Cytotoxic Activity Against Cancer Cell Lines from The Ethanolic Extracts and Its VLC Fractions of *Bauhinia strychnifolia* Leaves

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Background: *Cancer is a leading cause of morbidity and mortality worldwide. Bauhinia strychnifolia Craib leaves is used in Thai traditional medicine for detoxification. Its leaves contain total phenolic content and also exhibit high antioxidant activity. However, there has been no report on cytotoxicity testing from its leave extracts. Thus, the present study aims to investigate its cytotoxic activity against cancer cell lines.*
Objective: *To study cytotoxicity from the ethanolic extracts of B. strychnifolia leaves and its vacuum liquid chromatography fraction against cholangiocarcinoma cell line (KKU-M156) and two types of colon adenocarcinoma cell lines (SW480, LS174T).*
Material and Method: *In vitro cytotoxic activity of the ethanolic extracts against three human cancer cell lines were investigated by using sulforhodamine B (SRB) assay.*
Results: *The 95% ethanolic extract of dried leaves showed the higher cytotoxic activity against KKU-M156, SW480, and LS174T than 50% ethanolic extract of dried leaves. The chloroform fraction from the 95% EtOH extract of dried leaves showed the best cytotoxicity against KKU-M156 and SW480 with* IC_{50} *value of 5.79±0.47 and 6.90±0.14 µg/ml, respectively.*
Conclusion: *The chloroform fraction from the 95% ethanolic extract of dried leaves was the most effective fraction against bile duct and colon cancer cell lines, thus this extract should be further investigated for active compounds possessing those observed cytotoxic activity.*

Keywords: *Bauhinia strychnifolia, Detoxification, Thai traditional medicine, Cytotoxic activity, Sulforhodamine B (SRB) assay*

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Nowadays, cancer, a chronic disease, is a major problem of health leading to high morbidity and mortality rates worldwide[1]. Bile duct and colon cancers are among the most frequently found cancers of the body[2]. Prevention and treatment are important to reduce risk factors and retard the process of cancer disease. Eating a healthy diet such as fresh fruits and vegetables with high antioxidants[3-5], and detoxification are the physiological or medicinal removal of toxic substances from a living organism[6]. Thai traditional doctors used herbs to remove toxins from the body through urine and stool. Ethanolic or aqueous extracts from the leaves and vines of *Bauhinia strychnifolia* Craib have been widely used for long time in the north and northeast of Thailand to reduce toxicants from insecticides, fever relief, and diarrhea. *B. strychnifolia*, or Ya-nang-daeng in Thai, is a member of Fabaceae (Leguminosae-caesalpinioideae) family. Its leaves are astringent with sweet flavor[7-10]. It performs antimalarial[11,12], anti-inflammatory, antimicrobial and especially strong antioxidant which relate to high content of total phenolic constituents[13]. However, there have been no reports yet on its effectiveness against cancer cell lines. It is, therefore, of great interest to study in this aspect. All parts of *B. strychnifolia* are used as one ingredient among many other herbs mixed together for cancer treatment at Khampramong temple[14]. Its vines have ever been reported against

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breast, lung, and cervical cancer cells by using the MTT cytotoxic assay[15] but its leaves have no reported cytotoxic activity against any types of cancer. Thus, this investigation should support using this plant for cancer treatment in Khampramong temple.

Material and Method

Reagents and materials

Sulforhodamine B (SRB) assay

Dimethyl sulfoxide (DMSO) was purchased from RCI Labscan, Thailand. Fetal bovine serum (FBS), Minimum Essential Medium (MEM), RPMI medium 1640, Trypan blue stain 0.4% and Trypsin-EDTA were purchased from Gibco, USA. Hydrochloric acid (HCl), Sodium bicarbonate ($NaHCO_3$) and Sodium hydroxide (NaOH) were purchased from Univar, Australia. Phosphate buffered saline (PBS) was purchased from Amresco, USA. Nutrient mixture F-12 Ham medium, sulforhodamine B sodium salt ($C_{27}H_{29}N_2NaO_7S_2$) and Tris (hydroxymethyl) aminoethane were purchased from Sigma-Aldrich, USA. Trichloroacetic acid (TCA) was purchased from Merck, Germany. The antibiotics Penicillin-Streptomycin (P/S) was purchased from Biochem, Germany.

Silica gel 60 (0.063-0.200 nm) was purchased from Merck (Darmstadt, Germany). Hexane, chloroform, ethyl acetate, and methanol were purchased from RCI Labscan (Thailand).

Plant materials and extraction

Fresh leaves of *B. strychnifolia* were collected in June, 2013 from the home of a Thai traditional doctor, Mr. Sraupsin Thongnoppakhun, in Thailand. The voucher specimen (BKF No. 186113) was deposited at the herbarium of The Royal Forest Department, Chatuchak, Bangkok province, Thailand.

Leaves of this plant were cleaned, sliced thinly, dried in a hot air oven at 50°C, and ground to be coarsely powdered, then macerated at room temperature with 50% ethanol and 95% ethanol for 3 days and filtered through a Whatman No. 1 filter paper. The residue was further macerated with the same solvents two more times. The extracts were concentrated by using a rotary evaporator (Rotavapor R-205, Buchi, Switzerland). The extracts were dried to constant weight in a vacuum drier (Rocker, Taiwan) and kept in a freezer at -20°C until required.

Isolation of compounds from the 95% ethanolic extract of dried leaves

The 95% ethanolic extract of dried leaves (50 g) was separated into five fractions using vacuum liquid chromatography (VLC) on silica gel 60 (Merck, 70-230 mesh) by order of increasing polarity of solvents: hexane 2,000 ml, hexane: chloroform (1:1) 2,000 ml, chloroform 2,000 ml, chloroform: methanol (1:1) 2,000 ml and methanol 2,000 ml. Each fraction was dried and evaporated.

In vitro assay for cytotoxic activity

Cell culture

The cholangiocarcinoma cell line (KKU-M156) was obtained from Khon Kaen University, Thailand, cultured in nutrient mixture F-12 Ham medium (Sigma-Aldrich®). Two types of colon adenocarcinoma cell line (ATCC No. CL-188, LS174T and ATCC No. CCL-228, SW480) were cultured in Minimum Essential Media (MEM) and RPMI-1640 medium (GIBCO™), respectively. They were supplemented with 10% fetal bovine serum (GIBCO™), 50 IU/ml penicillin and 50 µg/ml streptomycin (Biochem®) at 37°C in an incubator with 5% CO_2 and 95% humidity.

Sulforhodamine B (SRB) assay

The SRB assay is used for cell density determination, based on the measurement of cellular protein content. This assay relies on the ability of SRB to bind with protein components of cells that have been fixed to tissue culture plate by ice-cold trichloroacetic acid (TCA). The amount of dye extracted from stained cells is directly proportional to cell mass[16].

Cytotoxicity testing

Growth inhibition of KKU-M156, SW480, and LS174T cell lines were determined by using the modified SRB assay as described by Skehan et al[14]. Briefly, cells were seeded at a density of $2x10^3$, $2x10^3$, $3x10^3$ cells/ml for KKU-M156, SW480 and LS174T, respectively, 100 µl of cell suspension was added into each well of 96-well plates and incubated in a 95% humidity incubator at 37°C with 5% CO_2. After 24 h, 100 µl of serial dilutions of samples (*B. strychnifilia* leaves of 50%, 95% ethanolic extracts and VLC fractions from 95% ethanolic extract leaves) in DMSO were added to each well for each concentration. For medium controls, only medium 100 µl was added and for solvent control wells, only 2% DMSO was added. The cells were exposed to test samples for continuous 72 h in incubator. After that, the medium was removed, the cells were washed with PBS, and 200 µl of fresh medium were then added. The plates were incubated for a recovery period of 72 h. For cell fixation, 100 µl of ice-

cold trichloroacetic acid (40%) was added to each well and incubated at 4°C for 1 h. Then the plates were washed five times with water and allowed to dry in the air. 50 µl sulforhodamine B (SRB) solution (0.4% w/v in 1% acetic acid) was added to each well of the dried 96-well plates and allowed to stain at room temperature for 30 min. Then the SRB solution was removed by washing the plates with 1% acetic acid, five times, to remove unbound dye. After air-drying at room temperature, SRB dye within cells was dissolved by adding 100 µl of 10 mM Tris base (pH 10.5) to each well and shaking gently for 15 min on a gyratory shaker to homogenize the dye solution. The optical density (OD) of SRB in each well was directly proportional to the cell number and measured with a power wave x plate reader at 492 nm as the percentage of inhibition. Percentage of inhibition was calculated by using the following equation and the 50% inhibitory concentration (IC_{50}) was calculated from the Prism program obtained by plotting the percentage of survival versus the concentration interpolated by cubic spine. According to National Cancer Institute extracts guidelines with IC_{50} values <20 µg/ml will be considered active[17]. All data are presented as means ± SEM of triplicates.

The percentage of inhibition = $[(OD_{control}-OD_{sample})/OD_{control}] \times 100$

$OD_{control}$ = Mean of control medium – mean of blank

OD_{sample} = Mean of sample – mean of blank

Statistical analysis

All determinations were carries out on using Excel (Microsoft Inc.) and Prism software. The obtained results were reported as mean ± standard error mean (SEM).

Results and Discussion

The percentage of yield and the cytotoxicity evaluation of all plant extracts as IC_{50} are shown in Table 1. The obtained results indicate that the maceration method with 50% ethanol gave higher yield than 95% ethanol of dried leaves and the chloroform: methanol (1:1) fraction gave the highest yield which was 51.58%. The percentage of yield showed the substance can dissolved in moderate polarity solution better than low and high polarity.

The cytotoxic activity of 50%, 95% ethanolic extracts from *B. strychnifolia* leaves and five fractions from 95% ethanolic extract leaves against cholangiocarcinoma cell line (KKU-M156) and two types of colon adenocarcinoma cell lines (SW480, LS174T) were determined by using SRB assay. The 95% ethanolic extract, which all of cell lines showed the cytotoxicity higher than 50% ethanolic extract with IC_{50} values of 37.22±2.92, 32.02±1.93, and 34.27±3.46 µg/ml for KKU-M156, SW480 and LS174T cell lines, respectively. The 95% ethanolic extract showed higher cytotoxicity against SW480 than LS174T colon cancer. Thus the 95% ethanol which showed higher cytotoxic activity was selected to separate five fractions. All fractions also tested cytotoxic activity against KKU-M156 cholangiocarcinoma and only one type of colon cancer cell which the extract showed the best cytotoxic activity as SW480. The chloroform fraction, which showed the highest cytotoxic activity against KKU-M156 cell line with IC_{50} value of 5.79±0.47 µg/ml and

Table 1. Percentage of yield and cytotoxic activity (IC_{50} mg/ml ± SEM) of ethanolic extracts and VLC fractions from *Bauhinia strychnifolia* Craib leaves against three types of human cancer cell lines (KKU-M156, SW480, and LS174T) by using SRB assay (n = 3)

Part used	Extract	% Yield	Cytotoxic activity (IC_{50} µg/ml ± SEM)*		
			KKU-M156	SW480	LS174T
Dried leaves	50% EtOH	27.60	82.80±4.83	54.73±3.32	45.08±2.48
	95% EtOH	17.35	37.22±2.92	32.02±1.93	34.27±3.46
	Hexane	0.42	>50	>50	NT
	Hexane: chloroform	1.12	37.37±1.35	48.41±3.56	NT
	Chloroform	10.52	5.79±0.47	6.90±0.14	NT
	Chloroform: methanol	51.58	38.93±1.91	69.10±4.42	NT
	Methanol	24.96	>50	>50	NT

* IC_{50} values are expressed as the mean ± SEM of 3 replicates.
SEM = Standard error mean; NT = not tested

SW480 cell line with IC_{50} value of 6.90±0.14 µg/ml. In terms of the deduced IC_{50} values, the ethanolic extracts, the hexane fraction, the hexane: chloroform (1:1) fraction, the chloroform: methanol (1:1) fraction, and the methanol fraction can be classified as "less active" and for the chloroform fraction, it can be classified as "active" according to the National Cancer Institute (NCI) guidelines[17]. The criteria of cytotoxicity for the crude extract as established by the NCI with an IC_{50} values <20 µg/ml will be considered active. This result is the first report of cytotoxic activity against these cancer cells from *B. strychnifolia* leaves. It was related to that previously reported in that the 95% and 50% ethanolic extracts of *B. strychnifolia* leaves possess anti-inflammatory with IC_{50} value of 74.45 mg/ml and 71.03 mg/ml, respectively[13]. For antimicrobial activity, the 95% ethanolic extract can be inhibiting both of the *S. aureus* and *B. subtilis* with MIC value 5 mg/ml and 2.5 mg/ml, respectively[13]. The 95% ethanolic crude extract of *B. strychnifolia* leaves has excellent *in vivo* antimalarial activities against *P. berghei* ANKA[11] that relate to Thai traditional doctors' use for fever relief and diarrhea[7-10]. In addition, its leaves were rich in phenolic content and demonstrated good antioxidant activity[13] that is the one factor to detoxify toxic from the body and good for prevention of cancer[3-5]. The present study also supported Thai traditional doctor use of its leaves for detoxification: to remove toxins from the body[7-10] and support using these plant leaves as ingredients in cancer preparation for cholangiocarcinoma treatment from Khampramong temple or Arokhayasala Hospital[14].

Conclusion

The results indicate that the chloroform fraction of *B. strychnifolia* leaves showed the highest specific in vitro cytotoxic activity against cholangiocarcinoma and colon adenocarcinoma cell lines; thus this extract should be further isolated to investigate the active compounds of *B. strychnifolia* leaves for treatment of bile duct and colon cancers. In addition, it should be studied in order to compare with the positive control for combination treatment and to be an alternative drug or supplement for cancer treatment in the future.

What is already known on this topic?

The leaves, vines and roots from *Bauhinia strychnifolia* Craib is widely used in Thai traditional medicine for detoxification. Its leaves possess antimalarial, anti-inflammatory, antimicrobial and especially strong antioxidant which relate to high content of total phenolic constituents. All parts of *B. strychnifolia* are used as one ingredient among many other herbs mixed together for cancer treatment at Khampramong temple. Its vines have been reported on cytotoxic activity against cancer cells, but its leaves have not been reported on for cytotoxic activity against all types of cancers.

What this study adds?

The present study shows the cytotoxic activity of *Bauhinia strychnifolia* Craib leaf extracts against bile duct and colon cancer cells. The results obtained shows that the extract from its leaves yield best cytotoxic activity against cholangiocarcinoma and colon adenocarcinoma cells that were related with the previously reported. In addition, the results from this study also support the use of *Bauhinia strychnifolia* Craib to treat cancer at Arokhayasala Khampramong temple and to detoxify toxic substances in Thai traditional medicine.

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Potential conflicts of interest

None.

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ฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งของสารสกัดที่หมักด้วยแอลกอฮอล์และสารสกัดย่อยจากใบย่านางแดง

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ภูมิหลัง: มะเร็งเป็นสาเหตุของการเจ็บป่วยและการเสียชีวิตของประชากรทั่วโลก ในปัจจุบันด้านการแพทย์แผนไทยมีการใช้ใบของสมุนไพรย่านางแดงเพื่อขับล้างสารพิษออกจากร่างกาย นอกจากนี้ใบของย่านางแดงยังมีฤทธิ์ต้านอนุมูลอิสระและมีปริมาณฟีนอลลิกสูง อย่างไรก็ตามยังไม่เคยมีการรายงานการศึกษาฤทธิ์ด้านความเป็นพิษต่อเซลล์มะเร็งจากใบย่านางแดง ดังนั้นงานวิจัยในครั้งนี้ทำการศึกษาความเป็นพิษต่อเซลล์มะเร็งจากสารสกัด ใบย่านางแดง

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งท่อน้ำดีและเซลล์มะเร็งลำไส้จากสารสกัดใบย่านางแดงที่หมักด้วยแอลกอฮอล์และส่วนของสารสกัดย่อย

วัสดุและวิธีการ: การทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งด้วยวิธี Sulforhodamine B (SRB) assay กับสารสกัดที่หมักด้วยแอลกอฮอล์และส่วนของสารสกัดย่อยจากใบย่านางแดง

ผลการศึกษา: สารสกัดจากใบย่านางแดงที่หมักด้วยแอลกอฮอล์ 95% แสดงฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งท่อน้ำดีและเซลล์มะเร็งลำไส้ได้ดีกว่าสารสกัดที่หมักด้วยแอลกอฮอล์ 50% เมื่อนำสารสกัดที่หมักด้วยแอลกอฮอล์ 95% มาแยกต่อด้วยวิธี Vacuum liquid chromatography (VLC) พบว่าสารสกัดย่อยส่วนคลอโรฟอร์มมีความเป็นพิษต่อเซลล์มะเร็งท่อน้ำดีและเซลล์มะเร็งลำไส้มากที่สุดที่ค่า IC_{50} เท่ากับ 5.79±0.47 ไมโครกรัม/มิลลิลิตร และ 6.90±0.14 ไมโครกรัม/มิลลิลิตร ตามลำดับ

สรุป: สารสกัดย่อยส่วนคลอโรฟอร์มจากใบย่านางแดงที่หมักด้วยแอลกอฮอล์ 95% มีฤทธิ์ดีที่สุดในการต้านเซลล์มะเร็งท่อน้ำดีและเซลล์มะเร็งลำไส้ ดังนั้นจึงควรมีการนำสารสกัดย่อยส่วนนี้ไปศึกษาสารออกฤทธิ์ในการต้านเซลล์มะเร็งท่อน้ำดีและลำไส้ต่อไป