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Anticancer effects of piperine-free Piper nigrum extract on cholangiocarcinoma cell lines

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Authors	Aman Tedasen, Araya Khoka, Siribhorn Madla, Somchai Sriwiriyajan, Potchanapond Graidist
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Description	Background: Black pepper (Piper nigrum L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free P. nigrum extract (PFPE) inhibited breast cancer in vitro and in vivo. Objective: In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA). Materials and Methods: 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation

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3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation followed by Western blot analysis were used.

Results:

PFPE composed of alkaloid, flavonoid, amide, lignans, opioid, and steroid. This crude extract represented cytotoxic effect against CCA cells which stronger than dichloromethane P. nigrum crude extract and piperine, especially on KKU-M213 (median inhibition concentration [IC 50] at 13.70 μg/ml ...

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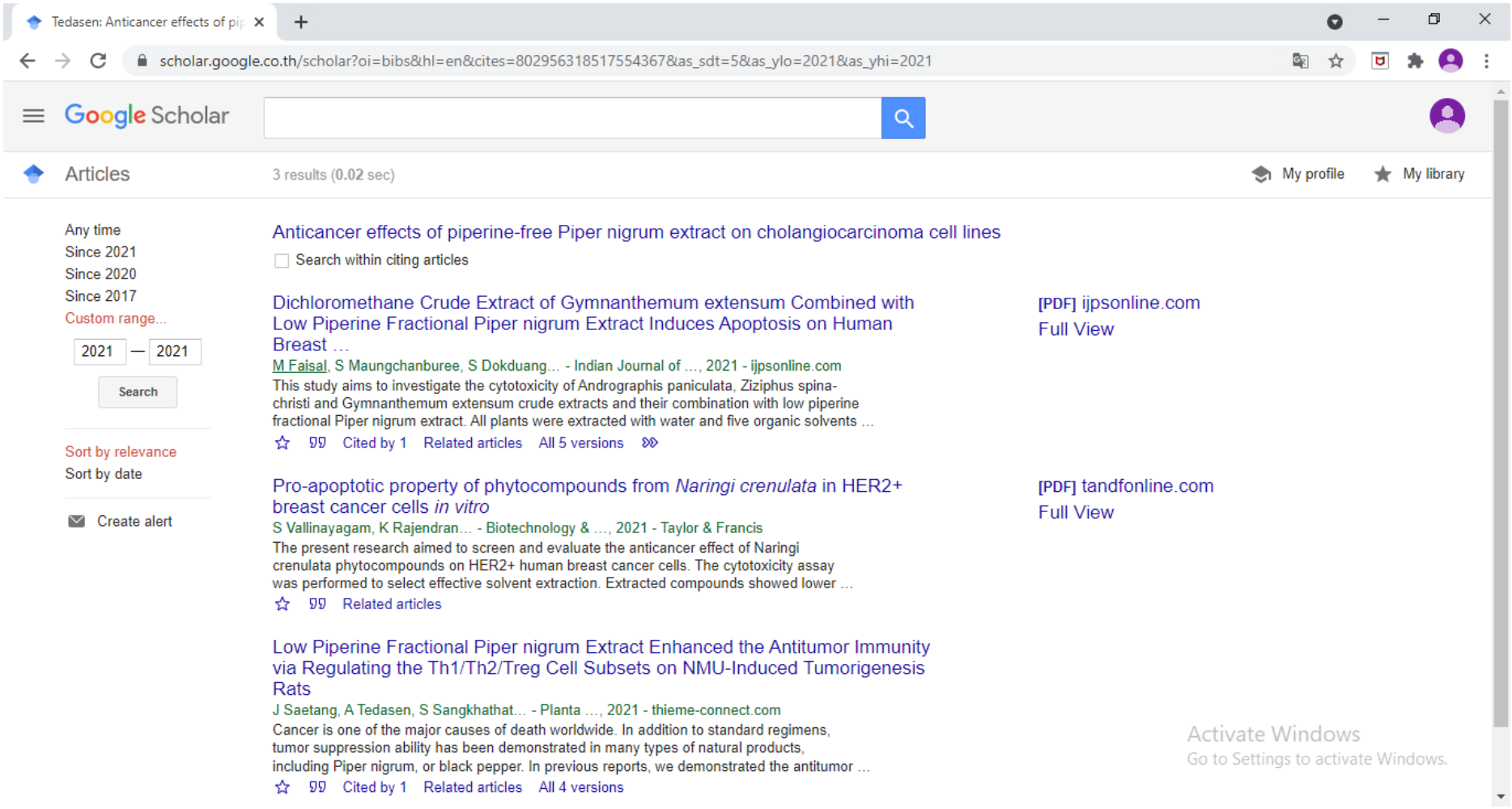
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Dichloromethane Crude Extract of *Gymnanthemum extensum* Combined with Low Piperine Fractional *Piper nigrum* Extract Induces Apoptosis on Human Breast Cancer Cells

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This study aims to investigate the cytotoxicity of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* crude extracts and their combination with low piperine fractional *Piper nigrum* extract. All plants were extracted with water and five organic solvents (methanol, ethanol, dichloromethane, ethyl acetate and hexane). Cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay on three cancer types (breast, colorectal and ovarian cancers) and two non-cancerous cells. The combination among extracts with low piperine fractional *Piper nigrum extract* was separately conducted in several tests including cytotoxicity, apoptosis and multi caspase activity. We found that, the dichloromethane crude extract of *Andrographis paniculata, Ziziphus spina-christi* and *Gymnanthemum extensum* exhibited the strongest cytotoxicity on colorectal cancer cells SW-620 (7.49±0.04 µg. ml^{-1}), breast cancer cells Michigan Cancer Foundation-7 (13.35±0.30 µg. ml^{-1}) and ovarian cancer cells A2780 (15.58±1.81 µg. ml^{-1}), respectively. Gas chromatography mass spectrometry study of dichloromethane crude extract of *Andrographis paniculata, Ziziphus spina-christi* and *Gymnanthemum extensum* identified major compounds including 1-heptatriacotanol (60.29 %) and palmitic acid (26.92 % for dichloromethane crude extract of *Ziziphus spina-christi* and 21.40 % for dichloromethane crude extract of *Gymnanthemum extensum*), respectively. The combination of dichloromethane crude extract of *Ziziphus spina-christi* and low piperine fractional *Piper nigrum* extract at ratio IC_{50}:0.5IC_{50} showed a moderate synergistic effect on Michigan Cancer Foundation-7 cells. Interestingly, the mixture of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract at ratio IC_{50}:IC_{50}, 0.5 IC_{50}:IC_{50} and IC_{50}:0.5 IC_{50} exhibited a synergistic effect on Michigan Cancer Foundation-7 cells. Moreover, combination of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract induced the apoptosis and multi caspase activity in a time dependent manner. In conclusion, dichloromethane crude extract of *Andrographis paniculata, Ziziphus spina-christi* and *Gymnanthemum extensum* displayed potent anticancer activities and the combination of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract can be a promising regimen for an alternative cancer treatment.

Key words: Apoptosis, combination, dichloromethane, Gymnanthemum extensum, Piper nigrum

The Global Cancer Observatory (GLOBOCAN) estimated more than 9 million death cases worldwide owing to cancer. Predictably, it would threaten people worldwide as 17 million cancer deaths per year in 2030[1]. Breast and colorectal cancer are the top three in death rates worldwide[2]. Moreover, ovarian cancer is the deadliest cancer type besides breast and colorectal cancers and causes over 50 % of the death rate[3]. Now a days, several therapies to remedy cancers have been harnessed by clinicians such as surgery, radiotherapy and chemotherapy. Indeed, chemotherapy has been

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acknowledged as the most commonly used therapy. Otherwise, chemotherapy somehow might lead to being ineffective therapy due to drug resistance and cancer recurrence[4].

To date, over 3000 plant species have been revealed their potency as an anticancer agent[5]. *Andrographis paniculata* (Acanthaceae) is a shrub plant grown annually and distributed to tropical and subtropical Asia and also Southeast Asia[6]. *Ziziphus spina-christi* (Rhamnaceae) is an endemic plant of the middle east and distributed from Saharan Oases to West Africa[7]. *Gymnanthemum extensum* (Asteraceae) is a bitter leaf tree distributed to Northeastern Thailand[8]. Previously, *A. paniculata, Z. spina-christi* and *G. extensum* leaves showed high cytotoxicity on several cancer cells, indicated by half-maximal inhibitory concentration (IC_{50}) lower than 20 µg/ml.

The methanolic crude extract of *A. paniculata* leaves exhibited a strong cytotoxicity effect on nasopharynx (KB) and leukemic (P388) cells[9] and colorectal cancer cells[10]. In addition, ethanolic crude extract of *A. paniculata* leaves also showed a high cytotoxicity effect on leukemic cells (HL-60)[11]. Ethyl acetate crude extract of *A. paniculata* leaves inhibited breast cancer (T-47D) and colorectal cancer (WiDr) cells[12]. Furthermore, *Z. spina-christi* leaves extracted in ethanol showed notable cytotoxicity on Michigan Cancer Foundation-7 (MCF-7) cells[13]. Methylene chloride crude extract of *G. extensum* showed high cytotoxicity on liver cancer (HepG2) cells[14].

According to these previous findings, we then carried out a study to investigate the cytotoxicity of these three medicinal plants in a different polarity of organic solvents. The selection of five different solvents used in this present study was referred to their polarity which correlated with the dielectric constant[15]. The polarity of solvent from highest to lowest are methanol (32.7), ethanol (24.6), dichloromethane (9.08), ethyl acetate (6.02) and hexane (1.9), respectively[16]. In addition, consideration of selective anticancer agents of these three medicinal plants was conducted on three cancer types and two non-cancerous cells (Vero and L-929). These normal cells were isolated from normal kidney cells of green African monkey and murine normal fibroblast cells, respectively. Due to the genetic stability and non tumorigenicity feature of Vero and L-929 cells, they are suitable to use in cytotoxicity tests[17,18].

Previously, we reported that low piperine fractional *Piper nigrum* extract (PFPE) exhibited the highest cytotoxicity compared to piperine and dichloromethane extract of *P. nigrum*[19]. This extract was able to inhibit various cancer cells including breast cancer (MCF-7, M.D. Anderson (MDA) Metastasis Breast cancer (MB)-468 and ZR-75-1) and cholangiocarcinoma (KKU-100, KKU-M213, KKU-M055 and human extrahepatic bile duct carcinoma cell line (TFK-1)[20]. Phytochemical compounds including kusunokinin and piperlongumine isolated from PFPE showed high cytotoxicity against breast cancer (MCF-7 and MDA-MB-468) and colorectal cancer (SW-620). PFPE degraded the proteins in cancer progression including signal transducer and activator of transcription 3 (STAT-3), cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF-κB), Topoisomerase II, Cellular myelocytomatosis oncogene (c-myc), cyclin D1 and B-cell lymphoma 2 (Bcl-2)). Moreover, PFPE contained pipericine, 2,4,14-eicosatrienamide, retrofractamide-a and piperanine showed cytoprotective on L-929 cells and hepatoprotective[20-22]. Furthermore, PFPE exhibited very low cytotoxicity against murine fibroblast cells (L-929 cells)[20]. Due to PFPE, showed high cytotoxicity on cancer cells and hepatoprotective activity, we here performed the combination of PFPE together with those three plant extracts to increase cytotoxicity which is higher than a single treatment.

A combination in cancer treatment has been recognized as an effective way to improve successful treatment by increasing the cytotoxicity[23]. The combination of *A. paniculata* with *Silybum marianum* showed a higher percentage of cell inhibition on breast (MCF-7), ovarian (Human cervical tumor cell-SiHa) and liver (human liver cancer cell line-HepG2) cancer cells than each individual treatment[24]. Moreover, *Z. spina-christi* with termite shelter extract concentration at 300 µg/ml exhibited stronger cytotoxicity on cervical cells (Henrietta Lacks-HeLa) than individual treatment[25]. Thus, we hypothesized that *A. paniculata*, *Z. spina-christi* and *G. extensum* would have stronger cytotoxicity once we combined them with PFPE rather than a single treatment. In this report, we conducted the cytotoxicity test of different extracts of *A. paniculata*, *Z. spina-christi* and *G. extensum* leaves individually and their combination with PFPE on cancer cells. Phytocompounds were observed by gas chromatography-mass spectrometry (GC-MS) and the cytotoxicity of three medicinal plants alone and their combination with PFPE on cancer cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Finally, to approach further results whether the regimen could inhibit precisely cancer cells growth only, apoptosis

and multi caspase analysis were performed by flow cytometry analysis.

MATERIALS AND METHODS

Plant materials collection

Dried leaves of *A. paniculata* and dried fruits *P. nigrum* were harvested from Banten Province, Indonesia and Songkhla Province, Thailand, respectively. Both plant specimens were authenticated by Assistant Professor Dr. Supreeya Yuenyongsawad and deposited in the Herbarium of Southern Centre of Thai Traditional Medicine, Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University (PSU), Thailand as a voucher specimen number SKP 001011601 and SKP 146161401, respectively. Dried leaves of *Z. spina-christi* was harvested from East Java Province, Indonesia and taxonomically authenticated by Dr. Nurainas (Chief of ANDA Herbarium, Department of Biology, Andalas University) with letter No. 305/K-ID/ANDA/IX/2018. Dried leaves of *G. extensum* were harvested from Phatthalung Province, Thailand and identified by Professor Dr. Suchada Sukrong and deposited as a voucher specimen (No. SS-PG-001) and kept at the Museum of Natural Medicine, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand.

Chemicals and reagents

In extraction protocol, ethanol (C_2H_5OH), methanol (CH_3OH), dichloromethane (CH_2Cl_2), ethyl acetate ($C_4H_8O_2$) and acetone (C_3H_6O) was purchased from J. T. Baker® (Phillipsburg, NJ, USA). Hexane (C_6H_{14}) was purchased from Reagent Chemical Industry (RCI) LabScan Ltd (Bangkok, Thailand). Furthermore, to make 1x phosphate buffered saline (PBS) for cell culture, the chemical substance such as sodium chloride (NaCl) was purchased in Omnipur® (Idaho, ID, USA). Moreover, sodium phosphate dibasic anhydrous (Na_2HPO_4), potassium chloride (KCl), potassium phosphate (KH_2PO_4) and dimethyl sulfoxide (C_2H_6OS) were purchased from Amresco® (Solon, OH, USA). In the cell culture study, the cell medium and supplements including Roswell Park Memorial Institute Medium (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) powder, L-Glutamine, Fetal Bovine Serum (FBS) and antibiotics (penicillin-streptomycin) were purchased from Gibco™ (Massachusetts, USA). MTT assay and dimethyl sulfoxide (C_2H_6OS) were purchased from GibThai (Bangkok, Thailand) and Amresco®, respectively. Apoptosis and multi caspase reagents were purchased from Becton, Dickinson (BD) Biosciences, Inc (San Jose, CA, USA).

Maceration extraction

3 g of pulverized dried leaves were soaked in 300 ml of five types of organic solvents consists of methanol, ethanol, dichloromethane, ethyl acetate and hexane. Afterward, the mixture was filtered using Whatman Paper No. 1 filter paper. The filtrates were evaporated using a rotor evaporator at 40-45º, 1-3 mbar of vacuum pressure and 40 rpm of rotation. Then, the crude extracts were stored at –20º until used. The stock solution was diluted with dimethyl sulfoxide (DMSO).

Decoction extraction and lyophilization

3 g of pulverized dried leaves were soaked in 300 ml of boiled water within 10 min. Next, the extracts were placed in room temperature. After the temperature of extracts was decreased, the extracts were lyophilized by the freeze-drying method as described by Seong and colleagues[26]. Then, the freeze- dried extracts were kept at –20º until used. The stock solution was diluted with dimethyl sulfoxide (DMSO).

Cell culture

Human breast adenocarcinoma MCF-7 and MDA-MB-231, colorectal (HT-29 and SW-620) and ovarian (A2780 and SKOV-3) were used as cancerous representative cells. MCF-7 (American Type Culture Collection (ATCC®) HTB-22), MDA-MB-231 (ATCC® HTB-26), SKOV-3 (ATCC® HTB-77) and Vero (ATCC® CCL-81) cells were purchased from American Type Culture Collection (Manassas, Virginia, USA). A2780 cells were purchased from AddexBio (San Diego, California, USA). SW-620 cells were kindly donated by Dr. Surasak Sangkhathat, MD from Department of Surgery, Faculty of Medicine, Prince of Songkla University (PSU). Meanwhile, HT-29 cells were kindly given by Dr. Ruedeekorn Wiwattanapatapee from Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, PSU. L-929 cells were kindly provided by Dr. Jasadee Kaewsichan (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, PSU). Vero, A2780 and SKOV-3 cells were cultured in RPMI-1640 culture medium. HT-29, SW-620, MDA-MB-231 and L-929 cells were grown in DMEM. Both media (RPMI-1640 and DMEM) were supplemented with 10 % fetal bovine serum and 1 % of penicillin and streptomycin. All cultures were

maintained at an incubation condition of 37º in 5 % carbon dioxide and at 95 % relative humidity.

Cytotoxicity of individual extract

To investigate the cytotoxicity in a single extract, an MTT colorimetric assay was conducted. Cells in the medium were seeded into 96 well plates (SDL Biosciences, Thailand). MCF-7, MDA-MB-231, SW-620 and SKOV-3 cells were seeded at a density of 2×10^3 cells/well. Vero, L-929 and A2780 cells were seeded at a density of 1×10^3 cells/well and HT-29 cells were seeded at a density of 1.5×10^3 cells/well. All seeded cells were incubated within 24 h for adhering. The cell medium was mixed with extracts with different concentrations (5, 10, 20, 40 and 80 µg/ml) and applied to the cells within 72 h. As a positive control, doxorubicin with different concentrations (0.625, 1.25, 2.5, 5 and 10 µM) was applied to the respective wells. Furthermore, 0.5 % v/v of DMSO (a final concentration) was added to the complete cell medium mentioned as a vehicle. A complete cell medium with cells was mentioned as a negative control. After 72 h, the cell medium was changed by 100 µl MTT for 30 min and then dissolved by 100 µl of DMSO per well to accumulate the formazan salt. The colour formation was generated and observed using a microplate reader spectrophotometer (Spectra Max M5, Molecular Devices) at 570 nm and 650 nm, then the IC_{50} values were calculated using the formula as previously described[19]. Refers to the National Cancer Institute (NCI), a plant extract is considered to possess *in vitro* activity with the IC_{50} value of ≤20 µg/ml[19]. The IC_{50} of the individual extract was used as the initial concentration of the combination study. The selective index (SI) was determined adapted by Sriwiriyajan and colleagues[27] which divided the IC_{50} values of extracts of non-cancerous cells with the IC_{50} values of extracts on cancer cells.

Gas Chromatography/Mass Spectrometry (GC/MS)

To confirm the presence of phytocompounds possessed of notable cytotoxicity, gas chromatography/mass spectrometry analysis was conducted using two analytical instruments, Gas chromatography Agilent 7890B and Agilent 5977A triple quadrupole mass spectrometer (Agilent Technologies Inc, USA). This protocol was performed as previously described[20].

Cytotoxicity of combination extract:

The present study was conducted to investigate the interaction of this combination exhibited synergism, additive, or antagonism in inhibiting cancer cells. The combination extract protocol was conducted by MTT colorimetric assay. This investigation was performed by four ratios including IC_{50} extract: IC_{50} PFPE; $0.5\times IC_{50}$ extract: IC_{50} PFPE; IC_{50} extract: $0.5\times IC_{50}$ PFPE and $0.5\times IC_{50}$ extract: $0.5\times IC_{50}$ PFPE. The combination treatment results were computerized by "CompuSyn" software to procure the combination index values[28]. Manually, the combination index (CI) values were calculated by the Chou-Talalay equation[29].

The interaction of combination was indicated by CI, which estimated from dose effect data, both single and combined drug treatments. The synergism was identified from a CI value based on the criteria as followed: CI<0.1 (synergism); 0.1-0.3 (strong synergism); 0.3-0.7 (synergism); 0.7-0.85 (moderate synergism); 0.85-0.9 (slight synergism); 0.90-1.10 (nearly additive); 1.10-1.45 (slight antagonism); 1.45-3.3 (antagonism); 3.3-10 (strong antagonism) and >10 (very strong antagonism)[30].

Apoptosis assay

In the present study, apoptotic and necrotic cells were observed using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) detection kit (Catalog No. MCH100105, Merck Millipore), following the manufacturer's instruction. Briefly, MCF-7 cells (2×10^5 cells/well) were seeded in 12 well plates. Afterward, the cells were incubated with the combination of dichloromethane crude extract of *Gymnanthemum extensum* DEGE with PFPE at ratio IC_{50}:IC_{50} at various times (0, 24, 48 and 72 h). Treated cells were harvested and cell pellets were incubated in 100 µl of Muse® FITC-Annexin V apoptosis staining kit and 100 µl of propidium iodide for 30 min. The signal of fluorescent was observed by flow cytometry using Muse® Cell Analyzer (Merck Millipore, Germany).

Multi caspase assay:

The observation of multi caspase activity was performed by Muse® multi caspase assay kit (Catalog No. MCH100109, Merck Millipore), following the manufacturer's instruction. The MCF-7 cells were seeded into 12 well plates at a density of 2×10^5 cells/well. Afterward, the cells were treated by the combination of DEGE and PFPE (ratio IC_{50}:IC_{50}) at various times (0, 24, 48 and 72 h). The cells were harvested and cell pellets were resuspended in 5 µl of Muse® Multi caspase assay for 30 min followed by

150 µl of 7-Aminoactinomycin D (7-AAD) within 15 min. The fluorescent marks of multi caspase and 7-AAD were completely identified by the Muse® Cell Analyzer (Merck Millipore, Germany).

Statistical analysis

All experiments were done in triplicate of each experiment and the data was calculated from three independent experiments. The IC_{50} values were expressed as the mean±standard deviation (SD). The percentages of apoptosis and multi caspase were presented as an average and (SD). All graphs were created by GraphPad Prism 8. The apoptosis and multi caspase data were statistically analyzed by the Student t-test to compare among the treated group and control group using Microsoft excel software. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cancer have been a health concern caused by free radicals and carcinogens, which have been naturally and artificially established to harm Deoxyribonucleic acid (DNA)[31]. Until now, many attempts for precise and effective cancer anticancer therapy were investigated and recognized as complementary alternative medicine (CAM),due to the fear of society against synthetic drugs detrimental effects. One of interesting CAM is to use plant extracts as an anticancer therapy. *A. paniculata* leaf was reported to possess of anticancer activity.

According to results in Table 1, we selected dichloromethane extract of *A. paniculata* (DEAP) for the next experiment due to its cytotoxicity on both HT-29 (8.93±0.52 µg. ml^{-1}) and SW-620 cells (7.49±0.04 µg. ml^{-1}). Even though methanolic extract of *A. paniculata* (MEAP) also showed strong cytotoxicity on SKOV-3 cells (7.60±0.72 µg. ml^{-1}), we rather considered the dichloromethane extract as the extract which can affect the two cell types, than methanolic one where only one cell type affected. In this present study, we also used high polar solvent such as methanol and ethanol. Empirically, organic solvents with higher dielectric constant prefer to dissolve and interact

TABLE 1: CYTOTOXICITY OF *A. paniculata* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS

Cell Line	IC_{50} value (µg/ml) (µM)[a]						
	FEAP (SI_{Vero}, SI_{L-929})	MEAP (SI_{Vero}, SI_{L-929})	EEAP (SI_{Vero}, SI_{L-929})	DEAP (SI_{Vero}, SI_{L-929})	EAAP (SI_{Vero}, SI_{L-929})	HEAP (SI_{Vero}, SI_{L-929})	DOX (SI_{Vero}, SI_{L-929})
Breast cancer							
MCF-7	Not inhibited (ND, ND)	14.97±1.56 (1.22, 0.72)	18.44±1.71 (0.77, 2.43)	26.77±2.18 (0.53, 0.93)	21.64±0.93 (0.69, 0.95)	47.46±0.93 (ND, ND)	1.15±0.07 (6.84, 1.38)
MDA- MB-231	Not inhibited (ND, ND)	12.54±1.38 (1.46, 0.86)	21.49±0.21 (0.66, 2.08)	24.03±0.40 (0.59, 1.04)	19.06±1.15 (0.79, 1.07)	Not inhibited (ND, ND)	1.57±0.23 (5.02, 1.01)
Colorectal cancer							
HT-29	Not inhibited (ND, ND)	13.77±1.61 (1.33, 0.78)	12.45±0.05 (1.13, 3.59)	8.93±0.52 (1.58, 2.80)	13.17± 0.52 (1.14, 1.56)	Not inhibited (ND, ND)	2.53±0.56 (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	12.39±1.61 (1.47, 0.87)	12.08±0.82 (1.17, 3.70)	7.49±0.04 (1.89, 3.34)	10.31±0.32 (1.46, 1.99)	39.40±0.42 (ND, ND)	3.15±0.62 (2.50, 0.5)
Ovarian cancer							
A2780	Not inhibited (ND, ND)	15.29±0.68 (1.19, 0.70)	20.51±1.60 (0.69, 2.18)	10.98±0.48 (1.29, 2.28)	13.53±1.28 (1.11, 1.51)	34.27±2.31	2.33±0.18 (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	7.60±0.72 (2.40, 1.41)	30.6±0.93 (0.46, 1.46)	19.34±0.15 (0.74, 1.29)	17.23±1.28 (0.87, 1.19)	Not inhibited (ND, ND)	1.64±0.19 (4.80, 0.97)
Non-cancerous							
Vero	Not inhibited	18.27±1.22	14.11 ±1.59	14.14 ±0.29	15.02 ±0.94	Not inhibited	7.88±0.43
L-929	Not inhibited	10.75 ±0.27	44.74 ±2.44	25.02 ±1.07	20.48 ±1.34	Not inhibited	1.59±0.18

Data represented mean±SD from three independent experiments. SI_{Vero} or SI_{L-929} cells are selectivity index, calculated by dividing IC_{50} of Vero or L-929 with IC_{50} cancer cells. [a]Doxorubicin concentration was in a unit of µM, Not inhibited: IC_{50} not observed at the maximum concentration at 80 µg/ml, ND: not determined, FEAP: freeze-dried extract of *A. paniculata*, MEAP: methanol extract of *A. paniculata*, EEAP: ethanol extract of *A. paniculata*, DEAP: dichloromethane extract of *A. paniculata*, EAAP: ethyl acetate extract of *A. paniculata*, HEAP: hexane extract of *A. paniculata*

with polar compounds. Correlating with the extracted phytocompounds, methanol is capable to dissolve antioxidant compounds such as phenol and polyphenol which is proved by the total antioxidant contents[32]. This information led to an implication that the antioxidant may play a role in cytotoxicity from MEAP.

Previously, the dichloromethane extract of *A. paniculata* leaves showed considerable cytotoxicity on HT-29 cells[33]. Another promising extract, dichloromethane extract of *Z. spina-christi* leaf (DEZSC), also showed notable cytotoxicity towards breast and ovarian cancer cells, MCF-7 cells (13.35±0.30 µg. ml^{-1}) and A2780 cells (14.64±1.51 µg. ml^{-1}), shown in Table 2. Apart from the dichloromethane extract, a previous investigation by Farmani and colleagues revealed that the methanol extract of *Z. spina-christi* exhibited notable cytotoxicity on MCF-7 cells[13]. In addition, an endemic plant of Northeastern Thailand, *G. extensum*, extracted by dichloromethane (abbreviated as DEGE) demonstrated strong cytotoxicity against MCF-7 (15.58±1.81 µg. ml^{-1}) and A2780 cells (10.08±0.04 µg. ml^{-1}) (Table 3). The dichloromethane extract of *Vernonia extensa* (synonym of *G. extensum*) was additionally reported for strong cytotoxicity on HepG2 (liver carcinoma cells), HuCCA-1 (cholangiocarcinoma cells), A549 (lung cancer cells) and MOLT-3 (leukemic cells)[14]. However, the freeze-dried extract of these three plants has no cytotoxic effect on tested cells, which could bencaused by freeze-drying process which damaged the phytocompounds structure and finally led to the losing activity of compounds after the rehydration process[34]. In this study, Vero and L-929

TABLE 2: CYTOTOXICITY OF *Z. spina-christi* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS

Cell Line	IC_{50} value (µg/ml) (µM)[a]						
	FEZSC (SI_{Vero}, SI_{L-929})	MEZSC (SI_{Vero}, SI_{L-929})	EEZSC (SI_{Vero}, SI_{L-929})	DEZSC (SI_{Vero}, SI_{L-929})	EAZSC (SI_{Vero}, SI_{L-929})	HEZSC (SI_{Vero}, SI_{L-929})	DOX (SI_{Vero}, SI_{L-929})
Breast cancer							
MCF-7	Not inhibited (ND, ND)	107.19±1.64	Not inhibited (ND, ND)	13.35±0.30 (1.58, 3.52)	57.63±0.25 (0.92, ND)	Not inhibited (ND, ND)	1.15±0.07 (6.84, 1.38)
MDA-MB-231	Not inhibited (ND, ND)	80.83±1.65 (ND, ND)	Not inhibited (ND, ND)	38.12±0.23 (0.55, 1.23)	55.13±0.44 (0.96, ND)	Not inhibited (ND, ND)	1.57±0.23 (5.02, 1.01)
Colorectal cancer							
HT-29	Not inhibited (ND, ND)	39.01±2.65 (ND, ND)	82.35±11.96 (ND, ND)	17.02±0.69 (1.24, 2.76)	96.46±12.08 (0.55, ND)	Not inhibited (ND, ND)	2.53±0.56 (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	77.91±2.04 (ND, ND)	40.73±0.73 (ND, ND)	23.2±1.08 (0.91, 2.03)	39.67±2.85 (1.34, ND)	Not inhibited (ND, ND)	3.15±0.62 (2.50, 0.5)
Ovarian cancer							
A2780	Not inhibited (ND, ND)	18.54±2.33 (ND, ND)	36.23±1.27 (ND, ND)	14.64±1.51 (1.44, 3.21)	53.86±2.46 (0.99, ND)	Not inhibited (ND, ND)	2.33±0.18 (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	44.45±0.79 (0.47, 1.06)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	1.64±0.19 (4.80, 0.97)
Non-cancerous							
Vero	Not inhibited	Not inhibited	Not inhibited	21.05±0.18	53.11±0.16	Not inhibited	7.88±0.43
L-929	Not inhibited	Not inhibited	Not inhibited	47.03±2.91	Not inhibited	Not inhibited	1.59±0.18

Data represented mean±SD from three independent experiments. SI_{Vero} or SI_{L-929} cells are selectivity index, calculated by dividing IC_{50} of Vero or L-929 with IC_{50} cancer cells. [a]Doxorubicin concentration was in a unit of µM, Not inhibited: IC_{50} not observed at the maximum concentration at 80 µg/ml, ND: not determined, FEZSC: freeze-dried extract of *Z. spina-christi*, MEZSC: methanol extract of *Z. spina-christi*, EEZSC: ethanol extract of *Z. spina-christi*, DEZSC: dichloromethane extract of *Z. spina-christi*, EAZSC: ethyl acetate extract of *Z. spina-christi*, HEZSC: hexane extract of *Z. spina-christi*

cells were used for determining SI score by dividing IC_{50} of non-cancerous cell lines with IC_{50} of cancer cell lines. Our results demonstrated SIL929 values of DEAP and DEZSC more than 2 on HT-29, SW-620, A2780 and MCF-7 cells. According to Koch and colleagues, SI score of less than 2.0 indicates toxicity to non-cancerous cell lines[35]. However, several extracts could not be calculated for the cytotoxicity at the maximum concentration because the extract did not show an inhibitory effect on cancer cells. These results were noted as "Not determined (ND)", shown in Tables 1-3.

Due to strong cytotoxicity exhibited by DEAP, DEZSC and DEGE, we decided to perform a phytochemical characterization. Two major DEAP compounds were 1-heptatriacotanol (60.29 %, alcohol) and androsta-1,4-dien-3-one,6,17-dihydroxy-,(6β,17β) (32.27 %, steroid). Furthermore, the highest amount in our DEZSC was palmitic acid (26.92 %, fatty acid). This chemical compound contained anticancer activity through intercalating with DNA topoisomerase-I[36]. DEGE displayed similar chemical composition with DEZSC, however, several compounds only found in DEGE were neophytadiene, phytol, linoleic acid, hentriacontane, phytol acetate and heneicosane, in which these compounds were reported for anticancer property[37-42]. The limitation of characterization was marked since GC-MS is able to detect only volatile compounds. However, other chemical contents can be analyzed through other spectroscopic methods with a linbrary of spectra to compare with the observed compounds[43]. We suggest phytochemical screening techniques such as UltraViolet-Visible spectroscopy[44], Infrared (IR) spectroscopy[45] and Liquid Chromatography Mass Spectroscopy (LC-MS) [46] to analyze other phytocompounds of DEAP, DEZSC and DEGE for further study.

Our individual extract cytotoxicity test revealed that DEAP, DEZSC and DEGE possessed high cytotoxicity. In this study, we attempted to enhance the cytotoxicity of DEAP, DEZSC and DEGE by combining them with PFPE. In the previous study using PFPE, *P. nigrum* dichloromethane crude extract and piperine showed IC_{50} values of 7.45, 23.46 and >20 μg. ml^{-1}, respectively[19]. Based on our previous results, *P. nigrum* extract containing piperine increased IC_{50} which attenuated the cytotoxic effect on cancer

TABLE 3: CYTOTOXICITY OF *G. extensum* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS

Cell Line	IC_{50} value (μg/ml) (μM)[a]						
	FEGE (SI_{Vero}, SI_{L-929})	MEGE (SI_{Vero}, SI_{L-929})	EEGE (SI_{Vero}, SI_{L-929})	DEGE (SI_{Vero}, SI_{L-929})	EAGE (SI_{Vero}, SI_{L-929})	HEGE (SI_{Vero}, SI_{L-929})	DOX (SI_{Vero}, SI_{L-929})
Breast cancer							
MCF-7	Not inhibited (ND, ND)	26.34±0.53 (1.27, 1.72)	23.67±1.81 (1.09, 2.01)	15.58±1.81 (0.59, 1.18)	25.56±0.60 (1.06, 1.20)	Not inhibited (ND, ND)	1.15±0.07 (6.84, 1.38)
MDA- MB-231	Not inhibited (ND, ND)	51.18±2.07 (0.65, 0.88)	57.84±1.31 (0.45, 0.82)	20.78±2.01 (0.44, 0.88)	23.15±1.57 (1.17, 1.33)	Not inhibited (ND, ND)	1.57±0.23 (5.02, 1.01)
Colorectal cancer							
HT-29	Not inhibited (ND, ND)	25.35±1.94 (1.32, 1.78)	29.68±2.04 (0.87, 1.60)	19.86±0.61 (0.46, 0.92)	33.63±2.16 (0.81, 0.91)	Not inhibited (ND, ND)	2.53±0.56 (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	23.27±1.54 (1.43, 1.94)	17.03±0.09 (1.52, 2.79)	20.09±2.03 (0.46, 0.91)	27.83±2.20 (0.97, 1.10)	Not inhibited (ND, ND)	3.15±0.62 (2.50, 0.5)
Ovarian cancer							
A2780	Not inhibited (ND, ND)	16.12±1.40 (2.07, 2.81)	41.21±1.23 (0.63, 1.15)	10.08±0.04 (0.91, 1.82)	15.22±0.58 (1.78, 2.02)	43.16±1.51 (ND, ND)	2.33±0.18 (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	29.95±1.12 (0.31, 0.61)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	1.64±0.19 (4.80, 0.97)
Non-cancerous							
Vero	Not inhibited	33.39±1.59	25.90±0.47	9.16±0.8	27.08 ±3.03	Not inhibited	7.88±0.43
L-929	Not inhibited	45.23±2.43	47.57 ±0.35	18.37±0.66	30.73 ±3.32	Not inhibited	1.59±0.18

Data represented mean±SD from three independent experiments. SI_{Vero} or SI_{L-929} cells are selectivity index, calculated by dividing IC_{50} of Vero or L-929 with IC_{50} cancer cells. [a]Doxorubicin concentration was in a unit of μM, Not inhibited: IC_{50} not observed at the maximum concentration at 80 μg/ml, ND: not determined, FEGE: freeze-dried extract of *G. extensum*, MEGE: methanol extract of *G. extensum*, EEGE: ethanol extract of *G. extensum*, DEGE: dichloromethane extract of *G. extensum*, EAGE: ethyl acetate extract of *G. extensum*, HEGE: hexane extract of *G. extensum*

cells. Moreover, phytocompounds of PFPE such as piperanine, pipericine, 2,4,14-eicosatrienamide and retrofractamide-A showed hepatoprotective activity which may protect non-cancerous cell lines from cytotoxic compounds[20]. Thus, we combined those three extracts with PFPE. The results showed that DEAP combination with PFPE provided antagonistic effect for the whole ratio against HT-29 and SW-620 cells. The combination of DEZSC with PFPE had an antagonistic effect on MCF-7 and A2780 cells. The observation on antagonistic effect emphasizes that phytocompounds of DEAP and DEZSC were not able to collaborate with compounds of PFPE. Steroids, fatty alcohols and fatty acids were detected in DEAP and DEZSC, from GC-MS results (fig. 1). Cholesterol is an initial form for synthesizing steroids. Meanwhile, fatty alcohols and fatty acids are the derivatives of lipids that can generate cholesterols. In contrast, caryophyllene which contained in PFPE exhibited anti-hypercholesterolemic or hyperlipid preventive activity[47]. Caryophyllene could additionally attenuate lipid accumulation on hepatic tissue which linked to the stimulation of AMP-activated protein kinase (AMPK) phosphorylation, a crucial enzyme in lipid metabolism. Caryophyllene also induces degradation of fatty acid synthase (FAS)[48]. The different roles in caryophyllene and lipid-derivatives (fatty acids, steroids and fatty alcohols) could pinpoint the antagonistic effect of this combination (Tables 4-7).

Surprisingly, our investigation revealed that a combination of DEGE with PFPE remarkably showed synergistic interaction on MCF-7 cells. We had tried to identify possible synergic chemical compounds. As we mentioned previously, palmitic acid and kusunokinin showed intercalation to DNA topoisomerase-I together with PFPE, enabling degradation of DNA topoisomerase-II[36,49]. Due to their important role as a DNA controller, DNA topoisomerase-I and DNA topoisomerase-II are targeted for promising anticancer treatment[50,51]. A synergistic effect against MCF-7 cells from a PFPE-combined DEGE led to a further study for the relevant information to strengthen our hypothesis that MCF-7 cells could not escape from cell death since it was attacked by several death pathways of this combination. These expected mechanisms accordingly convinced us to conduct a study regarding an apoptotic (cell death) process.

Apoptosis and multi caspase activity experiments were investigated using annexin V/propidium iodide assay and multi caspase/7-AAD assay. In the assay, the total apoptotic cells were obtained from a summation of early

TABLE 4: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *A. paniculata* (DEAP) IDENTIFIED THROUGH GC-MS ANALYSIS

RT (min)	Identified compound	Formula	Molecular mass (g mol^{-1})	Area (%)	Nature of compound
17.34	Furfuryl alcohol	$C_5H_{10}O_2$	102.13	0.01	Alcohol
21.28	Octadecane	$C_{18}H_{38}$	254.5	0.02	Hydrocarbon
29.72	Neophytadiene	$C_{20}H_{38}$	278.5	1.2	Terpene
29.83	6,10,14-trimethyl pentadecane-2-one	$C_{18}H_{36}O$	268.5	0.53	Terpene
31.24	2-Methyltetradecanal	$C_{15}H_{30}O$	226.4	0.03	Aldehyde
32.17	Palmitic acid	$C_{16}H_{32}O_2$	256.42	1.24	Fatty acid
33.3	2-octylacolein	$C_{11}H_{20}O$	168.28	0.01	Hydrocarbon
34.74	3,7-Dimethyl-2,3-epoxyoctanal	$C_{10}H_{18}O_2$	170.25	0.01	Aldehyde
38.16	Tricosane	$C_{23}H_{48}$	324.6	0.03	Hydrocarbon
42.09	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	$C_{24}H_{38}O_4$	390.6	0.25	Phthalate
47.09	2-oxobutanoic acid neopentyl ester	$C_9H_{16}O_3$	172.22	0.01	Carboxylic acid
49.91	1-Heptatriacotanol	$C_{37}H_{76}O$	537	60.29	Alcohol
50.14	α-Tocopherol	$C_{29}H_{50}O_2$	430.7	0.05	Tocopherol
50.45	Androsta-1,4-dien-3-one, 6,17-dihydroxy-, (6ß,17 ß)	$C_{19}H_{26}O_3$	302.4	32.27	Steroid
51.59	Stigmasterol	$C_{29}H_{48}O$	412.7	0.98	Steroid
52.29	γ-Sitosterol	$C_{29}H_{50}O$	414.7	3.04	Terpene
52.65	ß-Amyrin	$C_{30}H_{50}O$	426.7	0.02	Terpene

RT: Retention time

TABLE 5: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *Z. spina-christi* (DEZSC) IDENTIFIED THROUGH GC-MS ANALYSIS

RT (min)	Identified compound	Formula	Molecular mass (g mol-1)	Area (%)	Nature of compound
28.06	Myristic acid	$C_{14}H_{28}O_2$	228.37	1.33	Fatty acid
29.64	Neophytadiene	$C_{20}H_{38}$	278.52	7.91	Terpene
29.75	6,10,14-trimethyl pentadecane-2-one	$C_{18}H_{36}O$	268.48	1.79	Terpene
30.14	Phytol, acetate	$C_{22}H_{42}O_2$	338.57	1.28	Terpene
30.49	Phytol	$C_{20}H_{40}O$	296.53	4.07	Terpene
32.22	Palmitic acid	$C_{16}H_{32}O_2$	256.42	26.92	Fatty acid
35.31	Linoleic acid	$C_{18}H_{32}O_2$	280.46	6.76	Fatty acid
35.41	Linolenic acid	$C_{18}H_{30}O_2$	278.43	11.22	Fatty acid
35.81	Stearic acid	$C_{18}H_{36}O_2$	284.48	3.8	Fatty acid
38.93	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	324.54	0.71	Furan hydrocarbon
41.99	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.56	0.88	Phthalate
46.04	Squalene	$C_{30}H_{50}$	410.72	1.68	Terpene
46.37	α-Tocospiro A	$C_{29}H_{50}O_4$	462.7	1.34	Tocopherol
46.67	α-Tocospiro B	$C_{29}H_{50}O_4$	462.7	1.44	Tocopherol
46.99	n-Tetracosanol-1	$C_{24}H_{50}O$	354.65	6.07	Fatty alcohol
48.97	γ-Tocopherol	$C_{28}H_{48}O_2$	416.68	1.11	Tocopherol
49.63	Octacosanol	$C_{28}H_{58}O$	410.76	5.26	Fatty alcohol
50.01	Vitamin E	$C_{29}H_{50}O_2$	430.71	0.77	Tocopherol
51.08	Campesterol	$C_{28}H_{48}O$	400.68	1.04	Steroid
51.49	Stigmasterol	$C_{29}H_{48}O$	412.69	4.91	Steroid
52.19	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	414.7	5.24	Steroid
52.83	Lupenone	$C_{30}H_{48}O$	424.7	2.91	Terpene
53.09	Lupeol	$C_{30}H_{50}O$	426.72	1.91	Terpene

RT: Retention time

TABLE 6: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *G. extensum* (DEGE) IDENTIFIED THROUGH GC-MS ANALYSIS

RT (min)	Compound name	Formula	Molecular mass (g mol-1)	Area (%)	Compound nature
15.68	1,2-Di-tert-butylbenzene	$C_{14}H_{22}$	190.32	0.08	Phenylpropane
17.92	2-Chlorohexylacetate	$C_8H_{15}ClO_2$	178.66	0.02	Carboxylic acid
22.1	Pentadecane	$C_{15}H_{32}$	212.41	0.02	Hydrocarbon
22.49	5-Isopropyl-4-(trifluoromethyl)-1H-pyrimidin-2-one	$C_8H_9F_3N_2O$	206.17	0.04	Alkaloid
22.85	Dihydroactinidiolide	$C_{11}H_{16}O_2$	180.24	0.47	Benzofuran
27.44	Heptanoic acid, tridecafluoro-heptyl ester	$C_{14}H_{15}F_{13}O_2$	462.25	0.02	Fatty acid ester
28.14	Loliolide	$C_{11}H_{16}O_3$	196.24	1.64	Benzofuran
29.62	1-dodecanol	$C_{12}H_{26}O$	186.33	0.05	Fatty alcohol
29.72	Neophytadiene	$C_{20}H_{38}$	278.5	19.36	Terpene
29.83	6,10,14-trimethyl pentadecane-2-one	$C_{18}H_{36}O$	268.5	1.93	Terpene
30.21	Phytol	$C_{20}H_{40}O$	296.5	13.36	Terpene
31.23	2-Methyloctanal	$C_9H_{18}O$	142.24	0.04	Aldehyde
32.12	1,2-Benzenedicarboxylic acid, dibutyl ester	$C_{16}H_{22}O_4$	278.34	0.19	Benzoic acid ester
32.24	Palmitic acid	$C_{16}H_{32}O_2$	256.42	21.4	Fatty acid
34.42	Pentatriacontane	$C_{35}H_{72}$	492.9	0.34	Hydrocarbon
35.36	Linoleic acid	$C_{18}H_{32}O_2$	280.4	6.7	Fatty acid
35.48	Linolenic acid	$C_{18}H_{30}O_2$	278.4	12.08	Fatty acid
36.52	4-Tridecen-2-ynal, (Z)-	$C_{13}H_{20}O$	192.3	0.06	Hydrocarbon
43.99	3-Penten-2-one	C_5H_8O	84.12	0.05	Ketone

RT: Retention time

TABLE 7: COMBINATION INDEX OF EXTRACT WITH PFPE AGAINST CANCER CELL LINES

Combination	Cell line	Ratio Extract:PFPE	Extract Conc. (µg/ml)	PFPE Conc. (µg/ml)	% inhibition	CI	Interaction
DEAP+PFPE	HT-29	IC_{50}:IC_{50}	8.93	17.63	82.35±1.13	1.12±0.04	Slight antagonism
		0.5 IC_{50}:IC_{50}	4.46	17.63	54.89±1.34	1.86±0.05	Antagonism
		IC_{50}:0.5 IC_{50}	8.93	8.82	47.37±2.45	1.74±0.08	Antagonism
		0.5 IC_{50}:0.5 I C_{50}	4.46	8.82	34.21±0.24	1.73±0.01	Antagonism
	SW-620	IC_{50}:IC_{50}	7.49	11.3	81.20±1.55	1.09±0.06	Nearly additive
		0.5 IC_{50}:IC_{50}	3.74	11.3	67.68±1.25	1.29±0.04	Slight antagonism
		IC_{50}:0.5 IC_{50}	7.49	5.65	65.58±0.77	1.14±0.01	Slight antagonism
		0.5 IC_{50}:0.5 IC_{50}	3.74	5.65	43.00±1.2	1.33±0.03	Slight antagonism
DEZSC+PFPE	MCF-7	IC_{50}:IC_{50}	13.35	21.06	78.65±4.33	1.19±0.16	Slight antagonism
		0.5 IC_{50}:IC_{50}	6.675	21.06	50.4±2.65	1.86±0.11	Antagonism
		IC_{50}:0.5 IC_{50}	13.35	10.53	82.66±2.65	0.72±0.07	Moderate synergism
		0.5 IC_{50}:0.5 IC_{50}	6.675	10.53	37.07±1.05	1.52±0.04	Antagonism
	A2780	IC_{50}:IC_{50}	14.64	21.12	72.46±5.09	1.39±0.07	Slight antagonism
		0.5 IC_{50}:IC_{50}	7.32	21.12	13.59±4.36	4.77±0.91	Strong antagonism
		IC_{50}:0.5 IC_{50}	14.64	10.56	48.7±8.73	1.66±0.30	Antagonism
		0.5 IC_{50}:0.5 IC_{50}	7.32	10.56	14.15±9.69	3.21±1.36	Strong antagonism
DEGE+PFPE	MCF-7	IC_{50}:IC_{50}	15.58	21.06	97.29±0.33	0.36±0.03	Synergism
		0.5 IC_{50}:IC_{50}	7.79	21.06	93.65±3.72	0.44±0.14	Synergism
		IC_{50}:0.5 IC_{50}	15.58	10.53	95.32±1.5	0.34±0.06	Synergism
		0.5 IC_{50}:0.5 IC_{50}	7.79	10.53	37.69±4.12	1.46±0.13	Antagonism
	A2780	IC_{50}:IC_{50}	10.08	21.12	80.41±1.97	1.22±0.08	Slight antagonism
		0.5 IC_{50}:IC_{50}	5.04	21.12	10.11±4.19	6.70±1.61	Strong antagonism
		IC_{50}:0.5 IC_{50}	10.08	10.56	51.22±4.84	1.63±0.16	Antagonism
		0.5 IC_{50}:0.5 IC_{50}	5.04	10.56	5.54±2.14	5.55±1.18	Strong antagonism

Data represented mean±SD from three independent experiments. CI is combination index, analyzed by compusyn software. DEAP: dichloromethane extract of *A. paniculata*, DEZSC: dichloromethane extract of *Z. spina-christi*, DEGE: dichloromethane extract of *G. extensum*, PFPE: low piperine fractional *P. nigrum* extract

and late apoptotic cells. The early and later apoptosis stage could be distinguished by intercalation among Annexin V to Phosphatidylserine and intercalation of Annexin V together with PI inside permeable membrane of late apoptotic cells. Meanwhile, dead or necrotic cells were observed in this study. The different aspect of these two basic types of cell death is that the necrosis is caused by excessive external damage, while apoptosis occurs when the cells are aged and undergo progressive morphological alteration by internal molecular involvement[52]. Dot plots in fig. 2 reflected all cell conditions including live cells, early and late apoptotic cells and dead cells (necrotic cells). A significant decrease of live cells was found in MCF-7 cells at 72 h after the incubation with the combination of DEGE and PFPE. Moreover, late apoptotic and total apoptotic cells were increased statistically significantly at 72 h after the treatment and responded in a time-dependent manner.

The apoptosis insight can be harnessed as a key strategy for novel targeted therapies that lead to the arrest of cancer cell growth and spread[53]. Moreover, the correlation between apoptosis and caspase activation is the loss of mitochondrial membrane potential. An activation of cytochrome c (cyt-c) would be then followed by caspase activation[54]. The caspases are activated when the cell inactivates zymogens, provoking a low number of protease activity[55]. The dot plots, in

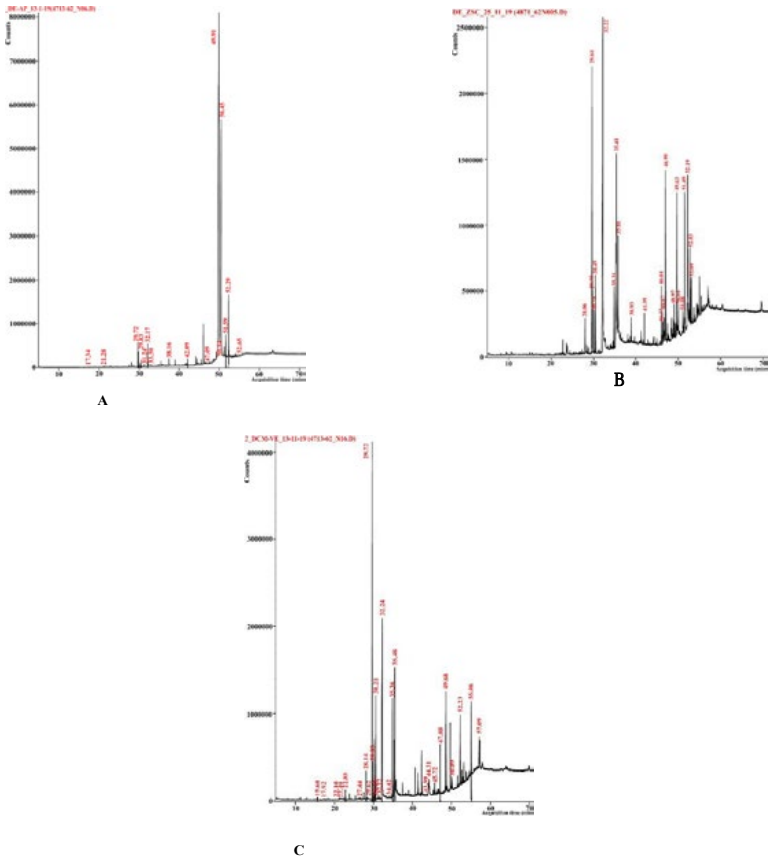

Fig. 1: GC-MS chromatographic profile of extracts: (A) DEAP, (B) DEZSC and (C) DEGE

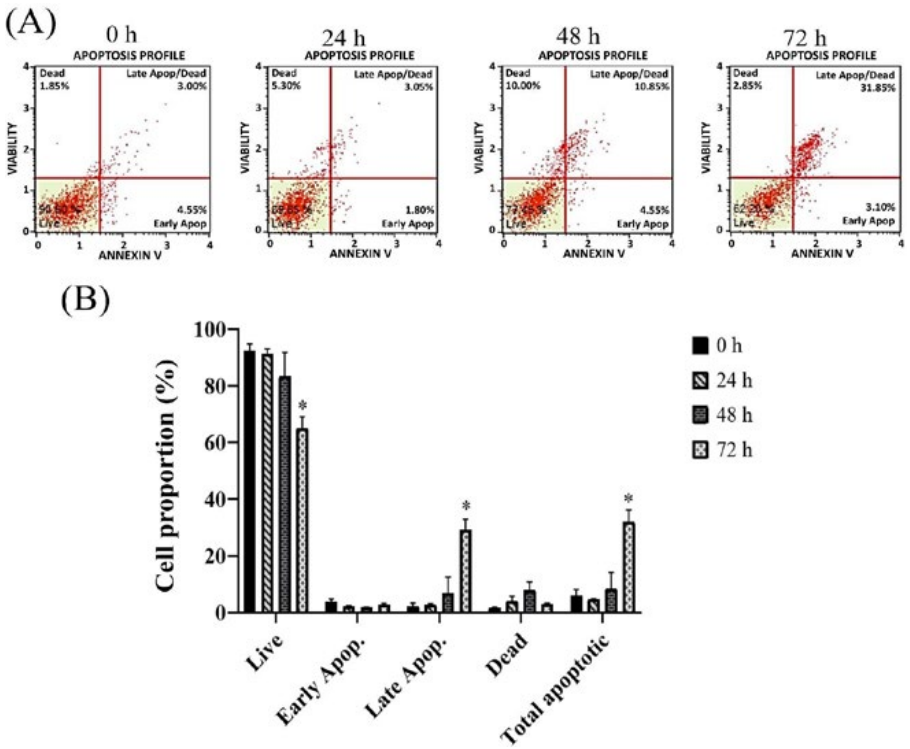

Fig. 2: The combination of DEGE with PFPE induces cell apoptosis ()
(A) MCF-7 cells were treated with the combination of DEGE with PFPE at IC_{50} concentration value of 15.58 µg/ml and 21.06 µg/ml, respectively and incubated for 24, 48 and 72 h. After treatment, apoptotic cells were analyzed by the Muse® Annexin V-FITC assay and Propidium Iodide (dead cell kit). (B) The graph represented the summary of average percentages±SD of live, early apoptotic, late apoptotic, dead and total apoptotic of three independent experiments. The statistical analysis of the data was tested by Student t test where p values less than 0.05 was statistically considered as significant differences compared to control group at 0 h

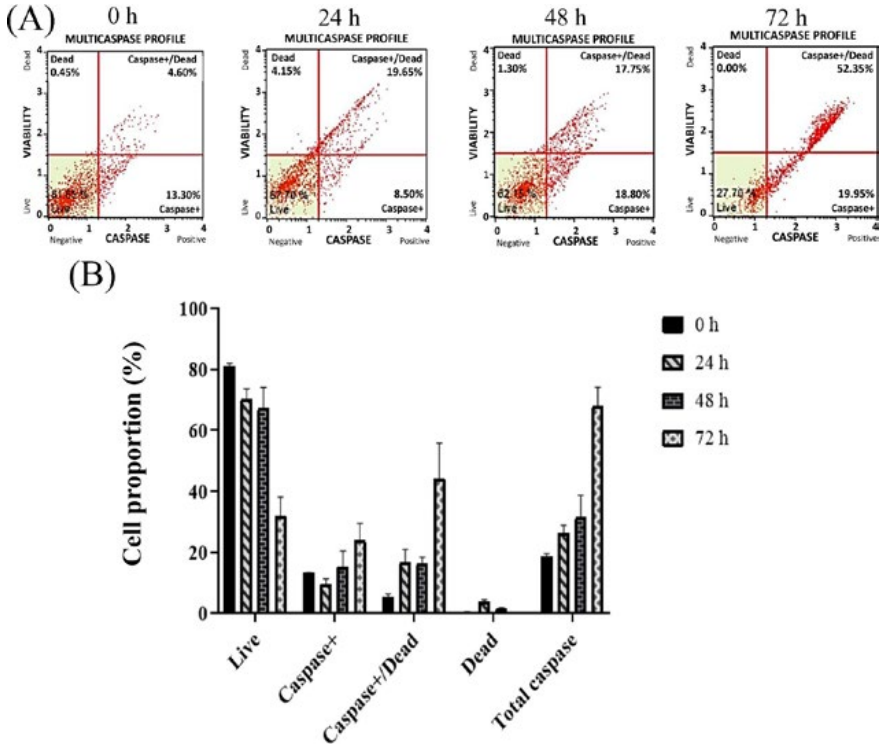

Fig. 3: The combination of DEGE with PFPE induces multi caspase activity
(A) MCF-7 cells were treated with the combination of DEGE with PFPE at IC_{50} concentration value of 15.58 µg/ml and 21.06 µg/ml, respectively and incubated for 24, 48 and 72 h. After treatment, multi caspase activity was analyzed by the Muse® Multi caspase assay and 7-AAD (dead cell kit). (B) The graph is represented the average percentages ± SD of live, caspase+, caspase+/dead and total caspase of three independent experiments. The statistical analysis of the data was tested by Student t-test where p values less than 0.05 was statistically considered as significant differences compared to control group at 0 h

fig. 3, represented the proportion of live, caspase+, caspase+/dead and dead cells of MCF-7 cells treated with the combination of DEGE and PFPE. We found that multi caspase activity was significantly increased at 72 h ($p<0.05$) after treatment and responded in a time-dependent manner similar to the apoptosis experiment. However, further analysis of proteins in the apoptosis pathway would be encouraged.

To the best of our insight, we analyzed and linked to the compounds observed by GC-MS. Several previous findings revealed that the chemical compositions of DEGE could affect apoptotic associated proteins and caspases. The most abundant phytocompound of DEGE was palmitic acid, which can promotes caspase -3, -8 and -9 activities in Pheochromocytoma cells (PC12)[56]. Furthermore, linoleic acid, phytol and stigmasterol up regulate apoptotic proteins, such as bax, caspase -3 and -9. These three compounds also down-regulate bcl-2 in colorectal, liver and Non-small cell lung cancer (NSCLC) cells, respectively[38,39,57]. Surprisingly, Western blot analysis of PFPE reflected that PFPE was able to regulate the apoptosis associated proteins including p53, bax and suppress bcl-2 expression on cholangiocarcinoma cells (KKU-M213 and TFK-1) [20]. Merged all results together, this report points out that DEGE and PFPE would be a potential regimen for breast cancer treatment in the future. Nevertheless, an *in vivo* study using breast cancer and clinical trials remain necessary to support supplementary information.

In conclusion, dichloromethane extract of *A. paniculata*, *Z. spina-christi* and *G. extensum* showed strong cytotoxicity against colorectal, breast and ovarian cancer, respectively, with an IC_{50} value less than 14 µg/ml. A combination of dichloromethane crude extract of *G. extensum* with *P. nigrum* crude extract interestingly enhanced a synergistic anticancer activity on breast cancer through the apoptosis induction.

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Conflict of Interests:

The authors declared no conflict of interest.

REFERENCES

1. Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: priorities for prevention. Carcinogenesis 2010;31(1):100-10.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68(6):394-424.
3. Coalition WO. The world ovarian cancer coalition atlas. Global Trends in Incidence, Mortality and Survival; 2018.
4. Wang X, Zhang H, Chen X. Drug resistance and combating drug resistance in cancer. Cancer Drug Resist 2019;2(2):141-60.
5. Solowey E, Lichtenstein M, Sallon S, Paavilainen H, Solowey E, Lorberboum-Galski H. Evaluating medicinal plants for anticancer activity. Sci World J; 2014.
6. Hossain MD, Urbi Z, Sule A, Rahman KM. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: a review of ethnobotany, phytochemistry and pharmacology. Sci World J; 2014.
7. Nkafamiya II, Shagal MH, Haruna M. Potential of *Ziziphus spina-christi* seed ethanolic extract on inhibition of microbial growth. Acad J Biotechnol 2013;1(4):53-6.
8. Bunwong S, Chantaranothai P, Keeley SC. Revisions and key to the *Vernonieae* (Compositae) of Thailand. PhytoKeys 2014;(37):25-101.
9. Siripong P, Kongkathip B, Preechanukool K, Picha P, Tunsuwan K, Taylor WC. Cytotoxic diterpenoid constituents from *Andrographis paniculata* Nees leaves. J Sci Soc Thailand 1992;18(4):187-94.
10. Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S. Anticancer and immunostimulatory compounds from *Andrographis paniculata*. J Ethnopharmacol 2004;92:291-5.
11. Cheung HY, Cheung SH, Li J, Cheung CS, Lai WP, Fong WF, *et al*. Andrographolide isolated from *Andrographis paniculata* induces cell cycle arrest and mitochondrial-mediated apoptosis in human leukemic HL-60 cells. Planta Med 2005;71(12):1106-11.
12. Sukardiman, Studiawan H, Rahman A, Santosa MH, Pratama FA. Ethyl acetate fraction of *Andrographis paniculata* Nees increases cytotoxic effect of 5-fluorouracil on human cancer cell lines. Int J Pharm Pharm Sci 2014;6(5):1-5.
13. Farmani F, Moein M, Amanzadeh A, Kandelous HM, Ehsanpour Z, Salimi M. Antiproliferative evaluation and apoptosis induction in MCF-7 cells by *Ziziphus spina christi* leaf extracts. Asian Pac J Cancer Prev 2016;17(1):315-21.
14. Thongnest S, Chawengrum P, Keeratichamroen S, Lirdprapamongkol K, Eurtivong C, Boonsombat J, *et al*. Vernodalidimer L, a sesquiterpene lactone dimer from *Vernonia extensa* and anti-tumor effects of vernodalin, vernolepin and vernolide on HepG2 liver cancer cells. Bioorg Chem 2019;92:1-10.
15. Moldoveanu SC, David V. Mobile phases and their properties. Essentials in Modern HPLC Separations 2013:363-447.
16. Abarca-Vargas R, Petricevich VL. Comparison of Different Extraction Methods for the Phenolic Compounds Recovery with the Antioxidant Activity of *Bougainvillea* x *buttiana*. Curr Anal Chem 2020;16(6):778-87.
17. Grachev V, Magrath D, Griffiths E, Petricciani JC. WHO requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (requirements for biological substances No. 50). Biologicals 1998;26(3):175-93.
18. Almeida JL, Hill CR, Cole KD. Mouse cell line authentication. Cytotechnology 2014;66(1):133-47.
19. Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus Piper in breast cancer cell lines. Trop J Pharm Res 2014;13(6):921-8.
20. Tedasen A, Khoka A, Madla S, Sriwiriyajan S, Graidist P. Anticancer effects of piperine-free *Piper nigrum* extract on cholangiocarcinoma cell lines. Pharmacogn Mag 2020;16(68):28.
21. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Hepatoprotective amide constituents from the fruit of *Piper chaba*: Structural requirements, mode of action and new amides. Bioorg Med Chem 2009;17(20):7313-23.
22. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Protective effects of amide constituents from the fruit of *Piper chaba* on d-galactosamine/TNF-α-induced cell death in mouse hepatocytes. Bioorg Med Chem Lett 2008;18(6):2038-42.
23. Eastman A. Improving anticancer drug development begins with cell culture: misinformation perpetrated by the misuse of cytotoxicity assays. Oncotarget 2017;8(5):8854-66.
24. Singh S, Mehta A, Baweja S, Ahirwal L, Mehta P. Anticancer activity of *Andrographis paniculata* and *Silybum marianum* on five human cancer cell lines. J Pharmacol Toxicol 2013;8(1):42-8.
25. Madlum KN, Galib RA, Ghazi FM, Ali ZH. The effects of *Zizyphus* stem bark and termite shelter tubes on HeLa cell growth. Res J Pharm BiolChem Sci 2015;6(6):620-6.
26. Seong J, Oyong GG, Cabrera EC. *Synsepalum dulcificum* extracts exhibit cytotoxic activity on human colorectal cancer cells and upregulate c-fos and c-jun early apoptotic gene expression. Asian Pac J Trop Biomed 2018;8(3):173-8.
27. Sriwiriyajan S, Tedasen A, Lailerd N, Boonyaphiphat P, Nitiruangjarat A, Deng Y, *et al*. Anticancer and cancer prevention effects of piperine free *Piper nigrum* extract on N-nitrosomethylurea-induced mammary tumorigenesis in rats. Cancer Prev Res 2016;9(1):74-82.
28. Chou T-C, Martin N. A computer program for quantitation of synergism and antagonism in drug combinations and the determination of IC_{50} and ED_{50} and LD_{50} values. Paramus, New Jersey: ComboSyn; 2005.
29. Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. Trends Pharmacol Sci 1983;4:450-4.
30. Chou TC. Theoretical basis, experimental design and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006;58(3):621-81.
31. Santos-Sanchez NF, Salas-Coronado R, Villanueva-Canongo C, Hernandez-Carlos B. Antioxidant compounds and their antioxidant mechanism. London, UK: IntechOpen; 2019. p. 1-28.
32. Widyawati PS, Budianta TD, Kusuma FA, Wijaya EL. Difference of solvent polarity to phytochemical content and antioxidant activity of *Pluchea indicia* less leaves extracts. Int J Pharmacogn Phytochem 2014;6(4):850-5.
33. Tan MC, Oyong GG, Shen CC, Ragasa CY. Cytotoxic activities of the dichloromethane extract from *Andrographis paniculata* (*Burm. f.)* nees. J Nat Sci Biol Med 2018;9(2):201-6.
34. Allison SD, Randolph TW, Manning MC, Middleton K, Davis A, Carpenter JF. Effects of drying methods and additives on structure and function of actin: mechanisms of dehydration-induced damage and its inhibition. Arch Biochem Biophys 1998;358(1):171-81.
35. Koch A, Tamez P, Pezzuto J, Soejarto D. Evaluation of plants

used for antimalarial treatment by the Maasai of Kenya. J Ethnopharmacol 2005;101(1):95-9.
36. Ravi L, Krishnan K. Cytotoxic potential of N-hexadecanoic acid extracted from *Kigelia pinnata* leaves. Asian J Cell Biol 2017;12(1):20-7.
37. Eswaraiah G, Peele KA, Krupanidhi S, Kumar RB, Venkateswarulu TC. Identification of bioactive compounds in leaf extract of *Avicennia alba* by GC-MS analysis and evaluation of its *in vitro* anticancer potential against MCF7 and HeLa cell lines. J King Saud Univ Sci 2020;32(1):740-4.
38. Sakthivel R, Malar DS, Devi KP. Phytol shows anti angiogenic activity and induces apoptosis in A549 cells by depolarizing the mitochondrial membrane potential. Biomed Pharmacother 2018;105:742-52.
39. Lu X, Yu H, Ma Q, Shen S, Das UN. Linoleic acid suppresses colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction. Lipids Health Dis 2010;9(1):106.
40. Kim SJ, Chung WS, Kim SS, Ko SG, Um JY. Antiinflammatory effect of *Oldenlandia diffusa* and its constituent, hentriacontane, through suppression of caspase-1 activation in mouse peritoneal macrophages. Phytother Res 2011;25(10):1537-46.
41. Farid MM, Hussein SR, Ibrahim LF, El Desouky MA, Elsayed AM, El Oqlah AA, *et al*. Cytotoxic activity and phytochemical analysis of *Arum palaestinum* Boiss. Asian Pac J Trop Biomed 2015;5(11):944-7.
42. Alkhalaf MI, Alansari WS, Ibrahim EA, ELhalwagy ME. Anti-oxidant, anti-inflammatory and anti-cancer activities of avocado (*Persea americana*) fruit and seed extract. J King Saud Univ Sci 2019;31(4):1358-62.
43. Want EJ, Cravatt BF, Siuzdak G. The expanding role of mass spectrometry in metabolite profiling and characterization. Chembiochem 2005;6(11):1941-51.
44. Jain PK, Soni A, Jain P, Bhawsar J. Phytochemical analysis of *Mentha spicata* plant extract using UV-VIS, FTIR and GC/MS technique. J Chem Pharm Res 2016;8(2):1-6.
45. Wulandari L, Retnaningtyas Y, Lukman H. Analysis of flavonoid in medicinal plant extracts using infrared spectroscopy and chemometrics. J Anal Methods Chem 2016;2016.
46. Irawan C, Rochaeni H, Sulistiawaty L, Roziafanto AN. Phytochemical Screening, LC-MS Studies and Antidiabetic Potential of Methanol Extracts of Seed Shells of *Archidendron bubalinum* (Jack) IC Nielson (Julang Jaling) from Lampung, Indonesia. Pharmacogn J 2018;10(6):S77-82.
47. Baldissera MD, Souza CF, Grando TH, Stefani LM, Monteiro SG. β-caryophyllene reduces atherogenic index and coronary risk index in hypercholesterolemic rats: The involvement of cardiac oxidative damage. Chem Biol Interact 2017;270:9-14.
48. Kamikubo R, Kai K, Tsuji-Naito K, Akagawa M. β-Caryophyllene attenuates palmitate-induced lipid accumulation through AMPK signaling by activating CB2 receptor in human HepG2 hepatocytes. Mol Nutr Food Res 2016;60(10):2228-42.
49. Sriwiriyajan S, Sukpondma Y, Srisawat T, Madla S, Graidist P. (-)-Kusunokinin and piperloguminine from *Piper nigrum*: An alternative option to treat breast cancer. Biomed Pharmacother 2017;92:732-43.
50. Skok Z, Zidar N, Kikelj D, Ilas J. Dual inhibitors of human DNA topoisomerase II and other cancer-related targets. J med chem 2019;63(3):884-904.
51. Sohretoglu D, Barut B, Sari S, Ozel A, Arroo R. *In vitro* and *in silico* assessment of DNA interaction, topoisomerase I and II inhibition properties of chrysosplenetin. Int J Biol Macromol 2020;163:1053-9.
52. Stepanek O, Brdicka T, Angelisova P, Horvath O, Spicka J, Stockbauer P, *et al*. Interaction of late apoptotic and necrotic cells with vitronectin. PLoS One 2011;6(5):e19243.
53. Rezadoost MH, Kumleh HH, Ghasempour A. Cytotoxicity and apoptosis induction in breast cancer, skin cancer and glioblastoma cells by plant extracts. Mol Biol Rep 2019;46(5):5131-42.
54. Li C, Jing H, Ma G, Liang P. Allicin induces apoptosis through activation of both intrinsic and extrinsic pathways in glioma cells. Mol Med Rep 2018;17(4):5976-81.
55. Parrish AB, Freel CD, Kornbluth S. Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol 2013;5(6):a008672.
56. Ulloth JE, Casiano CA, De Leon M. Palmitic and stearic fatty acids induce caspase-dependent and-independent cell death in nerve growth factor differentiated PC12 cells. J Neurochem 2003;84(4):655-68.
57. Kim YS, Li XF, Kang KH, Ryu B, Kim SK. Stigmasterol isolated from marine microalgae Navicula incerta induces apoptosis in human hepatoma HepG2 cells. BMB Rep 2014;47(8):433-8.

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ORIGINAL ARTICLE

Anticancer Effects of Piperine-Free *Piper nigrum* Extract on Cholangiocarcinoma Cell Lines

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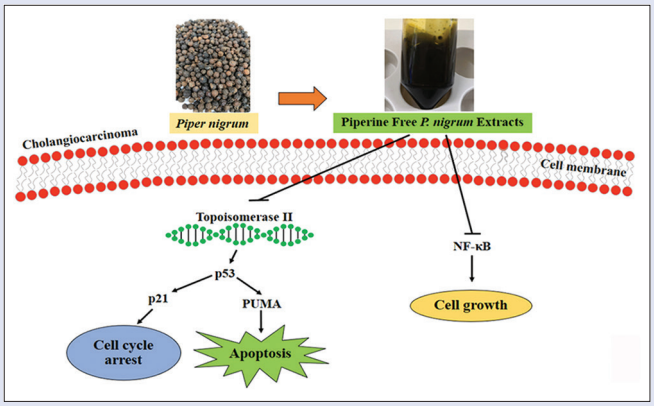
ABSTRACT

Background: Black pepper (*Piper nigrum* L.) is widely used as a traditional medicine, including usage for pain relief, fevers, as well as an anticancer agent. Previously, we reported that piperine-free *P. nigrum* extract (PFPE) inhibited breast cancer *in vitro* and *in vivo*. **Objective:** In this present study, we explored the anticancer effects of PFPE on cholangiocarcinoma (CCA). **Materials and Methods:** 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to analyze cytotoxic potential of PFPE whereas deoxyribonucleic acid (DNA) fragmentation followed by Western blot analysis were used. **Results:** PFPE composed of alkaloid, flavonoid, amide, lignans, opioid, and steroid. This crude extract represented cytotoxic effect against CCA cells which stronger than dichloromethane *P. nigrum* crude extract and piperine, especially on KKU-M213 (median inhibition concentration [IC_{50}] at 13.70 μg/ml) and TFK-1 (IC_{50} at 15.30 μg/ml). Interestingly, PFPE showed lower cytotoxicity against normal human cholangiocyte MMNK-1 cells (IC_{50} at 19.65 μg/ml) than KKU-M213 and TFK-1 cells. Then, the molecular mechanisms of PFPE were firstly evaluated by DNA fragmentation followed by Western blot analysis. The degradation of DNA was observed on KKU-M213 and TFK-1 cells after treatment with PFPE at day 2. Then, proliferation proteins including topoisomerase II, AKT8 virus oncogene cellular homolog, avian myelocytomatosis virus oncogene cellular homolog, cyclin D1, signal transducer and activator of transcription 3, cyclooxygenase-2, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) were decreased and p21 was increased. Furthermore, apoptotic proteins, such as tumor protein p53, Bcl-2-associated X protein, and p53 upregulated modulator of apoptosis were upregulated. Meanwhile, antiapoptotic protein B-cell lymphoma 2 was down-regulated. **Conclusion:** These results indicated that PFPE inhibited CCA through the down-regulation of cell proliferation and induction of apoptosis pathway.

Key words: Anticancer, apoptosis, cell proliferation, cholangiocarcinoma, *Piper nigrum*

SUMMARY

- piperine free *Piper nigrum* extract (PFPE) inhibited cholangiocarcinoma (CCA) cell lines
- PFPE induces CCA cells to undergo apoptosis and cell cycle arrest via the inhibition of topoisomerase II
- PFPE inhibit cell growth through the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells.



Abbreviations used: PFPE: Piperine free *Piper nigrum* extract; CCA: Cholangiocarcinoma; DPCE: dichloromethane *P. nigrum* crude extract; NMU: N-nitrosomethylurea; ER: Estrogen receptor; MMP-9: Matrix metalloproteinase-9; MMP-2: Matrix metalloproteinase-2; VEGF: Vascular endothelial growth factor; GC-MS: Gas chromatograph-mass spectrometer; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO: Dimethylsulfoxide; IC_{50}: Median inhibition concentration; MCLE: Methanol crude extract of *Curcuma longa*; DNA: Deoxyribonucleic acid; STAT-3: Signal transducer and activator of transcription 3; COX-2: Cyclooxygenase-2; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; c-Myc: Avian myelocytomatosis virus oncogene cellular homolog; Akt: AKT8 virus oncogene cellular homolog; Bcl-2: B-cell lymphoma 2; p53: Tumor protein p53; Bax: Bcl-2-associated X protein; PUMA: p53 upregulated modulator of apoptosis.

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INTRODUCTION

Cholangiocarcinoma (CCA) is an epithelial cancer originating from the bile ducts with features of cholangiocyte differentiation.[1] There are 2 types of CCA (based on its location) including intrahepatic and extrahepatic.[2] For over the past four decades, incidence of CCA has been increased in United States of America,[3] Australia, England,[4] and Northeastern Thailand.[5] There are several risk factors for CCA, including primary sclerosing cholangitis, liver fluke infections (*Clonorchis sinensis* and *Opisthorchis viverrini*), choledochal cysts, Caroli's disease, hepatitis B and C infection, obesity, cirrhosis and hepatolithiasis.[5,6] The therapeutic for CCA are limited and no current effective treatment because the majority of patients present with advanced stage disease.[7] Even treatments with advances in surgical

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techniques, chemotherapy and radiotherapy, the 5-year survival rate of patients after diagnosis still remain about 10%.[8] Although surgical resection has improved in the survival of most patients, the recurrent disease was found within 2 years after tumor resection.[9] Chemotherapy and radiation therapy are ineffective and show various side effects such as harmful to normal cells and bone marrow suppression.[10] Therefore, effective therapeutic and alternative treatments with no serious side effect for CCA are urgently needed.

P. nigrum L. belongs to family Piperaceae and can be used as antiapoptotic, antibacterial, anticolon toxin, antidepressant, antifungal, antidiarrhoeal, antiinflammatory, antimutagenic, antimetastatic, antioxidative, antipyretic, antispasmodic, antispermatogenic, antitumor, antithyroid, ciprofloxacin potentiator, cold extremities, gastric ailments, hepatoprotective, insecticidal, intermittent fever, and larvicidal activities.[11] The chemical constituents of *P. nigrum* are aromatic essential oils, alkaloids, amides, prophenylphenols, lignans, terpenes, flavones, and steroids.[12] Ethanolic crude extract of *P. nigrum* consists of high total phenol content shows antioxidant and anti-inflammation as well as cytotoxic property against colorectal carcinoma cell lines.[13] Using ethanol and high pressure (200 bar), *P. nigrum* crude extracts exhibits cytotoxicity against MCF-7 with median inhibition concentration (IC_{50}) of 14.40 ± 3.30 μg/ml and represents tumor inhibitory effect in mammary adenocarcinoma mouse.[14] Previously, we reported that piperine-free *P. nigrum* extract (PFPE) strongly inhibited breast cancer MCF-7 cells with IC_{50} value of 7.45 μg/ml. Moreover, PFPE inhibited tumor growth in *N*-nitrosomethylurea-induced mammary tumorigenesis rats without liver and kidney toxicity.[15] Interestingly, PFPE upregulated tumor protein p53 (p53) and downregulated estrogen receptor, E-cadherin, matrix metalloproteinase-9 (MMP-9), MMP-2, avian myelocytomatosis virus oncogene cellular homolog (c-Myc) and vascular endothelial growth factor (VEGF) *in vitro* and *in vivo*.[16] In this present research, we further explored the phytochemical component, investigated cytotoxicity and molecular mechanisms of PFPE on CCA cell lines.

MATERIALS AND METHODS

Preparation of piperine free *Piper nigrum* extract

Seeds of *P. nigrum* L. were collected from Songkhla province in Thailand. The plant specimen (voucher specimen number SKP 146161401) was identified by Asst. Prof. Dr. Supreeya Yuenyongsawad and deposited in the herbarium at the Southern Centre of Thai Traditional Medicine, Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University, Thailand. PFPE was prepared as previously described. Briefly, grounded 250 g of dried seeds of *P. nigrum* L. were soaked in 300 mL of dichloromethane and incubated at 35°C for 3 h in a shaking incubator. After filtration with Whatman filter paper No. 1 and concentration using rotary evaporator, the dark brown oil residue of extracts was obtained and then recrystallized with cold diethyl ether in an ice bath to get rich of yellow crystals (piperine) and obtain brown oil residue (PFPE).[15] PFPE was kept in a desiccator until used.

Phytochemical analysis and identification of bioactive constituents by gas chromatograph-mass spectrometer

The analysis of the phytochemical screening and composition of PFPE extracts were carried out using a Gas Chromatography-Agilent 7890B combination with an Agilent 5977A triple quadrupole mass spectrometer (Agilent Technologies Inc, USA). Gas chromatograph-mass spectrometer (GC-MS) analysis is a common confirmation test, which used to make an effective chemical analysis. The PFPE samples were evaluated phytochemicals such as a flavonoids, tannins, alkaloids, steroids, phenols, glycosides, lignans, and terpenoids. An inlet temperature of 280°C with the split ratio 7:1 was employed and the helium was used as the carried gas at the constant flow rate of 7 ml/min. The oven temperature was initially maintained at 60°C for 5 min and increase at a rate of 5°C/min to 315°C for 15 min. For MS detection, an electron ionization mode was used with an ionization energy of 70 eV, ion source temperature of 230°C, and scan mass range m/z 35–500. The components were identified based on a correlation of the recorded fragmentation patterns of mass spectra that provided in the GC-MS system software version Wiley10 and NIST14. All procedures were performed at Scientific Equipment Center, Prince of Songkla University, Songkhla, Thailand.

Measuring total phenolic, tannin, flavonoid content and radical scavenging activity

The total phenolic content was determined based on Folin–ciocalteu method. Gallic acid was used as the standard and total phenolics were expressed as mg gallic acid equivalent/mg extract (mg GAE/mg extract). Total condensed tannin was measured based on HCL-vanillin method and catechin was used as the standard. The total tannin was reported as mg catechin equivalent/mg extract (mg CE/mg extract). The total flavonoid content was determined by aluminum chloride solution ($AlCl_3$) colorimetric method. Quercetin was employed as the standard and expressed the total flavonoids as mg quercetin equivalent/mg extract (mg QE/mg extract). 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity was performed according to the DPPH trolox assay and reported as mg trolox equivalent antioxidant capacity/mg extract (mg TEAC/mg extract). All procedures were performed at Center of Excellence in Natural Products Innovation, Mae Fah Luang University, Chiang Rai, Thailand.

Cell lines and culture conditions

Three CCA (KKU-100, KKU-M213 and KKU-M055) and one cholangiocyte (MMNK-1) cells were kindly donated by Dr. Mutita Junking (Faculty of Medicine, Mahidol University, Bangkok, Thailand). TFK-1 cells were obtained from RIKEN BioResource Center and HuCC-T1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank. Mouse fibroblast, L-929 cells, were kindly donated by Associate Professor Dr. Jasadee Kaewsichan (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand).

KKU-100, KKU-M213, KKU-M055, MMNK-1 and L-929 cells were grown in DMEM medium (Invitrogen), which contained 10% of fetal bovine serum (Invitrogen), 2 mmol/L of L-glutamine (Invitrogen), and an antibiotic mixture of 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen). TFK-1 and HuCC-T1 cells were grown in RPMI 1640 (Invitrogen) supplemented with the same supplement as for DMEM. All cells were maintained by incubating in a 5% CO_2 atmosphere, at 37°C and 96% relative humidity.

In vitro cytotoxicity

The cytotoxicity assay was performed in 96-well plate. KKU-100, KKU-M055, and MMNK-1 cells were seeded at a density of 5×10^3 cells/well. KKU-M213, TFK-1, and HuCC-T1 cells were seeded at a density of 7.5×10^3 cells/well and L-929 cells were seeded at a density of 8×10^3 cells/well. After incubation for 24 h, cells were treated with PFPE at various concentration for 48 h. The cells were then washed with 1X PBS and incubated in 100 μl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution at 37°C for 30 min. Under light protection, the purple crystals of formazan or MTT metabolites were dissolved with 100 μl of dimethyl

sulfoxide and incubate at 37°C for 30 min. The absorbance was measured at 570 and 650 nm using a microplate reader spectrophotometer (Spectra Max M5, Molecular Devices), and the IC_{50} values were calculated.[17] According to US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with IC_{50} value ≤20 μg/ml.[18]

Deoxyribonucleic acid fragmentation analysis

KKU-M213 and TFK-1 cells in their exponential growth phase were seeded into 6 cm culture plate at a density of 2.5×10^5 cells/plate for 24 h and then treated with PFPE at 3 folds of IC_{50} values. After treatment for 96 h, cells were harvested by trypsinization. Cell pellets were lysed using the extraction buffer (containing 0.7 M NaCl, 17 mM SDS, 10 mM Tris-HCl (pH 8.0) and 2 mM EDTA (pH 8.0)) and fragmented deoxyribonucleic acid (DNA) in the supernatant was extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1). The DNA was precipitated with a two-thirds volume of cold isopropanol followed by centrifugation at 8,000 ×*g* and washed once in 70% ethanol. Finally, DNA pellet was resuspended in deionized water and analyzed by 1.5% agarose gel electrophoresis.[19]

Western blot analysis

KKU-M213 and TFK-1 cells were seeded into 6 cm culture plate at a density of 2.5×10^5 cells/plate for 24 h and then treated with PFPE at IC_{50} values. After treatment, cells were harvested every day for 4 days. Then, cell pellets were lysed using the RIPA buffer (containing 150 mM NaCl, 50 mM Tris, pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate and 1 mM EDTA). Total protein samples (150 mg) were loaded on 12% of SDS-polyacrylamide gel electrophoresis and transferred onto a 0.45 mm nitrocellulose membrane (Bio-Rad, 162-0115). Membrane was blocked at room temperature for 1 h with 5% non-fat milk in 1X TBS-T and then washed with 1% non-fat milk in 1X TBS-T. Membrane was incubated with primary antibodies against topoisomerase II, Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), p53 upregulated modulator of apoptosis (PUMA), p21, AKT8 virus oncogene cellular homolog (Akt), cyclooxygenase-2 (COX-2), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), signal transducer and activator of transcription 3 (STAT-3), cyclin D1 and p53 proteins. The membrane was then incubated with secondary horseradish peroxidase-conjugated antibodies. Bound antibodies were developed by a chemiluminescence detection kit using the SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific) and detected using a Fusion FX vilber lourmat, CCD camera (Fisher Biotechnology). GAPDH was used to normalize protein loading. Protein levels were expressed as a relative ratio to GPADH.

Statistical analysis

The median inhibition concentration (IC_{50}) data was acquired by SoftMax 1 Pro 5 program (MDS Analytical Technologies Inc., California, USA). Student's *t*-test was used to analyze intergroup differences. A $P < 0.05$ was considered to be statistically significant. All results were represented as the mean ± standard deviation (SD). The values were obtained from at least three independent experiments.

RESULTS

Total phenolic, tannin, and flavonoid contents

Phenolics, flavonoids, and tannins are one class of secondary plant metabolites which represented anticancer activity of plant. As present in Table 1, PFPE contained phenolic, tannin and flavonoid lower than methanol crude extract of *Curcuma longa* (MCLE). However, the cytotoxicity of PFPE against breast cancer MCF-7 cells (IC_{50} value at 7.45 ± 0.6 μg/ml) not significantly lower than MCLE (IC_{50} value at 5.74 ± 1.48 μg/ml). Therefore, we performed GC-MS in next experiment to identify the chemical compounds in PFPE.

Table 1: Total phenolic, tannin and flavonoid contents in piperine free *Piper nigrum* crude extract

Crude	Phenolics (mg GAE/g extract)[a]	Flavonoids (mg QE/mg extract)[b]	Tannins (mg CE/mg extract)[c]
PFPE	402.46±7.49	40.69±5.99	201.82±17.78
MCLE	2090.63±15.81	148.94±33.64	2373.75±92.77

[a]Mg of gallic acid equivalence by mg of extract; [b]Mg of quercetin equivalence by mg of extract; [c]Mg of catechin equivalence by mg of extract; *P. nigrum*: *Piper nigrum*; PFPE: Piperine free *P. nigrum* extract; *C. longa*: *Curcuma longa*; MCLE: Metanolic *C. longa* extract; GAE: Gallic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalent

Phytochemical screening

In this study, the phytochemical analysis using GC-MS was carried out. The chromatogram and predicted constituents are shown in Figure 1 and Table 2.

Results showed that PFPE contained five chemical groups including alkaloids, terpenes, amides, lignans, opioid and steroid with 17, 13, 7, 3, 1, and 1 compounds, respectively. The highest percentage of peak area of each group were pipercitine (21.66%, alkaloid), caryophyllene (13.28%, terpene), acrivastine (2.34%, amide), kusunokinin (1.28%, lignan), methyldihydromorphine (1.18%, opioid), and beta-stigmasterol (1.74%, steroid) which showed the anticancer activity.

Effect of piperine free *Piper nigrum* extract on the viability of cholangiocarcinoma, cholangiocyte and normal fibroblast cell lines

The cell viability of CCA and normal cell lines was measured using the MTT assay. All cell lines were incubated with extracts for 48 h. The IC_{50} values represented the mean ± SD of three different experiments. Among these cell lines, PFPE showed the highest cytotoxicity against KKU-M213 cells with IC_{50} value of 13.70 ± 1.14 μg/ml. Moreover, PFPE demonstrated cytotoxic effect stronger than dichloromethane *P. nigrum* crude extract (DPCE) (IC_{50} at 22.22 ± 0.26 μg/ml) and piperine (IC_{50} at 27.01 ± 0.36 μg/ml). The positive reference drug (doxorubicin) showed a very strong cytotoxic activity on normal and almost cancer cells. Surprisingly, doxorubicin showed same cytotoxic activity with PFPE against TFK-1 cells [Table 3].

Piperine free *Piper nigrum* extract induces deoxyribonucleic acid fragmentation on KKU-M213 and TFK-1 cells

A DNA fragmentation assay was used to determine whether the action of PFPE was associated with apoptosis or not. Apoptosis can be visualized as a ladder pattern of 180-200 base pairs due to DNA cleavage by the activation of a nuclear endonuclease enzyme. Since, PFPE demonstrated a strong cytotoxic effective on KKU-M213 and TFK-1 cells, both cell lines were used to determined DNA fragmentation. As shown in Figure 2, the DNA ladder pattern was observed at day 2 after exposure with 3 folds of IC_{50} concentration of PFPE.

Piperine free *Piper nigrum* extract inhibited proteins associated with inflammation that induces bile duct cancer

In this experiment, we determined proteins associated with inflammation that induced bile duct cancer including STAT-3, COX-2 and NF-kB using Western blot analysis. KKU-M213 cells were treated with 13.69 μg/ml of

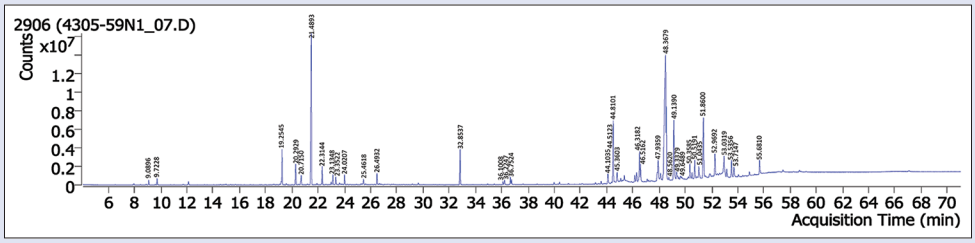

Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

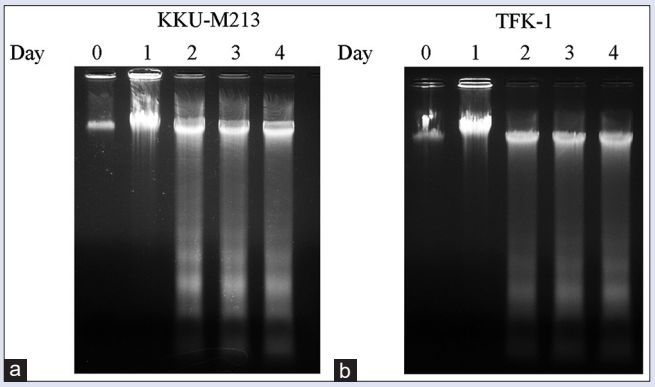

Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis and ethidium bromide staining. KKU-M213 (a) and TFK-1 (b) cells were treated with 41.10 and 45.90 μg/ml of piperine free *Piper nigrum* extract, respectively. The data are representative of three independent experiments carried out under the same conditions

PFPE and incubated for 96 h. The results showed that the STAT-3, COX-2 and NF-kB protein levels were reduced in a time dependent manner and significantly decreased at 48-96 h [Figure 3a and c]. Furthermore, TFK-1 cells were treated with 15.29 μg/ml of PFPE and incubated for 96 h cells. The STAT-3 and COX-2 protein levels were significantly reduced at 72-96 h in a time-dependent manner. The NF-kB protein was decreased significantly at 24 and 72 h [Figure 3b and d].

Piperine free *Piper nigrum* extract inhibited proteins involved in the cell proliferation and growth

Proteins related to cell proliferation and growth of bile duct cancer cells, including topoisomerase II, Akt, c-Myc, cyclin D1, and p21 were examined after treatment with PFPE using IC_{50} concentration of each cells. The result showed that topoisomerase II was significantly decreased at 24 h and p21 was increased at 96 h in KKU-M213 cells [Figure 4a and c]. Meanwhile, PFPE treated TFK-1 cells showed a significant decreased in topoisomerase II at 72 h and p21 was increased at 24 h [Figure 4b and d]. Then, Akt protein was decreased at 48 and 72 h in KKU-M213 and TFK-1 cells, respectively. Moreover, c-Myc and cyclin D1, a protein that worked after those proteins, were found significantly decreased at 48-96 h in both cell lines [Figure 4].

Piperine free *Piper nigrum* extract inhibited proteins associated with apoptosis

In this study, proteins associated with apoptosis pathway including antiapoptosis (Bcl-2) and apoptosis (p53, bax, and PUMA) were evaluated. After giving PFPE at IC_{50} concentration for 48 h, death cells were observed and Bcl-2 was decreased in both cells, KKU-M213 and TFK-1 [Figure 5]. In addition, the levels of p53 and Bax proteins were significantly increased at 96 h and PUMA protein was increased from 24 to 48 h in KKU-M213 cells [Figure 5a and c]. Moreover, p53, Bax and PUMA were increased significantly at 24 h TFK-1 cells [Figure 5b and d].

DISCUSSION

The incidence of bile duct cancer or CCA has increased in Thailand and chemotherapy is not sufficient to treat the aggressive type of this cancer.[5] Therefore, medicinal plants could be an alternative treatment for bile duct cancer. There are many medicinal plants that cause cell cycle arrest and apoptosis in CCA such as *Tripterygium wilfordii*, *Atractylodes lancea* (Thunb) DC., *Zingiber officinale* Roscoe, *Phyllanthus emblica*, *Terminalia chebula* Retz., *Moringa oleifera*, and *Curcuma longa* Linn.[20,21] *Piper* species is one of medicinal plant that also shows anticancer effect, such as *Piper sarmentosum,*[22] *Piper longum,*[23] *Piper chaba*[24] and *P. nigrum.*[17] In previous study, we reported that PFPE showed anticancer activity against breast cancer in *in vitro* and *in vivo*.[15,16] Here, we further explored the biological activity of PFPE on bile duct cancer and found that PFPE exhibited anticancer activity against CCA cell lines, especially TFK-1 and KKU-M213, a moderate differentiation with p53 mutation and well differentiation CCA cells, respectively. Using GC-MS technique, many active phytochemicals were founded in PFPE including alkaloids, terpenes, amides, lignans, opioid and steroids. Pipercitine, guineensine, and pipersintenamide, (an alkaloid compounds) represented percentage of peak area at 21.66, 10.17, and 5.65%, respectively. Pipercitine shows toxicity against larvae of *Aedes aegypti,*[25] and guineensine has an anticancer property against the mouse lymphoma cell line L5178Y with IC_{50} values of 17.0 μM.[26] Pipersintenamide, isolated from *Piper sintenense* Hatus, shows anticancer activity against leukamia P-388 and promyelocytic leukemia HL-60 cell lines with IC_{50} values of 3.78 and 3.80 μg/ml.[27,28] Moreover, caryophyllene (13.28% in PFPE), a bicyclic natural sesquiterpene, exhibits antiproliferative effects against colorectal cancer cells (IC_{50} 19 μM) though clonogenicity, migration, invasion and spheroid formation.[29] A beta-stigmasterol (1.74% in PFPE), a steroid compound, demonstrates inhibitory effects with IC_{50} values of 11.14 and 18.28 μM against human myeloid leukemia K562 and prostate cancer PC3 cell lines, respectively.[30] In this recent study, we found a very potent compounds in the PFPE including piperlonguminine (4.77%), kusunokinin (1.28%), and cubebin (0.28%), which have been reported as anticancer agents.(-)-Kusunokinin and piperlonguminine, a natural lignan and alkaloid compounds, inhibited breast cancer cells (MCF-7 and MDA-MB-468) and colorectal cells (SW-620) through down-regulation of topoisomerase II and up-regulation of of p53, p21 protein levels.[31] (-)-Cubebin, a lignan compound, represents anticancer effect against myeloid leukemia, lung and nasopharyngeal cancer.[32] Interestingly, we found that PFPE showed stronger cytotoxicity against CCA cells than DPCE and piperine [Table 3]. However, piperine, the major alkaloid compound in *P. nigrum*, still remained in the PFPE

Table 2: Chemical constituents in piperine free *Piper nigrum* extract

Identified compounds	Formula	Nature of compound	Molecular massb (g/mol)	Retention time	Area (%)	Biological activity
3-Carene	$C_{10}H_{16}$	Terpenes	136.24	9.0896	0.28	Antioxidant, antihyperuricemic and anti-inflammatory[33]
D-Limonene	$C_{10}H_{16}$	Terpenes	136.24	9.7228	0.39	Enhanced the antitumor effect of docetaxel against prostate cancer cells[34]
Clohexane, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans) 2,4-diisopropenyl-1-methyl-1-vinylcy clohexane (or beta-Elemene)	$C_{15}H_{24}$	Terpenes	204.36	19.2545	2.20	Cytotoxic effect on K562 (leukemic) cells by the induction of apoptosis[35]
Copaene	$C_{15}H_{24}$	Terpenes	204.36	20.2929	1.26	Antimicrobial activity against an anaerobic microorganism *Prevotella nigrescens*[36]
2,4-diisopropenyl-1-methyl-1-vinylcyclohexane (beta-Elemene)	$C_{15}H_{24}$	Terpenes	204.36	20.7150	0.73	Cytotoxic effect on K562 (leukemic) cells by the induction of apoptosis[35]
Caryophyllene	$C_{15}H_{24}$	Terpenes	204.36	21.4893	13.28	Antioxidant, preventing lipidic oxidative damage and prevention of atherosclerosis;[37] antigenotoxic and santioxidant[38]
1,4,7,-Cycloundecatriene, 1,5,9,9-tetra methyl-, Z, Z, Z-	$C_{15}H_{24}$	Terpenes	204.36	22.3144	1.15	No activity reported
Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-,[4aR-(4a.alpha.,7.alpha.,8a.beta.)]- (or β-helmiscapene, beta-Selinene)	$C_{15}H_{24}$	Terpenes	204.35	23.1348	0.60	Antioxidant and cytotoxic activity against HT29 (colon cancer) cells;[39] cytotoxicity against KB (oral cancer), MCF-7 (breast cancer) and NCI-H187 (small cell lung cancer) cells[40]
2-Isopropenyl-4a, 8-dimthyl-1,2,3,4,4a, 5,6,8a-octahydronapthalene (or 7-Epi-alpha-Selinene)	$C_{15}H_{24}$	Terpenes	204.36	23.3522	0.54	Antimicrobial activity against *Bacillus subtilis* and *Candida albicans*[41]
delta-Cadinene	$C_{15}H_{24}$	Terpenes	204.37	24.0207	0.61	Induction of apoptosis and cell cycle arrest on OVACR-3 (ovarian cancer) cells[42]
Caryophyllene oxide	$C_{15}H_{24}O$	Terpenes	220.36	25.4618	0.42	Chemosensitizing agents for doxorubicin chemotherapy;[43] anticancer;[44] increased the efficacy of DOX in MDA-MB-231 (breast cancer) cells;[45] inhibit STAT3 signaling pathway[46]
Isospathulenol	$C_{15}H_{24}O$	Terpenes	220.37	26.4932	0.71	Cytotoxic effects against *Aspergillus niger*, *Artemia salina* and *Caenorhabditis elegans*[47]
2,4-Decadienamide, N-isobutyl-, (E, E)- (or Pellitorine)	$C_{14}H_{25}NO$	Amides	223.36	32.8537	2.28	Antibacterial, anticancer and anti-inflammatory[48]
Piperidine, 1-(1-oxo-3-phenyl-2-prope nyl)- (or piperidine, 1-Cinnamoylpiperidine)	$C_{14}H_{17}NO$	Alkaloids	215.29	36.1008	0.22	No activity reported
(2E,4E)-1-(Pyrrolidin-1-yl) deca-2,4-dien-1-one (or Iyeremide A, sarmentine)	$C_{14}H_{23}NO$	Alkaloids	221.34	36.2247	0.37	Cytotoxicity against CCRF-CEM (acute lymphoblastic leukemia), HL-60 (acute promyelocytic leukemia), PC-3 (prostate carcinoma), and HA22T (hepatoma) cells;[27] inhibit lipoxygenase (5-LOX) and cyclooxygenase-1 (COX-1) [49]
(2E,4E)-N-Isobutyldodeca-2,4-dienamide (or Dodecatetraenoic acid isobutylamide)	$C_{16}H_{29}NO$	Amides	251.41	36.7524	0.48	Inhibit allergic and inflammatory[50]
N-Benzylidene-4-fluoroaniline	$C_{13}H_{10}FN$	Alkaloids	199.23	44.1035	0.34	No activity reported
(E)-5-(Benzo[d][1,3]dioxol-5-yl)-1-(pi peridin-1-yl) pent-2-en-1one (or piperanine)	$C_{17}H_{21}NO_3$	Alkaloids	287.359	44.5123	0.88	Hepatoprotective effect[51]
Piperlonguminine	$C_{16}H_{19}NO_3$	Alkaloids	273.33	44.8101	4.77	Anticancer against breast cancer cells[31]
(E)-1-(Piperidin-1-yl) hexadec-2-en-1-one	$C_{21}H_{39}NO$	Alkaloids	321.54	45.3603	0.79	No activity reported
Piperine	$C_{17}H_{19}NO_3$	Alkaloids	285.34	46.3182	5.09	Anticancer against Hep-G2 (hepatocellular carcinoma)[52] and Hela (cervical cancer) cells[53]
(2E,4E,10E)-N-Isobutylhexadeca-2,4,10-trienamide	$C_{20}H_{35}NO$	Amides	305.50	46.5162	0.48	No activity reported
(2E,4E)-N-Isobutyloctadeca-2,4-dienamide (or Pipericine)	$C_{22}H_{41}NO$	Amides	335.58	46.6004	0.85	Hepatoprotective effect[54]
1-Benzyl-2-(1-ethoxycarbonyl-2-phenylethyl)-4,5-dihydroimidazole (Acrivastine)	$C_{22}H_{24}N_2O_2$	Amides	348.45	46.6023	2.34	No activity reported
(E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl) hept-6-en-1 one (or Methyldihydromorphine)	$C_{18}H_{23}NO_3$	Opioid		47.8646	1.18	No activity reported

Contd...

Table 2: Contd...

Identified compounds	Formula	Nature of compound	Molecular massb (g/mol)	Retention time	Area (%)	Biological activity
Pyrrolidine, 1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]-, (E, E)- (or Pyrrolidine, Trichostachine, Piperyline)	$C_{16}H_{17}NO_3$	Alkaloids	271.32	47.9359	2.58	Antiproliferative effect, cycle arrest, induce apoptosis on MCF-7 cells and antitumor effect *in vivo*[55]
1H-Indene, 2-fluoro-2,3-dihydro-1-methoxy-, trans-(.+-.)-	$C_{10}H_{11}FO$	Amides		48.1182	0.66	No activity reported
(E)-1-(Piperidin-1-yl) octadec-2-en-1-one (or Pipercitine)	$C_{23}H_{43}NO$	Alkaloids	349.60	48.3679	21.66	Insecticidal activity[25]
(E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) hept-6-en-1one (or Piperolein A)	$C_{19}H_{25}NO_3$	Alkaloids	315.41	48.5620	0.24	No activity reported
(2E,6E)-7-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) hepta-2,6-dien-1-one (or Pipersintenamide)	$C_{19}H_{23}NO_3$	Alkaloids	313.39	49.1390	5.65	Cytotoxicity against CCRF-CEM (acute lymphoblastic leukemia), HL-60 (acute promyelocytic leukemia), PC-3 (prostate carcinoma), and HA22T (hepatoma) cells[27]
(2E,4E,14E)-N-Isobutylicosa-2,4,14-trienamide (or 2,4,14-Eicosatrienamide)	$C_{24}H_{43}NO$	Amides	361.61	49.3379	0.59	Cytoprotective activity on normal fibroblast L929 cells and hepatoprotective activity[54]
2-Furanol, 3,4-bis (1,3-benzodioxol-5-ylmethyl) tetrahydro- (or 2-Furanol, Cubebin)	$C_{20}H_{20}O_6$	Lignan	356.37	49.6489	0.28	Antiinflammatory;[56] anticancer[32]
Retrofractamide-A	$C_{20}H_{25}NO_3$	Alkaloids	327.42	50.3585	0.34	Larvicidal activity against *Culex pipiens* pallens, *Aedes aegypti* and *Aedes togoi*;[57] hepatoprotective effect[54]
2 (3H)-Furanone, 3,4-bis (1,3-benzodioxol-5-ylmethyl) dihydro-,(3R-trans)- (or (+)-Hinokinin, Cubebinolide)	$C_{20}H_{18}O_6$	Lignan	354.36	50.5191	1.13	Antiinflammatory;[58] antioxidant[59]
(E)-9-(Benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl) non-8-en-1-one (or Pyrrolidine, Tricholeine)	$C_{20}H_{27}NO_3$	Alkaloids	329.44	50.7269	0.42	Antiproliferative activity against various cancer cells[60]
(3R,4R)-3-(Benzo[d][1,3]dioxol-5-yl methyl)-4-(3,4-dimethoxybenzyl) dihydrofuran-2 (3H) one (or Kusunokinin)	$C_{21}H_{22}O_6$	Lignan	370.40	51.0435	1.28	Anticancer;[31] insecticidal activity against *Virola sebifera* and fungicidal activity against *Leucoagaricus gongylophorus*[61]
(E)-9-(Benzo[d][1,3]dioxol-5-yl)-1-(piperidin-1-yl) non-8-en-1-one (or Piperolein B)	$C_{21}H_{29}NO_3$	Alkaloids	343.47	51.3920	1.03	Inhibitor of acyl CoA: Diacylglycerol acyltransferase for potential therapy for the treatment of obesity and type 2 diabetes[62]
(2E,4E,12E)-13-(Benzo[d][1,3]dioxol-5-yl)-N-isobutyltrideca-2,4,12-trienamide (or Guineensine)	$C_{24}H_{33}NO_3$	Alkaloids	383.53	51.8600	10.17	Antiinflammatory[63]
(2E,4E,6E)-7-(Benzo[d][1,3] dioxol-5-yl)-1-(piperidin-1-yl) hepta-2,4,6-trien-1-one (or Piperettine)	$C_{19}H_{21}NO_3$	Alkaloids	311.38	52.9692	0.31	Trypanocidal effects against epimastigotes and amastigotes of *Trypanosoma cruzi*[64]
(22E)-Stigmasta-5,22-dien-3-ol (or beta-Stigmasterol, Poriferasterol)	$C_{29}H_{48}O$	Steroid	412.70	53.0319	1.74	Induce DNA damage and cell death[65]
(2E,4E,8E)-9-(Benzo[d] [1,3] dioxol-5-yl)-1-(piperidin-1-yl) nona-2,4,8-trien-1-one (or Dehydropipernonaline)	$C_{21}H_{25}NO_3$	Alkaloids	339.47	53.5356	2.32	Coronary vasodilating activity[66]
gamma-Sitosterol (or clionasterol)	$C_{29}H_{50}O$	Terpenes	414.72	53.7147	0.48	Cytotoxicity against P388 (murine lymphocytic leukaemia) and HL60 (leukemia) cells[67]
(2E,4E,12E)-13-(Benzo[d][1,3]dioxol-5-yl)-Nisobutyltrideca-2,4,12-trienamide (or Guineensine)	$C_{24}H_{33}NO_3$	Alkaloids	383.53	55.6810		Antiinflammatory[63]

at 5.09% [Table 2]. Similarly, CP2 (PFPE) exhibited IC_{50} values of 7.45 ± 1.59 μg/ml in MCF-7 cell lines, which was better than DPCE (IC_{50} at 23.46 ± 1.10 μg/ml).[17] These results indicate that PFPE, less piperine, was a potential crude extract in anticancer.

O. viverrini excretory/secretory products and *O. viverrini* antigen induce the expression of TLR4, IL-6, IL-8, TLR2, NF-κB, iNOS and COX-2 causing damage to biliary epithelium.[68] In this current study, PFPE showed down regulation of NF-kB, STAT-3 and COX-2 proteins [Figure 2]. In cancer cells, NF-kB and STAT-3 are major transcription factors that regulate proliferation, inflammatory, angiogenesis, invasive and apoptosis resistance by induction of several proteins, such as cyclin D, cyclin E1, CDK2, CDK4, CDK6, c-myc, tumor necrosis factor alpha, interleukin-1 (IL-1), IL-6, IL-8, VEGF and MMP-9.[69] NF-kB and STAT-3 proteins are induced by IL-6 to stimulate COX-2 expression in the inflammation process and cell cycle,[70,71] which associate to CCA progression. Therefore, suppression of NF-kB, STAT-3 and COX-2 proteins cause cancer growth inhibition. Piperlongumine, an alkaloid from *P. longum* reduces NF-kB and c-Myc protein levels and inhibits binding of NF-kB with DNA at promoters in lymphoma cancer cells.[72] Moreover, piperlongumine also reduced the phosphorylation of JAK-1, JAK-2 and STAT-3 in gastric cancer cells.[73] Matrine, an alkaloid from *Sophora flavescens* Ait., significantly inhibits the viability by reduction the phosphorylation levels of JAK-2 and STAT3 proteins in CCA cells.[74] Curcumin, a natural extracted polyphenol from *C. longa*, also suppresses proliferation in human biliary cancer cells through inhibition of NF-kB, STAT-3 and JAK1 proteins.[75]

Table 3: Cytotoxicity of piperine free *Piper nigrum* extract against cholangiocarcinoma, cholangiocyte and normal mouse fibroblast cell lines

Cell lines	IC_{50} value±SD (μg/ml)			
	DPCE	Piperine	PFPE	Doxorubicin
CCA				
KKU-100	22.88±0.43	46.53±0.09	17.79±0.88	0.78±0.03
KKU-M213	22.22±0.26	27.01±0.36	13.70±1.14	1.75±0.02
KKU-M055	46.66±0.48	55.32±0.22	16.74±0.61	0.69±0.09
TFK-1	23.25±0.45	29.38±0.07	15.30±0.18	15.19±0.12
HuCC-T1	37.17±0.03	35.02±0.12	20.72±0.75	2.53±0.04
Normal cholangiocyte				
MMNK-1	33.25±0.28	60.68±0.72	19.65±0.26	0.62±0.05
Normal fibroblast				
L-929	No effect	No effect	45.53±0.50	0.20±0.01

P. nigrum: *Piper nigrum*; DPCE: Dichloromethane *P. nigrum* crude extract; PFPE: Piperine free *P. nigrum* extract; CCA: Cholangiocarcinoma; SD: Standard deviation

There are many evidences on genes and proteins which relate to bile duct cancer growth and progression, such as p53 mutation, inactivation of p21 and activation of Ras and MAPKs proteins.[76] Here, we found that PFPE could inhibit CCA cancer proliferation by decreasing of topoisomerase II, Akt, c-Myc, cyclin D1, and increasing of p21 protein levels [Figure 4]. Topoisomerase II is an enzyme involved in the DNA replication process that controls cell cycle with peaking at G2/M phase.[77] Therefore, down regulation of topoisomerase II by PFPE could induced DNA damage, interrupted cell growth and caused cell death on KKU-M213 and TFK-1 cells. Most of the clinically active agents, including etoposide (lignan) and doxorubicin (alkaloid) are topoisomerase inhibitors.[78] Previously andrographolide analogue 3A.1 from *Andrographis paniculata*, a diterpenoid lactone, induces cell cycle arrest by down-regulation of CDK6 and cyclin D1 in KKU-M213 cell lines.[79] Surprisingly, PFPE also exerted a significant reduction of Akt protein leading to decreasing of c-Myc and cyclin D1 and increasing of p21 levels [Figure 6]. Akt and cyclin D1 stimulate the cell cycle progression from G1/S phase to G2/M phase.[80] β-caryophyllene oxide, a terpene compound from *P. nigrum*, shows down-regulation of downstream of AKT pathway, including cyclin D1, COX-2 and VEGF and also up-regulation of p53 and p21 proteins in human prostate and breast cancer cells.[81]

In this study, we founded that the PFPE induced cell death by causing DNA fragmentation, increasing apoptotic proteins (p53, Bax and PUMA) and decreasing Bcl-2 protein levels [Figure 5]. p53, a tumor suppressor and transcription factor, is initially induced when DNA

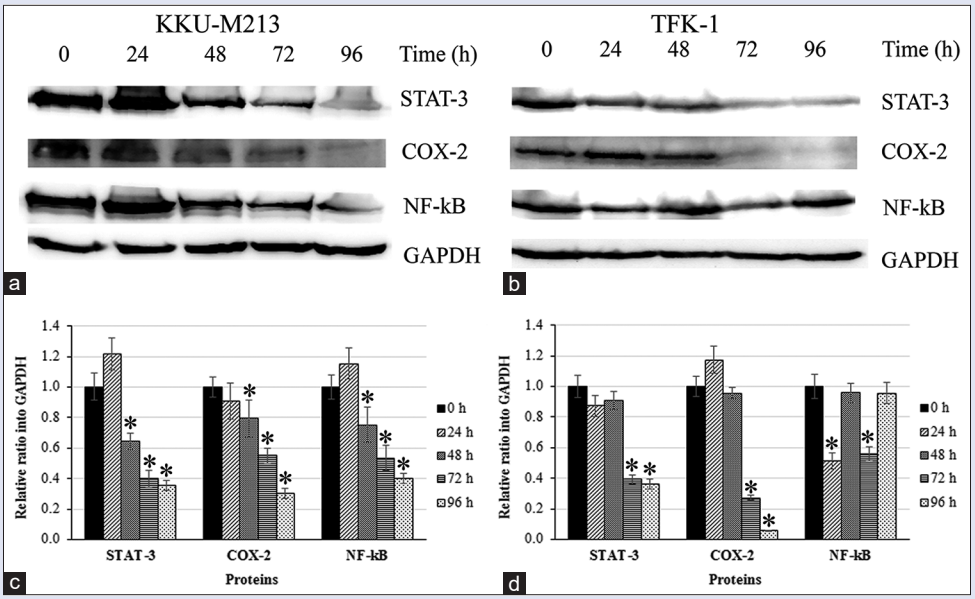

Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer of activated B cells and GAPDH proteins were measured using the Western blot analysis. Densitometric analysis normalized to GAPDH. Data were represented as mean ± standard deviation and three independent experiments were done. *$P < 0.05$ compared with control group (0 h)

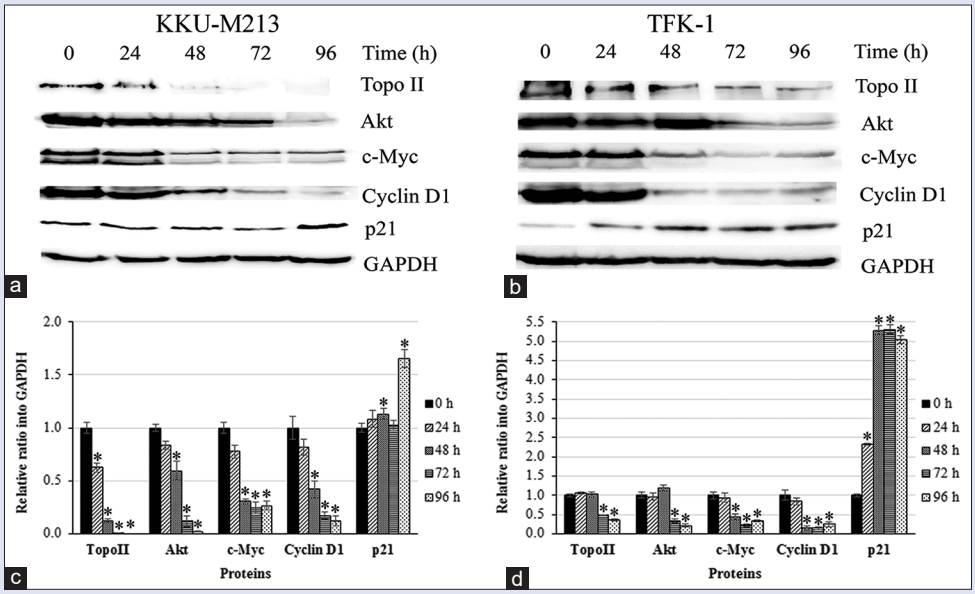

Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase II, AKT8 virus oncogene cellular homolog, avian myelocytomatosis virus oncogene cellular homolog, cyclin D1 and p21 proteins were investigated using Western blot analysis. Fold change of each protein was measured by densitometry quantitation using ImageJ software and normalized with GAPDH. $P < 0.05$ of three independent experiments was considered to indicate a statistically significant differences compared to control group (0 h)

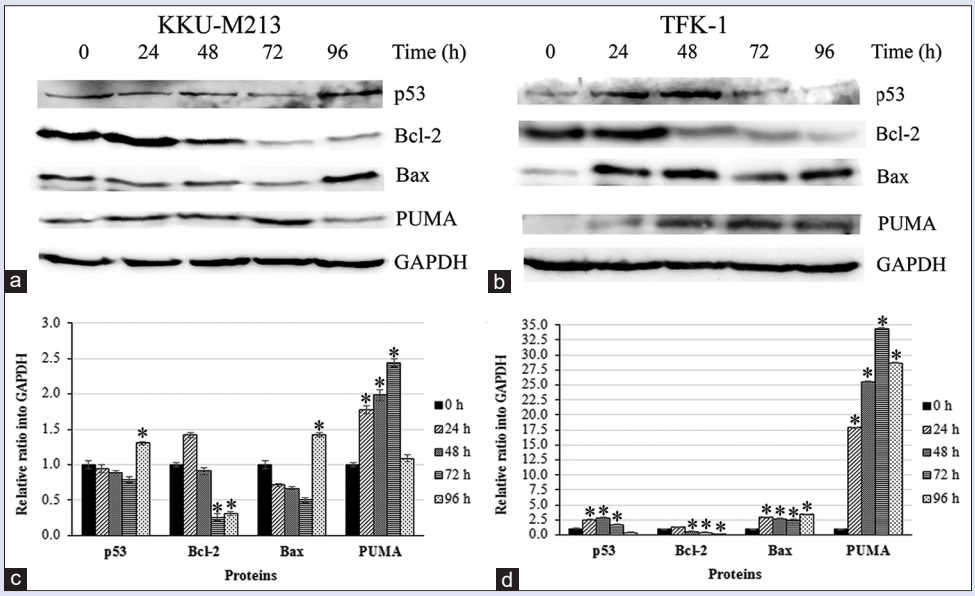

Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2, Bcl-2-associated X protein and PUMA proteins were investigated using Western blot analysis. Fold change of each protein was measured by densitometry quantitation using ImageJ software and normalized with GAPDH. $P < 0.05$ of three independent experiments was considered to indicate a statistically significant difference compared to control group (0 h)

damage and takes responsibility to activate several apoptotic genes, such as Bax, PUMA and NOXA.[82-84] Similarly, ethanolic extract of *P. nigrum* has antiproliferative effect on MCF-7 cells, antitumor effect *in vivo* and triggering apoptosis via p53 and Bax and decreasing of Bcl-2 proteins.[55] Curcumin effectively induces apoptosis in CCA (CCLP-1 and SG-231) cells by stimulation of Notch1, Hes-1 and survivin apoptotic proteins.[85] Andrographolide analog 3A.1 has cytotoxicity with IC_{50} of 8.0 μM on KKU-M213 cells at 24 h after treatment and induces apoptosis via induction of cleaved PARP-1, Bax, caspase-3, and p53.[79] Matrine stimulates apoptosis in CCA cells through induction of cytochrome c releasing from mitochondria and reduction of caspase-3 and-9 activity.[74] Taken together, PFPE can be a potential candidate for CCA treatment in future. However, study in CCA *in vivo* and clinical trial need to be carried out.

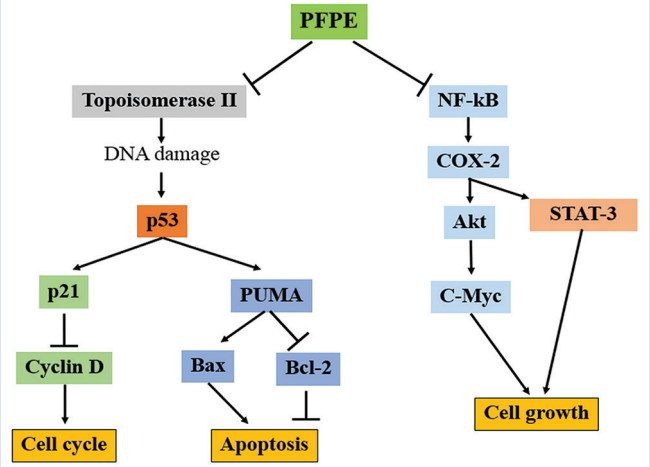

Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

CONCLUSION

PFPE showed strong cytotoxicity against KKU-M213 and TFK-1 cell lines with IC_{50} values of 13.70 ± 1.14 and 15.30 ± 0.18 µg/ml, respectively. PFPE suppressed inflammation through down-regulation of NF-kB, STAT-3 and COX-2. Moreover, PFPE inhibited CCA cells growth and proliferation by down-regulation of topoisomerase II, Akt, c-Myc and cyclin D and up-regulation of p21. Furthermore, PFPE triggered apoptosis through inhibition of Bcl-2 and induction of p53, Bax and PUMA levels as summarized in the Figure 5. In summary, PFPE can be served as a promising crude extract for CCA treatment.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Blechacz B, Gores GJ. Cholangiocarcinoma: Advances in pathogenesis, diagnosis, and treatment. Hepatology 2008;48:308-21.
2. Ghouri YA, Mian I, Blechacz B. Cancer review: Cholangiocarcinoma. J Carcinog 2015;14:1.
3. Saha SK, Zhu AX, Fuchs CS, Brooks GA. Forty-year trends in cholangiocarcinoma incidence in the U.S.: Intrahepatic disease on the rise. Oncologist 2016;21:594-9.
4. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J Hepatol 2002;37:806-13.
5. Sripa B, Pairojkul C. Cholangiocarcinoma: Lessons from Thailand. Curr Opin Gastroenterol 2008;24:349-56.
6. Plentz RR, Malek NP. Clinical presentation, risk factors and staging systems of cholangiocarcinoma. Best Pract Res Clin Gastroenterol 2015;29:245-52.
7. Gatto M, Alvaro D. New insights on cholangiocarcinoma. World J Gastrointest Oncol 2010;2:136-45.
8. Squadroni M, Tondulli L, Gatta G, Mosconi S, Beretta G, Labianca R. Cholangiocarcinoma. Crit Rev Oncol Hematol 2017;116:11-31.
9. van der Gaag NA, Kloek JJ, de Bakker JK, Musters B, Geskus RB, Busch OR, *et al.* Survival analysis and prognostic nomogram for patients undergoing resection of extrahepatic cholangiocarcinoma. Ann Oncol 2012;23:2642-9.
10. Barreto JN, McCullough KB, Ice LL, Smith JA. Antineoplastic agents and the associated myelosuppressive effects: A review. J Pharm Pract 2014;27:440-6.
11. Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA. Biological role of *Piper nigrum* L. (Black pepper): A review. Asian Pac J Trop Biomed 2012;2:S1945-53.
12. Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A, *et al.* Phytochemistry of the genus *Piper*. Phytochemistry 1997;46:597-673.
13. Prashant A, Rangaswamy C, Yadav AK, Reddy V, Sowmya MN, Madhunapantula S. Anticancer activity of ethanolic extracts of & against colorectal carcinoma cell lines. Int J Appl Basic Med Res 2017;7:67-72.
14. Grinevicius VM, Andrade KS, Ourique F, Micke GA, Ferreira SR, Pedrosa RC. Antitumor activity of conventional and supercritical extracts from *Piper nigrum* L. cultivar Bragantina through cell cycle arrest and apoptosis induction. J Supercrit Fluids 2017;128:94-101.
15. Sriwiriyajan S, Tedasen A, Lailerd N, Boonyaphiphat P, Nitiruangjarat A, Deng Y, *et al.* Anticancer and cancer prevention effects of piperine-free *Piper nigrum* extract on n-nitrosomethylurea-induced mammary tumorigenesis in rats. Cancer Prev Res (Phila) 2016;9:74-82.
16. Deng Y, Sriwiriyajan S, Tedasen A, Hiransai P, Graidist P. Anti-cancer effects of *Piper nigrum* via inducing multiple molecular signaling *in vivo* and *in vitro*. J Ethnopharmacol 2016;188:87-95.
17. Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus *Piper* in breast cancer cell lines. Trop J Pharm Res 2014;13:921-8.
18. Geran RI, Greenberg NH, McDonald MM, Schumacher AM, Abbot BJ. Protocol for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemoth Rep 1972;3:1-103.
19. Graidist P, Martla M, Sukpondma Y. Cytotoxic activity of *Piper cubeba* extract in breast cancer cell lines. Nutrients 2015;7:2707-18.
20. Plengsuriyakarn T, Thitapakorn V, Na-Bangchang K, Karbwang J. Thai medicinal plants: Potential sources of anticholangiocarcinoma drugs. Int J Pharm Pharmacol 2013;2:68-82.
21. Leelawat S, Leelawat K. Molecular mechanisms of cholangiocarcinoma cell inhibition by medicinal plants. Oncol Lett 2017;13:961-6.
22. Zainal Ariffin SH, Wan Omar WH, Zainal Ariffin Z, Safian MF, Senafi S, Megat Abdul Wahab R. Intrinsic anticarcinogenic effects of *Piper sarmentosum* ethanolic extract on a human hepatoma cell line. Cancer Cell Int 2009;9:6.
23. Ovadje P, Ma D, Tremblay P, Roma A, Steckle M, Guerrero JA, *et al.* Evaluation of the efficacy biochemical mechanism of cell death induction by *Piper longum* extract selectively in *in vitro* and *in vivo* models of human cancer cells. PLoS One 2014;9:e113250.
24. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells *in vitro*. BMC Complement Altern Med 2010;10:55.
25. Geris R, Ribeiro PR, Brandão MD, Da Silva HH, Silva IG. Bioactive natural products as potential candidates to control *Aedes aegypti*, the vector of dengue. In: Rahman A, editor. Studies in Natural Products Chemistry. Vol. 37. Oxford: Elsevier; 2012. p. 227-376.
26. Muharini R, Liu Z, Lin W, Proksch P. New amides from the fruits of *Piper retrofractum*. Tetrahedron Lett 2015;56:2521-5.
27. Chen JJ, Huang YC, Chen YC, Huang YT, Wang SW, Peng CY, *et al.* Cytotoxic amides from *Piper sintenense*. Planta Med 2002;68:980-5.
28. Chen JJ, Duh CY, Huang HY, Chen IS. Cytotoxic Constituents of *Piper sintenense*. Helv Chim Acta 2003;86:2058-64.
29. Dahham SS, Tabana YM, Iqbal MA, Ahamed MB, Ezzat MO, Majid AS, *et al.* The Anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. Molecules 2015;20:11808-29.
30. Shen T, Zhang L, Wang YY, Fan PH, Wang XN, Lin ZM, *et al.* Steroids from Commiphora mukul display antiproliferative effect against human prostate cancer PC3 cells via induction of apoptosis. Bioorg Med Chem Lett 2012;22:4801-6.
31. Sriwiriyajan S, Sukpondma Y, Srisawat T, Madla S, Graidist P. (-)-Kusunokinin and piperloguminine from *Piper nigrum*: An alternative option to treat breast cancer. Biomed Pharmacother 2017;92:732-43.
32. Rajalekshmi DS, Kabeer FA, Madhusoodhanan AR, Bahulayan AK, Prathapan R, Prakasan N, *et al.* Anticancer activity studies of cubebin isolated from *Piper cubeba* and its synthetic derivatives. Bioorg Med Chem Lett 2016;26:1767-71.
33. Kohoude MJ, Gbaguidi F, Agbani P, Ayedoun MA, Cazaux S, Bouajila J. Chemical composition

and biological activities of extracts and essential oil of Boswellia dalzielii leaves. Pharm Biol 2017;55:33-42.

34. Rabi T, Bishayee A. d -Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J Carcinog 2009;8:9.
35. Zou L, Liu W, Yu L. Beta-elemene induces apoptosis of K562 leukemia cells. Zhonghua Zhong Liu Za Zhi 2001;23:196-8.
36. Martins Cde M, do Nascimento EA, de Morais SA, de Oliveira A, Chang R, Cunha LC, *et al.* Chemical constituents and evaluation of antimicrobial and cytotoxic activities of *Kielmeyera coriacea* Mart. & Zucc. essential oils. Evid Based Complement Alternat Med 2015;2015:1-9. doi.org/10.1155/2015/842047.
37. Baldissera MD, Souza CF, Grando TH, Stefani LM, Monteiro SG. β-caryophyllene reduces atherogenic index and coronary risk index in hypercholesterolemic rats: The involvement of cardiac oxidative damage. Chem Biol Interact 2017;270:9-14.
38. Alvarez-González I, Madrigal-Bujaidar E, Castro-García S. Antigenotoxic capacity of beta-caryophyllene in mouse, and evaluation of its antioxidant and GST induction activities. J Toxicol Sci 2014;39:849-59.
39. Ali NA, Wursterb M, Denkert A, Arnold N, Fadail I, Al-Didamony G, *et al.* Chemical composition, antimicrobial, antioxidant and cytotoxic activity of essential oils of *Plectranthus cylindraceus* and *Meriandra benghalensis* from Yemen. Nat Prod Commun 2012;7:1099-102.
40. Keawsa-ard S, Liawruangrath B, Liawruangrath S, Teerawutgulrag A, Pyne SG. Chemical constituents and antioxidant and biological activities of the essential oil from leaves of *Solanum spirale*. Nat Prod Commun 2012;7:955-8.
41. Ali NAA, Chhetri BK, Dosoky NS, Shari K, Al-Fahad AJ, Wessjohann L, *et al.* Antimicrobial, antioxidant, and cytotoxic activities of *Ocimum forskolei* and *Teucrium yemense* (*Lamiaceae*) essential oils. Medicines (Basel) 2017;4:7.
42. Hui LM, Zhao GD, Zhao JJ. δ-Cadinene inhibits the growth of ovarian cancer cells via caspase-dependent apoptosis and cell cycle arrest. Int J Clin Exp Pathol 2015;8:6046-56.
43. Dl Giacomo S, Dl Sotto A, Mazzanti G, Wink M. Chemosensitizing properties of β-Caryophyllene and β-caryophyllene oxide in combination with doxorubicin in human cancer cells. Anticancer Res 2017;37:1191-6.
44. Fidyt K, Fiedorowicz A, Strządała L, Szumny A. β-caryophyllene and β-caryophyllene oxide-natural compounds of anticancer and analgesic properties. Cancer Med 2016;5:3007-17.
45. Hanušová V, Caltová K, Svobodová H, Ambrož M, Skarka A, Murínová N, *et al.* The effects of β-caryophyllene oxide and trans-nerolidol on the efficacy of doxorubicin in breast cancer cells and breast tumor-bearing mice. Biomed Pharmacother 2017;95:828-36.
46. Kim C, Cho SK, Kapoor S, Kumar A, Vali S, Abbasi T, *et al.* β-Caryophyllene oxide inhibits constitutive and inducible STAT3 signaling pathway through induction of the SHP-1 protein tyrosine phosphatase. Mol Carcinog 2014;53:793-806.
47. Dosoky NS, Satyal P, Gautam TP, Setzer WN. Composition and biological activities of & *Murraya paniculata* (L.) Jack essential oil from Nepal. Medicines (Basel) 2016;3:7.
48. Ku SK, Lee IC, Kim JA, Bae JS. Anti-septic effects of pellitorine in HMGB1-induced inflammatory responses *in vitro* and *in vivo*. Inflammation 2014;37:338-48.
49. Stöhr JR, Xiao PG, Bauer R. Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*. J Ethnopharmacol 2001;75:133-9.
50. Gulledge TV, Collette NM, Mackey E, Johnstone SE, Moazami Y, Todd DA, *et al.* Mast cell degranulation and calcium influx are inhibited by an *Echinacea purpurea* extract and the alkylamide dodeca-2E,4E-dienoic acid isobutylamide. J Ethnopharmacol 2018;212:166-74.
51. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Protective effects of amide constituents from the fruit of *Piper chaba* on D-galactosamine/TNF-alpha-induced cell death in mouse hepatocytes. Bioorg Med Chem Lett 2008;18:2038-42.
52. Gunasekaran V, Elangovan K, Niranjali Devaraj S. Targeting hepatocellular carcinoma with piperine by radical-mediated mitochondrial pathway of apoptosis: An *in vitro* and *in vivo* study. Food Chem Toxicol 2017;105:106-18.
53. Han SZ, Liu HX, Yang LQ, Cui LD, Xu Y. Piperine (PP) enhanced mitomycin-C (MMC) therapy of human cervical cancer through suppressing Bcl-2 signaling pathway via inactivating STAT3/NF-κB. Biomed Pharmacother 2017;96:1403-10.
54. Matsuda H, Ninomiya K, Morikawa T, Yasuda D, Yamaguchi I, Yoshikawa M. Hepatoprotective amide constituents from the fruit of *Piper chaba*: Structural requirements, mode of action, and new amides. Bioorg Med Chem 2009;17:7313-23.
55. de Souza Grinevicius VM, Kviecinski MR, Santos Mota NS, Ourique F, Porfirio Will Castro LS, Andreguetti RR, *et al. Piper nigrum* ethanolic extract rich in piperamides causes ROS overproduction, oxidative damage in DNA leading to cell cycle arrest and apoptosis in cancer cells. J Ethnopharmacol 2016;189:139-47.
56. Lima TC, Lucarini R, Volpe AC, de Andrade CQJ, Souza AM, Pauletti PM, *et al. In vivo* and *in silico* anti-inflammatory mechanism of action of the semisynthetic (-)-cubebin derivatives (-)-hinokinin and (-)-O-benzylcubebin. Bioorg Med Chem Lett 2017;27:176-9.
57. Park IK, Lee SG, Shin SC, Park JD, Ahn YJ. Larvicidal activity of isobutylamides identified in *Piper nigrum* fruits against three mosquito species. J Agric Food Chem 2002;50:1866-70.
58. Godoy de Lima R, Barros MT, da Silva Laurentiz R. Medicinal attributes of Lignans extracted from & *Piper cubeba*: Current developments. ChemistryOpen 2018;7:180-91.
59. Medola JF, Cintra VP, Pesqueira E Silva EP, de Andrade Royo V, da Silva R, Saraiva J, *et al.* (-)-Hinokinin causes antigenotoxicity but not genotoxicity in peripheral blood of Wistar rats. Food Chem Toxicol 2007;45:638-42.
60. Wang YH, Goto M, Wang LT, Hsieh KY, Morris-Natschke SL, Tang GH, *et al.* Multidrug resistance-selective antiproliferative activity of *Piper* amide alkaloids and synthetic analogues. Bioorg Med Chem Lett 2014;24:4818-21.
61. Bicalho KU, Terezan AP, Martins DC, Freitas TG, Fernandes JB, Silva MF, *et al.* Evaluation of the toxicity of *Virola sebifera* crude extracts, fractions and isolated compounds on the nest of leaf-cutting ants. Psyche 2012;2012:785424.
62. Lee SW, Rho MC, Park HR, Choi JH, Kang JY, Lee JW, *et al.* Inhibition of diacylglycerol acyltransferase by alkamides isolated from the fruits of *Piper longum* and *Piper nigrum*. J Agric Food Chem 2006;54:9759-63.
63. Reynoso-Moreno I, Najar-Guerrero I, Escareño N, Flores-Soto ME, Gertsch J, Viveros-Paredes JM. An endocannabinoid uptake inhibitor from black pepper exerts pronounced anti-inflammatory effects in mice. J Agric Food Chem 2017;65:9435-42.
64. Ribeiro TS, Freire-de-Lima L, Previato JO, Mendonça-Previato L, Heise N, de Lima ME. Toxic effects of natural piperine and its derivatives on epimastigotes and amastigotes of *Trypanosoma cruzi*. Bioorg Med Chem Lett 2004;14:3555-8.
65. Chaturvedula VS, Gao Z, Hecht SM, Jones SH, Kingston DG. A new acylated oleanane triterpenoid from *Couepia polyandra* that inhibits the lyase activity of DNA polymerase ß. J Nat Prod 2003;66:1463-65.
66. Shoji N, Umeyama A, Saito N, Takemoto T, Kajiwara A, Ohizumi Y. Dehydropipernonaline, an amide possessing coronary vasodilating activity, isolated from *Piper longum* L. J Pharm Sci 1986;75:1188-9.
67. Manoharan KP, Yang D, Hsu A, Huat BT. Evaluation of *Polygonum bistorta* for anticancer potential using selected cancer cell lines. Med Chem 2007;3:121-6.
68. Prueksapanich P, Piyachaturawat P, Aumpansub P, Ridtitid W, Chaiteerakij R, Rerknimitr R. Liver fluke-associated biliary tract cancer. Gut Liver 2018;12:236-45.
69. Fan Y, Mao R, Yang J. NF-κB and STAT3 signaling pathways collaboratively link inflammation to cancer. Protein Cell 2013;4:176-85.
70. Basu A, Das AS, Sharma M, Pathak MP, Chattopadhyay P, Biswas K, *et al.* STAT3 and NF-κB are common targets for kaempferol-mediated attenuation of COX-2 expression in IL-6-induced macrophages and carrageenan-induced mouse paw edema. Biochem Biophys Rep 2017;12:54-61.
71. Chen SC, Chang YL, Wang DL, Cheng JJ. Herbal remedy magnolol suppresses IL-6-induced STAT3 activation and gene expression in endothelial cells. Br J Pharmacol 2006;148:226-32.
72. Han SS, Son DJ, Yun H, Kamberos NL, Janz S. Piperlongumine inhibits proliferation and survival of Burkitt lymphoma *in vitro*. Leuk Res 2013;37:146-54.
73. Song B, Zhan H, Bian Q, Gu J. Piperlongumine inhibits gastric cancer cells via suppression of the JAK1,2/STAT3 signaling pathway. Mol Med Rep 2016;13:4475-80.
74. Yang N, Han F, Cui H, Huang J, Wang T, Zhou Y, *et al.* Matrine suppresses proliferation and induces apoptosis in human cholangiocarcinoma cells through suppression of JAK2/STAT3 signaling. Pharmacol Rep 2015;67:388-93.
75. Prakobwong S, Gupta SC, Kim JH, Sung B, Pinlaor P, Hiraku Y, *et al.* Curcumin suppresses proliferation and induces apoptosis in human biliary cancer cells through modulation of multiple cell signaling pathways. Carcinogenesis 2011;32:1372-80.
76. Fava G. Molecular mechanisms of cholangiocarcinoma. World J Gastrointest Pathophysiol 2010;1:12-22.
77. Larsen AK, Skladanowski A, Bojanowski K. The roles of DNA topoisomerase II during the cell cycle. Prog Cell Cycle Res 1996;2:229-39.
78. Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009;9:338-50.
79. Nateewattana J, Dutta S, Reabroi S, Saeeng R, Kasemsook S, Chairoungdua A, *et al.* Induction of apoptosis in cholangiocarcinoma by an andrographolide analogue is mediated through topoisomerase II alpha inhibition. Eur J Pharmacol 2014;723:148-55.

80. Xu N, Lao Y, Zhang Y, Gillespie DA. Akt: A double-edged sword in cell proliferation and genome stability. J Oncol 2012;2012:1-15. doi:10.1155/2012/951724.
81. Park KR, Nam D, Yun HM, Lee SG, Jang HJ, Sethi G, *et al*. β-Caryophyllene oxide inhibits growth and induces apoptosis through the suppression of PI3K/AKT/mTOR/S6K1 pathways and ROS-mediated MAPKs activation. Cancer Lett 2011;312:178-88.
82. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-9.
83. Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 2001;7:683-94.
84. Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 2001;7:673-82.
85. Koprowski S, Sokolowski K, Kunnimalaiyaan S, Gamblin TC, Kunnimalaiyaan M. Curcumin-mediated regulation of Notch1/hairy and enhancer of split-1/survivin: molecular targeting in cholangiocarcinoma. J Surg Res 2015;198:434-40.