

Molecular mechanisms of cholangiocarcinoma cell inhibition by medicinal plants

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Abstract. Cholangiocarcinoma (CCA) is one of the most common causes of cancer-associated mortality in Thailand. Certain phytochemicals have been demonstrated to modulate apoptotic signaling pathways, which may be targeted for the prevention and treatment of cancer. Therefore, the aim of the present study was to investigate the effect of specific medicinal plants on the inhibition of CCA cell proliferation, and to identify the molecular mechanisms underlying this. A WST-1 cell proliferation assay was performed using an RMCCA1 cell line, and apoptotic signaling pathways were also investigated using a PathScan Stress and Apoptosis Signaling Antibody Array Kit. The cell proliferation assay indicated that extracts from the *Phyllanthus emblica* fruit pulp (PEf), *Phyllanthus emblica* seed (PEs), *Terminalia chebula* fruit pulp (TCf), *Terminalia chebula* seed (TCs), *Areca catechu* seed (ACs), *Curcuma longa* (CL) and *Moringa oleifera* seed (MOs) exerted anti-proliferative activity in RMCCA1 cells. In addition, the PathScan assay revealed that certain pro-apoptotic molecules, including caspase-3, poly (ADP-ribose) polymerase, checkpoint kinase 2 and tumor protein 53, exhibited increased activity in RMCCA1 cells treated with the aforementioned selected plant extracts, with the exception of PEf. The mitogen-activated protein kinase (MAPK) pathways (including ERK1/2 and p38 MAPK) expression level was significantly increased in RMCCA1 cells pre-treated with extracts of PEs, TCf, CL and MOs. The activation of protein kinase B (Akt) was significantly demonstrated in RMCCA1 cells pre-treated with extracts of TCf, ACs and MOs. In summary, the present study demonstrated that extracts of PEs, TCf, TCs, ACs, CL and MOs exhibited anti-proliferative effects in CCA cells by inducing pro-apoptotic signals and modulating signal transduction molecules. Further studies

in vivo are required to demonstrate the potential applications of specific plant extracts for the treatment of human cancer.

Introduction

Cholangiocarcinoma (CCA) is one of the most common causes of cancer-associated mortality in Thailand, and is one of the most aggressive types of cancer due to its resistance to chemotherapy and propensity for local and distant invasion (1). Currently, no effective therapy has been identified for the disease, and alternative therapeutic options are urgently required. Certain dietary phytochemicals have been previously observed to exert chemopreventive and anticancer effects, and have been demonstrated to modulate apoptotic signaling pathways, which may be targeted for the prevention and treatment of CCA (2). To the best of our knowledge, the present study is the first to extensively examine the use of phytochemicals in CCA.

Ten different medicinal plants were selected for the current study and evaluated with regard to their cytotoxicity and cytotoxic mechanisms in a CCA cell line. The selected medicinal plants included *Phyllanthus emblica* fruit pulp (PEf) and seed (PEs), which contains polyphenols and hydrolysable tannin-derived compounds (3); *Phyllanthus* extracts have been demonstrated to trigger apoptosis and regulate multiple survival signal pathways in some cancer cell types (4,5). The plants also included *Curcuma longa* (CL), *Terminalia chebula* fruit pulp (TCf) and seed (TCs), *Moringa oleifera* seed (MOs), *Momordica charantia*, *Areca catechu* seed (ACs), *Brassica oleracea* var. *rubra* and *Tinospora crispa* (TiC). These extracts have previously demonstrated anti-proliferative effects via modulation of the apoptotic pathways (6-14).

Curcumin, derived from CL, has previously been shown to exhibit apoptotic effects in various cancer cell lines through the activation of caspase-3 and the downregulation of B-cell lymphoma-2 (BCL-2). Curcumin also activates the expression of tumor protein p53, peroxisome proliferator-activated receptor (PPAR), and other tumor suppressor genes, and downregulates specific oncogenes, including c-Myc, human epidermal growth factor receptor 2 and epidermal growth factor receptor (6). Chebulagic acid from the TCf has previously been demonstrated to induce G1 arrest, inhibit nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) and induce apoptosis in retinoblastoma cells (7). MOs and

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M. oleifera leaf extracts have been observed to induce CCA cell apoptosis by inducing reactive oxygen species accumulation and mitochondrial dysfunction (8). *M. oleifera* leaf extract inhibits pancreatic cancer cells by targeting the NF- κ B signaling pathway, increasing the efficacy of chemotherapeutic agents in human pancreatic cancer cells (9). Cucurbitae-type triterpene glycosides from *M. charantia* fruit (Mcf) inhibit NF- κ B and activate PPAR in HepG2 cells (10). In addition, *M. charantia* leaf extract induces apoptosis through caspase- and mitochondria-dependent pathways in human cancer cells (11). Arecoline from ACs has been demonstrated to induce HaCaT cell apoptosis by upregulating the expression and activation of cleaved-BH3 interacting-domain death agonist, cleaved-PARP and cleaved-caspase-3 (12). Sulforaphane from red cabbage [*B. oleracea* var. *capitata* L.f. *rubra* (BO)] has been demonstrated to reduce BCL-2 expression levels (antiapoptotic) and reduce the induction of p53, BCL-2-associated X protein (proapoptotic) and caspase-3 in HEp-2 cells (13). Methanol extract of the *T. crispa* stem has been observed to promote apoptosis by upregulating caspase-3 expression levels and insulin sensitization, through the inhibition of the insulin-like growth factor-1 receptor in HepG2 cells (14). Additionally, *Solanum torvum* (ST) and *Allium ascalonicum* (AA) have demonstrated cytotoxic effects in multiple cancer cell lines (15,16); however, the mechanisms underlying these effects require further study for elucidation.

The aim of the present study was to investigate the roles of specific medicinal plants that inhibit CCA cell proliferation and identify the molecular mechanisms underlying this.

Materials and methods

Plant materials and extraction procedures. All plant samples including Pef, PEs, TCf, TCs, ACs, BO, CL, ST, AA, Mcf pulp, *M. charantia* seed (MCs), TiC, *M. oleifera* fruit (MOF) pulp and MOs were purchased from Rangsit market in Amphoe Mueang, Pathum Thani province, Thailand. The fruits were separated into pulp and seed and all of the samples were dried at 55°C and ground into powder. Powder samples (15 g) were macerated with 300 ml 95% ethanol for 24 h. Extract solutions were subsequently dried by rotary evaporation and freeze-drying, and stored at -20°C until required. The sample extracts were freshly prepared in dimethyl sulfoxide (DMSO) to the concentration of 50 mg/ml then diluted with HAM's F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to the concentration of 1-500 μ g/ml.

Cell proliferation assay. The human CCA cell line RMCCA1 (16) was grown in HAM's F12 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO₂ humidified atmosphere. For the proliferation assays, cells were seeded into 96-well culture plates at a density of 10,000 cells per well, followed by the addition of sample extracts in various concentrations (1-500 μ g/ml) or control medium (Ham's F12 medium containing 0.01% DMSO). The cells were subsequently incubated for 48 h prior to the application of Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cell

viability was calculated as a percentage, relative to the control cells. The extracts that exhibited anti-proliferative effects were selected for further assays investigating the effects of stress and apoptosis on the RMCCA1 cell line.

Cellular stress and apoptosis signaling assay. RMCCA1 cells were treated with seven selected extracts (Pef, PEs, TCf, TCs, ACs, CL and MOs) prior to using the PathScan® Stress and Apoptosis Signaling Antibody Array kit with a chemiluminescent readout (cat. 12856; Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions. The kit facilitated the simultaneous detection of the phosphorylation of 13 signaling molecules, including extracellular signal-regulated kinase (ERK)-1/2 (Thr202/tyr204), protein kinase B (Akt; Ser473), BCL-2-associated death promoter (Bad; Ser136), heat shock protein 27 (HSP27; Ser82), Smad2 (Ser465/467), p53 (Ser15), p38 mitogen activated protein kinase (MAPK; Thr180/Tyr182), stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK; Thr183/Tyr185), checkpoint kinase (Chk) 1 (Ser345), Chk2 (Thr68), NF- κ B inhibitor α (I κ B α Ser32/36) eukaryotic translation initiation factor 2 subunit α (eIF2; Ser51) and transforming growth factor- β -activated kinase 1 (TAK1) (Ser412). In addition, the kit also allowed for simultaneous detection of 3 cleaved molecules, including poly (ADP-ribose) polymerase (PARP) (Asp214), caspase-3 (Asp175) and caspase-7 (Asp198), and detection of 3 total proteins, including I κ B α , survivin and α -tubulin. Cell lysates were incubated on the slide followed by a biotinylated detection antibody cocktail. Streptavidin-conjugated HRP and LumiGLO® Reagent were then used to visualize the bound detection antibody by chemiluminescence. Images were acquired by briefly exposing the slide to standard chemiluminescent film. The spot intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of each signal was calculated relative to the positive control signal of the array kit.

Statistical analysis. The experiments were performed in triplicate and the data are presented as the mean \pm standard deviation. Data between three or more groups were compared using one-way analysis of variance, followed by Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Effects of plant extracts on cell proliferation. Cell proliferation assays were performed in RMCCA1 cells treated with 14 extract samples at concentrations of 1, 10, 100 and 500 μ M or control medium. After 48 h of incubation, the results demonstrated that TCs (500 μ g/ml), Pef, PEs, TCf, CL and MOs (100 μ g/ml), and ACs (10 μ g/ml) significantly inhibited RMCCA1 cell proliferation ($P = 0.002$, < 0.001 , < 0.001 , < 0.001 , 0.002 , < 0.001 and 0.008 , respectively). However, BO, ST, AA, Mcf, TiC and MOF had no significant inhibitory effect on RMCCA1 cell proliferation, as compared with control cells ($P > 0.05$; Fig. 1). Therefore, Pef, PEs, TCf, TCs, ACs, CL and MOs were selected to further study the mechanisms underlying CCA cell proliferation inhibition.

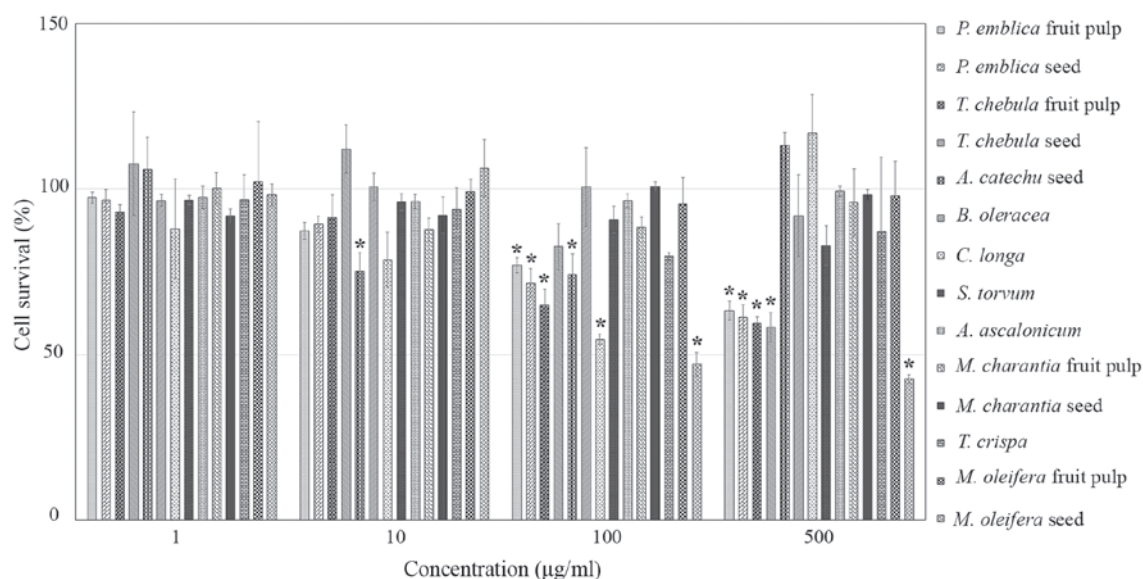


Figure 1. Cell proliferation assay of RMCCA1 cells treated with selected medicinal plants. The cells were treated with extracts of specific medicinal plants at various concentrations for 48 h. Cell proliferation was measured by WST-1 and analyzed by spectrophotometry (absorbance at 450 nm). The data are presented as a percentage of cell proliferation, for which the optical density value obtained from control cells, treated with HAM's F12 medium containing 0.01% Dimethyl sulfoxide, was set at 100%. The results are presented as the mean \pm standard deviation of three independent experiments. * P <0.05 vs. control.

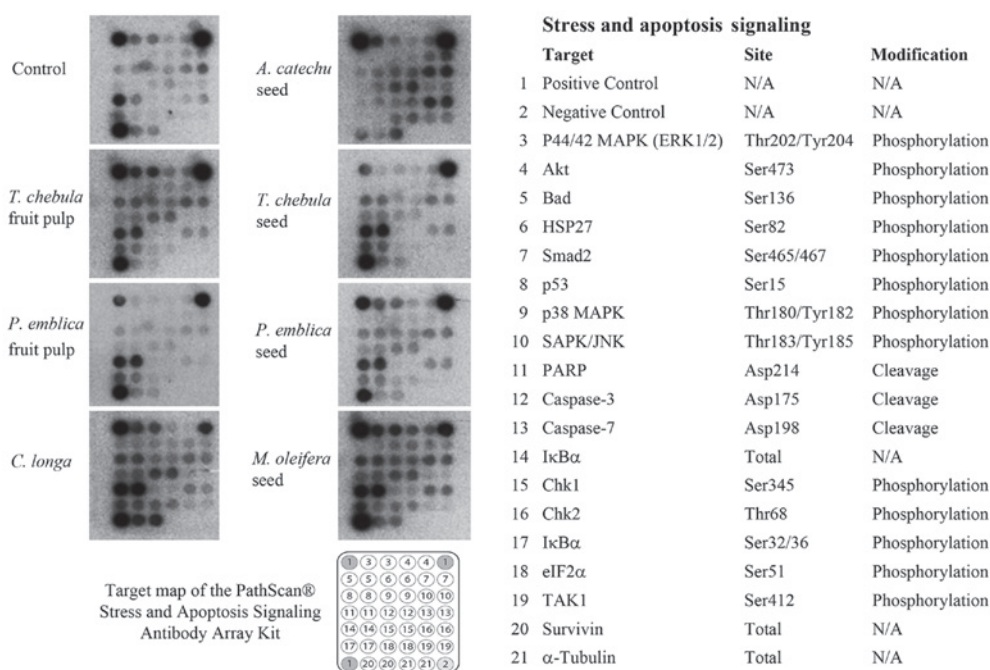


Figure 2. The effects of medicinal plants on the phosphorylation or cleavage of certain signaling proteins in RMCCA1 cells. Chemiluminescent array images obtained using the PathScan Stress and Apoptosis Signaling Antibody Array Kit allowed for the simultaneous detection of 19 signaling molecules that involved in the regulation of the stress response and apoptosis. Images were captured following brief exposure of the slide to a standard chemiluminescent film. Numbers on the target map correspond to the numbered targets shown on the right. The expression levels of α -tubulin were used to normalize the signals between various samples.

Effects of plant extracts on cellular stress and apoptosis signaling. A PathScan® Stress and Apoptosis Signaling Antibody Array kit with a chemiluminescent readout was used for the simultaneous detection of 19 signaling molecules that are involved in the regulation of the cellular stress response and apoptosis. The expression levels of α -tubulin were used to normalize the signals between various samples, and the α -tubulin levels were observed to be relatively constant between the samples.

The RMCCA1 cells treated with PEs, TCf, TCs, ACs, CL or MOs exhibited high intensity of the apoptotic signals p53, PARP and caspase-3 whereas Pef did not appear to induce apoptotic signals (Fig. 2). The intensity of each signal was measured by ImageJ software. The expression level was calculated relative to the positive control signal as intensity ratio.

Caspase-3 and -7 proteases exert pro-apoptotic functions through the cleavage of a number of cellular targets (18).

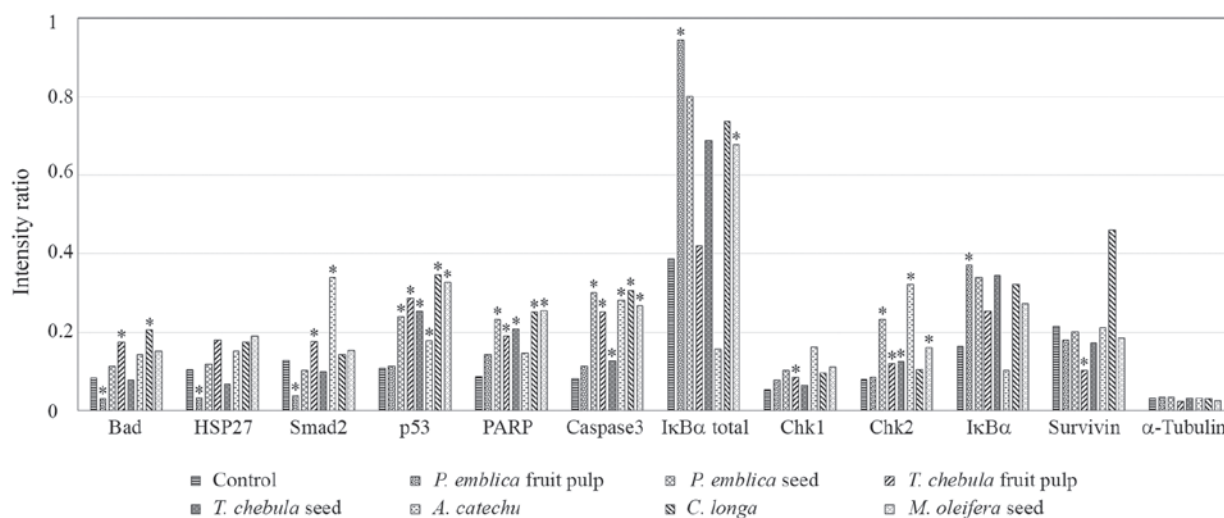


Figure 3. Array image pixel intensity ratio of phosphorylated/cleaved pro-apoptotic signaling molecules in RMCCA1 cells treated with medicinal plants. RMCCA1 cells were treated with seven selected plants that had exhibited apoptotic signals. The phosphorylation levels of Bad at Ser136, HSP27 at Ser82, Smad2 at Ser465/467, p53 at Ser15, IκBα at Ser32/36, Chk1 at Ser345 and Chk2 at Thr68, and the cleavage of PARP at Asp214 and caspase-3 at Asp175, were significantly altered. The expression of survivin decreased only in RMCCA1 cells treated with the extract of Tcf. The expression levels of α-tubulin were used to normalize the signals between various samples, and these were observed to be consistent. *P<0.05 vs. control. Bad, BCL-2-associated death promoter; HSP27, heat shock protein 27; PARP, poly (ADP-ribose) polymerase; IκBα, NF-κB inhibitor α; Chk, checkpoint kinase.

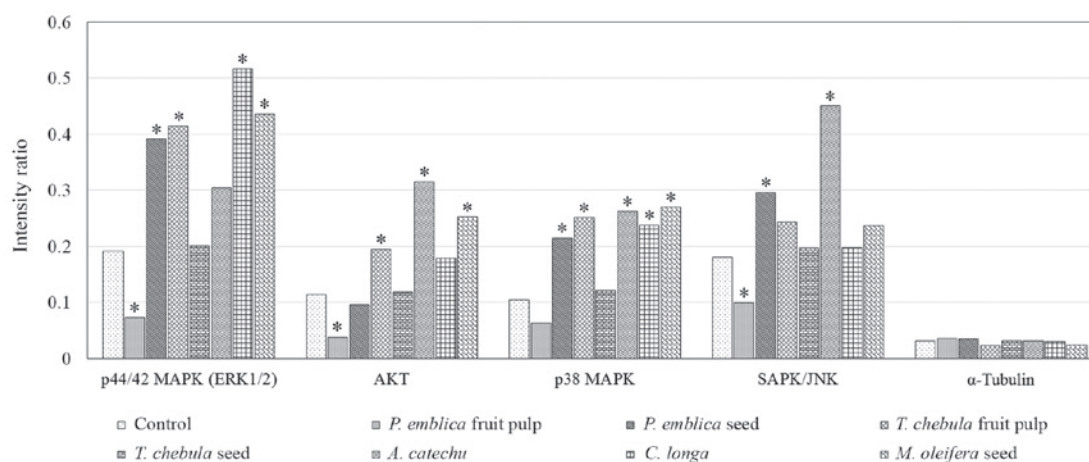


Figure 4. Detection of the phosphorylation of intracellular signal molecules in RMCCA1 cells treated with medicinal plant extracts. The phosphorylation levels of p44/42 MAPK (ERK1/2) at Thr202/Tyr204, Akt at Ser473, p38 MAPK at Thr180/Tyr182 and SAPK/JNK at Thr183/Tyr185 were significantly altered. The expression levels of α-tubulin were used to normalize the signals between various samples. *P<0.05 vs. control. MAPK, mitogen-activated protein kinase; Akt, protein kinase B; SAPK/JNK, stress-activated protein kinases/Jun amino-terminal kinases.

Increased activation of caspase-3 was demonstrated in RMCCA1 cells treated with the extracts of PEs, TCf, TCs, ACs, CL and MOs (P=0.018, <0.001, 0.018, 0.032, 0.029 and 0.006, respectively); however, this was not observed in RMCCA1 cells treated with the extract of Pef (P=0.183). No significant differences in caspase-7 expression levels were identified.

Other pro-apoptotic molecules, including cleaved-PARP, phosphorylated-Chk2 and phosphorylated-p53, were demonstrated to be upregulated in RMCCA1 cells treated with certain plant extracts. The expression level of cleaved-PARP was significantly increased in RMCCA1 cells treated with the extracts of PEs, TCf, TCs, CL and MOs (P=0.018, 0.014, 0.003, 0.039 and 0.005, respectively). Phosphorylated-Chk2 was significantly upregulated in RMCCA1 cells treated with the extracts of PEs, TCf, TCs, ACs and MOs (P=0.048, 0.005, 0.012, 0.029 and 0.007, respectively). The activation

of phosphorylated-p53 was demonstrated in RMCCA1 cells treated with the extracts of PEs, TCf, TCs, ACs, CL and MOs (P=0.014, 0.005, 0.001, 0.028, 0.041 and 0.002, respectively; Fig. 3).

Activation of the Akt and MAPK pathways, including ERK1/2 and p38 MAPK, were demonstrated in RMCCA1 cells. The expression level of phosphorylated-ERK1/2 was demonstrated to be upregulated in RMCCA1 cells treated with the extract of PEs, TCf, CL and MOs (P=0.010, 0.003, <0.001 and 0.001, respectively) while RMCCA1 cells treated with the extracts of PEs, TCf, ACs, CL and MOs exhibited significantly increased expression levels of phosphorylated-p38 MAPK (P=0.004, 0.002, 0.042, 0.037 and 0.002, respectively). The expression level of phosphorylated-AKT was significantly upregulated in RMCCA1 cells treated with the extract of TCf, ACs and MOs (P=0.001, 0.034 and <0.001, respectively).

However, the expression level of phosphorylated-ERK1/2 was significantly downregulated in the RMCCA1 cells treated with Pef extract ($P=0.005$), while the expression level of phosphorylated-p38 MAPK appeared downregulated but was not significant ($P=0.054$). In addition, the expression of phosphorylated-SAPK/JNK in the RMCCA1 cells treated with Pef extract was significantly downregulated ($P=0.004$). The activation of SAPK/JNK was demonstrated in RMCCA1 cells treated with the extracts of PEs and ACs ($P=0.043$ and 0.023 , respectively; Fig. 4).

Discussion

The present study investigated the inhibition of CCA cell proliferation by various types of medicinal plants. Each plant extract was composed of mixture of bioactive compounds (19); however, the current study evaluated the activity of the entire extract rather than the activities of the individual compounds. Inhibition of CCA cell proliferation was associated with the upregulation of numerous intracellular signaling molecules and pro-apoptotic molecules. A total of 19 signaling molecules involved in the regulation of the stress response and apoptosis were simultaneously detected in the present study.

Caspase-3 protease exerts a pro-apoptotic function through the cleavage of multiple cellular targets, and is activated in apoptotic cells by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (20). The present study demonstrated that caspase-3 is activated (via cleavage at Asp175) in CCA cells treated with the extracts of PEs, TCf, TCs, ACs, CL and MOs. The results revealed that these same extracts also inhibit PARP function via cleavage at Asp214. PARP is a DNA repair enzyme that is activated in response to DNA strand breaks (21). During apoptosis, cleavage of PARP-1 at Asp214 by caspase-3 is a useful marker of this type of cell death (21). The results of the current study suggested that the extracts of PEs, TCf, TCs, ACs, CL and MOs induced apoptosis in RMCCA1 cells.

The extracts of PEs, TCf, TCs, ACs and MOs were identified to increase Chk2 phosphorylation in CCA cells. Chk2 functions as a cell cycle checkpoint regulator and is a putative tumor suppressor protein; it is phosphorylated in response to DNA damage and prevents the cell from entering mitosis, thus arresting the cell cycle in the G1 phase (22). Previous studies have demonstrated that gallic acid (a hydrolysable tannin present in TCf) and quercetin induce cell cycle arrest in the G1 phase and induce apoptosis in certain cancer cells by inactivating the phosphorylation of Cdc25A/Cdc25C-Cdc2 via Chk2 activation (23,24). Therefore, the induction of Chk2 by the aforementioned plant extracts may have an important role in CCA apoptosis.

Accumulation of p53 has a crucial role in cell apoptosis. The phosphorylation of p53 underlies p53 accumulation and its subsequent prolonged protein half-life due to ubiquitination and degradation (25). The phosphorylation of p53 at Ser15 promotes the accumulation and functional activation of p53 in response to apoptotic stimuli or DNA damage (26). The current study demonstrated that p53 phosphorylation at Ser15 is increased in CCA cells treated with extracts of PEs, TCf, TCs, ACs, CL and MOs. Furthermore, it has previously been demonstrated that the phosphorylation of p53 at Ser15 may induce protein translocation to the mitochondria (intrinsic pathway), thereby inducing

apoptosis by the mitochondrial pathway (27). Consequently, the present study found that the induction of p53 phosphorylation at Ser15 in CCA cells treated with the extracts of PEs, TCf, TCs, ACs, CL and MOs, may also induce apoptosis by activating caspase-3 which serve a critical role in the induction of apoptosis in mitochondrial pathway and inhibiting PARP function. Wu *et al* (28) identified that the silencing of p53 by specific small interfering RNA reduced the effect of CL on the induction of nasopharyngeal carcinoma cell apoptosis. Therefore, p53 function may be important to the ability of the specified plant extracts to induce CCA cell apoptosis.

ERK1/2 and p38 kinase are activated in CCA cells that have been treated with the extracts of PEs, TCf, ACs, CL and MOs. Our previous study demonstrated that ERK1/2 functions to promote survival in CCA cells, whereas p38 kinase activation is a primary signal for apoptosis (29). ERK1/2 signaling is required for the proliferation of CCA cells and the inhibition of ERK1/2 contributes to cell apoptosis (30). A previous study suggested that the opposing effects of ERK1/2 and p38 kinase in apoptosis are regulated by the phosphorylation and accumulation of p53 (31). In addition, p38 kinase may physically interact with and phosphorylate p53 at Ser15 in response to ultraviolet B radiation, and induce apoptosis *in vitro* and *in vivo* (32). The present study demonstrated that the simultaneous activation of p38 kinase and p53 could be observed in CCA cells treated with extracts of PEs, TCf, ACs, CL and MOs, which suggests that the interaction of p53 and p38 MAPK induces apoptosis in CCA cells.

The current study also demonstrated that JNK is activated in CCA cells that have been treated with extracts of PEs and ACs. Consistently, a previous study revealed that curcumin induced osteoclastoma cell apoptosis by activating the JNK signaling pathway (33). JNK belongs to the superfamily of MAPKs and has a critical role in death receptor-initiated (extrinsic) and mitochondrial (intrinsic) apoptotic pathways (34). The induction of apoptosis by PEs and ACs extracts may be regulated by the JNK pathway.

In the present study, Pef extract was demonstrated to inhibit CCA cell proliferation; however, apoptotic signals in the cells treated with Pef were not observed. We hypothesized that the anti-proliferative effect of Pef occurs via the inhibition of Akt activation. Akt is a protein kinase that promotes cell growth and survival (35), and our previous study demonstrated that Akt is involved in the regulation of CCA cell proliferation and invasion (36). The results of the present study are concordant with another previous study, which demonstrated that *Phyllanthus* extracts inhibit prostate cancer cell proliferation by inhibiting Akt activation (37).

The present study investigated the interaction of certain signaling molecules in CCA cells that have important roles in the regulation of apoptosis by specific plant extracts. Certain plants exhibited anti-proliferative effects in CCA cells by inducing pro-apoptotic signals; however, they also exhibited anti-apoptotic signals, including the phosphorylation of Bad at Ser136 and ERK1/2. Furthermore, the concentrations of the extracts required to inhibit CCA cells are relatively high, as the selected samples were from dietary medicinal plants. Further studies must be performed *in vivo* to investigate the potential applications of these plant extracts in the clinical treatment of CCA.

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