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A Study on Cytotoxicity, Toxicity and Anticancer Activity of *Zingiber officinale* Roscoe Against Cholangiocarcinoma

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Abstract: Cholangiocarcinoma (CCA) is an uncommon adenocarcinoma which arises from the epithelial cells of the bile ducts. The aim of the study was to investigate the cytotoxicity, toxicity and anticancer activity of the crude ethanolic extract of ginger (*Zingiber officinale* Roscoe) against CCA. To investigate the anti-CCA activity, a total of 80 OV and nitrosamine (OV/DMN)-induced CCA hamsters were fed with the ginger extract at doses of 1000, 3000 and 5000 mg kg^{-1} body weight daily or every alternate day for 30 days. Control groups consisting of 10 hamsters for each group were fed with 5-fluorouracil (positive control) or distilled water (untreated control). Median IC_{50} values for cytotoxicity and anti-oxidant activities of the crude ethanolic extract of ginger were 10.95, 53.15 and 27.86 μg mL^{-1}, respectively. About >10 DNA fragments were visualized and up to 7-9 fold up-regulation of *MDR1* and *MRP3* genes were observed following the exposure to ethanolic extract of ginger. Acute and subacute toxicity tests indicated absence of any significant toxicity at the maximum dose of 5,000 mg kg^{-1} body weight given by intragastric gavage. The survival time and survival rate of the CCA-bearing hamsters were significantly prolonged compared to the control group (median of 54 vs 17 weeks). Results from the *in vitro* and *in vivo* studies indicate promising anticancer activity of the crude ethanolic extract of ginger against CCA with the absence of any significant toxicity. However, MDR1 and MRP3 may be involved in conferring resistance of CCA to the ginger extract.

Key words: Cholangiocarcinoma, cytotoxicity, ginger, *Zingiber officinale* Roscoe, toxicity, hamsters

INTRODUCTION

Cholangiocarcinoma (CCA) is a devastating cancer with increasing worldwide incidence and mortality rates. It is an uncommon adenocarcinoma which arises from the epithelial cells of the bile ducts anywhere along the intrahepatic and extrahepatic biliary tree excluding the papilla of vater and the gall bladder (Mosconi *et al*., 2009). *Opisthorchis Viverrini* (OV) infection is a high risk factor of CCA (Haswell-Elkins *et al*., 1994). The highest incidence rate in the world is observed in the Northeast region of Thailand where the prevalence of infection with OV is also highest (Haswell-Elkins *et al*., 1994; Sriamporn *et al*., 2004). The challenges posed by this often lethal biliary tract cancer are daunting with conventional treatment options being limited and the only hope for long-term survival being that of complete surgical resection of the tumor. Chemotherapeutics for CCA is largely ineffective and clinical efficacy of the standard treatment with 5-Fluorouracil (5-FU) is low. Furthermore, resistance of this type of cancer to chemotherapy and radiotherapy is a major problem (Hejna *et al*., 1998).

Chemotherapy with plant-derived compounds or dietary phytochemicals has emerged as an accessible and promising approach to cancer control and management (Surh, 2003). A growing trend among some cancer patients is to combine conventional therapy with some form of complementary therapy (Vapiwala *et al*., 2006). Ginger is a food plant known worldwide and is equally reputed for its medicinal properties (Shukla and Singh, 2007). It is a herbaceous, rhizomatous perennial plant widely distributed throughout the tropical and subtropical regions. The rhizome of *Zingiber officinale* Roscoe is widely used as a dietary condiment throughout the world. Besides its extensive utilization as a spice, ginger has also

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been used in traditional oriental medicine to ameliorate such symptoms as inflammation, rheumatic disorders, gastrointestinal discomforts, loss of appetite, travel sickness, hypercholesteremia and high level of triglyceride (Chrubasik *et al*., 2005). These diverse pharmacological activities of their major principles have already been confirmed (Surh *et al*., 2002). Several lines of evidence suggest that 6-gingerol is effective in the suppression of the transformation, hyperproliferation and inflammatory processes that initiate and promote carcinogenesis as well as the later steps of carcinogenesis, the angiogenesis and metastasis (Bode *et al*., 2001; Kim *et al*., 2005a, b; Lee *et al*., 2008; Suzuki *et al*., 1997).

It is regarded as a promising chemopreventive dietary agent exhibiting inhibition of cyclooxygenase and lipoxygenase activities (Huang *et al*., 1991; Kiuchi *et al*., 1982, 1992), apoptosis induction (Chauhan, 2002; Lee *et al*., 1998) and anti-tumorigenic effects (Park *et al*., 1998; Surh *et al*., 1999). The pungent vallinoids of ginger, [6]-gingerol and [6]-paradol, exhibit antiproliferation activity in liver, pancreatic, prostate, gastric and leukemia cancer cells (Chen *et al*., 2007; Lee *et al*., 1998; Shukla and Singh, 2007). Furthermore, [6]-shogaol has also been shown to exhibit anticancer activities against breast cancer through the inhibition of cell invasion reduction of matrix metalloproteinase-9 expression (via blockade of nuclear factor activation) (Ling *et al*., 2010), anti-proliferation activity (through disruption of microtubule network of non-small lung epithelium cancer) (Choudhury *et al*., 2010) and anti-invasion on human hepatocellular cell (Weng *et al*., 2010). To the knowledge, there has been no report on the anticancer activity of ginger against CCA. The previous study has demonstrated a promising cytotoxic activity of the ethanolic extract of ginger against CL-6 (CCA cell line obtained from human), HepG2 (Hepatocarcinoma) and Hep-2 (laryngeal carcinoma) cell lines *in vitro* with IC_{50} (concentration that inhibits cell growth by 50%) of <50 μg mL^{-1}.

The aim of the present study was to further investigate the cytotoxic activity of the crude ethanolic extract of ginger in other *in vitro* models (calcein-AM release and Hoechst 33342 assays) as well as its anti-oxidant activity, apoptotic activity and activity on inducing the expression of multidrug resistance genes. Finally, the *in vivo* anticancer activity and toxicity of the crude extract was evaluated in OV/Dimethylnitrosamine (DMN)-induced CCA in a Hamster Model.

MATERIALS AND METHODS

Chemicals and reagents: Commercial grade ethanol was purchased from Labscan Co. Ltd. The cell culture medium Ham-F12, Fetal Bovine Serum (FBS), L-glutamine, Dimethylsulfoxide (DMSO) and the antibiotics streptomycin and penicillin were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Renal epithelium cell growth medium and supplement pack were purchased from Promo cell Co. Ltd. (Germany). About 5-Fluorouracil (5-FU), DPPH (2, 2-Diphenyl-2-Picrylhydrazyl), L-ascorbic acid (vitamin C), Dimethylnitrosamine (DMN) and Tween-80 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of plant extract: Rhizomes of *Zingiber officinale* Roscoe (voucher No. SKP206261501) were obtained from the Applied Thai Traditional Medicine Center, Faculty of Medicine, Thammasat University, Thailand. Preparation of the ethanolic extract of ginger was performed according to the previously described method. The extract was standardized using high-performance liquid chromatography to examine the amount of 6-gingerol which was 243±1 mg g^{-1} of dried rhizome. Chromatographic separation condition used was as follows: Phenonemex™ Luna 5 μM C18 column; mobile phase: a mixture of water and acetonitrile with gradient elution 0 min (55:45), 8 min (50:50), 17 min (35:65), 32 min (0: 100) and 43 min (55:45) at follow rate of 1 mL min^{-1} (Fig. 1).

Cell lines and culture: The CCA cell line CL-6 was used for the *in vitro* assessment of cytotoxicity (calcein-AM

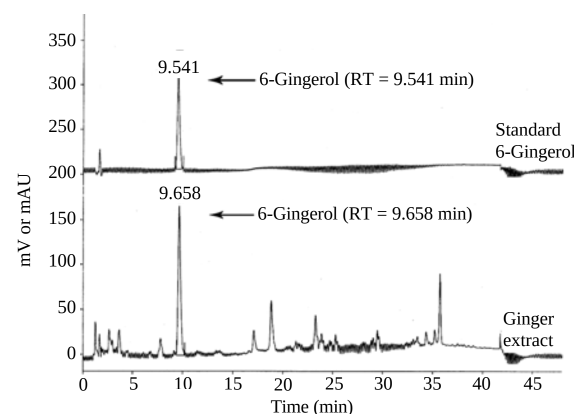

Fig. 1: Standardization using high-performance liquid chromatography to examine the amount of 6-gingerol. Chromatographic separation condition used was as follows: Phenonemex™ Luna 5 μM C18 column; mobile phase: a mixture of water and acetonitrile with gradient elution 0 min (55:45), 8 min (50:50), 17 min (35:65), 32 min (0: 100) and 43 min (55:45) at follow rate of 1 mL min^{-1}

release and Hoecst 33342 assays), anti-oxidant, apoptotic activity and inducing effect on resistance gene expression of the ethanolic extract of ginger. 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 Ham-12 medium supplemented with 10% heated fetal bovine serum and 100 IU mL^{-1} of anti-anti. Assessment of the cytotoxicity of ginger extract 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 medium supplemented with 10% fetal b ovine serum and 100 IU mL^{-1} pen-strep. HRE cell line was purchased from Promo cell Co. Ltd. (Germany) and was cultured in renal epithelial cell growth medium 2 with supplement pack. All cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity.

***In vitro* models for assessing cytotoxic, anti-oxidant and apoptotic activities**

Calcein-AM release assay: CL-6, HepG2 and HRE cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h of incubation, cells were incubated with various concentrations of ethanolic extract of ginger (1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125 and 250 μg mL^{-1}) at 37°C for 24 h. 5-FU (at concentrations of 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250 and 500 μg mL^{-1}) was used as positive control drug. Cells were then resuspended in complete medium and washed 3 times with PBS and incubated with 15 μM calcein-AM at 37°C for 30 min with occasional shaking. Calcein cellular fluorescence was read directly using a plate reader machine at excitation and emission wavelengths of 490 and 530 nm, respectively.

Hoechst 33342 assay: Inhibition of proliferation of CL-6, HepG2 and HRE cells by the ethanolic extract of ginger 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. Wells of H342 plates were washed twice with 250 μL PBS and tapped dry on a tissue paper. Plates were wrapped up and stored at -20°C for DNA quantification with Hoechst 33342. An amount of 100 μL of 0.01% SDS solution was added to each well.

The plates were shaken for 30 min and then refrozen at -70°C. The plates were thawed and 100 μL of a H342 solution (2 μg mL^{-1} Hoechst 33342, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 M NaCl) were added and plates were shaken at 37°C for 1 h (in the dark). The fluorescence intensity was monitored in a plate reader machine at excitation and emission wavelengths of 355 and 460 nm, respectively.

Anti-oxidant activity: The anti-oxidant activity of the ethanolic extract of ginger was determined by measuring radical-scavenging activity of DPPH (2, 2-Diphenyl-2-Picrylhydrazyl) (Szabo *et al*., 2007). Vitamin C (ascorbic acid) was used as a positive control reagent. The extract and vitamin C (at concentrations 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125 and 250 μg mL^{-1}) were added into each well of a 96-wells plate (100 μL each). DPPH solution (0.1 mL DPPH with 3.9 mL methanol) at final concentration of 6×10^{-5} M was then added. The decrease in absorbance compared with the control well was determined at a UV wavelength of 515 nm.

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 anti-oxidant) was calculated as follows:

$$\text{Inhibition}\,(\%) = \left[\frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}})}{\text{Absorbance}_{\text{control}}}\times 100\right]$$

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

Apoptosis assay: The apoptotic activity of the crude ethanolic extract of ginger was determined by electrophoresis of DNA (Hsu *et al*., 2008). CL-6 cells (1×10^6) were treated with various concentrations of the ethanolic extract of ginger (6.25, 12.5, 25, 50 and 100 μg mL^{-1}) at 37°C for 48 h and collected by trypsinization and centrifugation. Pellets were washed twice with PBS and lyzed by DNA lysis buffer (Tris EDTA, pH 8, 5 M NaCl and 0.5 M EDTA). Following centrifugation, supernatant was incubated overnight with proteinase K (0.1 mg mL^{-1}) and RNase (0.2 mg mL^{-1}) at 37°C for 2 h. DNA was collected by precipitation with two volumes of isopropanol in the presence of 3 M sodium acetate. After centrifugation, the DNA pellets were washed overnight with 70% ethanol and air-dried. DNA was separated on 1.8% agarose gel containing 1 mg mL^{-1} ethidium bromide and DNA fragmentation was visualized under a UV lamp. Negative control wells consisted of untreated and cells similarly treated with 50% ethanol. About 100 bp plus DNA ladder was used as a marker.

Induction of the expression of multidrug resistant genes: CL-6, HepG2 and HRE cells (1×10^6) were treated with the ethanolic extract of ginger at concentrations of 25, 50 and 100 μg μL^{-1} for 24 and 48 h. Total RNA was extracted by RNeasy Mini kit (Qiagen, Hilden, Germany) and quantified spectrophotometrically by Nanodrop machine (Thermo Scientific, Wilmington, USA). First-strand cDNA was synthesized from 100 ng total RNA by reverse transcription using oligo-dT primers and reverse transcriptase (Superscript III; Invitrogen, USA) according to the manufacturer's instructions. Relative quantitation of gene expression was measured by real-time PCR. Five sets of primers were used in all reactions to obtain amplification of housekeeping genes control, GAPDH and a specific target gene of interest, Multidrug Resistance 1 (*MDR1*), Multidrug Resistance Protein 1 (*MRP1*), Multidrug Resistance Protein 2 (*MRP2*) and Multidrug Resistance Protein 3 (*MRP3*) genes. Following an initial denaturation step at 94°C for 5 min, 35 cycles of PCR amplification were performed with each consisting of a denaturation step at 94°C for 30 sec, annealing at 62°C for 45 sec and extension at 72°C for 1 min. At the end of the 35 cycles, a 5 min extension phase at 72°C was included to provide complete synthesis. Primers were obtained from Eurofins MWG Operon (Huntsville, ALB, USA). The PCR primers used to amplify *MDR1*, *MRP1*, *MRP2* and *MRP3* genes were 20/20, 23/22, 19/20 and 21/22 nucleotide long oligonucleotides, respectively. The MDR1 sequences of sense and antisense strand primers were 5'GTCTTTGGTGCCATGGCCGT and 5'ATGTCCGG TCGG GTGGGATA, respectively. The MRP1 sequences of sense and antisense strand primers were 5'CTGACAAGCTAGACCATGAATGT and 5'CCTTTGT CCAAGACGATCACCC, respectively.

For MRP2 and MRP3, sequences of sense vs. antisense strand primers were 5'GCCAGATTGGCCCAGC AAA vs. 5'AATCTGACC ACCGGCAGCCT and 5'GGGA CCCTGCGCATGAACCTG vs. 5'TAGGCAAGTCCAGCA TCTCTGG, respectively. GAPDH, 22/21 long oligonucleotides was an internal standard for mRNA expression.

The sequences of sense and antisense strand primers were 5'CAACAGCCTCAAGATCATCAGC and 5'TTCTA GACG GCAGGTCAGGTC, respectively. The fluorescence threshold (Ct) was calculated from $2^{-\Delta\Delta CT}$. The absence of non-specific products was confirmed by the analysis of the melting-point curves.

In vivo model for evaluation of toxicity and anticancer activity

Animals: Syrian golden hamsters, 6-8 weeks of age, weighting 105-120 g used in all experiments were purchased from The National Laboratory Animal Centre of Thailand (Thamavit *et al*., 1978). They were housed under standard conditions and fed with a stock diet and water *ad libitum*. Approval of the study protocol was obtained from the Ethics Committee for Research in Animals, Thammasat University, Thailand.

Acute and subacute toxicity study: Acute and subacute toxicity tests were performed according to the OECD guideline for chemicals. A total of 60 hamsters (5 males and 5 females for each group) were fed (via gastric gavage) with three dose levels of ethanolic extract of ginger (resuspended in a mixture of distilled water and Tween-80, 4:1, v:v), i.e., 1000, 3000 and 5000 mg kg^{-1} body weight. The control hamsters were fed with the mixture of distilled water and Tween-80. Animals were closely observed for awareness, status of mood, motor activity, CNS excitation, posture, muscle tone, reflexes and autonomic signs during the first 30 min, periodically during the first 24 h and then daily for 14 days (acute toxicity) or 30 days (subacute toxicity). For subacute toxicity test, body weight and food and water consumption were recorded daily for 30 days. At the end of the observational period, all animals were sacrificed under ether anesthesia and vital organs (brain, heart, kidneys, liver, spleen, stomach, large and small intestine and lungs) were removed from all animals.

OV-DMN induced CCA in Hamster Model: Assessment of anticancer activity of the crude ethanolic extract of ginger against CCA was performed in 90 hamsters (45 males and 45 females) (Pinlaor *et al*., 2004). The metacercariae of *O. viverrini* were collected as the naturally infected cyprinoids fish captured from an endemic area of Khon Kaen, Northeast Thailand. The parasite species were confirmed under light microscope (Boonmars *et al*., 2009) and were minced and digested with pepsin-HCl then filtrated and washed with normal saline.

Animals were divided into 9 groups (5 males and 5 females each). The first 8 groups were treated as OV-infected groups. Development of CCA was induced by initial feeding of all animals (by gastric gavage) with 50 metacercariae of OV, followed four weeks later by drinking water containing 12.5 ppm of Dimethylnitrosamine (DMN) for eight weeks (Tesana *et al*., 2000). 5-FU was used as a positive control treatment (group 1) and the groups consisting of healthy hamsters (group 9) and OV/DMN-induced hamsters without any treatment (group 2) were served as normal control and untreated control groups, respectively. The occurrence and development of CCA was detected and confirmed by ultrasonography throughout the investigation period and finally by histopathology at autopsy.

Group 1 (5-FU treated, positive control): OV/DMN induced CCA hamsters treated with 5-FU (40 μg kg^{-1} body weight, single intravenous injection).

Group 2 (negative control): OV/DMN-induced CCA hamsters treated with vehicle (a mixture of distilled water and Tween-80) daily for 30 days.

Group 3 (high dose-1): OV/DMN-induced CCA hamsters treated with 5,000 mg kg^{-1} body weight crude ethanolic extract of ginger daily for 30 days.

Group 4 (high dose-2): OV/DMN-induced CCA hamsters, treated with 5,000 mg kg^{-1} body weight crude ethanolic extract of ginger every alternate day for 30 days.

Group 5 (medium dose-1): OV/DMN-induced CCA hamsters treated with 3,000 mg kg^{-1} body weight crude ethanolic extract of ginger daily for 30 days.

Group 6 (medium dose-2): OV/DMN-induced CCA hamsters, treated with 3,000 mg kg^{-1} body weight crude ethanolic extract of ginger every alternate day for 30 days.

Group 7 (low dose-1): OV/DMN-induced CCA hamsters, treated with 1,000 mg kg^{-1} body weight crude ethanolic extract of ginger daily for 30 days.

Group 8 (low dose-2): OV/DMN-induced CCA hamsters, treated with 1,000 mg kg^{-1} body weight crude ethanolic extract of ginger every alternate day for 30 days.

Group 9 (normal control): Healthy (non-CCA induced) hamsters treated with vehicle (a mixture of distilled water and Tween-80) daily for 30 days.

In groups 3-8, the crude extract of ginger was fed to the animals at 12 weeks after induction with OV metacercariae. Schematic diagram summarizing the experimental design is shown in Fig. 2. Body weight and food and water consumption were recorded daily for 30 days. At autopsy, livers and bile ducts were removed

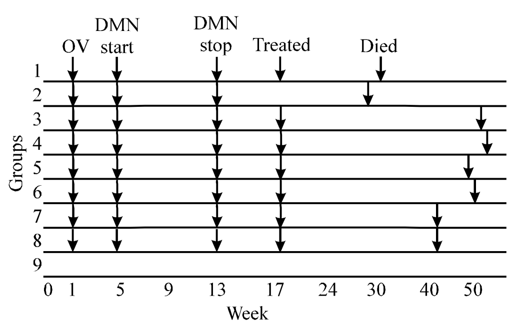

Fig. 2: Schematic diagram representing treatment sequence for the evaluation of the anticancer activity of the ethanolic extract of ginger against CCA in OV/DMN induced CCA in Hamster Model. OV = Infection with 50 *Opisthorchis viverrini* metacercariae; DMN start = Administration of Dimethylnitrosamine; DMN stop = Withdrawal of Dimethylnitrosamine; Treated = Administration of treatment (ethanolic extract of ginger or 5-FU); Died = Time of start of death of hamsters

from all animals. Survival time and survival rate were the primary endpoint parameters for the evaluation of the anticancer activity of the crude ethanolic extract of ginger against CCA.

Autopsy and histopathology: For both toxicity and anticancer activity evaluation, all organs were removed at autopsy and observed macroscopically. Samples were fixed with 10% formalin solution. Specimens were washed in phosphate buffer 3 times then dehydrated in an ascending series of ethanol for 15 min each and embedded in paraffin, followed by sectioning and staining with hematoxylin and eosin (Chaimuangraj *et al*., 2003).

Statistical analysis: Data are expressed as median (range) values. Significant difference between quantitative data of more than two data sets was performed by Kruskal-Wallis test. Significant difference between two quantitative data sets was performed by Mann-Whitney test. Statistical significance level was set at $\alpha = 0.05$ for all tests.

RESULTS

In vitro models for assessing cytotoxic, anti-oxidant and apoptotic activities

Cytotoxic activity: The cytotoxic activity of the ethanolic extract of ginger was investigated against the human CCA cell line CL-6 based on calcein-AM and Hoechst 33342 assays, in comparison with HepG2 and HRE cell lines. In both assays, the extract was found to inhibit cell viability in a dose-dependent manner following 48 h of exposure. The IC_{50} [median (range)] values of the extract in CL-6, HepG2 and HRE cell lines based on the calcein-AM and Hoechst assays are shown in Table 1. The extract exhibited about 1.5-6 times as potent as the positive control drug 5-FU against CL-6 cells in both assays. The cytotoxic activity against CL-6 cells was more specific when compared with HepG2 and HRE cells. The median values of Selectivity Index (SI) of the extract against CL-6, HepG2 and HRE cells were 18.09, 2.76 and 1 in the calcein-AM release assay, respectively. The corresponding SI values in the Hoechst 33342 assay were 4.63, 2.68 and 1, respectively.

Anti-oxidant activity: The *in vitro* DPPH assay demonstrated comparable anti-oxidant activity of the crude ethanolic extracts of ginger with ascorbic acid (positive control) with IC_{50} [median (range)] values of 27.86 (27.05-28.03) and 21.38 (21.25-21.49) μg mL^{-1}, respectively.

Apoptotic activity: The effect of the crude extract of ginger on the induction of apoptosis in CL-6 cells was assessed

Table 1: *In vitro* cytotoxic activity [median (range) values] and Selectivity Index (SI) of the crude ethanolic extract compared with 5-FU against CL-6, HepG2, and HRE cell lines. Data are presented as median (range) values

Cell line	Cytotoxicity assay	Potency/selectivity ($\mu g\ mL^{-1}$)	Ethanolic extract of ginger	5-FU
CL-6	Calcein-AM	IC_{50}	10.95 (10.87-11.12)	89.87 (89.57-90.84)
		SI	18.09	3.19
	Hoechst33342	IC_{50}	53.13 (48.25-55.13)	95.29 (92.84-98.24)
		SI	4.63	3.12
HepG2	Calcein-AM	IC_{50}	71.89 (69.88-73.14)	74.86 (73.42-77.96)
		SI	2.76	3.83
	Hoechst33342	IC_{50}	92.88 (87.15-94.26)	118.60 (115.67-120.19)
		SI	2.68	2.51
HRE	Calcein-AM	IC_{50}	198.15 (196.99-205.67)	286.74 (275.78-286.74)
		SI	1	1
	Hoechst33342	IC_{50}	245.91 (234.87-250.17)	297.39 (289.57-311.87)
		SI	1	1

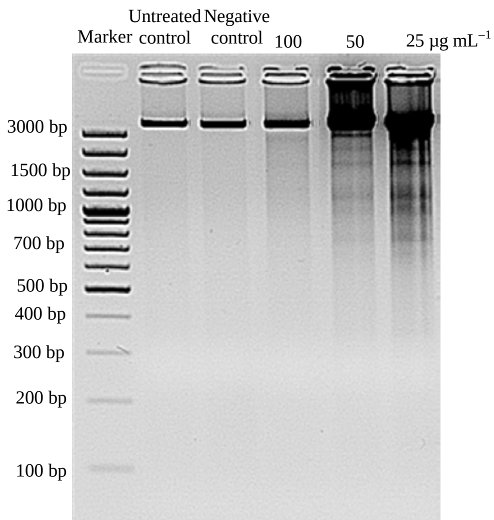

Fig. 3: DNA fragmentation of the ethanolic extract of ginger-treated CL-6 cells. DNA was extracted from untreated control or after treatment with 50% ethanol (negative control) or various concentration of the ethanolic extract of ginger (100, 50 and 25 $\mu g\ mL^{-1}$) for 48 h DNA was electrophoresed in 1.8% agarose and then stained with ethidium bromide. Marker represents the 100 bp plus DNA ladder

by DNA fragmentation assay. Agarose gel electrophoresis showed that treatment with ginger extract resulted in the formation of DNA fragments in CL-6 cells (Fig. 3). About >10 DNA fragments were visualized and the activity was concentration dependent as the proportion of apoptotic cells increased with higher concentrations of the extract.

Induction of the expression of multidrug resistant genes: The inducing effect of different concentrations (20, 50 and 100 $\mu g\ mL^{-1}$) of crude ethanolic extract of ginger on the mRNA expression of different multidrug resistance genes (*MDR1*, *MRP1*, *MRP2* and *MRP*) following exposure to CL-6, HepG2 and HRE cells for 24 and 48 h was investigated using real-time PCR. The expression of *MDR1* gene following 24 and 48 h exposure to the ethanolic extract clearly exhibited both time and concentration-dependency. A quantitative analysis showed that the copy number of *MDR1* and *MRP3* genes were found to be 7-9 folds up-regulated following exposure to the extract compared with the non-exposed CL-6 cell at time zero (Fig. 4). Compared with the non-exposed cells, the expression of MDR1 and MRP3 in HepG2 cells was 5-8 fold up-regulated following exposure to the extract whereas for HRE cells, only MRP3 was found to be about 5 fold up-regulated.

In vivo model for evaluation of toxicity and anticancer activity

Toxicity test: For the acute and subacute toxicity studies, single oral doses of crude ethanolic extract of ginger at all of the 3 levels (1000, 3000 and 5000 mg kg^{-1} body weight) did not cause mortality in any animal (0% mortality) during the investigation period. Only stomach irritation was observed in all animals immediately after feeding them with the extract.

The animals however, recovered from the symptom within 1 h of dosing. The average daily intake of water and food including the average body weight of animals were comparable in all groups. No abnormal histopathology was observed in any vital organ at autopsy.

Development of CCA: Compared with the normal control (group 9), the average body weights of the animals in groups 1-8 were significantly reduced after DMN withdrawal (Fig. 5). Morphology and histology of normal and cancerous hamster are shown in Fig. 6. Histopathological examination of all OV/DMN induced hamsters (groups 1-8) but not in the control group (group 9), confirmed adenocarcinoma and cholangiofibrosis.

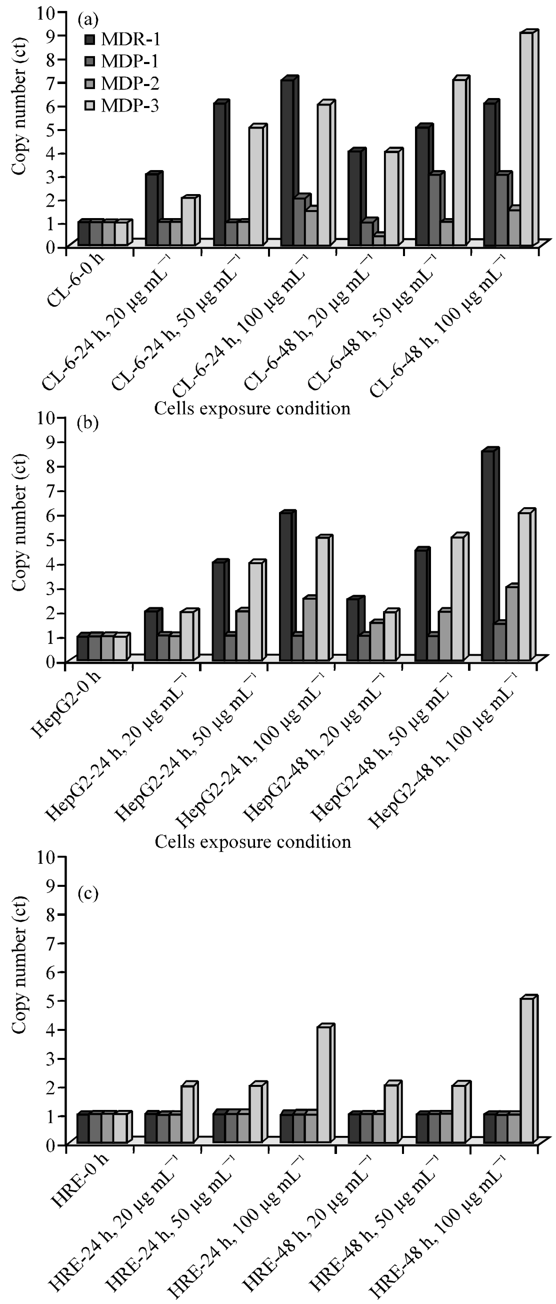

Fig. 4: Expression (gene copy number compared with baseline) of multidrug resistance genes MDR1, MRP1, MRP2 and MRP3 following 24 and 48 h exposure of: a) CL-6; b) Hep-G2 and c) HRE cell lines to the ethanolic extract of ginger at concentrations of 20, 50 and 100 μg mL^{-1}

Anticancer activity against CCA: Significant prolongation of survival time was observed in OV/DMN induced CCA hamsters which were treated with the ethanolic extract of ginger in all treatment regimens compared with both the 5-FU treated (group 1) and untreated control (group 2) group (Table 2). Animals started to die as early as 17 weeks in the untreated control group. Median (range) survival time of CCA-bearing hamsters following feeding with all the six treatment regimens was about 3- and 2-times of the untreated and 5-FU treated (positive control) groups, respectively. The extract when given at alternate days for 30 days was found to significantly prolong the survival time of animals compared with the daily dose regimens (Table 2). Survival rate at week 58 was 0% in all groups except the group treated with the highest dose level of 5,000 mg kg^{-1} every alternate day for 30 days (group 6) of which 2/10 (20%) of animals still survived (20% survival rate at week 58).

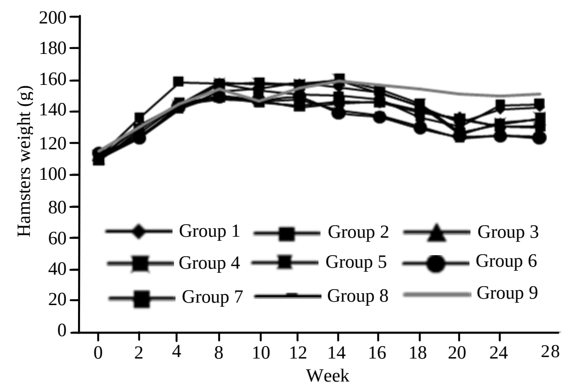

Fig. 5: Body weight (median values) change of the hamsters in the 9 groups: (Group 1: 5-FU treated; Group 2 = Untreated control; Group 3 = High dose-1; Group 4 = High dose-2; Group 5 = Medium dose-1; Group 6 = Medium dose-2; Group 7 = Low dose-1; Group 8 = Low dose-2; and Group 9 = Normal control)

Plant-derived compounds are gaining increasing interest as potential cancer therapeutics including for the treatment of refractory cancers such as CCA. The present study demonstrated the promising cytotoxic and anticancer activities of the crude ethanolic extract of ginger (rhizome of *Zingiber officinale* Roscoe) against CCA at tolerated dose levels. Results of toxicity tests suggest that the extract was well-tolerated when given by oral route even at a high dose of 5,000 mg kg^{-1} body weight to a total of 60 male and female hamsters. All animals survived during the investigation period with no overt sign of morbidity or abnormal locomotor activity. Only reversible gastrointestinal irritation occurred and hamsters recovered within one hour of drug administration.

Different *in vitro* cytotoxicity assays with different endpoints have been employed for screening of potential

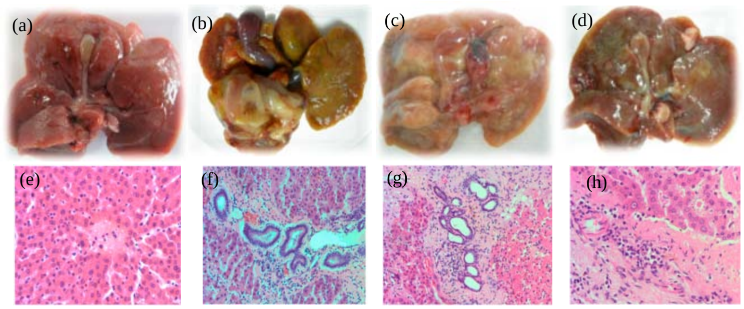

Fig. 6: Liver morphology and histopathological examination under light micrograph of small foci cancer stained with hematoxylin and eosin staining of the normal control (5a and e), 5-FU treated hamster (5b and f), untreated hamster (5c and g) and high dose treated hamster (5d and h)

Table 2: Survival time [median (range) values] of 5-FU treated OV/DMN-induced CCA hamsters (group 1) and treated OV/DMN induced CCA hamsters (groups 2-8)

Control hamsters		Treated hamsters					
		Daily regimen			Every alternate regimen		
5-FU treated[a] (group 1)	Untreated control[b] (group 2)	High dose (group 3)	Medium dose (group 5)	Low dose (group 7)	High dose (group 4)	Medium dose (group 6)	Low dose (group 8)
25.5 (22-28)	17 (16-20)	54 (52-57)	51.5 (49-53)	47 (44-49)	55 (53-58)	51 (50-55)	47 (44-49)

[a]Statistically significant difference with Group 2 ($p<0.001$), Group 3 ($p<0.001$), Group 4 ($p<0.001$), Group 5 ($p<0.001$), Group 7 ($p<0.001$), Group 2 ($p<0.001$), Group 4 ($p<0.001$) and Group 6 ($p<0.001$); [b]Statistically significant difference with Group 3 ($p<0.001$), Group 5 ($p<0.001$), Group 7 ($p<0.001$), Group 2 ($p<0.001$), Group 4 ($p<0.001$) and Group 6 ($p<0.001$)

compounds or plant-derived extract preparation for their anticancer activities. The most commonly used assays involve the use of dye stains that include MTT, calcein-AM and Hoecst 33342 assays. These dyes have some indicator properties allowing them to reveal ongoing cellular processes, providing indirect measure of mitochondria function (MTT), esterase activity (calcein-AM) and DNA binding (Hoecst 33342). In the previous study (Mahavorasirikul *et al*., 2010), the crude ethanolic extract of ginger was shown by MTT assay to exhibit cytotoxic activity against human CCA CL-6 cell line with IC_{50} of 34.26 μg mL. This activity was more selective to CL-6 cell compared with normal cell (HRE) with a selectivity index of 3.5. In the present study, the researchers applied two cytotoxicity assays, i.e., calcein-AM release and Hoecst 33342 to further obtain additional support for the cytotoxicity of the ethanolic extract of ginger.

The median IC_{50} values of the crude extract based on calcein-AM release and Hoecst 33342 assays were 10.95 and 53.15 μg mL^{-1}, respectively. This promising cytotoxic activity of the crude ethanolic extract of ginger was also reported with HepG2 (IC_{50} 9.67 μg mL^{-1}) and Hep2 (32.40 μg mL^{-1}) cell lines (Mahavorasirikul *et al*., 2010). The Selectivity Index (SI) of the extract for CL-6 cells in both assays were relatively high (18.09 and 4.63) compared with 5-FU (3.19 and 3.12). In addition, the extract was more selective to CL-6 cells compared with HepG2 cells (SI 2.76 and 2.68). In support of cytotoxic activity, the results also showed the apoptotic activity of the crude extract, a mechanism by which it induced cytotoxicity. A dose-dependent increase of DNA fragmentation (by DNA gel-electrophoresis) was observed in CL-6 cell line after exposure to the extract compared with control cells. DNA fragmentation and disintegrating apoptotic cells could be observed within 48 h of exposure to 12.5 μg mL^{-1} of the extract. It is interesting to further elucidate the mechanisms of induced apoptosis by major components of ginger, e.g., the possible involvement of caspase enzymes.

Evidence is accumulating which indicates that many chemotherapeutic agents may be selectively toxic to tumor cells because they increase oxidant stress and enhance these already stressed cells beyond their limit (Moungjaroen *et al*., 2006). A potent scavenger of these free radical species may therefore serve as a possible prevention intervention for free radical mediated cancer (Ames *et al*., 1995). The efficacies of various anti-oxidants have been associated with their ability to scavenge free radicals (Wang *et al*., 1999). In the present study, the crude ethanolic extract of ginger was found to exhibit radical scavenging activity with potency comparable to that of the standard compound ascorbic acid with median IC_{50} of 27.86 and 21.38 μg mL^{-1}, respectively.

Resistance of cancerous cells to chemotherapeutic drugs (multidrug resistance) is a major cause of the failure of cancer chemotherapy (Lonning, 2003). The drug resistant phenomenon seems particularly more obvious with CCA (Liu *et al*., 2011). Multiple mechanisms have been hypothesized to play a role in chemotherapeutic drug resistance in cancers and the most important ones are associated with the over-expression of various members of ATP-binding Cassette (ABC) MDR1 and MRPs (Gottesman *et al*., 2002), an increase in detoxification of chemotherapeutic drugs (e.g., Glutathione S-transferases: GST) and Dihydropyrimidine Dehydrogenase: DPD) (Nita *et al*., 1998; Tew, 1994) as well as an alteration of drug targets and suppression of drug-induced apoptosis (Stavrovskaya, 2000). Several lines of evidence suggest that both MDR1 and MRP1 are the major contributors of the multidrug resistance phenotypes observed in a number of tumor cells (Ambudkar *et al*., 2003; Larkin *et al*., 2004).

Many compounds have been investigated for their ability to inhibit the efflux protein function, thus leading to the development of several generations of MDR modulators (Lee, 2004). To assess the mechanism by which the crude ethanolic extract of ginger may modulate cellular efflux in CL-6 cells, the researchers evaluated the expression of the efflux transporters MDR1 and MRPs (MRP1, MRP2 and MRP3) which are the membrane pump proteins that were shown to be expressed in cholangiocytes (Cao *et al*., 1998; Courtois *et al*., 1999; Gigliozzi *et al*., 2000; Rost *et al*., 2001). The extract was found to induce mRNA expression of these genes at varying potencies and patterns of time and concentration-dependency. The expression level was found relatively higher with MDR1 and MRP3 and tended to be time and concentration-dependent. The copy number of cDNA was increased with prolonged exposure time and higher concentrations of the extract. Expression of MRP1 was unchanged following exposure of the cell for 24 h to the extract at concentrations of 20 and 50 μg mL^{-1} but the induction of expression occurred at 50 μg mL^{-1} when the exposure time was increased to 48 h. On the other hand, the mRNA expression of MRP2 was induced with 3 and 5 copies of genes expression when CL-6 cells were exposed to the extract at concentrations of 50 and 100 μg mL^{-1}. Interestingly, the expression was more or less stable when the exposure time was prolonged to 48 h. MRP2 is one of the expressed efflux protein on the apical membrane of hepatocytes and cholangiocytes.

It plays an important role in the biliary clearance of endogenous and exogenous toxic compounds. Association between a common MRP2 variant and CCA risk was reported (Hoblinger *et al*., 2009). In a previous study, MRP1 and MRP3 have been reported to be highly expressed in the five human CCA cell lines while MRP2 was only moderately expressed and MDR1 expression was detected only in one cell line. A strong correlation was also found between the level of MRP3 expression and the IC_{50} values of the anticancer drugs etopocide, doxorubicin and pirarubicin (Tepsiri *et al*., 2005). Consistent with this finding, a strong association between MRP3 mRNA level and response to doxorubicin has been reported in lung cancer (Young *et al*., 1999). The role of MDR1 and MRP3 in chemotherapeutic resistance in CCA patients needs to be further investigated. The data provide evidence that alteration in the expression or function of drug efflux pathways as a mechanism by which the ethanolic extract of ginger may modulate the response to chemotherapy. Increased expression of these membrane pumps enhances drug efflux and may be associated with chemo-resistance and subsequently with poor clinical response.

While recognizing the existence of numerous constituents of a ginger extract, further investigation on the *MDR1* and *MRP3* gene inducing potential of [6]-gingerol, [6]-shogaol and [6]-paradol is required. Several *in vivo* models have been described for assessing the anticancer activity of candidate compounds or extracts from natural products against CCA. These include subcutaneous xenograft model (Fava *et al*., 2005; Jimeno *et al*., 2005; Marienfeld *et al*., 2003) or hepatobiliary CCA model (Bibby, 2004; Johnson *et al*., 2001; Sausville and Burger, 2006; Voskoglou-Nomikos *et al*., 2003) in hamsters or rats after treatment with carcinogens [N-nitrosobis (2-oxopropyl) amine, methylazoxymethyl acetate, dimethylnitrosamine, furan, thioacetamide] or infection with *O. viverrini* (OV) (Iki *et al*., 1998; Imray *et al*., 1992; Jan *et al*., 2004; Maronpot *et al*., 1991; Tesana *et al*., 2000; Thamavit *et al*., 1993) and genetic CCA models (Lai *et al*., 2005; Sirica *et al*., 2008; Xu *et al*., 2006). In the study, OV/DMN induced CCA in Hamster Model was used. Metacercariae of OV was fed (gastric gavage) to animals 4 weeks before DMN. DMN was given in drinking water daily for 8 weeks before the start of treatment with the ethanolic extract of ginger at different dose schedules (groups 3-8) and standard drug 5-FU (group 1). Significant weight loss was observed in all groups induced with OV/DMN compared with the normal control (group 9). The crude ethanolic extract of ginger at all dose regimens markedly prolonged survival time and survival rate of the CCA-bearing hamsters compared with the untreated control group or even with 5-FU treated (positive control) group. The extract when given at highest dose of 5,000 mg kg^{-1} 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^{-1}) 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). A number of plant-derived compounds have been investigated for anticancer activity against CCA, notably triptolide from *Tripterygium wilfordii* (Tengchaisri *et al*., 1998) and the ubiquitous tannic acid (Naus *et al*., 2007). Anticancer activity of triptolide against CCA growth *in vitro* was reported with IC_{50} as low as 0.05 μg mL^{-1}. In addition, the compound showed a significant reduction of tumor growth when given at a total dose of 1.2 mg. Tannic acid, a plant-derived polyphenol was also shown to exert anticancer activity against CCA cells *in vitro* and *in vivo*. These effects include growth inhibition by blocking cell-cycle progression *in vitro* and decreased growth of xenografts in nude athymic mice. With regards to the anticancer activity of gingerol, [6]-gingerol, reputedly the most active ginger constituent has only been evaluated for its effect on various stages of carcinogenesis whereas [6]-paradol has been 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).

CONCLUSION

In this study, results from the present study suggest that the crude ethanolic extract of ginger exhibited *in vitro* cytotoxic, anti-oxidant and apoptotic activities. In addition, *in vivo* study showed promising anticancer activity of the extract against CCA. Its remarkable tumor inhibition effect *in vivo* offers an alternative treatment for CCA when either used alone or in conjunction with other anticancer or immune-modulating agents. Nevertheless, the inducing activity of the extract on the expression of drug resistant genes is of major concern. The molecular and cellular mechanisms of action and resistance of the active principle [6]-gingerol including [6]-shogaol and [6]-paradol in CCA should be further investigated.

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