Original Article

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Antiproliferative Activity against Various Cancer Cells and Phytochemical Components of Thai Herbal Formula

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Abstract

Recently, cancer has become a major disease causing death of Thai people. Herbal formulas are complementary choices used for cancer treatment. In this study, a traditional herbal formula, which has been used for lung and liver cancer treatment, was selected from Wang Nam Yen district in Sa Kaeo province. Aqueous extracts of the whole formula and of each component herb were investigated for antiproliferative activity by MTT colorimetric assay against four human cancer cells (KB, MCF-7, HepG2, SiHa) and a non-cancerous cell (Vero). The whole formula extract inhibited growth of hepatocellular carcinoma cell (HepG2) with IC_{50} value of 347.87 ± 55.06 µg/ml but showed no activity on other cancer cells. Furthermore, the aqueous extract of *phaya fai* (*Diospyros undulata* Well. Ex. G. Don. var. *cratericalyx* (Craib) Bakh.) and of *krajai* (*Caesalpinia hymenocarpa* (Prain) Hattink) showed the most potent antiproliferative effect against all cancer cell lines. Phytochemical screenings of aqueous extracts of the whole formula and of each herb were conducted. Flavonoids and lactone compounds were the major compounds found in the whole formula extract. Whereas, tannins and saponins were also found in each component extract, other anticancer activities and chemical compounds should be studied to consider the mechanism.

Key words: Herbal formula, Antiproliferative activity, *Stemona collinsiae*, *Caesalpinia hymenocarpa*, *Diospyros undulate* var. *cratericalyx, Celastrus sp.*

INTRODUCTION

Cancer is a serious health problem, and patients are confronted with undesirable side effects resulting from conventional treatments. Complementary and alternative medicines are optional choices[1]. Some herbal formulas from oriental medicines in China and Korea were found to show anti-cancer activities such as antiangiogenesis and apoptosis[2-4]. In Thailand, many herbal formulas have been used as complementary medicine without any proven evidence of their effectiveness. After interviewing traditional doctors in Sa Kaeo province, an herbal formula used to treat lung and liver cancers was selected. This formula merits investigation for the antiproliferative activity of its aqueous extracts and of each component herb against four human cancer cells (KB, MCF-7, HepG2, SiHa) and a non-cancerous cell (Vero). Phytochemical screening for major groups of compounds and thin layer chromatography were also carried out for qualitative analysis.

MATERIALS AND METHODS

Medicinal plant extraction

The herbal formula is composed of four herbs (Figure 1.) as follow: *non tai yak* whole plant *(Stemona collinsiae* Craib identified by using Census of *Stemona* (Stemonaceae) in Thailand[5]) (Figure 2.), *krajai* stem (*Caesalpinia hymenocarpa* (Prain) Hattink, voucher no. PBM 05105 and PBM

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05108) (Figure 3.), *phaya fai* (*Diospyros undulata* Well. Ex. G. Don. var. *cratericalyx* (Craib) Bakh., voucher no. PBM 05102) (Figure 4.) wood, and *mak taek* (*Celastrus sp.*) (Figure 5.) climbing stem. Following the traditional process, the whole formula and each component herb were extracted by boiling with water for 15 minutes. After filtering, the filtrates were then dried by lyophilization. The dried extracts were examined to determine antiproliferative activity and phytochemical components.

Figure 1. Crude drugs of the selected formula: *non tai yak* (A), *krajai* (B), *phaya fai* (C), and *mak teak* (D).

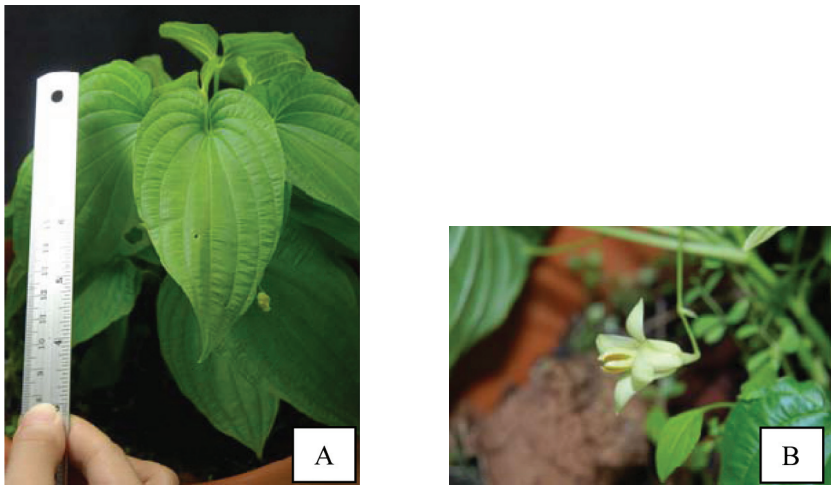

Figure 2. *Stemona collinsiae* whole plant (A) and flower (B).

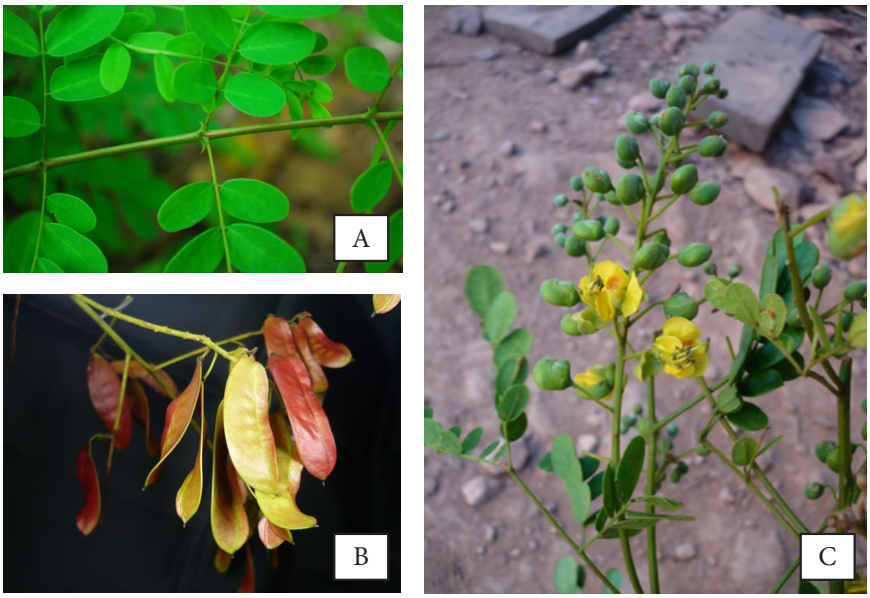

Figure 3. *Caesalpinia hymenocarpa* leaves (A), fruits (B), and flowers (C).

Figure 4. *Diospyros undulata* whole plant (A), fruits (B), and leaves (C).

Figure 5. *Celastrus sp*. stem (A) and leaves (B).

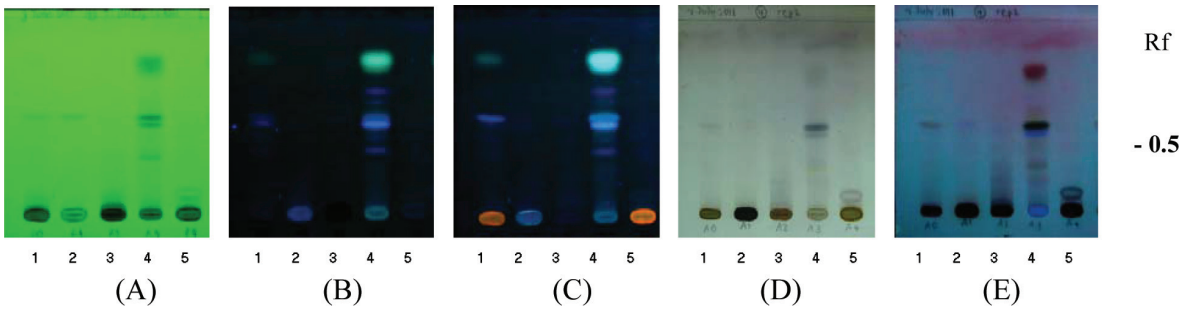

Figure 6. The TLC chromatograms of the herbal formula extracts: 1 = whole formula extract, 2 = *Stemona collinsiae* extract, 3 = *Caesalpinia hymenocarpa* extract, 4 = *Diospyros undulata* extract, and 5 = *Celastrus sp.* extract; stationary phase: silica gel GF254; mobile phase: toluene: chloroform: ethanol = 4: 4: 1; observed under ultra violet light 254 nm (A), 366 nm (B), 366 nm with NP/PEG spray reagent(C), visible light with anisaldehyde-sulphuric acid spray reagent (D), and 366 nm with anisaldehyde-sulphuric acid spray reagent (E).

Antiproliferative activity

Antiproliferative activity was measured by MTT colorimetric assay as described by Siripong *et. al*[6]. This assay was performed in 96-well plate at a cell density of 3 x 10^3 cells/well. KB, MCF-7, HepG2 cancer cells and Vero cells were precultured in Minimum Essential Medium (MEM) containting 10% Fetal Bovine Serum (FBS). SiHa cells was precultured in 10% FBS Dulbecco's Modified Eagle Medium (DMEM). All cell lines were precultured for 24 h before challenged with varying concentration of the extracts 10, 30, 100, 300, and 1,000 µg/ml. Phosphate buffer saline (PBS) was used as negative control and adriamycin was used as positive control. After a 72-h incubation period, the treated cells were added with MTT reagent then incubated again for 3 h and the formozan salts were dissolved with DMSO. The absorption was measured at 550 nm. The concentration that inhibited 50% cell growth (IC_{50}) was calculated using curve fitting. Each experiment was done in 5 replicates and reported as IC_{50} ± SD.

Phytochemical screening

The presences of tannins, flavonoids, alkaloids, saponins, lactone compounds, cardiac glycosides, and anthraquinone glycosides were detected using the methods as described by Farnsworth[7]. Thin layer chromotography (TLC) was performed; the chromatograms of those extracts were demonstrated using precoated silica gel aluminium $60F_{254}$ plates as a stationary phase, and toluene, chloroform and ethanol at the ratio of 4:4:1 was used as a mobile phase. Two plates were developed. The quenching and fluorescence bands were observed under short wavelength UV light (254 nm) and long wavelength UV (366 nm), respectively. After taking photographs, a plate was then sprayed with NP/PEG spray reagent and observed under long wavelength UV whereas the other was sprayed with anisaldehyde-sulphuric acid reagent (AS) and heated at 110°C for 5 min. The chromatograms were observed under visible light and long wavelength UV.

RESULTS

Antiproliferative activity

Concentrations of the extracts which exhibited 50 % growth inhibition (IC_{50} value) were shown in Table 1. The whole formula extract presented the most potent antiproliferative effect against hepatocellular carcinoma cells (HepG2) with IC_{50} value of 347.87± 55.06 µg/ml. For each component, the *phaya fai* (*Diospyros undulata*) aqueous extract exhibited the most potent activity against Vero, MCF-7 and SiHa cells but exhibited an inhibitory effect against KB cells at the

same concentration as *Krajai* (*Caesalpinia hymenocarpa*). In HepG2, *Krajai* (*Caesalpinia hymenocarpa*) showed the most potent inhibitory effect compared to other cell lines. These results indicate that *phaya fai* and *krajai* are the active components of this formula. In this case, the whole formula extract exhibited the most potent activity on a specific cancer cell – HepG2 cells which supported the traditional indication of this formula.

Table 1. Antiproliferative activity of the whole selected formula and each component against various cancer cells and a normal cell (n=5).

	IC50 Value (μg/ml)				
Extract / Cell line	SiHa	MCF-7	HepG2	KB	Vero
Whole formula	>1000	>1000	347.87±55.06	>1000	>1000
Stemona collinsiae	>1000	>1000	>1000	>1000	>1000
Caesalpinia hymenocarpa	572.96±36.20	511.34±13.16	72.60±4.39	191.49±3.52	551.54±39.79
Diospyros undulata	120.45±3.78	119.87±6.25	101.67±13.75	192.56±2.85	149.19±8.02
Celastrus sp.	>1000	>1000	>1000	>1000	>1000
Adriamycin (μM)	2.445±0.193	5.01±0.809	2.63±0.188	0.225±0.048	19.513±1.051

Phytochemical screening

The phytochemical groups present in the whole formula were flavonoids and lactone compounds as shown in Table 2. The tannin and flavonoid compounds were detected in *krajai* (*Caesalpinia hymenocarpa*) extract and the flavonoid, saponin, lactone compounds were detected in *phaya fai* (*Diospyros undulata*) extract. According to Liebermann Burchard's test, the saponin present in *phaya fai* extract would be steroidal saponins. However, alkaloids, cardiac glycosides, and anthraquinone glycosides were not detected among all selected extracts.

TLC chromatograms were developed using toluene, chloroform and ethanol. There were three bands of whole formula extract at Rf values of 0.46, 0.5, and 0.79. In *non tai yak* extract, a quenching band at Rf 0.5 was shown, and no band was detected in *krajai* extract. In *phaya fai* extract, 6 bands at Rf values as 0.29, 0.46, 0.5, 0.57, 0.63, and 0.79 were present. The band of *mak teak* extract showed a quenching at Rf 0.11.

Table 2. Phytochemical components in the herbal formula.

Sample	Tannins	Flavonoids	Alkaloids	Saponins	Lactone compounds	Cardiac glycosides	Anthraquinone glycosides
Whole formula		✓			✓		
Stemona collinsiae					✓		
Caesalpinia hymenocarpa	✓	✓					
Diospyros undulata		✓		✓	✓		
Celastrus sp.		✓			✓		

(✓ = presence)

DISCUSSION

In this study, *krajai* (*Caesalpinia hymenocarpa*) extract presented specifical antiproliferative activity against HepG2 and KB cells with IC_{50} at 72.60 ± 4.39 and 191.49 ± 3.52 µg/ml. Previously, two species of *Caesalpinia* had been reported for anticancer activity. Cassane diterpenes, isolated from *Caesalpinia bonduc*, demonstrated antiproliferative activity, and caesalpinolide-D showed potent activity against prostate and cervical carcinoma cells with IC_{50} at 68.52 and 58.2 µM. Furanoditerpene showed potent activity against breast and cervical carcinoma cells[8], and sappanchalcone, a flavonoid extracted from *Caesalpinia sappan*, showed antiproliferative activity against primary mouth carcinoma (HN_4) and metastatic carcinoma (HN_{12}) cells with IC_{50} at 5.2 and 25.3 µM, respectively[9]. The *phaya fai* (*Diospyros undulata*) extract selectively inhibited cell proliferation against SiHa, MCF-7, and HepG2 cells with IC_{50} about 100-120 µg/ml. Some species of *Diospyros* genus have been revealed to have antiproliferative activity against cancerous cells. Diosquinone, a napthoquinone epoxide isolated from *Diopyros mespiliformis* and *Diospyros tricolor* roots, showed potent cytotoxic activity at ED_{50} less than 5 µg/ml against various human cancer cells[10]. *Diospyros seychellarum* ethanol extract from leaves exhibited cytotoxic property at IC_{50} of 125 µg/ml against Jurkat cells[11]. The other two components, *mak taek* (*Celastrus sp.*) stem extract and *non tai yak* (*Stemona collinsiae*) extract showed no antiproliferative activity, but Akanitapichat *et al.* demonstrated cytotoxic activity of root extracts including dichloromethane-methanol, 95% methanol, and aqueous extracts of *non tai yak* (*Stemona collinsiae*) against KB and MCF-7 cells at IC_{50} of 85-270 µg/ml[12]. However, the aqueous extract of that study showed IC_{50} more than 250 µg/ml to both cancer cells and normal cells (Vero)[12]. The different results might be due to different collection sites of the sample plants that may have lead to different quantity or quality of chemical constituents of the plant.

Lactones found in the whole formula extract by the screening method and were confirmed with TLC chromatogram spraying with NP/PEG which showed blue-green fluorescence[13] at Rf 0.5. In *phaya fai* extract, lactones were detected when tested by 20% NaOH and Kedde's test. Also, the band at Rf values of 0.5 showed blue fluorescence after spraying NP/PEG. The yellow and violet bands at Rf values of 0.29 and 0.46 were evident after spraying with AS and, observed under visible light, showed the presence of saponins. Observation of the chromatogram confirmed the presence of saponins in a froth test. Flavonoids were found in *Phaya fai* extract by Shinoda's test together with TLC chromatogram which demonstrated a green fluorescence band at Rf 0.79 with NP/PEG spray. *Non tai yak* and *krajai* should further be studied to find out the appropriate mobile phases to obtain a TLC chromatogram.

In this study, the whole formula extract selectively inhibited proliferation of HepG2 which supports its traditional use as a liver cancer treatment. This formula has also been used to treat lung cancer. Further experimentation should be performed selectively with a well-known model such as LLC1 lung carcinoma cells.

CONCLUSIONS

An aqueous extract of the whole formula showed antiproliferative activity which might be due to the effects of *Diospyros undulata* and *Caesalpinia hymenocarpa*. Moreover, the specifically antiproliferative activity against HepG2 cells might affirm its traditional use to treat liver cancer. Using phytochemical screening and TLC chromatography, saponins were found in *phaya fai* extract, whereas flavonoids and lactone compounds were found in the whole formula extract. It may be worthwhile to investigate antimetastasis and other anticancer activities together with studying chemical compounds responsible for the activities further.

Table 3. TLC chromatogram of the whole formula and each component extracts with solvent system of toluene: chloroform: ethanol (4:4:1).

Extracts	Rf	UV (nm)		NP/PEG	AS	
		254	366		Visible	366 nm
Whole formula	0.46	-	✓ (blue)	-	-	-
	0.5	✓	✓ (blue)	✓ (blue-green)	✓ (violet)	✓ (red-violet)
	0.79	-	✓ (green)	✓ (green)	-	-
Non tai yak	0.5	✓	-	-	-	-
Krajai	-					
Phaya fai	0.29	✓	✓ (blue)	✓ (blue)	✓ (yellow)	✓ (blue-violet)
	0.46	✓	✓ (blue)	✓ (blue)	✓ (violet)	✓ (blue)
	0.5	✓	✓ (blue)	✓ (blue)	✓ (dark violet)	✓ (gray)
	0.57	-	✓ (blue)	✓ (blue)	-	-
	0.63	-	✓ (blue)	✓ (blue)	-	-
	0.79	✓	✓ (green)	✓ (blue-green)	-	✓ (red)
Mak teak	0.11	✓	-	-	✓ (violet)	✓ (gray)

(✓ = presence)

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