



# Hinesol, a compound isolated from the essential oils of *Atractylodes lancea* rhizome, inhibits cell growth and induces apoptosis in human leukemia HL-60 cells

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**Abstract** Hinesol is a unique sesquiterpenoid isolated from the Chinese traditional medicine, *Atractylodes lancea* rhizome. In a previous study, we screened various natural products in human leukemia HL-60 cells and identified an essential oil fraction from *A. lancea* rhizome that exhibited apoptosis-inducing activity in these cells; hinesol was subsequently shown to be the compound responsible for this apoptosis-inducing activity. In this study, we describe the cytotoxic effects and molecular mechanisms of hinesol in HL-60 cells. The antitumor effect of hinesol was associated with apoptosis. When HL-60 cells were treated with hinesol, characteristic features of apoptosis, such as nuclear fragmentation and DNA fragmentation, were observed. These growth-inhibitory and apoptosis-inducing activities of hinesol in leukemia cells were much stronger than those of  $\beta$ -eudesmol, another compound isolated from the essential oil fraction. Furthermore, hinesol induced activation of c-Jun N-terminal kinase (JNK), but not p38, prior to the onset of apoptosis. These results suggested that hinesol induced apoptosis through the JNK signaling pathway in HL-60 cells. Therefore, hinesol may represent a novel medicinal drug having indications in the treatment of various cancers, including leukemia.

**Keywords** *Atractylodes lancea* rhizome · Apoptosis · Essential oil · Hinesol · HL-60 cells · JNK

## Introduction

Apoptosis is characterized by specific changes in cell morphology [1], namely, reduction in cell volume, condensation of chromatin in the nucleus, and digestion of chromatin into DNA fragments. Apoptosis has been implicated in a variety of biological processes, such as the elimination of damaged cells [2], regulation of the immune system [3], and embryogenesis [4]. Several recent studies have demonstrated the importance of apoptosis in anti-cancer therapies, and various chemotherapeutic anticancer drugs, such as cisplatin [5], adriamycin [6], and taxol [7], have been shown to induce apoptosis in cancer cells. Cells that undergo apoptosis are recognized and engulfed by macrophages, thereby allowing the cells to be removed without inducing an inflammatory reaction. Therefore, the induction of apoptosis in cancer cells has become an indicator of the effect of anticancer agents, and agents with strong apoptosis-inducing activity are expected to have potential clinical applications. Interestingly, in our previous study, we identified a variety of apoptosis inducers from natural products in cancer cells, including bufalin [8],  $\beta$ -hydroxyisovalerylshikonin [9], and sophoranone [10].

Induction of apoptosis is regulated by a variety of extracellular stresses and intracellular signals. Our previous reports described several signaling cascades involved in the regulation of apoptosis induction. For example, inhibition of Bcr-Abl tyrosine kinase activity inhibits the kinase activity of PLK1 and sensitizes cancer cells to induction of apoptosis, suggesting the involvement of tyrosine kinases in the regulation of PLK1 kinase activity [11]. We also

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found that at low concentrations of bufalin, one of the components of bufadienolides in the traditional Chinese medicine chan'su (prepared from toad venom), is able to induce apoptosis in human leukemia cells. Bufalin caused the cooperative interaction of 2 different signal transduction pathways, leading to abnormal activation of mitogen-activated protein kinase (MAPK) [8], an important regulator of apoptosis. In addition to MAPK, 2 members of the MAPK superfamily, c-Jun N-terminal kinase (JNK) and p38 [also known as stress-activated protein kinase (SAPK)], are activated by a variety of extracellular stimuli, such as osmotic shock and ultraviolet (UV) light [12, 13]. Involvement of SAPK has been demonstrated in the apoptotic processes induced in mouse Pam 212 keratinocytes by cisplatin [14] and in Jurkat T cells by UV light and  $\gamma$ -irradiation [15].

We investigated the signaling pathways that lead to induction of apoptosis in response to compounds in the essential oils of *Atractylodes lancea* rhizome in HL-60 cells. *A. lancea* rhizome is an important traditional Chinese medicine and is listed in the Japanese pharmacopoeias (as so-jutsu). *A. lancea* rhizome has been prescribed in traditional medicine as a diuretic and stomachic drug, with  $\beta$ -eudesmol and hinesol as the main constituents of the essential oil from this medicinal plant [16]. Recently, there has been increasing interest in the use of essential oils as medical agents because essential oils have been reported to have antitumor potential, inducing apoptosis in various tumor cell lines [17, 18]. These observations suggest that the essential oils of *A. lancea* rhizome may be a useful apoptosis inducer in cancer cells.

In the present study, we found that hinesol, a compound isolated from the essential oils of *A. lancea* rhizome, had apoptosis-inducing activity in human leukemia cells. To the best of our knowledge, the apoptosis-inducing activity of hinesol has not previously been reported. We also demonstrated that hinesol activated the JNK/SAPK pathway without activation of other members of the MAPK family, such as p38. Our results suggested that hinesol may be a good candidate for a novel anticancer drug.

## Materials and methods

### Reagents, cell line, and cell culture

Hoechst 33342 and RPMI 1640 medium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phospho-p38 (Thr180/Tyr182) rabbit monoclonal antibodies (D3F9), anti-p38 (D13E1) rabbit monoclonal antibodies, anti-phospho-SAPK/JNK (Thr183/Tyr185) mouse monoclonal antibodies, anti-SAPK/JNK (56G8) rabbit monoclonal antibody, anti-phospho-p44/42 MAPK [extracellular signal-

regulated kinase (ERK) 1/2] (Thr202/Tyr204) mouse monoclonal antibodies, and anti-p44/42 MAPK (ERK1/2) mouse monoclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Human leukemia HL-60 cells were provided by the Japanese Cancer Resource Bank. The cells were maintained in RPMI 1640 medium, supplemented with 10 % heat-inactivated fetal calf serum, in an atmosphere of 5 % CO<sub>2</sub> at 37 °C. Cells were seeded at a concentration of 2–3 × 10<sup>5</sup> cells/mL, and logarithmic growth was maintained by passaging the cells every 2–3 days.

### Isolation of $\beta$ -eudesmol and hinesol

Commercial *A. lancea* Rhizome (the rhizomes of *A. lancea*; purchased from Uchida Wakanyaku Ltd., Lot No. 08M1145, 100 g) was placed in a 1-L glass-stoppered flask with 500 mL water. An apparatus was set up for the collection of essential oils, including a reflux condenser, and the contents of the flask were heated to 130–150 °C to boiling. The essential oil was captured in xylene (2 mL), as described in the essential oil content section of the Japanese Pharmacopoeia. After evaporation of the xylene, the essential oil (200 mg) was subjected to repeated high-performance liquid chromatography (HPLC) on an ODS Waters Symmetry PrepTMC18 column [7  $\mu$ m (7.8 × 300 mm) × 2] with CH<sub>3</sub>OH–H<sub>2</sub>O (9:1) as the solvent at a flow rate of 2 mL/min to yield  $\beta$ -eudesmol (*t*R: 20.37 min, 75 mg) and hinesol (*t*R: 21.06 min, 80 mg).

### Identification and assessment of the purity of isolated compounds

Identification and assessment of the purity of isolated compounds were carried out by analysis of NMR spectral data. The <sup>1</sup>H-NMR (600 MHz, CDCl<sub>3</sub>) spectra in Fig. 2 were obtained on an FT NMR system (Bruker). Hinesol: <sup>1</sup>H-NMR (Fig. 2a)  $\delta$ : 0.92 (3H, d, *J* = 6.0 Hz, H3-15), 1.20, 1.21 (each 3H, s, H3-12, H3-13), 1.68 (3H, br s, H3-14), 5.32 (1H, m, H-2).  $\beta$ -Eudesmol: <sup>1</sup>H-NMR (Fig. 2b)  $\delta$ : 0.70 (3H, s, H3-14), 1.21 (3H × 2, s, H3-12, H3-13), 4.45, 4.72 (each 1H, br s, H-15a, H-15b).

### Analysis of cell death

Apoptotic cells were assessed by examination of nuclear morphology after staining with Hoechst 33342. Cells were stained with 10  $\mu$ M Hoechst 33342 for 15 min at 4 °C and were examined under a fluorescence microscope. Analysis of DNA fragmentation by agarose gel electrophoresis was performed as follows. Drug-treated or untreated cells were collected by centrifugation and were washed with Dulbecco's phosphate-buffered saline (PBS, without Ca<sup>2+</sup> and Mg<sup>2+</sup>). The washed cells were suspended in lysis buffer

[10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5 % sodium dodecyl sulfate (SDS)] containing 0.1 % RNase A and were incubated for 60 min at 50 °C. The lysate was incubated for an additional 60 min at 50 °C in the presence of 1 mg/mL proteinase K, and the resulting preparation of DNA was analyzed by gel electrophoresis on a 1 % agarose gel in Tris acetate buffer (40 mM Tris acetate, 1 mM EDTA). After electrophoresis, DNA was visualized by staining with ethidium bromide.

### XTT assay

Cell viability was assessed using XTT assays [19]. A suspension of cells was treated with sample compounds. After culturing for 3 days at 37 °C, a solution of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS) was added, and the culture was incubated for an additional 4 h. The absorbance at 540 nm was measured on a 96-well plate with a micro plate reader.

### Preparation of cell lysates

Cells were washed twice with PBS and lysed in lysis buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 5 µg/mL pepstatin A, 5 µg/mL antipain, 50 mM NaF, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.15 M NaCl, 1 % Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride]. The lysate was centrifuged at 15,000g for 15 min, and the supernatant was subjected to Western blot analysis.

### Western blot analysis and assays of protein kinase activities

The cell lysate containing 30 µg of protein was fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), and proteins were then transferred to a nitrocellulose membrane. The membrane was first rinsed with TBST [20 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 0.05 % Tween 20] and then blocked with 5 % (w/v) skim milk in TBST for 1 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with primary antibodies at 1:200–1:1000 dilutions in blocking buffer. The membrane was washed 3 times with TBST and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibodies raised against rabbit or mouse IgG. The membrane was washed again with TBST, and protein bands were visualized with an enhanced chemiluminescence Western blotting detection kit (Perkin Elmer Life Sciences, Inc., Boston, MA, USA). The activities and expression of protein kinases were

determined by Western blotting with specific antibodies against phospho-p38, p38, phospho-ERK, ERK, phospho-JNK, and JNK.

### Statistical analysis

Data are expressed as mean  $\pm$  standard deviation. Results were analyzed using Student's *t* test, and statistical significance for all comparisons was assigned at  $P < 0.01$ .

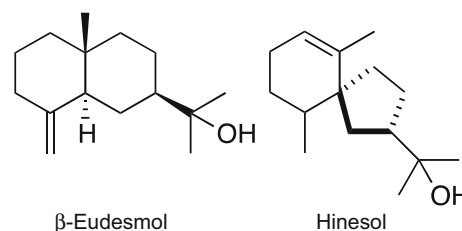
## Results

### Purification and isolation of compounds

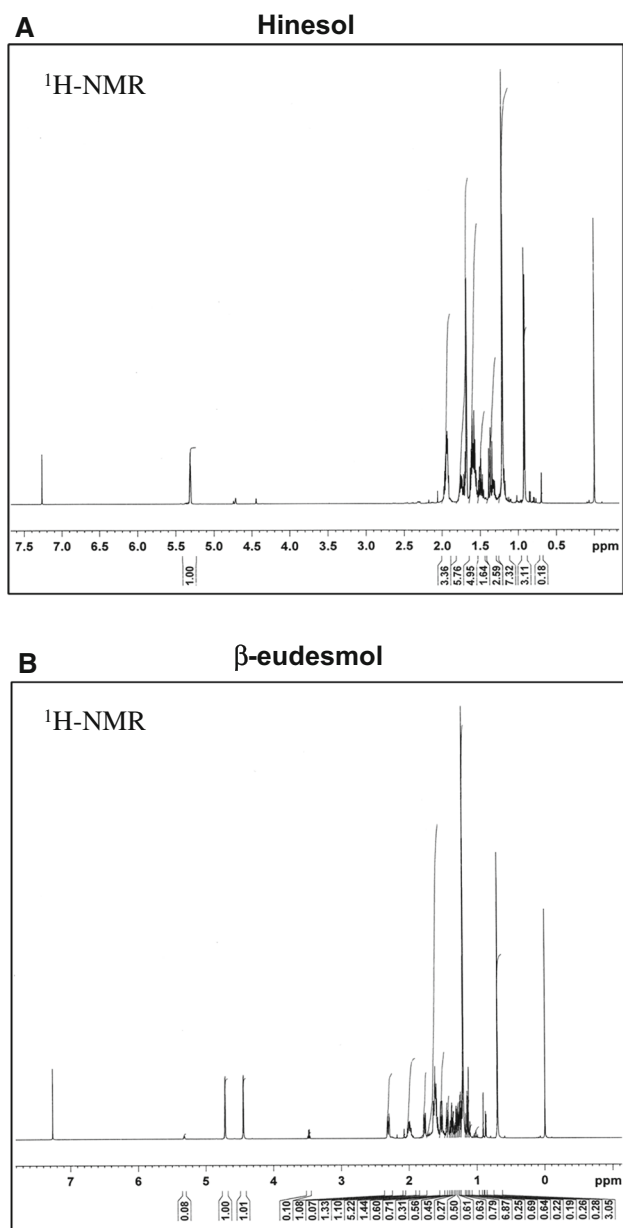
The essential oil extract of *A. lancea* rhizome was shown to have antitumor activity in HL-60 cells. To determine the effects of the components of *A. lancea* rhizome on the induction of apoptosis, the major components of the essential oil were isolated. The essential oil was subjected to repeated HPLC, yielding  $\beta$ -eudesmol (Fig. 1, left) and hinesol (Fig. 1, right), as described in “Materials and methods”. We identified hinesol and  $\beta$ -eudesmol by comparing the NMR spectra of the purified compound (Fig. 2) with previously reported spectra [20].

### Effects of essential oils on antitumor activity

Figure 3 shows the effects of the essential oil from *A. lancea* rhizomes on the viability of HL-60 cells. Cell viability was decreased in a dose-dependent manner in response to treatment with the essential oil fraction. The concentration of essential oil that decreased cell viability to 50 % (i.e., the  $IC_{50}$ ) was  $9.6 \pm 0.9$  µg/mL (Table 1). To evaluate the compounds involved in the induction of antitumor activity, the effects of the isolated compounds on cell viability were investigated. Hinesol exhibited antitumor activity with an  $IC_{50}$  of  $4.9 \pm 1.9$  µg/mL ( $22.1 \pm 8.6$  µM; Table 1). The growth-inhibitory activity of hinesol was stronger than that of  $\beta$ -eudesmol, another compound isolated from the essential oil fraction (Fig. 3). The  $IC_{50}$  of  $\beta$ -eudesmol was almost the same as that of the



**Fig. 1** Chemical structures of  $\beta$ -eudesmol and hinesol

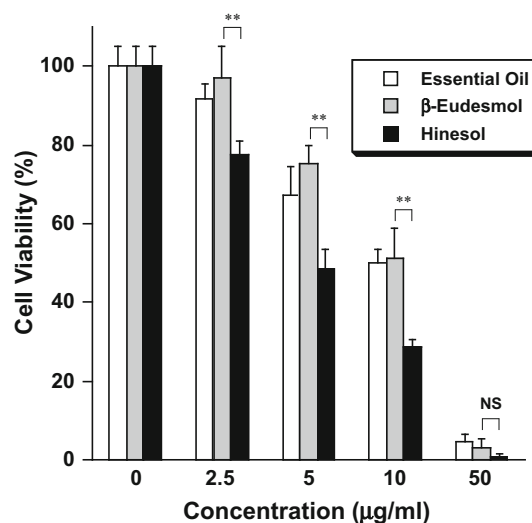


**Fig. 2** Identification of purified compounds. **a** <sup>1</sup>H-NMR (600 MHz, DCCl<sub>3</sub>) spectrum of hinesol. **b** <sup>1</sup>H-NMR (600 MHz, DCCl<sub>3</sub>) spectrum of β-eudesmol

essential oil fraction (10.4 ± 0.8 and 9.6 ± 0.9 μg/mL, respectively), and these IC<sub>50</sub> values were approximately 2.1- and 2.0-fold less than that of hinesol, respectively.

**Induction of apoptosis by hinesol and β-eudesmol**

To clarify whether the features of cell death induced by hinesol and β-eudesmol were from apoptosis or necrosis, the induction of morphological changes was investigated. Figure 4 shows morphological changes in HL-60 cells that had been treated with hinesol or β-eudesmol. The cells



**Fig. 3** Growth-inhibitory effects of hinesol and β-eudesmol from *Atractylodes lancea* rhizomes. HL-60 cells were treated with individual compounds at various concentrations for 3 days, and the cell viability was monitored by the XTT method, as described in “Materials and methods”. All results shown are means ± SDs from four independent experiments. \*\*Data with statistical significance against control: *P* < 0.01. NS not significant

**Table 1** Antitumor activity of essential oil from *Atractylodes lancea* rhizomes

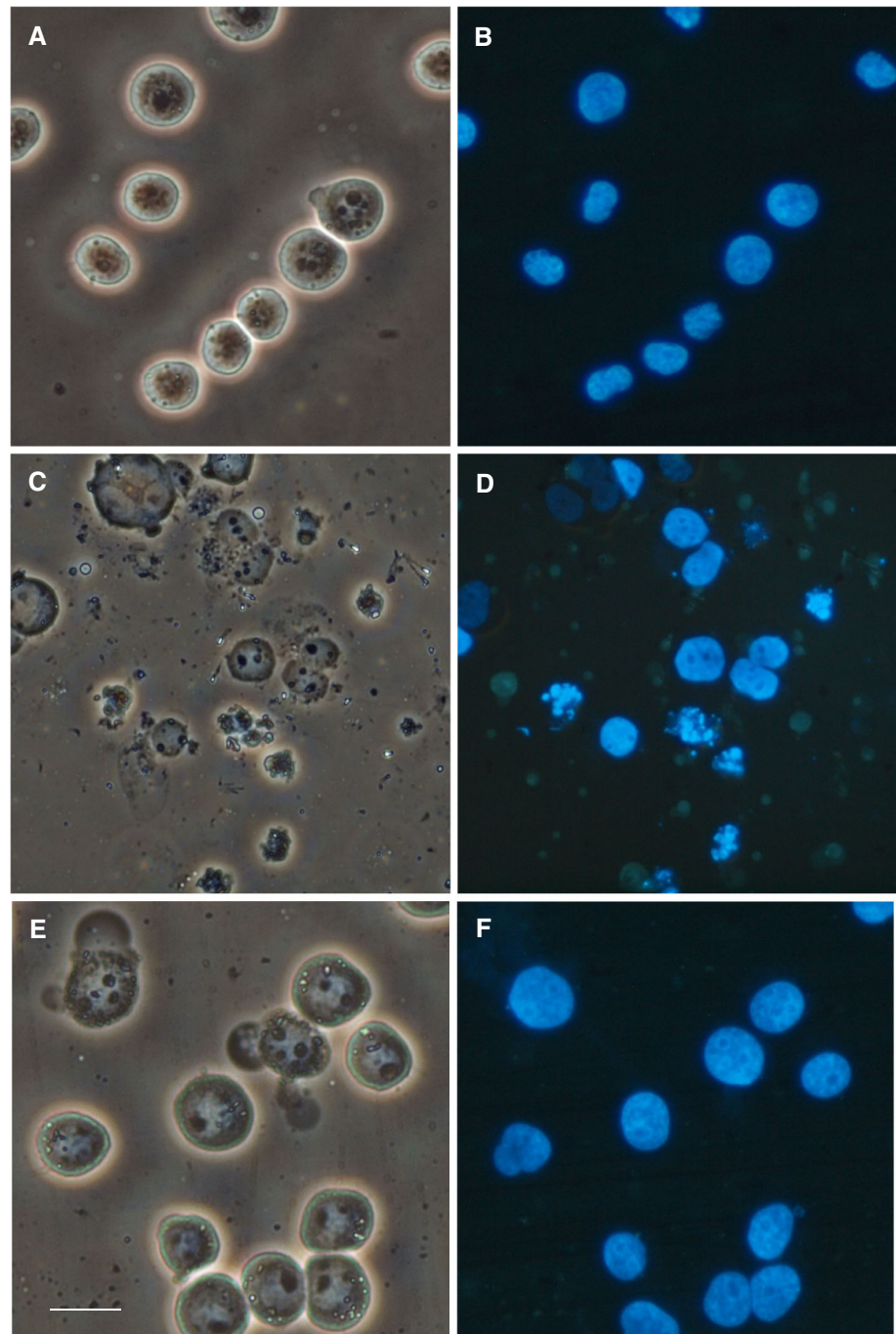
Compounds	IC <sub>50</sub> (μg/mL)	IC <sub>50</sub> (μM)
Essential oil	9.6 ± 0.9	ND
β-Eudesmol	10.4 ± 3.2	46.8 ± 3.2
Hinesol	4.9 ± 1.9	22.1 ± 8.6

HL-60 cells were treated with each compound for 3 days. The IC<sub>50</sub> is the concentration that gave 50 % inhibition of growth. Percentages of viable cells were determined by the XTT assay as described in “Materials and methods”. All results shown are means ± SDs from 3 independent experiments

ND not determined

developed obvious apoptotic features that included chromatin condensation and nuclear fragmentation by treatment with hinesol (Fig. 4c, d). In contrast, no morphological changes were evident in untreated control cells (Fig. 4a, b). Figure 4e, f show the effects of β-eudesmol on the induction of morphological changes associated with HL-60 cells. As is evident from the figure, almost no morphological changes were observed under the same experimental conditions (Fig. 4e, f). Since internucleosomal DNA fragmentation is a biochemical feature of the apoptotic process, we investigated the effects of hinesol and β-eudesmol on DNA fragmentation. HL-60 cells were treated with 0–100 μM of each compound for 8 h and subjected to agarose gel electrophoresis. A typical ladder pattern of internucleosomal fragmentation was observed in a dose-

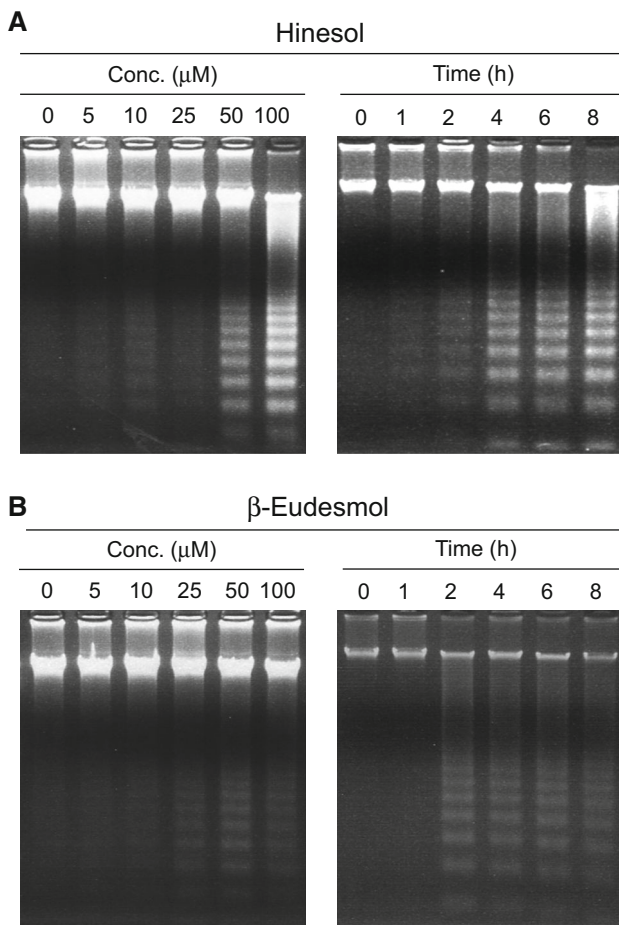
**Fig. 4** Analysis of morphological changes induced by hinesol or  $\beta$ -eudesmol. HL-60 cells were treated with 100  $\mu$ M hinesol (c, d) or 100  $\mu$ M  $\beta$ -eudesmol (e, f) for 8 h. Untreated cells were used as controls (a, b). After staining the cells with Hoechst 33258, morphological changes were analyzed under fluorescence microscopy (b, d, f) or assessed by phase contrast microscopy (a, c, e). The *bar* indicates 10  $\mu$ m



dependent manner (Fig. 5a, left panel). When the cells were treated with 100  $\mu$ M hinesol, the appearance of fragmented DNA was evident within 4 h of beginning treatment (Fig. 5a, right panel). In contrast,  $\beta$ -eudesmol had almost no effect on the induction of DNA fragmentation under the same experimental conditions (Fig. 5b). Moreover, even after treatment with a higher concentration of  $\beta$ -eudesmol (200  $\mu$ M) for 8 h, little or no induction of apoptosis was observed (data not shown). These results indicated that hinesol induced apoptosis in HL-60 cells.

#### Effects of hinesol on the activation of mitogen-activated protein (MAP) kinases

Various combinations of inhibition and activation of MAPKs have been proposed to play an important role in the regulation of apoptosis [21]. The activation of JNK and p38 promotes apoptosis, whereas the activation of ERK prevents apoptosis. When HL-60 cells were treated with 100  $\mu$ M hinesol, JNK activity increased markedly and reached a maximum after exposure to hinesol for 2 h. In

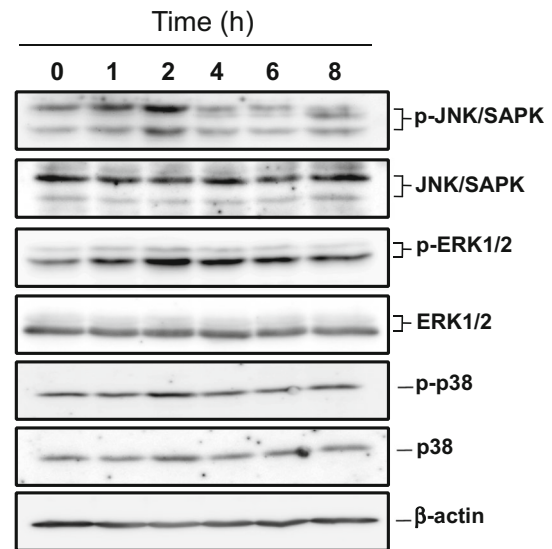


**Fig. 5** Analysis of hinesol- or  $\beta$ -eudesmol-induced DNA fragmentation in HL-60 cells. Cells were treated for 8 h with hinesol (**a**, left panel) or  $\beta$ -eudesmol (**b**, left panel) at various concentrations and with 100  $\mu$ M hinesol (**a**, right panel) or 100  $\mu$ M  $\beta$ -eudesmol (**b**, right panel) for the indicated times. DNA fragmentation was analyzed by agarose gel electrophoresis and staining with ethidium bromide, as described in “Materials and methods”

contrast, no significant alterations in p38 activity were observed (Fig. 6). Hinesol also induced a rapid and transient increase in the activity of ERK (Fig. 6). The maximum activation of ERK was detected at 2 h after the start of treatment with hinesol, after which ERK activity decreased slightly (Fig. 6). Because activation of ERK is involved in prevention of apoptosis, it was surprising that hinesol increased ERK activity. These results indicate that JNK and ERK were significantly activated by hinesol, but did not alter the activation of p38.

**Discussion**

The rhizomes of *A. lancea* have been used in traditional Chinese medicine for many years as stomatic and diuretic drugs. A recent report described the biological activity of



**Fig. 6** Effects of hinesol on MAPK activity. HL-60 cells were treated with 100  $\mu$ M hinesol for the indicated times. Activated forms of JNK, ERK, and p38 were detected by immunoblotting with antibodies against p-JNK, p-ERK, and p-p38. The amounts of JNK, ERK, and p38 were detected by immunoblotting with antibodies against JNK, ERK, and p38

the essential oil and its constituents from *A. lancea* rhizome. Additionally,  $\beta$ -eudesmol, which is contained in the rhizome of *A. lancea*, has been shown to exert anti-inflammatory effects through the suppression of caspase-1 activation in the mast cell-mediated inflammatory response [22]. *A. lancea* extracts and  $\beta$ -eudesmol may stimulate gastric emptying or small intestinal motility by inhibiting the dopamine D<sub>2</sub> and 5-HT<sub>3</sub> receptors [23]. Although the essential oil from *A. lancea* rhizome has multiple pharmacological effects, as described above, the apoptotic effects of the essential oil and the molecular mechanisms associated with these effects are unclear. Pharmacological agents that are able to induce apoptosis may be effective drugs against many cancers. One common strategy for developing novel chemotherapeutics is the evaluation of natural compounds. Indeed, a great number of clinically useful drugs that are used in cancer therapy are isolated from natural products [24]. Our present study demonstrated that the essential oil from *A. lancea* rhizome contained active components responsible for its antitumor activities, including hinesol, which was a potent apoptosis inducer in human leukemia cells.

In our present study, we isolated two compounds with potent growth-inhibitory effects on human leukemia cells (hinesol and  $\beta$ -eudesmol) from *A. lancea* rhizome. Recently, Li and coworkers demonstrated that  $\beta$ -eudesmol induces apoptosis in HL-60 cells via the mitochondrial apoptotic pathway, which is controlled through JNK signaling [25]. As shown in Fig. 3, our present findings

indicated that  $\beta$ -eudesmol had antitumor activity in HL-60 cells. However, in contrast to the results of Li et al.,  $\beta$ -eudesmol had minimal apoptotic activity in HL-60 cells. This difference may have been due to varying experimental conditions between our study and the previous study, particularly with regard to the duration of  $\beta$ -eudesmol treatment. Moreover, when HL-60 cells were treated with 100  $\mu$ M  $\beta$ -eudesmol, the duration of treatment was extended to 72 h or more. This may explain the differences in the induction of apoptosis. HL-60 cell death induced by hinesol was shown to be due to apoptosis, as evident by morphological changes, such as nuclear fragmentation and DNA fragmentation. One interesting feature of the effects of hinesol on HL-60 cells was that the concentrations needed to inhibit cell growth and to induce apoptosis were much lower than those of another constituent,  $\beta$ -eudesmol. The  $IC_{50}$  value of hinesol in HL-60 cells,  $22.1 \pm 8.6 \mu$ M, was 2.1-fold lower than that of  $\beta$ -eudesmol. Previously, we demonstrated that bufalin, one of the components of bufadienolides in the traditional Chinese medicine chan'su, induced typical apoptosis in HL-60 cells [8]. Cardiotonic steroids are well known to inhibit  $Na^+,K^+$ -ATPase activity, and bufalin showed potent inhibitory activity against a partially purified  $Na^+,K^+$ -ATPase from porcine cerebral cortex compared with cardiotonic steroids, such as ouabain and cinobufagin [26], indicating that inhibition of  $Na^+,K^+$ -ATPase activity in cancer cells was associated with the induction of apoptosis. Hinesol was recently shown to inhibit the activity of  $Na^+,K^+$ -ATPase [27]. Although the precise mechanism through which hinesol induces apoptosis is unknown,  $Na^+,K^+$ -ATPase may be a possible target for induction of apoptosis by hinesol.

MAPKs include several subfamilies, including ERK1/2, JNK, and p38, and play crucial roles in the regulation of apoptosis. Two members of the superfamily of MAPKs, JNK and p38 (a stress-activated protein kinase), are activated by a variety of extracellular apoptotic stimuli [28, 29]. In contrast, activation of ERK, which occurs primarily in response to stimulation by growth factors, prevents the initiation of apoptotic processes [21]. Therefore, the balance between the ERK and JNK-p38 pathways may be important for induction of apoptosis. Based on a recent report in which activation of JNK/SAPK MAPKs was observed in  $\beta$ -eudesmol-treated HL-60 cells and a JNK inhibitor blocked  $\beta$ -eudesmol-induced apoptosis [25], we investigated the activation of MAPK family proteins in hinesol-stimulated HL-60 cells. We found that treatment of HL-60 cells with hinesol induced preferential activation of JNK/SAPK and the growth factor-activated protein kinase, ERK, without activation of p38, suggesting the possible involvement of JNK in hinesol-induced apoptosis. Although the roles of JNK and p38 in the regulation of apoptosis by various stimuli have been extensively studied,

the activation of both kinases was not observed after treatment with  $\beta$ -eudesmol (data not shown). Interestingly, activation of ERK was also observed following treatment with hinesol. However, the mechanisms through which JNK and ERK regulate hinesol-induced apoptosis are still unclear. The balance between activation of the JNK and ERK pathways may play an important role in the regulation of hinesol-induced apoptosis.

In summary, our results indicated that hinesol induced apoptosis more potently than  $\beta$ -eudesmol in human leukemia HL-60 cells, suggesting the possibility that hinesol may be a useful anticancer drug that could enhance therapeutic efficacy. Further studies are needed to clarify the details of the molecular mechanisms of hinesol-induced apoptosis.

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**Conflict of interest** None declared.

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