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Original research article

Anticancer activity of the bark extract of *Phyllanthus emblica* on cholangiocarcinoma *in vitro*

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ABSTRACT

Cholangiocarcinoma (CCA) is the most common biliary epithelial malignancy in Northeast Thailand. The critical obstacle of CCA therapy is cancer chemotherapy resistance with intolerance of adverse drug reactions. A new approach has been the investigation of alternative herbal medicines for cancer therapy. The fruit of *Phyllanthus emblica* (PE) has several pharmacological properties, including potent anticancer properties. The present study aimed to evaluate the antiproliferative activity of the bark extract of *Phyllanthus emblica* in methanol on the KKU-452 CCA cell line. The cytotoxicity of Thai herbal plants, *Solanum torvum* (fruit), *Nephelium hypoleucum* (fruit), *Schleichera oleosa* (fruit), *Antidesma thwaitesianum* (fruit), *Tamarindus indica* (fruit, leaf, bark) and *Phyllanthus emblica* (fruit, leaf, bark), were screened against KKU-452. Cell proliferation, apoptosis and migration were evaluated using MTT-, Annexin V apoptosis- and wound healing assays, respectively. Reactive oxygen species (ROS) was determined by DCFH-DA fluorogenic dye staining. Bark of PE extracts was subjected to HPLC analysis. Results showed that the bark extract of *P. emblica* only had a potent cytotoxic effect on KKU-452 (IC_{50} of 52.2 µg/ml) and significant induction of apoptosis. Other plant extracts showed low potency of cytotoxic effects. Cell migration was significantly inhibited by *P. emblica* at 25 and 50 µg/ml by 42.8 and 32.9%, respectively. Moreover, the extract at 50 µg/ml induced oxidative stress *via* ROS production at 31% when compared with non-treated cells. Phenolic acids and flavonoids are the important

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phytochemical components in the bark extract of *P. emblica*. The cytotoxic effect of the bark extract of *P. emblica* was related to free radical generation.

Keywords: cholangiocarcinoma, anticancer, antiproliferation, bark *of Phyllanthus emblic*

1. Introduction

Cholangiocarcinoma (CCA) is an invasive epithelial bile duct cancer and is divided into two types based on the anatomical area, including intrahepatic- and extrahepatic-CCA. CCA is mostly found in Southeast Asia countries, particularly the Mekong sub-region and Northeast Thailand.[1] The incidence rate of CCA has been reported as 135.4 *per* 100,000 among men and 43.0 *per* 100,000 among women in Khon Kaen Province, Northeast of Thailand.[2] The incidence of CCA has been decreasing, but the survival time remains poor.[3] The primary risk factor of CCA is associated with *Opisthorchis viverrini* infection endemic in areas that cause chronic inflammation of bile ducts and induced malignancies.[4] The standard first-line regimens for CCA patients with unresectable tumors consists of gemcitabine and cisplatin alone or in combination.[5] Complications of CCA treatment are high rate of cancer recurrence, metastasis and systemic chemoresistance with a 5-year overall survival (OS) of only 10%.[6] Systemic chemotherapies for CCA are the only current approach. Dietary phytochemicals are therefore an attractive alternative approach in the treatment of CCA. Several phytochemicals exert an anticancer effect by inducing apoptotic, cell cycle arrest, alternated antioxidant redox cycling, modulating NF-κB and STAT3 signaling pathways, inhibition of cyclin-dependent kinase-like 3 (CDKL3) and PI3K/AKT pathway in CCA. These phytocehmicals include luteolin,[7,8] *Cratoxylum formosum* extracts,[9] curcumol,[10] kaempferol,[11] and *Scoparia dulcis* L. extracts.[12]

This study reports the cytotoxicity of six different Thai herbal plants in a CCA cell line. *Solanum torvum* and *Antidesma thwaitesianum* have been shown to exhibit antiproliferative activity.[13,14] *Nephelium hypoleucum* and *Schleichera oleosa* are tropical fruits in the family of *Sapindaceae*.[15] This family contains important phytochemicals such as flavonoids, terpenoids, botulin, and betulinic acid, with potent antioxidant and anticancer activities.[16,17] *Tamarindus indica* and *P. emblica* exert an antitumor effect and immunomodulatory activity.[18,19] Several studies have revealed that phytochemicals exert their anticancer activity through suppressing multiple signaling pathways, inducing apoptosis and generating reactive oxygen/nitrogen species.[19-21] Preliminary studies demonstrated that the bark extract of *Phyllanthus emblica* has potent cytotoxicity and other anti-cancer activities should be investigated.

P. emblica (PE: Thai-Makham Pom) is in the *Phyllanthaceae* family, which is a traditional Thai fruit wildly spreads in the tropical zone of Southeast Asia. The fruits of PE have been used for constipation, fever, inflammation, cancer, and for hepatic disorders.[22-25] The pharmacological effects include antioxidant,[26] gastroprotective,[27] antitussive,[28] hepatoprotective,[29] and antidiabetic activities.[30] The PE extracts from fruit pulp and seeds have been evaluated for cancer prevention and anticancer effects both *in vitro* and *in vivo*. The anticancer activity of the PE extracts involves cell apoptosis stimulation, modulation of inflammatory enzymes, alteration of cell cycle proteins, suppression of survival signal pathways and metastasis.[22,31-33] Its possible mechanisms of action for CCAs involve stimulation of the expression of caspase-3, poly (ADP-ribose) polymerase, checkpoint kinase 2, tumor protein 53, mitogen-activated protein kinase (MAPK) pathways (including ERK1/2 and p38 MAPK), and protein kinase B (Akt).[25] However, study on the PE extracts from the bark have been limited. The bark extract of PE could be hepatoprotective due to its antioxidant and free

radical scavenging effects.[24] Furthermore, the wound healing activity of the bark extract of PE has been evaluated *in vitro*.[34] Phytochemical compounds of the bark extract of PE contains polyphenols, flavonoids, and tannins.[6,24] Some studies demonstrated that plants with high amounts of phenolics and tannins have potent anticancer effects.[12,35,36]

The present study aimed to evaluate the antiproliferative activity against CCA of various Thai medicinal plants. Since the bark extract of PE showed the most potent cytotoxic activity against CCA cells, further investigations were focused on the molecular mechanisms as well as the phytochemical contents.

2. Materials and Methods

2.1 Cell cultures

KKU-452 is the human CCA cell line used in this study. Cells were kindly provided by Prof. Veerapol Kukong-viriyapan, Department of Pharmacology, Faculty of Medicine, and Cholangio-carcinoma Research Institute, Khon Kaen University, Thailand. The cell line was established from an extrahepatic CCA patient with liver fluke infection in Thailand. KKU-452 is a poorly differentiated adenocarcinoma with a 17.9 h doubling time, and fast migration and invasion properties with high expression of N-cadherin and p53 mutation.[37] Cells were grown in Ham's F12 medium supplemented with 12.5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.3), 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS), and were maintained under 5% CO_2 at 37 °C.[38] Cells were subcultured every three days until reaching confluence using 0.25% trypsin-EDTA, and the medium was changed after an overnight incubation.

2.2 Plant extracts

Solanum tovum (fruit), *Nephelium hypoleucum* (fruit), *Schleichera oleosa* (fruit), *Antidesma thwaitesianum* (fruit), *Tamarindus indica* (fruit, leaf, bark), and *Phyllanthus emblica* (fruit, leaf, bark) were collected from Lamtakhong Research Station, Thailand Institute of Scientific and Technological Research**,** Northeastern region of Thailand. *Ocimum santum* (leaf) was collected by Tropical Vegetable Research and Development Center, Kasetsart University, Thailand. The dried plant (100 g) was macerated with 95% methanol at room temperature. The solvent was renewed every 48 h for three times. The combined extracts were filtered and concentrated under the reduced pressure using a rotary evaporator at 50°C. Plant extracts were stored at -20°C until use.

2.3 MTT assay

KKU-452 cells were seeded in a 96-well plate and left overnight. Plant extracts were diluted in dimethyl sulfoxide (DMSO) and added to the medium at various concentrations. Final concentration of DMSO vehicle was 0.1 % in each experiment and control. Plant extracts at different concentrations were incubated with KKU-452 cells for 24 h. Cytotoxic activity was determined using the 3-(4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, KKU-452 was incubated with the MTT (5 mg/ml) solution at 37 ºC for 4 h. Then, MTT solution was removed, and formazan crystals were dissolved in DMSO (100 µl). The absorbance was measured at 540 nm (Spectra MaxM2 microplate reader). The control group was 100 % of cell viability. IC_{50} valves were calculated using nonlinear curve-fitting (Sigma Plot version 10 program).

2.4 Analysis of apoptosis

Apoptosis profiles (early, late apoptosis, and dead cells) were analyzed with Annexin V and a 7-AAD kit (Merk Millipore, Germany).[35] KKU-452 was cultured at a density of 2.5×10^5 cells/well into a 6-well plate overnight. The cells were treated with the bark extract of PE at the concentrations of 6.25, 12.5, 25, 50, 100 µg/ml for 24 h. After treatment, Annexin V and dead cell reagent were added in single-cell suspension for 20 min in the dark at room temperature. Total cell population was analyzed using flow cytometry (Guava EasyCyte HT, Millipore, Bedford,

MA, USA). Live cell and apoptosis profiles were compared with the non-treated cell as a control group.

2.5 Wound healing assay

The wound-healing assay was performed, as previously described.[35] Briefly, KKU-452 cells at a density of 2.5×10^5 cells/well were seeded into a 24-well plate and incubated overnight in a culture medium. A scratch wound was done with a sterile 200 µl pipette tip, and detached cells were removed by washing with PBS. Cells were treated with the bark extract of PE at the concentrations of 12.5, 25, 50 µg/ml for 24 h. Images of scratch wounds were taken at 0 and 24 h using Image-Pro Plus software (Media Cybernetics, LP, USA). The migration index was calculated as the ratio of net wound width at the given time and the initial time.

2.6 Reactive oxygen species assay

KKU-452 cells were seeded on an 8 well-chamber at density 2.5×10^4 cells/well and allowed adherent cell growing overnight. Intracellular reactive oxygen species (ROS) was detected by the fluorogenic dye 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) (Invitrogen, CA, USA) according. DCFH-DA diffuses into the cell and is deacetylated by cellular esterases as non-fluorescence and sub-sequently oxidized by ROS to 2′,7′-dichloro-fluorescein (DCF) to fluoresce.[39] Cells were incubated with the bark extract of PE and stained with DCFH-DA for 1 h (37 °C, 5% CO_2). The culture medium was removed and washed with PBS. The intensity of DCF fluorescence was measured using confocal laser scanning microscopy (Olympus FV10i, Tokyo, Japan) at excitation and emission wavelengths of 485 and 528 nm, respectively.

2.7 HPLC analysis

Phenolic and flavonoid contents of the bark extract of PE were analyzed by HPLC as previously described.[40] The bark extract of PE was filtered through 0.45 µm syringe with nylon filter and subjected to HPLC analysis (Waters® 2690 Separations Module, USA). The solutes were separated on a Reliant C18 HPLC column (4.6 mm × 250 mm, 5 µM particle size). The mobile phase consisted of acetonitrile (solvent A), and 0.5% trifluoroacetic acid (solvent B). The gradient elution program was 0-5 min with 100% B, 5-10 min with 5% A/95% B, 10-20 min with 10% A/90% B, 20-30 min with 15% A/85% B 30-51 min 25% A/75% B and 51 min with 100% B. There was a 5 min post-run at initial conditions for equilibration of the column. The injection volume was 10 µl. The flow rate of the mobile phase was 1 ml/min. The eluent was detected by UV-Vis at 366 nm for 60 min. Analysis of peak areas was performed using Empower3 chromatography data software and compared with the standard gallic acid, quercetin, and kaempferol.

2.8 Statistical analysis

Results are presented as the mean ± SEM of at least three separate experiments. Significant difference among groups was determined using One-way ANOVA. Statistical analyses were performed with GraphPad Prism 9 software (GraphPad, La Jolla, CA, USA). P-values of < 0.05 were considered statistically significance.

3. Results

3.1 Cytotoxicity of Thai plants

Results indicated that the bark extract of PE had the highest cytotoxic potency against KKU-452 with an IC_{50} of 52.2 ± 39.2 µg/ml (Table 1). Other plant extracts had IC_{50} values greater than 55 µg/ml, indicating low potencies for the cancer-killing effect. This suggested that the bark extract of PE was effective in inhibiting CCA cell proliferation.

Table 1. IC_{50} values of Thai plant extracts in KKU-452 cells following 24 h of exposure.

Agents	IC_{50} (µg/ml)
1. *Solanum torvum* (fruit)	> 100
2. *Nephelium hypoleucum* (fruit)	> 100
3. *Ocimum sanctum* (leaf)	67.7 ± 21.1
4. *Schleichera oleosa* (fruit)	> 100
5. *Antidesma thwaitesianum* (fruit)	> 100
6. *Tamarindus indica* (fruit)	> 100
7. *Tamarindus indica* (leaf)	67.5± 35.3
8. *Tamarindus indica* (bark)	> 100
9. *Phyllanthus emblica* (fruit)	> 100
10. *Phyllanthus emblica* (leaf)	> 100
11. *Phyllanthus emblica* (bark)	52.2 ± 39.2

Each value represents the mean ± SEM, each from three experiments (quadruplicate in each experiment), IC_{50} value represents the minimum concentration of samples that kills the cells for 50%.

3.2 Bark extract of PE induced apoptosis

The bark extract of PE significantly reduced live cells (Fig. 1A) and significantly induced the population of cell shift to early apoptosis at concentrations of 6.25, 12.5, 25, 50 µg/ml, as well as late apoptosis at a concentration of 100 µg/ml (Fig. 1B and C). The percentage of total apoptosis at the concentrations of 50 and 100 µg/ml bark extract of PE was 50.27 ± 1.94% and 78.03 ± 2.28%, respectively.

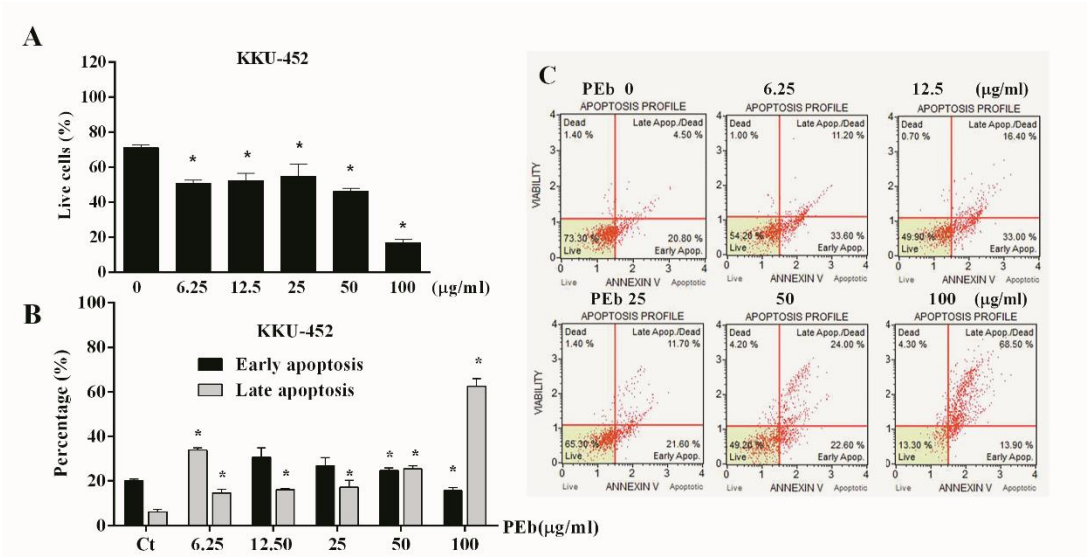

Fig. 1. Apoptosis-inducing effect of bark extract of PE (PEb) on KKU-452 cells. The numbers of live cell **(A)** and total apoptosis **(B)** are presented as percentages. Each bar is indicated as the mean ± SEM from three independent experiments. **(C)** Flow cytometry plots was used to show apoptosis (using annexin V & dead cell kit staining); *$p < 0.05$ *vs.* control group. PEb; bark extract of PE.

3.3 The effect of the bark extract of PE on cell migration

Cell migration is one of the characteristics of cancer that plays a role in cancer cell progression and metastasis. The wound healing assay was performed, as shown in Fig. 2A. The results indicated cell migration was significantly inhibited by the bark extract of PE at 25 and 50 µg/ml (42.8 and 32.9 %, respectively) compared with control (Fig. 2B). However, the extract at low concentrations had no effect on cell migration of KKU-452.

3.4 The effect of the bark extract of PE on ROS induction

One of the mechanisms of chemo-therapeutic agents is ROS generation, causing induced oxidative stress damage in cells. To investigate the ROS-generation-mediated cytotoxic activity of the bark extract of PE, cells were pretreated with the extract and stained with DCFH-DA permeant dye. The fluorescence intensity was determined in each separate cell (Fig. 3A). The results indicated that intracellular ROS in KKU-452 was significantly induced by 50 µg/ml of the bark extract of PE (31%) when compared with control (Fig. 3B).

3.5 HPLC analysis of the bark extract of PE

Phenolic acids and flavonoids have been shown to exhibit anticancer effects.[41, 42] To investigate the bioactive component in bark extract of PE, phytochemical compounds in bark extract of PE were analyzed by HPLC. The results revealed phenolic acids (gallic acid) and flavonoids (quercetin and kaempferol) as shown in Fig. 4.

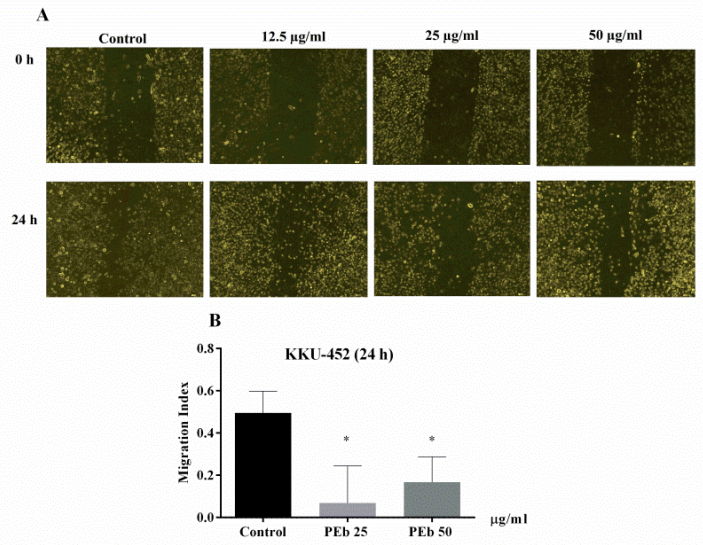

Fig. 2. The bark extract of PE suppressed KKU-452 cell migration. **(A)** a series of images of wound healing observed at 0 and 24 h after treatment with the bark extract of PE (one experiment), **(B)** migration index (mean ± SEM from four independent experiments); *p < 0.05 *vs.* control group.

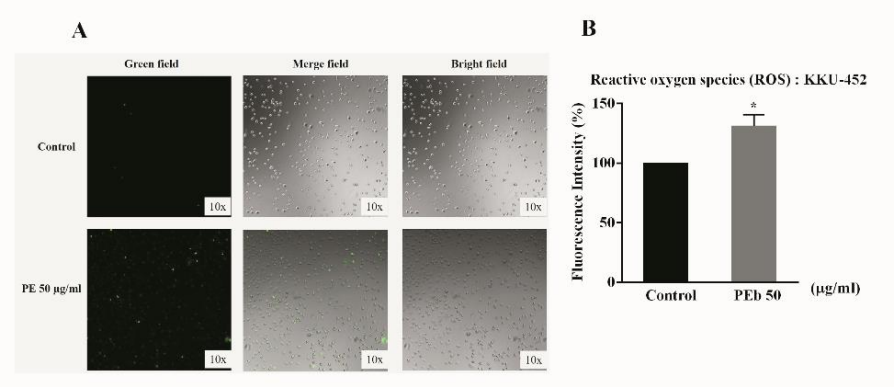

Fig. 3. Effects of the bark extract of PE (PEb) on inducing ROS generation in KKU-452. **(A)** a series of images showing KKU-452 cells with DCF fluorescence staining (green color) after exposing to the bark extract of PE (one experiment), **(B)** fluorescence intensity (percentage of control); each bar shows mean ± SEM from four independent experiments; *p < 0.05 *vs.* control group.

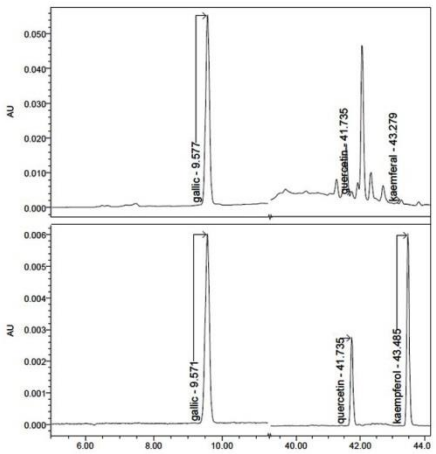

Fig. 4. HPLC analysis of bark extract of PE showing gallic acid, quercetin and kaempferol; X-axis: retention time/min. The upper and lower panels show the chromatograms of standard gallic acid, quercetin, kaemferol, and the bark extract of PE.

4. Discussion

The bark extract of PE exhibited cytotoxic effect on CCA (KKU-452 cells) through induction of cell apoptosis and inhibition of cell migration. Its phytochemical components included phenolic acids (gallic acid) and flavonoids (quercetin and kaempferol), which are consistent with the previous report.[24] In addition, tannin was also found in the bark extract of PE. It was noted that PEb induced a greater change to promote KKU-452 cell into late apoptosis. Cells in the late stage of apoptosis lost plasma membrane integrity and stimulate different mechanisms of the pro-inflammatory pathway.[43] Mechanisms of cell apoptosis induction involve both extrinsic and intrinsic pathways. For PE, possible mechanism that is likely to be involved is intrinsic pathway due to ROS-induced apoptosis. *C. orientalis, C. speciosum, P. kesiya, A. villosum, G. deltonii, A. tatarinowii* potentially induced late apoptosis, DNA alkylation, and as a consequence, DNA damage in HepG2 cells.[44] DNA fragmentation represents the late stage of apoptosis.[45] The antiproliferation of PE extract was involved with activation of

caspase-3 protease apoptotic cascades, p53 and G1 phase cell cycle arrest.[22,25] Moreover, PE extract was shown to inhibit angiogenesis.[46] The cytotoxic activity of the PE extract has been reported in various cancer cell lines including L929 cells,[47] human leukemic (HK63) cells,[48] MDA-MB-231 cells,[49] Hela cells,[49] MCF-7,[50] and RMCCA1.[25] Furthermore, combination treatment with conventional chemotherapy also enhanced the chemosensitivity in hepatocellular carcinoma and lung cancer cells,[51] and relieved the side effects of chemotherapy.[52] The results of this study suggested that PEb at 25 and 50 μg/ml exhibited antiproliferative and antimigration effects, the essential properties for chemotherapeutic agents. PEb may not directly affect cell migration and the molecular mechanism underlying this effect needs to be evaluated. In one study, the PE extract at 50-100 μg/ml was shown to suppress cancer cell growth by inducing DNA fragmentation, caspase3/7 activity, and extrinsic apoptosis pathway, while the extract at 25 and 50 μg/ml exhibited anti-invasive property.[49]

The bark extract of PE contains phytochemical components that are important for anticancer agents targeting apoptosis in cancer cells. These data suggest that antiproliferative and antimigration effects of the bark extract of PE may be attributed to the phenolic acids and flavonoids. Phenolic acids and flavonoids are known to interfere with several signal pathways of the process of carcinogenesis. The molecular targets may involve interference with several survival signaling pathways, such as NF-κB and STAT3, and induction of cell cycle arrest.[9] Gallic acid has been shown to possess cytotoxic activity against cancer cells without effect to the normal cells; moreover, it inhibits cell proliferation, invasion, and angiogenesis.[53] Quercetin inhibits cell proliferation by inducing the expression of P53, G2/M cell cycle arrest and apoptosis in HepG2, Hep3B, MDA-MA-231, HCT116 p53WT, and HCT116 p53KO cells.[54] Quercetin can induce both intrinsic and extrinsic mitochondrial apoptotic pathways,[55,56] by inhibition of growth signaling pathways, such as EGFR, PI3K and STAT3.[57-60] The proposed cytotoxic mechanisms of kaempferol against CCA include induction of the expression of pro-apoptotic proteins (Bax, Fas, cleaved-caspase 3, cleaved-caspase 8, cleaved-caspase 9, and cleaved-PARP), and suppression of phosphorylated AKT, TIMP2, and MMP2.[11] Tannins also have been reported to possess potent anticancer activities.[36] The synergizing effect of tannic acid on chemotherapeutic agents was shown to be by through downregulation of the expression calcein, PGP, MRP1, and MRP2 efflux pumps.[61]

The bark extract of PE (PEb) induced cytotoxic activity by ROS generation. Anticancer natural products have been reported to increase intracellular ROS levels and oxidative stress.[62] Some plant extracts have shown antiproliferation through changing the cellular redox balance by increased ROS and NO with decreased anti-oxidative activity.[63] Isothiocyanates can stimulate H_2O_2 formation and induce oxidative stress in cancer cells.[21] Sesquiterpene ferutinin and curcumin affect mitochondrial dysfunction, increase intracellular ROS, and stimulate of survival pathway.[64,65] The hepatoprotective effect of PEb has been shown to be due to potent antioxidant activity and sustaining redox homeostasis.[24] PEb exhibited cytotoxic activity against CCA cells. ROS generation and consequential irreparable DNA damage is one mechanism of anticancer agents. Normally, changing the level of intracellular ROS may lead to imbalance of antioxidant capacity. Together with genes initiating cell cycle arrest, apoptosis and oxidative stress are activated and upregulated.[21] It is likely that the bark extract of PE inhibited CCA cell growth through ROS-induction. Further studies are required to identify the molecular targets of action of PE on CCA cells.

4. Conclusion

The present study demonstrated that the anticancer properties of the bark extract of PE against CCA, including antiproliferation, induced apoptosis, antimigration, and ROS generation. The phytochemical compounds of the extract include phenolic acids and flavonoids which are thought to contribute to the anticancer properties of PE. The bark extract of PE could be used for further development of alternative medicine for cancer therapy in CCA patients.

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