นิพนธ์ต้นฉบับ • **Original Article**

ฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ของสารสกัดจากต้นรงทองต่อเซลล์มะเร็งท่อน้ำดี

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Growth Inhibitory Activity of *Garcinia Hanburyi* Extracts on Cholangiocarcinoma Cell Lines.

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หลักการและเหตุผล: มะเร็งท่อน้ำดีมีอุบัติการณ์สูงที่สุดในภาคตะวันออกเฉียงเหนือของประเทศไทย การรักษาผู้ป่วยด้วยวิธีต่างๆยังให้ผลที่ไม่ดีนัก แนวทางเลือกอีกทางหนึ่งในการรักษาคือ การค้นหายาตัวใหม่ๆ จากสารสกัดธรรมชาติที่มีฤทธิ์ยับยั้งการเติบโตของเซลล์มะเร็งท่อน้ำดี

วัตถุประสงค์: เพื่อตรวจหาฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ของสารสกัดหยาบและส่วนแยกจากเปลือกต้นและผลของต้นรงทองต่อเซลล์มะเร็งท่อน้ำดีของคน

วิธีการ: การตรวจหาฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์โดยใช้ Sulforhodamine B assay โดยรายงานผลเป็นค่าเฉลี่ยของค่า 50% inhibitory concentration (IC_{50}) จากการทดลอง 3 ครั้ง

ผลการศึกษา: การศึกษาฤทธิ์ในการยับยั้งการเพิ่มจำนวนของเซลล์ของสารสกัดหยาบจากเปลือกต้นและผลของต้นรงทอง 4 ชนิด ต่อเซลล์มะเร็งท่อน้ำดี 5 ชนิด พบว่าสารสกัดเอธิลแอซีเตตจากเปลือกต้น (VR12874) และผล (VR11626) มีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งท่อน้ำดี 5 ชนิด โดยมีค่าความเข้มข้นของสารสกัดที่สามารถยับยั้งเซลล์มะเร็งได้ 50% (IC_{50}) ในช่วง 1.84±0.10 ถึง 2.49±0.03 μg/ml และ 1.69±0.04 ถึง 4.41±0.10 μg/ml ตามลำดับ ในขณะที่สารสกัดเมธานอลจากเปลือกต้น (VR12875) และผล (VR11627) ไม่แสดงฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ จากผลการทดลอง

Background: Cholangiocarcinoma (CCA) is the highest incident of primary liver cancer in the northeastern Thailand. All of the treatment approaches remain poor. One attractive strategy is the discovery of new drugs from medicinal plants which have a potential growth inhibitory activity on cholangiocarcinoma cells.

Objective: To examine the growth inhibitory activity of crude extracts and fractions from barks and fruits of *Garcinia hanburyi* on human cholangiocarcinoma cell lines.

Method: Growth inhibitory activity was assessed by sulforhodamine B assay. The mean IC_{50} values of three independent experiments were reported.

Results: The growth inhibitory activity of four extracts from barks and fruits of *G. hanburyi* on five human CCA cell lines was performed. It was found that the ethyl acetate extracts from barks (VR12874) and fruits (VR11626) of *G. hanburyi* exhibited strong growth inhibitory activity against five CCA cell lines with IC_{50} values range from 1.84±0.10 to 2.49±0.03 μg/ml and 1.69±0.04 to 4.41±0.10 μg/ml, respectively whereas the methanol extracts from barks (VR12875) and fruits (VR11627) showed no growth inhibitory activity on CCA cell lines tested. Subsequent to these results eight fractions from VR12874 were examined for their growth inhibitory activities against

ดังกล่าวได้นำ 8 ส่วนแยกที่ได้จาก VR12874 มาตรวจสอบฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งท่อน้ำดี 5 ชนิด พบว่า 5 ส่วนแยกคือ VR12877, VR12878, VR12881, VR12882 และ VR12883 มีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ในระดับที่สูง ในขณะที่ VR12876 และ VR12879 มีฤทธิ์ในระดับที่ต่ำ และ VR12880 ไม่แสดงฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์

วิจารณ์และสรุป: ผลการศึกษาครั้งนี้สรุปว่า สารสกัดเอธิลแอซีเตตจากเปลือกต้นและผลของต้นรงทองมีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งท่อน้ำดีทั้ง 5 ชนิด ในขณะที่สารสกัดเมธานอลไม่มีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ จากผลที่ได้แสดงว่าสารสกัดเอธิลแอซีเตตน่าจะมีสารสำคัญซึ่งมีฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ ความแตกต่างในการแสดงฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์ในแต่ละส่วนแยกอาจขึ้นกับชนิดหรือปริมาณของสารสำคัญที่อยู่ในแต่ละส่วนแยกนั้น

คำสำคัญ: ต้นรงทอง, ฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์, เซลล์มะเร็งท่อน้ำดี

5 human CCA cell lines. It was found that five fractions including VR12877, VR12878, VR12881, VR12882 and VR12883 showed strong growth inhibitory activity whereas VR12876 and VR12879 exhibited low growth inhibitory activity and VR12880 showed no effect.

Discussion and conclusion: It could be concluded that the ethyl acetate extracts from barks and fruits of *G. hanburyi* showed strong growth inhibitory effect against 5 human CCA cell lines whereas the methanol extracts had no effect. These results indicated that the ethyl acetate extracts might contain active constituents. Variation in the growth inhibitory activities among fractions might depend on the type or concentration of active constituents containing in each fractions.

Key words: *Garcinia hanburyi*, growth inhibitory activity, cholangiocarcinoma cell lines.

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Introduction

Cholangiocarcinoma (CCA) is defined as an adenocarcinoma arising from cholangiocytes or bile duct epithelial lining cells anywhere in the biliary tree, excluding the gallbladder and the ampulla of Vater[1]. It is a rare primary liver cancer in the Western countries and worldwide, but it is a major malignancy in the northeastern Thailand where liver fluke *(Opisthorchis viverrini)* infection is endemic[2]. The CCA is considered to be a multidrug and radioresistant tumor. The results of palliative chemotherapy and /or radiotherapy remain poor. Only in a minority of cases can be cured by surgery and the response rate is not satisfactory. Based on these reason, alternative treatments should be considered. One attractive strategy is the use of medicinal plants which serve as a major source of drug in both primary health care and clinical therapy for centuries.

Garcinia hanburyi Hook. f. (Guttiferae) is a small to medium-sized tree found throughout Thailand. The latex is used as a dye and the folk medicine for potent purgative and infected wounds in Thai traditional medicine[3]. Previous phytochemical investigation on the latex, fruits and whole plant of *G. hanburyi* led to the identification of some cytotoxic caged xanthones.[4-8] Many caged xanthones have been reported to exhibit cytotoxic effects in several mammalian cancer cell lines,[5, 9-13] as well as anticancer and anti-tumor activities.[14,15]

This study was undertaken to examine the growth inhibitory activity of crude extracts from the barks and fruits of *G. hanburyi* on human cholangiocarcinoma cell lines.

Materials and methods

Human cancer cell lines

Five human CCA cell lines used in this study including KKU-100 (poorly-differentiated adenocarcinoma), KKU-M139 (squamous carcinoma), KKU-M156 (moderately-differentiated adenocarcinoma), KKU-M213 (adenosquamous carcinoma) and KKU-M214 (moderately-differentiated adenocarcinoma) were established in the Department of Pathology, Faculty of Medicine, Khon Kaen University. All cell lines were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 100 U/ml penicillin, 100 μg/ml streptomycin. All cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator and were subcultured weekly.

Plant extracts

The ethyl acetate extract (VR12874), methanol extract (VR12875) and eight fractions from ethyl acetate extracts (VR12876-VR12883) from barks and an ethyl acetate extract (VR11626) and methanolic extract (VR11627) from fruits of *G. hanburyi* were kindly provided by Professor Vichai Reutrakul, Department of Chemistry, Faculty of Science, Mahidol University, Thailand. Stock solution of the extracts were made in DMSO and diluted in culture media to the desired final concentration.

***In vitro* growth inhibitory assay**

The sulforhodamine B (SRB) assay was used in this study to estimate cell number indirectly by staining total cellular protein with the SRB. The protocol was based on that originally described by Skehan *et al.* (1990)[20] with slight modification. Briefly, cells at the exponential growth phase were detached with 0.25% trypsin-EDTA (Sigma) to make single-cell suspensions. The viable cells were counted by trypan blue exclusion in a haemocytometer and diluted with medium to give final concentrations about 0.5 to 1x10^5 cells/ml. Each cell suspension was seeded in 96-well microtiter plates in the volume of 190 µl/well and allowed to grow overnight. The cells were then treated with plant extracts by adding 10 µl/well of each concentration in triplicate to obtain final concentration of 0.8, 4 and 20 µg/ml. The cells with same final concentration of DMSO were used as the solvent-control wells. The plates were incubated for 72 h at 37 °C in a 5% CO_2 humidified incubator. At the end of drug treatment, the cells were then fixed with cold trichloroacetic acid (TCA), and washed 5 times with distilled water. The TCA-fixed cells were stained with SRB. The bound dye was solubilized with 10 mM Tris buffer (pH 10). The absorbance (OD) of each well (triplicate for each concentration) was measured by using ELISA plate reader (ELX-800; BIO-TEK INSTRUMENTS, INC.) at 510 nm. The intensity of color developed in each well was corresponded to the cell number. Percentage of cell survival will be calculated by using equation below. IC_{50} value was expressed as concentration of extract in microgram per milliliter that caused a 50% growth inhibition comparing with controls.

$$\% \text{ cell survival} = \frac{OD_{510} \text{ treated cells on day3} - OD_{510} \text{ starting cells} \times 100}{OD_{510} \text{ control on day3} - OD_{510} \text{ starting cells}}$$

Criteria activity: Crude extract having an IC50 <20 µg/ml = active

Crude extract having an IC50 >20 µg/ml = inactive

Statistical analysis

The statistical analysis was carried out by mean± SE.

Results

Effect of *G. hanburyi* extracts on cell growth

Of 4 crude extracts and 8 fractions from barks and fruits of *G. hanburyi*, the growth inhibitory activities were examined against five human CCA cell lines. After 72 h, cell viability was determined with SRB assay and the results are shown in Table1. The treatment of all cell lines with VR12874 markedly decreased cell viability with IC_{50} values ranging from 1.84±0.10 to 2.49±0.03 µg/ml (Table1), whereas the VR12875 showed no growth inhibitory çactivity on all cell lines with IC_{50} values >20 µg/ml. The extract VR11626 also showed strong growth inhibitory activity against all cell lines with IC_{50} values ranging from 1.69±0.04 to 4.41±0.10 µg/ml while the VR11627 showed no growth inhibitory activity on CCA cell lines tested (Table1).

Subsequent to these results 8 fractions from the VR12874 were also examined for their growth inhibitory activities against 5 human CCA cell lines. It was found that VR12877 and VR12881 were the two most effective extracts against these CCA cell lines with IC_{50} values ranging from 1.26±0.30 to 2.33±0.03 µg/ml.The VR12878, VR12882 and VR12883 also showed strong growth inhibitory activity against all cell lines with IC50 values ranging from 0.77±0.01 to 7.91±0.02 µg/ml. The low growth inhibitory activities were observed when VR12876 and VR12879 were tested against these cell lines with IC_{50} values ranging from 15.64±0.06 to 18.25±0.77 µg/ml (Table1).

Discussion and conclusion

The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and are nowadays used to treat human tumors. The ethnopharmacological knowledge is helpful in the searching for plants with potential cytotoxic activity.

Table 1 The IC_{50} values of *G.hanburyi* crude extracts and fractions on 5 human CCA cell lines. The results are expressed as mean±SE from 3 independent experiments.

Codes	Extraction solvents	Types of extraction	Plant parts	IC50 (µg/ml) of cell lines (mean±SE)				
				KKU-100	KKU-M139	KKU-M156	KKU-M213	KKU-M214
12874	EtOAC	Crude	Bark	1.84±0.10	2.26±0.03	2.28±0.13	2.33±0.02	2.49±0.03
12875	MeOH	Crude	Bark	NR	NR	NR	NR	NR
11626	EtOAC	Crude	Fruit	4.41±0.10	2.18±0.02	2.17±0.02	1.69±0.04	2.27±0.10
11627	MeOH	Crude	Fruit	NR	NR	NR	NR	NR
12876	EtOAC	Fraction	Bark	NR	NR	NR	16.57±1.10	18.25±0.77
12877	EtOAC	Fraction	Bark	1.56±0.01	2.16±0.02	2.30±0.11	2.31±0.03	2.31±0.02
12878	EtOAC	Fraction	Bark	5.91±1.70	7.44±0.51	7.27±0.03	3.13±0.04	7.54±0.41
12879	EtOAC	Fraction	Bark	NR	NR	NR	15.64±0.06	NR
12880	EtOAC	Fraction	Bark	NR	NR	NR	NR	NR
12881	EtOAC	Fraction	Bark	1.26±0.30	2.19±0.08	1.91±0.14	1.86±0.06	2.33±0.03
12882	EtOAC	Fraction	Bark	2.42±0.04	6.46±0.01	0.77±0.01	6.27±0.01	7.91±0.02
12883	EtOAC	Fraction	Bark	5.56±0.80	2.47±0.20	3.45±0.35	2.43±0.05	3.45±0.02

NR = None reactive

In the present study we analyzed the *in vitro* effect of *G. hanburyi* extracts on five CCA cell lines. Our results demonstrate that the ethyl acetate extracts from bark (VR12874) and fruit (VR11626) of *G. hanburyi* had marked growth inhibitory effects on the five human CCA cell lines whereas no effect was observed in methanol extract from bark (VR12875) and fruit (VR11627). In addition, seven fractions from VR12874 also showed strong to low growth inhibitory activities against five human CCA cell lines. These results indicated that the ethyl acetate extracts might contain active constituents. Variation in the growth inhibitory activities among fractions might be depending on the type or concentration of active constituents containing in each fraction. According to the previous reports several active components have been isolated from *G. hanburyi* for example desoxymorellin[16], isomorellin[17], isomorellinol[5], gambogic acid[10,18], and forbesione[19]. In addition, some cytotoxic caged xanthones isolated from resin, fruits and whole plant of *G. hanburyi* have been reported[4-8]. Many caged xanthones have been shown to exhibit cytotoxic activities in several mammalian cancer cell lines including human gastric cancer BGC-823, human lung carcinoma SPC-A1, human gastric carcinoma MGC-803 and HeLa cells[9,11,13,15], as well as anticancer and anti-tumor activities[14,15]. In the present study the crude extracts and fractions of *G. hanburyi* were used. The active components in these extracts have already been characterized. We are now in the process of studying the exact mechanism of the growth inhibitory activity and ability to induce apoptosis of these active components on human CCA cell lines. More research on the application of *G. hanburyi* in cancer treatment is warranted.

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