Journal of Medicinal Plants Research Vol. 5(10), pp. 1892-1899, 18 May, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

In vitro chemopreventive activity of an ethyl acetate fraction derived from hot water extract of *Orthosiphon stamineus* in HepG2 cells

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Accepted 16 February, 2011

Orthosiphon stamineus **(Lamiaceae) is a medicinal plant containing several biologically active components that have chemopreventive activity. To investigate the chemopreventive properties of *O. stamineus*, we studied the apoptotic activity of the ethyl acetate fraction (EAF) derived from the hot water extract of *O. stamineus* leaves on the human hepatocellular carcinoma cell line, HepG2. The sulforhodomine B assay indicated that the EAF inhibited the viability of HepG2 cells in a concentration dependent manner. Hoechst 33342 staining showed that EAF-treated cells exhibited typical apoptotic morphologic changes such as nuclear condensation and fragmentation. JC-1 assays indicated that the EAF disrupted the mitochondrial transmembrane potential of HepG2 cells in a dose-dependent manner. Western blot analysis revealed that the EAF activated caspase-3, caspase-8 and caspase-9, increased Bax expression, downregulated Bcl-2, decreased Cox-2 expression and decreased level of the NF-κB p65 in nucleus. HPLC-DAD analysis identified the major components in the EAF as rosmarinic acid (31.8%) and caffeic acid (20.2%). Taken together, our study suggests that the EAF has the potential to be developed as an agent for human liver cancer prevention.**

Key words: *Orthosiphon stamineus*, chemoprevention, apoptosis, HepG2, caspase.

INTRODUCTION

Cancer remains a major, world-wide public health problem (Jemal et al., 2009). In addition to chemotherapy and surgery, chemoprevention provides another method to fight against cancer (American Cancer Society, 2009). Chemoprevention is defined as the use of naturally occurring or synthetic substances to slow, retard or reverse the process of carcinogenesis before localized cancerous cells invade the surrounding tissue or/and metastasis to distant sites (Sporn and Suh, 2000). Chemopreventive agents prevent the transformation of pre-cancerous cells from reaching a final stage of carcinogenesis by stopping uncontrolled molecular events which activated by carcinogens. Thus, the net effect of chemopreventive agents on pre-cancerous or cancerous cells includes suppressing transformation, halting proliferation and finally inducing apoptotic cell death (Crowell, 2005; D'Agostini et al., 2005). The benefits and molecular targets of compounds from

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dietary and medicinal plants for use in cancer chemoprevention have been reviewed by several authors (Neergheen et al., 2009; Pan et al., 2008). These compounds include curcumin, resveratrol, geniestein, catechin, silymarin, ellergic acid and indole-3-carbinol (Neergheen et al., 2009). Interestingly, these compounds from plants origins targeted similar chemoprevention mechanisms in human cancer cell lines such as anti-proliferative effects, induction of apoptotic pathways (e.g., caspase-3,-9, -7, PARP, Bax, Bcl-2), transcription factors (e.g., NF-κB, AP-1, Nrf2, p53) and antimetastasis (e.g., Cox-2, MMP-9, 5-Lox) (Neergheen et al., 2009).

In Malaysia, the medicinal plant *Orthosiphon stamineus* (Lamiaceae) is commercially cultivated to make a tea (Akowuah et al., 2005). Local people use decoctions of fresh leaves to treat diabetes, hypertension and kidney-related diseases such as renal calculus and microbial infection of the urinary tract (Arafat et al., 2008; Englert and Harnischfeger, 1992; Olah et al., 2003; Sriplang et al., 2007). Several studies have reported that the bioactivity of compounds and extracts from *O. stamineus* leaves are related to cancer chemoprevention activity. For examples, diterpenes such as norstaminolactone A and norstaminol B and C showed anti-proliferative activity towards highly liver-metastatic colon 26-L5 carcinoma cells (Awale et al., 2002; Stampoulis et al., 1999), whereas neoorthoshiphonone, and siphonol A, B, C and D showed potent inhibitory effects on nitric oxide production in lipopolysaccharide-activated macrophage-like J774.1 cells (Awale et al., 2003, 2004). Several compounds with chemopreventive activities (*in vitro* and *in vivo*) are presence in *O. stamineus* leaves, these compounds are including caffeic acid, rosmarinic acid betulinic acid, oleanolic acid and ursolic acid (Cheung and Tai, 2007; Chung et al., 2004; Lin et al., 2007; Tezuka et al., 2000). In addition, water and organic solvents extracts of *O. stamineus* leaves have shown significant radical-scavenging activity in several assays system (Akowuah et. al., 2004, 2005; Masuda et al., 1992; Khamsah et al., 2006).

Therefore, to further elucidate the possible *in vitro* chemopreventive activity of *O. stamineus*, we examined the ethyl acetate fraction (EAF) derived from hot water extract of *O. stamineus* leaves on the human hepatocellular carcinoma cell line HepG2. We determined the effects of the EAF on cell proliferation, the induction of apoptosis and on proteins related to chemoprevention activity including Bax, Bcl-2, caspase-3, caspase-8, caspase-9, NF-κB p65 and Cox-2. We also determined the major components present in the EAF by HPLC-DAD.

MATERIALS AND METHODS

Materials

Ethyl acetate (HPLC grade), acetonitrile (HPLC grade) and triflouroacetic acid (analytical grade) were purchased from Merck, USA. Rosmarinic acid and caffeic acid standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Deionized water (18.2 MΩ cm) was produced using the Bio-Rad Water Purity System (Bio-Rad Laboratories, CA, USA). All of the materials for the experiments involving cultured cells were purchased from the GIBCO® Invitrogen Cell Culture (USA). For the assessment of apoptosis activity, all chemicals and reagents of the highest purity were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). All of the reagents for SDS-PAGE and western blotting were purchased from Bio-Rad Laboratories (CA, USA). Primary and secondary antibodies were obtained from BioVision Research Products (CA, USA).

Plant leaves

The leaves of *O. stamineus* were purchased from the Malaysian Agriculture Research and Development Institute (MARDI) in July 2008. The voucher specimen was deposited in the herbarium of Herbal Medicine Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia. The leaves were dried in an electric oven at 40°C for 3 days, powdered using an electric grinder, and stored at -20°C until used for extraction.

Extraction

Powdered plant material (300 g) was extracted in 1.5 L of deionized water for 2 h at 90°C. The hot water extract was cooled on ice and centrifuge at 500 g to remove particles, filtered, freeze-dried and was yielded the dry extract (11.96 g). The extract (5 g) was dissolved in 200 ml of deionized water and partitioned with ethyl acetate (3 X 300 ml) to yield the ethyl acetate fraction (EAF) (0.78 g). The stock solution of the EAF was prepared by dissolving in 100% DMSO at 100 mg/ml and kept at -80°C.

Cell culture

Human hepatoma cell line, HepG2 and a non-malignant human liver cell line, Chang Liver, were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 0.5% antibiotic-antimycotic solution at 37°C in 5% CO_2. At 70 to 90% confluences, cells were trypsinized, counted using a hemocytometer and adjusted to the particular densities for each of the assays.

Cell viability assay

Cell viability was determined by the sulforhodomine B (SRB) colorimetric assay (Vichai and Kirtikara, 2006). Briefly, HepG2 cells were seeded in 96-well flat-bottom microplates (1 x 10^4 cells/well in 100 µl of completed growth medium) and cultured overnight for adhesion in a humidified CO_2 incubator. The EAF was added to the cells in serial concentrations (0, 25, 50, 100, 200, 400 and 800 µg/l) and further incubated for 72 h. For comparison, the EAF was also tested on non-malignant human liver cell Chang Liver cells for 72 h. As a positive control, HepG2 and Chang Liver cells were treated with rosmarinic acid, an active compound present in the leaves of *O. stamineus*. At the end of the respective incubation periods, cells were fixed with 50 µl of pre-chilled 50% (w/v) trichloroacetic acid (TCA) per well, incubated at room temperature for 30 min, and washed with tap water. The TCA-fixed cells were stained for 20 min with 100 µl of 0.4% (w/v) SRB in 1% acetic acid. Plates were washed five times with 1% (v/v) acetic acid and air dried. Protein-bound dye was solubilized with 100 µl of 10 mM of unbuffered

Trizma base solution. The plates were read by a microplate reader at 492 nm. Results were expressed as IC_{50} (the dose that inhibited 50% of the cell population compared to untreated cells after the incubation period). The values were estimated from a plot of drug concentration (µg/ml) against the percentage of viable cells compared to control. The tests were repeated for three independent experiments.

Hoechst 33342 staining assay

Hoechst 33342 staining assay was used to determine an apoptotic cell, which based on the nuclear morphology (Ghavami et al., 2004; Zhivotovsky et al., 1999). HepG2 cells were growth on cover slips at a density 3 X 10^5 cells/well in 6-well plates. After incubation for 24 h, various concentrations of the EAF (0, 100, 200, 400 and 800 µg/ml) were added into individual wells and then further incubated for 48 h in a 5% CO_2 incubator. Cisplatin was used as a positive control. The cells were fixed with methanol/acetic acid (3:1) for 10 min at room temperature and stained with 1 µg/ml of Hoechst 33342 for 10 min. The cells were washed with PBS and examined using fluorescence microscopy (Olympus microscope BX51, Olympus Optical Co. Ltd). Apoptotic cells were scored on the basis of nuclear morphology changes, such as chromatin condensation and fragmentation.

JC-1 assay

A cationic dye, JC-1, is commonly used for the detection an early stage of apoptosis based on mitochondria dysfunction due to loss of mitochondrial transmembrane potential (MTP) (Kroemer et al., 2007; Ly et al., 2003). Briefly, HepG2 cells were seeded at a density of 3 X 10^5 cells into cell culture dishes (4 cm diameter) and incubated overnight. Various concentrations of the EAF (0, 100, 200, 400 and 800 µg/ml), camptothecin (4 µg/ml, positive control) and 0.01% DMSO (vehicle control), were added into individual culture dishes and then further incubated for 24 h in CO_2 incubator. At the end of assay, the old growth medium were removed, and replaced with 3 ml of medium containing 1 µg/ml JC-1, and incubated in CO_2 incubator at 37°C. After 30 min, the cells were examined under at fluorescence microscope (Olympus microscope BX51, Olympus Optical Co. Ltd). A total of ten images per sample were captured using analysis image processing software (Soft Imaging System GmbH, Germany). The numbers of green (loss of MTP or apoptotic) and red (normal MTP or healthy) cells were counted, with not less than a total number of 500 cells per sample.

Western blotting

HepG2 cells *(*2.5 X 10^6 cells) were seeded in 75 cm^2 culture flasks. After 24 h of incubation, cells were treated with various concentration of the EAF and incubated for the next 24 h. Whole cell extract from treated cells were prepared using the mammalian cell extraction kit (BioVision Research Product, CA, USA) or cells were fractionated into cytosolic and nuclear fractions using the Nuclear/Cytotsol fractionation kit (BioVision Research Product, CA, USA). The concentrations of lysate proteins were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Inc., USA) and bovine serum albumin as the protein standard. The lysates were mixed with 6X sample buffer (0.35 M Tris-HCL pH 6.8, 0.35 M SDS, 30% (v/v) glycerol, 0.6 M dithiothreitol and 0.175 mM bromophenol blue) and heated at 95°C for 4 min. Equal amounts (40 to 60 µg) of proteins were subjected to SDS-PAGE and electroblotting onto PVDF membranes using a Hoefer TE22 tank transfer unit (Hoefer, Inc., CA, USA). Membranes were blocked with 5% skim milk (Bio-Rad Laboratories, Inc. USA) in Tris-buffered saline/Tween-20 for 1 h and subsequently incubated with the corresponding primary antibodies for 90 min at room temperature. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody at 1:80,000 for 90 min. Antibody-bound proteins were detected by the Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore Corporation, USA) and exposed to X-ray film.

Identification of major compounds present in EAF by HPLC-DAD

The presence of the major components of the EAF was determined using HPLC-DAD as previously described by Akowuah et al. (2005) and Olah et al. (2003). The HPLC system consisted of Agilent 1100 HPLC coupled with a UV array detector (DAD). The UV wavelengths were monitored at 210, 220 and 254 nm. A Synergy Fusion-RP C18 (150 x 4.6 mm, Phenomenex, Torrance, CA, USA) was used to separate the EAF. The mobile phase was solvent A (water-0.1% TFA) and solvent B (acetonitrile-0.1% TFA). The gradient used was 0 to 20 min, linear gradient from 15 to 65% B. The flow rate was 1.0 ml/min, and the sample injection volume was 5 µl. Major components were identified based on peak area. For identification, we compared the retention time and UV spectra of major components with commercial standard compounds.

Statistical analysis

All values were expressed as means ± standard deviation, and statistical analysis was performed by analysis of variance (ANOVA). p values less than 0.05 were considered significant.

RESULTS

The effect of the EAF and rosmarinic acid on cell viability of HepG2 and Chang Liver cells

The effect of the EAF and rosmarinic acid on the viability of HepG2 and Chang Liver cells after 72 h incubation were assessed by the SRB assay. Figures 1A and B showed that EAF and rosmarinic acid were associated with decreased cell viability in HepG2 and Chang Liver cells in a dose-dependent manner. The IC_{50} values (concentrations required for 50% growth inhibition) of the EAF on HepG2 and Chang Liver cells were calculated as 130.82 ± 17.64 µg/ml and 497.52 ± 74.62 µg/ml, respectively (Figure 1A). The IC_{50} values of rosmarinic acid on HepG2 and Chang Liver were calculated as 270.14 ± 73.33 µg/ml and 190.00 ± 38.46 µg/ml, respectively (Figure 1B).

Morphological changes of HepG2 cells induced by the EAF

Figure 2 shows representative photographs of HepG2 cells treated with various concentrations of the EAF (0, 100, 200, 400 and 800 µg/ml) for 48 h and stained with Hoechst 33342. Results indicated that HepG2 cells treated with the EAF displayed a typical apoptotic form of

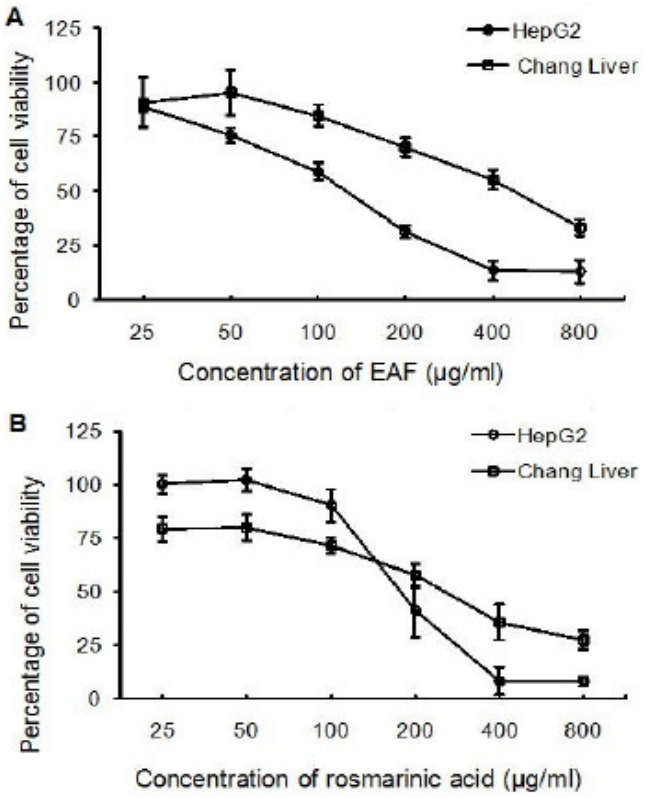

Figure 1. Effect of EAF (A) and rosmarinic acid (B) on the viability of HepG2 and Chang Liver cells after incubation for 72 h. Cells viability was determined by the SRB assay. Each data point represents the average value from three independent experiments (n=3).

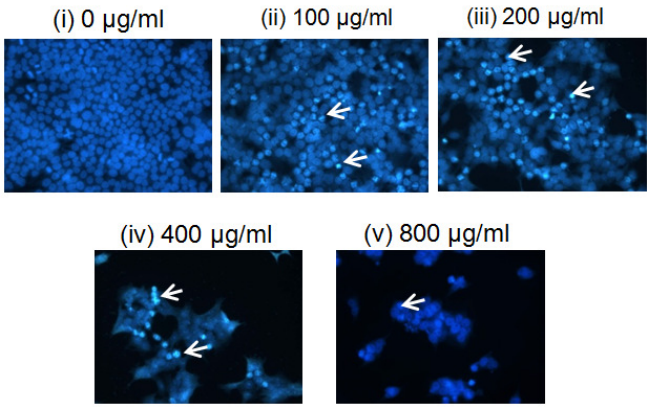

Figure 2. Morphological changes in the nuclei of HepG2 cells induced by the EAF. HepG2 cells were exposed with various concentrations of the EAF for 48 hrs and staining with Hoechst 33342. Cell with condensed chromatin and/or fragmented nuclei was defined as apoptotic (arrows, magnification x200).

nuclei that included chromatin condensation and fragmentation. The nuclei of untreated cells were stained homogenously.

EAF induced loss of mitochondrial transmembrane potential (MTP) in HepG2 cells

Disruption of the mitochondrial membrane is a hallmark of the early cellular events of apoptosis (Kroemer et al., 2007; Ly et al., 2003). Figure 3A shows representative photographs of JC-1 assays on HepG2 cells after treatment with or without the EAF for 24 h. The red (normal of MTP) and green (loss of MTP) fluorescent cells were counted as healthy cells and apoptotic cells, respectively. Figure 3B shows the percentage of apoptotic cells after in treated with various concentrations of the EAF and 4 µg/ml camptothecin (positive control). In vehicle control (0 µg/ml), the percentage of apoptotic cells was very low. However, after exposure to the EAF or camptothecin, the percentage of apoptotic cells was significantly increased ($p \leq 0.05$). These results suggested that the EAF induced apoptosis-associated loss of MTP in HepG2 cells in a dose-dependent manner.

Effects of EAF on Bax and Bcl-2 in HepG2

Figure 4A shows the level of Bax and Bcl-2 in the EAF-treated HepG2 cells. After exposure of HepG2 cells to the EAF (12.5 - 200 µg/ml) for 24 h, the expression level of Bax was increased consistently, but the expression level of Bcl-2 was slightly decreased. These results suggested that the expression levels of Bcl-2 family proteins modulated apoptosis induced by the EAF in Hep G2 cells.

Effects of EAF on caspases activation in HepG2 cells

After exposure to the EAF, the level of the active subunits of caspase-3 (17 kDa) and caspase-9 (37 kDa) were increased and the level of the procaspase-8 (55 kDa) was decreased (Figure 4A). These results suggested that the induction of apoptosis in HepG2 cells caused by the EAF was associated with activation of caspases-3,-7 and -8.

Effects of the EAF on NFκB p65 and Cox-2 in HepG2

Figure 4B shows the level of NFκB p65 and Cox-2 in HepG2 cells after exposure to the EAF at various concentrations for 24 h. In nuclear fractions, the levels of NFκB p65 were increased and this protein were nearly absent after exposure to 100 µg/ml of the EAF. In contrast, the levels of NFκB p65 from cytosolic fractions remained at the basal levels. The level of Cox-2 decreased as the EAF concentration increased. These results suggested that the induction of apoptosis in HepG2 cells induced by the EAF was associated with the activation of NFκB p65 and Cox-2.

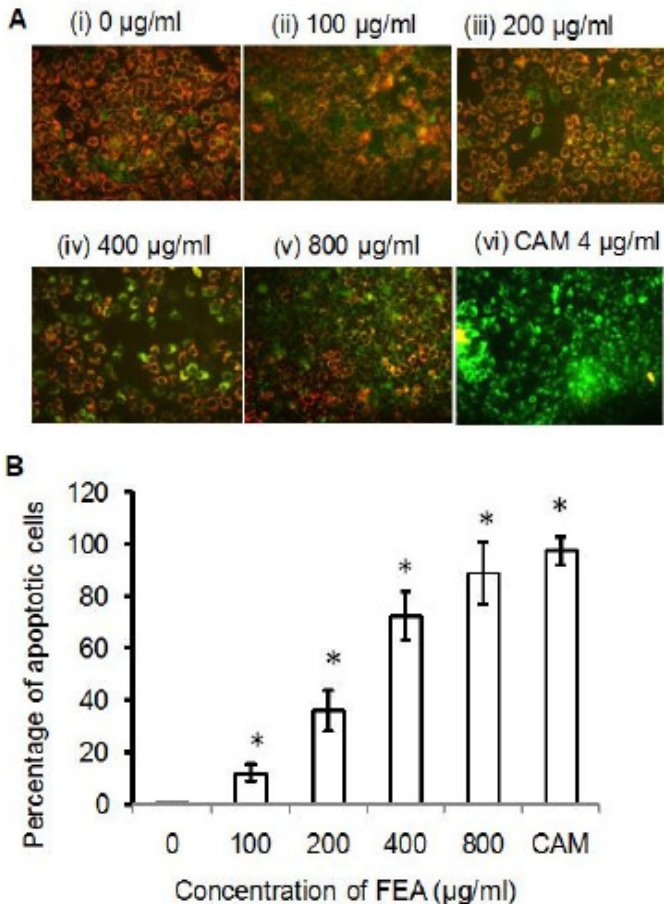

Figure 3. Analysis of mitochondrial transmembrane potential in HepG2 cells after treatment with various concentrations of the EAF for 24 h and staining with JC-1. Cells were photographed at a magnification of x200 with a fluorescence microscope. (A): Representative photographs of HepG2 cells after treatment without EAF (i) or with EAF (ii-v) and camptothecin (CAM, vi). Red fluorescent cells: cells with intact mitochondrial transmembrane potential, considered healthy cells. Green fluorescent cells: cells with a loss of mitochondria transmembrane potential, considered apoptotic cells. (B): Percentages of apoptotic cells at different concentrations of the EAF and camptothecin (CAM, 4 µg/ml). Each point represents the mean ± standard deviation of three independent experiments. *p < 0.05, significant differences compared to vehicle control.

High performance liquid chromatography (HPLC) analysis

Thirty-six peaks were detected in HPLC chromatogram of the EAF (Figure 5). Two major peaks were eluted at 5.620 and 7.714 min, which represented about 20.18 and 31.83% of chromatogram area, respectively. UV spectra analyses of the major peaks indicated that the compounds were from the phenols group. Therefore, we compared our results with standard water soluble polyphenols commonly present in *O. stamineus,* as reported elsewhere (Olah et al., 2003; Akowuah et al., 2005). The compounds eluted at 5.62 and 7.714 min were identified as caffeic acid and rosmarinic acid, respectively.

DISCUSSION

In this report, we presented the *in vitro* chemopreventive activity of the EAF in the human hepatoma cell line HepG2. The EAF was derived from the ethyl acetate fraction of the hot water extract of *O. stamineus* leaves. We have employed the HepG2 cell line because it is widely used for *in vitro* models evaluating chemopreventive agents and is also very well characterized (Slany et al., 2010). Anti-proliferative assays using human cancer cell lines are utilized as a first-line parameter in the evaluation of chemoprevention agents (Crowell, 2005; De Flora and Ferguson, 2005). Despite considerable work on the anti-proliferative activity of organic extracts of *O. stamineus*, a comparative study on water extract has not been conducted. In the present study, we have demonstrated, for the first time, the anti-proliferative activity of the EAF derived from hot water extract *O. stamineus* leaves. The results presented here showed that the EAF exhibited anti-proliferative activities in a concentration-dependent manner in HepG2. These results encouraged us to further investigate the chemopreventive activities of the EAF in HepG2 cells.

Apoptosis is a crucial target in the prevention of cancer (De Flora and Ferguson, 2005; Pan et al., 2008; Sun et al., 2004). Thus, the induction of apotosis in human cancer cell lines is considered a therapeutics parameter in evaluating candidate chemopreventive agents (Bode and Dong, 2004; Sun et al., 2004) such as curcumin and resveratrol (Bishayee et al., 2010; Reuter et al., 2008). In the present study, we demonstrated that EAF induced apoptosis in HepG2 cells, as determined by Hoechst 33342 and JC-1 assays. We also revealed that the induction of apoptosis by the EAF in HepG2 cells occurred in a concentration-dependent manner. To the best of our knowledge, this is the first report on apoptotic activities of extracts from *O. stamineus* leaves in HepG2 cells.

Caspases (cysteine aspartate-specific proteases) play important roles in signal transduction cascades that culminate in apoptosis. Caspases signaling converges with mitochondria-mediated pathways and death receptor-mediated pathways (Schultz and Harrington, 2003). The activation of mitochondria-mediated pathways is influenced of pro-apototic Bcl-2 family protein (e.g., Bax, Bok, Hrk and Bad) and anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2, Bcl-X_L and Mcl-1) (Antonsson and Martinou, 2000; Pellegrini and Strasser, 1999). Increases in pro-apoptotic proteins and decreases in anti-apoptotic proteins result in the releases of cyctochrome c from the mitochondria into the cytosol. The presence of free cytochrome c in the cytosol will lead to the formation of the apoptosome, which, in turn, activates an initiator,

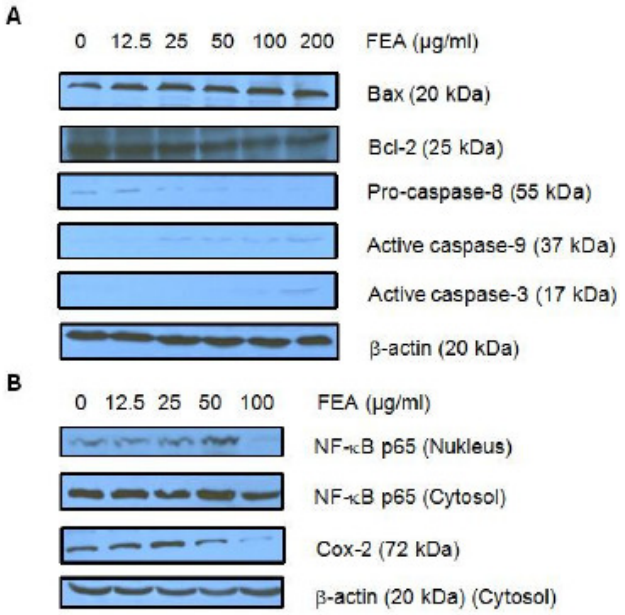

Figure 4. Western blotting analysis of apoptotic-related proteins in HepG2 cells after treatment with the EAF for 24 h. (A): Representative immunoblots of Bax, Bcl-2, procaspase-8, active caspase-3 and active caspase-9. (B): Representative immunoblots of NFκB p65 from cytosolic and nuclear fractions, and Cox-2. β-actin was used as a protein loading control.

caspase-9, subsequently activating downstream effector caspases (caspase-3, -6, or/and -7) and proceeding to an irreversible apoptotic cell-death mechanism (Debatin 2004). Death receptor-mediated pathways are triggered when death receptor ligands (e.g., tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand or Fas ligand) binds to corresponding death receptors, transmembrane receptors with ligands binding sites on the outer surface of cell membrane. Interactions of the death ligands with receptors can results in the activation of death-inducing signal complexes, which, in turn, activate caspase-8 and downstream caspases (Schultz and Harrington, 2003). In the present study, we demonstrated that in the EAF-treated cells, the level of the anti-apototic protein, Bcl-2, was decreased, and the level of pro-apototic Bax-2 protein was increased; caspase-8, capase-9 and caspase-3 were also activated. Therefore, we suggest that the mitochondria-mediated pathways and death receptor-mediated pathways are both involved in the EAF-induced apoptosis in HepG2 cells.

The NFκB family consists of p50, p52, p65, c-REL, and REL-B proteins, which are associated with various cellular processes, such as apoptosis resistance, oncogenesis, and cell-cycle activation, and are activates in a number of cancerous cells (Garg and Aggarwal, 2002). In the cytoplasm, NFκBs form inactive homodimers or heterodimers complexes that bind to IκB, and the predominant form of NFκB complex is p65/p50 heterodimers. In the presence of appropriate stimuli, such as inflammatory cytokines, bacterial lipopolysaccharide, viral infection or stress triggers the degradation of IκB, thereby allowing free NFκBs to enter the nucleus. In the nucleus, the binding of NFκBs to a specific sequence in a promoter region of genes triggers the transcriptional activation of NFκB-regulated genes (Vermeulen et al., 2002). NFκBs up-regulate of anti-apoptotic genes such as Bcl-2, Bcl-XL, Mcl-1 and inhibitor of apoptosis protein (IAP). NFκBs also up-regulate Cox-2, which promotes cell survival. In cancer cells, NFκBs are always activated, and the inhibition of NFκBs can induce cancer cells to undergo apoptotic cell death (Bharti and Aggarwal, 2002). In this study, we found that the level of NFκB p65 in the nucleus was markedly reduced in the EAF-treated HepG2, but the level of NFκB p65 in the cytosol was comparable to control. This observation suggested that the EAF may inhibit the translocation of NFκB p65 from the cytosol into the nucleus and thus contribute to apoptosis. The possible mechanisms of EAF-inhibited translocation of NFκB p65 from the cytosol into the nucleus in HepG2 cells remain to be studied.

Cox-2 is an enzyme that converts arachidonic acid into prostaglandins and thromboxanes; proteins involved in inflammations and mitogenenesis (Diaz and Arm, 2003). Cox-2 is always over-expresses in human solid cancers and this protein also plays a crucial role in cancer development (Hull, 2005). The roles of Cox-2 expression in cancer prevention have been reported elsewhere (Prescott and Fitzpatrick, 2000; Subbaramaiah and Dannenberg, 2003). For examples, the effects of chemopreventive agents such as curcumin, resveratrol, theaflavins and gingerol that induced apoptosis in human cancer cells lines are associated with a reduction in Cox-2 protein expression (Bode and Dong, 2004). In the present study, we demonstrated that the EAF was reduced the levels of Cox-2 protein in HepG2 cells. It is therefore reasonable to propose that a reduction in the level of Cox-2 might contribute to the induction of apoptosis in the EAF-treated HepG2 cells.

In this study, the components present in the EAF were analyzed by HPLC-DAD. Caffeic acid and rosmarinic acid were identified as the main chemical constituents of the EAF, representing 20.18 and 31.83% of the HPLC chromatogram area, respectively. Caffeic acid and rosmarinic acid are the most abundant polyphenols in the polar solvent extraction of *O. stamineus* leaf (Akowuah et al., 2005; Tezuka et al., 2000; Olah et al., 2003), and both compounds are also distributed in other Lamiaceae species, including *Rosmarinus officinalis*, *Salvia officianalis*, *Thymus vulgaris* and *Origanun heracleoticum* (Janicsak et al., 1999). Recent *in vitro* studies have shown that rosmarinic acid and caffeic acid exhibit chemopreventive activities in several cell lines (Cheung and Tai, 2007; Lin et al., 2007). A study by Chung et al.

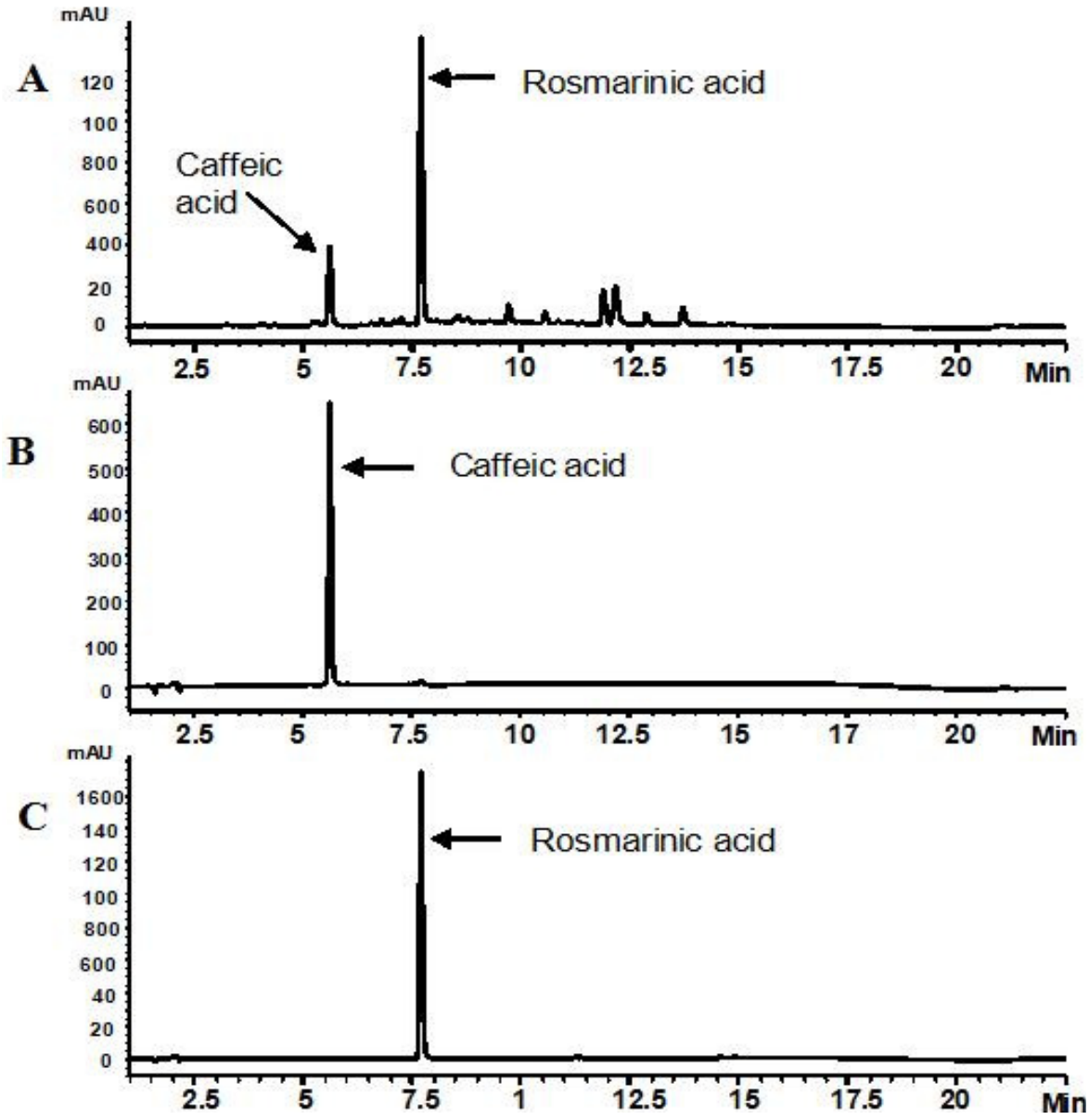

Figure 5. Representative HPLC chromatograms of the EAF (A), caffeic acid (B) and rosmarinic acid (C) standards.

(2004) showed that in HepG2 cells, caffeic acid inhibited cell proliferation, induced apoptotic cells death and inhibited NFκB p65 translocation into nucleus. In a nude mice tumor model, caffeic acid also exhibited anti-tumor activity. Hur et al. (2004) has found that rosmarinic acid-induced apoptosis in the Jurkat cell line was associated with the loss of MPT and activation of caspase-3, caspase-8 and caspase-9. Therefore, we suggest that rosmarinic acid and caffeic acid may synergistically contribute to apoptotic activity in EAF-treated cells.

In short, our study has demonstrated that the EAF-induced apoptosis in HepG2 cells occurs through the activation of the mitochondria-mediated and death receptor-mediated pathways, involves NFκB p65 and Cox-2. Our research on the mechanism of the EAF activity on HepG2 cells may lead to the development of traditional herbs for human liver cancer prevention.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Health, Malaysia (Reference number: JPP-IMR 07-029).

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