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SPICES- AN ALTERNATIVE THERAPY FOR CANCER TREATMENT

Ruchita Bhagwat*[1] and Renuka Bhagwat[2]

[1]Third Year B. Pharmacy, Shree Saraswati Institute of Pharmacy, Tondavali, Tal: Kankavali, Dist: Sindhudurg, India.

[2]Final Year B. Pharmacy, V P College of Pharmacy, Madkhol, Tal: Sawantwadi, Dist: Sindhudurg, India.

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***Corresponding Author**
Ruchita Bhagwat
Third year B. Pharmacy, Shree Saraswati Institute of Pharmacy, Tondavali, Tal: Kankavali, Dist: Sindhudurg, India.

ABSTRACT

Cancer is among the leading causes of morbidity and mortality worldwide. Current therapy available for cancer treatment is associated with number of side effects. The plant sources of India provide effective anticancer agents. Spice Herbs have a vital role in the prevention and treatment of cancer. ethnomedicinal spices used to treat cancer are considerably cheap. Herbal drug treatment can be given to poor people in the rural areas to treat different ancers effectively at an affordable cost. The main mechanisms of action include inducing apoptosis, inhibiting proliferation, migration and invasion of tumours, and sensitizing tumours to radiotherapy and chemotherapy. Some of the spice based active constituents which are widely known for their chemo preventive action against various malignancies are curcumin and curcuminoids (turmeric), allicin, allyl isothiocyanate (garlic), gingerol, zingiberene, zingiberene (ginger), piperidine piperine, (black pepper), crocetin, crocin and safranal (saffron) and others. The present review highlights the role of common spices in combating cancer.

KEYWORDS: Spices, Cancer, Carcinogens.

INTRODUCTION

Hippocrates has rightly said “Let food be thy medicine and medicine be thy food”. Continuous efforts are being made by various laboratories to find out the role food and its ingredients such as spices against various deadly diseases. Cancer is a disease in which some of the body’s cells grow uncontrollably and spread to other parts of the body. Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Normally,

human cells grow and multiply (through a process called cell division) to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place Sometimes this orderly process breaks down, and abnormal or damaged cells grow and multiply when they shouldn't. These cells may form tumors, which are lumps of tissue. Tumors can be cancerousor not cancerous (benign).

Spices can potentially inhibit the bioactivation of carcinogens, decrease free radical formation, suppress cell division and promote apoptosis in cancerous cells, regulate inflammation, and suppress microbial growth. Today, spices are increasingly appreciated not only for their culinary properties but also for their potential health benefits. The low toxicity may make them particularly useful as a subtle personal dietary change that may decrease the risk for several diseases. The phytochemicals in spices usually have antioxidant and anti-inflammatory properties. Inflammation is a natural process of the human immune system as a means of killingharmful pathogens. However, chronic inflammation can lead to cell damage and increased cell proliferation. Inflammation and other natural processes lead to free radical formation, which causes damage to cells and DNA. Bioactive components in spices have an antioxidant effect that can prevent free radical damage. modality. Researchers have studied the benefits and toxicity of various spices and found many phytochemicals are anticarcinogenic at low concentrations but may cause adverse reactions at higher concentrations.

Research carried out in different laboratories have demonstrated the role of phytochemicals derived from spices against various cancer cell lines including pancreatic, colon, breast andlung. The phytochemicals present in spices inhibit carcinogenesis through their bioactive components which block the activity of cytochrome P450, cyclooxygenase -2, and downregulate signal transducers. The bioactive components also influence the expression of proteins involved in cell cycle, activate caspases killers and supress kappa beta–activation.

GARLIC

Garlic (Allium sativum) is a widely used spice, and also a traditional remedy for a variety of ailments. Several epidemiological observation and a number of laboratory studies have indicated anticarcinogenic potential of garlic.[1] The anticarcinogenic action of garlic can also be attributed to its role in preventing DNA-carcinogen adduct formation and activation of carcinogen.[1] Several experimental studies on animals and cultured cells have demonstrated the anticarcinogenic effect of garlic and its chemical compounds.[1] Oral feeding of garlic

extracts has been shown to reduce the incidence and growth of transplantable and spontaneous tumours in experimental animals and the active components were found to influence a number of physiological and immunological functions which account for their anticarcinogenic and antitumour effects.[1]

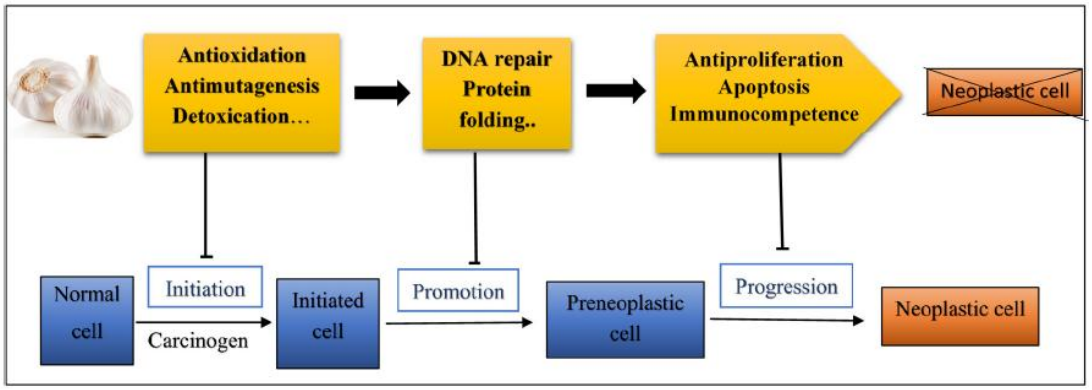

Fig.1 Anti-carcinogenic effect of garlic bioactive compounds in different stages of cancer progression.

Anti-carcinogenic effect of garlic bioactive compounds in different stages of cancer progression.[7] In the initiation stage, blocking phytochemicals prevents the bioactivation of carcinogens through antioxidation, antimutagenesis and detoxication. In the promotion stage, suppressing phytochemicals inhibits the proliferation of clonal cells by modulating protein folding and DNA repair.[7] In the progression stage, suppressing phytochemicals impedes the growth or metastasis of tumors by changing the cell behaviors, including antiproliferation, apoptosis and immunocompetence.[7]

Garlic has been demonstrated to exhibit anticancer activities via interfering with multiple stages of carcinogenesis.[7] However, the nutritional or chemopreventive roles of garlic go far beyond the notion that garlic has therapeutic effects against cancers.[7] More rationally designed experiments and trials are required to explore the novel properties of garlic.[7] It should be noted that preparation processing and administration methods may depress the anticancer effects of garlic when the effective components of garlic are isolated and analyzed.[7]

SAFFRON

Saffron is the dried stigmas of Crocus sativus L. Crocus sativus L belongs to the family of Iridaceas, the line of liliaceas and is mainly cultivated in several countries of mild and dry climate.[10] The extensive repertoire of traditional medicinal knowledge systems from various

parts of the world are being re-investigated for their healing properties Crocus sativus L., commonly known as saffron.[10] Saffron contains more than 150 volatile, non-volatile and aroma-yielding compounds which consist of lipophilic and hydrophilic carbohydrates, proteins, amino acids, minerals, musilage, vitamins (especially riboflavin and thiamine) and pigments including crocin, anthocianin, carotene, lycopene, zigzantin, flavonoids, starch, gums and other chemical compounds.[10]

- ✓ Saffron has a selective toxicity against cancer cells.[9]
- ✓ Crocin is the most important anticancer agent of saffron.[9]
- ✓ Crocetin inhibits the growth of cancer cells.[9]
- ✓ Saffron's toxicity against normal cells is very low at common doses.[9]

Saffron has selective toxicity against cancer cells, through inhibition of RNA and DNA synthesis and increasing apoptosis.[9] Crocin has been considered as the most important anticancer agent of saffron that plays a role in gene expression and apoptosis in cancer cells.[9] Crocetin has an inhibitory effect on the cancer cells growth that may be due to reduced synthesis of DNA, RNA and protein in neoplastic cells, RNA polymerase II inhibition, and interaction with histone H1 and H1-DNA structures.[9] Saffron and its crocin and crocetin have also shown anticancer and cancer-preventive effects in animal models of cancer.[9] Safranal also has shown antitumor activity with low toxicity.[9] On the other hand, the lethal dose of 50% (LD50) for the saffron and its constituents against normal cells can be very high.[9]

However, the scarcity and expense in obtaining large quantities of saffron may provide impediments to human chemoprevention and cancer treatment using this agent.

BLACK PAPPER

Black pepper (*Piper nigrum*) is a perennial climbing vine grown for its berries extensively used as spice and in medicine.[12] Black pepper (*Piper nigrum*) is a perennial climbing vine grown for its berries extensively used as spice and in medicine.[12] India is a leading producer, consumer and exporter of black pepper in the world.[12] Peperine is an alkaloid found naturally in Piper nigrum, this alkaloid is responsible for the pungency of Black pepper.[12] Manoharan et al. investigated the chemoprotective effect of piperine against dimethylbenz anthracene induced buccal pouch carcinoma of Syrian golden hamsters.[12] They observed that piperine completely prevented the formation of oral carcinoma.[12] El Hamss R et al. observed that when Drosophila melanogaster was exposed to mutation through promutagen

ethyl carbamate, in such induced situation the Piper nigrum is effective to reduce mutational events.[12] Duessel et al. observed that piperine displayed an anti-proliferation effect at 24 h and statistically significant inhibition at 48 and 72 h at 100-200 µm concentration against cultured human colon cancer cells (DLD-1).[12]

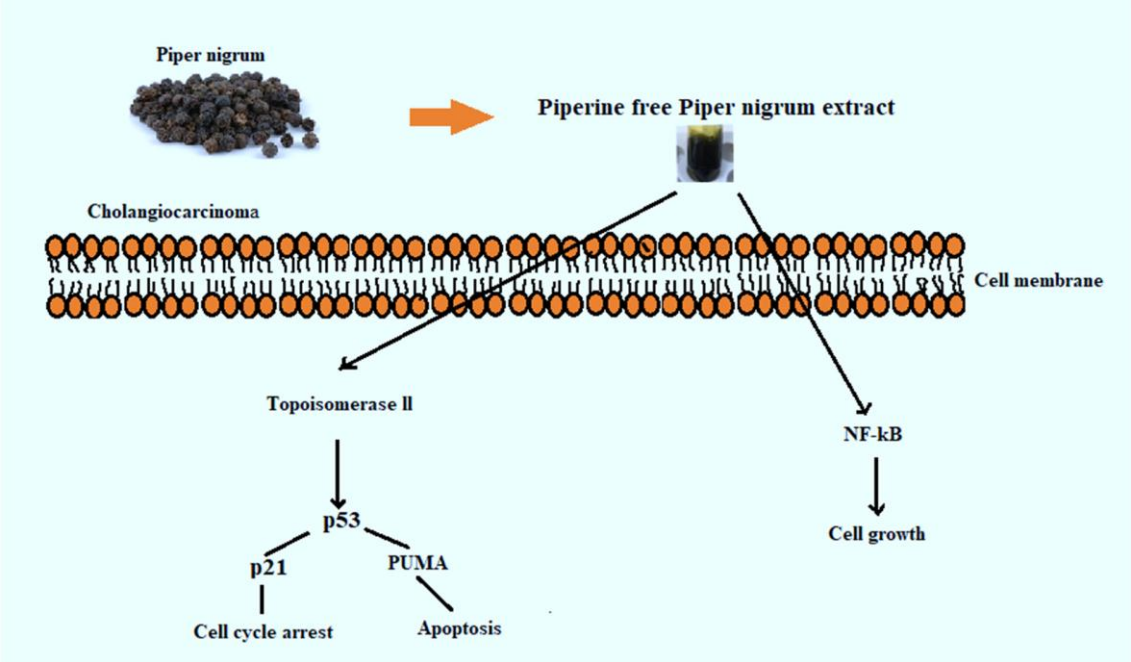

Fig.2: Activity of black paper on cholangiocarcinoma (CCA).

- ✓ Piperine free *Piper nigrum* extract (PFPE) inhibited cholangiocarcinoma (CCA) cell lines.[12]
- ✓ PFPE induces CCA cells to undergo apoptosis and cell cycle arrest via the inhibition of topoisomerase II.[12]
- ✓ PFPE inhibit cell growth through the inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells.[12]

These results indicated that PFPE inhibited CCA through the down-regulation of cell proliferation and induction of apoptosis pathway.[12]

TURMERIC

Turmeric is also known as Indian saffron, jiang huang, haridra and haldi. It is a spice grown in many Asian countries.[13] It belongs to the ginger family and is a main ingredient of curry powder.[13] The main active ingredient in turmeric is curcumin or diferuloyl methane.[13] Laboratory studies have shown curcumin has anticancer effects on cancer cells.[13]

The main mechanisms of action by which curcumin exhibits its unique anticancer activity include inducing apoptosis and inhibiting proliferation and invasion of tumors by suppressing a variety of cellular signaling pathways.[3] Several studies reported curcumin's antitumor activity on breast cancer, lung cancer, head and neck squamous cell carcinoma, prostate cancer, and brain tumors, showing its capability to target multiple cancer cell lines.[3] curcumin's applications are limited due to its low water solubility which results in poor oral bioavailability and also low chemical stability.[3] Another obstacle is the low cellular uptake of curcumin.[3] Due to its hydrophobicity, the curcumin molecule tends to penetrate into the cell membrane and bind to the fatty acyl chains of membrane lipids through hydrogen binding and hydrophobic interactions, resulting in low availability of curcumin inside the cytoplasm.[3] To overcome these obstacles and improve the overall anticancer activity of curcumin, several structural modifications have been suggested to enhance selective toxicity towards specific cancer cells, increase bioavailability, or enhance stability.[3] Another approach is to use different delivery systems to improve curcumin's physiochemical properties and anticancer activity.[3] This review focuses on the recent literature on the SAR of curcumin and its analogues and their anticancer activity in different cancer cell lines, animal models, and human clinical trials as well as different types of curcumin delivery systems that have been used for cancer therapy.[3]

GINGER

Ginger (*Zingiber officinale*), a common spice in foods and beverages worldwide, is rich in several bioactive phenolics, including non-volatile pungent compounds such as gingerols, paradols and shogaols, which possess antioxidant, anti-inflammatory, antifungal, anti-mycobacterial, and anticarcinogenic proprieties. Also, ginger leaf has long been used as a vegetable, tea and herbal medicine.[8]

Ginger and its components have been shown to modulate a wide range of signaling molecules. Ginger may upregulate or downregulate the gene expressions, depending on the target and cellular context.[5] Ginger extract increases antioxidant enzymes including GSH, SOD, and glutathione peroxidase.[5] Component of Asian ginger oil also targets to increase the phase II detoxification enzymes as well as nuclear localization of Nrf2/ARE.[5] A number of targets of ginger and its components have been documented in different cancer models.[5] These include transcription factors, enzymes, inflammatory mediators, protein kinases, drug resistance proteins, adhesion molecules, growth factors receptors, cell-cycle

regulatory proteins, cell-survival proteins, chemokines, and chemokine receptors.[5] In different GI cancers, ginger extract inhibits transcription factor NF-κB, inflammatory cytokine TNF-α and other enzymes and proteins, which include xanthine oxidase and myeloperoxidase, MDA, HMG CoA reductase, free fatty acids, triglycerides, phospholipase A, and phospholipase C.[5] The active ingredient of ginger, particularly, 6-gingerol and 6-shogaol targets several cellular molecules that contribute to tumorigenesis, cell survival, cell proliferation, invasion, and angiogenesis.[5] 6-Gingerol modulates NF-κB, STAT3, Rb, MAPK, PI3K, Akt, ERK, cIAP1, cyclin A, Cdk, cathepsin D, and caspase-3/7. Similarly, shogaol targets NF-κB, STAT3, MAPK, PI3k/Akt Ca^{2+} signals, COX-2, cyclin D1, survivin, cIAP-1, XIAP, Bcl-2, MMP-9, caspase activation, ER stress, and eIF2α. Besides these, Asian ginger component zerumbone modulates NF-κB, p53 VEGF, p21, and CXCR4 expression.[5] Thus these molecular targets of ginger components indicate that it may have the potential for preventing and treating the GI cancer.[5]

Ginger has been found to be effective against various GI cancers such as gastric cancer, pancreatic cancer, liver cancer, colorectal cancer, and cholangiocarcinoma.[5] However, its anticancer effects on other GI cancers like duodenal, esophageal, anal, GI carcinoid tumor and pancreatic islet cell cancer have yet not been established. Therefore, more extensive and well- controlled human studies are required to demonstrate its efficacy as an anticancer agent, as it is a safe and cost-effective alternative.[5]

Table no. 1: The anticancer activities of spices.[8]

Sites	Spices	Constituents	Anticancer Effects
Lung	Turmeric	Curcumin	Inducing apoptosis and DNA damage; inhibiting proliferation, migration, and the growth of cancer; decreasing cell growth and viability; inhibiting expression of DNA-repairassociated proteins
	Black cumin	Seed extract and seed oil; Thymoquinone	Reducing viability of human lung cancer; inhibiting proliferation, migration, and invasion of lung cancer cells
	Ginger	6-Shogaol	Decreasing tumorigenesis and the Metastasis
	Garlic	Thiacremonone	Inhibiting tumor growth
	Saffron	Ethanolic extract, aqueous extract	Inducing cell death and apoptosis, inhibiting the cell proliferation
	Red chili pepper	Capsaicin	Restraining angiogenesis, inducing apoptosis and oxidative DNA damage

Liver	Turmeric	Curcumin	Inhibiting the growth of hepatoma cells, inhibiting and reversing diethylnitrosamine-induced hepatocarcinogenesis
	Black cumin	Thymoquinone	Inhibiting cell proliferation
	Rosemary	Carnosic acid	Sensitizing TRAIL-mediated apoptosis, inducing autophagic cell death
	Clove	Eugenol	Improving the xenobioticmetabolizing Systems
	Galangal	Galangin	Inhibiting proliferation of cancer cells,
Breast	Turmeric	Curcumin	Inhibiting MCF-7 breast carcinoma cells, cell invasion, and sensitizing cancer cells to retinoic acid
	Black cumin	Thymoquinone	Anti-proliferative and pro-apoptotic effects
	Ginger	6-Shogaol	Decreasing tumorigenesis and the metastasis
	Garlic	Diallyl disulfide, Diallyl sulfide, Diallyl trisulfide, Sallyl mercaptocysteine	Inhibiting proliferation, cell growth, and metastasis; inhibiting diethylstilbestrol induced DNA damage; inducing apoptosis; immunomodulation; inhibiting estrogen receptor-α activity
	Saffron	Crocetin	Inhibiting invasiveness
	Black pepper	Piperine	Inhibiting proliferation, the growth and motility of cells, inducing apoptosis, enhancing the efficacy of TRAIL-based therapy
	Red chili pepper	Capsaicin	Inducing cell death, inhibiting invasion and migration
	Rosemary	Supercritical fluid rosemary extract	Downregulating estrogen receptor-α and HER2 receptors, sensitizing TRAIL-mediated apoptosis
	Clove	Eugenol	Inducing apoptosis
	Coriander	Ethyl acetate extract	Inhibiting DNA damage and Migration
	Wasabi	6-MITC	Inducing apoptosis
Stomach	Turmeric	Curcumin	Inhibiting proliferation and invasion, promoting apoptosis, suppressing lymphatic vessel density, inhibiting cell growth
	Garlic	Diallyl disulfide	Causing G2/M arrest, promoting apoptosis, suppressing xenograft tumors
	Saffron	Crocetin, crocin	antioxidant, anti-proliferative, and apoptotic activities
	Red chili pepper	Capsaicin	Inhibiting cell proliferation, inducing Apoptosis
	Cardamom	Not mentioned	Inhibiting Benzo(α)Pyrene-induced forestomach papillomagenesis

Colorectum	Turmeric	Curcumin	Preventing aberrant crypt foci, inducing apoptosis, inhibiting cell growth
	Black cumin	Thymoquinone	Attenuating tumor development and growth, inducing apoptosis, inducing autophagic cell death
	Ginger	Ginger root/leaf extract, 6-gingerol, shogaols	Reducing cell viability and proliferation, inducing apoptosis
	Garlic	Se-Methyl-lselenocysteine garlic extract	Inducing apoptosis, suppressing cell Proliferation
	Onion	Se-Methyl-lselenocysteine	Inducing apoptosis
	Scallion	Scallion extract	Inhibiting tumor growth
	Saffron	Crocin	Inducing apoptosis
	Black pepper	Piperine	Impairing cell cycle progression and inducing apoptosis
	Red chili Pepper	Capsaicin	Inhibiting cell proliferation and inducing apoptosis
	Rosemary	Rosemary extract, carnosic acid, diterpenes	Sensitizing cancer cells to 5-FU, inhibiting cell migration, inducing apoptosis
	Clove	Clove extract	Inhibiting tumor growth and promoting cell cycle arrest and apoptosis
	Galangal	Galangin	Inducing cell death
	Cinnamon	Cinnamaldehyde	Regulating drug-metabolizing genes
	Oregano	Carvacrol	Inhibiting proliferation and induces apoptosis
Cervix	Turmeric	Curcumin	Eradicating HPV+ cancer cells without affecting non-cancerous tissue, inhibiting the proliferation and inducing apoptosis, inhibiting tumor growth and angiogenesis
	Black cumin	Thymoquinone, methanolic extract	Inducing apoptosis and inhibiting proliferation
	Clove	Eugenol	Enhancing the effect of gemcitabine, anticarcinogenic and anti-inflammatory activity
Prostate	Turmeric	Curcumin	Targeting AR and histone modification, inhibiting the proliferation and growth
	Ginger	Ginger extract, 6-shogaol, 6-gingerol and 6-paradol	Inducing apoptosis, inhibiting prostate cancer cell proliferation and growth
	Saffron	Saffron extract	Antiproliferative properties, inhibiting cell invasion and migration
	Black pepper	Piperine	Reducing the androgen dependent and androgen independent tumor growth, inhibiting proliferation
	Red chili	Capsaicin	Reducing the metastatic burden, radio-

	pepper		sensitizing agent
	Rosemary	Rosemary extract	Promoting androgen receptor degradation and decreasing xenograft tumor growth

➢ BIOAVAILABILITY OF ACTIVE COMPOUND

The compounds derived from spices are usually with relative low bioavailability, such as curcumin, piperine.[8] Many methods have been proposed to enhance the bioavailability of these compounds in vivo.[8] For instance, the bioavailability of curcumin could be enhanced by molecular complexation of curcumin with pH sensitive cationic copolymer.[8] Moreover, nanocarrier loading and microparticles containing curcumin could improve bioavailability of curcumin.[8] Additionally, a novel curcumin analog showed anti-tumor activity and improved bioavailability.[8] As to thymoquinone, its absorption after administration was relatively slow, and novel analogues of thymoquinone might possess superior bioavailability and anti-tumor activity.[8] In a study about ginger, a suitably designed multiparticulate system containing ginger extract improved the therapeutic efficiency of colon cancer.[8] In addition, capsaicin-loaded microemulsion and liposomal nano formulation both enhanced oral bioavailability.[8] As for piperine, a self-emulsifying drug delivery system could enhance oral bioavailability of piperine.[8]

➢ SIDE EFFECT OF ACTIVE COMPOUNDS FROM SPICES

Spices are commonly consumed in human diets, and most varieties at appropriate doses are safe to humans.[8] Purified compounds are separated from spices for treatment of diseases.[8] A few studies reported the side effects of these bioactive compounds when they were used for treatment of cancers.[8] Some compounds might produce toxic and carcinogenic effects under specific conditions. For example, carcinogenic and toxic effects of curcumin were found in a long term study (2 years) in rats and mice, while no carcinogenic effect was observed in short term studies (3 months).[8] Curcumin might induce DNA damage in normal cells in the presence of Cu^{2+} in vitro and in vivo. In addition, safrole, a member of benzodioxoles, shows carcinogenic activities and is present naturally in essential oils of spices including black pepper,cumin, ginger, etc.[8] Thermal treatments such as drying (70 °C, 30 min) or boiling (5 min) during cooking could decrease dose of safrole to a safer level.[8] Besides, several compounds with anti-platelet effects from garlic, black cumin, ginger, fenugreek and turmeric might lead to excess bleeding in patients with bone marrow suppression.[8]

CONCLUSION

This review attempts to signify the role of spices and their constituents in therapeutic and pharmacological applications. From the description provided above, it is clear that spice-derived phytochemicals have an enormous potential in the prevention and treatment of cancer. These phytochemicals mediate their effects through multiple targets and yet pharmacologically they are highly safe. They can induce apoptosis, suppress proliferation of tumor cells, andinhibit invasion and angiogenesis. Spice-derived phytochemicals may be safer to use.

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ORIGINAL ARTICLE

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

Aman Tedasen[1,2], Araya Khoka[3], Siribhorn Madla[1], Somchai Sriwiriyajan[3], Potchanapond Graidist[1,2]

[1]Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, [2]The Excellent Research Laboratory of Cancer Molecular Biology, Prince of Songkla University, [3]Department of Pharmacology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand

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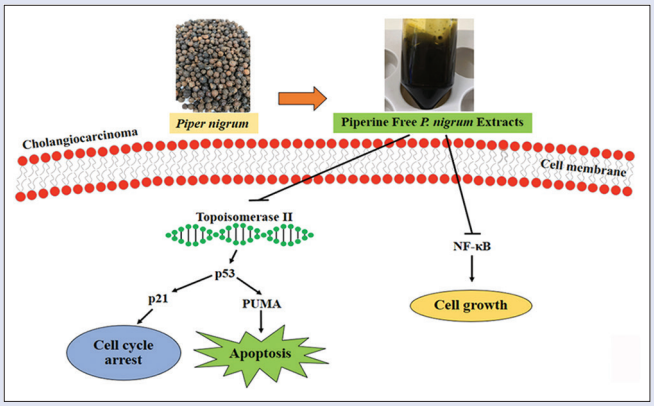
ABSTRACT

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.

Correspondence:

Dr. Potchanapond Graidist,
Department of Biomedical Sciences, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand.
E-mail: gpotchan@medicine.psu.ac.th

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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

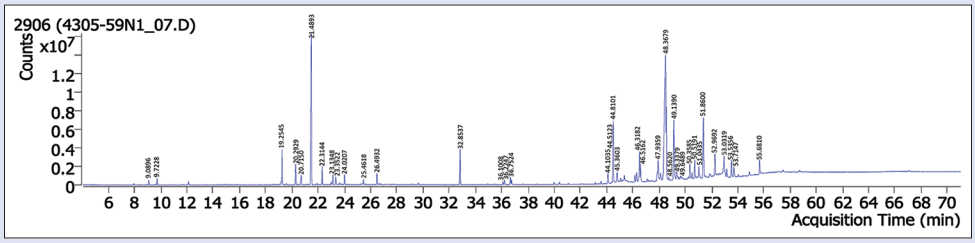

Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

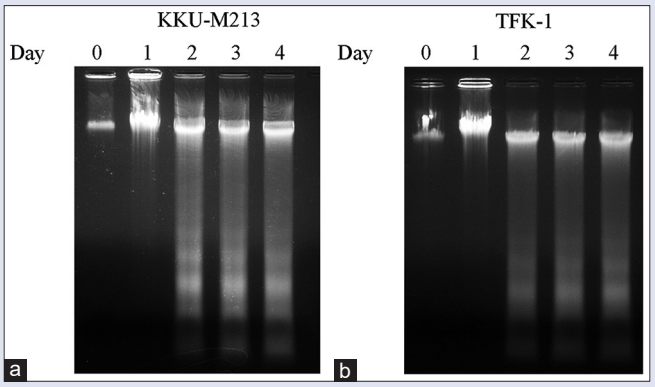

Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis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

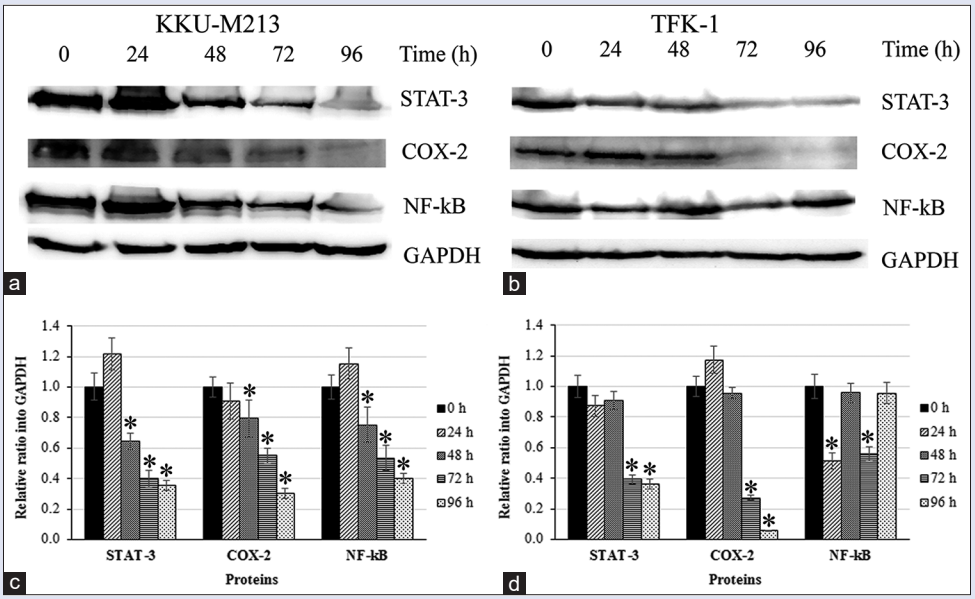

Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer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

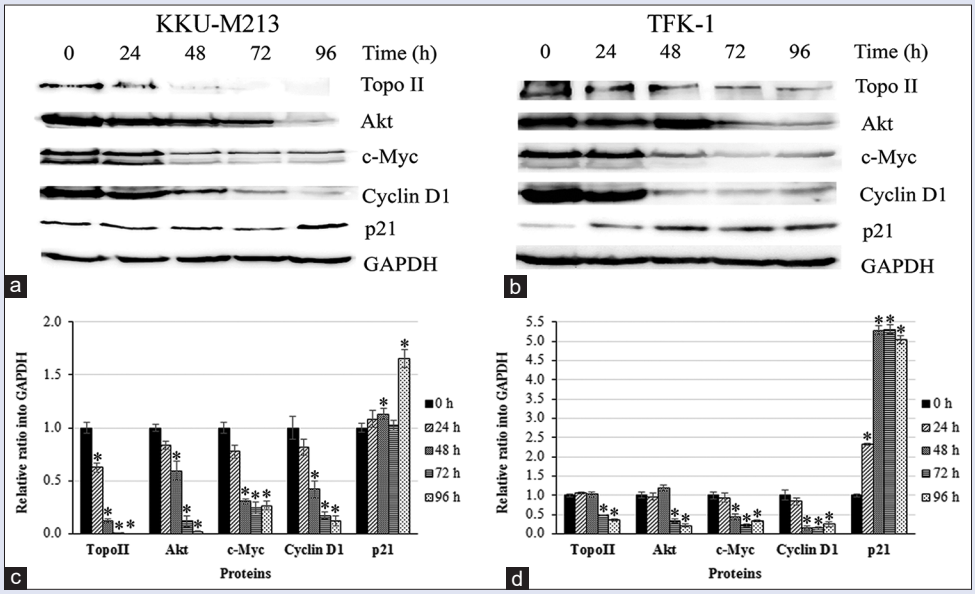

Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase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

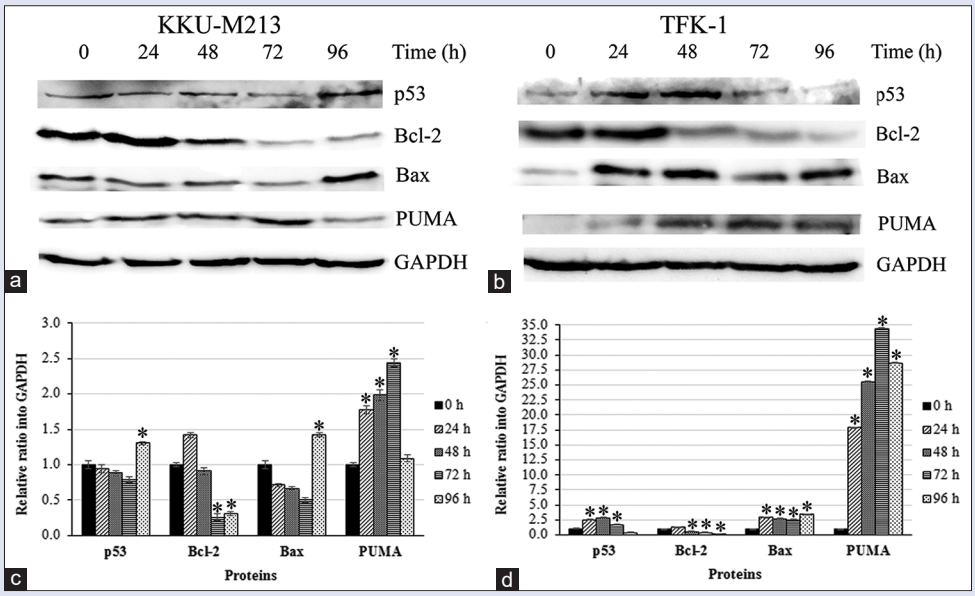

Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2,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

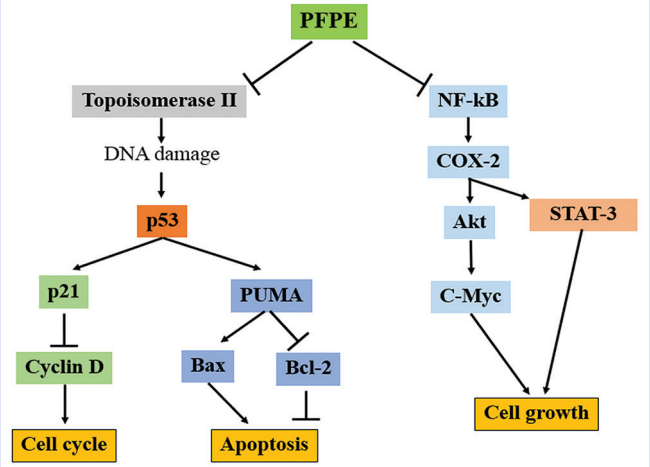

Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

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.

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