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Original Research Article

Ebracteolatain A and Ebracteolatain B Induce Apoptosis of Human Hepatoma Cell Line (HepG2)

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Abstract

Purpose: *To evaluate the effects of ebracteolatain A (EA) and ebracteolatain B (EB) from Euphorbia ebracteolata Hyata (Euphorbiaceae) on the proliferation of HepG2 cells and the possible mechanisms.*
Methods: *EA and EB from E. ebracteolata were obtained by column chromatography. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometry assays were used to study the cytotoxic and pro-apoptotic activities of EA and EB against HepG2 cells. Western blot assay was used to investigate the possible mechanisms of action.*
Results: *EA and EB were successfully isolated from E. ebracteolata by column chromatography. The results of MTT assay indicate that EA and EB have significant anti-proliferative activities against HepG2 cells in dose- and time-dependent manners with half maximal inhibitory concentration (IC_{50}) of 28.48 and 31.72 μg/mL. respectively. The results of flow cytometry assay suggest that EA and EB significantly ($p < 0.01$) induced the apoptosis of HepG2 cells at the levels of 47.45 and 42.26 %, respectively. Western blot data indicate that EA and EB significantly ($p < 0.05$ or 0.01) down-regulated the expression levels of anti-apoptotic proteins (survivin and Bcl-2) and up-regulated the expression levels of pro-apoptotic proteins (Smac, Bax, c-caspase-3 and c-caspase-9) in mitochondria-mediated apoptotic pathway.*
Conclusion: *EA and EB inhibit the proliferation of HepG2 cells, the probable mechanisms being associated with mitochondria-mediated apoptosis.*

Keywords: *Euphorbia ebracteolata, Phloroglucinol derivatives, Mitochondria-mediated apoptosis, Flow cytometry, Western blot*

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INTRODUCTION

Euphorbia ebracteolata Hyata (Euphorbiaceae) is cultivated in Japan, Korea and China [1] and its dried roots, named “Lang Du”, are usually used to treat ascites, edema, chronic tracheitis, pulmonary tuberculosis and cancer [2]. Currently, a few researches have reported the anti-cancer activities of *E. ebracteolata* [3-6]. It is reported that the water extract of *E. ebracteolata* induces the apoptosis of P388 lymphocytic leukemia cells by regulating the expression levels of Bax and Bcl-2 [3]. The 2, 4-dihydroxy-6-methoxy-3-methylacetophenone from *E. ebracteolata* has significant inhibitory effect on the growth of U14 cervical cancer cells [4]. Yuexiandajisu D from *E. ebracteolata* inhibits the proliferation of HCT-8, KB, A549, Bel-7402 and BGC-823 cells [5]. Jolkinolide B and ent-11α-hydroxyabieta-8(14),13(15)-dien-16,12α-olide from *E. ebracteolata* show cytotoxicity activities against ANA-1, B16 and Jurkat cells [6]. In addition, the

terpenoids from *Euphorbia* L. show cytotoxic activities against SMMC-7221, L342, MCc80-3, U937 and HeLa cells, and their anti-cancer mechanisms may be related to apoptosis [7,8].

Ebracteolatain A (EA) and ebracteolatain B (EB) from *E. ebracteolata* are phloroglucinol derivatives. Phloroglucinol derivatives such as dryofragin and 2,4-bis(2-fluorophenylacetyl) phloroglucinol exhibits anti-cancer effects [9-11]. Based on these reports, we supposed that EA and EB may have anti-cancer effects. In this work, we used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and flow cytometry assays to investigate the effect of EA and EB on the proliferation and apoptosis of HepG2 cells *in vitro* by determining their inhibition rates and apoptosis rates. Moreover, the Western blot assay was used to explore the possible mechanisms.

EXPERIMENTAL

Materials

The roots of *E. ebracteolata* were obtained from Zhong Yao Cai Tian Di Internet (www.zyctd.com) in March 2013, which were identified by Gang Peng from Suizhou Hospital, Hubei University of Medicine. A voucher specimen (Herbarium No. 201303154) of this plant is stored in Hubei University of Medicine for future reference.

Analytical grade reagents (petroleum, chloroform, n-butyl alcohol and methanol), silica gel, preparative TLC and Sephadex LH-20 were provided by Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China) or H&E Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) and RPMI-1640 medium were provided by Invitrogen (Carlsbad, CA, USA). MTT and DMSO were provided by Sigma (MO, USA). Enhanced BCA Protein Assay Kit and Annexin V-FITC/PI apoptosis assay kit were provided by Beyotime (Shanghai, China) and BD Bioscience (San Diego, CA, USA), respectively. β-actin, survivin, Bcl-xl, Bcl-2, Smac, Bax, cleaved-caspase-3 (c-caspase-3), cleaved-caspase-9 (c-caspase-9) antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody were purchased form Cell Signaling Technology (Beverly, MA, USA) and Abcam Biotechnology (Cambridge, MA, USA).

Isolation of EA and EB

The air-dried roots (10 kg) of *E. ebracteolata* were smashed and extracted thrice with 95 % 60 L of ethanol. After filtering, the combined ethanol solution was evaporated by rotary evaporator under high vacuum to obtain ethanol extract (1357 g), which was suspended in water and subsequently partitioned with petroleum, chloroform and n-butyl alcohol. The chloroform fraction (234 g) was subjected to 200 - 300 mesh silica gel column chromatography eluting with chloroform-methanol (19:1, 9:1, 7:3, 5:5) to obtain 7 fractions. Fraction 2 (17.5 g) was subjected to Sephadex LH-20 column chromatography eluting with chloroform-methanol (5:5) to obtain EA (121 mg). Fraction 6 (10.3 g) was separated on 200-300 mesh silica gel column and preparative TLC to obtain EB (53 mg). The chemical structures and purities of EA and EB were identified and analyzed by nuclear magnetic resonance (NMR) and high performance liquid chromatography (HPLC).

Cell culture

HepG2 cells were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). According to the existing literature [12], HepG2 cells were cultured in RPMI-1640 medium supplemented with 10 % FBS, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in 5 % CO_2/95 % air. HepG2 cells were sub-cultured to logarithmic phase of growth, and then assays were carried out on the re-cultured HepG2 cells.

Cell proliferation assay

The effects of EA and EB on the proliferation of HepG2 cells were evaluated using MTT assay according to the existing literature [13]. Briefly, HepG2 cells were seeded onto 96 well cell culture plates at 5000/well and incubated for 24 h at 37 °C [14]. Then HepG2 cells were treated with EA or EB at different concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μg/mL for 48 h or at same concentrations of 30 μg/mL for 0, 12, 24, 36 and 48 h. After treatments, 20 μL MTT solution (5 mg/mL, dissolved in PBS) was added into each well incubated at 37 °C in 5 % CO_2/95 % air for another 3 h to generate purple-blue formazan precipitate. Subsequently, DMSO (200 μL) was added into each well to dissolve the purple-blue formazan precipitate and the optical density (OD) of the DMSO solution was measured at 570 nm in a microplate reader (Bio Rad, Model 680). The inhibition rates were used to assess the effects of EA and EB on the proliferation of HepG2 cells and calculated using the following equation:

Inhibition rate (%) = (OD control – OD treatment)/OD control × 100.

Flow cytometry analysis

After treatment with EA or EB at concentration of 30 μg/mL for 48 h, HepG2 cells were harvested and washed thrice with PBS. Subsequently, the washed HepG2 cells were re-suspended and stained with Annexin V-FITC/PI, and then analyzed by flow cytometer according to the manufacturer's instructions. The early apoptotic cells were a kind of Annexin V-FITC-positivity and PI-negativity cells, and the late apoptotic cells were a kind of Annexin V-FITC-positivity and PI-positivity cells [15]. The numbers of apoptotic cells were calculated as the total numbers of the early and late apoptotic cells in flow cytometry.

Western blot analysis

HepG2 cells were collected to extract total proteins after treatment with EA or EB at different concentrations of 15, 30 and 45 μg/mL for 48 h. The Enhanced BCA Protein Assay Kit was used to determine the concentration of total proteins. Then equal amounts of total proteins (about 40 μg) were separated on 12 % sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and blotted on PVDF membrane. After blocking with 5 % fat-free milk in Tris buffered saline-Tween (TBS-T), the PVDF membranes were incubated with primary antibodies including anti-β-actin, anti-Survivin, anti-Bcl-xl, anti-Bcl-2, anti-Smac, anti-Bax, anti-c-caspase-3, anti-cleaved c-caspase-9 overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody for 1 h at room temperature. Chemiluminescence detection was used to detect all proteins. To assess the proteins loading, β-actin was selected as the internal control, and the expression levels of other proteins were expressed as proteins level/β-actin level.

Statistical analysis

The results are presented as mean ± standard deviation (SD) (n=3). Differences between different groups were analyzed by one-way ANOVA (Dunnett test) on SPSS 19.0. *P*-value less than 0.05 or 0.01 was taken as statistically significant.

RESULTS

Identifications and purities analysis of EA and EB

The target analytes were identified as EA and EB by comparing their NMR data with the literature [1] and their chemical structures are shown in Figure 1. The results of HPLC area normalization method indicated that the purity levels of EA and EB were 93.0 % and 92.8 %, respectively.

EA: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.24 (1H, s, H-5), 3.75 (3H, s, H-2-OMe), 2.48 (3H, s, H-3-Ar), 2.52 (3H, s, H-3'-Ac), 3.78 (3H, s, H-4'-OMe), 1.89 (3H, s, H-5'- CH_3), 3.62 (2H, s, H-Ar-CH_2-Ar); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 105.3 (C-1), 156.5 (C-2), 105.2 (C-3), 162.7 (C-4), 92.5 (C-5), 163.1 (C-6), 55.8 (C-2-OMe), 33.1 (C-3-Ac), 204.7 (C-3-Ac), 111.1 (C-1'), 162.5 (C-2'), 107.9 (C-3'), 163.5 (C-4'), 111.4 (C-5'), 160.4 (C-6'), 31.7 (C-3'-Ac), 202.4 (C-3'-Ac), 64.7 (C-4'-OMe), 8.3 (C-5'-CH_3), 16.5 (C-Ar-CH_2-Ar).

EB: ^{1}H-NMR (400 MHz, pyridine-d_5) δ: 2.47 (3H, s, H-1-Ac), 5.78 (2H, s, H-5', 5''), 3.21 (6H, s, H-4', 4''-OMe), 2.18 (6H, s, H-3', 3''-Ac), 4.03 (4H, s, H-Ar-CH_2-Ar); ^{13}C-NMR (100 MHz, pyridine-d_5) δ: 103.4 (C-1), 161.5 (C-2), 108.2 (C-3), 170.8 (C-4), 108.2 (C-5), 161.5 (C-6), 32.6 (C-1-Ac), 201.4 (C-1-Ac), 108.9 (C-1', 1''), 164.6 (C-2', 2''), 105.6 (C-3', 3''), 161.9 (C-4', 4''), 93.4 (C-5', 5''), 166.7 (C-6', 6''), 55.0 (C-4', 4''-OMe), 32.3 (C-3', 3''-Ac), 204.1 (C-3', 3''-Ac), 17.6 (C-Ar-CH_2-Ar).

Effects of EA and EB on the proliferation of HepG2 cells

As shown in Figure 2, after treatment with EA or EB at different concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 μg/mL for 48 h, the proliferation of HepG2 cells was significantly inhibited in dose-dependent manner and their half maximal inhibitory concentration (IC_{50}) values were 28.48 and 31.72 μg/mL, respectively. After treatment with EA or EB at same concentration of 30 μg/mL for 0, 12, 24, 36 and 48 h, the proliferation of HepG2 cells was significantly inhibited in time-dependent manner.

HepG2 cells apoptosis induced by EA and EB

The results of flow cytometry analysis (Figure 3) suggested that after treatment with EA or EB at concentration of 30 μg/mL for 48 h, the apoptosis of HepG2 cells were significantly ($p < 0.01$) induced, compared with the control, and their

apoptosis rates were 47.45 and 42.26 %, respectively.

EA and EB regulate the expression levels of apoptotic proteins

As depicted in Figures 4 and 5, EA and EB had similar effects on the expression levels of these apoptotic proteins (Survivin, Bcl-xl, Bcl-2, Smac, Bax, c-caspase-3 and c-caspase-9). After treatment with EA or EB at different concentrations of 15, 30 and 45μg/mL for 48 h, the expression levels of anti-apoptotic proteins (Survivin and Bcl-2) were significantly ($p < 0.01$) down-regulated and the expression levels of pro-apoptotic proteins (Smac, Bax, c-caspase-3 and c-caspase-9) were significantly ($p < 0.01$ or 0.05) up-regulated, compared with the control, whereas the expression level of anti-apoptotic protein (Bcl-xl) was not affected by the treatment.

DISCUSSION

Many study reports [3-6] have confirmed that *E. ebracteolata* can be used to treat cancer. In the present study, we studied the effects of EA and EB from *E. ebracteolata* on the proliferation of HepG2 cells and the possible mechanisms in vitro by MTT, flow cytometry and Western blot assays. The results showed that EA and EB inhibited the proliferation of HepG2 cells by inducing apoptosis via mitochondria-mediated apoptotic pathway.

MTT, a yellow dye, interacts with succinodehydrogenase (SDH) derived from mitochondria of living cells to generate the purple-blue formazan precipitate, but dead cells do not have the function [13]. Then formazan precipitate was dissolved in DMSO and the OD value of the DMSO solution was used to evaluate cell viability. The smaller the OD value is, the lower the cell viability is. Based on the theory, MTT assay is usually used to evaluate the effects of anticancer agents on proliferation of cancer cells [16]. The results of MTT assay (Figure 2) suggested that EA and EB significantly inhibited the proliferation of HepG2 cells.

The externalization of phosphatidylserine (PS) is observed in early apoptotic cells and the Annexin V can combine with the external PS. PI can combine with cell nucleus through the cell membrane in late apoptotic cells. After early or late apoptotic cells were stained with Annexin V-FITC/PI, they can be analyzed by flow cytometry [17]. The results of flow cytometry analysis (Figure 3) indicated that EA and EB induced the apoptosis of HepG2 cells. The results of MTT assay and flow cytometry analysis indicated that the anti-proliferative activities of EA and EB are associated with apoptosis.

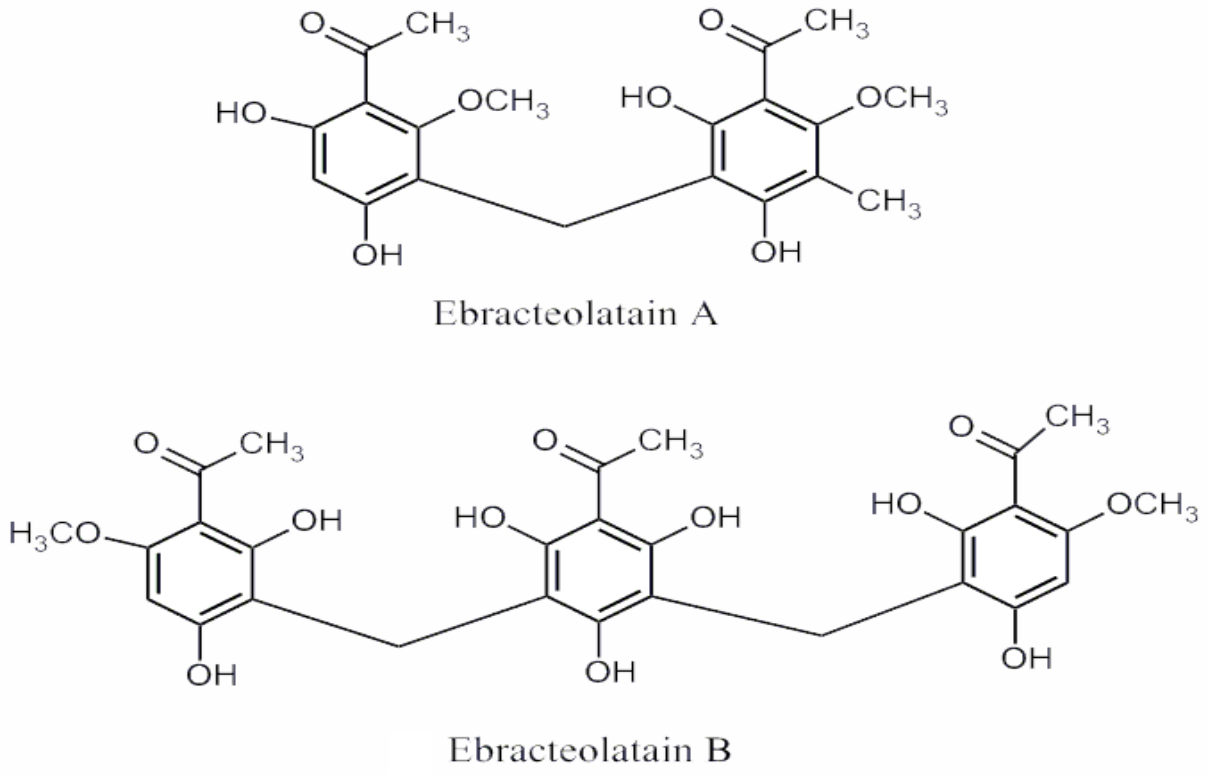

Figure 1: Chemical structures of ebracteolatain A (EA) and ebracteolatain B (EB)

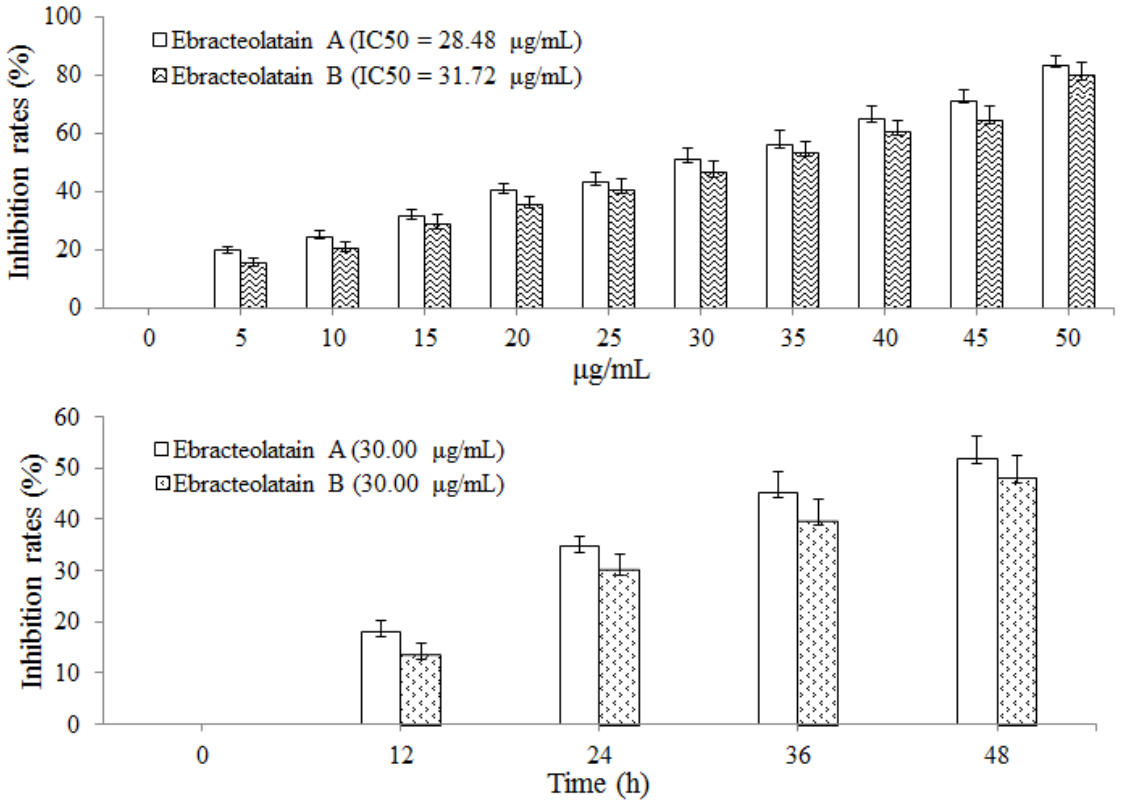

Figure 2: Inhibitory effects of ebracteolatain A (EA) and ebracteolatain B (EB) on the proliferation of HepG2 cells

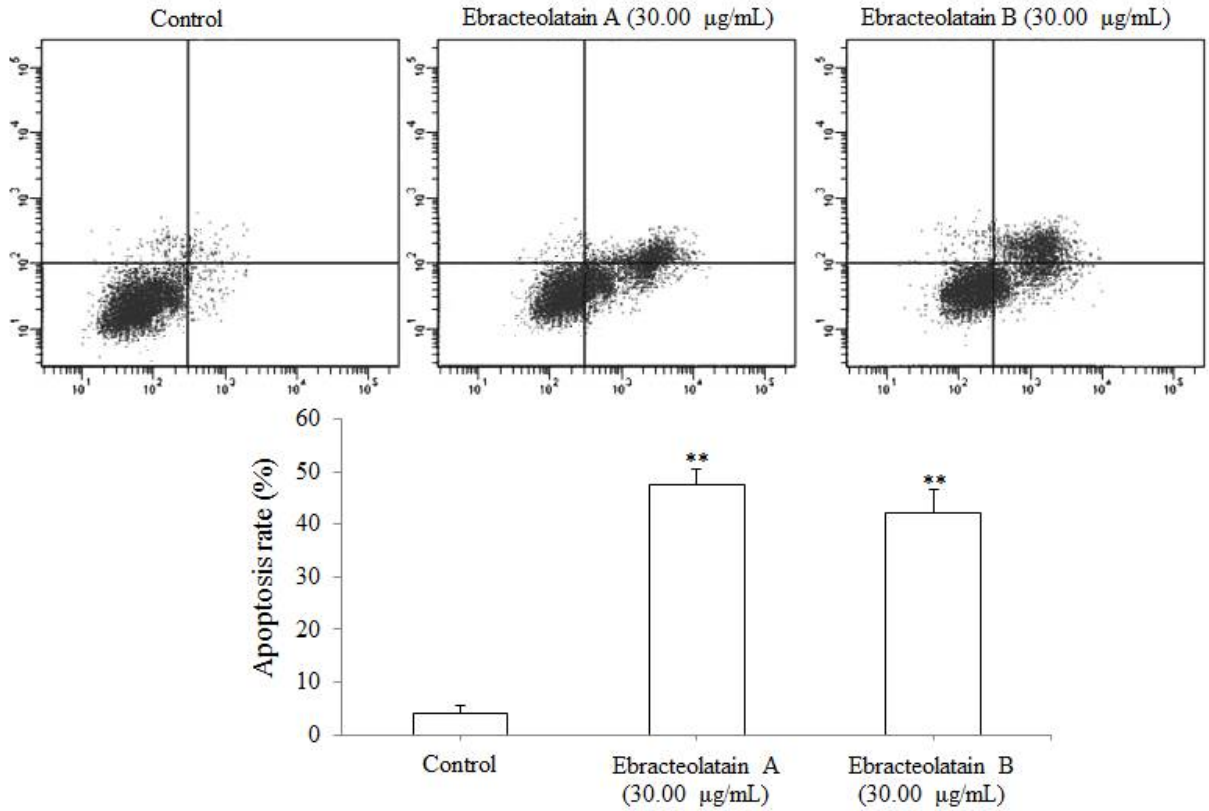

Figure 3: Effects of ebracteolatain A (EA) and ebracteolatain B (EB) on the apoptosis of HepG2 cells; $^{**}p < 0.01$, vs control

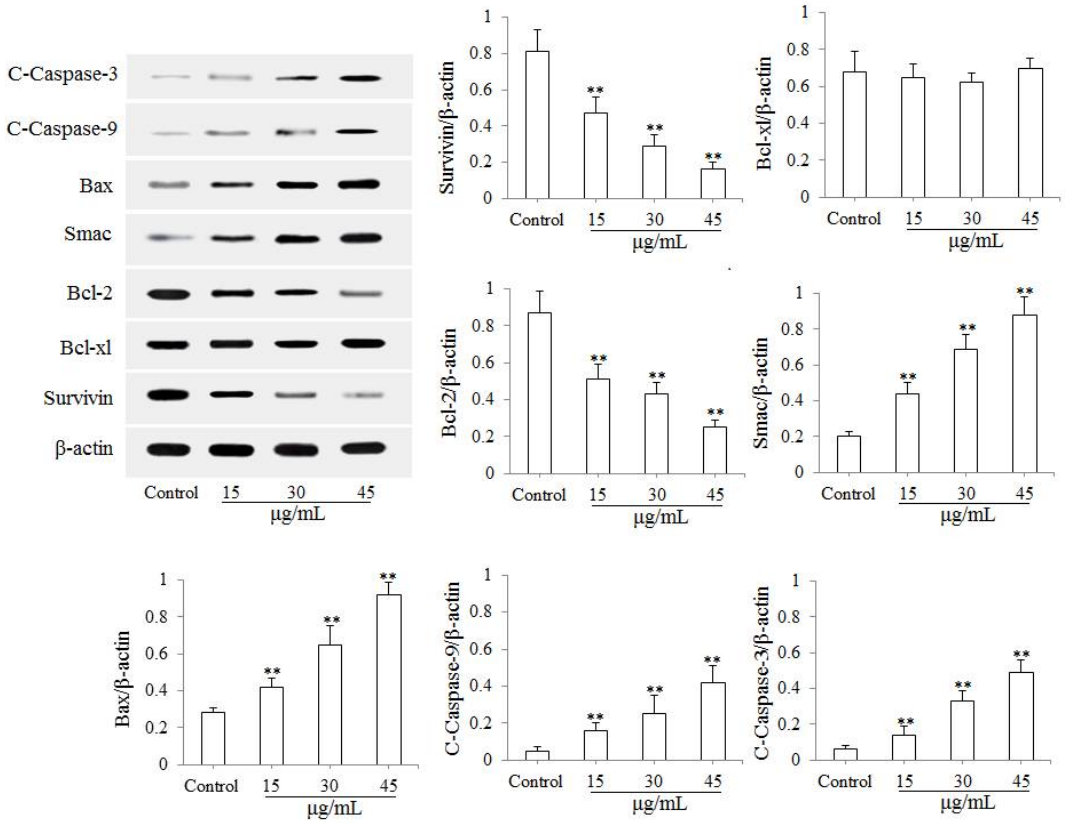

Figure 4: Effects of ebracteolatain A (EA) on the expression levels of anti-apoptotic proteins (Survivin, Bcl-xl and Bcl-2) and pro-apoptotic proteins (Smac, Bax, c-caspase-3 and c-caspase-9); ***p* < 0.01, vs control

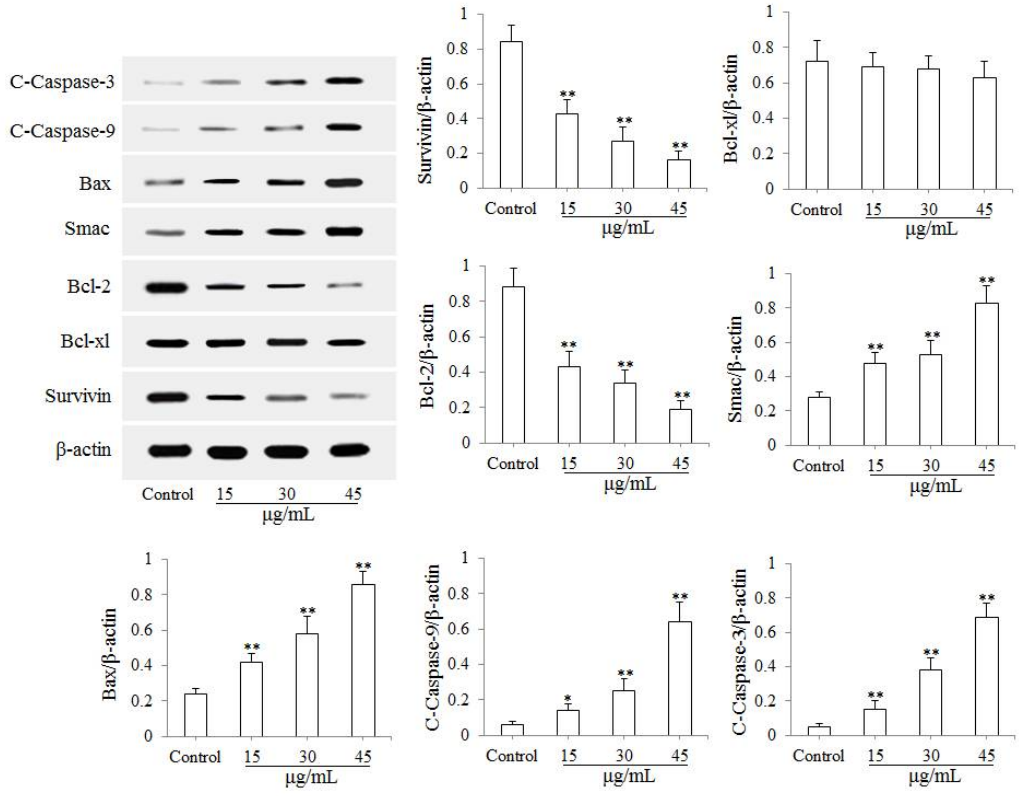

Figure 5: Effects of ebracteolatain B (EB) on the expression levels of anti-apoptotic proteins (Survivin, Bcl-xl and Bcl-2) and pro-apoptotic proteins (Smac, Bax, c-caspase-3 and c-caspase-9); **p* < 0.05, ***p* < 0.01, vs control

Mitochondria-mediated apoptosis is an important pathway that is used to induce the apoptosis of cells, and the apoptotic proteins (Bid, Bax, Bcl-2, Smac, survivin, cytochrome c, caspase-9 and caspase-3) play an important role in mitochondria-mediated apoptotic pathway [18,19]. When cells are stimulated by apoptotic stimuli, mitochondria will make a series of responses to reply the apoptotic stimuli. Firstly, the releases of Smac and cytochrome c from mitochondria to cytoplasm are up-regulated [20], but the releases are inhibited by Bcl-2, whose function can be suppressed by Bax and Bid [21]. Secondly, cytochrome c along with dATP, Apaf-1 and pro-caspase-9 form apotosome and then procaspase-9 is activated to generate c-caspase-9 [22]. Subsequently, caspase-3 is activated by c-caspase-9 to generate c-caspase-3 which induces the apoptosis of cells [23]. Meanwhile, survivin inhibits the generation and function of c-caspase-3 [24]. However, the inhibitory effect of survivin is eliminated by Smac [25]. The results of western blot assay (Figures 4 and 5) indicated that EA and EB significantly down-regulated the expression levels of anti-apoptotic proteins (Survivin and Bcl-2) and up-regulated the expression levels of pro-apoptotic proteins (Smac, Bax, c-caspase-3 and c-caspase-9) without effect on the expression level of anti-apoptotic protein (Bcl-xl), indicating that the pro-apoptotic mechanisms of EA and EB in HepG2 cells were related to mitochondria-mediated apoptosis.

CONCLUSION

The findings of this study indicate that EA and EB inhibit the proliferation of HepG2 cells and the possible mechanisms are associated with mitochondria-mediated apoptosis. The results also provide evidence to support the ethnomedical use of *E. ebracteolata* for the treatment of cancer and as a potential source of lead compounds for the development of anti-hepatoma drugs.

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