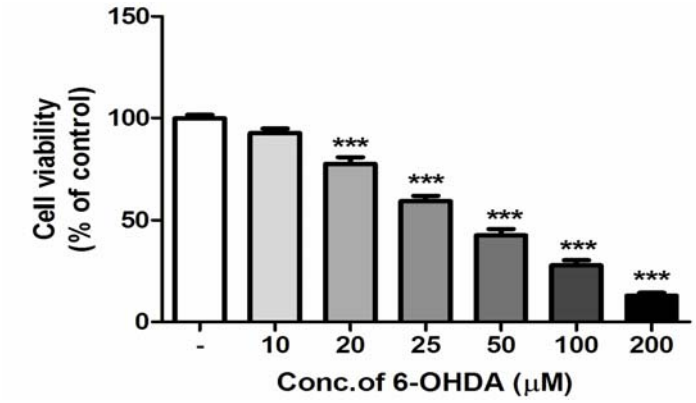

Figure 1. Effects of the various concentrations of 6-OHDA on the viability of SH-SY5Y cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with control.

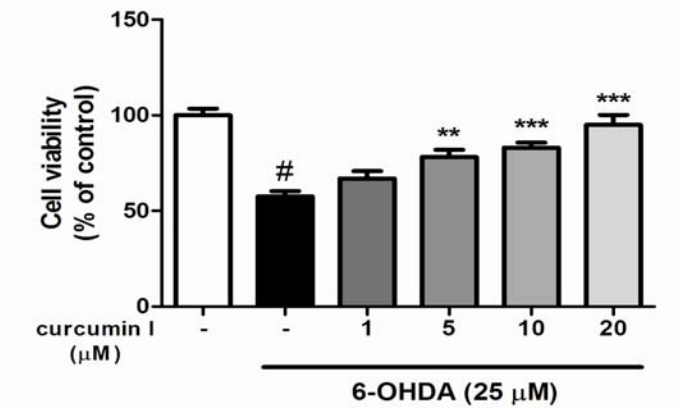

Figure 2. Effects of pretreatment with various concentration of curcumin on the viability of SH-SY5Y cells. #$p < 0.001$ as compared with the control; **$p < 0.01$, ***$p < 0.001$ as compared with 6-OHDA treated only.

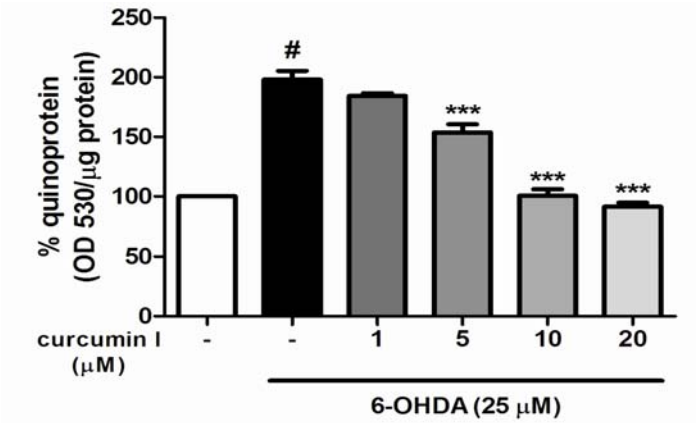

Figure 3. Effects of various concentrations of curcumin on the levels of quinoprotein production in 6-OHDA-treated SH-SY5Y. Data were represented as mean ± SEM of three separate experiments. $^{\#}p < 0.001$ as compared with the control; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with 6-OHDA treated cells.

Discussion

The results of our study demonstrated the protective effects of curcumin on 6-OHDA-induced neurotoxicity of SH-SY5Y cells. Previous study reported that 6-OHDA generated DAQ and quinoprotein (D. Blum et al., 2001). We demonstrated for the first time, that curcumin can decrease quinoprotein production in 6-OHDA-induced quinoprotein production.

Conclusion

We demonstrated that curcumin significantly increased cell viability and decreased quinoprotein productions in 6-OHDA-induced neurotoxicity. This study suggests that curcumin possesses neuroprotective effects and may have a therapeutic potential for the treatment of neurodegenerative diseases caused by oxidative stress.

Acknowledgements

We gratefully acknowledge Professor Pawinee Piyachaturawat for her help.

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Original article **P29**

Inhibitory effect of methanol and hexane extracts of *Moringa oleifera* Lam leaves on NO production in LPS-activated microglia

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Abstract

Moringa oleifera Lam(*M.oleifera*), is generally found in tropical areas, South Asia and Thailand. It has been used for the treatment of various diseases including inflammation. However, little is known about its anti-inflammatory activity. We, therefore studied the effect of crude extracts from leaves (Methanol and hexane fractions) of *M. oleifera* on the production of nitric oxide (NO), a pro-inflammatory substance in highly aggressively proliferating immortalized (HAPI) microglial cells activated by lipopolysaccharide(LPS) and found that the extracts from *M. oleifera* significantly suppressed nitric oxide production in a dose dependent manner. This preliminary result demonstrates that the anti-inflammatory activity of the crude extracts of *M. oleifera* leaves is due partly through the inhibition of NO production and suggests that *M. oleifera* may have neuroprotective potential in neurodegenerative diseases caused by neuroinflammation.

Keywords: *Moringa oleifera* Lam, Microglia , Nitric oxide .

Introduction

Central nervous system (CNS) composes of neurons and glia cells such as microglia and astrocytes. Microglia are known as residence brain immune system, responsible for homeostasis regulation and defense against injury. If microglia were chronically activated, they produced nitric oxide and other pro-inflammatory factors which caused damage to neurons and led to the development of neurodegenerative diseases.

Moringa oleifera Lam or in Thai "Maroom" a member of Moringaceae, has many pharmacological effects. Almost all parts of this plant have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, hematological and hepatorenal disorders.

The plant has been reported to be highly potent anti-inflammatory agent. Therefore, the objective of this study is to investigate the anti-inflammatory effects of crude extracts (methanol and hexane fractions) obtained from *Moringa oleifera* Lam leaves in the inflammation model, stimulating of HAPI microglial cell with lipopolysaccharide (LPS) ,on nitric oxide production.

Methods

Crude extracts (hexane and methanol fractions) of *Moringa oleifera* Lam leaves were obtained from Department of Chemistry, Faculty of Science, Mahidol University, Thailand.

Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) was used to maintain HAPI microglial cells at 37°C under humidified 5% CO_2 and 95% air atmosphere. In all experiments, cells were left to

acclimate for 24h before any treatments. Pretreatment of *M.oleifera* leaf extract was done by adding the extract 1 h before LPS treatment to HAPI cells.

The number of cell viability was determined by MTT assay. HAPI cells were cultured onto 96-well plate at a density of $2x10^4$ cells/well overnight. Then the cells were treated with various concentrations of crude extracts of *M.oleifera*. The medium was removed at 24 h and 10 mg/ml MTT in Hank's balanced salt solution was added to each well and further incubated for 4 h in a humidified atmosphere at 37 °C, 5% CO_2. After that MTT was removed and cells were lysed with 100 µl DMSO, microplate reader was used to determine the absorbance at the wavelength of 570 nm and at a reference wavelength of 665 nm.

Nitric oxide (NO) production from LPS-treated cells was determined by using Griess assay. HAPI cells ($5x10^5$ cells/well) were plated onto 6-well plate and treated with LPS (100 ng/ml) for 24 h. Then the cell culture supernatant from each sample was collected and the equal volume of Griess reagent was added. Optical density at 545 nm of the sample was determined by a microplate reader. Dilution of sodium nitrite with culture media at concentrations between 0 to 100 µM was used as standard curve.

Results

Effects of *M. oleifera* extracts on cell viability

The cells ($2x10^4$ cells/well) were treated with various concentrations of *M. oleifera* ranging from 10^{-12}g/ml to10^{-3}g/ml for 24 h then performed MTT assay. As shown in Fig. 1A, hexane extract at concentrations from 10^{-12}g/ml to 10^{-4}g/ml had no effect on cell survival. However, the viability of the cells was remarkably reduced to only 10% (of control) by high concentration (10^{-3}g/ml) of *M.oleifera* extract. Similar to hexane extract, (Fig.1B) methanol extract at concentrations from 10^{-12}g/ml to 10^{-5}g/ml had no effect on cell survival. However, the viability of the cells was remarkably reduced to 10% (of control) at 10^{-3}g/ml of *M. oleifera*.

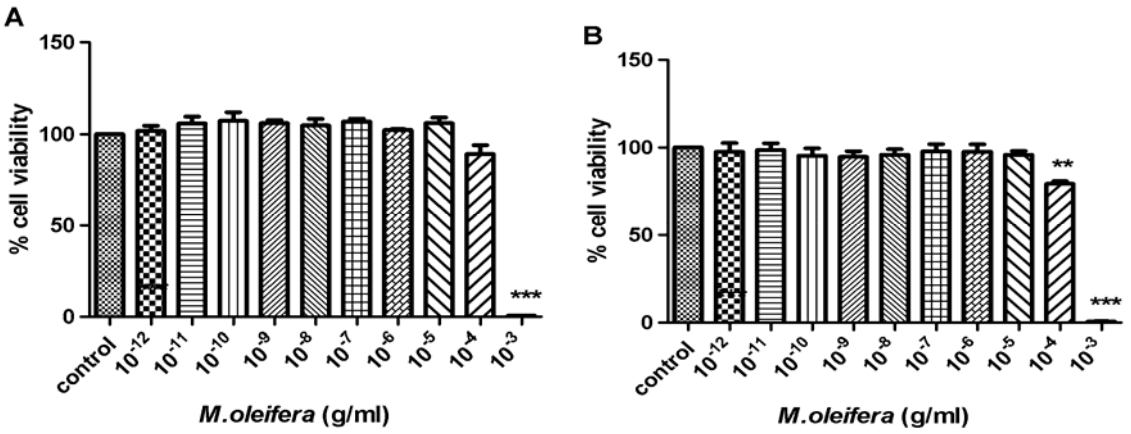

Fig 1. Effects of hexane (A) and methanol (B) extracts of *M.oleifera* leaves on HAPI cell viability. After 24-h incubation with various concentrations of the extract, cell viability was measured with MTT assay(%).

Inhibition of NO production by *M.oleifera* extracts

To determine the effect of *M.oleifera* extracts on NO production, The hexane and methanol extracts of *M.oleifera* at concentrations from 10^{-8}g/ml to 10^{-6}g/ml were added to HAPI cells for 1 h before adding LPS(100ng/ml). After stimulation with LPS for 24 hrs, the levels of NO in the culture media were determined with Griess assay. Both crude extracts of *M.oleifera* significantly reduced the levels of NO production from LPS-stimulated cells in a dose- dependent manner. In Fig. 2A, at concentration of 10^{-6}g/ml *M.oleifera* (hexane extract) was able to inhibit NO production more than 50 %, compared with LPS alone. As shown in

Fig. 2B, at concentration of 10^{-6}g/ml, *M.oleifera* (methanol) was able to inhibit NO production more than 70%, compared with cells activated only with LPS.

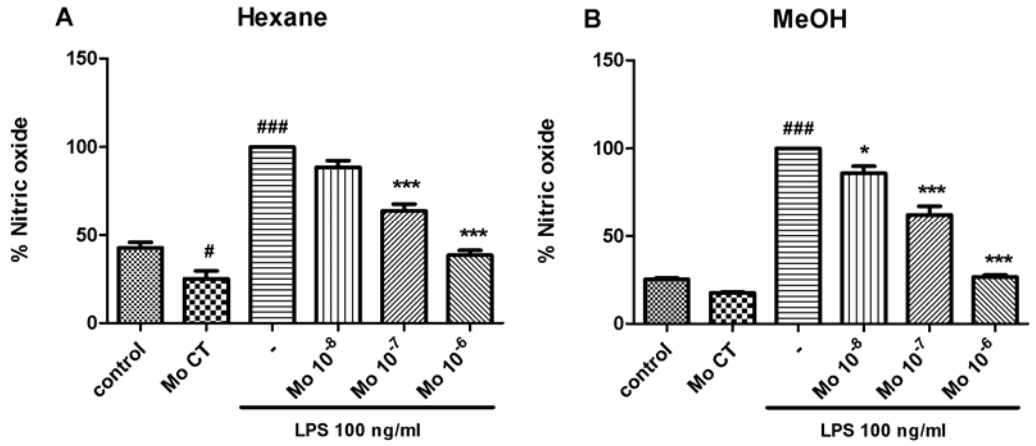

Fig. 2. Effects of hexane (A) and methanol (B) extracts of *M.oleifera leaves* on LPS-stimulated NO production in HAPI microglias.The cells ($5x10^5$ cells/well) were cultured with various concentrations of *M.oleifera* extract for 1 h before administration of 100 ng/ml LPS**.** After incubated with LPS for 24 h, the culture supernatants were assayed for NO production (%).

Discussion

The result from the present study demonstrates that both crude extracts (methanol and hexane fractions) of *M.Oleifera* Lam leaves have anti-inflammatory effect because both fractions significantly reduced NO production in HAPI microglial cells activated by LPS. It is well known that LPS stimulates microglia and macrophage to produce nitric oxide and other pro-inflammation cytokines such as interleukine-6(IL-6), and tumor necrosis factor-alpha etc. The reduction of NO production of *M. oleifera* leaf crude extracts on microglia may be due to the direct effect on inhibition of the inducible nitric oxide synthase (iNOS). Since upon activation the iNOS was expressed in microglia.

Conclusion

The results from the present study suggests that both crude extracts (methanol and hexane) obtained from leaves of *Moringa oleifera* Lam. have anti-inflammatory effect. It has been demonstrated that the active components from ethanol crude extract from *M.Oleifera* leaves contains isothiocyanate and thiocarbamate. The reduction of nitric oxide production in our experiments may come from the action of these 2 active components. However, further study is necessary.

Acknowledgement

This project was partly supported by Department of Chemistry, Faculty of Science, Mahidol University

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Original article **P30**

The hypoglycemic activity of Thai traditional medicine "Yahom"

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Abstract

Thai tradition medicines called Yahom, has been used for treatment in many chronic disease patients such as hypertension, heart disease, diabetes mellitus, hyperlipidemia. Ten plants are ingredient of this preparation. This study evaluated the ethanolic extract of Yahom preparation and each plant as ingredients of this preparation for promote the glucose transport. The uptake of radioactive 2-deoxyglucose in L6 myotube was used to test hypoglycemic activity assay. The antioxidant activity by DPPH assay was also tested. The extraction method is maceration in 95% ethanol. The result showed Yahom and *Syzgium aromaticum* exhibited good antioxidant activity, while *Syzgium aromaticum* and *Mesua ferrea* Linn. increase glucose uptake. The study conclude that ingredient in this preparation enhance the glucose transport.

Keywords: antioxidation activity, hypoglycemic activity, glucose uptake, L6 muscle cell, Diabetes mellitus

Introduction

Diabetes mellitus is chronic disease and there are going to be the large public health problem of Thailand and all developed country. Many Patients suffer from this disease. The prevalence and incidence of patients rapidly increase every year. Government budget on treatment and health serving of these patients have to increase follow to the number of patients to maintain quality of life of these population group.

Yahom, one of the most popular Thai tradition medicines, has been used for treatment of fainting, nausea, vomiting, flatulance and and unconcious. Containing of several herb in Yahom preparation make it's have many biological activity include anti-inflammation, antibiotic, antifungal, astringent, would healing, antioxidant, anti-glycemic and anti-hypertensive effect. The several pharmacology action indicated that yahom widely use in many chronic disease patients include hypertension, heart disease, diabetes mellitus, hyperlipidemia especially elderly which over 40 years persons. Thus, the aim of this study was focused to investigate the pharmacology activity of Yahom only on hypoglycemic effect by using cell based assay such as L6 muscle cells. The knowledges from this research could support using yahom of folk doctors to treatment Diabetic patients.

Methods

Plant materials and extraction method

Ten plants in Yahom preparation were collected from natural sources on all part of Thailand. All plants were dried in well ventilation open air and ground to give the powder. Each 100 gm. of the powder was macerated by 95 % ethanol for three days, filtrate and evaporate by evaporator and repeat for 3 times, filtrate was concentrated and dry by evaporator. All extracts were kept in -20 °C before reaction testing. The percentage of yield were showed in table 1

Glucose uptake assay [1-6]

L6 muscle cells is the most preferred cell lines to study insulin-stimulated glucose uptake .In this study , cell culture and glucose transport measurements by using monolayers of L6 muscle cell were grown at 37 ^{o}C . in 48-well culture dishes in dulbecco's modified Eagle's medium (DMEM, low glucose), containing 10% fetal bovine serum (FBS), penicillin (100 units ml.$^{-1}$) and streptomycin (100 μg/ml.$^{-1}$) in incubators equilibrated with 5% CO_2. At 95% confluence (day 0) and there after, differentiation was induced and maintained in the low serum (2% horse serum, HS) containing medium. Cells were used to investigate the effects of plant extracts on 2-DG transport between days 7 and 9 at which ~ 80-90% of myotubes were formed. The cells were stimulated in HEPES-buffered saline, pH 7.4 (HBS) solution with 2% HS, 15mM glucose with different concentrations of test agent for 1 h. Control cells were also performed in the absence of test sample for the same incubation periods. At the end of the incubation, the test media were washed out with HBS followed by incubation in HBS-2-dG solution (1 μCi ml^{-1},0.01 μM) for 10 min in the presence of absence of insulin. The radioactivity associated with the cells was determined by cell lysis in 0.05 N NaOH, and then subjected to liquid scintillation counting.

Results and discussion

The percentage of yield from Yahom extract and its ingradients showed in table 1. The highest percentage of yield is *Syzgium aromaticum* (Kaan Phluu), fallowed by *Mammea siamensis* Kosterm.(Saaraphee) and *Mesua ferrea* Linn.(Bunnaak) (33 %, 32% and 30 % respectively).

Table 1 The percentage of yield of the ethanolic extract Yahom preparation and its plant ingredients.

Scientific name	Thai name	% yield	EC_{50} (μg/ml)
Aquilaria crassna	Kritsanaa	4.3	29.36
Syzgium aromaticum	Kaan Phluu	32.55	5.57
Amomum krervanh Pierre.	Krawaan	2.96	82.79
Myristica fragrans Houtt.	Jaan Thet	10.25	23.65
Albizia myriophylla Benth.	Cha Em Thai	22.38	15.64
Jasminum sambac(Linn.) .Ait.	Mali Laa	13.36	100
Mammea siamensis Kosterm.	Saaraphee	31.99	9.12
Nelumbo nucifera Gaertn.	Bua Lung	10.88	100
Mimussops elengi Linn.	Pikun	19.86	74.68
Mesua ferrea Linn.	Bunnaak	30.27	29.01
Yahom		12.65	11.92
BHT			12.23

Yahom extract showed antioxidant activity evaluate by DPPH radical scavenging assay (EC_{50} = 12 μg/ml). The ingredients which exhibited the three highest antioxidant activity are *Syzgium aromaticum* (Kaan Phluu), *Mammea siamensis* Kosterm.(Saaraphee) *and Albizia myriophylla* Benth.(Cha Em Thai) by 5.57, 9.12 and 15.64 μg/ml respectively.

The results of glucose uptake in L6 myotubes evaluated to be fold increase of basal were found that *Mesua ferrea* Linn. (Bunnaak) extract at concentration 100 μg/ml showed the highest potent glucose uptake in cell as 1.5 , *Syzgium aromaticum* (Kaan Phluu) at 100 μg/ml is 1.3 , *Myristica fragrans* Houtt.(Jaan Thet) at 50 μg/ml is 1.3 and *Mimussops elengi* Linn.(Pikun) at 200 μg/ml. is 1.3.

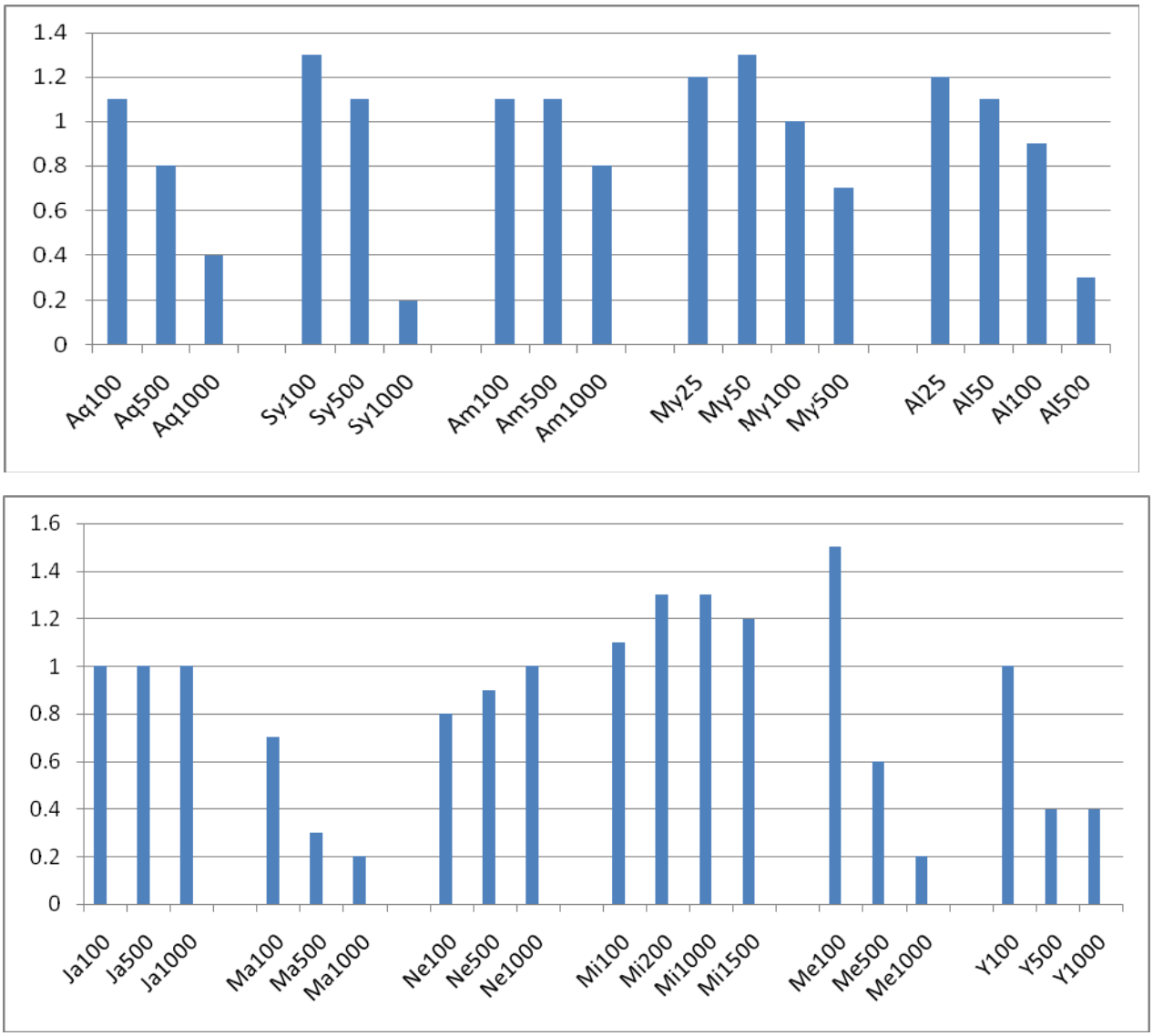

Figure 1, 2 Glucose uptake measure by fold of basal increasing by vary concentration in Yahom and single plants. *(Aq* = Kritsanaa *, Sy* = Kaan Phluu *, Am* = Krawaan *, My* = Jaan Thet , *Al* = Cha Em Thai , *Ja* = Mali Laa *, Ma*=Saaraphee , *Ne* = Bua Lung , *Mi* =Pikun , *Me* = Bunnaak, Y= Yahom)

From these investigation, they showed that *Syzgium aromaticum* (Kaan Phluu) have the hightest antioxidant activity by EC_{50} at 5.57 µg/ml and it also exhibit hypoglycemic effect at concentration 100 µg/ml. as 1.3 fold of basal. This results support theory of antioxidant can reduce glucose uptake or reduce blood sugar. However, the ethanolic extract of *Mesua ferrea* Linn.(Bunnaak) showed the highest hypoglycemic activity at dosage of 100 µg/ml by 1.5 fold of basal but it has low antioxidant activity (EC_{50} = 29 µg/ml.). However, Yahom , showed less active hypoglycemic activity because of this method is not appropriate for yahom which its ingredients have principle component as volatile oil . It should be change in study as adipose cells instead myotube or muscle cells., The result showed that low dose of *Myristica fragrans* extract exhibited more effective than high dose so it is benefit for find plant extract showed high activity by low dose using to treatment disease .

Conclusion

From the study ,*Syzgium aromaticum* (Kaan Phluu) extract enhance glucose transport and highest activity of antioxidant activity, while *Mesua ferrea* Linn.(Bunnaak) showed the highest hypoglycemic activity but low antioxidant activity.

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Original article **P31**

Formulation and stability test of Benjakul extract tablets: a preliminary study

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Abstract

Benjakul is a Thai Traditional medicine preparation, used for balanced health. In our previous study, the ethanolic extract of Benjakul preparation exhibited high cytotoxic activity against COR-L23. In this study, we formulated a Benjakul extract tablet and tested product stability under accelerated condition. A wet granulation method was used in developing the tablets. The suitable excipients were lactose, starch, Explotab® and magnesium stearate. The physical properties of tablets were evaluated following by the USP25 requirements. The results of stability testing found that stability of Benjakul extract tablet related with stability of Benjakul extract from our previous studied. It was indicated that plumbagin is unstable and the cytotoxic activity depend on plumbagin content. Thus, the cytotoxic activity against COR-L23 was also reduced.

Keywords: tablet formulation, stability test, Benjakul extract

Introduction

Benjakul is a Thai traditional medicine preparation which composed of five plants; *Piper longum* fruit, *Piper samentosum* root, *Piper interuptum* stem, *Plumbago indica* root and *Zingiber officinale* rhizome, in equal proportions (1). In our previous study, we found that the ethanolic extract of Bejakul preparation exhibited high cytotoxicity against lung cancer cell lines (COR-L23) with IC_{50} value of 19.80 μg/ml. Two compounds, piperine and plumbagin, were isolated from the extract. Plumbagin exhibited the highest cytotoxic activity against COR-L23 with IC_{50} value of 2.55 μM. The results from stability testing under accelerated condition of Benjakul extract, indicating that plumbagin was unstable (2).

Tablets are solid dosage forms containing medicinal substances that widely used. Plant extracts are often poorly flow ability, low compressible and very hygroscopic. In addition, tablets are appropriately containing high amount of extract show prolong disintegration times, affecting the release of active constituents (3). Thus, wet granulation method often used to improve the properties of products from plant extracts.

From this result, the development of appropriate dosage forms is necessary to increase the stability and to make them suitable for use. Thus, the aims of this study were preliminary study formulation of tablets from Benjakul extract using wet granulation method and investigation the tablet stability under accelerated condition.

Methods

Preparation of Benjakul extract

Plant materials were dried at 50°C and powdered. All plants in equal portion were mixed and extracted by macerated with 95% ethanol for 3 days, filtered and concentrated to dryness under pressure. The marc was macerated 2 times and dried by evaporator.

Tablet formulation

The tablet, containing 50% of extract, was prepared by conventional wet granulation method. Various excipients were screened before the tablet formulation study. According to quality control of product development, the physical properties that consisted of shape, color, weight variation, hardness, disintegration and friability were identified in accordance with the USP25 requirements.

Stability testing

The stability testing was performed according to Thai FDA guideline on stability testing of drug product (4). The tablets, packed in close amber glass containers, were stored under accelerated conditions (45 ± 2°C with 75 ± 5% RH) for 4 months and randomly sampled every 4 weeks interval for analyzing the percent remaining of pipierine and plumbagin contents by HPLC (2) and determining its cytotoxic activity against COR-L23 by SRB assay (5).

Results and discussion

Benjakul extract tablet formulation

From excipients screening, the suitable excipients were used: lactose as a diluent, starch as a binder, Explotab® (sodium starch glycolate and sodium carboxymethyl starch) as a disintegrant and magnesium stearate as a lubricant. Tablets were compressed by single punch machine and adjust weight about 500 mg/tab. Percentages of each ingredient showed in Table 1.

Table 1 Tablet formulation of Benjakul extract by wet granulation.

Ingredients	Percent (%)
Benjakul ethanolic extract	50
Lactose	q.s.
Starch	q.s.
Starch for paste	q.s.
Explotab®	4
Magnesium stearate	1.2

The physical properties of Benjakul extract tablet

Benjakul extract tablets were analyzed by physical properties, the data showed in Table 2. The tablet characteristics were smooth, shiny surface and round in shape. Because of the extracted was dark brown in color, so the tablet was darker brown in color. This prepared tablet had a weight variation of 491.1 ± 9.5 mg, a hardness of 6.7 ± 0.6 kg, a percentage friability of 0.02% and a disintegration of 10.7 ± 1.2 minutes. All the physical properties were allowed in the requirements of the USP 25 standard.

Table 2 Physical properties of Bejakul extract tablet formulation.

Physical properties	Benjakul extract tablet (500 mg/tab)
Shape	Round
Color	Dark brown
Weight variation (mg)	491.1 ± 9.5
Hardness (kg)	6.7 ± 0.6
Friability (%)	0.02
Disintegration time (min)	10.7 ± 1.2

All data are mean±SD as obtained by triplicate analyses.

Stability of Benjakul extract tablet

The results of piperine and plumbagin contenting and cytotoxic activity against human lung cancer cell (COR-L23) were showed in Table 3 and Figure 1. Piperine was slightly reduced (about 15.09% from day 0) and it remained to be 27.71 mg/g after day 120. By the contrast of piperine, plumbagin content was reduced quickly and it could not be detected after day 120. In addition, the IC_{50} value of Benjakul extract tablet was also change from 39.77 µg/ml to be 61.66 and 91.56 µg/ml after day 60 and 120. Its results indicated that cytotoxic activity of this preparation depend on plumbagin content which this compound can evaporate in high temperature.

This result related with stability of Benjakul extract from our previous studied which indicating that plumbagin was unstable but piperine exhibited as a stable compound. It's illustrated that the amount of plumbagin was significantly reduced under high temperature due to its low melting point of 78-79 °C and can be sublimated easily. Therefore, the cytotoxic activity was also reduced due to increasing of IC_{50} value. Thus, the uncoated tablet formulation cannot improve the stability of Benjakul extract.

Table 3 Piperine and plumbagin content (mean ± SD) of Benjakul extract tablet after stored under accelerated condition (n=3)

Day	Piperine content (mg/g)[a]	Plumbagin content (mg/g)[b]
0	30.04 ± 0.17	2.77 ± 0.03
30	29.22 ± 0.70	1.02 ± 0.09
60	26.86 ± 1.58	0.76 ± 0.03
90	28.08 ± 0.14	0.47 ± 0.01
120	27.71 ± 0.57	ND

ND = cannot detected

[a] Calculated as the linear equation: $y = 19388x - 5076.5$, $r^2 = 0.9999$ (**y** = peak area, x = conc. of sample)

[b] Calculated as the linear equation: $y = 28888x - 39265$, $r^2 = 0.999$ (**y** = peak area, x = conc. of sample)

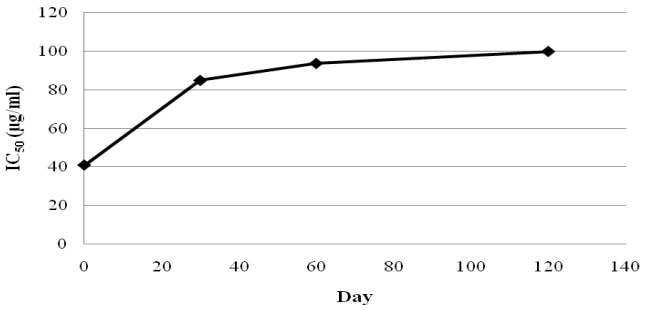

Figure 2 Cytotoxic activity (IC_{50}) against COR-L23 of Benjakul extract tablet after stability testing.

Conclusion

In summary, Benjakul extract tablet was prepared by wet granulation and the suitable excipients were used lactose, starch, Explotab® and magnesium stearate. All the physical properties of tablet were accepted by the requirements of the USP 25 standard. For stability testing, the Benjakul extract tablet was unstable both chemical and biological activities.

Because of its unstable, Benjakul extract tablet should be further developed by using another tabletting technique, such as film coated tablet, to improve the stability of product or avoid the method of preparing tablets which had to used high temperature.

Acknowledgements

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Original article **P32**

Evaluation of antinociceptive activity of the ethanolic extract from *Scaphium lychnophorum* (Hance) Pierre fruit in mice

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Abstract

The antinociceptive activity of the ethanolic extract from the fruit of *Scaphium lychnophorum* was assessed in mice using *in vivo* animal models included the acetic acid-induced writhings, formalin, and hot plate tests. The acute toxicity of the ethanolic extract was also performed in mice. The results demonstrated that the ethanolic extract from the fruit of *Scaphium lychnophorum* markedly showed the antinociceptive activity at doses of 50, 100 and 200 mg/kg, po when compared with the control ($p < 0.05$). The estimated LD_{50} in mice was more than 5 g/kg.

Keywords: *Scaphium lychnophorum*, Antinociceptive, Ethanolic extract

Introduction

Scaphium lychnophorum (Hance) Pierre is in Sterculiaceae family which is commonly known in Thai as Samrong. This plant is mainly distributed in Vietnam, Thailand (Ubon Ratchathani and Chanthaburi), Malaysia, Indonesia, as well as South China (1). It has been used in folk medicine for relieving various symptoms such as pain, cough and clear phlegm, and used as antipyretics**.** This study examined the antinocciceptive potential of ethanol extract in mice using different concentrations (50, 100 and 200 mg/kg). The aim of this study was to investigate the antinociceptive activity from fruits of *Scaphium lychnophorum* (Hance) Pierre in animal models.

Materials and methods

Plant material: The plant collected from Chanthaburi province which is located in the East of Thailand.

Experimental animals: Male Swiss albino mice were used in the experiments. All animal obtained from the southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand, were kept in room of controlled conditions of 24 ± 1ºC and 12 h light – 12 h dark cycles. All experiments were approved by Animal Ethics Committees, Prince of Songkla University, Thailand.

Preparation of the plant extract and reference drugs**:** 2.6 kg of the dried fruit *Scaphium lychnophorum* (Hance) Pierre was pulverized to give 2.6 kg of coarse powder. The powder obtained was macerated 2 times with 7.5 L and 5.5 L of ethanol, respectively, and left for 7 days at room temperature. The combined filtrate was filtered, and the filtrate was evaporated under reduced pressure and lyophilized to give a total semi-solid brownish-green residue of 41.57 g (yield 1.6%, w/w) which was stored and kept in temperature below 4 ºC until tested. The ethanolic extract of *Scaphium lychnophorum* (Hance) Pierre (EESL) at doses of 50, 100

and 200 mg/kg were prepared in cosolvent (distilled water: Tween 80: propylene glycol; 5:1:4). Aspirin, morphine sulphate and naloxone were used as reference drugs.

Assessment of antinociceptive activity

Acetic acid-induced writhings: 0.6% acetic acid in 0.9% normal saline was intraperitoneally injected in mice (10 ml/kg). The EESL at doses of 50, 100 and 200 mg/kg were given orally to the test groups. Cosolvent (10 ml/kg, po) and aspirin (200 mg/kg, po) were given to mice in the control group. The mice were observed and counted for the number of abdominal constrictions and stretchings in a period of 0-20 min. This method was done as previous described (2).

Formalin test: The control and reference groups received cosolvent (10 ml/kg, po) and aspirin (200 mg/kg, po), respectively. The EESL at doses of 50, 100 and 200 mg/kg were given orally to the test groups. After 30 min of treatment (except only 15 min for morphine), 20µl of 2.5% formalin in saline was injected subcutaneously into hind paw of each mouse. The times spent in the licking hind paw in early phase (0-5 min) and late phase (15-30 min) were recorded. This method was done as previous described (3).

Hot plate test: The animals were placed on a hot plate at temperature of 55 ± 0.5 ºC of maximum time of 45 sec. The animal test groups were treated with different doses (50, 100 and 200 mg/kg, po) of the extract. Reaction times were recorded when the animals licked and flicked of hind paw or jumping at 30, 45, 60, 75 and 90 min after oral administration of the extract. This method was done as previous described (4).

Evaluation of acute toxicity: The up and down procedure for acute toxicity (LD_{50}) testing was carried out as previously described (5). Using strategy for acute toxicity testing, the animal is dosed one at a time. If an animal survives, the dose of next animal is increased. But if it died, the dose is decreased. Behavior parameters observed after administration were convulsion, hyperactivity, sedation, grooming and loss of righting reflex. Food and water were provided *ad libitum*.

***Statistical analysis*:** The data obtained were analysed as a mean ± SEM. Statistically significant differences between groups were calculated by the application of analysis of variance (ANOVA). *p*-Values less than 0.05 ($p<0.05$) were used as the significance level.

Results

Antinociceptive activity

1.1 Acetic acid-induced writhings: In acetic acid induced writhing response test, aspirin at dose 200 mg/kg and EESL at all doses used in this experiment significantly inhibited writhing compared to the control ($p<0.05$) (Fig.1). The percentage of inhibition treated with aspirin (200 mg/kg, po) was 64.6%, and with EESL at the concentrations of 50, 100 and 200 mg/kg were 13.9, 35.4 and 62.79%, respectively.

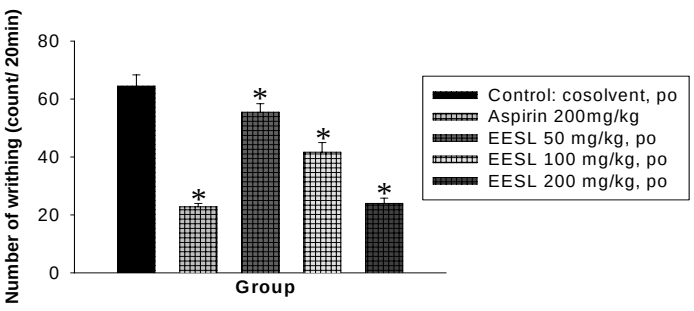

Figure 1: The antinociceptive activity of *S. lychnophorum* on acetic acid-induced writhing responses in mice. Each value presents mean ± S.E.M (n=6); *$p<0.05$

1.2 Formalin test: EESL dose-dependently decreased the licking activity in early phase and late phase. In early phase, all doses of the extract, 50, 100 and 200 mg/kg significantly inhibited by 10.69, 23.5 and 26.43% respectively, and late phase they significantly inhibited by 12.61, 30.37 and 67.62%, respectively.

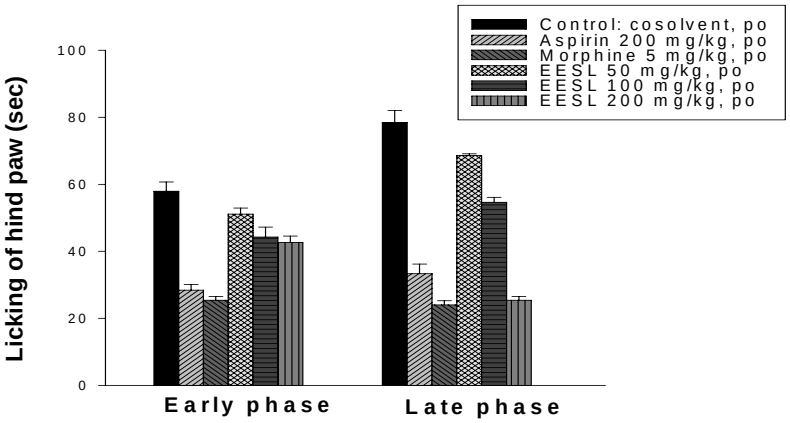

Figure 2: The antinociceptive activity of *S. lychnophorum* on formalin-induced paw licking in mice. Each value presents mean ± SEM (n=6); **p*<0.05

1.3 Hot plate test: EESL (50, 100 and 200 mg/kg, po) significantly exerted protective effect on heat-induced pain in mice. Naloxone (2 mg/kg, ip) before EESL (200 mg/kg, po) significantly decreased latency of nociceptive response (***p*<0.01) (Fig. 3B).

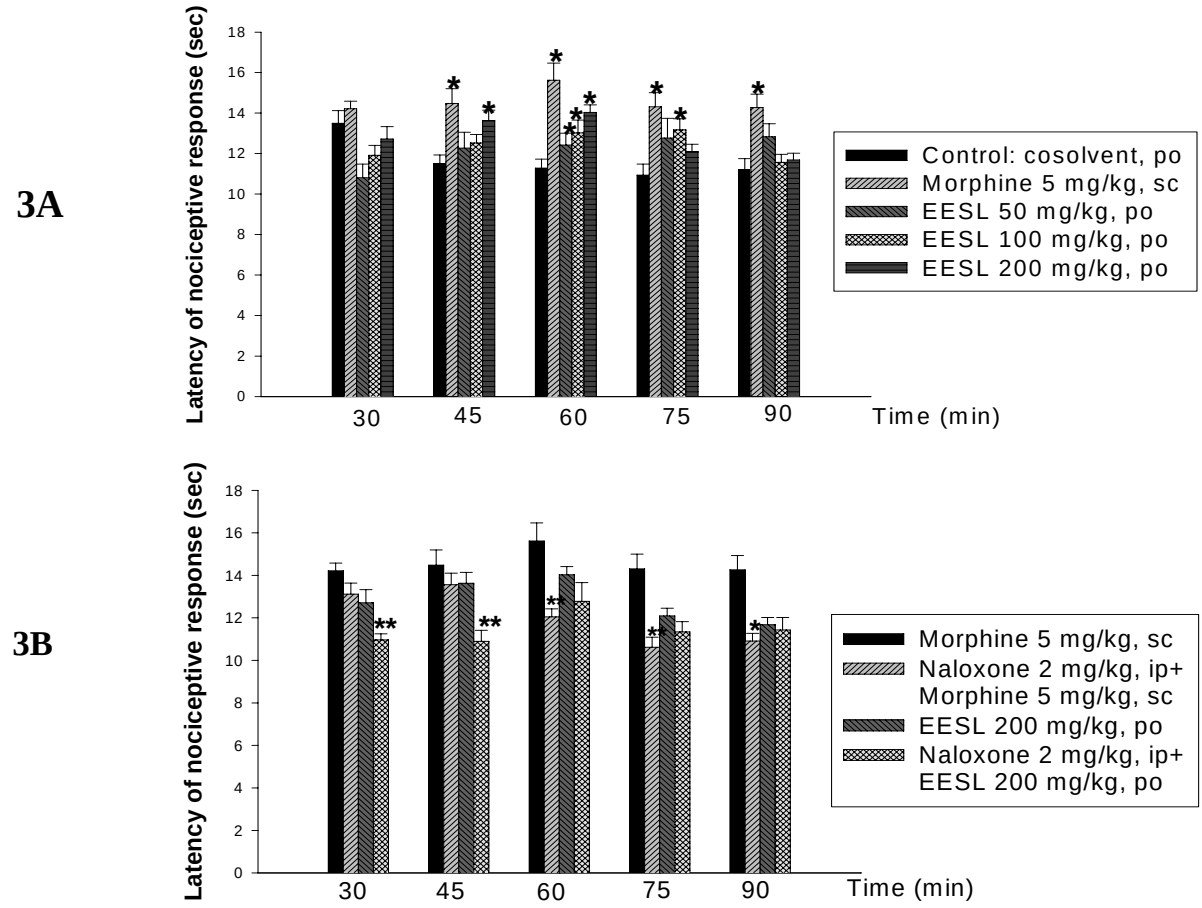

Figure 3: **3A:** The antinociceptive activity of *S. lychnophorum* on heat-induced pain in mice. Each value presents mean ± SEM (n=6); **p*<0.05. **3B:** Antagonist effects of naloxone (2 mg/kg, ip.) on morphine (5 mg/kg, sc.) and ethanol extract of *Scaphium lychnophorum* (EESL) at dose 200 mg/kg, po on heat-induced pain in mice. Each value presents mean ± SEM (n=6)**;***p<0.05, **p<0.01

Acute toxicity: EESL at the dose of 5g/kg, po given to mice had no affect on their behavioral responses and did not cause the mortality in mice during the observation period of 8 h and 7 days after administration. Estimated LD_{50} in mice was more than 5 g/kg.

Discussion and conclusion

The hot plate and formalin tests have been extensively used for evaluation of centrally acting analgesic activity. The mediator such as substance P involved in this mechanism and it acts as a neurotransmitter released from C fibers found within nociceptive primary afferent neurons into the spinal cord and mediates a part of the excitatory synaptic input to nociceptive neurons at this level (6). The writhing test generally used for screening of antinociceptive activity of various drugs. In acetic acid induced abdominal writhing test is the visceral pain model involved in the process of release arachidonic acid via cyclooxgenase and prostaglandin biosynthesis which play an important role in nociceptive mechanisms (7). In the present study, The extract at tested doses were shown to possess the antinociceptive activity in writhing and formalin and hot plate tests in dose-dependent manners. In conclusion, The EESL markedly posseses antinociceptive activity which supports the traditional uses of *S. lychnophorum* for the treatment of pain in folk medicine preparations.

Acknowledgement

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Original article **P33**

Inhibitory effect of *Harrisonia perforata* root extract on macrophage activation

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Abstract

Root of *Harrisonia perforata* Merr. (*Simaroubaceae*) is one of compositions in Thai herbal remedy named Bencha-Loga-Wichien which has been used as an alternative medicine for the treatment of pyresis. The direct effect of *H.perforata* on LPS-activated macrophage, J774A.1 cells, were investigated in this study. The ethanol extract of *H.perforata* root inhibited LPS-activated nitric oxide (NO) production in a concentration-dependent manner. This inhibition was corresponded to the decrease in mRNA expression of inducible nitric oxide systhase (iNOS) in the extract treated LPS-activated J774A.1 cells. The extract also decrease the mRNA expression of the cyclooxygenase-2 (COX-2) which is the enzyme required for large amount of prostaglandin synthesis in activated macrophage during inflammatory process. These results indicated that the ethanol extract of *H.perforata* root has direct inhibitory effect on macrophage activation. It may have not only antipyretic effect but also anti-inflammatory activity.

Keywords: *Harrisonia Perforata,* inflammation, NO, iNOS, COX-2.

Introduction

Inflammation is a protective response of host against phathogen, chemical or physical stimuli. Several immune cells involve in the inflammatory process as well as macrophages. Macrophages in the inflammation area are activated by the components of pathogenic microorganism invading the host such as LPS which is an endotoxin derived from the cell wall of Gram negative bacteria. Activated macrophages express various components on their cell surface and synthesize several intracellular and extracellular mediators involve inflammation such as pro-inflammatory cytokines (TNF-α, IL-1, IL-6 and IL-8), NO, superoxide free radicals and PG_s. These mediators lead to cardinal signs of inflammation; pain, edema, red and fever. Many anti-inflammatory agents inhibit synthesis or functions of these pro-inflammatory and inflammatory mediators[1]. Several alternative medicine as well as herbal medicines are also used to treat inflammation. *Harrisonia perforata* (khon-thaa) root is one in five roots in Bencha-Loga-Wichien remedy which is used in Thai traditional medicine as antipyretics. Several compounds from this plant roots were identified including 2-hydroxymethyl-3-methylalloptaeroxylin, heteropeucenin-7-methylather, perforatic acid, lupeol, 5-hydroxy-6-7-dimethoxycoumin, β-sitosterol, campesterol, stigmasterol, β-sitosteryl-3-0-glucopyranoside, stigmasteryl-3-0-glucopyranoside, chloresteryl-3-0-glucopyranoside[2]. The ethanol extract of *H.perforata* has been reported to have anti-asthmatic, anti-infective and antipyretic activities[3][4]. In this study we investigate the direct inhibitory effect of the ethanol extract from roots of *H.perforata* on LPS-activated macrophages, J774A.1.

Methods

Plant extract: The 95% ethanol extract from dried root powder of *H.perforata* was dissolved in dimethylsulfoxide (DMSO) as the stock solution. The final concentration of this extract were prepared in DMSO 0.2%.

Cells: The murine macrophage, J774A.1, were obtained from ATCC. The cells were maintained in the completed DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37^{o}C in 5% CO_2 / 95% air.

Effect of the extract on NO production:

J774A.1 cells (2x105 cells/ml) were treated with the ethanol extract of *H.perforata* roots at 3.125-50 μg/ml (5 concentrations) for 24 h. Ten μM dexamethasone was used as the positive control. The treated cells were activated with 100 μg/ml LPS for 24 h. The supernatants were used to measure the amount of released NO in nitrite form by Griess reagent. Nitrite concentration was determined by a standard curve prepared with sodium nitrite. The percentage of NO inhibition of the ethanol extract was compared to the LPS – activated cells without the extract. The treated cells were determined for their viability by staining with rezasurin(blue dye) which is reduced in viable cells to its substrate, rezorufin(red product). The amount of rezorufin production was determined at 570 and 600 nm. The percentage of cells viable of the extract-treated cells was compared to the LPS– activated cells without the extract

Effect of the extract on the mRNA expression of iNOS and COX-2 :

J774A.1 cells (2x105 cells/ml) were treated with the ethanol extract at the concentrations of 12.5,25 and 50 μg/ml for 24 h. Ten μM dexamethasone was used as the positive control. The treated cells were then activated with 100 ng/ml LPS for 24 h. Total RNA was isolated from the treated cells using Trizol reagents and then reversed to cDNA using reverse transcription system kit. The cDNA was used as the template to amplified mRNA of iNOS and COX-2 with specific primers for iNOS and COX-2 genes. The PCR products were run on 1.5% agarose gel electrophoresis and measured their densities by gel documentation.

Statistical analysis:

Data were presented as means.±.S.E.M. One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was used to determine the statistical significance analysis. The p-value<0.05 was considered as statisticaly significance.

Result

Effects of *the ethanol extract* on NO production in LPS-stimulated J774A.1 cells

When compared to the untreated LPS-stimulated J774A.1, the extract suppressed NO production in LPS-activated cells in a concentration-dependent manner with its IC_{50} 23.14 μg/ml (Fig. 1A). It didn't have any effect on J774A.1 cell viability at all concentrations used in the study (Fig.1B).

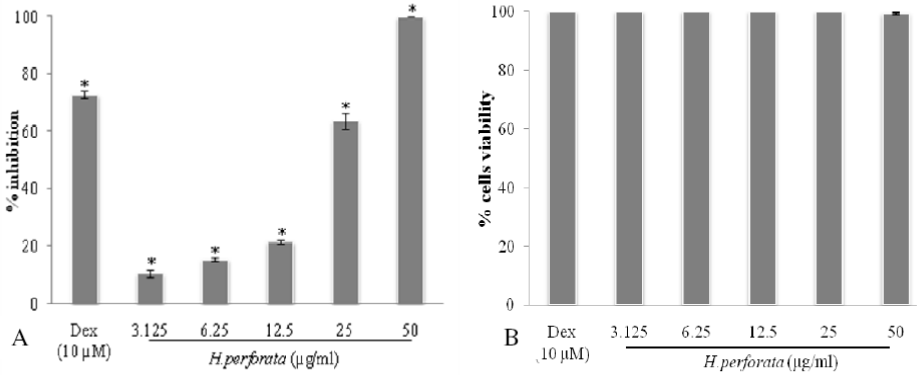

Figure1. Effects of the ethanol extract from *H.perforata* roots at the concentrations 3.125-50 µg/ml on NO production(A) and on cell viability (B) in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 4 independent experiments (n=4). *$P < 0.05$ compared to untreated cells.

Effects of the extract on the mRNA expression of iNOS and COX-2.

The extract decreased the mRNA expression of iNOS and COX-2 in a concentration dependent manner (Fig.2.). The inhibitory effect of the ethanol extract on iNOS mRNA expression is correlated to its effect on NO production.

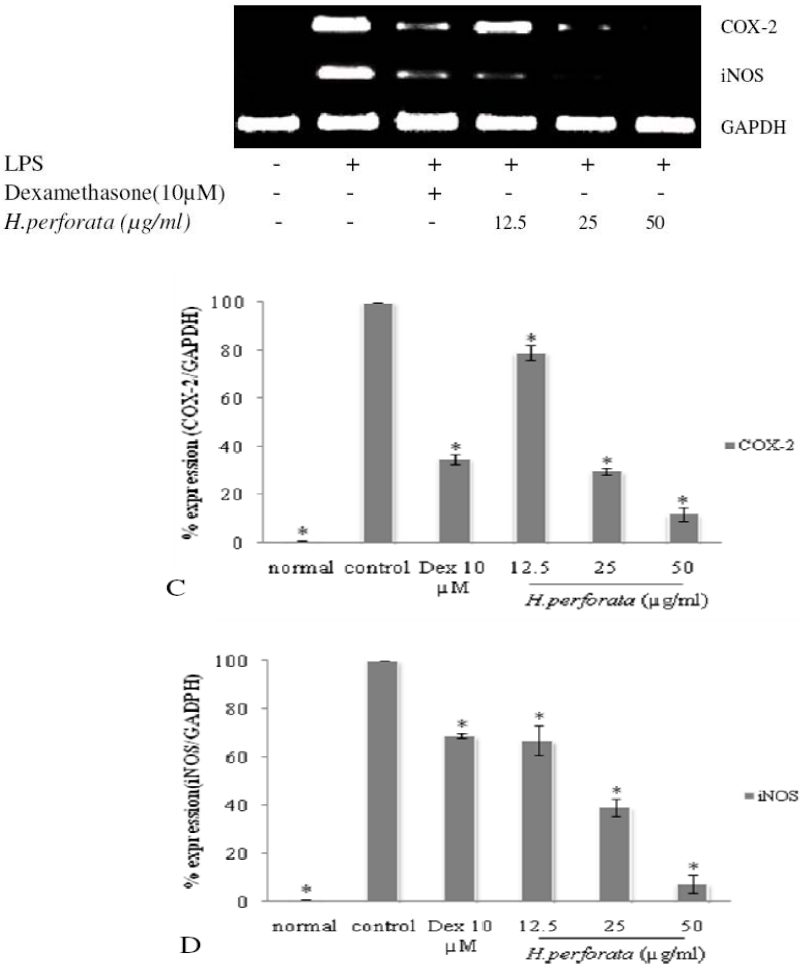

Figure2. Effects of the ethanol extract from *H. perforata* roots on mRNA expression of COX-2 (C) and iNOS (D) in LPS-stimulated J774A.1 macrophage cells. The data are expressed as mean ± S.E.M from 3 independent experiments (n=3). *$P < 0.05$ compared to untreated cells.

Discussion

Harrisonia perforata root is used one of the components in Thai traditional medicine, Bencha-Loga-Wichien, which has been used as antipyretics. Fever is one of the cardinal signs of inflammation and infection. Activated macrophages are the source of many pro-inflammatory cytokines and several mediators involve inflammatory process. In this study, we examined the effect of the ethanol extract of *Harrisonia perforata* root on macrophage activation. We demonstrated that the ethanol extract inhibited LPS-activated murine macrophage, J774A.1 cells. It suppressed NO production as well as iNOS mRNA expression in a concentration dependent manner in LPS-activated J774A.1 cells. It also significantly decreased the mRNA expression of COX-2 in the activated cells. It is known that iNOS and COX-2 are responsible to the large amounts of NO and prostaglandin E2 production, respectively. Both NO and PGE2 are the important inflammatory mediators in fever and inflammatory diseases. These results suggest that *H.perforata* extract may have therapeutic potential in reducing fever and inflammation.

Conclusion

Our results demonstrated that the ethanol extract of *H.perforata* roots decreased the expression of iNOS and COX-2 which are responsible for NO and PGE2 production. *H.perforata* roots might the source of an effective therapeutic agent for not only fever but also various inflammatory diseases.

Acknowledgements

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Original article **P34**

Effect of *Curcuma comosa* on the expression of cytokine genes in rabbits fed with high cholesterol diet

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Abstract

Curcuma comosa Roxb. (Zingiberaceae), an indigenous plant in Thailand, has been used as an anti-inflammatory agent in post-partum uterine bleeding. It was shown to lower plasma lipid levels thus potentially be used in cardiovascular disease. Lipid lowering drugs like HMG-CoA reductase inhibitor including simvastatin were implicated with manifestation of liver toxicity. In the present study, we therefore investigated the possibility of liver toxicity of *C. comosa* by assessing the expression of pro-inflammatory cytokine genes as well as the serum liver enzymes in rabbits fed with high cholesterol diet combined with *C. comosa* compared to the rabbits fed with high cholesterol diet either alone or combined with simvastatin for three months. The results showed that serum liver enzymes and pro-inflammatory cytokine expression of *C. comosa* treatment group were not significantly different as compared to the high cholesterol diet control group. On the other hand, rabbits in high cholesterol diet with simvastatin treatment group significantly demonstrated an increase of alanine aminotransferase level and the expression of pro-inflammatory cytokines, MCP-1 and TNF-α as compared to the high cholesterol diet group. The pharmacological activity reported earlier and the safety regarding liver toxicity shown in this study suggested that *C. comosa* is a potentially promising candidate to be developed as an alternative agent for cardiovascular disease therapy.

Keywords: *Curcuma comosa*, liver toxicity, cytokine, IL-1, MCP-1, TNF-α

Introduction

Cardiovascular disease is the leading cause of death worldwide. Primary goal of the treatment is to prevent further morbidity and mortality from coronary heart disease (CHD) and the associated conditions. HMG-CoA reductase inhibitor is the drug of choice for lowering serum lipid in CHD treatments because of its low cost and capability to reduce the risk of ischemia in CHD patients. However, the main hindrance of drugs in this group is its liver toxicity as indicated by elevated serum alanine aminotransferase level. Therefore, efforts were tried to develop more efficient CHD drug with lower or no toxicity. *Curcuma comosa* Roxb. (Zingiberaceae) is an indigenous plant in Thailand with a common name in Thai as Wan Chak Mot Luk. It has been used traditionally as an anti-inflammatory agent in post-partum uterine bleeding. Besides the estrogenic activity, it was also found to process choleretic effect, hypocholesterolemic effect (1) and anti-atherogenic effect (2). These beneficial effects of *C. comosa* is promising to be developed as an alternative agent for CHD therapy. In addition to the pharmacological effects, safety information in term of toxicities especially liver toxicity is needed. Therefore, the aim of this study was to investigate the

possibility of liver toxicity of *C. comosa* by assessing the expression of pro-inflammatory cytokine genes as well as the serum liver enzymes in rabbits fed with high cholesterol diet combined with *C. comosa* compared to the rabbits fed with high cholesterol diet either alone or combined with simvastatin.

Materials and Methods

Materials: *C. comosa* powder was kindly provided by Professor Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkamhaeng University. Simvastatin was purchased from an accredited drug store (Bangkok, Thailand).

Animals and treatment: Twelve male New Zealand white rabbits of body weight between 1.5 – 2.0 kg were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed one per cage at the Faculty of Medicine, Srinakharinwirot University, Thailand. All animals were in a controlled humidify room at a constant temperature of 25 ± 2 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to food (C.P. Company, Thailand) and drinking water. Prior to the experiment, they were randomly divided into three treatment groups of 4 rabbit each. All treatment groups were given orally with 0.1% cholesterol for 1 month. After 1 month, rabbits in group 1, 2, 3 were given orally for 3 months with 0.5% cholesterol, 0.5% cholesterol combined simvastatin at the dosage of 5 mg/day and 0.5% cholesterol combined *C. comosa* at the dosage of 400 mg/kg/day, respectively.

Blood and liver sample collection: Blood samples were collected from 12 hours fasted rabbit at the end of treatment. Plasma were separated and analyzed for liver function parameters (alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline phosphatase, ALP) using auto-analyzer (Hitachi 917) at Professsional Laboratory Management Corp Co., Ltd., Bangkok. Liver samples were kept at -80 °C until assay.

RNA extraction and reverse transcription: RNA was extracted using Trizol® reagent according to the manufacturer's instruction (Invitrogen, USA). RNA quantity was measured by spectrophotometer at 260 nm absorbance and stored at -80 °C. Process of reverse transcription was described in RT script kit's manual protocol (USB, USA). cDNA was synthesized by MMLV-reverse transcriptase and using random hexamer as probe. The condition for reverse transcription was 42 °C for an hour.

Real-time RT PCR: The expression of cytokine genes analysis was performed with iQ™ multicolor real-time PCR detector system and iQ™ SYBR® Green Supermix as recommended by the manufacturer. The cDNA templates were probed with the specific primers (Table 1) designed by PerlPrimer version 1.1.18 for IL-1 (Accession No M26295.1), MCP-1 (Accession No NM_001082294.1) and TNF-α (Accession No NM_001082263.1) while GAPDH (Accession No NM_001082253.1), an internal control gene, was used as previously reported (3). Amplification was performed under the condition using thermocycler for 40 cycles at 95 °C 30 seconds, specific annealing temperature (Table 1) 30 seconds and 72 °C 30 seconds. The calculation of the Threshold cycle (Ct) was performed using iQ™ optical system software version 2.0. Amplification's specificity was verified by melt curve analysis and gel electrophoresis.

Statistical analysis: The data were analyzed by using one-way analysis of variance (one-way ANOVA) calculated from SPSS version 16.0 software program (SPSS Inc., USA). The significant different between group is at the level of p-value <0.05.

Table 1 Primer and specific annealing temperature for PCR amplification

Cytokines	Primer sequences	Annealing temperature
IL-1	sense: 5'-CAA CAA GTG GTG TTC TCC AT-3' anti-sense: 5'-GAG GTG CTG ATG TAC CAGT-3'	55.0 °c
MCP-1	sense: 5'-CTT CTG TGC CTG CTG CTC ATA G-3' anti-sense: 5'-TGC TTG GGG TCA GCA CAG AT-3'	57.1 °c
TNF-α	sense: 5'-AGA TGG TCA CCC TCA GAT CAG-3' anti-sense: 5'-GAA GAG AAC CTG GGA GTA GAT GAG-3'	61.4 °c
GAPDH	sense: 5'-CAT CAT CCC TGC CTC CAC T-3' anti-sense: 5'-GCC TGC TTCA CCA CCT TCT T-3'	65.0 °c

Figure 1 showed that rabbit-fed high cholesterol diet with simvastatin significantly increased the expression of MCP-1 and TNF-α as compared to the rabbit-fed high cholesterol control and the rabbit-fed high cholesterol diet with *C. comosa*. The rabbit-fed high cholesterol diet with *C. comosa* did not affect all pro-inflammatory cytokines when compared to the rabbit-fed high cholesterol control.

Table 2 Liver functions at 4 months of rabbits

Groups	Liver functions		
	AST (U/L)	ALT (U/L)	ALP (U/L)
0.5% cholesterol	51.00 ± 8.93	34.00 ± 4.76	65.75 ± 14.59
0.5% cholesterol + 5 mg/day simvastatin	73.00 ± 7.70	102.50 ± 26.21*	81.25 ± 19.29
0.5% cholesterol + 400 mg/kg/day *C. comosa*	53.75 ± 4.97	28.00 + 1.00	47.75 ± 3.25

Value were shown as the mean ± SEM (n = 4). *p<0.05 significant difference from high cholesterol-fed control group. ALT = alanine aminotransferase, AST = aspartate amino transferase, ALP = alkaline phosphatase.

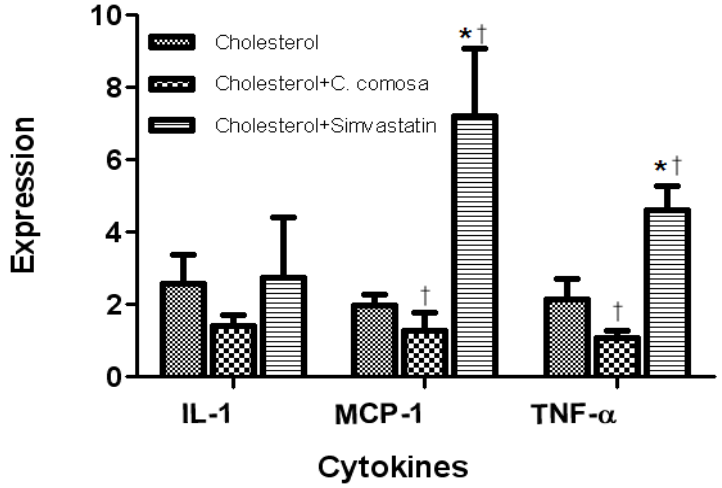

Figure 1 Effect of *C. comosa* on the expression of cytokine genes

Data were shown as the mean ± SEM (n = 4). *p<0.05 significantly different from high cholesterol diet fed control group.
† p<0.05 high cholesterol-diet with *C. comosa* vs high cholesterol-diet with simvastatin.

Discussion

Among the lipid lowering drugs used in Thai patients in term of cost-effectiveness, simvastatin is the most popular as compared to atorvastatin and rosuvastatin (4). Simvastatin not only reduces LDL cholesterol but also possesses pleiotropic effect on other cellular functions including anti-inflammatory effect and anti-oxidation. These effects not only provide the advantageous effects on blood vessel but the pleiotropic effect can also induce liver injury by inhibition of selenoproteins (5). Most cases with liver injury were associated with an increase of serum ALT (6). Rabbit-fed high cholesterol diet with *C. comosa* at 400

mg/kg/day for 3 months was previously found to reduce plasma LDL-cholesterol (7). Serum and liver samples from these groups of animals were used in this study to investigate the toxicity of *C. comosa* and simvastatin in the livers. The results from this study showed that serum ALT was significantly increased in rabbit-fed high cholesterol diet with simvastatin indicating the liver toxicity as previously seen in human (8) while *C. comosa* did not affect ALT level.

To further investigate the cytokine gene expression to explain the liver toxicity data for *C. comosa* and simvastatin, in this study we found that simvastatin treatment induced pro-inflammatory cytokines, MCP-1 and TNF-α, gene expression while *C. comosa* did not. This implied that there was a direct injury on hepatocyte to induce cytokines expression and thus overcome the anti-inflammatory effect of simvastatin. In contrast, *C. comosa* treatement did not significantly increase serum liver enzymes. Consistently, pro-inflammatory cytokine expression did not significantly increase but tend to diminish instead.

Conclusion

In conclusion, *C. comosa* treatment at 400 mg/kg/day in rabbits fed with high cholesterol diet did not change serum liver enzymes after three months of the treatment. Consistently, the expression of pro-inflammatory cytokines including IL-1, MCP-1 and TNF-α were not affected. These findings may provide the possibility that *C. comosa* is a promising candidate to be developed for coronary heart disease therapy without liver toxicity. However, the benefits and toxicities of *C. comosa* require further experimental and clinical studies.

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Original article **P35**

Effects of curcuminoid on ethanol-induced toxicity in hepatic cells and rats

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Abstract

Alcoholic liver disease (ALD) is caused by excessive consumption of alcohol. The pathological progress of ALD involves in increasing reactive oxygen species (ROS) and nitric oxide (NO) production, cytokines secretions, and inflammatory reactions. Curcuminoid, a mixture of active substance derived from turmeric compound, has exhibited an antioxidant and anti-inflammatory properties. This study aimed to examine effect of curcuminoid on NO production in ethanol-stimulated hepatic cells and on hepatoprotective effect in ethanol-induced toxicity rats. We found that curcuminoid at lower concentration (0.313 and 0.625 μg/ml) tended to reduce NO production in the ethanol-stimulated cells. In addition, curcuminoid at concentration 500 and 750 μg/ml decreased the liver function enzymes significantly in the ethanol-induced toxicity rats. The results suggested that curcuminoid had a potential property to be use as a hepatoprotective agent in ethanol-induced hepatic toxicity.

Keywords: alcoholic liver disease, ethanol, nitric oxide, hepatoprotective agent, curcuminoid

Introduction

The excessive consumption of alcohol has been identified as the leading cause of ALD. The pathological changes of ALD range from fatty liver (steatosis), hepatic inflammation and cell injury (hepatitis), cell fibrosis (cirrhosis) and finally, hepatocellular carcinoma. (1) On the early stages, alcohol increases fatty molecule in the hepatic cells, lipid peroxidation and ROS that resulting in initiation of inflammatory process. (2) On the other hand, the direct alcohol toxicity is contributed from its metabolism pathway to ROS production. (3) Several studies have shown that NO, an interesting ROS generated from hepatic cells involve in both physiological and pathological roles in the liver. (4, 5) NO, an endogenous gas with short half-life (< 10 seconds) is synthesized from L-arginine by enzyme nitric oxide synthase (NOS). (6) Under normal condition, the hepatic cells produce low level NO to regulate vascular perfusion, however, in ALD, the large amounts of NO are produced. (7) The mechanisms of alcohol induced hepatic toxicity are complex with diverse consequences in different cell types and tissues. In addition, there is no pharmacological agent for ALD treatment. Now a day, one idea of developing hepatoprotective agent from herbal plants to reduce production of ROS is remarkably under investigation. Curcuminoid, a yellow pigment in turmeric compound is derived from *Curcuma Longa Linn* in ginger family. (8) Curcuminoid has been shown variety of pharmacological actions such as anti-inflammatory, antimicrobial, antioxidant and anticarcinogenic properties. (9, 10) Curcuminoid interacts with numerous target molecules; enzymes, transcription factors, growth factors, receptors or metals, that supports the notion of its influence of many biological cascades. (10) We are interested in the effect of curcuminoid as a hepatorprotective agent against alcohol-induced toxicity. The aim of this study was to investigate effects of curcuminoid on NO production in ethanol-stimulated cells and on aminotransferase enzymes in rats.

Methods

Cell culture: The human liver cell line, Hep G2 cells were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum and 1% penicillin-streptomycin to 90% confluence.

Effect of curcuminoid on nitric oxide production: All cells were plated into 96-black well plates at density $3x10^4$ cell/well for 24 hours. Then, the medium was removed and cells were pre-treated with various concentrations of curcuminoid in serum free medium for 2 hours. After that cells were added with10% (V/V) of ethanol for 22 hours. The diaminofluorescein-2 diacetate (DAF-2 DA) was added to the wells and incubated for 30 minutes in dark. Fluorescence was read at excitation 485 nm, emission 535 nm. The cells were lysed and measured protein content by BCAkits®. (Bio-rad, Philadelphia)

Animals: Sprague-Dawley rats (weight 180-220 g) were obtained from National laboratory animal center, Mahidol University, Nakornpathom. All rats were rested 7 days before experiments. The rats were fed with regular diet and water ad libitum.

Effect of curcuminoid on ethano- induced toxicity rats: Rats were divided into 6 groups of six rats in each group. Group I was the control animal. Group II was the rats received vehicle. Group III was the rat received isocaloric 60% glucose. Group IV, V, and VI, VII, the rats were received ethanol (6 g/kg /day p.o.) for 14 weeks and on week 8th the rats were received sylimarin ((Legalon®) 100 mg/kg/day or curcuminoid 250,500 and 750 mg/kgday respectively. Serums from the animals were analyzed for alnine aminotransferase (ALT) and aspartate aminotransferase (AST) by LFEkits® (S.E. supply, Bangkok)

Results

Effect of curcuminoid on NO production in ethanol stimulated Hep G2 cells

Hep G2 cells, stimulated with various concentrations of ethanol for 24 hours, increased NO production as a dose-dependent manger. The ethanol concentration at 7.5% and 10% v/v could induce the cells to generate amounts of NO significantly, comparing with the control cells (figure 1A.) When 10% ethanol-stimulated Hep G2 cells were pre-incubated with curcuminoid, we found that curcuminoid at lower concentration (0.313 and 0.625 μg/ml) trends to decrease NO productions. However, curcuminoid at higher concentration could enhance NO productions in the stimulated cells. (figure 1B.)

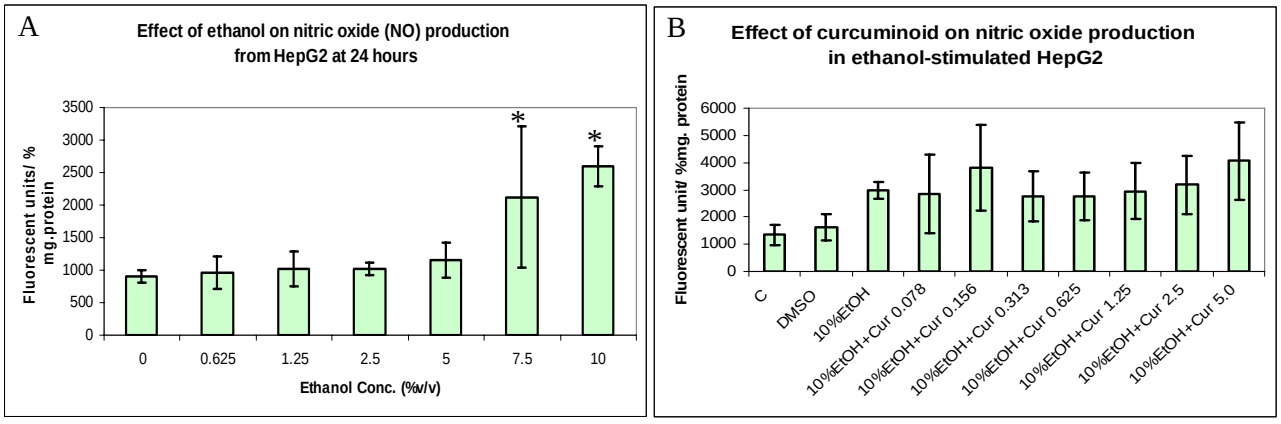

Figure 1. Nitric oxide (NO) production in ethanol stimulated Hep G2 cells. Cells were stimulated with ethanol with various concentration (0.625, 1.25, 2.5, 5.0, 7.5, and 10 % v/v) of ethanol for 24 hours (A.) The ethanol stimulated cells were pre-incubated with curcumiond (B.) NO productions were measured by DAF-2DA reagent. Data were from 3 separated experiments (n=3) and shown as mean ± SD of fluorescent unit/%mg. protein. Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$).

Effect of curcuminoid on aminotransferases in ethanol-induced toxicity rats

Serum ALT and AST levels were increased significantly from all groups received ethanol, comparing to the control group ($p \leq 0.05$). (figure 2A, 2B.) After that the ethanol-induced toxicity rats were received a vehicle, sylimarin (hepatoprotective agent) or various doses of curcuminoid for another 6 weeks. The results showed that curcuminoid concentration at 750 and 1000 μg/ml decrease the serum ALT and AST levels significantly ($p \leq 0.05$) and this effect of curcuminoid seemed to be similar to sylimarin. (figure 2A, 2B.) The serum level of ALT and AST showed fewer changes in the rats received only the vehicle.

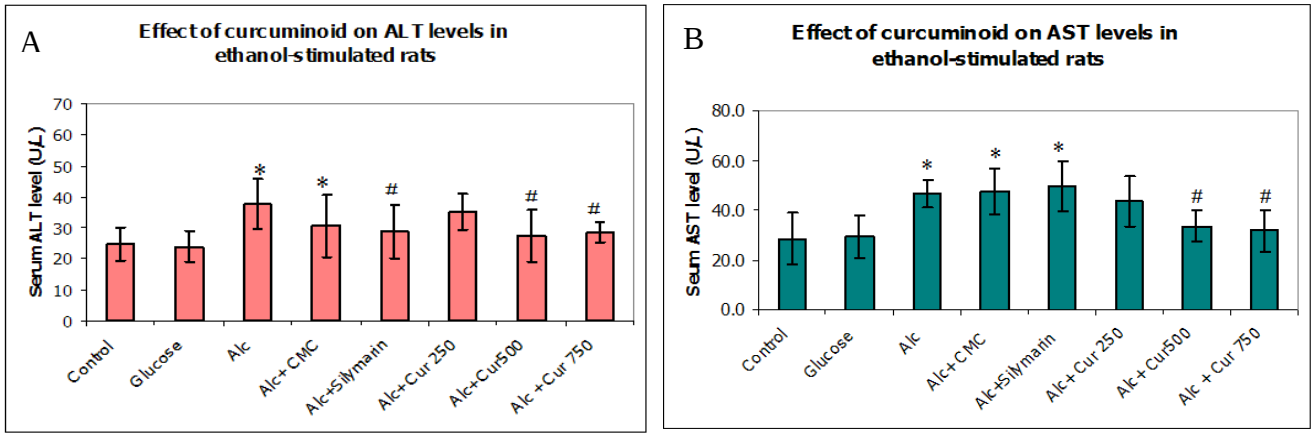

Figure 2. Effects of curcuminoid on serum ALT and AST in ethanol-induced hepatic toxicity rats. Rats were received ethanol (6 g/kg/day) for 8 weeks and then were given carboxymethylcellulose (CMC), sylimarin or curcuminoid (250, 500, 750 μg/ml) for another 6 weeks. Serum ALT and AST levels were measured with the liver function enzyme kits. Data were from 6 animals (n=6) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA ($p \leq 0.05$), comparing to the control group(*) or comparing to the ethanol induced hepatic toxicity baseline (#).

Discussions

It has been demonstrated that enzyme NOS are upregulated in cirrhosis livers and they involve in pathological process. (11, 12) NO modulates different inflammatory cells and prolong cytokines secretions such as TNF-α, resulting in hepatitis. (5) In this present study the ethanol-stimulated hepatic cells enhance NO production. When we tested the effects of curcuminoid on ethanol-stimulated cells, the results showed that curcuminoid at lower concentration trends to reduce NO production. However, curcuminoid at high concentration seemed to accelerate NO production in the cells. We also studied the effect of curcuminoid in long term ethanol-induced rats. Curcuminoid could significantly reduce the enzyme ALT and AST which are the markers for chronic hepatitis. Many studies before have been demonstrated that curcuminoid contains anti-inflammatory and antioxidant effects. (10) For example, curcuminoid has protective effect on iron-induce hepatotoxicity (13) and ethanol induced pancreatitis. (14) Our studies confimed that curcuminoid decrease ethanol-induce hepatic toxicity in rats as well as reduce NO production in ethanol-stimulated hepatic cells

Conclusion

In this study, we showed that curcuminoid in lower concentration reduce NO production in the ethanol-stimulated hepatic cells. In chronic ethanol-induced rats, curcuminoid also decreased the liver function enzymes, ALT and AST significantly. These results suggest that curcuminoid might delay the inflammatory progression in chronic hepatitis and be use as hepatoprotective agents. However, further investigation in mechanism of actions and toxicity of curcuminoid on ethanol-stimulated in vitro and in vivo are need to be done.

Acknowledgements

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Original article **P36**

Analgesic activity of the hexane extract fraction from the *Abroma augusta* Linn. seed in mice

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Abstract

The effects of the n-hexane extract fraction from the seeds of *Abroma augusta* Linn. (Sterculiaceae) were assessed for its analgesic activity in mice using acetic acid-induced writhing, formalin and hot plate tests as the tested models. The extract at doses of 100, 200 and 400 mg/kg orally administered in all tests showed markedly significant antinociceptive activity when compared to control or standard drug groups.

Keywords: *Abroma augusta* Linn., Analgesic, Hexane extract fraction

Introduction

Abroma augusta Linn.(Sterculiaceae), commonly known as devils cotton, is a small tree growing wild in India, China and Thailand. The aerial parts of *Abroma augusta* has been used in traditional medicine and reported that it is used to treat joint pain, asthma, sinusitis, tonsillitis, back pain, diarrhea, influenza, and to be as antifungal and antibacterial (1). Chemical constituents that have been identified from seeds extracted with n-hexane include fatty acid composition (2). From the point of view of its traditional medicine uses, the *Abroma augusta* seeds extract (AALE) may posses an analgesic activity. Thus this study was done to evaluate the analgesic effect of the n-hexane extract fraction of *Abroma augusta* seeds in mice.

Materials and methods

Plant material and extraction: The seeds of *Abroma augusta* were used in the study. The seeds (3 kg) were air-dried at room temperature, and pulverized to powder. The powder was macerated with 6 L of n-hexane for 7 days at room temperature. The extraction process was repeated 2 times and the combined extracts were filtrated. The solvent was then evaporated under reduced pressure to give oil-like brownish extract (yield 23.02% w/w). The obtained extract was stored in a closed bottle and kept in a refrigerator at temperature below 4 °C until use.

Chemicals and Drugs: 0.6% acetic acid, aspirin, 2.5% formalin, morphine, naloxone and n-hexane were used in this study.

Animals: Male Swiss albino mice (30-40 g), obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand, were used and kept in a room maintained under the condition of 24±1 °C and 12 h light-12 h dark cycles. All animals had free access to water and standard diets. The animal Ethics Committees, Prince of Songkla University approved all experimental protocols.

Assessment of analgesic effect:

Writhing test*:* The experiment was done according to the previous described method (3). Male mice were divided into five groups of six animals. The standard drug aspirin and AALE at doses of 200 mg/kg, and 100, 200 and 400 mg/kg, respectively were orally administered to each mouse 30 min before intraperitoneal injection with 0.6% acetic acid (10 ml/kg). The number of writhes was counted for 0-20 min.

Formalin test*:* The method was done according to a previous described (4). Male mice were divided into six groups of six animals. Standard drugs (aspirin 200 mg/kg, po and morphine 5 mg/kg, sc), and AALE at doses of 100, 200 and 400 mg/kg were orally administered to each mouse. After 30 min of treatment (except only 15 min for morphine), 20 µl of 2.5% formalin was subcutaneously injected into a hind paw of each mouse. The total licking time in early phase (0-5 min) and late phase (15-30 min) after formalin injection were recorded.

Hot plate test*:* The method was used as previous described (5). Male mice were divided into eight groups of six animals. Standard drugs (morphine 5 mg/kg, sc and naloxone 2 mg/kg, ip), and AALE at doses of 100, 200 and 400 mg/kg were orally administered to each mouse. After 30 min of treatment (except only 15 min for morphine and 10 min for naloxone), mice were placed on a hot plate maintained at 55±1 °C. The latency of nociceptive responses such as licking of the hind limb or jumping were recorded at 30, 45, 60, 75 and 90 min after administration.

Evaluation of acute toxicity of the AALE : The method was done according to as previous described (6). In this study procedure, the animal received a single dose of the extract. If an animal died, the dose for the next is decreased while if it survives, the dose for the next is increased. Each animal is then observed for 2 day before dosing the next animal. The first dose was begun at 300 mg/kg to 5 g/kg adjusted by a constant multiplicative factor of 1.5 up. Behavior parameters were observed such as convulsion, hyperactivity, sedation, loss of righting reflex and increased or decreased respiration during a period of 8 h and 7 day. Food and water were given *ad libitum*.

Statistical analysis : The data are expressed as mean±S.E.M and analyzed statistically by one-way ANOVA followed by LSD test. $P<0.05$ was considered significant in all cases.

Results

Writhing test

The AALE at doses of 100, 200 and 400 mg/kg significantly inhibited writhings compared to the control ($p<0.05$). (Fig 1). The percentage of inhibition of aspirin 200 mg/kg and AALE at doses of 100, 200 and 400 mg/kg were 55.32% and 35.65, 49.74, and 57.99%, respectively

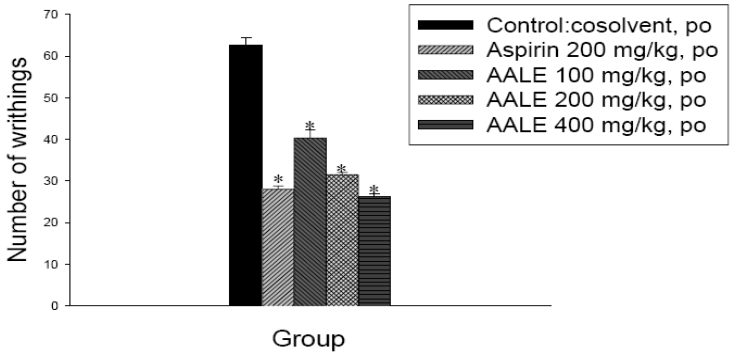

Figure 1: Effects of the n-hexane extract of *Abroma augusta* Linn**.** (AALE) and aspirin on acetic acid-induced writhing in mice. ***** $p<0.05$**,** significant different from control

Formalin test

The effects of AALE on early and late phase of the formalin test were shown in Fig 2. AALE at doses of 100, 200 and 400 mg/kg significantly inhibited both phases

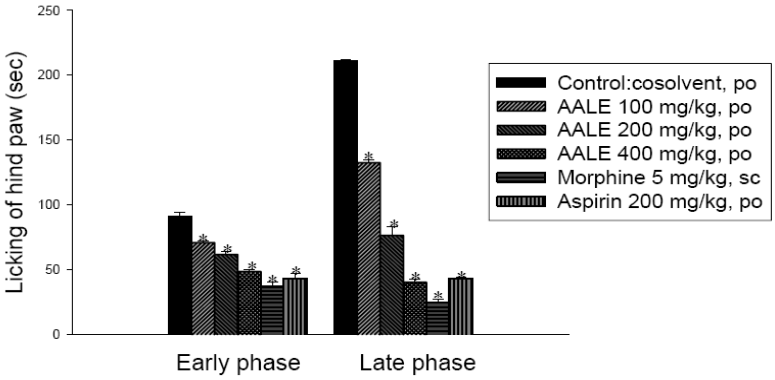

Figure 2: Effects of the n-hexane extract of *Abroma augusta* Linn**.** (AALE), morphine and aspirin on 2.5% formalin-induced paw licking in mice.
***** $p<0.05$**,** significant different from control

Hot plate test

AALE at doses of 100, 200 and 400 mg/kg increased in the reaction time to the thermal stimulus compared to control (Fig 3A). Naloxone (2 mg/kg, ip) give before the morphine (5 mg/kg, sc) or AALE (400 mg/kg, po) abolished the latency of the nociceptive response (Fig 3B).

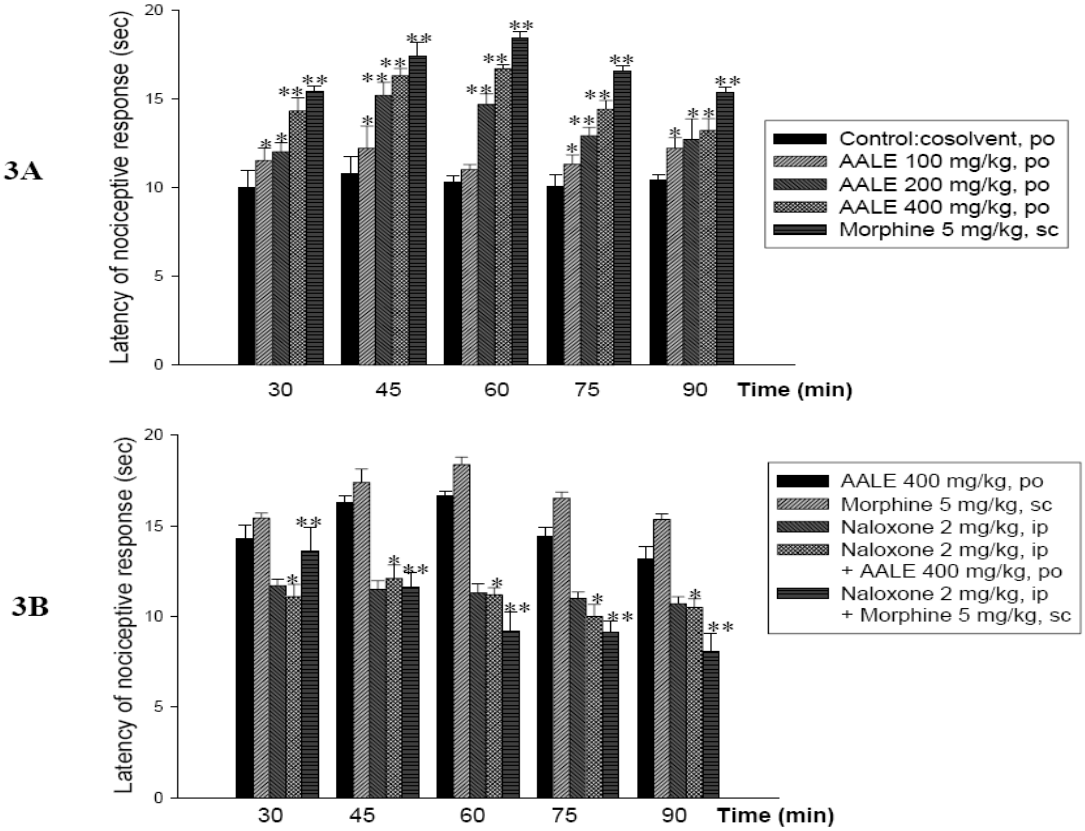

Figure 3 : Effects of AALE, morphine and naloxone on the reaction time of mice in the hot plate test. **3A**:***** $p<0.05$**,**** $p<0.01$,significant different from control; **3B**: ***** $p<0.01$**,** significant different from AALE 400 mg/kg , ****** $p<0.01$, significant different from morphine 5 mg/kg

Acute toxicity

AALE at the dose of 5 g/kg, po given to three male mice had no effect on their behavioral response and no mortality. The LD_{50} value of the extract in mice was therefore estimated to more than 5 g/kg, po. In this study, the doses of 100, 200 and 400 mg/kg, po given to mice is safe.

Discussion and conclusion

The hot plate and formalin tests have been used for investigation of centrally acting analgesic effect, whereas the acetic acid-induced method is widely used for the evaluation of peripheral antinociceptive activity (7). Many endogenous mediators such as substance P, histamine, serotonin, bradikinin and prostaglandind play an important role in the nociceptive mechanisms (8). The AALE at dose of 400 mg/kg marked significantly increased the latency time in hot plate test and decreased the licking time in formalin test. For the writhing test, the AALE at doses of 100, 200 and 400 mg/kg significantly inhibited writhing with dose-dependent manner. As the present evidence in this study, the antinociceptive activity of AALE was likely to have the mechanisms both the peripheral and central pathways.

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Original article **P37**

Effects of the hexane extract from *Glycosmis parva* on LPS-induced macrophage activation

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Abstract

The *in vitro* effect of the hexane extract from branches of *Glycosmis prava* on LPS-induced macrophage activation was investigated. Murine macrophages, J774A.1cells, were used in this study. The extract suppressed nitric oxide production from LPS-activated J774A.1 cells in a concentration dependent manner with IC_{50} 44.70 μg/ml. It decreased the mRNA expression of the inducible nitric oxide synthase (iNOS) which is responsible for NO production as well as the cyclo-oxygenase 2 (COX-2) which is responsible for prostaglandins (PGs) synthesis in these activated macrophages. The extract also inhibited the mRNA expression of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6. The results from this study indicated that the hexane extract from *G. parva* may have anti-inflammatory activities against activated macrophages which play a significant role during inflammatory process.

Keywords**:** *Glycosmis parva*, macrophage, inflammatory.

Introduction

Macrophages play an essential role in the innate and adaptive immunities as well as the inflammatory process. These cells are activated by directly recognizing pathogens via pattern recognition receptors (PRRs) such as toll like receptors (TLRs) or by several cytokines from immune cells such as interferon-γ from Th1 cells. Activated macrophages express and secrete several enzymes and mediators that involve in immune response and inflammatory process. They express inducible nitric oxide synthase (iNOS) for nitric oxide (NO) synthesis, cyclooxygenase-2 (COX-2) for prostaglandin (PG) synthesis. The cells also express several cytokines and chemokines as well as pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) that activate iNOS and COX-2 expression. They also play a key role in many inflammatory diseases such as rheumatoid arthritis and atherosclerosis (1). Some mediators of activated macrophages including PGs and pro-inflammatory cytokines are targets of clinically used anti-inflammatory agents including corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), selective COX-2 inhibitors and cytokines inhibitors (anti-TNF-α drugs and IL1 receptor antagonist).

Many medicinal plants have been used as alternative medicines for treatment of various inflammatory diseases . *Glycosmis parva* Craib is a small shrub widely distributes in Thailand. Acridone alkaloids and sulfur-containing propanamides are major compounds found in plants of genus *Glycosmis*. Anti-infective and anticancer activities of these compounds have been reported. This study aimed to investigate anti-inflammatory activity of the hexane extract of *G.parva* on LPS-induced J774A.1 cells activation.

Materials and methods

Plants extract: The precipitated pellet from methanol extract from branches of *G .parva* was partition in hexane. The hexane extract was dissolved in dimethylsulfoxide (DMSO) and further diluted to various final concentrations in DMSO 0.2%.

Cells: The murine macrophage cells J774A.1 were obtained from American Type Culture Collection (ATCC). The cells were maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37°C in 5% CO_2/95% air.

Other materials: Reagents for cell culture, TRIzol and Taq polymerase were obtained from Gibco, USA. Reverse transcription system kit was purchased from Promega, USA. Other chemical reagents were from Sigma USA.

Determination of nitric oxide production

J774A.1 cells ($2x10^5$cells/ml) were treated with the hexane extract of *G. parva* at the concentrations 6.25-50 µg/ml for 24 h, and then activated with 100 ng/ml LPS for 24 h. The amount of NO as nitrite form in the supernatants of the treated cells was determined using Griess reagent.

Identification of mRNA expressions of pro-inflammatory cytokines, iNOS and COX2

J774A.1 cells ($2x10^5$ cells/ml) were treated with the hexane extract of *G. parva* at the concentrations of 25 and 50 µg/ml for 24 h, and then activated with 100 ng/ml LPS for 6 h or 24 h to determine cytokines or iNOS and COX-2 expression, respectively. Total RNA was isolated from the treated cells and then reversed to cDNA. The cDNA was used as the template to amplified mRNA of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), iNOS and COX-2 with suitable primers. The PCR products were run on 1.5% agarose gel electrophoresis and semi-quantitatively determined by gel documentation.

Statistical analysis

Data were expressed as mean ± standard deviation. One-way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test was used to determine the statistical significance of differences between the values for the various experimental and control groups. The p-value < 0.05 was considered as statistically significance.

Results

Effect of *Glycosmis parva* hexane extracts on LPS stimulated-macrophages

The extract inhibited NO production in LPS-stimulated J774A.1 cells in a concentration-dependent manner with IC_{50} values at 44.70µg/ml (Fig.1)

Effect of the extract on the expressions of cytokines, iNOS and COX-2 in LPS activated J774A.1

The hexane extract of *G.parva* at 50 µg/ml significantly inhibit the mRNA expression of pro-inflammatory cytokines; TNF-α, IL-1β, IL-6, as well as iNOS and COX-2 (Fig.2).

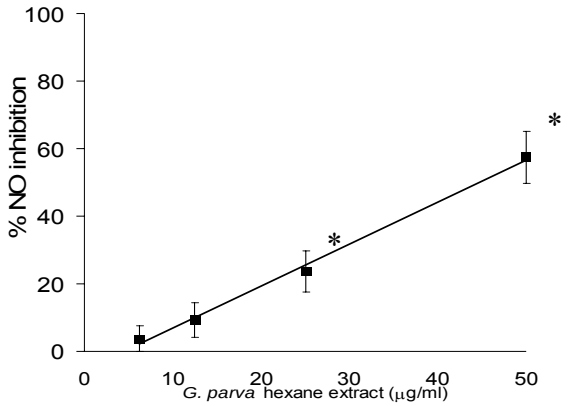

Figure 1. Inhibitory effects of the hexane extract of *G.parva* on NO production of LPS-activated J774A.1 cells. The percentage of inhibition is expressed as mean ± SD of 5 independent experiments.
* significantly difference between the untreated and the extract-treated cells at p <0.001.

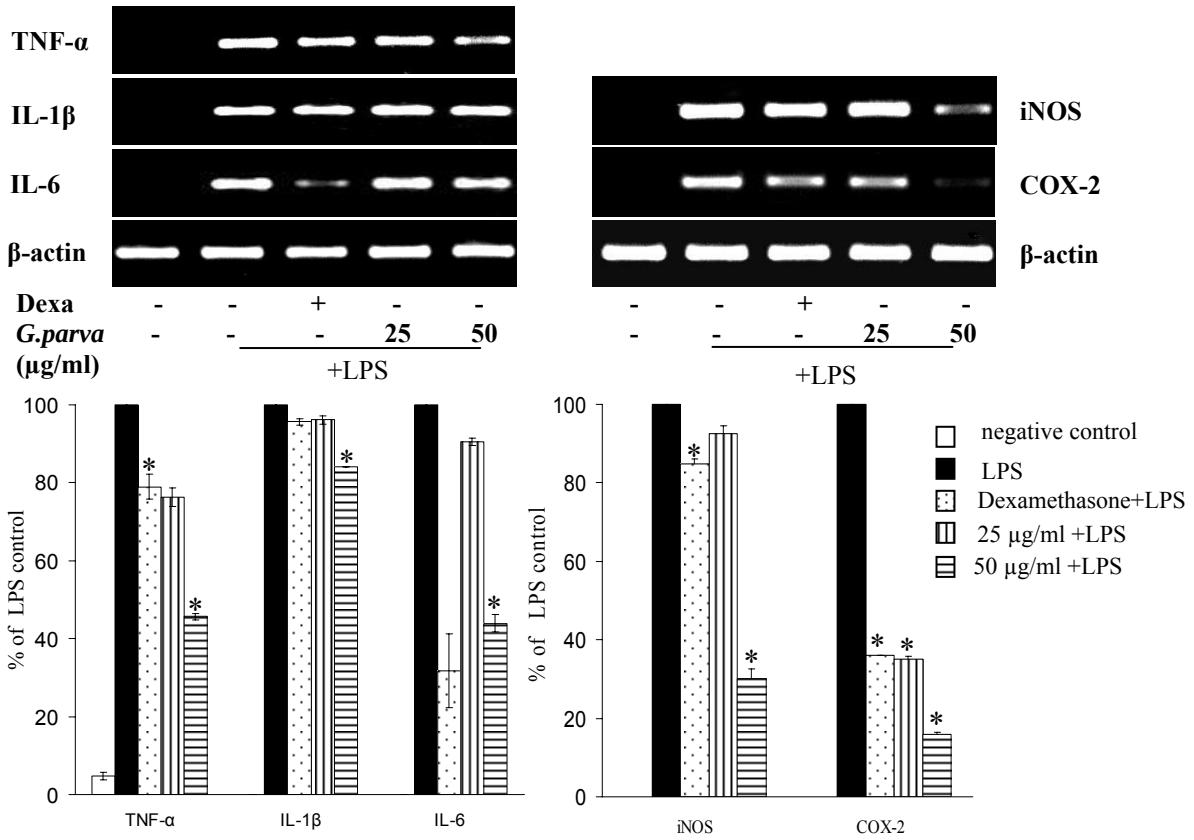

Figure 2. Inhibitory effect of the hexane extract on the mRNA expressions of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), iNOS and COX-2 in LPS-activated J774A.1 cells. The percentage of inhibition is expressed as mean ± SD of two independent experiments.
* significantly difference between the untreated and the extract treated cells at p<0.001

Discussion

The hexane extract from branches of *G. parva* on activated macrophages was investigated in this study. The results demonstrated that the extract inhibited several mediators in LPS-activated J774A.1 cells. It suppressed the mRNA expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2 as well as NO production. These mediators not only play roles in innate immune response but also initiate and induce neighbor tissue injury and lead to inflammatory diseases such as rheumatoid arthritis (1). Inhibition of these mediators is

current or trend therapeutic strategy for treatment of inflammation. TNF-α is the major pro-inflammatory cytokine that induces the expression of other pro-inflammatory cytokines, iNOS and COX-2. Inducible NOS is responsible to excess NO production (2). NO in activated macrophages can interact with superoxide anion to generate powerful free radical, peroxynitrite. This free radical can cause tissue injury during inflammation (3). COX-2 is the inducible COX that induces large amount of PG synthesis in activated macrophages. PGs are the important mediators of inflammation response (4). Our study suggests that *G. parva* contains compound(s) which suppress the production of inflammatory mediators in activated macrophages. It has been reported that acridone alkaloids and sulfur-containing propanamides are major compounds found in *G. parva* (5). This plant also contains β-sitosterol and stigmasteriol which have been demonstrated to suppress the production of inflammatory mediators in LPS-activated macrophage (6). It needs further investigation to identify the compound that has anti-inflammatory activity in this medicinal plant.

Conclusion

This study demonstrated that the hexane extract from the branches of *G. parva* inhibits the production of several inflammatory mediators in LPS-activated macrophages. *G. parva* might be a source for development of anti-inflammatory agent(s).

Acknowledgments

This study was supported by a grant from the Graduate School, Chulalongkorn University.

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Original article **P38**

Nitric oxide inhibitory activity of Thai medicinal plants called Kod-Tung-Ha

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Abstract

Thai herbal medicine called *Kod-Tung-Ha* composes of five plants: *Angelica dahurica*, *Angelica sinensis*, *Artemisia annua*, *Atractylodes lancea* and *Ligusticum sinense*. These plants were examined for their inhibitory activities against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW 264.7 cell lines. These plant extracts were also tested for the inhibitory effect on LPS-induced TNF-α release in RAW 264.7 cell lines. The extraction methods imitated by Thai folk doctors using such as maceration in ethanol or ethanolic extract and boiling by water or water extract. The results were found that the ethanolic extract of *Atractylodes lancea* exhibited the most potent inhibitory activity nitric oxide production (IC_{50} = 9.70 μg/ml) an also possessed potent activity against TNF-α release with an IC_{50} value of 24.35 μg/ml., followed by an ethanolic extract of *Angelica sinensis* (IC_{50} = 12.52 μg/ml). The water extract of all plants were apparently inactive. These results can support the use of these plants in combination as *Kod-Tung-Ha* for treatment of inflammatory-related diseases through the inhibition of NO and TNF-α release.

Keywords: Nitric oxide, Lipopolysaccharide, TNF-α release, RAW 264.7, Kot-Tung-Ha.

Introduction

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs. This inorganic free radical has been implicated in physiologic and pathologic processes, such as vasodilation, non-specific host defense, ischemic stroke and acute or chronic inflammation[1)]. NO is produced from L-arginine by a chemical reaction catalyzed by the enzyme inducible nitric oxide synthase (iNOS) in living systems. After stimulation with bacterial lipopolysaccharide (LPS), many cells including macrophages express the iNOS which is responsible for the production of large amount of NO[2)]. Kot-Tung-Ha is composed of five plants: *Angelica dahurica* root, *Angelica sinensis* root, *Artemisia annua* aerial, *Atractylodes lancea* rhizome and *Ligusticum sinense* rhizome. These plants have been used for antipyretic[3,4)], analgesic[3)], anti-inflammatory[3,5,6,7)], cardiovascular diseases[8,9)], digestive disorders[6)] and cold treatment[10)]. In spite of the fact that all of these plants were reported to be tested for anti-inflammatory activity, but NO-inhibitory activity of some plants were not reported.

The aim of this study was to test for inhibitory activity of Kot-Tung-Ha against LPS-induced NO production in RAW 264.7 cell lines.

Materials and Methods

Plant material preparation and extraction

The plants were purchased from China. The voucher specimens are deposited at the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. Plants material were washed and then dried at 50°C, powdered and extracted by methods similar to those used by Thai traditional

doctors. In brief, for ethanolic extracts, dried plant material (300 g) was macerated by 95% ethanol for 3 days, 2 times, filtered and dried by using an evaporator. For water extracts, dried plant material (100 g) were boiled in distilled water for 30 minutes, filtered and dried by using a lyophilizer.

Anti-inflammation by nitric oxide inhibitory assay

In these experiments, RAW 264.7 murine macrophage leukemia cell lines used in this study were received from Assoc. Prof. Dr. Supinya Tewtrakul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. RPMI-1640 medium, Fetal bovine serum (FBS) and Penicillin-streptomycin (P/S) were purchased from Gibco. Lipopolysaccharide (LPS) and 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma. 96-well microplates were purchased from Costar Corning. ELISA test kit was purchased from R&D Systems Inc.

Inhibitory effects on NO production by murine macrophage-like RAW 264.7 cells were evaluated by the following method[1,11]. The RAW 264.7 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% P/S in 96-well plates with 1×10^5 cells/well for 1 h. The cells were stimulated with 5 μg/ml LPS together with test samples at various concentration for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric method. The absorbance at 570 nm was measured.

Inhibitory effects on the release of TNF-α from RAW 264.7 cells were evaluated using Quantikine mouse TNF-α ELISA test kit.

The inhibition of NO production and TNF-α production were calculated using the following equation and IC_{50} values was calculated from the Prism program.

$$\text{Inhibition (\%)} = \frac{A - B}{A - C} \times 100$$

A–C: NO_2^- concentration (μM) for NO production and TNF-α concentration (pg/ml) for TNF-α production [A: LPS (+), sample (-); B:LPS (+), sample (+); C: LPS (-), sample (-)]

Results and Discussion

Ethanolic and water extracts form five plants of Kot-Tung-Ha were investigated for their inhibitory activities against LPS induced NO production in RAW 264.7 cell lines. Among these plants, an ethanolic extract of *A. lancea* exhibited the highest inhibitory activity against NO inhibitory effect with IC_{50} value of 9.70 μg/ml (Table 1), whereas other plants possessed high and moderate activity. The inhibitory activity of these plants were weaker than that of positive control, indomethacin (non-steroidal anti-inflammatory drug, NSAID, IC_{50} = 25.0 μM or 8.95 μg/ml). These plants were also tested for the inhibitory effect on LPS-induced TNF-α release in RAW 264.7 cells. The result revealed that ethanolic extract of *A. lancea* also possessed the most potent activity against TNF-α release with an IC_{50} value of 24.35 μg/ml, while other plants exhibited inactive. From this study, *A. lancea* showed strong inhibition on both NO and TNF-α releases. Regarding the biological activities, it has been reported that *A. lancea* exhibited potent inhibitory activities in 5-lipoxygenase, cyclooxygenase-1 and anti-tumor activity[6,12].

Table 1. Inhibitory effect of Kot-Tung-Ha on LPS-induced NO production and TNF-α release from RAW 264.7 cells.

Plant	Solvent	Inhibition of NO production		Inhibition of TNF-α release	
		% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)	% Inhibition at conc. 100 μg/ml	IC_{50}±SEM (μg/ml)
Angelica dahurica	Ethanol	88.33±1.76*	44.23±2.71	-	-
	Water	13.50±0.35*	>100	-	-
Angelica sinensis	Ethanol	95.60±0.71*	12.52±2.31	86.59±0.17*	-
	Water	42.13±4.56	>100	-	-
Artemisia annua	Ethanol	96.47±2.23*	17.06±2.69	87.68±0.92*	-
	Water	35.17±3.08*	>100	-	-
Atractylodes lancea	Ethanol	94.03±3.22*	9.70±0.54	81.62±0.69*	24.35±1.19
	Water	39.27±4.41*	>100	-	-
Ligusticum sinense	Ethanol	92.00±4.70*	16.48±2.03	3.66±1.15	>100
	Water	20.43±5.06	>100	-	-
Indomethacin		80.30±1.50 (μM)	25.0 (μM) (8.95 μg/ml)	-	-

* Cytotoxic effect was observed.

Conclusion

In summary, the result obtained in this work indicated that the ingredients or plants in Kot-Tung-Ha, which were used to treat fever and cold, possessed strong active against LPS induced NO production in RAW 264.7 cell lines such as *Angelica sinensis*, *Artemisia annua*, *Atractylodes lancea* and *Ligusticum sinense*. The ethanolic extracts of *A. lancea* showed strong inhibition on both NO and TNF-α releases. These result supports using these plants for treatment of fever, cold and inflammatory-related diseases by Thai folk medicine.

Acknowledgements

We are grateful to the Faculty of Medicine, Thammasat University, for financial support.

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Original article **P39**

Antispasmodic effects of alcoholic extracts from polyherbal formulation "Prasaplai" on isolated rat uterine horn

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Abstract

This study investigated the spasmolytic effect of the alcoholic extracts from polyherbal formulation "Prasaplai" (PSP01 and PSP02), in comparison with the alcoholic extract from *Zingiber cassumunar* Roxb. (PSPoil), using in the *in vitro* model of isolated rat uterine horn. Our result indicated that all of the test materials significantly relaxed the muscle tension pretreated with oxytocin (2 nM) and KCl (50 mM) in concentration-dependent manner. The IC_{50} (inhibition of force) of PSP01, PSP02 and PSPoil on oxytocin-induced contraction were 27.48±2.89, 30.26±4.44 and 32.51±3.05 µg/ml, respectively. The IC_{50} of these extracts on KCl-induced contraction were 20.11±2.72, 19.67±2.99 and 27.80±4.62 µg/ml, respectively. This study demonstrated that the alcoholic extracts of polyherbal formulation "Prasaplai" contained spasmolytic activity on the uterus muscle. In addition, the prasaplai extracts were more potent than the extract of its major herbal ingredient.

Keywords: Uterine relaxation, *Zingiber cassumunar***,** Prasaplai, polyherbal medicine

Introduction

Prasaplai preparation has been used orally in Thailand for a long time. Currently, it is on the list of the Thai Traditional Common Household Drug, announced by the Ministry of Public Health for relieving muscle pain, postpartum uterine involution and abnormal menstrual cycle (1). This preparation composes of ten medicinal plants and two minerals, with *Zingiber cassumunar* Roxb. or "plai" as a major ingredient (approximately 80%). Although there were certain reports of the spasmolytic activity of Prasaplai extract in the *in vitro* model of isolated rat uterus, there was uncertain whether the pharmacological actions of major herbal ingredient and the whole preparation would be equivalent. Thus, this study aimed to investigate the spasmolytic effect of the alcoholic extract of polyherbal formulation "Prasaplai" in comparison with the alcoholic extract of *Zingiber cassumunar* Roxb. (plai), in the *in vitro* model of isolated rat uterine horn.

Materials and methods

Chemicals and test materials

The alcoholic extracts of Prasaplai formulation were commercially available from two different sources (PSP01, PSP02) and the alcoholic extract of *Zingiber cassumunar* Roxb. (PSPoil). All the extracts were kindly provided by Thailand Institute of Scientific and Technological Research.

Tissue preparations

The animals were pretreated with estradiol-17-benzoate (1 mg/kg, i.p.) 24-48 hours before the experiments (2). On the day of experiment, the rat was sacrificed, and removed uterine horn. Then, the uterine horns were cut in to 4 segments (approximately 1.0-1.5 cm long), and suspended in Locke-Ringer solution at 37 ^{o}C gassed with carbogen (95% O_2 and

5% CO_2). The contractile response was recorded isometrically with a force transducer UFI 1030 (ADInstruments, Australia) connected to PowerLab/4SP and computer equipped with program SCOPE CHART 5 V.2.0 (ADInstruments, Australia).

The study protocols were approved by the Ethics Committee on Animal Experiment, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Experimental protocol

Effects of the test materials on oxytocin and KCl- induced contraction

This experiment used oxytocin (2 nM) and KCl (50 mM) to induce uterine contraction. When the contraction reached plateau state, the test materials (10-50 µg/ml) were added cumulatively to the organ bath. The tension was recorded and expressed as percentage of the agonist-induced contraction. In separated experiments, the effects of DMSO were also determined as a control.

Statistical analysis

Results were expressed as mean±S.E.M. Statistical analysis was carried out by one-way ANOVA, followed by Dunnett's test post hoc comparison. $P<0.05$ indicated statistical significance.

Results

Effects of the test materials on oxytocin and KCl- induced contraction

As shown in Figure 1**,** oxytocin (2 nM) caused rhythmic contractions of isolated rat uterus with the developed tension of 7.12±0.53 g and frequency of 7.85±0.48 stroke/10 min (n=16). The cumulative addition of the test materials (PSP01, PSP02 and PSPoil) 10-50 µg/ml significantly inhibited both force and frequency of oxytocin-induced uterine contraction in the concentration dependent manner (Figure 2) with the IC_{50} (inhibition of force) were 27.48±2.89, 30.26±4.44 and 32.51±3.05 µg/ml, respectively.

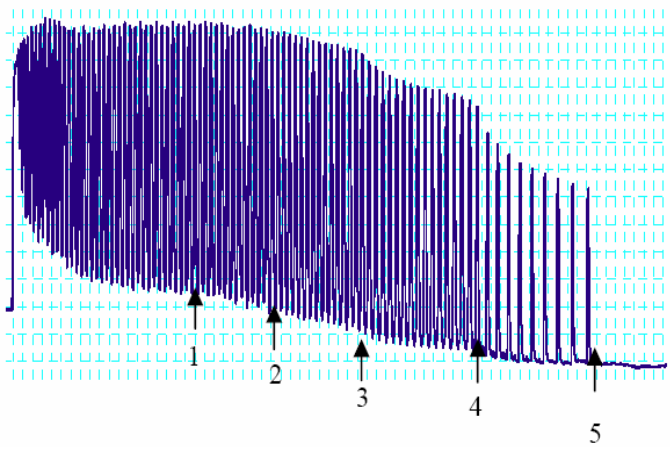

Figure 1 Representative tracing showed effect of the test materials (PSP01) on oxytocin-induced contraction at cumulative concentrations 1=10, 2=20, 3=30, 4=40 and 5=50 µg/ml in Locke-Ringer solution.

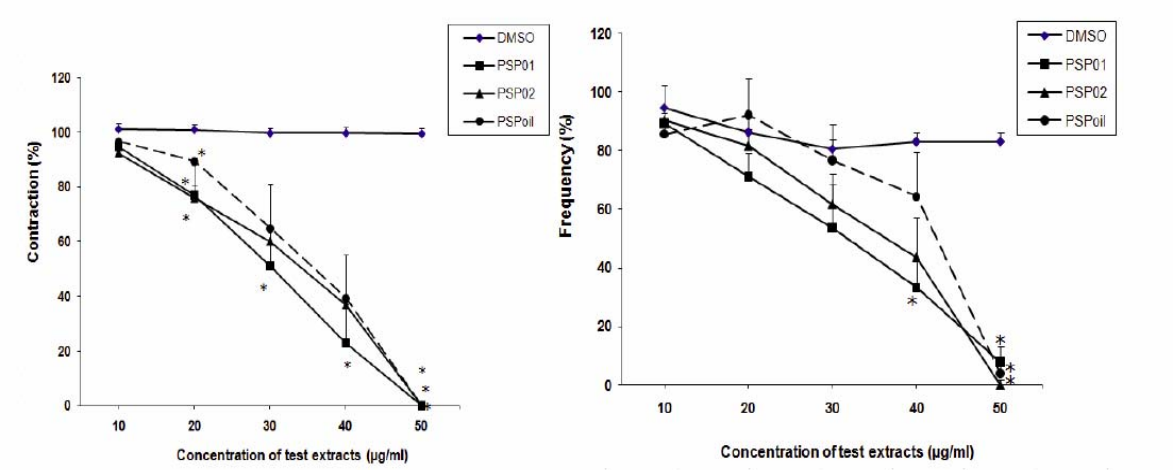

Figure 2 Concentration-response curve of test extracts (10-50 µg/ml) on the contractile amplitudes (A) and frequency (B) of oxytocin-induced contraction on rat isolated uterine horns. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test, *$p < 0.05$ denotes statistically significant difference from DMSO.

The contractile response of uterus toward KCl (50mM) contain two phase including initial rapid, phasic contraction followed by a sustained tonic contraction (Figure 3). The baseline values of the contractile amplitude were 4.63±0.40 g (n=20). Cumulative addition of each alcoholic extracts (PSP01, PSP02 and PSPoil) 10-50 µg/ml in the sustained tonic contraction inhibited the concentration in dose dependent manner (Figure 4). The IC_{50} (inhibition of force) were 20.11±2.72, 19.67±2.99 and 27.80±4.62 µg/ml, respectively.

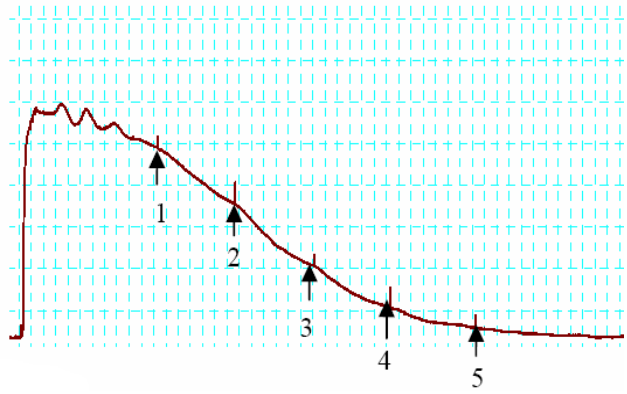

Figure 3 Representative tracing showed effect of the test materials (PSP02) on KCl-induced contraction at cumulative concentrations 1=10, 2=20, 3=30, 4=40 and 5=50 µg/ml in Locke-Ringer solution.

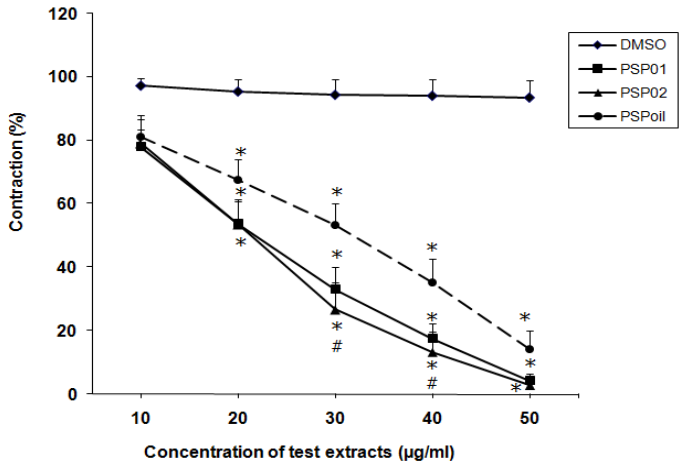

Figure 4 Concentration-response curve of the test extracts (10-50 µg/ml) on KCl-induced rat isolated uterine horns. Each point represents mean±S.E.M. of 4-6 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.*$p < 0.05$ denotes statistically significant from DMSO. $^{\#}p < 0.05$ denotes statistically significant from PSPoil.

Discussions and Conclusions

Our finding showed that the alcoholic extract of Prasaplai formulation inhibited uterus contractions produced by oxytocin and KCl in a concentration-related manner. At the equivalent concentration, the spasmolytic action of the extracts of the whole prasaplai preparation was higher than the extract of the major ingredient. All of the test materials inhibited the contraction induced by either oxytocin or KCl. As known, potassium chloride induced contraction of smooth muscles via mechanism involved with an increase in Ca^{2+} influx through voltage-operated Ca^{2+} channels (3). In contrast, oxytocin causes myometrium contractions by acting on oxytocin receptors (4). Hence, our results suggested that the uterine relaxant effect of Prasaplai was probably mediated through a non-specific, spasmolytic mechanism. Further works would be in need to investigate the mechanisms involved.

Acknowledgement

This project was partly supported by Thailand Institute of Scientific and Technological Research.

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Original article **P40**

Inhibitory effect of *Derris reticulata* ethanol extract on LPS-induced macrophage activation

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Abstract

Derris reticulata Craib is a plant in Leguminosae family which has flavonoids as its major active compounds similar to other plants in genus *Derris*. It has been used as expectorant and thirst relief. In this study, we studied the effect of ethanol extract from stem of *D. reticulata* on LPS-induced macrophage activation. Murine macrophage, J774A.1 cells, were pretreated with 6.25-100 μg/ml the extract for 24 h and then stimulated with 100 ng/ml lipopolysaccharide (LPS) for 24 h. The extract inhibited nitric oxide production in LPS-activated J774A.1 cells in concentration-dependent manner with IC_{50} at 62.5 μg/ml. The effect of this extract on phagocytosis activity of LPS-activated J774A.1 cells was also investigated. The extract at the concentrations of 50 and 100 μg/ml significantly inhibited zymosan phagocytosis of LPS-activated cells in a concentration dependent manner. These results demonstrated that flavonoids from *D. reticulata* may have potential to be an inflammatory agent.

Keywords: *Derris reticulata*, LPS-activated macrophage, phagocytosis, nitric oxide

Introduction

Activated macrophages play important roles in innate and adaptive immune responses against a wide range of microorganisms as well as inflammatory response against infectious and non-infectious stimuli. These activated cells express and release a numerous molecules involving in those responses. They express several cytokine receptors, adhesion molecules, and various accessory molecules for immune responses on their cell surface. These cells increase ability to get rid of pathogens, debris cells and apoptotic cells by phagocytosis. They also generate several free radicals such as hydrogen peroxide, super oxide anion and nitric oxide (NO) for intracellular destroying pathogen. Activated macrophages express inducible nitric oxide synthase (iNOS) that catalyzes L-arginine to large amount of NO. They also produce and release more than 30 types of cytokines. These include pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8 and IL-12) that initiate to generate various inflammatory mediators such as prostaglandins (PGs) and leukotrienes (LTs) which cause the signs of inflammation; redness, swelling, fever and pain. Many clinically used anti-inflammatory agents target on PGs production or inhibit pro-inflammatory cytokine functions.

Many medicinal plants containing flavonoid compounds are used as traditional medicine for inflammation or fever treatment. Several plants in genus *Derris* are identified to have flavonoids as their major constituent (1). *Derris reticulata* Craib or "Cha-aem-nuea" is a plant in Leguminosae family that widely distributes throughout Thailand. Thai people use its stems and roots as a sweetening agent, an expectorant, an antitussive, a remedy for throat diseases and as a tonic agent (2). This study intended to investigate potential anti-inflammatory effect of the ethanol extract from stems of this plant.

Materials and Methods

Plant extract

Air-dried and grounded stems of *D. reticulata* was extracted with dichloromethane and then with absolute ethanol. The ethanol extract was dissolved in dimethylsulfoxide (DMSO). The constant final concentration of DMSO in this study was 0.2%.

Cells

Murine macrophages J774A.1 were obtained from ATCC. The cells were subcultured 3 times weekly and maintained in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37 ^{0}C in 5%CO_2/ 95% air.

Chemicals

The following reagents were obtained from Sigma, USA; nitroblue tetrazolium (NBT), LPS, zymosan A from *Saccharomyces cervisiae*, DMSO, 0.4% trypan blue dye. Chemicals and reagents for cell culture were from Gibco, USA. Griess reagent for nitric oxide was obtained from Promega, USA.

Determination of NO production

1x10^5 cell/well J774A.1 cells in 96-well plates were treated with the ethanol extract at 6.25, 12.5, 25, 50 and 100 µg/ml for 24 h. The treated cells were stimulated with 100 ng/ml LPS for 24 h. The amount of NO release into the supernatant was determined in nitrite form by using Griess reagent. The reaction mixture was detected with a microplate reader at 540 nm. The amount of nitrite was calculated from sodium nitrite standard curve. The viability of the treated cells was determined by staining with resazurin for 2-4 h at 37 ^{0}C in 5%CO_2/ 95% air. The reduction product resorufin in viable cells was detected by a microplate reader at 570 and 600 nm. The percentage of cell viability was calculated by comparing with LPS-activated condition.

Determination of phagocytosis activity

1x10^5 cell/well J774A.1 cells in 96-well plates were treated with 25, 50 and 100 µg/ml ethanol extract for 24 h and then activated with 100 ng/ml LPS for 24 h. The treated cells were carefully washed twice with DMEM and then incubated with 800 µg/ml of zymosan and 600 µg/ml of NBT for 1h. The cells were washed 3 times with methanol, air-dried, and lyses in 120 µl of 2M KOH and 140 µl of DMSO. The oxidized NBT product in blue color was detected at 570 nm. The percentage of phagocytosis inhibition was determined by comparing to LPS-activated condition.

Statistical analysis

All data were presented as mean ± S.E.M. Data analysis was performed on SPSS 17.0. Statistical comparisons were made by one-way ANOVA followed by Turkey's post hoc test. The p-value<0.05 was considered statistically significant.

Results

Effect of the ethanol extract on NO production

D. reticulata ethanol extract decreased NO production in LPS-stimulated J774A.1 cells in a concentration-dependent manner with IC_{50} 62.5 µg/ml (Fig.1). The extract at the concentration of 6.25, 12.5, 25, 50 and 100 µg/ml inhibited NO production by 5.1%, 10.9%, 24.9%, 46.1% and 74.8%, respectively. It didn't affect cell viability of the treated macrophages (Fig.2).

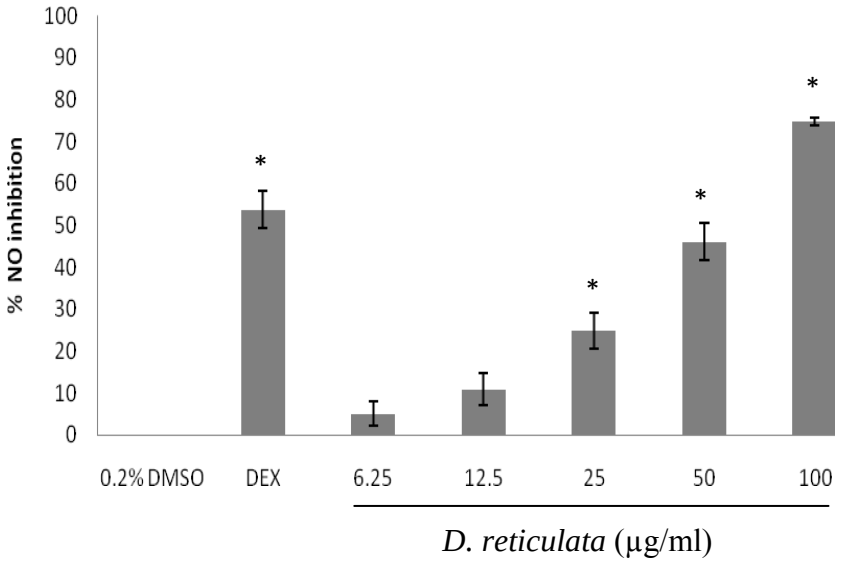

Figure1: Inhibitory effect of *D. reticulata* ethanol extract on NO production in LPS stimulated-J774A.1 cells. The cells were treated with 6.25-100 µg/ml extract and then stimulated with 100 ng/ml. Five µg/ml dexamethasone (DEX) was used as the positive control. The NO production was determined by Griess reagent. The percentage of NO inhibition compared to LPS-activated condition is presented as mean ± S.E.M. of four independent experiments (n=4); * $p<0.05$ compared to untreated control (LPS-activated cells).

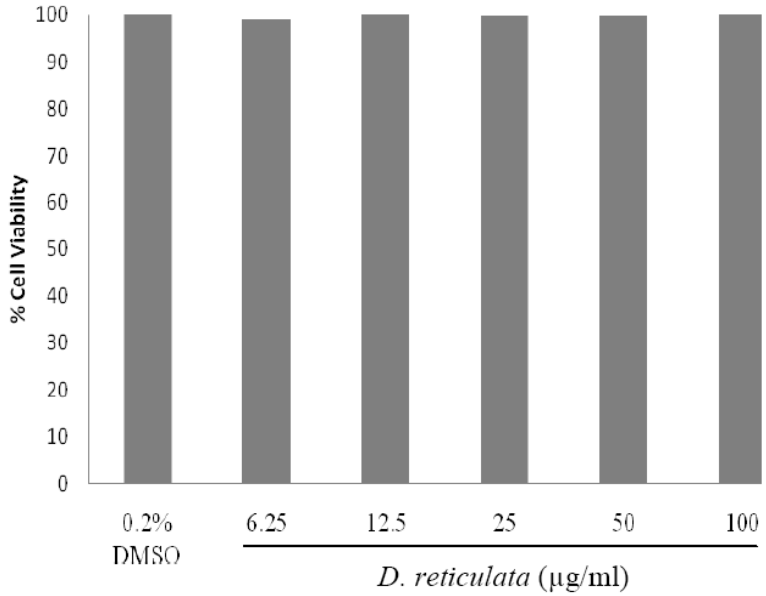

Figure 2: Effect of the ethanol on cell viability. Viability of the treated cells from figure 1 was determined by resazurin assay. The data are presented as mean ± S.E.M.

Effect of the ethanol extract on phagocytosis

When compared to the LPS-activated condition, the extract at 25, 50, and 100 µg/ml inhibited phagocytosis in a concentration-dependent manner.

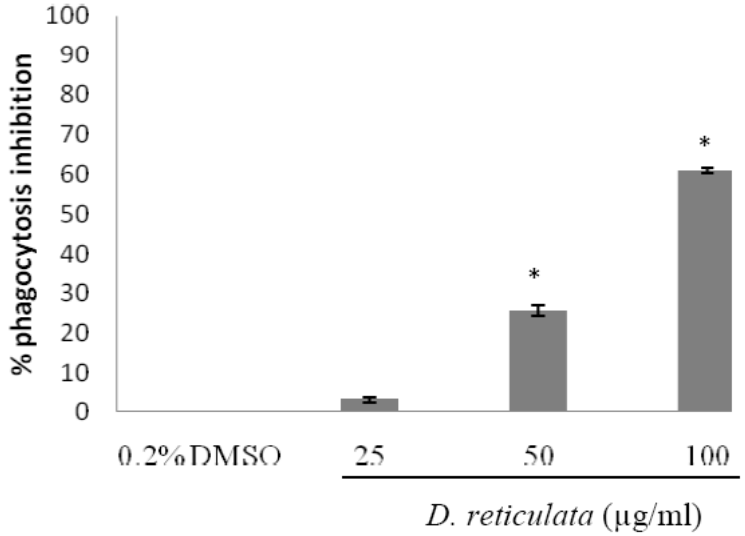

Figure 3: Effect of *D. reticulata* ethanol extract on phagocytosis in LPS stimulated-J774A.1 cells. The cells were treated at dose 25-100 µg/ml extract. Phagocytosis of the treated cells was determined by zymosan-NBT assay. Data are presented as mean ± S.E.M. of three independent experiments (n=3). * $p<0.05$ compared to LPS-activated condition (0.2% DMSO).

Discussion and conclusion

This study intended to investigate the pharmacological effects of the ethanol extract from stems of *Derris reticulata* on macrophage activation. Activated macrophages are important immune cells that generate and release numerous mediators involving in inflammatory response against infectious and non-infectious stimuli (3). These mediators include pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8), oxygen free radicals, NO and prostaglandins. They play important roles in inflammatory process and the signs of inflammation. Many clinically used anti-inflammatory agents target on both synthesis and functions of these mediators such as NSAIDs, corticosteroids, and TNF-α and IL-1 inhibitors. We demonstrated that the ethanol extract from stems of *D. retculata* suppressed the production of NO in LPS-activated J774A1.1 cells in a concentration-dependent manner. This extract also decreased phagocytosis activity of activated macrophages which can lead to generation of inflammatory mediators in these cells. These results demonstrate the anti-inflammatory potential of compounds presenting in *D. reticulata* ethanol extract.

Acknowledgements

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Original article **P41**

A high-throughput spectroscopic-based bioassay method for determination of fosmidomycin in plasma: application for pharmacokinetic study

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Abstract

A simple, sensitive, selective and reproducible bioassay based on spectroscopic method was developed for the determination of fosmidomycin in human plasma. Enterobacter cloacae ATCC 23355 strain was used as a test organism. Inhibition of bacterial growth was assessed using MTT assay. Calibration curves were prepared from concentration response curves in plasma (0, 0.5, 1, 2.5, 5, 12.5, 25, 50 ng/μl) and were all linear with correlation coefficients better than 0.995. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 10% (% coefficient of variations: % C.V.) Good accuracy was observed for both the intra-day and inter-day assays, as indicated by the minimal deviation of mean values found between the measured samples and the theoretical values (below $\pm$10%). Limit of quantification (L.O.Q.) was accepted as 0.5 ng using 20 μl plasma or 10 μl urine sample. The mean recovery for fosmidomycin was greater than 99%. The method was free from interference from other commonly used antibiotics including azithromycin. The method appears to be robust and has been applied to a pharmacokinetic study to determine the plasma of fosmidomycin in a Thai patient with acute uncomplicated falciparum malaria following oral doses of 1,800 mg fosmidomycin given every twelve hours for three days, in combination with azithromycin at the dose of 750 mg given every twelve hours for three days.

Keywords: *Plasmodium falciparum*, fosmidomycin, bioassay, spectroscopy.

Introduction

Fosmidomycin [3-(formylhydroxy-amino)-propylphosphonic acid mono-sodium salt,3-(*N*-formyl-*N*-hydroxy-amino)-propylphosphonic acid mono-sodium salt, FR-31564) is a phosphonic acid derivative originally isolated as a natural antibiotic from *Streptomyces lavendulae*. It acts as a potent inhibitor of 1-deoxy-*D*-xylulose 5-phosphate (DOXP) reductoisomerase, an essential enzyme of the non-mevalonate pathway and therefore, selectively blocks the biosynthesis of isopentenyl diphosphate and the subsequent development of isoprenoids in *Plasmodium falciparum* (1). The drug has been shown *in vitro* and *in vivo* in animal studies to be a potential antimalarial, but the development of recrudescence found in early phase of clinical trial precludes its use as monotherapy (2). Recently pharmacokinetics and pharmacodynamics of fosmidomycin monotherapy and combination therapy with clindamycin have been evaluated in Thai patients with multi-drug resistance falciparum malaria (3-4). Pharmacokinetic investigation to determine the minimum inhibitory concentration (MIC) is essential for optimal dose adjustment particularly in the event of multidrug resistant *P.falciparum*. Recently, we have reported a bioassay method based on agar diffusion disk assay (5). The method is sensitive with limit of quantification (L.O.Q.) of 1 ng using 40-μl plasma. Nevertheless, it requires tedious and long procedure of 3-4 days for bacterial culture and assessment of growth inhibition. In the present study, we develop a high throughput bioassay method for determination of fosmidomycin in plasma and urine based on spectrophotometrric assessment of growth by MTT assay (6).

Methods

The test organism used was *Enterobacter cloacae* ATCC 23355 strain. For each assay, plasma (20 µl) containing different concentrations of fosmidomycin (0, 0.5, 1, 2.5, 5, 12.5, 25, 50 and 100 ng/µl (triplicate wells each) was added into each well of the 96-well microtiter plate containing 150 µl of the prepared bacterial suspension in LB broth (2.1×10^5 CFU) per ml). The plate was then incubated at 37 °C for 18-24 h, and thereafter, 5 µl of the 5-mg/ml stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (20 µl of 5 mg/ml) was added to each well of the plate. The pate was left at room temperature for 5 min and optical density reflecting bacterial growth (formation of formazan crystals) was measured at 595 nm using a microplate reader. The blank well consisted of LB Both and MTT. A nonlinear regression analysis of sigmoidal dose-response (variable slope) was performed using automated curve fitting software (GraphPad Prism 5.0™, CA, USA.). The developed method was validated for linearity, recovery, sensitivity, specificity, precision, and accuracy (7). The validated assay method was applied to the investigation of the pharmacokinetics of fosmidomycin in plasma and urine in a Thai patient with acute uncomplicated falciparum malaria (aged 22 years and weighing 50 kg) who received treatment with fosmidomycin (Jomaa Pharm Co. Ltd., Germany) at a dose of 1,800 mg given every twelve hours for 3 days, in combination with azithromycin (Zithromax™, Pfzer Pharm Co. Ltd.) at a dose of 750 mg given every twelve hours for 3 days. Venous blood samples (3 ml) were collected into heparinised-coated plastic tubes at the following time points: 0, 1, 2, 3, 4, 6, 8, 12, 14, 18, 24, 26, 30, 36, 38, 42, 48, 50, 54, 60, 62, 66, 72, 78 and 84 hours after the first dose of fosmidomycin.

Results

Selectivity of the bioassay system was demonstrated by the absence of interferences from endogenous substances and commonly used antibiotics. The calibration ranges yielded linear relationships with correlation coefficients of 0.995 or better (Fig 1). Good precision and accuracy for both the intra-assay (within-day) and inter-assay (day-to-day) was obtained (Table 1). The limit of quantification (L.O.Q.) in human plasma for fosmidomycin was accepted as 0.5 ng using 20 µl plasma. The pharmacokinetics of fosmidomycin accorded with those described in Thai patients with malaria following mono- or combination therapy of fosmidomycin with clindamycin (12-13). The mean (SD) peak plasma concentration of 4.59 (2.55) µg/ml was achieved at 3.0 h following the first dose. Trough concentrations at 12, 24, 36, 48, 60, 72 and 84 h were 2.18 (1.29), 1.62 (0.74), 1.62 (0.8), 2.20 (0.6), 2.09 (1.2) and 1.15 (0.43) µg/ml respectively.

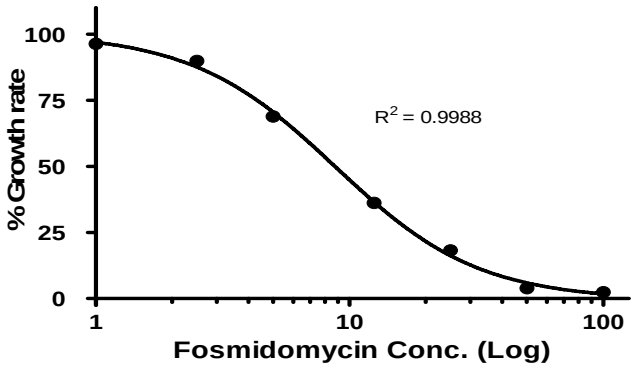

Figure 1 Calibration curves of fosmidomycin in plasma over the concentration ranges of 0-50 ng/µl.

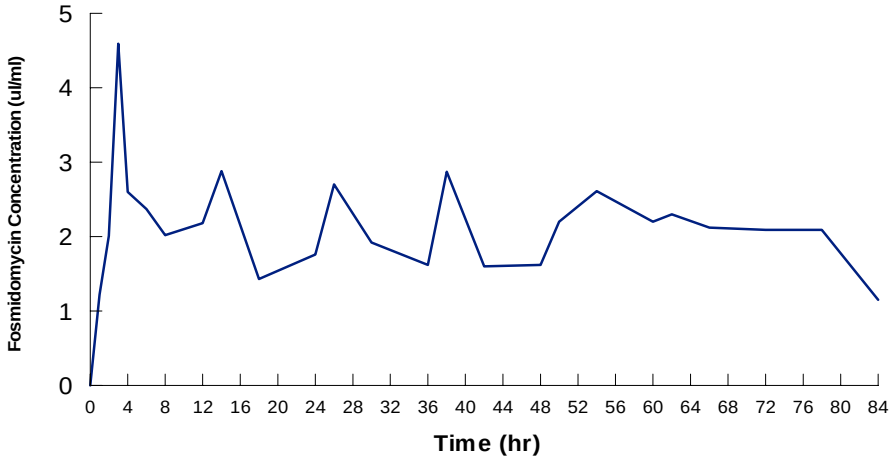

Figure 2 Plasma concentration-time profile of fosmidomycin in a Thai patient with acute uncomplicated falciparum malaria following treatment with 1,800 mg fosmidomycin given every 12 hours for 3 days, in combination with 750 azithromycin given every 12 hours for 3 days.

Table 1 Summary of assay precision and accuracy (intra- assay and inter-assay) for fosmidomycin assay in plasma.

Concentration added (ng/µl)	Precision (%C.V.)[b]		Accuracy (%D.M.V.)[a]	
	Intra-assay (n=6)	Inter-assay (n=6)	Intra-assay (n=6)	Inter-assay (n=6)
0.5	3.68	6.94	-8.00	+6.94
1	8.44	5.63	-6.00	+5.63
2.5	6.29	9.07	+4.40	+9.07
5	4.53	3.11	+2.60	+3.11
12.5	1.07	4.73	-1.76	+4.73
25.0	3.91	3.86	-5.56	+3.86
50.0	6.20	5.47	+7.76	+5.47

[a] %CV = coefficient of variation (%)
[b] %DMV = deviation of mean value from theoretical value (%)

Conclusion

The analytical method established in this study meets the criteria of simplicity, high sensitivity, accuracy and reproducibility for routine use in pharmacokinetic studies. The method has advantages over the recently developed bioassay (5) in its simplicity, accuracy

(end point of measurement was inhibition of bacterial growth assessed spectroscopically by MTT assay *vs* manual measurement clear zone diameter), short analysis time (1 day *vs* 3-4 days), good sensitivity (0.5 *vs* 1 ng), and the requirement for smaller volumes of samples (20 *vs* 40 μl).

Acknowledgement

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Original article **P42**

Liquid chromatography mass-spectrometry for determination of azithromycin in plasma and application for pharmacokinetic study

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Abstract

A simple, sensitive, selective and reproducible method based on high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/MS) was developed for the determination of azithromycin (AZM) in human plasma. The internal standard (IS: roxithromycin) was separated from AZM on a Hypersil Gold C_{18} column, with retention times of 10.71 and 13.67 min, respectively. The mobile phase consisted of a mixture of 20 mM ammonium acetate buffer (pH 5.2), acetonitrile and methanol (50:40:10, v/v/v), running through the column at a flow rate of 0.3 mL/min. Sample preparation was prepared by liquid-liquid extraction with a mixture of 7:3 (v/v) diethylether: dichloromethane. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 5% (% coefficient of variations: % CV). Good accuracy was observed for both the intra-day or inter-day assays. Limit of quantification was accepted as 0.5 ng using 200 μL plasma samples. The mean recoveries for AZM and the IS were greater than 85%. The method was applied successfully to the investigation of the pharmacokinetics of AZM when given as oral doses of 750 mg twelve hourly for 3 days in a total of 5 Thai male patients with acute uncomplicated falciparum malaria.

Keywords: Azithromycin, liquid chromatography mass-spectrometry, pharmacokinetics

Introduction

The antibiotic azithromycin (AZM: Figure 1a), is an inhibitor of protein synthesis by specifically binding to the 50S subunit of the ribosomes in the apicoplast (1). It has been successfully used in combination with artemisinin derivatives and quinine for prophylaxis and treatment of malaria (2). The combination of AZM with fosmidomycin represents another innovative approach to malaria chemotherapy through novel modes of action, coupled with the benefit of additive activity against *Plasmodium falciparum in vitro and in vivo* (3) and there are grounds for anticipating lack of cross resistance with existing drugs and protection against the development of resistance. Pharmacokinetic study of AZM is therefore, essential for dose optimization of the combination. A number of analytical methods have been reported for the determination of AZM in biological fluids including bioassay (3), high performance liquid chromatography (HPLC) with ultraviolet (4), electrochemical (5), fluorescence (6), and HPLC with mass-spectrometry (LC/MS) (7) detection. In the present study, we propose an alternative simple and sensitive LC/MS method with electrospray ionization for determination of AZM. The method was applied for the investigation of the pharmacokinetics of AZM in Thai patients with acute uncomplicated falciparum malaria fallowing a 3-days combination regimen of AZM and fosmidomycin.

Methods

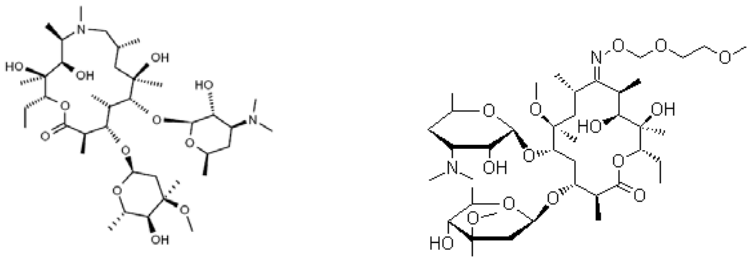
AZM and the internal standard roxithromycin (IS: Figure 1b) were separated on a Hypersil Gold C_{18} reversed phase column (Thermo, 4.6 x 150 mm, 5 μm particle size) with

the mobile phase consisting of a mixture of 20 mM ammonium acetate buffer (pH 5.2), acetonitrile and methanol at a ratio of 50:40:10 (v/v/v), running at a flow-rate of 0.3 mL/min. The mass spectrometer consisted of a Finnigan LCQ Deca XP Max plus ion trap detector equipped with the positive electron spray ionization (ESI) interface (temperature 300 °C, pressure 551 kPa; nitrogen gas flow 70 and 15 arb). Mass results were plotted and processed by the LcQuan^{TM} 2.0 (Thermo Electron Corporation, California, USA). Ions monitored in the selected reaction monitoring (SRM) mode were *m/z* 749.6 *m/z* for AZM; and 837.6 *m/z* for IS. Sample preparation was performed by liquid-liquid extraction as follow: 200 µL plasma, 25 ng IS, 50 µL methanol, 250 µL of 0.25 M carbonate-bicarbonate buffer pH 9.5, 3 mL of the mixture of 7:3 (v/v). Concentrations of AZM were determined from the peak height (PH) (Millennium 2000 Chromatograph$^{™}$) ratios (PH of AZM/PH of IS), which corresponded to the known AZM concentrations in a calibration curve. The developed method was validated for linearity, recovery, sensitivity, specificity, precision, and accuracy (8).

The validated method was applied for the investigation of pharmacokinetics of AZM when given in combination with fosmidomycin at 750 mg (250 mg *per* capsule Zithromax$^{™}$, Pfizer, USA) given every twelve hours for three days in 5 Thai male patients with acute uncomplicated falciparum malaria (aged 25-42 years). Blood samples were collected from all patients at 0, 1, 2, 3, 4, 6, 8, 12, 14, 18, 24, 26, 30, 36, 38, 42, 48, 50, 54, 60, 62, 66, 72, 78, 84, 90, 96 and 108 hours after the first dose. The study was conducted at Hospital for Tropical Diseases and the study protocol was approved by the Ethics Committees of the Faculty of Tropical Diseases, Mahidol University, Thailand. Written informed consents for study participation were obtained from all subjects who had been informed of the study protocol.

Results and discussion

Selectivity of the bioassay system was demonstrated by the absence of interferences from endogenous substances and commonly used drugs. The retention time of AZM and IS were 10.71 and 13.67 min, respectively (Figure 2a, b). The calibration ranges yielded linear relationships with correlation coefficients of 0.990 or better. Good precision and accuracy for both the intra-assay (within-day) and inter-assay (day-to-day) was obtained (Table 1). The limit of quantification (L.O.Q.) in human plasma for AZM was accepted as 0.5 ng using 20 µl plasma. The mean recoveries for AZM and the IS were greater than 85%. AZM was stable when stored at -20^{o} C for up to 3 months. Figure 2 shows mean concentration-time profiles of AZM in 5 male patients. The advantage features of the developed method over the previously reported method include the higher sensitivity, simplicity and rapidity (single step liquid-liquid extraction), and requirement of relatively small extraction volume of 250 µL. Pharmacokinetics of AZM (mean$\pm$SD) following a 3-days multiple dosing are as follows: maximum concentration (C_{max}) 303 $\pm$12 ng/mL, time to maximum concentration (t_{max}) 2$\pm$0.1 hours, and terminal phase elimination half-life ($t_{1/2z}$) 33.2$\pm$2.3 hours.



(a) (b)

Figure 1 Chemical structures of (a) AZM and (b) the IS

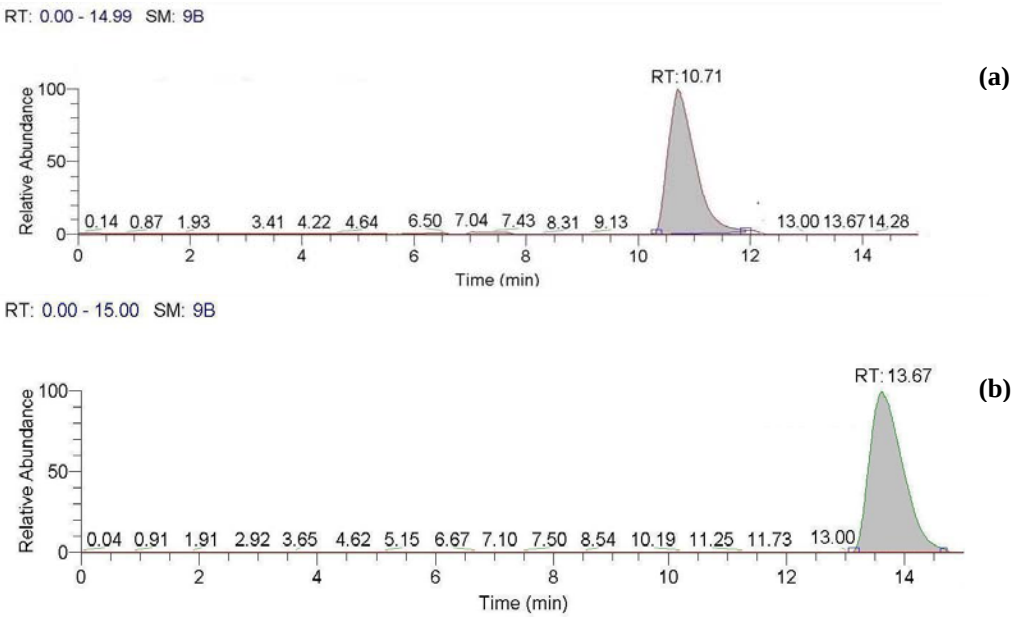


Figure 2 Chromatograms of plasma spiked with (a) 50 ng/mL AZM and (b) 250 ng/mL IS. The retention times for AZM and IS are 10.71 and 13.67 min, respectively.

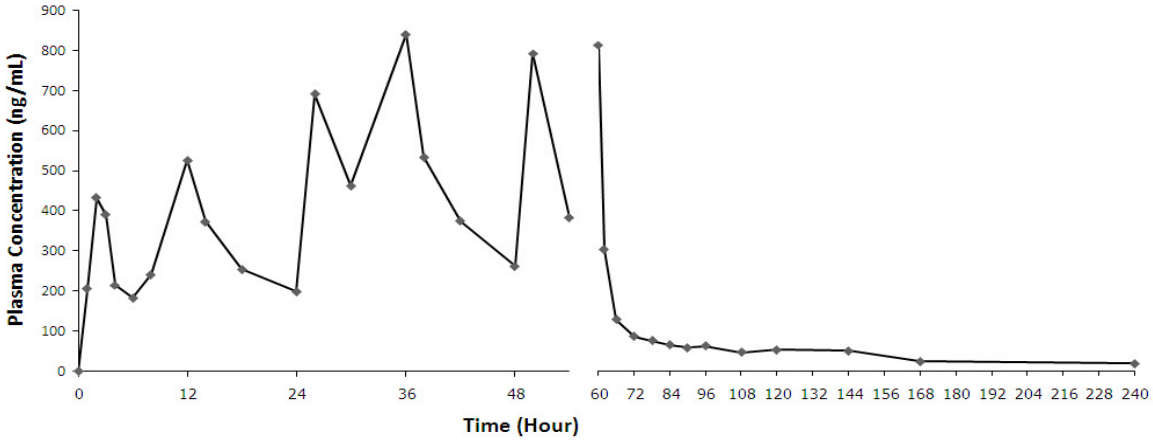


Figure 3 Mean plasma concentration-time profiles of AZM in 5 Thai male patients following a 3-days multiple dosing of 750 mg AZM given every 12 hours

Table 1 Inter-day (between day) and intra-day (within day) validation of AZM concentrations

Concentration added (ng/mL)	Intra-day precision (n = 6)		Accuracy (% DMV)	Inter-day precision (n = 6)		Accuracy (% DMV)
	Concentration measured (mean ± SD; ng/mL)	% CV		Concentration measured (mean ± SD; ng/mL)	% CV	
5.00	5.14 ± 0.06	1.17	2.83	5.34 ± 0.11	2.06	6.80
50.00	48.79 ± 0.51	1.05	-2.41	47.87 ± 0.60	1.25	-4.26
200.00	191.41±2.76	1.44	-4.29	189±189.49	0.89	-5.26
1,000.00	976.70 ± 5.48	0.56	-2.33	980.90 ± 3.44	0.35	-1.91

% CV: coefficient of variation; % DMV: deviation of mean value from the theoretical value.

Conclusion

The LC/MS method developed for determination of AZM in plasma is simple, accurate, sensitive, selective and reproducible for application for pharmacokinetics study of AZM in patients with malaria.

Acknowledgement

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Original article **P43**

Correlation of FcγRIIIa polymorphisms and the response to rituximab in Thai population

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Abstract

It has been reported that polymorphism in Fc gamma IIIa receptor (FcγRIIIa) associates with antibody-dependent cellular cytotoxicity (ADCC) activity of rituximab, the chimeric IgG_1 monoclonal antibody against CD20. This antibody has been used for treatment of several non-Hodgkin's lymphomas originate from B cells and has ADCC as an important mechanism of action. It is also known that genotype frequencies of FcγR polymorphisms depend on race and ethnicity. In this study, we investigated Fc gamma IIIa receptor (FcγRIIIa) genetic polymorphism at amino acid position 158 in Thai population. The nested polymerase chain reaction-restriction fragment length polymorphism (nested PCR-RFLP) was used to identify the FcγRIIIa genotypes of 60 healthy Thai male volunteers with informed consent. The distributions of FcγRIIIa-158 polymorphisms in these subjects were as follows: high binding genotypes (VV and VF), 55%; and low binding genotypes (FF), 45%. The different response of these genotypes to ADCC activity of rituximab was also evaluated. Rituximab-induced ADCC was performed by using PBMCs from 60 volunteers mentioned before as effect cells and ramos cells which express CD20 as target cells in the present of rituximab. High rituximab-induced Ramos cell cytotoxicity (mean rank 37.8%) was observed in the present of PBMCs from subjects with VV and VF genotypes while lower cytotoxicity (mean rank 21.6%) was determined in the present of PBMCs from subjects with FF genotypes. Our results provide the distribution of FcγRIIIa polymorphisms in Thai population which seems to differ from other Asian countries. This information should be useful for considering to use many IgG therapeutic antibodies in Thai population.

Keywords: Anti CD20, FcγRIIIa polymorphisms, Non-Hodgkin's lymphoma, Rituximab.

Introduction

Several monoclonal antibodies have been used for treatment cancers during the past decade. Most of them are IgG_1 cytotoxic antibody specific to tumor antigen on tumor cells. Rituximab, the chimeric mouse/human IgG_1 monoclonal antibody has been approved either alone or in combination with chemotherapeutic agents to treat several non-Hodgkin's lymphomas (NHLs) originate from B-cell since 1997 (1). This antibody targets CD20 which specifically express on B lymphoma cells. ADCC by rituximab-activated NK cells has been suggested to be an important mechanism of rituximab (2). This antibody uses its Fab part for recognizing CD20 on the surface of B lymphoma cells and its Fc part for binding to its receptors, Fc gamma IIIa receptor (FcγRIIIa), on natural killer (NK) cells. This leads to NK cell activation and follows by destruction of the CD20 positive cancer cells by ADCC mechanism. FcγRIIIa on macrophages and NK cells has two expressed alleles that differ at amino acid position 158 in the extracellular domain, valine (V158) and phenylalanine (F158) (3). These allelic variants have been demonstrated to differ in IgG_1 binding and ADCC. VV homozygotes and V/F homozygotes bind IgG high affinity than FF homozygotes (2). Genotype frequencies of FcγRIIIa polymorphisms depend on race and ethinicity. The low binding FF allele is high among Asian population (68%) than Europes or Africa population

(58%) (4, 5). In this study, we identified the distribution of FcγRIIIa polymorphisms in Thai population and correlated these polymorphisms to rituximab-induced ADCC activity of NK cells.

Materials and Methods

Materials:

Human peripheral blood mononuclear cells (PBMCs) were used as effector cells in ADCC assay. The PBMCs were isolated by Ficoll gradient centrifugation of buffy coats from 8 ml-whole blood of healthy male blood donors with informed consent from the National Blood Bank, Thai Red Cross Society. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°c in 5% CO_2/ 95% air. One ml whole blood from each donor was used for FcγRIIIa genotyping.

Human B lymphoma cells, Ramos, which express CD20 from American Type Culture Collection (ATCC) were used as target cells. The cells were stained with 5μM carboxyfluorescein succinimidyl ester (CFSE) for 5 min and then washed with complete RPMI 1640 medium. The CFSE-stained cells were maintained in complete RPMI 1640 medium for 24h before ADCC assay

Rituximab, chimeric mouse-human IgG_1 monoclonal antibody against human CD20, was obtained from Chulalongkorn memorial hospital.

FcγRIIIa genotyping:

The genomic DNA was isolated from one ml whole blood by a blood DNA extraction kits (VIvantis, Malaysia). The FcγRIIIa G559T genotype was determined by nested PCR-RFLP with specific primers for FcγRIIIa gene and *Nla*III restriction enzyme. The PCR-RFLP products were identifies by 3% agarose gel electrophoresis, ethidium bromide staining and UV exposure. PCR products from G (V158) allele were digested by to 2 fragments by the restriction enzyme, *Nla*III, while the products from T (F158) allele were not digested by *Nla*III. The data of genotypes were presented as the percentage of high binding- (VV, VF) and low binding- (FF) genotypes.

FcγRIIIa-induced NK cell activation:

Rituximab (IgG_1 monoclonal antibody) was used as an antibody to activate NK cells in PBMCs via binding to FcγRIIIa on NK cell surface. Activated NK cells can release perforin and granzyme to kill their target cells, Ramos cells, which specifically recognized by rituximab.

Ramos cells, as target cells, were distinguished from PBMCs effector cells by staining with CFSE. CFSE-stained Ramos cells were treated with 10 μg/ml of rituximab for 1 h and then co-cultured with human PBMCs, at PBMCs:Ramos cells ratio of 10:1, for 4 h. These cella were washed with PBS and stained with 1 μg/ml propidium iodide (PI). Rituximab-mediated Ramos cell cytotoxicity ($CFSE^+/PI^+$ cells) was analyzed by flow cytometer. The percentage of rituximab-mediated Ramos cell cytotoxicity was calculated.

The correlation between FcγRIIIa genotype and ADCC response were presented as individual dot plot with mean rank values. The difference of rituximab-mediated cytotoxicity in each genotyping group was compared by using the Mann-Whitney u test to determine.

Results

The frequencies of FcγRIIIa -158 polymorphisms from 60 healthy Thai male volunteers were 55% V carrier (VV, VF) and 45% homozygous FF (Table 1). Polymorphism

at position 158 of FcγRIIIa have been reported influence human IgG1 binding and ADCC. It has been demonstrated that patients with follicular-NHL who were VV homozygous and VF heterozygous had higher response to rituximab than FF homozygous (6). We investigated the correlation of FcγRIIIa polymorphism among the volunteers in this study to FcγRIIIa-induced NK cells from these volunteers were used as effector cells and separated from target cells, Ramos cells, by staining Ramos cells with CFSE. Rituximab-mediated Ramos cell death was higher in VV and VF subjects than in FF subjects. The mean rank of the percentage of cytotoxicity in the former group (37.8%) was statistically different the latter group (21.6%), as shown in Fig 1.

Table 1: Distribution of FcγRIIIa genotyping from 60 normal volunteers

	High binding genotypes (VV, VF)	Low binding genotype (FF)
Normal volunteer (60n)	33	27
Percent (%)	55%	45%

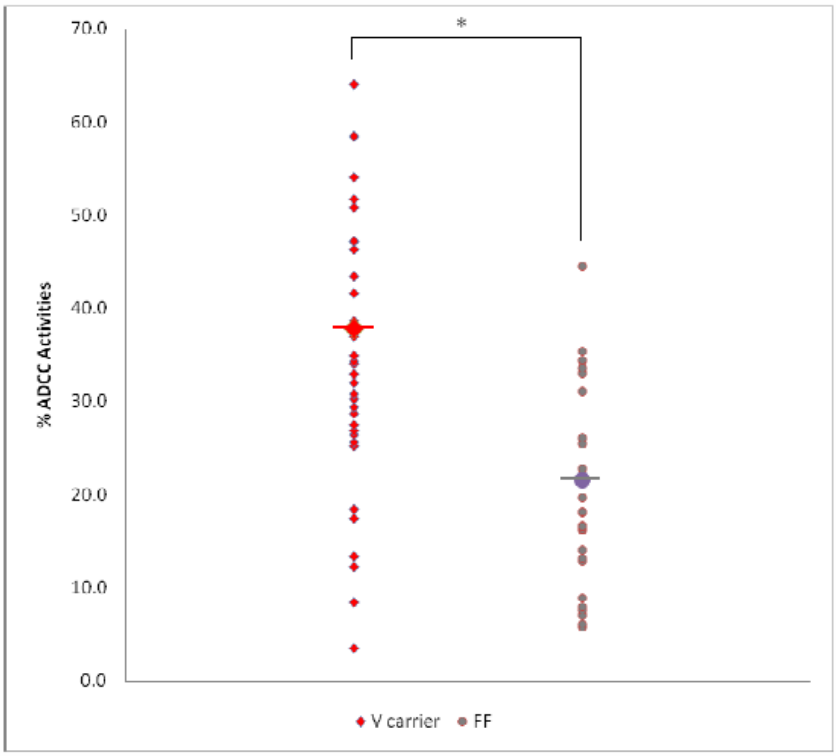

Figure 1: Correlation of the FcγRIIIa genotype to rituximab-induced ADC activity of NKc ells in PBMCs from 60 healthy Thai male volunteers. Data are presented as individual dot plot and the mean rank values of both polymorphisms.

Discussion

Rituximab is used for treatment of various B cell lymphomas. It has been demonstrated that this anti-CD20 antibody induces lymphoma cell death *in vitro* by ADCC, complement-mediated cytotoxicity or apoptosis (2). ADCC is an important rituximab cytotoxic mechanism against tumor cells. The implication of FcγRIIIa in the anti-tumor

effects of rituximab against human lymphoma cell lines has been demonstrated in murine models. Dimorphism of this antibody receptor at amino acid position 158 (V or F) has an in fluence on IgG binding and ADCC. Human IgG_1 binds more strongly to homozygous FcγRIIIa-158V NK cells than to homozygous FcγRIIIa-158F NK cells (5). It has been also reported that NHL patients with FcγRIIIa –FF have lower clinical response than patients with V homozygous (6). We indentified the distribution of FcγRIIIa polymorphisms in Thai population. Our results demonstrated that the percentage of FF homozygous in Thai populationis lower than Asian Population (68%) and Europes or Africa population (58%) (4, 5). The association between the FcγRIIIa genotype and the response to rituximab in Thai patients with NHL is ongoing. Because this polymorphism depends on race and ethinicity, these results may provide useful information to understand beneficial response of rituximab as well as other IgG_1 therapeutic antibody in Thai patients.

Acknowledgements

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Original article **P44**

Possible role of heme oxygenase-1 and prostaglandins in pathogenesis of cerebral malaria: induction of heme oxygenase-1 by prostaglandin D2 and metabolite by human astrocyte CCF-STTG1 cells *in vitro*

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Abstract

Astrocytes are the most abundant cells in central nervous system that play role in maintaining the blood-brain-barrier and in neural injury, including cerebral malaria, a severe complication of *Plasmodium falciparum.* Prostaglandin D_2 (PGD_2) is abundantly produced in the brain and regulates the sleep response. Moreover, PGD_2 is a potential factor derived from *P.falciparum* within erythrocytes. Heme oxygenase-1 (HO-1) is catalyzing enzyme in heme breakdown process to release iron, carbon monoxide and biliverdin/bilirubin, and may influence iron supply to the *falciparum* parasites. Here, we showed that treatment of human astrocyte cell, CCF-STTG1, with PGD_2 and its metabolite 15d-PGJ_2 significantly increased the expression levels of HO-1 mRNA by RT-PCR. Western blot analysis showed that PGD_2 and 15d-PGJ_2 treatment increased the level of HO-1 protein, in a dose- and time-dependent manner. Thus, both prostaglandins may be involved in the pathogenesis of cerebral malaria by inducing HO-1 expression in malaria patients.

Keywords: astrocyte cell, heme oxygenase, iron, malaria, prostaglandin D_2

Introduction

Malaria is a worldwide protozoan infection, and most malignant malaria is caused by *Plasmodium falciparum*. Cerebral malaria is one of the most severe complications of *P. falciparum* infection (1). The parasite does not enter the brain parenchyma, but staying in the intravascular circulation, which is responsible for changes at the BBB. *In vitro* and *in vivo* studies support the role of astrocytes in controlling blood-brain-barrier (BBB) maintenance and regulation through their interaction with cerebral endothelial cells (2). The molecular basis underlying cerebral malaria nevertheless, remains unclear. Recently, the microsomal enzyme heme oxygenase-1 (HO-1) has been proposed as one of the factors that play significant role in pathogenesis of this malaria complication (3). It is the rate-limiting enzymes in heme catabolism to generate biliverdin IXα/bilirubin IXα, carbon monoxide, and ferrous iron (3). The expression levels of HO-1 is inducible or repressible, depending on cells types or cellular microenvironments, but expression levels of HO-2 are fairly constant (3). We have recently reported that the short $(GT)_n$ repeats ($n < 28$) in the HO-1 gene promoter are associated with higher incidence of cerebral malaria in the Karen ethnic minority group who live near the border between Myanmar and Thailand (4). The cell homogenates of *P. falciparum* have been shown to contain the activity that produces PGD_2 and PGE_2 after incubation with arachidonic acid (5). Moreover, PGD_2 was maintained at a higher level in serum of falciparum malaria patients than control serum (6). We also found that PGD_2 and 15d-PGJ_2, a minor species of PGD_2 metabolites, increased HO-1 gene promoter, mRNA levels and protein levels in retinal pigment epithelial cells that may associate with the pathogenesis of malarial retinopathy in malaria patients with cerebral complication (7). In the present study, we provide additional data to support the link between HO-1 and pathogenesis of cerebral malaria through the induction by PGD_2 in human astrocytes.

Methods

To examine the effects of PGD_2 and 15d-PGJ_2 (Cayman chemicals) on the expression levels of HO-1 protein and mRNA, CCF-STTG1 cells(ATCC®, # CRL-1718, USA) were grown to 70-80% confluence (RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin and 0.1 mg/ml streptomycin) before they were incubated with vehicle (ethanol), PGD_2 or 15d-PGJ_2. The Western blots were probed with HO-1 (SPA-895, StressGen Biotechnologies) and β-actin (Sigma) antibodies (7), followed by incubation with AP-goat anti-rabbit and AP-goat anti-mouse, respectively (ZyMax™, Invitrogen) for detection of HO-1 protein.

Total RNA was extracted from treated CCF-STTG1 cells using RNeasy® Mini Kit (Qiagen) and was transcribed to cDNA using Omniscript® RT Kit (Qiagen). Then HO-1 cDNA levels were determined by RT-PCR. Primers were designed according to the published cDNA sequences for human HO-1 (8).

Results

Effects of PGD_2 and 15d-PGJ_2 on the expression of HO-1 protein in CCF-STTG1 cells: Western blot analysis revealed that the expression of HO-1 protein was induced in a dose- and time-dependent manner by PGD_2 and 15d-PGJ_2 at final concentrations of 5 and 10 µM after 24-h and 6-h treatment, respectively (Figure 1A, 1B). The onset of the HO-1 induction with 15d-PGJ_2 was earlier than with PGD_2.

Increased expression of HO-1 mRNA in CCF-STTG1 cells treated with PGD_2 and 15d-PGJ_2: The expression of HO-1 mRNA was induced by PGD_2 or 15d-PGJ_2 in a dose-dependent manner (Figure 2A, B). The expression levels of HO-1 mRNA were increased after 6 h of the treatment with PGD_2 of 5 µM or 10 µM, and continuously increased at maximum level after 12 h (Figure 2A). For 15d-PGJ_2, the expression levels of HO-1 mRNA were detected at 3 h of treatment (Figure 2B). The maximum induction of HO-1 mRNA was achieved after 6 h of treatment, but it was decreased at 24 h (Figure 2B). The induction profiles of HO-1 mRNA were in good agreement with those of HO-1 protein (Figure 1A, B).

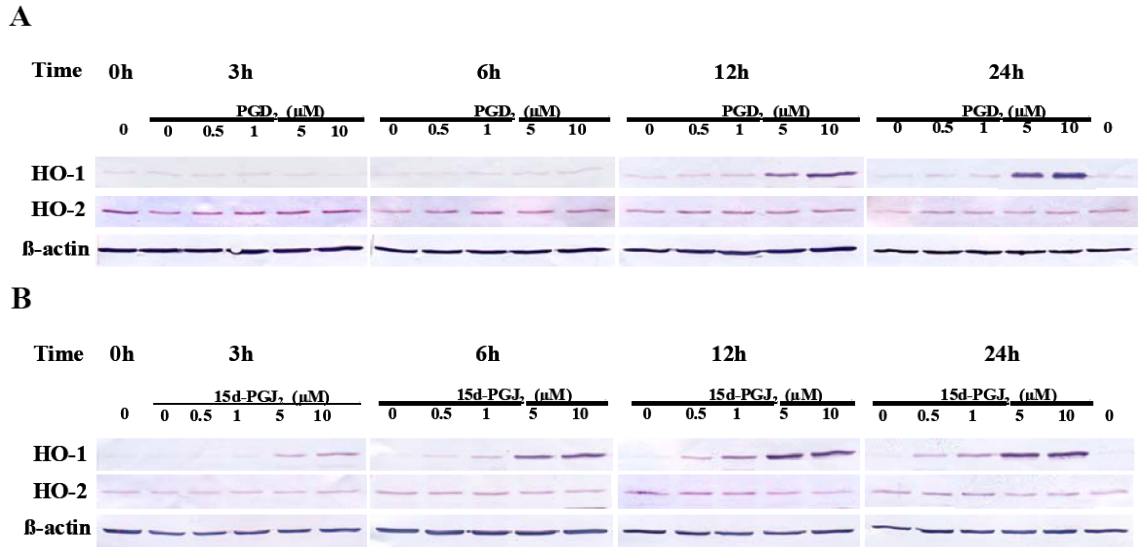

Figure 1. Effects of (A) PGD_2 and (B) 15dPGJ_2 on HO-1 protein in CCF-STTG1 cells. Data shown are from one of two independent experiments.

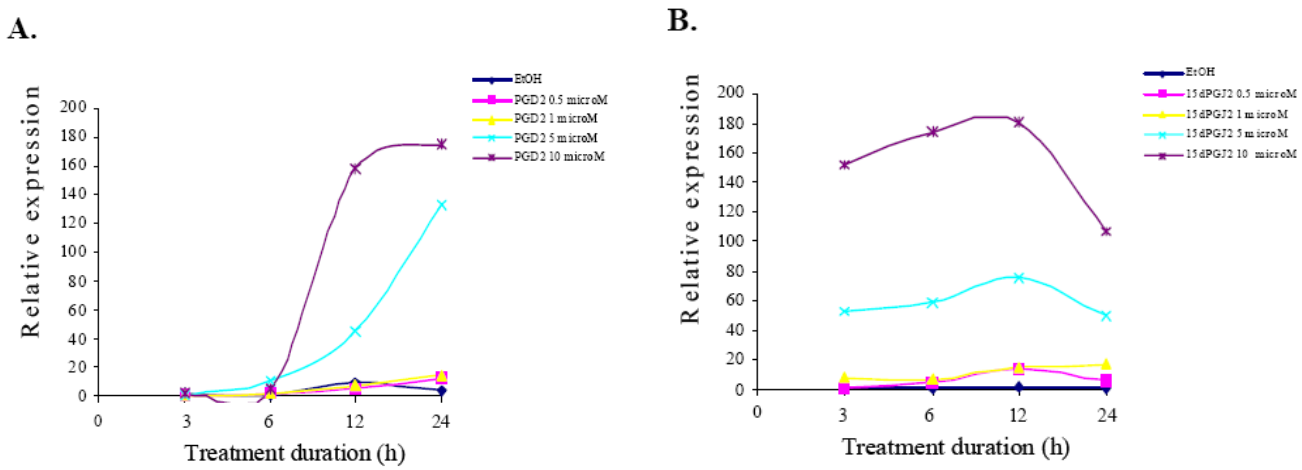

Figure 2. Expression of HO-1 mRNA by (A) PGD_2 and (B) $15dPGJ_2$ in CCF-STTG1 astrocyte cells. Data were obtained by dividing the intensity value for each sample with 0-h untreated control cells, which reflected basal expression level.

Discussion

The current result showing that treatment of the astrocyte cells CCF-STTG1 with exogenous PGD_2 consistently induced HO-1 expression, is in agreement with our recent report in retinal pigment epithelial cells (7). The induction profile of HO-1 expression with PGD_2 is essentially similar to that with 15d-PGJ_2, although the onset of the induction by PGD_2 is later than by 15d-PGJ_2. It remains to be explored whether PGD_2 by itself or a specific PGD_2 metabolite other than 15d-PGJ_2 is responsible for the induction of HO-1 expression. Apart from astrocyte cells themselves, falciparum parasites may also release PGD_2 that may hasten the expression of HO-1. This consequence additively results in the increase in HO-1 activity to catalyse heme to end products, especially, iron. Malaria growth and proliferation depend on iron supply from host cells, such as endothelial cells or neuron cells near the sequestration site. Therefore, PGD_2 might enhance the growth of parasites by modulating iron availability from host. The stimulation of HO-1 by PGD_2 and the metabolite 15d-PGJ_2 observed in this study is critical since excessive heme degradation may result in toxic levels of iron similar to that by carbonmonoxide, and bilirubin/biliverdin.

Conclusion

PGD_2 and 15d-PGJ_2 may be involved in the pathogenesis of cerebral malaria by inducing HO-1 expression in malaria patients. This information could be exploited for development of new antimalarial drugs acing as inhibitors of HO-1 to prevent the progression to severe cerebral malaria. Further studies are require to obtain supporting evidence in patients with malaria.

Acknowledgements

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Original article **P45**

The association between genetic polymorphisms of heme oxygenase-1, tumor necrosis factor-α (TNF-α), and malaria severity in different ethnic group of patients

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Abstract

Malaria is one of the most important public health problems in several countries in the world. The knowledge on pathogenesis of severe malaria particularly cerebral malaria remains controversial and debatable. Heme oxygenase (HO) enzyme has been proposed as one of the factors that may play significant role in pathogenesis including susceptibility and severity of malaria infection. TNF-α is a cytokine produced primarily by monocytes and macrophages in many inflammatory diseases. The polymorphisms of the TNF promoter have been reported to be associated with susceptibility to severe malaria. In this study, we analyzed $(GT)_n$ repeat polymorphism in the promoter region of the inducible HO-1 and six mutations of TNF-α from malaria patients (Thais, Burmeses and Karens), to determine the association between genetic polymorphisms of HO-1, TNF-α and severity of malaria infection. Our result indicated that the genotype of $(GT)_n$ repeat between ethnic group of patients was significantly different.

Keywords**:** heme oxygenase, malaria, *Plasmodium falciparum*, disease severity

Introduction

Malaria remains health problem in Thailand. Cerebral malaria (CM) is one of the major pathological complications of *Plasmodium falciparum* infection in humans manifesting as coma that can lead to death. The pathogenesis of CM remains controversial but major factors involved, *i.e.*,cytokines and adhesion molecules are well documented (1). Malaria is problematic along international borders of Thailand where there is significant population movement. The highest cases of malaria in 2009 were reported from Mae Sot District, Tak Province. Heme oxygenase (HO) enzyme has been proposed as one of the factors that may play significant role in pathogenesis including susceptibility and severity of malaria infection (2, 3). The two isiforms, HO-1 and HO-2, are microsomal enzymes that play important role in heme catabolism to produce biliverdin/bilirubin, carbonmonoxide (CO) and iron (2, 4,). The polymorphism of human HO-1 gene promoter may contribute to the fine tuning of the transcription. Long $(GT)_n$ alleles have been found associated with susceptibility to several diseases, while they may be linked to resistance to CM (3). TNF-α is a cytokine produced in many inflammatory diseases. Polymorphism in TNF promoters have been reported to be associated with susceptibility to severe malaria, and TNFP-D allele has been shown significantly associated with cerebral malaria in Karen and Burmese populations (5). The objective of this study was to investigate the association between the genetic polymorphisms of HO-1, tumor necrosis factor-α (TNF-α) and severity of malaria infection in three ethnic

groups of patients. This information would hopefully be exploited for development of antimalarial drug targets that interrupt the progress of malaria pathogenesis.

Methods

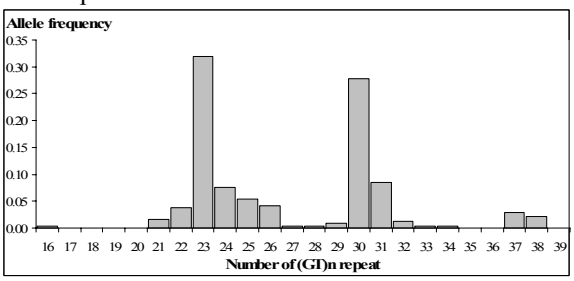
Blood samples were collected from 488 malaria patients (mean age 28 years, parasitemia 77-1,840,000/µl) who were present at Mae Sot General Hospital, Mae Sot, Tak Province, Thailand. The study protocol was approved by the Ethics Committee of the Ministry of Public Health, Thailand. All patients gave informed consents for study participation prior to the study enrolment. Severity of malaria pathology was classified based on the type and parasitemia as non-severe (uncomplicated falciparum malaria and vivax malaria) and severe malaria (falciparum malaria with hyperparasitemia). Microsatellite polymorphisms were used for analysis of HO-1 and direct sequencing was used for analysis of TNF-α. The association of malaria severity, polymorphisms of HO-1 and TNF-α genotypes in different ethnic groups was analyzed using chi-square test at a statistical significance level (α) of 0.05.

Results

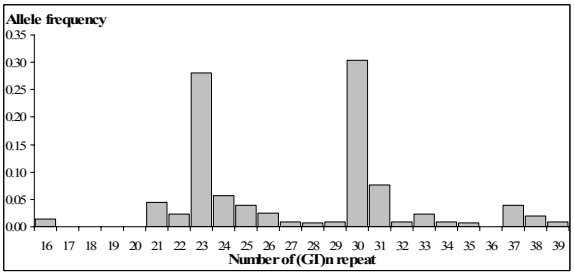
The polymorphism of HO-1 in three ethnic groups of malaria patients

The number of $(GT)_n$ repeats of the HO-1 gene varied between 16 and 39 in all patients (Figure 1). The allele frequencies of $(GT)_n$ allele were similar in the three ethnic groups. With regards to the frequencies of the genotypes (S/S, S/M, S/L, M/M, M/L, L/L) of $(GT)_n$ repeats (Table 1), significantly lower frequency of S/L but higher frequency of MM genotype was found in Thai patients compared with Burmese patients. Burmease patients however carried S/S and M/L genotypes at significantly lower frequencies than Karen patients. No association between disease severity and HO-1 genotype including L and non-L allele frequencies were observed.

a. Thai patients



b. Burmese patients



c. Karen patients

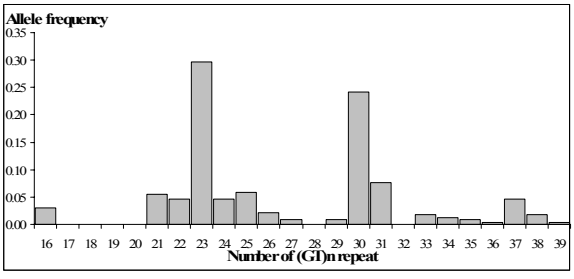


Figure 1. Frequency distribution of $(GT)_n$ repeats in three ethnic groups of patients

	Ethnic		
	Thai	Burmese	Karen
n	119	240	118
Alleles, *n* (%)			
S	132 (55.5%)	236 (49.2%)	133 (56.4%)
M	93 (39.1%)	205 (42.7%)	81 (34.3%)
L	13 (5.5%)	39 (8.1%)	22 (9.3%)
Genotypes, *n*			
S/S	39 (32.8%)	57 (23.8%)[a]	40 (33.9%)
S/M	50 (42.0%)	97 (40.4%)	45 (38.1%)
S/L	4 (3.4%)[b]	25 (10.4%)	8 (6.8%)
M/M	18 (15.1%)	49 (20.4%)[c]	12 (10.2%)
M/L	7 (5.9%)	10 (4.2%)[d]	12 (10.2%)
L/L	1 (0.8%)	2 (0.8%)	1 (0.8%)

Table 1. Distribution of HO-1 promoter genotypes and allele frequencies of the malaria patients

[a] $p = 0.042$, 95%CI: 0.3746-0.9849 for Burmese vs Karen
[b] $p = 0.021$, 95%CI: 0.1016-0.8804 for Thai vs Burmese
[c] $p = 0.015$, 95%CI: 1.1545-4.4483 for Burmese vs Karen
[d] $p = 0.026$, 95%CI: 0.109-0.9169 for Burmese vs Karen

The polymorphism of TNF-α alleles in three ethnic groups of malaria patients

The patterns of SNPs in TNF-α alleles in the three ethnic groups were observed based on five (5) and three (6) point mutations as shown in Table 2 and 3. For the five SNPs of biallelic polymorphism sites (-1031, -863, -857, -308 and -238), 6 types of TNF promoter (TNFP) alleles were found, *i.e.,* TNFP-A, TNFP-B, TNFP-C, TNFP-D, TNFP-E and TNFP-F. For the pattern of three SNPs of biallelic polymorphism sites (-1031, -863, -857), 11 types of TNFP alleles were observed, *i.e.,* TNFP1, TNFP-2, TNFP-3, TNFP-4, TNFP-5, TNFP-6, TNFP-7, TNFP-8, TNFP-9, TNFP-10 and TNFP-11. There was no significant difference in the frequencies of each mutation among all the three ethnic groups. In addition, lack of association between disease severity and SNP in TNF-α alleles was found.

Table 2. Five TNF- α alleles detected in Thai, Burmese and Karen patients with malaria diseases

TNF type	Polymorphic sites of the promoter region of TNF-α gene					Ethnic		
	-238	-308	-857	-863	-1031	Thai (%)	Burmese (%)	Karen (%)
TNFP-A	G	G	C	C	T	49.4	50.3	51.0
TNFP-B	G	G	C	A	C	37.0	33.8	36.5
TNFP-C	R	G	C	M	C	1.3	4.7	3.1
TNFP-D	G	G	Y	M	Y	6.5	4.1	3.1
TNFP-E	G	R	C	M	Y	5.2	5.6	4.2
TNFP-F	G	G	C	A	T	0.6	1.6	2.1

Table 3. Eleven TNF- α alleles detected in Thai, Burmese and Karen patients with malaria diseases

TNF type	Polymorphic sites of the promoter region of TNF-α gene			Ethnic		
	-857	-863	-1031	Thai (%)	Burmese (%)	Karen (%)
TNFP-1	C	C	T	28.6	31.1	37.5
TNFP-2	C	A	C	11.7	9.9	16.7
TNFP-3	C	M	C	1.3	3.7	4.2
TNFP-4	Y	M	Y	3.9	3.1	2.1
TNFP-5	C	M	Y	44.2	42.2	31.3
TNFP-6	C	A	T	0.0	0.6	0.0
TNFP-7	C	C	Y	0.0	3.7	2.1
TNFP-8	Y	C	T	7.8	4.3	2.1
TNFP-9	C	A	Y	1.3	0.6	2.1
TNFP-10	Y	M	T	0.0	0.0	2.1
TNFP-11	Y	C	Y	1.3	0.6	0.0

Discussion

The $(GT)_n$ repeat polymorphism in the HO-1 gene promoter has been demonstrated to link with different diseases in various studies with different ethnic backgrounds (7, 8, 9). In malaria, the association between the microsatellite polymorphism of HO-1 gene promoter and malaria disease was previously reported in Burmese and Karen patients, where a significant association between short $(GT)_n$ alleles (S/S) and high incidence of cerebral malaria was found (10). This observation could be confounded by the influence of ethnics. Nevertheless, in this study, the different in HO-1 genotype among the three ethnic groups of malaria patients was observed with S/S, S/L, M/M and M/L, but not with S/M, and L/L genotypes. Individuals with HO-1 genotypes with S allele would be expected to have increased HO-1 transcription and enzyme activity, with the activity in descending order as follow: S/S > S/M > S/L > M/M > M/L > L/L. Unfortunately, the activity of HO-1 could not directly be determined to support this conclusion.

TNF-α is one of candidate genes to determine the susceptibility for severity of malaria. In Thailand, the level of TNF-α and severe malaria have been reported. Three single nucleotide polymorphisms (SNPs) of the tumor necrosis factor alpha (TNF) promoter -1031, -863, and -857 have been studied and the frequency of TNF U04 allele was found to be significantly higher in patients with cerebral malaria than that with mild malaria (6). The biallelic polymorphic sites at nucleotides -238, -308, -857, -863, and -1031, and seven alleles have been identified in patients from Burma who lived near Thai-Burmese border (5) and TNF promoter (TNFP)-D allele was significantly associated with cerebral malaria in Karen and Burmese patients.

Conclusion

The findings support the hypothesis for the contribution of ethnicity and genetic polymorphism of HO-1 to the severity of malaria infection. The knowledge could be exploited for developing of drugs that might interrupt the progression of severity of disease pathogenesis.

Acknowledgments

This work was supported by The Commission on Higher Education, Ministry of Education of Thailand. We thank staff of Maesot General Hospital for their assistance in sample collection.

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Original article **P46**

Compliance with a three-day course of artesunate-mefloquine combination in an area of Thailand with highly multidrug resistant falciparum malaria

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Abstract

The compliance of a three-day course of artesunate (ARS)-mefloquine (MQ) (4 mg/kg body weight ARS once daily for three consecutive days, plus 15 and 10 mg/kg body weight MQ on the first and second days) was evaluated in a total of 240 patients (196 males and 44 females) who were attending the malaria clinics in Mae-Sot, Tak Province and presenting with symptomatic acute uncomplicated *Plasmodium falciparum* malaria. The gametocytocydal anti-malarial drug primaquine (PQ) is administered at the dose of 30 mg (0.6 mg/kg) on the last day. The first dose of the treatment was given to the patients under direct supervision. All patients were given the medication for self-treatment at home and were requested to come back for follow-up on day 3 of the initial treatment. The Kaplan-Meier estimate of the 42-day efficacy rate of this combination regimen was 99.2% (238/240). A three-day combination regimen of ARS-MQ provides excellent patient compliance with good efficacy and tolerability in the treatment of highly multidrug resistance falciparum malaria Based on whole blood MQ and plasma PQ concentrations on day 3 of the initial treatment, compliance with MQ and PQ in this three-day ARS-MQ combination regimen were 96.3% (207/215), and 98.5% (197/200), respectively.

Keywords**:** *Plasmodium falciparum*, artesunate-mefloquine combination, compliance

Introduction

Malaria chemotherapy is under constant threat from the emergence and spread of multidrug resistance of *Plasmodium falciparum.* In Thailand, where multidrug resistant *P. falciparum* is at high level, the National Malaria Control Programme has adopted artesunate (ARS) in combination with mefloquine (MQ) as first-line treatment for uncomplicated falciparum malaria since 1995 (1). The current regimen is a 3-day course of a total dose of 600 mg ARS and 1,250 mg MQ (given as two split doses of 750 and 500 mg). The gametocytocydal anti-malarial drug primaquine (PQ) is administered at the dose of 30 mg (0.6 mg/kg) on the last day. It is believed that this regimen will improve the cure rate and delay anti-malarial drug resistance. The concern is however, patient compliance when applied to field condition. The main purpose of the present study was to investigate patient compliance of the current three-day course of artesunate-mefloquine as first-line treatment for uncomplicated falciparum malaria in Thailand.

Methods

The study was conducted at malaria clinics, Tak Provinces, during March 2008 – February 2009. The study was approved by the Ethics Committee of the Department of Disease Control, Ministry of Public Health of Thailand. A total of 240 patients (196 males and 44 females) presenting with symptomatic acute uncomplicated falciparum malaria (asexual form parasitaemia over 1,000 *per* microliter blood), who had no history of liver and

kidney disease and no previous anti-malarial treatment during the previous four weeks, were recruited into this study. Written informed consents were obtained from all patients before study participation. Patients were treated with a 3-day combination regimen of ARS and MQ. The initial dose of 200 mg ARS (4 tablets; Atlantic Pharmaceutical Company, Thailand) and 750 mg MQ (3 tablets; Atlantic Pharmaceutical Company, Thailand) were given under supervision on the first day (day 0). Then patients were given ARS and MQ tablets for self-treatment at home. The dose regimen on day 2 was 200 mg ARS and 500 mg MQ. On day 3, 200 mg ARS was given with 15 mg (2 tablets; Government Pharmaceutical Organization of Thailand). Patients were requested to return for follow-up in the morning of the third day of treatment (day 3), and on days 7, 14, 21, 28 and 42 or at any time if fever or symptoms suggestive of malaria developed. Blood samples were taken (3 ml) for determination of MQ and PQ concentrations on day 3 to assess patient complaince. MQ and plasma PQ concentrations were measured by high performance liquid chromatography (2, 3).

Results

All patients had a rapid initial response to treatment with parasites cleared from peripheral blood within 3 days of an initial dose of artesunate and mefloquine. The Kaplan-Meier estimate of the 42-day efficacy rate was 99.2% (95% CI 99.0-99.8). No serious adverse event (SAE) was reported during the study. Figure 1 and 2 represent Box and Whisker plot of whole blood MQ and plasma PQ concentrations on day 3 in 215 and 200 cases, respectively. For MQ, the upper, mid and lower lines which represent 1st, 2nd and 3rd quatiles were 1,716, 2,359, and 3,059 ng/ml, respectively. Median (range) concentrations on day 3 were 2,359 (27-10,965) ng/ml, with a 95% confidence interval of 1,977-2,353 ng/ml. There were 10 patients with outlier MQ concentrations (1.5-3 box length apart from the upper or lower limit line). Two had concentrations above the upper limit (10,000 ng/ml), 7 had concentrations below the lower limit (27, 90, 141, 172, 215, 247, 695 ng/ml), and one had undetectable level of MQ (lower than quantification limit). Based on MQ concentrations on day 3, complaince with MQ in this 3-day regimen was 96.3% (207/215). For PQ, the upper, mid and lower lines which represent 1st, 2nd and 3rd quatiles were 1,716, 2,359, and 3,059 ng/ml, respectively. Median (range) concentrations on day 3 were 89 (6-640) ng/ml, with a 95% confidence interval of 75-106 ng/ml. There was no patient with outlier PQ concentrations, but undetecable concentration was observed in three cases. Based on PQ concentrations on day 3, compliance with PQ in this combination regimen was 98.5% (197/200).

Figure 1 Box and Whisker plot of whole blood MQ concentrations on day 3 after the initial treatment. Median, 1st and 3rd quatiles = 2,359, 1,716 and 3,059 ng/ml, respectively. Each individual dots represents the case with outlier concentration below or above 1.5-3 box length

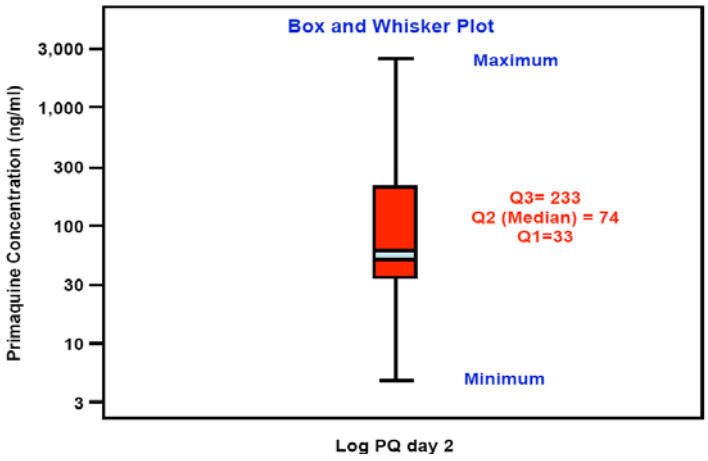

Figure 2 Box and Whisker plot of plasma PQ concentrations on day 3 after the initial treatment. Median, 1st and 3rd quatiles = 33, 74 and 223 ng/ml, respectively. Each individual dots represents the case with outlier concentration below or above 1.5-3 box length

Discussion

The present combination regimen of artesunate-mefloquine was shown to improve the cure rate from approximately 87% with the 2 day course to 96.3% in the same study area (Tak province) during the year 2001 to 2002 (4). As the combination is not a fixed dose regimen, assessment of blood concentration of only one combination partner, *i.e.* mefloquine with long half-life of 14-21 days (5) may not reflect the real full compliance of the combination regimen. Furthermore, since the half-lives of ARS and its active plasma metabolite (dihydroartemisinin) are very short (0.5-2 hr), the drug would have been cleared from blood before 24 hours until patients returned for follow-up on the third day of treatment (day 3). Apart from MQ, plasma concentrations of PQ on day 3 was, therefore, also used as a marker of patient's adherence to the 3-day regimen with the assumption that if patients took primaquine tablets, it was likely that they would have also taken artesunate tablets on the second and third day. Based on mefloquine and primaquine concentrations on day 3 after the intial treatment, patient compliance of as high as 96-98%. This is considered excellent when comparing with compliance to long treatment courses of other regimens, such as a seven-day course of quinine-tetracycine, where prolonged drug administration or a relatively high incidence of cinchonism contributes to about 71.7% compliance in the field trials (6). MQ is a long half-life drug, therefore, whole blood mefloquine levels on day 3 after the initial treatment could be applied for monitoring of compliance to this combination regimen with good accuracy. For PQ on the other hand, plasma concentration on day 3 may not be absolutely a suitable marker for monitoring of compliance to this combination regimen in this field setting as the half-life of primaquine is relatively short (3.7-9.5 hr) (3).

Conclusion

The current first-line treatment three-day combination regimen of ARS-MQ provides excellent patient compliance with good efficacy and tolerability in the treatment of multidrug resistance falciparum malaria in field setting.

Acknowledgements

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Original article **P47**

Genetic polymorphism of heme-oxygenase-1 in Thai population with low environmental cadmium exposure

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Abstract

The aims of the study were to investigate (i) the effects of environmental cadmium (Cd) on high blood pressure development, (ii) the association between genetic polymorphism of heme oxygenase-1 (HO-1) and high blood pressure development, and cadmium-induced renal injury in exposed Thai population. The study was a case-control study in adults residing in a Cd contaminated area (Mae Sot District, Thailand). All subjects were randomly selected and consistently distributed for sex, age and residential areas. Blood and urinary Cd levels were not significantly different between the case (hypertensive subjects) and control (healthy subjects) groups. While other kidney dysfunction parameters were comparable between the two groups, microalbumin and serum creatinine showed significant difference (p=0.008 and p=0.02, respectively). With respect to HO-1 polymorphism, the frequencies of S (short), M (medium) and L (long) alleles in this group of subjects were 49.7, 44.4 and 5.9%, respectively. The frequencies of S, M and L alleles between the matched pair case and the control groups were not significantly different.

Keywords: Cadmium, hypertension, heme oxygenase-1, genetic polymorphism, renal biomarkers

Introduction

Cadmium (Cd) is an environmental pollutant. Food and smoking is the major sources of Cd exposure from the environment for general population. Cd-induced hypertension and renal diseases are the health problems in population that reside in Cd-contaminated areas. In individuals with chronic exposure, Cd accumulates, particularly in the kidney and can cause renal toxicity. The early sign of Cd induced kidney damage is tubular dysfunction demonstrated by an increased excretion of low molecular weight proteins, such as β_2-microglobulin, retinol binding protein (RBP), α_1-microglobulin, or *N*-acetyl-β-glucosaminidase (NAG) enzyme (1, 2, 3). Several animal studies have demonstrated the increase in blood pressure levels in animal exposing to chronic low-dose of Cd. Effects of Cd on blood pressure depend on Cd dose levels. In rats, 0.1-5 *ppm* of cadmium induces hypertension, but 10-50 *ppm* does not or only does during the early exposure before toxicity becomes evident (4). Nomiyama *et al*. reported that chronic Cd exposure may slightly elevate blood pressure, whereas a higher dose may depress blood pressure only in hypertensive humans and animals (5). Study on relationship between Cd and hypertension in human population is however, limited and has several methodological errors (6).

Heme oxygenase 1 (HO-1) enzyme has also been proposed to play important role in modulating Cd -induced renal toxicity and hypertension. It is a renal protector from a wide variety of cellular stressors including Cd, and on the other hand, also possesses depressure ability. Several single nucleotide polymorphisms (SNPs) in human HO-1 gene result in a

variety of HO-1 expression. Individuals with reduced HO-1 activities are more susceptible to Cd-induced renal toxicity and severe hypertension than those with normal HO-1 activities (7). The aims of the study were to investigate (i) the effects of environmental Cd on high blood pressure development, (ii) the association between genetic polymorphism of HO-1 and high blood pressure development, and Cd-induced renal injury in exposed Thai population.

Methods

The study protocol was approved by the Ethics Committee of the Ministry of Public Health of Thailand. The study was conducted in local residents of 10 Cd-contaminated villages in Mae Sot District, Tak province (Tambon Mae Tao, Mae Ku and Prathadphadaeng). These Cd contaminated areas were defined based on urinary cadmium levels in a total of 7,697 subjects (8). All subjects were randomly selected and consistently distributed for sex, age and residential areas, and were divided into three groups as follows: control (healthy subjects), pre-hypertensive and hypertensive groups. Hypertensive group was classified based on clinical diagnosis, and/or the level of systolic blood pressure (SBP) of more than or equal to 140 mmHg, and/or diastolic blood pressure (DBP) of more than or equal to 90 mmHg. Those genetic-inherited hypertensive cases or those hypertensive cases from other diseases especially renal dysfunction were excluded. The pre-hypertensive group was classified based on the level of systolic blood pressure (SBP) between 120-139 mmHg, and/or diastolic blood pressure (DBP) between 80-89 mmHg (9). Blood and second morning urine samples were collected from each subject for analysis of blood and urinary Cd concentrations, renal toxicity biomarkers [β_2-microglobulin, *N*-acetyl-β-glucosaminidase (NAG), microalbumin and creatinine]. Cadmium concentrations in blood and urine were determined by electrothermal (graphite furnace) atomic absorption spectrometer. Urinary NAG was determined by a colorimetric assay using NAG test kit. gDNA was prepared from buffy coat of blood samples for analysis of $(GT)_n$ repeat of HO-1 by using laser-based automate DNA sequencer.

Results

Table 1 shows demographic characteristics, smoking status, blood and urinary Cd levels, and biomarkers of kidney dysfunction. Blood cadmium concentration was significantly different between the control and hypertensive group, whereas urinary Cd concentrations were similar in the two groups. It is noted however that the normatensive group had higher propertion of current smokers than the hypertensive group. Current smokers had significantly higher blood Cd concentrations than non-smokers and ex-smokers. Kidney dysfunction biomarkers including serum creatinine, β_2-microglobulin and NAG in the three groups were similar, but the level of microalbumin was significantly higher in the hypertensive group. When the hypertensive subjects (cases) were matched for sex, age, residential areas and smoking habit with normatensive subjects (control), no differences in blood (median 2.87 *vs* 3.08 μg/l) or urinary Cd (median 0.39 *vs* 0.43 μg/g creatinine) levels was found. The levels of microalbumin (median 0.66 *vs* 0.33 mg/g creatinine) was however, significantly higher in the case group. The levels of other kidney dysfunction parameters were similar between the two groups.

The number of $(GT)_n$ repeats of HO-1 gene in all subjects varied between 16-39. HO-1 gene allele was categorized into three groups as: short (S) with 21-27 repeats, intermediate (M) with 28-33 repaets and long (L) alleles with 34-39 repreats (10). The frequencies of S, M and L alleles were 49.7, 44.4 and 5.9% respectively (Table 3). The frequencies of S, M and L alleles in the case and control matched pair groups were similar. The frequencies of genotypes (SS, SM, SL, MM, ML, LL) of $(GT)_n$ repeat in all subjects and case-control matched pair groups were shown in Table 2.

Table 1 Demographic data, smoking habits, blood and urinary cadmium levels, and markers of kidney dysfunction in all subjects included in the study.

	Hypertension	Pre-hypertension	Normatension
Males:Females (n:n)	35:112	17:40	35:76
Age (years)[b]	47.34 ± 4.50	47.68 ± 4.79	46.86 ± 4.91
BMI (kg/m^2)[b]	25.11 ± 3.60 (n=147)	23.85 ± 3.38 (n=55)	23.91 ± 3.35 (n=109)*
Smoking status[a]			
Non-smoke**rs**	95 (50.8%)	30 (16.0%)	62 (33.2%)
Current smokers	21 (28.8%)	20 (27.4%)	32 (43.8%)
Former smokers	31 (56.4%)	7 (12.7%)	55 (30.9%)
Cadmium levels [c]			
Blood cadmium (μg/l)	2.35 (0.47-14.64)	2.97 (0.52-17.95)	3.11 (0.40-19.50)**
Urinary cadmium (μg/g creatinine)	0.38 (0.08-1.83)	0.40 (0.03-2.93)	0.42 (0.09-3.12)
Markers of kidney dysfunction[c]			
Serum creatinine (mg/dl)	0.80 (0.50-2.30)	0.80 (0.50-1.20)	0.90 (0.50-1.30)
Microalbumin (mg/g creatinine)	0.63 (0.08-42.73) (n=129)	0.47 (0.06-19.98) (n=49)	0.37 (0.06-29.34) (n=91)**
NAG (U/ g creatinine)	1.06 (0.16-8.21)	1.22 (0.00-11.33)	0.95 (0.44-4.22)
β2-MG (ug/g creatinine)	4.64 (0.00-1728.35)	4.31 (0.04-4035.14)	6.33 (0.06-1766.76)

[a]Data are presented as number (percentage).; [b]Data are presented as mean ± SD.; [c]Data are presented as median (range).
*Statistically significant difference between normatensive group and hypertension group with $p<0.05$ (One-way ANOVA)
**Statistically significant difference between normatensive group and hypertension group with $p<0.05$ (Kruskal-Wallis test)

Table 2 Distribution of HO-1 promoter genotypes and allele frequecies

Characteristic	Total	Case-control match paired	
		Case	Control
HO-1 phenotype (allenes), n (%)			
- **S**	313 (49.7%)	79 (51.3%)	78 (50.6%)
- **M**	280 (44.4%)	67 (43.5%)	68 (44.2%)
- **L**	37 (5.9%)	8 (5.2%)	8 (5.2%)
Total	630 (100%)	154 (100%)	154 (100%)
HO-1 phenotype, n (%)			
- **SS**	73 (23.2%)	19 (24.7%)	17 (22.1%)
- **SM**	147 (46.7%)	41 (53.2%)	36 (46.8%)
- **SL**	20 (6.3%)	0	8 (10.4%)*
- **MM**	60 (19.0%)	10 (13.0%)	16 (20.8%)
- **ML**	13 (4.1%)	6 (7.8%)	0*
- **LL**	2 (0.6%)	1 (1.3%)	0
Total	315 (100%)	77 (100%)	77 (100%)

*Statistically significant difference between case and control with $p<0.05$ (Chi-square test)

Discussion

Despite the abundance of evidences showing the inductive effect of Cd to high blood pressure in animals, there are limited information on Cd-exposed workers (1). Moreover, available data on the relationship between Cd and hypertension in human population are controversial and debatable due to methodological errors (6). Several confounding factors such as smoking status, other air pollutants, as well as other environmental factors make it difficult to draw conclusions concerning the effects of Cd to hypertension (1). In 1984, Staessen *et al*., showed negative correlation between blood pressure and urinary Cd or $ß_2$-microglobulin in some groups after the confounding variables age, sex, body weight, and cigarette smoking were adjusted in a multiple regression analysis. As there was a very strong age effect on both blood pressure and urinary Cd, the meaning of the negative correlation is not clear (1, 11). In the present study, although hypertensive subjects were matched with the normatensive subjects for sex, age, residential areas of Cd-exposure and smoking status, Cd concentrations in blood and urine in both groups were similar. This could be due to

inadequate sample size. Apart from microalbumin, the levels of biomarkers for kidney dysfunction in both hypertensive and normatensive subjects were comparable and lied within the range of expected value for a healthy population. A wide ranges of tests with different sensitivities and levels of significance have been used to determine Cd nephrotoxicity. For occupationally populations, 2 μg/g creatinine of urinary Cd is associated with biochemical alterations. The albumin level in urine as a marker of glomerular dysfunction and urinary NAG as a marker of renal tubule injury are associated with 4 μg/g creatinine of urine cadmium. The 10 μg/g creatinine of urinary Cd is associated with β_2-MG: a tubular proteinuria resulting from an impaired tubular reabsorption (12).

HO-1 can be induced by of Cd and act as renal protector. It also has depressure effect *via* ability of HO-1 to generate CO which cause vasodilation. Several polymorphisms have been found in HO-1 genes of human which lead to a variety of HO-1 expression. A $(GT)_n$ repeat in the 5'flanking region of the HO-1 gene is highly polymorphic and its expression of may be altered according to the number of $(GT)_n$ repeat. The large size of a $(GT)_n$ repeats in the HO-1 gene promoter may reduce HO-1 activity (13). Therefore, individuals with reduced HO-1 activities are expected to be more susceptible to Cd-induced renal toxicity and severe hypertension than those with normal HO-1 activities. Nevertheless, no such association was observed in this study.

Conclusion

Results from the present study suggest no clear evidence on the effects of environmental Cd nor HO-1 polymorphism on high blood pressure development, and Cd-induced renal injury in exposed Thai population.

Acknowledgements

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Original article **P48**

Gender-specific distribution of mefloquine in the blood of healthy volunteers following the administration of therapeutic doses

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Abstract

The distribution of the antimalarial drug mefloquine (MQ) following the administration of standard therapeutic doses (1,250 mg mefloquine in split dose) to 22 healthy Caucasian volunteers was assessed in whole blood, serum, plasma, RBCs, WBCs, and platelets using high performance liquid chromatography. Plasma MQ levels after 14, 48, and 168 hours were considerably higher in female subjects than in males, concordant with a significantly higher frequency, duration, and severity of adverse reactions. However, mean drug concentrations of RBCs tended to be higher in male volunteers. The concentrations in other blood components were similar in both genders. No correlation was seen between MQ concentrations in plasma and RBCs. Since the observations relate to healthy individuals, they do not take into account t selective uptake of MQ by *Plasmodium*-infected RBCs as in the case of therapeutic drug use. Although plasma MQ levels in female healthy volunteers are considerably higher and the concentrations of the RBC are initially lower as compared to males they do not seem to justify an adjustment of treatment guidelines for MQ in female Caucasian individuals.

Keywords: Mefloquine, pharmacokinetics, gender-specific distribution, plasma, red blood cell

Introduction

The apparent incidence of adverse events (AEs) following the oral administration of the antimalarial drug mefloquine (MQ) is high, with reports of 47 to 90% of adults experiencing some type of AE (1-2). Previous studies with MQ have shown that a significantly higher frequency and severity of treatment- and prophylaxis-related adverse events (AEs) occur in female patients [14-16]. The use of high doses of MQ is also associated with higher frequencies of AEs, particularly in female patients (3-4). MQ also inhibits acetylcholinesterase and butyrylcholinesterase, the likely cause for the frequent gastrointestinal and CNS-related AEs which occur at high dosages of the drug (5). The gender-specific differences in the frequency and severity of AEs experienced following the administration of MQ in prophylactic as well as therapeutic dosages may at least in part be attributable to different distribution patterns in liquid and cellular blood compartments. The aim of the present study was to elucidate the gender-specific distribution of MQ in these compartments at therapeutic dosages of the drug in order to assess any relationship with the occurrence of treatment-related AEs. Furthermore, the eventual necessity of adjusting treatment guidelines in female patients was explored.

Methods

The study was conducted with 22 healthy Caucasian volunteers (10 males, 12 females) aged 20 to 45 years (median age of 26) at the Department of Specific Prophylaxis and Tropical Medicine, University of Vienna. Written informed consent was obtained from all study participants and the study protocol was approved by the ethical review board at the University of Vienna. All subjects received 1,250 mg Lariam® each (Hoffmann-la Roche

Pharmaceuticals, Basel, Switzerland) as split dosages of 750 mg, followed by another 500 mg at 6 hours apart. CBCs were conducted at 14, 48, and 168 hours after the administration of the first MQ dose. All volunteers were monitored for AEs during the 21 days after the first dose had been administered. Venous blood samples were collected at 0, 14 (the estimated time to reach peak plasma concentrations, 8 hours after the administration of the second MQ dose), 48 (estimated to be the beginning of the log-linear elimination phase), and 168 (the minimum time required for therapeutic drug levels to eliminate malaria parasites) hours. MQ concentrations in whole blood, plasma, serum, RBC, WBC, and platelets) were measured using high performance liquid chromatography (HPLC) (6).Statistical analysis was performed by Non-parametric test at $\alpha = 0.05$ for all tests.

Results

A significant higher plasma MQ concentrations was observed in female subjects at all time points, whereas RBC concentrations tended to be higher in males compared with females at 14 and 48 hr. The concentration in whole blood, serum, WBC, and platelet were similar for both genders (Table 1). This resulted in significantly higher plasma/RBC concentration in females than in males at 14 and 48 hr. Figure 1 is a graphical representation of the changes observed in the concentrations in RBCs *vs* plasma in males and females. MQ concentrations in RBC in both genders were significantly lower than in whole blood and serum. Platelet and WBC MQ levels were approximately 6 times higher and 20 times higher than in whole blood, respectively. No correlation was seen between plasma drug concentrations and RBC levels, suggesting that plasma levels are a poor predictor of RBC drug levels. The most commonly reported AEs were vertigo (96%), followed by nausea (82%), headache (73%), sleeping disturbances (59%), and diarrhea (41%). The overall symptom scores (OSS) reflecting the frequency, duration, and severity of drug-related AEs were significantly higher in female subjects (20.8 in males *vs* 43.3 in females; p=0.003). Frequency, duration, and severity of AEs were directly correlated (r=0.519; p=0.016) with plasma drug levels (Figure 2).

Table 1 Mean MQ concentrations, in ng/mL, in male (n = 10) and female (n = 12) healthy volunteers at 14 hours (H14), 48 hours (H48) and 168 hours (H168) after administration of the first drug dose

Substrate	H14		H48		H168	
	Female	Male	Female	Male	Female	Male
Whole Blood	1,360	1,648	1,437	1,272	896	885
Serum	1,570	1,623	1,244	847	811	1,304
Plasma*	2,778	1,017	2,106	1,214	1,353	666
RBC*	719	857	633	827	389	375
WBC	35,641	33,885	32,414	29,449	17,584	16,214
Platelets	9,212	9,002	9,710	7,825	5,538	5,808

* Statistically significant difference between males and females ($p < 0.01$; Mann-Whitey U test)

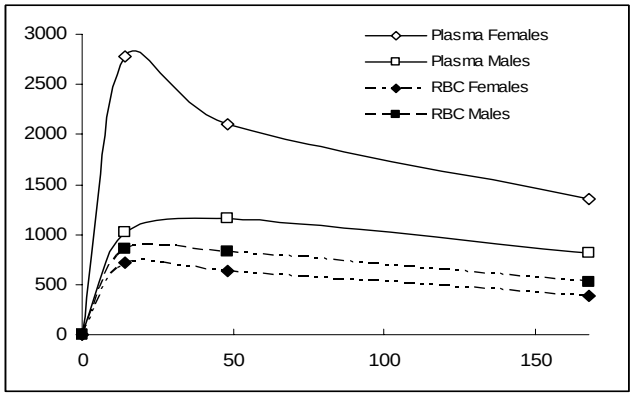

Figure 1 Mean MQ concentrations, in ng/ml, in (A) the serum and plasma, and (B) plasma and RBC of male and female healthy volunteers at 14, 48 and 168 hours after the administration of the first dose

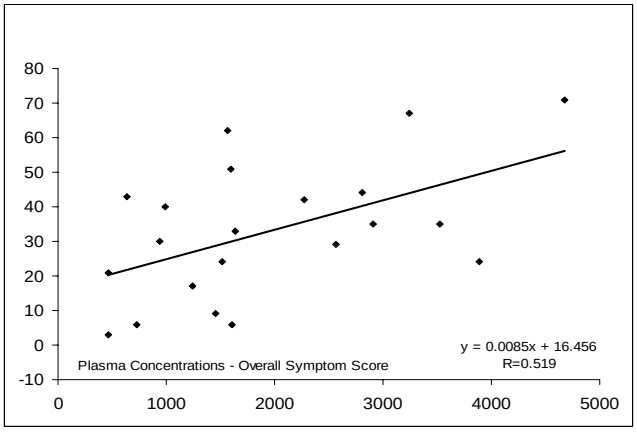

Figure 2 Scatter plot and regression line for MQ plasma concentrations (ng/mL) and overall symptom score (OSS). The frequency, duration and severity of adverse events (represented by OSS) exhibited significant correlation with mefloquine plasma levels (Spearman Rank test: $r = 0.519$; $p = 0.016$)

Discussion

Our data suggest that plasma concentrations are significantly higher in females and this correlated with higher frequency and severity of AEs in females compared with males. RBC drug levels at H14 and H48 are however, lower in females than in males. Since the RBCs are the site of malarial infection, this may be interpreted as a potential shortfall of therapeutic activity. However, recent investigations (7) have shown that RBCs infected with *P.falciparum* contain >4 times as much MQ as compared to uninfected RBCs (8). These observations would also explain the equivalence of the therapeutic efficacy of MQ in uncomplicated infections with MQ-sensitive *P. falciparum* in both genders. The lack of any significant correlation between plasma and RBC drug concentrations suggests that MQ plasma levels may not truly represent the amount of drug reaching uninfected or parasitized RBCs. However, this would also mean that low plasma concentrations, as observed in predominantly male populations, do not automatically indicate sub-therapeutic RBC drug concentrations (9). MQ concentrations were much higher in WBCs and platelets, suggesting an active uptake of the drug into these cells. However, these levels appear to have little relevance for the treatment of malaria.

Conclusion

The higher AE frequencies and severities caused by higher plasma levels in females in combination with lower RBC drug concentrations create speculation about the risks and benefits of MQ treatment for female patients. In spite of the considerably higher number of AEs in females, we would not recommend a down-adjustment of mefloquine treatment guidelines for these individuals.

Acknowledgements

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Original article **P49**

Effect of itraconazole on the pharmacokinetics of ciprofloxacin in healthy volunteers

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Abstract

Itraconazole, a triazole antifungal agent, is a potent cytochrome P-450 inhibitor and also a P-glycoprotein (P-gp) inhibitor. Ciprofloxacin, a fluoroquinolone compound with broad spectrum antimicrobial activity, is often coadministered with an antifungal drug in HIV-infected patients. The drug-drug interaction between itraconazole and ciprofloxacin may occur in these patients. Therefore, the aim of this study was to investigate the effect of itraconazole on the pharmacokinetics of ciprofloxacin following a multiple oral dose in healthy volunteers. Two-phase crossover study with a 2 week washout period was designed to study in six healthy volunteers. In phase 1, each volunteer received 500 mg of ciprofloxacin twice daily for 7 days whereas in phase 2, they also received 500 mg ciprofloxacin and 200 mg itraconazole twice daily for 7 days. The results revealed that there was no significant difference in each ciprofloxacin pharmacokinetic parameter between 2 phases of study in six healthy volunteers. In conclusion, itraconazole co-administration with ciprofloxacin did not alter the ciprofloxacin pharmacokinetics in healthy volunteers.

Keywords: Ciprofloxacin, Itraconazole, Pharmacokinetics, Drug interaction

Introduction

Ciprofloxacin, a nalidixic acid derivatives and a fluoroquinolone drug with broad spectrum antimicrobial activity, is widely used both in human and veterinary medicine to treat infectious diseases. It is metabolized in the liver and eliminated via the kidney including tubular secretion (1).

Itraconazole, an azole derivative, has been found to be effective agents with a variety of fungal infections. It is widely used for the treatment of superficial and systemic fungal infections. Itraconazole is reported to be both an inhibitor of CYP3A4 and P-glycoprotein (P-gp) (2). Ciprofloxacin and itraconazole are usually combined together in the treatment of patients coinfected with bacteria and fungi. In previous study, it was shown that ketoconazole and itraconazole reduced ciprofloxacin urinary excretion in mice (3). So, the pharmacokinetic interaction between itraconazole and ciprofloxacin in human could be possible. The aim of this study was to investigate the effect of itraconazole on the pharmacokinetics of ciprofloxacin following a multiple oral dose in healthy volunteers.

Materials and methods

Subjects and drugs

Six healthy, nonsmoking, nonalcoholic and nonobese male volunteers were registered in this study. Their mean age and body mass index were 24.33 ± 4.46 years and 21.05 ± 2.70 kg/m^2, respectively. The study was approved by the ethics committee of the Faculty of Science, Prince of Songkla University and the written informed consent was obtained from each volunteer. Each subject underwent a pre-study evaluation that they had no underlying illness and were not currently on any medication. All subjects had normal biochemical and hematological laboratory profiles.

Ciprofloxacin tablet (Ciprobay®) was purchased from Bayer, Thailand and itraconazole capsules (Sporal®) was purchased from Olic, Thailand.

Study design and sample collection

The study was an open-labeled, randomized, two-phase crossover design with a 2 week washout period. The first phases, all subjects received 500 mg (1 tablet) of ciprofloxacin twice daily for 7 days. The second phases, all subjects received 500 mg ciprofloxacin (1 tablet) and 200 mg itraconazole (2 capsules) twice daily for 7 days. For each phase, blood samples were obtained at the following time; before (time 0), and up to 48 h after drug administration. The plasma was collected and stored at -70 ºC until analysis.

Ciprofloxacin assay

The concentration of ciprofloxacin was determined by the reverse-phase HPLC method which was modified from Jim *et al.*(1992) (4). Briefly, the plasma sample was deproteinized with acetonitrile. The 200 µl of supernatant was transferred to 800 µl of mobile phase. The 20 µl of the mixture was injected onto HPLC system. The mobile phase consisted of methanol, phosphate buffer adjusted to pH 3 with 85% H_3PO_4 and tetrahydrofuran (21.2: 78: 0.8 v/v/v). The flow rate was 1.2 $ml.min^{-1}$. The column effluent was monitored at 50 °C by fluorescence detection at λ_{ex}277 and λ_{em}440 nm. The standard curve of ciprofloxacin was done on concentrations ranging from 31.25 to 8,000 $ng.ml^{-1}$. The lower limit of quantification of ciprofloxacin was 31.25 $ng.ml^{-1}$.

Pharmacokinetic and statistical analysis

All pharmacokinetic parameters were analyzed by non-compartment model with the use of WinNonlin Professional Software Version 1.1. Results were expressed as mean ± SD and statistical comparisons were made using Paired *t-test.* The significance level was set at *p*-value of less than 0.05.

Results

The mean plasma ciprofloxacin concentration time data for phase 1 and phase 2 in healthy normal volunteers were shown in Fig. 1. The mean pharmacokinetic parameters of ciprofloxacin for phase 1 and phase 2 were shown in table 1. The results in the present study showed that there were no significant differences in each ciprofloxacin pharmacokinetic parameter when compared between phase 1 and 2.

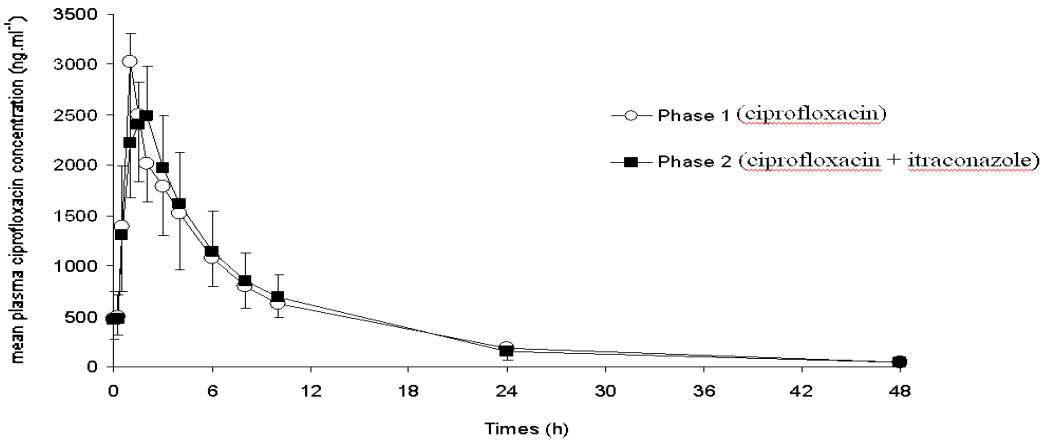

Fig. 1 The mean plasma concentration-time curves of ciprofloxacin in six healthy male volunteers after a multiple oral dose of 500 mg ciprofloxacin alone (Phase 1), and combination with 200 mg itraconazole (Phase 2) twice daily for 7 days.

Table 1 Pharmacokinetic parameters of ciprofloxacin in subjects after receiving a multiple oral dose of 500 mg ciprofloxacin alone (Phase 1), and combination with 200 mg itraconazole (Phase 2) twice daily for 7 days. Data are shown as mean ± SD and 95% confidence interval

Parameter (units)	Phase 1	Phase 2	*P*-value
C_{max} (ng.ml^{-1})	3,330.74 ± 883.95	2,997.40 ± 582.52	0.086
AUC_{0-48} (ng.h.ml^{-1})	21,908.08 ± 3,715.54	22,257.50 ± 5,033.84	0.875
$AUC_{0-\infty}$ (ng.h.ml^{-1})	22,490.41 ± 4,010.32	22,912.01 ± 5,091.33	0.856
$t_{1/2}$ (h)	8.66 ± 2.10	9.02 ± 2.02	0.252
Cl (L.h^{-1})	0.022 ± 0.004	0.023 ± 0.005	0.541

Discussion

The pharmacokinetic drug interactions between agents used as parts of multidrug therapy are importance and may influence drug efficacy and safety. Ciprofloxacin is mainly eliminated via the kidney both glomerular filtration and tubular secretion, and trans-intestinal elimination and biliary clearance around at one-third of total ciprofloxacin clearance (1).

Itraconazole is known to be potent inhibitor of CYP3A4 and P-gp (2). Co-administration of drugs with CYP3A4 and P-gp substrates can result an increased toxicity. Measured pharmacokinetic parameters of ciprofloxacin in our study were C_{max}, AUC_{0-48}, $AUC_{0-\infty}$, $t_{1/2}$ and *Cl*. The results in this investigation were found that there were no significant differences of these parameters when compared between phase 1 and phase 2. Since there were not reported the metabolism pathway of ciprofloxacin via cytochrome P-450, therefore it could be implied that ciprofloxacin is not a substrate of CYP3A4. In addition, there was demonstrated that the secretion of cholic acid across the apical membrane was not inhibited by ciprofloxacin in Caco-2 and T84 monolayers, suggesting the ciprofloxacin is not a substrate of MDR1 and MRP2 in humans (5). On the contrary, some studies have reported ciprofloxacin was secreted via P-gp and other pathway. For example, Abou-Auda *et al.* (2008) found that there was a reduction of ciprofloxacin urinary excretion in mice, owing to inhibition of its P-gp at renal tubular cells by ketoconazole and itraconazole (3). Dautrey *et al.* (1999) reported ciprofloxacin intestinal elimination in rats seems to be mediated by organic anion and/or cation transporters (6). The differences of ciprofloxacin pharmacokinetics in humans, rats and mice may be explained by their different species.

In conclusion, this study indicated that itraconazole co-administration with ciprofloxacin did not alter the ciprofloxacin pharmacokinetic parameters in healthy volunteers. Further studies are needed to clarify the effect of ciprofloxacin on the pharmacokinetics of itraconazole in healthy volunteers.

Acknowledgements

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Original article **P50**

Effects of *Phyllantus Amarus* aqueous extract and *Phyllanthus Emblica* aqueous extract on human CYP2D6 and CYP3A4

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Abstract

Phyllanthus amarus Schum. & Thonn. and *Phyllanthus emblica* Linn. are well-known as herbal medicine with many pharmacological effects. Inhibitory effects of the aqueous extracts of both plants on cytochrome P450 (CYP) including CYP2D6 and CYP3A4, were investigated using recombinant human CYP. The results demonstrated that *P. amarus* aqueous extract and *P. emblica* aqueous extract possessed inhibitory effects on CYP2D6 with IC_{50} of 180.02 μg/mL and 599.34 μg/mL, respectively. Inhibitory effects of *P. amarus* aqueous extract and *P. emblica* aqueous extract on CYP3A4 were shown by the IC_{50} of 2.11 μg/mL and 321.89 μg/mL, respectively. Results from this study provide an information indicating the possibilities of herb-drug interaction if these extracts are co-administered with the prescribing drugs that are metabolizing by CYP2D6 and CYP3A4.

Keywords: *Phyllanthus amarus* , *Phyllanthus emblica*, CYP2D6, CYP3A4, Herb-Drug interaction

Introduction

Herbal medicines have currently become popular as an alternative medicine [1]. Pharmacological activities of several plant species are interesting. *Phyllanthus amarus* Schum. & Thonn. and *Phyllanthus emblica* Linn., the plants in family Euphorbiaceae, have been found in many tropical countries including Thailand. Both herbal-plants are well-known as traditional medicine with various pharmacology activities [2]. Their common activities include antioxidant [3,4], antimicrobial [5,6] as well as hepatoprotective activity from ethanol [7,8] and *N*-nitrosodiethylamine [9] etc. Besides the toxicity data of both plants in animals [10,11], herb-drug interaction investigation is also needed to ensure the safety of using these plants in human. CYP, the phase I enzyme system in the liver, normally plays a key role in metabolism of most currently used medicines. Among CYP isoforms, CYP2D6 and CYP3A4 involve in metabolism of drugs for more than 80% [12]. Modification of CYP has been shown to be one of the most important etiology of drug-drug interaction. Therefore, the aim of the present study was to investigate the inhibition effect of *P. amarus* and *P. emblica* aqueous extracts on CYP2D6 and CYP3A4 by using recombinant human CYPs *in vitro*.

Materials & Methods

The inhibition of the extracts on CYP2D6 and CYP3A4 activities was determined according to the Vivid® CYP450 Screening Kits Protocol (www.invitrogen.com). The principle of this assay was characterized by the inhibition effect of the compound of interest with the specific CYP isoform. Thus, the reaction of changing the Vivid® substrates (7-benzyloxymethyloxy-3-cyanocoumarian; BOMCC or ethyloxymethyloxy-3-cyanocoumarian;

EOMCC) by that specific CYP isoform into a highly fluorescent product was reduced as compared to the reaction without the compound of interest.

The reaction was performed in 96-multiwell black plates. The mixture of the NADPH-regeneration system (comprised Glucose-6-phosphate and Glucose-6-phosphate dehydrogenase in potassium phosphate buffer pH 8.0) and the CYP450 BACULOSOMES® reagent were prepared and added in each wells with 40 μL of the test compound at various concentrations. The plate was pre-incubated for 20 minutes at room temperature. The reaction was started by adding 50 μL of the mixture of $NADP^+$ and the Vivid® substrate solution and incubated for 30-60 min at room temperature. Ten microliters of 0.5 M Tris-base buffer (pH 10.5) was added to stop the reaction. Fluorescence intensity was measured by fluorescence microplate reader ($VICTOR^3$; PerkinElmer, USA) with the excitation wavelength of 405 ± 10 nm and the emission wavelength of 460 ± 20 nm. Validation of the protocol was performed before analyzing the test compounds by using the known selective CYP2D6 or CYP3A4 inhibitors. Miconazole was used as the selective CYP2D6 inhibitor whereas ketoconazole was used as the selective inhibitor of CYP3A4.

Statistical analysis

Percentage of inhibition of each concentration of the test compounds was calculated by using the following equation.

$$\% \text{ Inhibition} = 1 - \left[\frac{(RFU_{\text{test compound}} - RFU_{\text{background of test compound}})}{(RFU_{\text{solvent control}} - RFU_{\text{background of solvent control}})} \right] \times 100$$

Median inhibition concentration (IC_{50}) was calculated by plotting concentrations of the test compound against the corresponding percent inhibition. Data were analyzed using probit analysis of SPSS software version 16.

Results

IC_{50} of miconazole on CYP2D6 was shown to be 0.67 μM whereas the IC_{50} of ketoconazole on CYP3A4 was 0.11 μM (Table 1). The data were consistant to the IC_{50} of both selective inhibitors of the particular CYP reported earlier [13,14].

IC_{50} of *P. amarus* aqueous extract on CYP2D6 and CYP3A4 was shown to be 180.02 μg/mL and 2.11 μg/mL, respectively (Table 1, Figure 1 and 2). While IC_{50} of *P. emblica* aqueous extract on CYP2D6 and CYP3A4 was shown to be 599.34 μg/mL and 321.89 μg/mL, respectively (Table 1, Figure 3 and 4).

Table 1: IC_{50} values of the selective inhibitors, *P. amarus* aqueous extract and *P.emblica* aqueous extract on CYP2D6 and CYP3A4

CYP isoforms	Selective inhibitor	IC_{50} (μM) of selective inhibitor	IC50 (μg/mL) of selective inhibitor	IC_{50} (μg/mL)	
				P. amarus aqueous extract	*P. emblica* aqueous extract
CYP2D6	**miconazole**	**0.67**	**0.32**	**180.02**	**599.34**
CYP3A4	**ketoconazole**	**0.11**	**0.06**	**2.11**	**321.89**

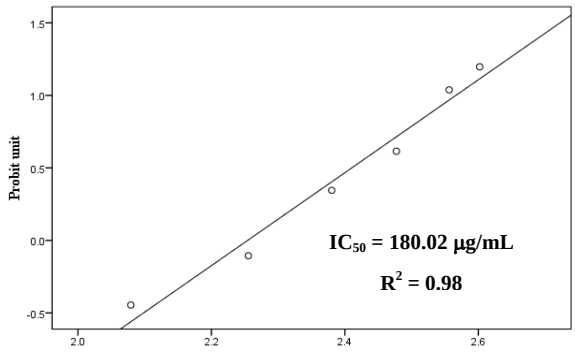

Figure 1: Inhibitory effect of *P. amarus* aqueous extract on CYP2D6. (Each data point represented the mean of triplicate analysis)

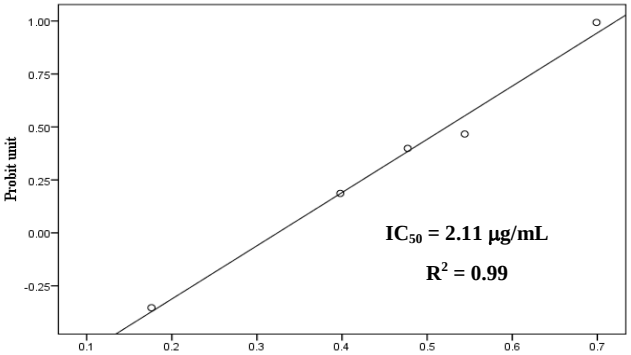

Figure 2: Inhibitory effect of *P. amarus* aqueous extract on CYP3A4. (Each data point represented the mean of triplicate analysis)

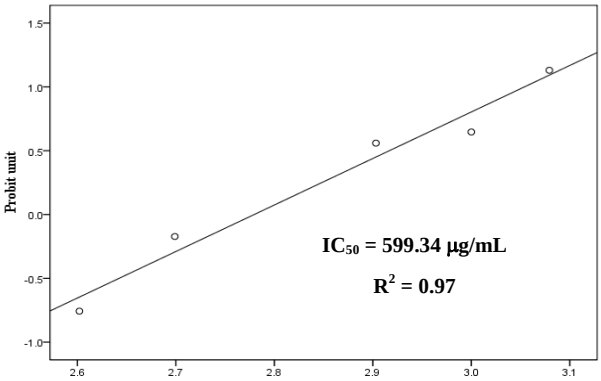

Figure 3: Inhibitory effect of *P. emblica* aqueous extract on CYP2D6. (Each data point represented the mean of triplicate analysis)

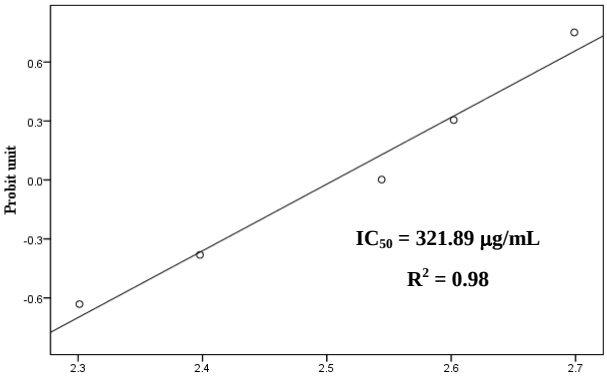

Figure 4: Inhibitory effect of *P. emblica* aqueous extract on CYP3A4. (Each data point represented the mean of triplicate analysis)

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Original article **P51**

Carbamazepine- but not phenytoin-induced severe cutaneous adverse drug reactions are associated with *HLA-B*1502* in a Thai population

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Abstract

Antiepileptic drugs (AED) containing aromatic rings such as carbamazepine (CBZ) and phenytoin (PHT) have been reported as the most common culprit drug for severe cutaneous adverse drug reactions (SCADR) in several Asian countries including Thailand. A strong association between *HLA-B*1502* and CBZ-induced SJS/TEN has been reported in Han Chinese but not in Caucasian and Japanese populations. A case-control study was therefore conducted to determine whether *HLA-B*1502* is a valid pharmacogenetic test for SCADR caused by CBZ and PHT in a Thai population.

Among 49 CBZ-induced SCADR patients, 45 (89.58%) patients carried the *HLA-B*1502* while only 5 (10.42%) of the CBZ-tolerant controls had this allele. The risk of CBZ-induced SCADR was significantly higher in the patients with *HLA-B*1502* with an odds ratio (OR) of 74 (95%CI 17–341, $p<0.001$). Among the 18 PHT-induced SCADR patients, the *HLA-B*1502* allele was present in only 5 (27.8%) of these patients, whereas 7 (19.4%) of the PHT-tolerant controls carried this allele. The risk of PHT-induced SCADR was not significantly higher in the patients with *HLA-B*1502* (OR= 1.6; 95%CI 0.3-7.1, p =0.50). Results from this study suggest that *HLA-B*1502* is a good marker for CBZ-induced SCADR but not for PHT-induced SCADR in a Thai population.

Keywords: *HLA-B*1502*, carbamazepine, phentyoin, severe cutaneous adverse drug reactions (SCADR)

Introduction

Antiepileptic drugs (AED) containing aromatic rings such as carbamazepine (CBZ) and phenytoin (PHT) are the most common culprit drugs of severe cutaneous adverse drug reactions (SCADR). These SCADR include hypersensitivity syndrome (HSS), Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) [1].

Although the pathogenesis of SCADR is not fully understood but there is some evidence that genetic may plays importance role [2]. Recent studies in Han Chinese and Thais showed that the *HLA-B*1502* allele is strongly associated with CBZ and this allele may also associated with PHT-induced SJS/TEN [3-5]. In contrast, the association between *HLA-B*1502* and CBZ- and PHT-induced SJS/TEN could not be demonstrated in Caucasian and Japanese populations[6].

Given the serious and life-threatening consequences of SJS/TEN and its extremely strong association with *HLA-B*1502*, the US-FDA has recently released a warning to health professionals and patients that these SCADR may occur in patients with *HLA-B*1502* and has also recommended genetic screening for patients of Asian ancestry prior to initiation of CBZ or PHT therapy. It should be noted that the FDA alert is not specific about the

definition of Asian [7]. In fact, the frequency of *HLA-B*1502* allele among Asian populations are vary, ranging from 10–15% in Han Chinese and South East-Asian populations to less than 1% in Japanese, Koreans [6, 7]. It should also be noted that the prevalence of CBZ-and PHT-induced SJS/TEN is positively correlated with the frequency of *HLA-B*1502* in different populations [8]. The objectives of this study were to determine the association between the *HLA-B*1502* allele and CBZ-and PHT-induced SCADR in a Thai population.

Materials and Methods

Study design: The retrospective case–control study was conducted in 10 local hospitals in Thailand.

Patients: CBZ and PHT-induced SCADR were identified from their medical records. The diagnosis of SCADR were confirmed by either a dermatologist or an internist based on the clinical morphology of the patients' skin according to Roujeau *et al.* [1]. CBZ and PHT were identified as the culprit drug if the symptoms occurred within the first 3 months of exposure and the symptoms resolved upon withdrawal of this drug. Patients who had used CBZ/PHT for $\geq$ 6 months without evidence of any cutaneous reactions were recruited as controls. Subjects were informed both verbally and in writing about the experimental procedures and the purpose of the study. The study protocol was approved by the institutional review boards.

HLA-B*1502 genotyping: Analysis for the presence of the *HLA-B*1502* allele was performed using a PG1502 DNA detection kit (Pharm*i*Gene, Inc., Taipei, Taiwan).

Statistical analysis:Data are expressed as positive or negative for *HLA-B*1502.* Chi-square test and Fisher's exact test were used to analyze the association between AED-induced SCADR and *HLA-B*1502* status. *P*-values $\leq$ 0.05 (two-sided) were considered statistically significant.

Results

Forty-eight patients who had been diagnosed with CBZ-induced SJS/TEN and 48 patients who received CBZ for at least 6 months without any evidence of cutaneous reactions were enrolled as cases and controls. Whereas eighteen patients who had been diagnosed with PHT-induced SCADR and 36 patients who received PHT for at least 6 months without any evidence of any cutaneous reactions were enrolled as cases and controls.

Among CBZ-patients, the *HLA-B*1502* allele was present in 43/48 (89.6%) of CBZ-induced SJS/TEN patients, whereas only 5/48 (10.4%) of the CBZ-tolerant controls carried this allele. The risk of CBZ-induced SJS/TEN was significantly higher in the patients with *HLA-B*1502* with OR of 74 (95% CI 17 – 341, $p<0.001$).

Among PHT-patients, the *HLA-B*1502* allele was present in only 5/18 (27.8%) of PHT-induced SCADR patients, whereas 7/36 (19.4%) of the PHT-tolerant controls carried this allele. The risk of PHT-induced SCAR was not significantly higher in the patients with *HLA-B*1502* (OR =1.6 ; 95% CI 0.3-7.1, $p=0.50$).

Table 1. Risk of CBZ-induced SJS/TEN in patients with *HLA-B*1502* compare to CBZ-tolerant controls

HLA-B*1502	**Number of patient SJS/TEN**	**Tolerant control**	**Odds ratio (95%CI)**	***P* value**
Positive	43(89.58%)	5(10.42%)	**73.96**	
Negative	5(10.42%)	43(89.58%)	***[17.46-340.70]***	<0.001
Total number	48	48		

Table 2. Risk of PHT-induced SCADR in patient with *HLA-B*1502* compare to PHT-tolerant control

HLA-B*1502	**Number of patient SJS/TEN**	**Tolerant control**	**Odds ratio (95%CI)**	***P* value**
Positive	5(27.78%)	7(19.44%)	**1.59**	
Negative	13(72.22%)	29(80.56%)	***[0.32-7.13]***	0.50
Total number	18	36		

Conclusion

Consistence with those reports in Han Chinese and Thai epileptic patients, strong association between *HLA-B*1502* and CBZ-induced SJS/TEN was observed in the present study. The use of *HLA-B*1502* as a screening test before prescribing CBZ will help to prevent CBZ-induced SJS/TEN in Thailand.

Based on the fact that there is a strong association of the *HLA-B*1502* and CBZ-induced SJS/TEN and the structure of PHT is very similar to CBZ, the FDA promptly alert the physicians and patients to aware of the genetic link between *HLA-B*1502* and PHT-induced SJS/TEN. Although recent studies reported a high frequency of *HLA-B*1502* in PHT-induced SJS/TEN in Han Chinese and Thai epileptic patients, the number of reported cases were very small (only 1 Chinese and 4 Thais) [5, 9]. Results from our study reveal that the risk of PHT-induced SCAR was not significantly higher in the patients with *HLA-B*1502*. This lack of association between *HLA-B*1502* and PHT-induced SCADR may suggest that the *HLA-B*1502* screening test not be so useful for prediction of PHT-induced SCADR. However, larger number of patients may need to be confirmed about the lack of this association.

Acknowledgement

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Original article **P52**

Effect of albumin on human Cytochromes P450 kinetics: extrapolation of *in vivo* clearance from *in vitro* data

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Abstract

It has been reported that addition of bovine serum albumin (BSA) to *in vitro* incubations improves estimation of kinetic parameters, intrinsic clearance ($CL_{int} = V_{max}/K_m$), by decreasing the K_m for drugs metabolised by cytochrome P450 2C9 (CYP2C9). However, the effect of BSA on other CYP isozymes was unclear. The aims of this study were to characterize effect of BSA on kinetics of specific pathways for CYP2C8, 2C19, and 3A4 whether the addition of BSA could improve the prediction of *in vivo* clearance by using human hepatic microsomal 6α-hydroxypaclitaxel, 5-hydroxyomeprazole, and omeprazole sulfone formations as markers for CYP2C8, 2C19, and 3A4 pathways, respectively. The metabolite formations were determined by HPLC. In the presence of 2% BSA, rate of CYP2C8-mediated 6α-hydroxypaclitaxel formation was well described by single enzyme Michaelis-Menten kinetic. Mean CL_{int} was significantly increased about 4 fold. Thus the extrapolation of *in vitro* CL_{int} to *in vivo* clearance for paclitaxel 6α-hydroxylation was more accurate in the presence of BSA in the incubation. 5-Hydroxyomeprazole formation in the presence of 2% BSA followed two enzyme Michaelis-Menten kinetics. In the presence of 2% BSA, the mean K_{m1} and V_{max1} for omeprazole 5-hydroxylation were decreased 4 and 2 fold, respectively which resulted in 2-fold increases in the mean CL_{int1}. Moreover, the mean K_m and V_{max} for CYP3A4-mediated omeprazole sulfoxidation were increased (15-19 fold) in the presence of 2% BSA therefore the mean CL_{int} was not significantly changed. BSA converted omeprazole sulfoxidation from two enzyme to single enzyme Michaelis-Menten kinetic. In conclusion, the addition of 2% BSA is likely to improve *in vitro* clearance prediction for CYP2C8-mediated paclitaxel 6α-hydroxylation. Moreover, BSA showed minor effect on the CYP2C19-mediated omeprazole 5-hydroxylation whereas had no effect on CYP3A4-mediated omeprazole sulfoxidation. The effect of albumin on individual CYP isoforms was variable; the use of BSA to improve prediction of *in vivo* intrinsic clearance seems to be possible with only CYP2C8.

Keywords: bovine serum albumin, cytochromes P450, *in vitro-in vivo* extrapolation

Introduction

In vitro approaches have been used to predict drug clearance in humans during drug development process. An *in vitro* intrinsic clearance (CL_{int}), generally determined from kinetic data of human liver microsomes (HLM) may be extrapolated to hepatic clearance using a mathematical equation. However, the microsomal CL_{int} values underpredict the *in vivo* CL_{int} and hepatic clearance (CL_H) of drug metabolized by cytochrome P450 (CYP).

Polyunsaturated fatty acids (PUFAs) released from the membranes of enzymes during the course of an incubation act as the inhibitors of several drug metabolizing enzymes (i.e. CYP2C9) (Rowland et al., 2008) resulting in overestimation of the K_m value. Consequently,

CL_{int} based on this value may underpredict *in vivo* CL_H. This effect was reversed by adding bovine serum albumin (BSA) to *in vitro* incubations. BSA improves estimation of kinetic parameters, CL_{int}, by decreasing the K_m for drugs metabolized by CYP2C9 (Rowland et al., 2008). In this regard, the universality of the albumin effect on other CYP isozymes is still arguable.

Therefore, the aims of present study were (i) to investigate effect of BSA on the kinetics of specific pathways for CYP2C8-mediated paclitaxel (PAC) 6α- hydroxylation, CYP2C19-mediated omeprazole (OMP) 5-hydroxylation, and CYP3A4-mediated OMP sulfoxidation using HLM as enzyme source and (ii) to investigate the effect of BSA on the clearance prediction.

Methods

Kinetics of PAC 6α-hydroxylation, OMP 5-hydroxylation, and OMP sulfoxidation.

Incubation samples contained phosphate buffer (0.1 M, pH 7.4), HLM, BSA (0 or 2%), NADPH generating system, and selective substrates (PAC or OMP). Reactions were performed at 37°C and terminated. The metabolite concentrations were determined by validated HPLC techniques. Incubation conditions were optimized to ensure linearity with respect to protein concentration and incubation time for each reaction.

Data Analysis

Kinetic parameters for all metabolic pathways in the absence and presence of 2% BSA were generated by fitting experimental data to the single or two Michaelis-Menten or substrate inhibition kinetics. Fitting was performed with EnzFitter (version 2.0.18.0; Biosoft, Cambridge, UK) based on the unbound concentration present in incubations. Statistical analysis (*t*-test or Mann-Whitney Rank Sum test) was performed using SigmaStat version 3.11. Values of *p* less than 0.05 were significant.

Intrinsic clearances (CL_{int}) were determined as V_{max}/K_m and subsequently scaled to the whole liver CL_{int} assuming microsomal yield of 40 mg microsomal protein/g of liver, and a liver weight of 1500 g. *In vivo* CL_H was then predicted using expression for the well-stirred model.

Results

Kinetics of PAC 6α-hydroxylation, OMP 5-hydroxylation, and OMP sulfoxidation by HLM in the absence and presence of 2% BSA.

In the absence of 2% BSA, kinetic results for 6α-hydroxypaclitaxel formation by HLM were well described by Michaelis-Menten or substrate inhibition models while in the presence of 2% BSA exhibited single enzyme Michaelis-Menten kinetic (Fig. 1A). 5-Hydroxyomeprazole formation in the absence and presence of 2% BSA followed two enzyme Michaelis-Menten kinetics (Fig. 1B). In the absence of BSA, OMP sulfoxidation exhibited biphasic kinetics (Fig. 1C). Addition of 2% BSA to incubations resulted in Michaelis-Menten kinetic (Fig. 1C).

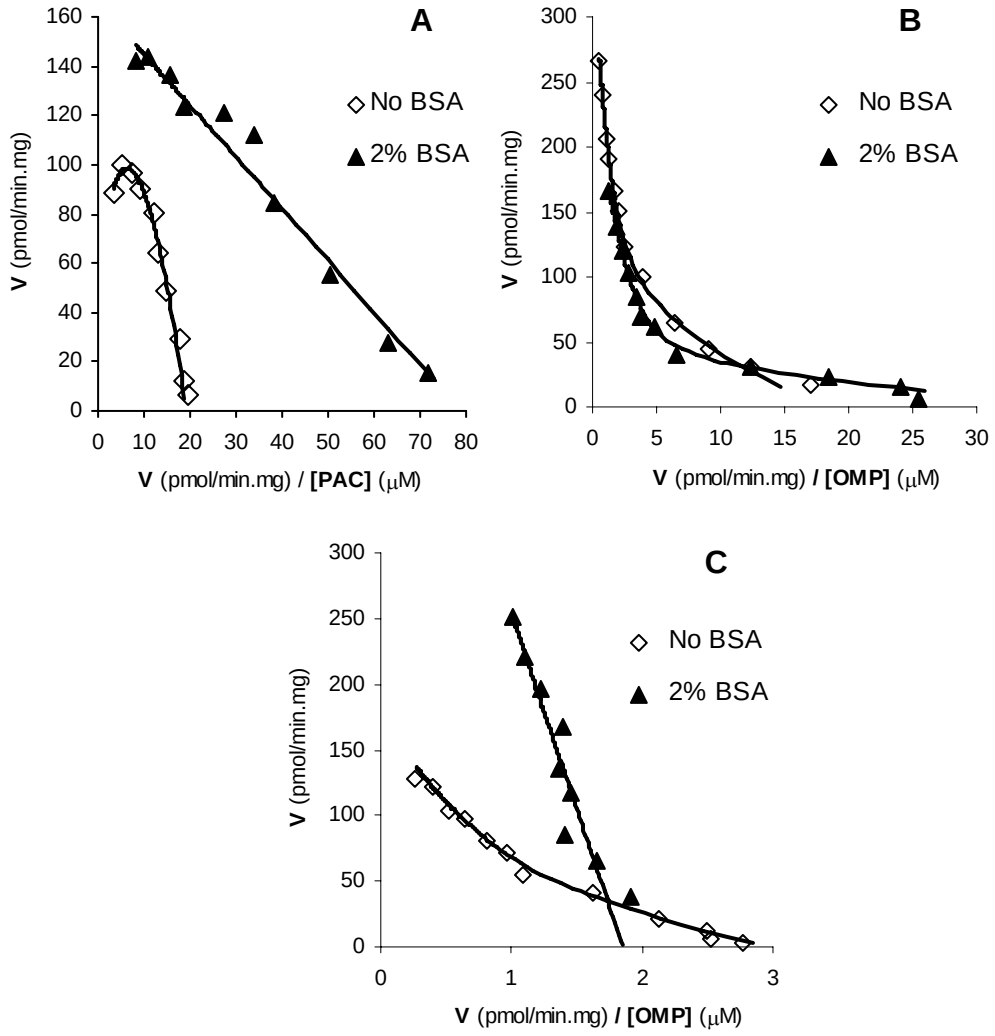

Figure 1. Representative Eadie Hofstee plots for PAC 6α-hydroxylation (A), OMP 5-hydroxylation (B), and OMP sulfoxidation (C) by HLM in the absence and presence of 2% BSA.

BSA increased mean CL_{int} for PAC 6α-hydroxylation about 4 fold due to significantly decreasing mean K_m with a minor effect on mean V_{max} (Table 1). In addition, BSA increased mean CL_{int1} (2 fold) for OMP 5-hydroxylation. Although BSA reduced the K_m (4 fold) for OMP 5-hydroxylation, V_{max} was also decreased (2 fold) (Table 1). For OMP sulfoxidation, BSA increased both of K_{m1} and V_{max1} for this pathway (15-19 fold) thus the mean CL_{int1} was not significantly changed (Table 1).

Comparison of predicted CL_H to reported *in vivo* CL_H for PAC 6α-hydroxylation.

In the absence of 2% BSA, mean predicted CL_H of PAC 6α-hydroxylation by HLM was under-predicted from reported *in vivo* CL_H around 17-fold. Whereas the mean predicted CL_H of PAC 6α-hydroxylation in the presence of 2% BSA resulted in 5-fold underprediction (12.3 vs 65.2 L/h). The *in vivo* CL_H was determined to be 65.2 L/hr (data from Smorenburg et al. 2003; the approximately administrated dosage was 175 mg/m^2 3 hour infusion).

Table 1. Effect of 2% BSA on the kinetic parameters of PAC 6α-hydroxylation, OMP 5-hydroxylation, and OMP sulfoxidation.

Metabolic pathways	K_m[a] (μM)	K_{si}[b] (μM)	V_{max}[a] (pmol/min/mg)	CL_{int}[a] (μL/min/mg)
CYP2C8-mediated paclitaxel 6α-hydroxylation				
without BSA	8.66±4.15	41.2±26.6	167±78.1	19.6±3.17
with 2% BSA	2.23±0.34*		154±22.8	70.6±16.6*
CYP2C19-mediated omeprazole 5-hydroxylation				
without BSA	8.63±6.83		97.3±46.7	13.8±8.39
with 2% BSA	2.39±1.80		49.7±22.4	27.1±12.4
CYP3A4-mediated omeprazole sulfoxidation				
without BSA	16.5±11.5		63.0 ± 60.2	3.16±1.50
with 2% BSA	307±133*		965±272*	3.62±1.92

Data are mean ± S.D. ([a] n = 5, [b]n = 3), * Significant difference ($p \leq 0.05$) to control.

Conclusions

The present study reveals that BSA shows variable effects on each CYP isoform. The prediction of *in vivo* clearance from *in vitro* kinetic data for CYP2C8-catalysed PAC 6α-hydroxylation is likely to be improved in the presence of 2% BSA. In addition, BSA had minor effect on the CYP2C19-mediated OMP 5-hydroxylation while no effect on CYP3A4-mediated OMP sulfoxidation for improving *in vitro* CL_{int} prediction.

Acknowledgements

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Original article **P53**

A preliminary study on possible existence of cytochrome P450 (CYP) gene in *Plasmodium falciparum*

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Abstract

The existence of cytochrome P450 (CYP) enzymes in *Plasmodium falciparum* as well as other protozoa may imply the role/function of CYP in parasite survival. Successful hybridization of *CYP*-specific probes (*CYP 2B1/B2*) to *P. falciparum* mRNA extracted from sensitive strains was reported. This evidence supported the existence of *CYP* genes in *P. falciparum* malaria. In the present study, we characterized *CYP* gene in *P. falciparum* and correlated CYP activities to antimalarial sensitivity *in vitro*. At the first step, an array of bioinformatic technique was used to analyze all currently sequenced protozoan genomes (http://www.sanger.ac.uk/ Projects/ Protozoa/) including *Plasmodium spp.*, and with *P. falciparum* in particular. The initial BLAST sequences with the conserved *CYP* gene were performed, followed by more specific BLAST searches for sequences possessing *CYP* homology of human and protozoan database. Results from BLAST analyses showed no sequence alignments which would ascertain conserved nucleotide regions to be *CYP* gene in *P. falciparum*. This contradictory finding confirmed the previous report. Phenobarbital was added at 1 □M concentration to the *in vitro* culture of early trophozoite stage *P. falciparum* for both chloroquine-sensitive (3D7) and -resistant (K1) clones and the culture was incubated at 37^{o} C for 12 hr. Total RNA was isolated from the parasites and dot blot hybridization was performed using a rat cDNA probe covering exon 6-9 of the phenobabitone inducible *CYP 2B1/B2* gene. The negative result was shown in this experiment. Based on this result together with the bioinformatics analyses, we suggested the absence of *CYP 2B1/B2* gene in *P. falciparum* genome.

Keywords: *Plasmodium falciparum*, cytochrome P450, bioinformatics, hybridization

Introduction

CYP belongs to a superfamily of proteins ubiquitously found across the phylogenetic tree and are known to catalyze a multitude of enzymatic reaction types. There have been a number of reports in the literature indicating the existence of enzyme activity in *P.falciparum* that is attributable to cytochrome P450 (CYP) enzymes. The CYP-dependent activity was shown to be enhanced in *P. falciparum* resistant strains, and could also be induced by known classical CYP inducers (1-2). Successful hybridization of CYP-specific probes (CYP 2B1/B2) to *P.falciparum* mRNA extracted from sensitive strains, and with an increased level of binding in resistant strains, lends support to the existence of CYP-related genes in this malarial parasite (1). The possible existence of CYP or CYP-like enzymes in *P.falciparum* as well as that shown in other protozoa may imply the role/function of CYP in parasite survival (3).

In the present study, we characterized CYP or CYP-like gene in *P.falciparum* and investigated the association between CYP activity in *P.falciparum* to sensitivity of the parasite to antimalarial drugs *in vitro*. We used array of bioinformatic techniques to analyze all currently sequenced protozoan genomes (http://www.sanger.ac.uk/ Projects/Protozoa/) including *Plasmodium spp.*, and with *P.falciparum* in particular. The initial blast sequences with the conserved CYP gene were performed, followed by more specific blast searches for

sequences possessing CYP homology of human and protozoan database. Results from blast analyses showed no sequence alignments which would ascertain conserved nucleotide regions of CYP gene in *P.falciparum*. Due this contradictory observation, we carried out the experiment to confirm the previous finding by Surolia et al. for the existence of CYP (CYP 2B1/B2) genes in *P.falciparum* malaria (1).

Method

Male rats of Sprague-Dawley strain (90-100 g) were given single injections of phenobarbital (8 mg/100 g, intraperitoneally). Rats were sacrificed after 24 hr and livers were collected for total RNA extraction by RNeasy Mini kit (QIAGEN). cDNA was prepared by using Omniscript Reverse Transcription (QIAGEN), and used as a template in polymerase chain reaction (PCR) for exon 6-9 of CYP 2B1/B2 gene. The forward and reverse primers used were as follows:

Forward primer: GATTCAGGAGGAAGCCCAAT

Reverse primer: TTTTTCCAATGCCACTCTCC

PCR reactions were carried out in 25 µl volumes comprising 2 µl template, 1.0 µM primers, 1.0 mM magnesium chloride, 200 µM dNTP, 1X PCR Buffer and 0.1U of Taq DNA polymerase. PCR annealing cycles consisted of an initial four minutes denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55.5°C for one minute and extension at 72°C for one minute. Final extension was at 72°C for five minutes. The PCR amplifications were performed in a Bio-Rad thermal cycler. CYP 2B1/B2 PCR product size was 1,004 basepairs resolved by electrophoresis on 1.0% agarose gel and visualized by UV transillumination. PCR product was extracted from agarose gel using QIA quick spin kit (QIAGEN), then sequenced and aligned to exon 6-9 of rat CYP 2B1/B2 gene. PCR DIG labeling probe for rat cDNA CYP 2B1/B2 gene was prepared after compatible alignment of CYP 2B1/B2 was observed.

P.falciparum, chloroquine-sensitive (3D7) and chloroquine-resistant (K1) clones were cultured continuously *in vitro* by a modification of the methods of Trager and Jensen (4). Synchronization was done when 5% parasitemia was achieved to obtain only ring stage parasite. Phenobarbital (CYP-inducer) was added at 1.0 □M concentration to the culture at early trophozoite stage and incubation carried out for 12 hr (1). Total RNA was isolated from washed parasite pellet by using TRIZOL® reagent (Invitrogen). Dot blot analysis was carried out using standard procedure (5) with 10 and 25 □g of total RNA, respectively. A rat cDNA probe covering exon 6 to 9 of the phenobarbitone inducible CYP2B1/B2 gene was used (1). Hybridization was carried out in 50% formamide at 42°C and final washing included 0.2x SSC in 0.1% SDS at 65°C for 15 min (6).

Results

Total RNA of Sprague Dawley rat with good quality especially 28S and 18S band was obtained, with a huge PCR product of CYP 2B1/B2 (product size 1004 bps.) before extraction and sequencing it from gel. After sequencing, the alignment result showed that PCR product was the CYP 2B1/B2 fragment. CYP 2B1/B2 DIG labeling probe resulted in a little bigger size than its fragment which was none labeling. Based on the results of blot hybridization of CYP 2B1/B2 DIG labeling probe on total RNA of *P.falciparum* (K1 and 3D7 clones) in both control and 1 □M Phenobarbital, a negative result of CYP 2B1/B2 probe hybridization was shown with total RNA from both *P.falciparum* clones, whereas a positive result was obtained with total RNA from rat liver treated with phenobarbital.

Discussion

Results shown in the present study contradict with that reported previously by Surolia et al (1). The negative hybridization of CYP 2B1/B2 DIG labeling probe observed in total

RNA of *P.falciparum* in all groups suggests the absence of CYP 2B1/B2 gene in *P.falciparum*. The positive results previously reported could be explained by contamination of rat RNA with *P.falciparum* RNA. It was noted however that even with the absence of CYP-gene, metabolic activity was found in *Plasmodium* spp. (7). It is possible that other protein that acts like CYP enzyme (CYP-like protein) may contribute to this metabolic activity. Proteomics of the *P.falciparum* CYP-like protein is being investigated and correlate its activity with sensitivity of the parasites to antimalarial drugs.

Conclusion

Based on our results from bioinformatic analysis of *P.falciparum* genomes, together with that from hybridization experiment using CYP-specific probes (CYP 2B1/B2) from mRNA obtained from rat liver, with mRNA obtained from *P.falciparum* clones, we conclude for the absence of CYP gene sequences in *P.falciparum* genome.

Acknowledgement

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Original article **P54**

Bioequivalence of 400 mg moxifloxacin tablet in healthy Thai volunteers

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Abstract

Moxifloxacin-HCl, a fluoroquinolone, is a broad spectrum antibacterial agent against respiratory tract pathogens, including Gram-positive and Gram-negative bacteria, anaerobic bacteria and atypical respiratory tract pathogens. In order to ensure the efficacy and safety of generic moxifloxacin formulations, the bioequivalence study of these products need to be evaluated. Thus the aim of this study was to compare the rate and extent of absorption of a new generic moxifloxacin formulation (Rapiflox®, Atlantic Laboratories Corporation Ltd., Bangkok, Thailand) with that of a Reference formulation (Avelox®, Bayer HealthCare AG, Leverkusen, Germany) when given at a dose of 400 mg. A single dose randomized two treatments, crossover with 2 weeks washout period was performed in twenty health Thai volunteers. The subjects received either 400 mg of the Reference or Test formulation. Blood samples were collected at predose (0 hr) and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 34 hr post dose. Moxifloxacin plasma level was measured by HPLC with fluorescence detector. Pharmacokinetic parameters were calculated using non-compartmental model. The mean C_{max} $\pm$ SD for the Test and Reference formulations were 4,279.1 $\pm$ 1,186.5 and 4,302.5 $\pm$ 976.9 ng/ml, respectively. Furthermore, the mean $\pm$ SD of AUC_{0-t} for the Test and Reference formulations were 50,686.4 $\pm$ 9,148.7 and 52,535.3 $\pm$ 8,548.9 ng.hr.ml^{-1} whereas $AUC_{0-\infty}$ were 52,777.5 $\pm$ 9,016.5 and 55,048.9 $\pm$ 8,615.7, ng.hr.ml^{-1}, respectively. The mean T_{max} for Test and Reference formulations were 2.01 $\pm$ 1.57 and 2.24 $\pm$ 1.87 hr, respectively. The mean ratios (90% confidence intervals) for pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were 0.9963 (0.8321-1.1219), 0.9962 (0.8707-1.0534) and 0.9957 (0.8652-1.0485), respectively. Therefore, it can be concluded that two moxifloxacin tablets (Test and Reference formulation) were bioequivalent in healthy Thai volunteers under fasting condition.

Keywords: bioequivalence, moxifloxacin, pharmacokinetic parameters

Introduction

Moxifloxacin hydrochloride is a synthetic broad spectrum antibacterial agent and is available as AVELOX Tablets for oral administration and as Avelox I.V. for intravenous administration. Moxifloxacin, a fluoroquinolone, is available as the monohydrochloride salt of 1-cyclopropyl-7-[(S, S)-2,8-diazabicyclo[4.3.0]non-8-yl]-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinoline carboxylic acid.

The bactericidal action of moxifloxacin results from inhibition of the topoisomerase II (DNA gyrase) and topoisomerase IV required for bacterial DNA replication, transcription, repair, and recombination. Moxifloxacin is an extended spectrum of antimicrobial activity. Its activity normally kills gram-negative pathogens, including β-lactamase-negative and β-lactamase-positive, *Haemophilus influenzae*, *Moraxella catarrhalis*, and Enterobacteriaceae. Furthermore, moxifloxacin has extended activity against gram positive cocci, including penicillin-resistant strains of *Streptococcus pneumoniae*, anaerobic and intracellular bacteria,

and atypical organisms such as *Legionella*, *Mycoplasma*, and *Chlamydia.* Moxifloxacin has a broad range of activity against clinically significant pathogens, it is a potentially useful therapeutic agent for the treatment of respiratory tract and other infections. Moxifloxacin is now commercially available in several formulations. However, the efficacy of these generic formulations is of great concerned. To ensure the efficacy of these generic formulations, the bioequivalence study of these products need to be evaluated. The aim of this study was to compare the rate and extent of absorption of a new generic moxifloxacin formulation (Rapiflox®, Atlantic Laboratories Corporation Ltd., Bangkok, Thailand) with that of a reference formulation (Avelox®, Bayer HealthCare AG, Leverkusen, Germany) when given at a dose of 400 mg.

Methods

Drug and Chemicals:

Reference formulation used in the present study was Avelox® (Bayer HealthCare AG, Leverkusen, Germany, Batch No. BXF5112) and the Test product was Rapiflox® (Atlantic Laboratories Corporation Ltd., Bangkok, Thailand, Batch No. PD090019). The Test and Reference formulations were pharmaceutical equivalent according to the regulatory criteria.

Subjects:

Twenty healthy Thai male and female volunteers were included in the study. All subjects participating in the study were healthy under medical criteria from medical histories, physical examinations and standard clinical laboratory tests. Subjects were excluded if they had liver, kidney or cardiovascular diseases, including allergic history related to moxifloxacin, or other drugs in the medical history. All volunteers had signed the informed consent to participate in the experiment. This project was approved by The Khon Kaen University Ethics Committee for Human Research (HE511062).

Study Design:

The study was conducted using a single-dose, randomized, two-way, crossover design with two weeks wash-out period. The subjects received either 400 mg of the Reference or Test formulation. Blood sample of about 10 ml were collected at predose (0 hr) and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 34 hr after drug administration.

Determination of drug levels in plasma:

Plasma concentrations of moxifloxacin were measured by a pre-validated analytical method. Moxifloxacin plasma level was measured by HPLC with fluorescence detector.

Results

1) The mean plasma concentrations versus time profiles of moxifloxacin in healthy subjects (N = 20) after oral administration of the Test formulation and the Reference formulation are shown as linear plot in Figure 1.

2) The mean C_{max} of both formulations were similar, with mean moxifloxacin concentration of 4,302.5 ± 976.9 ng/ml and 4,279.1 ± 1,186.5 ng/ml for the Reference and Test formulations, respectively. The mean AUC_{0-t} for the Test formulation (50,686.4 ± 9,148.7 ng.hr.ml^{-1}) was also similar to that of the Reference (52,535.3 ± 8,548.9 ng.hr.ml^{-1}). Also, the mean $AUC_{0-\alpha}$ for the Test formulation (52,777.5 ± 9,016.5 ng.hr.ml^{-1}) was similar to that of the Reference (55,048.9 ± 8,615.7 ng.hr.ml^{-1}) .

3) The C_{max}, AUC_{0-t} and $AUC_{0-\alpha}$ were transformed to logarithmic values (Ln), the relative bioavailability was calculated as the ratio of Ln C_{max}, Ln AUC_{0-t} and Ln $AUC_{0-\alpha}$

between the Test and Reference (Test/Reference) (Table 1). The mean ratios (90% confidence interval) for Test/Reference of the Ln C_{max}, Ln AUC_{0-t} and Ln $AUC_{0-\alpha}$ were 0.9963 (0.8321-1.1219), 0.9962 (0.8707-1.0534) and 0.9957 (0.8652-1.0485), respectively.

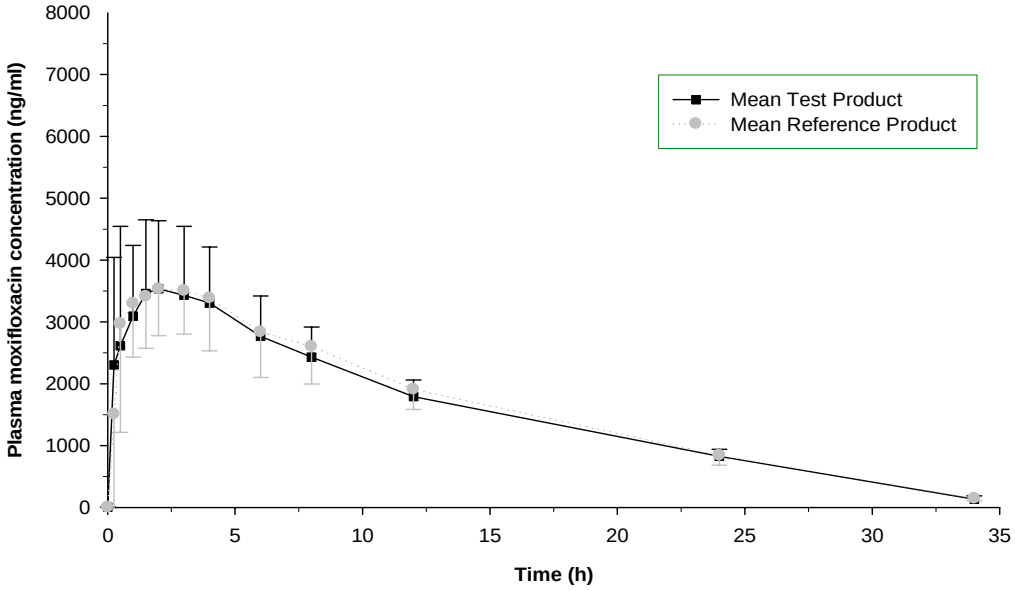

Figure 1 Linear plot of mean plasma Moxifloxacin concentrations versus time in healthy subjects (N = 20)

Conclusion

All volunteers participated in this study were well tolerated to both the Test formulation and the Reference formulation. The pharmacokinetic parameters of both the Test formulation and the Reference formulation were determined, with mean C_{max} of the Test formulation and the Reference formulation were 4,279.1 and 4,302.5 ng/ml, respectively. Mean AUC_{0-t} for the Test and Reference formulations were 50,686.4 and 52,535.3 $ng.hr.ml^{-1}$ whereas mean $AUC_{0-\infty}$ were 52,777.5 and 55,048.9 $ng.hr.ml^{-1}$, respectively. The 90% confidence interval of Ln ratios of either C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ between the Test formulation and the Reference formulation were in FDA acceptance range of 0.80-1.25. Therefore, the Test formulation, Rapiflox® 400 mg tablet of Atlantic Laboratories Corporation Ltd., Bangkok, Thailand is bioequivalent to the Reference formulation, Avelox® 400 mg tablet of Bayer HealthCare AG, Leverkusen, Germany in healthy Thai volunteers under fasting condition.

Acknowledgements

This study was supported by Atlantic Laboratories Corporation Ltd., Thailand.

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Original article **P55**

Selective inhibition of human cytochrome P450 1A2 by *Moringa oleifera*

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Abstract

The use of *Moringa oleifera* Lam as a healthcare product in Thailand becomes very popular during a past few years. Since medicinal plants constituents may interact with drug metabolizing enzymes, particular cytochromes P450 (CYP) raises the potential of herb-drug interactions. The aim of the present study was to determine the inhibitory effects of *M. oleifera* extractions on human CYP activities including CYP1A2, CYP2D6, CYP2E1 and CYP3A4 using selective substrate of these enzymes. The results revealed that both ethanolic and aqueous extracts of *M. oleifera* inhibited CYP1A2, CYP2D6, CYP2E1 and CYP3A4 activities in a dose-dependent manner. Moreover ethanolic extract of *M. oleifera* exhibited selective inhibition of CYP1A2 with IC_{50} values of 13.8 $\pm$ 9.8 μg/mL incubation. The results obtained from the present study suggested the possibility of potential herb and drug interactions of *M. oleifera*. Therefore, healthcare professionals and patients should be aware when using this medicinal plant with some prescribed drugs especially drugs that metabolized by CYP1A2.

Keywords: *Moringa oleifera*, Cytochromes P450, Herb-Drug interaction

Introduction

Moringa oleifera Lam (*M. oleifera*), commonly known in Thai as "ma-room", has long been used as foods, nutritional supplements, or medicines in several countries, including Thailand. *M. oleifera* is a tree belongs to Moringaceae family. It has been reported to have various pharmacological properties such as antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities (1). *M. oleifera* contains several compounds such as carotenoids, flavonoids, phenolics and ascorbic acid (2, 3). Several medicinal plants have been reported to inhibit the activities of cytochromes P450 (CYP) which are the most important enzymes involved in the metabolism of thousands of clinically used drugs. Major isoforms of human CYP isoforms that involved in drug metabolism are CYP3A4, CYP2D6, CYP2E1 and CYP1A2 (4). The potential interaction of medicinal plants with clinically used drugs is a major safety concern, especially for drugs with narrow therapeutic index (e.g. warfarin and theophylline) and may lead to treatment failure or life threatening adverse reactions (5).

Although *M. oleifera* has been used since ancient times in Thailand, data concerning the interaction between this plant and CYPs is still limited. The aim of the present study was to determine the inhibition effects of *M. oleifera* both ethanolic and aqueous extracts on human CYP1A2, CYP2D6, CYP2E1 and CYP3A4 activities.

Materials & Methods

Preparation of M. oleifera ethanolic and aqueous extract: Dried leaves of *M. oleifera* was grounded and extracted with 95% ethanol or water. Ethanol or water was evaporated under low pressure at 60°C and freeze dry, respectively.

Inhibitory effect of M. oleifera extracts on human CYP activities: Human hepatic phenacetin O-deethylase, dextromethorphan O-demethylase, chlorzoxazone 6-β hydroxylase and testosterone 6β-hydroxylase were used as a selective marker of CYP1A2, CYP2D6, CYP2E1 and CYP3A4, respectively (6, 7). An incubation mixture (500 μl) contained the ethanolic or aqueous extract of *M. oleifera*, a selective CYP substrate, human liver microsomes in 0.1 M phosphate buffer, pH 7.4. An equal volume of DMSO or water was also added in controls. The reaction was initiated by the addition of β-NADPH then incubated at 37 °C for a specific period. The amounts of metabolites were measured by HPLC.

Data analysis: IC_{50} values (concentrations of inhibitor causing 50% reduction in activity relative to the control) were calculated by linear regression analysis of the log inhibitor concentration versus percentage control activity plots.

Results

The ethanolic and aqueous leaves extracts of *M. oleifera* inhibited human CYP1A2, CYP2D6, CYP2E1 and CYP3A4 activities in dose-dependent manner with varied IC_{50} values range from 13.8 to 1,500 μg/mL incubation (Table 1).

Ethanolic extract of *M. oleifera* showed selective inhibition of CYP1A2 with IC_{50} value of 13.8 μg/mL incubation. Comparing to the known CYP1A2 inhibitors, the inhibitory potency of *M. oleifera* ethanolic extract on CYP1A2 was less than furafylline but more potent than cimetidine.

CYP 3A4 was also inhibited by the extracts of *M. oleifera* which the ethanolic extract exhibited comparatively stronger inhibitory activity towards CYP3A4 than clarithromycin, with IC_{50} value of 101 μg/mL incubation.

Table 1: IC_{50} values of *M. oleifera* ethanolic and aqueous extract on human CYP1A2, CYP2D6, CYP2E1 and CYP3A4 activities.

	IC_{50} values (μg/mL incubation)			
	CYP1A2	CYP2D6	CYP2E1	CYP3A4
Known CYP1A2 inhibitor				
Furafylline	0.08 ± 0.05			
Cimetidine	71.0 ± 52.8			
Known CYP2D6 inhibitor				
Quinidine		0.97 ± 0.06		
Fluoxetine		0.04 ± 0.01		
Paroxetine		0.02 ± 0.01		
Known CYP2E1 inhibitor				
Diethyldithiocarbamate			0.18 ± 0.05	
Disulfiram			0.83 ± 0.21	
Known CYP3A4 inhibitor				
Ketoconazole				0.10 ± 0.08
Erythromycin				83.3 ± 61.1
Clarithromycin				730 ± 233
Moringa oleifera				
Ethanolic extract	13.8 ± 9.8	219 ± 114	> 400[a]	101 ± 40
Aqueous extract	630 ± 324	> 1000[b]	725 ± 243	1500 ± 258

Data presents as mean ± S.D. from four microsomal preparations.

[a] The data was limited by the solubility of *M. oleifera* ethanolic extract.

[b] The peak of dextromethorphan O-demethylation was interfered by the high concentration of *M. oleifera* aqueous extract.

Discussion and conclusion

Both ethanolic and aqueous extracts of *M. oleifera* inhibited human CYP1A2, CYP2D6, CYP2E1 and CYP3A4 activities *in vitro* in a dose-dependent manner. *M. oleifera* particularly the ethanolic extract was a strong inhibitor of human CYP1A2. CYP1A2 is the main hepatic CYP1A which involved in the metabolisms of a number of clinically used drugs such as acetaminophen, alosetron, theophylline, tarcrine, tizanidine etc.

This results obtained from the present study suggest the possibility of potential herb-drug interaction if *M. oleifera* was administrated concomitantly with medicines that are metabolizes by CYP1A2. The effects of this medicinal plant *in vivo* need further investigation.

Acknowledgements

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Original article **P56**

Method validation for simultaneous determination methamphetamine and its metabolite amphetamine in rat liver by using GC-FID

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Abstract

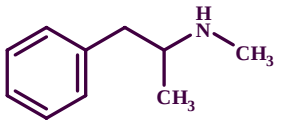
The purpose of these studies was to develop method for simultaneous determination of methamphetamine (MA) and its metabolite amphetamine (AM) in rat liver. Quantification of MA and AM in rat liver by using gas chromatography with a flame ionization detector (GC-FID) was validated. The chromatography used a VertiBond^{TM} 5 capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness). Sample of rat liver was extracted by liquid-liquid extraction. MA and AM were well separated within 12.8 min. The calibration curves were linear with good correlation coefficient. The intra- and inter-day precisions of the method were 8.35-10.97%RSD and 4.79-8.58%RSD for AM, 7.94-9.58%RSD and 8.51-12.21%RSD for MA. The accuracy ranged from (-) 14.77 to (+) 5.41%DEV for AM and (-) 6.98 to (+) 4.11%DEV for MA. The results were shown good recovery both AM and MA. The limit of detection and limit of quantification of AM and MA were 6.25 μg/g and 9.375 μg/g, respectively. It was shown to be useful for the study of pharmacokinetic and forensic toxicology.

Keywords: methamphetamine, amphetamine, GC-FID, liver.

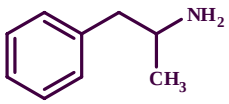
Introduction

Methamphetamine (MA; Fig.1A) and its metabolite amphetamine (AM; Fig.1B) are powerful stimulants of central nervous system and are abuse in many countries including Thailand. MA is a dangerous narcotic drug which causes health problems. MA produces many toxic effects such as, wakefulness, hyperthermia, hypertension and euphoria (1). The death is a hallmark of toxic effect from MA. Therefore, it is important to indicate level of MA and its metabolite AM in metabolic organ, liver. Gas chromatography (GC) (2,3,4) and liquid chromatography (LC) (5) are successful technique to detect MA and its metabolite AM in tissues after derivertization to minimized tailing peak or unsuccessful baseline separation. However, derivertization has several inherent disadvantages over direct analysis. In addition, MA and AM have been determined by using GC-MS which is a powerful technique. However, this technique has been limited since this running cost is very expensive. The gas chromatography-flame ionization detector (GC-FID) is considered to be more possible for determining the MA and its metabolite AM in liver.

Therefore, the aim of this study was to develop suitable condition of GC-FID for determination of MA and its metabolite AM in rat liver without derivertization.



Methamphetamine (MA)
(1A)



Amphetamine (AM)
(1B)

Figure 1 Chemical structure of MA and AM

Methods

Chemicals and reagents

Reference standard methamphetamine hydrochloride was obtained from the Regional Forensic Science Division 4, Office of the Police Forensic Science, Thailand. Amphetamine was provided by Department of Pharmacology, Faculty of Science, Prince of Songkla University.

Preparation of standard solutions

The stock solution of 1,000 μg/mL of MA and AM standards were prepared in pure methanol. Working standard solutions were prepared by diluting the stock solutions to a concentration range of 2.5-50 μg/mL as a mixture of the standard MA and AM.

Chromatographic instrument and condition

A gas chromatography model GC 6890 (HP, U.S.A.) equipped with a flame ionization detector, capillary column VertiBond™ 5 (5% diphenyl and 95% dimethypolysiloxane, 30 m x 0.32 mm i.d., 0.25 μm film thickness) (Vertical®, Thailand). Determination of MA and its metabolite AM in liver were modified (6). Temperature of the column was programmed initially at 70 °C (2 min) and increased with a rate of 30 °C/min to 120 °C and then increased to 150 °C at a rate of 5 °C/min. Finally, the temperature was increased at a rate of 70 °C/min to 300 °C where it was held for 1 min. 1 μL of sample was injected in a splitless injection mode (splitless injection with inlet insert purge time of 0.3 min and split vent flow rate of 80 mL/min). Helium gas was used as the carrier gas with a flow rate of 2.5 mL/min. Fuel gas (H_2) and make-up gas (N_2) flow rate were 30 mL/min. Oxidant gas (O_2) flow rate was 300 mL/min. Injector and detector temperature were 200 °C and 300 °C, respectively.

Liver preparation and extraction procedure

Rat liver was quickly removed after euthanized with diethyl ether and perfused with 0.9% normal saline. Prior to extraction procedure, MA and AM free-liver was homogenized using a homogenizer (Kinematica CH-6010 Kriens-LU, Polytron®, Switzerland) and sonicated with ultrasonic processor (Branson Sonifier 450, U.S.A.) in an ice-chilled tube containing an ice. Homogenized tissue was divided into 2 portions. One was used as blank and the others were used for mixture standard MA and AM spiked tissue. The extraction procedure was modified by method of Moriya and Hashimoto (7). The final MA and AM concentration in the liver were in a range of 6.25-125 μg/g. Finally, 1μL of the reconstituted volume was injected into the GC-FID.

Method validation

The method of analysis was validated in accordance with the Guidance for Industry: Bioanalytical Method Validation (8).

Linearity was evaluated by preparing eight standard concentrations (6.25-125 μg/g; 5 replicates each concentration) of MA and AM in liver. The calibration curve was constructed by plotting the peak area of the analyte versus its concentrations. Regression analysis for each calibration curve was performed to obtain the calibration equation and correlation coefficient (r).

Precision was evaluated from intra- and inter-day precision by using 3 quality control samples (5 replicates for each concentration) into liver. Precision is frequently expressed as the percentage of the relative standard deviation (%RSD). The level of acceptance for precision is 15% RSD value.

The accuracy was also determined using QC with five replicates for each concentration. Accuracy was expressed as the deviation (DEV) and is acceptable when the DEV is within ±15%.

Recovery was determined in three concentration (15.625, 31.25, 125 μg/g; 5 replicates of each concentration).

The limit of detection (LOD), signal to noise ratio equal to or greater than 3, and the limit of quantification (LOQ), signal to noise ratio equal to or greater than 10, were using concentrations of MA and AM (6.25, 9.375, 12.5 μg/g; 5 replicates of each concentration).

Results

The chromatographic separation of MA and AM in rat liver is shown in Fig. 2. MA and AM were well separated with no interference. The retention times of MA and AM were 4.66, and 5.18 min, respectively within a run time of 12.8 min. Regression analysis results showed that the calibration curves were linear over the concentration ranges of 9.375-125 μg/g for MA and AM. The regression equation was y = (8.23 ± 0.69) *x* – (2.03 ± 0.95), *r* = 0.9996 for AM and y = (10.25 ± 0.80) *x* – (2.30 ± 0.79), *r* = 0.9997 for MA. The accuracy (%DEV) for determination of all analytes ranged between ±15%. Intra- and inter-day precisions for determining AM and MA are shown in Table 1. Both values for all analytes were found to be within the acceptable value (15%RSD). The LOD and LOQ of AM and MA were 6.25 μg/g and 9.375 μg/g, respectively. The mean percentages of recovery were 86-114% for AM and 88-110 % for MA

Table 1 Precision and accuracy of the method for determination of MA and AM in rat liver (n=5)

Analyte	Concentration (ug/g)	Precision (%RSD)		Accuracy (%DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
AM	125	8.35	8.55	+5.41	-6.23
	31.25	8.47	8.58	-2.88	-10.51
	18.75	10.97	4.79	-13.80	-14.77
MA	125	7.94	8.51	+4.11	+1.26
	31.25	9.58	8.51	-2.46	-3.40
	18.75	9.39	12.21	-6.98	+0.37

.

Discussion

The present liquid-liquid extraction method which was modified is more available. This study is present a good recovery and cost saving apparatus. In addition, without derivertization the chromatogram are symmetry and shorter run time than previous reported (6). Therefore, this study may be useful for study of pharmacokinetic and forensic toxicology.

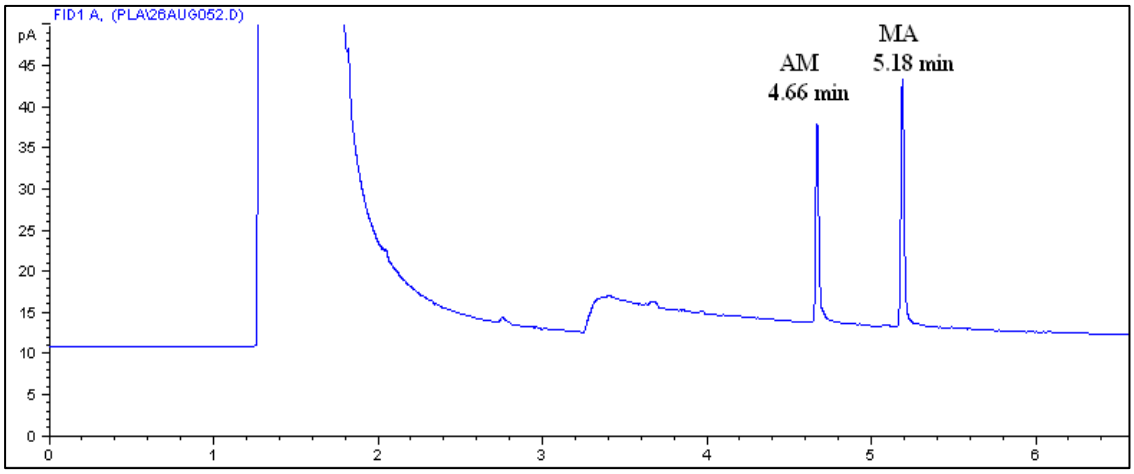


Figure 2 Representative chromatograms of AM and MA in rat liver (62.5 µg/g)

Conclusion

GC-FID technique is universal, laboratories can afford, cost saving technique, simple, sensitive, precise and accurate to determine MA and AM in tissue.

Acknowledgements

This project was partly supported by Forensic Science Program, Faculty of Science and the Graduate School, Prince of Songkla University. The Regional Forensic Science Division 4, Office of the Police Forensic Science, Songkhla.

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Original article **P57**

Fasciola gigantica: molecular analysis of a water channel protein (Aquaporin)

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Abstract

Fasciolosis caused by *Fasciola gigantica* is an important disease of cattle in Thailand. In order to develop new drugs and vaccines we have recently started to conduct research on aquaporins in *Fasciola*. Aquaporins (AQPs) are essential for the maintenance of water homeostasis in all organisms including animals, plants, and bacteria. Structure, function, and pathology of AQPs have been extensively studied in vertebrates but data for AQPs of trematodes is still limited. In the present study, a cDNA encoding an aquaporin (FgAQP-1) was molecular cloned from a metacercarial stage cDNA library of *F. gigantica*. The FgAQP-1 cDNA contained the complete coding sequence for a protein of 299 amino acid residues. Comparison of the deduced amino acid sequence with protein sequences in public databases using NCBI-BLASTP showed highest similarity to aquaporin-1 of *Bos taurus*. Expression and distribution of FgAQP-1 has been characterized at the nucleic acid and protein level in the adult parasite. Functional data of FgAQP-1 will be obtained after transformation of yeast with a plasmid carrying FgAQP-1 DNA by stopped flow analysis. Furthermore, it is planned to introduce mutations into the original FgAQP-1 sequence to analyze the importance of single amino acid residues for the functional integrity of the protein.

Keywords: *Fasciola gigantica*, aquaporin, water, *in vitro* mutagenesis.

Introduction

Fasciolosis caused by *Fasciola gigantica* is an important disease of cattle in Thailand. The successful development of a vaccine for *Fasciola* spp. will require several issues to be addressed [1]. Drug resistance is not a major problem at present, but that is not a reason for complacency, constant vigilance and monitoring are needed to avoid the problems that bedevil the control of *Fasciola* spp. [2]. Aquaporins (AQPs) are essential for the maintenance of water homeostasis in all organisms including animals, plants, and bacteria. Structure, function, and pathology of AQPs have been extensively studied in vertebrates but not in detail in trematodes. Currently, there are two examples of molecules that pass through aquaporins and kill protozoan parasites, hydroxyurea [3] and the hydroxide of tervalent antimony [4]. AQPs could possibly be exploited for the transport of novel drugs into the parasite.

The aim of this study was to identify and characterize the molecular biological properties of a water channel protein (Aquaporin) in *F. gigantica*.

Methods

The following experiments were used in this study:

1. Screening of *F. gigantica* cDNA libraries with a specific AQP probe and sequence analysis of the obtained FgAQP-1 cDNA.
2. Characterization of FgAQP-1 nucleic acids by Southern and Northern analyses.
3. Expression and purification of a C-terminal rFgAQP peptide in a prokaryotic system.

4. Production of polyclonal immune sera against the C-terminal rFgAQP-1 peptide.

Results

1. cDNA Screening, Sequencing and sequence analysis of FgAQP-1

A full-length aquaporin (FgAQP-1) cDNA was isolated from a metacercarial stage *F. gigantica* cDNA library with a partial gene-specific DIG labeled DNA probe. The FgAQP-1 cDNA contained the complete coding sequence for a novel aquaporin. A homology search of the GeneBank database by NCBI-BLASTP showed the highest identity to aquaporin-1 of *Bos taurus*.

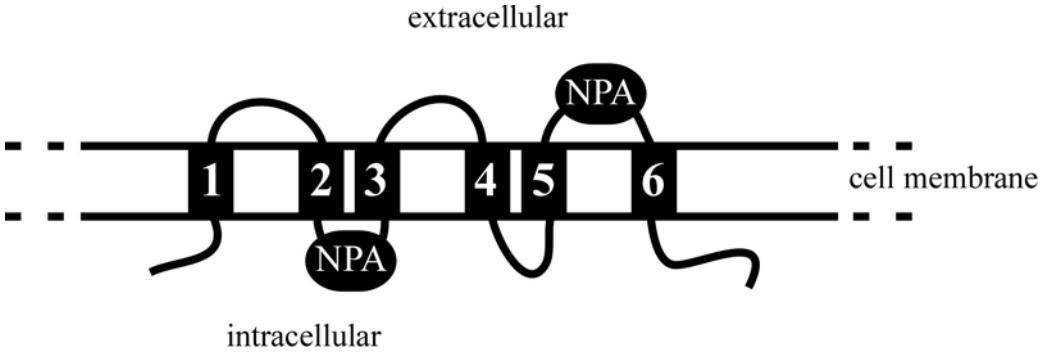

Figure 1 Schematic structure of FgAQP-1. It is a typical aquaporin which contains six-transmembrane segments and cytoplasmic located N- and C-termini. The two family-specific NPA boxes are located in the B- and E-loops.

2. Southern and Northern analysis

Southern and Northern analyses were performed using a FgAQP-1 DNA probe. For Southern analysis, genomic DNA was digested with restriction endonuclease *Hin*d III, *Xho* I and combination of *Hin*d III and *Xho* I. The DIG-labeled FgAQP-1 probe detected several DNA fragments with sizes up to 20,000 bp. For Northern analysis the total RNA of adults was probed with a FgAQP-1 DNA probe and showed a single hybridizing transcript at a size of 1900 nucleotides.

3. Expression and purification of a C-terminal rFgAQP peptide and antibody production against it.

Recombinant C-terminal FgAQP-1 peptide was expressed in *E. coli* BL21pLysS as a 40/41 kDa fusion protein after induction with IPTG. The expressed protein was purified by Zn^{2+}-Sepharose affinity chromatography. The C-terminal FgAQP-1 peptide was released from the fusion protein by chemical cleavage with hydroxylamine and was used for production of polyclonal antisera in mice.

Discussion

FgAQP-1 belongs to the major intrinsic protein (MIP) superfamily. AQPs have two usually conserved NPA motifs that are important for the function of the channel pore [5]. In FgAQP-1 the first NPA box showed an unusual motif while the second box was fully conserved. Aquaporins are encoded by multi-family genes in many organisms, e.g. there are at least 13 human aquaporins [6] and 23 plant aquaporins [7]. More than one DNA fragment was detected by Southern blot analysis, this result may imply that not only a single FgAQP gene exists.

Conclusion

A full length FgAQP-1 cDNA was cloned from a metacercarial cDNA library of *F. gigantica.* The deduced amino acid sequence of FgAQP-1 contains 299 residues and showed the best match to aquaporin-1 of *Bos taurus* when using NCBI-BLASTP. Functional analysis of this protein will be done in *Xenopus* oocytes and yeast systems. Furthermore, it is planned to introduce mutations into the original FgAQP-1 sequence to analyze the importance of single amino acid residues for the functional integrity of the protein.

Acknowledgements

This research was supported by the Commission on Higher Education, Ministry of Education, Thailand and the Faculty of Allied Health Sciences, Thammasat University, Thailand.

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Original article **P58**

Production of monoclonal antibodies against cathepsin-L and cystatin of *Faasciola gigantica*

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Abstract

Fasciolosis caused by *Fasciola gigantica* is an important disease in cattle and buffaloes in Thailand. At present, an efficient molecular diagnostic test for fasciolosis is not available. This study aims to produce monoclonal antibodies specific to two important parasite antigens, cathepsin L and type 1 cystatin (FgStefin-1) and to evaluate their efficacy as diagnostic tools. Purified native cathepsin L and recombinant cystatin produced in *E. coli* will be used for immunization of BALB/c mice for three times in 3-week intervals using Freund's adjuvant. The spleen cells of the mice will be collected and fused with myeloma cells to obtain clones that produce antigen-specific monoclonal antibodies. These monoclonal antibodies will be analyzed for their sensitivity and specificity.

Keywords**:** *Fasciola gigantica*, cathepsin L, cystatin, monoclonal antibody, diagnosis

Introduction

Fasciola gigantica is the most common cause of liver fluke disease in cattle and buffalo in Thailand. The detection of parasite eggs in faecal samples is the most commonly used diagnostic method but it has relatively low sensitivity. Therefore, new diagnostic tools with higher diagnostic sensitivity are needed to detect the infection. The present study aims to produce monoclonal antibodies specific to two important and abundant parasite antigens, cathepsin L and type 1 cystatin (FgStefin-1) and to evaluate their efficacy as diagnostic tools.

Materials and methods

Expression of recombinant FgStefin-1 in *E.coli*

The protein was expressed in *E.coli* M15 transformed with pQE30-FgStefin-1 after induction with IPTG (1 mM final concentration). The soluble protein was purified by Ni-NTA affinity chromatography under native conditions.

Collection of excretion/secretion product and purification of cathepsin L

Adult *F. gigantica* were incubated at 37°C in RPMI-1640 medium, pH 7.3 containing 2% (W/V) glucose, 30 mM HEPES and 25 mg/l gentamycin. After 4 h incubation, the culture medium was centrifuge at 10,000 × g for 1 h at 4°C and the supernatant was used to precipitate cysteine proteinases. Chilled ethanol was added drop by drop to a final concentration of 60% (v/v) and the mixture incubated at -20°C for 24 h. After centrifugation at 6,000 × g (40 min, 4°C), the supernatant was mixed with 75% ethanol and incubated at -20°C overnight. After centrifugation at 6,000 × g (40 min, 4°C) the supernatant was discarded and the pellet air dried and resuspended in PBS, pH 7.2.

Zymography analysis

The proteins were incubate with reaction buffer (0.1 M sodium acetate, 1 mM EDTA adjusted to pH 5.5) before performing gel electrophoresis under non-reducing conditions on 10% polyacrylamide gels containing 0.1% gelatin from porcine skin. After electrophoresis,

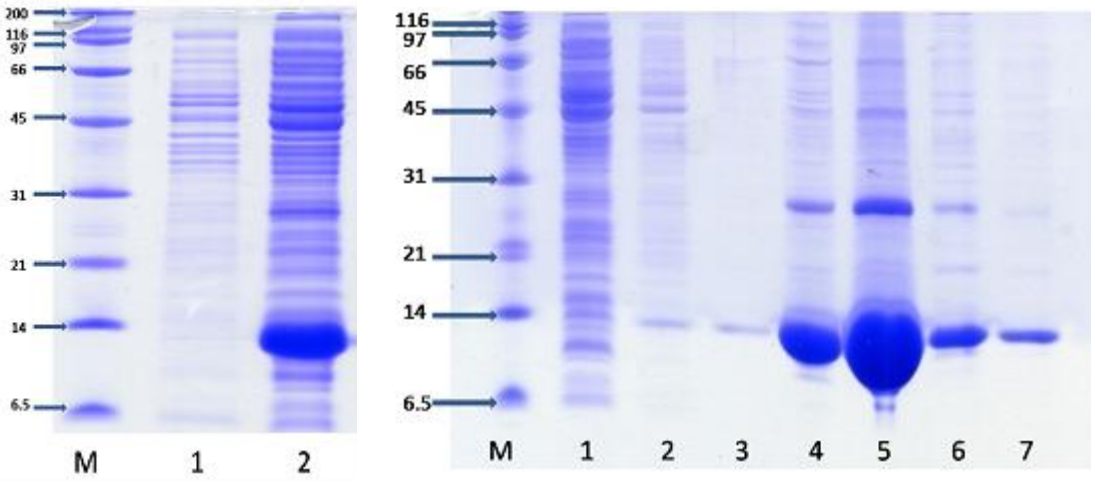
the gel was washed in 2.5% Triton-X 100 with agitation and then washed with developing buffer (0.1 M Sodium acetate, 1 mM EDTA, 2 mM DTT, adjusted to pH 5.5). The gel was incubated with developing buffer overnight at 37°C. The zymographic gel was then stained in 0.5% Coomassie Brilliant Blue R-250 and de-stained.

Fluorometric assay

The substrate Z-Arg-Arg-AMC (Calbiochem) was added to a final concentration of 400 µM and cathepsin L and cysteine protease activity in ES product were measured by a modified method from Barrett and Kirschke. Cathepsin L (1 ng/reaction) and ES product (10 ng/reaction) were incubated in 340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA, 8 mM DTT, pH 5.5 for activation of the enzyme. E-64 a specific cysteine proteases inhibitor was used as positive control. All samples had a 100 µl final reaction volume and were incubated for 30 min at 37°C in black 96-well microtiter plates before measurement of activity at wavelengths 355 nm and 460 nm.

Results

Expression of recombinant FgStefin-1 in *E.coli* M 15



(A) **(B)**

Figure1. SDS-PAGE analysis of the expression and purification of FgStefin-1. A, non-induced (1) and IPTG induced (2) bacterial protein. B, purification by Ni-NTA affinity chromatography flow through (1) wash (2, 3) and elution (4, 5, 6, 7) fractions.

Purification of cathepsin L

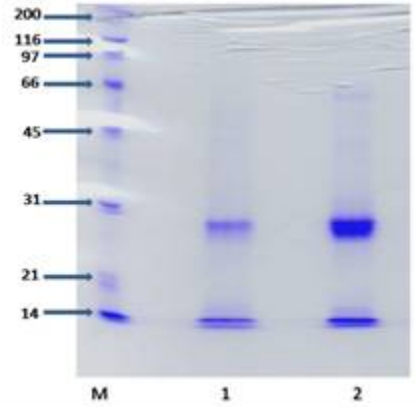


Figure 2. Purification of cathepsin L: ES (1), cathepsin L (2)

Figure 3. Zymography analysis: (1) ES and cysteine protease inhibitor E64, (2) ES, (3) cathepsin L and cysteine protease inhibitor E64, (4) cathepsin L

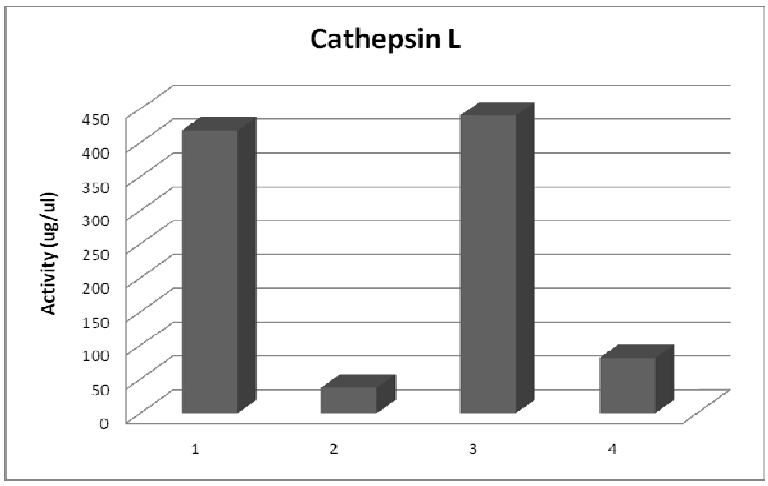


Figure 4. Fluorometric assay: (1) ES activity was detected using the substrate Z-Arg-Arg-AMC. (2) ES and cysteine protease inhibitor E64. (3) Cathepsin L activity was detected using the substrate Z-Arg-Arg-AMC. (4) Cathepsin L and cysteine protease inhibitor E64.

Discussion

Type 1 cystatin (FgStefin-1) and cathepsin L are important proteins in *F. gigantica*. They are secreted in large amounts by the parasite and induce a strong immune response in the infected host. Both proteins are also stable. These properties make them interesting candidates for diagnostic application. Monoclonal antibodies against these proteins are preferable to polyclonal antisera as they may show higher specificity.

Conclusion

Type 1 cystatin (FgStefin-1) and cathepsin L were purified as recombinant or native protein, respectively. Expression of recombinant FgStefin-1 in *E. coli* was induced by IPTG and the protein was purified by Ni-NTA affinity chromatography. Native cathepsin L was precipitated from excretion/secretion product by ethanol and tested by zymography analysis and fluorometric assay for its activity. Both proteins will be used for immunization of BALB/c mice to produce monoclonal antibodies that can be applied for diagnosis of fasciolosis.

Acknowledgements

This research was financially supported by Thammasat University and the Commission on Higher Education of Thailand.

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Original article **P59**

Molecular and biochemical characterization of type I cystatin (Stefin) of *Fasciola gigantica*

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Abstract

In the present study we describe type 1 cystatin, a cysteine protease inhibitor, as a major released antigen of the tropical liver fluke *Fasciola gigantica* (FgStefin-1). Immunohistochemical analysis showed that FgStefin-1 is abundant in tissue of tegumental type and the intestinal epithelium. Faint staining was observed in the epithelia of ovary and proximal uterus. Immunoblots showed the presence of FgStefin-1 in the parasite's excretion/secretion (ES) product. Sera of experimentally infected sheep reacted with recombinant FgStefin-1 starting 8 weeks postinfection. Activity analyses of recombinant FgStefin-1 showed nanomolar inhibition constants for mammalian host and parasite cysteine proteases. Our results suggest protective functions of FgStefin-1, regulating intracellular cysteine protease activity, and possibly protection against extracellular proteolytic damage to the parasite's intestinal and tegumental surface proteins.

Keywords**:** *Fasciola gigantica,* Cysteine protease inhibitor, Cystatin, Immunolocalization

Introduction

Cathepsin B and L cysteine proteases are established as important and abundantly produced antigens in the trematode genus *Fasciola* and are implicated in parasite nutrition, protection, and host invasion [1]. Expression patterns and biological functions of their counterparts, cysteine protease inhibitors of the cystatin family (MEROPS [2] inhibitor family I25, clan IH) have been less well researched in *Fasciola* and in trematodes in general. At present, neither immunohistochemical data demonstrating the distribution of cystatin in parasite tissues nor data regarding possible effects of trematode cystatins on the host immune system exist. In contrast, substantial immunomodulatory effects of type 2 cystatins from parasitic nematodes have been demonstrated in the last years [3-5]. They were found to decrease T cell responses by inhibition of proteases participating in MHC class II antigen processing and presentation and to increase interleukin 10 and nitric oxide production [6].

The purpose of this study was to characterize the molecular biological properties of a cysteine protease inhibitor from *F. gigantica* including its inhibition properties against mammalian and endogenous cysteine proteases. This work should lead to a better understanding of the biological function of type 1 cystatin in *Fasciola* and trematodes in general and in the future we will perform additional analyses to investigate whether FgStefin-1 has immunomodulatory properties as has been demonstrated for nematode cystatins.

Methods

A cDNA encoding a type 1 cystatin (FgStefin-1) was isolated from a *F. gigantica* cDNA library and used to produce recombinant FgStefin-1 (rFgStefin-1) in functional form

in *E. coli*. Polyclonal antibody against rFgStefin-1 was produced and used to study the distribution of native FgStefin-1 in *F. gigantica*. The inhibitory properties of rFgStefin-1 against cysteine proteases were characterized using fluorogenic substrates. The immune response of infected animals against FgStefin-1 was analyzed by ELISA.

Results

FgStefin-1 is present in the parasite from the metacercarial to the adult stage. It reacted with mouse anti-rFgStefin-1 antiserum as an 11 kDa antigen in immunoblotted crude worm (CW) extract, excretion/secretion (ES) product and tegumental antigen extract of the adult parasite. Immunohistochemical analysis showed that FgStefin-1 is abundant in tissue of tegumental type and the intestinal epithelium. Faint staining was observed in the epithelia of ovary and proximal uterus (Fig. 1). Purified rFgStefin-1 was tested for inhibitory activity against native human cathepsins B and L, recombinant *F. hepatica* cathepsin B and L. The protein was able to inhibit all tested proteases. FgStefin-1 was able to stimulate a host immune response in infected sheep before the parasites reached maturity.

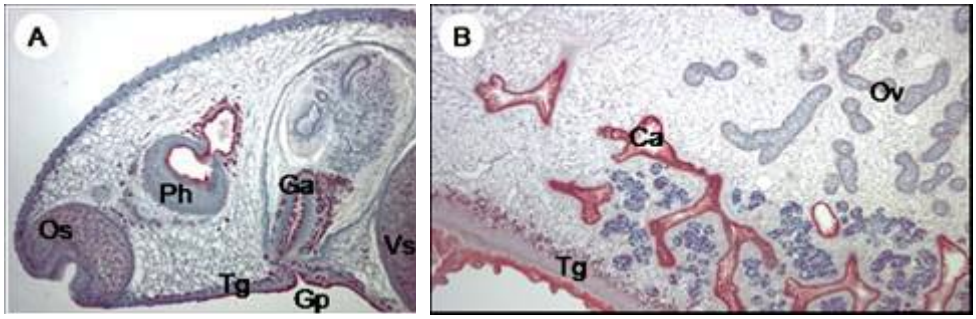

Figure 1. Immunohistochemical detection of FgStefin-1 in adult *F. gigantica* by mouse anti-rFgStefin-1 antiserum. (A) sagittal sections of the anterior region. (B) longitudinal section. Ca, caecum; Ga, genital atrium; Gp, genital pore; Os, oral sucker; Ov, ovary; Ph, pharynx; Tg, tegument; Vs, ventral sucker.

Discussion

FgStefin-1 has inhibitory properties comparable to homologs described from other organisms with higher activity against cathepsin L than cathepsin B. The occluding loop of cathepsin B might protect the active site of this protease from inhibition by cystatins. FgStefin-1 is present in tissues that constitute external and internal surfaces, tegument and gastrodermis. Cathepsin B is not only found in these tissues but also in vitelline cells through which it becomes incorporated in the parasite's eggs, Mehlis' gland, prostate gland, and male and female germ line cells while cathepsin L is a major gut-specific protease. An obvious function of cytoplasmic located FgStefin-1 is, therefore, inhibition of these proteases when leaking from their storage vesicles. The presence of FgStefin-1 in several tissues and its release in the ES product indicates that it has intracellular and extracellular functions. FgStefin-1 stimulates an immune response in *F. gigantica* infected sheep that could be detected 8 weeks postinfection. This finding suggests that FgStefin-1 cannot be applied for early diagnosis of fasciolosis.

Conclusion

We have demonstrated that FgStefin-1 is an abundantly expressed, highly stable protein in juvenile and adult *F. gigantica* that has intracellular and extracellular functions and is able to inhibit parasite and host cysteine proteases. Infected sheep showed an immune response against rFgStefin-1 when the juvenile parasites were still migrating through the liver

parenchyma. In the future we would like to investigate whether FgStefin-1 is active in the modulation of the host immune response.

Acknowledgements

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Original article **P60**

Protective effects of captopril against hydrogen peroxide-induced disruption of tight junctions in ECV304 monolayers

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Abstract

Angiotensin-converting enzyme (ACE) inhibitors demonstrated various beneficial actions on vascular structure and function beyond their blood pressure-lowering effects. In this study, we investigated the protective effect of captopril on hydrogen peroxide (H_2O_2)-induced dysfunction of endothelial barriers, using an *in vitro* model of ECV304 cells. Treatment the ECV304 monolayers with H_2O_2 for 4 hr at the noncytotoxic concentration of 200 micromolar, resulting in the loss of TEER values and tight junction proteins occludin and zonular occluden (ZO)-1. Pretreatment the cells with captopril for 30 min prior to H_2O_2 attenuated the loss of TEER values in concentration dependent manner. The immunofluorescent visualization revealed that captopril prevented the H_2O_2-mediated loss of expression and localization of occludin and ZO-1 at the cell borders. Our results suggested that captopril could protect the barrier function of ECV304 monolayers from oxidative stress through preventing the loss of tight junction proteins. Consequently, the integrity of tight junction structure was preserved during oxidative assault.

Keywords: captopril, tight junction, oxidative stress, ECV304 cells

Introduction

Tight junction is a paracellular structural component to restrict the paracellular flux of ions and solutes through the epithelial and endothelial monolayers. Formation of tight junction structure between adjacent cells is attributed to the protective barrier function of endothelial monolayers against blood-borne pathogens. The tight junction is highly dynamic structure, and the degree of "tightness" sealing depends upon external stimuli, physiological and pathological conditions (1). Disturbance of the tight junction is linked to several pathological conditions including stroke, proteinuria, inflammatory bowel disease (2).

Angiotensin-converting enzyme (ACE) inhibitors exerted their actions through interference with the rennin-angiotensin system. In addition, this drug groups proved their beneficial actions in reduction the risk of stroke, myocardial infarction and cardiovascular death which were beyond their blood pressure-lowering effects (3). Moreover, ACE inhibitors was reported to improve endothelial functions (4). In this study, we investigated the plausible action of ACE inhibitors in preventing the disruption of endothelial barriers from oxidative assaults. We evaluated the capability of captopril, a known ACE inhibitors, to preserve the integrity and function of tight junctions that were damaged by treatment with H_2O_2 in the in vitro model of ECV304 cells.

Materials and Methods

Culture of ECV304 cells

The ECV304 cells were maintained in M199 supplemented with 10% FBS, 1% combined penicillin and streptomycin (100 units/100 µg/ml) at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were counted and seeded onto polycarbonate

membranes with 0.4 µm in pore size, 12 mm in diameter Transwell insert (Costar®, Corning, NY, USA) at a density of $2x10^5$cells/transwell for 12 days before experiments.

Transendothelial electrical resistance (TEER) measurement

ECV304 cells were counted and seeded onto Transwell inserts. TEER was measured using a Millicell®-ERS potentiometer (Millipore, MA, USA) before seeding. After 12 days, cells treated with captopril (0-500 µM) for 30 min, followed by H_2O_2 (200 µM) for another 4 hr. The TEER value was obtained by multiplying the measured electrical resistance (Ω) with the surface area of the monolayer (cm^2).

Immunofluorescent staining measurement

ECV304 cells on Transwell inserts were treated with captopril (100 µM) for 30 min, followed by H_2O_2 (200 µM) for 4 hr. The localization of tight junction proteins were visualized by an immunofluorescent staining method (5) with the use of fluorescence microscopy (BX-FLA, Olympus, Tokyo, Japan) at the absorbance of 490 nm.

Statistical analysis

All values were presented as mean ± standard error (SEM). One-way ANOVA followed by the Tukey's test was performed for statistical comparisons, $p<0.05$ was considered significant.

Results

As shown in Fig 1, H_2O_2 (200 µM) significantly decreased TEER values approximately by 70%, whereas captopril (up to 500 µM in this study) had no effect on the TEER values. Pretreatment the cells with captopril for 30 min prior to H_2O_2 could prevent the TEER reduction. Our results demonstrated that the protective effect of captopril was concentration- dependent, with the maximum effect at the concentration of 100 µM.

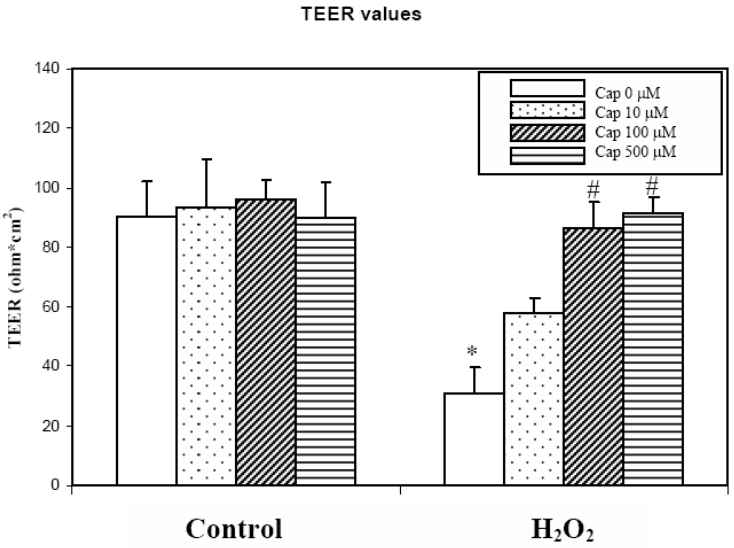

Figure 1 Effects of captopril (cap) on TEER values of ECV304 monolayers treated with H_2O_2 200 µM. Captopril was added at concentrations ranging from 0-500 µM for 30 min prior to addition of H_2O_2 (200 µM) for 4 hr. Each bar represents the mean±SEM with *$p<0.05$ compared with control and #$p<0.05$ compared with H_2O_2 only (n=4).

Next, we investigated the integrity of tight junction structure through immunofluorescent visualization of tight junction proteins occludin and ZO-1. Under the normal condition, ECV304 monolayers expressed the continuous distribution pattern of occludin and ZO-1 proteins at the cell boundaries, as shown in Fig 2A and 2E, respectively. Captopril had no influence on the localization of these proteins (Fig 2D and 2H). In this study, treatment of H_2O_2 (200 µM) for 4 hr apparently caused dislocalization of occludin and ZO-1 at the cell border as seen in Fig 2B and 2F, respectively. Interestingly, our results revealed that pretreatment the ECV304 monolayers with captopril (100 µM) for 30 min could

prevent the H_2O_2-induced disruption of the junctional lining at the cell border, indicating the localization of occludin and ZO-1 remained intact (Fig 2C and 2G).

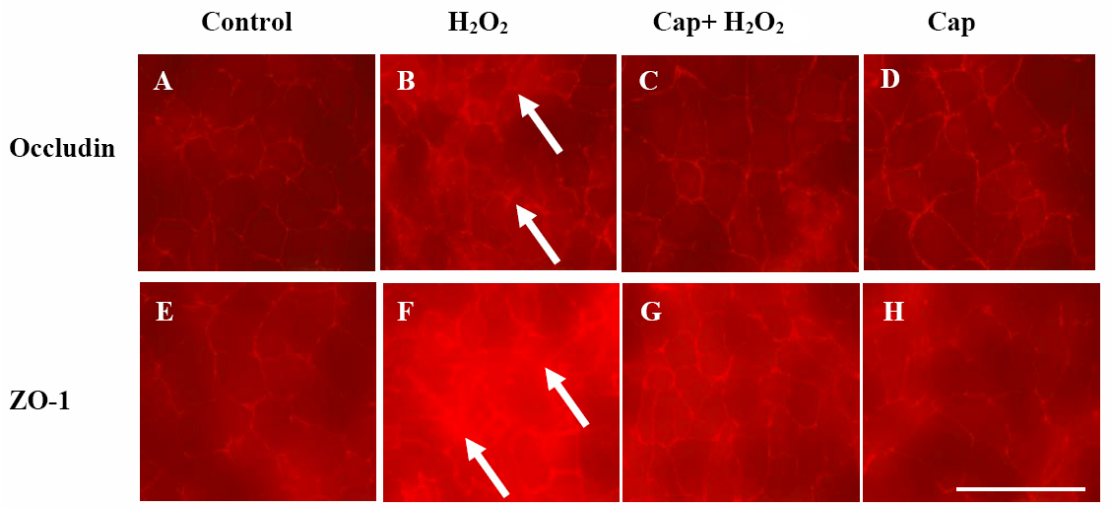

Figure 2 Immunofluorescent staining of ECV304 monolayers showing tight junction proteins occludin and ZO-1 after incubated with captopril in the presence or absence of H_2O_2. Occludin and ZO-1 as control (A, E), H_2O_2 (B, F), captopril plus H_2O_2 (C, G) and captopril only (D, H) are shown. Bar 100 µm.

Discussion & Conclusion

This study was the first to demonstrate the protective effect of captopril against H_2O_2-mediated tight junction disruption. Treatment the ECV304 monolayers with H_2O_2 at 200 µM for 4 hr directly disrupted expression and localization of tight junction proteins occluding and ZO-1 at the cell borders without any influence on cell viability. Consequently, the loss of junctional proteins compromised the integrity and function of tight junction complexes. This model of oxidative stress induced tight junction disruption was in agreement with others reported in the literature (5). As expected, the TEER values decreased in the presence of H_2O_2, indicating the loss of barrier function of the ECV304 monolayers. This dysfunction of ECV304 monolayers as restrictive barrier could be prevented by pretreatment the cells with captopril before H_2O_2 exposure. In this study, we demonstrated that captopril was able to retain the expression and localization of occludin and ZO-1 at the cell borders during the oxidative assault. We anticipated that this protective feature of captopril on tight junction disruption might take part in preventing the barrier leakage of endothelium or epithelium monolayers in various pathological conditions such as stroke. Several substances were shown to protect the tight junction damage from oxidative assaults via various mechanisms including alteration of nitric oxide level (6) and inhibition of MAP kinase signaling pathways (7). In addition to its inhibitory effect on ACE, captopril was reported to inhibit the activity of MAP kinases (8). It was possible that the effects of captopril on tight junction proteins might relate to this action. The molecular protective mechanism of captopril against H_2O_2-mediated tight junction disruption would be in need for further investigation.

Acknowledgements

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Original article **P61**

Characterization of estrogen receptor mediating neutrophil function

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Abstract

Estrogen and its receptors play important roles in the prevention of cardiovascular disease in women. 17β-estradiol (E2) has a direct role in the modulation of the innate immune function. 17β-E2 attenuates the production of pro-inflammatory cytokines including IL-1, IL-6 and TNF-α. IL-8 production is also decreased by 17β-E2 in activated monocytes. Estrogen also has significant effects on neutrophil functions. Estrogen effects are mediated principally by two receptor subtypes, ERα and ERβ; both are expressed in endothelial, vascular smooth muscle cells, and immune cells. Both estrogen receptor subtypes are also expressed on human neutrophils, but their roles in mediating estrogen effects on neutrophil functions are remained to be elucidated. The purpose of this study was, therefore, to characterize the estrogen receptor subtypes that mediate estrogen effects on neutrophil functions. Neutrophils were isolated from post-menopausal women venous blood by Dextran and Percoll gradient centrifugation. All compounds were primarily investigated for their cytotoxic effects on neutrophils using XTT assay. Neutrophils were pre-incubated with 17β-E2, PPT (a selective ERα agonist), and DPN (a selective ERβ agonist), then activated by LPS or fMLP for the study of IL-8 production or adhesion molecule expression, respectively. The IL-8 production of leukocytes was assayed using ELISA. Neutrophil surface adhesion molecule expression was measured using flow cytometry. Neutrophil chemotaxis and superoxide anion generation (SAG) were also investigated. The results showed that 17β-E2 and PPT inhibited fMLP-induced CD62L-selectin shedding, while DPN did not. All drugs did not affect fMLP-induced MAC-1 expression. Both 17β-E2 and PPT attenuated IL-8 production in a dose-dependent manner. These results suggest the role of ERα on L-selectin shedding and IL-8 production in neutrophils. In addition, neutrophil chemotaxis-induced by rh IL-8 was inhibited by 17β-E2 and PPT, whereas SAG was inhibited by 17β-E2, PPT, and DPN. These results suggest that the inhibitory effect of 17β-E2 on neutrophil chemotaxis is mediated via ERα while both ERα and ERβ play roles in SAG.

Introduction

Inflammation plays an important role in the pathogenesis of various cardiovascular diseases. Estrogen plays important roles in the prevention of cardiovascular diseases in women. 17β-E2 has a direct role in the modulation of innate immune function and mediates profound effects on monocyte and macrophage immune function (1). It attenuates the production of pro-inflammatory cytokines including IL-6 and TNFα. Interleukin-8 (IL-8) plays a critical role in the recruitment of leukocytes to areas of vascular injury and its production is also decreased by 17β-E2 in activated monocytes. 17β-E2 also has significant effects on neutrophil functions (2,3). Estrogen effects are mediated principally by two receptor subtypes; the ERα and the ERβ, both are expressed in endothelial cells, vascular smooth muscle cells and immune cells. Human neutrophils are known to express both estrogen receptors subtypes. The role of estrogen receptor subtypes in mediating estrogen effects on neutrophil functions are remained to be elucidated. The purpose of this study was, therefore, to investigate the estrogen receptor subtypes involving estrogen effects on

neutrophil functions including, surface adhesion molecule expression, IL-8 production, rh IL-8 -induced neutrophil chemotaxis, and superoxide anion generation.

Materials and methods

Isolation of human neutrophils

Peripheral venous blood was drawn from healthy postmenopausal woman using heparin as an anticoagulant. Human neutrophils (PMN) were isolated by Percoll density gradient centrifugation. Briefly, venous blood was mixed with an equal volume of Percoll, and the mixture centrifuged at room temperature. After centrifugation, PMN were washed with PBS. Any contaminating red cells were removed by hypotoniclysis. The cells were >99% viable as determined by trypan blue exclusion and were resuspended as required.

Cytotoxic assay

Neutrophils were incubated with 17β-E2 or PPT or DPN in a 96-well plate for 4 h. XTT was added into the plate and then incubated for 3 h. The absorbance was measured spectrophotometrically at 450/650 nm.

In Vitro culture of neutrophils for IL-8 assay

Neutrophils were resuspended in Iscoves's IMDM containing pen/strep. They were incubated with 0.01-10 μM of 17β-E2 or PPT or DPN for 1 h, further activated with LPS 10 ng/ml for 7 h. Supernatant were collected for IL-8 ELISA assay as manufacturer.

Assessment of neutrophils L-selectin shedding and CD 11 b expression (4)

Neutrophils resuspended in PBS were incubated with 0.01-10 μM of 17β-E2 or PPT or DPN for 4 hrs, and then further activated with fMLP 10^{-7} M for 30 min then incubated with human CD26L and CD11b antibodies for 30 min. CD62L and CD11b expression were measured using flow cytometry.

Determination of Neutrophil Chemotaxis (5)

Neutrophil chemotaxis was measured in a 96 well chemotaxis chamber. The bottom wells of the chamber were filled with fMLP. The upper wells with the installed filter were filled with neutrophils which had been treated with the various concentrations of 17β-E2 or PPT or DPN for 10 min. The Chamber was incubated for 45 min at 37°C. The filter was then removed, washed, fixed and stained. Chemotaxis was quantified spectrophotometrically measuring absorbance at 550 nm

Determination of Superoxide Anion Generation (SAG) (6)

Neutrophil SAG was determined by spectrophotometric evaluation of the reduction of ferricytochrome C (Fe^{3+}) to ferrocytochrome C (Fe^{2+}) in the presence of cytochalasin B. Neutrophils were resuspended in PBS containing cytochrome C and cytochalasin B and were preincubated with various concentrations of 17β-E2 or PPT or DPN or PBS, for 10 minutes, then further incubated for 10 minutes with fMLP. After terminating the reaction, aliquots were dispensed into 96-well plate and the absorbance at 550 nm was measured.

Results

1. Cytotoxicity

Incubation of human neutrophils with 17β-E2, PPT, DPN (0.01-10 μM) for 4 h and 8 h caused no cytotoxic effect.

2. IL-8 production of LPS-activated neutrophils from post-menopausal woman

Both 17β-E2 and PPT at the concentrations of 0.01-1μM attenuated LPS-induced IL-8 production by human neutrophils in a dose-dependence manner (Fig.1). Dexamethasone, a

reference compound, exhibited strong inhibition of IL-8 production in LPS-activated neutrophils. However, DPN had no effects on LPS-activated IL-8 production in human neutrophils.

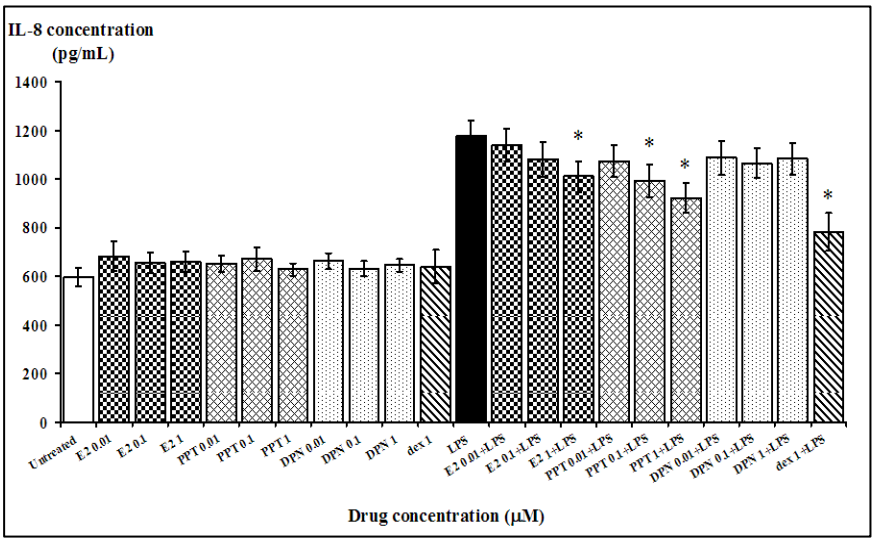


Figure 1. The effects of 17β-E2, PPT, DPN (0.01-1μM) on IL-8 levels in LPS-activated neutrophils from post-menopausal women. The values are expressed as means ± S.E.M. of six different donors. *p<0.05 indicates a significant difference from LPS-treated neutrophils.

3. Neutrophil adhesion molecule expression

17β-E2 and PPT at the concentrations of 0.1-1 μM inhibited fMLP-induced CD62L-selectin shedding in neutrophils, while PPT at the concentration of 10 μM tended to enhanced the shedding of CD62L-selectin. In contrast, DPN did not affect CD62L-selectin shedding in neutrophils (Fig.2A). All drugs did not inhibited fMLP-induced an increase in CD11b expression (Fig.2B).

Fig.2A

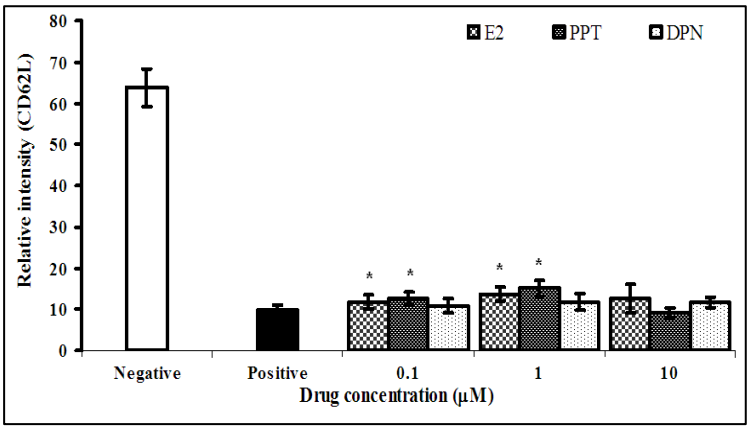
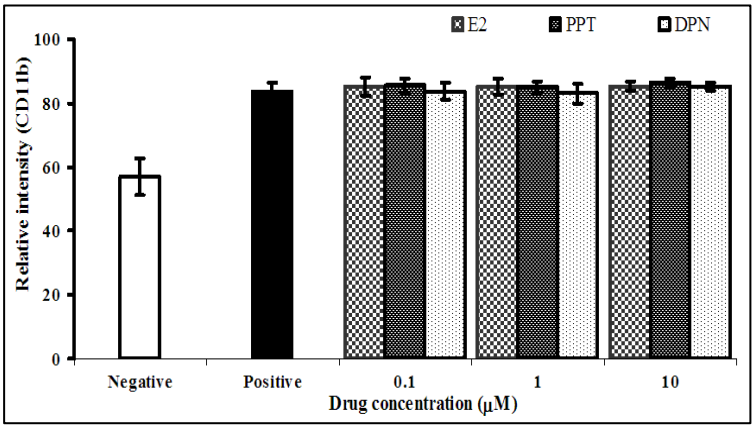


Figure 2. Effects of 17β-E2, PPT, DPN (0.1-10 μM) on fMLP-induced CD62L-selectin shedding of human neutrophils (Fig.2A) and $CD_{11}b$ (MAC-1) expression on surface of human neutrophils (Fig.2B). The values are expressed as means±S.E.M. of five different donors. *p<0.05 indicates a significant difference from positive control.

Fig.2B



4. Neutrophil Chemotaxis

Both 17β-E2 and PPT inhibited rh IL-8-induced neutrophil chemotaxis of which PPT exerted higher potency than 17β-E2, whereas, DPN had a very low potency (Fig 3). Indomethacin exhibited strong inhibition of neutrophil chemotaxis. It appears that the inhibitory effects of 17β-E2 on neutrophil chemotaxis-induced by rh IL-8 is mediated through the estrogen receptor alpha subtype (ERα).

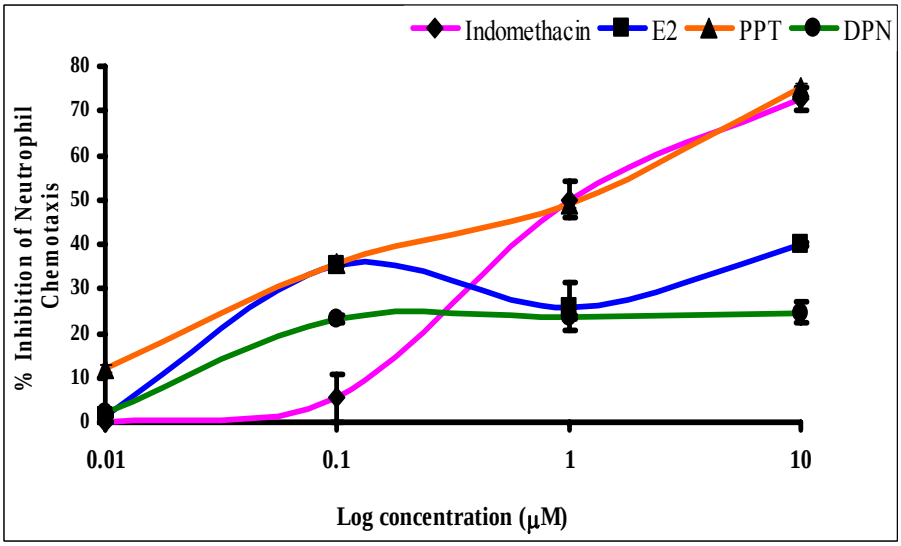


Figure 3. Log concentration-effect curves for inhibition of rh IL-8 (100 nM)-induced human neutrophil chemotaxis of 17β-E2, PPT and DPN. Indomethacin was used as a reference compound. The values are expressed as means±S.E.M. from five independent experiments.

5. Superoxide anion generation (SAG)

All drugs inhibited fMLP-induced human neutrophil SAG of which PPT exerted the greatest inhibitory effect (Fig.4). It demonstrates that the inhibitory effects of 17β-E2 on fMLP-induced human neutrophil SAG are mediated through both estrogen receptor alpha and beta subtypes (ERα and ERβ).

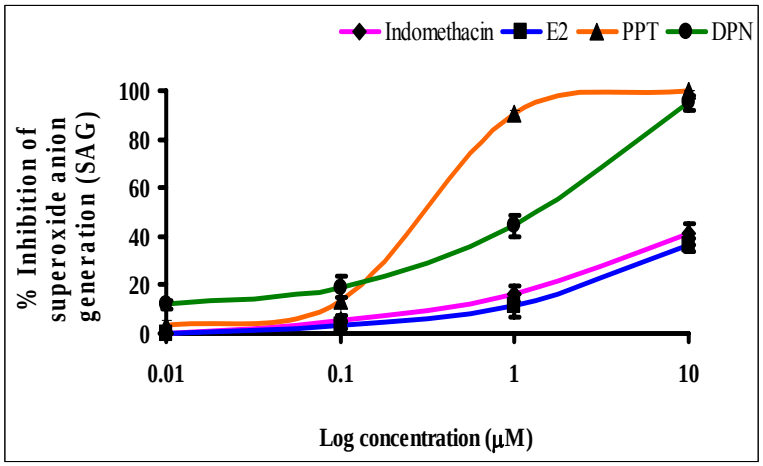


Figure 4. Log concentration-effect curves for inhibition of fMLP (100 nM)-induced human neutrophil SAG of 17β-E2, PPT and DPN. Indomethacin was used as a reference compound. The values are expressed as means±S.E.M. from five independent experiments.

Discussion

IL-8 is a chemotactic for neutrophils to sites of inflammation. It also induces shedding of L-selectin, expression of CD18 on PMNs, up-regulation of LFA-1 and transendothelial migration of neutrophils. The IL-8 production of neutrophils induced by LPS was inhibited in a dose-dependent manner by 17β-E2 and PPT, whereas DPN had no effects. The results reveal that the inhibitory effect of 17β-E2 on LPS-induced IL-8 production by human neutrophils is mediated via ERα. L-selectin plays a crucial role in leukocyte rolling and adhesion on endothelial cell surface. It has been reported that estrogen attenuates recruitment and adhesion of leukocytes to the endothelium offering a possible mechanism by which estrogens exert an anti-inflammatory effect (**7**). The results from this study showed that 17β-E2 and PPT (0.1-1μM) inhibited fMLP-induced CD62 L-selectin shedding in neutrophils, while DPN did not, suggesting that 17β-E2 and PPT mediated their inhibitory effects on CD62L-selectin shedding via ERα. While both 17β-E2 and PPT strongly inhibited rh IL-8-induced neutrophil chemotaxis, DPN possessed weak inhibitory effect. It seems that the inhibitory effects of 17β-E2 on neutrophil chemotaxis-induced by rh IL-8 is mediated via ERα. Both PPT and DPN inhibited fMLP-induced human neutrophil SAG stronger than 17β-E2 and indomethacin. The results showed that both ERα and ERβ involved in the inhibitory effects of 17β-E2 on fMLP-induced human neutrophil SAG. Taken together, these findings demonstrate that 17β-E2 exerts its inhibitory effects on LPS-induced IL-8 production by human neutrophils, CD62 L -selectin shedding, and rh IL-8-induced neutrophil chemotaxis via ERα, whereas its inhibitory effects on fMLP-induced neutrophil SAG is mediated via both estrogen receptor subtypes (ERα and ERβ).

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Original article **P62**

Identification and characterization of novel antigens from *Opisthorchis viverrini*

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Abstract

Opisthorchis viverrini is the causative agent of human opisthorchiasis in Thailand. Long lasting infection with the parasite has been correlated to the development of cholangiocarcinoma. This kind of cancer is a major public health problem in North- and Northeast-Thailand. At present, the knowledge which parasite antigens are involved in the establishment of infection and tumorigenesis is quite limited. We have, therefore, screened the published parasite transcriptome sequences to identify new antigens which might be involved in the host/parasite interaction. Two related transcripts were selected due to a suspected function of the encoded proteins (OvDM9A, OvDM9B) in the transport of hydrophobic molecules. The corresponding cDNAs were isolated from an adult stage *O. viverrini* cDNA library and subcloned into bacterial expression vectors. Recombinant proteins were produced in *E. coli* but were insoluble. Only OvDM9A was selected for further analyses and the purified recombinant protein has been used for production of polyclonal antisera in mice. The antisera will be used to localize native OvDM9A in parasite antigen extracts and tissues. To study its biochemical function OvDM9A will be expressed in yeast to obtain soluble protein which will be tested in binding assays with hydrophobic molecules. The biological function will be studied in vivo using RNAi to analyze if the protein is essential for parasite survival in the host. If a role in binding and transport of hydrophobic molecules can be established these novel proteins could be useful for drug targeting.

Keywords**:** *Opisthorchis viverrini*, cholangiocarcinoma, transcriptome, transport, drug targeting

Introduction

Opisthorchiasis caused by *Opisthorchis viverrini* is mainly prevalent in Thailand, Lao People's Democratic Republic, and Cambodia. It is highly endemic in Northeast Thailand, where the occurrence of cholangiocarcinoma (CCA) is the highest in the world (IARC 1994, 1997). Infection occurs when raw or inadequately cooked infected freshwater fish are ingested. The fluke is residing in the bile ducts and gall bladder. Infection with many parasites can produce morbidity including abdominal pain, dyspepsia, and fatigue and in very heavily infected cases, pyogenic cholangitis, biliary calculi, obstructive jaundice, and even cholangiocarcinoma in long lasting infections (Harinasuta et al., 1984; Pungpak et al., 1994; Schwartz, 1980). Since the early 1980s, when the good safety and therapeutic profile of praziquantel against opisthorchiasis had been established, treatment and control of opisthorchiasis relies on this drug (Keiser and Utzinger, 2007). Although treatment failures have not yet been reported for praziquantel in *O. viverrini* infected patients, the search for alternative trematocidal drugs is warranted. The aim of this study is discover novel antigens which are involved in host/parasite interaction and could be applied for drug targeting.

Methods

Database analysis

The published parasite transcriptome sequences were searched to identify novel proteins and to design primer pairs for cloning of their cDNAs

Cloning and sequence analysis of OvDM9 genes

We isolated cDNAs for OvDM9A, OvDM9B from an adult stage *O. viverrini* cDNA library and subcloned them into bacterial expression vectors

Expression and purification of recombinant OvDM9

Recombinant proteins were produced in *E. coli* and purified by Ni-NTA affinity chromatography

Results

Database analysis

Two sequence related transcripts were selected due to a suspected function of the encoded proteins (OvDM9A, OvDM9B) in the transport of hydrophobic molecules.

Cloning and sequence analysis of OvDM9 genes

The size of both OvDM9 cDNAs is 470 bp.

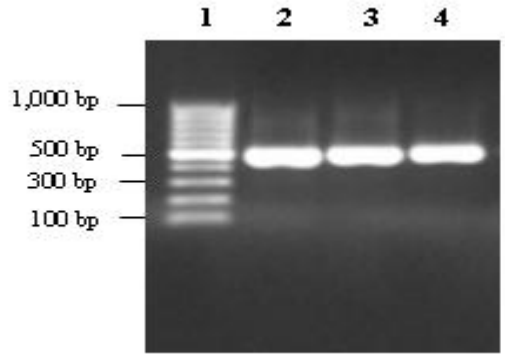

Figure 1. PCR products of the OvDM9 cDNAs subcloned into the expression vector, lane 1: 100 bp DNA ladder, lane 2-3: OvDM9B cDNA, lane 4: OvDM9A cDNA.

Expression and purification of recombinant OvDM9A protein

The molecular mass of rOvDM9 is approximately 17 kDa

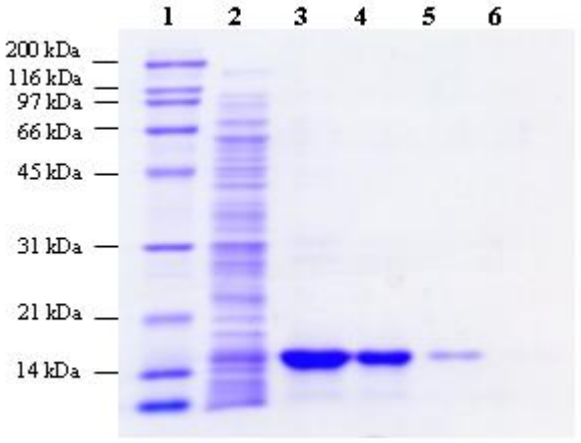

Figure 2. Purification of rOvDM9A under denaturing conditions, lane1: broad range marker, lane 2: flow through, lanes 3-6: elution fractions.

Discussion and conclusion

The cDNAs of two related genes from *O. viverrini* were cloned. They encode proteins which may act as transporters of hydrophobic molecules analogous to cytosolic fatty acid binding proteins. The recombinant OvDM9 proteins were not correctly folded in *E. coli* and insoluble. Only rOvDM9A was purified and used for production of polyclonal antisera in mice. These sera will be used for immunohistochemical analyses and in immunoblots. To analyze the biochemical function of the protein it will be expressed in yeast to obtain it in soluble form which can be used in binding assays with hydrophobic molecules. The biological function will be studied in cultured parasites using RNAi to analyze if the protein is essential for parasite survival. If a role in binding and transport of hydrophobic molecules can be established these novel proteins could be useful for drug targeting.

Acknowledgement

This research was supported by a research grant from Thammasat University and the Commission on Higher Education, Ministry of Education, Thailand.

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Original article **P63**

Pfatp6 polymorphisms of *Plasmodium falciparum* isolates in Thailand and association with *in vitro* sensitivity to artesunate and mefloquine

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Abstract

The sarco/endoplasmic reticulum Ca^{2+}- ATPase of *Plasmodium falciparum* or *pfatp6* has been proposed to be a target for artemisinin and derivatives which are currently used worldwide to combat the emergence of multi-drug resistance *P. falciparum*. Nevertheless, reports of clinical treatment failure with supplemented data on single-nucleotide polymorphisms (SNPs) of *P.falciparum* genes associated with resistance have been increasing in malaria endemic areas including Thailand. In this study, we investigated the association between *Pfatp6* polymorphisms, and *in vitro* sensitivity in a total of 63 *P. falciparum* isolates collected from the Thai-Myanmar border, to artesunate and mefloquine. All isolates were adapted to continuous culture *in vitro* and assessed for their susceptibility to artesunate and mefloquine. Malarial parasite DNA was extracted from blood samples using Chelex-100 assay. Polymorphism of *pfatp6* at codons R37K, G639D, S769N and I898I were analyzed based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Gene copy number of all isolates was analyzed by quantitative real time-polymerase chain reaction (qRT-PCR). Results showed no mutation of *pf*ATP6 gene at any codon investigated. In this limited number of isolates under investigation, no association between SNP and amplification of *pfatp6* gene and *in vitro* sensitivity of *P.falciparum* isolates to artesunate and mefloquine.

Keywords**:** *Plasmodium falciparum*, *pfATPase6*, single-nucleotide polymorphism, gene amplification, artemisinin, drug resistance, PCR-RFLP

Introduction

Multidrug resistance *Plasmodium falciparum* including resistance to structurally related antimalarials such as chloroquine, quinine and mefloquine, is still problematic along the border areas of Thailand, especially Thai-Myanmar border (1). A 3-day artesunate (ARS)-mefloquine (MQ) combination is currently being used as the first-line treatment of uncomplicated falciparum malaria to cope with the situation of multi-drug resistance (1). Recently however, there has been a report of modest increase in resistance of this combination in areas along the Thai-Cambodian and Thai-Myanmar borders (2-5). Apart from assessment of clinical response, *in vitro* sensitivity test together with molecular surveillance system can help target *in vivo* studies to where they are needed the most. Polymorphisms of genes involved in vial process of malaria parasite are suggested as the key factor contributing to drug resistance. Among these, the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) PfATP6 is proposed to be an important target for artemisinin and derivatives. Nevertheless, recent observations revealed that polymorphisms (single nucleotide polymorphism: SNP and gene amplification) in this gene were associated with *in vitro* resistance of *P. falciparum* to artemisinin derivatives (6). In addition, amplification of this gene has also been linked with both artesunate and mefloquine resistance (6). In the present study, we investigated the association between *in vitro* sensitivity to artesunate and mefloquine, and the polymorphisms of *pfatp6* gene in in a total of 63 *P. falciparum* isolates collected from an area along the Thai-Myanmar border during 2007-2009.

Methods

Venous blood samples (3 ml) were collected from patients attending malaria clinics in areas along the Thai-Myanmar border after microscopically confirmation of *P.falciparum* mono-infection. Approval of the study protocol was obtained from the Ethics Committee of Ministry of Public Health of Thailand. Written informed consents were obtained from all patients prior to blood collection. *P.falciparum* isolates were adapted to culture *in vitro* (7) for sensitivity testing to artesunate and mefloquine, as well as for investigation of single nucleotide polymorphism (SNP) and amplification of the *pfatp6* gene. *In vitro* sensitivity testing (3 independent experiments, triplicate each) of all isolates to artesunate (concentration range 0.39-50 nM), and mefloquine (concentration range 1.56-200 nM) was performed in a 96-well microtiter pate based on SYBR green I-based Assay (8), in order to obtain the IC_{50} values (concentrations that produce 50% inhibition of parasite growth). SNPs of *pfatp6* gene at codons S769N, R37K, G639D and I898I were examined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (9). DNA sequencing of *pf*ATP6 and quantitative real time- PCR (10) were performed in 5 isolates with different degree of *in vitro* sensitivity to artesunate.

Results

***In vitro* Sensitivity Testing:** *In vitro* sensitivity test was successful in a total of 50 out of 63 isolates collected. Mean ± SD IC_{50} values for artesunate and mefloquine were 2.21±0.99 and 43.12±26.12 nM, respectively. All were classified as sensitive to artesunate and 68% (34/50) were classified as resistance to mefloquine (IC_{50} >24 nM) (11). There were 9 isolates with marked decline in sensitivity to artesunate (mean $\pm$ SD IC_{50} =3.66$\pm$0.46 nM); mean$\pm$SD IC_{50} of 26 and 15 isolates were between 1.42$\pm$0.27, and 2.50$\pm$0.28 nM, respectively.

SNPs and Amplification of pfatp6: All isolates carried wild-type allele *pfatp6* (Table 1). All of the five isolates selected based on *in vitro* sensitivity to artesunate (IC_{50} =4.41, 3.10, 2.62, 2.46 and 1.22 nM) carried only a single copy of the *pfatp6* gene.

Table 1 Prevalence of SNP in *pfatp6* gene

Gene	***pfatp6* Codon**							
Target allele	**37R**	**37K**	**693G**	**693D**	**769S**	**769N**	**898I**	**898I**
Target SNP	110G	110A	1916G	1916A	2306G	2306A	2694A	2694T
%Prevalence (*N*)	100 (22)	0	100 (63)	0	100 (63)	0	100 (63)	0

Discussion

Our results show the increase in prevalence of mefloquine resistant *P.falciparum* isolates (68%) collected from the multi-drug resistance area of Thailand compared with the isolates collected during 1998-2005 (32%) (10) and 2007 (46%) (11). Sensitivity to artesunate is also gradually declining with about 14.3% (9 isolates) exhibiting IC_{50} of greater than 3 nM. The IC_{50} range observed for artesunate in this study is similar to that reported in Africa (12). Nevertheless, at the molecular level, no genetic changes (SNP and amplification) in the *pfatp6* gene which is proposed to be linked with resistance of artemisinins or mefloquine were found. Continuous monitoring of these genetic changes in parallel with *in vitro* and *in vivo* sensitivity of *P.falciparum* isolates collected from various endemic areas are required to definitely conclude on the involvement of this gene in conferring resistance of the parasite to artemisinins. This would assist in policy making in the malaria control program of the country.

Conclusion

With limited number of isolates (63) under investigation, lack of association between *in vitro* sensitivity of *P.falciparum* isolates to artesunate and mefloquine, and genetic polymorphism (SNP and gene amplification) were observed.

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Original article **P64**

Host-parasite interactions at the hormonal level in Fasciolosis

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Abstract

Fasciolosis is an important disease in ruminants caused by liver flukes of the genus *Fasciola*. For the development of new drugs and vaccines it is important to understand the host/parasite interactions at the molecular level. Vertebrates release a variety of hormones by which they trigger responses in distant tissues. We investigated whether *Fasciola* is sensitive to host hormones and shows specific responses. More specifically, we were interested in peptide hormones and their receptors which belong to the type II G-protein coupled receptor family. Structure, function, and pathology of this receptor family have been extensively studied in vertebrates, especially human and porcine. In contrast, data for host/trematode interactions at the hormone level are very limited. The latest study dated back 20 years and focused on changes in muscle contractions of the parasite. Proteomic and nucleic acid analysis techniques developed in the last decades will allow us to study the effects of hormones at the molecular level and to identify putative parasite hormone receptors. In the present study a cDNA encoding a putative type II G-protein coupled receptor of *Fasciola* was isolated as a partial product by cDNA library screening and completed by a rapid amplification of cDNA 5'-end (5'-RACE) method. The deduced amino acid sequence was compared to the sequences of other members in the type II G-protein couple receptor family and showed conserved key features of this family. In further studies, the protein will be localized in the parasite tissue with specific polyclonal antibodies and we would like to determine which ligand will bind to it and what its biological role is.

Keywords: *Fasciola gigantica*, type II G-protein coupled receptor, peptide hormones, and rapid amplification of cDNA ends (RACE)

Introduction

Fasciolosis, caused by the trematode species *Fasciola gigantica and F. hepatica*, is an important, worldwide disease in ruminants and leads to severe pathology in the infected animals. For the development of new drugs and vaccines it is essential to understand the host/parasite interaction at the molecular level. Few attempts were made to analyze to which extent host hormones influence the behavior of *Fasciola* in the mammalian host. The latest of these studies dates back 20 years and focused on changes in the muscle contractions of the parasite. Modern proteomic and nucleic acid analysis techniques will allow us to study the effects of hormones at the molecular level and to identify putative parasite hormone receptors.

The aim of this study is to identify a parasite hormone receptor, to determine its ligand, and to establish its biological role. The obtained primary data should be of use for further investigation of the receptor as a drug target.

Methods

In the already performed experiments, a hormone receptor encoding cDNA was isolated using cDNA library screening and rapid amplification of cDNA ends (RACE) methods. DNA sequencing and analysis were used to confirm that a sought cDNA had been isolated. In ongoing experiments, the temporal and spatial expression patterns of the receptor

will be analyzed using polyclonal antiserum raised against a N-terminal peptide of the receptor. Mammalian peptide hormones will be studied for their interaction with the receptor and knock-out experiments will be performed to identify its biological function. Depending on promising results the use of the receptor as a drug target will be studied by structure modeling to identify putative disruptive ligands.

Results

A full-length cDNA encoding a type II G-protein coupled receptor of *Fasciola gigantica* was molecular cloned by cDNA library screening using a DIG-labeled specific DNA probe and the rapid amplification of cDNA ends (RACE) method. The deduced amino acid sequence was compared with members of the human type II G-protein coupled receptor family and clearly shows conserved features of this family. For the present, the protein has been named FgSCTR because of a somewhat higher similarity to the secretin receptor, this is preliminary and may change depending on its true ligand.

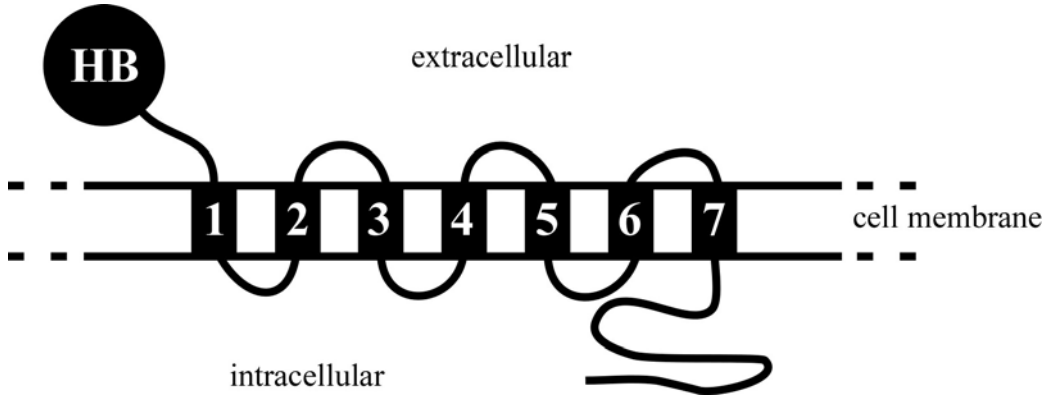

Figure 1: Schematic drawing of FgSCTR with the putative extracellular hormone binding (HB) domain at the N-terminus, seven transmembrane regions and a C-terminal end which might interact with an unidentified G-protein.

Discussion

For many years already, the knowledge about host/parasite interaction in *Fasciola* has been limited with a focus on only a small number of identified antigens. We considered the even smaller number of studies concerning host hormone/parasite interaction as insufficient and began to investigate the possibility of a molecular analysis. Starting point was a partial cDNA (EST) spanning two transmembrane regions of a predicted type II G-protein coupled receptor in *Fasciola*. So far, type II G-protein coupled receptors have not been described from trematodes. They are of interest as several of their known ligands are gastrointestinal hormones in mammals and potentially affect the parasite. Using the partial EST as a probe we isolated a full length cDNA by the described methods. As the alignment demonstrates the deduced amino acid sequence has all characteristics of a II G-protein coupled receptor and it will be important to identify first, its distribution in the parasite tissue and, secondly, the ligand in the next analyses.

Conclusion

We have molecular cloned the first full length cDNA of a type II G-protein coupled receptor in trematodes. In the following studies, we would like to determine its ligand and its biological role which might be important for its use as a drug target.

Acknowledgements

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Original article **P65**

Quantitative real-time PCR with *TaqMan* and SYBR green for estimation of *Pfmdr1* copy number and application for monitoring of antimalarial drug resistance

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Abstract

Amplification of *Plasmodium falciparum* multi-drug resistant gene 1 (*Pfmdr-1*) has been linked with resistance of antimalarial drugs, particularly mefloquine and artemisinin derivatives. This could be used as a molecular marker for monitoring and surveillance of antimalarial drug resistance. The objective of the study was to compare the correlation between copy number of *Pfmdr-1* gene analyzed by two different techniques, *i.e.*, real-time polymerase chain reaction (PCR) with Taqman and and real-time PCR with SYBR green assay. Gene copy number was determined in of a total of 18 dried blood spot samples collected onto filter paper from patients infected with *P.falciparum* malaria. DNA extraction Kit was used to extract genomic *P.falciparum* DNA. The results showed good correlation of the copy number analyzed by the two methods (Spearman correlation test, $r = 0.852$). Further study should be performed with more number of *P.falciparum* isolates, together with clinical (*in vivo*) response, to confirm the association between *Pfmdr-1* amplification and resistance to mefloquine-artemisinin combination.

Keywords: *Pfmdr-1*copy number, SYBR green1, *Taqman*, drug resistance

Introduction

Multidrug resistance *Plasmodium falciparum* is a serious problem throughout the world particularly in Southeast Asia where strains are mostly resistant to chloroquine, antifolates, quinine, and mefloquine . Surveillance for drug-resistant malaria is based on *in vivo* criteria for treatment failure, measurement of the activities of antimalarial drugs against cultured parasites *in vitro* and more effectively by using molecular markers (1). Recently, *Pfmdr-1* (*P. falciparum* multidrug resistance gene-1) copy number, has proved as useful molecular marker in assessing resistance to mefloquine and artemisi1nin derivertives (2). The *Pfmdr-1*, a member of the ATP-binding cassette(ABC) superfamily, has been proposed to function as drug transporter, using the energy from ATP hydrolysis to catalyze drug efflux from a cell or cellular compartment containing the relevant drug target, thereby promoting drug resistance (3). Two techniques are being used for DNA quantification, real-time PCR has been developed using either TaqMan probe with reporter and quencher dye, or SYBR Green dye were used for sources of the fluorescence (4). The first method has been more widely used as a standard method due to its high sensitivity. However, the technique is relatively expensive and requires PCR machine with filter with specific wavelength to detect fluorescence signal. In addition, it also requires specific probe in PCR reaction. The objective of this study was to preliminarily investigate the correlation between *Pfmdr-1* gene copy number analyzed by these two methods in a total of 18 *P.falciparum* isolates.

Methods

DNA extraction from dried blood spot on filter paper

A total of 18 of dried blood spot samples collected from patients infected with *P. falciparum* from different malaria endemic areas of Thailand along the Thai-Mynamar border (14 from Kanchanaburi, 2 from Mae Hongson and 2 from Ranong) during 2009, were included in the study. Parasite genomic DNA was extracted from all samples by QIAmp® DNA extraction kit (Qiagen, Crawley, UK).

Quantification of *Pfmdr-1* by real-time PCR

The amplification of *Pf mdr-1* was determined by Taqman and SYBR green real-time PCR as follows:

Taqman: Amplification reactions were done in 25 µL, containing *TaqMan* buffer (8% glycerol, 0.625 U DNA polymerase, 5.5 mM $MgCl_2$, 300 µM dNTP, 600 nM passive reference dye ROX(5-carboxy-X-rhodamine), pH 8.3), 300 nM of each forward and reverse primer, 100 nM of each probe and 5 µL of template DNA (5).

SYBR green: Individual PCR reaction was prepared in a total volume of 25 µL containing 0.5x of Platinum®SYBR® Green qPCR Supermix-UDG (Invitrogen®), 10µM of each forward and reverse primer, and 2 µl of DNA template.

For each experiment, *Taqman* and SYBR green real-time PCR , *Pfmdr-1* copy numbers of DNA of the *P. falciparum* clone Dd2 and 3D7 were determined in parallel as control strains. Cycle threshold (Ct) and melting curve were generated at the end of each reaction for further data analysis. The results were analyzed by a comparative C_t method (5), based on the tested assumption that the target (*pfmdr1*) and reference gene were amplified with the same efficiency within an appropriate range of DNA concentrations.

Statistical an\alysis

Correlation of *Pfmdr-1* copy number analyzed by the two methods were determined using Spearman Correlation Test at a statistical significance level of p =0.05.

Results

Total of 18 DNA samples of *P. falciparum* isolates were successfully analyzed for *Pfmdr-1* copy number by Taqman and SYBR green assay and results are shown in Table 1.

Table 1 Analysis of *Pfmdr-1* copy number by Taqman and SYBR green assay. Data are presented as number of *P.falciparum* isolates.

***Pfmdr-1* copy number**		**Taqman**			**Total**
		1.00	**2.00**	**3.00**	
SYBR green	**1.00**	14	1	0	15
	2.00	0	2	1	3
Total		14	3	1	**18**

Statistical analysis of *Pfmdr-1* copy number by Spearman Correlation Test showed good correlation with r = 0.852.

Discussion and Conclusion

The preliminary results from this study, based a limited number of *P.falciparum* isolates included, showed a good and significant correlation between the two techniques currently being used for estimation of *Pfmdr-1* copy number (quantitative real-time PCR with *Tagman* and SYBR green). Inconsistent results were found only in 2 samples where higher number of gene copy number was obtained with the Taqman method (3 *vs* 2 and 2 *vs* 1 copies for *Taqman* vs SYBR green method). It was noted however that the sampling procedure (4) including sample collection and DNA concentration in each reaction (6) could greatly affect to the results. In one report, Taqman assay was found to be more specific than SYBR green in general since it needs hybridization with fluorescence probe (4). Further study should be performed with larger number of *P.falciparum* isolates to confirm this finding. Real-time PCR with SYBR green would thus be used to replace Taqman to investigate the association between *Pfmdr-1* amplification and resistance to mefloquine-artemisinin combination. In this preliminary study, two isolates collected from Kanchanaburi and Ranong Provinces were observed to carry *Pfmdr-1* copy number greater than 1 (2 and 3 copies). It is interesting to investigate the relationship between clinical response and amplification of this gene in large number of patients throughout the endemic areas of Thailand where mefloquine –artesunate combination therapy has been used as first-line treatment.

Acknowledgement

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Original article **P66**

Identification of protein targets for development of diagnostics and chemotherapeutics for cholanigiocarcinoma

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Abstract

Cholangiocarcinoma (CA) is a rare but devastating neoplasm that accounts for about 3% of all gastrointestinal cancers and 15% of all primary liver cancers worldwide. The lack of early detection and limited therapeutic options are major problems in controlling CA.
This study was aimed to identify differentially expressed proteins in plasma obtained from CA patients (n=30) and healthy subjects (n=30). Protein patterns and spots were identified in pooled and each individual sample from each group using two-dimensional gel electrophoresis (2-DE) and mass spectrometry. There were a number of protein spots which were either up- or down-regulated in pooled and individual plasma samples from CA patients. Five spots with up-regulation of proteins were focused on for further analysis, one of which was identified as α_1-antitrypsin. The up-regulation of α_1-antitrypsin was observed in all CA patients (10/10), and the spot density was significantly higher in these patients than the control group (75.49 *vs* 19.98). Apart from α_1-antitrypsin, it is noteworthy that among the down-regulated protein spots, one spot identified as transferrin, had markedly low expression in CA patients. Study to investigate the association between the up- and down-regulation of α_1-antitrypsin and transferrin, and the levels of both proteins in plasma/serum of patients is underway.

Keywords: Cholangiocarcinoma, proteomics, two-dimensional gel electrophoresis

Introduction

Cholangiocarcinoma (CA) is an adenocarcinoma arising from cholangiocyte, the epithelial cell lining the bile duct apparatus. Several epidemiologic studies have demonstrated an increase in the incidence of CA in Southeast Asia (1). Infection with liver fluke *Opisthorchis viverini* (OV) has been identified as one significant factor associated with the risk of development of CA (2). OV is endemic in Southeast Asia, particularly in the northeastern part of Thailand, where the daily habit of eating raw and salt-fermented freshwater fish repeatedly exposed this local population to both OV and nitrosamine-contaminated food. The prevalence of OV infection is up to 70.8%, and the incidence of CA in this region is up to 317.6 *per* 100,000 person-years (3). The lack of early detection and limited therapeutic options are major problems ib controlling the disease. Even those with operable tumor, the recurrence rate was extremely high, with a 5-year survival rate of less than 40% (4). At present, there is no effective tool or specific biomarkers that can predict the early stage and status of CA. Established serum tumor markers include carbohydrate antigen 19.9 (CA19.9) and carcinoembryonic antigen (CEA); however, these markers are not always helpful, with sensitivities of approximately 70% and 50%, respectively. A specific protein marker for either early detection or monitoring of the tumor would significantly improve the prognosis and therapeutic management of such patients. In addition, information of the pattern of proteins that were up- or down-regulated in CA patients would be exploited for

development of chemotherapeutics that act to inhibit the growth of this cancer. The objective of this study was to identify differentially expressed proteins in plasma obtained from CA patients and healthy subjects by using two-dimensional gel electrophoresis (2-DE) and mass spectrometry.

Methods

Patient Samples: A total of 60 plasma samples collected from patients with CA (n=30) and healthy subjects (n=30) were included in the analysis. Sample collection was performed at The National Cancer Institute of Thailand. The study protocol was approved by the ethics committee of the ministry of public health of Thailand. Written informed consents were obtained from all subjects before sample collection. The diagnosis of CA was based on abdominal ultrasound and serological. We firstly investigated protein patterns from the pooled plasma samples to provide an overall picture of the protein profiles of samples from both groups. Equal volume of plasma from each subject was pooled for each group (case and control). The pooled plasma samples were depleted for removal of high abundant protein by using a Proteome Lab IgY-12 Spin Column from Beckman Coulter. The depleted pooled plasma sample was then applied to a Microcon YM-3 (YM-3, MWCO 3 KD, Millipore MA) and centrifuged at 5,000 rpm ($4^{O}C$). The supernatant was then filtered by using 2-D Clean-Up**.** Total protein content of depleted plasma samples was determined using the DC Protein assay (Bio-Rad) with bovine serum albumin (BSA) as a standard protein. In the second step, proteomic profile of each individual plasma sample in both groups (10 each) was examined. The procedures followed that described above for the pooled plasma samples.

Two-Dimensional Gel Electrophoresis (2-DE): For 2-DE, each 100 µg of depleted plasma was exchanged with sample buffer (8 M urea, 2 M thiourea, 2%CHAPS, 65 mM DTT, 0.5% ampholyte). After dilution to 125 µl with rehydration buffer (8 M urea, 4% CHAPS, 65 mM DTT, 0.001% bromphenol blue, 1% Bio-Lyte ampholytes), the samples were loaded onto IPG strips (7 cm, covering the pH ranges 3-10 NL; Bio-Rad) for isoelectric focusing using the PROTEAN IEF cell. After rehydration overnight at room temperature, the voltage/time profile applied as as follows: 250 V for 15 min, 4,000 V for 1 hour, and then 4,000 V to total of 15,000 V-hr. After equilibration with a solution containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2%(w/v) DTT, IPG strips were equilibrated again with the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. IPG strips were placed on top of 12% SDS- PAGE for second dimension separation will follow by 200 V for 45 min with Mini-PROTEAN 3 Electrophoresis.

Protein identification by MALDI-TOF-MS: The protein spots from either the pooled plasma or individual plasma sample from both groups were used for identification of the proteins by peptide mass fingerprinting by MALDI-TOF-MS. Analysis of selected protein spots was performed using PDQuest^{TM} software version 7 (BioRad, USA). Peptides identified was investigated with the MASCOT search engines (http://www. matrixscience.com).

Results

Figure 1 shows protein patterns separated by 2-DE, of pooled plasma samples of healthy subjects (control group) and CA patients. There were a number of protein spots which were either up- or down-regulated in pooled plasma samples from CA patients. Table 1 shows examples of six protein spots which were identified by MALDI-TOF. Figure 2 presents the analysis of separated spots from pooled plasma samples of CA patients using PDQuest^{TM} software. Among these, five spots (ID 1375, 1427, 1512, 1324, 1368) with up-regulation of proteins were focused on for further analysis (Table 2). The first spot was identified as α_1-antitrypsin, whereas for the other four spots, identification was not possible

due to poor resolution from the nearby spots. The ratio of protein density in pooled plasma collected from patients to that from healthy subjects were 4.39, 2.51, 2.11, 4.18, and 6.09, respectively. Table 2 shows the mean density of protein spots in each individual plasma samples of both groups. The number in parenthesis indicate the proportion of spots with up-regulation in each group. The up-regulation of the protein spot number 1375 was observed in all samples obtained from patients with CA, and in addition, the spot density was significantly higher ($p < 0.00001$; Mann-Whitney U test) in the patient group than the control group (75.49 *vs* 19.98).

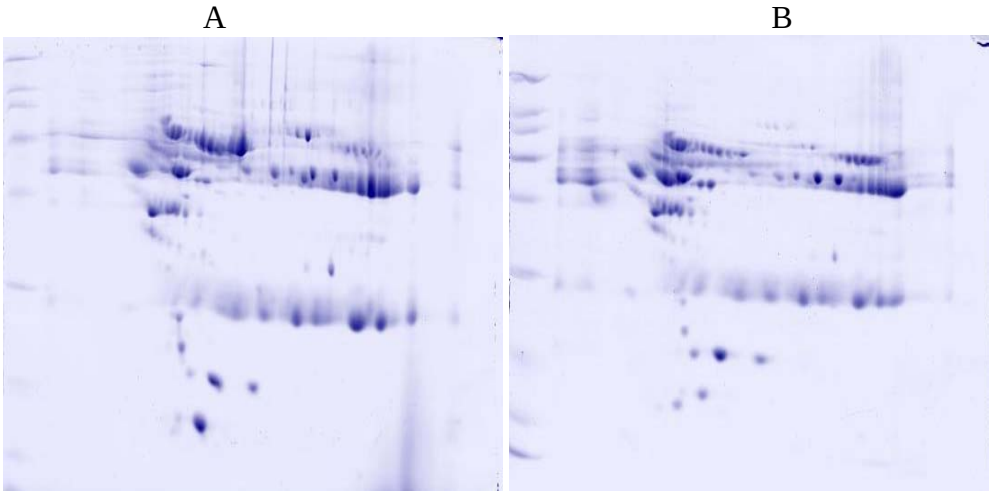

Figure 1 Protein patterns separated from pooled plasma samples of (A) healthy subjects (control group) and (B) CA patients by 2DE

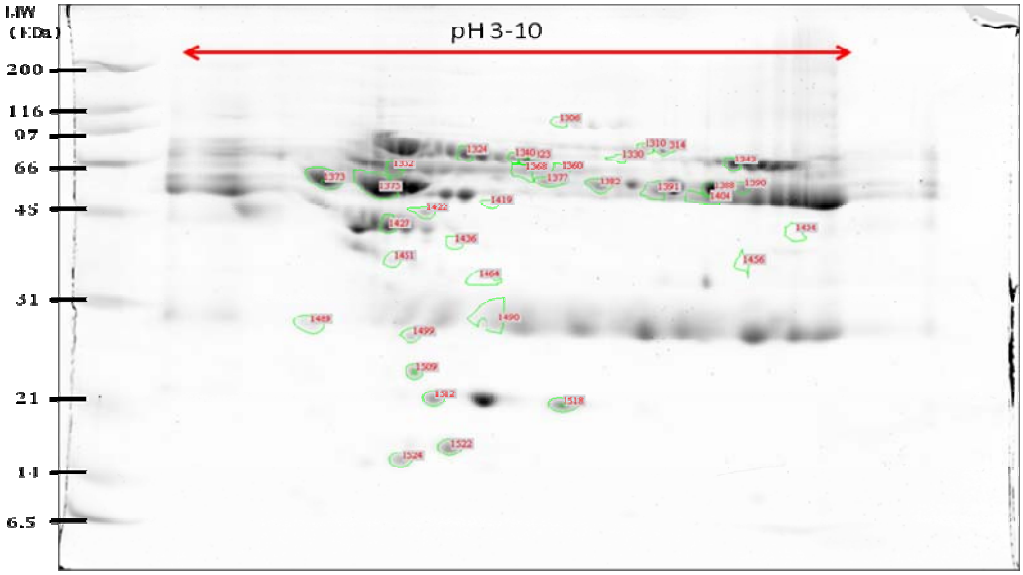

Figure 2 Analysis of separated spots by PDQuest^{TM} software.

Discussion

Our preliminary results demonstrate significantly different protein patterns of plasma from CA patients with a number of protein spots which were either up- or down-regulated. Spot ID 1375 seems prominent in its up-regulation in all plasma samples (10/10), with significantly higher protein density (75.49 *vs* 19.98). This spot was identified by MALDI-TOF as α_1-antitrypsin (A1AT). α_1-antitrypsin is a glycoprotein and generally known as

serum trypsin inhibitor or α_1-proteinase inhibitor (A1PI). It is a serine protease inhibitor (serpin) which inhibits a wide variety of proteases (5). α_1-antitrypsin protects tissues from enzymes of inflammatory cells, especially elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter (in US the reference range is generally expressed as mg/dL or micromoles), but the concentration can rise many folds upon acute inflammation (6). Marked increase in α_1-antitrypsin was also previously reported in CA (7).

Table 1 Examples of spots identified by MALDI-TOF

Spot ID	Protein	Accession No NCBI	Number of matched peptides	MALDITOF-MS sequence coverage (%)	Protein score	MW (kDa)	Pi	Expression
1375	α_1-antitrypsin	151302818	13/27	34	82	46978	53.5	+4.4
1314	Transferrin	553788	12/39	33	90	55207	6.0	-3.2
1321	Hemopexin	11321561	11/41	29	79	52385	6.55	-3.3
1340	Chain A, crystal structure of human serum albumin	3212456	18/53	31	117	68425	5.67	-4.5
1330	RAB39 member RAS oncogene family	39930371	8/40	52	68	25390	7.57	-3.4
1352	Kimimogen 1 isoform 2	4504893	12/51	28	81	48936	6.29	-5.0

Table 2 Mean density of protein spots in each individual plasma samples of both groups.

Spot ID	Density of Protein Spot (Number of sample found/Total sample)	
	Control (n=10)	Cancer (n=10)
1375 (α_1–antitrypsin)	19.98 (8/10)	75.49 (10/10)
1427	16.93 (6/10)	30.0 (6/10)
1512	2.2 (5/10)	8.2 (5/10)
1524	1.6 (5/10)	8.2 (5/10)
1368	1.46 (2/10)	7.59 (2/10)

Apart from α_1-antitrypsin, it is noteworthy that among the down-regulated protein spots, spot ID 1314 identified as transferrin, the iron binding protein, which had markedly low expression in plasma of CA patients. High rate of iron intake has been observed in most cancer cells (8). In addition, the cells also express high cell surface concentration of transferrin receptors (9) which allow binding of iron to the iron transporter holotransferrin. Iron then enters into the cells *via* a receptor-mediated endocytosis process (10). Therefore, the iron storage of tumor cells is generally greater in tumor cells than in normal cells (8). Holo-transferrin and other iron sources have clearly been shown to increase the potency of artemisinin (antimalarial drug with anticancer activity) in killing cancer cells (11). In our recent study however, a controversial result was observed. Pretreatment with holo-transferrin did not alter the cytotoxicity of artemisinin and its derivatives against the CA cell line-- CL6 (12). The possible explanation is that expression of transferrin receptor in CA may be lower than normal cell. In a previous study, the level of transferrin was shown to be significantly lower in intrahepatic CA cell lines when compared with normal liver tissues which is specific for this protein (12). Study to investigate the association between the up- and down-

regulation of α_1-antitrypsin and transferrin, and the levels of both proteins in plasma/serum of patients is underway. Further study to investigate the expression of transferrin receptor in CA cells is required to support this supposition.

Conclusion

Our preliminary results demonstrate significantly different protein patterns of plasma from CA patients with a number of protein spots which were either up- or down-regulated. The roles of the two proteins identified as α_1-antitrypsin (up-regulated) and transferrin (down-regulated) in CA are under investigation.

Acknowledgement

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Original article **P67**

Investigation on the involvement of glutathione (GSH) system in the mechanism of antimalarial action and its possible role in conferring resistance of *Plasmodium falciparum* to antimalarials

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Abstract

The oxidant enzymes of *Plasmodium falciparum* such as glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) may play important role in survival of malaria parasites including their possible involvement in conferring antimalarial drug resistance. This study was aimed to investigate the mutations of regulatory gene controlling glutathione level in a total of 34 *P. falciparum* isolates collected during 2006-2009 from endemic areas of multidrug resistance *P. falciparum* in Thailand. *In vitro* sensitivity testing of each *P. falciparum* isolate to the four antimalarial drugs, chloroquine (CQ), quinine (QN), mefloquine (MQ) and artesunate (ARS) was performed by using SYBR green modified assay, and 50% inhibitory concentration (IC_{50}) of each drug was determined. Real-time quantitative PCR was performed in DNA extracted from all parasite isolates using IQ SYBR green supermix. Allele-specific oligonucleotides from the gene encoding *Pf*GR and *Pf*GST were selected based on the coding sequence alignment of 3D7 parasites. The amplification of copy number of *Pf*GR and *Pf*GST gene were determined. Median (range) IC_{50} of CQ, QN, MQ and ARS were 86.33 (39.18-189.86), 387.79 (75.92-870.80), 45.87 (9.26-101.56) and 2.26 (0.83-5.21) nM, respectively. All isolates were resistant to CQ. Median (range) IC_{50} of the 24 QN-sensitive (70.6%) and 10 QN-resistant (29.4%) isolates were 262.63 (75.92-491.43) and 688.19 (520-870) nM, respectively. Median (range) IC_{50} of the 16 MQ-sensitive (47.1%) and 18 MQ-resistant (52.9%) isolates were 21.17 (9.26-32.75) and 67.82 (36.82-101.56) nM, respectively. None had more than one copy number of either *Pf*GR or *Pf*GST gene. No associations were observed between sensitivity of parasite isolates to the four antimalarials, and amplification of both *Pf*GR and *Pf*GST genes. Larger number of parasite isolates is required to increase power of the hypothesis testing in order to confirm this finding.

Keywords : *Plasmodium falciparum*, SYBR green assay, real-time PCR, glutathione reductase, glutathione S-transferase, gene amplification

Introduction

Malaria-infected erythrocytes are under oxidative stress produced by the digestion of host's hemoglobin. The oxidant enzymes such as glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) are detected in malaria-infected cells to reduce oxidative stress in the cell (1). Glutathione reductase (GR) belongs to the pyridine nucleotide-disulfide oxidoreductase family of homodimeric flavoenzymes which include lipoamide dehydrogenase and thioredoxin reductase. Both human GR (hGR) and *P.falciparum* GR (*PfGR*) are essential for the survival of the malarial parasite within the human erythrocyte. In addition, glutathione *S*-transferases (GST) also serves as the intracellular detoxification enzyme; it detoxifies lipid peroxidation products which lead to the inactivation and immobilization of these products (2). Recently, the association between the level of these enzymes in *P.falciparum* isolates and sensitivity of the parasites to chloroquine has been reported (3-4). This may imply the importance of these enzymes in survival of malaria

parasites, as well as their possible involvement in conferring antimalarial drug resistance (5). Compounds which act on these enzyme targets would eventually lead to parasite death, and thus, would be promising antimalarial drug candidates.

The aim of the study was to investigate the association between genetic polymorphisms of glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) in *P.falciparum* isolates collected from different malaria endemic areas of Thailand in 2009 and susceptibility of the parasites to currently available antimalarial drugs.

Methods

The mutations of regulatory gene controlling glutathione level was investigated in a total of 34 *P.falciparum* isolates collected from endemic areas of multidrug resistance in Thailand (Tak and Ranong Provinces) during the year 2006-2009. Prior to the study, approval of the study protocol was obtained from the Ethics Committee of Ministry of Public Health of Thailand. All *P.falciparum* isolates were adapted to culture *in vitro* according to the method of Trager and Jensen (6). *In vitro* sensitivity testing of each *P.falciparum* isolate to the four antimalarial drugs, chloroquine (CQ), quinine (QN), mefloquine (MQ) and artesunate (ARS) was performed using SYBR green modified assay (7), and 50% inhibitory concentration (IC_{50}) of each drug was determined. Threshold IC_{50} value of CQ, QN and MQ resistance were 30, 500 and 35 nM, respectively. There is no defined threshold IC_{50} value for ARS (8).

DNA sequences of gene controlling GSH level in K1 clone *P.falciparum* (CQ resistant clone) were determined. Comparison of these sequences with 3D7 *P.falciparum* clone from malaria genome project database were performed in order to search for allele-specific sequence(s) involving *Pf*GR and *Pf*GST gene. Allele-specific oligonucleotides from the gene encoding *Pf*GR and *Pf*GST were selected based on the coding sequence alignment of 3D7 parasites. Real-time quantitative PCR was performed in DNA extracted from all parasite isolates using IQ SYBR green supermix and amplification of copy number of *Pf*GR and *Pf*GST gene was performed. The $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy numbers of *Pf*GR and *Pf*GR in *P.falciparum* genes (9). Chi-square test was used to assess significant association between sensitivity of parasite isolates and amplification of *Pf*GR and *Pf*GR genes.

Results

Median (range) IC_{50} values of CQ, QN, MQ and ARS were 86.33 (39.18-189.86), 387.79 (75.92-870.80), 45.87 (9.26-101.56) and 2.26 (0.83-5.21) nM, respectively. All isolates (100%) were resistant to CQ. Twenty-four isolates [70.6%: IC_{50} 262.63 (75.92-491.43) nM] and 10 isolates [29.4%: IC_{50} 688.19 (520-870) nM] were defined as QN-sensitive and QN-resistant, respectively. For sensitivity to MQ, 16 isolates [47.1%: IC_{50} 21.17 (9.26-32.75) nM] and 18 isolates [52.9%: IC_{50} 67.82 (36.82-101.56) nM] were categorized as MQ-resistant and MQ-sensitive, respectively (Table 1). All isolates carried only one copy number of either *Pf*GR or *Pf*GST gene.

Discussion

Results from *in vitro* sensitivity test suggest virtually 100% of CQ-resistant isolates. It is noted however for the improved sensitivity of CQ in these isolates compared with that was previously reported in isolates collected from the same area during 1994-1999. [median (range) IC_{50} 157.05 (105.75-198.73 nM)] (10-11). Sensitivity of parasite isolates to QN was more or less stable since 1994 [median IC_{50} 403.87 (312.88-493.54) nM] (10-11). Interestingly, sensitivity to MQ was markedly improved, with the IC_{50} reduced by about 50% since 1994 [median (range) IC_{50} 95.48 (75.90-122.42) nM] (10-11). For ARS, median (range) IC_{50} values were comparable with that reported during 1991-1992, 1994, and 1999 [2.58 (1.80-3.71), 3.39 (2.67-4.29), and 2.19 (1.67-2.87) nM, respectively] (10-11). No

isolate carried more than one copy number of *Pf*GR and *Pf*GST genes. No association was observed between sensitivity of parasite isolates to the four antimalarials, and amplification of both *Pf*GR and *Pf*GST genes. However, larger number of parasite isolates is required to increase power of the hypothesis testing in order to confirm this finding.

Table 1 Number and percentage of sensitive and resistant groups classified based on results from *in vitro* sensitivity test

Drug	Drug susceptibility test			
	Sensitive group		Resistant group	
	Number (%)	Median (Range) (nM)	Number (%)	Median (Range) (nM)
CQ	0 (0%)	-	34 (100%)	86.33 (39.18-189.86)
QN	24 (70.6%)	262.63 (75.92-491.43)	10 (29.4%)	688.19 (520-870)
MQ	16 (47.1%)	21.17 (9.26-32.75)	18 (52.9%)	67.82 (36.82-101.56)

Conclusion

In this study with limited number of *P.falciparum* isolates under investigation, lack of association between amplification of either *Pf*GR or *Pf*GST gene was observed. CQ resistance was virtually 100%, whereas MQ and QN resistance were 52.9 and 29.4%, respectively.

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Original article **P68**

Effect of TGFβ1 and TGFβ1-inhibitor on limbal stem cell properties

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Abstract

Corneal epithelium is constantly renewing itself from stem cells located at the limbus, the transition zone between the corneal and neighboring conjunctival epithelium. The maintenance of limbal stem cells (LSCs) is widely believed to be controlled by various cues present in their niches. The aim of the present study was to investigate the effect of TGF-β, a key cytokine found during corneal wound healing, on cultivated LSCs. We found that when LSCs were grown at clonal density on 3T3 feeder, TGF-β1 treatment results in the clonal transition from cobblestone-like colonies to spindle-shaped colonies with migrating protrusions. Within spindle-shaped colonies there was marked reduction in putative stem cell marker ΔNp63α expression. In contrast, treatment with SB431542, TGFβ-inhibitor, promotes colony forming efficiency of LSCs as well as increase a proliferation rate of LSCs colonies. Our results suggest that TGFβ signaling induces limbal epithelial stem cells to transdifferentiate into fibroblast-like cells as described in epithelial-to-mesenchymal transition (EMT). TGFβ-inhibitor may be useful in promoting corneal wound healing and improve corneal transparency in patient with corneal injury.

Keywords: limbal stem cells, TGFβ, Epithelial-to-mesenchymal transition.

Introduction

Stem cells of the corneal epithelial system are thought to be slow cycling cells localized to the epithelial basal layer of the limbus, the transitional zone between the cornea and the conjunctiva (*Schlotzer-Schrehardt U. et al., 2005*). Upon activation by corneal wounding LSCs enter the cell-cycle and show high proliferative potential. It is still unclear which signals in the limbal niche play a role in regulating self-renewal and fate decision of limbal epithelial stem cells (*Walker MR. et al., 2009*). One candidate pathway is the transforming growth factor β (TGF-β) signaling. TGF-β is known to play critical roles during corneal wound healing as well as control numerous cellular processes, such as proliferation, apoptosis, and differentiation (*Goumans MJ., 2003*). Both transforming growth factor-beta (TGF-b) receptors types I and II were reported to be weakly expressed in the central corneal epithelium of human eyes, but were presented at much higher levels in the basal cells of the limbus (*Zieske JD et al., 2001*). Nevertheless, the effects of TGF-β1 on LSCs properties have not been fully clarified.

The aim of this study was to investigate the effect of TGFβ1 and TGFβ-inhibitor (SB431542) on clonogenic potential and proliferation rate of cultured limbal stem cells.

Material and Methods

Human limbal rims taken from organ donors were provided by the Department of Ophthalmology of Thai Red Cross. After careful removal of excessive sclera and conjunctiva tissue, the limbal rings were exposed to dispase II (1.2 IU/mL in PBS) at 37°C under humidified 5% CO_2 for 1 hour. Following one rinse with DMEM containing 10% FBS The loosened tissues were cut into cubes of approximately 2x2 mm^2. Limbal tissues were cultured on the laminin coated plate containing limbal complete media; 1:1 mixture of Dulbecco's

modified Eagle's medium (DMEM) and Ham's F12, supplemented with 10% fetal bovine serum USA grade (FBS), 1% Pen-Strep, 1.25 μg/mL amphotericin, 1% L-glutamine, 5 μg/mL insulin (Invitrogen), 20 ng/mL EGF (R&D), 0.5 μg/mL hydrocortisone, 2.5 mg/ml $NaHCO_3$. Culture Media was changed every 1-2 days. After 5 days of cells growth from the explant, limbal epithelial cells were separated into single cells by 0.25% trypsin-0.02% EDTA for 2 minutes. For clonal analysis, single cells were inoculated onto multiwell plates/cultured dishes containing 3T3 feeder layers. TGF-β1 (R&D systems) or TGFβ-inhibitor SB431542 (Stemgent) were added at the first day of seeding. After 10 days clones were stained with rhodamine B then counted for determination of the colony-forming efficiency. Immunofluorescence staining with ΔNp63α antibody (Cell Signaling) was performed after 10 days of culture on 3T3 cells for putative LSCs identification and characterization.

Results

Human limbal epitheilium when cultured on mitomycin C (MMC)–treated 3T3 feeder cells give rise to three types of colonies: Large colonies consist mainly of small undifferentiated cells (SU), small colonies consist of large differentiated cells (LD), and abortive colonies (AB). When 20 ng/ml TGF-β1 were added to culture medium, all colonies were transformed into fibroblat-like colony (FL) that composed of more extended and elongated shape cells with migratory capacity (Fig. 1). After culture for 10 days, the migrating fibroblast-like cells covered an entire surface of the tissue culture plate. In contrast, 10 μM SB431542 (TGFβ-inhibitor) increases colony-forming efficiency (CFE) of limbal epithelium (Table. 1). Moreover, all of colonies cultured in the presence of SB431542 were large colonies with smooth perimeter, contained mainly small and rapidly growing cells (SU). Whereas in normal condition (untreat) had many of small colony containing large and terminally differentiated epithelium (LD), (Fig. 2). Immunofluorescence analysis showed that TGF-β1 treatment decreases a number of ΔNp63α-positive cells in their colonies.

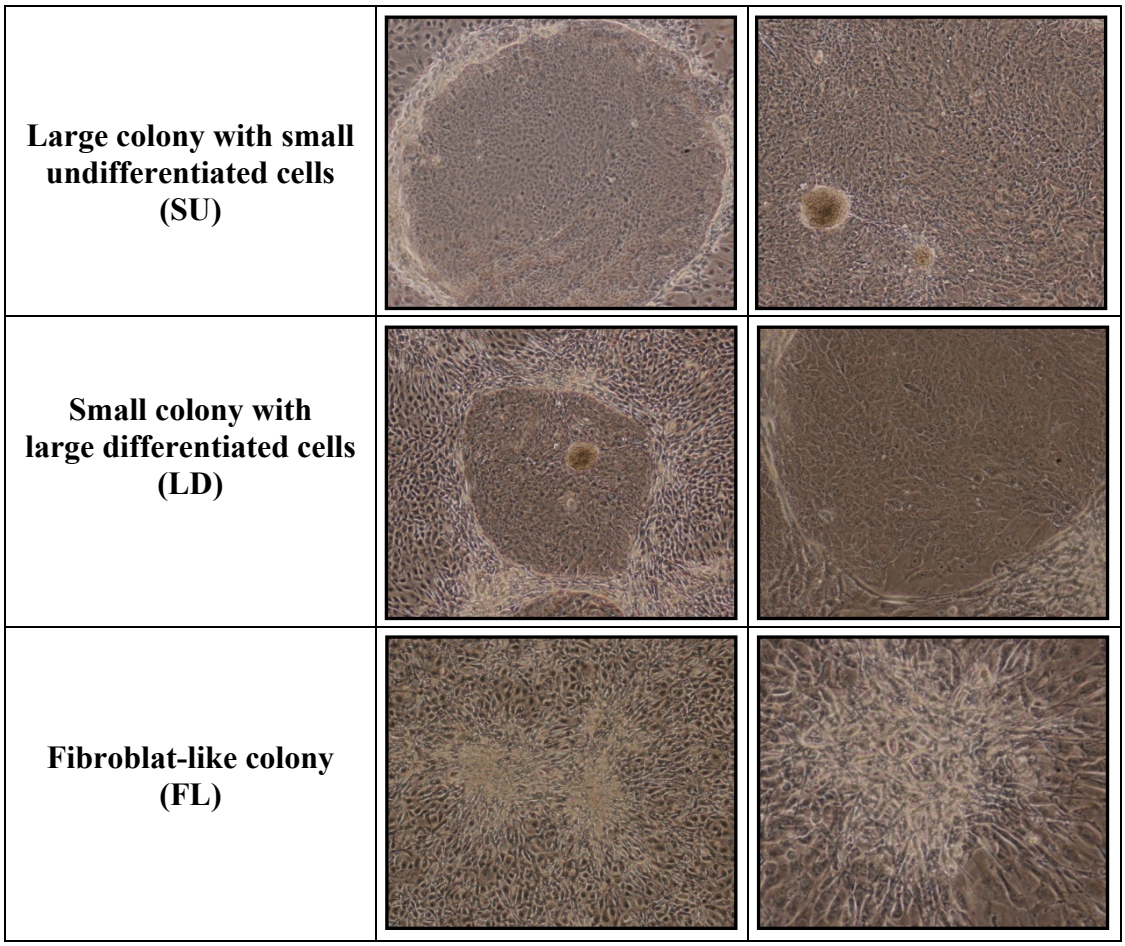

Figure 1: Morphology of human limbal epithelial stem cell colony formation. Both of two colony types (SU and LD) were observed in untreat condition (control). Culture with SB431542 treatment was found only SU-clones, whereas FL-clones were observed in 20 ng/ml TGF-β1 treatment. Magnification: (left) x4; (right) 10x.

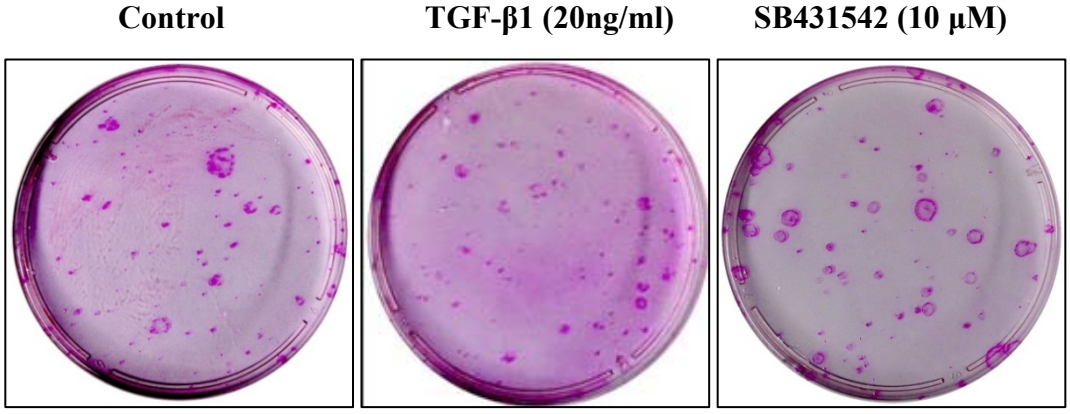

Figure 2: Determination of the clonogenic ability. 300 limbal epithelial stem cells were plated onto inactivated 3T3 feeder layers. Dishes were stained 10 days later with rhodamine B. 10 μM SB431542 treatment increases clonogenic ability and promotes limbal epithelial stem cell proliferation to form the large colony with a smooth perimeter.

Table 1: Clonogenicity of human limbal epithelial stem cells in each culture condition system were taken from the eye of a female, 69-yr-old organ donor. Colony-forming efficiency (CFE = number of colonies/number of cells seeded x100%). TGF-β1 treatment induces LSCs transition to fibroblast-like colony. There was more the large colony with small undifferentiated cells (SU) in 10μM SB431542 treatment significantly (* $p<0.008$) when compared to untreated condition (control). And the cultivated human limbal clones in SB431542 treated condition were also formed more quickly.

Clonogenicity of human limbal epithelial stem cells				
Colony type	Culture system, (% CFE)			
	Untreat (control)	TGF-β1 (20ng/ml)	SB (2μM)	SB (10μM)
Small colony with large differentiated cells (LD)	8.17 ± 0.73	* 0.00	* 00.00	* 0.00
Large colony with small undifferentiated cells (SU)	17.5 ± 1.65	* 0.00	22.72 ± 0.92	* 26.44 ± 0.67
Fibroblast-like colony (FL)	0.00	* 6.94 ±0.54	0.00	0.00

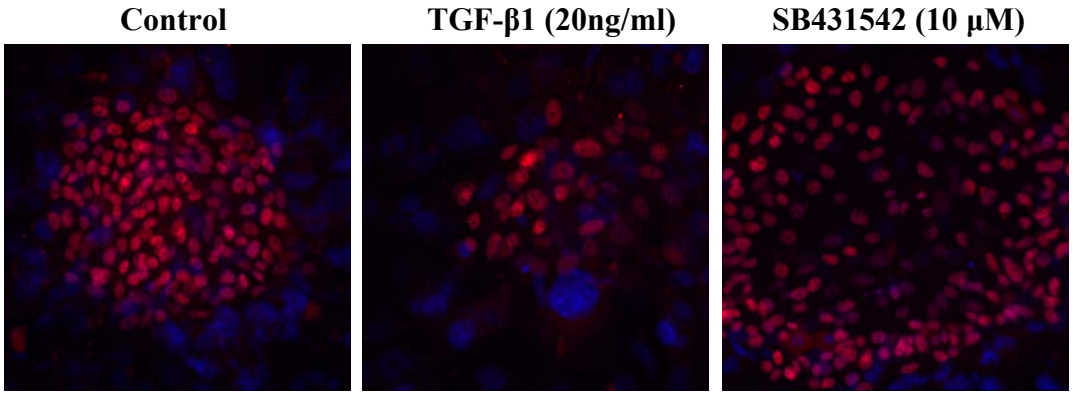

Figure 3. Expression of ΔNp63α in LSCs colonies. To confirm the clone of limbal epithelial stem cells and progenitor cells. Note the reducton of ΔNp63α expression in fibroblast-like colonies observed from TGF-β1 treatment, whereas the size of ΔNp63α-positive colony increased considerably in SB431542 treatment. (Red fluorescence: Alexa Fluor 546-conjugated goat antirabbit IgG; blue fluorescence: nuclear counterstaining with Dap; magnification: X40)

Discussion and Conclusion

In our experiment, we demonstrated that TGF-β1 treatment results in the clonal transition from limbal epithelial colony containing LSCs to migrating fibroblast-like colony corresponding with the reduction in the number of putative stem cells based on ΔNp63α expression. These results suggest that during injury, and perhaps in culture condition, TGF-β may play a role in promoting LSCs differentiation and migration. Because the clinical success of cultivated LSCs therapy depends on whether they contained a sufficient number of stem cells essential for long-term epithelial renewal, our finding that SB431542 inhibits fibroblastic transformation and promote LSCs maintenance may be applied to improve the method for ex vivo expansion of LSCs used in clinical applications

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Original article **P69**

Antioxidant and antimutagenic of Thai *Ganodema lucidum*

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Abstract

Thai *Ganoderma lucidum* (G2), which has been grown in Thailand as part of the Royal Project since 1988 was studied for its safety and efficacy. The present study aimed at comparing the fruiting body (whole fruiting) and mycelium of *GL* hot water extracts with regards to their *in vitro* mutagenic and antimutagenic potential by bacterial reverse mutation assay, antioxidant activity by free radical scavenging activity (DPPH assay), and iron chelating activity. The results showed that both *GL* extracts ranging from 0.15 to 3.0 mg/ml was neither mutagenic nor antimutagenic in bacterial system. Higher antioxidant activity in term of DPPH scavenging free radicals was found in fruiting body extract when compared with the mycelium extract. Moreover, both *GL* extracts showed a slight effect of chelating activity on Fe^{2+}. Significantly, the antioxidant capacity correlated with the total phenolic content. While no tested concentrations of *GL* extracts were toxic to TA98 and TA100 *salmonella typhimurium,* the highest non-mutagenic concentration was cytotoxic to lung carcinoma cell lines (A549) as determined by Trypan blue assay. This is the first comparative study on the antimutagenic and antioxidant activities of Thai *Ganoderma* fruiting body and mycelium extracts. The information obtained from the study will provide useful information for safety profile of Thai *GL* and idea to select the *GL* parts to develop *GL* products that promote health and longevity or disease prevention.

Keywords: Thai *Ganoderma lucidum;* antioxidant; antimutagenic; Ames'test; cytotoxicity

Introduction

Recently there has been greater interest in investigating compounds originating from plants and their effects on DNA. The actions of these compounds may be involved in maintaining the balance between the consumption of mutagenic and antimutagenic substances, thus contributing to increases or reductions in the incidence of cancer in the population (Ames, 1971). Compounds from plants could act as protective agents with respect to human carcinogenesis, acting against the initiation, promotion or progression stages of this process or, perhaps, destroying or blocking the DNA-damaging mutagens outside the cells, thus avoiding cell mutations. On the other hand, it is known oxidative stress is involved in variety of disorders such as cancer; hypertension, neurodegenerative (Alzheimer's and Parkinson's disease) and autoimmune diseases, and thus many antioxidant ingredients from foods or other natural sources are being challenged for diseases protection and treatment.

Ganoderma lucidum (Lingzhi) is a woody mushroom, which is highly regarded in Asian traditional medicine and is widely consumed in the belief that it promotes health and longevity. Scientific evidences shown *G. Lucidum* therapeutic effects, which including anti-inflammatory, anti-tumor, anti-viral (e.g. Anti-HIV), anti-bacterial and anti-parasitic, blood pressure regulation, cardiovascular disorder, kidney tonic, nerve tonic, sexual potentiator, chronic bronchitis, proteinuria in nephoresis. These benefits are thought to arise partly from *GL*'s role as an antioxidant. A variety of chemical ingredients of *GL* includes triterpenes, polysaccharides, nucleosides, steroids, fatty acids, alkaloids, proteins, peptides, amino acids,

and inorganic elements, which vary among geographic cultivation, species and part of the mushroom.

In this study, Thai *Ganoderma lucidum* (G2 species), which has been grown in Thailand as part of the Royal Project since 1988 was studied for its safety and efficacy. The purpose of this study was to evaluate the mutagenic and antimutagenic potential, cytotoxic, antioxidant activities and total polyphenol contents of hot water extracts from fruiting body and mycelium of Thai *GL*.

Material and methods

Fruiting body and mycelium of *GL* obtained from the Royal Project were extracted with hot water and dried by lyophilizer.

Determination of mutagenic/antimutagenic potential

The bacterial reverse mutation test (Ames' test) was used to detect mutagenicity of *GL* extracts. Five concentrations of *GL* (0.15, 0.3, 1.0, 1.5 and 3.0 mg/plate) were tested, with and without S9 supernatant, the number of revertant colonies were counted and compared to negative and positive control plates. Antimutagenicity was determined against known mutagenic substances (4-oxide-1-nitroquinoline, and 3,4 benz(a)pyrene), using the modified. Ames'test.

Determination of antioxidant activity and total polyphenol content

The DPPH radical scavenging activity of *GL* extracts was determined by spectrophotometer in terms of hydrogen donating or radical scavenging ability. The change in color was measured at 515 nm on an automated micro-plate reader. The radical scavenger activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50 % (EC 50 value). The chelating activity on Fe^{2+} was measured after the *GL* extract was reacted with $FeSO_4$ and ferrozine at 562 nm. Chelating activity was calculated as % = [(A562 nm of blank - A562 nm of sample)/A562 nm of blank] x 100. The effective concentration value (EC50) is the plot extrapolated concentration at which ferrous ions were chelated by 50 %.

The amount of total phenolics in *GL* extracts was determined with the Folin-Ciocalteu reagent using the method of Singleton and Rossi, 1965. *GL* extract was reacted with Folin-Ciocalteau's reagent and Na_2CO_3. The absorbance of sample was measured at 765 nm. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g).

Determination of cytotoxicity

The *GL* extracts were evaluated for their cytotoxic potential using lung carcinoma cell lines (A549). Cells ($5x10^4$ cells per well) were incubated at 37 ºC for 24 h. before exposed to different concentration of *GL* extracts for 9 h. Viability of cells were determined by Trypan blue assay.

Statistical analysis

All data are presented as mean ±SD from three or more independent experiments. Statistical comparison between different groups was done by one-way ANOVA. Differences were considered significant at $p<0.05$.

Results and Discussion

No mutagenic activity was found for base-pair substitution (TA100) and frame-shift mutations (TA98) in *GL* fruiting body and mycelium hot water extracts studied. However, the extracts of these two parts against the mutagen 4NQO and Benz(a)pyrene were unable to inhibit mutagenic activity. The anti-oxidative effectt of plants is mainly due to phenolic components and the antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals,

quenching singlet and triplet oxygen, or decomposing peroxides. The amount of total phenolics varied in *GL* parts and ranged from 6.65 to 31.77 mg GAE/g of dry material. In this study, higher antioxidant activity in term of DPPH scavenging free radicals was found in fruiting body extract when compared with the mycelium extract. Moreover, both *GL* extracts showed a slight effect of chelating activity on Fe^{2+}. The correlation between free radical scavenging capacity and total phenolic contents of *GL* extracts was observed. Both *GL* extracts ranging from 0.15 to 3.0 mg/ml were not cytotoxic to TA98 and TA100 *Salmonella typhimurium*, while the highest non-mutagenic concentration was cytotoxic to lung carcinoma cell lines (A549) as determined by Trypan blue assay.

Conclusion

For 4000 years *G. lucidum* has been used as a part of Chinese and Japanese medicine especially for the treatment of most of the human ailments. *Ganoderma lucidum* (G2 species) was selected to cultivate by the Royal Project because of its pharmaceutical functionality. This is the first comparative study on the antimutagenic and antioxidant activities of Thai *GL* fruiting body and mycelium extracts. The results revealed that *GL* hot water extracts showed antioxidant activity, with no mutagenicity/antimutagenicity. The present study also showed that hot water extract of *GL* fruiting body, which are often present in many *GL* products, are stronger radical scavengers and can be considered as good sources of natural antioxidants for medicinal and commercial use.

Acknowledgements

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Original article **P70**

Is the resistance transporter gene *pfcrt* linked with susceptibility of *Plasmodium falciparum* to chloroquine?

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Abstract

Recent studies have definitively demonstrated a link between mutations in the gene *pfcrt* and resistance to the antimalarial chloroquine (CQ) in *Plasmodium falciparum*. Although these mutations are predictive of CQ resistance they are not quantitatively predictive of the degree of resistance. We had undertaken analysis of a total of 95 recently adapted *P. falciparum* isolates from Thailand. Parasites had been characterized for their drug susceptibility phenotypes and genotypes with respect to *pfcrt.* From the original 95 isolates, 20 were selected for complete *pfcrt* sequence analysis. Almost all of the parasites characterized carried the previously reported mutations K76T, A220S, Q271E, N326S, I356T and R371I. On complete sequencing, isolates were identified with novel mutations at K76A and E198K. The isolates carrying E198K were less resistant to CQ than those that were not. Data from the present study suggested that other genes may also contribute to the degree of resistance once the resistance phenotype is established through mutations in *pfcrt* gene.

Keywords*: Plasmodium falciparum; pfcrt, pfmdr1,* quinoline antimalarials, drug resistance

Introduction

Multidrug resistant *Plasmodium falciparum* including resistance to structurally related antimalarial aminoquinolines such as chloroquine (CQ), quinine (QN) and mefloquine (MQ), is still problematic along the border areas of Thailand, especially Thai-Myanmar border. Reduced dug accumulation is a phenotypic feature of CQ resistance (CQR) which can be partially reversed by the calcium channel blocker verapamil, an agent that also reverses CQ resistance. Two genes have been linked with this phenotype namely *pfmdr1* and *pfcrt*. The *pfmdr1* gene is located on chromosome 5 and *pfcrt* gene is located on chromosome 7. The weight of molecular evidence suggests that while *pfmdr1* may exert a modulatory effect in parasite susceptibility to CQ (1), mutation in *pfcrt* is the principal determinant of CQR (2). Although it is generally accepted that PfCRT is the principal determinant of CQR, it is not possible to predict the degree of CQR based on the *pfcrt* genotype alone or even in combination with *pfmdr1* genotype. It is clear form our own surveillance studies and those of others that parasites considered to be CQR in actual fact, display a broad range of sensitivity to the drug. In this study we have characterized the phenotype-genotype relationship between a total of 95 recently adapted isolates of *P. falciparum* from Thailand. Furthermore, 20 selected isolates were fully sequenced for *pfcrt* gene, in order to look for novel mutations that might be implicated in the degree of CQR.

Methods

In vitro **drug susceptibility testing:** A total of 95 *P. falciparum* field isolates were collected from malaria endemic area of Thailand. All were adapted to continuous culture

according to the methods of Trager and Jensen (3) with modifications. Laboratory strains of G112 (CQ-sensitive) and K1 (CQ-resistant) clones were used as positive control. Susceptibilities of *P. falciparum* isolates to CQ, QN, and MQ were assessed using the radio-isotopic technique based on the uptake of [^{3}H]Hypoxanthine (4). IC_{50} value (drug concentration that inhibits the parasite growth by 50%) was used as an indicator for antimalarial susceptibilities of the tested drugs, and was determined from a log-dose response curve plotted using the Grafit™ computer program (Erithacus Software Ltd., U.K). Sensitivity of the isolates to CQ, QN and MQ was categorized based on IC_{50} values described by Cerutti *et al*, (5).

Detection of *pfcrt* and *pfmdr1* polymorphisms: Genomic DNA was extracted using Chelex-resin (Biorad Co. Ltd., USA). Previously published nested and PCR/RFLP methods were employed to detect *pfmdr1* at the codons 86 (6), and *pfcrt* mutations at the codons 76, 220, 271, 326, 356 and 371 (2). The primers and reaction conditions used were according to the previously described methods (2).

***Pfcrt* DNA sequencing:** The *pfcrt* gene was amplified from genomic DNA using a nested PCR strategy. Exons 1-2, 3-8 and 9-13 were sequenced independently using the primers designed by our group. Each amplicon was then cloned and the positive clones were picked from white or blue colonies following overnight incubation in selection media, S-Gal (Sigma, USA). The plasmid DNA was purified and digested with the restriction enzyme *EcoRI* to confirm the correct insertion. Finally, sequencing was carried out using the M13 forward and reverse primers and DNA*star* (Lasergene) was utilized for sequence analysis.

Statistical analysis: All data are expressed as mean ± standard deviation. Data were assessed for normality using a Shapiro-Wilk statistical test. A Kruskal-Wallace statistical test was utilized to assess the relationship between mutations in *pfcrt* and *pfmdr1* and drug IC_{50}. Correlations were assessed by Spearman Rank test.

Results

***In vitro* drug susceptibility testing:** A total of 30 (32%), 61 (64%), and 4 (4%) isolates were categorized as highly CQ-resistance, moderately CQ-resistance, and CQ-sensitive, respectively [geometric mean (95% CI) IC_{50}: 130.9 (122.1-140.2), 60.8 (56.7-65.3), and 23.0 (20.5-25.5) nM, respectively] (Figure 1A). For QN, 1 (1%), and 92 (99%) isolates were categorized as QN-resistant and QN-sensitive, respectively [geometric mean (95% CI) IC_{50}: 654.4 and 144.4 (129.4-161) nM, respectively]. For MQ, 44 (32%), and 47 (68%) isolates were categorized as MQ-resistant and MQ-sensitive, respectively [geometric mean (95% CI) IC_{50}: 38.2 (34.4-42.4), and 13.3 (11-16) nM, respectively] (Figure 1B).

Genetic polymorphisms: Genotyping for *pfcrt* revealed a 100% prevalence of the 76T, 220S, 271E, 326S and 371I mutations (95 of 95 samples) with a 94% prevalence of the 356T Mutation (89 of 95 samples). The K1-type mutation of the *pfmdr1* was seen in only 5% (5 of 95) of the samples with almost all isolates containing the wild type Asn at position 86.

Novel sequence polymorphisms in *pfcrt*: The sequencing results, one isolate carried the novel mutation at codon 76 with a different amino acid substitution K76A. This is a unique position 76 mutation, which has never been reported before in field isolates. Four of the field isolates categorized as displaying moderate CQ resistance carried a E198K mutation which may be functionally relevant.

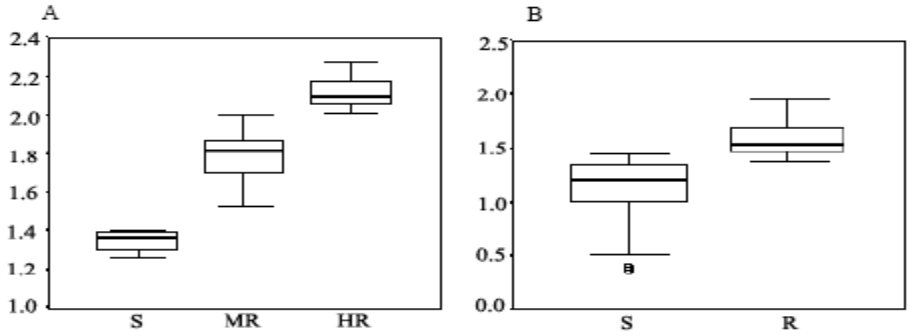

Figure 1 Box plot between logIC_{50} and the order of drug susceptibility. (A) CQ was categorized into highly resistant (HR: IC_{50} > 101 nM), moderately resistant (MR: 30.9 < IC_{50} ≤ 100.9 nM) and sensitive (S: IC_{50} ≤ 30.9 nM). (B) MQ was categorized into 2 groups, resistant (R; IC_{50} >24 nM and sensitive (S; IC_{50} ≤ 24 nM).

Discussion

The K76T mutation of *pfcrt* is reported to be highly associated with CQ-resistance. The frequency of this mutation is almost 100% in Thailand. Consequently there seems to be no link between the frequency of this gene mutation and CQ sensitivity. Parasites carrying the K76T mutation with comparable patterns of other *pfcrt* gene mutations still showed a variable degree of CQ-resistance. Data reported from other South East Asian countries, *i.e*., the Philippines, showed different mutations carrying K76T and N326D without 220S residue, together with the novel mutations A144T and L160Y (7). Furthermore, four novel mutations A144F, L148I, I194T and T333S were observed from Cambodian isolates (8). These mutation patterns were not observed in Thailand. From our results, revealed the novel 76 haplotype K76A in one isolate. Previously, the substitution of other amino acids at this codon, *i.e*., K76I and/or K76N has been reported only in laboratory strains. This allelic type replaces lysine (positively charged amino acid) with alanine (non-charged amino acid), similar to the charge loss found with K76T, K76I and K76N mutations. In addition, the new mutation at position E198K was detected in 4 out of 20 isolates with high CQ sensitivity (low IC_{50}). These findings may support the proposed hypothesis that CQR results from a "charged drug leak", in which the loss of positive charge in the channel of PfCRT might allow the protonated species of CQ to leak out of the digestive vacuole, thus reducing vacuolar CQ concentration and ultimately conferring resistance (9).

Conclusion

Data from the present study suggest that, apart from the reported mutations of *pfcrt* gene--K76T, A220S, Q271E, N326S, I356T and R371I, other genes may also contribute to the degree of resistance once the resistance phenotype is established through mutations in *pfcrt* gene.

Acknowledgements

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Original article **P71**

Leucine aminopeptidase of *Fasciola gigantica:* functional characterization and evaluation as a vaccine in animal fasciolosis

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Abstract

The infection of livestock animals with the parasite *Fasciola gigantica* causes economic loss due to mortality, weight loss, reduced productivity and poor milk production. An effective vaccine to overcome these problems would be a viable alternative to the currently used drugs. Leucine aminopeptidase (LAP) plays an important role in the parasite's biology, such as in processing, maturation, activation or degradation of substrates and is, therefore, considered as a candidate for development of a vaccine. In the presented work the protein coding fragment of a *FgLAP* cDNA was subcloned into the pThioHisB bacterial expression vector. Recombinant *FgLAP* was expressed and purified from *Escherichia coli* TOP10. The purified *rFgLAP* has and will be used in further studies such as proteolytic activity assays, immunohistochemistry and vaccine development. FgLAP protein- and DNA-based immunization trials have been performed in mice to test the antigen's vaccine potential. In addition to the recombinant FgLAP, a complex of synthetic FgLAP peptides (spFgLAP) was designed from the active site of FgLAP and has also been used for protein-based immunization. DNA-based immunization has been performed with the pSecTag2A mammalian expression vector containing the FgLAP coding DNA.

Keywords: *Fasciola gigantica*, leucine aminopeptidase, vaccine, molecular cloning

Introduction

Fasciolosis is a helminth disease caused by trematodes in the genus *Fasciola* (*F. hepatica* and *F. gigantica*) and belongs to the plant-borne zoonoses. *F. gigantica* is the common *Fasciola* species infecting ruminants in the tropical parts of Asia. The infection of livestock animals with the parasite causes economic loss due to, weight loss, reduced productivity and poor milk production. Triclabendazole is the standard drug for treatment of animal fasciolosis, but the cost of treatment with this drug is a problem in developing countries. An effective vaccine to overcome these problems would be a viable alternative to Triclabendazole and other currently used drugs.

Methods

In the present study a cDNA encoding leucine aminopeptidase from *Fasciola gigantica* (FgLAP) was synthesized and isolated from total RNA using reverse transcription and polymerase chain reaction with oligonucleotide primers specific to the leucine aminopeptidase DNA sequence from *F. hepatica*. The sequence of the obtained FgLAP cDNA was determined by Sanger dideoxy sequencing. The coding DNA fragment of the FgLAP cDNA was then inserted into the pThioHisB bacterial expression vector and *Escherichia coli* TOP10 was transformed with the plasmid for production of recombinant FgLAP (rFgLAP) fused to bacterial thioredoxin encoded by the plasmid. Expression of rFgLAP was induced by addition of 1 mM IPTG to the medium.

Both protein- and DNA-based immunization trials have been started to test the vaccine potential in mice. Recombinant FgLAP and synthetic peptides (spFgLAP) which

cover parts of the active site of FgLAP have been used in protein-based immunization. DNA-based immunization has been performed with mammalian expression vectors carrying the FgLAP encoding sequence with and without additional signal peptide encoding sequences.

Results

The deduced amino acid sequence of FgLAP is closely related to the homologous leucine aminopeptidase of *F. hepatica*. Both proteins contain 523 amino acid residues and have 98% sequence identity while LAP from *Clonorchis sinensis* has only 70% identity (Fig. 1). The bacterially expressed and purified rFgLAP-thioredoxin fusion protein was soluble and migrated as a single band at a molecular mass of 66 kDa on SDS-PAGE (Fig. 2).

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                10        20        30        40        50        60        70        80        90       100
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  M.A..V.V.DL.D.RFD.VIFIND...E...K.AAV.E.L.SFSKINP.L..ELSIVPFP.HPSGRLIYSPTGALNTD.ADIRNVYDAAC..VKRALS.G
FhLAP  M.A..V.V.DL.D.RFD.VIFIND...E...K.AAV.E.L.SFSKINP.L..ELSIVPFP.HPSGRLIYSPTGALNTD.ADIRNVYDAAC..VKRALS.G
CsLAP  M......V..L.D.RFD...F.ND...E......A..E.L.SFSK.NP....EL.IVPFP.HPSGRLI.SPTG.LNTD.AD.RNVYDA.C...KRA...G

               110       120       130       140       150       160       170       180       190       200
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  ....L.YL.S-.R.......WMQRKHLLLN.LLGAYHA.Y.PLEVREM.P....K..HLGV.......DE.V.RLA.ALEEGRWLARDIGGSDPERMAAP
FhLAP  ....L.YL.S-.R.......WMQRKHLLLN.LLGAYHA.Y.PLEVREM.P....K..HLGV.......DE.V.RLA.ALEEGRWLARDIGGSDPERMAAP
CsLAP  ....L.Y.............WM.R...LLN.LLGAYHA.Y.PLEVREM.P....K..H.G....----D......A.A.EEGRW.ARDIGGSDPERMAAP

               210       220       230       240       250       260       270       280       290       300
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  .IV.YLKT.L.G.KGITM.VEKVD.QKYPLMAAVNRAASVVARHDGRVVHL.Y.PPN...VDTTLYLIGKGITYDTGGADIKA.GVMAGMHRDKCGAAAI
FhLAP  .IV.YLKT.L.G.KGITM.VEKVD.QKYPLMAAVNRAASVVARHDGRVVHL.Y.PPN...VDTTLYLIGKGITYDTGGADIKA.GVMAGMHRDKCGAAAI
CsLAP  ..V.YL...L.G..GITM.VEKVD..KYPLMAAVNRAASVV.RHDGRVVHL.Y.PPN....DTTLYLIGKGITYDTGGAD.KA.GVMAGMHRDKCGAAAI

               310       320       330       340       350       360       370       380       390       400
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  AG.F.TL.QLQPP.LSVSAALAFVRNSVG.DSYVADEILVARSGQRIRVGNTDAEGRMVM.DLLCEAKEKAV.ATNPFLFTIATLTGHVVRAYKHYTAVM
FhLAP  AG.F.TL..LQPP.LSVSAALAFVRNSVG.DSYVADEILVARSGQRIRVGNTDAEGRMVM.DLLCEAKEKA..ATNPFLFTIATLTGHVVRAYKHYTAVM
CsLAP  AG.F.TL..LQPP.LSVSA.LAFVRNS.G.DSYV.DEI.V.R.G.R.R.GNTDAEGRMVM.DLLCEAKEKAV.ATNPFLFTIATLTGHVVRAYKH.TAVM

               410       420       430       440       450       460       470       480       490       500
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  DNGP.RI..VSQ.LQ.AGD.ISDMAEISTVRKEDYEFNRGKTEYED.LQCNNLPSSAT.RGHQIPAAFM.LASGLDKHGL.S.KPIPYTHVDVAGSA.EI
FhLAP  DNGP.RI..VSQ.LQ.AGD.ISDMAEISTVRKEDYEFNRGKTEYED.LQCNNLPSSAT.RGHQIPAAFM.LASGLDKHGL.S.KPIPYTHVDVAGSA.EI
CsLAP  DNGP.R...VSQ.LQ.AGD..SD.AEISTVRKEDYEFN.G.TEYED.LQCNNLPSSAT.RGHQ.PAAF...ASGLDKHG..S.KP.PYTHVDVAGSA.EI

               510       520       530       540       550       560       570       580       590       600
       ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
FgLAP  HVLPTAAPLLMFASRYVLPRLGF.
FhLAP  HVLPTAAPLLMFASRYVLPRLGF.
CsLAP  HVLPTAAPL.MFASRY.LPR.GF.
```

Figure 1. The sequence alignment of the *F. gigantica* leucine aminopeptidase (FgLAP) with the LAP sequences from *F. hepatica* (AAV59016) and *Clonorchis sinensis* (ABK91810).

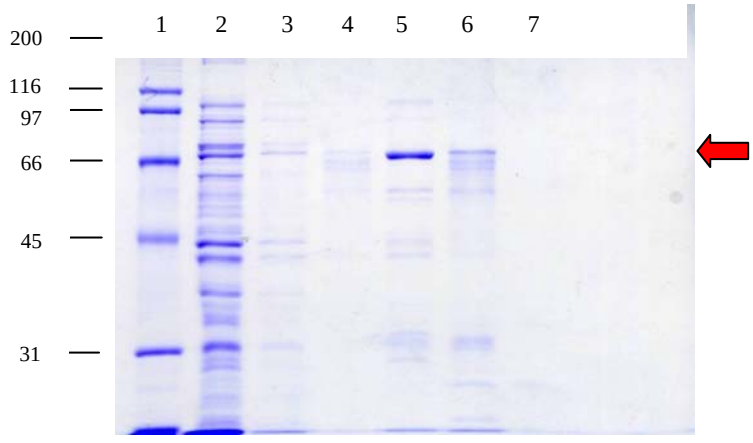

Figure 2. SDS-PAGE of rFgLAP: purified under denaturing conditions by Ni-NTA affinity chromatography. Lane 1: flow through, lanes 2, 3: washes, lanes 4-7: elution fractions. The arrow indicates the rFgLAP-thioredoxin fusion protein at 66 kDa.

Discussion

The purified rFgLAP fused with thioredoxin will be used for further studies such as functional analysis of rFgLAP, production of polyclonal anti-rFgLAP antiserum, analysis of tissue-specific distribution of native LAP, vaccine development approaches. Sera from immunized mice will be used for analysis of specific antibody responses by ELISA and Western blot.

Conclusion

The FgLAP protein is highly conserved in the genus *Fasciola* (98% identity between the two species) and also shows high conservation to LAP from *C. sinensis* with 70% identity. Recombinant FgLAP could be expressed as a 66 kDa soluble fusion protein in *E. coli*.

Acknowledgements

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Original article **P72**

Genotyping of polymorphic marker (*MSP3α* and *MSP3β*) genes of *Plasmodium vivax* field isolates from malaria endemic of Thailand

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Abstract

Two polymorphic marker genes; merozoite surface protein 3α (*MSP3α*) and merozoite surface protein 3β (*MSP3β*) of *Plasmodium vivax* 100 field isolates have been investigated using PCR-RFLP. Genotyping of *PvMSP3α* and *PvMSP3β* exhibited high polymorphism in length and sequence. Three major types of *PvMSP3α* (Type A, B, and C) and two major types of *PvMSP3β* (Type A, and B) were detected based on length of PCR products. Fourteen alleles of both genes were distinguished by restriction fragment length polymorphism, with difference frequencies. These results strongly support that *P. vivax* isolates are highly diverse. *PvMSP3α* and *PvMSP3β* are reliable polymorphic markers for population genetic analysis of *P. vivax* and PCR-RFLP provide a powerful method for genotyping and identification of mixed parasite infections without the need for sequencing

Keywords*:* polymorphic marker, genotyping, merozoite surface protein, *Plasmodium vivax*

Introduction

The widespread of drug resistant parasites has major consequences for future malaria control. Recently, *Plasmodium vivax* infections have become more prevent than *Plasmodium falciparum* in some regions of Thailand. Increasing trend of *P. vivax* malaria and the emergence of drug resistant parasites are a major concern for future malaria control. Both *P. falciparum* and *P. vivax* infection in malaria endemic region are consisting of multiple genotypes (Bruce *et al.,* 2000). Different genotypes exhibit distinctive biological characteristics. Polymorphic molecular markers such as Circumsporozoite Surface Protein (CSP) (Imwong *et al.,* 2005), Apical Membrane Antigen 1 (*AMA1*), Merozoite Surface Protein (*MSP*), Duffy Binding Protein (*DBP*) and microsatellites have been the powerful and easily deployable tool for assessing parasite genetic variation in *P. falciparum* and also for *P. vivax*. However, some polymorphic markers exhibit limited polymorphism. Molecular epidemiological studies require reliable polymorphic markers to understand the population structure of parasite and disease transmission. Molecular techniques have become more widely apply for field studies with limited resources. Using a combination of molecular markers to investigate the parasite genetic diversity of field populations displayed high level of genetic diversity. Understanding genetic diversity of *P. vivax* field isolate is essential for developing effective antimalarial drugs and vaccine.

The objective of this work was to characterize the polymorphic molecular markers in *P. vivax* isolates collected from malaria endemic areas of Thailand. These selected polymorphic marker genes were extensively investigate for genetic diversity study in *P. vivax* population.

Materials and Methods

1. Sample collection

P. vivax infected blood used for genotyping were collected from patients attending the Malaria Clinics in different geographical malaria endemic areas along the international border of Thailand-Myanmar and Thailand-Cambodia (Tak, Kanchanaburi, Ranong, Ratchaburi, and Chanthaburi provinces). Written inform consent for study participation was

obtained from all patients. This study protocol was reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. Following microscopic confirmation of blood films, approximately 200 ul of infected blood sample, irrespective of parasitaemia, were collected from finger-prick and doted onto the filter paper (Whatman 3MM). The dried filter paper samples were stored in small plastic zip lock bags prior to the extraction of parasite DNA and analysis of parasite genotypes by the polymerase chain reaction and Restriction fragment length polymorphism (PCR/RFLP). Extraction of parasite genomic DNA from individual dried blood spots on filter paper was carried out using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification.

2. Genotyping of *P. vivax MSP3α* and *MSP3β*

MSP3α and *MSP3β* primers and PCR condition were used according to the previous studies (Bruce et al., 1999, Cui et al., 2003, Yang *et al.,* 2006) with some modification. The amplified PCR products were then analyzed on 1.5% agarose gel. Restriction digestion was carried out in 20 ul reaction. The amplified PCR product of *MSP3α* and *MSP3β* were digested with enzymes *Hha I* and *Pst I* (5 units of enzyme/reaction; Promega) respectively, in buffer supplied with the enzymes at 37^{O}C for 4–5 hr. Restriction fragments were analyzed on 2% agarose gel.

Results

Genotyping of *P. vivax MSP-3α* locus.

Polymorphism of the *PvMSP3α* was studied using the PCR-RFLP method The *PvMSP3α* was amplified by a nested PCR and RFLP were analyzed using *Hha* I. The restriction patterns of PCR/RFLP were analyzed by 2% agarose gel electrophoresis. Based on the size of the PCR products, three major types, A (1.9 kb), B (1.5 kb), and C (1.1 kb), were identified with frequency 86%, 12% and 2%, respectively. A total of 100 isolates were classified into 14 distinguishable genotypes by restriction pattern (named subtype A1-A11, B1-B2, and C) (Figure 1, Table 1).

Genotyping of *P. vivax MSP-3β* locus.

PvMSP3 β fragments were amplified from 100 field samples collected from different malaria endemic areas in Thailand. The PCR products showed size polymorphism with two alleles size which were categorized as type A= 1.7-2.2 kb and type B = 1.4-1.5 kb. Type A is a predominant type, accounting for 81% while type B accounting for only 19%. RFLP typing by *PstI* digestion showed the 14 allele subtypes by restriction pattern (named subtype A1-A6, and B1-B8 (Figure 2, Table 1).

Table 1. *PvMSP3α,* and *PvMSP3 β* RFLP alleles types and their frequency

PvMSP3α	A1=19	A2=3	***PvMSP3 β***	A1=18	A2=9
	A3=4	A4=3		A3=14	A4=11
	A5=5	A6=3		A5=21	A6=8
	A7=26	A8=2		B1=2	B2=1
	A9=14	A10=1		B3= 1	B4=3
	A11=6	B1=10		B5=4	B6=3
	B2=2	C=2		B7= 3	B8=2

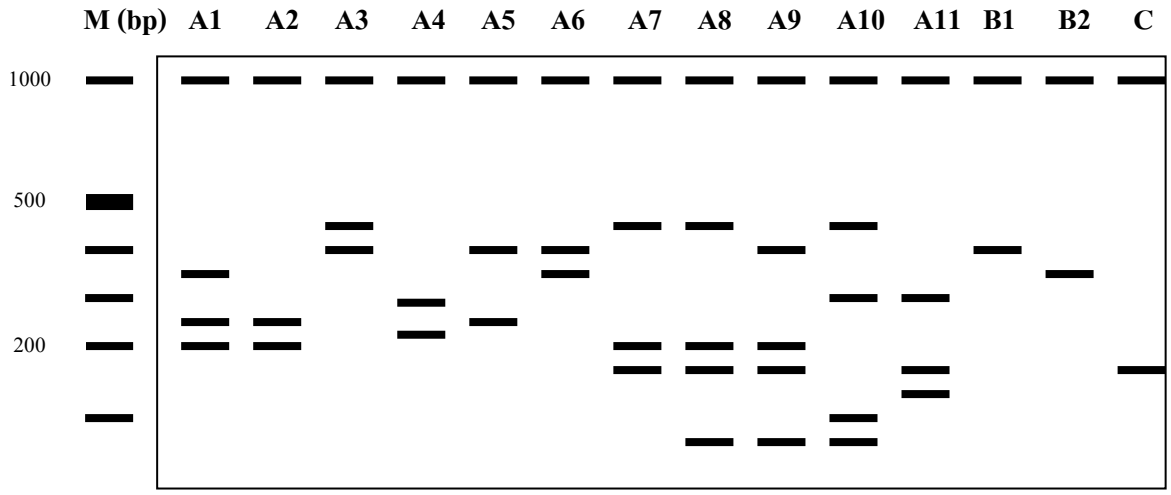

Figure 1. Schematic representation of *PvMSP3α* restriction patterns digested with *HhaI*

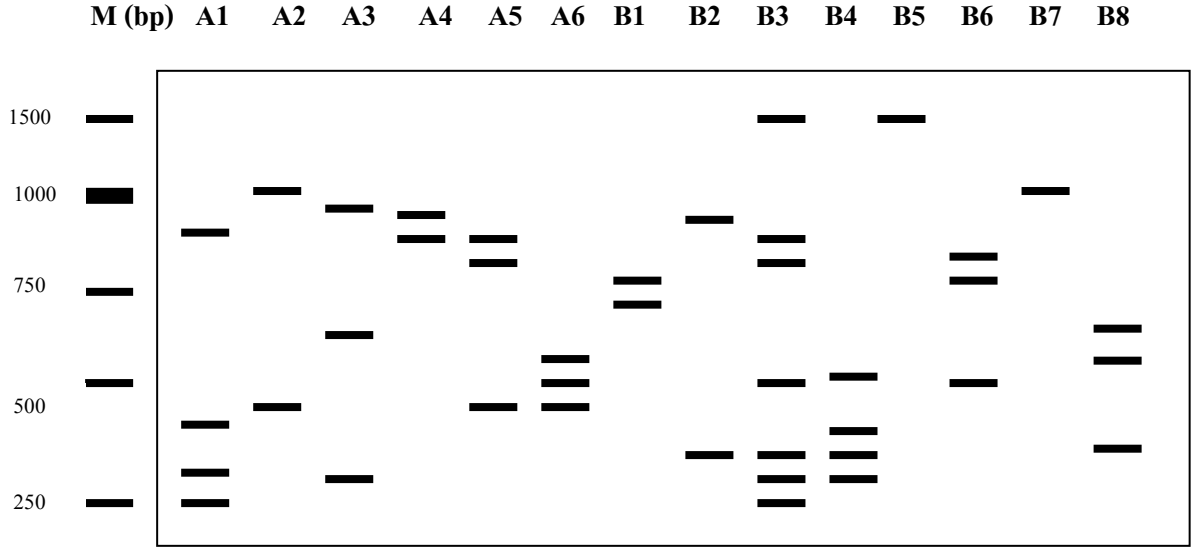

Figure 2. Schematic representation of *PvMSP3β* restriction patterns digested with *PstI*

Discussion

Polymorphic molecular markers have been described in malaria parasite, particularly polymorphic markers encoding parasite surface antigens that play an importance role in vaccine development. In addition, polymorphic molecular markers have been employed to evaluate genetic directly and distinguish parasite isolates for differentiating relapse and re-infections. PCR-RFLP methods are simple and available for parasite genotyping such as *PvMSP1, PvMSP3α, PvMSP3β*. and *PvCSP* genes.

Acknowledgement

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Original article **P73**

Vasorelaxant activity and inhibitory effects on leukocyte function of a flavanoid compound from *Kaempferia Parviflora*

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Abstract

The rhizomes of *Kaempferia parviflora (K. parviflora)* (Zingiberaceae) have been used in Thai traditional medicine for gastrointestinal disorders, pain, allergy, and inflammation. The alcoholic infusion of its rhizome has also been used as a tonic for rectifying male impotence. Ethanolic extract of *K. parviflora* was reported to exhibit acute anti-inflammatory activities in the rat paw edema model (unpublished data) and vasorelaxant activity in isolated rat aortic rings. The purposes of this study were to investigate the effects of a flavanoid compound from *K. Parviflora*, (VR-F6/1) on vasorelaxant activity, the expression of ICAM-1 on human umbilical vein endothelial cells (HUVEC), CD62-L and Mac-1 on neutrophils, human neutrophil functional responsiveness and T-lymphocyte proliferation. VR-F6/1 was primarily investigated for its cytotoxic effects on HUVECs, neutrophils and T-lymphocytes using XTT cytotoxicity assay. The vasorelaxant activity of VR-F6/1 was assessed using myography technique. The effects of VR-F6/1 on fMLP-induced L-selectin shedding and Mac-1 expression on neutrophils were assessed using flow cytometry. Human neutrophil functional responsiveness was determined by measuring fMLP-induced chemotaxis, superoxide anion generation (SAG), and MPO release. PHA-induced-T-lymphocyte proliferation was quantified by [H^3] thymidine incorporation. Viability of HUVECs, neutrophils, and T-lymphocytes were not significantly affected by VR- F6/1 (1-100 μg/ml) (IC_{50}>1000 μM). VR-F6/1 concentration-dependently caused acute relaxation of isolated mouse mesenteric arteries. VR-F6/1 (10 μM) inhibited ICAM-1 expression on HUVECs, and inhibited fMLP-induced L-selectin shedding and Mac-1 surface expression on neutrophils. VR-F6/1 concentration-dependently inhibited fMLP-induced chemotaxis, SAG, and MPO production in neutrophils. VR-F6/1 also significantly inhibited PHA-induced T-lymphocyte proliferation in a concentration-dependent manner. These findings suggest that anti-inflammatory activity of VR-F6/1 might be, in part, attributable to inhibition of ICAM-1 expression on HUVEC, inhibition of CD62L shedding and Mac-1 surface expression on neutrophils, neutrophil chemotaxis and SAG as well as T-lymphocyte proliferation. This study also demonstrates an acute vasorelaxant activity of VR-F6/1 where nitric oxide may mediate the effect.

Keywords: *K.Parviflora*, vasorelaxation, ICAM-1, neutrophil chemotaxis, superoxide anion generation, MPO, T-lymphocyte proliferation.

Introduction

The rhizomes of *Kaempferia parviflora* (Zingiberaceae) have been used in Thai traditional medicine for gastrointestinal disorders, pain, allergy, and inflammation. The alcoholic infusion of its rhizome has also been used as a tonic for rectifying male impotence. Ethanolic extract of *K. parviflora* was reported to exhibit acute anti-inflammatory activities in the rat paw edema model (unpublished data) and vasorelaxant activity in isolated rat aortic rings. *K. parviflora* contains many flavonoids that possess anti-inflammatory, anti-allergic and anti-oxidant activities. In this study, therefore, the effects of VR-F6/1 on TNFα-induced ICAM-1 expression on human umbilical vein endothelial cells (HUVEC), neutrophil

functional responsiveness including, fMLP-induced L-selectin shedding and Mac-1 expression, chemotaxis, SAG, and MPO production as well as PHA-induced T-lymphocyte proliferation were investigated in order to elucidate the cellular mechanisms of its anti-inflammatory activity. In addition, the effect of VR-F6/1 on vasorelaxant activity was also investigated.

Materials and Methods

Plant

The isolation of VR-F6/1 from ethyl acetate extract of *K. Parviflora* was performed by silicagel column chromatography using ethyl acetate and hexane as eluents. The derived fractions isolated from column chromatography were re-chromatographed and followed by crystallization in order to obtain the pure compounds.

Isolation of human neutrophils

Human neutrophils (PMNs) were isolated by Percoll density gradient centrifugation. Venous blood obtained from healthy donors, was mixed with an equal volume of Percoll, and the mixture centrifuged at 280*g* for 20 min. After centrifugation, PMN were harvested and washed. Any contaminating red cells were removed. The cells were >99% viable as determined by trypan blue exclusion and were resuspended as required.

Isolation of human T-lymphocytes

Human PBMC were isolated by Ficoll-Hypaque centrifugation. T-lymphocytes were isolated from PBMC by Rosetting with neuraminidase-treated sheep red blood cells (SRBC). PBMC were mixed with neuraminidase-treated SRBC and FCS, incubated then centrifuged. PBMC/FCS/SRBC mixture were layered over Ficoll-Hypaque and centrifuged. The E-rosette-positive population (T-cells) was separated from SRBC by hypotonic lysis. The cells were > 95% viable as determined by trypan blue exclusion.

Cytotoxicity assay (1)

VR-6/1 was primarily investigated for its cytotoxic effects on neutrophils, T-lymphocytes and HUVECs. Human neutrophils, T-lymphocytes and HUVECs were incubated with VR-F6/1 (0.1-10 μM) for 4 h, 3 days and 12h, respectively in 96-well plates, then XTT was added and incubated at 37°C for an additional 4 h. The XTT formazan product was measured spectrophotometrically at 450 nm.

Determination of Neutrophil Chemotaxis (2)

Human neutrophil chemotaxis can be assessed by measuring of neutrophil migration across the polyvinylcarbonate filter using chemotaxis chamber. The bottom wells of the chamber were filled with fMLP. The top plate with the installed filter was placed onto the filled bottom plate, and upper wells were filled with neutrophils treated with VR-F6/1 (0.1-10 μM). After incubation, the filter removed, washed, fixed and stained with DiffQuik TM. Chemotaxis was quantified spectrophotometrically by measuring absorbance at 550 nm. Indomethacin was used as a reference compound.

Determination of Superoxide Anion Generation (SAG) (3)

Neutrophil SAG is determined by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C in the presence of cytochalasin B**.** Neutrophils resuspended in PBS containing cytochrome C/cytochalasin B were preincubated with VR-F6/1 (0.1-10 μM) before stimulating with fMLP. The reaction was terminated followed by centrifugation. The absorbance of the supernatant from each tube was measured at 550 nm. Indomethacin was used as a standard reference compound.

Determination of Myeloperoxidase (MPO) production (4)

Neutrophils were preincubated with VR-F6/1 (0.1-10 μM) before stimulating with fMLP. After centrifugation, supernatants were incubated with the reaction mixture of

3,3',5,5'-Tetramethylbenzidine (TMB) supplemented with H_2O_2. The reaction was then stopped by adding H_2SO_4 and absorbance was measured spectrophotometrically at 450 nm. Indomethacin was used as a reference compound.

Determination of adhesion molecule expression on human neutrophils (5)

Neutrophils were preincubated with VR-F6/1 (0.1-10 μM) in incubator before stimulating with fMLP. After incubation, neutrophils were stained with CD62L-PE antibody and CD11b/Mac-1-APC antibody. The analysis of immunofluorescence as a measure of L-selectin (CD62L) and Mac-1 (CD11b/CD18) surface expression was performed on a FACS Calibur flow cytometer.

In vitro culture of human umbilical vein endothelial cells (HUVECs) and determination of ICAM-1 expression

HUVECs were cultures in 6-well plate in endothelial cell growth medium at 37ºC with 35% CO_2 in a humidified incubator. HUVECs incubated with VR-F6/1 (0.1-10 μM) for 10 h, then activated with TNF-α (50 ng/ml). Cells were trypsinized and counted, and then incubated with 2μl of antibody CD54-PE. The reaction was stopped by adding PBS. Cells were washed and the expression of ICAM-1 was measured using flow cytometry.

T-lymphocyte proliferation assay

T-lymphocytes were incubated with VR F6/1(0.1-100 μM) in the presence of 0.001% (w/v) PHA for 3 days at 37ºC with 5% CO_2. After incubation, the cells were labeled with [^{3}H] thymidine (0.5mCi/well). After a further incubation, the cells were harvested, washed, dried using Cell Harvester and radioactivity thymidine incorporation was determined by liquid scintillation counting.

Vasorelaxant activity

The vasorelaxant activity of VR-F6/1 was investigated using myography technique. Male mice were killed by cervical dislocation. The segments of third order branches of the mouse superior mesenteric arteries were removed, cut into rings (1.5 mm long) and mounted in a myograph chamber for measuring the isometric wall tension. The chamber was filled with physiological salt solution maintained at 37ºC and continuously bubbled with a 95% O_2 and 5% CO_2 mixture. After equilibration, the arteries were set to a circumference 90% of that obtained when the arteries were stretched. The rings were precontracted with phenylephrine and tested for the prescence of functional endothelium. The vasorelaxant effect of VR-F6/1 was tested by precontracting arteries with phenylephrine and then cumulatively adding VR-F6/1 ($3x10^{-8}$–$3x10^{-5}$M) to induce a concentration-dependent response. Relaxation is expressed as the percentage of decrease in the maximal tension induced by phenylephrine.

Results

Cytotoxic effects

The viability of neutrophils, T-lymphocytes and HUVECs was not affected by VR-F6/1 (0.1-10 μM) (IC_{50} > 1000μM, n=6).

Neutrophil Superoxide Anion Generation (SAG)

VR-F6/1 exerted strong inhibitory effects on fMLP-induced human neutrophil SAG (IC_{50} of 4.1±1.3 μM) compare with that of indomethacin (0.1-10μM), IC_{50} of 33.8± 6.2 μM (Fig.1).

Neutrophil chemotaxis

VR-F6/1 significantly suppressed fMLP-induced human neutrophil chemotaxis in a concentration-dependent manner with an IC_{50} of 3.23±1.1 μM. Indomethacin (0.1-100μM) exerted strong inhibition of fMLP-induced human neutrophil chemotaxis, with IC_{50} of 1.1 ± 0.3 μM (Fig.2)

Neutrophil MPO production

VR-F6/1 exerted very weak inhibitory effect on MPO production in human neutrophils induced by fMLP (IC_{50}>1000μM). Indomethacin (0.1-10μM) caused strong inhibition of fMLP-induced MPO production in human neutrophil, with IC_{50} of 42.9± 5.2 μM.

Neutrophil adhesion molecules expression

Activation of neutrophil by fMLP caused L-selectin shedding and increase in Mac-1 surface expression. VR-F6/1 at 10 μM statistically significant inhibited shedding of L-selectin and Mac-1 surface expression (Table 1).

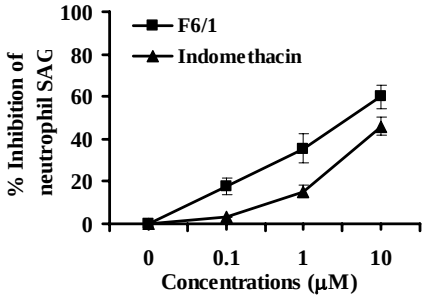

Figure 1. Inhibitory effects of VR-F6/1 (0.1-10μM) on fMLP- induced-SAG in human neutrophils Results are mean±S.E.M of 5 experiments using cells from different donors.

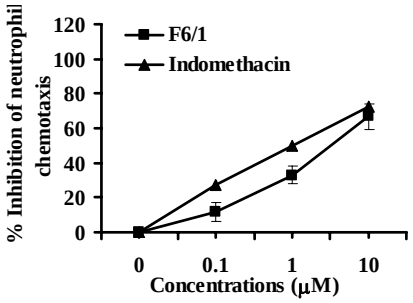

Figure 2 Inhibitory effects of VR-F6/1(0.1-10μM) on fMLP-induced chemotaxis in human neutrophils.Results are mean± S.E.M of 5 experiments using cells from different donors.

Intercellular adhesion molecule-1 (ICAM-1)

Activation of HUVECs by TNF-α (50ng/ml) caused an increase in ICAM-1 expression and this increase was suppressed by VR-F6/1 (1-10 μM) in a concentration–dependent manner (Table 2).

Table 1. Inhibitory effects of VR-F6/1 on fMLP-stimulated shedding of CD-62L (A) and Mac-1 surface expression on neutrophils (B). Results are expressed as means±S.E.M. of 3 independent experiments. *p<0.05 indicates a significant difference from fMLP- stimulated cells.

Concentration (μM)	A. Relative intensity (CD62L)			B. Relative intensity (Mac-1)		
	Negative	Positive	VR-F6/1	Negative	Positive	VR-F6/1
-	30.3±2.1	5.3±0.8	-	4.3±0.9	51.1± 3.4	-
1	-	-	6.8±1.6	-	-	47.1±2.4
10	-	-	12.0±3.2*	-	-	40.9± 3.4*

Table 2. Inhibitory effects of VR-F6/1 on TNF-α stimulated ICAM-1 expression in HUVECs. Results are expressed as means±S.E.M. of 3 independent experiments. *p<0.05 indicates a significant difference from fMLP- stimulated cells.

Concentration (μM)	Relative intensity (ICAM-1)		
	Negative	Positive	VR-F6/1
-	2.1±0.4	51.2±4.6	-
1	-		40.2± 4.4*
10	-		25.2± 3.8*

T-lymphocyte proliferation

VR-F6/1(0.1-100 μM) caused a concentration-dependent inhibition of PHA-induced T-lymphocyte proliferation with IC50 of 6.3 ± 0.4 μM (Fig. 3).

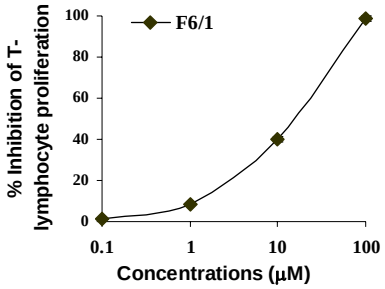

Figure 3. Log concentration-effect curves for inhibition of PHA-induced T-lymphocyte proliferation by VR-F6/1. Results are the mean ± S.E. mean of 5 different donors.

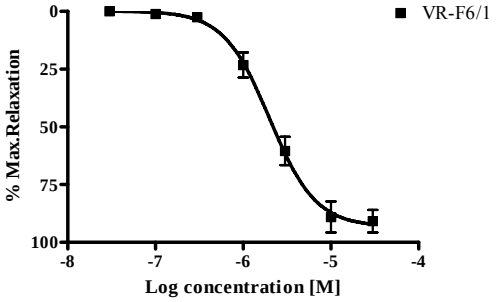

Figure 4. The concentration–dependent relaxant effect of VR-F6/1 on mesenteric artery precontracted by phenylephrine (10^{-5}M). Relaxation is expressed as the percentage of decrease in the maximal tension induced by phenylephrine. Results are the mean ± S.E. mean (N=4).

Vasorelaxant activity

VR-F6/1 ($3x10^{-8}$–$3x10^{-5}$M) produced a concentration-dependent relaxation of the phenylephrine-preconstricted mouse mesenteric arterial rings with endothelium-intact. The maximal relaxation and EC_{50} of VR-F6/1 were 90.7±3.8 % and 2.06±0.3μM, respectively, n=4, (Fig.4).

Discussion and Conclusion

In acute inflammation, neutrophils rapidly migrate to the site of injury and die through apoptosis during the resolution of inflammation. Chronic inflammation is characterized by infiltration of mononuclear cells. The activation of lymphocytes causes cytokine production and produces persistent inflammatory response. Neutrophil recruitment, into inflamed tissue proceeds via multi-step processes. Neutrophil emigration through endothelial cells involves several adhesion processes including cell rolling, arrest, and transmigration. Rolling is mediated by selectins, while arrest and transmigration both require activated integrins (LFA-1 (CD11 a/CD18) and Mac-1 (CD11 b/CD18). During neutrophil activation, L-selectin, which initially has a high basal expression, is rapidly down-regulated and causes up-regulated Mac-1 surface expression. Integrins and selectin ligands are also able to signal into the cell, where they initiate neutrophil extravasation, promote cytoskeletal rearrangement and ultimately induce superoxide production and degranulation.

VR-F6/1 (10μM) significantly inhibited fMLP-induced L-selectin shedding and an increase in Mac-1 surface expression. TNF-α –induced ICAM-1 expression on HUVECs was also inhibited by VR-F6/1. Pre-treatment of neutrophils with VR-F6/1 (0.1-10μM) significantly inhibited fMLP-induced chemotaxis and SAG. VR-F6/1 (0.1-100μM) also inhibited PHA-induced T-lymphocyte proliferation in a concentration-dependent manner. These findings suggest that the anti-inflammatory activity of VR-F6/1 from *K. Parviflora* extract may be attributed to its inhibitory effects on neutrophil functions and T-lymphocyte proliferation. However, further investigation is required to define the underlying molecular mechanisms of the inhibitory effects of this compound on neutrophil and T-lymphocyte function. Penile erection involves relaxation of smooth muscle of the corpus cavernosum and its associated arterioles. The alcoholic infusion of its rhizome has been used as a tonic for rectifying male impotence. This study demonstrated an acute, significant vasorelaxant activity of VR-F6/1 where nitric oxide may mediate the effect. Interestingly, its vasorelaxant activity may be one of the contributing factors for improving impotence problem in men.

However, further investigation to elucidate molecular mechanisms for its vasorelaxant activity is required.

Acknowledgements

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Original article **P74**

Comparative biological activities of five Thai medicinal plants called Hua-Khao-Yen

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Abstract

Plants named 'Hua-Khao-Yen' are common ingredients in traditional cancer remedies in Thailand and are best-selling medicinal plants in traditional drugstores. From the selective interview found that Hua-Khao-Yen was found to comprise at least five species such as *Dioscorea membranacea* Pierre (Dioscoreaceae) (DM), *D. burmanica* Prain ex Burkill (Dioscoreaceae) (DB), *Smilax corbularia* Kunth (Smilacaceae) (SC), *S. glabra* Roxb. (Smilacaceae) (SG), or *Pygmeopremna herbacea* Prain et Burkill (Verbenaceae)(PM). Three *in vitro* bioassay were used to compare activities of these plants such as cytotoxic , antioxidant and antimicrobial activities.The extract procedures used were similar to those practiced by Thai traditional doctors (water and ethanolic extraction). The SRB assay used to test cytotoxicity against two types of human liver cancer cell line (HepG2). The lipid peroxidation of liposomes assay was used to test for antioxidant activity of the 5 plant extracts. Disc diffusion method were used for antimicrobial activity. The results found that The ethanolic extracts of DB showed the highest cytotoxic activity against Hela (IC_{50}=28.060±6.338 μg/ml) and the ethanolic extracts of DM showed the highest cytotoxic activity against HepG2 (IC_{50}=18.539±2.371 μg/ml). The water extracts of these plants showed no cytotoxic activity against two types of human cancer cell line. The ethanolic extract of *Dioscorea membranacea* rhizome showed highest antioxidant activity (EC_{50}= 8.09 μg/ml). and also showed the highest activity against with *S. aureus* , *B.subtilis* and *E. floccosum*

Keywords**:** Hua-Khao-Yen, cytotoxic, antimicrobial, antioxidant.

Introduction

In Thai traditional medicines, herbal drugs named "Hua-Khao-Yen" have been long used as common ingredients in several preparations, including those used in the treatments of lymphopathy, dermopathy, venereal diseases, leprosy, and cancers. Interestingly, despite their close resemblance, the drugs available in traditional drug stores throughout the country are in fact rhizomes from different plant species from at least 3 genera and 5 species, *Dioscorea membranacea* Pierre (Dioscoreaceae) (DM), *D. burmanica* Prain ex Burkill (Dioscoreaceae) (DB), *Smilax corbularia* Kunth (Smilacaceae) (SC), *S. glabra* Roxb. (Smilacaceae) (SG), and *Pygmeopremna herbacea* Prain et Burkill (Verbenaceae)(PM). Among these, we found that the EtOH extract from the rhizome of *D. membranacea* Pierre was potently cytotoxic against various cancer cell lines, including COR-L23, LS-174T, MCF-7, and SVK-14[1]. However, no report about antimicrobial, antioxidant and cytotoxic activities of these plants against liver and cervical cancer cells.

The aim of this study was to compare antimicrobial, antioxidant and cytotoxic activities of five plant extracts against two types of human liver (HepG2) and cervical cancer cells.

Methods

Plant material and Preparation of extract

The part of plants, which were reported to be used against anticancer by folk doctors in Thailand, were collected from all parts of Thailand. Place of collection were all of part of Thailand, *Dioscorea membranacea* Pierre. (Chumporn), *Dioscorea burmanica* Prain ext Burkill. (Chuntaburee), *Smilax corbularia* Kunth.(Chiengmai), *Smilax glabra* Roxb.(Loui), *Pregmeopremna herbacea* Prain et Burkill (Ubonrajatanee). Authentications of plant materials were carried out at the herbarium of the Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept.

The plants material were dried at 50^{o}C, powdered and divided into two portions. The first portion (100 g of each plant) was boiled for 30 min in water and the filtrated was freeze dried to obtain water extract of each plant. The second portion (100 g of each plant) was macerated with 95% ethanol and the filtrated was evaporated to dryness under reduced pressure to obtain the ethanolic extract of each plants.

Antioxidant Assay by Lipid peroxidation of liposome

This assay are foolowed the method of Uchiyama and Mihara[2]. This assay used liposomes which were prepared from a bovine brain extract suspension in phosphate buffered saline (PBS) (5mg/ml), 0.1 ml $FeCl_3$, 0.1ml ascorbic acid (1mM), 0.5 ml PBS and 0.1 ml of ethanolic or water extract to be assessed. Propyl gallate ($1x10^{-4}$M) is positive control. All test tubes were incubated at 37°C for 20 minutes. The lipid peroxidation of liposomes should occur within that incubation period, unless the test substance exerted a protective antioxidant effect. The extract was prepared as 0.5, 0.1, 0.05, 0.01 and 0.05 mg/ml. Four replicates were carried out for each mixture. The TBA test was performed after the 20 minute incubation at the end of this incubation period at 85°C, thiobarbituric acid should have formed a coloured adduct with malonaldehyde and measure by spectroscopy at 532 nm .

In vitro assay for cytotoxic activity

The cytotoxicity assay was carried out using sulphorhodamine B (SRB) assay[3] (Skehan *et al*., 1990). Two different types of human cell lines were used i.e. liver carcinoma (HepG2) and cervical carcinoma (Hela) . The monolayered culture of each cell line were seeded in 96-well microtiter plate and incubated to allow for cell attachment (18-24 hours). Then treated cell with 4 serial dilution and 6 replications. The plates were incubated for the exposure time at 72 hours, then the medium was removed and added the new medium. The plates were incubated for recovery period of 72 hours. The survival percentage was measured colorimetrically using SRB assay and the IC_{50} values (effective concentration of sample required to inhibit cell growth by 50%) was calculated from dose-response curves plotting between %inhibition and concentrations by Prism program. According to American National Cancer Institute (NCI) guidelines [4](Suffness and Pezzuto, 1990) crude extract with an IC_{50} values < 30 μg/ml were considered active.

Antimicrobial assay [5,6]

In the preliminary studies, all extracts were evaluated for antibacterial activity by disc diffusion method. All extracts were tested against two types of gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), two type of gram negative bacteria (*Escherichia coli* and *Pseudomonas aeroginosa*) and two type of fungi (*Candida albicans* and *Epidermophyton floccosum*). The active plant extracts were diluted to determine the minimum inhibitory concentration (MIC).

Results and discussion

Water and ethanolic extracts from five species of Thai medicinal plants named Hua-Khao-Yen were investigated antioxidant and cytotoxic activities (Table 1, Fig.1 and Fig. 2).

The results indicated that the ethanolic extract of *Dioscorea membranacea* possessed the highest antioxidant activity by DPPH assay with an EC_{50} value of 8.1±1.21 µg/ml, followed by the ethanolic extract of *Dioscorea burmanica* and *Smilax glabra* (EC_{50} = 16.13 ±3.70, 32.91± 2.02µg/ml respectively). Two ethanolic extract of *Dioscorea membranacea and Dioscorea birmanica* exhibited high cytotoxic against both cancer cells

Table 1 % yield, antioxidant as EC_{50} (µg/ml) ± SEM, cytotoxic as IC_{50} (µg/ml) ± SEM of extracts from five plants called Hua-Khao-Yen against cancer cell lines at exposure time 72 hours (n=3)

Plant extract	% yield	Antioxidant	Cytotoxic	
		Lipid Peroxidation	HepG2	Hela
DM(W)	21.9	742.17± 0.05	>100	>100
DM(Et)	3.4	8.10±1.21	18.539±2.37	32.435±3.152
DB (W)	15.0	334.57±0.05	>100	>100
DB(Et)	3.1	16.13 ±3.70	33.67±0.37	28.060±6.338
SC(W)	9.2	185.65 ± 0.05	>100	>100
SC(Et)	12.03	46.87± 7.50	85.78±1.45	>100
SG(W)	7.2	94.95 ± 0.01	>100	>100
SG(Et)	6.4	32.91± 2.02	>100	>100
PM(W)	6.4	2470.73±0.90	>100	>100
PM(Et)	7.2	754.12 ± 2.40	>100	>100

Note: DM=*Dioscorea membranacea*, DB=*Dioscorea burmanica*, SC=*Smilax corbularia*, SG=*Smilax glabra*, PM=,*Pygmaeopremna herbacea*, W.= Water extract, Et.= Ethanolic extract

Table 2: Antibacterial and antifugal activity screening of the crude extract of all kinds of Hua-Khao Yen at concentration of extract 25mg/ml

Plant meterial	Inhibition zone diameter (mm)					
	E.c.	*B.s.*	*S.a.*	*P.a.*	*C.a.*	*E.f.*
DM(E)	-	14±0.2	14±0.1	-	-	+
DM(W)	-	-	-	-	-	+
DB(E)	-	-	9	-	-	+
DB(W)	-	-	-	-	-	+
SMC(E)	-	10±0.3	13±0.5	-	-	-
SMC(W)	-	-	13±0.0	-	-	+
SMG(E)	-	10±0.1	12±0.4	-	-	-
SMG(W)	-	-	-	-	-	-
PM(E)	-	-	-	-	-	-
PM(W)	-	-	-	-	-	-
Ampicillin(0.003mg/ml)	16±0.0	25.0±0.1	30±0.3	-	NT	NT

Note: Diameter of inhibition zones in millimeters are means of triplicates;-no inhibition,+ inhibit fungi growing ;*E.c.: Escherichia coli; B.s:.Bacillus subtilis; S.a.: Staphylococcus aureus; P.a.:Pseudomonas aeroginosa.; C.a:Candida albican; E.f:Epidermotophytum floccosum* NT= not test, DM : *Dioscorea membranacea*, DB: *Dioscorea birmanica*, SMC: *Smilax corbularia*, SG: *Smilax glabra*, PM: *Pygmaeopremna herbacea*, DMC, DMM, and DMW are chloroform , methanol and water fraction of *Dioscorea membranacea*; E: ethanolic extract, W: water extract.

The results of antibacterial and antifungal screening shown in table 2 indicate that the ethanolic extract of the two genera of *Dioscorea* and *Smilax* showed antibacterial effects

against gram positive bacteria (*S.aureus* and *B.subtilis*) with *D. membrancea* having the highest activity. The result of screening, which was confirmed testing for minimum inhibition concentration (MIC) (Table 3) found that the ethanolic extract of *Dioscorea membranacea* showed the highest antimicrobial activity against gram positive bacteria (*S. aureus*) and fungi (*E. floccosum*). For the two *Smilax* species it was found that the ethanolic extract of *Smilax glabra* exhibited higher antibacterial against *S. aureus, B. subtilis* and *E. coli* than *S. corbularia.* Conversely the water extract of *Smilax corbularia* showed higher antimicrobial activity than *Smilax glabra* and it exhibited antifungal activity against with *E. floccosum*

Table 3: MIC (mg/ml) for extracts of all kinds of Hua-Khao-Yen on antibacterial and antifungal activity

Plant extracts	*S.aureus*	*B.subtilis*	*E.coli*	*C.albicans*	*E.floccosum*
DM(E)	<1.25	<1.25	2.5	>10	<1.25
DB(E)	<1.25	<2.5	5	>10	>10
SC(E)	<1.25	5	2.5	>10	>10
SC(W)	<1.25	5	5	>10	<1.25
SG(E)	<1.25	<5	2.5	>10	>10
SG(W)	<1.25	>10	5	>10	>10

Note: MIC was regarded as the lowest concentration showing no bacterial growth after 24 hours and fungal growth after 7 days.

Conclusion

Cytotoxic activity screening of five species extracts by using the SRB assay was carried out against three human cell lines i.e. HepG2 and Hela. The results found that the ethanolic extracts of Dioscorea birmanica showed the highest cytotoxic activity against cervical cancer and the ethanolic extracts of Dioscore membranacea showed the highest cytotoxic activity against liver cancer cells. The water extracts of all plants showed no cytotoxic activity against two types of human cancer cell line. The ethanolic extract of *Dioscorea membranacea* rhizome showed highest antioxidant activity and also showed the highest activity against with *S. aureus*, *B.subtilis* and *E. floccosum*

This result conclude that *Dioscorea membranacea* showed cytotoxic against cancer cell and also have antioxidant and antimicrobial activity. Thus *this plant was supported* for using of folk doctors for treatment cancer.

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