

# Kuromoji (*Lindera umbellata*) essential oil-induced apoptosis and differentiation in human leukemia HL-60 cells

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**Abstract.** Essential oils diluted from certain plants have been shown to have antitumor activity against several human tumor cell lines. Kuromoji (*Lindera umbellata*) essential oil (KEO) has long been used in Japan as a traditional medicine. KEO and its major chemical constituent, linalool, were investigated in this study for their ability to induce apoptosis and differentiation in human leukemia HL-60 cells. HL-60 cells were treated with 5 or 50  $\mu\text{g/ml}$  KEO or linalool for 24 or 48 h. Then, cell proliferation and apoptosis induction were estimated. In addition, HL-60 cells are known to differentiate into granulocyte or monocytes by a variety of compounds. Therefore, the effect of KEO or linalool on differentiation of HL-60 cells was assessed by Giemsa stain and a nitroblue tetrazolium reduction assay. Cells treated with KEO or linalool for 48 h showed significantly suppressed cell proliferation, with induced apoptosis. Moreover, KEO and linalool promoted cell differentiation. Treatment with KEO cells at the same dose as linalool showed an almost identical effect on HL-60 cells. These results suggest that KEO and linalool have efficacy as anticancer therapeutic products.

## Introduction

Second to cardiovascular disease, cancer is the leading cause of death throughout the world. Cancer cells evade self-demise through apoptosis by the accumulation of several genetic and epigenetic changes (1). Therefore, agents that trigger apoptosis in cancer cells are regarded as important for the development of anticancer chemotherapeutics (2). In addition, cancer cells, including leukemia cells, exhibit a defect in their capacity to mature to non-replicating adult cells, therefore remaining in a highly proliferative state and outgrowing their normal cellular counterparts (3,4). Induction of terminal differentiation, leading to the eventual elimination of tumorigenic cells and

reestablishment of normal cellular homeostasis, represents an alternative approach to cancer treatment using conventional antitumor agents.

Essential oils diluted from several plants have been used for a long time in perfumery, aromatherapy, food and flavors. Many essential oils are known to be potent antibacterial and antifungal agents. Certain essential oil components have been reported to have anticancer activity (5). The advantage of these components in anticancer therapy is their low or negligible toxicity. Therefore, such components can be used for the long-term treatment of chemoprevention and chemotherapy of cancer (6).

Kuromoji (*Lindera umbellata*) is a deciduous shrubby tree that is native to cool or warm temperate areas of Japan. It is known as a spicebush because it contains aromatic components in its twigs and leaves. Consequently, Kuromoji branches have been used for indoor decorations and to produce shavings for aromatic baths. Furthermore, Kuromoji essential oil (KEO) obtained through steam distillation has been used traditionally as a medicine for neuralgia, stiff neck and back pain. The main KEO components are terpenes and alcohol esters; linalool is the most abundant component. Geranyl acetate, an ester contained in KEO contributes a relaxing and calming effect on humans. Other terpenes (limonene and  $\alpha$ -pinene) and alcohols (geraniol and cineol) in essential oil have beneficial activities for various diseases, supporting anti-inflammatory and anti-obesity effects.

Linalool reportedly possesses strong activity against U937 histiocytic lymphoma cells and P3HR1 Burkitt lymphoma cells (7). Moreover, several recent reports have revealed that linalool reverses doxorubicin resistance in human breast adenocarcinoma cells (8) and exhibits antiproliferative activity against certain solid tumor cells, such as melanoma and renal cell adenocarcinoma cells (9), and HepG2 (10). Particularly, linalool induces apoptosis in human leukemia cells without affecting normal hematopoietic cell growth (11). These terpenoid components become ligands in nuclear receptors, regulate expression of various genes and ameliorate conditions related to these diseases (12).

Human myeloid leukemia cells, specifically HL-60 cells, are known to differentiate into granulocytes or monocytes when treated with a variety of compounds, such as all-trans-retinoic acid (ATRA), dimethyl sulfoxide and vitamin D3 (13,14). In particular, ATRA is a class of chemical compounds that are structurally related to vitamin A; ATRA is one class

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of compounds known as retinoids, which are used for clinical therapy for acute promyelocytic leukemia (APL) (13). These retinoid components become ligands of nuclear receptors; they regulate cell proliferation and differentiation (15).

This study was undertaken to investigate whether KEO induces apoptosis and promotes differentiation in human leukemia HL-60 cells. The results suggest that KEO may be available for use as a natural therapeutic agent of APL.

## Materials and methods

**Chemical reagents and cells.** For use in this study, HL-60 cells (ATCC CCL240) were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries Ltd. (Kibbutz, Beit, Israel). RPMI-1640 medium was purchased from Sigma-Aldrich Japan (Tokyo, Japan). ATRA was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A sandwich ELISA kit used to estimate the levels of histone-associated DNA fragments was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals, guaranteed to be of reagent or tissue-culture grade, were obtained from Sigma-Aldrich Japan or Wako Pure Chemical Industries, Ltd.

**Isolation of Kuromoji essential oil.** KEO was donated from Senjyakudo (Aomori, Japan). Fresh Kuromoji leaves and branches were collected in the mountains near Aomori city. These ingredients were heated to boiling temperature. The hydro-distilled volatile part was subsequently collected and separated (upper layer) from the aqueous portion.

Analysis of KEO constituents was carried out using gas chromatography-MS at the Aomori Prefectural Industrial Technology Research Center. The GC-MS system (GC-17A/QP-5000; Shimadzu Corp.) was equipped with a DB-1701 column (30 mm x 0.25 mm x 0.25  $\mu$ m; J&W Scientific Inc.). The carrier gas was helium. The program was operated with the following oven temperature program: 50°C, held for 5 min, rising at 5°C/min to 100°C, rising at 2°C/min to 150°C, rising at 10°C/min to 250°C; the injection temperature and volume were 270°C and 1.0  $\mu$ l, respectively. Constituents were identified by computer matching of mass spectral data with data from the NIST62 mass spectral database (Shimadzu Corp.). The major constituents of KEO were linalool (65.78%), geranyl acetate (17.59%), geraniol (5.29%), cineol (2.34%), limonene (2.11%), 3-carene (1.78%),  $\alpha$ -pinene (1.43%) and carvone (1.18%).

**Cell culture.** HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin at 37°C in a 5% CO<sub>2</sub> humidified incubator. For each experiment, HL-60 cells were seeded at a density of 5x10<sup>4</sup> cells/ml. As an ethanol solution, KEO, ATRA and linalool were added to the culture medium. The final concentration of ethanol was <0.1% (v/v).

**Proliferation of HL-60 cells.** HL-60 cells (5x10<sup>4</sup> cells/ml) were incubated in the culture medium containing KEO, ATRA or linalool. The cell number was determined using a hemacytometer, and cell viability was determined with a dye exclusion test using trypan blue.

**ELISA analysis.** DNA fragmentation was estimated using a biotin-labeled anti-histone antibody and a peroxidase-conjugated anti-DNA-antibody in a sandwich ELISA kit. First, HL-60 cells (5x10<sup>4</sup> cells/ml) were treated with KEO or linalool. Then, cells were washed with PBS and lysed with 200  $\mu$ l or lysis buffer for 30 min at room temperature. The lysate was centrifuged at 200 x g for 10 min, and 20  $\mu$ l of supernatant was transferred onto a streptavidin-coated microtiter plate. The presence of histone-associated DNA fragments in the cell lysates was determined using a sandwich ELISA kit. The level of DNA fragmentation of mononucleosomes and oligonucleosomes in HL-60 cells was expressed as an enrichment factor, as calculated using the following formula: Enrichment factor = mU of the sample (HL-60 cells treated with sample)/mU of the corresponding control (HL-60 cells without sample treatment); mU = absorbance (A<sub>405nm</sub>).

**Staining and nitroblue tetrazolium reduction assay.** As an indicator of HL-60 cell differentiation, a nitroblue tetrazolium (NBT) reduction assay was utilized (16). After HL-60 cells were treated with the samples for 72 h, they were suspended in PBS; Giemsa stain results were then observed using microscopy (x400). Subsequently, the cell pellet was centrifuged at 500 x g for 5 min and rinsed three times with PBS. The cells were suspended in 0.5 ml of NBT solution containing 2 mg/ml of NBT and 200 ng/ml TPA in PBS. They were then incubated for 30 min at 37°C. The cells were washed with aliquots of PBS and resuspended in 0.1 ml of PBS. HL-60 cells do not produce superoxide anions (O<sup>2-</sup>), but when they differentiate, they start to produce O<sup>2-</sup>. They then form blue-black formazan deposits by reducing the NBT reagent. For each determination, at least 200 cells were counted using a hemacytometer. The number of NBT-positive cells containing intracellular formazan deposits was expressed as a percentage of the viable cell number.

**Statistical analysis.** The results are expressed as the means  $\pm$  standard deviation (SD). Statistical analyses between multiple groups were performed using ANOVA.

Statistical comparisons were carried out using Dunnett's multiple comparison test. Differences were considered significant at  $p < 0.05$  or  $p < 0.01$ . Analyses were performed using StatView-J software ver. 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA).

## Results

**Kuromoji essential oil inhibits cell proliferation.** The effect of KEO on the growth of HL-60 cells was examined (Fig. 1). The HL-60 cells were treated with 5 or 50  $\mu$ g/ml KEO or linalool for 24 or 48 h, and cell proliferation was assessed using a dye exclusion test using trypan blue. The number of viable cells treated with linalool at 50  $\mu$ g/ml for 24 h was significantly ( $p < 0.05$ ) suppressed compared to the control cells. After 48 h of treatment with KEO or linalool, the cell growth was inhibited significantly in a dose-dependent manner.

Fig. 2 presents an illustration of the apoptosis-inducing activity in the HL-60 cells treated with KEO or linalool for 48 h. Enrichment of mononucleosomes and oligonucleosomes in the HL-60 cells was estimated using sandwich ELISA after

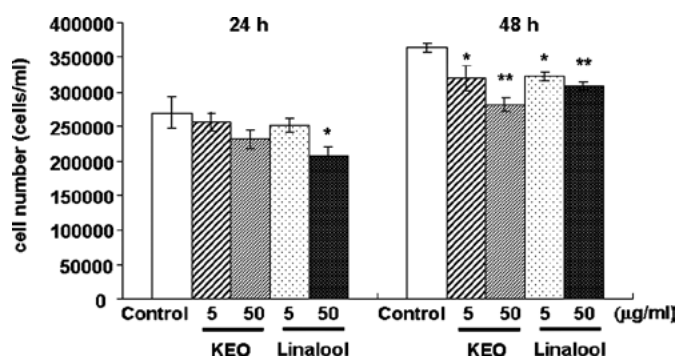


Figure 1. Effects of KEO or linalool on the viability of HL-60 cells. HL-60 cells ( $5 \times 10^4$  cells/ml) were incubated with KEO or linalool at 5 or 50  $\mu\text{g/ml}$  for 24 or 48 h. \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control.

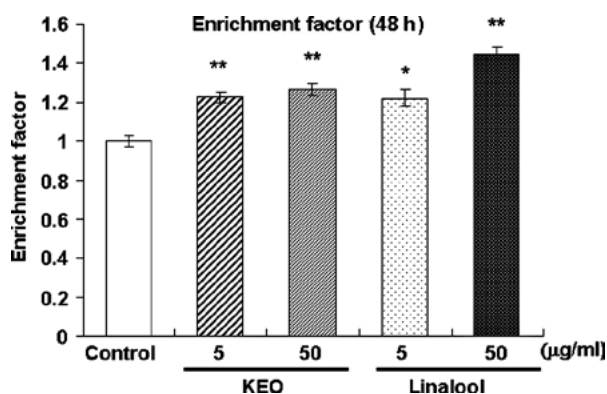


Figure 2. Effects of KEO or linalool on DNA fragmentation in HL-60 cells ( $5 \times 10^4$  cells/ml) incubated with KEO or linalool for 48 h. DNA fragmentation was analyzed using sandwich ELISA with the biotin-labeled anti-histone antibody and peroxidase-conjugated anti-DNA antibody. The level of DNA fragmentation was expressed as an enrichment factor relative to the untreated cells (control). \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control.

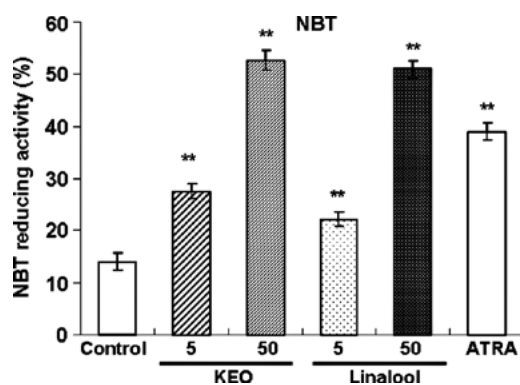


Figure 3. Effect of KEO or linalool on NBT reduction in HL-60 cells. HL-60 cells were cultured with KEO or linalool at 5 and 50  $\mu\text{g/ml}$ , or ATRA at 1  $\mu\text{M}$  for 72 h, and the NBT reducing activity was determined. \*\* $p < 0.01$  vs. control.

treatment with KEO or linalool for 48 h. The fragmented DNA content, designated as an enrichment factor, increased ( $p < 0.05$  or  $p < 0.01$ ) in the HL-60 cells treated with KEO and linalool compared to the control cells. These results suggest that KEO and linalool suppress HL-60 cell proliferation by inducing apoptosis.

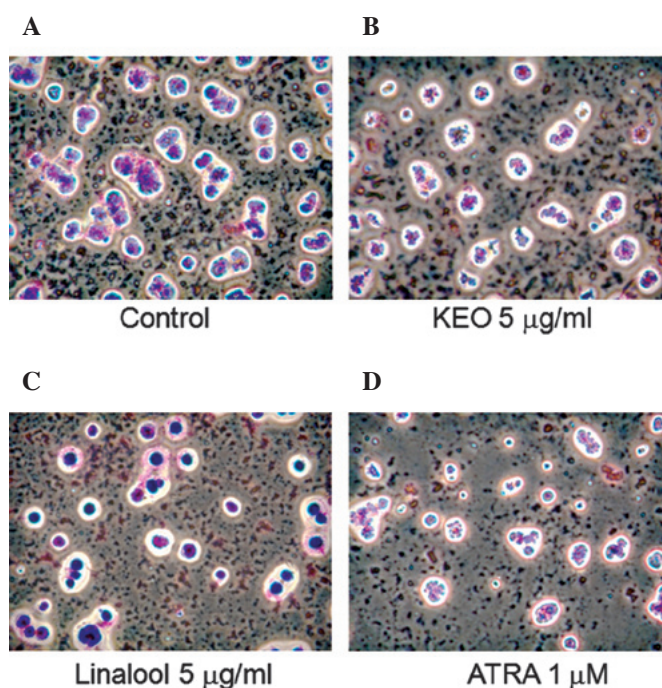


Figure 4. Effect of KEO on morphological changes in HL-60 cells.

*Effect of KEO or linalool on the differentiation of HL-60 cells.* Treatment with KEO or linalool enhanced the NBT-reducing activity in the HL-60 cells (Fig. 3). Treatment with 1  $\mu\text{M}$  ATRA increased the NBT-reducing activity. Treatment of the HL-60 cells with KEO at 5 and 50  $\mu\text{g/ml}$  promoted the NBT-reducing activity in a dose-dependent manner. Effects of KEO and linalool treatment on the HL-60 cells were almost equal for equivalent concentrations.

Furthermore, the morphology of cells treated with KEO was observed using the Giemsa staining method (Fig. 4). Control cells were round and the ratio of the nucleus to cytoplasm was high (Fig. 4A). By contrast, irregular and small nuclei, which are typical shapes of differentiation-induced cells, were observed in cells treated with KEO (5  $\mu\text{g/ml}$ ), linalool (5  $\mu\text{g/ml}$ ) or ATRA (1  $\mu\text{M}$ ) (Fig. 4B-D).

## Discussion

This study demonstrated that KEO and its major chemical constituent linalool exhibit anticancer therapeutic effects in HL-60 cells. Treatment with 5 or 50  $\mu\text{g/ml}$  KEO suppressed the growth of HL-60 cells after 48 h. Treatment with linalool cells at the same dose of KEO showed an almost identical effect on HL-60 cells. Cell proliferation is a key to the promotion and progression of carcinogenesis. Therefore, we investigated the apoptosis-inducing activity of KEO and linalool. The fragmented DNA content increased in cells treated with KEO and linalool for 48 h. This result suggests that KEO has leukemia cell apoptosis-inducing activity. Gu *et al* observed that linalool arrested growth arrest and caused apoptosis of various human leukemia cells, but noted that it spared normal hematopoietic cells (11). Linalool is the predominant component of KEO (65.78%). Therefore, it was presumed that linalool was the most active component of KEO in inducing apoptosis of HL-60 cells. Reportedly, cineole, another KEO component, was also

found to exhibit a suppressive effect on leukemia cell lines resulting from the induction of apoptosis (17). Consequently, co-efficiency of these components has been considered. Thus, further studies are necessary to clarify the active component.

The present study demonstrated that KEO exhibits apoptotic effects in HL-60 cells. However, the effect was not strong when compared to effects reported in a previous study (11). Reportedly, many natural compounds enhance the effects of leukemia cell differentiation (18). Treatment with KEO increased the differentiation of mature cells, similar to that noted in ATRA-treated cells. Nuclear receptors are known to mediate the actions of ATRA on cell differentiation (19). Nuclear receptor families of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) are associated with the differentiation transcription of various genes related to the differentiation of HL-60 cells. Actually, ATRA is known to induce the differentiation of HL-60 cells to granulocytes as mediated by RARs (20). Presumably, KEO and linalool regulate the expression of related differentiation genes as mediated by RARs or RXRs. Therefore, detailed mechanisms of the differentiation effects of KEO and linalool should be examined further.

ATRA was found to induce complete remission in a high percentage of patients with APL, although the response is sometimes quite slow (21). Furthermore, relapse and resistance to treatment often occur despite continued treatment with ATRA (22). Moreover, chemotherapy with ATRA infrequently induces Sweet's syndrome, which is characterized by acute onset of inflammatory skin nodules associated with systemic features which include malaise, fever and neutrophilia (23). Consequently, combination treatment strategies have been suggested to circumvent these problems.

In conclusion, results of this study revealed that KEO as well as its major chemical linalool induce apoptosis and differentiation in human leukemia HL-60 cells. The possibility exists that KEO and linalool, currently applied as an essential oil in aromatherapy, can be developed into an anticancer therapeutic product.

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