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Keywords: Zuo-Jin-Wan; Hepatocellular carcinoma; Activator protein 1; Nuclear factor-κB; Chinese herbs

Corresponding Author: Prof. Tin-Yun Ho,

Corresponding Author's Institution: China Medical University

First Author: De-Cheng Chao

Order of Authors: De-Cheng Chao; Li-Jen Lin; Shung-Te Kao; Hui-Chi Huang; Chih-Shiang Chang; Ji-An Liang; Shih-Lu Wu; Chien-Yun Hsiang; Tin-Yun Ho

Abstract: Zuo-Jin-Wan (ZJW) has been used to treat hepatocellular carcinoma in Asia. This study was to determine whether ZJW and its components blocked activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) activities as well as tumor promotion in hepatoblastoma HepG2 cells. ZJW and its components, Coptis chinensis and Evodia rutaecarpa, inhibited AP-1 and NF-κB activities, and suppressed anchorage-independent growth of HepG2 cells. The major alkaloidal ingredients, berberine and evodiamine, inhibited AP-1 activities and/or NF-κB activation, and further suppressed hepatocellular transformation. In conclusion, ZJW and its constituents, berberine and evodiamine, suppressed tumor promotion primarily through AP-1 and/or NF-κB pathways in HepG2 cells.

Dear Dr. Appendino,

We have revised our manuscript (FITOTE-D-11-00020) according to reviewers' comments. Our point-by-point reply to reviewers' comments is described as follows.

We thank you for your consideration of this matter and hope that our manuscript will be acceptable for publication in Fitoterapia.

Yours sincerely,

Tin-Yun Ho and Chien-Yun Hsiang

Reviewer #1

1. The English style could be improved, especially in the introduction

   The English style has been edited and revised by Dr. Chiang.

2. Both alkaloids are pleiotropic agents, and this information should be clearly stated.

   We have supplemented this information in the "Discussion" section (page 13, 3rd paragraph). This information is described as follows.

   Evodiamine and berberine are pleiotropic agents that display apoptotic, anti-differential, and anti-proliferative properties [36, 37]. They also display therapeutic potentials for central nervous system disorders [38, 39].

Reviewer #2

1. Some references on the antitumoral activity of berberine must be incorporated in the manuscript.

   We have cited the references on the antitumor activity of berberine in the "Discussion" section (page 13, lines -9~-10).

2. The NF-<kappa>B transactivation without cytotoxic effects of berberine is known and these must be cited.

   We have cited this reference in the "Results" section (page 11, line -7).

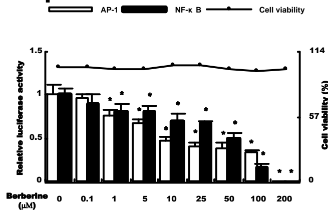
3. What is HCC (page 5). Use abbreviations, but define them at the first instant.

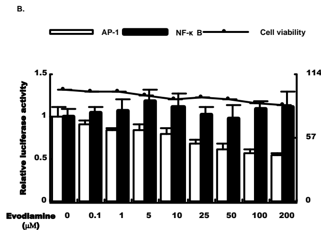
   We have defined the abbreviations at the first instant.

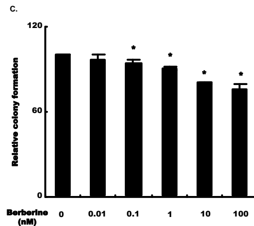
4. ZJW might suppress hepatocellular transformation. What is the mechanism is not clear.

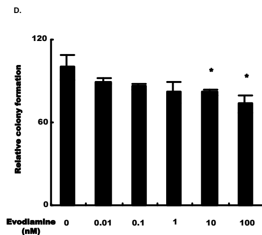
   Our data showed that ZJW significantly inhibited TPA-induced AP-1 and NF-κB activations in HepG2 cells. Moreover, TPA-induced anchorage-independent cellular growth was significantly inhibited by ZJW. Therefore, these findings suggested that ZJW might suppress hepatocellular transformation through inhibition of TPA-induced AP-1 and NF-κB activations in HepG2 cells.

*Graphical Abstract









These data suggested that ZJW and its likely active constituents, berberine and evodiamine, suppressed tumor promotion primarily through AP-1 and/or NF-κB pathways in HepG2 cells.

*Manuscript

Inhibitory effects of Zuo-Jin-Wan and its alkaloidal ingredients on activator protein 1, nuclear factor-κB, and cellular transformation in HepG2 cells

De-Cheng Chao[a], Li-Jen Lin[b], Shung-Te Kao[b], Hui-Chi Huang[c], Chih-Shiang Chang[d], Ji-An Liang[e], Shih-Lu Wu[f], Chien-Yun Hsiang[g,1], Tin-Yun Ho[a,*,1]

[a] Graduate Institute of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

[b] School of Chinese Medicine, China Medical University, Taichung 40402, Taiwan

[c] School of Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 40402, Taiwan

[d] Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 40402, Taiwan

[e] Department of Radiation Therapy and Oncology, China Medical University Hospital, Taichung 40447, Taiwan

[f] Department of Biochemistry, China Medical University, Taichung 40402, Taiwan

[g] Department of Microbiology, China Medical University, Taichung 40402, Taiwan

Corresponding author. Prof. Tin-Yun Ho, Graduate Institute of Chinese Medicine, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan. Tel.: +886 4 22053366 x 3302; fax: +886 4 22032295. *E-mail address:* cyhsiang@mail.cmu.edu.tw

[1] These authors contributed equally to this work.

Abbreviations: AP-1, activator protein 1; CC, *Coptis chinensis*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; ER, *Evodia rutaecarpa*; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibition concentration; NF-κB, nuclear factor-κB; [poly-(HEMA)], poly (2-hydroxyethyl methacrylate); RLU, relative light unit; TPA, 12-*O*-Tetradecanoylphorbol-13-acetate; ZJW, Zuo-Jin-Wan

ABSTRACT

Zuo-Jin-Wan (ZJW) has been used to treat hepatocellular carcinoma in Asia. This study was to determine whether ZJW and its components blocked activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) activities as well as tumor promotion in hepatoblastoma HepG2 cells. ZJW and its components, *Coptis chinensis* and *Evodia rutaecarpa*, inhibited AP-1 and NF-κB activities, and suppressed anchorage-independent growth of HepG2 cells. The major alkaloidal ingredients, berberine and evodiamine, inhibited AP-1 activities and/or NF-κB activation, and further suppressed hepatocellular transformation. In conclusion, ZJW and its constituents, berberine and evodiamine, suppressed tumor promotion primarily through AP-1 and/or NF-κB pathways in HepG2 cells.

Keywords: Zuo-Jin-Wan; Hepatocellular carcinoma; Activator protein 1; Nuclear factor-κB; Chinese herbs

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death and the fifth most common human cancer worldwide [1]. Poor survival rates after surgical procedures and chemotherapy are still major problems in the treatment of HCC [2, 3]. Therefore, it is important to clarify the molecular events involved in hepatocarcinogenesis and to identify potential targets for the treatment of HCC.

Clinical findings have implicated the constitutive activation of transcription factors activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) in the hepatocarcinogenesis [4, 5]. 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 [6]. AP-1 binds to the promoter regions of DNA containing 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive elements to activate the transcription of genes involved in cellular proliferation, apoptosis, and transformation [6-9]. For examples, exposure of JB6 P^+ cells to TPA or epidermal growth factor induces the anchorage-independent growth and tumorigenicity [7, 9]. Increased AP-1 activity has been shown to be involved in tumor promotion and progression of various types of cancers, such as skin, lung, and breast cancers [10-13]. Moreover, inhibition of AP-1 activity by some food products, such as omega- 3 fatty acids and glycosides, blocks the cell transformation in JB6 cells [14]. NF-κB is a heterodimer consisting of RelA (p65), c-Rel, RelB, p50, and p52. NF-κB plays a critical role in the immune response and inflammation. It also influences cell growth and survival by regulating genes involved in cell growth, apoptosis, and carcinogenesis [15]. Furthermore, NF-κB is activated in various chronic liver diseases, such as cholestasis and HCC [16].

Various studies have demonstrated the involvement of AP-1 or NF-κB in cancer

initiation, promotion, progression, and metastasis *in vitro* and *in vivo* [17, 18]. The critical roles of AP-1 and NF-κB in carcinogenesis imply that AP-1 and NF-κB can be served as the targets for chemopreventive agents [19]. For examples, hepatitis C virus establishes persistent infection by suppressing interleukin-12 synthesis in human macrophages via AP-1 activation [20]. Moreover, inhibition of NF-κB activation markedly reduces tumorigenesis in both colitis-associated cancer and inflammation-promoted cholestatic carcinoma [21, 22]. Therefore, we propose that down-regulation of AP-1 and/or NF-κB activity by herb extracts can be an effective way to inhibit tumor promotion.

Zuo-Jin-Wan (ZJW), a traditional Chinese medicinal formula, consists of two herbal components, *Coptis chinensis* (CC) and *Evodia rutaecarpa* (ER), at a ratio of 6: 1. ZJW has been used clinically for centuries in Asia to treat gastroenterological disorders and HCC [23]. However, it is still unclear how ZJW and its herbal components exert anti-tumor effects in hepatocytes. Here we studied the effects of ZJW on TPA-induced hepatocellular transformation and further clarified the mechanisms involved in the anti-tumor effects of ZJW and its constituents.

2. Materials and methods

2.1. Cell line and chemicals

The human hepatoblastoma cell line (HepG2) 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 37^0C under 5% CO_2 in air. 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, evodiamine was dissolved in dimethyl sulfoxide at 100 mM, and berberine sulfate was dissolved in 50% methanol at 100 mM.

2.2. Preparation of aqueous extracts of ZJW, CC, and ER

ZJW, CC, and ER were purchased from Hsien-Lu Pharmaceutical Co., Ltd. (Taiwan). The aqueous extracts of these herbs were prepared by boiling the herbs in water to extract water-soluble heat-resistant compounds. Briefly, each herb (20 g) was soaked in 200 ml distilled water and boiled in a flask. This flask was attached to a reflux apparatus to cool the vapor and to return it back to the flask as a liquid. The mixture was then centrifuged at 715 xg for 20 min. The supernatant was collected and vacuum-dried at room temperature. The dried powder was then resuspended in distilled water to 1 mg/ml before use.

2.3. Quantitative analysis of active compounds in the extracts of ZJW, CC, and ER

High-performance liquid chromatography (HPLC) analysis was performed on a Waters 2695 Alliance HPLC instrument (Waters, Milford, MA, USA) equipped with an auto-sampler and a 2996 photo diode array detector. Evodiamine was separated by acetonitrile/5 mM phosphoric acid (50:50, v/v) at a flow rate of 1 ml/min. The column used for evodiamine was the Merck Purospher® STAR RP-18e (250 mm×4.6 mm, 5 μm), and evodiamine was detected at 254 nm. The mobile phase for berberine was comprised of acetonitrile/water (16:84, v/v). The solvent flow rate was 0.8 ml/min and berberine was detected at 280 nm. A Waters SunFire™ C 18 column (150 mm ×4.6 mm, 5 μm) was used. Contents of evodiamine and berberine in extracts were determined by referring to the calibration curve established by running standard at

varying concentrations under the same conditions.

2.4. Cell culture and TPA treatment

Recombinant HepG2/AP-1 and HepG2/NF-κB cells containing the luciferase gene driven by AP-1- and NF-κB-responsive element, respectively, were constructed as described previously [24, 25]. HepG2/AP-1 and HepG2/NF-κB cells were maintained in DMEM supplemented with 10% FBS. For TPA treatment, cells were cultured in 96-well plates at 37^0C. After a 24-h incubation, cells were washed and starved with DMEM for another 24 h. Various amounts of TPA were then used to treat cells for 16 or 24 h.

2.5. Luciferase assay

Luciferase assay was performed as described previously [24]. Briefly, HepG2/AP-1 or HepG2/NF-κB cells were cultured in 25-cm^2 flasks at 37^0C 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 TPA or compounds and incubated at 37^0C for 16 h. Cells were washed with 4 ml of ice-cold phosphate-buffered saline (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2), lysed with 400 μl reporter lysis buffer, and centrifuged at 12,000 xg for 2 min at 4^0C. Luciferase activity was measured by mixing 20 μl of cell lysate with 100 μl of Luciferase Assay Substrate (Promega, Madison, WI, USA), followed by determination with a luminometer (FB15, Zylux, Huntsville, AL, USA). Relative luciferase activity was calculated by dividing the relative light unit (RLU) value of treated cells by the RLU of solvent-treated cells. The 50% inhibition concentration (IC_{50}) was determined as the concentration of

compounds required to inhibit luciferase activity at 50%.

2.6. Cytotoxicity Assay

Cells were cultivated in 96-well culture plates. After a 24-h incubation at 37^0C, various amounts of chemicals were added to confluent cell monolayers and incubated for another 24 h. Cytotoxicity was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, one tenth volume of 5 mg/ml MTT was added to the culture medium. After a 4-h incubation at 37^0C, an equal volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan, and the absorbance value was measured at 570 nm using a microplate reader. Cell viability (%) was calculated as (OD of compound-treated cells/OD of solvent-treated cells)×100.

2.7. Anchorage-independent growth assay

Anchorage-independent growth assays were performed as described previously [26]. 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 37^0C for at least two days. 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 MTT (5 mg/ml) and incubated at 37^0C for 4 h. The MTT formazan was then dissolved in 0.04 N HCl/isopropanol, and the absorbance values were measured at 570 nm using a microplate reader. The relative colony formation was calculated as (OD value of compounds-treated cells/OD value of solvent-treated cells)×100.

2.8. Statistical analysis

Data are represented as the mean ± standard error. Student's *t* test was used for comparisons between groups. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Determination of alkaloidal ingredients in ZJW, CC, and ER by HPLC

The evodiamine and berberine constituents were analyzed and identified by comparing the retention time and ultraviolet absorption with standard samples. As shown in Fig. 1, the contents of evodiamine and berberine in ZJW were 0.03% and 2.9%, respectively. The contents of evodiamine and berberine in ER and CC were 0.05% and 3.34%, respectively.

3.2. AP-1 and NF-κB were activated by TPA in HepG2 cells

To investigate whether AP-1 and NF-κB activities were induced by TPA, we seeded HepG2/AP-1 or HepG2/NF-κB cells in 96-well plates and treated with various concentrations of TPA. As shown in Fig. 2, TPA induced AP-1 and NF-κB activities in a dose-dependent manner. No cytotoxicity was observed during treatment. NF-κB activity was increased by 3-fold at 1 ng/ml TPA, whereas AP-1 activity was increased by 3-fold at 100 ng/ml TPA. These findings suggested that TPA induced NF-κB and AP-1 activities in HepG2 cells.

3.3. TPA induced anchorage-independent growth in HepG2 cells

To further examine whether anchorage-independent cellular growth was promoted

by TPA, we seeded HepG2 cells in poly(HEMA)-coated 96-well plates and treated with various amounts of TPA. Anchorage-independent growth of cells was then evaluated by a MTT colorimetric assay. Fig. 3 shows that TPA significantly stimulated the anchorage-independent growth of HepG2 cells. Maximal induction of anchorage-independent growth was observed at 20 ng/ml TPA. Therefore, 20 ng/ml TPA was used in following experiments.

3.4. ZJW suppressed TPA-induced AP-1 and NF-κB activations in HepG2 cells

Because increased AP-1 and NF-κB activations may play important roles in carcinogenesis [4, 5], we explored the effects of ZJW on TPA-induced AP-1 and NF-κB activities in HepG2 cells. ZJW inhibited TPA-induced AP-1 and NF-κB activities in HepG2 cells in a dose-dependent manner (Fig. 4). The IC_{50} values of ZJW for AP-1 and NF-κB activations were >200 and 22.9 μg/ml, respectively. No significant cytotoxic effect was observed during ZJW treatment. These data suggested that ZJW significantly inhibited TPA-induced AP-1 and NF-κB activations in HepG2 cells.

3.5. TPA-induced colony transformation was inhibited by ZJW

The growth abilities of transformed cells are typically correlated with their tumorigenicity [27]. Fig. 5 shows that TPA-induced anchorage-independent cellular growth was significantly inhibited by ZJW. ZJW reduced the HepG2 cell growth by 20% at 50 μg/ml. These findings suggested that ZJW might suppress hepatocellular transformation through inhibition of TPA-induced AP-1 and NF-κB activations in HepG2 cells.

3.6. TPA-induced AP-1 activation was inhibited by CC and ER in HepG2 cells

We further analyzed the effects of CC and ER on TPA-induced AP-1 and NF-κB activities. CC was able to cause a dose-dependent decrease on TPA-induced AP-1 and NF-κB activities (Fig. 6A). The IC_{50} values of CC for AP-1 and NF-κB activations were 23.2 and >200 μg/ml, respectively. ER inhibited AP-1 in a dose-response manner with an IC_{50} value of 200 μg/ml (Fig. 6B). However, ER did not affect the NF-κB activity. No cytotoxic effect was observed during treatment. Therefore, these findings indicated that CC inhibited TPA-induced AP-1 and NF-κB activations, while ER only suppressed AP-1 activity in HepG2 cells.

3.7. TPA-induced AP-1 activation was inhibited by berberine and evodiamine in HepG2 cells

The major ingredients of CC and ER are berberine and evodiamine, respectively [28, 29]. We further determined whether the effects of these components were consistent with those of herbs on AP-1 and NF-κB activities in HepG2 cells. Like CC, berberine suppressed both AP-1 and NF-κB activities in a dose-dependent manner with IC_{50} values of 9.5 and 50 μM, respectively (Fig. 7A). Like ER, evodiamine inhibited AP-1 but not NF-κB activity in a dose-response manner (Fig. 7B). No cytotoxic effect was observed during treatment [24]. These findings suggested that berberine and evodiamine might be the active compounds in CC and ER, respectively. In addition, TPA-induced AP-1 activation was inhibited by berberine and evodiamine in HepG2 cells.

3.8. Berberine and evodiamine inhibited TPA-induced hepatocellular transformation

The inhibition of hepatocellular transformation by berberine and evodiamine were

evaluated by anchorage-dependent growth assays. The growth of transformed cells is anchorage-independent, and their growth ability is correlated with their tumorigenicity [27]. Therefore, inhibition of anchorage-independent growth of transformed cells may represent an effective strategy for cancer chemotherapy. As shown in Fig. 8, treatment with 100 nM berberine and evodiamine inhibited the TPA-treated HepG2 cellular growth by 25% and 26 %, respectively. Taken together, we concluded that berberine and evodiamine, the likely active compounds of CC and ER, respectively, were anti-tumor transformation agents that inhibited AP-1 and/or NF-κB activities in hepatocytes.

4. Discussion

Herbal medicine is becoming popular among people seeking for alternative therapy. The interest in herbal medicine has led to more researches on this field for better understanding the effects of herbal medicine. For examples, Sho-Saiko-To (TJ-9) has been shown to prevent the development of HCC in patients with cirrhosis, particularly in patients without hepatitis B virus surface antigen [30]. Herbal formula MSSM-002 is a useful complementary approach for the treatment of allergic asthma [31]. Bai-Hu-Tang, the herbal formula used in patients with diabetes, potentiates the insulin-stimulated glucose uptake in 3T3-L1 adipocytes via peroxisome proliferator-activated receptors-γ signaling pathway [32].

ZJW, the traditional Chinese herbal medicine used in this study, has been used to treat gastrointestinal disorders and cancers. This study sought to examine the effect of ZJW on the chemoprevention against HCC. ZJW inhibited TPA-induced AP-1 and NF-κB activities as well as anchorage-independent cellular growth in HepG2 cells in a dose-dependent manner. These results suggested that ZJW might suppress

hepatocellular transformation.

Previous studies have found that transactivation of AP-1 is required for tumor promotion and blockage of tumor promoter-induced AP-1 activation inhibits neoplastic transformation [7, 17, 33]. NF-κB can regulate the expression of specific genes typically involved in inflammatory responses [34]. In addition, activation of NF-κB is frequently detected in apoptosis, angiogenesis, cell proliferation, and cellular transformation, suggesting a potential link between inflammation and cancer [5, 18, 35]. Therefore, effective strategies against hepatocarcinogenesis may be achieved through suppression of AP-1 and/or NF-κB activity.

Further investigations were designed to characterize the active constituents in ZJW that were responsible for anti-tumor effects in hepatocytes. Our findings showed that berberine and evodiamine, the major alkaloid ingredients of CC and ER, respectively, inhibited AP-1 and/or NF-κB activities, and suppressed anchorage-independent growth of HepG2 cells. Evodiamine and berberine are pleiotropic agents that display apoptotic, anti-differential, and anti-proliferative properties [36, 37]. They also display therapeutic potentials for central nervous system disorders [38, 39]. In this study, we found that both compounds effectively reduced AP-1 activation and influenced cell transformation in a dose-dependent manner in HepG2 cells.

In conclusion, this study first demonstrated that ZJW and its constituents inhibited TPA-induced hepatocellular transformation by suppressing AP-1 and/or NF-κB transactivation without cytotoxic effects. These findings suggested that ZJW and its constituents were potential agents for the treatment of tumor promotion in human hepatocytes.

Acknowledgments

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Figure captions

Fig. 1. HPLC chromatographs. Panel A is the chromatogram and structure of evodiamine standard. Panels B and E are the chromatograms of ZJW samples in different analysis. Panel C is the chromatogram of ER sample. Panel D is the chromatogram and structure of berberine standard. Panel F is the chromatogram of CC sample. Arrows indicate the peaks representing evodiamine or berberine.

Fig. 2. Effect of TPA on AP-1 and NF-κB activities in HepG2 cells. HepG2 cells were treated without or with various concentrations of TPA. Luciferase activity and cell viability were determined at 16 h. Bars represent relative luciferase activity, which is presented in comparison to RLU of untreated cells. Lines represent cell viability during treatment. Values are mean ± standard error of triplicate assays. $*p < 0.05$, compared to the control.

Fig. 3. Effect of TPA on the anchorage-independent transformation in HepG2 cells. Cells were seeded in poly-(HEMA)-coated wells and treated without or with various amounts of TPA. Values are mean ± standard error of triplicate assays. $*p < 0.05$, compared to the control group.

Fig. 4. Inhibitory effect of ZJW on TPA-induced AP-1 and NF-κB activations. HepG2 cells were pretreated for 1 h with various concentrations of ZJW and then exposed to 20 ng/ml TPA. After culturing the cells at 37^0C for 48 h, AP-1 or NF-κB activity of the cell extract was measured by luciferase assay. Bars represent relative luciferase activity, which is presented in comparison to RLU of TPA-treated cells. Lines represent cell viability during treatment. Values are mean ± standard error of triplicate

assays. *$p < 0.05$, compared to the TPA-treated group without ZJW.

Fig. 5. Inhibitory effect of ZJW on the TPA-induced anchorage-independent transformation in HepG2 cells. HepG2 cells were treated with 20 ng/ml TPA and/or various amounts of ZJW. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group without ZJW.

Fig. 6. Inhibitory effects of CC and ER on TPA-induced AP-1 and NF-κB activations in HepG2 cells. HepG2 cells were pretreated for 1 h with various concentrations of CC (A) or ER (B) and then exposed to 20 ng/ml TPA. After culturing the cells at 37^0C for 48 h, AP-1 or NF-κB activity of the cell extract was measured by luciferase assay. Bars represent relative luciferase activity, which is presented in comparison to RLU of TPA-treated cells. Lines represent cell viability during treatment. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group without CC and ER.

Fig. 7. Inhibitory effects of berberine and evodiamine on TPA-induced AP-1 and NF-κB activations in HepG2 cells. HepG2 cells were pretreated for 1 h with various concentrations of berberine (A) or evodiamine (B) and then exposed to 20 ng/ml TPA. After culturing the cells at 37^0C for 48 h, AP-1 or NF-κB activity of the cell extract was measured by luciferase assay. Bars represent relative luciferase activity, which is presented in comparison to RLU of TPA-treated cells. Lines represent cell viability during treatment. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group without berberine and evodiamine.

Fig. 8. Inhibitory effects of berberine and evodiamine on TPA-induced colony formation in HepG2 cells. HepG2 cells were treated with 20 ng/ml TPA and/or berberine (A) and evodiamine (B) at indicated concentrations. Values are mean ± standard error of triplicate assays. *$p < 0.05$, compared to the TPA-treated group without berberine and evodiamine.

Figure 1

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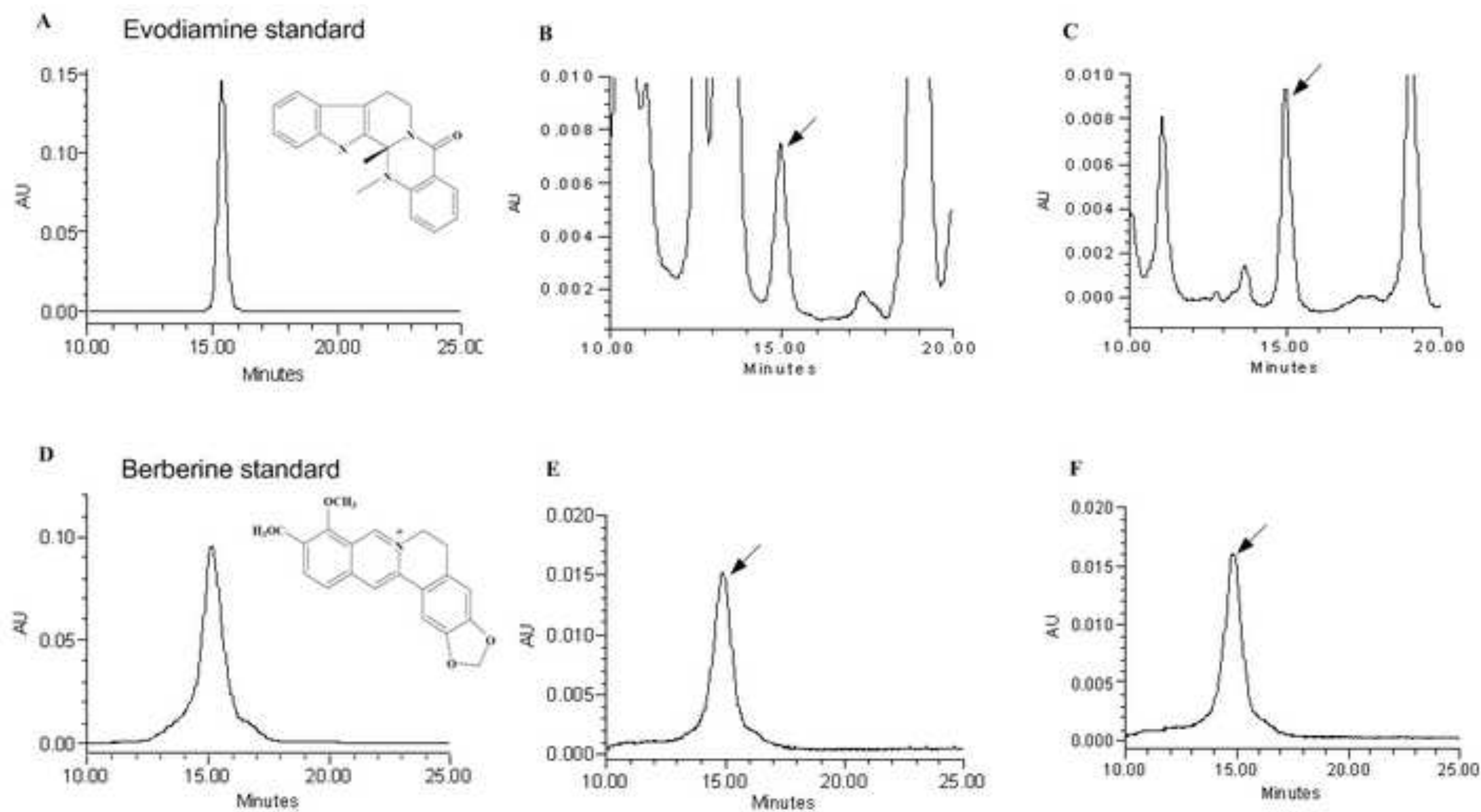

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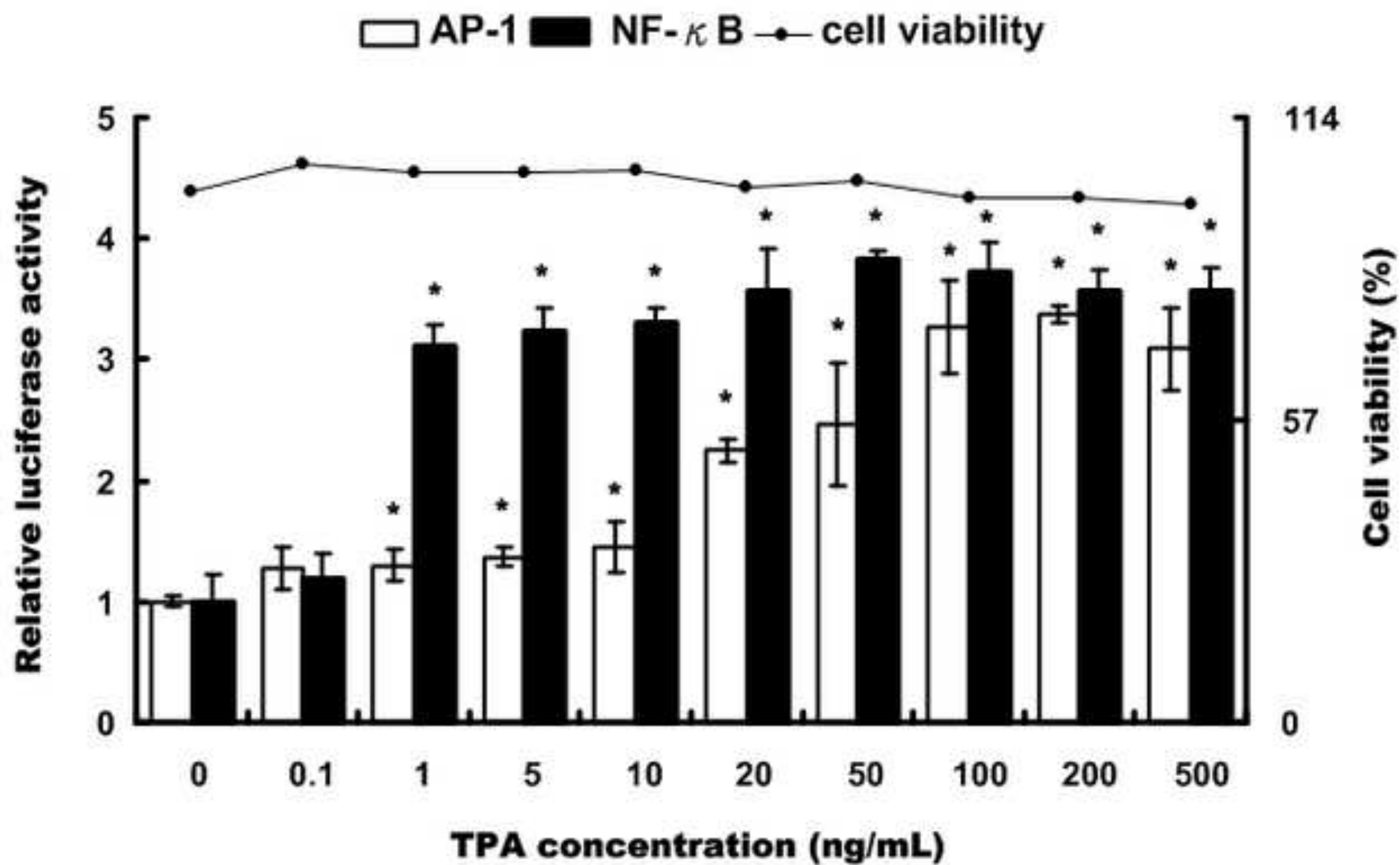

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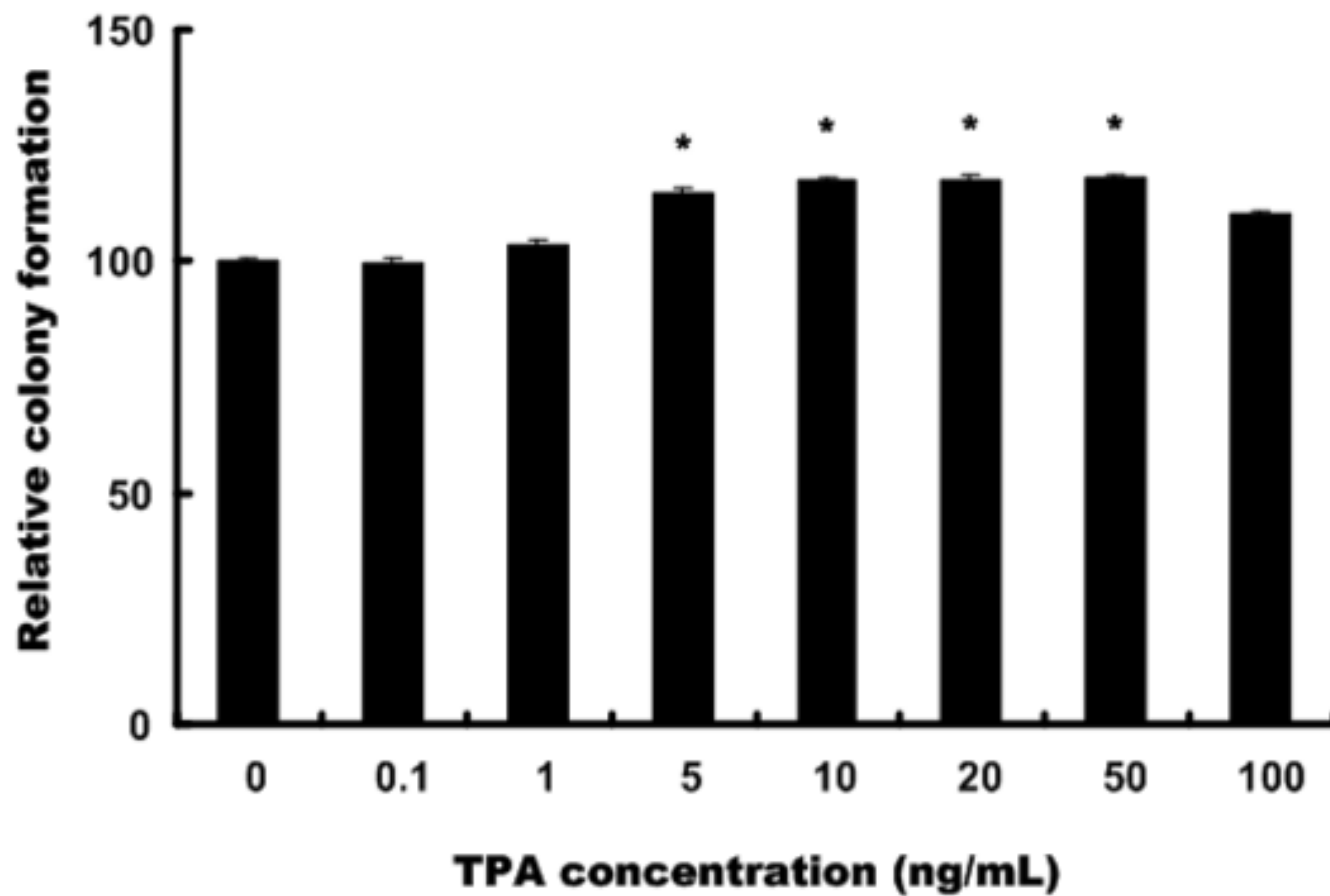

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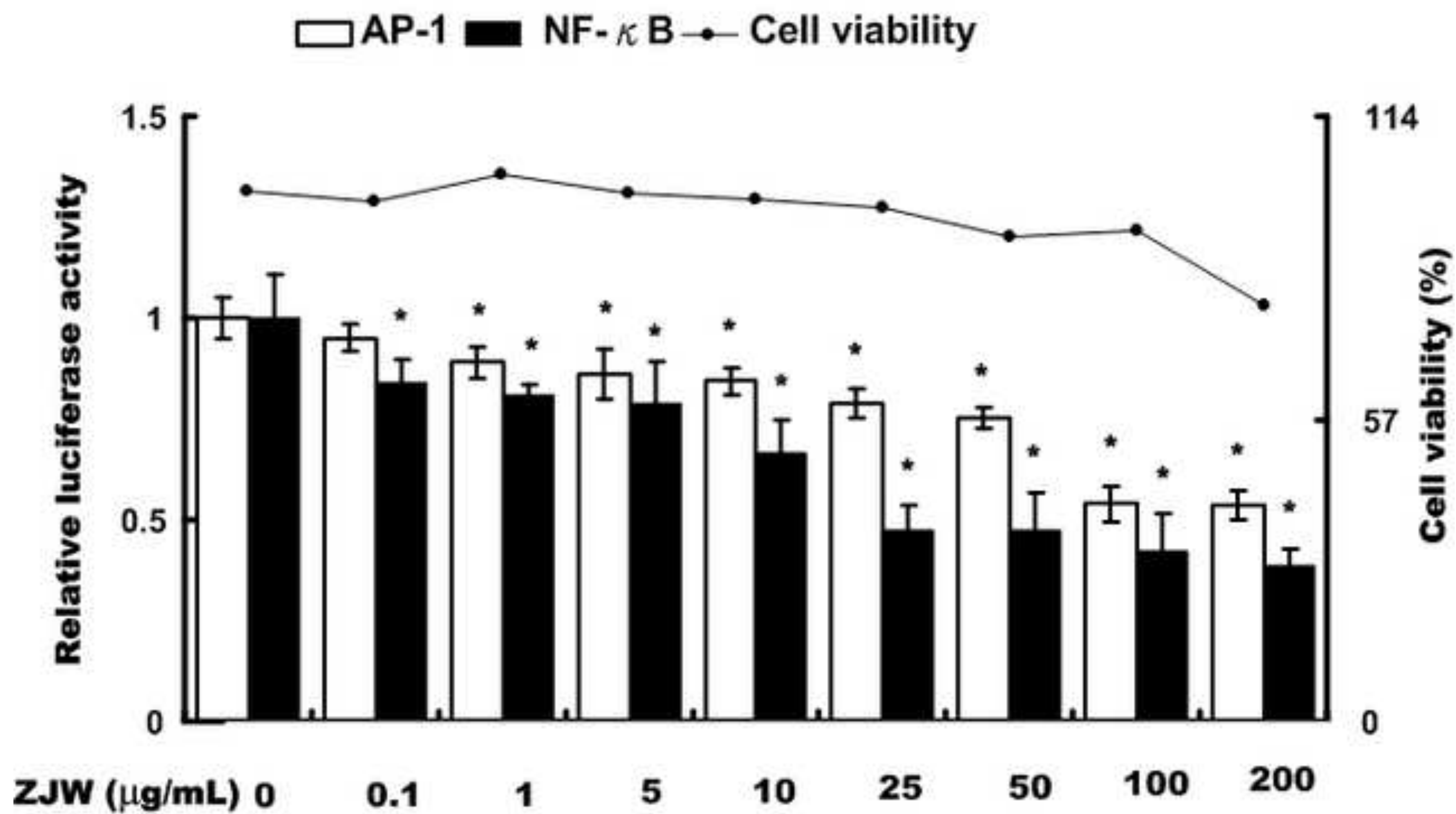

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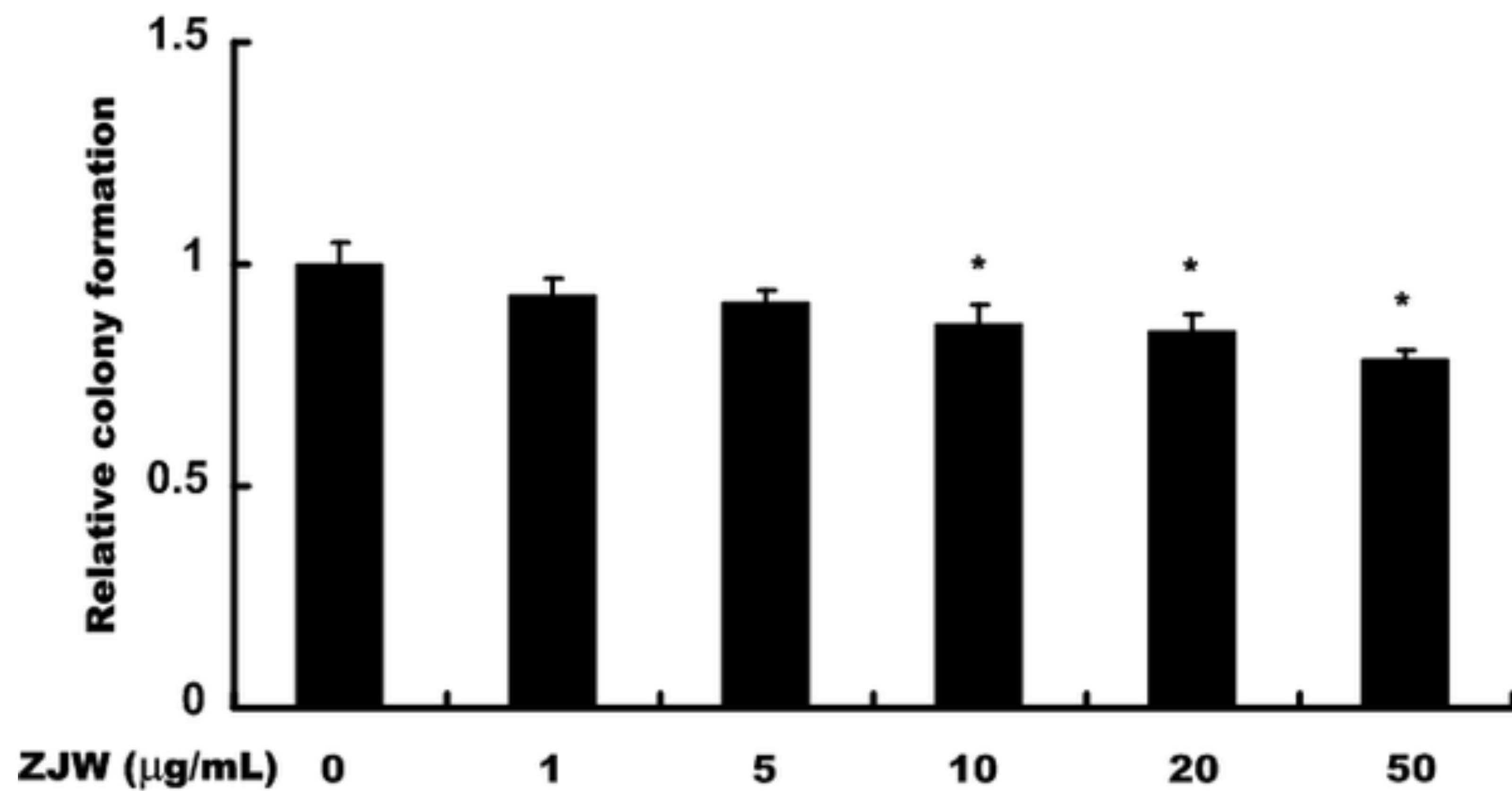

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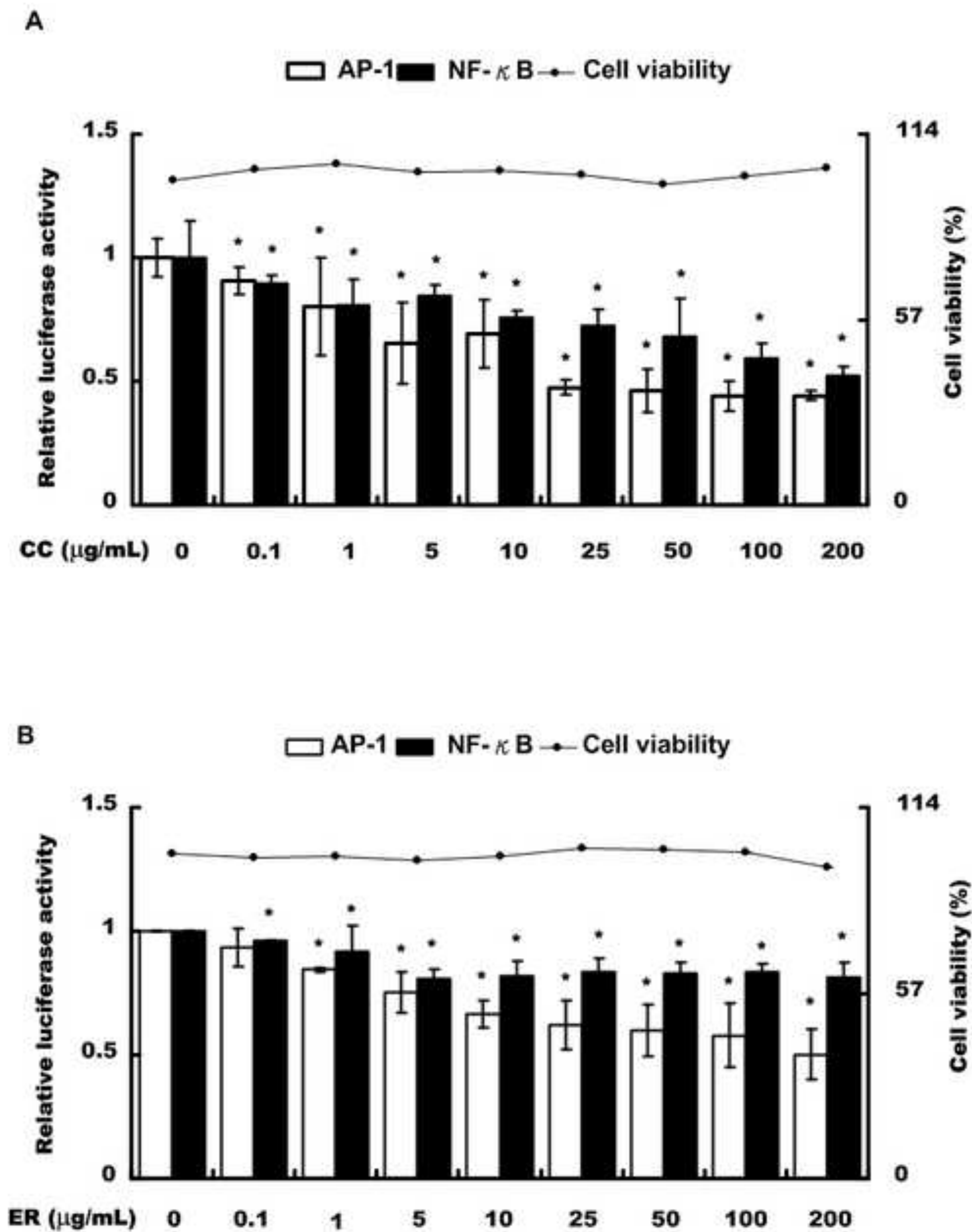

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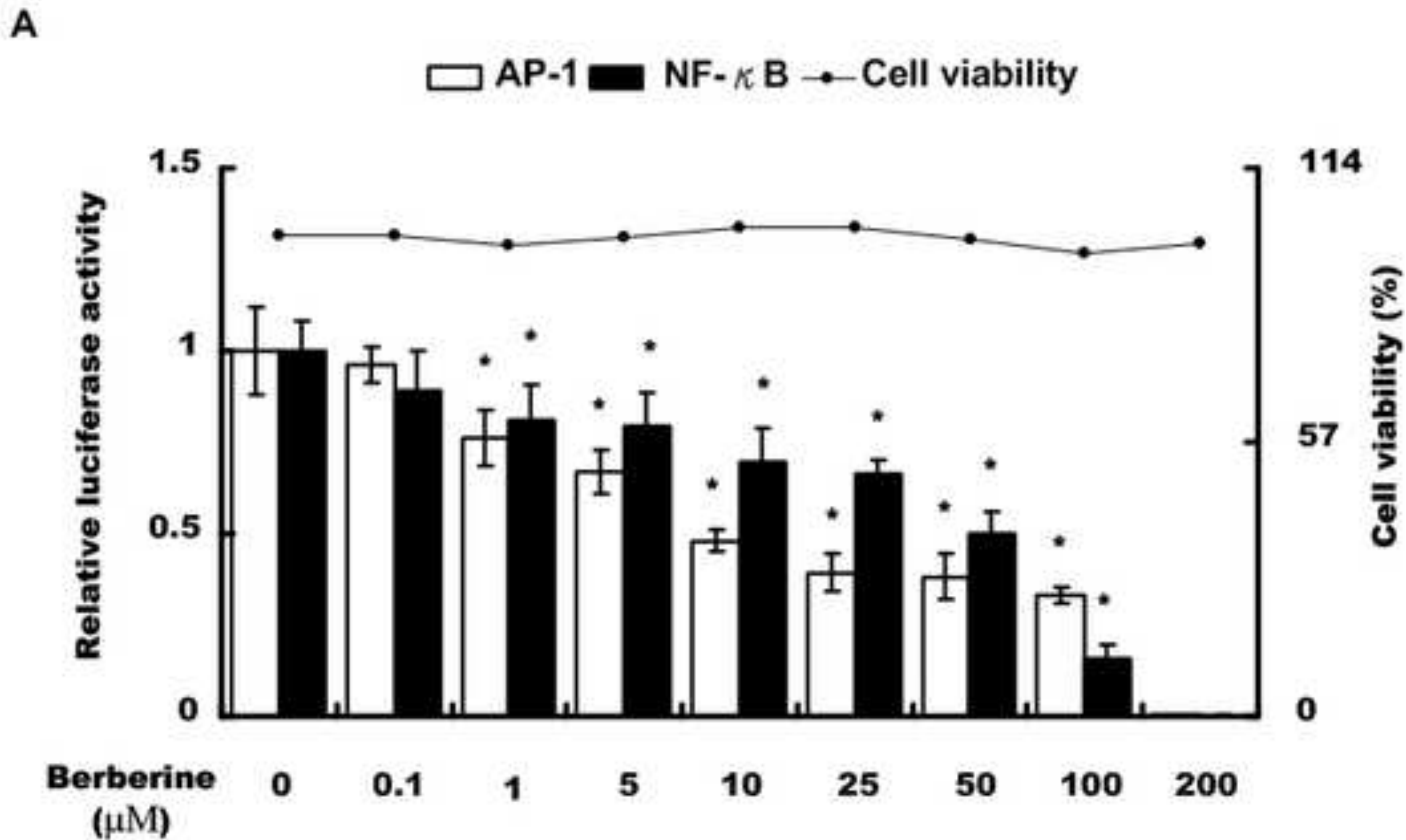

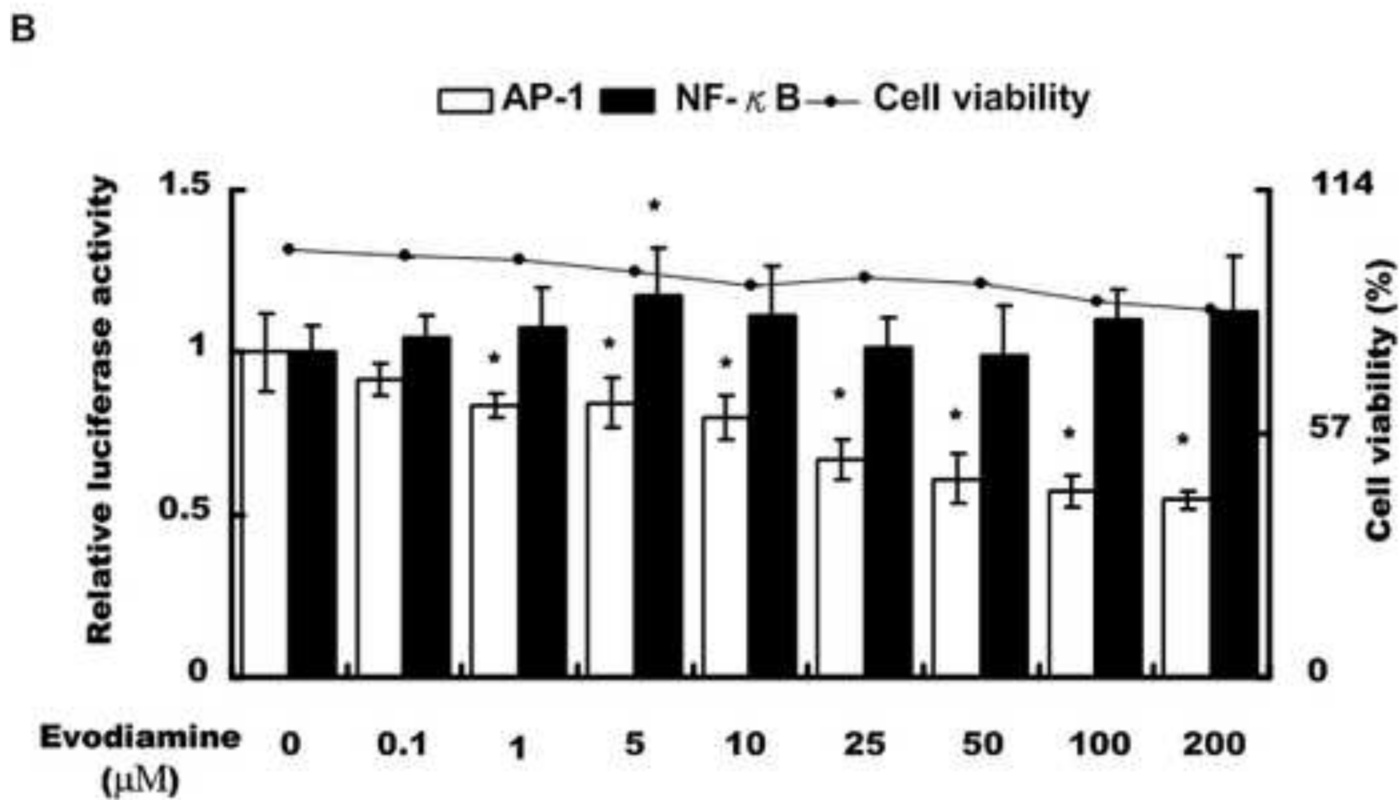

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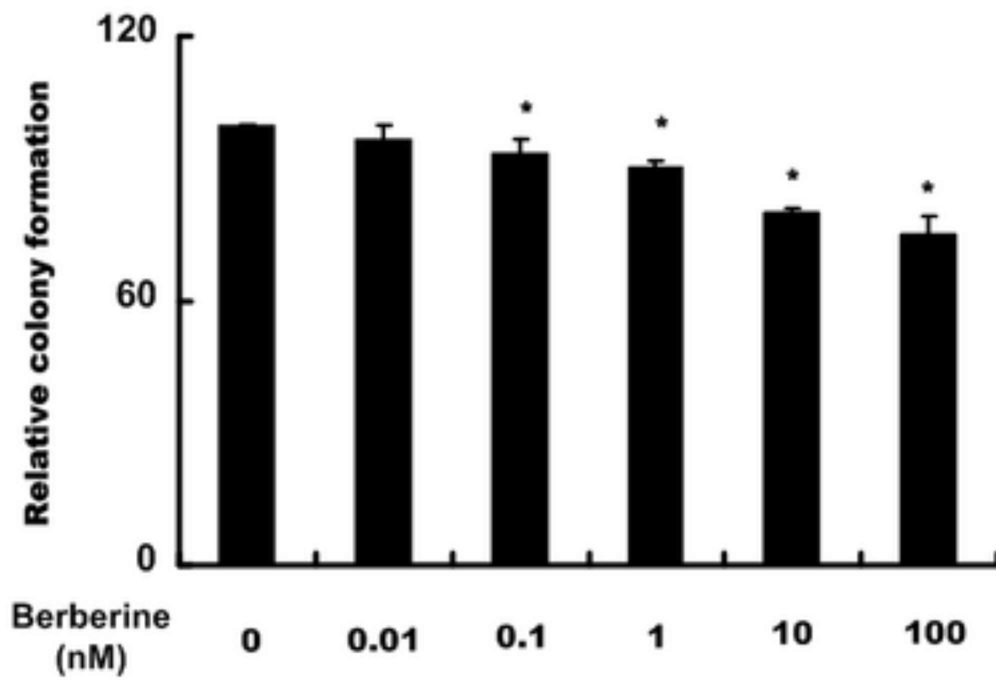

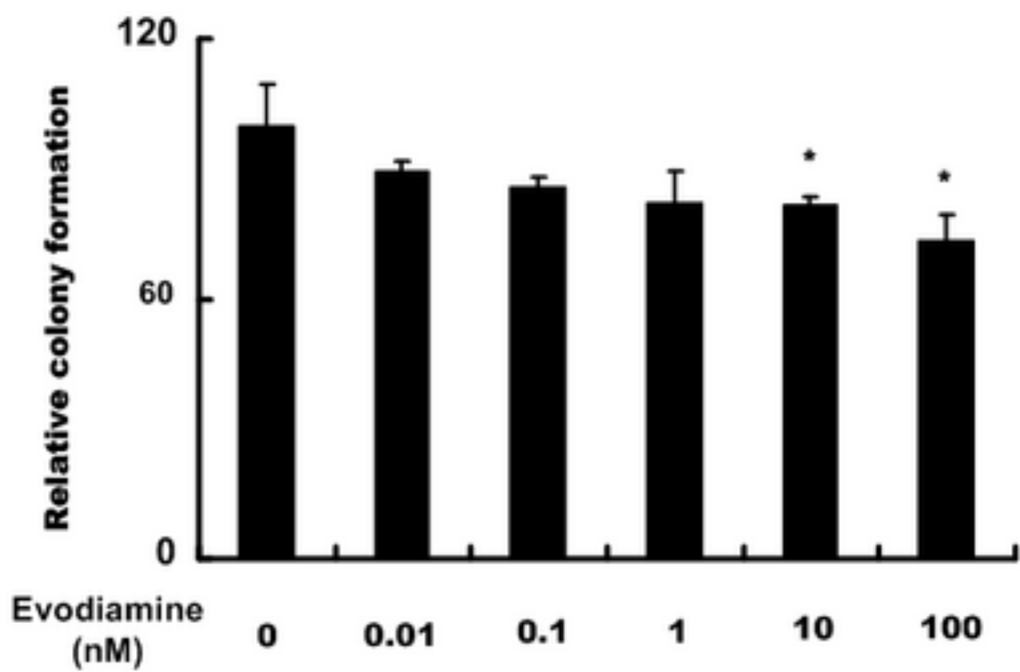

Fig. 8.