In vitro investigations on the potential roles of Thai medicinal plants in treatment of cholangiocarcinoma

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The aim of the present study was to investigate the potential roles of the crude ethanolic extracts of rhizomes of *Zingiber Officinale* Roscoe (Ginger, ZO) and *Atractylodes Lancea* (Thung.) DC. (Khod-Kha-Mao, AL), fruits of *Piper chaba* Hunt. (Dee-Plee, PC), Pra-Sa-Prao-Yhai formulation (a mixture of parts of 18 Thai medicinal plants, PPF), and curcumin compound (CUR) for treatment of cholangiocarcinoma (CCA) in different *in vitro* models (cytotoxic, antioxidant, anticlonogenic activities, and inhibitory effects on cell invasion and angiogenesis). The cytotoxic activity of the test materials against the CCA cell line CL-6 (selectivity index: SI = 3.93-18.26) was found to be more specific when compared with HepG2 (SI = 2.17-6.35). All test materials were significantly more potent than the reference drug 5-FU in CL-6 for both cytotoxic assays. CUR compound produced the most potent antioxidant activity with potency of about 2-3 times of 5-FU. AL produced the most potent inhibitory effect on clonogenic survival of CL-6 cells compared with the reference drug 5-FU and control. The test materials at all concentration levels significantly inhibited tube formation and inhibitory effects on cell invasion. Altogether, results suggest the potential roles of some Thai medicinal plants in treatment of CCA.

Keywords: Cholangiocarcinoma, Anticancer, Antioxidant, Cell invasion, Angiogenesis, Thai medicinal plants

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor of the bile duct epithelium associated with a high mortality rate with increasing incidence worldwide (Minami and Kudo, 2010). It is an important public health problem in several parts of Southeast Asia, particularly the northeastern region of Thailand. The major contributing factor of CCA in Thailand is consumption of improper cooked and fermented fresh water cyprinoids fish called 'Pla-ra' or 'Pla-som', which contains *Opisthorchis viverrini* (OV) and nitrosamine (Sripa et al., 2011). Lack of effective diagnostic tool and chemotherapeutics are major constraints for controlling CCA. Chemotherapy of CCA is largely ineffective; clinical efficacy of the standard treatment with 5-fluorouracil (5-FU) is low. Furthermore, resistance of CCA to chemotherapy and radiotherapy is a major problem. Discovery and development of chemotherapeutics that are effective for treatment and control of this type of cancer is urgently needed.

Among the natural products, plants were the most widely and diverse sources of medicines against a great variety of ailments including the treatment of refractory cancers such as CCA. In our previous study (Mahavorasirikul et al., 2010), the ethanolic extracts of rhizomes of *Zingiber officinale* Roscoe (ZO) and *Atractylodes lancea* (Thung.) DC. (AL), fruits of *Piper chaba* Hunt. (PC), and Pra-Sa-Prao-Yhai formulation (PPF) were shown to exhibit promising activity against the human CCA cell line CL-6, with IC_{50} (concentration that inhibits cell growth by 50%) of less than 50 μg/ml. Further in *vivo* investigation in CCA-xenografted nude mice revealed promising anti-CCA activity of the

ethanolic extract of AL at all dose levels (1,000, 3,000, and 5,000 mg/kg body weight), as well as ZO and PPF at the highest dose level (5,000 and 4,000 mg/kg body weight, respectively). PC produced no significant anti-CCA activity. Results from acute and subacute toxicity tests both in mice and rats indicated their safety profiles in a broad range of dose levels. The aim of the present study was to further investigate the anticancer potential of ZO, AL, PC and PPF against CCA in different *in vitro* models (cytotoxic, antioxidant, anticlonogenic activities, and inhibitory effects on cell invasion and angiogenesis). Due to potential therapeutic interest for treatment of cancer, the *in vitro* anti-CCA potential of curcumin (CUR), the phenolic compound extracted from rhizome of *Curcuma longa* Linn. also merits exploration. ZO, known as ginger, is a common condiment for various foods and beverages and is used in folk medicine in Asia and tropical areas for various purposes such as colds, fevers, digestive problems, and a treatment for nausea and vomiting, as well as for arthritis (White, 2007). AL, the dried rhizome of *A. lancea* (Thung.) DC. or "Khod-Kha-Mao", has been used in Thai traditional medicine for treatment of fever and cold (Chayamarit, 1995). PC, the fruit of *P. chaba* Hunt. commonly called "Dee-Plee", has been used in Thai traditional medicine as an antiflatulent, expectorant, carminative, antitussive, antifungal, uterus contracting agent, sedative-hypnotic, appetizer, counter-irritant, and is also useful in asthma, bronchitis, fever, and inflammation (Patra and Ghosh, 1974). PPF is a Thai traditional medicine used for treatment of fever in children (Chayamarit, 1995). This remedy consists of a mixture of various parts of eighteen medicinal plants including *Amomum testaceum* Ridl. (seed), *Angelica dahurica* Benth. (rhizome), *Angelica sinensis* (Oliv.) Diels (rhizome), *Anethum graveolens* Linn. (rhizome), *Artemisia annua* Linn. (rhizome), *Atractylodes lancea* (Thung.) DC. (rhizome), *Asclepias curassavica* Linn. (flower), *Cuminum cyminum* Linn. (seed), *Dracaena loureiri* Gagnep. (stem bark), *Foeniculum vulgare* Mill. var. dulce Alef (seed), *Kaempferia galangal* (leaf and fruit), *Ligusticum sinens*e Oliv. cv. Chuanxiong (rhizome), *Mammea siamensis* Kosterm. (flower), *Mesua ferrea* Linn. (flower), *Mimusops elengi* Linn. (flower), *Myristica fragrans* Houtt. (seed), *Nigella sativa* Linn. (seed), and *Syzygium aromaticum* (L.) Merr. and L.M. Perry (flower).

MATERIALS AND METHODS

Chemicals and reagents

Commercial grade ethanol was purchased from Labscan Co. Ltd. (Pathumwan, BKK, THA). The cell culture medium RPMI, fetal bovine serum (FBS), L-glutamine, dimethylsulfoxide (DMSO), the antibiotics streptomycin and penicillin, antibiotics-antimycotics (anti-anti) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Renal epithelium cell growth medium and SupplementPack were purchased from Promocell Co. Ltd. (Germany). 5-fluorouracil (5-FU), DPPH (2,2- diphenyl-2-picrylhydrazyl), L-ascorbic acid (vitamin C), curcumin compound (CUR) and giemsa staining kit were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of plant extract

Plant materials under investigation included rhizomes of *Zingiber officinale* Roscoe (ZO) and *Atractylodes Lancea* (Thung.) DC. (AL), fruits of *Piper chaba* Hunt. (PC), and Pra-Sa-Prao-Yhai formulation (PPF). PPF consisted of parts obtained from eighteen plants as described above. Information on plant species, parts used, voucher number, including their uses in Thai traditional medicine are summarized in Table 1. Plant materials were collected from various parts of Thailand and some were purchased from city markets. Authentication of plant materials were carried out at the herbarium of the Department of Forestry, Bangkok, Thailand, where the herbarium vouchers have been kept. A duplicate set has also been deposited in the herbarium of Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Songkhla, Thailand. Preparation of the ethanolic extracts of all plant materials were performed according to the previously described method (Mahavorasirikul et al. 2010). All extracts were standardized using high performance liquid chromatography to examine the amounts of active ingredients.

Cell lines and culture

The CCA cell line CL-6, was used for the *in vitro* assessment of cytotoxic (calcein-AM release and Hoechst 33342 assays), anticlonogenic, and inhibitory activities on cell invasion of the ethanolic extracts of ZO, AL, PC, PPF and CUR compound. CL-6 cell line was established and kindly provided by Associate Professor Dr. Adisak Wongkajornsilp of the Department of Pharmacology, Faculty of Medicine (Siriraj Hospital), Mahidol University, and were cultured in RPMI medium supplemented with 10% heated fetal bovine serum and 100 IU/ml of anti-anti. Assessment of the cytotoxicity of test material against CL-6 cell line was performed in comparison with HepG2 (hepatocarcinoma) and HRE (normal human renal epithelium) cell lines. HepG2 cell line was purchased from the Cell Line Service Co. Ltd. (Germany) and was cultured in a complete RPMI

Table 1. Medicinal plants and herbal formulation under investigation

Family	Plant	Part used	Voucher Specimen	Thai Traditional use
Zingiberaceae	Zingiber Oficinal Roscoe	Rh	SKP 206261501	Treatment of hypercholesteremia
Compositae	Atractilodes lancea thung. DC	Rh	SKP 051011201	Treatment of fever, cold, flu, sore throat
Piperacae	Piper chaba hunt	Fr	SKP 146160301	Used as caminative, antidiarrheal
Composition of pra-sa prao Yhai formulation:				
Composite	Artemisia annua L.	Rh	SKP 051010101	Treatment of fever and hemorrhoids
Cruciferae	Asclepias curassavica L	Fl	SKP 057121901	Use as analgesic
Dracaenaceae	Dracaena loureiri Gagnep	St, Ba	SKP 056041201	Treatment of cough, fever, inflamation
Guttifarae	Manmae siamensis kosterm	Fl	SKP 083131901	Restorative
Guttifarae	Mesua ferea L	Fl	SKP 083130601	Treatment of Dyspepsia
Myristicaceae	Myristica fragrans Houtt.	Sd	SKP 121130601	Treatment of uterus pain, Diarrhae
Mytaceae	Syzygium aromaticum (L) merr & L.M Perry	fl	SKP 123190101	Treatment of toothache, bacteria infection
Nelumbonaceae	Nigella sativa Linn	Sd	SKP 160141901	Treatment of joundice
Sapotadeae	Mimusops elengi L.	Fl	SKP 171130501	Use as cordil, tonic, Treatment of syncope
Umbeliferae	Angelica dalurica benth.	Rt	SKP 199010401	Use as antipyretic, antiasthema, anticough
Umbeliferae	Angelica sinensis (Oliv.) Diels	Rh	SKP 199010901	Treatment of bronchitis, pleurisy
Umbeliferae	Anetum graveoplens L.	Rt, fr	SKP 199010701	Use as carminative, treatment of eye pain
Umbeliferae	Cuninum cyminum linn.	Sd	SKP 199030301	Treatment of dyspepsia,diarrhoae, jaundice
Umbeliferae	Foeniculum vulgare mill. Var. dulce alef	Sd	SKP 199062201	Use as analeptic
Umbeliferae	Ligusticum sinence oliv. Cv Chuanxiong[1]	Rh	SKP 199121901	Treatment of urinary bladder channel, headache, neurodematitis
Zingiberaceae	Amonnum testaceum Ridl	Sd	SKP 206011101	Use as carminative antibacterial
Zingiberaceae	Curcuma longa Linn.	Rh	SKP 206012101	Treatment of cancer, high cholesterol, dyspepsia, gullstone
Zingiberaceae	Kaempferia	Lf	SKP 206110701	Anti-nociceptive anti-inflammatory

medium supplemented with 10% fetal bovine serum and 100 IU/ml pen-strep. HRE cell line was purchased from Promocell Co. Ltd. (Germany) and was cultured in renal epithelial cell growth medium 2 with SupplementPack. All cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity.

In vitro models for assessing cytotoxic, antioxidant and clonogenic survival activities

Calcein-AM release assay

The assay was modified from Neri et al. (2001). CL-6,

\HepG2, and HRE cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 hours of incubation, cells were incubated with various concentrations of each test material (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, and 250 µg/ml) at 37°C for 24 hours. 5-FU (at concentrations of 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µg/ml) was used as positive control drug.

Hoechst 33342 assay

Inhibition of proliferation of CL-6, HepG2, and HRE cells by the test materials was measured by Hoechst 33342 assay (Schoonen et al., 2005) using the same seeding cells and concentration ranges as that used in calcein-AM release assay.

Antioxidant activity assay

The antioxidant activities of the test materials were determined by measuring radical-scavenging activity of DPPH (2,2-diphenyl-1-picrylhydrazyl radical) (Szabo et al., 2007). Vitamin C (ascorbic acid) was used as a positive control reagent. The concentrations of the test materials and vitamin C used in the experiment were 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, and 250 µg/ml.

For all of the above mentioned assays, results were generated from three independent experiments, triplicate each. Percentage of inhibition of the activity (cytotoxic, anti-proliferation, and antioxidant) was calculated as follows:

$\% \text{ Inhibition} = [(\text{Absorbance}_{control} - \text{Absorbance}_{test}) / \text{Absorbance}_{control}] \times 100$

The IC_{50} (concentration that inhibits the activity by 50%) values were calculated using CalcuSyn™ software (Biosoft, UK).

Clonogenic survival assay

Clonogenic survival assay was used to elucidate the long-term cytotoxic effects of the test materials on CL-6 cell line (Hsu et al., 2008). CL-6 cells were plated on 35 cm^3 dish and treated with plants extracts (in appropriate RPMI-complete medium) or CUR compound or various concentrations (12.5, 25, 50 and 100 µg/ml) for 48 hours.

In vitro models for assessing inhibitory effects on angiogenesis and metastasis

Angiogenesis assay

The inhibitory effects of the plant extracts and CURcompound on angiogenesis were assessed according to the method of Vaio et al (2011) using Angiogenesis Assay KIT (Millipore, MA, USA). The development of tube formation of endothelial cells (cellular network structure) was examined under an inverted light microscope at 40x-200x magnification.

Cell invasion assay

The inhibitory effects of the plant extracts and CUR compound on cell invasion were assessed in QCM™ 96-well cell invasion chambers (8 µm; Millipore, USA) according to the method of Liang et al(2008). The concentrations of each plant extract and CUR compound used were 12.5, 25, 50, 100 and 150 µg/ml.

Statistical analysis

All quantitative variables were presented as mean ± SEM values of results obtained from three independent experiments. Comparison of all quantitative variables between the groups treated with test materials and control or reference drugs was performed using student t-test. Statistical significance level was set at $\alpha = 0.05$ for all tests.

RESULTS

Cytotoxic, antioxidant and clonogenic survival activities

Cytotoxic activity

The cytotoxic activity of CUR compound and ethanolic extracts of ZO, AL, PC and PPF against the human CCA cell line CL-6, HepG2 and HRE cell lines were investigated using calcein-AM and Hoechst 33342 cytotoxic assays. The cytotoxic assays calcein-AM and Hoechst 33342 assays provide indirect measure of esterase activity and DNA binding, respectively. In both assays, all test materials were found to inhibit cell viability in a dose-dependent manner following 48 hours exposure. The IC_{50} (mean ± SEM) values of the test materials in CL-6, HepG2 and HRE cell lines including their selectivity index (SI) in both assays are summarized in Table 2 and 3. The cytotoxic activity of the test materials against CL-6 cells (SI 3.93 – 18.26) was found to be more specific when compared with HepG2 (SI = 2.17-6.35). All test materials were significantly more potent than the reference drug 5-FU in CL-6 for both assays. The comparative potencies in descending for calcein-AM and Hoechst 33342 assays were ZO > AL > PPF > CUR > PC > 5-FU, and AL > PPF > ZO > PC > CUR > 5-FU, respectively.

Table 2. *In vitro* cytotoxic activity of CUR compound and ethanolic extracts of ZO, AL, PC, PPF, and 5-FU against CL-6, HepG2 and HRE cell lines in calcein-AM assay. Data are presented as selectivity index (SI) and mean ± SEM values of IC_{50}.

Cell line	Potency/ selectivity	5-FU	CUR	ZO	AL	PC	PPF
CL-6	IC_{50} (µg/ml)	89.75 ± 0.36	44.36 ± 0.42	10.88 ± 0.32	23.24 ± 0.35	45.69 ± 0.41	30.4 ± 0.44
	SI	3.20	7.23	18.26	10.98	5.34	8.71
HepG2	IC_{50} (µg/ml)	75.62 ± 0.40	67.33 ± 0.38	71.67 ± 0.41	48.33 ± 0.33	70.59 ± 0.39	69.41 ± 0.35
	SI	3.80	4.76	2.77	5.28	3.46	3.81
HRE	IC_{50} (µg/ml)	287.35 ± 1.61	320.82 ± 1.68	198.63 ± 1.45	255.19 ± 1.55	244.06 ± 1.73	264.87 ± 1.39
	SI	1	1	1	1	1	1

Significantly lower for CUR compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for ZO compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for AL compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for PC compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for PPF compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)

Table 3. *In vitro* cytotoxic activity of CUR compound and ethanolic extracts of ZO, AL, PC, PPF, and 5-FU against CL-6, HepG2 and HRE cell lines in Hoechst 33342 assay. Data are presented as selectivity index (SI) and mean ± SEM values of IC_{50}.

Cell line	Potency/ selectivity	5-FU	CUR	ZO	AL	PC	PPF
CL-6	IC_{50} (µg/ml)	94.63 ± 0.41	55.42 ± 0.35	49.89 ± 0.34	27.16 ± 0.37	50.72 ± 0.43	36.91 ± 0.32
	SI	3.05	5.71	3.93	10.16	4.67	6.93
HepG2	IC_{50} (µg/ml)	75.08 ± 0.46	70.92 ± 0.39	90.38 ± 0.48	43.35 ± 0.31	67.11 ± 0.35	66.46 ± 0.38
	SI	3.83	4.46	2.17	6.36	3.53	3.85
HRE	IC_{50} (µg/ml)	287.65 ± 1.34	316.38 ± 1.52	196.23 ± 1.45	275.87 ± 1.39	237.14 ± 1.44	255.67 ± 1.42
	SI	1	1	1	1	1	1

Significantly lower for CUR compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for ZO compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for AL compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for PC compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)
Significantly lower for PPF compared with control and 5-FU in CL-6 ($p < 0.001$, student t-test)

Antioxidant activity

CUR compound produced the most potent antioxidant activity with potency of about 2-3 times of 5-FU. IC_{50} (mean ± SEM) values of ascorbic acid, CUR, ZO, AL, PC and PPF were 16.95 ± 0.35, 6.95 ± 0.22, 26.68 ± 0.38, 154.78 ± 0.84, 266.6 ± 0.76, and 57.03 ± 0.39 µg/ml, respectively (Figure 1).

Clonogenic survival activity

All test materials produced significant inhibitory effects on clonogenic survival of CL-6 cells compared with the

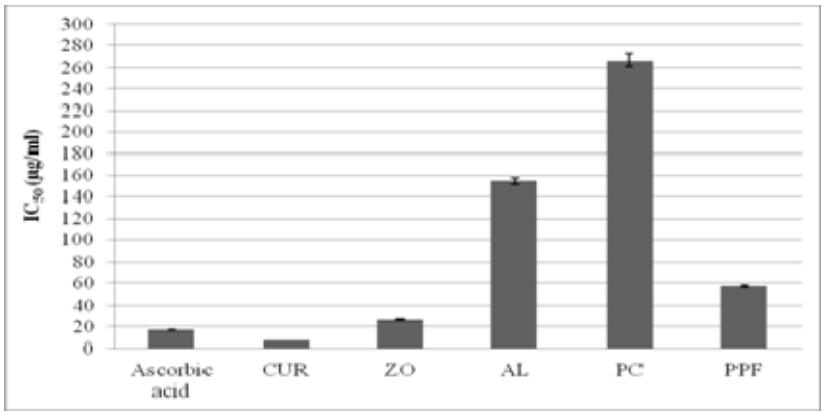

Figure 1. *In vitro* DPPH scavenging activity (represented by IC_{50} values) of CUR compound and the ethanolic extracts of ZO, AL, PC, PPF, in comparison with ascorbic acid (reference control). Data are presented as mean ± SEM values.
Significantly lower for CUR compared with ascorbic acid (p < 0.001, student t-test)

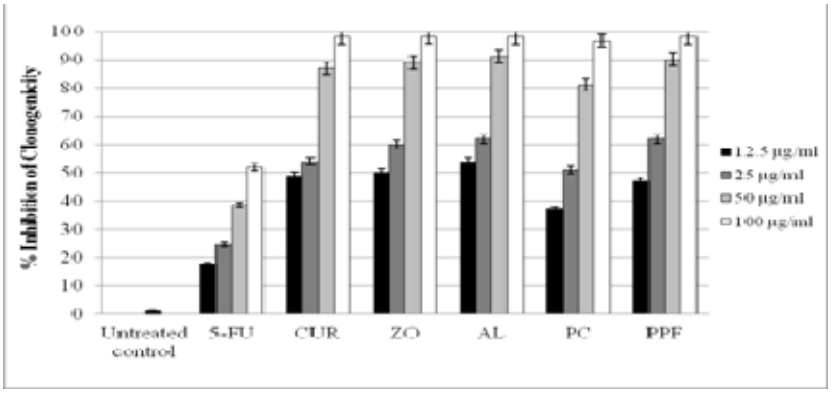

Figure 2. Inhibitory effects of CUR compound and ZO, AL, PC, PPF at various concentrations (12.5, 25, 50 and 100 µg/ml), in comparison with control and 5-FU (reference drug) on colony-forming in CL-6 cells. Data are presented as mean ± SEM values.
Significantly lower for CUR compared with control and 5-FU at each concentration (p < 0.001, student t-test)
Significantly lower for ZO compared with control and 5-FU at each concentration (p < 0.001, student t-test)
Significantly lower for AL compared with control and 5-FU at each concentration (p < 0.001, student t-test)
Significantly lower for PC compared with control and 5-FU at each concentration (p < 0.001, student t-test)
Significantly lower for PPF compared with control and 5-FU at each concentration (p < 0.001, student t-test)

reference drug 5-FU and control (Figure 2).
Clonogenicity of the CL-6 cell lines was reduced in a dose-dependent manner after exposure to 5-FU and all test materials. The inhibitory effects of all test materials were significantly higher than 5-FU at all concentrations. AL produced the most potent inhibitory effect at 12.5, 25 and 50 µg/ml (53.5, 61.6 and 91.2% of control, respectively).

Inhibitory effects on angiogenesis and cell invasion

Angiogenesis assay

Tube formation of endothelial cells was observed microscopically following incubation of CL-6 cells (37°C) with 5-FU, CUR compound and plant extracts at various concentrations on ECMatrix™ plate for 12-18 hours

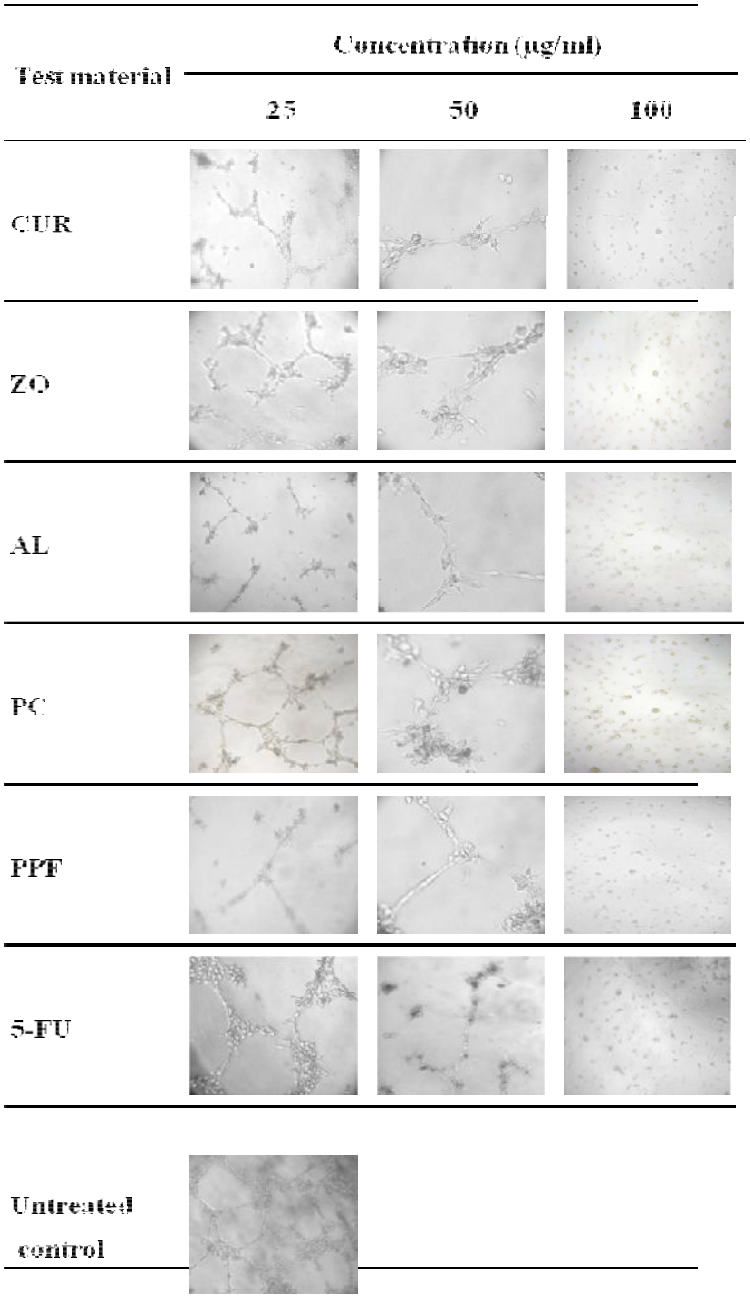

Figure 3. Morphology of tube formation of epithelial cells following exposure to CUR compound and ZO, AL, PC, PPF at various concentrations (25, 50, 100 µg/ml), in comparison with control and 5-FU (reference drug), on angiogenesis.

(Figure 3). CL-6 cells were rapidly reformed to form capillary-like structure of cellular network (Figure 3). Tube formation of cells exposed to 5-FU and the test materials were however incomplete, whereas complete tube formation was observed in the control cells (Figure 3). 5-FU and test materials at all concentration levels significantly inhibited tube formation compared with control (Figure 4).

Cell invasion assay

The cell invasion assay provides an efficient system for evaluating the invasion of tumor cells through a

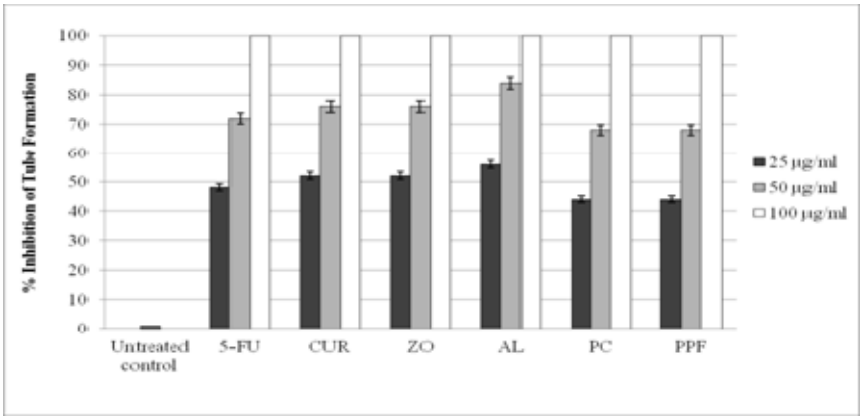

Figure 4. Inhibitory effects of CUR compound and ZO, AL, PC, PPF at various concentrations (25, 50, 100 µg/ml), in comparison with control and 5-FU (reference drug), on angiogenesis. Data are presented as mean ± SEM values.
Significantly higher for 5-FU at all concentrations compared with control ($p < 0.001$, student t-test)
Significantly higher for CUR compound at all concentrations compared with control ($p < 0.001$, student t-test)
Significantly higher for ZO at all concentrations compared with control ($p < 0.001$, student t-test)
Significantly higher for AL at all concentrations compared with control ($p < 0.001$, student t-test)
Significantly higher for PC at all concentrations compared with control ($p < 0.001$, student t-test)
Significantly higher for PPF at all concentrations compared with control ($p < 0.001$, student t-test)

basement membrane model. The inhibitory effects of 5-FU and the test materials on cell invasion were investigated in CL-6 and HRE cells. Invasion of CL-6 cells was significantly reduced following exposure to 5-FU and all test materials in a dose-dependent manner (Figure 5). The effects were significantly higher with the test materials than 5-FU at each concentration. For HRE cell, the effects of all test materials at all concentrations on cell invasion were not significantly different.

DISCUSSION

A number of plant-derived compounds have been investigated for anti-CCA, notably triptolide from *Tripterygium wilfordii* (Tengchaisri et al., 1998) and the ubiquitous tannic acid (Naus et al., 2007) in *in vitro* and animal models. In the present study, we explored the anti-CCA potential of medicinal plants used in Thai traditional medicine. Results from a series of *in vitro* experiments in the current study are in line to support that medicinal plants used in Thai traditional medicine constitute a promising source of anticancer repository. All the test materials were shown to produce significantly greater potency of cytotoxic, anticlonogenic, and inhibitory activities on cell invasion than the reference drug 5-FU, while their antiangiogenic activities were similar to 5-FU. All were considered safer than 5-FU based on their selectivity index values. Tumor growth involves various cellular processes that ultimately lead to its establishment. Metastasis (spread of tumor cells to other tissues through cell invasion) and angiogenesis (a process of new blood vessel development) are the two important characteristic features of malignant tumors. CCA is a malignant tumor of biliary epithelium associated with a high metastatic and mortality rate (Sirica, 2005). The incidence of lymph node and remote organ metastasis were reported to be 75% and 71%, respectively (Nakajima et al., 1988). Angiogenesis plays an important role in cancer growth; tumors induce blood vessel growth by secreting various growth factors particularly vascular endothelial growth factor (VEGF) (Folkman and Cotran, 1976). Cancer cells within the tumor will then use the newly formed blood vessels as a port to metastasize to other localities (Weidner et al., 1991). Since the interdependency and a close relationship between angiogenesis, cancer growth and metastasis has been well-established, much effort have been invested into development or discovery of antiangiogenic compounds to target cancer and variety of other angiogenic related ailments. To the best of our

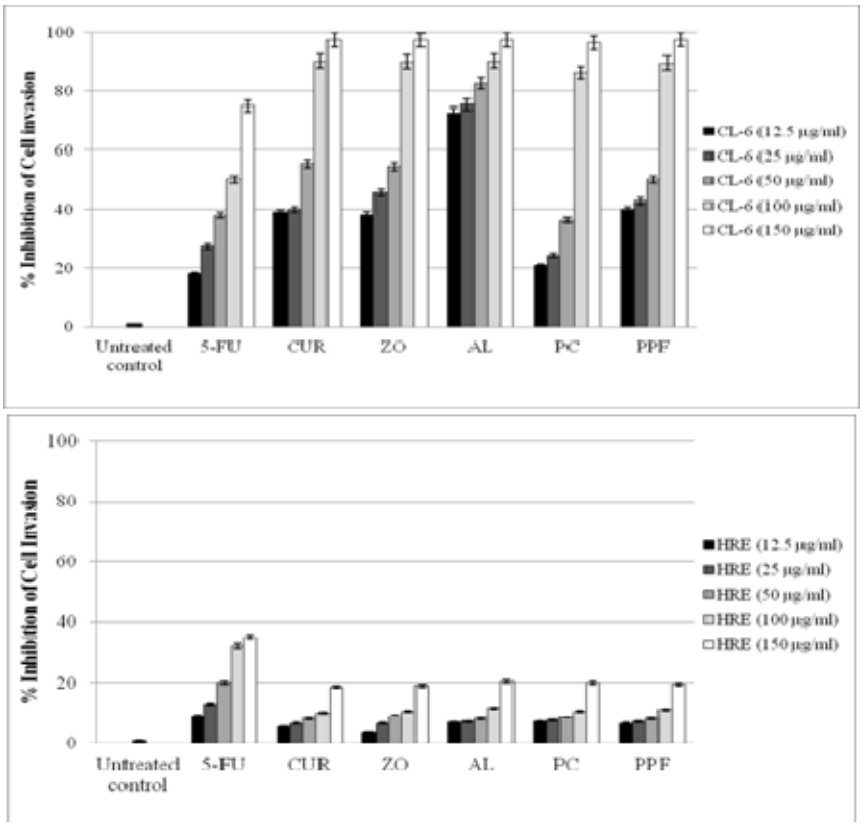

Figure 5. (a) Inhibitory effects of CUR compound and ZO, AL, PC, PPF at various concentrations, in comparison with control and 5-FU (reference drug) on cell invasion in (a) CL-6 and (b) HRE cells. Data are presented as mean ± SEM values.
Significantly lower for CUR compared with control and 5-FU at all concentrations in CL-6 cells ($p < 0.001$, student t-test)
Significant lower for ZO compared with control and 5-FU at all concentrations in CL-6 cells ($p < 0.001$, student t-test)
Significant lower for AL compared with control and 5-FU at all concentrations in CL-6 cells ($p < 0.001$, student t-test)
Significant lower for PC compared with control and 5-FU at all concentrations in CL-6 cells ($p < 0.001$, student t-test)
Significant lower for PPF compared with control and 5-FU at all concentrations in CL-6 cells ($p < 0.001$, student t-test

knowledge, the present study demonstrated for the first time, the potential roles of ZO, AL, PC, and PPF on tumor metastasis and angiogenesis.

Altogether, the results correlate well with those our previously reported *in vivo* studies in animal models with regards to anti-CCA potential of ZO, AL, PC, PPF and CUR (Plengsuriyakarn et al., 2012a,b). In a CCA-xenografted nude mouse model, the ethanolic extract of AL at all dose levels (1000, 3000, 5000 mg/kg body weight), as well as ZO, PPF and CUR compound at the highest dose level (5000 mg/kg body weight) produced significant prolongation of survival time of animals and in addition, significant reduction of tumor volume. Among all the test materials under current investigation, the ethanolic extracts of ZO and AL were shown to produce the most potent anti-CCA activity *in vitro*. Cytotoxicity tests in both assays showed the potency of about 2-8 times of 5-FU, and with significant selective toxicity to CL-6 cell. Their inhibitory activities on CL-6 colony forming was about 2-fold of 5-FU at each concentration. Furthermore, the extracts of both plants demonstrated marked inhibitory activities on cell invasion and angiogenesis. Interestingly, results from cell invasion and angiogenesis assays support the observation of a significant reduction of lung metastasis (to 5% of total lung mass) in the CCA-xenografted mice treated with AL, whereas metastasis of more than 90% of total lung mass was found in the untreated mice (Plengsuriyakarn et al., 2012a). The hepatocyte growth factor HGF/Met has been shown in a previous study to

play role in cell invasion by promoting CCA cell invasiveness through dys-localization of E-cadherin and induction of cell motility by distinct signaling pathways (Menakongka and Suthiphongchai, 2010). Moreover, revision-inducing-cysteine-rich protein with Kazal motifs (RECK) has been implicated in the attenuation of CCA tumor metastasis by negatively regulating metalloproteinase levels (Namwat et al., 2011). Further investigations into mechanisms of inhibitory effects of CUR, ZO and AL on these molecular targets in the metastasis and angiogenesis processes is encouraging. In addition, efforts should also be made on the exploration of the role of PC as a non-cytotoxic agent with anti-angiogenetic activity. This treatment approach would be expected to decrease the side effects that accompany the classical chemotherapeutics drugs (Nassar et al., 2011).

Since potent scavengers of free radical species that produce tumor cell oxidative stress may serve as a possible prevention intervention for free radical mediated cancer (Ames et al., 1995), we also examined the antioxidant potential of the test materials. ZO was found to possess the most significant radical scavenging activity with potency comparable to ascorbic acid. The promising cytotoxic activity of the crude ethanolic extract of ZO has recently been reported in HepG2 (IC_{50} 9.67 μg/ml) and Hep2 (IC_{50} 32.40 μg/ml) cancer cell lines (Mahavorasirikul et al., 2010). In our previous study in OV/dimethylnitrosamine-induced CCA in hamster model (Plengsuriyakarn et al., 2012b), the extract when given at the highest dose of 5000 mg/kg body weight daily for 30 days resulted in prolongation of survival time (median of 54 weeks) which was about 2- and 3- times of 5-FU (median of 25.5 weeks) and untreated (median of 17 weeks) control groups, respectively. When the extract was given at the same dose levels (1000, 3000, and 5000 mg/kg) but at every alternate day, survival time appeared to be significantly more prolonged (about 2- and 3- times of the 5-FU treated and untreated control groups). Some pungent constituents present in ZO were shown to exhibit antioxidant cancer preventive activity in experimental carcinogenesis (Keum et al., 2002; Lee and Surh, 1998). [6]-gingerol, reputedly the most active ginger constituent, was only evaluated for its effect on various stages of carcinogenesis, whereas [6]-paradol was demonstrated for antiproliferation activity in liver, pancreatic, prostate, gastric and leukemia cancer cells, and [6]-shogaol (dehydrated [6]-gingerol) for anticancer activity against breast cancer (Pereira et al., 2011).

The anticancer activity of PPF is likely to be due to the activities of various components in the formulation. Many traditional medicine are commonly used as a mixture of parts of various plants for additive or synergistic pharmacological activities. The cytotoxic or antitumor activities of most components of the formulation have previously been reported reported (Cao et al., 2010; Mohamad et al., 2011; Ngo et al., 2010; Padhye et al., 2008). The cytotoxic activities against CL-6, of the two components of PPF, were demonstrated: *Mimusops elengi* Linn. (Flower) (IC_{50} = 48.53 μg/ml), and *Kaempferia galangal* ("Proh hom" in Thai; leaf) (IC_{50} = 37.36 μg/ml) (Mahavorasirikul et al., 2010). The anti-CCA activity of PPF was shown to be moderate in CCA-xenograft nude mouse model (Plengsuriyakarn et al., 2012a). Thymoquinone, the bioactive compound derived from *Nigella sativa* Linn. (commonly known as black seed or black cumin) was shown to exhibit antitumor activities, including antiproliferative and pro-apoptotic effects on cell lines derived from breast, colon, ovary, larynx, lung, myeloblastic leukemia and osteosarcoma (El-Mahdy et al., 2005; Gali-Muhtasib et al., 2004; Roepke et al., 2007). APS-1d, a novel polysaccharide isolated from rhizome of *A. sinensis* (Oliv.) Diels, was shown to exhibit cytotoxic activity towards several cell lines *in vitro* including apoptotic activity was shown (Cao et al., 2010). Theraphin C, a coumarin isolated from the bark of *M. siamensis* Kosterm was shown to exhibit the strongest inhibitory activity on cell proliferation in DLD-1 (colon cancer), MCF-7 (breast adenocarcinoma), HeLa (human cervical cancer), and NCI-H460 (human lung cancer) cell lines (Ngo et al., 2010). Furthermore, several of the formulation components including *Foeniculum vulgare* Mill. Var. dulce Alef (Malo et al., 2011), *Angelica sinensis* (Oliv.) Diels (Wu et al., 2004), *Atractylodes lancea* (Thung.) DC. (Sakurai et al., 1994), *Mesua ferrea* Linn. (Yadav and Bhatnagar, 2010), *Mimuscops elengi* Linn. (Ashok et al., 2010), *Myrisrica fragrans* Houtt. (Akinboro et al., 2011), *Cuminum cyminum* Linn. (El-Ghorab et al., 2010), *Anethum graveolens* Linn. (Shyu et al., 2009), and *Artemisia annua* Linn. (Singh et al., 2011) were reported to possess antioxidant activities. An exhaustive survey of literature revealed that the different species of Artemisia have a vast range of biological activities including antimalarial, cytotoxic, antihepatotoxic, antibacterial, antifungal and antioxidant activity. The antioxidant activity and toxicity towards Molt-4 cells were shown with ethanolic extract of *Artemisia annua* Linn. (Singh et al., 2011). Several flavonoids isolated from plants were also shown possess the potential antioxidant activities. Flavonoids and phenolics are most important groups of plant secondary metabolites with antioxidant activity capable of scavenging free superoxide radicals, anti-aging and reducing the risk of cancer.

CONCLUSION

In conclusion, results obtained from the current study suggest that plants used in Thai traditional medicine for treatment of various ailments may provide reservoirs of promising candidate chemotherapeutics for treatment of

CCA. It seems to hold great potential for in-depth investigation of the active ingredients and molecular mechanisms of action of all the test materials which are responsible for their anti-CCA activities.

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