Evodiamine Inhibits 12-*O*-Tetradecanoylphorbol-13-Acetate-Induced Activator Protein 1 Transactivation and Cell Transformation in Human Hepatocytes

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Evodiamine Inhibits 12-*O*-Tetradecanoylphorbol-13-Acetate-Induced Activator Protein 1 Transactivation and Cell Transformation in Human Hepatocytes

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ABSTRACT

Evodia rutaecarpa has been used to treat inflammatory digestive disorders in Asian countries. However, little is known about the anti-tumor activities of *E. rutaecarpa* and its bioactive constituent evodiamine (EVO). The aim of this study was to characterize the anti-tumor mechanisms of *E. rutaecarpa* and EVO in human hepatocytes. Human Chang liver cells were transfected with activator protein 1 (AP-1)-luciferase reporter gene and designated as Chang/AP-1 cells. The Chang/AP-1 cells were treated with *E. rutaecarpa* and its bioactive constituents, and challenged with AP-1 stimulator 12-*O*-tetradecanoylphorbol-13- acetate (TPA). The present study showed that methanol extract of *E. rutaecarpa* decreased the TPA-induced AP-1 transactivation in Chang/AP-1 cells, with the EC_{50} value of 24.72 μg/ml. EVO inhibited the TPA-induced AP-1 transactivation and colony formation, with the EC_{50} values of 82 μM and 8.2 μM, respectively. Moreover, EVO significantly diminished the TPA-induced phosphorylation of extracellular signal-regulated kinases (ERKs). These results suggested that EVO treatment suppressed the TPA-induced AP-1 activity via ERKs pathway. In conclusion, EVO inhibited the AP-1 activity and cellular transformation in human hepatocytes, suggesting that EVO was a potential agent for anti-tumor therapy.

Keywords: evodiamine; 12-*O*-tetradecanoylphorbol-13-acetate; activator protein 1; transformation; hepatocellular carcinoma; Chinese Herb

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of death and the fifth most common cause of cancer worldwide. The mortality of HCC has been estimated at more than 500,000 cases per year (Parkin, 2001). Although both diagnostic and surgical techniques have led to a considerable progress in decreasing morbidity and mortality rates, the overall outcome of HCC therapy remains unsatisfied (Giannelli and Antonaci, 2006). Therefore, it is important to clarify the molecular events involved in hepatocarcinogenesis and to identify potential targets for the treatment of HCC.

Inhibition of tumor promotion, a rate-limiting phase in carcinogenesis, has been known to be a potential strategy for preventing carcinogenesis (Trosko, 2001). The transcription factor activator protein 1 (AP-1) is required for the tumor promotion (Angel and Karin, 1991; Hsiang *et al*., 2004). AP-1 is a dimeric protein typically consisting of JUN (c-Jun, JunB, and JunD), FOS (c-Fos, FosB, Fra-1, and Fra-2), and ATF families of proteins (Angel and Karin, 1991). AP-1 dimers bind promoter regions of DNA containing 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive elements to activate the transcription of genes involved in cellular proliferation, transformation, and apoptosis (Angel and Karin, 1991; Dong *et al*., 1994; Sawai *et al*., 1995). For example, exposure of JB6 P^+ cells to TPA or epidermal growth factor induces the anchorage-independent growth and tumorigenicity *in vivo* (Dong *et al*., 1994). Highly AP-1 activity has been shown to be involved in tumor promotion and progression of many types of cancers, such as skin, lung, and breast cancers *in vitro* and *in vivo* (Saez *et al*., 1995; Risse-Hackl *et al*., 1998; Dumont *et al*., 1996). Therefore, downregulation of AP-1 activity by herb extracts may be an effective way to inhibit the tumor promotion (Cheng *et al*., 2007; Hsiang *et al*., 2002).

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Mitogen-activated protein (MAP) kinases are essential members of intracellular signal transduction pathways that regulate cell proliferation and apoptosis. There are three subgroups of MAP kinases: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase. A variety of stimuli, such as TPA (Sawai *et al*., 1995), UV radiation (Lamb *et al*., 1997), growth factors (Lamb *et al*., 1997) and oxidative agents (Pinkus *et al*., 1996), can stimulate AP-1 activity by activating MAP kinases.

Evodia rutaecarpa has been used clinically for centuries in Asian countries to treat inflammatory digestive disorders (ChPC, 2005). Evodiamine (EVO), the major active constituent isolated from *E. rutaecarpa*, is a kind of quinazolinocarboline alkaloids (Chiou *et al*., 1996). EVO increases arterial pressure *in vivo* and has anti-nociceptive, vasorelaxant, (Chiou *et al*., 1992), and anti-tumor activities (Ogasawara *et al*., 2001). However, effects of EVO on tumor promotion remain to be elucidated.

Here, we studied the effects of *E. rutaecarpa* extracts and EVO on TPA-induced hepatocellular transformation *in vitro* and clarified their mechanisms of action in respect to AP-1 activity and MAP kinase/ERKs phosphorylation

MATERIALS AND METHODS

Cell culture and materials. The human Chang liver cell line was purchased from Bioresource Collection and Research Center (Hsin-Chu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified CO_2 ANTITUMOR EFFECT OF EVODIAMINE

atmosphere (5% CO_2). All chemicals were purchased from Sigma (St. Louis, MO, USA) unless indicated. TPA was dissolved in ethanol at 0.5 mg/ml. Neomycin (G-418; Promega, Madison, WI, USA) was dissolved in water. EVO, rutaecarpine, and limonin were dissolved in dimethyl sulfoxide at 100 mM. Plasmids pAP1-Luc and pSV3-neo were purchased from Stratagene (La Jolla, CA, USA) and ATCC, respectively. The plasmid DNA was prepared using the Qiagen plasmid kit (Qiagen, Valencia, CA, USA). Dried fruits of *E. rutaecarpa* were purchased from a Chinese herbal drug store in Taichung. The methanol extract was prepared by mixing herb powder (3 g) in 10 ml of 100% methanol with shaking at 4°C overnight.

Stable transfection. Plasmid pAP1-Luc, containing the luciferase gene driven by AP-1-responsive element, was linearized by *Alw*NI as described previously (Hsiang *et al*., 2002). Cells were co-transfected with 2.5 μg linear pAP1-Luc DNA and 2.5 μg *Eco*RI-linearized pSV3-neo DNA using the SuperFect® transfection reagent (Qiagen). Forty-eight hours later, cells were selected with G-418 (400 μg/ml). A single clone was obtained by limiting dilution and tested using a luciferase assay. A clone that showed a high luciferase activity was selected and designated as Chang/AP-1 cells. The recombinant cell line was maintained in DMEM supplemented with 10% FBS and 400 μg/ml G-418.

Anchorage-independent growth assay. Anchorage-independent growth assay was performed as described previously (Fukazawa *et al*., 1995). Briefly, 96-well plates were coated with poly (2-hydroxyethyl methacrylate) (poly-HEMA) by adding 50 μl of 5 mg/ml poly-HEMA to each well and drying at 37°C for at least 2 d. Cells (1×10^3 - 2.5×10^3)

were cultivated in poly-HEMA-coated 96-well plates for 24 h and then treated with compounds. After a 24-h incubation, cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml) and incubated at 37°C for 4 h. The MTT formazan was then dissolved in 0.04 N HCl/isopropanol, and absorbance values were measured at 570 nm using a microplate reader. The relative colony formation was calculated as (OD value of compound-treated cells/OD value of solvent-treated cells)×100.

Luciferase assay. Chang/AP-1 cells (1 x 10^4) were cultured in 96-well plates at 37°C for 24 h, washed with DMEM, and starved by culturing in DMEM supplemented with 0.1% FBS for an additional 24 h. The cells were then treated with various amounts of compounds and incubated at 37°C for 48 h. Cells were washed with 4 ml ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2), lysed in 350 µl Triton lysis buffer (50 mM Tris-HCl, 1% Triton X-100, and 1 mM dithiothreitol, pH 7.8), and centrifuged at 12,000 *g* for 2 min at 4°C. Luciferase activity was measured by mixing 20 µl cell lysate with 100 µl luciferase assay substrate (Promega, Madison, WI, USA) and read using a luminometer (FB15, Zylux, Maryville, Tenn., USA). The relative AP-1 activity was calculated by dividing the relative light unit (RLU) value of compound-treated cells by the RLU of solvent-treated cells. The 50% inhibition concentration (EC_{50}) was determined as the concentration of compound required to inhibit luciferase activity by 50%.

Cytotoxicity assay. Cells were cultivated in 96-well culture plates. After a 24-h incubation at 37°C. Various amounts of compounds were added to confluent cell monolayers and incubated for another 24 h. Cytotoxicity was measured using the MTT colorimetric assay. Cell viability (%) was calculated as (OD of compound-treated cells/OD of solvent-treated cells) X 100.

Western blot analysis. Chang/AP-1 cells were cultured in 25-cm^2 flasks at 37°C for 24 h, washed with DMEM, and starved in DMEM supplemented with 0.1% FBS for an additional 24 h. Cells were then treated with TPA for various periods, washed with PBS, and lysed with 250 μl sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). The protein concentration of cell lysate was determined using the Bradford method. Proteins (15–20 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked in blocking buffer (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween-20, and 5% skim milk powder) and probed with anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-ERK, or anti-phospho-ERK antibodies (Cell Signaling, Beverly, MA, USA). The bound antibody was detected using a horseradish peroxidase-conjugated anti-mouse antibody followed by chemiluminescence (ECL System, Amersham, Buckinghamshire, UK) and exposed by autoradiography.

Statistical analysis. Data are presented as mean ± standard error. Student's *t* test was used for comparisons between groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

AP-1 was activated by TPA in human hepatocytes

To investigate whether AP-1 activity was induced by TPA in human hepatocytes, we constructed a stable AP-1/luciferase transfectant as previously described (Hsiang et al., 2004). As shown in Fig. 1, TPA significantly increased AP-1 activity in a dose-dependent manner. Cell viability was consistent during TPA treatment. AP-1 activity was increased by 4.1-fold in response to 25 ng/ml TPA. This result indicated that TPA induced AP-1 activity in human hepatocytes.

The methanol extract of *E. rutaecarpa* suppressed TPA-induced AP-1 activation and hepatocellular transformation in human hepatocytes

To evaluate whether *E. rutaecarpa* suppressed cellular transformation and to determine the effective constituents of *E. rutaecarpa*, we extracted *E. rutaecarpa* using various solvents. We analyzed the effects of various solvent extracts of *E. rutaecarpa* on the suppression of TPA-induced AP-1 activity, and the results indicated that potent constituents were present in the methanol extract (Table 1). The EC_{50} value of *E. rutaecarpa* methanol extract for the suppression of TPA-induced AP-1 activation was 24.72 μg/ml. As shown in Fig. 2A,

methanol extract of *E. rutaecarpa* significantly suppressed the TPA-induced AP-1 activity without cytotoxic effect at 25 μg/ml. These findings suggested that *E. rutaecarpa* methanol extract inhibited the TPA-induced AP-1 activity in Chang liver cells. It was also noted that methanol extract of *E. rutaecarpa* gradually activated the TPA-induced AP-1 activity at 0.2-5 μg/ml. This activation may be the stress response of cells because of the complexity of herbal extract. Because AP-1 mediates cell transformation and tumorigenesis (Dong *et al*., 1994), we further examined whether the suppression of AP-1 activation by *E. rutaecarpa* methanol extract resulted in an inhibition of colony formation. As shown in Fig. 2B, *E. rutaecarpa* methanol extract inhibited TPA-induced cellular transformation in a dose-dependent manner. These findings suggested that the methanol extract of *E. rutaecarpa* inhibited TPA-induced AP-1 activity and, in turn, suppressed TPA-induced cellular transformation in Chang liver cells.

EVO downregulated TPA-induced AP-1 activation in human hepatocytes

EVO, rutaecarpine, and limonin are major components of *E. rutaecarpa* methanol extract (Matsuda *et al*., 1998) (Fig. 3A). Therefore, we wanted to identify which bioactive component was responsible for the anti-tumor effect of *E. rutaecarpa.* As shown in Fig. 3B, TPA-induced AP-1 activation was effectively suppressed in a dose-dependent manner by EVO. AP-1 activity was reduced by approximately 40% at 10 μM EVO. The EC_{50} value of EVO was 82 μM. AP-1 activity was increased by 2.5-fold in response to 10 μM rutaecarpine, but AP-1 activity was declined to basal level in response to 100 μM rutaecarpine (Fig. 3C). Additionally, we did not observe an obvious change after limonin

treatment (Fig. 3D). Thus, EVO was the bioactive compound of *E. rutaecarpa* for the suppression of TPA-induced AP-1 activity.

EVO inhibited TPA-induced hepatocellular transformation and regulated TPA-induced AP-1 activation via ERKs pathway

To confirm the anti-tumor effect of EVO, we used an anchorage-independent assay. As shown in Fig. 4A, EVO decreased the number of colonies in a dose-dependent manner. The EC_{50} value of EVO for the inhibition of TPA-induced colony formation in Chang/AP-1 cells was 8.2 μM. Thus, EVO inhibited TPA-induced human hepatocellular transformation *in vitro*.

All three MAP kinase cascades, including ERKs, JNK and p38 pathways, have been shown to mediate AP-1 induction in response to extracellular signals (Shaulian and Karin, 2001). Therefore, we analyzed which pathway was inhibited following exposure of EVO. The MAP kinase proteins and their phosphorylated (activated) forms in hepatocytes were analyzed using Western blotting (Fig. 4B). Phosphorylation of ERKs, p38, and JNK was induced by TPA treatment. However, EVO significantly diminished the TPA-induced phosphorylation of ERK kinases, slightly inhibited the phosphorylation of p38, and did not inhibit the phosphorylation of JNK. These data indicated that ERKs were the target of EVO, which led to the suppression of TPA-induced AP-1 activation in hepatocytes.

DISCUSSION

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E. rutaecarpa has been used to treat gastrointestinal disorders and liver diseases for thousands of years (Li SC, 1596; Wu *et al*., 2002). Previous studies have shown that *E. rutaecarpa* and its constituents evodiamine and rutaecarpine may be effective against IgE-induced allergic diseases, such as atopic dermatitis and rhinitis (Shin *et al*., 2007). The ethanol extracts of *E. rutaecarpa* have also been shown to inhibit lipopolysaccharide-induced nitric oxide production and nitric oxide synthase upregulation in microglial cells (Ko *et al*., 2007). However, there is no direct correlation between *E. rutaecarpa* or evodiamine and anti-tumor or anti-cellular transformation activities. In the present study, we identified for the first time that *E. rutaecarpa* methanol extract inhibited TPA-induced human hepatocellular transformation by blocking AP-1 transactivation. Moreover, EVO was the likely bioactive component of *E. rutaecarpa* that suppressed AP-1 activity in a dose-dependent manner via ERKs pathway.

AP-1 is a pivotal transcription factor that modulates biological functions, such as cell proliferation, differentiation, apoptosis, and neoplastic transformation (Karin *et al*., 1997). Previous studies have indicated that transactivation of AP-1 is required for tumor promotion, and inhibition of AP-1 activity inhibits neoplastic transformation (Hsiang et al., 2004; Dong *et al*., 1994). In this study, our results showed that TPA-induced AP-1 activity and colony formation were decreased in cells treated with methanol extract of *E. rutaecarpa*. We suggested that the *E. rutaecarpa* methanol extract suppressed TPA-induced AP-1 activation and inhibited cell transformation.

EVO, rutaecarpine, and limonin are the major bioactive constituents of *E. rutaecarpa* (Matsuda et al., 1998). Our data showed that evodiamine suppressed the TPA-induced AP-1 activity in a dose-dependent manner, while rutaecarpine gradually induced the

TPA-induced AP-1 activation and the activation was reduced to the basal level at 100 μM. Although evodiamine and rutaecarpine exhibited opposite effects on the TPA-induced AP-1 activity, we speculated that evodiamine was the likely compound responsible for the antitumor effect of *E. rutaecarpa* because evodiamine exhibited similar effect with *E. rutaecarpa*. Previous studies have demonstrated that EVO, rutaecarpine, and limonin exhibit anti-tumor, anti-inflammatory (Matsuda et al., 1998), anti-allergic (Shin *et al*., 2007), and anti-angiogenesis effects (Shyu *et al*., 2006). However, there is no direct link between AP-1 activity and these compounds. In the present study, we showed that EVO but not rutaecarpine or limonin was the major component of *E. rutaecarpa* that suppressed AP-1 activation in a dose-dependent manner. It has been demonstrated that EVO exhibits a more potent anti-migratory activity than rutaecarpine (Ogasawara et al., 2002). Moreover, EVO but not rutaecarpine inhibits cell proliferation and invasion (Ogasawara et al., 2004). A comparison of structural and functional relationships suggested that the presence of a methyl group at N-14 and the configuration of hydrogen at C-13β of EVO may be responsible for its inhibitory activities.

AP-1 activation is mediated via MAP kinase pathways, including ERKs, p38, and JNK. A significant increase in MAP kinase expression and activity occurs in human HCC, indicating that the MAP kinase pathway may be critical for the formation and maintenance of HCC (Schmidt *et al*., 1997). Another observation in human breast cancer has suggested that oncogene-mediated MAP kinase activation contributes to anti-estrogen resistance via p27 deregulation, and blockage of MAP kinase activity restores p27 inhibitory function and estrogen sensitivity (Donovan *et al*., 2001). Our data showed that EVO inhibited the

phosphorylation of ERKs but not JNK or p38 kinase. The colony formation assay was also inhibited by EVO. Therefore, inhibition of ERKs phosphorylation was a possible mechanism contributing to the suppression of TPA-induced AP-1 activity and cell transformation by EVO.

In conclusion, we speculated that AP-1 was a potential target of EVO for its anti-tumor promotion activity in Chang liver cells. Moreover, our data suggested that EVO may be a potent chemoprevention agent for treating human HCC.

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ABBREVIATIONS：

HCC, hepatocellular carcinoma; AP-1, activator protein 1; TPA, 12-O-tetradecanoylphorbol-13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RLU, relative luciferase unit; JNKs, c-Jun N-terminal kinases; ERKs, extracellular signal-regulated kinases; MAP, mitogen-activated protein; EVO, evodiamine

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Table 1. Comparison of EC_{50} and TC_{50} values of various solvent extracts of *E. rutaecarpa*

	Acetic ethanol	Ethanol	Methanol	H_2O_2	Boiled water
EC_{50}[a]	24.8	24.44	24.72	—	—
TC_{50}[b]	90.42	79.77	122.51	>625	>625
SI[c]	3.65	3.26	4.96	—	—

[a]EC_{50} (μg/ml) is the concentration of compound required to inhibit TPA-induced AP-1 activity by 50%.

[b]TC_{50} (μg/ml) is the concentration of compound required to reduce cell viability by 50%.

[c]SI (selective index) = TC_{50} / EC_{50}

Figure Legends

Figure 1. Effect of TPA o the AP-1 activity in human hepatocytes. Luciferase activity and cell viability were measured after TPA treatment. Bars represent relative AP-1 activity, which is presented as a comparison with the RLU relative to solvent-treated cells. Lines represent the cell viability. Values are the mean ± standard error of triplicate assays.*$p <$ 0.05, compared to the control.

Figure 2. Effects of *E. rutaecarpa* methanol extract on the TPA-induced AP-1 activity and TPA-induced anchorage-independent transformation of human hepatocytes. (A) AP-1 activity assay. Chang/AP-1 cells were pretreated for 1 h with the indicated concentrations of *E. rutaecarpa* methanol extract and then exposed to 20 ng/ml TPA. (B) Anchorage-independent transformation assay. Chang/AP-1 cells were treated with 20 ng/ml TPA and methanol extracts of *E. rutaecarpa* at the indicated concentrations. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group.

Figure 3. Effects of EVO, rutaecarpine, and limonin on the TPA-induced AP-1 activities in hepatocytes. (A) Chemical structures of compounds of used in this study. Chang/AP-1 cells were pretreated for 1 h with the indicated concentrations of EVO (B), rutaecarpine (C), or limonin (D), and then exposed to 20 ng/ml TPA. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group.

Figure 4. Effects of EVO on the TPA-induced colony formation and MAP kinase activation. (A) Anchorage-independent transformation assay. Chang/AP-1 cells were ANTITUMOR EFFECT OF EVODIAMINE

treated with 20 ng/ml TPA and EVO at the indicated concentrations. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group. (B) Western blot analysis. Chang/AP-1 cells were starved in DMEM containing 0.1% FBS for 24 h and then exposed to 20 ng/ml TPA and EVO at indicated concentration for 30 min. This experiment was replicated three times and photos are representative data.

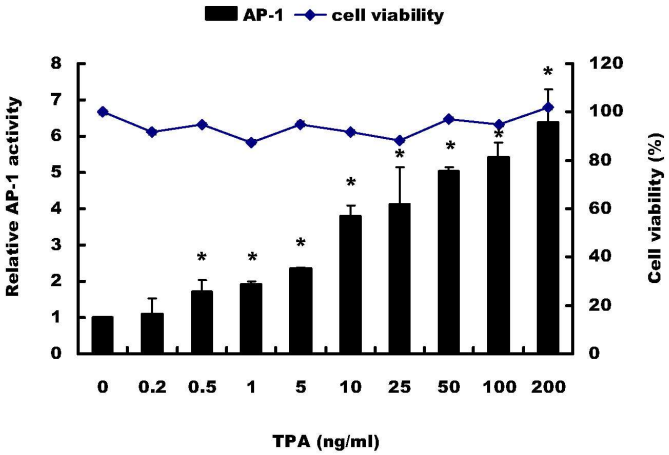

Figure 1.

Figure 1. Effect of TPA o the AP-1 activity in human hepatocytes. Luciferase activity and cell viability were measured after TPA treatment. Bars represent relative AP-1 activity, which is presented as a comparison with the RLU relative to solvent-treated cells. Lines represent the cell viability. Values are the mean ± standard error of triplicate assays.*$p < 0.05$, compared to the control.
209x297mm (200 x 200 DPI)

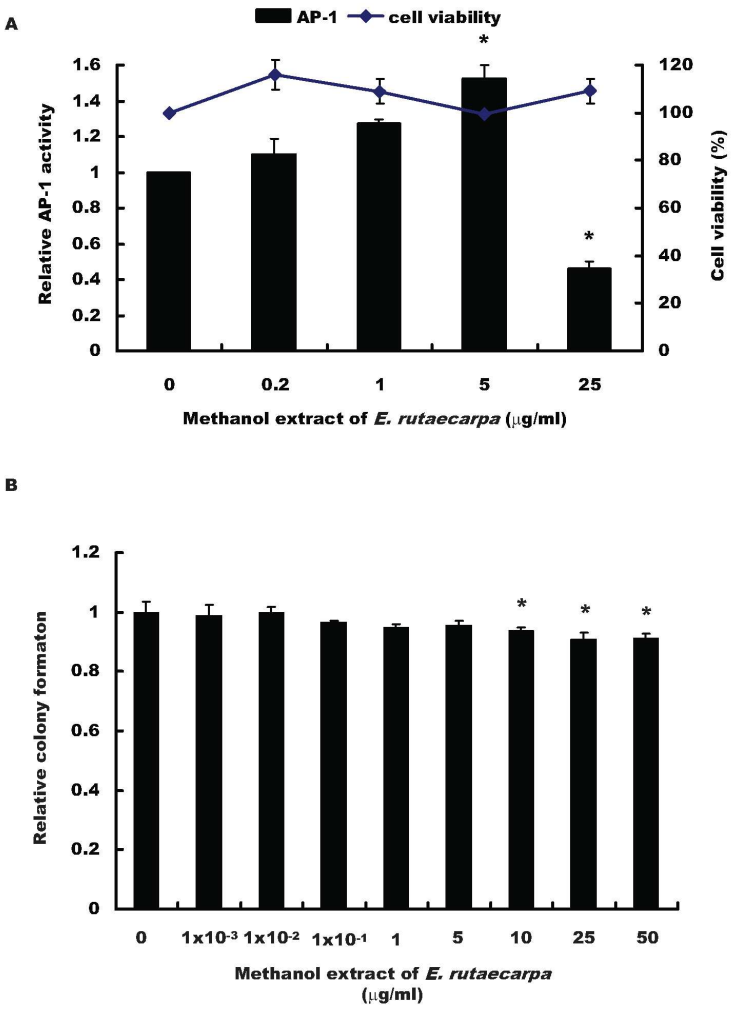

Figure 2.

Figure 2. Effects of *E. rutaecarpa* methanol extract on the TPA-induced AP-1 activity and TPA-induced anchorage-independent transformation of human hepatocytes. (A) AP-1 activity assay. Chang/AP-1 cells were pretreated for 1 h with the indicated concentrations of *E. rutaecarpa* methanol extract and then exposed to 20 ng/ml TPA. (B) Anchorage-independent transformation assay. Chang/AP-1 cells were treated with 20 ng/ml TPA and methanol extracts of *E. rutaecarpa* at the indicated concentrations. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group.
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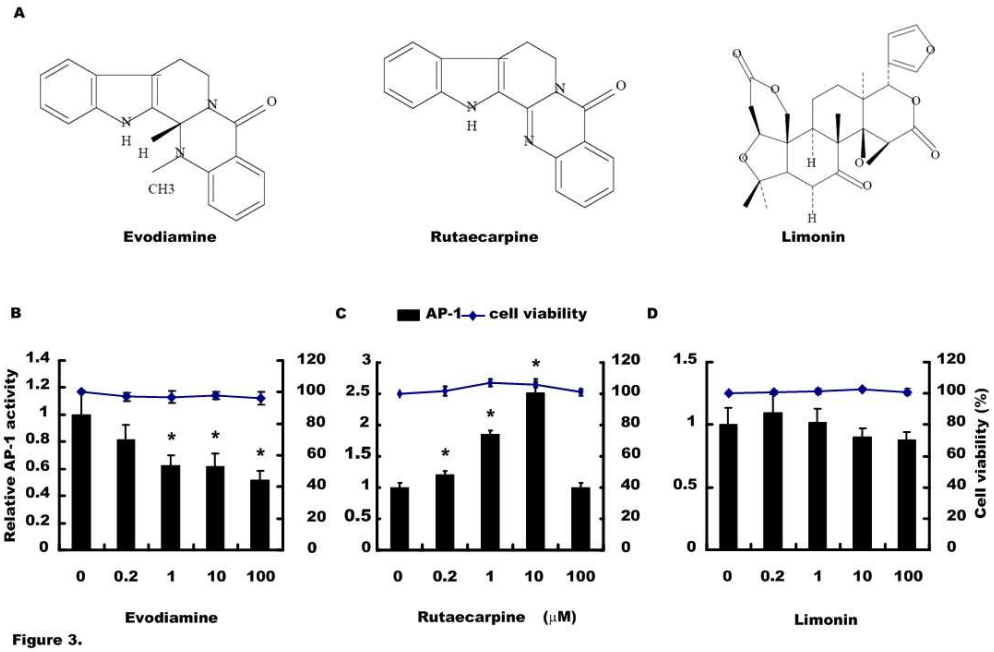

Figure 3. Effects of EVO, rutaecarpine, and limonin on the TPA-induced AP-1 activities in hepatocytes. (A) Chemical structures of compounds of used in this study. Chang/AP-1 cells were pretreated for 1 h with the indicated concentrations of EVO (B), rutaecarpine (C), or limonin (D), and then exposed to 20 ng/ml TPA. Values are mean ± standard error of triplicate assays. *$p <$ 0.05, compared to the TPA-treated group.
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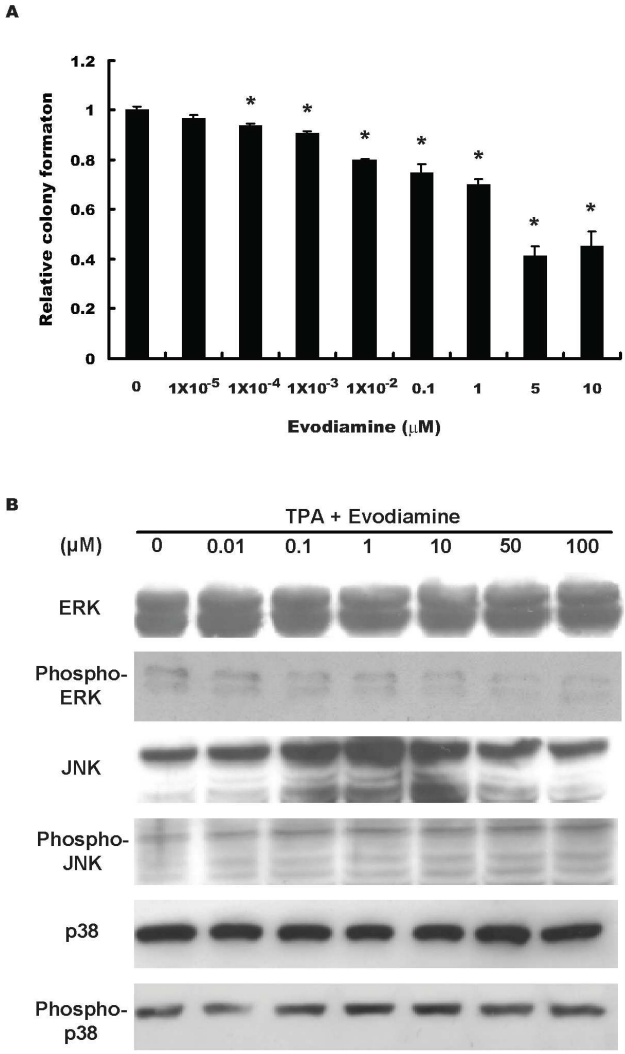

Figure 4.

Figure 4. Effects of EVO on the TPA-induced colony formation and MAP kinase activation. (A) Anchorage-independent transformation assay. Chang/AP-1 cells were treated with 20 ng/ml TPA and EVO at the indicated concentrations. Values are mean ± standard error of triplicate assays. *p < 0.05, compared to the TPA-treated group. (B) Western blot analysis. Chang/AP-1 cells were starved in DMEM containing 0.1% FBS for 24 h and then exposed to 20 ng/ml TPA and EVO at indicated concentration for 30 min. This experiment was replicated three times and photos are representative data.
209x297mm (200 x 200 DPI)