Antiprolifrative Activities of Three Thai Medicinal Plants on Human Cancer Cells

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ABSTRACT

In Thailand, the stems and leaves of *Pouzolzia pentandra* Benn., stems of *Erycibe elliptilimba* Merr.& Chun., and *Gelonium multiflorum* A. Juss. have long been used as traditional medicines for cancer therapy. The ethanol, water, methanol:water and ethyl acetate extracts of these three plants were tested in vitro against four different human breast cancer cell lines and two human lung cancer cell lines using MTT screening method. The methanol extract of *Erycibe elliptilimba* Merr. & Chun. showed the most potent antiproliferative activities against these specific human cancer cells. The results, therefore, suggested to some extent the benefit of the traditional use of *Erycibe elliptilimba* Merr.& Chun. in the treatment of malignancies.

Keywords: Antiproliferative activities; *Pouzolzia pentandra* Benn.; *Erycibe elliptilimba* Merr. & Chun.; *Gelonium multiflorum* A. Juss.

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Breast cancer is one of the most prevalent malignancies in women in many developed and developing countries. It is a complex disease that occurs in heterogeneous population. In Thailand, breast cancer is the second in frequency after cervical cancer, with an estimated incidence rate of 17.2 per 100,000 which has been increasing in all parts of the country during the past decade.[1] Although the breast cancer therapy which is usually a multimodality treatment is advanced, cytotoxic drugs still play important roles in increasing the survival rate together with good quality of life.[2,3] However, as the treatment of breast cancer has become sophisticated and many cytotoxic drugs have been introduced, the acquired resistance of cancer has been increasing. Therefore, the development and search for novel and effective anticancer agents have become very important issues.[4] To date, many cytotoxic agents including natural products isolated from plant sources have been investigated for the discovery of novel potential anticancer drugs.

Thailand has many natural medicinal resources especially from higher plants which have been used to treat various diseases for many decades. Among these potential Thai plants that have been widely prescribed for cancer therapy, three plants named *Pouzolzia pentandra* Benn., *Erycibe elliptilimba* Merr. & Chun., and *Gelonium multiflorum* A. Juss have been well documented in Thai traditional medicine.

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Pouzolzia pentandra Benn., *Erycibe elliptilimba* Merr. & Chun., and *Gelonium multiflorum* A. Juss. are Thai traditional plants belonging to the Urticaceae, Convolvulaceae and Euphobiaceae families, respectively. They grow naturally in tropical and subtropical East Asia.[5] Their stems and leaves have been used in treatment of various diseases including malignancies.[6] Since the plants have been taken for granted for their uses in Thai medicinal therapies for cancers, there is no scientific reference to confirm their pharmacological activities. This prompted us to determine the selectivity of their antiproliferative activities against four human breast cancer cell lines: SKBR3, MCF7, T47D and MDA-MB435 including two human lung cancer cell lines: A549 and SK-LU1.

MATERIALS AND METHODS

Plant material and extraction procedures

Dried stems and leaves of *Pouzolzia pentandra* Benn. and dried stems of *Erycibe elliptilimba* Merr. & Chun. and *Gelonium multiflorum* A. Juss. were purchased from a Thai Herbal Pharmacy (Chao-Krom-Po). They were cut into small pieces and ground into powder. The powder of each extract was macerated thrice with 4 liters of 95% ethanol for seven days. The extracts were then concentrated under reduced pressure, resulting in crude extract.

Diaion® HP-20 chromatography

The ethanol extract was chromatographed on an Diaion® HP-20 column (Misubishi Chemical Corp., Japan), an ion-exchange resins open column, which was dry packed using a glass column (inner diameter 4 cm,

TABLE 1. Antiproliferative activities of 3 Thai medicinal plants (15 extracts) against 4 human breast cancer cell lines and 2 human lung cancer cell lines. Cell proliferation was determined by MTT assay. Results are reported as ED50 value (μg/ml.) which means the 50% inhibition of cell growth. The data shown are from three independent experiments, each with triplicate wells.

Plant name	Part used	Local name	Fraction	Cell lines					
				SKBR3	MCF7	T47D	MDA-MB 435	A549	LU1
Pouzolzia	stems	ขอบชะ	crude	> 500	> 500	> 500	> 500	> 500	> 500
pentandra	leaves	นาง	1	> 500	> 500	> 500	> 500	> 500	> 500
			2	> 500	> 500	> 500	> 500	> 500	> 500
			3	320	200	250	250	> 500	280
			4	> 500	> 500	350	250	> 500	> 500
Erycibe	stems	พระขรร	crude	450	> 500	450	350	> 500	370
elliptilimba		ไชยศรี	1	> 500	> 500	> 500	> 500	> 500	> 500
			2	> 500	> 500	> 500	> 500	> 500	> 500
			3	56	26	20	30	70	35
			4	> 500	> 500	> 500	> 500	> 500	> 500
Gelonium	stems	ขันทอง	crude	> 500	> 500	> 500	> 500	> 500	> 500
multiflorum		พยาบาท	1	> 500	> 500	> 500	> 500	> 500	> 500
			2	> 500	> 500	> 500	> 500	> 500	> 500
			3	> 500	> 500	> 500	> 500	> 500	> 500
			4	> 500	> 500	> 500	380	> 500	> 500

Note: Crude = ethanol extract, Fraction 1 = water extract, Fraction 2 = water:methanol extract, Fraction 3 = methanol extract, Fraction 4 = ethyl acetate extract

60 cm long) and equilibrated with water (100 ml). The ethanol extract was dissolved in distilled water (100 ml) and sonicated for 15 minutes in an ultrasonic bath to get the water soluble fraction. The suspension was centrifuged at 20,000 rpm (about 42,000 g) for 30 minutes. The supernatant was applied onto the Diaion® HP20 column and the eluent was collected (fraction 1). The precipitate was dissolved in methanol:water (1:1/100) and methanol (150 ml.) to get the fraction 2 and fraction 3, respectively. Each supernatant was repeatedly performed as described above. The precipitate was dissolved in ethyl acetate (fraction 4). The chemical composition of each fraction was monitored on thin layer chromatography.

Preparation of the plants extracts for cell proliferation assay

All plants extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma, St.Louis, USA) except for the water fraction (fraction 1) which was dissolved in water. In all experiments, final concentrations of the tested compound were prepared by diluting the stock with the culture medium.

Cell lines and culture

Human breast cancer cell lines, SKBR3, MCF7, T47D and MDA-MB435, were kindly provided by Dr. Pornchai O-charoenrat (Mahidol University, Bangkok). SKBR3 is human breast cancer cell line with over-expression of HER2/neu receptor, absence of ER receptor. T47D and MCF7 are breast cancer cell lines with the ER receptor positive and HER2/neu negative whereas MDA-MB435 breast cancer cell line is absent ER receptor and also HER2/neu expression.[7] Human lung cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). A549 is cisplatin-resistant adenocarcinoma lung cancer cell, whereas SK-LU1 is adenocarcinoma cell type of lung cancer.[8] Cells except SK-LU1 were cultured in DMEM (PromoCell, Germany) supplemented with 10% heat-inactivated fetal bovine serum (PromoCell, Germany) and 1% Penicillin-streptomycin (PromoCell, Germany). SK-LU1 cells were cultured in MEM in Earle's BSS (PromoCell, Germany) with non-essential amino acid (PromoCell, Germany) and 1mM Sodium pyruvate (US Biological, USA) supplemented with 10% heat-inactivated fetal bovine serum and also antibiotics. The cells were incubated at 37°C in a humidified atmosphere with 5%CO_2.

Cell proliferation assay

A total of 1x 104 cells/well were seeded in a 96-well plate. After 24 hours of incubation at 37°C with 5% CO_2 in a humidified atmosphere, various concentrations of herb extracts were added to the wells to get the final concentration of 1, 10, 100, and 500 μg/ml. Control groups were added with DMSO to get the final concentration of 1% and vinblastine 30 μg/ml and Taxol 10 μg/ml were used as positive controls since these chemotherapeutic agents are derived from the plants. Then cells were incubated for an additional 48 hours. After two days, 50 μl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) 1 mg/ml in PBS was added to each well and incubated for 4 hours at 37°C. Cell proliferation assay was measured at 590 nm using a Bi0-assay reader.[9] The growth inhibition was determined by: Growth inhibition (%) = (control OD - sample OD)/ control OD x 100

Statistical analysis

All experiments were performed in triplication with at least three independent experiments. The data were expressed as means ± standard deviation. The statistical difference was analyzed using the student's t-test. Values of $p < 0.05$ were considered significant.

RESULTS

Yield of extracts from plant materials

The powder of *Pouzolzia pentandra* Benn., *Erycibe elliptilimba* Merr. & Chun., and *Gelonium multiflorum* A. Juss. Approximately 1.8 kg of each was extracted

Pouzolzia pentandra **Benn.**

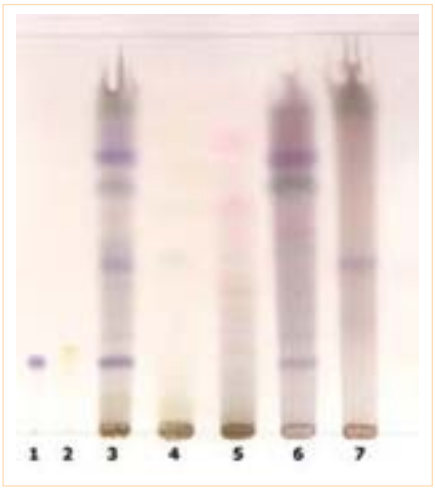

Fig 1. Thin-layer chromatogram of *Pouzolzia pentandra* ethanol (crude) extract and fractions from Diaion® HP-20.

Adsorbent	:	Silica gel GF254 Alufolien, Merck, Germany		
Solvent system	:	Chloroform : Methanol (90 : 10)		
Detection	:	Spray with anisaldehyde-sulfuric acid, heat at 100°C, 1-2 min. in oven.		
Reference	:	1 = 1% Gla (Phytosteryl glucoside) in ethanol	3	µl
		2 = 1% quercetin in ethanol	3	µl
Sample*	:	3 = Ethanol extract	30	µl
		4 = Water fraction	30	µl
		5 = Water/methanol fraction	30	µl
		6 = Methanol fraction	30	µl
		7 = Ethyl acetate fraction	30	µl

*sample concentration = 140 mg/ml

Erycibe elliptilimba **Merr. & Chun.**

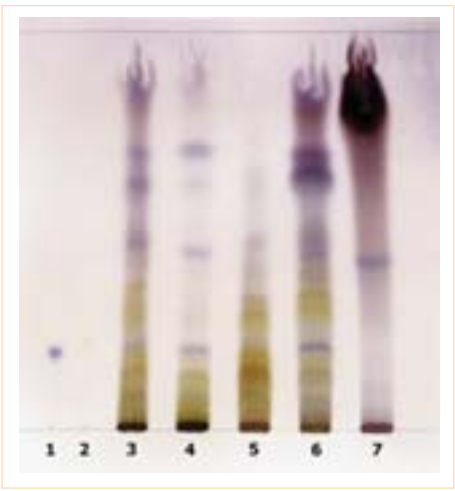

Fig 2. Thin-layer chromatogram of *Erycibe elliptilimba* ethanol (crude) extract and fractions from Diaion® HP-20.

Adsorbent	:	Silica gel GF254 Alufolien, Merck, Germany		
Solvent system	:	Chloroform : Methanol (90 : 10)		
Detection	:	Spray with anisaldehyde-sulfuric acid, heat at 100°C, 1-2 min in oven.		
Reference	:	1 = 1% Gla (Phytosteryl glucoside) in ethanol	3	µl
		2 = 1% quercetin in ethanol	3	µl
Sample*	:	3 = Ethanol extract	30	µl
		4 = Water fraction	30	µl
		5 = Water/methanol fraction	30	µl
		6 = Methanol fraction	30	µl
		7 = Ethyl acetate fraction	30	µl

*sample concentration = 140 mg/ml

successively as described in the extraction procedure. Yield of *Pouzolzia pentandra* Benn. fraction 1,2,3, and 4 from 35 gm of crude extract was 11.15 gm, 3.14 gm, 10.37 gm, and 2.4 gm, respectively. For *Erycibe elliptilimba* Merr. & Chun., 70 gm of crude extract resulted in 8.59 gm, 6.15 gm, 3.9 gm, and 0.3 gm of fractions 1,2,3, and 4, whereas the 35 gm of crude extract from *Gelonium multiflorum* A. Juss. yielded 6.8 gm, 3.5 gm, 8.6 gm, and 0.6 gm of fractions 1,2,3, and 4, respectively. The thin layer chromatography of the herb extracts from the plants was demonstrated in Fig 1. through Fig 3.

Growth inhibition of the plant extracts against human tumor cell lines

The results of 15 kinds of plant extracts against human cancer cell lines are shown in Table 1. Five of the extracts showed antiproliferative activities in a concentration-dependent manner with ED50 values less than 500 µg/ml: methanol extract (fraction 3) of *Pouzolzia pentandra* Benn. and *Erycibe elliptilimba* Merr. & Chun., crude extract of *Erycibe elliptilimba* Merr. & Chun. and ethyl acetate extracts (fraction 4) of *Pouzolzia pentandra* Benn., and *Gelonium multiflorum* A. Juss. These five kinds of extracts had varying degrees of antiproliferative activities with the methanol extract of *Erycibe elliptilimba* Merr. & Chun. showed strong antiproliferative activities against SKBR3, MCF7, T47D and MDA-MB435 with ED50 values at 56.07, 26.65, 20.06 and 30.61 µg/ml, respectively. Regarding the human lung cancer cell lines, the methanol extract of *Erycibe elliptilimba* Merr. & Chun. also showed potent antiproliferative activity with the ED50 values at 70.44 and 35 µg/ml. for A549 and SK-LU1, respectively; whereas the other extracts showed weak activities against specific human tumor cell lines with ED50 values ranging from over 200 µg/ml. In Fig 4, the proliferative inhibitory effects of *Erycibe elliptilimba* methanol extract was observed to be in a dose-dependent manner.

DISCUSSION

Thai traditional plants have long been used for many decades as anti-cancer agents. However, many of these herbal plants which might be the very useful sources in tumor therapy have not yet been demonstrated of these unique properties. Stems and leaves from *Pouzolzia pentandra* Benn., stems from *Erycibe elliptilimba* Merr. & Chun., and stems from *Gelonium multiflorum* A. Juss. are the parts of these special herbal plants which have been used as certain ingredients in Thai anti-cancer remedies for a long time. Lee-Huang S, et al. has reported the inhibitory effects of the purified compound from seeds of *Gelonium multiflorum* called GAP31 on human breast tumor cells, MDA-MB231.[10] Interestingly, in our findings,

Gelonium multiflorum **A. Juss.**

Fig 3. Thin-layer chromatogram of *Gelonium multiflorum* ethanol (crude) extract and fractions from Diaion® HP-20.

Adsorbent	:	Silica gel GF254 Alufolien, Merck, Germany		
Solvent system	:	Chloroform : Methanol (90 : 10)		
Detection	:	Spray with anisaldehyde-sulfuric acid, heat at oven 100°C, 1-2 min in oven		
Reference	:	1 = 1% Gla (Phytosteryl glucoside) in ethanol	3	µl
		2 = 1% quercetin in ethanol	3	µl
Sample*	:	3 = Ethanol extract	30	µl
		4 = Water fraction	30	µl
		5 = Water/methanol fraction	30	µl
		6 = Methanol fraction	30	µl
		7 = Ethyl acetate fraction	30	µl

*sample concentration = 140 mg/ml

the stems of *Gelonium multiflorum* A. Juss. which have been used traditionally as anticancer medicine exhibited very low activities against specific human tumor cell lines in contrast to the antiproliferative activities of the extract from the seeds itself.[11,12] Other two plants have never been demonstrated for these special anticancer activities, although they are widely used. Based on our findings, five of the fifteen herbal extracts have antiproliferative effects in these specific human cancer cell lines with the greatest activities from *Erycibe elliptilimba* methanol extract. As demonstrated in Fig 4, the antiproliferative effects of this plant seemed to inhibit growth against all tumor cells in a dose-dependent manner. Hence, the results suggested that an effective ingredient against specific human cancer cells should be in the methanol fraction of *Erycibe elliptilimba* Merr. & Chun. which has never been investigated.

Erycibe elliptilimba Merr. & Chun. is called in Thai language "phra khan chai sri" (พระขรรไชยศรี).[6] Its leaf blades are broad circular in shape. Its shrubs are covered with rough brown bark and dotted with scales when cut through.[13] In the course of our next research, this plant which grows in the tropical and subtropical forest will be collected and examined in fresh preparation for further identification.

In summary, from the three examined plants, the emethanol extract of *Erycibe elliptilimba* Merr. & Chun. suppressed the proliferation of human cancer cells which displayed in a dose-dependent manner. It is interesting to

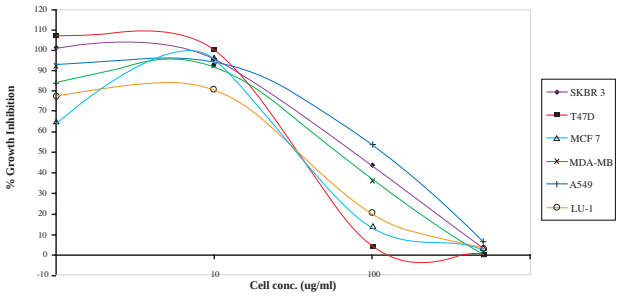

Fig 4. The antiproliferative effect of *Erycibe elliptilimba* methanol extract in 4 human breast cancer cells and 2 human lung cancer cells. Adherent cells that plated in 96-well plates (1x104 cells /well) were incubated with different concentrations of the herb extract at 48 hours. Cell proliferation was determined by MTT assay.

find that only this specific extract is effective against highly metastatic and resistant cell lines. This reveals the therapeutic potentiality of herb extract against specific human tumor cells. Further investigation is carried on in our laboratory to determine in more details the active compounds from the stem of this plant that has the inhibitory effects including the molecular mechanisms involving these activities before applying this herb extract into the treatment of cancers in a clinical setting in future.

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REFERENCES

1. Martin N, Cheirsilpa A. Breast. Chapter 11. Cancer in Thailand. Vol III, 1995-1997, Bangkok, Ministry of Public Health, 2003:47-8.
2. Hebert-Croteau N, Brisson J, Latreille J, Rivard M, Abdelaziz N, Martin G. Compliance with consensus recommendation for systemic therapy is associated with improved survival of women with node-negative breast cancer. J Clin Oncol 2004;22:3685-93.
3. Fargeot P, Bonneterre J, Roche H, Lortholary A, Campone M, Praagh IV, et al. Disease free survival advantage of weekly epirubicin plus tamoxifen versus tamoxifen alone as adjuvant treatment of operable, node-positive, elderly breast cancer patients: 6 - year follow up results of the French adjuvant study group 8 trial. J Clin Oncol 2004;22:4622-30.
4. Cameron D, Bell R. Optimizing treatment for patients with breast cancer. Sem Oncol 2004;31(Suppl):4-5.
5. Bunyaprapat N, Chokchaicharoenporn A. Thai traditional medicinal plants 1-5. 1st ed, Prachachaon printing, Bangkok, 1996-2000.
6. Sintusarn U. Thai traditional medicinal plants of Chao-Krom-Po. 2nd ed. Thammasarn Publishing company, Bangkok, 2002.
7. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249-89.
8. Liang CH, Liu LF, Shiu LY, Huang YS, Chang LC, Kuo KW. Action of solamargine on TNFs and cisplatin-resistant human lung cancer cells. Biochem Biophys Res Commun 2004;322:751-8.
9. Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, et al. Comparison of in vitro anticancer-drug-screening data generated with a Tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Cancer Inst 1990;82:111-6.
10. Lee-Huang S, Huang PL, Sun Y, Chen HC, Kung HF, Huang PL, Murphy WJ. Inhibition of MDA-MB231 human breast tumor xenografts and HER2 expression by Anti-tumor agents GAP31 and MAP30. Anticancer Res 2000;20:653-9.
11. Murakami A, Ohigashi H, Koshimizu K. Possible antitumor promoting properties of traditional Thai food items and some of their active constituents. Asia Pacific J Clin Nutr 1994;3:185-91.
12. Bourinbaiar AS, Lee-Huang S. The activity of plant-derived antiretroviral proteins MAP30 and GAP31 against hepes simplex virus in vitro. Biochem Biophys Res Commun 1996;219:923-9.
13. Pongbungrod S. The properties of Thai medicinal plants (Part 2). 1st ed. Prachettupol temple, Bangkok, 1978:227.

บทคัดย่อ

ฤทธิ์ยับยั้งการเจริญเติบโตของสมุนไพรไทย 3 ชนิดต่อเซลล์มะเร็ง

ธนวรรณ กุมมาลือ พ.บ.*, พรชัย โอเจริญรัตน์ พ.บ., ปร.ด., วีณา จิรัจฉริยากูล ภ.บ., ปร.ด.***, มลฤดี จันทร์ฉาย วท.ม.***, สุรัตน เอี่ยมศรี วท.บ.***

**ภาควิชาพยาธิวิทยาคลินิก, **ภาควิชาศัลยศาสตร์, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กทม. 10700, ***คณะเภสัชศาสตร์, มหาวิทยาลัยมหิดล, กทม.10400, ประเทศไทย.*

ในสูตรตำรับสมุนไพรไทยสำหรับใช้ในการรักษาโรคมะเร็งต่าง ๆ นั้น สมุนไพรไทย 3 ชนิดได้แก่ ต้นและใบของขอบชะนาง ต้นของพระขรรไชยศรีและต้นของขันทองพยาบาท ได้ถูกนำมาใช้เป็นส่วนประกอบหลักในสูตรตำรับ ผู้วิจัยได้นำสิ่งสกัดเอธานอล น้ำ เมธานอล:น้ำ เมธานอลและเอธิลอะซิเตทรวม 5 สารสกัดจากสมุนไพรแต่ละชนิดทั้ง 3 ชนิดมาทำการทดสอบกับเซลล์มะเร็งเต้านม 4 ชนิดและเซลล์มะเร็งปอด 2 ชนิดโดยใช้สาร MTT พบว่าสิ่งสกัดจากเมธานอลของต้นพระขรรไชยศรีให้ผลในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งชนิดต่าง ๆ ได้ดีกว่าสิ่งสกัดจากสมุนไพรอีก 2 ชนิด บ่งชี้ว่าสมุนไพรไทยที่ถูกนำมาใช้ในการรักษาโรคมะเร็งในตำรายาแผนโบราณนั้นมีฤทธิ์ต่อเซลล์มะเร็งบางอย่างจริง