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"LIVING THE NEW NORMAL: *ACHIEVING RESILIENCE AND ENSURING SUSTAINABLE FUTURE*"

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Living the New Normal:
Achieving Resilience & Ensuring Sustainable Future

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Living the New Normal:
Achieving Resilience & Ensuring Sustainable Future

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Preface

Om Swastyastu

It gives me great pleasure to extend to you all a proceeding book of the 7th International Conference of Interreligious and Intercultural Studies. Universitas Hindu Indonesia would like to say how grateful we are to the scientist, scholar, and researcher who have contributed in the 7 th ICIIS with an insightful theme: Living The New Normal: Achieving Resilience And Ensuring Sustainable Future on 30 September, 2021.

On this proceeding book, there are 10 papers presented organized by Universitas Hindu Indonesia in collaboration with International Consortium for Religious Studies-Universitas Gadjah Mada (ICRS UGM Yogyakarta), Research Center for Area Studies-The Indonesian Institute of Sciences (PSW-LIPI Jakarta), and International Federation of the Social Sciences Organisation (IFSSO). The greatest academic issues that discussed are the general and specifics issues in Achieving Resilience And Ensuring Sustainable Future during the pandemic. How faith, religion, tourism, economic, political aspects and also culture in the broaden sense could be functioned as support systems in dealing with the new challenges after the experience of hardship with the pandemic that has ravaged religious practices, and has disturbed economic as well as political and cultural aspects of life. Reformulation of worthy elements from cultural values rooted in the society could be practiced or repracticed to deal with a new normal life or even a normal life again. Lessons learned from different countries in dealing with the pandemic could be shared in this conference so that any weeknesses of previous life with pandemic, shall not be repeated by others

In this precious moment, I would like to express our gratitude Hilmar Farid, Ph. D.(the General Director of Culture-the Ministry of Education and Culture-the Republic of Indonesia who gave a valuable speech at this conference. I would like also to convey my appreciation to all invited speakers, both local and broad scholars. We consider that the papers contribution of participants and speakers is exactly the main thing. Through these articles, we explore and develop smart ideas to deal with the threat to the social and culture resiliencies. There are many strategies could be applied by lessons learned from the bad impacts of the pandemic in reviving to the new normal life or even a normal life.

We sincerely hope that this book could be an academic references for scholars from various fields of interest.

Om santih, santih, santih, Om

Denpasar, September 2021

Prof. Dr. drh. I Made Damriyasa, M.S
Rector Of Universitas Hindu Indonesia

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BAB. VIII

MEDICINE PLANTS IN THE *LONTAR* MANUSCRIPT "*TARU PRAMANA*" AND IT USES FOR COUGH MEDICINE

I Nyoman Arsana[1*] and Ni Ketut Ayu Juliasih[1]

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The *lontar* manuscript "*Taru Pramana*" describes the use of plant species to treat various diseases according to the traditional Balinese medicine system. This study aims to identify plants in the *lontar* manuscript "*Taru Pramana*" which are used for cough medicine. The research uses the library method. The unit of analysis is the *lontar* manuscript "*Taru Pramana*". Data were analyzed descriptively. An emic approach combined with an ethical perspective is used in the analysis. A total of 11 species of plant were recorded to be used for cough medicine including; bilimbi (*Averrhoa bilimbi*), carambola (*Averrhoa carambola* L.), fig (*Ficus sp*), yams (*Dioscorea sp*), galangal (*Alpinia galanga*), calamus (*Acorus calamus*), garlic (*Allium sativum L*), coriander (*Coriandrum sativum* L), turmeric (*Curcuma domestica*), White Pepper (*Piper nigrum* L), *Temu tis* (*Curcuma purpurascens* Blume). In addition, other ingredients are also used, such as black chicken eggs, stingless bee honey. These materials are used singly or in the form of a mixture (Polyherbal). The herb is used by drinking or in scrub in the chest area. In conclusion, the practice of using plants as medicinal ingredients is based on knowledge and belief.

Keywords: Medicinal plants, Traditional Balinese Medicine, *Lontar* manuscript "*Taru Pramana*"

INTRODUCTION

The emergence of a new virus known as Severe acute respiratory coronavirus-2 (SARS-Cov-2) and the sickness known as Coronavirus disease 2019 (COVID-19) shook the globe at the start of 2020. This virus first appeared in Wuhan, China, at the end of 2019. Currently (August 10, 2021), 223 countries have been infected with COVID-19 with a total of 202,144,929 confirmed cases and 4,285,421 have been declared dead [1]. Although the Covid-19 vaccine has been found and many countries have worked hard to contain it, the signs of the end of this pandemic are still unclear.

Efforts to prevent, treat, and treat COVID-19 using traditional medicine systems have also been carried out. China, the United States, Italy, and India are countries that apply a lot of traditional medicine systems. The types of treatment are very diverse, including using herbal ingredients. Consumption of supplements such as vitamins C, D, zinc, omega-3, and herbs such as garlic, ginger, turmeric tends to increase during a pandemic. The consumption is mainly for the reasons of increasing immunity [2].

The main clinical manifestations of SARS-Cov-2 infection are fever, shortness of breath, and cough. Coughing is also a symptom of respiratory disease, shortness

of breath, or wheezing due to a blockage in the respiratory tract, such as in the case of asthma. However, coughing is also needed to clean the respiratory tract from particles, dust, germs, and secretions that cover the airways.

Various types of plants to prevent and treat coughs have been known and used by traditional Balinese people. These various types of plants are recorded in ancient manuscripts known as *lontar*, one of which is *lontar* "*Taru Pramana*". These plants are made in the form of herbal concoctions consisting of various types of plants and used in various ways such as drinking (loloh) or a scrub (*boreh*). This study aims to identify the types of plants in the *lontar* manuscript "*Taru Pramana*" and its uses for cough medicine.

METHOD

The research uses the library method. The unit of analysis is the *lontar* manuscript "*Taru Pramana*" which has been translated from Balinese script to Latin script. Several types of *lontar* manuscripts analyzed include; *lontar* manuscript from Bugbug Village, Karangasem District which was transliterated by I Dewa Ayu Puspita Padmi, typed on December 31, 1995; *lontar* belongs to Wayan Catra from Pandak Gede, Kediri, Tabanan which was copied by AAKetut Rai, and typed on September 4, 1993; *lontar* manuscript belongs to I Ketut Sengod from Banjar Pidpid Kaler, Abang Subdistrict, Karangasem, which was copied by Ida I Dewa Catra and typed on December 10, 1990.

The types of plants used as cough medicine were recorded and identified to determine their scientific names. Data were analyzed descriptively. An emic approach combined with an ethical perspective is used in the analysis. The emic approach is a local community perspective related to the practice, knowledge, and belief in plants as cough medicine. The emic perspective is then combined with a scientific perspective based on scientific data according to published research results.

RESULTS AND DISCUSSION

The results of the study found that as many as 11 plant species were used for cough medicine including; bilimbi (*Averrhoa bilimbi*), carambola (*Averrhoa carambola* L.), fig (*Ficus* sp), yams (*Dioscorea* sp), galangal (*Alpinia galanga*), calamus (*Acorus calamus*), garlic (*Allium sativum* L), coriander (*Coriandrum sativum* L), turmeric (*Curcuma domestica*), White Pepper (*Piper nigrum* L), *Temu tis* (*Curcuma purpurascens* Blume) as presented in Table 1. In addition, other ingredients are also used, such as black chicken eggs, stingless bee honey.

Table 1. Medicinal Plants in *Lontar* Manuscript "*Taru Pramana*" which is used for cough medicine

No	Species	Ways of making	How to use
1	Leaves of Bilimbi (*Averrhoa bilimbi*), galangal (*Alpinia galanga*) 3 slices.	chewed	Spout (*sembar*)
2	The stem bark of Bilimbi (*Averrhoa bilimbi*), Coriander (*Coriandrum sativum* L) 5 seeds, Temu tis (*Curcuma purpurascens* Blume).	crushed and filtered	Drink (*loloh*)
3	Carambola fruit (*Averrhoa carambola* L.), White Pepper (*Piper nigrum* L) 11 seeds	crushed and filtered	Drink (*loloh*)
4	Carambola leaves (*Averrhoa carambola* L.), galangal (*Alpinia galanga*), Turmeric (*Curcuma domestica*) 3 slices,	chewed	Spout (*sembar*)

5	Stem bark of Carambola (*Averrhoa carambola* L.), Coriander (*Coriandrum sativum* L), 5 seeds	crushed and filtered	Drink (*Loloh*)
6	Leaf fig (*Ficus* sp) 11 strands, garlic (*Allium sativum* L), calamus (*Acorus calamus*),	chewed	Spout (*Sembar)*
7	The sap of Yams (*Dioscorea* sp), black chicken eggs, stingless bee honey, temu tis (*Curcuma purpurascens* Blume), Coriander (*Coriandrum sativum* L) 15 seeds	crushed and filtered	Drink (*Loloh*)

From an emic perspective, the practice of using plants as medicinal ingredients is based on local community knowledge about plants. Plants with white, yellow, or green flowers have heat properties, plants with red or blue flowers have *tis* (cool) properties, and plants with colorful flowers have *dumalada* (moderate) properties. In addition, plants with reddish-white sap will have heat properties. Plants with yellowish-white sap are also hot, greenish-white sap is *dumalada* (medium), blackish white is *dumalada* (medium), green sap is *tis* (cool), black is tis (cool), blue gummy is also *tis* (cool).

From an emic perspective, there are only three pains, namely fever, cold, and moderate, therefore the treatment is related to the nature of the plant. Fever pain can be treated with plants that are efficacious *tis*, cold sick are treated with warm plants, and moderate are treated with plants that have *dumalada* (medium) properties.

The use of plants as medicinal ingredients is inseparable from the public trust. Local people believe that health-illness is a combination of *stula sarira* (body)-*suksma sarira* (mind)-*antahkarana sarira* (spirit), in other words, that health-illness is a balance between body, mind, and soul (spirit). If there is no balance between body-mind-spirit then a person is said to be sick.

Meanwhile, from an ethical perspective, the practice of using plants is associated with the active compounds of the plant so it has many pharmacological effects. For example, *Averrhoa bilimbi* leaves are known to have saponins, tannins, steroids, flavonoids, and alkaloids, which function as very strong antioxidants and as anti-inflammatory [3]. Meanwhile, bilimbi fruit is known to have the ability as antidiabetic by reducing hyperglycemia and reducing oxidative stress due to the presence of the compound of quercetin [4], as an antibacterial, especially multi-drug resistant bacteria [5], as an antioxidant that can reduce the formation of free radicals so that it can be used in cardiotoxicity treatment [6].

Averrhoa bilimbi locally known as *belimbing buluh* is a member of the Oxalidaceae family which has fruit with a sour taste. Aside from being a medicinal ingredient, local people also use bilimbi fruit as a cooking mixture to get rid of the fishy smell of fish. The parts used as cough medicine are the leaves and bark.

Averrhoa carambola is locally known as *belimbing manis* because it has ripe fruit with a sweet taste. Besides being a medicinal ingredient, local people also use carambola leaves as *lawar* (traditional Balinese food). The parts used as cough medicine are the stem bark, fruit, and leaves.

Carambola leaves are known to contain several active compounds that play an important role in medicine, including; Butane, 1,1-diethoxy-3 methyl-(CAS) methyl; Dodecanoic acid, methyl ester (CAS); Octadecanoic acid methyl ester (CAS); Octadecanoic acid methyl ester; and 9-Octadecanoic acid ethyl ester [7].

Carambola leaf methanol extract has the potential as an antihyperlipidemic by preventing the accumulation of liver lipids and inhibiting the activity of HMG-CoA reductase and lipase enzymes, as well as antioxidants [8]. The HMG-CoA reductase enzyme plays a role in converting HMG-CoA into mevalonate, which is the first stage of cholesterol synthesis in cells, thereby preventing the production of endogenous cholesterol. Low plasma cholesterol will trigger the expression of LDL receptors thereby increasing LDL uptake.

Alpinia galanga is a member of the Zingiberaceae family which is widespread in Asia and is used as a spice in cooking and as a medicinal ingredient. These medicinal ingredients are associated with compounds, especially the terpene and phenolic groups. Phenolic compounds and their derivatives include ferulic acid, apigenin, vanillic acid, kaempferol, kaempferol-3-O-methyl ether, luteolin, chrysin, 1'-acetoxyeugenol acetic acid, and p-hydroxybenzoic acid. These compounds are mainly found in rhizomes. Meanwhile, the terpene group compounds include -pinene, -terpineol, and 1,8-cineole. *Alpinia galanga* is used as an antimicrobial, antifungal, anti-inflammatory, anti-hepatotoxic, antioxidant, immunomodulatory, antidiabetic [9].

Acorus calamus is a member of the Acoraceae family. Local people call it by the name *Jangu* and it is used for various purposes such as cooking spices, Hindu religious rituals, ornamental plants, as well as medicinal ingredients. Rhizome calamus is known to contain neo-acorane A, acorid acid, and calamusin D compounds. These compounds have the potential to protect nerve cells [10]. The rhizome is also known to contain essential oils, especially α-asarone, (E)-methyl isoeugenol, methyl eugenol, β-asarone, α-cedrene, and camphor.

Allium sativum L by local people is referred to as *kesuna* and is used as a cooking spice, medicinal ingredient, and Hindu religious rituals. The main secondary metabolites contained in *Allium sativum* are mainly organosulfur compounds. These compounds are rich in sulfur so that *Allium sativum* has a distinctive smell and taste as well as pharmacological functions in health. Mainly organosulfur compounds are; alliin, allicin, allyl trisulfide, E1 propenyl allyl disulfide, 2 propenyl 1 propenyl disulfide, 2 phenyl 4H 1,3 dithiin, 3 vinyl 4H 1,2 dithiin, and ajoene[11]. Organosulfur compounds are known to act as immunomodulators so that they have the potential to prevent SARS-CoV-2 [12].

Coriander (*Coriandrum sativum* L) is locally known as coriander. The part of the plant that is used is the seed, used as a spice in cooking, in Hindu religious rituals, and as a medicinal ingredient. These medicinal ingredients are associated with the presence of secondary metabolites in the form of essential oils, including; α–pinene, camphene, 1-Limonene, and Camphor. These compounds cause coriander to act as gram-negative and gram-positive antibacterials [13]. In addition, there are linalool, geraniol, terpinen-4-ol, α-terpineol, γ-terpinene, r-cymene, camphene, myrcene, geranyl acetate, and linalyl acetate [14].

Turmeric (*Curcuma domestica* L) is locally known as *kunyit* and is used as a spice in cooking, in Hindu religious rituals, and as an ingredient in traditional medicine. These medicinal ingredients are associated with the presence of secondary metabolites including; saponins 3.73 %w/v, alkaloids 0.24 %w/v, steroids 1.55 %w/v, flavonoids 1.99 %w/v, tannin 41.33 %w/v, and phenol 1.71 %w/v. Turmeric also has good

antioxidant activity, with an IC_{50} of 363 g/ml at 1,1-diphenyl-2-picrylhydrazyl (DPPH).

Pepper (*Piper nigrum* L) has long been used as a spice in cooking to get a spicy taste. The taste comes from piperine compounds which reach 26% [15]. *Piper nigrum* has important pharmacological functions including treating colon cancer [16], inhibit bile duct cancer through the pathway of down-regulation of cell proliferation and induction of apoptosis [17], as an herbal medicine for anti-cancer and migratory activity through the mevalonate pathway [18], as an antibacterial in both gram-positive and gram-negative species [19], as an antioxidant [24], as a hepatoprotective against drugs [20], as an effective nutraceutical in overcoming oxidative stress and anti-inflammatory.

Curcuma purpurascens locally is known as *temu tis*, used in traditional medicine and Hindu religious rituals. Essential oils found in rhizome include turmerone (13.5%), germacrone (13.2%), ar-turmerone (9.4%), germacrene-B (8.8%), curlone (6.2%), curzerene (5.8%), camphor (5.8%), and ar-turmerone (9.4%). (4%). The presence of these essential oils may act as a strong antiproliferative in human colon carcinoma cells (HT29) lines [21]. Hexane rhizome extract [22]. C-elemene, 6-ethenyl-4,5,6,7-tetrahydro-3,6-dimethyl-5-isopropenyl, benzofuran, 3,7-cyclodecadiene-1-one, 3,7 dimethyl-10-(1-methylethylidene), turmerone, and curlone are among the active compounds found in *Curcuma purpurascens*. Because of the presence of these active substances, it can protect the stomach from harm.

These herbs were used in the mixed form (Table 1). Such mixed forms are known as polyherbal [23]. The polyherbal form is generally better than the single form because the compounds contained in a single plant are not sufficient to achieve the desired effect. Polyherbal forms will work synergistically to achieve a better effect [24]. For example, essential oil made in the form of a mixture of Caraway (*Carum carvi* L) with coriander produces antibacterial activity, as well as better antioxidant and antidiabetic activity than single form [25]. Ayurvedic polyherbal, *Dhanwantaram kashyam*, which consists of 40 kinds of herbal ingredients, can reduce free radicals and can restore normal lipid profiles in diabetic rats [26]

The polyherbal is used by drinking (*loloh*) or spout (*sembar*) on the chest area (Table 1). *Loloh* is a starch extract made by grinding all the ingredients and adding a little water, filtering it, then drinking it. *Sembar* is made by chewing all the ingredients and then spout it on the chest. However, due to the COVID-19 pandemic, the use in the form of *sembar* can be replaced with the form of *boreh. Boreh* is obtained by grinding all the ingredients until smooth using a mortar and use by srub in the chest area.

CONCLUSION

The practice of using plants as medicinal ingredients is based on knowledge and belief. A total of 11 species were recorded as being used for cough medicine, and this finding opens the opportunity to explore further to obtain scientific evidence so that it can be integrated into conventional medicine systems.

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ORIGINAL ARTICLE

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

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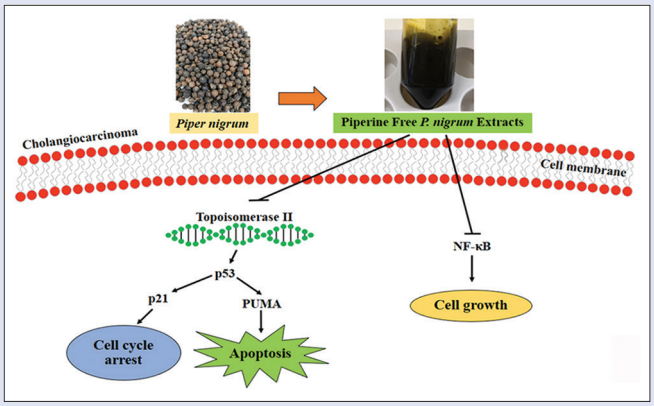
ABSTRACT

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

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

SUMMARY

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



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

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INTRODUCTION

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

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

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

MATERIALS AND METHODS

Preparation of piperine free *Piper nigrum* extract

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

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

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

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

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

Cell lines and culture conditions

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

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

In vitro cytotoxicity

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

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

Deoxyribonucleic acid fragmentation analysis

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

Western blot analysis

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

Statistical analysis

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

RESULTS

Total phenolic, tannin, and flavonoid contents

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

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

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

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

Phytochemical screening

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

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

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

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

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

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

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

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

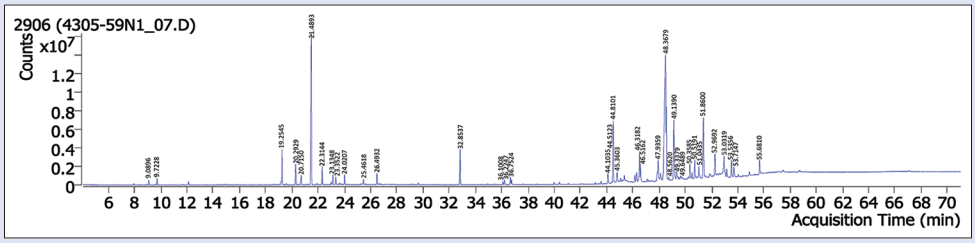

Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

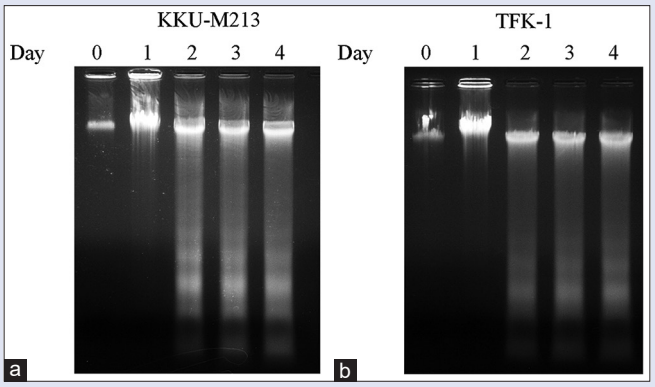

Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis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

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

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

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

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

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

DISCUSSION

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

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

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

Contd...

Table 2: Contd...

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

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

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

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

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

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

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

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

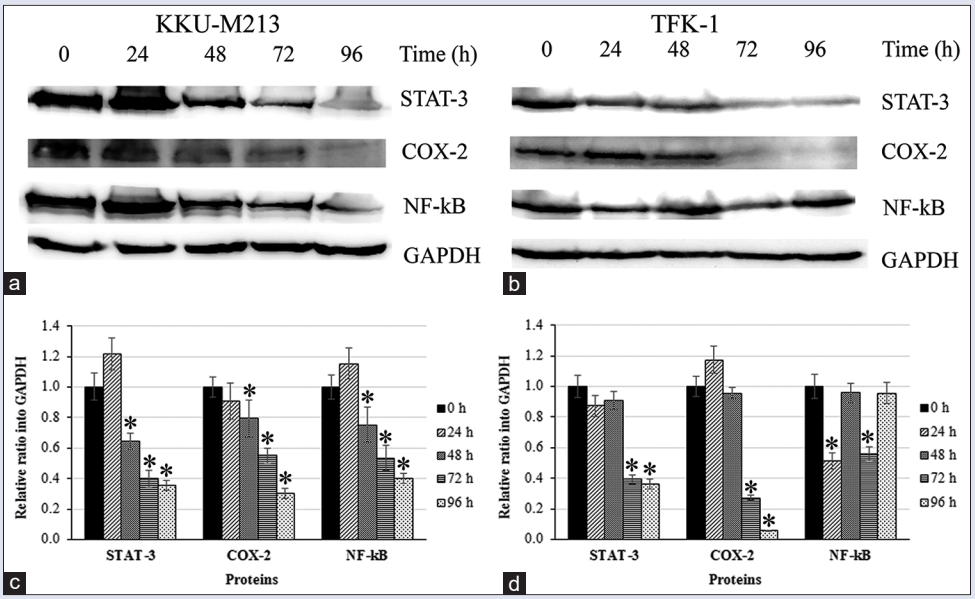

Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer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

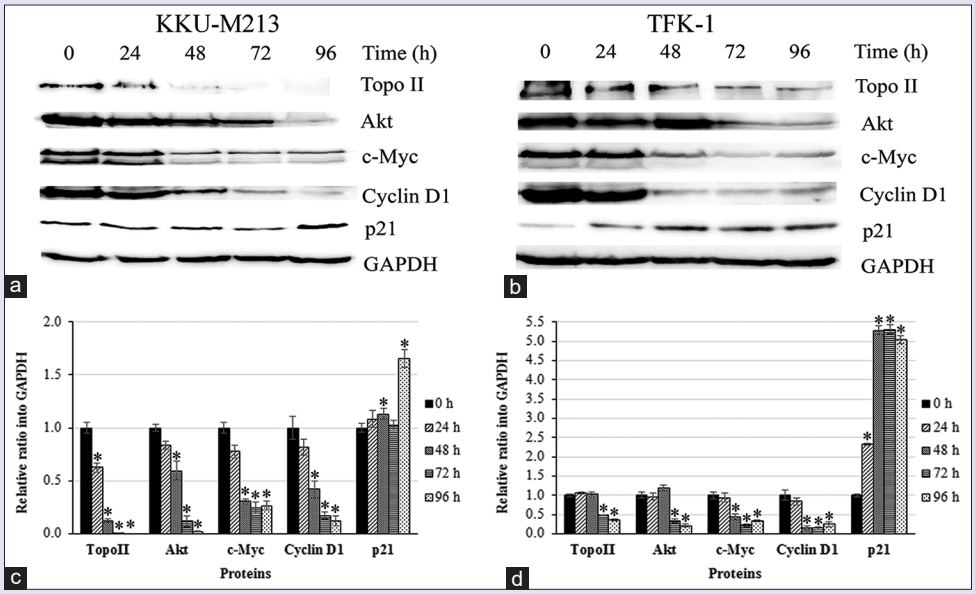

Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase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

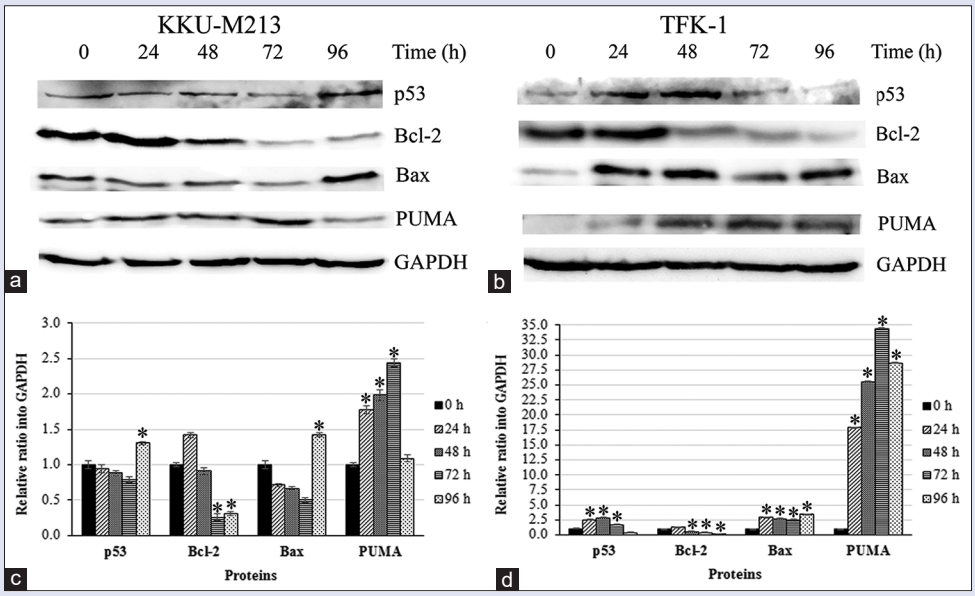

Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2,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

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

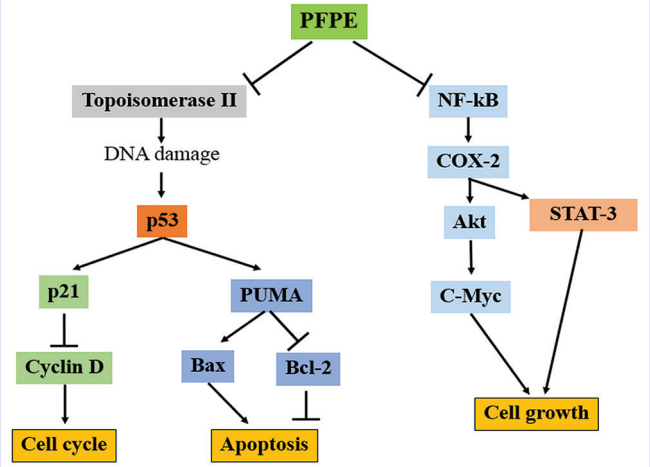

Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

CONCLUSION

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

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Conflicts of interest

There are no conflicts of interest.

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