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บรรณาธิการแถลง

เรียนสมาชิกทุกท่าน

วารสาร Thai Journal of Pharmacology โดยสมาคมเภสัชวิทยาแห่งประเทศไทยฉบับนี้มีเนื้อหาหลากหลายครอบคลุมหลายด้าน โดยเฉพาะในเรื่องสมุนไพรและพืชพื้นบ้านไม่ว่าจะเป็น Ling Zhi, Malva Nut, Mangosteen, Curcuminoids, *Centella asiatica*, *Senna alata*, *Passiflora foetida*, *Mitragyna speciosa*, *Vernonia cinerea* Less., *Elephantopus Scaber* Linn., *Aegle marmelos* (L.) Correa ex Roxb., *Ocimum gratissimum* Linn., Citrus Peels, *Derris scandens Benth*, *Citrus reticulata* Blanco และอีกมากมาย หากรวมกับฉบับที่แล้วคิดว่าน่าจะประมาณ 30-40 ชนิดทีเดียว หากได้มีการประสานงาน ร่วมมือแลกเปลี่ยนในเชิงเทคนิค รูปแบบ model ต่างๆ ในการวิจัย ก็น่าจะนำไปสู่ประโยชน์และความสำเร็จของการวิจัยได้มากยิ่งขึ้น นอกจากเรื่องดังกล่าวแล้วยังมีหัวข้ออื่นที่เกี่ยวข้องเช่น pharmacogenetics, pharmacokinetics, pharmacodynamics]h, molecular pharmacology ซึ่งน่าสนใจเป็นอย่างยิ่ง

เป็นที่น่ายินดีว่าวารสาร Thai Journal of Pharmacology ได้รับการยอมรับในฐานข้อมูลของศูนย์ดัชนีการอ้างอิงวารสารไทย (Thai citation index) ดังนั้นในก้าวต่อไปจึงเป็นความพยายามที่จะยกระดับมาตรฐานให้สูงขึ้นเพื่อให้มี impact factor ที่ดี จึงหวังเป็นอย่างยิ่งว่าจะได้รับความร่วมมือจากทุกท่านอย่างเช่นเคยและตลอดไป หากมีข้อเสนอแนะใดๆ คณะบรรณาธิการวารสารขอน้อมรับด้วยความยินดียิ่ง

รองศาสตราจารย์ ดร.ลัดดาวัลย์ ผิวทองงาม

บรรณาธิการวารสาร

RESEARCH ARTICLE

Content of Ganoderic Acids A and F in Ling Zhi Preperations Available in Thailand

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Abstract

The objectives of this study were to determine and compare the content of ganoderic acids A and F in various Ling Zhi preparations available in Thailand. Seventeen samples of commercial Ling Zhi preparations in various brands and dosage forms were randomly purchased from different stores in Chiang Mai and Bangkok, Thailand. Each Ling Zhi preparation was given the sample code instead of its trade name. The investigated preparations included the sliced fruiting bodies (MG2FB) and the water extract of fruiting bodies of MG2-strain (MG2FB-WE) kindly provided from Muang Ngai Special Agricultural Project. The content of ganoderic acids A and F in all Ling Zhi preparations was determined by high performance liquid chromatography (HPLC). The limits of quantification (LOQ) of ganoderic acids A and F were 2.21 and 2.03 μg/mL, respectively. In 19 investigated Ling Zhi preparations, NPN capsule had the highest content of total ganoderic acids A and F (8723.10 ± 146.53 μg/g), followed by MG2FB-WE (3980.01 ± 28.34 μg/g) and DXN-r (2625.77 ± 26.04 μg/g), respectively. GNO had the lowest content of total ganoderic acids A and F (233.80 ± 33.33 μg/g). Ganoderic acid A was the major compound in most Ling Zhi preparations, except NPN capsule in which ganoderic acid F was the major compound. Neither ganoderic acid A nor F was detected in GEC, DXN-g capsules and powder of instant BNR. The total content of ganoderic acids in commercially available Ling Zhi preparations was not statistically correlated with their price. The content of ganoderic acids A and F varied considerably among investigated Ling Zhi preparations. It ranged from below the LOQ to a remarkably high content.

Keywords Ganoderic acid A, Ganoderic acid F, Ling Zhi preparations

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ปริมาณของกาโนเดอริกแอซิดเอและเอฟ ในผลิตภัณฑ์เห็ดหลินจือที่จำหน่ายในประเทศไทย

ศศินันท์ สัจจา[1], ณัฏฐกานติ์ จิรัณธนัฐ[1,2], ชัยชาญ แสงดี[1], บุญเยี่ยม คำสอน[1], ศุภนิมิต ทีฆชุณหเถียร[1,2]

[1]ภาควิชาเภสัชวิทยา, [2]ศูนย์การแพทย์แผนไทยและการแพทย์ผสมผสาน คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ เชียงใหม่

บทคัดย่อ

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อตรวจวัดและเปรียบเทียบปริมาณของกาโนเดอริกแอซิดเอและเอฟ ในผลิตภัณฑ์เห็ดหลินจือต่าง ๆ ที่จำหน่ายในประเทศไทย โดยสุ่มซื้อตัวอย่างผลิตภัณฑ์เห็ดหลินจือที่เตรียมในรูปแบบแตกต่างกันจำนวน 17 ผลิตภัณฑ์จากแหล่งจำหน่ายในจังหวัดเชียงใหม่และกรุงเทพฯ ประเทศไทย ซึ่งแต่ละผลิตภัณฑ์จะได้รับการตั้งรหัสเพื่อใช้เรียกแทนชื่อการค้า นอกจากนี้ยังได้นำตัวอย่างผลิตภัณฑ์รูปแบบดอกเห็ดอบแห้งฝาน (MG2FB) และสารสกัดน้ำจากดอกเห็ดหลินจือสายพันธุ์ MG2 (MG2FB-WE) ของโครงการพิเศษสวนเกษตรเมืองงายฯ มารวมไว้ในการศึกษาครั้งนี้ด้วย จากนั้นนำผลิตภัณฑ์เห็ดหลินจือแต่ละตัวอย่างมาทำการตรวจวัดปริมาณของกาโนเดอริกแอซิดเอและเอฟโดยใช้วิธีโครมาโตกราฟีเหลวสมรรถนะสูง (High performance liquid chromatography, HPLC) ซึ่งเทคนิคการตรวจวัดดังกล่าวมีค่าขีดจำกัดของการวิเคราะห์ปริมาณ (Limit of quantification, LOQ) ของกาโนเดอริกแอซิดเอและเอฟเท่ากับ 2.21 และ 2.03 μg/mL ตามลำดับ จาก 19 ตัวอย่างผลิตภัณฑ์เห็ดหลินจือที่ทำการศึกษาพบว่า NPN แคปซูลมีปริมาณรวมของกาโนเดอริกแอซิดเอและเอฟสูงที่สุด (8723.10 ± 146.53 μg/g) รองลงมาคือสารสกัดน้ำจากดอกเห็ดหลินจือสายพันธุ์ MG2 (3980.01 ± 28.34 μg/g) และ DXN-r แคปซูล (2625.77 ± 26.04 μg/g) ตามลำดับ ขณะที่ชาชงผสมเห็ดหลินจือ GNO มีปริมาณรวมของกาโนเดอริกแอซิดเอและเอฟต่ำที่สุด (233.80 ± 33.33 μg/g) ทั้งนี้กาโนเดอริกแอซิดเอเป็นสารหลักที่พบในผลิตภัณฑ์เห็ดหลินจือที่ทำการศึกษา ยกเว้น NPN แคปซูลที่มีปริมาณของกาโนเดอริกแอซิดเอฟสูงกว่า อย่างไรก็ตามไม่พบสารสำคัญทั้งสองชนิดในแคปซูล GEC, DXN-g และเครื่องดื่มเห็ดหลินจือผงสำเร็จ BNR ทั้งนี้ปริมาณรวมของกาโนเดอริกแอซิดดังกล่าวไม่มีความสัมพันธ์กับราคาของผลิตภัณฑ์อย่างมีนัยสำคัญทางสถิติ กาโนเดอริกแอซิดเอและเอฟในผลิตภัณฑ์เห็ดหลินจือต่าง ๆ ที่นำมาศึกษามีปริมาณที่แตกต่างกันอย่างมาก กล่าวคือมีตั้งแต่ระดับที่ต่ำกว่าค่า LOQ ของการตรวจวัดด้วยวิธี HPLC ไปถึงระดับที่สูงมาก

คำสำคัญ กาโนเดอริกแอซิดเอ, กาโนเดอริกแอซิดเอฟ, ผลิตภัณฑ์เห็ดหลินจือ

Introduction

The fruiting bodies of *Ganoderma lucidum* (Fr.) Karst (Ganodermataceae) known as Ling Zhi in China, one of the most famous traditional Chinese medicinal mushrooms, has been used extensively to preserve human vitality and to promote longevity in China and other eastern Asian countries for thousands of years[1-3]. Although it is still not clear about Ling Zhi's mechanism on human vitality and health promotion, Ling Zhi has been used for the prevention or treatment of various conditions and diseases such as anorexia, neurasthenia, insomnia, migraine, asthma, allergy, bronchitis, gastritis, hepatitis, nephritis, arthritis, lupus erythematosus, hypertension, diabetes, hypercholesterolemia, cardio-vascular problems, as well as cancers[2-4].

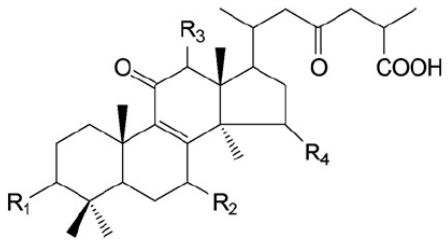
Modern researchers have revealed that Ling Zhi contains a variety of phyto-chemical compounds. One of the potent biologically active compounds that has been shown to possess diverse and potentially significant pharmacological activities is the bitter triterpenes[3-5]. Since the first discovery of ganoderic acids A and B, more than 130 types of triterpenes have been isolated from various parts of Ling Zhi[3-5], among which ganoderic acids A and F (Fig. 1) have received considerable attention due to their conspicuous pharmacological activities, e.g., antihypertensive activity[6], antinociceptive activity[7], antioxidative activity[8], enzyme-inhibitory activity on farnesyl protein transferase[9], hepatoprotective activity[10,11], especially anticancer activity[12-15] which is the most attractive character of this medicinal mushroom.

Owing to these potential medical values, Ling Zhi has been increasingly cultivated and used as a health supplement and herbal medicine worldwide including Thailand[2,4]. There are several commercial Ling Zhi preparations available in various dosage forms such as Ling Zhi extract, spore, tea bag, instant tea, and sliced fruiting bodies. Although several lines of scientific data supporting various *in vitro* and *in vivo* pharmacological activities of Ling Zhi have been extensively documented, quantitative analysis of its biologically active compounds in their preparations has not yet been widely investigated. Therefore, the purposes of this study were to determine and compare the amount of ganoderic acids A and F, the potent biologically active compounds, in various Ling Zhi preparations available in Thailand.

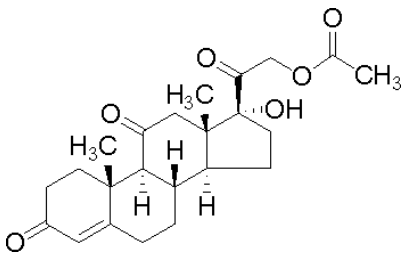
Materials and methods

Ling Zhi preparations

Seventeen commercial Ling Zhi preparations in various brands and dosage forms including Ling Zhi capsules (8 preparations), tea (5 preparations) and sliced fruiting bodies (4 preparations) were randomly purchased from different stores in Chiang Mai and Bangkok, Thailand. Each preparation was given the sample code instead of its trade name. In addition, the sliced fruiting bodies (MG2FB) and the water extract of fruiting bodies of MG2-strain (MG2FB-WE) kindly provided by Muang Ngai Special Agricultural Project under the patronage of Her Majesty Queen Sirikit, Chiang Mai, were also included in the determination of ganoderic acids A and F.



Ganoderic acid A: R_1=O, R_2=β-OH, R_3=H, R_4=α-OH
Ganoderic acid F: R_1=R_2=R_4=O, R_3=β-OAc



Cortisone 21-acetate
(Internal standard)

Figure 1 Structures of ganoderic acids A and F, as well as cortisone 21-acetate used as internal standard.

Chromatographic system and conditions

A Shimadzu (Shimadzu Ltd., Kyoto, Japan) HPLC system equipped with two LC-10ADvp pumps, DGU-14A degasser, SIL-10ADvp auto injector, CTO-10ASvp column oven, SPD-M10Avp diode array detector, and SCL-10Avp system controller was used. Its stationary phase consisted of an Inertsil ODS-3-C_{18} analytical column (250×4.6 mm, 5 µm) connected to an Inertsil ODS-3 guard column (50×4 mm, 5 µm) and maintained at 50 ˚C. The detecting wavelength was set at 252 nm. The mobile phase consisted of solvent A [20 mM ammonium acetate and 0.2 mM perchloric acid in deionized water/ acetonitrile/methanol (250/60/1.5, v/v/v)] and solvent B [20 mM ammonium acetate and 0.2 mM perchloric acid in deionized water/ acetonitrile/methanol (250/150/1.5, v/v/v)]. A gradient elution of 35% B for 15 min, 60% B at 15-35 min and 100% B at 35-55 min was scheduled. The flow rate was maintained at 1.0 mL/min. Chromatographic data were analyzed using the Shimadzu Class-VP software.

Preparation of standard solutions

The stock solution of ganoderic acids A and F was prepared by dissolving each reference substance in 90% methanol to the final concentration of 500,000 ng/mL, and then the stock solution was subsequently diluted in the same diluents to give the seven respective concentrations (2.5, 5, 10, 25, 50, 100 and 200 µg/mL) for establishment of calibration curves. The internal standard (IS) solution was prepared by dissolving cortisone 21-acetate (Fig. 1) in the same diluents at a concentration of 100,000 ng/mL. All the stock solutions were stored at -20 ˚C.

Sample preparation of Ling Zhi products

The sample extraction was modified from the method described by Wang *et al*[16]. Briefly, 50 mg of Ling Zhi from each preparation was extracted with 1 mL of 95% methanol in an ultrasonic water bath for 60 min. The extraction solution was then centrifuged at 14,000 rpm for 5 min at room temperature. Thereafter, 10 µL of clear supernatant was spiked with 10 µL of IS (20.00 µg/mL of cortisone 21-acetate) and diluted with 30 µL of mobile phase B. Aliquot of 10 µL of each sample solution was injected onto the HPLC system. The content of ganoderic acids A and F in each Ling Zhi preparation was determined from a calibration curve and linear regression of the seven known concentrations of ganoderic acids A or F, *versus* the peak area ratios of corresponding ganoderic acids and IS.

Assay validation

The intra- and inter-day assay validation was performed to verify the precision of HPLC method. Intra-day assay validation was determined by analyzing five repetitions of the standard mixtures of ganoderic acids A and F at three different concentrations (7.5, 90, 180 µg/mL) on the same day. Inter-day assay validation was determined by analyzing five repetitions of these standards on the three independent

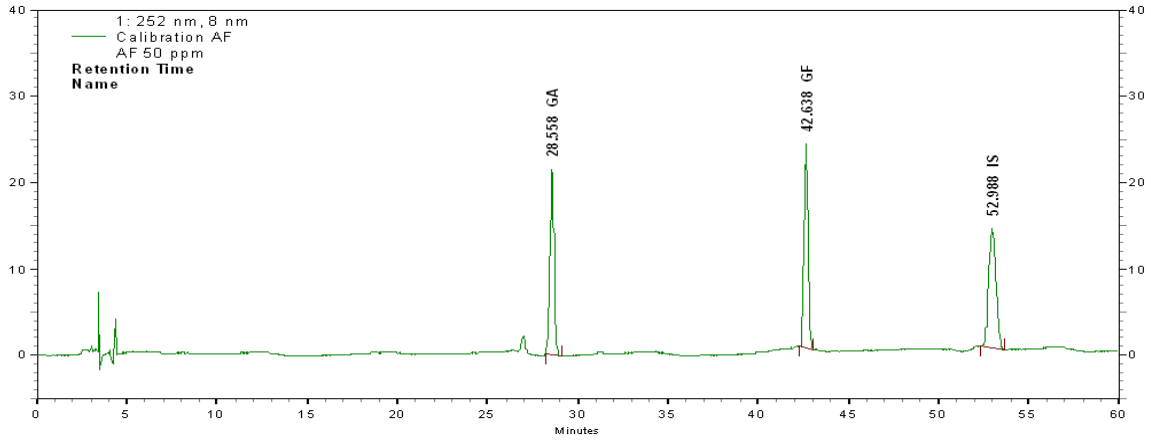


Figure 2 HPLC chromatogram from standard mixture of ganoderic acid A (GA, 50 µg/mL), ganoderic acid F (GF, 50 µg/mL) and IS (20 µg/mL).

Table 1 Regression equations, correlation coefficients (r^2), linear ranges, limits of detection (LOD) and limits of quantification (LOQ) of ganoderic acids A and F under the chromatographic condition used in this study.

Compound	Regression equation	r^2	Linear range (µg/mL)	LOD[#] (µg/mL)	LOQ[§] (µg/mL)
Ganoderic acid A	y = 20.4114x - 0.1285	1.0000	2.5-200	0.73	2.21
Ganoderic acid F	y = 18.0097x + 3.2104	1.0000	2.5-200	0.67	2.03

[#] LOD was determined based on signal-to-noise ratio (S/N) of 3:1
[§] LOQ was determined based on S/N of 10:1

days. The percentage of relative standard deviation (% RSD), calculated from (standard deviation/mean of the measurements) x100, was taken as a measure of precision.

Statistical analysis

The individual and total content of ganoderic acids A and F in each Ling Zhi preparation was presented as mean ± standard deviation (SD) and descriptive analysis was used to summarize the data and consolidate a mass of numerical data into significant information. Correlation coefficient value calculated by linear regression analysis was used to evaluate the correlation between total content of ganoderic acids A and F in 17 commercially available Ling Zhi preparations and their prices.

Results

The HPLC chromatogram obtained from the standard mixture of ganoderic acids A, F and IS is shown in Figure 2. Ganoderic acid A was eluted first from the HPLC system, followed by ganoderic acid F and IS with the retention time of 28.56, 42.64 and 52.99 min, respectively. All peaks were clearly separated and no peak interference was observed at the retention times of ganoderic acids A and F as well as IS. The regression equations, correlation coefficients (r^2), linear ranges, limits of detection (LOD) and limits of quantification (LOQ) of ganoderic acids A and F under the chromatographic conditions used in this study are presented in Table 1. The linear regression of both ganoderic acids A and F exhibited good linearity within the test range. The % RSD of intra- and inter-day assay validation for ganoderic acids A and F were all less than 2% (Table 2), indicating acceptable precision of the developed analytical method.

The content of ganoderic acids A and F in the 19 investigated Ling Zhi preparations including MG2FB and MG2FB-WE is shown in Table 3. Ganoderic acids A and/or F were detected in 16 out of 19 preparations.

The ratios of ganoderic acids A to F content in almost all preparations were approximately 1.1-3.5, indicating that ganoderic acid A was the major compound in comparison to ganoderic acid F, except for NPN in which ganoderic acid F was the major compound. However, only ganoderic acid A was detected in GNO. Among the 16 Ling Zhi preparations in which ganoderic acid(s) existed, the first 3 preparations containing the highest content of total ganoderic acids were those of 100% Ling Zhi extract namely NPN, MG2FB-WE and DXN-r, respectively. The HPLC chromatograms of these preparations are shown in Figure 3.

It is worth noting that the different dosage forms of 100% crushed Ling Zhi or 100% Ling Zhi fruiting bodies under the same trade name (DHP-c, DHP-t and DHP-s as well as DTG-t and DTG-s) demonstrated the comparable content of total ganoderic acids, as well as the comparable ratios of ganoderic acids A to F content (i.e., 2.0-2.2 for DHP and 2.3-2.6 for DTG). In contrast, the total ganoderic acids content of different dosage forms of 100% Ling Zhi extract *versus* 100% Ling Zhi mycelium and sprout extract under the same trade name (DXN-r *versus* DXN-g) were considerably different, since DXN-r possessed a remarkably high ganoderic content whereas neither ganoderic acid A nor F was detected in DXN-g.

Table 2 Intra- and inter-day assay validation of ganoderic acids A and F.

Compound	Concentration (μg/mL)	Intra-day precision		Inter-day precision				
				Day 1	Day 2	Day 3	Overall	
		Mean ± SD[1]	% RSD[§]	Mean ± SD[1]	Mean ± SD[1]	Mean ± SD[1]	Mean ± SD[2]	% RSD[§]
Ganoderic acid A	7.5	7.33 ± 0.05	0.74	7.33 ± 0.12	7.47 ± 0.13	7.33 ± 0.05	7.38 ± 0.08	1.10
	90	89.04 ± 0.59	0.66	88.61 ± 0.88	89.47 ± 0.56	89.04 ± 0.59	89.04 ± 0.43	0.48
	180	183.36 ± 1.31	0.71	179.73 ± 1.79	180.82 ± 1.54	183.36 ± 1.31	181.30 ± 1.86	1.03
Ganoderic acid F	7.5	7.31 ± 0.07	0.89	7.12 ± 0.10	7.35 ± 0.14	7.31 ± 0.07	7.26 ± 0.12	1.69
	90	87.58 ± 0.79	0.90	87.25 ± 0.86	88.18 ± 0.62	87.58 ± 0.79	87.67 ± 0.47	0.54
	180	180.34 ± 1.43	0.80	177.29 ± 1.35	178.81 ± 1.87	180.34 ± 1.43	178.81 ± 1.53	0.85

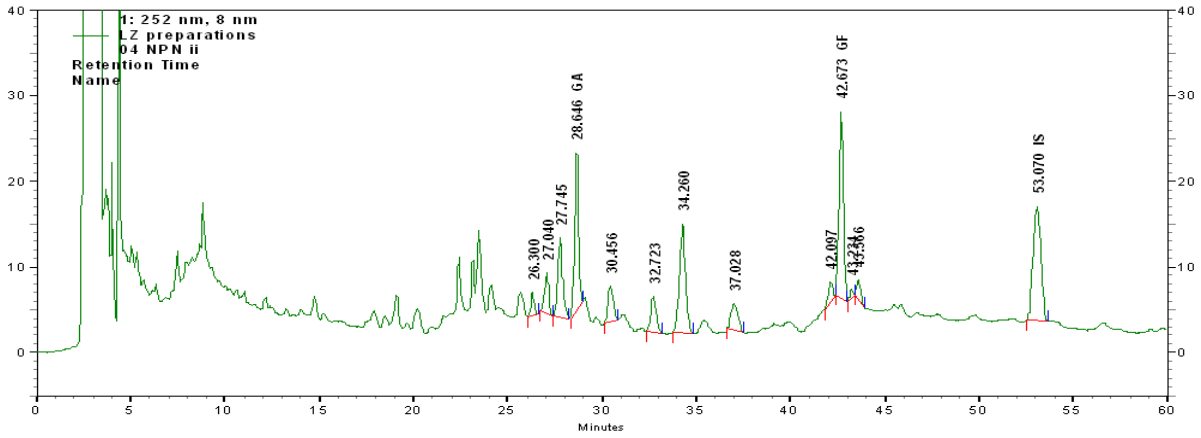
[§] Percentage of relative standard deviation (% RSD) = (Standard deviation/mean of the measurements) x100
[1] Data represents mean ± SD of the five repetitions of the corresponding concentrations of ganoderic acids analyzed on the specific day
[2] Data represents the overall mean ± SD averaged from the mean values of day 1, 2 and 3

Table 3 The content of ganoderic acids A and F in Ling Zhi preparations investigated in this study[§].

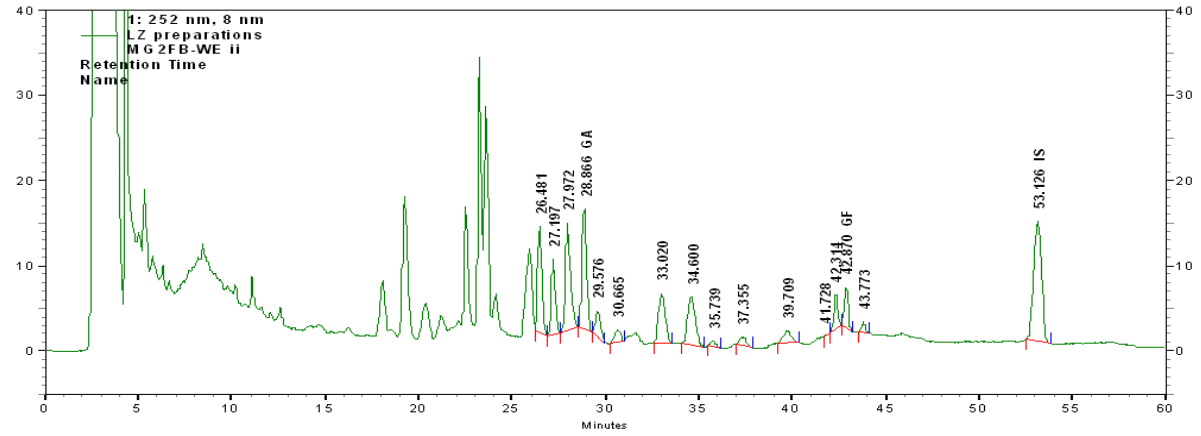
Preparation	Sample No	Sample code	Constituents	Price (baht/g)	Ganoderic acid A (µg/g)	Ganoderic acid F (µg/g)	Total ganoderic acids[δ] (µg/g)
Powder from capsule or granule from extract	1	NPN	100% Ling Zhi extract	66.67	3992.88 ± 63.17	4730.21 ± 84.43	8723.10 ± 146.53
	2	MG2FB-WE	100% Ling Zhi extract	*	3100.63 ± 62.11	879.39 ± 84.52	3980.01 ± 28.34
	3	DXN-r	100% Ling Zhi extract	38.27	1361.55 ± 11.23	1264.22 ± 14.84	2625.77 ± 26.04
	4	DHP-c	100% crushed Ling Zhi	6.67	802.83 ± 5.84	369.51 ± 7.02	1172.31 ± 7.07
	5	HAV	50% broken spore, 50% Ling Zhi water extract	160.00	791.92 ± 26.35	274.62 ± 8.31	1066.55 ± 21.32
	6	TYP	100% crushed Ling Zhi	3.75	699.57 ± 39.36	359.71 ± 27.65	1059.28 ± 65.06
	7	OTH	100% crushed Ling Zhi	14.35	737.51 ± 6.32	301.82 ± 5.76	1039.33 ± 11.13
	8	DXN-g	100% Ling Zhi extract (mycelium and sprout)	22.96	-	-	-
	9	GEC	100% Ling Zhi extract (mycelium)	18.67	-	-	-
			Median (Range)		802.83 (699.57-3992.88)	369.51 (274.62-4730.21)	1172.31 (1039.33-8723.10)
Materials from tea bag or powder from instant tea	10	DHP-t	100% crushed Ling Zhi	1.25	1175.55 ± 25.88	563.30 ± 13.76	1738.84 ± 36.89
	11	PTA	100% crushed Ling Zhi	3.33	840.10 ± 23.06	554.89 ± 14.19	1395.00 ± 36.77
	12	DTG-t	100% crushed Ling Zhi	3.00	794.18 ± 40.23	310.91 ± 14.89	1105.08 ± 54.58
	13	GNO	20% crushed Ling Zhi, 80% tea powder	6.50	233.80 ± 33.33	-	233.80 ± 33.33
	14	BNR	60% Ling Zhi water extract, 40% sugar	0.26	-	-	-
			Median (Range)		317.14 (233.80-1175.55)	554.89 (310.91-563.30)	1250.04 (233.80-1738.84)
Sliced fruiting bodies	15	DTG-s	100% Ling Zhi fruiting bodies	1.30	1159.11 ± 12.84	519.96 ± 7.05	1679.07 ± 11.72
	16	DHP-s	100% Ling Zhi fruiting bodies	2.50	908.38 ± 31.29	459.82 ± 3.94	1368.20 ± 30.12
	17	JLD	100% Ling Zhi fruiting bodies	3.33	834.26 ± 22.43	282.82 ± 13.34	1117.08 ± 29.32
	18	MG2FB	100% Ling Zhi fruiting bodies	*	764.43 ± 1.14	240.09 ± 2.80	1004.52 ± 1.66
	19	MKI	100% Ling Zhi fruiting bodies	10.00	518.40 ± 33.06	468.74 ± 6.91	987.14 ± 29.88
			Median (Range)		834.26 (518.40-1159.11)	459.82 (240.09-519.96)	1117.08 (987.14-1679.07)

[§] Data represents mean ± SD of three repetitions of measurement; [δ] Summative content of ganoderic acids A and F; * The products kindly provided from Muang Ngai special agricultural project under the patronage of Her Majesty Queen Sirikit, Chiang Mai; (-) means lower than limit of quantification (LOQ)

A. NPN



B. MG2FB-WE



C. DXN-r

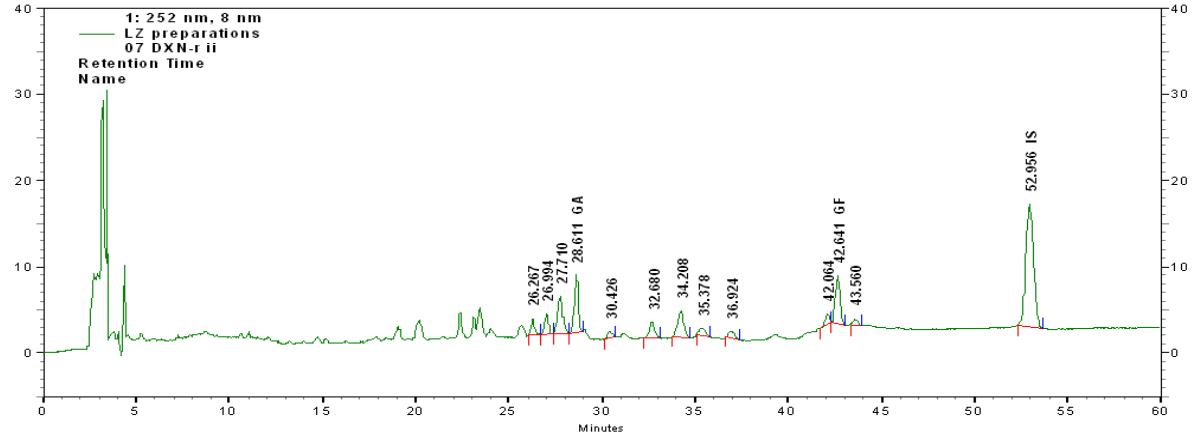


Figure 3 HPLC chromatograms of the first three Ling Zhi preparations containing the highest total content of ganoderic acids A and F.

Neither ganoderic acid A nor F was detected in 3 out of 19 preparations (GEC, DXN-g and BNR). Among these preparations, the constituent of 2 preparations (GEC and DXN-g) were 100% Ling Zhi extract, whereas the remaining preparation (BNR) was 60% Ling Zhi water extract.

Interestingly, the total content of ganoderic acids in the 17 commercially available Ling Zhi preparations was not statistically correlated with their prices (Table 3, $r^2 = 0.086$).

Discussion

This is the first study to evaluate and compare ganoderic acids A and F content in various Ling Zhi preparations available in Thailand. The content of ganoderic acids A and F varied considerably among investigated Ling Zhi preparations. It ranged from below the limit of quantification to a remarkably high content. On the basis of the extraction method, the content of active ingredients in the extract is generally significantly greater than that in raw materials. Therefore, it was not surprising that highest content of total ganoderic acids were those of 100% Ling Zhi extract namely NPN, MG2FB-WE, and DXN-r, respectively. However, this is not always true, since some Ling Zhi extract preparations in this study, e.g., GEC (100% Ling Zhi mycelium extract), DXN-g (100% Ling Zhi mycelium and sprout extract) and BNR (60% Ling Zhi water extract) demonstrated only a negligible content of ganoderic acids. These findings might result from at least two possibilities. Firstly, Ling Zhi mycelium and/or sprout might contain a low level of ganoderic acids compared to Ling Zhi fruiting bodies. Therefore, study to determine the content of ganoderic acids in the different parts of Ling Zhi should be further investigated. Secondly, variation in content of ganoderic acids might depend on Ling Zhi strains, cultivating conditions and areas, extraction procedure, or manufacturing processes[17]. Nonetheless, our data mandate that the pharmaceutical manufacturer should pay more attention to quality control of biologically active compounds presented in raw materials. Indeed, raw materials should be screened not only for total ganoderic acids or triterpenes, but also for total polysaccharides if the immunomodulating effect of Ling Zhi is the main marketing issue. Furthermore, the content of biologically active compounds should be labeled on the packaging of commercial Ling Zhi products in order to help consumers to make decisions base on cost-effective context.

The pharmacological activities of ganoderic acid A are different from ganoderic acid F in many ways. Ganoderic acid A has been reported to exert antinociceptive activity[7], antioxidative activity[8], enzyme-inhibitory activity on farnesyl protein transferase[9], hepato-protective activity[10,11], as well as anticancer activity[13,14], whereas gaoderic acid F demonstrates antihypertensive activity[6] and anticancer activity[12,13,15]. The present study revealed that ganoderic acid A was the major compound in most Ling Zhi preparations, except NPN in which ganoderic acid F was the major compound. The discrepancy in major biologically active compounds might contribute to the different in pharmacological effects as well as clinical applications of each Ling Zhi preparation.

Dosage forms of Ling Zhi are also an important factor affecting orally administered doses of ganoderic acids. Based on the equivalent dose of ganoderic acids present in different Ling Zhi preparations, the dosage forms of capsule, granule, and solution prepared from instant tea powder theoretically seem to provide a greater oral dose of ganoderic acids compared to a solution prepared from a tea bag or decoction prepared from fruiting bodies since preparation of tea (using tea bag) or decoction usually causes incomplete dissolution of ganoderic acids into the solutions. In our preliminary experiments, immersion of 1 sachet of DHP-t tea bag (2 g) containing 3,477 µg of ganoderic acids (A and F) in 100 mL hot water for 4 min yielded a tea solution containing only 1,600 µg of ganoderic acids per serving. Similarly, decoction of 10 g of sliced MG2FB containing 10,045 µg of ganoderic acids in 500 mL hot water for 30 min provided a decoction containing 5,227 µg of total ganoderic acids per 500 mL serving size. This finding indicates that only approximately 50% of ganoderic acids in the tea bag or sliced fruiting bodies could be

dissolved into the solutions during the preparation process. Therefore, in some clinical setting in which a high dose of ganoderic acids is warranted (such as in treatment of cancers), oral administration of Ling Zhi extract in the dosage form of capsule, granule or instant tea is preferred.

Although the pharmacokinetic data of ganoderic acids in humans has not been available as of yet, Gao *et al*[18] demonstrated that an oral administration of purified ganoderic acid A at a dose of 5 mg/kg in rats yields approximately 10% absorption of ganoderic acid A into the systemic circulation. Nonetheless, we are now conducting the study to evaluate the pharmacokinetics of ganoderic acids A and F after a single oral administration of MG2FB-WE in healthy Thai male volunteers. The pharmacokinetic data of ganoderic acids A and F will then be used to formulate an appropriate dosing regimen for orally administered Ling Zhi preparations in clinical trials, regarding treatment of allergic rhinitis as well as various types of cancers, which will be initiated at Faculty of Medicine, Chiang Mai University in the very near future.

In conclusion, ganoderic acids A and/or F were detected in 16 out of 19 preparations. The content of ganoderic acids A and F varied considerably among these preparations. Ganoderic acid A was the major compound in most Ling Zhi preparations, except one preparation of which ganoderic acid F was the major compound. Neither ganoderic acid A nor F was detected in 3 out of 19 preparations. Additionally, the total content of ganoderic acids in commercially available Ling Zhi preparations did not correlate with their prices.

Acknowledgements

The authors would like to acknowledge Muang Ngai special agricultural project under the patronage of Her Majesty Queen Sirikit, Chiang Mai for providing the samples of sliced fruiting bodies and water extract of fruiting bodies of MG2-strain Ling Zhi. Furthermore, we also would like to express our special gratitude to Assoc. Prof. Noppamas Soonthornchareonnon, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University for providing the reference standards of ganoderic acids A, F and IS. Finally, grateful acknowledgement is made for financial support by the Thai Traditional Medical Knowledge Fund, Department for Development of Thai Traditional and Alternative Medicine, Ministry of Public Health, Thailand.

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RESEARCH ARTICLE

Are Renal Transplant Recipients with Sirolimus-base Regimen with Low Risk for Cardiovascular Disease?

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Abstract

Renal transplant recipients have increased risk of cardiovascular disease (CVD) compared to the general population and other groups of renal disease. In fact, CVD remains the leading cause of death in renal transplantation. Homocysteine is the established CVD risk marker. This study aimed to assess whether renal transplant recipients who received sirolimus (SRL) as their main immunosuppressive therapy had lower risk for CVD as evidenced by a CVD surrogate, homocysteine, compared to those with calcinuerin inhibitor (CNI). Sixty five renal transplant recipients (55 with CNI-based regimen and 10 with SRL-based regimen) and 34 healthy controls were recruited. Plasma homocysteine levels were measured using ARCHITECT® assay. Plasma homocysteine levels were higher in patients (15.43±1.88 μmol/L) compared to those of the controls (10.91±3.67 μmol/L, $p < 0.01$). Plasma homocysteine levels of the CNI-based patients were higher than the SRL-based but this did not reach significance (15.62±8.70 and 14.38±4.47 μmol/L, respectively). These results suggested the higher CVD risk in renal transplant recipients and may support the role of SRL-based regimen in reducing the CVD risk in this group of patients.

Keywords homocysteine, sirolimus, renal transplant recipients

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Introduction

Cardiovascular disease (CVD) is recognized as the major cause of death in renal transplant patients*[1]*. Immunosuppressive therapies which are associated with a reduction of the risk for CVD would therefore greatly reduce post-transplantation morbidity and mortality*[2]*. The most widely used immunosuppressive regimen comprises with calcineurin inhibitor (CNI) which itself is associated with nephrotoxic effects and has pro-atherogenic property. Sirolimus (SRL), the more recent immunosuppressive agent with a unique mechanism of action, offers an alternative to CNI*[3]*. The absence of nephrotoxic effects and the potent immunosuppressive efficacy in animal models had led to SRL use in protocols that spare or avoid CNI*[4]*. This may indicate a better long-term outcome in SRL-treated patients. Thus, total CNI avoidance with SRL substitution appears to be promising in renal transplantation. Homocysteine were a prognostic marker for CVD risk and CVD mortality in renal transplant recipients*[5]*. Homocysteine is an amino acid produced by the body, usually as a byproduct of meat consumption*[6]*. Elevated levels of homocysteine in plasma is associated with an increased risk of CVD*[7]*. This study aimed to investigate whether renal transplant recipients who received SRL as their main immunosuppressive agent had lower risk for CVD as evidenced by a CVD surrogate, homocysteine, compared to those with CNI-based regimen.

Methods

Subject

The study was approved by the Ethic Committee of the Faculty of Medicine, Chulalongkorn University. All subjects provided their written informed consent. The total of 65 renal transplant recipients was enrolled into the study. Of these, 55 patients were receiving CNI-based regimen and 10 patients were receiving SRL-based regimen as their maintenance post-transplantation immunosuppressive therapies. Doses of the treatments were stable for at least 3 months prior to study. Thirty four healthy subjects from the community were also recruited as the representative of Thai general population. All of these healthy subjects were healthy by medical history, physical examination and screening laboratories. Venous blood samples were collected into EDTA-containing tubes and centrifuged at 500*g*, 4°C for 10 minutes. Plasma was then transferred into aliquots and stored at -70°C until analysis.

Determination of homocysteine in human plasma[8]

The ARCHITECT homocysteine, a chemiluminescent microparticle immunoassay (CMIA), was used to analyze plasma homocysteine levels as previously describe*[8]*. In brief, 50 µl of sample was used. Bound or dimerised homocysteine (oxidized form) were reduced by dithiothreitol (DTT) to free homocysteine, which was then converted to S-adenosyl homocysteine (SAH) by the action of recombinant enzyme S-adenosyl homocysteine hydrolase (rSAHHase) in the presence of excess adenosine. The intra- and inter-assay variability were 3.20% and 4.32% respectively.

Statistical analysis

Statistical analyses were performed using SPSS version 17.0. Descriptive data were presented as mean±standard deviation (SD) unless otherwise indicated. Means of plasma homocysteine levels between controls and patients were compared using unpaired Student *t* test. Means of plasma homocysteine levels of the 3 groups (controls, CNI-based and SRL-based patients) were compared using one-way analysis of variance (ANOVA). P value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of the subjects were presented in Table 1. Age was lower in healthy controls. As expected, patients' hemoglobin (Hb) and hematocrit (Hct) were decreased while serum creatinine (Cr) and blood urea nitrogen (BUN) were increased compared to controls. CNI-based and SRL-based groups had comparable baseline parameters. Plasma homocysteine levels were higher in patients (15.43±1.88 µmol/L) compared to those of the controls (10.91 ± 3.67 µmol/L, $p < 0.01$). Plasma

homocysteine levels of the CNI-based group were higher than the SRL-based but this did not reach significance (15.62±8.70 µmol/L and 14.38±4.47 µmol/L, respectively, Table 1, Figure 1).

Table 1 Baseline characteristics and plasma homocysteine levels.

Baseline characteristics	Control *(n=34)*	CNI-based *(n=55)*	SRL-based *(n=10)*	*P value*
Age (year)	30.68±4.97	49.53±10.95	51.7±9.52	.000*
Hb (g/dL)	14.94±0.99	12.71±1.77	13.29±1.18	.000*
Hct (%)	43.51±2.80	39.21±5.30	41.26±3.29	.001*
Cr (mg/dL)	1.00±0.11	1.99±3.14	1.13±0.48	.200
BUN (mg/dL)	13.12±2.42	20.21±9.50	14.99±5.74	.001*
HT (n(%))	N/A	9(16.4)	1(10.0)	N/A
DM (n(%))	N/A	49(89.1)	7(70.0)	N/A
IHD (n(%))	N/A	50(90.9)	10(100.0)	N/A
Dyslipidemia (n(%))	N/A	48(82.3)	6(60.0)	N/A
Homocysteine (µmol/L)	10.91±3.67	15.62 ± 8.70	14.38±4.47	.011*

Values are presented in mean±SD
*P values by one-way ANOVA.

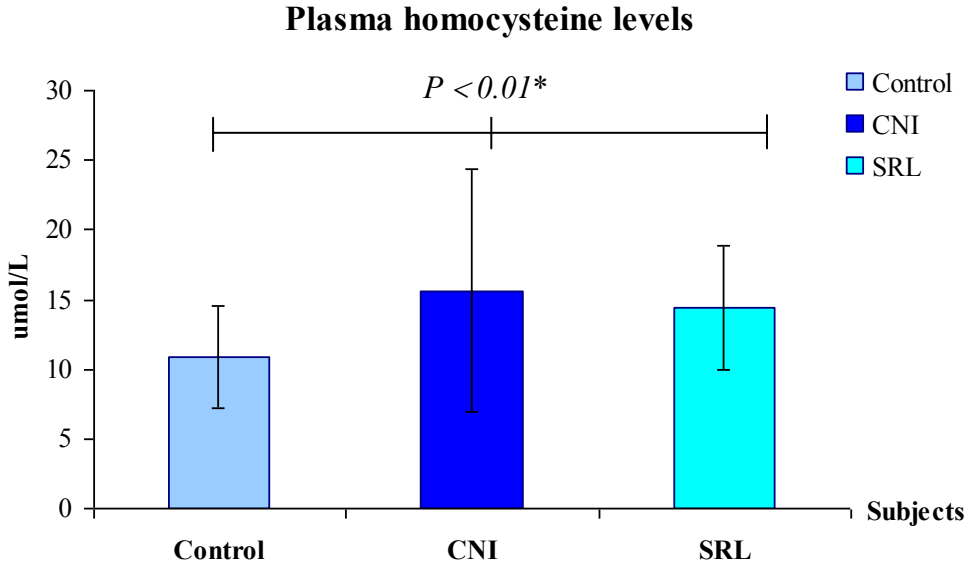

Figure 1 Plasma homocysteine levels.
*P values by one-way ANOVA.

Discussion

This study have shown that renal transplant recipients had increased plasma homocysteine, the surrogate for CVD, and those with SRL-based tended to have lower homocysteine levels. Avoidance of CNI has become a challenge in renal transplant recipients as this drug has long-term nephrotoxicity*[9]*. Results of this study are in keeping with this as shown in Table 1 that the CNI-based group has lightly worse renal function compared to the SRL-based group. Importantly, CNI may enhance CVD risk by increasing the levels of hyperlipidemia, diabetes and hypertension, as well as the levels of nonconventional risk factor of vascular and renal injury*[10]*. Homocysteine is a naturally occurring amino acid. High levels of homocysteine are associated with coronary artery disease, stroke and peripheral vascular disease. Homocysteinemia has emerged as a prevalent and strong risk factor for atherosclerotic vascular disease in the coronary, cerebral, and peripheral vessels, and for arterial and venous thromboembolism*[11]*. Although not significant, the finding those levels of plasma homocysteine in CNI-based group were slightly higher than of SRL-based may support the fact that CNI increase CVD risk and stresses the benefit of using SRL as an alternative immunosuppressive agent. This data was also supported by the findings in Table 1 that both groups have comparable evidence of concomitant co-morbidity, yet the SRL group presents with lower plasma homocysteine concentrations.

Conclusion

Treatment with SRL-based regimen might lower the CVD risk in renal transplant recipients. Further longitudinal studies with larger sample size are needed.

Acknowledgements

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RESEARCH ARTICLE

Molecular Characterization of *Plasmodium Vivax Dihydrofolate Reductase (Pvdhfr)* in *Plasmodium Vivax* Isolates from Thailand

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Abstract

Malaria is the most important public health problems in several countries. In Thailand, co-infections of *Plasmodium vivax* and *P. falciparum* are common. The prevalence and patterns of mutations of *Pvdhfr* was investigated in a total of 60 blood samples collected from patients with *P. vivax* infection who attended the malaria clinic in Mae Sot, Tak Province in 2009. SNP-haplotypes at amino acid positions 13, 33, 57, 58, 61, 117 and 173 of *Pvdhfr* was examined by nested PCR-RFLP. All parasite isolates carried triple mutant *Pvdhfr* alleles (100%). The most common *Pvdhfr* alleles were 57I/58R/117T (81.7%), 57L/58R/117T (8.3%), 58R/61M/117N (8.3%), and 57I/58R/117T/N (1.7%). Results suggest that all *P. vivax* isolates from Mae sot carried mutant alleles of *Pvdhfr*. The development of new alternative antifolates drugs that are effective against sulfadoxine-pyrimethamine resistant *P. vivax* is required.

Keywords *Plasmodium vivax*, *Plasmodium vivax dihydrofolate reductase (Pvdhfr)* and antifolate

Introduction

Antimalarial drug resistance is a major public health problem in tropical and sub-tropical countries. In Thailand, malaria disease is endemic throughout the country, with the highest incidence reported from Tak Province (Na-Bangchang & Congpuong 2007). The antimalarial combination sulfadoxine-pyrimethamine (SP, Fansidar^{TM}) was introduced to Thailand as the first-line treatment of chloroquine resistant *P. falciparum* malaria. Rapidly after its introduction for clinical use, resistance of the parasite to this drug was reported and widespread throughout the country (Pinichpongse et al. 1982). The molecular targets of action of sulfadoxine and pyrimethamine are dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), respectively. Although in Thailand, SP has never been used for treatment of *P. vivax* infections, *P. vivax* often co-exists with *P. falciparum* with relative equal frequencies (Snounou & White 2004). Therefore, *P. vivax* has often been exposed unintentionally to SP during treatment of *P. falciparum*, and this has caused a progressive selection of SP-resistant alleles in *P.vivax* (Imwong et al. 2003). The distribution of point mutations in the *dhfr* and *dhps* alleles varies among different geographical regions and is related to the intensity of SP use for treatment of *P. falciparum* in the past. In the present study, we determined the prevalence and diversity of *Pvdhfr* mutant alleles in *P. vivax* isolates collected from Mae Sot District, endemic areas of Thailand. This information will assist in development of new effective antimalarial drugs with antifolate action.

Materials and methods

Study areas and sample collection

A total of 60 blood samples were collected from patients attending the malaria clinics in Mae Sot, Tak Province in 2009. Approval of the study protocol was obtained from the Ethics Committees of Mae Sot General Hospital, Tak province, Thailand. Two-hundred to 300 µl finger-prick blood samples were collected onto filter paper (Whatman No. 3). Giemsa-stained thin and thick blood smears were prepared and examined microscopically for the presence of *P. vivax* parasites.

Extraction of parasite genomic DNA

Parasite genomic DNA was extracted using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification.

Table 1 The frequencies of *Pvdhfr* single nucleotide polymorphisms in 60 *P. vivax* isolates

Gene	Amino acid position	SNPs	Number of isolates (%)
Pvdhfr	13	I (wild-type)	60 (100%)
		L (mutant)	0 (0%)
	33	P (wild-type)	60 (100%)
		L (mutant)	0 (0%)
	57	F (wild-type)	5 (8.3%)
		I (mutant)	50 (83.4%)
		L (mutant)	5 (8.3%)
	58	S (wild-type)	0 (0%)
		R (mutant)	60 (100%)
	61	T (wild-type)	55 (91.7%)
		M (mutant)	5 (8.3%)
	117	S (wild-type)	0 (0%)
		N (mutant)	5 (8.3%)
		T (mutant)	54 (90.0%)
		N/T (mutant)	1 (1.7%)
	173	I (wild-type)	60 (100%)
		L (mutant)	0 (0%)

Table 2 Frequency distribution of *Pvdhfr* mutation alleles in 60 *P. vivax* isolates

Genotype	Number of isolates (%)
57I/58R/117T	49 (81.7)
57I/58R/117T/N	1 (1.7)
57L/58R/117T	5 (8.3)
58R/61M/117N	5 (8.3)

Amplification of Pvdhfr

Point mutations of *Pvdhfr* (codons: 13, 33, 57, 58, 61, 117 and 173) in all *P. vivax* isolates were investigated by nested PCR-RFLP according to the previously described methods (Imwong et al. 2003, Rungsihirunrat et al. 2008, Snounou et al. 2005).

Results

Detection of mutations in the Pvdhfr

The frequencies of *Pvdhfr* mutations are summarized in Table 1. Point mutations were detected in 4 (codons 57, 58, 61 and 117) out of 7 codons investigated, while wild-type alleles were detected at 3 codons (13, 33 and 173). Among the multation alleles, all isolates carried mutations at codons 58 (58R) and 117 (117N, 117T, 117N/T).

Distribution of Pvdhfr alleles

The distribution of four different mutation types of *Pvdhfr* in all isolates was summarized in table 2.

Discussion

The prevalence of mutations in *Pvdhfr* genes of *P. vivax* isolates collected from Mae Sot district were investigated in this study. A total of four type mutations of *Pvdhfr* alleles were detected. All prevalent (100.0%) alleles carried triple mutant *Pvdhfr* alleles. The common mutant *Pvdhfr* allele 117N has been reported in isolates carrying other mutant alleles (Imwong et al. 2001, Lu et al. 2010, Rungsihirunrat et al. 2008), but in our study this mutant allele was only found with isolates carrying 61M mutant allele. Our previous study conducted in the same area in 2005 in 32 isolates revealed that the mutant alleles of *Pvdhfr* were quadruple (81.3%), triple (9.4%) and double (9.4%) mutant alleles (Rungsihirunrat et al. 2007). In both studies, no isolate carrying wild-type alleles of *Pvdhfr* alleles was detected, while mutant alleles at codons 58 and 117 were found in all isolates (100%). In another recent study (Lu et al. 2010) conducted in 30 *P. vivax* isolates collected from the same area, the most prevalent (71.4%) *Pvdhfr* mutant allele was 57I/58R/61M/117T (Lu et al. 2010). Of the five *Pvdhfr* codons (57, 58, 61, 117, 173) under investigation, single (57L), double (58R, 117N), and quadruple (57I/L, 58R, 61M, 117T) mutations were found in 3.6, 10.7 and 85.7% of the isolates, respectively. All these data reveal that all *P. vivax* isolates from Mae sot, Thailand carried mutant alleles of *Pvdhfr* and the mutant *Pvdhfr* alleles may be changed over certain time period under varying degree of drug pressure. Increasing treatment failure with SP for uncomplicated *P. falciparium* malaria in several areas has led to the development of new combinations of antifolates, sulfas and dihydrofolate inhibitors such as LapDap™ and WR99210.

Acknowledgements

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RESEARCH ARTICLE

Study on Laxative, Antihyperglycemic, and Lipid Lowering Effects of Malva Nut (*Scaphium Lychnophorum* (Hance) Piere.) in Rats Fed with High Fat Diet Compare with Konjac (*Amorphophallus* sp.)

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Abtract

Dietary fiber has many beneficial effects on human health. Malva nut tree (*Scaphium lychnophorum*) is the indigenous plant of Thailand known as Sumrong. Large amount of mucilaginous substance can be extracted from the fruit. The aim of this study was to investigate the laxative, antihyperglycemic and lipid lowering effects of malva nut in rats fed with high-fat diet compare with konjac. The animals were fed with normal diet, high-fat diet, high-fat diet + malva nut pulp 5% and 10%, high-fat diet + konjac powder 5% and 10%, for 3 months. The results showed that body weight and food intake were not different among all groups. The fecal weight of high-fat diet + malva nut pulp 5%, 10% were significantly increase (68% and 89%, respectively) when compared with high-fat diet group. In all treated rats, their fasting blood glucose, oral glucose tolerance, blood cholesterol, triglyceride, HDL and LDL were not significantly different form high-fat diet group. It is concluded that malva nut has marked laxative effect, but lack of antiobesity, antihyperglycemic and lipid lowering effects.

Keywords *Scaphium lychnophorum*, laxative, lipid lowering, glucose tolerance

Introduction

Dietary fibers, the indigestible portion of plant foods, have various beneficial effects on human health such as laxative, weight controlling, lipid and blood glucose lowering effects (1). Malva nut tree (*Scaphium lychnophorum* (Hance) Piere., Sterculiaceae) is the native plant known in Thailand as Sumrong. Large amount of mucilaginous substance can be extracted from the fruit by soaking in water. The mucilage, when sweetened, can be consumed as dessert, but its principal uses are relief of canker sore and cough, prevention of pharyngitis, treatment of tussis and constipation (2). It is believed that malva nut mucilage is useful for weight control, blood glucose and lipid lowering. Moreover, there were some *in vitro* data showing the glucose entrapment and alpha- glucosidase inhibitory effects of malva nut mucilage (3). However, limited *in vivo* data has been reported. This study aims to investigate the laxative, antihyperglycemic and lipid lowering effects of malva nut pulp in rats fed with high-fat diet compare with konjac.

Materials and Methods

Animals

Sixty male Wistar rats, weighing 150-250 g were acclimated in a room maintained at 25±2°C, 12 h light/dark cycle for 1 week. Rats had free access to food and water.

Treatments

Rats were divided into 6 groups of 10 each. Group 1 : normal diet (C.P. Rat Feed containing 4.5% fat), Group 2 : high-fat diet (15% fat), Group 3,4 : high-fat diet with dried malva nut pulp 5% and 10%, Group 5,6 : high-fat diet with konjac powder 5% and 10%.

Experimental protocol

Feces of each rat were collected weekly. The body weight and food intake of each rat were recorded weekly for 3 month. At the end of the experiment, oral glucose tolerance test were evaluated by using glucometer (GlucoDr^{TM}, All Medicus) and blood cholesterol, triglyceride, HDL and LDL were determined by automatic analyzer (COBAS MIRA). The experimental protocol was approved by the Institutional Committee for Ethical Use of Animals, Prince of Songkla University, Thailand.

Statistical Analysis

The data were expressed as mean values ± SEM. The statistical analysis were done by one-way ANOVA followed by LSD test. $P < 0.05$ was considered statistically significant.

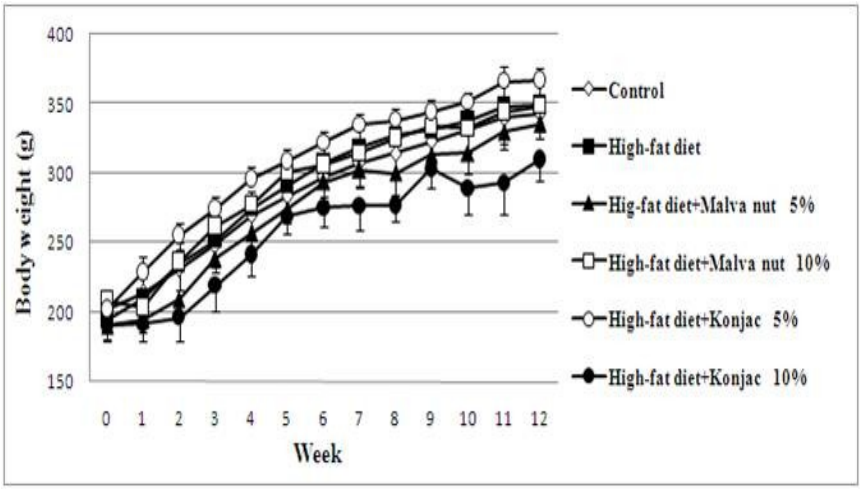

Figure 1 Body weight of rats received normal diet, high-fat diet, high-fat diet + malva nut 5% and 10%, high-fat diet + konjac 5% and 10%.

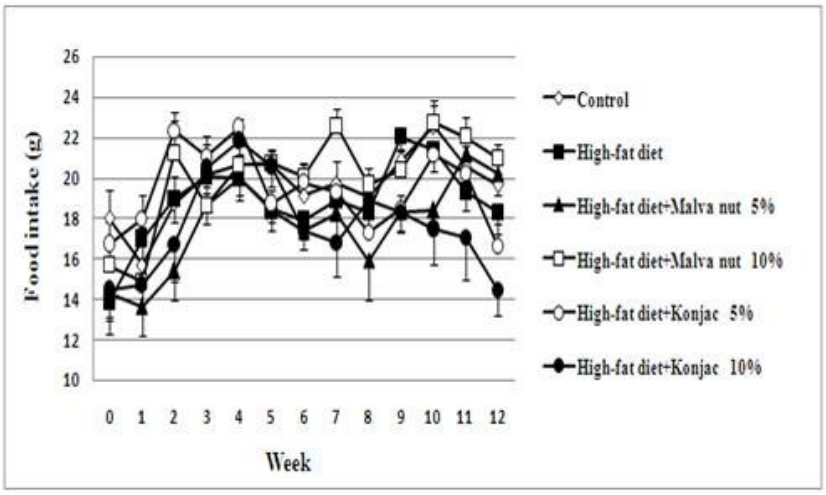

Figure 2 Food intake of rats received normal diet, high-fat diet, high-fat diet + malva nut 5% and 10%, high-fat diet + konjac 5% and 10%.

Results

Body weight

After 3 month, the percentage increase in body weight of rats fed with high-fat diet + malva nut pulp 10% and high-fat diet + konjac powder 10% were 12% and 17% less than high-fat diet group, respectively, but not statistically significant. While negligible changes were observed in the other groups (Fig.1).

Food intake

The percentage increase in food intake of rats fed with high-fat diet + malva nut pulp 5% and 10% were not different from high-fat group. It was noted that the food intake of rats fed with high-fat diet + konjac 5% and 10% were 40% and 30%, less than high-fat diet group, respectively, but not statistically significant (Fig. 2).

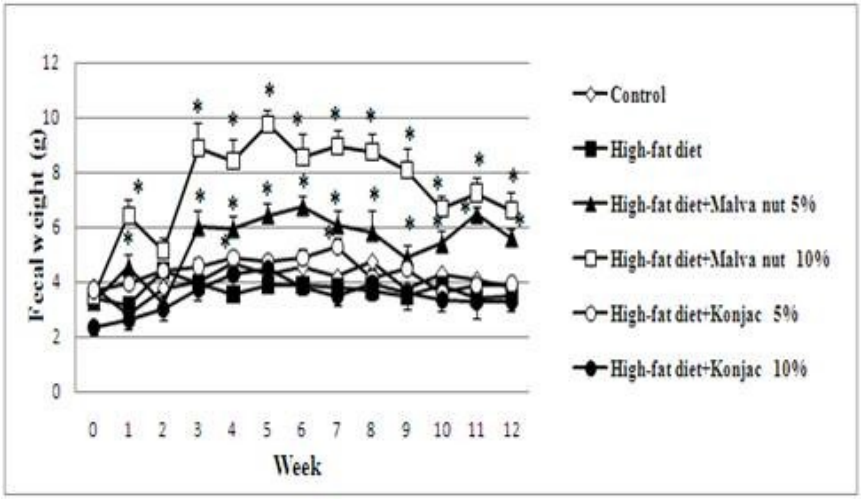

Figure 3 Fecal weight of rats received normal diet, high-fat diet, high-fat diet + malva nut 5% and 10%, high-fat diet + konjac 5% and 10%. $P<0.05$.

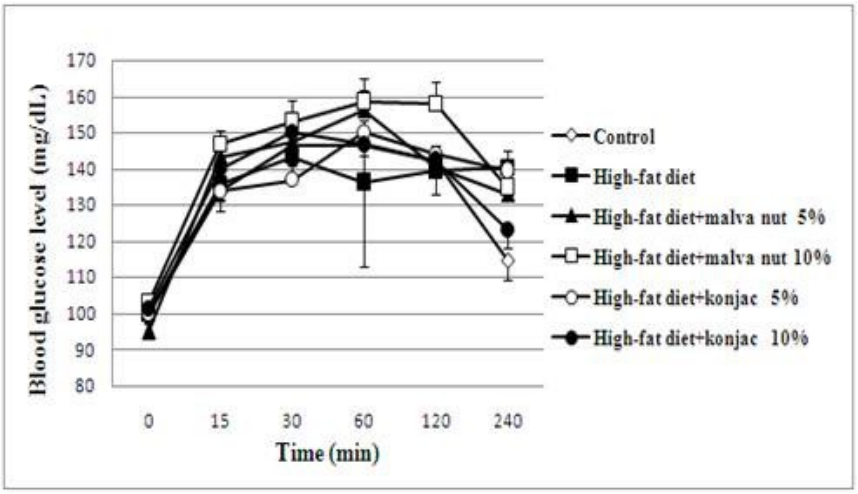

Figure 4 Oral glucose tolerance test of rats received normal diet, high-fat diet, high-fat diet + malva nut 5% and 10%, high-fat diet + konjac 5% and 10%.

Table 1 Serum lipid profile in rats received normal diet, high-fat diet, high-fat diet + malva nut 5% and 10%, high-fat diet + konjac 5% and 10%.

Treatment	Cholesterols	% change	Triglyceride	% change	HDL	% change	LDL	% change
High fat diet	56.70 ± 1.97	-	46.40 ± 5.42	-	42.10 ± 2.19	-	9.80 ± 1.91	-
High fat diet +Malva nut 5%	57.20 ± 2.30	0.88	43.00 ± 4.22	-7.33	43.30 ± 1.88	2.59	8.40 ± 1.12	-14.29
High fat diet +Malva nut 10%	58.56 ± 3.29	3.28	52.00 ± 5.19	12.07	41.22 ± 2.90	-1.90	9.11 ± 1.36	-7.04
High fat diet +Konjac 5%	58.70 ± 4.08	3.53	37.90 ± 2.66	-18.32	47.70 ± 3.67	12.07	7.00 ± 0.82	-28.57
High fat diet +Konjac 10%	52.71 ± 3.24	-7.04	41.71 ± 4.31	-10.11	41.00 ± 1.90	-2.37	6.71 ± 1.90	-31.53

Fecal weight

The fecal weight of rats fed with high-fat diet + malva nut pulp 5% and 10% were significantly increase (68% and 89%, respectively) when compared with high-fat diet group, where as those of high-fat diet + konjac 5% and 10% were not different (Fig. 3).

Fasting blood glucose and oral glucose tolerance test

Fasting blood glucose and oral glucose tolerance did not differ significantly among all groups after 3 month-treatment (Fig. 4).

Lipid profile

There were no significant differences in the average serum cholesterol, triglyceride, HDL and LDL among all groups. However, in rats fed with konjac, serum triglyceride and LDL tended to decrease (10-18% and 28-31%, respectively) (Table 1).

Discussion and conclusion

In the present study, we demonstrated that rats fed with high fat diet + malva nut pulp 5 - 10% for 3 month showed marked increase in fecal weight whereas body weight, food intake, fasting blood glucose, oral glucose tolerance, and lipid profile (cholesterol, triglyceride, HDL, LDL) did not differ from the control group or those of rats fed with high fat diet. Body weight, food intake, serum triglyceride, and LDL in rats fed with high fat diet + konjac 5% and 10% tended to be decreased, though

not significantly, compared to the high fat diet group which is consistent with previous report (4). Malva nut pulp (dry powder of *Scaphium lychnophorum* seed mucilage) contained 97.55, 7.67, 89.88% w/w of total, soluble, and insoluble dietary fiber, respectively, with the water holding capacity of 92.13 times of the dry powder weight (5). With these properties, its laxative effect is obviously demonstrated in this study as it was suggested in traditional medicine (2). The mechanism (s) responsible for the laxative effect of most dietary fibers include the increase of colonic content leading to colonic propulsion which promotes defecation, the stimulation of colonic motility by fibers and end products of fiber fermentation as well as an increase in bowel movement (6). Although the laxative effect of malva nut pulp has been shown, the mechanism by which it relieves constipation should be further investigated. In contrast to the believe that malva nut pulp possess the body weight, blood glucose, and lipid lowering effects, the present study could not reveal those properties. From our study above, malva nut pulp should be considered as one of those good laxatives.

Acknowledgements

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RESEARCH ARTICLE

Population Pharmacokinetics-Pharmacodynamics of Mefloquine When Used in Combination with Artesunate as a 3-day Combination Regimen in the Treatment of Highly Multidrug Resistance *Plasmodium falciparum* in Thailand

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Abstract

Declining in clinical efficacy of artesunate-mefloquine combination for treatment has been documented in areas along the eastern border (Thai-Cambodian) of Thailand. In the present study, we investigated the population pharmacokinetics of MQ when used in combination with artesunate as a three day combination regimen in relation to treatment response in a total of 150 Burmese patients with acute uncomplicated falciparum malaria. The study was conducted at Mae Tao clinic for migrant workers, Tak Province, Thailand. A total of 996 blood samples were collected from 150 (85 males, 65 females) Burmese patients aged over 15 years with acute uncomplicated *Plasmodium falciparum* malaria following a three day combination regimen of artesunate-mefloquine. Whole blood mefloquine concentrations were determined by high performance liquid chromatography (HPLC). The basic pharmacokinetic model, 2 –compartment model for mefloquine absorption and disposition were best fit with mefloquine concentration-time data (NONMEM). Recrudescence was observed in 34 during days 7 and 42; 5 and 5 cases, respectively had reinfection with *P. falciparum* and reappearance of *P. vivax* in their peripheral blood during follow-up. The 42-day efficacy rate of the combination regimen was 72.58%. The pharmacokinetics of mefloquine in patients with sensitive (n=116) and recrudescence (n=34) responses were similar. There appears to be no association between treatment response [parasite clearance time (PCT), fever clearance time (FCT), occurrence of recrudescence] between patients with sensitive and recrudescence responses. This suggests that intrinsic parasite factor, *i.e.,* development of parasite resistance to antimalarial drug, may play important role in determining treatment response of the patients.

Keywords *Plasmodium falciparum*, multi-drug resistance, mefloquine, artesunate, combination therapy, population pharmacokinetics-pharmacodynamics

Introduction

Malaria remains a substantial public health problem in several tropical areas. The development and spreading of multidrug-resistant *Plasmodium falciparum*, particularly the previous mainstay antimalarial drug chloroquine, sulphadoxine-pyrimethamine and mefloquine is further aggravating the situation [1]. To deal with the threat of resistance of *P. falciparum* to monotherapies, combinations of anti-malarials are now recommended by the World Health Organization (WHO). Artemisinin-based combination therapy (ACT) is widely promoted as a strategy to counteract the increasing resistance of *P. falciparum* to anti-malarials, as well as to prevent disease transmission and reduce the risk of drug resistance [1]. Thailand was the first country to use monotherapy of mefloquine for the treatment of uncomplicated malaria, but resistance to mefloquine developed rapidly on both borders [1]. In 2007, the malaria control programme of Thailand switched to a three-day treatment course in accordance with WHO recommendation. In the present study, we investigated the population pharmacokinetics of MQ when used in combination with artesunate as a three day combination regimen in relation to treatment response in a total of 150 Burmese patients with acute uncomplicated falciparum malaria.

Materials and methods

Sample collection

The study was conducted at Mae Tao clinic for migrant workers, Tak Province during April 2009 – July 2010. The study was approved by the Ethics Committee of Ministry of Public Health of Thailand. A total of 996 blood samples (4 points from each patients at various time points from 0 hr to 42 days) were collected from 150 (85 males, 65 females) Burmese patients aged over 15 years with acute uncomplicated *P. falciparum* malaria. Written informed consents were obtained from all patients before study participation. Patients were treated with a three-day combination regimen of artesunate and mefloquine [4 mg/kg body weight artesunate (200 mg) and 15 mg/kg body weight mefloquine (750 mg) were given on the first day (day 0); 4 mg/kg body weight artesunate and 10 mg/kg body weight mefloquine (500 mg) on day 2; 4 mg/kg body weight artesunate given with 0.6 mg/kg body weight primaquine (15 mg) on day 3]. Reinfection and recrudescence of *P. falciparum* were differentiated by genotyping of three polymorphic genes merozoite surface protein 1 (*msp1*), *msp2*, and glutamate-rich protein in paired blood spot samples (pre-treatment and the day of recrudescence) using nested PCR [2].

Efficacy assessment

The primary end point of efficacy assessment of a 3 day artesunate-mefloquine was the PCR-corrected 42-day cure rate (proportion of patients with clinical and parasitological after 42 days of follow-up). Secondary endpoint parameters included parasite clearance time (PCT), proportions of patients with clearance of parasitemia by 24 (PCT_{24hr}) and 48 (PCT_{48hr}) hours, fever clearance time (FCT), proportions of patients with clearance of fever by 24 (FCT_{24hr}) and 48 (FCT_{48hr}) hours.

Drug analysis

Concentrations of mefloquine in whole blood were measured by high performance liquid chromatography with UV-detection (HPLC-UV) [3], with quantification limit of 1 ng/ml.

Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed using nonlinear mixed-effect modeling (NONMEM version VI). Two compartmental models were fitted to the ln-transformed mefloquine concentration data using the FOCE (first-order conditional estimation) method with interaction. Softwares Census (v 1.1) and Xpose (v 4) module for R was used for diagnostics. In addition, the program Perl speaks NONMEM was used to facilitate modelling and for some diagnostics. To evaluate the residual error, combined additive and proportional error model were applied. All parameters were estimated with interindividual variability (IIV) added. Discrimination between competing models was done with the help of the objective function value (OFV) outputted by NONMEM which corresponds to the -2xlog-likelihood and can be assumed to be

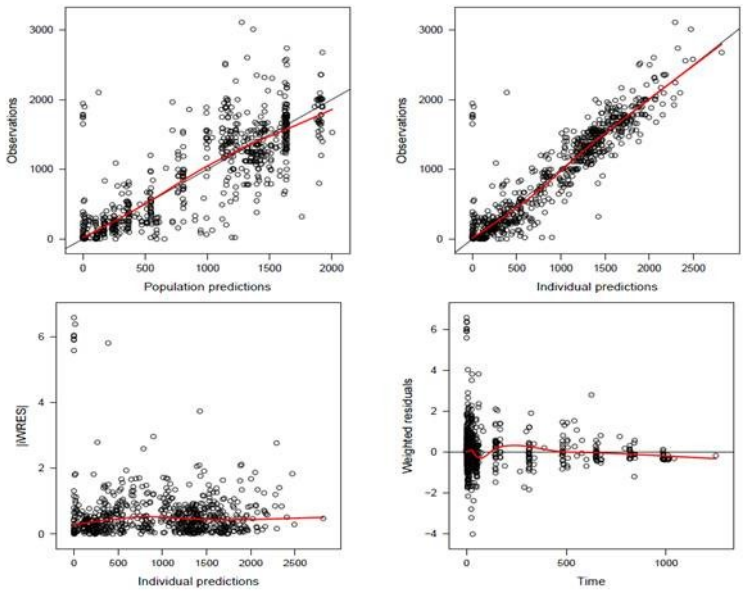

Figure 1 Basic goodness-of-fit plots describing the pharmacokinetics of mefloquine in the population

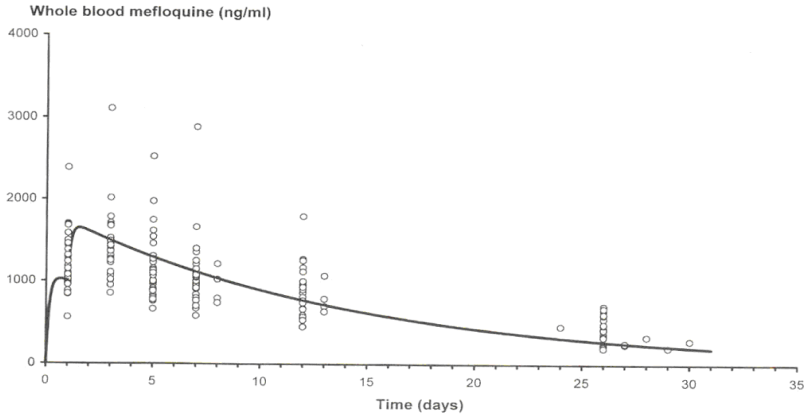

Figure 2 Poppulation estimates of whole blood mefloquine concentration-time profiles in patients with sensitive and recrudescence responses

Chi-square distributed. Thus, if one parameter (one degree of freedom) is added and the OFV drops with 3.84 it is significant at $p<0.05$ while a drop of 6.635 is significant at $p<0.01$. Model diagnostics were done using goodness-of-fit plots.

Results

Mefloquine plasma concentrations during oral treatment were best described by a 2 compartment disposition model linked to a sequential, zero and first order, drug absorption model. The population estimates of mefloquine pharmacokinetics are presented with their relative standard errors (RSE), interindividual and residual variabilities. The goodness of fit plots is presented in Figure 1. Thirty-four cases had recrudescence during days 7 and 42. Five and 5 cases, respectively had reinfection with *P. falciparum* and reappearance of *P. vivax* in their peripheral blood during follow-up. The 42-day efficacy rate of this combination regimen was 72.58% (95% CI: 63.20-79.07%). Parasite clearance time (PCT) and fever clearance time (FCT) were significantly prolonged in patients with

Table 1 Population estimates of mefloquine pharmacokinetics

	Population estimate [RSE%]		IIV (CV%) [RSE%]	
	Sensitive (n=106)	**Recrudescence (n=34)**	**Sensitive (n=106)**	**Recrudescence (n=34)**
CL/F (L/h)	2.46 [6.30]	2.37 [5.6]	-	-
V (L)	261 [9.31]	250 [8.91]	-	-
Q (L/h)	15.7 [7.96]	16.1 [8.08]	40.2 [66]	37.12 [54.1]
V2 (L)	712 [6.11]	700 [8.02]	-	-
Ka (1/h)	0.197 [14.5]	0.201 [12.1]	30.4 [56.9]	32.4 [60.1]
Duration (h)	5.42 [4.96]	5.22 [3.89]	33 [18.1]	30.4 [15.4]
F	1 fix	1 fix	16.2 [41.7]	17.0 [35.2]
σ_{add} (mg/L)	296 [10.2]	300 [11.12]	-	-

treatment failure compared with those with sensitive response [median (95% CI) values for PCT 32.0 (20.0-48.0) *vs* 24.0 (14.0-32.0) hr; and FCT 30.0 (22.0-42.0) *vs* 26.0 (18.0-36.0) hr]. The pharmacokinetics of mefloquine in patients with sensitive (n=116) and recrudescence (n=34) responses were similar (Fig 2). There appears to be no association between treatment response (PCT, FCT, occurrence of recrudescence) between patients with sensitive and recrudescence responses. This suggests that intrinsic parasite factor, i.e., development of parasite resistance to antimalarial drug, may play important role in determining treatment response of the patients.

Discussion and Conclusion

The basic pharmacokinetic model, 2 –compartment model for mefloquine absorption and disposition in the studied population has thus been established. Subsequent refinement of the model will include testing and inclusion of demographic and disease-specific covariates included in the dataset. There appears to be no association between treatment response (PCT, FCT, occurrence of recrudescence) between patients with sensitive and recrudescence responses. This suggests that intrinsic parasite factor, i.e., development of parasite resistance to antimalarial drug, may play important role in determining treatment response of the patients.

Acknowledgements

The study was supported by The Commission on Higher Education, Ministry of Education of Thailand and The National Research University Project of Thailand Office of Higher Education Commission of Thailand.

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RESEARCH ARTICLE

In vitro Antioxidative Synergy of Mangosteen Crude Extracts

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Abstract

An ethanolic crude extract obtained from the ripe fruit rind of mangosteen (*Garcinia mangostana* L.) (GmEt) possesses antioxidative effect, of which is potentiated when tested in combination with the aqueous crude extract (GmAq). Their synergistic *in vitro* profile is similar to that of the combination between GmEt and the aqueous extract of green tea (GtAq).

Key words mangosteen crude extract, *Garcinia mangostana*, antioxidant, synergistic effect

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Introduction

Mangosteen (*Garcinia mangostana* L., GUTTIFERAE) has received attention not only from the economic value of its edible fruit, but also the traditional medicinal applications among the Southeast Asian countries. The medicinal records include, for example, the use on treatment of stomachache, diarrhea, skin disorders, and infectious wounds[1]. The phytochemicals of concern are basically referred to the group of xanthones, the majority of which is α-mangostin (Figure 1), which are responsible for the antioxidant property of mangosteen products[1, 2]. In this study, we compare this bioactive effect with that of the green tea. Whereas the compounds of interest in green tea are known to be the group of catechins, specifically epigallocatechin gallate (EGCG) (Figure 2), the activity profiles of their extracts in combination are also investigated.

Materials and Methods

The fruit rind, dried and coarsely ground, was extracted with 95% ethanol and water to yield ethanolic extract (GmEt) and aqueous extract (GmAq), respectively. These crude extracts were diluted to 10, 1, and 0.1 concentration part as proportional to the initial concentration which was regarded as 100. Aqueous green tea extract was freshly prepared and serially diluted in similar fashion.

Assay for antioxidative effect was based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. Each effective concentration was reported as an EC_{50} value. The experiment and calculation on combination study were modified from those described by Berenbaum (1978)[3]. The degree of synergism and antagonism were justified based on their combination index (FIC_{50}), as well as by the correlating appearance of an concave or convex isobole, respectively[3, 4].

Results

The DPPH radical scavenging ability of GmEt was less than that of GmAq, the latter of which provided approximately a similar level to that of GtAq.

In combination, GmEt and GmAq (1:1 ratio) was synergistic on their antioxidative activity, as displayed by a concave isobole (Figure 3) with the sum of FEC_{50} equaled 0.6208. In similar, antioxidant combination of GmEt and GtAq was also synergistic (Figure 4) at 1:1 ratio of each EC_{50} (sum of FEC_{50} = 0.5008).

However, the 1:1 combination of GmAq and GtAq was likely an additive, as reflected by an apparent straight line of the isobole with the sum of FEC_{50} equaled 0.9286 (Figure 5).

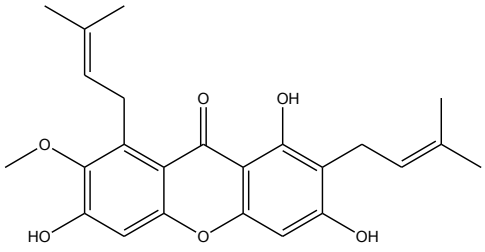

Figure 1 α-mangostin

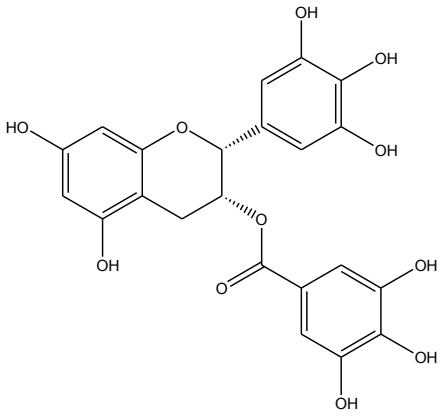

Figure 2 EGCG

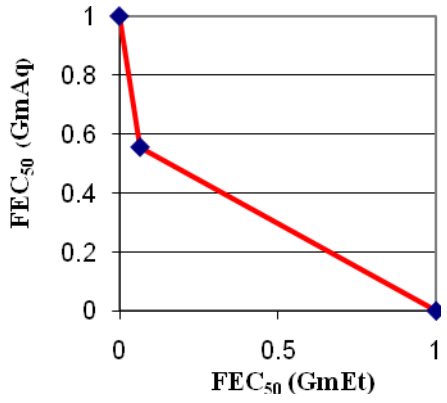

Figure 3 Isobologram of 1:1 GmEt/GmAq

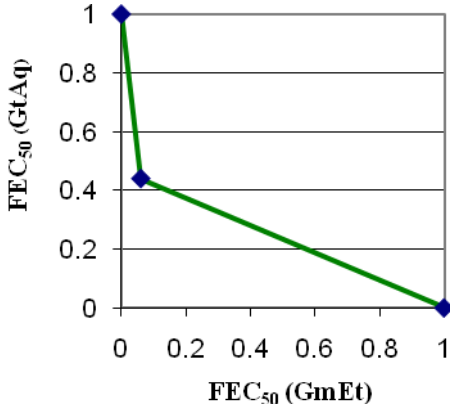

Figure 4 Isobologram of 1:1 GmEt/GtA

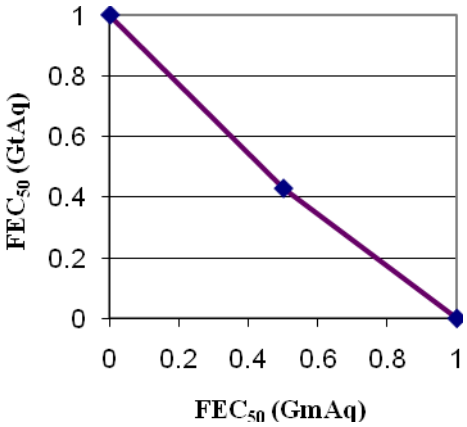

Figure 5 Isobologram of 1:1 GmAq/GtAq

Discussion

As mentioned in the introduction, the major antioxidants in mangosteen and in green tea that are of concern are different types of phytochemicals. Xanthones are more soluble in ethanolic solution, whereas the EGCG group does better in water. However, mangosteen also contains catechins that are extractable with water. The presence of catechins in the aqueous crude extract of mangosteen fruit rind could simply be quantitative detected by UV-spectroscopy in correlation with DPPH radical scavenging assay[5]. Synergism, if not antagonism, is more explainable among different class of bioactive chemicals, as it is common approaches to treat infectious diseases[6]. Likewise, the same principle was speculative on the case of mangosteen crude extracts. The result from GmEt+GmAq (Figure 3), therefore, indicates the possibility to share with this principle.

Replacing GmAq by GtAq in the combination with GmEt gives the similar pattern of interaction (Figure 4) as confirmative of the speculation. Further, GmAq+GtAq yields no beneficial mark on the antioxidant effect but an additive in combined (Figure 5), thus does concur the principle recently discussed herein.

Conclusion

Synergism between the alcoholic and aqueous extracts of mangosteen fruit rind appears to be dependent on an *in vitro* combination interaction of the different classes of phytochemicals, the xanthones and catechins. In other words, the effect of

certain antioxidants could be enhanced when combined with other common antioxidants, thus the less quantity of each components is required to achieve biological effectiveness.

The finding from mangosteen and green tea also appears to benefit the creative formulation of functional beverages, which is becoming more popular among health concerning consumers.

Implication from this *in vitro* study also supports the application of natural products in the form of crude extracts or mixtures that are well defined for their desirable components and bioactivity.

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RESEARCH ARTICLE

Effect of Curcuminoids Extract Capsules on Oxidative Stress in Diabetes Mellitus Type II

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Abstract

Oxidative stress plays a major role in the complications of diabetes mellitus. Free radical was greatly increased due to prolonged exposure to hyperglycemia and impairment of oxidant/antioxidant equilibrium. Curcuminoids have been claimed to represent a potent antioxidant properties. The purpose of this study was to investigate the effects of curcuminoids extract capsules on oxidative stress and antioxidant enzyme activities in diabetes mellitus type II. Two hundred diabetes mellitus patients were participated in this study. Patients received two capsules of either curcuminoids extract (250 mg) or placebo capsules three times a day for 6 months. The glutathione (GSH) levels and antioxidant enzymes activities in red blood cell were measured at 0, 3 and 6 month. The results showed that superoxide dismutase (SOD) activity and GSH were significantly increased in curcuminoids group when compare with placebo group (p<0.05). This study demonstrated that supplementation with curcuminoids extract capsules have a potential role in boosting superoxide dismutase activity and total glutathione which are antioxidant-related defenses in diabetes mellitus type II patients.

Keywords oxidative stress, antioxidant enzyme, diabetes mellitus, curcuminoid

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Introduction

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and defects of secretion or action of endogenous insulin (1). Several studies have indicated that diabetes mellitus is associated with increased formation of free radicals and decrease in antioxidant potential (2). Hyperglycemia accelerates generation of reactive oxygen species (ROS) and increase in oxidative chemical modification of lipids, DNA and proteins in various tissues induce multiple cellular change leading to complications (3) such as the development of cardiovascular disease (4). The use of antioxidant may attenuate oxidative stress related tissue damage and pathophysiological complications (5). Curcuminoids, a group of phenolic compounds of turmeric extract, are well known with potential antioxidant and may be used to ameliorate oxidative damage in patients such as thalassemia and Alzheimer's disease (6,7).

The aim of this study was to investigate the effect of oral intake of curcuminoids extract capsules on antioxidant defense in diabetes mellitus type II patients.

Materials and methods

Subjects

Two hundred patients with diabetes mellitus type II more than 35 years of age were recruited from the HRN Princess Maha Chakri Sirindhorn Medical Center. Exclusion criteria were kidney, liver or cardiovascular diseases and ongoing herbal treatment. This study protocol was approved by the Srinakharinwirot University Ethics Committee for Human Research. In the study, all patients with inform consents were treated with the appropriate oral hypoglycemic drugs. In addition, subjects received 2 capsules of either curcuminoids or placebo three times a day for 6 months, according to which group they were randomized to. Both curcuminoids extract and placebo capsules were produced and standardized by the Government Pharmaceutical Organization. Blood sample were collected at 0, 3 and 6 months for measurement of antioxidant enzyme and glutathione (GSH) levels in red blood cells.

Sample collection

Blood samples were obtained after a 12 hour overnight fasting into the EDTA tubes, then plasma was immediately separated by centrifugation at 3250 rpm at 4^{o}C for 15 min. The buffy coat was discarded and the remaining erythrocytes were washed in saline three times. The erythrocytes was hemolysed by adding equal volume of ice-cold deionized water to yield a 50% hemolysate and was frozen at -80^{o}C for later analysis.

Determination of superoxide dismutase

The activity of SOD was measured by using SOD assay commercial kit (Sigma-Aldrich). Samples and standards are added to wells of a 96-well plate. Add WST working solution to each well, and mix. Enzyme working solution is added to the wells to initiate a reaction. The absorbance readings were taken at 450 nm every minute for 10 minutes. SOD activity is calculated from the % inhibition of the reaction.

Determination of catalase activity

To determine the catalase activity by catalyzed decomposition of H_2O_2. Add 3 ml of 10 mM H_2O_2 in 50 mM potassium phosphate buffer to cuvet and pre-warm at 25^{o}C for 5 min. After that add 20 µl of hemolysate and record the change in absorbance at 240 nm between 30 and 210 sec. One unit of activity is defined arbitrarily as the amount of enzyme, which induces a change in A_{240} of 0.43 during the 3 min incubation.

Determination of total glutathione

Preparation of sample by adding 0.5 ml of 4% sulfosalicylic acid into 0.5 ml of hemolysate, then centrifuging at 12,000 rpm for 15 min at 4^{o}C. Supernatant were transferred to 96-well microplate. Then 80 µl of 0.01 sodium phosphate buffer with 1 mM EDTA pH 7.5 was added. Subsequently, 100 µl of reaction mixture (containing 1 mM of DTNB, 0.5 mM of NADPH, 1 iu of GSH reductase dissolved in 0.01 M of sodium phosphate buffer containing 1 mM of EDTA pH 7.5) was added immediately. After addition of the reagent, color development was recorded at 405 nm for 4 min.

Determination of glutathione peroxidase activity

The hemolysate was added to the reaction mixture (consisted of 5 mM EDTA-Na salt, 0.1 M GSH, 10 unit/ml glutathione reductase, Tris-HCl buffer pH 8.0) and allowed to incubate for 5 min at 37°C. Then 7 mM cumene hydroperoxide was added as a starting reagent and the absorbance was monitored at 340 nm. The difference of absorbance per minute was used to calculate the enzyme activity by using an extinction coefficient of NADPH at 6.22 x 10^3 $M^{-1}cm^{-1}$

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Data were analyzed by Student's *t* test and repeated measures one-way analysis of variance (ANOVA) to determine differences between groups. Values of $p<0.05$ were considered to be statistically significant.

Results

The effect of curcuminoids extract or placebo capsules on antioxidant enzyme activities in diabetes mellitus at 0, 3 and 6 months are shown in figure 1. The results show a significant increase of SOD activities and GSH in curcuminoids group when compared with placebo group ($p<0.05$). However catalase and glutathione peroxidase were not significant difference between two groups.

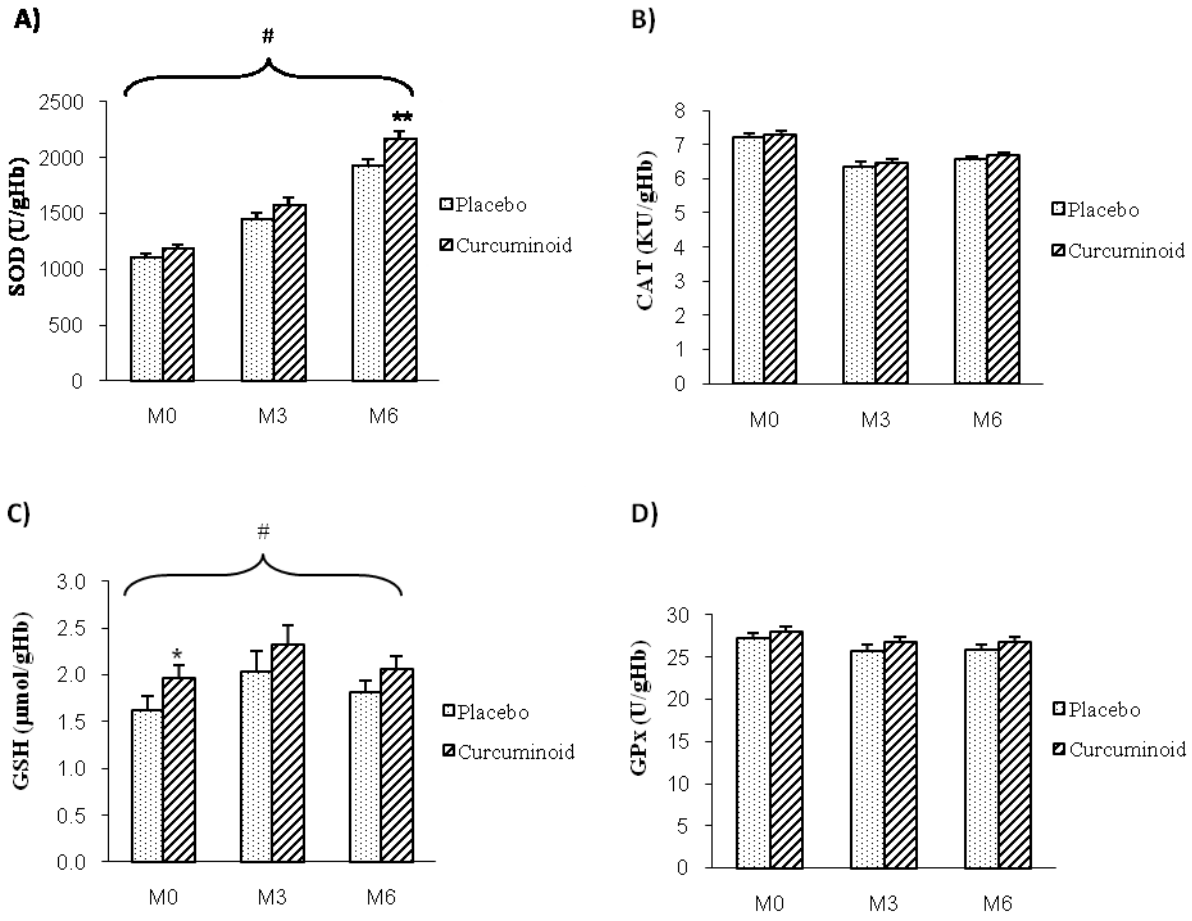

Figure 1 Levels of (A) superoxide dismutase (SOD) activity, (B) catalase (CAT) activity, (C) total glutathione (GSH) and (D) glutathione peroxidase (GPx) activity (mean ± SEM) in red blood cell of diabetic patients receiving curcuminoids extract and placebo capsules at 0, 3 and 6 month.
*$P < 0.05$, **$P < 0.01$ different from placebo group in the same month. (Student *t* test)
#$P < 0.05$ different from placebo group. (repeated measures ANOVA)

Discussion and Conclusion

Our data show that SOD and GSH in curcuminoids group of diabetic patients were significantly higher than those in placebo group. The previous finding of hyperglycemia induces over production of oxygen free radicals in diabetes to cause the levels of enzymatic (GPx, SOD, catalase in RBC) and non enzymatic antioxidants (β-carotene, retinol, vitamin C & E and uric acid) of RBC decrease in NIDDM patients (Ramakrishna V and Jailkhani R., 2008). Glutathione functions as a direct free-radical scavenger and SOD converts O_2^- to hydrogen peroxide, a less reactive ROS. These endogenous antioxidant enzymes provide a first line of defense against superoxide and hydrogen peroxides. Diabetic patients may have elevated requirement for antioxidants which may help to reduce damage brought about by free radical toxicity.

In conclusion, this study demonstrated that supplementation with curcuminoids extract capsules have a potential role in boosting superoxide dismutase activity and total glutathione which are antioxidant-related defenses in diabetic. Adjunct therapy with curcuminoids may represent a useful ancillary pharmacologic approach to the management of diabetes mellitus type II patients.

Acknowledgements

We wish to thank the HRN Princess Maha Chakri Sirindhorn Medical Center for this clinical trial study, the Government Pharmaceutical Organization for supporting curcuminoids capsules and placebo capsules, and the Interdisciplinary Program in Pharmacology, Graduated School, Chulalongkorn University, Thailand for facility of analysis.

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RESEARCH ARTICLE

Effects of Phyllanthin and Hypophyllanthin on Vascular Tension of Isolated Rat Aorta

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Abstract

The purpose of this study was to investigate the modulating effects of phyllanthin and hypophyllanthin on vascular tension, using in the *in vitro* model of isolated rat aorta. Our results indicated that both phyllanthin and hypophyllanthin significantly relaxed the sustained contraction induced by phenylephrine (PE) in a concentration-dependent manner. In addition, endothelial removal had no significant influence on the vasorelaxation responses of the aortic rings toward these two compounds. In comparison to hypophyllanthin, phyllanthin was a more potent vasorelaxant with the apparent EC_{50} values of 55.4 ± 5.5 μM (for the endothelium-denuded rings). Our data also demonstrated that both compounds were able to inhibit the contraction of vascular smooth muscle provoked by either PE (1 μM) or KCl (40 mM). In high K^+ - Ca^{2+} free solution, phyllanthin (100 μM), but not hypophyllanthin, significantly inhibited the contractile responses upon cumulative addition of $CaCl_2$. These findings suggested that phyllanthin and hypophyllanthin could modulate the vascular tension via the endothelium-independent mechanisms. The modulating effect of phyllanthin, in part, was through the inhibition of Ca^{2+} influx to vascular smooth muscle cells.

Keywords vasorelaxation, phyllanthin, hypophyllanthin

Introduction

Phyllanthin and hypophyllanthin are major active components found in *Phyllantus amarus* Schum. and Thonn. (Euphorbiaceae) [also known as "Luk Tai Bai" in Thai] (1). In Thailand, this plant has been used in traditional medicine for a number of diseases in cardiovascular and gastrointestinal tract systems. It has been reported that the alcohol extract of *P. amarus* induced relaxation on smooth muscle including vascular smooth muscle, uterus, intestine, stomach and trachea (2). In addition, the aqueous extract from its leaves exerted its myocardial suppression and hypotensive effects in rabbits, which was possibly linked to muscarinic receptor-mediated mechanism and calcium channel ion blockade (3). It was likely that either phyllanthin or hypophyllanthin, the two major lignans isolated from this plant, possessed the intrinsic pharmacological actions similar to those of the *P. amarus* extracts. However, a few researches have been conducted to investigate the modulating effects of these two lignans on vascular tension and the involved mechanism.

The purpose of this study was to investigate the modulating effects of phyllanthin and hypophyllanthin on vascular tension. Furthermore, we also examined the influence of endothelium on the vascular actions of these two compounds.

Materials and Methods

Test compounds

Phyllanthin and hypophyllanthin were authenticated by Assoc.Prof. Chaiyo Chaichantipyuth, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Preparation of aortic rings

Adult male Wistar rats (250-300 g) were anaesthetized by CO_2 and sacrificed by cervical dislocation. The thoracic aorta were carefully removed and cut into 2-3 mm rings. Then, the rings were suspended in Krebs-Henseleit solution (KHS) bubbled with carbogen gas, pH 7.4 ± 0.5 at 37 °C. The vascular tension was recorded with an isometric force transducer (Harvard Apparatus Ltd. England and MLT 050/A, ADInstruments, Australia) that was connected to computer equipped Software Chart 5.0 of PowerLab 4/SP data acquisition system (ADInstruments, Australia). In some preparations, the endothelium were removed by gently rubbing the lumen with cotton swab. The aortic rings were tested for functional endothelium by addition of acetylcholine (Ach 10 μM). The preparations were considered as endothelium-intact when the relaxation responses were greater than 60%. The endothelium-denuded preparations were applied when the relaxation responses to Ach were less 10%.

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

Experimental protocols

Vasorelaxation effects

The aortic rings were pre-contracted with PE (1 μM). Then, at the plateau state, either phyllanthin or hypophyllanthin was added cumulatively in the concentration ranges of 1-100 μM. The relaxation responses were calculated and expressed as the percentage of the PE-induced contraction.

Effects on agonist-induced contraction

The inhibitory effect of our test compounds on contractility of vascular smooth muscle was also examined by pre-incubating the compound with the tissue in KHS for 20 min. Then, either PE (1 μM) or KCl (40 mM) was added to induce the contraction. In parallel experiments, the preincubation of the test compounds and aortic tissues were performed in high K^+ - Ca^{2+} free depolarizing solution. After 10 min of preincubation period, $CaCl_2$ in the concentration ranges of 10 μM-10 mM was added cumulatively to induce the contraction. The contractile responses were expressed as the percentages of the maximal value of certain agonist-induced contraction.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (S.E.M.). Statistical significances were tested by one-way analysis of variance (ANOVA),

followed by post-hoc Dunnett's. $p<0.05$ was statistically significant.

Result

Vasorelaxation profile

We demonstrated that either phyllanthin or hypophyllanthin (1-100 µM) significantly induced vasorelaxation in both endothelium-intact and -denuded aortic rings (Figure 1, 2). The vasorelaxant activities of these two compounds were concentration-dependent with the calculated EC_{50} (effective concentration) values shown in table 1.

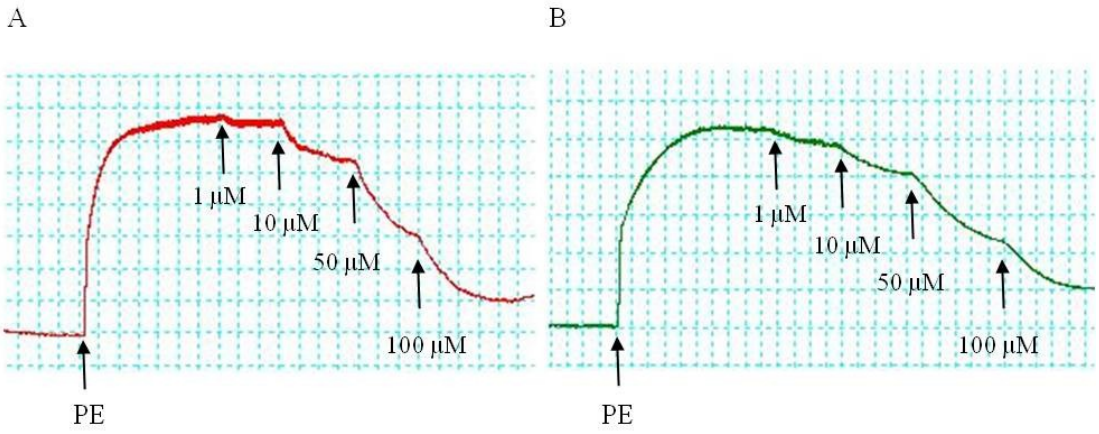

Figure 1 Representative tracing showed the vasorelaxation effect of phyllanthin at cumulative concentration in endothelium-intact (A) and endothelium-denuded aortic rings (B).

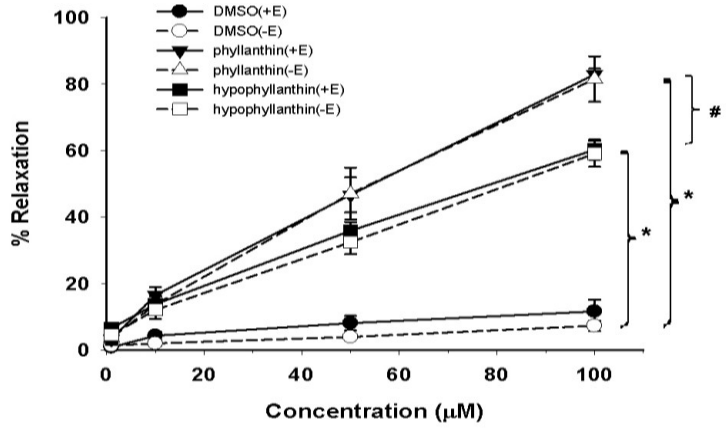

Figure 2 Relaxation effect of phyllanthin and hypophyllanthin in endothelium-intact and -denuded aortic rings that were pre-contracted with PE (1 µM). *$p<0.05$ showed significant difference between test compounds and DMSO. #$p<0.05$ showed significant difference between phyllanthin and hypophyllanthin.

Table 1 The apparent EC_{50} of the test compounds on vasorelaxation

Test compounds	EC_{50} values (μM)	
	Endothelium-intact	Endothelium-denuded
phyllanthin	56.55 ± 2.91	55.40 ± 5.5
hypophyllanthin	80.08 ± 5.05	84.02 ± 5.93

Data were presented as mean ± S.E.M., n=6

Effects on agonist-induced contraction.

Phyllanthin and hypophyllanthin were able to inhibit the aortic contraction provoked by either PE or KCl (Figure 3A and B). The inhibition profiles suggested that phyllanthin was more potent than hypophyllanthin in suppressing the contractile responses of vascular smooth muscle upon challenges with either PE or KCl. The apparent IC_{50} values of phyllanthin were 57.67 ± 8.85 μM for PE-mediated contraction, and 63.30 ± 2.69 μM for KCl-mediated contraction.

In high K^+- Ca^{2+} free depolarizing solution, phyllanthin (100 μM) significantly inhibited the $CaCl_2$-induced contraction with the pD_2 value of 3.49 ± 0.12 (Figure 4). By contrast, the contractile responses in the presence of hypophyllanthin at the equimolar concentration were not significantly different from the DMSO control group. These findings suggested that phyllanthin, but not hypophyllanthin, was able to directly inhibit Ca^{2+} influx to the smooth muscle through VOC.

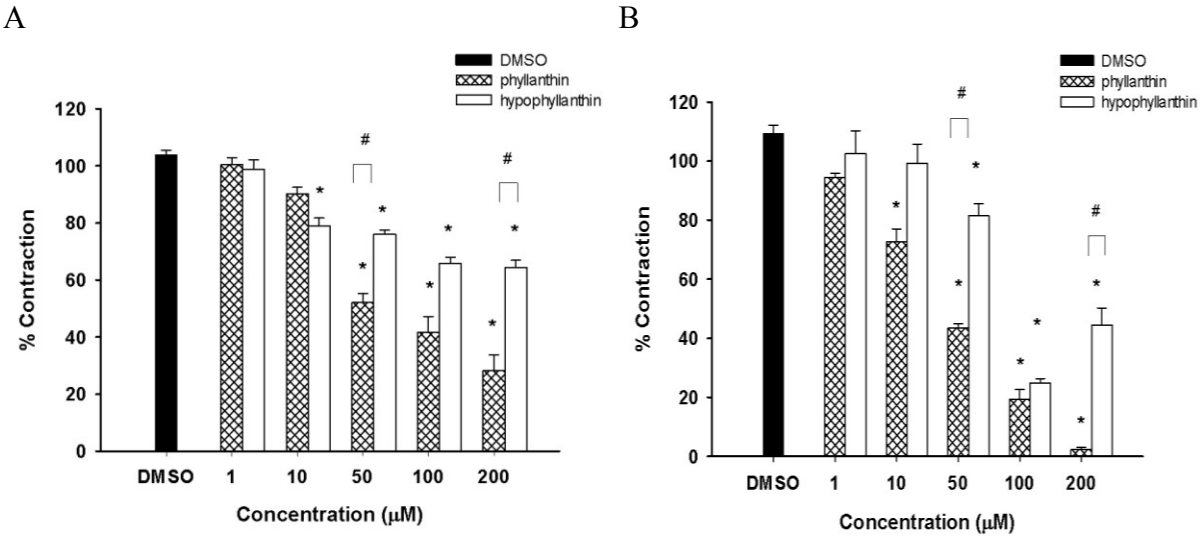

Figure 3 Effects of phyllanthin and hypophyllanthin on contraction of endothelium-denuded aortic rings induced by PE (1 μM, A) or KCl (40 mM, B) in KHS. *p<0.05 showed significant difference form DMSO control group. #p<0.05 showed significant difference between phyllanthin and hypophyllanthin (n=4-6).

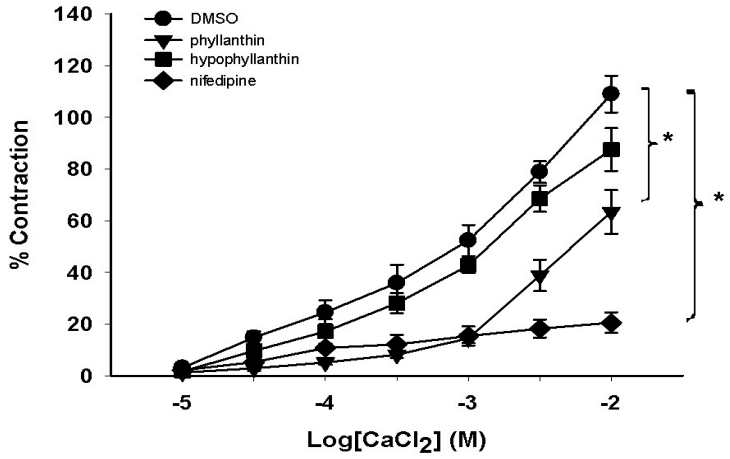

Figure 4 Effect of phyllanthin and hypophyllanthin on contraction of endothelium-denuded aortic rings induced by $CaCl_2$ (10 μM-10 mM) in high K^+ - Ca^{2+} free depolarizing solution. *p<0.05 showed significant difference from DMSO control group (n=6).

Discussion and conclusions

Our findings demonstrated that endothelium had no significant influence on the vasorelaxation induced by either phyllanthin or hypophyllanthin. These results suggested that the mechanisms of vasorelaxation of these two compounds did not involve with the NO-cGMP pathway. It was unlikely that these two compounds bound to the muscarinic receptors on the endothelial cells and activated the synthesis of NO. On the contrary, these two compounds exerted its vasorelaxation effect by directly modulate the function of vascular smooth muscle. One possibility was that the two compounds might affect extracellular Ca^{2+} influx through Ca^{2+} channels. The inhibition profiles against PE- and KCl-mediated contraction suggested that these two lignans might be able to interfere a rising of cytosolic Ca^{2+} which is a key element of muscle contraction. As known, activation of α_1- receptors eventually results in activation of receptor-operated Ca^{2+} channel whereas KCl-induced depolarization of plasma membrane causes an opening of voltage-gated Ca^{2+} channel (VOC) (4, 5). Although these two compounds showed a comparable relaxation profiles, phyllanthin was more potent than hypophyllanthin in inhibiting contraction of the aortic muscle in KHS as well as in high K^+ depolarizing solution. These findings suggested that phyllanthin and hypophyllanthin exerted its vascular effects via different mechanisms. The modulating action of phyllanthin on vascular tension might relate to inhibition of Ca^{2+} influx through VOC

In conclusion, phyllanthin or hypophyllanthin induced aortic relaxation endothelium-independent mechanism. The effect of phyllanthin, in part, involved with the inhibition of Ca^{2+} influx to vascular smooth muscle cells. Further works would be in need to investigate another mechanism involved.

Acknowledgements

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RESEARCH ARTICLE

Comparison the Efficacy Between Intranasal Corticosteroids Mometasone Furoate with Fluticasone Furoate in Persistent Allergic Rhinitis

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Abstract

Allergic rhinitis (AR) is an extremely common health problem. Patients with AR suffer from both nasal and ocular symptoms. The new intranasal corticosteroids (INCs) mometasone and fluticasone with furoate ester side chain are highly potent with minimal systemic absorption. This study purposed to compare the efficacy, tolerability and safety of mometasone furoate (MF) and fluticasone furoate (FF) in Thai patients with persistent allergic rhinitis (PER). The study was a randomized, open-label and parallel group study. Ninety seven patients with PER and nasal symptoms with or without eye symptoms were enrolled into the study. The patients were randomized into 2 groups receiving 2 sprays/nostril once daily either MF or FF for 4 weeks. Total nasal symptom scores (TNSSs), total ocular symptom scores (TOSSs) and nasal airway resistance (NAR) were assessed at baseline and after 4 weeks of treatment. After 4 weeks treatment, MF and FF produced statistically significant improvement in TNSSs, TOSSs and individual symptoms ($P < 0.0001$). Furthermore, both MF and FF produced a similar significant improvement in total NAR from baseline at 75 Pascal (Pa) ($P = 0.009$ and $P < 0.0001$, respectively) and at 150 Pa ($P = 0.002$ and $P < 0.0001$, respectively). However, the difference between treatments was not statically significant. MF was as effective as FF in relieving nasal symptoms and ocular symptoms and in improving nasal airflow.

Keywords persistent allergic rhinitis, mometasone furoate, fluticasone furoate, symptom scores, nasal airway resistance.

Introduction

AR is an extremely common health problem, affecting 10-25% of the population worldwide [1]. Patients with AR suffer from both nasal symptoms (rhinorrhea, sneezing, itching and congestion) and ocular symptoms (redness, itching and tearing). INCs are recommended as first-line therapy for patients with moderate-to-severe AR, especially when nasal congestion is a major component of symptoms [2]. MF and FF are highly lipophilic and highly potent, and have high affinity for glucocorticoid receptor (GR) with minimal systemic absorption [3]. Both are highly effective in reducing nasal and ocular symptoms [4, 5] without causing sedation and side effects associated with systemic corticosteroids [6]. However, no direct comparison of the efficacy and safety of both INCs have been performed in Thai patients with PER.

The aim of this study was to compare the efficacy, tolerability and safety of MF and FF in Thai patients with PER by using both subjective (TNSSs and TOSSs) and objective (Rhinomanometry; RMM) assessments.

Methods

This study was a randomized, open-label and parallel group study. It was approved by the Human Research Ethic Committee of the Faculty of Medicine, Chiang Mai University, and all participants provided written informed consents. A total of 97 patients with a minimum of 6-month history of PER, with positive skin prick test response to 1 or more allergens and had TNSSs ≥ 6, with or without TOSSs of ≥ 4 at baseline were enrolled into the study. Patients were excluded if they had received systemic or INCs 4 weeks before the study; a history of hypersensitivity to glucocorticoids, asthma, structural abnormalities of the nose, acute or chronic upper respiratory infections within the last 4 weeks before the study, chronic illness; and pregnant or nursing women. Patients entered a 1-week run-in period without any medication. Patients were then randomized into 2 groups receiving 2 sprays/nostril once daily either MF or FF for 4 weeks. Patients were asked to record the 24-hour reflective symptoms of TNSSs and TOSSs in daily diary cards according to the 4-point scale; 0 = no symptom, 1 = mild symptoms (present but not troublesome), 2 = moderate symptoms (frequently troublesome, but not sufficient to interfere with normal daily activity or night-time sleep), 3 = severe symptoms (sufficiently troublesome to interfere with normal daily activity or night-time sleep). NAR was measured bilaterally by anterior RMM using the ATMOS rhinomanometer 300 (Lenzkirch, Germany). Nasal airflow was reported as the sum of recorded airflow through the right and left nostrils at a transnasal pressure of 75 and 150 Pa.

Unpaired *t*-test and paired *t*-test were used for comparison between treatments and for comparison within treatment, respectively. The statistical software used to process the data was SPSS 16.0. All comparisons were performed as two-sided tests, *P* values of less than 0.05 were considered significant.

Table 1 Demographic data of patients in both treatment groups

Characteristic	Treatment group		*P* value
	MF (n = 51)	FF (n = 46)	
Mean age in years (range)	33.35 ± 11.82 (18-56)	33.93 ± 11.75 (18-57)	0.809
Sex (n) -Female -Male	 33 18	 27 19	 0.676 0.676
Mean duration of AR in years (range)	8.12 ± 6.40 (1-30)	7.73 ± 6.38 (1-30)	0.765

Values are mean ± SD, n= number

Results

Baseline demographic and disease characteristics of the 97 patients were comparable for both treatment groups (Table 1). Two patients in each of the two groups discontinued the study; 2 with unknown reason and 2 with incomplete treatment.

Subjective assessment

At baseline, TNSSs and TOSSs were comparable for both MF and FF treatment groups (P = 0.316 and P = 0.241, respectively). After 4-weeks, TNSSs, TOSSs and individual symptom scores of both treatments were improved significantly and comparably as shown in Figure 1a and 1b. Figure 1c and 1d compare the efficacy of 4-week treatments on TNSSs, TOSSs and individual symptom scores between MF and FF. Each score was significantly decreased with both treatments. However, the differences between treatments were not statically significant.

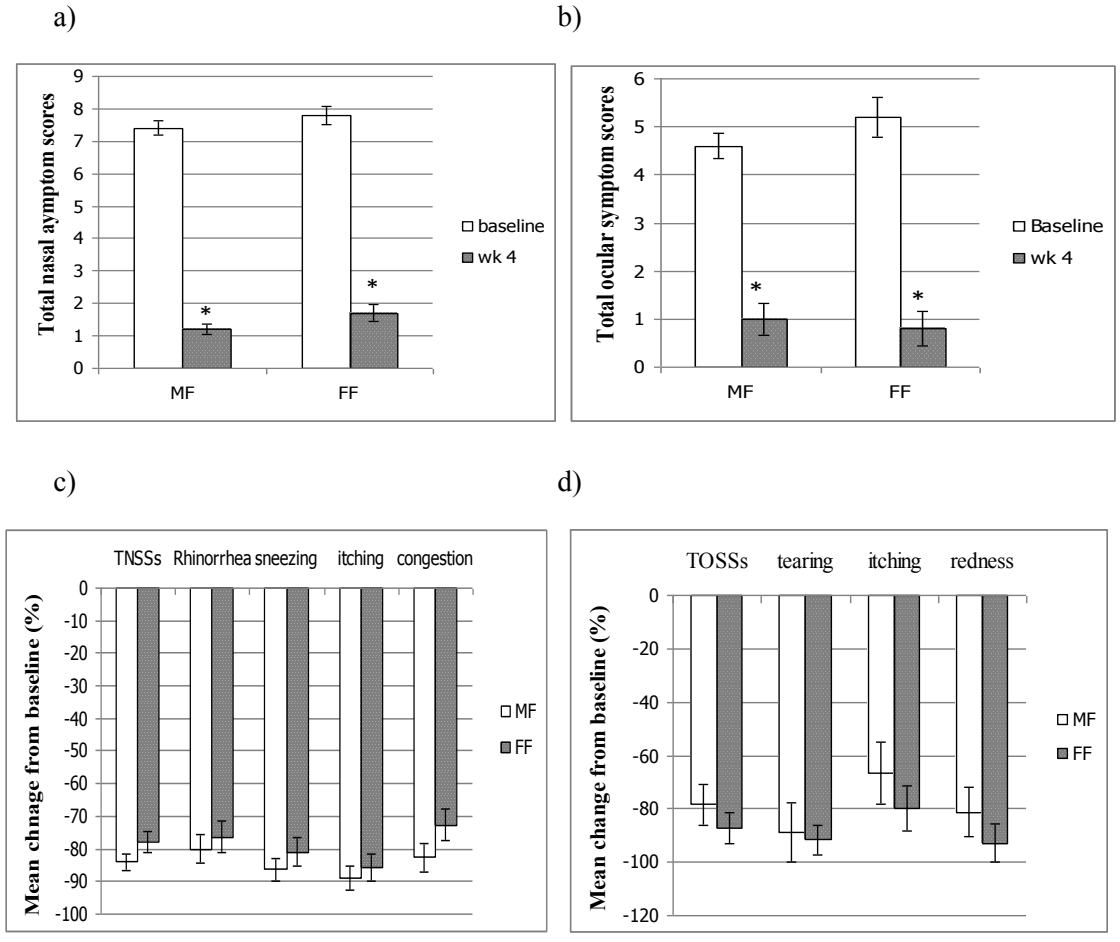

Figure 1 Effects of MF and FF on TNSSs (a), TOSSs (b), and individual symptom scores (c and d) after 4 weeks of treatment. Data are shown as mean ± S.E.M. * $P < 0.0001$. TNSSs: MF, n = 49, FF, n = 44; and TOSSs: MF, n = 10, FF, n = 10.

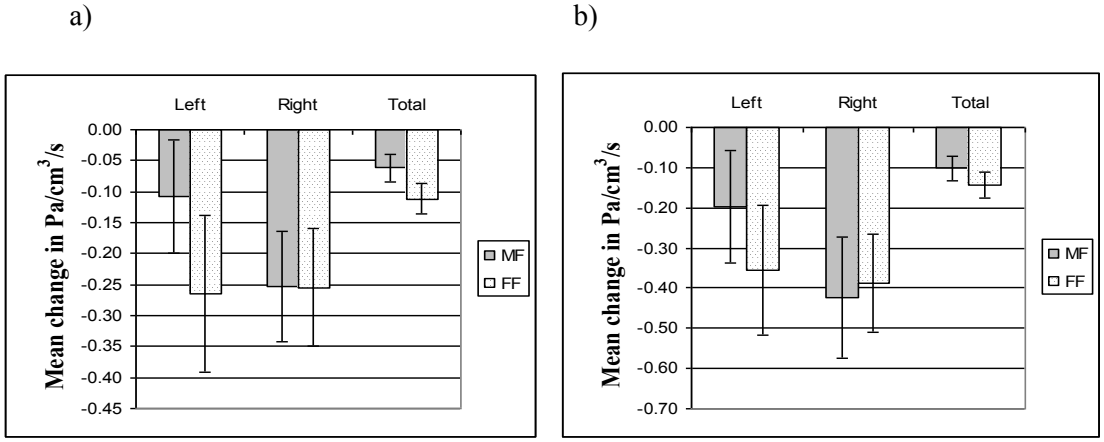

Figure 2 Effects of MF and FF on the unilateral and total NAR (Pa/cm^3/s) after 4 weeks of treatment at 75 Pa (a) and 150 Pa (b). Data are shown as mean ± S.E.M.

Objective assessment

Unilateral and total NAR were similar in both treatment groups at baseline. Only FF but not MF significantly decreased NAR of the left side. However, both MF and FF produced similar statistically significant improvement in total NAR from baseline at 75 Pa (P = 0.009 and P < 0.0001, respectively) and at 150 Pa (P = 0.002 and P < 0.0001, respectively). The comparative mean changes of MF and FF on the unilateral and total NAR from baseline are depicted in Figure 2. These changes were not significant difference between the two drug treatments.

Both drug treatments were well tolerated. Adverse events were reported by 41.24% of patients. Most adverse events were mild and moderate in intensity and no serious adverse events were reported. Nasal irritation and pharyngitis were the most common adverse events reported. The incidence of adverse events was similar and was not significantly different between the two drugs.

Discussion

This study was the first trial comparison between MF and FF in Thai patients with PER. Traditionally, clinical trials in AR have focused on nasal symptoms; however, recent studies have highlighted the significance of ocular symptoms. Although ocular symptoms are common in AR patients, their severity is variable. Only 20% of patients in this study had symptoms severity that met the inclusion criteria. The mechanism of action by which INCs relieving nasal symptoms of AR is their potent anti-inflammatory effects to suppress the production of multiple pro-inflammatory mediators such as cytokines and leukotrienes and also to inhibit the action, recruitment, and migration of inflammatory cells [1]. However, the mechanism of action of INCs in relieving ocular symptoms is not well understood. Recent study proposed that the INCs could affect the nasal-ocular reflex to reduce ocular symptoms [7]. RMM provides objective information of nasal patency that has been used for studying efficacy of INCs in alleviating nasal obstruction [8]. Both MF and FF are potent INCs and share the same mechanism of action, thereby producing equal efficacy as measured by TNSSs , TOSSs and total NAR and producing similar adverse events profile in the present study. The minor difference was that only FF but not MF that produced significant decrease in NAR of the left nostril. In conclusion, MF is as effective as FF in relieving nasal and ocular symptoms, in improving nasal airflow and they produce similar adverse events profile.

Acknowledgements

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RESEARCH ARTICLE

Effects of the Standardized Extract of *Centella asiatica* ECa233 on the Respiration of Mitochondria Isolated from Rat Brain

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Abstract

The effects of standardized extract of *Centella asiatica* ECa233 on the respiration of mitochondria isolated from rat brain were studied. The rat brain mitochondria were incubated with ECa233 5 concentrations (0.01, 0.1, 1, 10, and 100 mg/ml). High concentration of ECa233 (100 mg/ml) decreased the rate of oxygen consumption by 26 and 30% while using glutamate plus malate and succinate as substrate, respectively. However there was no significant difference in the rate of oxygen consumption of the brain mitochondria incubated with all ECa233 concentrations compared with the control. The finding suggested no toxic effect of ECa233 on mitochondria. The data supported further investigation on the protective effects of ECa233 on brain mitochondria.

Keywords standardized extract of *Centella asiatica* ECa233, brain mitochondria, oxygen consumption, respiratory control index

Introduction

Mitochondria play the important roles in energy production, cell signalling and regulation of cell morphology, mobility, multiplication, and apoptosis. Dysfunction of mitochondria leads to decreased of ATP production, disrupted calcium buffering and promoted the generation of reactive oxygen species (ROS) (1). Neurons are susceptible to the alterations of mitochondria functions, thus mitochondrial dysfunction has been hypothesized to be involved in aging and neurodegenerative diseases (2).

Centella asiatica (Linn.) has been traditionally used to improve cognitive function (3). Clinical studies showed the benefits of *Centella asiatica* extract in age-related decline in cognitive function (4), elderly with mild cognitive impairment (5), generalized anxiety disorder and depression (6). *In vivo* studies revealed that *Centella asiatica* extract improved cognitive behavior in pentylenetetrazole-induced kindled seizure rats (7), protected against 3-nitropropionic acid-induced mitochondrial dysfunction in mice brain (8) and attenuated MPTP-induced oxidative stress in aged rats (9). The standardized extract of *Centella asiatica* ECa233 is established by researchers from the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The extract contained not less than 80% triterpenoids and the ratio between madecassoside and asiaticoside should be within 1.50 ± 0.50. Oral administration (10-30 mg/kg) exhibited ameliorating effects on memory deficits induced by either transient bilateral occlusion of common carotid arteries or i.c.v. injection of β-amyloid mice suggesting a promising neuroprotective effect of the extract in stroke and Alzheimer's disease (10). The aim of the present study was to investigate the effect of ECa233 on the mitochondria isolated from rat brain.

Materials and Methods

Male Wistar rats weighing 200–250 g (National Laboratory Animal Center, Mahidol University, Nakornpathom) were housed at a constant ambient temperature (25±2 °C) and humidity (45–65%) on a 12-h light/dark cycle with free access to food and water. The standardized extract of *Centella asiatica* ECa233 were provided by Associate Professor Chamnan Patarapanich and co-workers, Faculty of Pharmaceutical Sciences, Chulalongkorn University. ECa233 used in this experiment was dissolved in DMSO to 5 concentrations; 0.01, 0.1, 1, 10, and 100 mg/ml.

Method for isolation of rat brain mitochondria was modified from Zhan *et al.* (11). Briefly, rat was sacrificed using chloral hydrate (400 mg/kg i.p.) and decapitated. The brain was quickly removed and placed in ice-cold isolation buffer containing 250 mM sucrose, 10 mM Tris, 2 mM EDTA, and 1 mg/ml of BSA (pH 7.4). The cerebellum was removed and the cerebrum was homogenized in 20 ml of the isolation buffer. Rat brain homogenate was centrifuged at 2000 g for 3 min. The supernatant was collected and added 20 µl of 0.02% digitonin then centrifuged twice at 2000 g for 3 min and 12000 g for 10 min. The pellet was resuspended in the isolation buffer and centrifuged at 12000 g for 10 min. The synaptosome layer was washed with ice-cold incubation buffer containing 300 mM mannitol, 75 mM sucrose, 5 mM KCl, 10 mM Tris, and 5 mM KH_2PO_4 (pH 7.4). Finally, the mitochondria pellets were collected and resuspended in 2 ml incubation buffer. Protein concentrations were determined using Lowry method.

Effects of ECa233 on the respiration of rat brain mitochondria were investigated using Gilson oxygraph apparatus. Mitochondrial suspension was incubated with the incubation buffer in a Gilson reaction chamber at the controlled temperature of 37 ^{o}C. Either 10 µl of 1 M glutamate plus 1 M malate or 10 µl of 1 M succinate, the substrates for mitochondrial electron transport chain complex I and complex II respectively, was added (state 4 respiration) and then added 10 µl of ECa233 to the reaction chamber for 1 min. Four µl of 0.3 M ADP plus 0.6 M phosphate were then added to initiate mitochondrial oxidative phosphorylation reaction (state 3 respiration). The oxygen concentration in the reaction chamber was measured using Clark oxygen electrode and calculated as natoms oxygen/minute/mg protein. The respiratory control index (RCI) was used to evaluate mitochondria function and only the mitochondria suspension which RCI = 4

were used. Data were presented as mean ± SEM (n = 4/group). One-way ANOVA was performed for statistical comparisons between each concentration of ECa233 and control. $P \leq 0.05$ was considered as significant difference.

Results

The mean respiratory control index (RCI) of mitochondria suspension (±SEM) used in the present study was 5.64 ± 0.28 (n = 8) when using glutamate plus malate as mitochondria respiratory chain substrates indicating a good condition of mitochondria before incubating with ECa233. The range of ECa233 concentration used in this experiment was 0.01-100 mg/ml to examine the toxic effect of ECa233 from low to high concentrations. It was demonstrated that ECa233 tended to reduce rate of oxygen consumption of mitochondrial state 3 respiration when using either glutamate plus malate (figure 1A) or succinate (figure 1B) as substrates. ECa233 100 mg/ml (1,000 µg in chamber) decreased rate of oxygen consumption of mitochondrial state 3 respiration by 26.11 and 29.88% when using glutamate plus malate and succinate as substrates, respectively, but these changes were not significantly different from control. In addition ECa233 had no effect on rate of oxygen consumption of mitochondrial state 4 respiration.

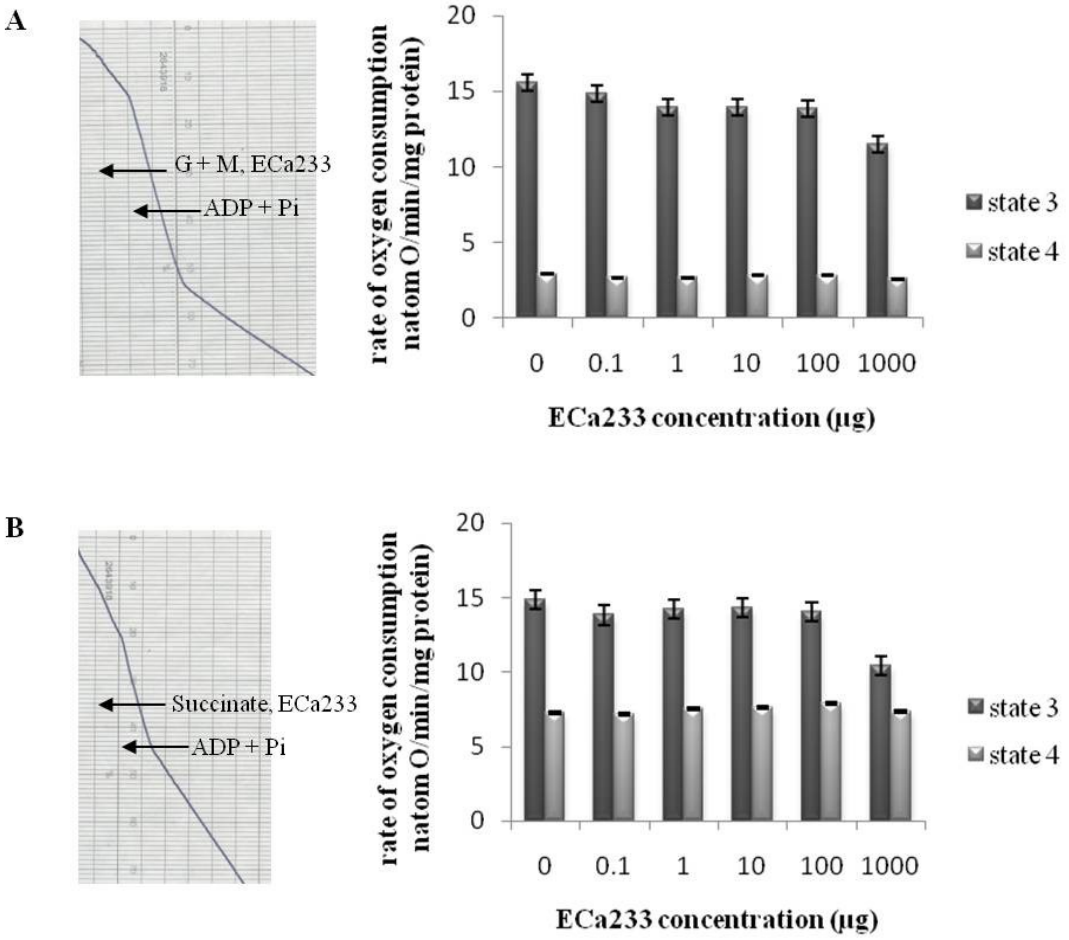

Figure 1 The examples of oxygen monitor tracing and rate of mitochondrial oxygen consumption of rat brain mitochondria in incubated medium containing glutamate plus malate, ADP + Pi and ECa233 (A) and succinate, ADP + Pi and ECa233 (B)

Discussion and Conclusion

The present study showed no toxic effect of the standardized extract of ECa233 on the respiration of rat brain mitochondria, even though brain mitochondria were incubated with very high concentration of ECa233 (1000 μg). This finding is in agreement with earlier *in vivo* studies showing that pre-treatment of ECa233 (10 and 30 mg/kg) significantly decreased cerebral lipid peroxidation indicating an antioxidant property of ECa233 [10]. As the pathogenesis of Parkinson's disease (PD) and Alzheimer's disease (AD) related to the inhibition of complex I and complex IV in mitochondrial electron transport chain, respectively, it is worth to investigate the neuroprotective effect of ECa233 on brain mitochondria models implicated PD and AD *in vitro*.

Acknowledgment

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RESEARCH ARTICLE

Effect of Anthraquinone Glycosides Extracted from *Senna alata* Leaves on the Contractions of Rat Isolated Gastric Fundus

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Abstract

Anthraquinone glycosides extracted from the leaves of *Senna alata* Linn. (Chum-Het-Thet) were investigated on the motility of rat isolated gastric fundus. The glycosides produced dose-dependently and significantly increase in the force of contraction of the fundus. The contractions were partially inhibited (50-60%) by the muscarinic receptor blocker, atropine (10^{-6} M), and were almost or completely abolished by other receptor blockers, histamine H_1-receptor antagonist, chlorpheniramine (2.56×10^{-4}M), serotonin-receptor antagonist, cyproheptadine (10^{-5} M) and also by a calcium channel blocker, verapamil 10^{-4} M. Thus, it is suggested that the contractile effect of the glycosides involved the activation of muscarinic, histamine and serotonin receptors which caused the contraction by the increase in the intracellular Ca^{2+} which partly due to the influx of extracellular Ca^{2+}. It is likely that the extract might be a useful gastrokinetic agent.

Key words anthraquinone glycoside, *Senna alata*, rat isolated gastic fundus, gastrokinetic, contraction

Introduction

Senna alata Linn. (Fam. Caesalpinioideae) is commonly known as "Chum-Het-Thet". Its leaves and flowers are commonly used as laxative. The active ingredient, which contributes to the laxative effect, is anthraquinone (rhein, emodin, aloe-emodin, chrysopherol and physione). They are present mainly as glycosidic form, which are inactive products. After ingestion, the glycosides are activated by bacterial β-glucosidase in the large intestine into their pharmacological active aglycones and reduced to anthrone or anthranone[1]). It is interesting to see whether the anthraquinone glycosides will act on the upper part of GI tract, such as stomach which might be a potential gastrokinetic agent.

Materials and Methods

Plant material

The mature fresh leaves of *S. alata* were harvested in May from Songkhla and Trang Provinces, Thailand. The voucher specimen (PSU No. 0012977) was identified by Assoc. Prof. Chaothip Purintaravarakul, and is kept at the Herbarium of the Department of Biology, Faculty of Science, Prince of Songkla University, Thailand. The leaves were dried at 50°C in hot air oven for 24 hours, and then pulverized and kept in air tight container.

Plant extraction

Extraction of glycosides

The dried leaf powder of *S. alata* (0.6 kg) was extracted twice with water under reflux condition for 30 min. The extract was then partitioned between water and ethyl acetate. The water fraction was collected and freeze dried (102.42 g; yield 17.07 %). The extract was kept in light protected container and placed in desiccator at 4^{o} C until used.

Experimental animals

Wistar rats (200-250 g) of either sex were supplied by the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai campus, Songkhla, Thailand. They were housed in air-conditioned room (temperature 24-26^{o}C, humidity 50%) with a 12 hr. light/dark cycle. The animals were fed with rodent laboratory chow and water *ad libitum*. The study protocol was approved by the Ethics Committee on Animal Experiment, Prince of Songkla University, Thailand.

Experimental procedures

Preparation of isolated rat gastric fundus

The preparations were based on the methods of the Staff of the Department of Pharmacology, University of Edinburgh[2]. Rats were sacrificed by cervical dislocation and exsanguinations. The abdomen was opened; the whole stomach was removed, the fundal part of the stomach was separated from the pyrolic part. The fundus was opened out longitudinally, placed in a dish containing Krebs-Henseleit (Krebs') solution and made into a strip about 4-5 cm long and 0.3 cm wide by transverse cut.

The fundus was then set up in an organ bath filled with 20 ml Krebs' solution and aerated with 95% O_2 and 5% CO_2. The preparation was loaded with 2 g tension and allowed to equilibrate for 30 minutes before commencement of the experiment. During the equilibration period, the tissues was washed with fresh Krebs' solution every 10 minutes. The contractions of fundus was recorded isometrically with a force FT03 displacement transducer connected to a Grass Model 7H polygraph (Grass International Co., Quincy, Mass, USA).

Experimental protocols

The glycosides (0.1-10 mg/kg) were studied on the contraction of rat gastric fundus. The contractile responses were compared with those produced by the standard spasmogens: acetylcholine (10^{-6} M, histamine (10^{-6} M), serotonin (10^{-7} M), and potassium chloride (40 mM) and the inhibition of the contractions by their corresponding antagonists: atropine (10^{-6} M), chlorpheniramine (2.56×10^{-4} M), cyproheptadine (10^{-7} M), and verapamil.

Statistical analysis

Data were expressed as mean ± standard error of mean. Differences between means were analyzed using analysis of variance (ANOVA). This was followed by LSD to determine individual differences. A probability of less than 0.05 was taken to indicate statistical significance.

Results

The glycosides (0.3-10 mg/ml) caused dose-dependently and significantly increase in the force of contraction of isolated rat gastric fundus. The contractions were partially inhibited (50-60%) by atropine 10^{-6} M whereas it blocked ACh (10^{-6} M)-induced contraction by 82% (Figure 1), while the parallel time control experiment did not significantly change. The glycoside-induced contractions were almost or completely abolished by other receptor blockers, histamine H_1-receptor blocker, chlorpheniramine (2.56 x 10^{-4} M), and serotonin $HT_{2A/2B}$-receptor antagonists, cyproheptadine (10^{-5} M). These two receptor antagonists completely blocked the contraction-induced by histamine 10^{-4} M and serotonin 10^{-7} M , respectively The glycoside-induced contractions were also completely abolished by a calcium channel blocker, verapamil 10^{-4} M. Data were summarized in Table 1.

The glycoside content of the extract (analyzed according to Thai Herbal Pharmacopoeia 1998) calculated as rhein-8 glycoside = 0.84 % (w/w).

Discussion

This study demonstrated that the anthraquinone glycosides of the *S. alata* leaves had significant contractile effect on the rat isolated gastric fundus. There are evidences revealing that the muscarinic, histamine and serotonin receptors of rat gastric fundus are M_1 and M_2[3]; H_1[4] and $5HT_{2B}$[5] subtypes, respectively. The glycoside-induced contraction was blocked by the nonselective muscarinic blocker, atropine; the H_1-blocker, chlorpheniramine and the $5HT_{2A}/_{2B}$ blockers, cyproheptadine. Thus it is suggested that the contractile effect of the glycoside was due to the activation of these receptors. The stimulation of the receptors caused the increase in intracellular Ca^{2+} through the stimulation of Gq-PLC-IP_3 pathway, resulted in the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} [6]. This was substantiated to our results that the contractile effect of the extract was blocked by the L-type Ca^{2+} blocker, verapamil. The results also suggested that the extract can be used as a gastrokinetic, however, in vivo study should be performed.

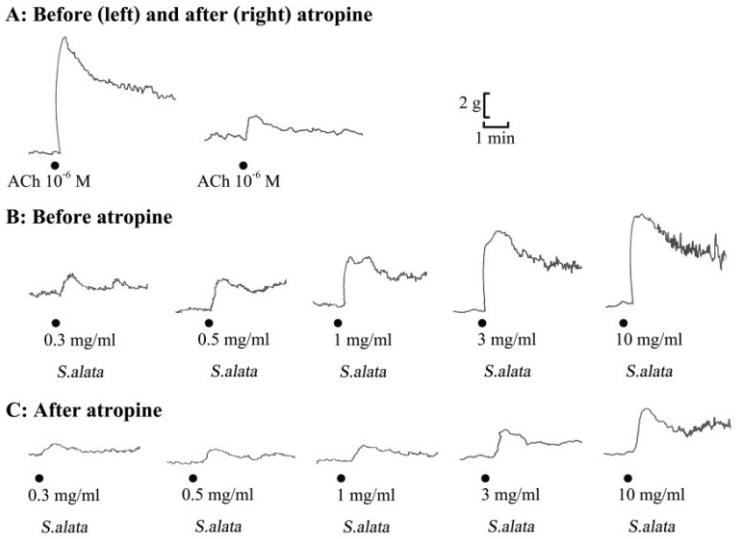

Figure 1 Typical traces of the effects of atropine (10^{-6} M) on the contraction of rat isolated gastric fundus induced by *S. alata* anthraqinone glycoside as compared to those induced by the references drugs, acetylcholine (ACh).

(Table 1 P53)

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RESEARCH ARTICLE

Protective Effects of Silk Lutein Extract and Vitamin E on UV-B Induced Oxidative Stress in Retinal Pigment Epithelial Cell Damage

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Abstract

UV-B induced oxidative stress of retinal pigment epithelial cells plays an important role in the development of age-related macular degeneration (AMD). This study was aimed to investigate the protective effect of silk lutein extract (SLE) and vitamin E on UV-B induced retinal epithelial cells damage. Oxidative stress in ARPE-19 cells was evaluated by measuring the level of intracellular reactive oxygen species (ROS) and lipid peroxidation. The results showed that SLE and vitamin E cloud significantly reduce the effect of UV-B on the formation of intracellular ROS and lipid peroxidation, and these two substances however slightly increased cell viability. The combination of SLE and vitamin E exhibits more antioxidative effect than that of individual compound. These data suggest that lutein from silk cocoon and vitamin E exhibit a partial protective effect against UV-B induced oxidative stress.

Keywords Oxidative stress; Silk lutein extracted; vitamin E; UV-B

Introduction

Age-related macular degeneration (AMD) is one of the most common causes of severe visual loss in the elderly population (1). Oxidative damage is thought to play an important role in the pathogenesis of AMD (2). Oxidative stress by UV radiation may cause retinal pigment epithelial (RPE) cells damage (3). Among several antioxidants that are involved in the protection of membrane lipids against peroxidation, vitamin E and carotenoids may be particular important (4-5). Lutein is the major caroteniods that present in the macular region of human eyes (6). It is the pigments that absorbs light and directly scavenge free radical to prevent RPE cells damage. Yellow pigments of silk worm cocoon are mainly composed of flavoniods and caroteniods (7). Lutein is a one of a caroteniods found in pigment extract of yellow silk cocoon (8). In this study, we hypothesized that lutein in silk yellow cocoon, and vitamin E can protect RPE cells from UV-B induced cell death through its antioxidative effects. The possible mechanisms include inhibition of the UV-B mediated intracellular ROS production and lipid peroxidation.

Methods

Cell culture

The human retinal epithelial cells, ARPE-19 cells line was obtained from American Type Culture Collection (ATCC). Cells were grown in DMEM/F12 containing 10% fetal bovine serum and 1% penicillin-streptomycin. Cells ($1x10^5$ cells/well) were plated into 24-well plates for 24 h and pre-treated with silk lutein extract (SLE) and/or vitamin E, before exposure to UV-B radiation.

Cell viability assay

The viability of cells was determined by tryphan blue assay. After UV-B exposures, ARPE cells were trypsinized, suspended in 0.4 % trypan blue, and counted on a hemacytometer under a microscope.

Intracellular ROS measurement

Cells were incubated with 50 μM 2',7'-dichlor- fluorescein-diacetate (DCFH-DA) for 30 min before UV-B exposure. After UV-B irradiation, medium were replaced with fresh serum free medium without phenol red. The fluorescence was measured at 30 min after UV-B exposure using a fluorescence multi-well plate reader at Ex 485 nm and Em 535 nm.

Determination of lipid peroxidation

The lipid peroxidation was assessed by the thiobarbituric acid (TBA) reaction. The lipid peroxidation was expressed as nanomoles of malondialdehyde (MDA) per $1x10^4$ cells. Briefly, after 24 h of UV-B irradiation, TBARs reagent consisting of 0.4% TBA, 40% TCA and 8% HCl was added to cells and incubated at 90 °C for 1 h. The absorbance was measured fluorescence at Ex 485 nm and Em 535 nm.

Results

SLE and vitamin E inhibits UV-B-induced ARPE-19 cell death

UV-B exposures decrease of cell viability of ARPE-19 cells in a dose dependent pattern (figure 1). Pretreatment cells with SLE and vitamin E at 50 μM could not completely increase cell viability to the control level. SLE and vitamin E showed moderate protection against UV-B at 80 mJ/cm^2. This result suggests that SLE and vitamin E can slightly prevent retinal cells damage mediated by UV-B irradiation.

SLE and vitamin E decrease UV-B-induced intracellular ROS production and lipid peroxidation.

The exposure of ARPE-19 cells to UV-B increased intracellular ROS production and lipid peroxidation compared to non UV-B exposed cells (figure 2). These increases were dependent on the intensity of UV-B. Pre-incubating cells with SLE or vitamin E (50 μM) significantly decreased UV-B-induced intracellular ROS production at all of UV-B doses (figure 2A). Combination of SLE and vitamin E exhibited higher ROS production suppression than that of individual substance. The inhibitory effect of SLE and vitamin E on lipid peroxidation (figure 2B) was similar to that of ROS production.

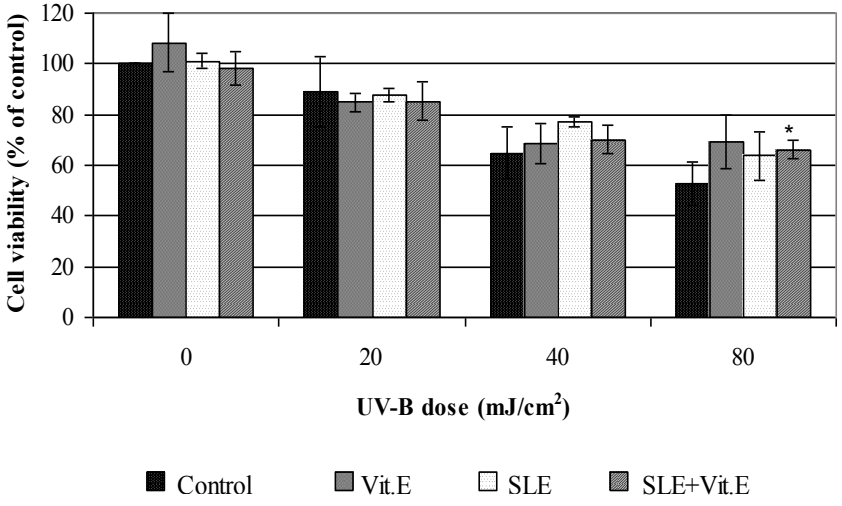

Figure 1 Effect of SLE and vitamin E on UV-B induced cell death. ARPE-19 cells were pre-incubated for 24 h with SLE and vitamin E at concentration 50 μM, and then exposed to UV-B radiation (20, 40, and 80 mJ/cm^2). The data represent mean ± SEM of three experiments. * P<0.05 compared with no treatment.

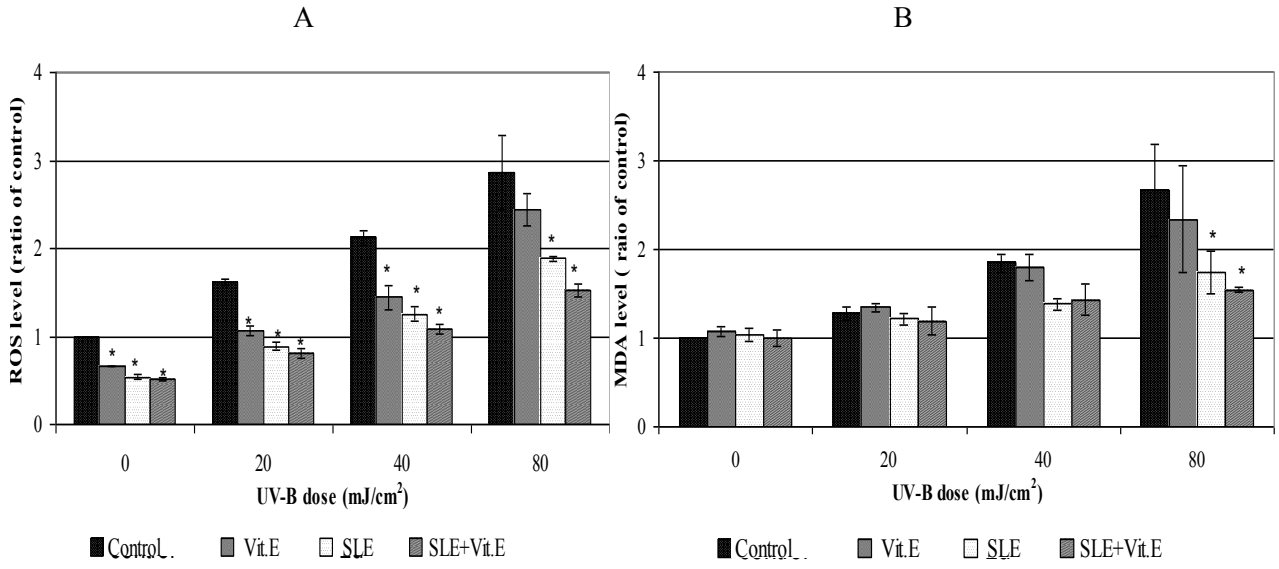

Figure 2 Effect of SLE and vitamin E on UV-B induced ROS production (A) and lipid peroxidation (B). ARPE-19 cell were pre-incubated for 24 h with SLE and vitamin E at concentration 50 μM, and then exposed to UV-B radiation (20, 40, and 80 mJ/cm^2). The data represent mean ± SEM of three experiments. *P<0.05 compared with no SLE and/or vitamin E treatment.

Discussions

We investigated the protective effects of a silk lutein extract (SLE) and vitamin E on UV-B induced oxidative damage in RPE cells. This study demonstrates that the combination of SLE and vitamin E is most efficient to protect RPE damage mediated by UV-B induced oxidative stress. The possible protection mechanisms against this oxidative stress could be the result of quenching singlet

oxygen and scavenging lipid free radicals (9). Vitamin E was showed to reduce the rate of zeaxanthin depletion in cells culture medium (10). This evidence may supports the additive effect of the combined SLE and vitamin E in the present study. The synergistic of antioxidant such as lycopene and vitamin E was also demonstrated on the inhibition of LDL oxidation because of ability of vitamin E to scavenge the lycopene derrived pyroxyl radical therefore enhancing lycopene antioxidant activity (11). Since lutein is an unstable compound, reducing its degradation rate or restoring lutein derived radicals by vitamin E is likely to promote the antioxidant activity of lutein.

Conclusion

In conclusion, UV-B irradiation induces cell oxidative damage in ARPE-19 cells by mediating the production of intracellular ROS and induction of lipid peroxidation. Silk lutein extracte (SLE) in the present of vitamin E provides protection against UV-B induced cell damage through the suppression of intracellular ROS and lipid peroxidation. Therefore, the use of lutein in the combination with vitamin E may be beneficial for attenuation of oxidation induced retinal epithelial cells damage that occurs in AMD.

Acknowledgements

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RESEARCH ARTICLE

Effect of the Ethanolic Extract of *Passiflora foetida* on Conditioned Place Preference

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Abstract

In the traditional medicine of many countries, *Passiflora incarnata* was proved to be useful in drug addiction therapy. In this study, we investigated the effect of *Passiflora foetida,* a plant in the same genus, on drug addiction. We evaluated the effect of the ethanolic extract of *P. foetida* (PF) on locomotor activity and conditioned place preference (CPP). The results showed that PF at doses of 25, 50, 100 and 200 mg/kg produced no significant effects on locomotor activity as compared to control animals. The reinforcing effect of PF was tested using CPP paradigm. All doses of PF did not show any significant effects of CPP. These results suggest that PF may be useful in the prevention and treatment of drug addiction.

Keywords Addiction, *Passiflora foetida*, Morphine, Conditioned Place Preference

Introduction

Various species of plants in the genus *Passiflora* (family Passifloraceae) have been used extensively in the traditional medicine in many countries (1). One of the species which is found abundant in Thailand is *Passiflora foetida* (Ka-tok-rok). In Thai traditional medicine and India, this plant has been used for the treatments of cough, cold, fever, headache and asthma (1). From the facts that the ethanolic extract of *P. foetida* was found to act on D_1 dopaminergic receptors using receptor binding study (Meksuriyen et al., personal communication); and the methanolic extract of *P. incarnata* was found to reduce the naloxone-precipitated withdrawal jumps in mice after morphine injection and to render tolerance due to chronic treatment with 10 mg/kg of morphine (2). Therefore, this plant should be tested for its abuse potential before testing for other potential uses in clinical purposes including drug addiction treatment. Thus, the purpose of this study was to investigate the reinforcing effect of the ethanolic extract of *P. foetida* (PF) using conditioned place preference model in rats.

Materials and Methods

Chemicals

Morphine sulfate 5 mg/kg (Temad, Iran) was dissolved in 0.9% normal saline solution (NSS) as a positive control. 0.5% Carboxymethylcellulose (CMC; Sigma, USA) was dissolved in distilled water as a vehicle control. The PF was dissolved in 0.5% CMC. PF and vehicle were orally administered and morphine was administered by intraperitoneal injection. The solutions *were freshly prepared* immediately before use.

Animals

Male ICR mice weighing 18-25 g and Wistar rats weighing 200-250 g purchased from the National Laboratory Animal Centre (Salaya campus, Mahidol University, Nakhonprathom, Thailand). Animals were housed under a 12-h light-dark cycle at a temperature of 25 ± 2 ^{o}C with free access to food and water for at least 1 week prior to testing. All procedures were approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Locomotor activity test

The effect of PF on locomotor activity in mice was tested using an activity cage (UGO Basile, Comerio, Italy). Mice were treated with NSS, 0.5% CMC, morphine 5 mg/kg or PF 25, 50, 100 and 200 mg/kg. Immediately after administration of tested compound, a mouse was placed individually in the activity cage. The locomotor activity of the animal was continuously recorded at 5 min intervals for 75 min.

Conditioned place preference (CPP)

CPP paradigm has been used as a model for studying the reinforcing effects of dependence-liable drugs. The CPP apparatus was consisted of 3 different compartments. Two equal-sized compartments (length 25 cm, width 34 cm) were separated by guillotine doors from central compartment. One compartment was painted white on each wall, while the other was painted with black and white vertical stripes and had mesh floor. These lateral compartments offered distinct stimuli in odor, color and texture. The middle compartment (length 25 cm, width 11 cm) was painted with grey. Removal of the guillotine doors allowed animal's free access to all compartments. Time spent by animals in each of the two compartments was recorded for 15 min. CPP test consisted of a 12 day schedule with three phases: preconditioning (3 days), conditioning (8 days) and test phases (1 day). This protocol was described previously by Spyraki et al. (3). Conditioned place preference was evaluated as the difference in pre-conditioning and post-conditioning time spent in the drug-paired compartment. An increase in the time spent in the drug-paired compartment after conditioning phase suggests the presence of the positive reinforcing effect.

Statistical analysis

Results were expressed as mean ± SEM. Differences among means were tested by one-way ANOVA followed by Dunnett's test, $P < 0.05$ was considered significant.

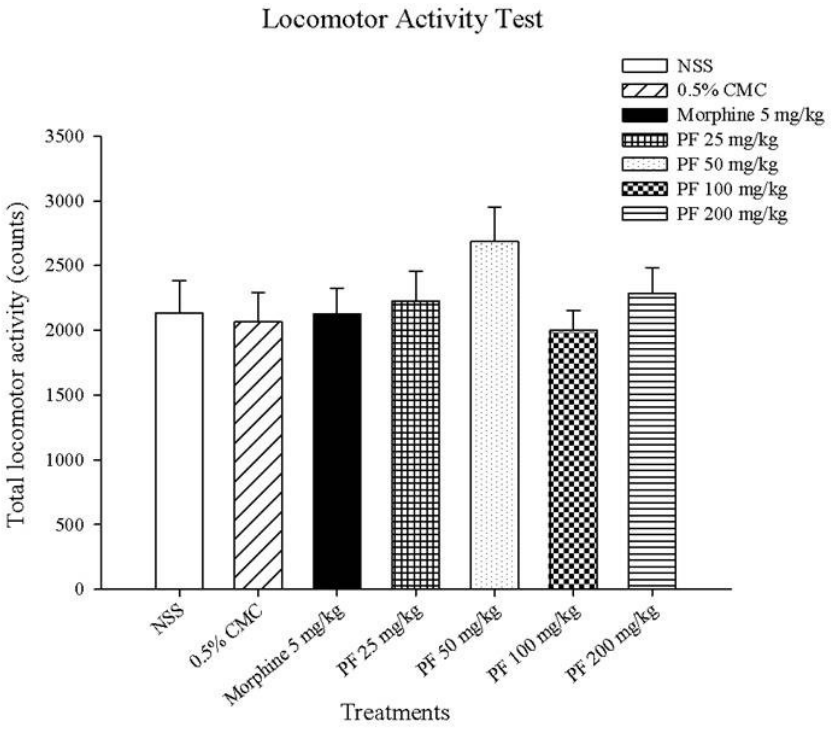

Figure 1 Locomotor activity of mice receiving NSS, morphine 5 mg/kg (i.p.), 0.5% CMC, and various doses of PF (25-200 mg/kg; p.o.). N=8 for all groups. Each value represents the mean ± S.E.M.

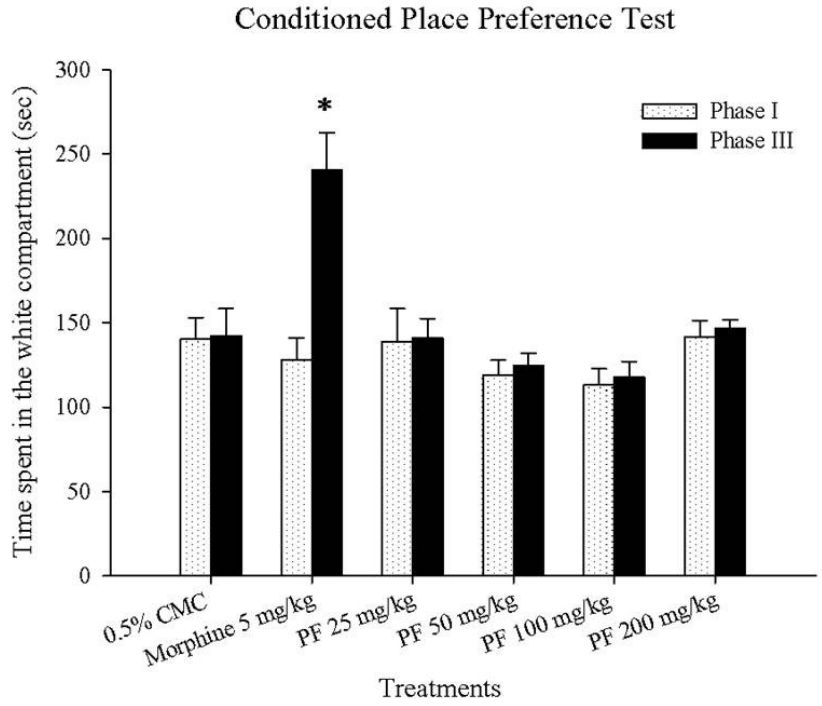

Figure 2 Conditioned place preference of rats receiving 0.5% CMC, morphine 5 mg/kg (i.p.), and various doses of PF (25-200 mg/kg; p.o.). N=8 for all groups. Each value represents the mean ± S.E.M. *$P < 0.05$, significantly different compared with that of phase I (pre-conditioning phase) time spent in the drug-paired compartment.

Results

Locomotor activity

As shown in Fig. 1, morphine (5 mg/kg) and all doses of PF produced no significant effects on locomotor activity as compared with the vehicle group.

Conditioned place preference

Rats treated with 5 mg/kg of morphine showed a significant effect on CPP. Meanwhile, rats treated with all doses of PF did not produce any CPP (Fig. 2).

Discussion and Conclusion

The present study evaluated the effect of PF on locomotor activity and CPP. From the results, no stimulant/sedative effects of PF on locomotor activity were found in any doses tested. Therefore, all doses of PF were further tested in CPP paradigm. PF did not produce any CPP. Meanwhile, morphine produced significant effect of CPP. Since PF did not show any stimulant/sedative or rewarding effects, further studies may be needed to determine the other pharmacological effects of this plant.

Acknowledgements

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RESEARCH ARTICLE

Opisthorchis viverrini: Molecular Analysis of a Gene Encoding Vitelline B Eggshell Precursor Protein

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Abstract

Eggshell proteins are essential and highly abundant antigens of trematodes as each adult parasite produces a large number of eggs every day. In this study, an adult stage cDNA library of *Opisthorchis viverrini* was constructed and screened for abundant transcripts using a differential filter hybridization approach. The cDNA prepared from adult stage RNA of the parasite was used as ^{32}P-labeled probe and several hybridizing clones were isolated and had their cDNA inserts sequenced. The deduced amino acid sequence of the obtained cDNA from clone C2A2 showed significant similarity to vitelline B eggshell precursor proteins of other trematodes. This cDNA had a size of 893 bp and encoded a protein of 247 amino acid residues with a calculated molecular mass of 27.6 kDa (OVVPB). BLASTP results showed significant identity values to eggshell precursor proteins from *Clonorchis sinensis* (63%), *Fasciola hepatica* (37%) and *Schistosoma japonicum* (46%). OVVPB contains 17 tyrosine-glycine motifs (-YG-) which are known to be the site for tyrosine oxidation to DOPA in quinone-tanning during eggshell formation. Detection of the gene's transcripts by Northern and RNA *in situ* hybridization showed a 900 nucleotides transcript size and a location in vitelline cells, respectively. In ongoing analyses the OVVPB protein will be characterized for its application in diagnosis and/or vaccine approaches.

Keywords Eggshell, vitelline, *Opisthorchis viverrini*

Introduction

The eggshell formation in Digenea has been thoroughly analyzed and reviewed[1]. Eggshell proteins form a rigid shell around the fertilized egg which protects its content from damage, firstly, during the passage in the host feces into a natural water source and, secondly, during ongoing embryogenesis until the miracidium hatches from the egg. Therefore, eggshell formation has been extensively studied with the aim to control parasite transmission and the application of these proteins for diagnosis. At present, the transmission of trematodes is mainly controlled through eradicating their intermediate snail host with molluscicides and by educating the population in endemic areas in safe food preparation and consumption. Vaccines would also be a valuable tool to control parasite infection. Unfortunately, an efficacy vaccine is still not available although several candidate proteins have been analyzed for this purpose. The study of eggshell proteins will provide crucial basic knowledge about their effect on fecundity that can be applied to control the parasite transmission. In this study, we have isolated a vitelline B eggshell precursor protein (OVVPB) encoding cDNA from *O.viverrini* and ongoing analyses will show whether this protein can be applied for development of an anti-fecundity vaccine or diagnosis.

Materials and Methods

Construction of cDNA library: Total RNA was isolated from adult *O. viverrini* by homogenization in TRIzol reagent (Life Technologies) with an Ultra-Turrax T25 (IKA). The total RNA was used for construction of a cDNA library by using a SMART™ cDNA synthesis kit (CLONTECH). Briefly, the cDNA was synthesized, directionally cloned into the *Sfi* IA and *Sfi* IB sites of the λTriplEx2 vector and packaged into bacteriophage particles by using packaging extracts (Gigapack® III Gold Packaging Extract, Stratagene).

Screening of cDNA library: The cDNA library was screened for abundant transcripts by using differential filter hybridization. Total adult stage cDNA was labeled with ^{32}P-dCTP (Hexalabel™ DNA labeling, MBI Fermentas) and used as hybridization probe. Screening of cDNA library was done at 50,000 plaques/150 mm plate. Nitrocellulose membranes (Schleicher &Schuell) were used for plaque lifts and the phage DNA was fixed to the membranes by baking at 80°C for 1 h. Hybridization was performed at 55°C in 5×SSPE, 5× Denhardt's solution, 30% formamide, 0.5% SDS, 1 µg/ml Herring sperm DNA for 16 h. Hybridization signals were recorded on X-ray film by exposure to the membrane for an appropriate length of time at –70°C. Positive plaques were isolated and screened at low density (100-200 plaques/90 mm plate) to isolate pure clones. Conversion of isolated positive λTriplEx2 to pTriplEx2 was done by Cre recombinase-mediated site specific recombination at the lox P sites flanking the embedded plasmid.

Sequence analysis of cDNA: Plasmid DNA was prepared by using a plasmid midi kit (Jet Star Kit, GENOMED Inc.) For DNA sequencing the service of MWG AG Biotech, Germany was used. Sequence analysis of the cDNA was done in MacMolly Lite (Softgene, Germany). The amino acid sequence was deduced from the cDNA sequence and used to search for homologous proteins in the NCBI non-redundant protein database by BLASTP. EMBOSS-matcher was used to calculate protein identity values.

Northern hybridization: Total RNA, 20 µg was size separated on a 1.5% agarose gel containing 1×MOPS, 2.2 M formaldehyde. The separated RNA was blotted onto a nylon membrane (Schleicher&Schuell), fixed on the membrane by baking at 80°C for 1 h and hybridized at 65°C with a DIG-labeled antisense OVVPB RNA probe (DIG-RNA labeling kit, Roche) in 5×SSC, 2% blocking solution, 50% formamide, 0.02% (w/v) SDS for 16 h. After hybridization, the immunological detection using alkaline phosphatase anti-DIG antibody conjugate with NBT/BCIP substrates was done following the DIG-detection kit protocol (Roche).

RNA *in situ* hybridization: The localization of OVVPB RNA in parasite tissue was done by RNA *in situ* hybridization. Briefly, adult worms were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4,

overnight, dehydrated through a series of ethanol dilutions for 30 min each and embedded in paraplast. Sections of the embedded tissue were cut at 6 µm thick using a Leica microtome and dried at 42°C, overnight. The sections were rehydrated, post-fixed in 4% paraformaldehyde in PBS, treated in active 0.1% DEPC in PBS for 15 min each for two times, and equilibrated in 5×SSC (0.75 M NaCl, 75 mM Na citrate). The tissue sections were hybridized at 60°C with a DIG-labeled antisense OVVPB RNA probe (DIG-RNA labeling kit, Boerhinger Manheim) in hybridization buffer (5×SSC, 2% blocking solution, 50% formamide, 0.02% (w/v) SDS) for 12-16 h. After hybridization, the tissue sections were washed in 2×SSC for 30 min at room temperature and then at 65°C for 1 h. Final wash was done in 0.1×SSC at 65°C for 1 h. The signal was detected as described for the Northern hybridization procedure.

Results and Discussion

Cloning and characterization of OVVPB: Several positive plaques were obtained in the primary screening. One of these contained a cDNA insert encoding glutathione S-transferase[2]. The isolated recombinant λTriplEx2 clone C2A2 was selected in the secondary screening and its cDNA insert was sequenced and analyzed. The C2A2 cDNA has a size of 893 bp (Fig. 1) and contains an 741 bp open reading frame encoding a protein of 246 amino acid residues and a calculated molecular mass of 27.6 kDa. The BLASTP result demonstrates that it is a homolog of previously analyzed eggshell proteins from *Clonorchis sinensis*, *Fasciola hepatica*, and *Schistosoma japonicum*. The identity values (%) of OVVPB to these homologs are shown in Table 1. OVVPB contains 17 tyrosine-glycine motifs (-YG-) spread throughout its amino acid sequence. These motifs are known to be the site for oxidation of tyrosine to DOPA in quinone-tanning during eggshell formation. Cysteine residues are absent in OVVPB, therefore disulfide bonds are not required for its folding or interaction with other eggshell proteins. OVVPB is a glycine, tyrosine, lysine and aspartic acid rich protein comparable with other trematode eggshell proteins (Table 2).

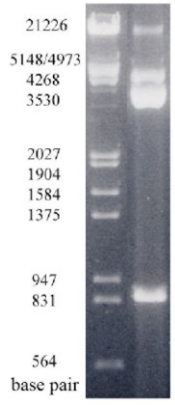

Figure 1 The C2A2 fragment

Table 1. Identity values (%) of OVVPB with homologous proteins from other trematodes.

	OVVPB					
CSVPB	63	CSVPB				
FHVPB I	37	38	FHVPB I			
FHVPB II	37	39	86	FHVPB II		
FHFGC	39	42	96	79	FHFGC	
Sj	46	39	41	37	38	Sj
Sj34ESH	49	41	49	43	49	100

Table 2 Percentage of abundant amino acid residues in OVVPB, *Fasciola hepatica* VPB1 and VPB2.

	OVVPB (246 aa)	FHVPB1 (272 aa)	FHVPB2 (272 aa)
Glycine	16% (40)	14% (38)	14% (39)
Tyrosine	13% (31)	13% (34)	13% (35)
Lysine	11% (31)	11% (31)	13% (34)
Aspartic acid	10% (24)	8% (23)	8% (22)

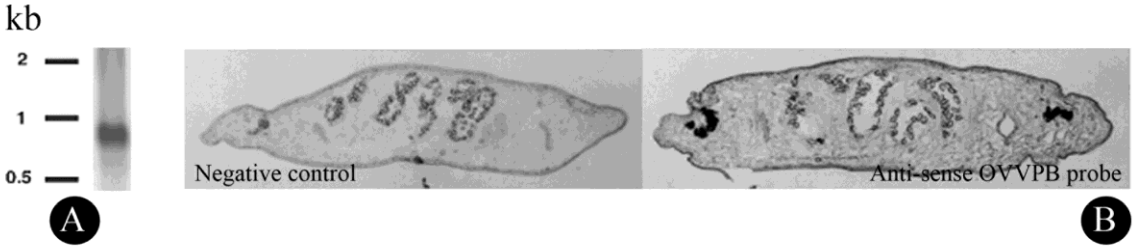

Figure 2 A: Northern hybridization B: RNA *in situ* hybridization, notice the lateral located, prominent stained vitelline cells.

Northern hybridization and RNA *in situ* hybridization of OVVPB: The Northern hybridization detected a OVVPB transcription product of approximately 900 nucleotides size (Figure 2) consistent with the isolated OVVPB cDNA. RNA *in situ* hybridization to tissue sections of adult worms detected the OVVPB transcript only in vitelline cells. This supports the function of OVVPB as an eggshell precursor protein as all known eggshell proteins are produced in these cells. Due to their high transcript number in this parasite, the differential filter hybridization method could be successfully applied to isolate this cDNA from an adult *O. viverrini* cDNA library. Further analyses of OVVPB may be useful for diagnosis and vaccine aspects.

Conclusion

In this study, a OVVPB cDNA was isolated and partially characterized. Characterization of OVVPB mRNA and protein may be useful for development of diagnosis and/or vaccine aspects.

Acknowledgements

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RESEARCH ARTICLE

Preliminary Investigation on the Application of Ultrasonography as a Tool for Monitoring the Development and Progress of Cholangiocarcinoma in *Opisthorchis viverrini* /Dimethylnitrosamine-Induced Hamsters

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Abstract

Cholangiocarcinoma (CCA) is the bile duct cancer which is the most common cancer in Thailand particularly in northeastern region. Effective diagnosis of CCA either in human or animals is not currently available. Diagnostic tool for monitoring the development and progress of CCA in animal models is essential for research and development of new promising chemotherapeutics for CCA. In this study, we preliminarily investigate the application of ultrasonography to monitor the development and progress of CCA in 10 hamsters induced by *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN) administration. Control group (10 hamsters) received a mixture of water and Tween-80 during the same period. Ultrasonography was performed once every four weeks starting from week 12 until week 24. Results of histopathological examination (at autopsy) and ultrasonography images of liver and gall bladder were in agreement. Although ultrasonography does directly detect the occurrence of CCA, it reflects the thickening of bile ducts and abnormality of liver tissues. Ultrasonography may be used as a reliable tool to monitor the development and progress of CCA in animal models used in research and development of new promising chemotherapeutics for CCA.

Keywords Ultrasonography, Cholangiocarcinoma, Diagnosis, Hamster, *Opisthorchis viverrini*, dimethylnitrosamine

Introduction

Cholangiocarcinoma (CCA) is the bile duct cancer which is the most common cancer in Thailand particularly in northeastern region. The major cause of CCA in Thailand is the consumption of Pla-ra or Pla-som which contains *Opisthorchis viverrini* (OV) and nitrosamine (a preservation)[1]. Lack of effective diagnostic tool and chemotherapeutics are major constraints for controlling this type of cancer. Chemotherapy and radiotherapy are only effective in patients with early stage, whilst the majority of patients come to receive treatment when cancer progresses to advanced stage. Early diagnosis is therefore crucial for effective treatment of CCA. Several diagnostic tools have been used to detect clinical development and to monitor the progress of CCA, but each of which has shortcoming and limitation. These include the use of blood biochemistry or serum tumor markers, computed thermography (CT) scan, magnetic resonance imaging (MRI) and ultrasonography. CT scan and MRI are effective but are too expensive for routine application[2]. The non-invasive diagnosis by abdominal ultrasonography provides low sensitivity result, but is a useful tool to rule out liver diseases due to other causes. Ultrasonography can differentiate CCA from gallstone biliary obstruction. Furthermore, dilation of intra- but not extrahepatic bile duct imaged by ultrasonography is a definitive diagnosis of CCA. For research and development of new promising chemotherapeutics for CCA, validity of animal models which closely mimic the pathogenicity of human CCA is a pre-requisite component. In all cases, the development and progress of CCA in animals (hamsters) can only be confirmed by histopathological examination of liver and gallbladder at autopsy[3]. This may obscure or misinterpret therapeutic efficacy of the test substances. The aim of the study was to preliminarily investigate on the applicability of ultrasonography to monitor the development and progress of CCA in hamsters following induction of CCA by OV and DMN.

Materials and Methods

Induction of CCA in hamsters

A total of 20 hamsters (10 males and 10 females), golden syrian hamsters (aged 6-8 weeks), the susceptible animal model for development of CCA, were used in the study. CCA was induced in 10 hamsters (5 males, 5 females) by an initial intragastric administration of 50 OV metacercariae (from infected fishes obtained from Khon Kaen Province, Thailand), followed by 12.5 ppm dimethyl nitrosamine (DMN, Sigma) in drinking water *ad libitum* starting from week 4 to 12. Control group (5 males, 5 females) received a mixture of water and Tween-80 during the same period.

Ultrasonography

Ultrasonography (HS-2000V Veterinary Ultrasound System, Honda electronics) was applied to detect the development and progress of CCA in all hamsters at weeks 12, 16, 20, 24 and 28 after OV infection. Animals were fasted for three hours before ultrasonography and were anesthesized with isofurane (Minrad Inc.). The lubricant gel was applied before insertion of microconvex probe (HCS-4710M 9.0MHz 10R). Histopathology of liver, bile ducts and gall bladders was examined after sacrification. The development and progress of CCA was classified into four grades as follow:

Table 1 Classification of CCA pathology by ultrasonography

CCA Grade	Bile duct	Liver
0	Normal	Normal
1	Thickening	Mild
2	Thickening	Moderate
3	Thickening	Severe

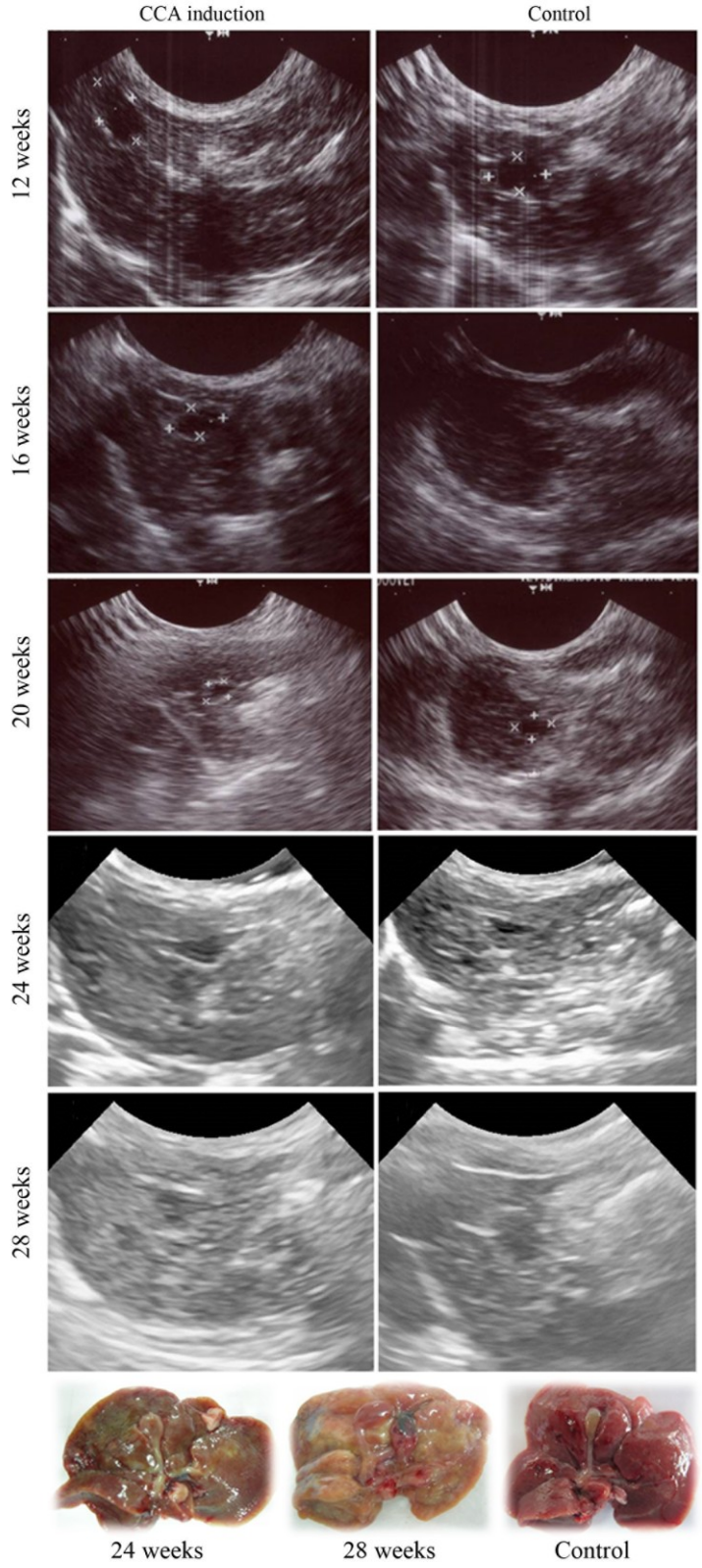

Figure 1 The ultrasonography image of CCA induction group and control at week 12, 16, 20, 24 and 28 and liver from sacrificed hamster at week 24, 28 and normal control.

Results and Discussion

The development and progression of CCA was monitored by ultrasonography at 12, 16, 20, 24 and 28 weeks after OV administration in both animal groups. Based on ultrasonographic results, none of the control hamsters developed CCA (CCA grade 0). Liver and gallbladders of the group induced by OV/DMN from week 0 to 28 showed abnormal changes (1+ to 2+) (Figure 1). Furthermore, death of hamster was observed at week 28. Although ultrasonography does directly detect the occurrence of CCA, it reflects the thickening of bile ducts and abnormality of liver tissues. The abnormal change in liver tissue (tumor and pus) from sacrificed hamster showed development of tumor and pus in liver.

Conclusion

Ultrasonography may be used as a reliable tool to monitor the development and progress of CCA in animal models used in research and development of new promising chemotherapeutics for CCA.

Acknowledgements

This project was supported by the Office of Commission on Higher Education, Ministry of Education of Thailand, Thailand National Research University (NRU) Project, and Thammasat University. We thank staff of Radiology Unit, Kasetsart Veterinary Teaching Hospital, Kasetsart University for technical support in ultrasonography.

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RESEARCH ARTICLE

Relationships between Topiramate Concentrations in Serum and Saliva of Thai Epileptic Patients

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Abstract

The objective of this study was to determine the correlation between serum and saliva topiramate concentrations in Thai epileptic patients. The study was conducted in the Department of Medicine Neurology Unit, Epilepsy Clinic, Pramongkutklao Hospital. Patients aged between 15-60 years old and receiving topiramate were included into this study. The correlation equation between serum and saliva topiramate concentrations was constructed from 10 patients, receiving topiramate monotherapy. The blood and saliva samples were collected at the time before the morning dose and at 1, 2, 4, 6 and 8 hours after topiramate ingestion. Topiramate concentrations in blood and saliva samples were measured by turbidimetric immunoassay technique. The results showed that serum and saliva topiramate concentrations were closely correlated with a correlation coefficient of 0.919 ($n=60$, $p<0.001$). The describing equation of this relationship was $Y= 0.962X + 1.197$. The correlations between serum and saliva concentrations were closely correlated with the correlation coefficient of 0.992, 0.929, 0.873, 0.915, 0.933 and 0.993 (all data were from $n=10$, $p<0.001$) at the time point of 0, 1, 2, 4, 6 and 8 hours after topiramate ingestion, respectively. The results of this study support the use of saliva as an alternative to serum for monitoring topiramate therapy. And the most appropriate time of saliva collection is the time before or at least 8 hours after topiramate ingestion.

Keywords topiramate, serum, saliva.

Introduction

Epilepsies are among the most common serious neurological disorder worldwide. Antiepileptic drugs are effective in 60-70% of individuals.[1] Treatment of epilepsy is one of the areas where therapeutic drug monitoring (TDM) has made the most significant contributions. The goal of TDM is to optimize patient's clinical outcome by managing their medication regimen with the assistance of measured drug concentrations. Determination of serum concentrations of the conventional antiepileptic drugs; phenobarbital, phenytoin, carbamazepine, valproate and ethosuximide are widely accepted in clinical practice.[2]

Topiramate a newer antiepileptic drug has been demonstrated to be effective for the treatment of partial and generalized seizures in adults and children. Several factors can affect serum topiramate concentration such as age, co-administered enzyme inducing drugs, metabolic enzyme capacity as well as renal function status of the patients. These factors can cause marked variability in the correlation between topiramate dosage and topiramate serum concentration. [3,4] Thus, topiramate monitoring would be useful for optimizing the dosing regimen in individual patient besides assessing medication noncompliance, which is an important issue in patients with epilepsy.[5]

There are several studies reported that saliva serves as an alternative medium to serum for monitoring of many conventional antiepileptic drugs such as phenobarbital, phenytoin, carbamazepine, etc.[6,7] However, few data exist for newer antiepileptic drugs, such as lamotrigine, oxcarbamazepine, gabapentin and topiramate. Regarding topiramate, there is one study examining the relationship between serum and saliva concentrations using the specimens collected from 31 epileptic patients. Strong correlation exists between serum and saliva topiramate concentration, supporting the use of saliva as an alternative to serum for monitoring topiramate therapy.[8] Due to the limited data from only one study mostly using specimens of children in a western country treated with topiramate both monotherapy and co-therapy with other antiepileptic drugs, more additional study is encouraged. Thus, the objective of this study is to determine the correlation between serum and saliva topiramate concentrations in Thai adult epileptic patients.

Material and methods

Subjects

Ten Thai epileptic patients, aged between 15 to 60 years old receiving topiramate monotherapy for at least 1 month, at the Department of Medicine Neurology Unit, Epilepsy Clinic, Pramongkutklao Hospital, Bangkok were recruited into the study. The study protocol was approved by the ethical committee on the protection of rights of human subjects of the Pramongkutklao Hospital. (Approval # 1748/2551, December 15, 2008)

Study Design

Serum and saliva of each patient were collected at the time before topiramate ingestion (0 hour) and at 1, 2, 4, 6 and 8 hours after topiramate ingestion.

Blood and saliva samples were centrifuged at 3,000 *g* for 10 minutes at room temperature and the clear supernatants were stored at -80 °C until analysis using Turbidimetric immunoassay by automated clinical chemistry analyzer at Laboratory unit of Bangsai Hospital, Ayutthaya.

Data Analysis

The correlation between serum and saliva topiramate concentrations was assessed by simple linear regression and correlation analysis. The correlation was tested by Pearson's correlation with $p < 0.05$.

Results

The average (mean ± SD) age and weight of patients were 36.20 ± 10.40 years old and 56.00 ± 6.77 kilograms. Three patients were male and 7 patients were female. The average dose of topiramate prescribed in recruited patients was 125.00 ± 83.33 mg/day.

The concentrations of serum and saliva samples of ten patients, collected at the time before topiramate ingestion (0 hour) and at 1, 2, 4, 6 and 8 hours after topiramate ingestion were shown to be closely correlated with a correlation coefficient of

0.919 (n=60, p<0.001) and the correlation equation was Y= 0.962X + 1.197 (Figure 1). The correlations between serum and saliva concentrations of ten patients, collected at each time point after ingestion, were also determined. It was show that the correlations between serum and saliva concentrations were closely correlated with the correlation coefficient of 0.992, 0.929, 0.873, 0.915, 0.933 and 0.993 (all data were from n=10, p<0.001) at the time point of 0, 1, 2, 4, 6 and 8 hours after topiramate ingestion, respectively (Figure 2). The results of this study support the use of saliva as an alternative to serum for monitoring topiramate therapy. And the most appropriate time of saliva collection is the time before or at least 8 hours after topiramate ingestion, respectively.

Discussion and Conclusion

The results of this study support the use of saliva as an alternative to serum for monitoring topiramate therapy. And the most appropriate time of saliva collection is the time before or least 8 hours after topiramate ingestion.

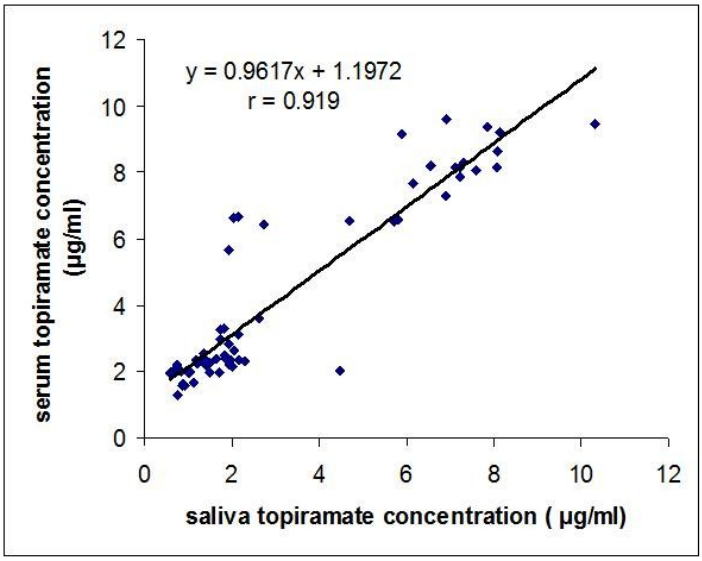

Figure 1 Correlation between serum and saliva topiramate concentrations at the time before topiramate ingestion (0 hr) and 1, 2, 4, 6 and 8 hour after topiramate ingestion (n = 60)

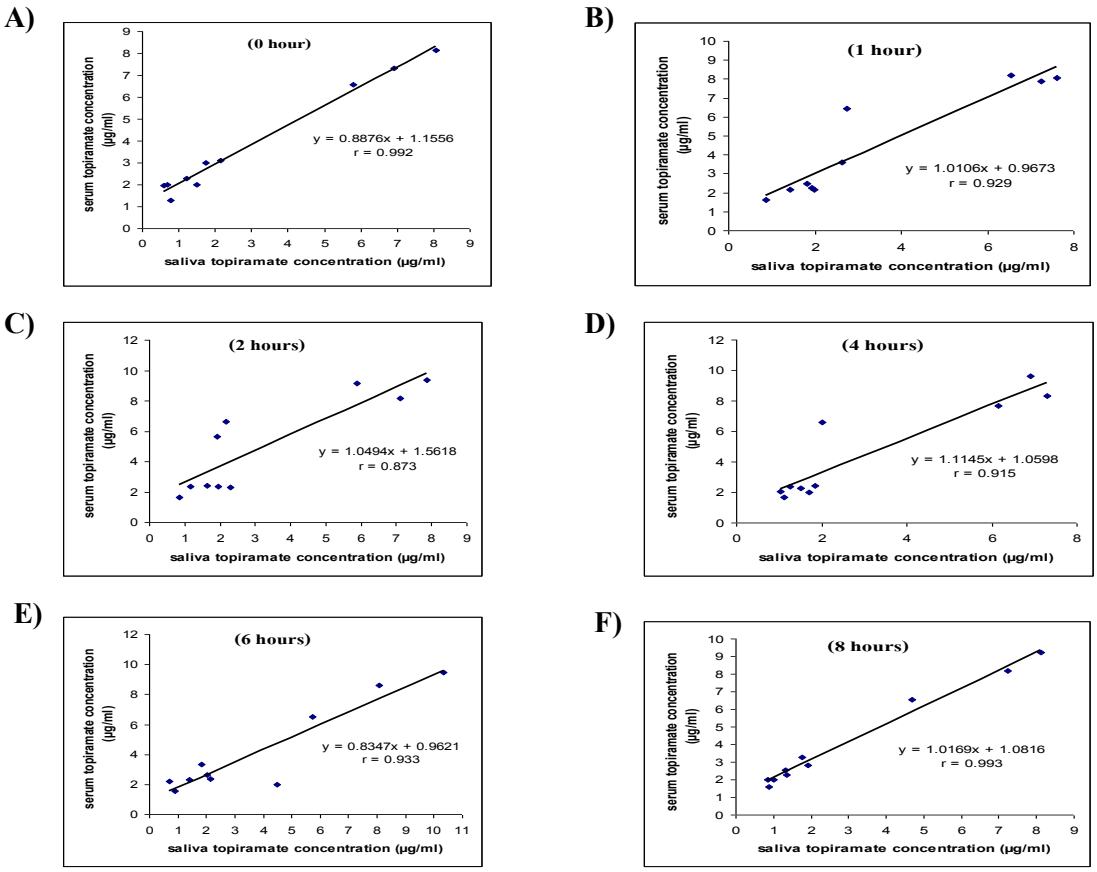

Figure 2 Correlation between serum and saliva topiramate concentrations at the time before topiramate ingestion (0 hour) (A) and at 1 (B), 2 (C), 4 (D), 6 (E) and 8 (F) hours after topiramate ingestion (n=10)

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RESEARCH ARTICLE

Effect of the Ethanolic Extract of *Mitragyna speciosa* Leaves on Conditioned Place Preference

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Abstract

Mitragyna speciosa Korth. has long been used in Thai traditional medicine for the treatments of pain, fever, cough, diarrhea, opioid-addiction and for enhancing the labor work efficiency and tolerance. However, there is no clear evidence of the rewarding effect of *M. speciosa* in animal models. Thus, this study was aimed to investigate the rewarding effect of the ethanolic extract of *M. speciosa* leaves (MS) using conditioned place preference (CPP) model in rats. Various doses of MS (50-400 mg/kg, p.o.) show neither stimulating nor sedative effects using locomotor activity test in mice. In CPP test, the same doses of MS also did not showed reinforcing effect compared to vehicle and morphine positive-control groups. The results from this study demonstrated that MS were not found to have stimulating, sedative and rewarding effects.

Keywords Addiction, *Mitragyna speciosa*, Morphine, Conditioned Place Preference, Kratom

Introduction

Mitragyna speciosa Korth., called kratom in Thai, is a tropical plant found in the Southeast Asia countries including Thailand, Malaysia, Indonesia and Myanmar. In Thai traditional medicine, kratom leaves have been used for the treatment of pain, fever, wound, cough, and diarrhea**.** Furthermore, it has also been used to increase labor work efficiency and tolerance under the hot sunshine atmosphere (1). Additionally, it was often used for opiate-addiction treatment, self treatment of opioid withdrawal and as a replacement for opium when opium is unavailable (1, 2). The major constituent of *M. speciosa* leaves, mitragynine, has been shown to act on noradrenergic and serotonergic systems and has an opioid-liked effect (3).

The ethanolic extract of *M. speciosa* leaves (MS) has been shown to possess numerous pharmacological effects including antinociception, anti-inflammatory in rats and inhibit ethanol withdrawal in mice (3, 4, and 5). Although *M. speciosa* leaves are a controlled substance listed in the Thailand Narcotic Act since 1943, there is no clear evidence of their rewarding effect in animal models. Thus, the aim of this study was to investigate the rewarding effect of MS using conditioned place preference (CPP) model in rats.

Materials and Methods

Chemicals

Morphine sulfate 5 mg/kg (Temad, Iran) was dissolved in 0.9% normal saline solution (NSS) as a positive control. 0.5% Carboxymethylcellulose (CMC; Sigma, USA) was dissolved in distilled water as a vehicle control. The MS was dissolved in 0.5% CMC solution. The doses of MS used in this experiment were 50, 100, 200 and 400 mg/kg. MS and vehicle were orally administered and morphine was administered by intraperitoneal injection. In locomotor activity test, the experiments were started immediately after administering various treatments while in the CPP test all, treatments were pretreated 30 min before starting the experiments.

Animals

Male Wistar rat weighing 200-250 g and male ICR weighing 18-25 g from the National Laboratory Animal Centre, Mahidol University, Nakhonprathom, Thailand served as experimental animals in this study. The animals were kept in the animal facility of Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions for one week prior to the start of the experiments and allowed food and water *ad libitum*. The study protocol was approved by the Institutional Animal Care and Use Committee, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Locomotor activity test

The effect of MS on locomotor activity in mice was examined in an activity cage (UGO Basile, Comerico, Italy). Mice were placed in the activity cage immediately after administration of the test substances. The locomotor activity of animals was continuously recorded in each 5 min intervals for 75 min.

Conditioned place preference

CPP paradigm has been used in animals model for evaluate the motivation properties such as rewarding or aversive effects of drugs. The CPP studies were conducted using CPP apparatus (length 25 cm, width 80 cm, height 36 cm) consisted of a three compartments separated by guillotine doors. The middle compartment consisted of an (length 25 cm, width 11 cm) area painted grey. The lateral compartments (length 25 cm, width 34 cm) offered distinct stimuli in odor, color and texture. One compartment wall was painted white with a smooth floor. The opposite lateral compartment wall was painted black and white as vertical stripped with a mesh floor and painted with 2% acetic acid. The animals were observed though VDO camera. The CPP protocol consisted of a 12 day schedule with three phases: preconditioning (3 days), conditioning (8 days) and test (1 day) phases. This protocol was described previously by Spyraki et al. (6). An increase in the time spent in the drug-paired compartment after conditioning suggests the presence of the positive reinforcing effect.

Statistical analysis

The data were presented as the mean ± S.E.M. Statistical analyses were performed with One way analysis of variance (ANOVA) and follow by Dunnett's test or Student's paired t-test where applicable. Values of $P<0.05$ was considered statistically significantly.

Results

Locomotor activity

MS had no significant effect on the locomotor activity when compared to the vehicle group (Figure 1).

Conditioned place preference

Morphine 5 mg/kg significantly produced CPP but MS failed to induce CPP when compared to vehicle group (Figure 2).

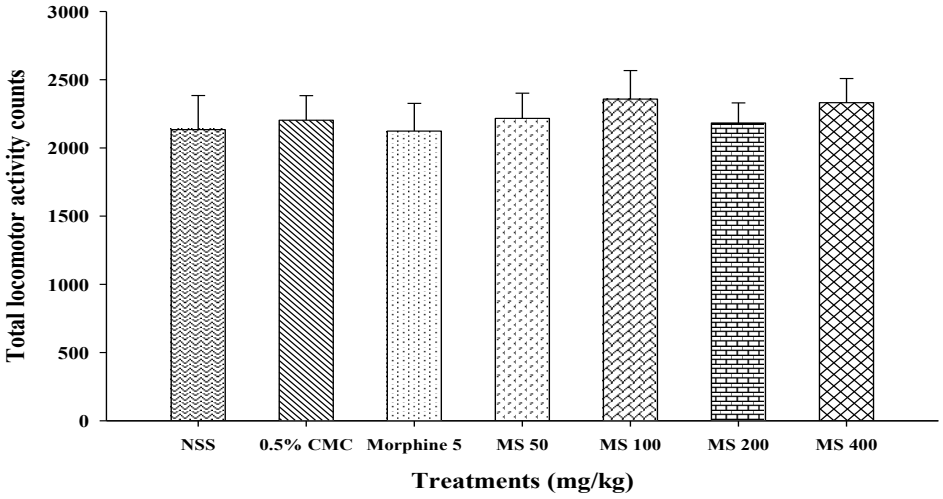

Figure 1 Locomotor activity in mice produced by NSS, morphine (5 mg/kg; i.p), 0.5% CMC, and various doses of MS (50-400 mg/kg; p.o.) Each value represents mean ± S.E.M. (N=8).

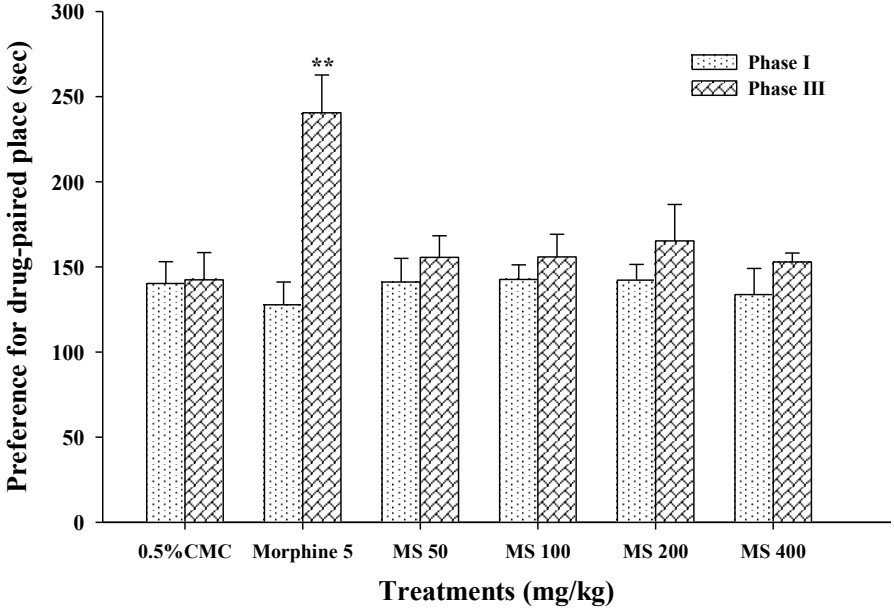

Figure 2 Conditioned place preference in rats produced by 0.5% CMC, morphine (5 mg/kg i.p) and various doses of MS (50-400 mg/kg; p.o.) Each value represents mean ± S.E.M. (N=8). $^{**}P<0.01$ significantly different compared to control animals.

Discussion and Conclusion

The present study attempted to investigate the effects of MS on locomotor activity and rewarding system. The results showed that all doses of MS (50-400 mg/kg p.o.) did not affect the locomotor activity in mice. The similar results were previously reported with the methanolic extract and the aqueous extract of *M. speciosa* leaves (4, 7). All doses of MS also demonstrated no positive reinforcing effect using CPP model in rats while morphine (5 mg/kg) showed a strong rewarding effect in the same animal model. It is known that morphine acts through μ-opioid receptor which produces rewarding effect via activation of the mesolimbic dopaminergic system. Since MS failed to produce CPP, it may be concluded that the major constituents in this extract may not have μ-opioid receptor agonist property strong enough to produce CPP or they may antagonize each other to reduce the rewarding effect. However, further investigations using other rewarding models may be needed to confirm this negative result observation.

In conclusion, the results from this study demonstrated that MS were not found to have stimulating, sedative and rewarding effects. Since MS has been shown to possess several pharmacological properties without rewarding effect, it may have a potential to be developed for clinical purposes.

Acknowledgements

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RESEARCH ARTICLE

Hypoglycemic Effect of Standardized *Centella asiatica* Extract ECa 233 in Streptozotocin-induced Diabetic Rats

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Abstract

Centella asiatica (*C.asiatica*), locally known as Bua-bok, is a local Thai herb used as CNS depressant, antibacterial, antiinflammatory, antiproliferant, antiulcer, and wound healing. Recently, the ethanolic and methanolic extracts of *C.asiatica* were found to exert hypoglycemic effect in Alloxan-induced diabetic rats. Thus, this study was designed to examine the hypoglycemic effects of ECa 233, which is a standardized *C.asiatica* extract, on Streptozotocin-induced diabetic rats by measuring body weight , food intake, and levels of plasma glucose at day 0, 7, 10, 14, 21 and 28 days after the intraperitoneal injection of 50 mg/kg B.W. STZ. In contrast to gradually increasing in weight observed in normal rats, diabetic rats receiving orally given distilled water or ECa 233 showed no increment of body weight whereas the food intake had increased from that of day 7 in all groups. Plasma glucose in diabetic group treated with distilled water and ECa 233 at the dose of 10 mg/kg B.W. gradually increased and significantly different from those of their respective day 7 at day 21 and 28. However, anti-hyperglycemic effect of ECa 233 was demonstrated at the dose of 30 and 60 mg/kg. B.W. in which the plasma glucose at the late phase of the experiment did not show significant elevation from their value at day 7. Our findings clearly reveal hypoglycemic effect of ECa 233 in STZ-induced diabetic rats and suggest the possibility to develop the test compound to a food supplement or adjunctive medication for diabetic patients.

Keywords hypoglycemic effects, the standardized extract of *Centella asiatica* ECa 233, diabetic rats

Introduction

Centella asiatica (Linn.) Urban is a herbal medicine that has long been used in many Asian countries for hundreds of years (1). In the course of pharmacological studies, the plants showed CNS depressant activity (2), antibacterial activity (3), antiinflammatory activity (4), antiproliferant effects (5), antiulcer activity (6) as well as wound healing effects (7). Recently, the ethanolic and methanolic extracts of *C.asiatica* were found to exert hypoglycemic effect in alloxan-induced diabetic rats (8). Therefore, it is our interest to investigate the hypoglycemic effect of ECa 233, a standardized extract of *C.asiatica* recently established by researchers at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. ECa 233 was prepared by a well-controlled and patented method to contain triterpenoid at least 80% and the ratio of masdecassoside and asiaticoside was kept at 1.5 ± 0.5 : 1 (1). In addition to attenuation of β-amyloid-induced deficit in learning and memory in mice, wound healing effect of ECa 233 has been clearly demonstrated in incision and second degree burn wound in both non-diabetic and diabetic rats (9-11). However, no study on its effect on blood sugar has been conducted. Thus, we herein report anti-hyperglycemic effect of orally given ECa 233 in streptozotocin-induced diabetic rats.

Materials & Methods

Preparation and administration of the test compound

ECa 233 was kindly provided by Dr. Chamnan Patarapanich and co-workers, Faculty of Pharmaceutical Sciences, Chulalongkorn University. It was dissolved in 100 ml drinking water which was freely accessible to the animals. The water has to be totally consumed by each animal within 24 hours and the daily doses of ECa 233 given were 10, 30 and 60 mg/kg, B.W.

Animals and induction of diabetes

Male Wistar rats weighing 250-300 g (National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom Province, Thailand) were housed in groups for four to five rats under controlled environmental conditions of a 12 h light/dark cycle at 25 ± 2 ^{o}C for at least a week prior to the experiments. They were processed according to the ethics of using animals in experiment by the National Research Council of Thailand (NRCT).

Diabetes was induced by a single intravenous injection of 50 mg/kg streptozotocin (STZ, Sigma Aldrich, USA) prepared in citrate buffer (0.1 M, pH 4.5) into the animals after overnight fasting. Seven days later, a blood glucose was determined from tail-vein blood using Glucometer (Accu-CHEK advantage, USA). Animals with plasma glucose levels >200 mg/dL were considered as diabetic rats to be used in the present study (12).

Experimental protocal

The diabetic animals were randomly divided into four experimental groups with 6-8 animals each ; group I : diabetic rats receiving di-distilled water (DDW), groups II, III and IV were diabetic rats receiving ECa 233 at dose of 10, 30 and 60 mg/kg, respectively. In addition one group of non-diabetic rats was included as normal control. Body weight, food intake and blood glucose of the animals at 0, 7, 10, 14, 21 and 28 days after injection of STZ were determined (12-13).

Statistical analysis

Results are presented as mean ± standard error of mean (S.E.M.). Blood glucose at each time point (at day 10, 14, 21 and 28 after injection of STZ) was compared with its respective initial value at day 7. Student Pair “t” test and unpair “t” test were used for comparison within group and between group, respectively. Statistical significance was considered when P value was less than 0.05.

Results

Body weight and food intake

The initial body weight at day 0 was rather similar in all groups. The mean body weight of non-diabetic rats gradually increased from 286.33±5.17 g at day 0 to the 449.5±5.72 g at day 28 whereas the body weight of all diabetic rats, either with or without ECa 233 treatment, showed no increment but a reduction of body weight

(Fig. 1). The mean body weight of 10, 30 and 60 mg ECa 233-treated groups at day 28 were found to be 272±16.53, 275.67±19.73 and 265±13.32, respectively. In contrast, the food intake of all diabetic at any time points of observation progressively increased from that of day 7 whereas an increase of food intake in non-diabetic rats was noted only at day 10 and then no further increases were observed (Fig. 2).

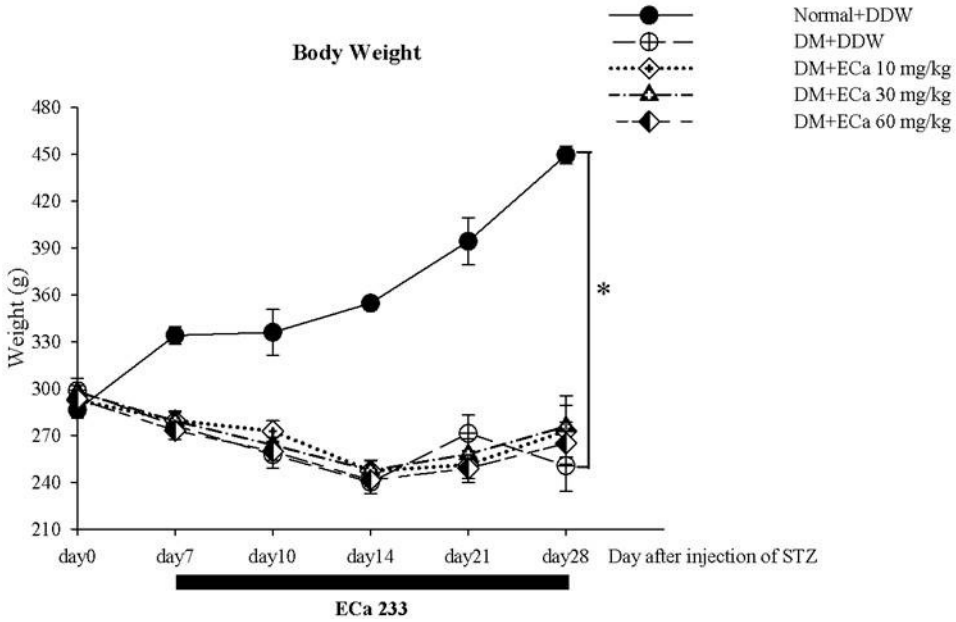

Figure 1 Effect of orally given ECa 233 on the body weight of STZ-induced diabetic rats at 7, 10, 14, 21 and 28 days after the injection of STZ. Data represent the mean±S.E.M. (n = 6-8), **P* < 0.05 *versus* DDw-treated normal group (Student's "t" test).

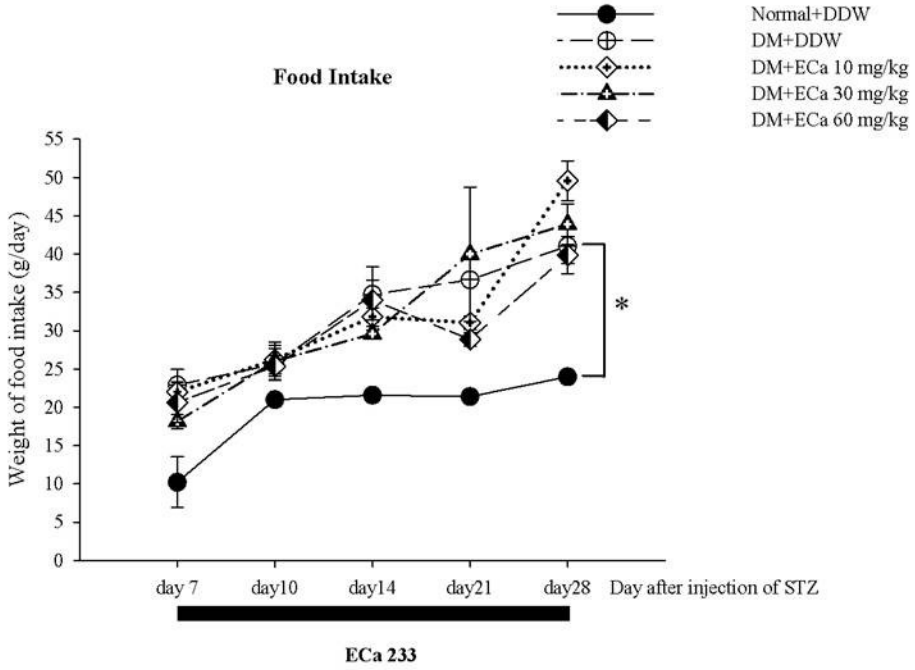

Figure 2 Effect of orally given of ECa 233 on the food intake of STZ-induced diabetic rats at 7, 10, 14, 21 and 28 days after the injection of STZ. Data represent the mean±S.E.M. (n = 6-8), **P* < 0.05 *versus* DDW-treated normal group (Student's "t" test).

Plasma glucose level

As shown in Figure 3, plasma glucose in diabetic rats receiving di-distilled water (DDW) was progressively increased over time. However, significant increases in mean plasma glucose compared to that of day 7 (271.6±28.41 mg/dL) were observed at day 21 (403±28.64 mg/dL) and 28 (411.8±11.25 mg/dL) after injection of STZ (Fig. 3C and 3D). Similarly, significant increases of plasma glucose in relation to that of day 7 were also observed in diabetic rats receiving 10 mg/kg. ECa 233. In general, except for a small but significant increase of plasma glucose at day 28 in 60 mg/kg. ECa 233-treated DM group, no significant increase of plasma glucose from that of day 7 was observed at various time points of observation in diabetic rats receiving ECa 233 at the doses of 30 and 60 mg/kg. Apparently anti-hyperglycemic effect of ECa 233 at these two doses was demonstrated.

Discussion and conclusion

The present study demonstrated that STZ which selectively destroyed the pancreatic insulin secreting β-cells, significantly induced hyperglycemia indicating diabetes in experimental animals (12). Significant weight loss in the face of increasing food intake has been previously reported in STZ-induced diabetic animals and this might accounted by decreasing of serum insulin level concurrently observed (13-14). In association with the weight loss, plasma glucose in diabetic rats receiving distilled water gradually increased over time and significant difference from the initial value at day 7 was observed at day 21 and

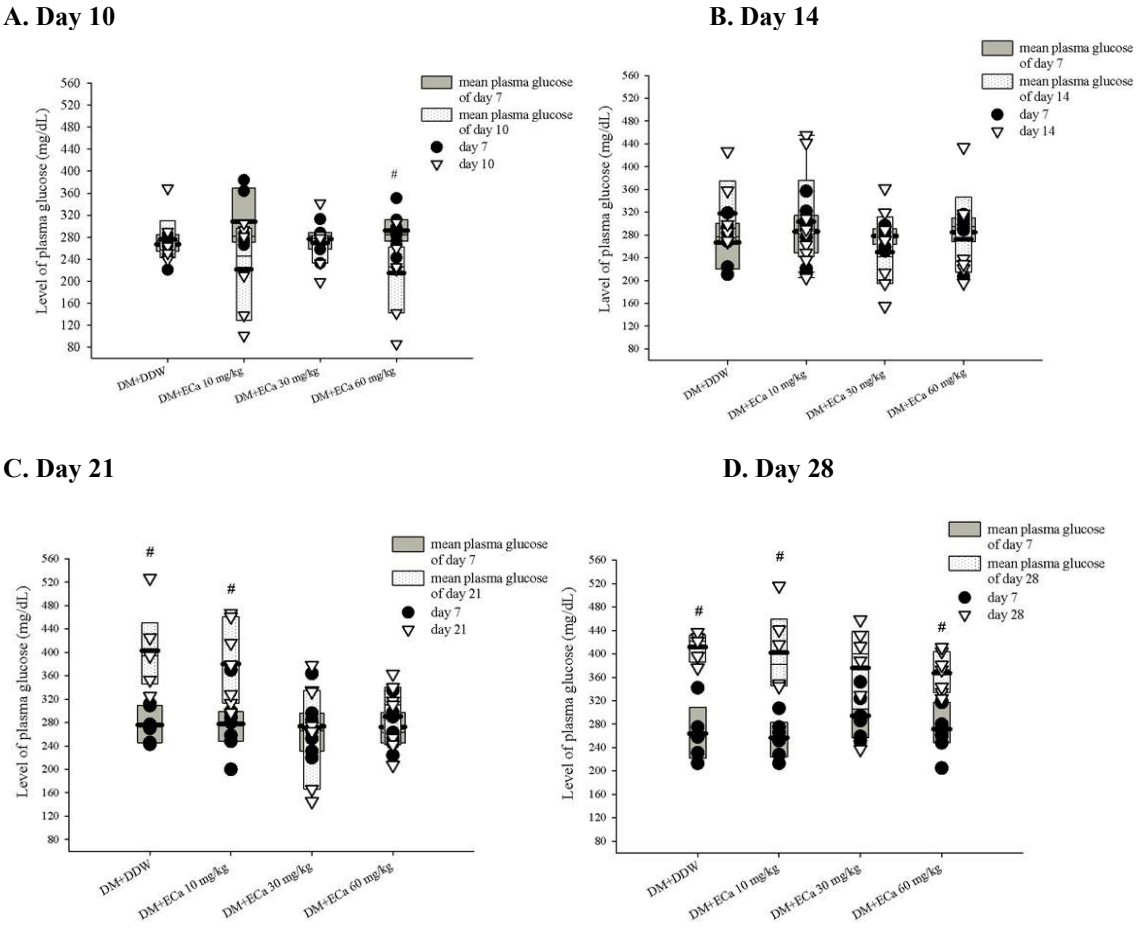

Figure 3 Effect of the oral administration of ECa 233 on the plasma glucose levels in STZ-induced diabetic rats on day 10 (A), 14 (B), 21 (C) and 28 (D). Each data points represents the mean ± S.E.M. # $P < 0.05$ denotes statistically significant difference from respective value at day 7 (Student's "t" test).

28. Orally administered ECa 233 at the dose of 10 mg/kg/day did not demonstrate any improvement of all parameters observed as the mean plasma glucose level in this group significantly increased since day 21. In contrast, anti-hyperglycemic effect of ECa 233 at the dose of 30 and 60 mg/kg/day was demonstrated. The mean plasma glucose level of these two groups of diabetic animals, at any time point of observation except a small increase at day 28 of 60 mg/kg/day ECa 233-treated group, showed no significant increase from it respective value at day 7. A number of mechanisms have been found to underlie hypoglycemic effect of herbs. *Artemisia campestris* has been proposed to mitigate pathogenesis of alloxan-induced diabetes by antioxidant property (15). Stimulation of insulin release has been demonstrated by chloroform extract of *C. zeylanicum*[15]. Though antioxidant properties of ECa 233 has been previously reported (17), underlying mechanism of its hypoglycemic effect observed in the present study is a subject for further investigation. Taking into consideration of a favorable safety profiles of ECa 233, our findings do support the development of ECa 233 to a food supplement or adjunctive medication for diabetic patients.

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RESEARCH ARTICLE

Effect of Water Extracts of *Vernonia cinerea* Less. on Nicotine Withdrawal Mice.

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Abstract

Nicotine is considered to be the primary component of tobacco smoke. It exhibits dopamine release in core structure of reward system, ventral tegmental area (VTA) and nucleus accumbens (NAc), and causes tobacco addiction. *Vernonia cinerea* Less. has been reported to have many medicinal properties. Different parts of the plant have different therapeutic values such as analgesic, antipyretic, anti-inflammation, and smoking cessation. The aims of this study were to evaluate effect of *V. cinerea* on nicotine withdrawal mice and to determine the mechanism of action of *V. cinerea* extracts (VE) in alteration of nicotinic and muscarinic receptors protein expression on western blot analysis. We found that VE at high concentration (500 mg/kg) significantly decreased total abstinence signs (TAS) and exhibited no changes in locomotion and anxiety-like behaviors. Moreover, VE had no differences on nicotinic and muscarinic receptor protein expression. These results suggested that VE might be involved with reduction of nicotine withdrawal symptoms in other mechanisms which are not be related to nicotinic and muscarinic receptors.

Keywords Nicotine, Withdrawal, *Vernonia cinerea* Less., Nicotinic receptor, Muscarinic receptor

Introduction

Tobacco smoking and tobacco addiction is a major worldwide health problem involved with health and economic impact on society [1, 2]. The leading causes of death from smoking are cardiovascular diseases, chronic obstructive pulmonary disease and lung cancer [3, 4], making it necessary to develop strategies for reducing tobacco use and treating nicotine dependence. Nicotine is considered to be the primary component of tobacco smoke that causes tobacco dependence [5]. Successful smoking cessation is difficult because physiological and psychological dependence and withdrawal symptoms are developed after long-term smoking. Currently available smoking cessation agents (i.e., nicotine replacement therapy, bupropion and varenicline) have limited efficacy and relapse rates are reported to be high, revealing a continuing need for the development of alternative, and more efficacious smoking cessation pharmacotherapies [6, 7]. *Vernonia cinerea* Less. is an annual herb that has been reported to have many medicinal properties. Different parts of the plant have different therapeutic values. The plant has been used for analgesic [8, 9], antipyretic [8, 9], anti-inflammation [8, 10, 11], and treatment malaria fever [12]. In a recent study *V. cinerea* extracts (VE) showed efficacy in reduction of smoking rate [13] and supplementation with VE provided benefit related to reduced smoking rate in smokers [17]. However, the mechanism of action of VE in smoking cessation is not well understood. Thus, this study will provide information of the effect of VE on nicotine withdrawal symptoms for the further studies of developing of therapeutic agent for smoking cessation.

Materials & Methods

Animals

The experiment was performed using ICR mouse (National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand.) with weights of 28-32 g at the beginning of the experiments. The animals were maintained under standard laboratory conditions (25 ^{o}C and 12-h light/dark cycle, food and water ad libitum).

Treatments

Animals were randomly allocated to six different groups as follows: control; C, NIC withdrawal; N, mecamylamine (MEC); M, VE 125 mg/kg; VE125, VE 250 mg/kg; VE250, and VE 500 mg/kg; VE500. VE, MEC, nicotine were dissolved in normal saline before administration. All groups of the mice except C group were injected with nicotine 2 mg/kg SC (4 injections daily, 4 hrs apart, starting at 08.00 hours) for 14 days to induce withdrawal symptoms. For treatment period (day 15–21), N group was received normal saline intraperitoneally while M, VE125, VE250, and VE500 groups were intraperitoneally received MEC 2 mg/kg, VE 125 mg/kg, VE 250 mg/kg, and VE 500 mg/kg, respectively. C group was injected with normal saline throughout the experiment in the same volume of nicotine.

Behavioral evaluations

All behavioral evaluation were performed after first nicotine withdrawal (day 15) and at the end of experiment (day 21). Total abstinence signs (TAS) were evaluated for 30 minutes using The Nicotine Abstinence Scale for scoring the frequency of the following signs: rearing, body lifting, abdominal constrictions, nose scratching, ear scratching, dog shaking, body shaking, body scratching, and chewing. Anxiety-like behavior were measured in the elevated plus maze (EPM) test which mice were allowed to freely explore the maze for 5 minutes. Total entries into open arms was measured by an observer blind to the drug treatment. Locomotors activity (LMA) was measured with an open-field apparatus. The bottom of this apparatus was divided into 25 blocks, (10x10 cm per block). Ambulatory locomotion of the animal was scored as number of squares entered within 5 minutes.

Western blot analysis

Brains the animals were collected after behavioral evaluations were terminated and were homogenized in 1 ml of lyses buffer (0.01 M Tris-HCl, 0.05 M EDTA, 5% SDS, 7.5% DOC, 1 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.88% Triton X-100, 75 mM NaCl, 1.25 mM NaF) containing 1% cocktail protease inhibitor at 4 ^{o}C in a 10 ml glass tissue

grinder. Whole tissue homogenate was centrifuged for 60 minutes with 15,000 g at 4 ^{o}C and supernatant were collected. The MicroBCA^{TM} protein assay kit (Pierce, IL, USA) was used for determined supernatant protein concentration. Changes in expression of nicotinic and muscarinic receptors were determined by western blot using specific antibodies for α7 nicotinic receptors (nAChRα7, H-302, Santa Cruz) and M5 muscarinic receptor (mAChRM5, H-197, Santa Cruz), respectively. An equal amount of protein (75 µg) in each sample was separated by SDS–PAGE (10%, w/w, gel) and electrotransferred to polyvinyllidene fluoride (PVDF) membrane. The PVDF membranes were blocked with 5% skim milk, washed, and then incubate with primary antibody (1:300 dilutions) in 2.5% skim milk in washing buffer for overnight. After three 10 minutes washes in washing buffer, secondary antibody (1:10000 dilutions) in 2.5 % skim milk were incubated for 60 minutes. After washing, proteins were visualized using the ECL detection kit (Pierce, IL, USA) and exposed to X-ray film. Band intensity were measured using a Quantity One® software package (Bio-Rad, CA, USA). The expression levels were calculated from specific band intensity.

Results

Effect of VE on TAS score

First day of discontinuing intermittent of nicotine administration (day 15), all groups had no differences in TAS. However, VE500 group showed significantly decrease of TAS score compare with N group ($p \leq 0.05$) at the end of experiment (day 21), VE exhibited to decrease TAS score in dose dependence manner (Table1). N group is a nicotine withdrawal showed minor changes in TAS with respect to C group.

Effect of VE on LMA

At day 15, VE500 group showed significantly decrease of LMA compared with N, M and VE125 groups ($p \leq 0.05$). Different doses of VE decreased LMA in dose dependence manner. However, day 21, all groups of mice had no differences in LMA (Table2).

Effect of VE on the EPM test

At day 15, Total entries into open arm of VE500 group significantly decreased compared with C and M groups whereas mice received VE 250 mg/kg entered open arm less often than M group. Total open arm entry of N groups was significant lower than M group (Table3) and also nearly significant lower than C group.

Effect of VE on α7 nicotinic and M5 muscarinic receptors protein expression

VE did not affect of both α7 nicotinic and M5 muscarinic receptor protein expression. Nicotine withdrawal and MEC also did not show any effects on the two receptor expression (Table 4).

Table1 Mean value of total abstinence signs

Treatments	Mean value of total abstinence signs	
	Day 15	Day 21
C	215.40±19.77	178.80±12.50
N	250.83±26.81	254.50±19.86
M	193.83±23.21	213.83±6.77
VE125	195.75±32.85	234.50±25.72
VE250	231.33±12.19	105.50±34.28
VE500	289.80±27.72	47.60±41.44[a]

Values represent mean ± S.E.M. (n = 4-6), a $P<0.05$ vs N.

Table2 Mean value of locomotor activity

Treatments	Mean value of locomotor activity	
	Day 15	Day 21
C	167.40±26.90	143.60±16.40
N	154.67±18.22	179.83±9.63
M	193.17±17.27	207.83±15.71
VE125	197.00±16.81	175.00±19.08
VE250	153.50±19.44	149.83±10.41
VE500	65.00±13.68[a, b, c]	153.20±20.30

Values represent mean ± S.E.M. (*n* =4-6), a=*P*<0.05 vs N, b=*P*<0.05 vs M, c=*P*<0.05 vs VE125.

Table3 Mean value of open arms entries

Treatments	Mean value of open arms entries	
	Day 15	Day 21
C	7.00±2.58	7.00±1.50
N	4.33±2.67	8.00±2.65
M	8.67±2.77[b]	9.17±1.59
VE125	6.50±2.78	3.75±0.85[c]
VE250	5.33±2.36[c]	6.00±2.93
VE500	3.20±1.21[a, c]	6.60±2.67

Values represent mean ± S.E.M. (*n* = 4-6), a=*P*<0.05 vs C, b=*P*<0.05 vs N, c =*P*<0.05 vs M. Each symbol denotes significance when compared in the same day.

Table4 Mean value of intensity

Treatments	Mean value of intensity	
	α7	M5
C	192.46±5.72	158.11±18.93
N	191.37±7.91	159.27±21.76
M	198.50±5.69	152.06±14.91
VE125	196.59±9.56	160.71±23.83
VE250	198.67±6.76	155.65±20.91
VE500	186.59±13.84	159.29±17.85

Values represent mean ± S.E.M. (*n* = 3).

Discussion

Nicotine withdrawal symptoms contain both somatic and emotional components. Somatic nicotine withdrawal symptoms which are observed in humans can be induced in mice [14]. *V. cinerea* has been used for smoking cessation in several forms in Thailand such as coffee, tea, and sprays. It has been demonstrated that VE improved smoking cessation in human [13, 15]. The results of the present study indicated that nicotine-induced withdrawal symptoms in mice were significant attenuated by VE. This result is supported by a study in human that show efficacy of *V. cinerea* tea in decrease smoking rate [13, 15]. Withdrawals from chronic nicotine exposure can stimulate mice to express anxiety-like behaviors [16]. VE showed significant decrease of open arm entry in EPM. This decrease may involve with sedative effect of VE [13] which is related to calming effect and reduce movement rather than anxiety reduction. From behavioral evaluation, the results exhibit that VE has an action on decrease nicotine addiction through actions in central nervous system (CNS). Control and regulation of behaviors such as cognition, motivation, reinforcement or reward system have been found to be involved with nicotinic [17] and also muscarinic [18] receptors. Form western blot analysis, VE has no any effects on nicotinic and muscarinic receptor expression in mice brains. The results suggest that VE to play a role in reduction of nicotine abstinence signs through other CNS mechanisms.

Conclusion

In this study, we found that, *V. cinerea* has potential useful as therapeutic agent for smoking cessation by reduced abstinence signs, anxiety, and locomotor activity. Its action may occur on CNS through other mechanisms that might not be related to nicotinic and muscarinic receptors. However, further investigations in mechanism of actions of VE on dopamine and glutamate receptors are needed to be done to assure effects of *V. cinerea* on nicotine withdrawal symptoms.

Acknowledgements

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RESEARCH ARTICLE

Acute Oral Toxicity of *Aegle marmelos* (L.) Correa ex Roxb. Ethanolic Extract in Rats

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Abstract

Aegle marmelos (L.) Correa ex Roxb is a tree from the family Rutaceae. Many parts of the plant were designed for different biological activity study such as anti-diarrhea, anti-flatulent and anti-asthma. However the toxicity data of this plant is still unavailable. Acute oral toxicity of *Aegle marmelos* (L.) Correa ex Roxb ethanolic extract was investigated by using OECD guideline No.423, 2001. Both sexes of Wistar rats were oral administered at dose 2,000 and 15,000 mg/kg bw of suspension extracted solution and observed for 14 days. The result showed that no mortality, abnormal toxicity signs and gross pathology in rats were found. Therefore, the oral LD_{50} of the ethanolic extract in rats is higher than 15,000 mg/kg bw.

Introduction

Aegle marmelos (L.) Correa ex Roxb. is commonly named as bale. The plant has been used in Thai traditional medicine for relief from gastrointestinal disorder such as diarrhea and anti-flatulent. The ethanolic extract contains various chemical components namely mucilage, pectin, tannin and volatile oil. The objective of this study is to determine the toxicity of 95% ethanolic extract of *Aegle marmelos* (L.) Correa ex Roxb. in rats.

Material and method

Animals

Female Wistar rats (200 ± 20 g) and Male Wistar rats (230 ± 20 g) were obtained from National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom. The rats were acclimatized at 24 ± 2°C in 12 h light/dark cycle for 7 days. All rats were fasted for 16 hrs prior to dosing the test sample while drinking water was available *ad libitum*

Method

Rats were divided into three groups each group containing five rats of both sex. Test group1 and 2 received the extract at dose 2,000 mg/kg and 15,000 mg/kg respectively. Control group received distilled water as equivalent volume to the test group. After dosing, food was withheld for a further 3-4 hrs. Toxic signs were observed at 0.5, 1, 3 hr. and once daily thereafter for 14 days, including rats' body weight was recorded weekly and at the end of the test. Pathology necropsy finding was then performed after rat-euthanasia by CO_2 asphyxiation. The mean of body weight gain of the animals in the test groups was calculated in comparison to the rats of the control group using Student's t-Test ($p \leq 0.05$).

Results

Both high doses of bale ethanolic extract at 2,000 and 15,000 mg/kg caused no toxic sings, symptoms and death throughout the observation period. The body weight gain of the rats did not show the difference from the control group. Necropsy findings shown normal appearance and no macroscopic pathological lesions of visceral organ.

Conclusion

The LD_{50} of 95% ethanolic extract of *Aegle marmelos* (L.) Correa ex Roxb. was higher than 15,000 mg/kg body weight. (OECD Guideline No. 423: 2001). This study reveals that this extract could be safe in use as material source for herbal drug development. However, the repeated dose toxicity evaluation is still necessary in drug developing program.

Acknowledgement

The author would like to gratitude thanks to Thailand Institute of Scientific and Technological Research for their financial support.

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RESEARCH ARTICLE

Phytochemicals and Cytotoxicity of *Elephantopus Scaber* Linn. Leaves Extracts

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Abstract

Elephantopus scaber Linn. (Asteraceae) is commonly called "Doe-mai-rue-lom" and has been used for medicinal plant. The present study was performed to phytochemical screening and evaluate cytotoxic activity of this herb using MTT assay. The *E scaber* leaves was extracted with 95% ethanol to give yield 8.9 % w/w. It was composed of high antioxidant activity compound, such as chlorogenic acid and luteolin. The ATCC CRL-1474 (dermal human fibroblast:NHFF) and ATCC CRL-6475 (Melanoma cell:B16-F10) were chosen for cosmetic application. The IC_{50} value were 0.33 and 0.20 mg/ml. for 24 hr treatment. It would be interesting to do further study for developing in cosmetic products.

Keywords *Elephantopus scaber* Linn., cytotoxicity , phytochemical

Introduction

Elephantopus scaber Linn. (Asteraceae) is a aromatic herb distributed in the moist deciduous forests of northern part of Thailand. This plant is known to contain bioactive compound. Phytochemically the plant has been reported to contain sesquiterpene lactones deoxyelephantopin, isodeoxy-elephantopin, and scabertopin. The pharmacological properties of the leaf extracts have been evaluated for diuretic, antiinflammatory, and hepatoprotective properties. The previous study, it contained flavonoid compounds which was exhibited antioxidant activity. It was claimed the biological active in protecting the body, the skin collagen and elastic tissue against damaging by reactive oxygen species. The previous study was used *E.scaber* as whitening agent in cosmetic products. The cytotoxicity testing in this study is very necessary in cosmetic application. Thus, the objective of this study was performed to evaluate cytotoxicity of ethanolic extract of *E. scaber* using MTT assay that was a part of scientific aspects.

Materials and Methods

Preparation of plant extracts

The leaves of *E.scaber* were dried at 40 ^{o}C, ground into powder and extracted 10 times with 95% ethanol at room temperature. The combined filtrates of ethanol solution were evaporated under reduced pressure at room temperature to give yield 8.9 % w/w ethanolic extract.

Phytochemical Screening

Phytochemical screening was performed using TLC chromatography technique. TLC tanks were allowed to equilibrate for at least 30 min. Crude extract (10 mg) was dissolved in 1 ml of 50 % ethanol and partition with 1 ml of ethyl acetate. The amount of 15 µl from ethyl acetate fraction were applied to Silica gel 60 F_{254} TLC plates and developed in toluene : ethyl acetate : formic acid (3:17:3), identified by co-TLC with authentic flavonoids standards (chloroginic acid, apiginin, luteolin and rutin). Visualization of the compounds was attained by spraying the sheets with 1% methanolic diphenylboryloxyethylamine, followed by 5% ethanolic polyethylene glycol 4000. The chromatograms were evaluated in at 366 nm UV light.

Sample preparation

The ethanolic extract of *E. scaber* was weighed and dissolved in 1% EtOH as stock concentration of 1,000 µg/ml. The samples were then filtrated through a 0.2 µm filter and prepared as serial dilution in the culture medium at 8 concentrations.

Cell culture

The human dermal fibroblast (ATCC CRL-1474: NHFF) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated for 72 h. at 37°C in a fully humidified, 5% CO_2: air atmosphere.

The mouse skin melanoma (ATCC CRL-6475: B16-F10) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated for 72 h. at 37°C in a fully humidified, 5% CO_2: air atmosphere.

MTT cytotoxicity test

The cells were seeded in a 96-well plate at a density 10^5cells/ml, and incubated for 24 h. The range of samples were added to the cells and incubated for 24 h., and were then removed from the cells. MTT (5 mg/ml) 50 µl was added to the medium (150 µl) in each well and incubated for 4 h. at 37^{o}C 5% CO_2 for 4 h. Add 100 µl of DMSO to replace old medium and agitation for 5 min. The survival cells were measured and calculated from the absorbance at 590 nm.

Results

Phytochemical Screening

TLC profile of ethanolic extract of *E.scaber* leaves is present in high concentrations of bioflavonoids compound (Fig 1). TLC chromatogram are composed of chlorogenic acid (R_f = 0.18), apiginin (R_f=0.70) and luteolin (R_f= 0.65).

Cytotixicity Testing

The cytotoxicity test was showed in Fig 2. The treatment NHFF and B16-F10 cell line with various of concentration of ethanolic extract of *E scaber. Which* indicated by IC_{50} value was 0.33 and 0.20 mg/ml for 24 hr treatment.

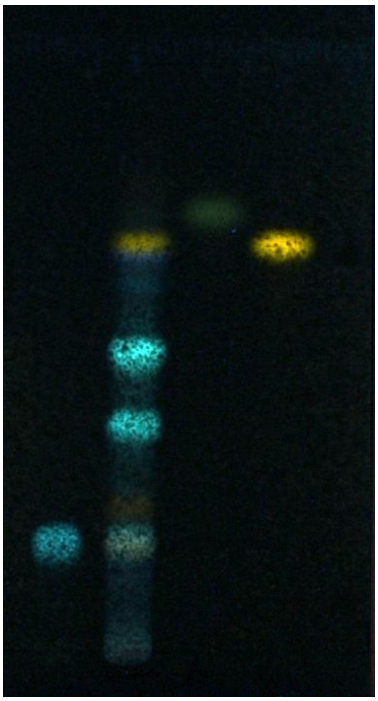

Figure 1 TLC of ethanolic extract of *E.scaber.* leave was developed using solvent system toluene : ethyl acetate : formic acid (3:17:3), co-TLC with authentic flavonoids standards (chloroginic acid, apiginin, and luteolin), spraying reagent with 1% methanolic diphenylboryloxyethylamine, followed by 5% ethanolic polyethylene glycol 4000 and observe under 366 nm UV light.

Discussion and Conclusion

These results show that the ethanolic extract of *E. scaber* leaves are strong antioxidant agent. It was composed of high antioxidant activity compound, such as chlorogenic acid and luteolin. Regarding IC_{50} values of ethanolic extract *of E. scaber* leaves illustrate on NHFF and B16-F10 cell line was 0.33 and 0.20 mg/ml. for 24 hr treatment. The ethanolic extract *of E.scaber* leaves could be potential sources of antioxidant activity and high concentration of bioflavonoid and phenolic compound. It would be protecting the body, the skin collagen and elastic tissue against damaging by UV and reactive oxygen species.

Acknowledgement

The author would like to gratitude thanks to Thailand Institute of Scientific and Technological Research for their financial support.

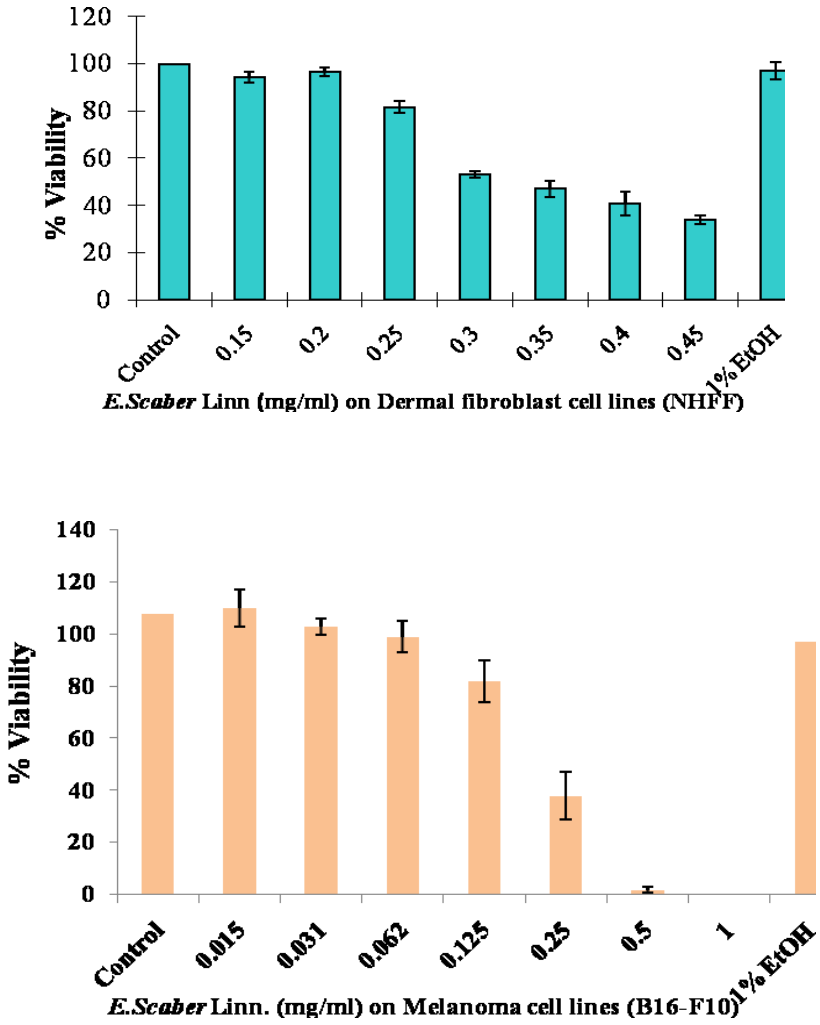

Figure 2 The viability of treated NHFF and B16-F10 with various of concentration of ethanolic extract of *E.scaber*

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RESEARCH ARTICLE

Anti-stress Effect of *Ocimum gratissimum* Linn. Ethanolic Extract in Cold Restraint-Induced Stress Rats

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Abstract

Ocimum gratissimum Linn**.** (*O. gratissimum*) is a native plant grown in Thailand, so called "Ka-prao-chang" or "Yee-ra". The pharmacological properties of the plant have been known as anti-bacterial, laxative, analgesic and muscle relaxant. The anti-stress study of *O. gratissimum* ethanolic extract (OGE) was investigated using cold restraint model in rats. The blood cortisol level was measured for stress status consideration. The result showed that this extract could reduce blood cortisol in rats which under stress. This indicated that OGE has a tendency to use as anti-stress agent.

Keywords *Ocimum gratissimum* Linn**.**, Anti-stress, Cold restraint stress

Introduction

"Ka-prao-chang" or "Yee-ra" (*Ocimum gratissimum* Linn.) is a shrub in family Labiatae and 1-3 meter tall. The medicinal properties of *O. gratissimum* plant are anti-bacterial, laxative, analgesic and muscle relaxant. Aim of this study was to investigate the anti-stress effect of *O. gratissimum* ethanolic extract (OGE) in Cold restraint stress rats.

Methods

Plant material and extraction

The plant's fresh leaves were purchased from Nakhon Pathom province and identified voucher specimen as TISTR No. 250310. The plant was dried at 50 °C and then was pulverized into powder. The power was extracted with 95% ethanol by maceration. The rotary dried-viscous dark green extract was obtained and the yield was 6.75% (w/w).

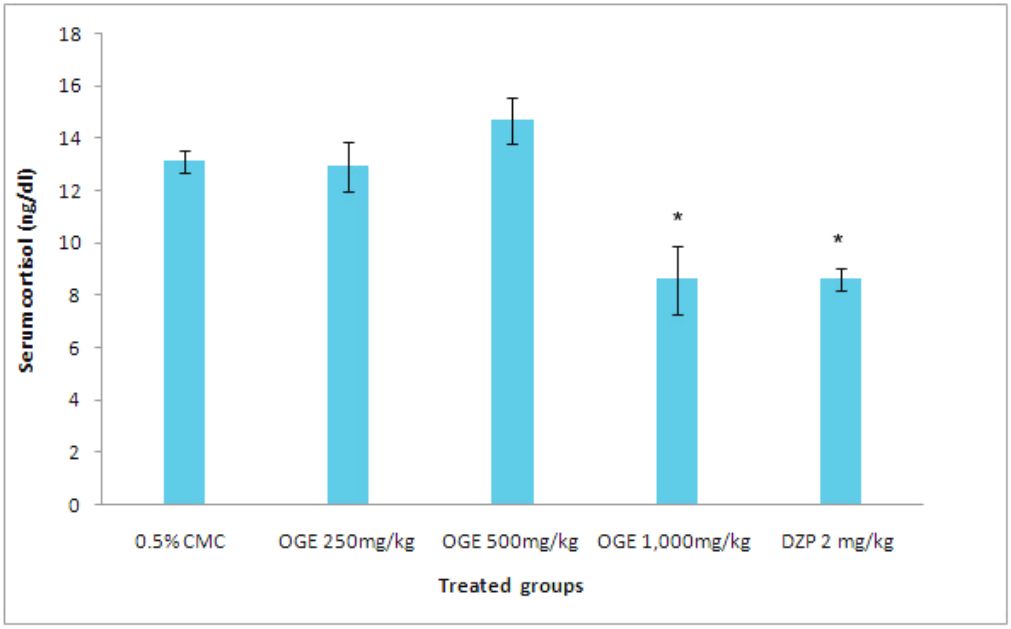
Animals study

Twelve male Wistar rats (250-300g) were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn pathom. All rats were housed in animal care facility building at Thailand Institute of Scientific and Technological Research for 1 week prior to use.

The rats were given orally with OGE suspension in 0.5% CMC (control group) at dose 250, 500 and 1,000 mg/kg bw for 7 consecutive days. The positive control group was administered diazepam (DZP) at dose of 2 mg/kg bw orally. After 1 hr, each rat was then individually placed in fix plastic bottle as normal position using adhesive tape and soaked in cold water that was maintained at 10°C for 1 hr. On the 8th day, rats were fasted at least for 18 hr. before blood collection by tail vein. Plasma cortisol level of each rat was measured using Cortisol EIA Kit Assay (Cortisol EIA Kit,Assay Designs, Inc.,USA) . After blood collection, all rats were euthanasia by CO_2 asphyxiation.

Result

OGE at dose of 1,000 mg/kg bw could significantly reduce plasma cortisol levels in cold restraint stress rats as shown in Fig.1.



*p<0.05= significantly different from the 0.5%CMC (control group)

Figure 1 Shown the plasma cortisol level in cold restraint-induced stress rat 1 h. for 7 days of control and treatment rats (n=5)

Conclusion and Discussion

After cold restraint-induced stress rat 1 h. for 7 consecutive days, OGE at dose 1,000 mg/kg bw could reduce plasma cortisol level in comparison with control group (Fig.1) Basically, an increment of plasma cortisol levels are reversed by anti-stress agents (Sen *et. al*., 1992). Thus, this study indicated that the "Ka-prao-chang" or "Yee-ra" (*Ocimum gratissimum* Linn**.**) ethanolic extract exhibited promise as an anti-stress agent.

Acknowledgement

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RESEARCH ARTICLE

The Comparison of Bioflavonoid Compounds and Anti-oxidant Activity from Citrus Peels Extract

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Abstract

The ethyl acetate extracts was obtained from fruits peels of *Citrus reticulata* Blanco cv. Sainampueng, *Citrus aurantifolia* Swingle and *Citrus hystrix* DC. The active ingredient was analyzed using thin layer chromatography (TLC) techniques comparing to authentic standard. It contained spots equivalent to some bioflavonoid compounds including rutin, hesperidin, hesperitin, and chlorogenic acid. The crude extract of *C. hystrix* has hesperidin and rutin more than *C. aurantifolia* and *C. reticulate*. While *C. reticulate* has unknown phenolic compound (blue spot) which strong antioxidant, but *C. aurantifolia* and *C. hystrix* are less. The antioxidant activity was evaluated using DPPH assay. The EC_{50} of crude extract of *C. hystrix*, *C. aurantifolia* and *C.reticulata* are 41.74 μg/ml, 157.54 μg/ml and 26.14 μg/ml, respectively. The crude extracts had lower activity than vitamin C and rutin, which have EC_{50} 1.90 μg/ml and 0.03 μg/ml, respectively. From these results showed that ethyl acetate extract from *C.reticulata* peels has high content of some phenolic compound equivalent to bioflavonoid which was high potent antioxidant activity more than *C. hystrix* and *C. aurantifolia*.

Keywords *Citrus reticulata* Blanco cv. Sainampueng, *Citrus aurantifolia* Swingle, *Citrus hystrix* DC, bioflavonoid compounds

Introduction

Citrus reticulata Blanco cv. Sainampueng, *Citrus aurantifolia* Swingle and *Citrus hystrix* DC are belonging to Rutaceae family. The peels of these fruits are contained of bioactive compounds including flavonoid, carotenoids and limonoids with potential health promoting properties. The citrus bioflavonoids are natural antioxidant, antiviral, anti-allergy and anti-inflammatory properties. The flavanones (hesperidin and narirutin) and polymethoxyflavones (nobiletin, tangeretin, and sinensetin) were shown strong antioxidant and radical scavenging activity. It was appeared to be associated with reduced risk of certain chronic diseases, the prevention of some cardiovascular disorders, and certain types of cancerous processes.

Materials and Methods

Preparation of Plant Extracts

The fresh fruits peel of *C. hystrix*, *C. aurantifolia* and *C.reticulata* were extracted 3 times with 50 % ethanol at room temperature and then follow extraction with ethyl acetate for 10 times. The combined filtrates of ethyl acetate solution were evaporated under reduced pressure at room temperature.

Phytocemical Screening

Phytochemical screening was performed using TLC chromatography technique. TLC tanks were allowed to equilibrate for at least 30 min. Crude extract (10 mg) was dissolved in 1 ml of ethyl acetate. The amount of 15 µl solution were applied to Silica gel 60 F_{254} TLC plates and developed in dichloromethane-diethyl ether-methanol-formic acid-water (12:16:4:3:1), identified by co-TLC with authentic flavonoids standards (hesperidin, hesperitin, chloroginic acid, and rutin). Visualization of the compounds was attained by spraying the sheets with 1% methanolic diphenylboryloxyethylamine, followed by bismuth nitrate and 10 % potassium hydroxide. The chromatograms were evaluated in at 366 nm UV light.

Scavenging of Diphenyl-picrylhydrazyl (DPPH) Radicals Assay

The free radical scavenging activity of ethyl acetate extracts was analyzed by the DPPH assay [1]. The amount of 100 µl of various concentrations sample were reacted with 100 µl of $6x10^{-3}$ M DPPH ethanolic solution in a 96-well plate, incubated at 37 ^{o}C for 30 min. The absorbance was measured at 517 nm using a UV–VIS microplate reader. All experiments were carried out in triplicates.

Results, Discussion and Conclusion

The fresh fruits peel of *C. hystrix*, *C. aurantifolia* and *C.reticulata* were extracted to give 0.56, 0.46 and 1.024 % yield (w/w), respectively. The ethyl acetate extracts TLC profile of *C. hystrix, C. aurantifolia* and *C. reticulata*. are present high concentration of bioflavonoids compound. The TLC fingerprints were showed green-blue band of hesperidin R_f= 0.17, pale green-blue band of chloroginic acid R_f= 0.21 and orange band of rutin R_f = 0.11, when using NP/$BiNO_3$/KOH spray reagent and observe at 366 nm. TLC plate of *C. hystrix* is present in higher concentrations of hesperidin and rutin than *C. aurantifolia* and *C. reticulata*. While concentration of blue band of unknown compound (R_f = 0.74 and 0.78), which show strong antioxidant activity were found only in *C. reticulate*.

The concentration of antioxidants to quench DPPH radical (EC_{50}) of *C. reticulate* crude extract are showed stronger activity than *C. hystrix* and *C. aurantifolia*. It was contained high content of unknown compounds, which showed strong antioxidant. The activity of crude extract compared to authentic standard was lower activity than Vitamin C and Rutin**.** Result from Table 1 showed that ethyl acetate extract from *C. reticulate* peels has high potent anti-oxidant activity similar to authentic standard. It could be potential sources of antioxidant for using in nutraceutical product for prevention of chronic diseases, some cardiovascular disorders, and certain types of cancerous processes.

Table1 Antioxidant (EC_{50}) activity of ethyl acetate extract from *C. hystrix*, *C. reticulata* and *C. aurantifolia* peels compared with standard.

sample	Antioxidant (EC_{50}, ppm)
Rutin	0.03
Vitamin C	1.90
crude extracts of *C. hystrix*	41.74
crude extracts of *C. aurantifolia*	157.54
crude extracts *C. reticulata*	26.11

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RESEARCH ARTICLE

Effects of curcuminoids on lipid peroxidation and antioxidant enzyme in rat microsome and HepG2 cells

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Abstract

Alcohol consumption enhances reactive oxygen specie (ROS), lipid peroxidation or decreases the level of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) in alcoholic liver disease (ALD). ALD cause oxidative stress. Curcuminoids, a complex compounds derived from turmeric extract, have shown antioxidant activities. The present study was evaluated the effects of curcuminoids against ethanol-induced lipid peroxidation in rat microsomal extraction and cells culture. We found that curcuminoids in the dose dependent manner decrease lipid peroxidation as represented with malondialdehyde (MDA) levels in ethanol induced toxicity HepG2 cells supplemented with various concentrations curcuminoids. In addition, curcuminoids at 500 and 750 mg/kg/day decreased the MDA levels significantly in liver microsomes from the ethanol induced toxicity rats. However, the superoxide dismutase (SOD) enzyme activities did not change in rat microsomal extractions and curcuminoids did not enhance the enzymes activity. Therefore, curcuminoids have a potential property to protect lipid peroxide production in ethanol-stimulated HepG2 cells and in microsomal extraction from ethanol induced toxicity rats.

Keywords alcoholic liver disease, ethanol, lipid peroxidation, superoxide dismutase, curcuminoids

Introduction

Alcoholic liver disease (ALD) is a major disease of morbidity and mortality worldwide. The dose and time dependence of excess alcohol consumption involve in the progression of ALD. Toxic by-products of alcohol metabolism mainly generate cell damages by producing reactive oxygen specie (ROS) and lipid peroxidation that results in initiation of inflammatory process (1). Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by ethanol and leads to membranes dysfunction (2). On the other hand, cellular protections against ROS and lipid peroxidation are related to complex antioxidant defense system. There are 3 major antioxidant enzymes that protect the cell during ethanol-induced oxidative stress, including catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) (3). The increased formation of ROS and lipid peroxidation reduces levels of antioxidant enzymes. Microsome, a high-speed centrifugation particle preparation has been isolated from liver tissues. The microsome fraction of various contains fat, steroid and various enzymes including antioxidant enzymes (4).

At this time, there is still no effective treatment for ALD treatment. The principle of treatment ALD is to protect the progression of liver cells damage. Therefore, one idea of developing hepatoprotective agent from herbal plants to reduce production of ROS is remarkably under investigation. Curcuminoids are polyphenol substances from the colored extract of dried powder from turmeric rhizome. Curcuminoids have been shown variety of pharmacological actions such as anti-inflammatory, antimicrobial, and antioxidant properties (5). Traditionally, many countries have been applied turmeric and natural curcuminoids in a therapeutic preparation for many ailments. It is used to treat diseases associated with gastrointestinal tract such as dyspepsia, peptic ulcer, and liver disorders (6). Curcumin, an active constituent from turmeric protect animal liver from a variety of hepatotoxic substances like galactosamine, carbon tetrachloride acetaminophen and ethanol (7). We are interested in the effect of curcuminoids as a hepatorprotective agent against alcohol-induced toxicity. The aim of this study was to evaluate effects of curcuminoids on lipid peroxidation in ethanol-stimulated cells and antioxidant enzymes in rat microsomes.

Methods

Cell culture

The human liver cell line, HepG2 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.

Lipid peroxidation

All cells were plated into 24- well plates at density $1x10^5$ 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 with 10% (v/v) of ethanol for 22 hours. The thiobarbituric acid reactive substances (TBARs) reagent was added to the wells and incubated at 90 ˚C for 1 hour. Fluorescences were read at excitation 485 nm, emission 535 nm.

Animals and treatment

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. Rats were divided into 7 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 8^{th} the rats were received sylimarin (Legalon®) 100 mg/kg/day or curcuminoids 250, 500 and 750 mg/kg/day respectively. Rats were sacrificed by 50 mg/kg pentobarbital and liver were collected for microsomal preparation.

Microsomal preparation

One gram of liver was cut into pieces and homogenized with 3 ml of phosphate buffer, pH 7.4. The liver homogenate was centrifuged at 10,000 g for 30 minutes at 4 ˚C. The supernatant was transferred into

ultracentrifuge tubes and centrifuged at 100,000 g for 60 minutes at 4 °C. The pellet microsomal fraction was suspended in phosphate buffer, pH 7.4, containing 20% v/v glycerol and stored at -80 ˚C

Superoxide dismutase assay

Superoxide dismutase (SOD) in microsomal extraction was measured by SOD kit® (Sigma Aldrich). The enzyme activity in which the enzyme decreases the reduction of water-soluble tetrazolium salt (WST) by superoxide radical generated from xanthine and xanthine oxidase was monitored at 450 nm.

Results

Effects of curcuminoids on ethanol-induced lipid peroxidation and superoxide dismutase in microsomal extraction.

Ethanol is known to generate oxidative stress in cell which can be measured from lipid peroxidation in microsomal extraction. The thiobabituric acid reactive substance (TBARs) calculated as malondialdehyde (MDA) content in microsomal extraction was assessed (Figure 1A.) The MDA levels trended to increase in the microsomes from the ethanol induced toxicity rat supplemented with vehicle (carboxymethylcellulose, CMC) However, curcuminoids at concentration of 500, 750 mg/kg/day could attenuate the MDA levels significantly in the ethanol-induced lipid peroxidation group,compared to the ethanol induced lipid peroxidation supplement with CMC and the control groups. When we measured the SOD enzyme activity in the microsomal extraction, we found that the enzyme activities were not changed among groups, (Figure 1B).

Effect of curcuminoids on lipid peroxidation in ethanol stimulated HepG2 cells

HepG2 cells, stimulated with various concentrations of ethanol for 24 hours, increase MDA levels as a dose-dependent manger. The ethanol concentration at 7.5% and 10% v/v could induce the cells to produce amounts of MDA levels significantly, comparing with the control cells (figure 1A.) When 10% ethanol-stimulated HepG2 cells were pre-incubated with curcuminoids, we found that curcuminoids trends to decrease MDA levels.

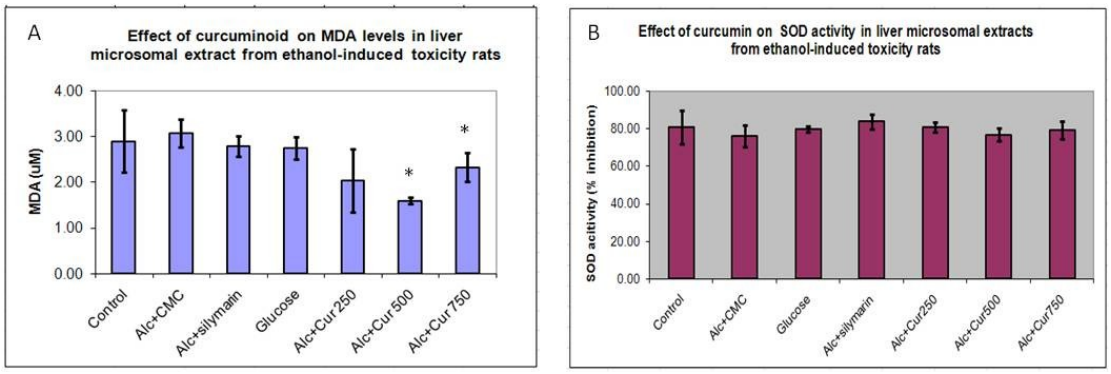

Figure 1 The effects of curcuminoids on lipid peroxidation (A) and superoxide dismutase (B) in liver microsomal extraction from ethanol-induced toxicity rats. Data were from 3 separated experiments (n=3) and shown as mean ± SD of MDA level , Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$)**.** Alc = Alcohol, Cur = Curcuminoids (mg/kg/day), CMC = carboxymethylcellulose

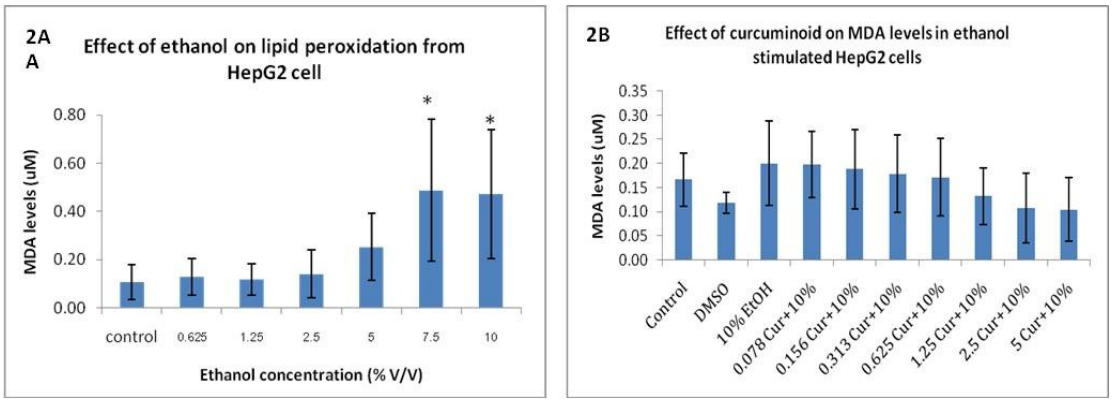

Figure 2 The effects of curcuminoids on lipid peroxidation in ethanol stimulated HepG2 cells (2A) and ethanol treated HepG2 cells combination with various concentrations of curcuminoids (2B). Data were from 4 separated experiments (n=4) and shown as mean ± SD of MDA level , Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$).EtOH= ethanol, Cur = Curcuminoids (ug/ml)

Discussions

Oxidative stress is one major role in the pathogenesis of ALD. The increase of ROS production and the decrease of antioxidant activity, including other harmful effect, cause lipid peroxidation which lead to the damage of the liver cells (1). Our data confirmed that the MDA levels trended to increase in the ethanol induced toxicity rat microsomes. Curcuminoids at concentration 500, 750 mg/kg/day reduced the ethanol-induced lipid peroxidation Furthermore, in this present study, we demonstrated that the ethanol-stimulated HepG2 cells enhanced lipid peroxidation. When we tested the effects of curcuminoids on ethanol-stimulated the HepG2 cells, the results showed that curcuminoids at various concentrations trends to attenuate the lipid peroxidation. The level of lipid peroxidation was taken as an index for oxidative stress (8) Several studies have showed the antioxidative effects of curcuminoids in hepatotoxicity (5). Our study has informed this possible mechanism of curcuminoids as an antioxidant, by reducing lipid peroxidation, in the ethanol induced toxicity. However, when we investigated the changes of antioxidant enzyme in microsomal extraction, our results showed that the hepatic SOD activity were not different between groups. Many reviews suggested that hepatic cells have variety of antioxidant enzymes including SOD, catalase and glutathione peroxidase (9). The effects of chronic ethanol exposure on activity of the antioxidant enzymes are controversial. These may depend on many pathological factors. This study revealed that curcuminoids diminished ethanol-induced lipid peroxidation. Curcuminoid functions as an antioxidant to scavenge free radicals and inhibit the propagating chain of lipid peroxidation (10).

Conclusion

Results from this current experiments demonstrated that curcuminoids have protective effect on lipid peroxidation in microsomal extraction from the ethanol induced toxicity rats as well as decrease lipid peroxidation in HepG2 cells. However ethanol and ethanol supplemented with curcuminoids did not change SOD enzyme activity levels in our study. Thus, the prevention of lipid peroxidation generation in ethanol induced toxicity rats and cell culture via curcuminoids may be considered for a potential strategy in ALD treatment.

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RESEARCH ARTICLE

ฤทธิ์ของสารสกัดจากหญ้าดอกขาว (*Vernonia cinerea* Less.) ต่อการสร้างไนตริกออกไซด์ ในภาวะตับอักเสบ

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[1]หน่วยปฏิบัติการวิจัยเภสัชวิทยา คณะเภสัชศาสตร์, [2]ภาควิชาเภสัชกรรมปฏิบัติ คณะเภสัชศาสตร์ มหาวิทยาลัยนเรศวร พิษณุโลก 65000

บทคัดย่อ

โรคตับเป็นหนึ่งในโรคที่เป็นสาเหตุการตายของประชากรไทยและยังไม่สามารถรักษาให้หายขาดได้ เกิดได้จากหลายสาเหตุ เมื่อเซลล์ตับเกิดการอักเสบจะกระตุ้นให้เกิดการสร้างอนุมูลอิสระรวมถึง ไนตริกออกไซด์ (nitric oxide, NO) ในปริมาณที่มากกว่าปกติส่งผลให้เซลล์เกิดการอักเสบอย่างต่อเนื่อง ในปัจจุบันมีการนำสมุนไพรไทยมาใช้รักษาโรคต่าง ๆมากมาย หญ้าดอกขาว (*Vernonia cinerea* Less.) เป็นพืชสมุนไพรที่มีการศึกษาว่ามีฤทธิ์ยับยั้งการอักเสบได้ดี การศึกษาครั้งนี้จึงมุ่งเน้นศึกษาฤทธิ์ของสารสกัดหญ้าดอกขาวต่อการสร้าง NO และปริมาณเอนไซม์ inducible nitric oxide synthase (iNOS) ในภาวะตับอักเสบโดยให้สารก่อการอักเสบ lipopolysaccharide (LPS)1.0 μg/m, tumor necrosis factor-alpha (TNF-α) 400 ng/ml, interleukin-1 beta (IL-1β) 400 ng/ml ร่วมกับสารสกัดหญ้าดอกขาวในเซลล์ตับ HepG2 ที่ความเข้มข้น 62.5, 125, 250 และ 500 μg/ml เป็นเวลา 24 ชั่วโมง วัดการสร้าง NO ด้วยสารเรืองแสง diaminofluorescein -2 diacetate และตรวจสอบการแสดงออกของเอนไซม์ iNOS ด้วยวิธี immuno blot พบว่า HepG2 ที่ได้รับสารก่อการอักเสบมีการเพิ่มการสร้าง NO และปริมาณเอนไซม์ iNOS เมื่อเปรียบเทียบกับกลุ่มควบคุม ในเซลล์ตับที่เกิดภาวะอักเสบพบว่า สารสกัดหญ้าดอกขาวมีแนวโน้มที่จะผลลดการสร้าง NO และปริมาณเอนไซม์ iNOS เมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับสารสกัด อย่างไรก็ตามผลการทดลองไม่แสดงนัยสำคัญทางสถิติ จากผลการศึกษาแสดงในเบื้องต้น สารสกัดหญ้าดอกขาวมีแนวโน้มที่จะลดปริมาณและการสร้าง NO ของเซลล์ตับ HepG2 ที่กระตุ้นให้เกิดการอักเสบได้

คำสำคัญ Nitric oxide, Anti-inflammation, *Vernonia cinerea* Less., Hepatitis

บทนำ

โรคตับอักเสบเกิดจากภาวะที่มีการอักเสบและทำลายเซลล์ตับส่งผลให้ตับมีการทำงานผิดปกติ และเป็นปัญหาที่สำคัญทางด้านสาธารณสุขของประเทศไทย โรคตับเกิดจากสาเหตุหลายประการ ที่พบได้บ่อย คือ การติดเชื้อไวรัสและการดื่มสุราในปริมาณมาก โรคตับอักเสบยังไม่มียาที่รักษาโรคได้โดยตรง วิธีการรักษาในปัจจุบันคือ ชะลอการอักเสบของเซลล์ตับ ผ่าตัดเปลี่ยนตับ ใช้ยาเคมีบำบัด (1) เมื่อเซลล์ตับอยู่ในภาวะอักเสบจะส่งผลให้มีการสร้างสารอนุมูลอิสระต่าง ๆในปริมาณที่มากกว่าปกติ โดยเฉพาะ NO ซึ่งจัดอยู่ในกลุ่ม reactive nitrogen species (RNS) สร้างจากเอนไซม์ nitric oxide synthase (NOS) อย่างต่อเนื่อง โดยเฉพาะเอนไซม์ iNOS ซึ่งเป็นเอนไซม์ที่จะถูกสร้างขึ้นและตอบสนองต่อการอักเสบเป็นสำคัญ ซึ่งพบได้ในเซลล์เกือบทุกชนิดเมื่อถูกกระตุ้นด้วยสารก่อการอักเสบ เช่น LPS, TNF-α, IL-1β, Interferon-gamma (IFN-γ) เป็นต้น (2) ดังนั้นการลดการอักเสบของเซลล์ตับจึงเป็นกลไกหนึ่งที่ช่วยชะลอภาวะโรคตับอักเสบ ปัจจุบันพบว่า พืชสมุนไพรหลายชนิดมีฤทธิ์ยับยั้งการอักเสบได้ดีและมีผลข้างเคียงน้อย จึงเป็นที่น่าสนใจค้นคว้าและพัฒนายาจากสมุนไพรในท้องถิ่น หญ้าดอกขาว (*Vernonia cinerea* Less.) เป็นพืชสมุนไพรพื้นบ้านที่ขึ้นกระจายทั่วไปในประเทศไทย มีสรรพคุณใช้เป็นยารักษาอาการหรือโรคต่าง ๆมากมาย มีรายงานการศึกษาพบว่า สารสกัดหญ้าดอกขาวจากเมทานอลมีฤทธิ์ยับยั้งการอักเสบในหนูที่ชักนำให้เกิดการบวมของอุ้งเท้า และลดไข้ในหนูขาว (3, 4) รวมถึงมีฤทธิ์ยับยั้งเชื้อแบคทีเรีย (4) และปัจจุบันมีการนำหญ้าดอกขาวในรูปชาชงมาใช้ในทางคลินิกเพื่อบำบัดภาวะติดบุหรี่ (5) อย่างแพร่หลาย ดังนั้นการวิจัยนี้จึงมีเป้าหมายหลักในการศึกษาฤทธิ์ของสารสกัดหญ้าดอกขาวจากน้ำต่อการสร้าง NO และ ปริมาณเอนไซม์ iNOS ในเซลล์ตับ HepG2 ที่ถูกกระตุ้นด้วยสารก่อการอักเสบ เพื่อเข้าใจฤทธิ์ต้านการอักเสบและหรือต้านอนุมูลอิสระของสารสกัดหญ้าดอกขาวและนำไปสู่การพัฒนายารักษาโรคตับต่อไป

วิธีดำเนินการวิจัย

การเตรียมสารสกัดหญ้าดอกขาว

นำส่วนลำต้นและดอกของหญ้าดอกขาวแห้งมาสกัดด้วยวิธีการหมักโดยใช้น้ำที่อุณหภูมิประมาณ 60 °C ทิ้งไว้ 16 ชั่วโมง จากนั้นกรองและหมักซ้ำ สารที่ได้จากการกรองนำมาสกัดแห้งด้วยเครื่องทำให้แห้งด้วยวิธีเยือกแข็ง (freeze dry) เป็นเวลา 3 วัน สารสกัดแห้งที่ได้จะเก็บใส่ขวดที่ปิดสนิทและเก็บไว้ที่อุณหภูมิ -20°C

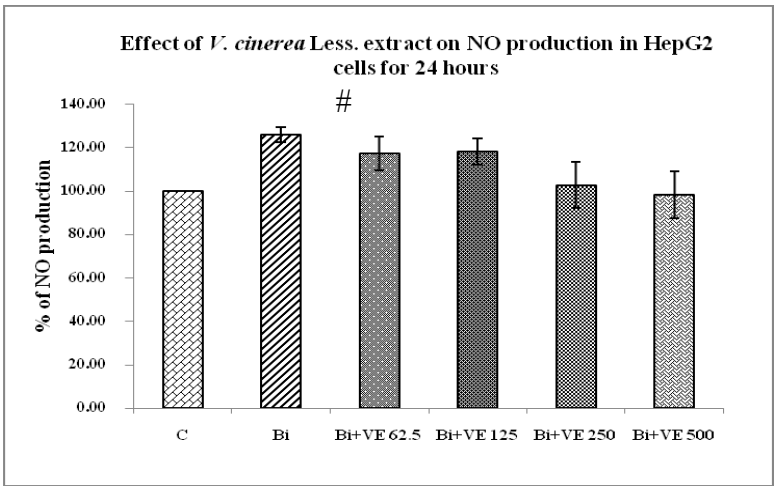
การเพาะเลี้ยงเซลล์ตับ HepG2 (Human hepatocellular liver carcinoma cell line)

เซลล์ตับ HepG2 จะนำมาเพาะเลี้ยงในอาหารเลี้ยงเซลล์ชนิด Dulbecco's Modified Eagle Medium (DMEM) ที่ประกอบด้วย fetal bovine serum (FBS) 10%, penicillin/streptomycin 1% เซลล์จะเปลี่ยนอาหารทุก ๆ 3 วัน เมื่อเซลล์เจริญหนาแน่นแล้วจะถ่ายเลี้ยง (subculture) เพื่อใช้ทดลองต่อไป

การศึกษาผลของสารก่อการอักเสบและสารสกัดหญ้าดอกขาวต่อการสร้าง NO

เซลล์ตับ HepG2 เพาะเลี้ยงในถาดหลุม (96-well-plate) ที่ความหนาแน่น $3x10^4$ เซลล์ต่อหลุม เป็นเวลา 24 ชั่วโมง จากนั้นเปลี่ยนเป็นอาหารที่ปราศจากซีรัมและเติมสารก่อการอักเสบ LPS 1.0 µg/ml, TNF-α 400 ng/ml, IL-1β 400 ng/ml และสารสกัดหญ้าดอกขาว 62.5, 125, 250 และ 500 µg/ml เลี้ยงเซลล์ต่อไปจนครบ 24 ชั่วโมง

การวัดปริมาณ NO โดยเติมสาร diaminofluorescein-2 diacetate (DAF-2DA) นำไปบ่มในตู้เลี้ยงเซลล์ 30 นาที จากนั้นล้างออกเติมสารละลาย phosphate buffer saline (PBS) นำไปวัดค่าเรืองแสงที่เกิดจาก NO ทำปฏิกิริยากับสาร DAF-2DA ในอัตราส่วน 1:1 ได้เป็นสารเรืองแสงในระยะเวลา 30 นาทีด้วยเครื่องสเปกโตรโฟโตมิเตอร์ ที่ความยาวคลื่น 485 นาโนเมตร ปริมาณ NO แสดงในรูป fluorescence เซลล์ตับ HepG2 จะนำไปหา



รูปที่ 1 ผลของสารสกัดหญ้าดอกขาวต่อร้อยละการสร้าง NO ของเซลล์ตับ HepG2 ผลการทดลองนำเสนอเป็นค่า Mean±SEM, C คือ เซลล์กลุ่มควบคุม, Bi คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบ, Bi+VE 62.5, Bi+VE 125, Bi+VE 250, Bi+VE 500 คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml ตามลำดับ, * แสดงนัยสำคัญทางสถิติด้วย one way ANOVA, # แสดงนัยสำคัญทางสถิติด้วย student t-test ($p< 0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม

ปริมาณโปรตีนด้วยวิธีเบรดฟอร์ด (Bradford assay) ผลการศึกษานำเสนอในรูปของ % NO production

การศึกษาผลของสารก่อการอักเสบและสารสกัดหญ้าดอกขาวต่อปริมาณเอนไซม์ iNOS

เซลล์ตับ HepG2 ที่ทดสอบด้วยสารก่อการอักเสบเมื่อครบ 24 ชั่วโมง ขูดเซลล์ในสารละลาย PBS นำไปปั่นเหวี่ยงที่ความเร็วรอบ 12,000g เป็นเวลา 10 นาที จากนั้นเติมบัฟเฟอร์สำหรับแยกโปรตีนแล้วนำไปทำให้เซลล์แตกด้วยการใช้คลื่นเสียง นำมาปั่นเหวี่ยงที่ความเร็วรอบ 15,000g เป็นเวลา 15 นาที เก็บส่วนใส (cell lysate) ที่อุณหภูมิ -20 °C นำ cell lysate ที่เตรียมได้ในปริมาณ 75 µg/well ไปแยกโปรตีนด้วย electrophoresis และถ่ายโปรตีนลงบน PVDF membrane นำบ่มกับ polyclonal rabbit anti-iNOS อัตราส่วน 1: 200 ที่อุณหภูมิ 4 °C เป็นเวลา 16 ชั่วโมงและบ่มกับ polyclonal goat anti-rabbit อัตราส่วน 1:10,000 เป็นเวลา 1 ชั่วโมง ตรวจวัดโปรตีนด้วย chemiluminescence reagent ได้เป็นแถบโปรตีนบนแผ่นฟิล์ม แล้วจึงวิเคราะห์หาปริมาณโปรตีนจากแถบบนฟิล์มด้วยโปรแกรม Quantity One®

การวิเคราะห์ข้อมูล

การศึกษาทำซ้ำอย่างน้อย 3 ครั้งแสดงผลเป็น mean ± SEM และวิเคราะห์ผลทางสถิติเปรียบเทียบความแตกต่างระหว่างกลุ่มโดยใช้สถิติ one way ANOVA ($p< 0.05$), หรือ student t-test ($p< 0.05$)

ผลการทดลอง

ผลของสารก่อการอักเสบและสารสกัดหญ้าดอกขาวที่มีต่อการสร้าง NO

จากการทดสอบสารก่อการอักเสบ LPS, IL-1β และTNF-α และสารสกัดหญ้าดอกขาวที่ความเข้มข้น 62.5, 125, 250 และ 500 µg/ml ต่อการสร้าง NO ของเซลล์ HepG2 พบว่า เซลล์ที่ได้รับสารก่อการอักเสบมีการสร้าง NO เพิ่มขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุม บ่งชี้การเกิดเซลล์ตับอักเสบและเมื่อเซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว พบว่าการสร้าง NO มีแนวโน้มลดลงตามความเข้มข้น อย่างไรก็ตามผลไม่แสดงนัยสำคัญทางสถิติ (รูปที่ 1) ส่วนเซลล์ HepG2 ที่ได้รับสารสกัดหญ้าดอกขาวโดยตรง พบว่า มีการ

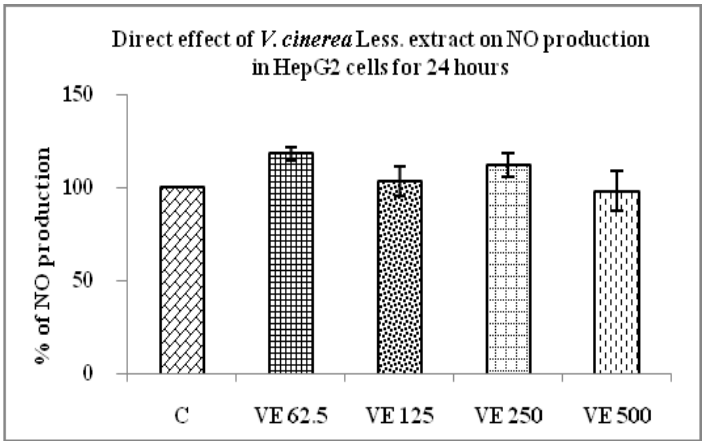
สร้าง NO เพิ่มขึ้นเล็กน้อยเมื่อเปรียบเทียบกับกลุ่มควบคุม แต่ไม่แสดงนัยสำคัญทางสถิติ (รูปที่ 2)

ผลของสารก่อการอักเสบและสารสกัดหญ้าดอกขาวที่มีต่อปริมาณเอนไซม์ iNOS

จากการทดสอบสารก่อการอักเสบและสารสกัดหญ้าดอกขาวที่ความเข้มข้น 125, 250 และ 500 μg/ml ต่อปริมาณเอนไซม์ iNOS ของเซลล์ตับ HepG2 พบว่าเมื่อเปรียบเทียบปริมาณเอนไซม์ iNOS ของเซลล์กลุ่มที่ให้สารก่อการอักเสบและกลุ่มควบคุม พบว่า มีแนวโน้มเพิ่มขึ้น และเมื่อกลุ่มเซลล์ที่ให้สารก่อการอักเสบแล้วได้รับสารสกัดหญ้าดอกขาวในขนาดต่าง ๆพบว่าปริมาณเอนไซม์ iNOS มีแนวโน้มลดลงโดยเฉพาะที่ความเข้มข้น 125 μg/ml เมื่อเปรียบเทียบกับกลุ่มให้สารก่อการอักเสบ อย่างไรก็ตามผลไม่แสดงนัยสำคัญทางสถิติ (รูปที่ 3)

อภิปรายผลการทดลอง

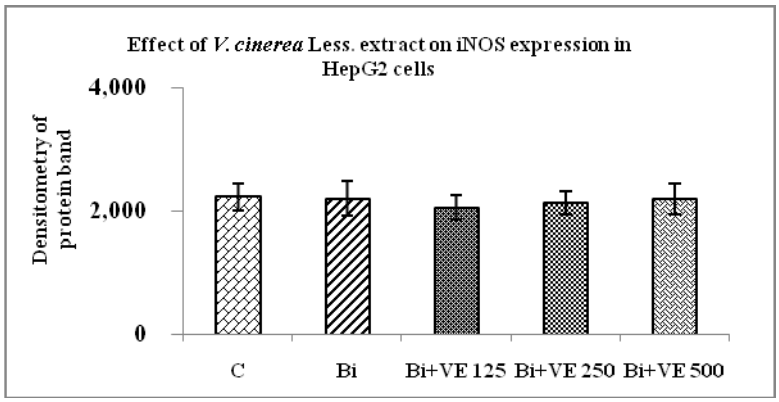
ในภาวะเซลล์ตับอักเสบซึ่งเกิดจากการกระตุ้นด้วยสารก่อการอักเสบหลายชนิดที่มาจากการตอบสนองในระบบภูมิคุ้มกันจะกระตุ้นให้เกิดการสร้างอนุมูลอิสระรวมทั้ง NO (2) ในการศึกษานี้แสดงให้เห็นว่า เมื่อเซลล์ได้รับสารก่อการอักเสบหลายชนิดร่วมกัน (LPS 1.0 μg/ml, TNF-α 400 ng/ml, IL-1β 400 ng/ml)เซลล์ HepG2 จะเพิ่มการสร้าง NO และมีปริมาณเอนไซม์ iNOS เพิ่มขึ้น จากการศึกษาเบื้องต้นเราพบว่า การใช้สารก่อการอักเสบเพียงชนิดเดียวหรือในขนาดที่ไม่เหมาะสมมีผลน้อยมากต่อการกระตุ้นการสร้าง NO (ไม่รายงานผล) การศึกษานี้เป็นการศึกษาแรกที่ทดลองใช้สารสกัดหญ้าดอกขาวจากการสกัดด้วยน้ำคล้ายกับการใช้หญ้าดอกขาวในรูปแบบชาชงในทางคลินิก ผลการทดลองครั้งนี้สอดคล้องกับรายงานการศึกษาก่อนหน้าที่ได้ศึกษาสารสกัดหญ้าดอกขาวจากเมทานอล พบว่ามีฤทธิ์ในการยับยั้งการเกิดภาวะ oxidative stress โดยสามารถกำจัดสารอนุมูลอิสระ superoxide, hydroxyl radical, NO ใน serum ของหนูขนาดเล็ก ร่วมถึงยับยั้งการเกิด lipid peroxidation และเพิ่มสารต้านอนุมูลอิสระ catalase, superoxide, dismutase, glutathione, glutathione peroxidase และ glutathione-S transferase ในเลือดและตับของหนูขาวเล็ก (6, 7) ร่วมถึงมีฤทธิ์ยับยั้งการอักเสบจากการให้ carrageenin ที่ชักนำให้เกิดการบวมของอุ้งเท้าของหนู (paw edema) โดยไปลดระดับสารก่อการอักเสบ TNF-α, IL-1β, IL-6 (6, 8) และจากการให้ cyclophosphamide (CTX) ทำให้เกิดความเป็น



รูปที่ 2 ผลของสารสกัดหญ้าดอกขาวต่อร้อยละการสร้าง NO ของเซลล์ตับ HepG2 โดยตรง ผลการทดลองนำเสนอเป็นค่า Mean±SEM, C คือ เซลล์กลุ่มควบคุม, Bi คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบ, Bi+VE 62.5, Bi+VE 125, Bi+VE 250, Bi+VE 500 คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว 62.5 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml ตามลำดับ

A

B



รูปที่ 3 A แถบโปรตีนเอนไซม์ iNOS, B กราฟแสดงปริมาณเอนไซม์ iNOS ของเซลล์ตับ HepG2 ผลการทดลองนำเสนอเป็นค่า Mean±SEM, C คือ เซลล์กลุ่มควบคุม, Bi คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบ, Bi+VE 125 คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว 125 μg/ml, Bi+VE 250 คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว 250 μg/ml, Bi+VE 500 คือ เซลล์กลุ่มที่ได้รับสารก่อการอักเสบและได้สารสกัดหญ้าดอกขาว 500 μg/ml

พิษอย่างรุนแรงต่อเซลล์ สารสกัดหญ้าดอกขาวสามารถลดสารก่อการอักเสบ TNF-α, IFN-γ, IL-2 และลดความเสียหายของเซลล์ลำไส้เล็กในหนูที่ให้ CTX (7) จากการศึกษาผลของสารสกัดหญ้าดอกจากการสกัดน้ำมีแนวโน้มที่จะสามารถลดการอักเสบของเซลล์ตับได้ในเบื้องต้น อย่างไรก็ตามผลการศึกษาไม่แสดงนัยสำคัญทางสถิติ ฉะนั้นจำเป็นจะต้องมีการศึกษาต่อ ๆไปถึงผลการต้านการอักเสบในภาวะตับอักเสบในสัตว์ทดลองร่วมทั้งความเป็นพิษของหญ้าดอกขาว

กิตติกรรมประกาศ

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RESEARCH ARTICLE

Derris scandens Benth Extract Induces Necrosis Rather Than Apoptosis of SW480 Colon Cancer Cells

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Abstract

The extract from *Derris scandens Benth* was previously shown to have anti-proliferative effect against SW480 colon cancer cells. Therefore, the present study was aim to investigate the mechanism of action of the anti-proliferative effect of *D. scandens* extract. Several apoptotic signaling pathways were determined following *D. scandens* treatment. Caspase-3 activity and the expression of Bax pro-apoptotic and Bcl-2 anti-apoptotic proteins were determined. The result showed that *D. scandens* (5-10 μg/ml) slightly increased caspase-3 activity, as well as up-regulated Bax and down-regulated Bcl-2 proteins of SW480 cells. However, these changes were not statistically significant. *D. scandens* extract significantly induced cell necrosis determined by the release of LDH. These results suggest that *D. scandens* primarily mediate SW480 cell death through necrotic rather than apoptotic process.

Keywords *Derris scandens Benth*, apoptosis, colon cancer, SW480 cells

Introduction

Colorectal cancer is a common disease that remains the major cause of cancer-related mortality in developed countries. The incidence rate of colorectal cancer in Thailand is low when compared with other countries and the highest incidence is seen in Bangkok (1). This rate is expected to be rapidly increased in the next decade probably due to the acquisition of Western lifestyle. Diet with high levels of fat and red meat, and low dietary fiber is the major risk factor of colorectal cancer (2). Since diet is definitely important for colon cancer development, dietary interventions are received much attention as one of approaches to prevent this type of cancer. The protective effects of diets rich in fruits and vegetables against colon carcinogenesis are thought to be due to their content of anti-oxidant vitamins and fibers (3,4). Several traditional Thai herbal medicines are believed to have anti-cancer activity but there is limited scientific evident to support their effectiveness. *D. scandens* is one of Asian medicinal plant, local Thai name, Tao-Wan-Priang. Its dried stem has been used as an expectorant, anti-tussive, diuretic and agent for the treatment of muscle aches (5). Based on our previous study, the extract from *D. scandens* showed an effective anti-proliferative activity against SW480 colon cancer cells (IC_{50} = 4.86 μg/ml) (unpublished data). Thus, the aim of this study was to investigate whether *D. scandens* extract drives colon cancer cells to undergo necrosis or apoptosis cell death pathway.

Methods

Preparation of plant extract:

The *D. scandens* powder was prepared and provided by Bangkratum Hospital, Phitsanulok. The dried powder was macerated with 95% methanol for 3 days. The aqueous extract was subsequently filtered and evaporated in a rotavapor at 55-60°C under pressure. The plants extract was kept at -20°C.

Cell culture

The human colorectal cancer cells (SW480) was purchased from the American Type Culture Collection (ATCC). SW480 cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell viability assay

Cells were exposed to various concentrations of *D. scandens* extract for 24 h. Cells were incubated with 0.5 mg/ml of MTT 2 h before the end of treatment period. Then cells were lysed with DMSO:ethanol (1:1) and the absorbance was read at 595 nm. Lactate dehydrogenase (LDH) released into cultured medium was measured by using pyruvate and NADH as substrates. The reduction of NADH was determined at 340 nm.

Caspase-3 activity

The cells were harvested by trypsinization before the detection of caspase-3 activity by using EnzChek® Caspase-3 assay kit (Molecular Probes). According to the manufacturer's instruction, caspase-3 activity was determined by using rhodamine 110 bis-(*N*-CBZ-L-aspertyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) as a substrate. The fluorescence of rhodamine 110 (R110) was measured at Ex 488 nm and Em 535 nm.

Expression of Bcl-2 and Bax

Immunoblotting was used to determine the expression of Bcl-2 and Bax proteins. Briefly, proteins in cell lysate were separated on SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membrane. The membrane was then incubated with specific antibody against Bcl-2 or Bax and subsequently secondary antibody conjugated with alkaline phosphatase. The activity was assessed by using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate.

Results

Effect of D. scandens on colon cancer cell viability

The cell viability of SW480 cells in the presence of various concentrations of *D. scandens* was examined. As shown in

Figure 1A, *D. scandens* at 5 and 10 μg/ml dramatically decreased cell viability. At the same concentrations, *D. scandens* induced the substantial release of LDH enzyme indicating necrotic cell death (Figure 1B). These results suggest that *D. scandens* mediates SW480 colon cancer cell death via cell necrotic pathway.

Effect of D. scandens on caspase-3 activity

Caspase-3 is one of executioner caspases which its activity is increased when cell decides to undergo apoptosis (6). The result showed that *D. scandens* extract tended to increase caspase-3 activity in a dose-dependent manner, but no significant difference was observed.

Effect of D. scandens on expression of Bcl-2 and Bax

Apoptosis pathway is controlled by Bcl-2 family proteins. Bcl-2 is the member of a large family of proteins that can be divided into two groups: pro- and anti-apoptotic members such as Bax and/or Bak and Bcl-2 and/or Bcl-X_L respectively (7). After treating cells with *D. scandens* at 10 μg/ml, there was a slight down-regulation of Bcl-2, whereas up-regulation of Bax was observed. However, there was no marked difference in the expression of these two proteins compared to control cells.

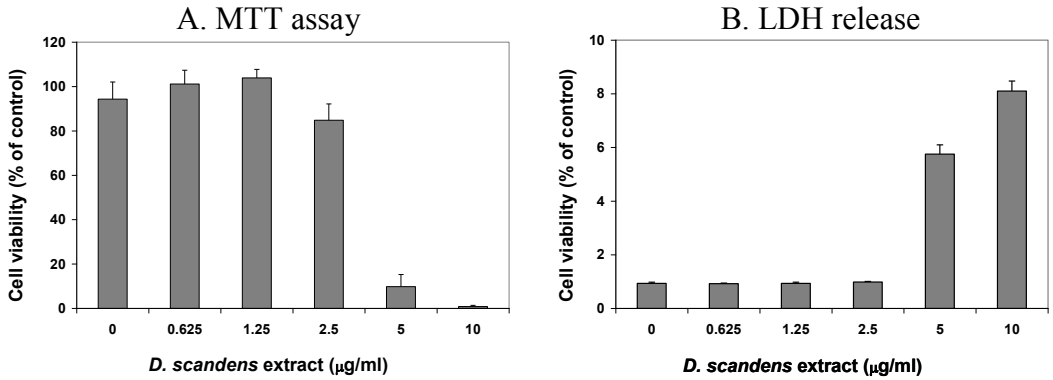

Figure 1 Effect of *D. scandens* extract on cell viability of SW480 colon cancer cells. SW480 cells were treated with various concentrations of the extract for 24 h, cell lysates were prepared for the MTT assay (A) and cultured medium were collected to measure LDH activity (B).

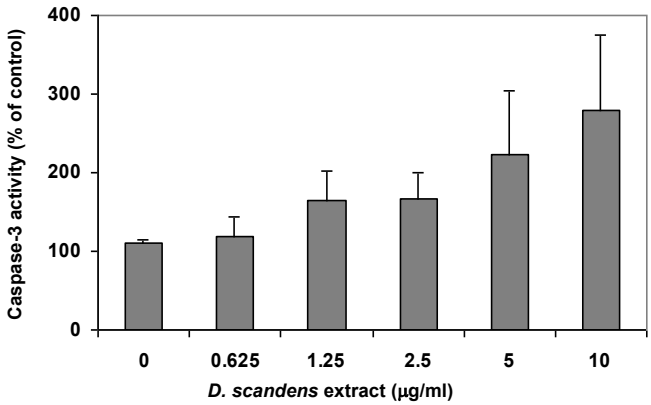

Figure 2 Effect of *D. scandens* extract on caspase-3 activity of SW480 colon cancer cells. Cell lysates were prepared for measuring caspase-3 activity. The data represent mean ± SE from 5 experiments.

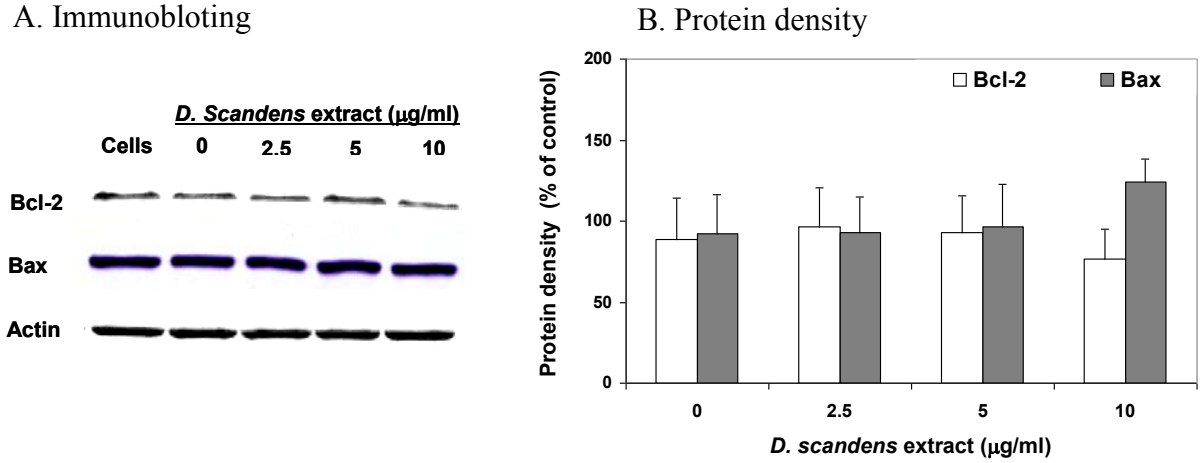

Figure 3 Effect of *D. scandens* extract on the expression of Bcl-2 and Bax in SW480 colon cancer cells. A; immunoblotting and B; the averaged density of protein bands from at least three experiments.

Discussions and Conclusion

From our previous study, the extract from *D. scandens* showed an effective anti-proliferative activity against SW480 colon cancer cells with IC_{50} 4.86 µg/ml (unpublished results). This anti-proliferative effect may be due to certain compounds found in *D. scandens* such as coumarins, isoflavones and isoflavone glycosides which previously showed to have the anti-migration of cancer cells (8). In the present study, we demonstrated that this extract slightly increased caspase-3 activity, up-regulation of Bax pro-apoptotic protein and down-regulation of Bcl-2 anti-apoptotic protein. *D. scandens* leads to substantially release of LDH from SW480 colon cancer cells. Taken all data together, cell necrosis is the major pathway of *D. scandens*-induced cell death. Our finding suggests that the extract of *D. scandens* decreased colon cancer cell viability by induction of cell necrosis rather than cell apoptosis.

Acknowledgements

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RESEARCH ARTICLE

Acridone Alkaloids from the Root of *Citrus reticulata* Blanco

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Abstract

Chemical investigation of the dichloromethane extract from the root of *Citrus reticulata* Blanco (Rutaceae) resulted in the isolation of four known acridone alkaloids, citracridone-I (1), 5-hydroxynoracronycine (2), citrusinine-I (3) and citbrasine (4). Their structures were elucidated by spectroscopic analyses as well as comparison their spectral data to those reported in the literatures. Their antimicrobial activity was evaluated.

Keywords *Citrus reticulata* Blanco, acridone alkaloids, antimicrobial

Introduction

Citrus reticulate is in the family of Rutaceae. Its fruit peels has been used to help relieve stress and digestive problems. Some of the compounds from the fruit peels were reported to show activity against cancer cells (Du *et al*., 2010). With the aim of searching for the biologically active compounds, we therefore investigated the chemical constituents of this plant and evaluated for their antimicrobial activity.

Methods

Ground-dried root (5.2 kg) of *Citrus reticulata* were immersed twice in dichloromethane at room temperature (each extract time of 3 days). After removal of the solvent, the dark-brown gum (39.87 g) was obtained. The extract (39.87 g) was dissolved in methanol to give soluble (15.90 g) and insoluble (23.92 g) fractions. The insoluble (23.92 g) fraction was subjected to a quick column chromatography over silica gel 60H using gradient solvent systems of acetone-hexane as eluents. On the basis of their TLC characteristics, fractions which contained the same major components were combined to give twenty-two fractions (CD1-CD22). Fraction CD10 yielded a yellow solid of 1 (12.30 mg). Fraction CD7-9 (3.5605 g) was further separated by CC and eluted with 60% dichloromethane in hexane to afford subfractions CD7-9A to CD7-9G. Subfraction CD7-9D (1.36 g) was further separated by CC eluting with 10% acetone in hexane to yield a orange solid of 2 (6.30 mg). Fraction CD13-14 (0.9501g) was further separated by CC and eluted with 60% dichloromethane in hexane to afford subfractions CD13-14A to CD13-14O. Subfraction CD13-14F was a yellow solid of 3 (51.8 mg). Subfraction CD13-14I (0.2666 g) was further separated by CC eluting with 20% acetone in hexane to afford 5 subfractions (CD13-14I1 to CD13-14I5). Subfraction CD13-14I1 yielded a orange solid of 4 (21 mg) .

Compounds 1-4 were screened for antimicrobial activity at a concentration of 200 µg/mL by a broth microdilution method against *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* NCPF3153 and *Cryptococcus neoformans* ATCC90113.

Results and Discussion

Four compounds (1-4) were obtained from the dichloromethane extract of *C. reticulate*.

Compound 1

Compound 1 was obtained as a yellow solid. Its ^{1}H NMR spectral data (Table 1) showed the resonances of a chelated hydroxyl proton 1-OH at δ 14.23, *N*-methyl proton at δ 3.70, methoxyl proton at δ 3.90, an aromatic proton H-2 at δ 6.26 and ortho-aromatic protons H-7 and H-8 at δ 6.99 (*d*, *J*=8.7 Hz) and δ 8.06 (1H, *d*, *J*=8.7 Hz). Proton H-8 was confirmed to be at peri position to carbonyl group by HMBC correlations of H-8 to carbonyl carbon (C-9, δ 181.48). The correlations of H-2 to C-1, C-3, C-4, C-9a supported location of H-2. The presence of dimethyl chromene ring was indicated from the resonances of methyl protons at δ 1.52 (6H) and olefinic protons at δ 6.54 (*d*, *J*=9.9) and δ 5.58 (*d*, *J*=9.9). The HMBC correlations of H-1' to C-3 and C-4a suggested that the chromene ring was at C-3 and C-4. Therefor 1 was assigned as 1,6-dihydroxy-5-methoxy-10,3',3'-trimethylpyrano[2,3-c]acridin-9-one which was known as citracridone-I (Wu et al., 1983).

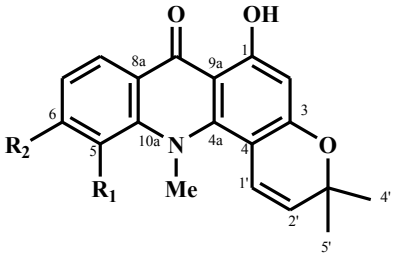
Compound 2

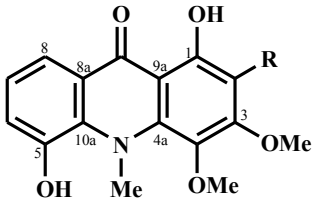
Compound 2 was obtained as an orange solid. Its ^{1}H NMR spectrum (Table 1) indicated that it was acridone possessed of *N*-methyl group (δ 3.81), a chelated hydroxyl proton 1-OH at δ 14.43, hydroxyl proton 5-OH (δ 9.98), dimethyl chromene ring (H-1', δ 6.68; H-2', δ 5.56; the rasonances of a chelated hydroxyl proton 1-OH at δ 14.43, H-4′/5′δ 1.51) and aromatic proton H-2 (δ 6.13) as for 1. The splitting pattern as ABM system of aromatic protons H-7, H-6 and H-8 was shown at δ 7.14 (*t*), δ 7.26 (*d*) and δ 7.75 (*d*), respectively. Proton H-8 was confirmed at peri position to carbonyl group by HMBC correlations of H-8 to carbonyl carbon (δ 186.64). This compound was thus identified as 1,5- dihydroxy-10,3',3'-trimethyl-10,3'-dihydro3H–pyrano[2,3-c]acridin-9-one. It

was identical to 5-hydroxynoracronycine (Wu et al., 1983).

Compound 3

Compound 3 was obtained as a yellow solid. Its ^{1}H NMR spectrum (Table 2) showed the resonances of a hydrogen bonded hydroxyl group (δ 14.24), a hydroxyl group (δ 9.36), an *N*-methyl group (δ 3.84), aromatic proton H-2 (δ 6.36) and trisubstituted aromatic protons (H-6, δ 7.22; H-7, δ 7.11; H-8, δ 7.82) as those of 2. A methoxy resonance at δ 3.79 was assigned for 4-OMe according to the HMBC correlations of H-2 and OMe (δ 3.79) to C-4. Consequently, a methoxy resonance at δ 3.96 was assigned for 3-OMe. On the basis of its spectroscopic data and comparison with the previously reported data, 3 then was identified as 1,5-dihydroxy-3,4-dimethoxy-10-methylacridin-9-one which was identical to citrusinine-I (Wu *et al*., 1983)





1: R_1 = OMe , R_2 = OH **3: R = H**
2: R_2 = OH , R_2 = H **4: R = OMe**

Table 1 ^{1}H, ^{13}C NMR and HMBC spectral data of compound 1 and 2

Position	Compound 1			Compound 2		
	δ_H mult, *J*(Hz)	δ_C	HMBC	δ_H mult, *J*(Hz)	δ_C	HMBC
1	-	164.7	-	-	169.07	-
2	6.26 (*s*)	98.7	C-1, C-3, C-4, C-9a	6.13 (*s*)	102.25	C-1, C-4, C-4a
3	-	161.1	-	-	152.53	-
4	-	102.1	-	-	106.98	-
5	-	135.8	-	-	152.53	-
6	-	154.4	-	7.26 (*d*, 7.8)	124.90	C-5, C-10a, C-8
7	6.99 (*d*, 8.7)	112.0	C-5, C-8a	7.14 (*t*, 7.8)	128.10	C-5, C-8a
8	8.06 (*d*, 8.7)	123.4	C-10a, C-9	7.75 (*d*, 7.8)	120.70	C-10a, C-9
9	-	181.5	-	-	186.64	-
4a	-	147.2	-	-	165.88	-
8a	-	118.5	-	-	129.50	-
9a	-	106.8	-	-	111.67	-
10a	-	141.5	-	-	141.75	-
1′	6.54 (*d*, 9.9)	120.4	C-3, C-4a, C-3′	6.68 (*d*, 9.8)	125.79	C-4a, C-3, C-9a, C-3′
2′	5.58 (*d*, 9.9)	124.7	C-4, C-3′, C-4′/5′	5.56 (*d*, 9.8)	128.45	C-4, C-3′
3′	-	77.2	-	-	81.37	-
4′/5′	1.52 (*s*)	27.2	C-3′, C-2′	1.51 (*s*)	31.85	C-2′, C-3′, C-5′
1-OH	14.23 (*s*)	-	C-1, C-2, C-9a	14.43 (*s*)	-	-
5-OH	-	-	-	9.98 (*s*)	-	-
5-OMe	3.90 (*s*)	60.0	-	-	-	-
10-NMe	3.70 (*s*)	47.9	C-4a, C-10a	3.81 (*s*)	53.39	C-5, C-10a

Taken in $CDCl_3$ (compound 1) and in $CDCl_3$+DMSO (compound 2).

Compound 4

Compound 4 was obtained as an orange solid. Its ^{1}H and ^{13}C NMR spectrum (Table 2) were similar to those of 3. The difference was the replacement of signal of aromatic proton H-2 by that of methoxyl group at δ 3.80. Therefore, 4 was assigned to be 1,5-dihydroxy-2,3,4-trimethoxy-10-methyl-10H-acridin-9-one which was known as citbrasine (Wu *et al*., 1983).

Compounds 1-4 were evaluated for the antimicrobial activity against *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *C.albicans* NCPF3153 and *C. neoformans* ATCC90113. The result indicated that they have no activity at MIC 200 μg/mL.

Table 2 ^{1}H, ^{13}C NMR and HMBC spectral data of compound 3 and 4

Position	Compound 3			Compound 4		
	δ_H mult, *J*(Hz)	δ_C	HMBC	δ_H mult, *J*(Hz)	δ_C	HMBC
1	-	160.17	-	-	151.2	-
2	6.36 (*s*)	93.32	C-1, C-4, C-9a	-	134.2	-
3	-	159.38	-	-	154.5	-
4	-	125	-	-	133.9	-
5	-	147.95	-	-	147.2	-
6	7.24 (*dd*, J=1.5, 7.8)	119.93	C-8, C-10a	7.24 (*d*, J=6)	120.32	C-8, C-10a
7	7.11 (*t*, J=7.8)	122.45	C-5, C-8a	7.12 (*t*, J=6)	122.86	C-5, C-8a
8	7.82 (*dd*, J=1.5, 7.8)	116.25	C-6, C-9, C-10a	7.89 (*d*, J=6)	117.46	C-6, C-9, C-10a
9	-	182.34	-	-	183	-
4a	-	137	-	-	110	-
8a	-	124.36	-	-	124.2	-
9a	-	106.17	-	-	108	-
10a	-	133	-	-	137.8	-
1-OH	14.24 (*s*)	-	C-1, C-2, C-9a	14.1 (*s*)	-	C-1, C-2, C-9a
5-OH	9.36 (*s*)	-	C-5, C-10a	-	-	-
2-OMe	-	-	-	3.80 (*s*)	60.87	C-2
3-OMe	3.96 (*s*)	56.01	C-3	4.16 (*s*)	61.79	C-3
4-OMe	3.79 (*s*)	60.16	C-4	3.97 (*s*)	60.20	C-4
10-NMe	3.84 (*s*)	46.03	C-4a, C-10a	3.88 (*s*)	46.45	C-4a, C-10a

Taken in $CDCl_3$ (compound**1** and **2**).

Conclusion

Four known acridone alkaloids, citracridone-I (1), 5-hydroxynoracronycine (2), citrusinine-I (3) and citbrasine (4) were isolated from the root of this plant. All of them showed no antimicrobial activity.

Acknowledgements

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Instruction for Authors
Thai Journal of Pharmacology

วารสารไทยเภสัชวิทยา (Thai Journal of Pharmacology) เป็นวารสารทางวิชาการระดับชาติ ที่มี impact factor และอยู่ในฐานข้อมูลของศูนย์ดัชนีการอ้างอิงวารสารไทย (Thai citation index) กองบรรณาธิการวารสารยินดีรับผลงานทางวิชาการในสาขาเภสัชวิทยาและสาขาอื่นที่เกี่ยวข้อง ได้แก่ งานวิจัย (Research article), Short communication, รายงานผู้ป่วย (Case report), จดหมายถึงบรรณาธิการ (Letter to editor), บทความปริทรรศน์ (Reviews), และ New drugs profile เพื่อตีพิมพ์และเผยแพร่ในวารสาร โดยผลงานวิชาการที่ส่งมา ต้องไม่ได้รับการเผยแพร่ที่ใดมาก่อนและผู้นิพนธ์จะต้องไม่ส่งบทความเพื่อไปตีพิมพ์ในวารสารฉบับอื่นในเวลาเดียวกัน เพื่อความสะดวกในการพิจารณาจึงขอแนะแนวทางการเตรียมต้นฉบับและการส่งต้นฉบับดังนี้

คำแนะนำสำหรับการเขียนต้นฉบับ

1. การส่งต้นฉบับ ต้องส่งเป็นไฟล์ Microsoft word เวอร์ชั่น 97-2003 และ ไฟล์ PDF อย่างละ 1 ชุด
2. การเขียนต้นฉบับ (Manuscript) สามารถเขียนเป็นภาษาอังกฤษหรือภาษาไทยก็ได้ แต่บทคัดย่อ (Abstract) ต้องมีทั้งภาษาอังกฤษและภาษาไทย
3. การพิมพ์ต้นฉบับ (Manuscript) ภาษาอังกฤษใช้ตัวอักษร Times New Roman ขนาด 10 ภาษาไทยใช้ตัวอักษร EucrosiaUPC ขนาด 16 เอกสารอ้างอิงไม่เกิน 45 เรื่อง
4. รูปภาพและตารางประกอบ (Figures and tables) ควรเป็นภาพที่ชัดเจน รูปภาพทุกรูปต้องมีหมายเลขและเขียนเรียงตามลำดับ พร้อมกับมีคำบรรยายใต้ภาพ โดยใช้ชื่อรูปภาพเป็น “Figure 1” ส่วนการเขียนหมายเลขตารางใช้เลขอารบิกและให้เรียงตามลำดับที่ของตารางอย่างต่อเนื่องจาก 1 ,2 ,3 , ...
5. ชื่อเรื่อง ควรสั้นแต่ได้ใจความ ใช้อักษรใหญ่ capital letter ในตัวหน้าทุกคำ เน้นประโยคด้วยตัวหนา (Bold) ตัวอย่างเช่น

   The Study of *UGT1A1* Polymorphism in Neonate at Songkhla Hospital

งานวิจัย (Research article)

1. **หน้าชื่อเรื่อง (Title page)** ควรมีชื่อเรื่องและชื่อผู้นิพนธ์ โดยชื่อผู้นิพนธ์เป็นภาษาอังกฤษ ใช้ตัวเต็ม ชื่อ สกุล โดยไม่มีคำนำหน้าหรือต่อท้าย ให้ใส่ตัวเลขยกกำลังท้ายนามสกุล เพื่อระบุถึงสถานที่ทำงาน ตัวอย่างเช่น

   Peerapon Sornying[1], Sopen Chunuan[2], Wandee Udomuksorn[1]

   [1]*Medical Science Program, Faculty of Medicine, Chulalongkorn University, Thailand*

   [2]*Laboratory of Chemistry, Chulabhorn Research Institute, Thailand.*

   [3]*Department of Anatomy, Faculty of Medicine, Chulalongkorn University.*

   ส่วนด้านล่างของหน้าให้ระบุผู้รับผิดชอบบทความ (correspondence) พร้อม E-mail และสถานที่ทำงาน
2. **บทคัดย่อ (Abstract)** ต้องระบุถึงความสำคัญของเรื่อง วัตถุประสงค์ วิธีการศึกษา ผลการศึกษา และบทสรุป ความยาวไม่เกิน 250 คำ เป็น single paragraph และระบุคำสำคัญของเรื่อง (Keywords) จำนวนไม่เกิน 5 คำ
3. **บทนำ (Introduction)** ควรระบุความสำคัญของที่มาและปัญหาของงานวิจัย ภูมิหลังของงานวิจัยที่เกี่ยวข้องกับสมมติฐานที่นำไปสู่เหตุผลของการวิจัยและวัตถุประสงค์ของการวิจัย
4. **วิธีการ (Methods)** อธิบายรายละเอียดของวิธีการศึกษาและแบบจำลองการศึกษาที่ชัดเจนและสมบูรณ์ ถ้าวิธีการศึกษามีผู้เผยแพร่มาก่อนควรมีการอ้างอิง มีการระบุถึงรายละเอียดของโปรแกรมสถิติวิเคราะห์และค่าความแตกต่างอย่างมีนัยสำคัญทางสถิติที่ใช้
5. **ผลการทดลอง (Results)** บรรยายผลการศึกษาวิจัย พร้อมเสนอข้อมูลในรูปแบบ ตารางหรือภาพประกอบ
6. **วิจารณ์ (Discussion)** ควรเชื่อมโยงกับผลการศึกษาว่าสอดคล้องกับสมมุติฐาน หรือแตกต่างไปจากผลงานวิจัยที่ผู้รายงานไว้ก่อนหรือไม่อย่างไรและด้วยเหตุผลใด โดยมีพื้นฐานการอ้างอิงที่เชื่อถือได้

7. **สรุป (Conclusion)** ควรสรุปผลที่ได้รับจากการศึกษาวิจัยว่าเป็นไปตามวัตถุประสงค์หรือไม่ พร้อมให้ข้อเสนอแนะหรือระบุอุปสรรคและแผนงานวิจัยที่จะดำเนินการต่อไป
8. **กิตติกรรมประกาศ (Acknowledgement)** เป็นการแสดงความขอบคุณแก่แหล่งทุนและผู้ที่ช่วยในงานวิจัย
9. **การอ้างอิง (References)** มีรูปแบบดังนี้

1) การอ้างอิงในส่วนเนื้อหา ตาราง และรูปภาพต่างๆ ต้องระบุ แบบนาม-ปี (author-date in-text citation) โดยชื่อผู้วิจัยและปีที่พิมพ์ของเอกสาร ไว้ข้างหลังข้อความที่ต้องการอ้าง ถ้ากรณีมีผู้วิจัยตั้งแต่ 1-2 คนให้ระบุชื่อผู้วิจัยทั้งหมด แต่ถ้ามีผู้วิจัยมากกว่า 3 คน ให้ระบุเฉพาะผู้วิจัยคนแรกและตามด้วยคณะผู้วิจัย

ตัวอย่าง Morgan 2001 , Jones and Miller 2008, Hatano et al. 2009

2) ไม่ควรใช้บทคัดย่อ (Abstracts) เป็นเอกสารอ้างอิง โดยถ้าเป็นเอกสาร (papers) ที่ได้รับการยอมรับแล้ว แต่รอการตีพิมพ์ ควรระบุด้วยคำว่า "in press" หรือ "forthcoming" ส่วนเอกสารต้นฉบับ (manuscripts) ที่ไม่ได้ตีพิมพ์แม้บรรณาธิการจะรับไว้พิจารณาก็ตาม ก็ควรเขียนระบุด้วยคำว่า "unpublished observations"

3) การเขียนเอกสารอ้างอิงท้ายบทความ การอ้างอิงวารสารท้ายบทความให้ใช้แบบ The Vancouver style

3.1) ผู้เขียนเป็นบุคคล (Author(s))

Format: Author (s). Article Title. Journal Title. Date of Publication;Volume(Issue):Pagination.

*หมายเหตุ การเขียนชื่อวารสารให้ใช้ชื่อย่อตามมาตรฐานสากลที่อยู่ใน Pubmed

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3.5) หนังสือ

3.5.1) ผู้เขียนหนังสือ (Personal author(s))

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บทความปริทรรศน์ (Reviews)

บทความปริทรรศน์ (Reviews) ใช้รูปแบบการเขียนเหมือนนิพนธ์ต้นฉบับ

Short communication

Short communication ควรเป็นองค์ความรู้ใหม่ ความยาวไม่เกิน 2 หน้ากระดาษ A4 อาจจะมีรูปภาพและตารางรวมกันไม่เกิน 2 รูป

การส่งต้นฉบับ (Manuscript submission)

การส่งผลงานทางวิชาการให้ส่งถึงบรรณาธิการวารสารสมาคมเภสัชวิทยาแห่งประเทศไทย (Thai Journal of Pharmacology) ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ 114 ถนนสุขุมวิท 23 แขวงคลองเตยเหนือ เขตวัฒนา กรุงเทพฯ 10110 ประเทศไทย หรือ E-mail: laddawal@gmail.com ผลงานทางวิชาการทุกฉบับจะได้รับการประเมินโดยผู้ทรงคุณวุฒิ (peer-review journal) เพื่อประเมินคุณภาพความเหมาะสมก่อนการตีพิมพ์ ในกรณีที่ผลการประเมินระบุให้ต้องปรับปรุงหรือแก้ไข ผู้นิพนธ์จะต้องดำเนินการให้แล้วเสร็จภายในระยะเวลาที่กำหนดนับจากวันที่ได้รับผลการประเมินบทความ

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