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Full Length Research Paper

Ethanol extract of *Hedyotis diffusa* Willd. induction of apoptosis via PI3K/Akt and XIAP pathways in human leukemic THP-1 cells

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***Hedyotis diffusa* (H. diffusa) Willd. is known to induce apoptosis in cancer cells. However, the molecular mechanism of its anti-cancer activity has not been fully elucidated. In this study, we found that the ethanol extract of H. diffusa Willd. (EEHDW) reduced cell viability and induced apoptosis in a dose- and time-dependent manner in human leukemic THP-1 cells. The induction of apoptosis was also accompanied by the down regulation of PI3K/Akt and the inhibitor of apoptosis protein (IAP) family proteins. Moreover, we observed that EEHDW treatment resulted in activation of caspase-3, which may partly explain the anti-cancer activity of EEHDW.**

Key words: *Hedyotis diffusa* Willd., THP-1, apoptosis, caspase.

INTRODUCTION

Medicinal plants are widely used in the treatment of various cancers in many Asian countries and are recognized as an attractive alternative to surgical therapy and radiotherapy (Xie et al., 2009). The herb of *Hedyotis diffusa* Willd. (synonym *Oldenlandia diffusa* Willd., family Rubiaceae), an annual herb distributed in northeastern Asia, has been widely used to treat cancer, appendicitis, hepatitis, furunculosis, enteritis and bleeding in China (Zhang et al., 2010; Xu et al., 2010). Recently, this herb has gained increasingly attention to its usage as an antitumor herb, such as therapy in liver, lung, colon, brain, pancreas and other cancers (Fang et al., 2004). Previous studies have shown that the herb has the ability to inhibit the proliferation of tumor cells and that the primary extract of the herb could induce apoptosis in human breast cancer cell line SPC-A (Zhang et al., 2007). However, the exact mechanism and signaling pathway involved in EEHDW-induced apoptosis have not been fully elucidate.

The regulation of apoptosis in both normal and malignant cells has become an area of extensive study in

cancer research. 20 years ago, several cell lines derived from patients with leukemia and blocked at various stages of differentiation were intensively used to study proliferation, apoptosis and differentiation processes (Champelovier et al., 2008). The human monocytic cell line THP-1 cell was first derived from the peripheral blood of a one year old male with acute monocytic leukaemia (Tsuchiya et al., 1980).

The cellular decision to undergo either cell death or cell survival is a very complex process, which depends on the integration of multiple survival and death signals (Galluzzi et al., 2009). Phosphatidylinositol 3-kinases (PI3K) are a family of related intracellular signal transducer enzymes that have been linked to a diversity of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking (Guan et al., 2009; Cardoso et al., 2008). The anti-apoptotic effects of PI3K are mediated by its downstream target Akt, which can regulate the expression of several apoptosis-related genes, such as Bcl-2/Bax (Zheng et al., 2008). The activated Akt phosphorylates target molecules including mammalian target of rapamycin (mTOR), which modulates cell proliferation in part by the regulation of initiation of translation (Bjornsti et al., 2004; Hay and Sonenberg,

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2004). In addition to Akt, inhibitor of apoptosis protein (IAP) families of proteins also plays a critical role. The inhibitor of apoptosis (IAP) family of proteins is the potent natural factors that function by directly inhibiting the activity of caspase, the principal effectors of apoptosis (Turner et al., 2007).

In this study, we evaluated the antitumor potential of EEHDW in leukemia-derived cell lines, THP-1 cells. With the aim to clarify the mechanisms underlying *H. diffusa* Willd. cell growth inhibition activity, we analyzed the effect of the drug on cell death and apoptosis. The contribution of caspase, PI3K/AKT pathway and XIAP families in the *Hedyotis diffusa* Willd. induced cell death was also investigated.

MATERIALS AND METHODS

Reagents

Monoclonal anti- β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Phospho-specific Akt and Akt; anti-phospho-specific mTOR and mTOR; Anti-XIAP; anti-cIAP1; Anti-cIAP2; anti-smac; anti-survivin were obtained from New England Biolabs, Beverly, MA; stocks of the selective PI3K/Akt inhibitor LY294002; stocks of the selective XIAP inhibitor Embelin were obtained from Calbio-chem-Behring, La Jolla, CA, USA. RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco BIL Company (Gibco, NY, USA). An annexin V apoptosis detection kit was purchased from RD Systems (Abingdon, U.K.). Cell isolation and tissue culture reagents were obtained from Invitrogen life Technologies (Lidingo, Sweden). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

THP-1 cells culture

THP-1 cells obtained from Sun Yat-sen University were used in all the experiments and they were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin (RPMI medium) and incubated at 37°C in a humidified air containing 5% CO₂. The exponentially growing THP-1 cells were seeded into 24-well flat-bottomed plates at a density of 5×10^5 /ml. The cells were collected at different treated-time points. Before induction with EEHDW, the cells were washed once with PBS to remove dead cells, and incubated in tissue-culture plates for 30 min at 37°C. THP-1 cells in multiwell tissue-culture plates were incubated with EEHDW at different concentrations (0, 1, 2 and 4 mg/ml) for 2 days or at a concentration of 2 mg/ml for 0, 1, 2, 3 and 4 days, respectively. In some experiments, 50 μ M LY294002 (PI3K inhibitor) or 20 μ M Embelin (XIAP inhibitor) were used at 30 min before EEHDW induction.

Cell viability

To assess the overall viability of THP-1 cells following EEHDW treatment, the cells were treated as described earlier. At particular time points, the THP-1 cells were washed two times with PBS and treated with a 0.4% solution of trypan blue and visualized as clear cells under the microscope. THP-1 cells that were no longer viable, which had damaged membranes allowed entry of the dye; were stained blue. Assays were performed in triplicate and repeated at least three times. The number of intact viable cells was expressed

as a percentage of total cells and was assessed at different times post-infection.

Flow cytometry analysis

THP-1 apoptosis was quantified by flow cytometry using FITC-conjugated annexin V and propidium iodide (PI). Specific binding of annexin V was achieved by incubating 10^6 cells in 60 μ l of the binding buffer saturated with annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and PI before analysis. The binding of annexin V-FITC and PI to the cells was measured by flow cytometry (FACS Calibur, BD Biosciences) using CellQuest software. At least 10 000 cells were counted in each sample. Experiments were performed and interpreted as follows: cells that were Annexin V (-)/ PI (-) (lower left quadrant) were considered as living cells, the Annexin V (+)/ PI (-) cells (lower right quadrant) as apoptotic cells, Annexin V (+)/ PI (+) (upper right quadrant) as necrotic or advanced apoptotic cells and Annexin V (-) / PI (+) (upper left quadrant) may be bare nuclei, cells in late necrosis or cellular debris.

Western blot analysis

After treatment, briefly, cells were washed once with ice-cold phosphate buffered saline containing 1 mM Na₂VO₄ and extracted with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5% Glycerol, 1% Triton X-100, 25 mM NaF, 2 mM Na₂VO₄, 10 μ g/ml of each aprotinin, leupeptin and pepstatin). The preparation of cytoplasmic was conducted using the NE-PER cytoplasmic extraction reagents (Pierce). The cell lysates were frozen and thawed three times and were further centrifuged at 14 000 \times g for 10 min at 4°C to pellet insoluble material. The supernatant of cell extracts was analyzed for protein concentration by using Lowry's technique (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (50 μ g) from each sample were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to PVDF membranes (MSI, Westborough, MA, USA). Membranes were blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) and then incubated with rabbit polyclonal for Phospho-specific Akt (Ser 473) and Akt (1:2000 dilution); Phospho-specific mTOR (289KD) and mTOR (1:2000 dilution); XIAP(57KD, 1:2000 dilution); cIAP1 (70 KD, 1:2000 dilution); cIAP2 (68 KD, 1:2000 dilution); Smac (22 KD, 1:2000 dilution) and Survivin (16 KD, 1:2000 dilution). β -actin (42 KD, 1:2000) was used to control equal protein loading. The immunoblots were then washed three times with TBS-T buffer, incubated with a horseradish peroxidaseconjugated secondary antibody (goat anti-rabbit IgM, Santa Cruz, CA, USA) and developed using chemiluminescent substrate (Pierce, Rockford, IL, USA). To quantify and compare levels of proteins, the density of each band was measured by densitometry.

Measurement of caspase-3 activity

THP-1 cells were harvested and centrifuged at 1500 rpm for 10 min. Cells were washed two times with PBS (pH 7.4) and then resuspended with 50 μ l lysis buffer at 4°C and incubated on ice for 10 min. All subsequent steps were performed on ice. After centrifugation, cell extracts were transferred to fresh tubes and protein concentrations were measured. Each 50 μ l cell extract containing 100 μ g of protein were combined with equal volumes of 2 \times reaction buffer in a microplate followed by the addition of 5 μ l of peptide substrates of caspase-3. After overnight incubation in the dark at 37°C, samples were read in a microplate reader at 405 nm. Caspase-3 activity were evaluated by the absorbance ratio of

treated/control samples. In some experiments, caspase-3 inhibitor (Z-DEVD-FMK) was added into fresh medium of THP-1 cells at 1 h before EEHDW was added.

Statistical analysis

Each experiment was carried out in duplicate or triplicate and three or four independent experiments were performed. Results were expressed as means \pm standard deviation (SD) and analyzed with SPSS 11.5 software. Results were compared using analysis of variance (ANOVA). When ANOVA showed a statistically significant difference, a group-by-group comparison was performed using a *t*-test with Tukey's correction for multiple comparison. Statistical significance was set at $P < 0.05$.

RESULTS

EEHDW inhibition of cell viability in human THP-1 cells

We performed a trypan blue exclusion assay. Trypan blue staining showed that $98.23 \pm 1.74\%$ of the cells incubated with medium retained an integrated cell membrane (that is, resisted trypan blue staining) (means \pm standard errors of the means: $n = 5$). The percentage of necrosis cells rose with the increasing of time and the concentrations of EEHDW (Figure 1).

EEHDW induction of apoptosis in human THP-1 cells

Flow cytometry using FITC-conjugated annexin V revealed that THP-1 cells exposed to EEHDW underwent rapid apoptosis. This effect was positively correlated with exposure time and the concentrations of EEHDW (Figure 2) and excessive apoptosis was associated with loss of membrane integrity in an increased portion of THP-1, which indicates necrosis or late apoptosis.

Involvement of PI3K in the apoptosis of THP-1 cells

It was found that EEHDW treatment significantly decreased P-Akt and P-mTOR production in a time-dependent manner (Figure 3). These experiments support the conclusion that EEHDW-induced apoptosis is mediated by P-Akt down regulation. LY294002, a potent inhibitor of Akt, reduced the levels of P-Akt in a dose-dependent manner without affecting basal Akt (data not shown).

EEHDW induction of THP-1 apoptotic cell death via modulation of XIAP family proteins

Expressions of antiapoptotic protein cIAP1/2, XIAP and Survivin were decreased in a time-dependent manner (Figure 4). However, the expression of Smac was increased in a time-dependent manner (Figure 4). These

results suggest that the changes in expression of cIAP1/2, Survivin, Smac and XIAP may contribute to EEHDW-induced apoptosis in THP-1 cells.

Effects of inhibitors of Akt or XIAP on EEHDW-induced THP-1 apoptosis

To identify the relevance of Akt and XIAP signaling pathways in controlling the apoptotic cell death by EEHDW, inhibition assays were performed with LY294002 (a specific inhibitor of PI3K) or Embelin (a specific inhibitor of XIAP). The percentage of apoptosis was determined by flow cytometry. THP-1 cells were pretreated with 50 μ M LY294002 or 20 μ M Embelin for 30 min and then cultured with EEHDW for 2 days. The results showed that LY294002 or Embelin significantly reduced the apoptosis rate (Figure 5).

Expression of caspase-3 activity

The expression of caspase-3 activity in THP-1 cells incubated in the presence of EEHDW is presented in Figure 6. Treatment of THP-1 cells with EEHDW for different days at a concentration of 2 mg/ml or for 2 days at the concentrations of 0, 1, 2 and 4 mg/ml, respectively showed marked increase of caspase-3 activation. Activity of caspase-3 in THP-1 cells with EEHDW treatment showed dose- and time-dependent up-regulation. Inhibition of PI3K pathway with LY294002 or XIAP with Embelin potentiated the EEHDW-induced caspase-3 activity. To characterize the pathway of apoptosis execution, experiments were carried out using the caspase-3 inhibitor Z-DEVD-FMK. Apoptosis was greatly reduced by Z-DEVD-FMK (Data not shown), whereas, caspase inhibitors had no effect on PI3K, XIAP and Smac activation in THP-1 cells (data not shown). Together, our data demonstrated that caspase-3 mediates EEHDW-induced THP-1 cells apoptosis.

DISCUSSION

Previous studies demonstrated that EEHDW induces apoptosis in human breast cancer cells (Liu et al., 2010). In this study, we investigated the *in vitro* effects of EEHDW on cell growth and death in THP-1 cells, a human acute monocytic leukemia cell line and examined the mechanisms underlying its actions. To the best of our knowledge, this study for the first time demonstrated that EEHDW induced THP-1 cell apoptosis in a time- and dose-dependent manner.

Apoptosis signaling is regulated by various pro- and anti-apoptotic proteins (Ahn et al., 2010). Akt promotes cell survival by inhibiting apoptosis and its phosphorylation has been considered a critical factor in the aggressiveness of cancer (Lee et al., 2008). Although

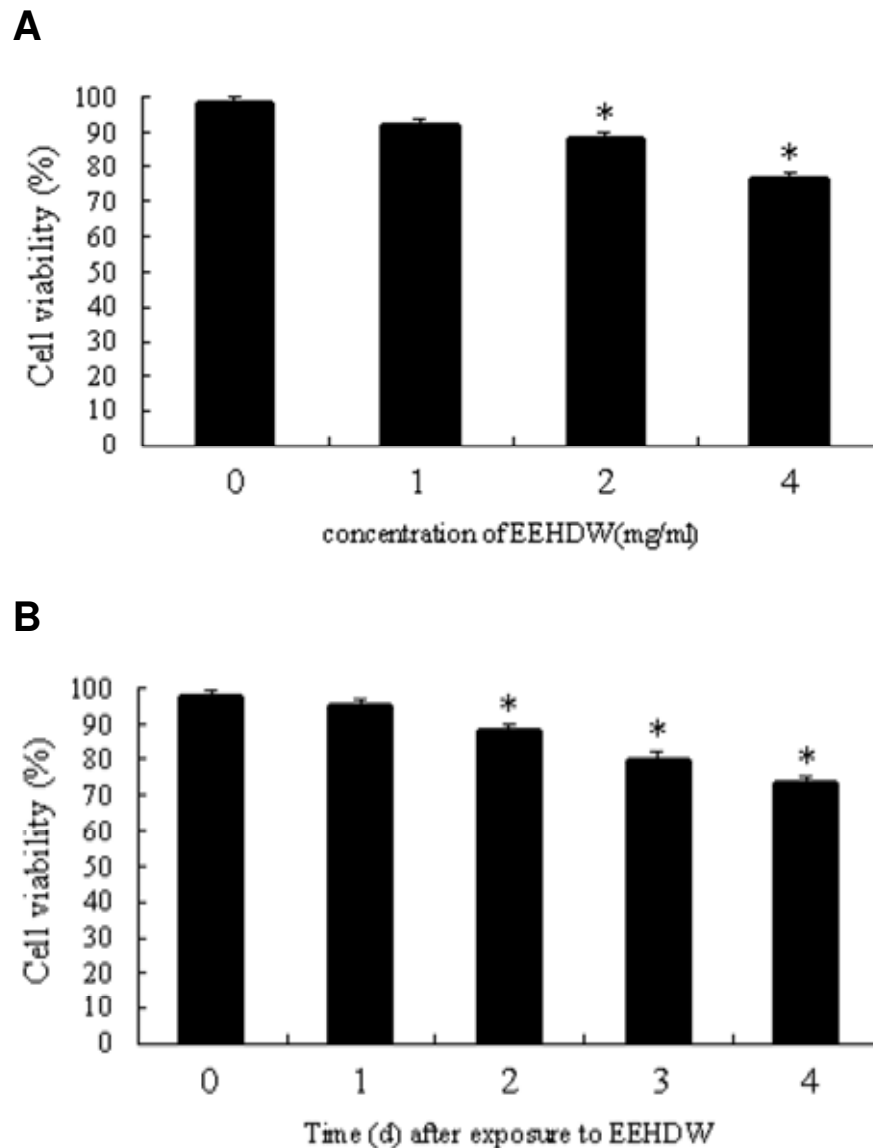


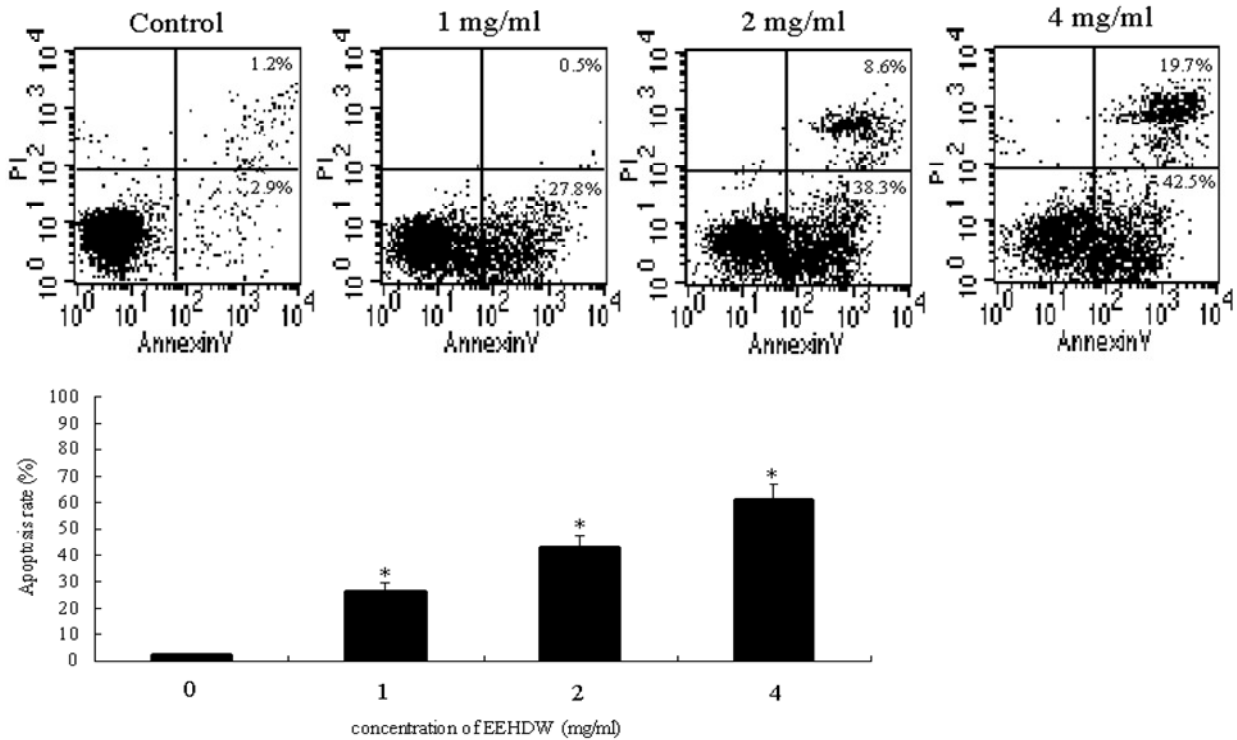
Figure 1. Effect of EEHDW on cell cytotoxicity of THP-1 cells. THP-1 cells were incubated with different concentrations of EEHDW for 2 days (A) or 2 mg/ml EEHDW for different time (B). Viability of THP-1 cells was determined by trypan blue assay. Note that the number of dead cells was increased with EEHDW concentration and infection time used. Data represent means \pm SD of three determinations (* $P < 0.05$) compared with that of the control.

the precise anti-apoptotic effects of Akt are still unclear, Akt directly phosphorylates and inactivates procaspase-9 and blocks caspase-9-mediated apoptosis. Therefore, we investigated whether EEHDW induces down regulation of PI3K/Akt and whether pretreatment with LY294002 could enhance EEHDW-induced apoptosis in THP-1 cells. We reported EEHDW induced inactivation of Akt by decreasing the level of phosphorylated Akt in a concentration-dependent manner, contributing to the promotion of apoptosis. In this study, we also found that the pharmacological inhibitor of PI3K, LY294002, dramati-

cally exerted caspase-3 activity under EEHDW treatment condition.

Another family of apoptosis-regulatory proteins, inhibitors of apoptosis proteins (IAPs), is recently considered a valuable target to modulate apoptotic cell death in many cancer cells (Ahn et al., 2010). Members of the mammalian IAP family include: XIAP, cIAP-1, cIAP-2 and others, which directly inhibit caspase-3, caspase -7 and caspase-9 (Roy et al., 1997). The main antagonist of XIAP is the second mitochondria derived activator of caspases (Smac). Dimeric Smac sterically and/or

A



B

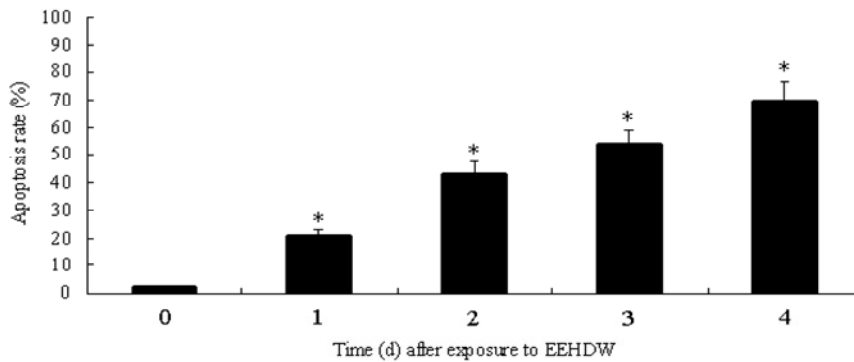


Figure 2. EEHDW-induced apoptosis in THP-1 cells. THP-1 cells were cultured without or with EEHDW. THP-1 cells were harvested for 2 days with different concentration of EEHDW (A) or 2 mg/ml EEHDW for different time (B) and incubated with FITC-conjugated annexin V (AV) and propidium iodide (PI) double staining. Flow cytometric analysis was performed and the data shown are representative of three separate experiments. The lower right quadrants represent early apoptotic cells that were stained by annexin V but not by propidium iodide. The upper right quadrants represent late apoptotic cells that were stained by both annexin V and propidium iodide. * $P < 0.01$ compared with that of THP-1 alone.



Figure 3. The expression of phosphorylated and total Akt protein and phosphorylated and total mTOR protein in THP-1 cells following treatment of 2 mg/ml EEHDW for the time periods indicated. Representative data of three independent experiments are shown.

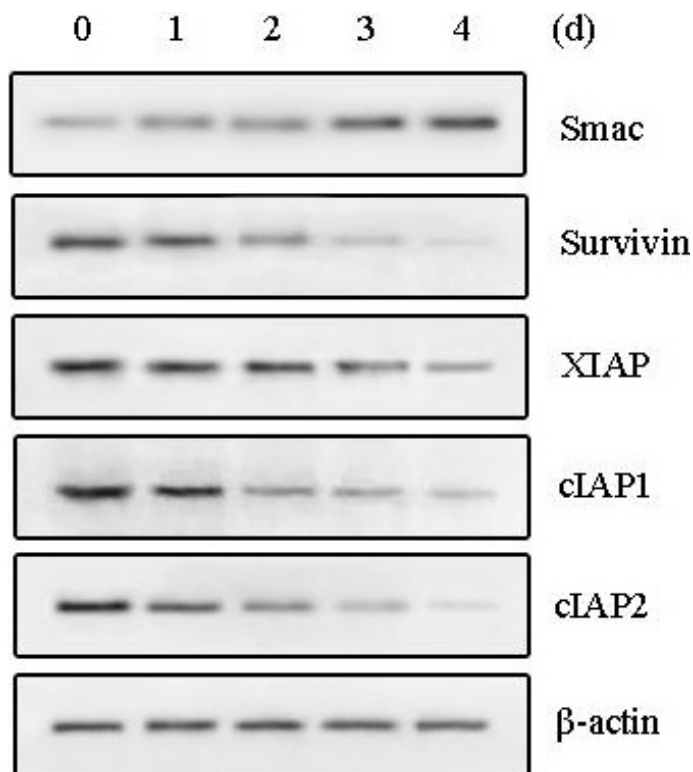


Figure 4. The expressions of XIAP family proteins. Whole cell extracts were prepared and analyzed by Western blotting analysis using Abs against XIAP, cIAP1/2, Survivin, Smac and β -actin in THP-1 cells following treatment of 2 mg/ml EEHDW for the time periods indicated. Representative data of three independent experiments are shown. β -actin was used as a loading control.

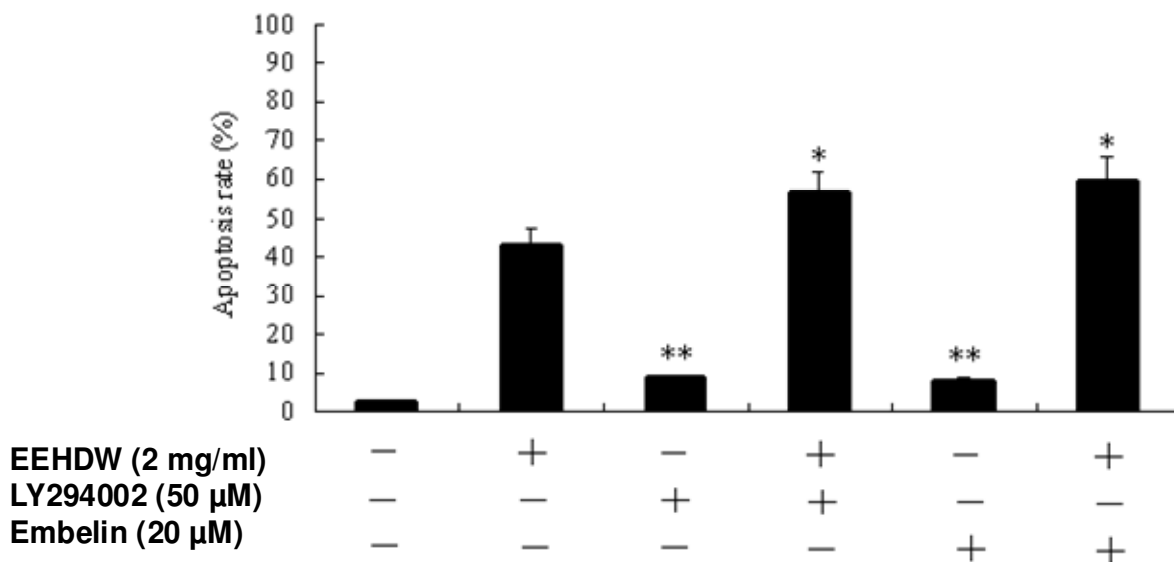


Figure 5. Effect of Akt or XIAP inhibitors on EEHDW-induced THP-1 cell apoptosis. THP-1 cells were treated with 2 mg/ml EEHDW and incubated for 2 days with the indicated concentrations of Akt and XIAP inhibitors: 50 μ M LY294002 or 20 μ M Embelin for 30 min. Values represent means \pm SD of five experiments performed in duplicate. * $P < 0.05$; ** $P < 0.001$ compared with that of EEHDW alone.

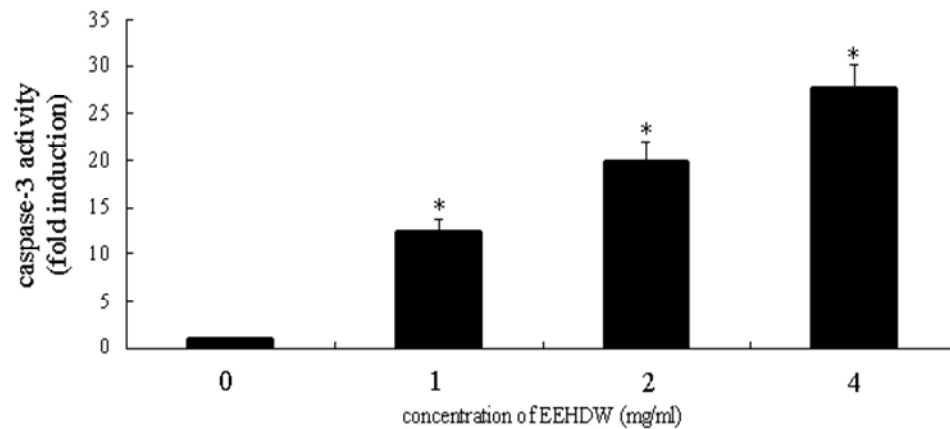
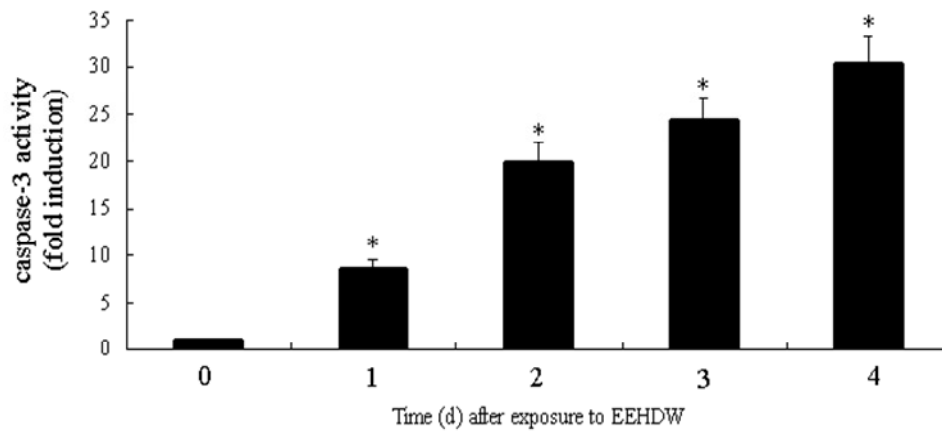
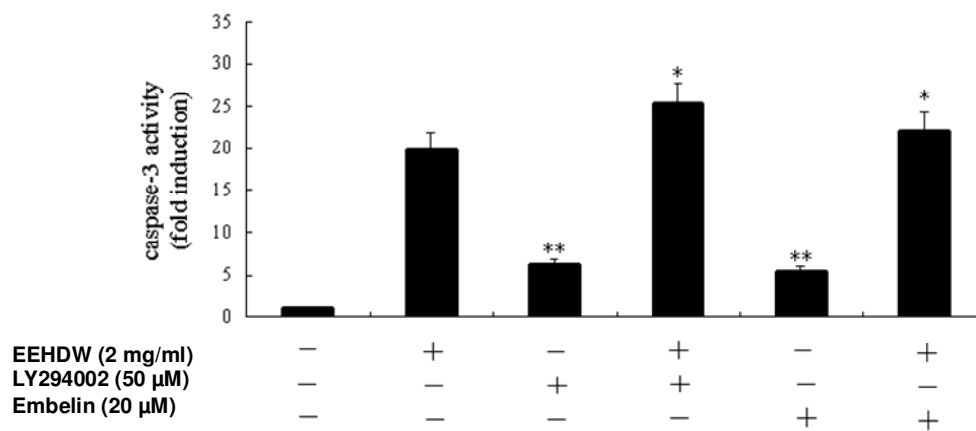
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Figure 6. Effect of EEHDW, Akt inhibitor or XIAP inhibitors on the activity of caspase-3. (A) Dose-dependency of EEHDW-induced caspase-3 activity; *P < 0.01 compared with that of 0 min; (B) time-dependency of EEHDW-induced caspase-3 activity. *P < 0.01 compared with that of 0 min; (C) THP-1 cells were treated with 2 mg/ml EEHDW and incubated for 2 days, with the indicated concentrations of LY294002 or Embelin. Values represent means \pm SD of five experiments performed in duplicate. *P < 0.05, **P < 0.001 compared with that of control.

competitively occludes the caspase 3, 7 and 9 binding sites of XIAP and thereby, can pave the way for efficient cell death execution (Wu et al., 2000; Hao et al., 2004). Smac also binds to other IAPs, such as livin/ML-IAP, Bruce/Apollon, cIAPs 1 and 2 (Bartke et al., 2004; Vucic et al., 2002), which interfere with caspase-mediated cell death and/or have additional roles in pro-survival and proliferation signaling. Nevertheless, it is not currently known whether EEHDW-induced apoptosis is related to down regulation of the IAP family proteins. In this study, there were a tendency of alterations with a decreased expression level of cIAP1/2, Survivin and XIAP and also with an increased expression level of Smac and activation of caspase-3.

Caspase family, aspartate-specific cysteine proteases, plays a central role in regulation of apoptosis (Salvesen and Stennicke 1998; Filip et al., 2011; Souza et al., 2010; Wei et al., 2010). Caspase-3 in particular, when activated, has many cellular targets that when served and/or activated, produce the morphologic features of apoptosis (Cohen, 1997). Recent studies revealed that the modulation of caspases is a complex process and involves a number of regulatory proteins, including PI3K and IAP family proteins (Roccaro et al., 2010; Chauhan, 2007). Study of caspases, which are also important regulators of apoptosis, revealed that exposure of THP-1 cells to EEHDW increased caspase-3 activity. To more directly link PI3K/Akt and XIAP signaling pathway with caspase-3 activation, we examined EEHDW-mediated caspase-3 activation in cells treated with PI3K/Akt or XIAP inhibitors LY294002 or Embelin. Inhibition of PI3K/Akt or XIAP significantly increased caspase-3 activity. In our studies, pretreatment with caspase-3 specific inhibitor significantly blocked caspase-3 activity. These results suggest that in EEHDW-induced THP-1 cells, LY294002 or Embelin induced apoptosis, which are dependent on the activation of caspase-3.

In conclusion, the data presented in this study support the hypothesis that EEHDW are potential and selective anti-cancer agents by modulating apoptosis and proliferation in leukemia cells. Further studies should test the usefulness (efficacy and toxicity) of these compounds in other cell lines.

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