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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
Publication date	2020/3/1
Journal	Pharmacognosy Magazine
Volume	16
Issue	68
Pages	28
Publisher	Medknow Publications
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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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.

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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 ...

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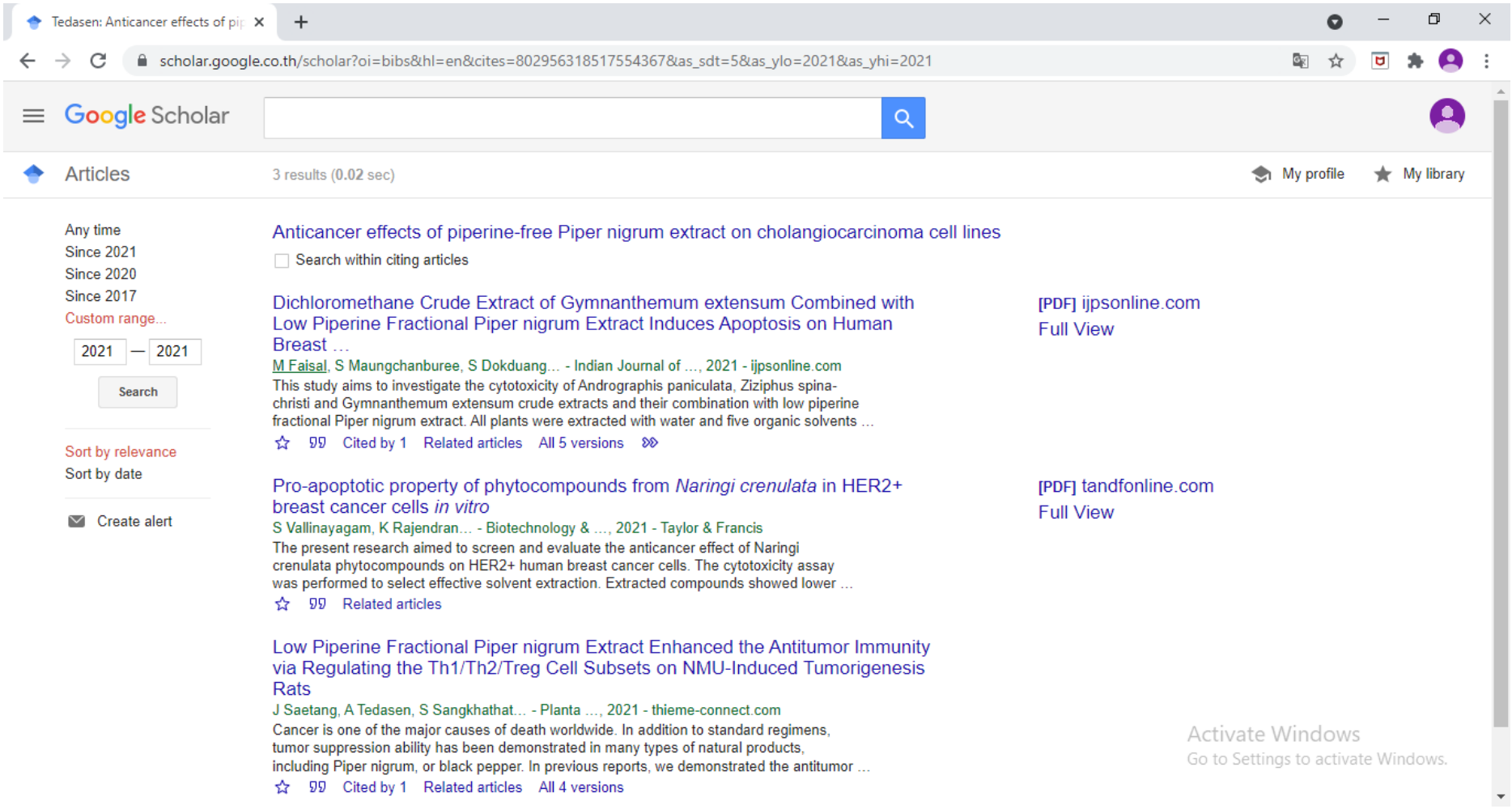
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Pro-apoptotic property of phytocompounds from *Naringi crenulata* in HER2+ breast cancer cells *in vitro*

Sugumari Vallinayagam , Karthikeyan Rajendran & Vigneshkumar Sekar

To cite this article: Sugumari Vallinayagam , Karthikeyan Rajendran & Vigneshkumar Sekar (2021) Pro-apoptotic property of phytocompounds from *Naringi crenulata* in HER2+ breast cancer cells *in vitro* , Biotechnology & Biotechnological Equipment, 35:1, 311-322, DOI: 10.1080/13102818.2020.1868333

To link to this article: https://doi.org/10.1080/13102818.2020.1868333

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Pro-apoptotic property of phytocompounds from *Naringi crenulata* in HER2+ breast cancer cells *in vitro*

Sugumari Vallinayagam, Karthikeyan Rajendran and Vigneshkumar Sekar

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ABSTRACT

The present research aimed to screen and evaluate the anticancer effect of *Naringi crenulata* phytocompounds on HER2+ human breast cancer cells. The cytotoxicity assay was performed to select effective solvent extraction. Extracted compounds showed lower toxicity on normal breast cells and significant cytotoxicity on human HER2+ breast cancer cells (SK-BR3) with an IC_{50} value of 24.59 μg/mL. The results indicate that the *Naringi crenulata* extract (NCE) shows anticancer potential via stimulating the cellular death of cancerous cells. The findings of the present study thus suggest that NCE supplementation might help in the treatment of HER2+ breast cancer.

ARTICLE HISTORY
Received 5 October 2020
Accepted 20 December 2020

KEYWORDS
HER2+ breast cancer; *Naringi crenulata* extract; ultrasound-assisted extraction; apoptosis

Introduction

Breast cancers, in general, are differentiated into 5 different types such us HER2+, a normal-like tumor, basal-like tumor, luminal A and luminal B, based on the gene expression of the tumor cells. Studies have identified that breast cancer is the second leading fatal cancer in the world, and is said to be malignant [1]. Earlier researchers investigated various chemotherapies and radiotherapies to treat hormone-responsive breast cancer, but therapy for HER2+ breast cancer is still required [2]. The HER2 is expressed and amplified on the surface of the cell, and is involved in cell signaling pathways. Endocrine therapies were reported to show less effect on HER2+ breast cancer cells [3]. The HER2 receptor inhibitor trastuzumab can suppress the HER2 receptor and significantly inhibit the development of breast tumors. Lapatinib (LPT) prevents tumor growth *via* inhibiting two tyrosine kinases (EGFR and HER2) by modifying the intracellular domain of HER2 [4]. Binding of inhibitor at the ATP-binding site of the HER2 protein kinase inactivates the phosphorylation and also inhibits the ERK-1/2 and PI3K downstream signaling cascades. LPT, being an FDA-approved drug, has been utilized for the treatment of breast cancer since 2007 [5]. LPT has low solubility and high permeability in bodily fluids due to its poor solubility of 0.007 mg/mL in water [6]. The major downside of LPT is the dosage restriction (the minimum appropriate dose is 1250 mg/day), due to poor bioavailability in the oral setting. Numerous side effects have been witnessed with LPT, which include cardiotoxicity, hepatotoxicity and diarrhea. Chemotherapy can cause toxicity in patients including various side effects and risks, as well as immune suppression by affecting bone marrow cells and increasing susceptibility to diseases. Consequently, an alternative therapeutic method must resolve the side effects, which exhibit greater efficacy against cancer [7].

It is well known from ancient times that medicinal plant extracts are used systematically in the treatment and prevention of various diseases. Surprisingly, traditional reports explained that the utilization of medicinal plants in the treatment of cancer did not cause any side effects. There are now 121 medicinal products used for cancer treatment, 90 are plant-derived [8]. About 74% of these drug compounds were discovered by testing a reference to folk medicine. Guggulsterone is extracted from the *Commiphora mukul* plant and suppresses the DNA binding of NF-κB, which is involved in proliferative, anti-apoptosis, and metastatic pathways. The rhizome of turmeric (*Curcuma longa*) is a source for curcumin, utilized to inhibit carcinogenesis, to obstruct proliferation, and to encourage apoptosis [9–11]. Several studies on human

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breast cancer cells have reported the anti-cancer activities of many phytocompounds. Indeed, the researchers discovered that HER2 suppression boosted the antiproliferative activity and stimulated apoptotic activity [12,13]. Many ayurvedic medicines propose epidemiological examinations to prevent cancer. Ayurvedic medicines such as curcumin, vinblastine, withanolide, etc. downregulate HER2, induce cell apoptosis, and can inhibit protein tyrosine kinases [14,15]. Therefore, supplementation of *Naringi crenulata* extracts can help to suppress HER2 expression and to treat HER2+ breast cancer.

Naringi crenulata is a branched tree with a wide distribution in South India and Myanmar [16]. Different tropical diseases have been treated with the help of extracts of the fruits, leaves and roots in folk medicinal practice. Modern research described that the plant extract of *Naringi crenulata* has anti-inflammatory activity [17], anti-oxidative effect [17] and hepatoprotective property [18], but the pharmacological activities of this plants are not fully studied. The utilization of antioxidants along with anticancer drugs will reduce the adverse outcomes of reactive species. The presence of higher content of antioxidants in *Naringi crenulata* makes it likely to find an anticancer compound in this plant. There are hopes that this plant's anticancer compounds can overcome the side effects. So far, the anticancer properties of *Naringi crenulata* leaf extract have yet to be investigated in HER2+ breast cancer. While the action mechanisms of several phytocompounds currently remain unknown, several studies report the bioactivity of certain compounds.

In the present study, we aimed to screen and evaluate the anticancer effect of phytocompounds of *Naringi crenulata* on HER2+ human breast cancer cells (SK-BR3) *in vitro* as well as to explore the fundamental anticancer mechanism of the extracted phytocompounds *in silico*. We found that some phytocompounds of *Naringi crenulata,* such as 6,7-Dimethoxy-1-methyl-3,4-dihydroiso-quinoline, Hesperetin, Hesperetin 7-rhamnoglucoside, and Tanakamine, exhibited the properties of anticancer activity *via* stimulating the apoptosis in cancer cells.

Materials and methods

Reagents and chemicals

McCoy's 5A growth medium, Dulbecco's modified Eagle's medium (DMEM), and trypsin EDTA (1×), RPMI-1640 medium was acquired from Hi-Media. The kit for JC-1 mitochondrial membrane potential assay was acquired from Cayman Chemicals. Fetal bovine serum (FBS) was also acquired from Hi-Media. 3-(4,5-Di-methylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide, piperazine-*N*,*N*′-*bis*(2-ethane sulfonic acid) (PIPES), Triton-X, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and most other standard analytical grade reagents used in the present study were acquired from Sigma-Aldrich.

Plant sampling and extraction

The plant *Naringi crenulata* was collected from in and around Western Ghats, Tamil Nadu, India, at the Botanical Survey of India located in the Southern Regional Centre, Coimbatore, Tamil Nadu, India, where the plant material was described and authenticated. A voucher specimen was preserved in the Ethnopharmacology Unit, Department of Biotechnology, Mepco Schlenk Engineering College, Tamil Nadu. India.

Collected leaves were cut into small pieces and allowed to dry at 60 °C for 6 h and the leaf mass was ground into powder. The powder was divided into 250 aliquots. Phytocompounds were extracted using different polar solvents (distilled water, absolute ethanol, hexane) by ultra-sonication (Solid: Solvent: 1:30) with parameters 60 amplitude, 0.5 cycle for 120 s [19]. The collected extracts were dried to obtain the concentrated sample and were used in the cytotoxicity assay. After the solvent selection, a mixed solvent system was optimized with standard parameters for extraction.

Compound separation

The extract that showed the highest cytotoxic activity was subjected to column chromatography (Silica Gel mesh 200, column height – 20 cm, diameter – 1.5 cm) using various solvents: hexane, chloroform, ethyl acetate, ethanol and water, eluent with a gradual increase of polarity to hexane and ethanol [20]. Different fractions were collected and named as F1–F15. The collected fractions were then subjected to MTT assay. The efficient fraction was separated and analyzed by gas chromatography–mass spectrometry (GC–MS).

Cell culture and cell viability assay

The human HER2 positive breast cancer cell line (SK-BR3 cell line) was acquired from NCCS (National Centre for Cell Science), Pune, India. The obtained cells were kept enriched in DMEM with 10% (*v/v*) FBS, 10 μg/mL penstrp. Cell lines were grown at 37 °C

humidified atmosphere incubator with 5% CO_2. We seeded of 2×10^4 SK-BR3 cells in 96 well plates. After 24 h the cells were treated with the extracts (5 μmol/L) and incubated for various time (8–48 h) followed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Vinblastine (Natural remedies, Bangalore) was used as a positive control (100 nmol/L). SK-BR3 cells kept for incubation at different concentrations of NCE (hexane to ethanol ratio of 6:4) for about 24 h were observed under an inverted light microscope. Absorbance was read with an ELISA microplate reader at 570 and 620 nm. Cell viability was calculated and thereby expressed in terms of percent of control (nontreated cells). After screening and fractionation of the anticancer compound, IC50 of the compounds on cancer cell lines was predicted from the concentration–effect curves [21].

Phytochemical profile of Naringi crenulata extracts by GC–MS

Naringi crenulata extracts were analyzed by an Agilent 7890 BGCMS system, equipped with an autosampler G4513A Agilent and splitless injector. The column used SLB-5MS (Supelco, Milan, Italy), 30 m × 0.25 mm i.d., 0.25 μm df, coated with 5% diphenyl-95% polydimethylsiloxane. Mass data were handled by using the software Mass Hunter Qualitative Analysis B. 07.00 (Agilent). Isolated compounds were discovered based on computer matching with the MS libraries (NIST 14 Search 2.2 and Wiley).

Cell cycle arrest assessment

The effect of the extract on the cell cycle was analyzed using the propidium iodide staining method in SK-BR3 cells. Briefly, the cultured SK-BR3 cells were trypsinized and then pooled in a 15-mL centrifuge tube. Then, the cells were plated at a density of 1×10^6 cells/mL into a 6-well tissue culture plate in McCoy's 5 A medium containing 10% FBS and 1% antibiotic–antimycotic (penicillin–streptomycin purchased from Sigma Aldrich) solution for 24 to 48 h at a temperature of about 37 °C. The wells were gently rinsed with the help of sterile PBS followed by treatment with 24.59 μg/mL of extracted compound sample in serum-free McCoy's 5 A medium, which was then incubated at a room temperature of 37 °C in a 5% CO_2 incubator for 24 h. Again, the cells were trypsinized after the completion of the incubation process and washed in PBS for 5 min by centrifuging at 300*g*. The cells extracted after PBS wash were then fixed for 30 min at 4 °C using cold 70% ethanol by gentle addition in a drop wise manner to the cell pellet. Following the incubation process, the cells were washed by centrifuging at 300*g* for about 5 min with sterile PBS twice, and then the obtained supernatant was decanted. Consequently, the cell pellet was treated with RNase 50 μL (100 μg/mL) and PI 500 μL (100 μg/mL) and held at 4 °C before the evaluation using flow cytometry equipped with argon lasers (L1) and red diode (L2) [22].

Mitochondrial membrane potential ($\Delta\Psi m$)

The SK-BR3 cells (30,000–50,000 cells/well) were plated to a 24 well plate and incubated for 24 h in McCoy's 5A growth medium. After incubation, the plate was gently rinsed with PBS and treated with 24.59 μg/mL of extracted compound sample in serum-free McCoy's 5A medium based on previous research on other extracts [23]. Again, the plate was kept for incubation at a temperature of about 37 °C in a humidified 5% CO_2 incubator for about 24 h. The mitochondrial membrane potential measurement for the treated and control cells was done according to the manufacturer's instruction. In particular, the cells were incubated with 100 μL/well of JC-10 dye loading solution, and the plate was protected from the light. The plate was incubated at a temperature of 37 °C for 30 to 60 min in a 5% CO_2 atmosphere. After incubation, 100 μL/well assay buffer was added to each sample/well. Finally, the plate was centrifuged at 300*g* for 2 min and the fluorescence was observed at 490/525 and 540/590 ratio. All imaging was performed at 40x objective magnification under fluorescence Microscope (EVOS™ FL Color, AMEFC4300) [22].

DNA fragmentation assay

SK-BR3 cells were coated in a six-well plate at about a density of 1×10^6 cells/well and were kept for incubation at a temperature of about 37 °C in a humidified 5% CO_2 incubator for about 24 h [24]. The cells/wells were rinsed gently with sterile PBS and treated with the extracted compound sample at the concentration of 24.59 μg/mL in serum-free McCoy's 5A medium to induce apoptosis and incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. The cells were extracted by the process of trypsinization in a 1.5 mL tube and centrifuged at 300*g* for 10 min. After centrifugation, the supernatant obtained was decanted and 500 μL of lysis buffer was then added to the cell pellet, incubated at 37 °C for 1 h. Then 700 μL of

phenol–chloroform–isoamyl alcohol were added and dissolved by inversion, and then centrifuged at 300*g* for about 5 min. The aquatic (upper) phase was then moved into the new Eppendorf tubes. Then, an equal amount of cold isopropanol was taken into the tubes, and then gently dissolved well by inversion. Further, the tubes were centrifuged at 300*g* for centrifugation for 5 min and the procured supernatant was discarded. The pellet was kept for air drying for 30 min. Finally, the dried DNA was then gently dissolved in 50 µL of distilled water. Besides, the DNA that was extracted was quantified at an optical density (OD) of 260 nm and 280 nm using a UV spectrophotometer. The OD value from 1.7 to 1.8 indicated that the quality of DNA was good, without protein/RNA contamination. Then, an equal amount of each DNA sample was run at 0.8% of agarose gel electrophoresis accompanied with a 100-bp molecular size ladder. Later, the image was obtained utilizing a gel documentation system (BioRad USA).

Statistical analysis

The results are depicted as mean values with standard errors of means (±SEM). The statistical analysis was carried out utilizing GraphPad (GraphPad Prism Software Inc. of Version 5.01, San Diego, California, USA). Tukey's test (each treatment compared to control) was used to test the statistical differences after the one-way analysis of variance (ANOVA). Differences were considered statistically significant at a level of $p < 0.05$.

Results and discussion

Effects of Naringi crenulata extracts (NCE) on SK-BR3

Phytocompounds from *Naringi crenulata* leaves were extracted with water, ethanol and hexane. The anticancer effect of extracts was tested in SK-BR3 cells. SK-BR3 cells were treated with different extracts, for 22–24 h, after which the cell viability was assessed in the MTT assay. The results showed a varied cytotoxicity pattern in SK-BR3 cells incubated with different solvent extracts (Figure 1a). The hexane and ethanol extracts showed maximal effect at 24 h of exposure. Akrout et al. [25] reported that the ethanol extract of *Artemisia campestris* (wormwood) has revealed a higher cytotoxic effect. SK-BR3 cells exposure to both extracts showed a similar cell survival curve pattern, although with a distinct reduction in the cell viability when compared to the water extract. Due to the loss of cells, differences in the cell number could also be visualized in the culture dish following treatment with ethanol and hexane extracts for 24 h in comparison to the control dish (nontreated cells). These results showed that the extracts in various solvents exhibited a potent cytotoxic activity in the cancer cells (Figure 1b). In the next step, we applied a series of different ratios of hexane and ethanol-containing solvent systems in the extraction and evaluated the cell viability. Marwa et al. [26] explored the anticancer effect of *Orobanche crenata* methanolic extract on cancer cell lines, where the study identified that most of the cells were diminished, and the remaining cells were reduced in size with obvious cell shrinkage and further monolayer destruction and chromatin clumping. Kanoh et al. [27] used hexane and ethanol mixed solvent for the extraction of phenylahistin (anticancer compound). Compared to all the assayed proportions of Hexane: Ethanol, the ratio of 60:40 showed the highest cell growth inhibition activity (Figure 2). It is inferred that the content of the cytotoxic compound in the hexane extract was higher than in the ethanol extract, which was reflected in the different ratios. Cell viability decreased with the gradual increase in hexane concentration up to 60%. The ethanol extract also showed cytotoxic activity, but some highly active compounds were probably missing in the extract.

Effect of extract fractions on the viability of SK-BR3 cells

The obtained crude extract contains numerous phytocompounds; therefore, column fractionation was performed to screen for the effective cytotoxic compound. Brinda et al. [28] investigated the antiproliferative activity of L-methioninase, where the crude L-methioninase was purified by acetone precipitation and diethylaminoethyl column methods. Upon column purification, the activity of L-methioninase in the 6th fraction of the column purified sample was 11.07-fold higher than in the crude extract. As compared to the present study, five different polar solvents were used in the elution, for each solvent 3 fractions were collected from the column [29]. The polarity of hexane is 0, so the higher polarity solvents such as chloroform and ethyl acetate can purify compounds eluted by the hexane. Compounds with increased polarity have been indicated to possess good anticancer activity [30,31]. Thus, fractions $1-9$ with less polarity cannot effectively interact with proteins involved in cancer cell signaling [32,33]. Fractions $10-12$ contain the

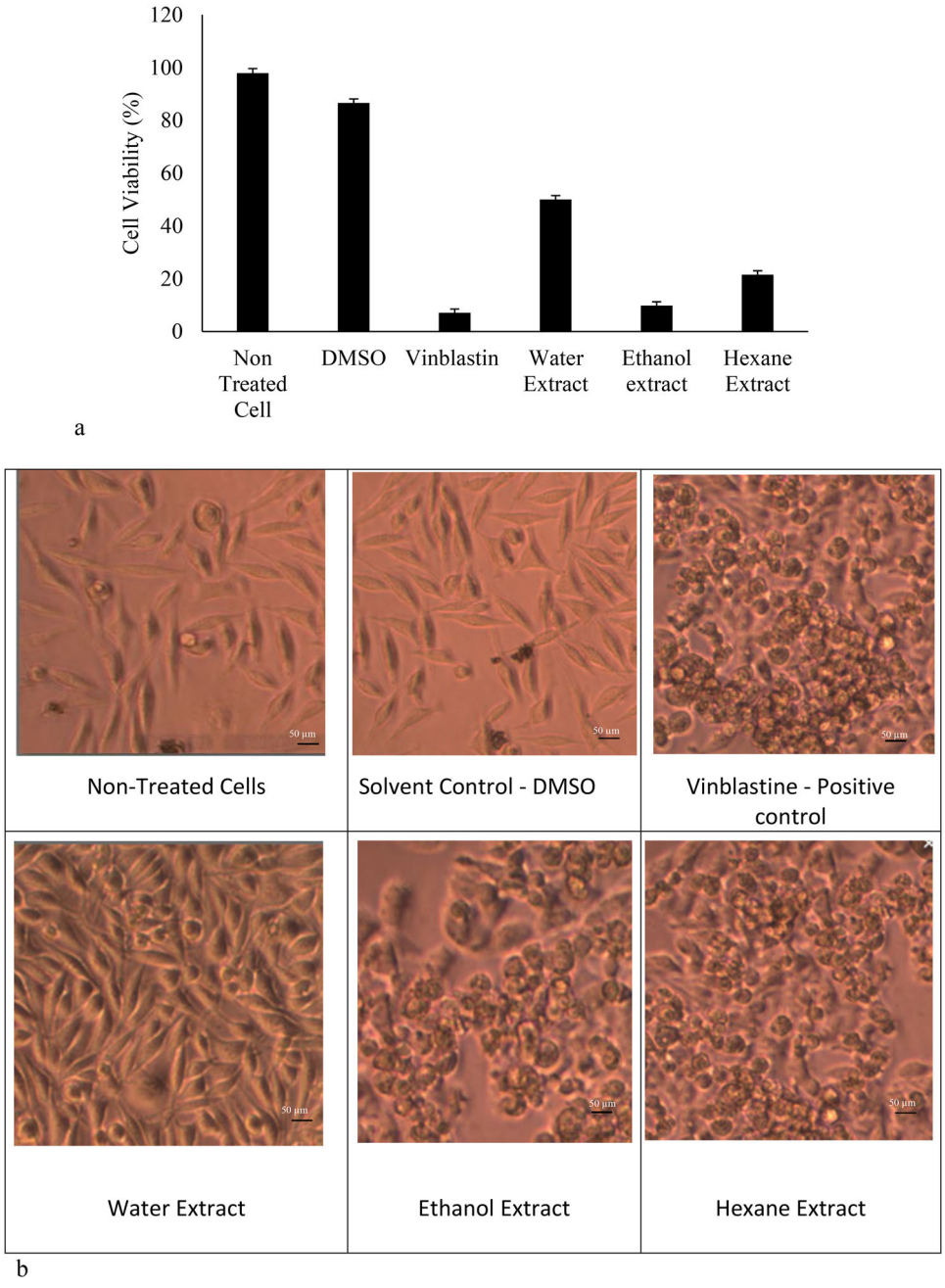

Figure 1. Effects of *Naringi crenulata* extract on SK-BR3 cells *in vitro*. (a) Cell viability and cell morphology (b) following treatment with water, ethanol and hexane extracts compared with the control (nontreated). Images were captured with a 40x objective on an inverted light microscope. *Note.* (a) The cell viability in the nontreated control was taken as 100%. (b) The cell viability was varied thereby cells lost their adhesion property. Scale bars correspond to 50 µm.

mid polar compounds and fractions 13 – 15 contain the highly polar compounds. Fraction 11 (i.e. eluted with ethanol) showed higher cytotoxic activity in SK-BR3 cells; indicating that the presence of active compounds in the fraction is higher. These compounds were separated by column fractionation (Figure 3). Other compounds are eluted with respective polar solvent elution. Screened compounds were subjected to GC–MS analysis.

Phytochemical profile of screened extract of Naringi cranulata

We used GC–MS to distinguish the compounds in the bioactive extract and characterize each component both qualitatively and quantitatively [34–37]. In the present study, GC–MS analysis showed multiple peaks for a purified fraction (Figure 4a), and the compounds were matched against the database. Based on the GC–MS analysis 6,7-Dimethoxy-1-methyl-3,4-dihydro

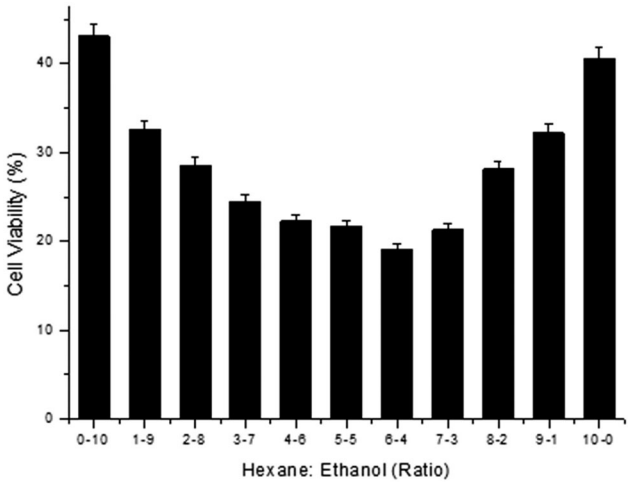

Figure 2. Effects of Hexane: Ethanol ratio for extraction of the cytotoxic compound(s) from the leaves of *Naringi crenulata*. *Note*. The cell viability in the nontreated control was taken as 100%.

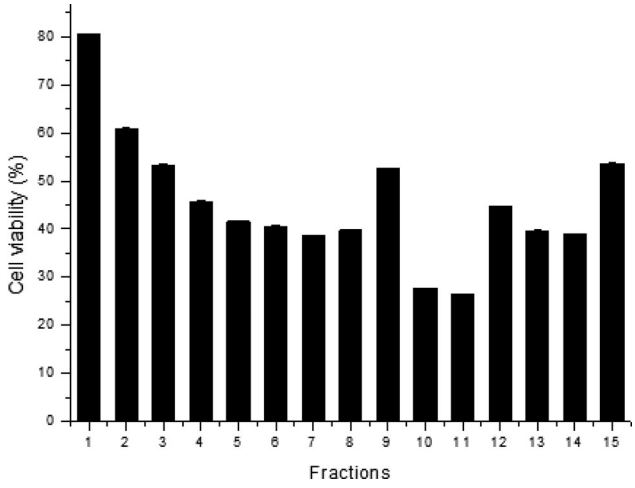

Figure 3. Cell viability following treatment with compounds in different fractions collected from the column fractionation process. *Note*. The cell viability in the nontreated control was taken as 100%.

isoquinoline, 2,4-Dimethylbenzo[h] quinoline, 7,8-Dihydroneopterin, Naringetol, Hesperetin, Hesperetin 7-rhamnoglucoside, Methyl limonilate, Tanakamine, Tanariflavanone B, Chloroquine, 4a,8a-Methanophthalazine-1,4-dicarboxylic acid, Naringenin, and Estragole were identified in fraction 11. Figure 4b shows the structures of all of the compounds. Their structural properties were evaluated with the help of DruLito software and their structure was acquired utilizing PubChem (Table 1).

Cytotoxicity assay

The ayurvedic medicine system suggests that the crude extract for any treatment contains compounds from the same plants having antioxidant and anti-inflammatory properties which help to avoid certain side effects [38,39]. Considering the adverse effects, toxicity and higher costs of common cancer treatment modalities, the use of novel herbal medicine-based therapies can provide promising health outcomes [40]. Purified NCE has many active compounds; the drug-likeness property was analyzed by the DruLito, so NCE was taken for further analysis. The effect of NCE on SK-BR3 cells was analyzed by utilizing a cell viability assay. Figure 5 indicates that treated cells gradually grew relative to untreated cells, which explains that NCE (hexane to ethanol ratio of 6:4) has lower cytotoxicity to cancer cells with an anti-proliferative effect compared to other phytocompounds. Cells that undergo apoptosis exhibit several morphological and biochemical changes, such as aberrant chromatin condensation and/or fragmentation, and the formation of apoptotic bodies. This biochemical change hypothesis was examined using specific assays. Talib and Mahasneh [41] obtained 27 μg/mL IC_{50} for Methanol fractions of *Ononis hirta*. At the concentration of 24.5 μg/mL ($p < 0.01$), approximately 50% cell growth inhibition was witnessed in NCE. From the outcomes of the present study, there was no significant difference between the solvent control and cells treated with NCE.

NCE compounds induced cell cycle arrest

Cancerous cells bypass several cell cycle safeguards as well as checkpoints, which could prevent the division of cells with aneuploidy and other defects, to allow unrestricted proliferation. This phenotype is the result of acquiring numerous genetic and epigenetic molecular modifications that hyper activate or inactivate the main cell cycle components. It imposes specific cellular dependencies upon cancer cells to sustain aberrant proliferation [42,43]. Şimşek et al. [44] demonstrated that DNA-damage and cell cycle arrest initiated anticancer potency of super tiny carbon dots on MCF7 cell line, where the study identified cell cycle arrest in the G0/G1 phase and the induction of cell cycle arrest was reported to occur in a concentration-dependent manner. NCE compounds induce cell cycle arrest in SB RK3 cells, which is evaluated by cell cycle distribution by utilizing flow cytometry. Upon 24 h of the treatment period, NCE caused a significant accumulation of cells in the G1 phase followed by a logical decrease of the cells in G0/G1 phase and a parallel induction in the M1 phase (Figure 6a). The results indicate that NCE induces cellular apoptosis. Figure 6b shows the cell cycle distributions. These findings indicate that the NCE exercised its growth-suppressive activity by

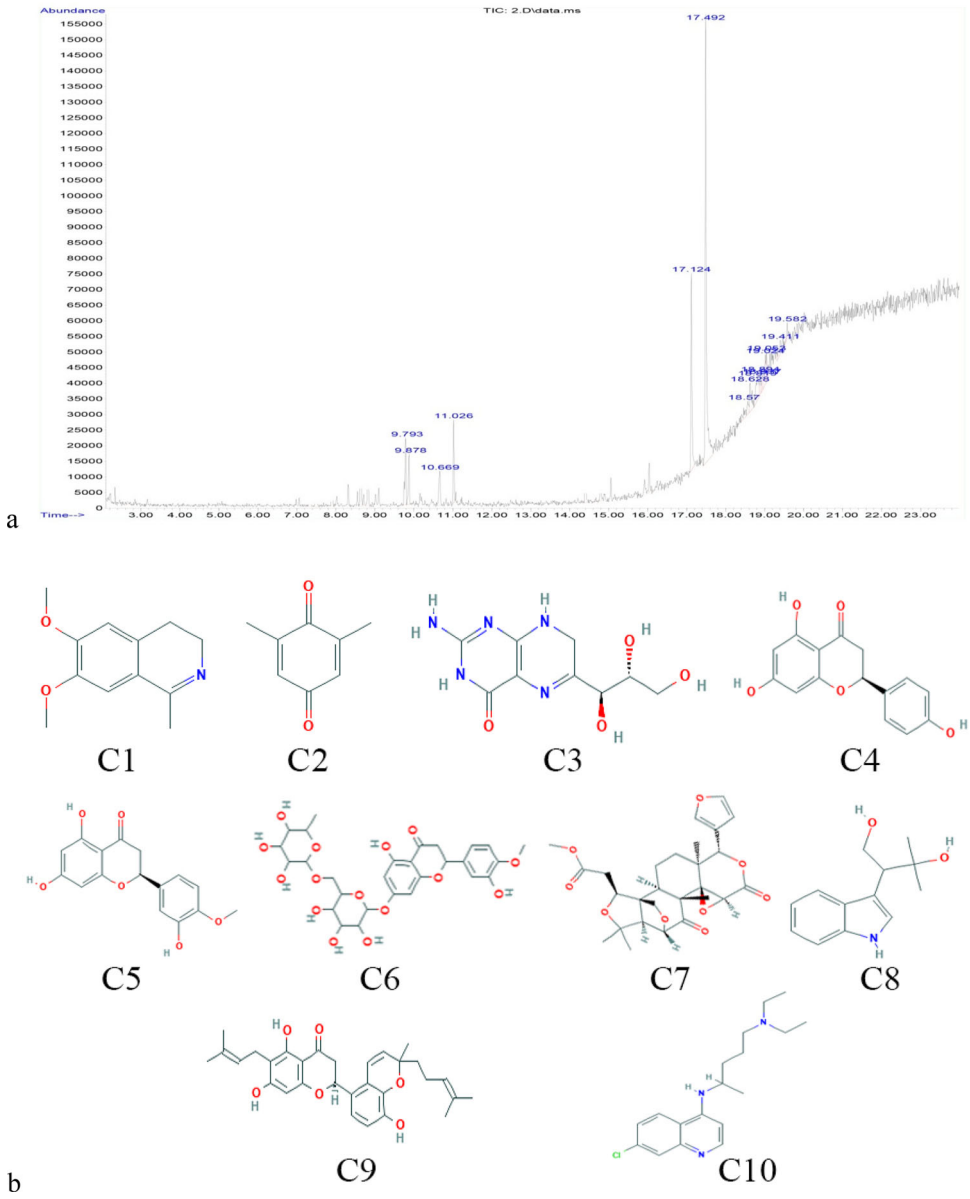

Figure 4. Identified phytocompounds in *Naringi crenulata* extract. GC–MS analysis (a) and chemical structures (b) of phytocompounds present in the higher activity of fraction 11 obtained from column fractionation. *Note*. Compound codes are listed in Table 1. The solvent ratio of 6:4 (hexane: ethanol) was used to obtain the extract analyzed subsequently.

Table 1. Phytochemicals identified from the extract of *Naringi crenulata* by GC–MS.

Compound label	*Rf*	Name	Peak area (%)
C1	9.793	6,7-Dimethoxy-1-methyl-3,4-dihydroisoquinoline	3.54
C2	9.878	2,4-Dimethylbenzo[h]quinoline	3.01
C3	10.609	7,8-Dihydroneopterin	1.58
C4	11.026	Naringetol	3.97
C5	16.125	Hesperetin	1.57
C6	17.124	Hesperetin 7-rhamnoglucoside	10.55
C7	17.492	Methyl limonilate	49.72
C8	18.57	Tanakamine	3.11
C9	19.087	Tanariflavanone B	2.94
C10	19.562	Chloroquine	3.11
		Impurities	16.9

disrupting the progression of the cell cycle and triggering cell death by inducing apoptosis. Extracted compounds are majorly involved in the apoptotic induction pathway, so further, the apoptotic activity of the extract was studied in the further *in-vitro* experimental part.

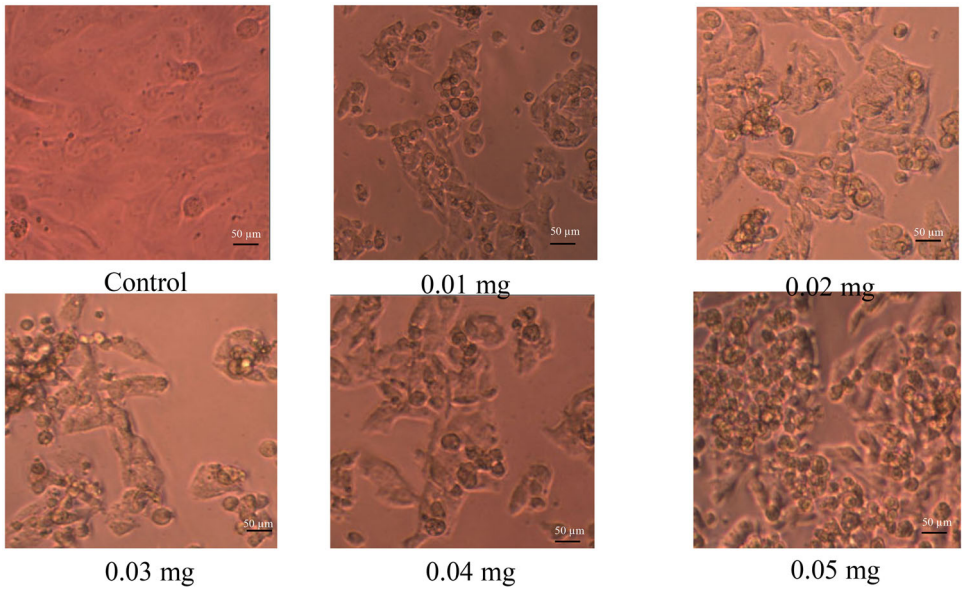

Figure 5. SK-BR3 cells kept for incubation at different concentrations of NCE (hexane to ethanol ratio of 6:4) for about 24 h and observed under an inverted light microscope. Note. Images were captured with a 40× objective on an inverted light microscope. Scale bars correspond to 50 µm.

NCE effect on mitochondrial membrane potential (ΔΨm)

We explored another primary marker of apoptosis to further elucidate the mechanisms by which NCE induced cell death in cancerous cell lines. Mitochondria are instrumental in inducing apoptosis in mammalian cells. Ngoc et al. [45] reported that compounds from *Adenosma bracteosum* induced significant increase in the production of reactive oxygen species accompanied by attenuation of mitochondrial membrane potential, thus inducing the activation of caspase-3 activity in human cancer cells. To determine whether treatment with NCE affects the permeabilization of the mitochondrial membrane and subsequently induces cell death, the MMP was evaluated before and after exposure to the IC_{50} concentration of NCE (Figure 7). Caspase-3/7 activity was investigated to examine the possible effects of NCE on mitochondrial-mediated apoptosis. The mitochondrion is one of the main regulators in controlling cell death and survival mechanisms in cells. The JC-1 green fluorescence ratio signals measured in SK-BR3 cells with and without NCE treatment are illustrated in Figure 7. Studies have reported that when JC-1 is introduced to living cells, it gets localized predominantly in the mitochondria [46–48]. In such organelles, the JC-1 accumulation results in the production of J-aggregates (especially red fluorescence emission maximum at 590 nm), in addition to the unique green fluorescence of J-monomers (emission maximum of ∼529 nm). The lack of mitochondrial (ΔΨ) results in the reduction of J-aggregate formation as well as the depression of JC-1 mitochondrial accumulation. Thus, the ratio of red and green fluorescence of cells loaded with JC-1 is commonly utilized for detecting the mitochondrial membrane potential [46–48]. The results showed that NCE treated cells exhibited a higher elevated reduction in the JC-1 fluorescence ratio by 76% relative to untreated cells ($p < 0.05$). The mitochondrial-mediated apoptotic intrinsic pathway leads to consequent cytochrome C release *via* depolarizing the membrane of the mitochondria, and this in turn leads to complex formation with caspase-9 and Apaf-1. Further, the caspase-9 contributes to the stimulation of the caspase-3/7 executioner. The results indicate that the NCE induced intrinsic apoptotic pathway by mitochondrial mediation.

DNA fragmentation

The inter-nucleosomal fragmentation of genomic DNA is widely utilized as one of the biochemical indicators of apoptosis [49]. A previous study identified the anticancer effect of piperine-free *Piper nigrum* extract and reported that the anticancer effect was associated with apoptosis, where the apoptosis was visualized using DNA fragmentation, thereby indicating the DNA cleavage by the activation of a nuclear endonuclease enzyme [50]. Similarly, in the present study, a conventional agarose gel electrophoresis was performed to identify the presence of DNA fragmentation in SK-BR3 cells after treatment with NCE at the IC_{50} (24.5 µg/ml)

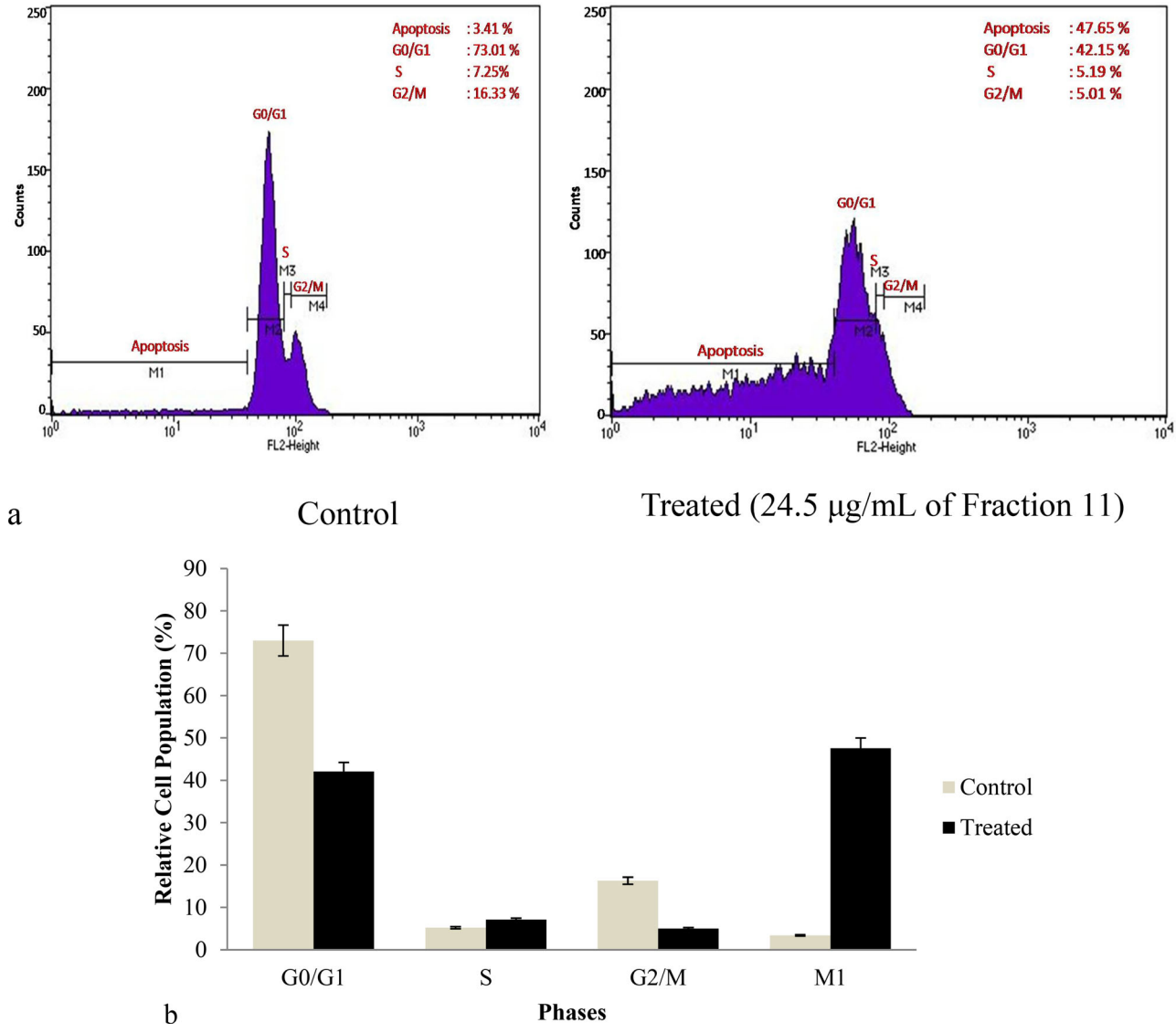

Figure 6. Effects of NCE on cell cycle distribution. Representative results (a) and histograms (b). SK-BR3 human breast cancer cells were treated with 24.5 µmol/L NCE for 24 h and then harvested and stained with PI, and analyzed using flow cytometry.

for about 24 h. The untreated cells retained the intact genomic DNA without internucleosomal degradation (lane 1 and 2, Figure 8). The NCE treated SK-BR3 cells displayed a laddering pattern at IC50 (24.5 µg/mL) (lane 2). The results indicated the association with the enhancement in caspase-3/7 activity in SK-BR3 cells treated with the NCE. There is a clear expectation that the DNA fragmentation is strongly linked with the activation of caspase-3 as an apoptosis endpoint feature. A DNA smearing pattern was detected in the cells treated with NCE (lane 2). It, therefore, indicates that the apoptotic cells gain entry into the later stage of apoptosis due to a lack of cell remnant engulfment by the phagocytes. DNA fragmentation was observed in the cells treated with NCE. Activation of caspases results in apoptotic morphological changes and DNA fragmentation by selective cleavage of essential cellular substrates. Correspondingly, identification of DNA fragmentation in NCE treated cells indicates that NCE contributes to the activation of caspase 3. The cell activates DNase enzymes after the activation of caspase-3, resulting in DNA fragmentation and ultimately apoptosis.

Conclusions

The results of the present study indicate that *Naringi crenulata* extracts could be investigated further as promising anti-cancer phytochemicals against breast cancer. It is hypothesized that NCE could reduce cancer cell resistance by triggering cytotoxicity. NCE also deepens awareness about the potential of cellular models in studies on natural bioactive compounds, e.g. in this study NCE demonstrated anti-proliferative

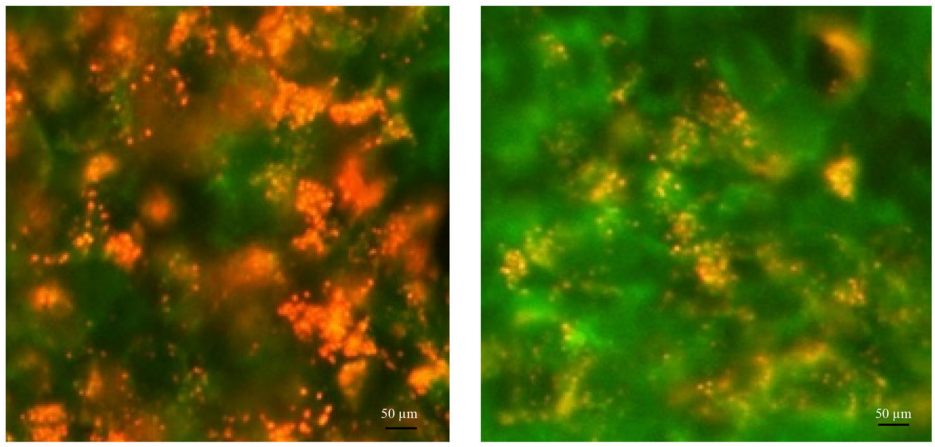

Figure 7. JC-1 green fluorescence ratio signals in SK-BR3 cells with and without treatment with Fraction 11. Note: SK-BR3 human breast cancer cells were treated with 24.5 µmol/L of Fraction 11 for 24 h. Images were captured with a 40× objective on an inverted imaging system.

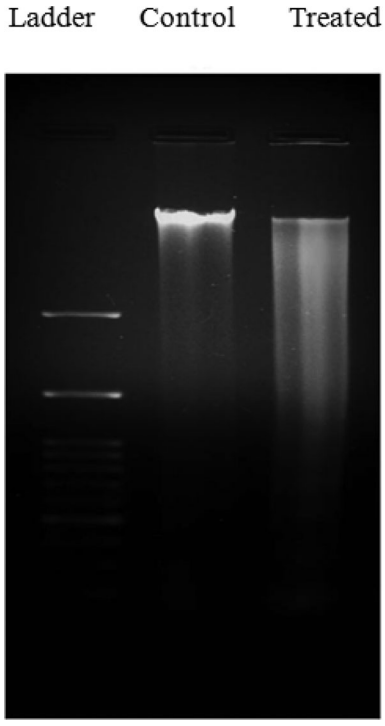

Figure 8. DNA fragmentation in SK-BR3 cells after treatment with NCE. The cells are treated with the NCE at IC_{50} for 24 h. Lane 1: untreated SK-BR3 cells; Lane 2: SK-BR3 cells treated with 24.5 µg/mL.

potential against SK-BR3 cells. More interestingly, SK-BR3 cells exposure to NCE induced cell cycle arrest (in S and G2/M phases; in G0/G1 phase). Thus, suggesting that NCE might exhibit anti-proliferative potential and pro-apoptotic potential. *Naringi crenulata* extract which exhibits various medicinal properties might serve as an outstanding candidate for anti-cancer treatment.

Disclosure statement

We have no conflicts of interest to disclose.

Data availability statement

The data that support this study are available from the corresponding author upon reasonable request.

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ORIGINAL ARTICLE

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

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Submitted: 01-08-2019 Revised: 30-09-2019 Published: 31-03-2020

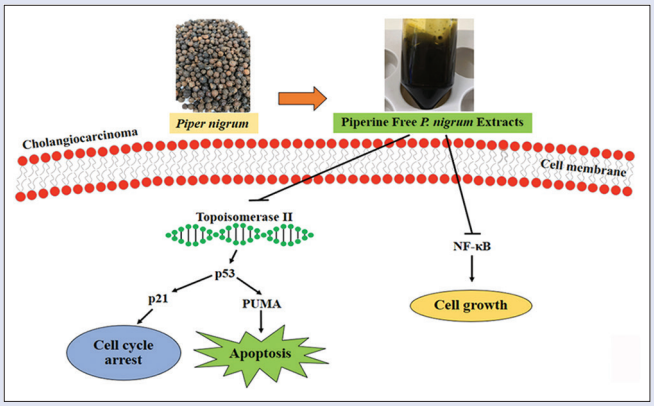
ABSTRACT

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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DOI: 10.4103/pm.pm_288_19

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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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Cite this article as: Tedasen A, Khoka A, Madla S, Sriwiriyajan S, Graidist P. Anticancer effects of piperine-free *Piper nigrum* extract on cholangiocarcinoma cell lines. Phcog Mag 2020;16:S28-38.

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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

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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

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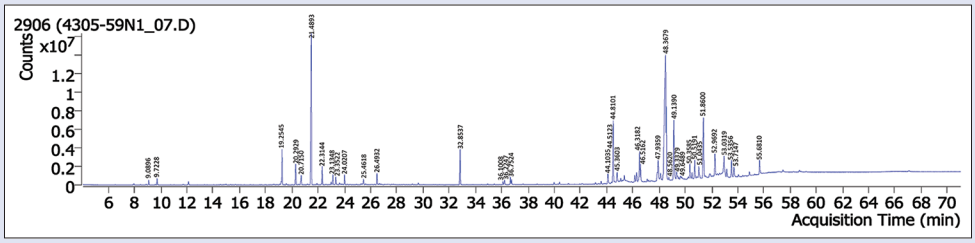

Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

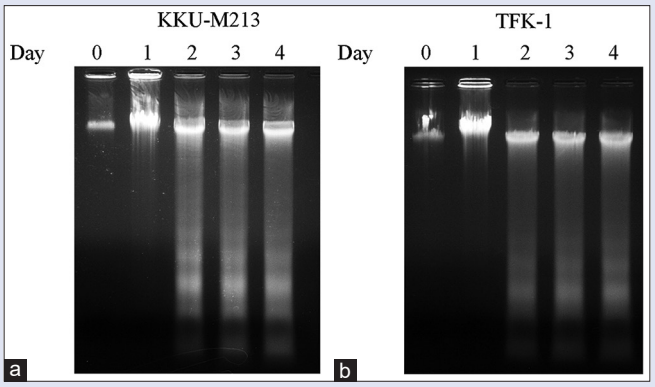

Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis 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

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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

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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]

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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

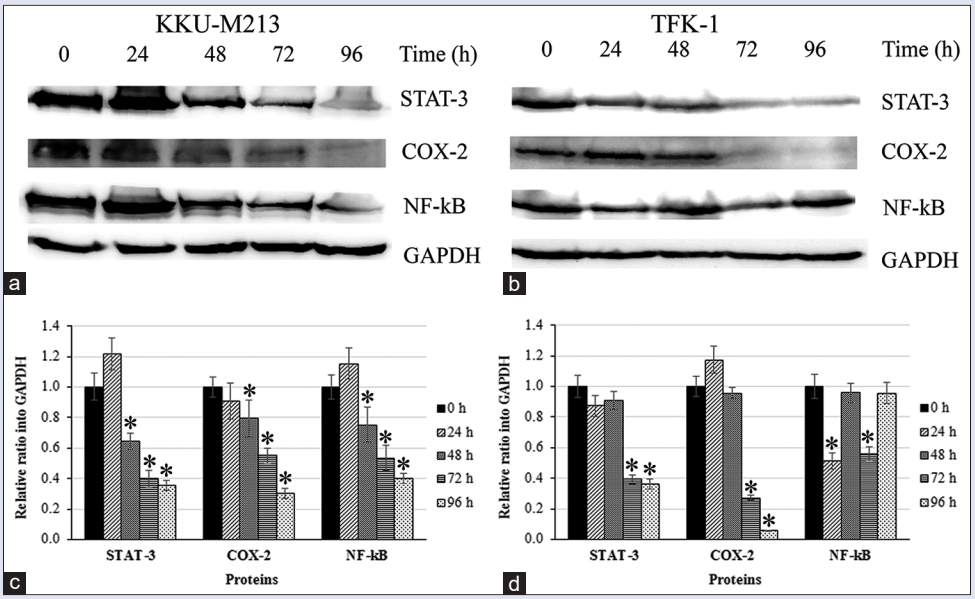

Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer 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)

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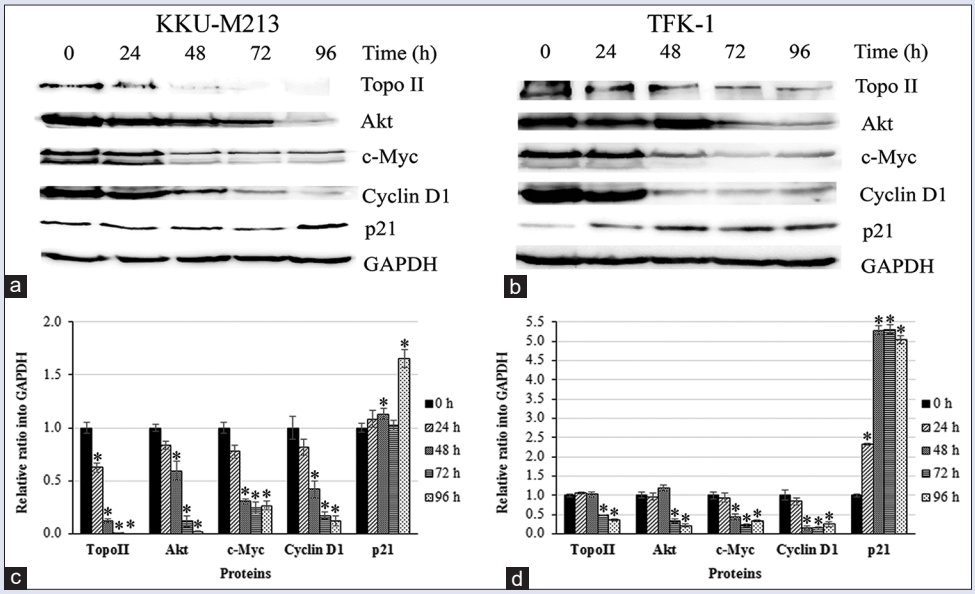

Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase 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)

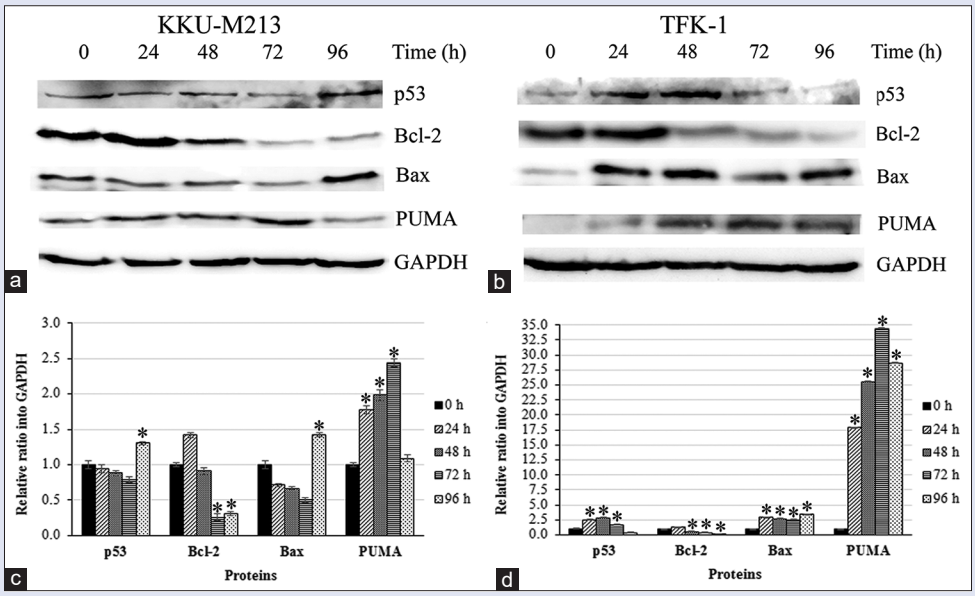

Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2, 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.

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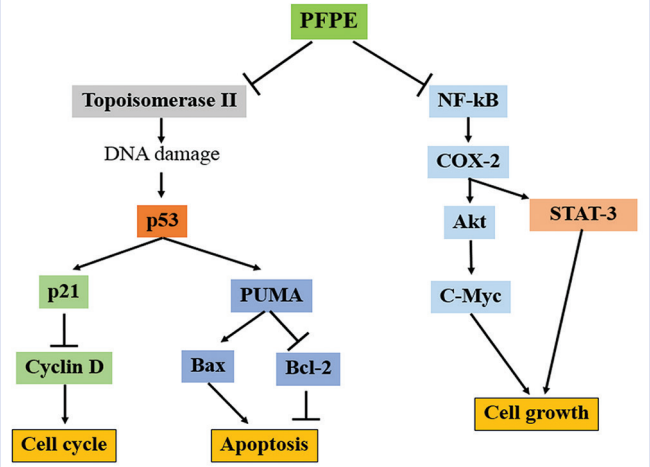

Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

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.

Acknowledgement

The authors thank Prince of Songkla University for the equipment funding (the Excellent Research Laboratory (ExLab004)).

Financial support and sponsorship

This work was supported by the research fund (REC 60-279-04-2) Faculty of Medicine and the Postdoctoral Fellowship, Prince of Songkla University.

Conflicts of interest

There are no conflicts of interest.

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