The Role of Herbal Medicine in Cholangiocarcinoma Control: A Systematic Review

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ABSTRACT

The growing incidence of cholangiocarcinoma (bile duct cancer) and limited treatment options stimulate a pressing demand for research and the development of new chemotherapeutics against cholangiocarcinoma. This study aimed to systematically review herbs and herb-derived compounds or herbal formulations that have been investigated for their anti-cholangiocarcinoma potential. Systematic literature searches were conducted in three electronic databases: PubMed, ScienceDirect, and Scopus. One hundred and twenty-three research articles fulfilled the eligibility critera and were included in the analysis (68 herbs, isolated compounds and/or synthetic analogs, 9 herbal formulations, and 119 compounds that are commonly found in several plant species). The most investigated herbs were *Atractylodes lancea* (Thunb.) DC. (Compositae) and *Curcuma longa* L. (Zingiberaceae). Only *A. lancea* (Thunb.) DC. (Compositae) has undergone the full process of nonclinical and clinical development to deliver the final product for clinical use. The extracts of *A. lancea* (Thunb.) DC. (Compositae), *Garcinia hanburyi* Hook.f. (Clusiaceae), and *Piper nigrum* L. (Piperaceae) exhibit antiproliferative activities against human cholangiocarcinoma cells (IC_{50} < 15 µg/mL). Cucurbitacin B and triptolide are herbal isolated compounds that exhibit the most promising activities (IC_{50} < 1 µM). A series of experimental studies (*in vitro, in vivo*, and humans) confirmed the anti-cholangiocarcinoma potential and safety profile of *A. lancea* (Thunb.) DC. (Compositae) and its active compounds atractylodin and *β*-eudesmol, including the capsule pharmaceutical of the standardized *A. lancea* (Thunb.) DC. (Compositae) extract. Future research should be focused on the full development of the candidate herbs to deliver products that are safe and effective for cholangiocarcinoma control.

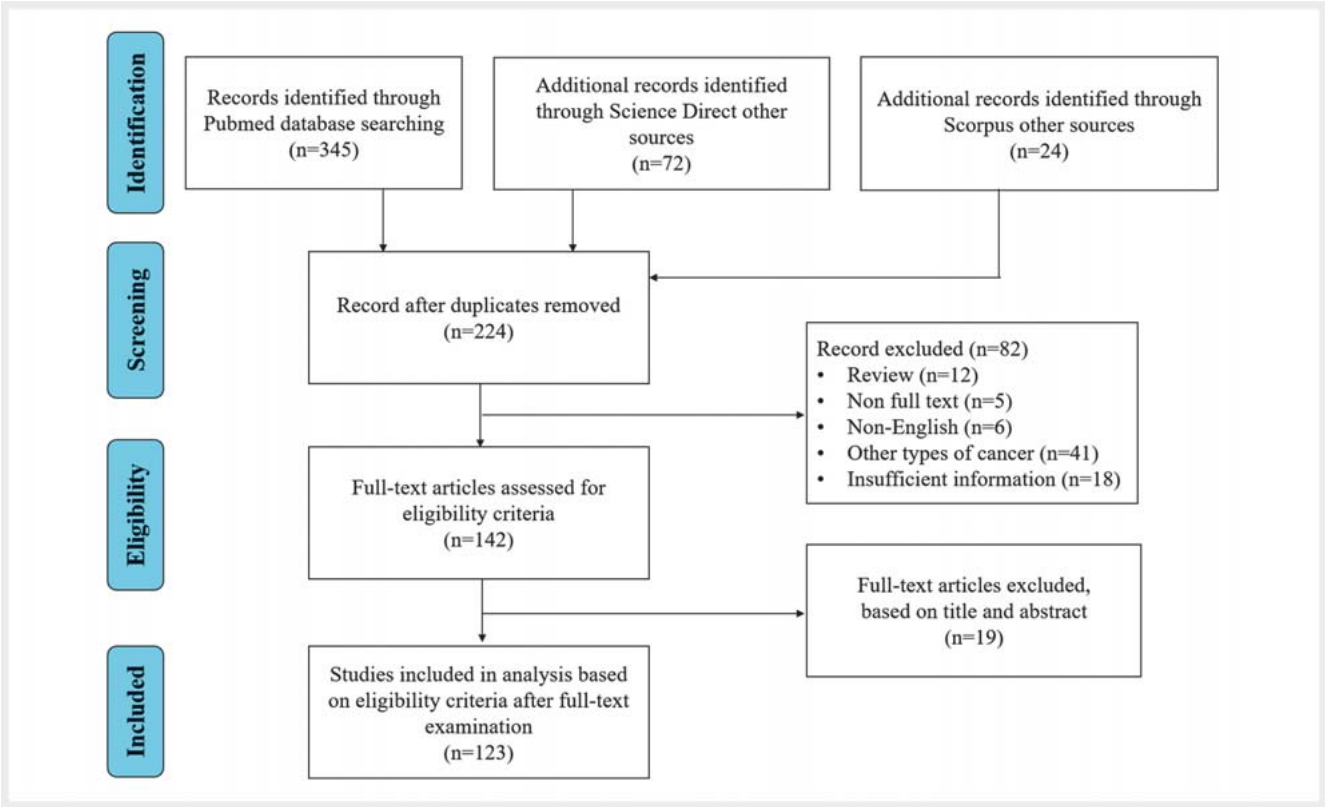
ABBREVIATIONS

5-FU	5-fluorouracil
ADP	adenosine diphosphate
AKT	protein kinase B
ALNP	atractylodin-loaded PLGA nanoparticle
AMPK	5′ AMP-activated protein kinase
AP1	activator protein 1
Apaf-1	apoptotic protease-activating factor 1
AT	atractylodin
Bax	Bcl2-associated X protein
Bcl2	B-cell lymphoma 2
BE	beta-eudesmol
BID	twice per day
CAF	cancer-associated fibroblast
CCA	cholangiocarcinoma
Cdk	cyclin-dependent kinase
CHOP	C/EBP homologues protein
cIAP	cellular inhibitor of apoptosis protein
COVID-19	coronavirus disease of 2019
COX2	cyclooxygenase 2
DAPK1	death-associated protein kinase 1
DMN	dimethylnitrosamine
DR	death receptors
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF2α	eukaryotic initiation factor 2
E_{max}	maximum drug effect
EMSA	electrophoresis mobility shift assay
EPMC	ethyl-*p*-methoxycinnamate
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
GLI1	glioma-associated oncogene homologue 1
GSKβ	glycogen synthase kinase beta
HO1	heme oxygenase 1
HS	hinesol
ICAM1	intercellular adhesion molecule 1
IFNγ	interferon gamma
IL6	interleukin 6
JAK	Janus kinase
JNK	Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCL-1	myeloid cell leukemia-1
MDR	multidrug resistance
MMP	matrix metalloproteinases
MRP	multidrug resistance associated protein
MTD	maximum tolerated dose
mTOR	mammalian target of rapamycin
NFκB	nuclear factor kappa light chain enhancer of activated B cells
NK	natural killer
OV	Opisthorchis viverrini
p38	38-kilodalton protein kinase
PBMC	peripheral blood mononuclear cell
PI3K	phosphoinositide 3-kinase
PLGA	poly lactic-co-glycolic acid
RAS	rat sarcoma
Rb	retinoblastoma
ROS	reactive oxygen species
RT-PCR	real-time PCR
SRB	sulphorhodamine B
STAT	signal transducer and activator of transcription
TEM	transmission electron microscope
TR1	type 1 regulatory T cells
TRAF1	tumor necrosis factor receptor associated factor 1
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VEGFR	vascular endothelial growth factor receptor
WST	water-soluble tetrazolium salts
XIAP	X-linked inhibitor of apoptosis protein

Introduction

CCA is a malignant bile duct cancer of epithelial cells with high morbidity and mortality. The world's highest incidence is reported from the northeastern part of Thailand, with an age-standardized incidence rate of 33.4 per 100 000 in males and 12.3 per 100 000 in females. It is the second most common hepatic malignancy in the world after hepatocellular CCA. Increasing incidence and mortality from CCA have been reported globally [1]. Several risk factors are associated with CCA development, including primary sclerosing cholangitis, cirrhosis, fibropolycystic hepatic disease, hepatolithiasis, congenital intrahepatic biliary stones, viral hepatitis, and liver fluke infection (*Opisthorchis viverrini* and *Clonorchis sinensis*). Infection with *O. viverrini* is a risk factor for almost all cases of CCA in Thailand [2]. Treatment and control of CCA remain unsatisfactory due to the lack of sensitive and specific diagnostic tools for early detection, as well as effective drugs. The overall 5-year survival rate of CCA patients is less than 5%. Surgical resection is the curative treatment option eligible for patients with an early-stage tumor. Gemcitabine and/or cisplatin-based chemotherapy is the first-line treatment option for patients with advanced or metastatic disease. However, the effectiveness of these drugs is limited, with the median overall survival of less than 1 year [3]. The growing incidence of CCA and limited treatment options hasten a pressing demand for research and development of new chemotherapeutics against CCA.

In recent years, natural products and the research and development of herbs for cancer chemotherapy have been an intensive area of research. This is due to the diverse chemical structures and bioactivities of herbs that could be exploited as promising drug candidates for various types of cancer. Numerous studies have been carried out to discover effective cancer chemotherapeutic agents from plant sources with low toxicity. Examples of successful drugs for cancer include vincristine, vinblastine, etoposide, teniposide, paclitaxel, vinorelbine, docetaxel, topotecan, camptothecin, and irinotecan [4]. The aim of this study was to systematically review herbs and herb-derived compounds that have been investigated for their anti-CCA potential both *in vitro, in vivo*, and humans. Information obtained was analyzed to facilitate further



▶ **Fig. 1** Flow chart of the article selection process.

development of effective and safe anti-CCA drugs in a systematic approach.

Results and Discussion

A total of 224 articles from PubMed, ScienceDirect, and Scopus databases were downloaded to the EndNote database. Eighty-two articles were excluded, and further analysis of the titles and abstracts of the remaining 142 articles led to the exclusion of 19 articles (excluded based on title and abstract). Finally, 123 articles were included in the analysis. The flow diagram of the study inclusion and exclusion is presented in ▶ **Fig. 1**. Antiproliferative activities of plant extracts or active compounds are summarized in ▶ **Table 1** and results of clinical studies of some herbal formulations are summarized in ▶ **Table 2**. Mechanisms of antiproliferative activities including *in vivo* studies in animals are provided in the Supporting Information. The included articles involve 68 herbs, isolated compounds, and/or synthetic analogs, 9 herbal formulations, and 199 compounds that are commonly found in several plant species. The most investigated plant was *Atractylodes lancea* (Thunb.) DC. (Compositae) (n = 17), followed by *Curcuma longa* L. (Zingiberaceae) (n = 15), *Garcinia hanburyi* Hook.f. (Clusiaceae) (n = 6), *Artemisia annua* L. (Compositae) (n = 5), *Zingiber officinale* Roscoe (Zingiberaceae) (n = 5), *Andrographis paniculata* (Burm.f.) Nees (Acanthaceae) (n = 4), *Capsicum* spp. (Solanaceae) (n = 3), *Derris indica* (Lamk.) Benn. (Leguminosae) (n = 3), *Piper longum* L. (Piperaceae) (n = 3), and *Tripterygium wilfordii* Hook. f. (Celastraceae) (n = 3). Other plants were reported in one or two research articles. Pra-Sa-Prao-Yai was the most investigated formulation (n = 2). Resveratrol (n = 5) and capsaicin (n = 3) derived from several plants was the most investigated compounds for anticancer activity against CCA. Most studies reported the antiproliferative activities using different *in vitro* tests (n = 108), including MTT, SRB, WST-1, Hoechst, neutral red, acridine orange/ethidium bromide, cell counting kit-8, crystal violet, PrestoBlue, calcein-AM, tryphan blue, cell titer 96 aqueous, IncuCyte zoom, morphological examination, flow cytometry, and clonogenic assays. *In vivo* evaluation of anti-CCA activity in animal models [xenograft mouse model, OV/DMN-induced CCA hamster model, and allograft hamster model] was reported in 26 articles. Mechanisms or targets of action at the molecular or cellular level were reported in 95 studies. Others involved studies on antioxidative (n = 3) and immunomodulatory activities (n = 2), as well as their inhibitory activities on cell migration (n = 22) and cell invasion (n = 17), pharmacokinetic studies (n = 2), clinical studies (safety and/or efficacy) (n = 3), development of nanoformulations (n = 2), and synergizing effects on chemotherapeutic drugs (n = 5).

The potential role of herbs/herbal medicines for CCA control has been one of the focuses in CCA research, as seen by a relatively large number of research articles published during the years 2000 to 2021. Evidence-based knowledge is provided by scientific support from *in vitro, in vivo*, and clinical studies in a total of 68 herbs, 9 herbal formulations, and 199 isolated compounds or syn-

▶ **Table 1** Plants/isolated compounds/symthetc compounds (underlined) under investigation and available antiproliferative activity against CCA cell lines.

References	Plants/Active compounds (Family)	Antiproliferative activity
[5]	Crude ethanol extracts: *Amomum testaceum* Ridl. (Zingiberaceae), *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. (Apiaceae), *Angelica sinensis* (Oliv.) Diels. (Apiaceae), *Anethum graveolens* L. (Apiaceae), *Artemisia annua* L. (Compositae), *Asclepias curassavica* L. (Apocynaceae), *Atractylodes lancea* (Thunb.) DC. (Compositae), *Cuminum cyminum* L. (Apiaceae), *Curcuma longa* L. (Zingiberaceae), *Dioscorea membranacea* Pierre ex Prain & Burkill (Dioscoreaceae), *Dracaena loureirin* Gagnep. (Asparagaceae), Foeniculum vulgare Mill. (Apiaceae), *Kaempferia galanga* L. (Zingiberaceae), Ligusticum sinense Oliv. (Apiaceae), *Mammea siamensis* Kosterm. (Guttiferae), *Mesua ferrea* L. (Calophyllaceae), *Mimusops elengi* L. (Sapotaceae), *Myristica fragrans* Houtt. (Myristicaceae), *Nigella sativa* L. (Ranunculaceae), *Piper chaba* Hunt. (Piperaceae), *Piper interruptum* Opiz. (Piperaceae), *Piper sarmentosum* Roxb. (Piperaceae), *Plumbago indica* L. (Plumbaginaceae), *Smilax corbularia* Kunth (Smilacaceae), *Syzygium aromaticum* (L.) Merr. & L.M.Perry (Myrtaceae), *Zingiber officinale* Roscoe (Zingiberaceae), *Zingiber ligulatum* Roxb. (Zingiberaceae), Ben-ja-Kul 1 formulation, Ben-ja-Kul 2 formulation, Pra-Sa-Prao-Yhai formulation, Tein-5 formulation	*A. lancea* (Thunb.) DC. (Compositae): most potent and selective against CL6 cells (IC_{50} = 24.09 µg/mL, SI = 8.6); five others with promising activity (< 50 % cell survival at 50 µg/mL) = *K. galanga* L. (Zingiberaceae), *Z. officinal* Roscoe (Zingiberaceae), *P. chaba* Hunt. (Piperaceae), *M. ferrea* L. (Calophyllaceae), and Pra-Sa-Prao-Yhai formulation (IC_{50}s of 37.36, 34.26, 40.74, 48.23, 44.12 µg/mL, respectively)
[33]	*Cardiospermum halicacabum* L. (Sapindaceae), *Gomphrena celosioides* Mart. (Amaranthaceae), *Scoparia dulcis* L. (Plantaginaceae) (ethanolic extracts)	*S. dulcis* L. (Plantaginaceae): most potent (56–75 % growth inhibition on KKU-100 and KKI-213 cells at 250 µg/mL for 72 h)
[17]	*Andrographis paniculata* (Burm.f.) Nees (Acanthaceae)/Semisynthetic andrographolide analog (19-triphenylmethyl ether andrographolide, AG 050)	Excellent activity against KKU-M213 cells (IC_{50} = 3.33 µM)
[18]	*Andrographis paniculata* (Burm.f.) Nees (Acanthaceae)/14-deoxy-11,12-didehydroandrographolide analogs	Analogs 5a, 5b: most potent and selective against KKU-M213 cells (IC_{50} = 3.37, 3.08 µM); KKU-100 = 2.93, 3.27 µM
[19]	*Andrographis paniculata* (Burm.f.) Nees (Acanthaceae)/Andrographolide	Significant activity against KKU-100 cells (IC_{50} ~ 120 µM)
[34]	*Aesculus hippocastanum* L. (Sapindaceae)/*β*-escin	IC_{50} Mz-ChA1 cells: 34.21 µM (24 h), 28.48 µM (48 h), 22.1 µM (72 h); SK-ChA1 cells: 59.04 µM (24 h), 41.69 µM (48 h), 33.3 µM (72 h); QBC939 cells: 63.3 µM (24 h), 44.36 µM (48 h), 34.06 µM (72 h)
[35]	Anthocyanin complex [from cobs of purple way corn Zeamays, certina Kulesh, and petals of blue butterfly pes *Clitoria ternatea* L. (Leguminosae)]	IC_{50} for KKU-213 cells = 620 µg/mL
[36]	*Arachis hypogaea* L. (Leguminosae)/Peanut testa extract, KK4 and ICG15042	Potent activity against KKU-M214 cells (KK4: IC_{50} = 38.28 µg/mL; ICG15042: IC_{50} = 43.91 µg/mL) and KKU-100 cells: (KK4: IC_{50} = 78.40 µg/mL; ICG15042: IC_{50} = 82.77 µg/mL) at 72 h
[37]	*Artemisia annua* L. (Compositae)/Artemisinins	Potent activity against CL6 cells: IC_{50} = 339 µM (artemisinin), 131 µM (artesunate), 354 µM (*β*-artemeter), 75 µM (dihydroartemisinin)

continued

▶ **Table 1** *Continued*

References	Plants/Active compounds (Family)	Antiproliferative activity
[21]	*Atalantia monophylla* DC. (Rutaceae)/7 new benzoyltyramines, atalantums A–G (1–7) and 5 known compounds	Compound **5**: most potent activity against KKU-M156 (IC_{50} = 1.97 µM), 4.7-fold higher than ellipticine standard. Compound **1**: potent activity against KKU-M214 (IC_{50} = 3.06 µM), comparable with 5-FU. Compounds **2, 4, 11**: more potent activity against KKU-M213 than ellipticine (IC_{50} = 2.36, 5.63, 2.71 µM). Compounds **1, 5, 7**: activity against KKU-M214 (IC_{50} = 3.06, 8.44, 7.37 µM, respectively).
[22]	*Atalantia monophylla* DC. (Rutaceae)/limonophyllines A–C (1, 4, 5), limonoids (2, 3), acridone alkaloids (6–16)	Compounds **12, 14, 16**: activity against KKU-M156 cells (IC_{50} = 3.39–4.1 µg/mL)
[38]	*Atractylodes lancea* (Thunb.) DC. (Compositae)/Atractylodin	IC_{50} = 216.8 µM for CL6 cells
[39]	*Atractylodes lancea* (Thunb.) DC. (Compositae)/Atractylodin (AT) and Atractylodin-loaded PLGA nanoparticle (ALNPs)	IC_{50} for CL6 cells, ALNPs vs. AT: 29.28 vs. 56.36 µM (24 h), 35.06 vs. 37.66 µM (48 h), 50.74 vs. 52.02 µM (72 h) µg/mL; IC_{50} for HuCC-T1 cells: ALNPs vs. AT: 47.68 vs. 53.66 µg/mL (24 h), 66.09 vs. 59.74 µM (48 h), 71.3 vs. 76.15 µg/mL (72 h)
[40]	*Atractylodes lancea* (Thunb.) DC. (Compositae)/Atractylodin (AT) Atractylodin-loaded PLGA nanoparticle (ALNPs)	IC_{50} for CL-6 cells: ALNPs vs. AT: 15 vs. 43 (24 h), 23 vs. 40 (48 h), 43 vs. 40 (72 h) µg/mL; IC_{50} for HuCC-T1 cells: ALNPs-1 vs. AT: 9 vs. 65 (24 h), 16 vs. 42 (48 h), 39 vs. 65 (72 h) µg/mL
[41]	*Atractylodes lancea* (Thunb.) DC. (Compositae)/Atractylodin and *β*-eudesmol	IC_{50} for CL6 cells: atractylodin = 41.66 µg/mL, *β*-eudesmol = 39.33 µg/mL
[42]	*Atractylodes lancea* (Thunb.) DC. (Compositae)/*β*-eudesmol	IC_{50} for CL6 cells = 166 µM
[43]	*Caesalpinia mimosoides* Lam. (Leguminosae) ethylacetate extract/ Gallic acid (natural: nGA, commercial: cGA)	IC_{50}: nGA = 120 µM (M213 cells) and 124 µM (M214 cells cGA), 119 µM (M213 cells), and 147 µM (M214 cells)
[44]	*Clausena harmandiana* (Pierre) Pierre ex Guillaumin (Rutaceae) hexane, ethyl acetate, methanol extracts/isolated and purified 12 azarbazoles and coumarins	7-hydroxy-heptaphylline and nordentatin: potent activity against KKU-OCA17 cells (IC_{50} = 88.7, 46.1 µM, respectively and KKU-214 cells (IC_{50} = 43.7, 39.1 µM, respectively)
[45]	Corilagin (natural plant polyphenol tannic acid)	IC_{50} for QBC9939 and MZ-Cha-1 cells = 39.73 and 36.88 µM, respectively
[46]	*Cratoxylum formosum* (Jack) Benth. & Hook. f. ex Dyer (Hypericaceae) aqueous and ethanolic Dyer leaf extract	Potent activity (IC_{50} for the aqueuous extract = 11.3 µg/mL, ethanol extract = 12.1 µg/mL)
[24]	*Curcuma longa* L. (Zingiberaceae)/Curcumin	IC_{50} = 5–17 µM (sensitive) for KKU-100, KKU-214, and KKU-OCA17 cells
[25]	*Curcuma longa* L. (Zingiberaceae)/Curcumin	IC_{50} = 5.9 µM for KKU-214 cells
[26]	*Curcuma longa* L. (Zingiberaceae)/Curcumin	Activity against CCLP-1 cells (10, 48, and 56% growth inhibition) and SG-231 cells (13, 25, and 50%) at 7.5, 10, and 15 mM, repectively
[27]	*Curcuma longa* L. (Zingiberaceae) New allylated mono-carbonyl curcumin analogs (MACs)	Compound **6c**: potent activity (IC_{50} for HuCCA cells = 8.7 µM, QBC-939 cells = 9.3 µM, and RBE = 8.9 µM)
[31]	*Derris indica* (Lamk.) Benn. (Leguminosae)/Candidione	Potent activity against KKU-M156 cells: IC_{50} = 6 µg/mL (17 µM) and 4.24 µg/mL (12.03 µM) at 8 and 24 h, respectively; KKU-M213 cells: IC_{50} = 5.7 µg/mL (16.17 µM) and 5.74 µg/mL (15.28 µM) at 8 and 24 h, respectively
[47]	*Derris indica* (Lamk.) Benn. (Leguminosae) ethylacetate extract/ a new furanoflavonoid derivative, 4′-hydroxypinnatin (1) and 5 known compounds	Pinnatin: potent activity against KKU-100 cells (IC_{50} = 6.0 µg/mL), E_{max} of 88–90% Flavone 5: highest activity against KKU-100 cells (IC_{50} = 1.3 µg/mL), but with moderate efficacy (E_{max} of 50.7%)
[48]	*Derris indica* (Lamk.) Benn. (Leguminosae) hexane extract/isolated Derrivanone (1) and Derrischalcone + 14 known compounds	Potent activity against KKU-M156 cells: Chalcones 2, 3, 4: IC_{50} = 7.0, 0.73, 0.59 µg/mL, respectively; Flavanones 14, 15, 16: IC_{50} = 0.59, 7.8, 2.4 µg/mL, respectively
[49]	*Derris malaccensis* (Benth.) Prain (Leguminosae)/Pomiferin (prenylated isoflavonoid)	IC_{50} for HuCCA-1 cells = 0.9 µg/mL

continued

▶ **Table 1** *Continued*

References	Plants/Active compounds (Family)	Antiproliferative activity
[50]	*Derris malaccensis* (Benth.) Prain (Leguminosae)/Pomiferin-4′-O-methyl ether, and a new prenylated chalcone, 2′,4′-dihydroxy-4-methoxy-3′-(2-hydroxy-3-methylbut-3-enyl)chalcone, 4 known flavonoids	Compounds **2** and **3**: potent activity against HuCCA-1 cells (IC_{50} = 4.8 and 3.8 µg/mL, respectively) Compounds **1, 4, 5, 6**: weak activity against HuCCT-1 cells (IC_{50} = 10.5, 14.0, 24.0, and 25.0 µg/mL, respectively)
[8]	*Dioscorea membranacea* Pierre ex Prain & Burkill (Dioscoreaceae) ethanol extract/7 isolated compounds	Crude extract: weak but selective activity against KKU-M156 cells (IC_{50} = 30.49 µg/mL); Compound **5**: selective activity against KKU-M156 cells (IC_{50} = 3.46 µM); Compounds **1**–**3**: no activity against KKU-156 cells (IC_{50} = 4100 µM)
[6]	*Garcinia hanburyi* Hook.f. (Clusiaceae) ethyl acetate and methanol extracts & fractions	Ethyl acetate extracts from bark (VR12874) and fruits (VR11626): potent activity (IC_{50} = 1.84–2.49 and 1.69–4.41 µg/mL); VR12876 and VR12879: weak activity; VR12880: no activity
[51]	*Garcinia hanburyi* Hook.f. (Clusiaceae)/4 caged xanthones: isomorellin, isomorellinol, forbesione gambogic acid	IC_{50}: Isomorellin: KKU-100 cells = 0.11 µM, KKU-156 cells = 0.12 µM; Isomorellinol: KKU-100 cells = 2.2 µM, KKU-M156 cells = 0.43 µM; Forbesione: KKU-100 cells = 0.15 µM, KKU-M156 cells = 0.02 µM; Gambogic acid: KKU-100 cells = 2.64 µM, KKU-M156 cells = 0.03 µM)
[52]	*Garcinia hanburyi* Hook.f. (Clusiaceae)/isomorellin	IC_{50} for KKU-100 cells vs. KKU-M156 cells: 6.2 vs. 1.9 µM (24 h), 5.1 vs. 1.7 µM (48 h), 3.5 vs. 1.5 µM (72 h)
[53]	*Holothuria scabra* Jaeger (sea cucumber)/Scabraside D (sulfated triterpene glycoside)	Significant activity against CL6 cells (IC_{50} = 12.8 µg/mL at 24 h)
[9]	*Kaempferia galanga* L. (Zingiberaceae) ethanol extract/Ethyl-p-methoxycinnamate (EPMC)	Extract and EPMC: moderate activity against CL6 cells (IC_{50} = 64.2, 49.19 µg/mL; SI = 2.2, 2.09)
[10]	*Kaempferia galanga* L. (Zingiberaceae) ethanol extract/Ethyl-p-methoxycinnamate (EPMC)	Moderate activity against CL6 cells: extract IC_{50} for CL6 cells = 78.41 µg/mL, SI = 4.44; EPMC: IC_{50} = 100.76 µg/mL, SI = 2.2; moderate activity against HuCCT1 cells: extract IC_{50} = 66.03 µg/mL, SI = 6.04; EPMC IC_{50} = 156.6 µg/mL, SI = 2.23
[54]	*Kaempferia parviflora* Wall. ex Baker (Zingiberaceae) (crude ethanol extract)/5,7,4- trimethoxyflavone (KP.8.10)	Flavonoid component in *K. parviflora* Wall. Ex Baker extract (KP.8.10): potent activity against HuCCA1 cells (IC_{50} = 46.1 µg/mL) and RMCCA-1 cells (IC_{50} = 62 µg/mL)
[20]	*Mylabris phalerata* (Pallas) or *Mylabris cichorii* (Laeus)/Cantharidin, Norcantharidin	Cantharidin: most sensitive (IC_{50}: RBE cells = 2 µM, QBC939 cells = 3 µM, HCCC9810 cells = 3 µM)
[55]	Phenformin and Quercetin and Myricetin (from several plant species)	Quercetin: enhancement of activity of phenformin against KKU-256 cells (IC_{50} = 1363 µM)
[56]	*Phomopsis archeri* B. Sutton (*fungus*)/phomoarcherins A–C (sesquiterpenes), kampanol A, R-mevalonolactone, ergosterol, ergosterol peroxide	Compounds **1**–**4**: IC_{50} = 0.1–19.6 µg/mL (KKU-100, KKU-M139, KKU-M156, KKU-M213, and KKU-M214 cells)
[57]	*Pinellia ternata* (Thunb.) Makino (Araceae/Banxia: polysaccharide (PTPA)	Sk-ChA-1 cells: most sensitive (IC_{50}: SNU-245, CL-6, Sk-ChA-1, and MZ-ChA-1 cells = 194, 76.9, 57.2, and 29.2 mg/mL, respectively)
[28]	*Piper longum* L. (Piperaceae)/Piperlongumine	IC_{50} for KKU-055, KKU-213, KKU-214, KKU-139, KKU-100, MMNK1, and NIH3T3 cells = 4.2, 5.2, 6.2, 8.8, 15.9, 5.7 and, 12.7 µM, respectively
[29]	*Piper longum* L. (Piperaceae)/Piperlongumine	IC_{50} for HuCCT-1–1 cells = 24.8 and 4.2 µM at 24 and 48 h, respectively
[7]	*Piper nigrum* L. (Piperaceae)/Piperine, Piperine-free *Piper nigrum* (black pepper) dichloroqmethane extract (PFPE)	PFPE: most potent and selective, especially on KKU-M213 cells (IC_{50} = 13.70 µg/mL) and TFK-1 cells (IC_{50} = 15.30 µg/mL)
[58]	*Pistacia atlantica* Desf. (Anacardiaceae)/Mastic gum resin	Activity against KMBC cells: IC_{50} = 15.34 µg/mL
[32]	*Plumbago indica* L. (Plumbaginaceae)/Plumbagin	IC_{50} for CL6 cells = 24.00 µM, SI = 2.28 (low)

continued

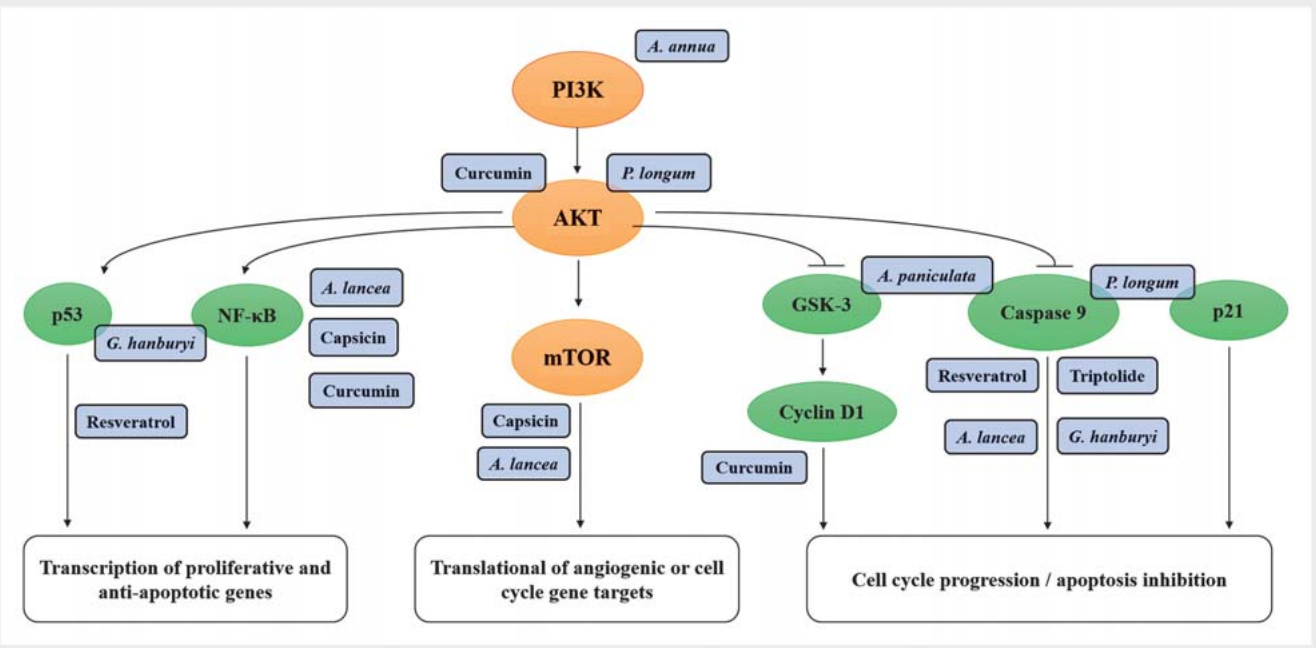
▶ **Table 1** *Continued*

References	Plants/Active compounds (Family)	Antiproliferative activity
[30]	*Reseda luteola* L. (Resedaceae)/Luteolin	Potent activity against KKU-M156 cells (IC_{50} = 10.5 and 8.7 µM at 24 and 48 h, respectively)
[16]	*Rhinacanthus nasutus* (L.) Kurz (Acanthaceae)/Rhinacanthin-C	Potent activity against KKU-M256 cells (IC_{50} = 1.50 µM)
[59]	*Tanacetum parthenium* L. (Compositae)/Parthenolide	IC_{50} for SCK cells = 10 µM
[23]	*Tiliacora triandra* (Colebr.) Diels (Menispermaceae)/Tiliacorinine	Significant activity against KKU-M055, KKU-100, KKU-M213, and KKU-M214 cells (IC_{50} = 4.5–7 µM)
[13]	*Trichosanthes cucumerina* L. (Cucurbitaceae)/Cucurbitacin B (CuB) (natural tetracyclic triterpene)	Potent activity against KKU-213: IC_{50} = 0.048 µM (24 h), 0.036 µM (48 h), and 0.032 µM (72 h) µM; KKU-214: IC_{50} = 0.088 µM (24 h), 0.053 µM (48 h), and 0.04 µM (72 h)
[14]	*Tripterygium wilfordii* Hook. f. (Celastraceae)/Triptolide	Potent activity against HaLCCA-1.1, HaLcca-2, HaTCCA-1.1 cells (IC_{50} = 0.05 mg/mL for all cells)
[15]	*Tripterygium wilfordii* Hook. f. (Celastraceae)/Triptolide	IC_{50} for HuCCT1, QBC939, and FRH0201 cells = 12.6, 20.5, 18.5 nM at 48 h, respectively
[11]	*Zingiber officinale* Roscoe (ginger) (Zingiberaceae) ethanol extract	Promising activity against CL6 cells (IC_{50} for each assay = 10.95, 53.15 µg/mL, SI = 18.09, 3.19)

SI = selectivity index

▶ **Table 2** Clinical studies of potential herbs and herbal formulations for CCA.

Ref	Plants/Active compounds	Methodology	Key findings
[124]	*Atractylodes lancea* (Thunb.) DC. (Compositae) ethanol standardized extract (CMC capsule formulation)	Clinical study: Phase I study, 48 healthy participants. Thais: *Group 1*: single oral dose of 1000 mg of *A. lancea* or placebo (20 : 4 participants). *Group 2*: daily oral doses of 1000 mg *A. lancea* or placebo daily for 21 days (20 : 4 participants). Clinical parameters: assessment of safety and tolerability. Pharmacokinetics: model-dependent and model-independent analysis.	Well tolerated in both groups. Atractylodin: rapidly absorbed but with low systemic exposure and residence time. No difference in the pharmacokinetics following a single or multiple dosing, suggesting the absence of accumulation and dose dependency in human plasma after continuous dosing for 21 days.
[137]	*Atractylodes lancea* (Thunb.) DC. (Compositae) ethanol standardized extract (CMC formulation)/*β*-eudesmol and atractylodin	Antiproliferation of PBMCs against CCA (CL6) (flow cytometry-based NY cytotoxic assay). Clinical study: Phase I study, 48 healthy participants. Thais receiving a single (1000 mg) or multiple oral dosing (1000 mg for 21 days) or placebo. Immunomodulation: cytokine levels (cytokine bead assay) and expression (RT-PCR); lymphocyte subpopulations (flow cytometry).	Immunomodulatory activity of *A. lancea* (Thunb.) DC. and compounds in complement with the direct action on apoptosis induction. Atractylodin: significant inhibition of IL6, TNF-*α*; *A. lancea* at a single dose: suppression of IFN*γ* and IL10, increase of B cells, increase of NK, CD4+, CD8+ cells, and a trend of increased antiproliferation activity of PBMCs at 24 h. *A. lancea* (Thunb.) DC. at multiple dosing: suppression of all cytokine production, increase of CD4+ and CD8+, increase of antiproliferative activity of PBMCs at 24 h (terminated at 48 h of dosing).
[155]	PHY906 formulation	Clinical study: open-label phase I trial (800 mg BID on days 1–4 + escalating doses of capecitabine (1000, 1250, 1500, 1750 mg/m^2), orally twice daily on days 1–7 of a 14-day cycle (7/7 schedule) in CCA (n = 1), pancreatic cancer (n = 15), colon cancer (n = 6), esophageal cancer (n = 1), unknown primary cancer (n = 1).	Well-tolerated at MTD of 1500 mg/m^2 BID administered in a 7/7 schedule, in combination with PHY906 800 mg BID on days 1–4; partial response (n = 1), stable disease > 6 weeks (n = 13).



▶ **Fig. 2** Proposed molecular targets and signaling pathways of potential herbs and isolated compounds/synthetic analogs on human CCA.

thetic analogs. The plants that were investigated the most were *A. lancea* (Thunb.) DC. and *C. longa* L. Other plants with more than three research articles published on antiproliferative activities included *G. hanburyi* Hook.f., *A. annua* L., *Z. officinale* Roscoe, and *A. paniculata* (Burm.f.) Nees. The previously reported studies of various potential herbs (extracts or isolated compounds/synthelic analogs) for CCA focused on their antiproliferative activities against CCA cell lines or antitumor activities in animal models, activities on cell invasion and migration, and underlying mechanisms or targets of their actions [5–155]. None of these herbs/isolated compounds/synthetic analogs, except *A. lancea* (Thunb.) DC., has undergone the full process of nonclinical, clinical, and pharmaceutical development to deliver final products for clinical use. The IC_{50} (concentration that inhibits cell growth by 50 %) values indicating the potency of activities were not reported for most herbs/isolated compounds/synthetic analogs/herbal formulations investigated. The potency of activity of the antiproliferative activity against human CCA cells was classified according to the IC_{50} as (i) weak activity (IC_{50} > 100 µg/mL for the herbal extract and > 100 µM for the isolated compounds/synthetic analogs), (ii) moderate activity (IC_{50} 10–100 µg/mL for the herbal extract and 10–100 µM for the isolated compounds/synthetic analogs), and (iii) relatively potent (IC_{50} < 10 µg/mL for the herbal extract and < 10 µM for the isolated compounds/synthetic analogs). Based on available published data, the antiproliferative activities of the extracts of *A. lancea* (Thunb.) DC., *G. hanburyi* Hook.f., and *Piper nigrum* L. (Piperaceae) are classified as potent [5–7], while those of *Dioscorea membranacea* Pierre ex Prain & Burkill (Dioscoreaceae), *Kaempferia galanga* L. (Zingiberaceae), *Mesua ferrea* L. (Calophyllaceae), *Piper chaba* Hunt. (Piperaceae), *Z. officinale* Roscoe, and Pra-Sa-Prao-Yhai formulation are classified as moderate [5, 8–11], and that of sho-saiko-to is classified as weak activity [12]. For the isolated compounds/synthetic analogs, those with the most potent activity are cucurbitacin B and triptolide [from *T. wilfordii* Hook. f.: IC_{50} < 1 µM] [13–15], followed by rhinacanthin C [from *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae): IC_{50} = 1.5 µM] [16], compounds from *D. membranacea* Pierre ex Prain & Burkill: IC_{50} = 1–2 µM [8], andrographolide and analogs [from *A. paniculata* (Burm.f.) Nees: IC_{50} = 3 µM] [17–19], cantharidin and norcantharidin [from *Mylabris phalerata* (Pallas): IC_{50} = 2–3 µM] [20], isolated/synthetic compounds from *Atalantia monophylla* DC. (Rutaceae): IC_{50} = 3–5 µM [21, 22], tiliacorinine [from *Tiliacora triandra* (Colebr.) Diels (Menispermaceae): IC_{50} = 4–7 µM] [23]. Curcumin and analogs (Zingiberaceae): IC_{50} 3–17 µM [24–27], piperlongumine [from *P. longum* L.: IC_{50} = 4–15 µM] [28, 29], luteolin [from *Reseda luteola* L. (Resedaceae): IC_{50} = 10 µM] [30], candidione [from *Derris indica* (Lamk.) Benn.: IC_{50} = 12–17 µM] [31], and plumbagin [from *Plumbago indica* L. (Plumbaginaceae): IC_{50} = 24 µM] [32] showed moderated to potent activities (▶ **Table 1**). Possible molecular mechanisms of these herbs and/or isolated compounds/synthetic analogs on CCA cells involve induction of apoptosis, autophagy, and cell cycle arrest (at G_0/G_1, G_1, G_1/S, or G_2/M phases) through suppression of proinflammatory cytokines and growth factors (IL6, EGF, VEGF, etc.) [10, 13, 23, 27–30, 32, 34, 36, 41, 43, 46, 51–53, 57–85], suppression of expression of cell surface receptors (*Vegfr2*, EGFR peroxisome proliferator-activated receptor gamma, DR4 and DR5, and TRAIL) [69, 86–88], and deregulation of intracellular pathways (JAK/STAT3, RAS/MAPK, PI3K/AKT, GSKβ/β-catenin, NFκB/AMPK, ERK, p38/MAPK, HO1, ROS/JNK, EGFR, VEGF, COX2, FAK, MMP2, MMP9, ICAM1, caspase-3, -8, and − 9, TR1, MDR1, MRP1, 2, and 3, TRAF1, XIAP, p21, p53, p65, and CHOP dependent) (▶ **Fig. 2**) [7, 11, 16, 19, 25, 26, 30, 31, 37, 38, 42, 45, 49, 54, 66, 70, 78, 79, 89–120].

The most advanced development of a potential herb as a chemotherapeutic agent for CCA is *A. lancea* (Thunb.) DC. A series of studies on the research and development of *A. lancea* (Thunb.) DC. was systematically conducted by our research group [121]. *A. lancea* (Thunb.) DC. is a medicinal plant growing in tropical and subtropical zones of East Asia such as China and Japan. Its dried rhizome is commonly used in Chinese ("Cang Zhu"), Japanese campo ("So-jutsu"), and Thai ("Khod-Kha-Mao") traditional medicines for fever, colds, flu, sore throat, rheumatic diseases, digestive disorders, night blindness, influenza, rheumatic diseases, digestive disorders, night blindness, and cancers. Modern pharmacological studies also support the broad pharmacological effects of *A. lancea* (Thunb.) DC. in various diseases [122]. Phytochemical investigations reveal a series of sesquiterpenoids, monoterpenes, polyacetylenes, phenolic acids, and steroids from *A. lancea* (Thunb.) DC. rhizomes [123]. The major constituents are AT (14%), BE (6%), atractylon (2%), and HS (1%). The potential of *A. lancea* (Thunb.) DC. and the two major compounds AT and BE for treatment and control of CCA has extensively been evaluated both *in vitro* (human CCA cell lines) and *in vivo* (xenograft mouse model and OV/DMN-induced CCA hamster model) [121, 124, 125]. Results confirm anti-CCA potential and safety profiles of both the crude *A. lancea* (Thunb.) DC. extract, as well as AT and BE and the finished product [capsule pharmaceutical formulation of the standardized *A. lancea* (Thunb.) DC. extract] [126, 127]. *A. lancea* (Thunb.) DC. and both compounds exhibit potent and selective antiproliferative activities against CCA cells. The IC_{50} values range from 20 to 30 µg/mL, with a selectivity index of 3–5 [128, 129]. The potencies of activity of *A. lancea* (Thunb.) DC. and both compounds on CCA cell growth is about 3- to 4-fold of the standard drug 5-FU. Furthermore, *A. lancea* (Thunb.) DC. extract, AT, and BE inhibit CCA cell invasion and migration and formation of new blood vessels [86, 128, 130–132], suggesting a potential role as an antimetastasis and antiangiogenesis agent for CCA. The potential anticancer and antiangiogenesis properties of *A. lancea* (Thunb.) DC. extract and its major constituents have been demonstrated in various types of cancer, e.g., murine blastoma cells HeLa (human cervical cells), SGC-7901 (human gastric cancer cells), BEL-7402 (human liver cancer cells), H33, S180, HL-60, leukemic cells, and gastric cancer [131–135]. The underlying mechanisms of the antiproliferative effects of *A. lancea* (Thunb.) DC., AT, and BE against CCA cells mainly involve the induction of cell cycle arrest (at G_1 phase) and apoptosis through activation or suppression of molecular targets/signaling pathways involved in CCA pathogenesis. These include the activation of caspase-3/7 and suppression of HO1 production, activation of STAT1/2 and JAK/STAT signaling cascades, suppression of NFκB, and suppression of cytoprotective enzymes and key growth regulatory transcription factors [38, 41, 42, 62, 98–100]. The first-in-human starting dose was estimated from the MRSD (maximum recommended starting dose) from toxicology testing in animals [136], which was 2400 mg for a person weighing 60 kg. Despite the concern of bleeding (antiplatelet aggregation) and adverse effect on the nervous system previously reported *in vitro* and in animals [123], results of phase I clinical trials using 1 g *A. lancea* (Thunb.) DC. (about 50% of the estimated maximum dose in humans) confirmed the safety profile in healthy Thai subjects [124]. The pharmacokinetics of AT was investigated in healthy Thai subjects following a single (1 g) or daily (1 g for 21 days) administration of the capsule formulation of the standardized *A. lancea* (Thunb.) DC. extract [124]. AT was rapidly absorbed but with low systemic bioavailability and a short residence time (within 8 h). The immunostimulatory activity of the standardized *A. lancea* (Thunb.) DC. extract was linked with suppression of the production of TNF-α and IL6 cytokines, which are involved in the pathogenesis and severity of CCA [137]. A phase II dose-finding study is underway to confirm efficacy, tolerability, and immunomodulatory activity of *A. lancea* (Thunb.) DC. in patients with advanced-stage CCA. It is noted for the toxic effect of AT and BE on zebrafish embryo development [86]. Although the results may imply similar toxicity in humans, considering the much more sensitivity of the zebrafish model compared with mammalian cells and rodent models, high intensity of the effect would not be expected in humans. Further studies are needed to confirm this finding.

Apart from *A. lancea* (Thunb.) DC., *C. longa* L., *G. hanburyi* Hook.f., *A. annua* L., *Z. officinale* Roscoe, and *A. paniculata* (Burm. f.) Nees are among the herbs that have been of research interest for anti-CCA development. Curcumin is a major component of *C. longa* or turmeric. It is a dietary constituent with tumor-suppressing potential by inhibiting multiple molecular targets/signaling pathways involved in carcinogenesis, including CCA. Curcumin and synthetic analogs exhibit potent antiprolifertive activities against human CCA cells with IC_{50} values of 3–17 µM [24, 27]. However, clinical uses of curcumin in CCA and other types of cancer may be limited due to its low systemic bioavailability [138]. It inhibits cell migration and induces cell cycle arrest at the G_2/M phase [66]. The action of curcumin in CCA involves multiple molecular targets/signaling pathways, including transcription factors (NFκB, STAT3, and AP1.16), peroxisome proliferator-activated receptor, AKT activation pathway, B-cell lymphoma 2, B-cell lymphoma-extra large, cell survival proteins (cIAP1, cIAP2, and surviving 15), and Notch1 signaling [25, 26, 66, 83, 106, 107, 109, 110].

The anticancer potentials of *G. hanburyi* Hook.f. extract and isolated compounds/synthetic analogs have been well demonstrated in various types of cancer [139]. *G. hanburyi* Hook.f. and its isolated caged xanthones (gambogic acid, forbesione, isomorellin, and isomorellinol, etc.) from the resin and fruits have been used widely in Thai traditional medicine [51]. Gambogic acid was shown to have a favorable safety profile in a phase II a trial in patients with advanced malignant tumors, i.e., lung, gastrointestinal, liver, breast, and renal adenocarcinoma [140]. Neverthess, no clinical study was conducted in patients with advanced-stage CCA. The antiproliferative activity of both the extract (IC_{50} = 2–3 µg/mL) and isolated compounds/synthetic analogs (IC_{50} = 0.03–3 µM) is considered potent [6, 51]. The extract and caged xanthones induce apoptosis via the mitochondrial pathway [51] and induction of G_0/G_1-phase cell cycle arrest through p53 and NFκB signaling pathways [52]. Combinations of isomorellin or forbesione with doxorubicin exhibited a significant synergistic effect on CCA cells through suppression of MRP1, activation of NFκB, enhancement of Bcl2-like protein 4 (Bax)/Bcl2, activation of caspase-9 and caspase-3, and suppression of the expression of survivin, procaspase-9, and procaspase-3 [112]. The combination of forbesione with 5-FU strongly suppressed the expression of

Bcl2 and procaspase-3 while enhancing the expression of p53, Bax, Apaf-1, caspase-9 and caspase-3 compared with single-drug treatment [111]. The safety profile of gambogic acid in humans together with its potent antiproliferative activity against CCA make this compound a strong candidate for further development as a CCA chemotherapeutic agent. In addition, gambonic acid is available in the parenteral formulation, which is suitable for CCA patients.

The sesquiterpene lactones artemisinin and derivatives (artemether, artesunate, arteether, and dihydroartemisinin) derived from *A. annua* L. constitute a unique class of antimalarial drugs with significant potential for drug repurposing for a wide range of diseases, including cancer [141]. The antiproliferative activities of artemisinins against CCA cells are relatively weak (IC_{50} = 75–377 µM) [37]. The mechanisms of their action against CCA have been reported to involve multiple critical biological targets/signaling pathways of CCA pathogenesis, i.e., DAPK1, BECLIN1, Bcl2, PI3KC3, and MCL-1 [61, 96, 97]. The anti-CCA activities have been shown to be through induction of both apoptosis and autophagy-dependent caspase-independent cell death and cell cycle arrest at phases S, G_0/G_1, and G_2/M.

Z. officinale Roscoe, or ginger, is a popular spice used globally, especially in most Asian countries. It has been used as a pain relief for arthritis, muscle soreness, chest pain, low back pain, stomach pain, and menstrual pain. The rhizomes contain over 400 different compounds. The phenolic compounds gingerol and shogaol are found in higher quantities than others. Evidence from *in vitro*, animal, and epidemiological studies suggest that ginger and its active constituents suppress the growth and induce apoptosis of a variety of cancer types, including skin, ovarian, colon, breast, cervical, oral, renal, prostate, gastric, pancreatic, liver, and brain cancer. The active ingredients of ginger, mainly, 6-gingerol and 6-shogaol, target several cellular molecules that contribute to tumorigenesis, cell survival, cell proliferation, invasion, and angiogenesis (NFκB, STAT3, Rb, MAPK, PI3k/Akt Ca^{2+} signals, Akt, ERK, cIAP1, cyclin A, cyclin D1, Cdk, cathepsin D, caspase-3/7, survivin, cIAP1, XIAP, Bcl2, MMP9, ER stress, and eIF2α) [142]. *In vitro* studies showed that ginger has promising antiprolifertive and antioxidant activities against human CCA cells by inducing programmed cell death [11, 84]. The ethanolic extract of ginger exhibits significant tumor growth inhibition, prolongs survival time, and increases survival rate in CCA-xenografted mice and OV/DMN-induced CCA in hamsters. In the xenograft model, the crude extract of ginger produced significant anti-CCA activity compared with cisplatin and the untreated control. The extract at medium (1 g/kg body weight) and high (2 g/kg body weight) dose levels (oral daily dose for 30 days) significantly inhibited tumor growth to about 55.6 and 51.1 % of the untreated control, respectively, while cisplatin inhibited tumor growth to 60 % of the control [84]. Interestingly, significant reduction of lung metastasis was observed in the xenografted mice treated with the crude extract of ginger and cisplatin compared with the untreated control. In OV/DMN-induced CCA hamsters, promising anti-CCA activity of the crude extract of ginger was observed at all dose levels, particularly at the highest oral dose level of 5 g/kg body weight for 30 days [143]. The median survival rate and survival time were significantly prolonged (about two times) in hamsters treated with the extract at all dose levels compared with 5-FU-treated and untreated control groups during the 4–6 months observation period. At week 36, all hamsters except those treated with the highest ginger dose died (1 hamster died, 80 % survival rate). The untreated control animals started to die as early as 14 weeks.

A. paniculata (Burm.f.) Nees is an important herbal medicine widely used in several Asian countries, including China, India, and Thailand, for the treatment of respiratory infection, inflammation, immunostimulation, hepatoprotective, cardioprotective, cold, fever, bacterial dysentery, diarrhea, and hypoglycemic and anticancer activities [144–149]. Recently, the Ministry of Public Health of Thailand has approved *A. paniculata* (Burm.f.) Nees for the treatment of COVID-19 [150]. *A. paniculata* (Burm.f.) Nees and its active compound andrographolide have been shown to inhibit cancer cell migration and invasion, including CCA. Due to their low potencies of activity and requirement of a large dose [151], a number of andrographolide analogs, particularly C19 triphenylmethyl ether substitution (AG050) and its nanoencapsulated formulation, have recently been developed with improved activities against CCA (IC_{50} = 3 µM) [17, 18]. These analogs and nanoformulation exhibit potent activity against CCA cells. The inhibitory effect on CCA cell proliferation is through induction of apoptosis and cell cycle arrest at the G_o/G_1 and G_2/M phases through downregulation of cyclin D1, Bcl2, and caspase-3, while the upregulation of proapoptotic protein Bax and cleavage of poly (ADP-ribose) polymerase occurs [60]. Andrographolide was also shown to inhibit CCA cell invasion and migration via suppression of claudin 1 through the activation of p38 MAPK signaling [19]. The long history of use and relatively safe profile [152] together with evidence of the potency of antiproliferative activity against human CCA cells make *A. paniculata* (Burm.f.) Nees extract or andrographolide a candidate as a repurposed drug for CCA.

Resveratrol and capsaicin are among other reported compounds derived from several plant species that have been investigated for anti-CCA activities [73, 75–77]. Resveratrol is a polyphenol found naturally in red wine, grapes, mulberries, cranberries, and peanuts. The compound exhibits cancer chemopreventive activity through inhibition of tumor initiation, promotion, and progression. In CCA cell lines, resveratrol was shown to interfere with cell cycle progression, resulting in arresting different phases of the cell cycle (G_o/G_1, S, and G_2 phases) to induce apoptosis via the mitochondrial-dependent pathway (caspase-dependent and -independent) [75], to stimulate autophagy, and to suppress IL6 by CAFs secretory product [76]. It also produces the chemosensitizing effect of 5-FU on CCA growth inhibition [73]. Capsaicin, found in hot red chilli peppers [*Capsicum* spp. (Solanaceae)], possesses several pharmacological activities, i.e., analgesic, anti-inflammation, and antiproliferative effects, on different gastrointestinal cancer cells [154]. The anti-CCA activity of capsaicin was shown to be associated with the induction of apoptosis and attenuation of the GLI1 and GLI2 targets of the Hedgehog signaling pathway (role in carcinogenesis) [101–102]. The use of capsaicin as a food supplement to inhibit Hedgehog signaling might therefore be of additional therapeutic benefit in patients with CCA. In the xenograft mouse model, a combination of capsaicin with 5-FU was synergistic and significantly suppressed tumor growth compared with 5-FU alone. Further investigation revealed that

the autophagy induced by 5-FU was inhibited by capsaicin. The mechanism of action was shown to be through the inhibition of 5-FU-induced autophagy by activating the PI3K/AKT/mTOR signaling pathway [103].

Herbs constitute a promising source of medicine for CCA control. The anti-CCA potential of several herbs and isolated compound/synthetic analogs have been demonstrated in different experimental models in conjunction with their underlying mechanisms of action at the molecular and cellular levels. As herbal medicines usually contain several pharmacologically active compounds, their multi-ingredient characteristics may make the evaluation of clinically useful products more complex than synthetic drugs. With regard to the therapeutic aspect, however, using the whole herbal extract would be expected to provide more therapeutic benefit compared to synthetic drugs concerning efficacy (synergistic action) and tolerability (buffering effect). The limitation of the current study includes only articles published in English were included in the analysis and the number of the reported articles may therefore be underestimated. Comparison of the potencies of antiproliferative activities of the investigated plants/isolated or synthetic compounds/herbal formulations was made based on only available data on the IC_{50} values, which were not reported in some studies. Some reported the antiproliferative activity potencies as the percentage of inhibitory effects on cell growth at specified concentrations. In addition, different CCA cell lines and assay methods for assessment of antiproliferative activities were used in different studies.

In conclusion, a number of plants, isolated compounds, synthetic analogs, and herbal formulations have been demonstrated for their potential to control CCA. However, only *A. lancea* (Thunb.) DC. was fully developed based on the reverse pharmacology approach. Future research should be geared toward the full development of the candidate herbs until delivery of final products that are safe and effective for CCA control. Other targets of their action should be further investigated. Research targeting inflammatory, proliferative, and angiogenesis processes, development, and progression has been an extensive area. Blocking the generation of an inflammatory infiltrate by interfering with critical molecules of the adhesion process is an attractive strategy to control CCA.

Materials and Methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [156].

Database and search strategy

The literature search was conducted from three databases, i.e., PubMed, ScienceDirect, and Scopus in March-June 2021. The search terms applied were “Cholangiocarcinoma” AND “Herbs” AND/OR “Herbal medicine” AND/OR “Traditional medicine” AND/OR “Plants”. All articles were retrieved and downloaded to the EndNote X9 database (Thomson Reuters Company) for further analysis.

Study selection

Study selection was performed independently by two reviewers. The studies were initially screened by titles and abstracts to exclude irrelevant articles and duplication. Full-text articles included after the screening were further evaluated by applying the predefined eligibility criteria. Studies were eligible if they met the following criteria: (i) published up to May 2021; (ii) available as full text in English; and (iii) with *in vitro*/*in vivo*/clinical studies related to the investigation of the anti-CCA activity of herbal or traditional medicine. The articles were excluded if: (i) there was unclear methodology or insufficient information or (ii) if they were review articles, letters to the editor, editorials, a systematic analysis, or a meta-analysis.

Data extraction

Two reviewers extracted data independently and resolved the disparity by discussion and suggestion from the third reviewer. The following information was extracted: first author’s name and year of publication, name of herbs/herbal extract/herbal medicine or isolated/synthetic analog(s), type of study (*in vitro*/*in vivo*/clinical), objective(s) of the study [investigation of antiproliferative activity alone or with antimetastasis or antiangiogenesis or antioxidative, anti-CCA activity, and mechanism/target(s) of action], and key findings.

Supporting information

Mechanisms of antiproliferative activities in animals are available in the Supporting Information.

Contributors’ Statement

Data collection and analysis: K. Na-Bangchang, T. Plengsuriyakarn; design of the study: K. Na-Bangchang, J. Karbwang; drafting the manuscript: K. Na-Bangchang; critical revision of the manuscript: K. Na-Bangchang, T. Plengsuriyakarn, J. Karbwang.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

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

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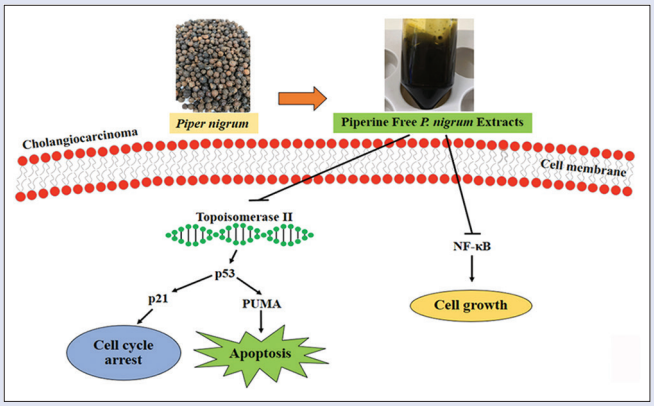
ABSTRACT

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

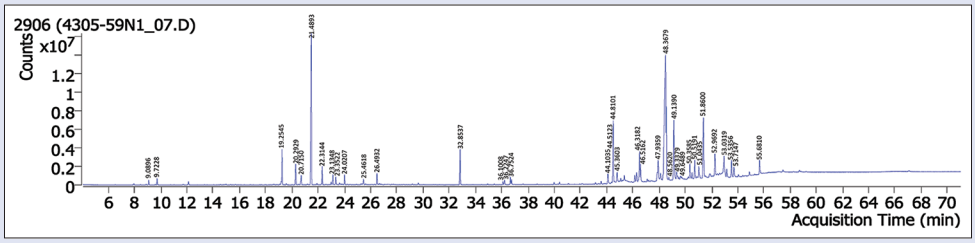

Figure 1: Gas chromatograph-mass spectrometer chromatogram of piperine free *Piper nigrum* extract

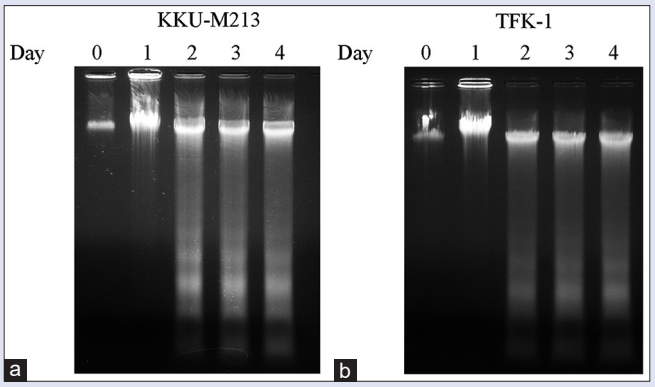

Figure 2: Analysis of Deoxyribonucleic acid fragmentation induced by piperine free *Piper nigrum* extract in KKU-M213 and TFK-1 cell lines. Cells were treated with piperine free *Piper nigrum* extract for 4 days and Deoxyribonucleic acid fragmentation was assessed by 1.5% agarose gel electrophoresis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

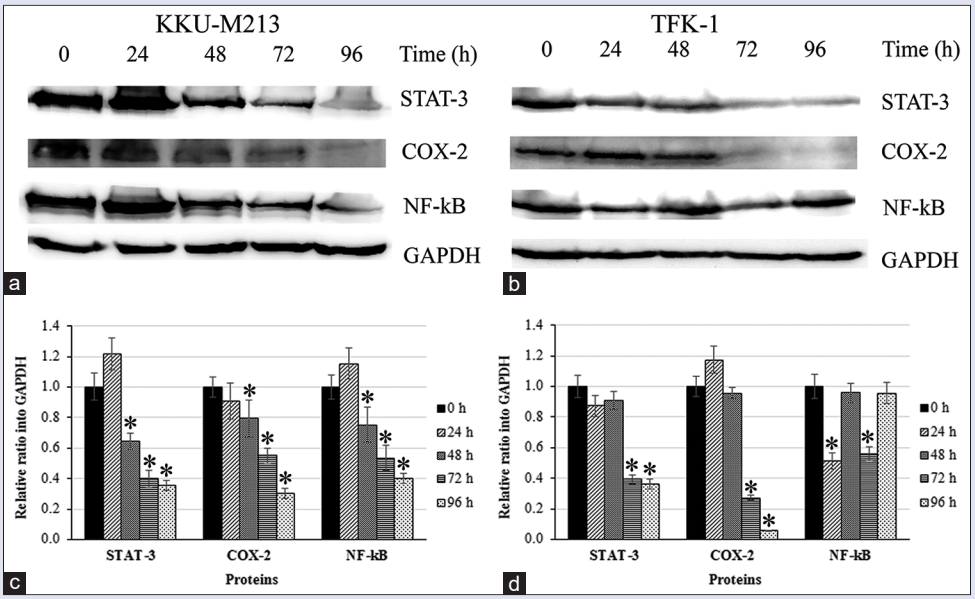

Figure 3: Expression of inflammation-related proteins in KKU-M213 (a and c) and TFK-1 (b and d) cells treated with piperine free *Piper nigrum* extract at 24, 48, 72 and 96 h. The levels of signal transducer and activator of transcription 3, cyclooxygenase-2 and Nuclear factor kappa-light-chain-enhancer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

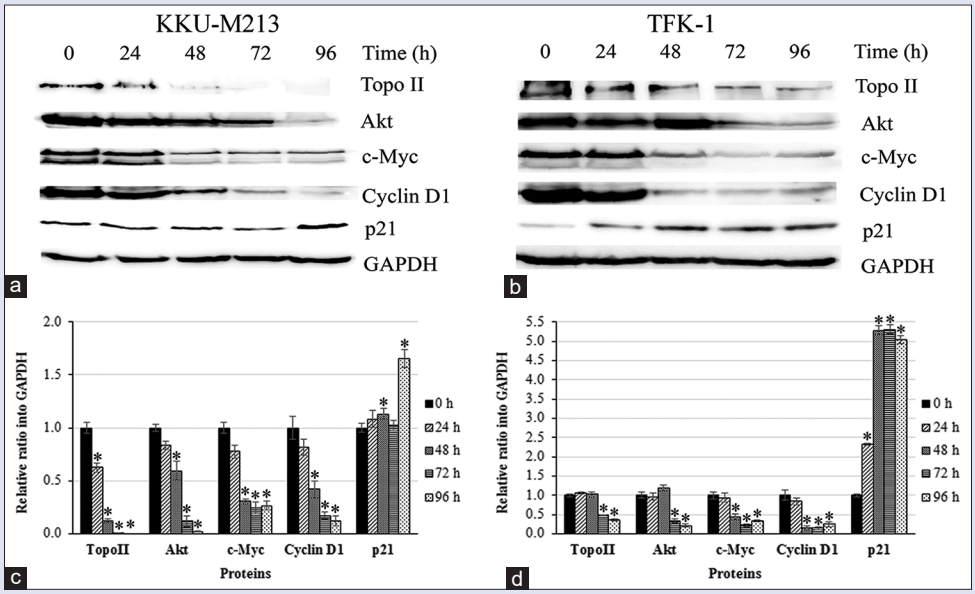

Figure 4: Effect of piperine free *Piper nigrum* extract on cell growth and cell cycle arrest. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of topoisomerase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

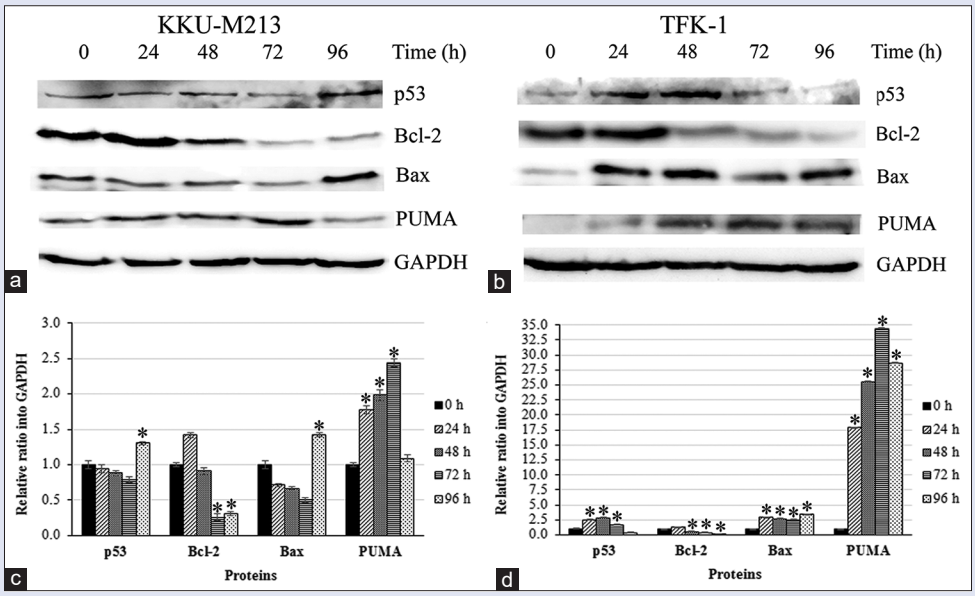

Figure 5: Effect of piperine free *Piper nigrum* extract on apoptosis. KKU-M213 (a and c) and TFK-1 (b and d) cells were treated with Median inhibition concentration concentration of piperine free *Piper nigrum* extract for 24, 48, 72 and 96 h. Then, the levels of tumor protein p53, B-cell lymphoma 2,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

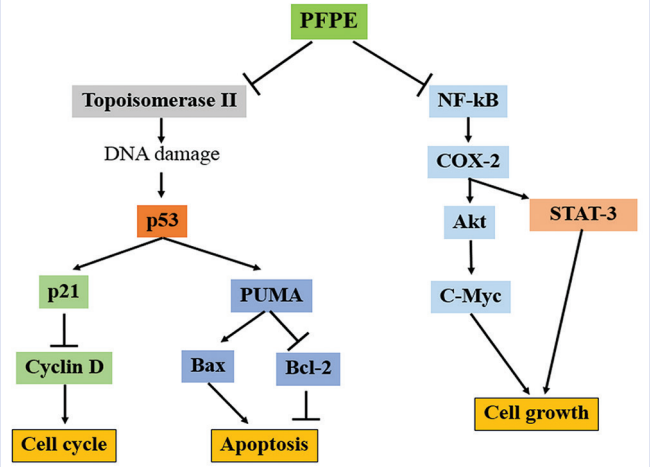

Figure 6: The anticancer mechanism of piperine free *Piper nigrum* extract in cholangiocarcinoma

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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