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Research Article

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Anti-tumor activity of 13(*E*)-Labd-13-ene-8α,15-diol inducing apoptosis in human breast cancer cells

Jung-Hee Lee and Hwa-Jung Choi*

Department of Beauty Science, Gwangju Women's University, 165 Sanjeong-dong, Gwangsan-gu, Kwangju, Korea

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ABSTRACT

Our previous report showed that 13(E)-labd-13-ene-8α,15-diol [13(E)] isolated from Brachyglottis monroi possessed anticancer activity on human cancer cells (A549 and Hep2). In this study, we examined the inhibitory effect of 13(E) on the proliferation of human breast cancer (MDA-MB-231) cells and its anti-tumor activity in MDA-MB-231 cells xenografted mice. Also, effect of 13(E) on biochemical markers (LPO, lipid peroxidation; GSH, glutathione; ALT, alanine aminotransferase; AST, Aspartate aminotransferase) was assessed in same mice. The 13(E) was cytotoxic to the MDA-MB-231 cells. The mode of cell death induced by 13(E) was found to be apoptosis, which was judged by the morphological alteration of the cells as well as by the checking of nuclear condensation and nuclear fragmentation by 4',6-diamidino-2-phenylindole (DAPI) staining. 13(E) treatment resulted in significant decreases in tumor volume without acute side effects, including body weight loss and mortality. Biochemical parameters such as LPO, GSH, ALT and AST also significantly reverted to normal level in 13(E) treated mice (p>0.05). The results showed that 13(E) is effective in inhibiting the tumor growth in ascetic models. 13(E) has potential in the development of anti-tumor therapy.

Key words: Apoptosis, *Brachyglottis monroi*; Anti-tumor; 13(*E*)-labd-13-ene-8α,15-diol

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INTRODUCTION

Herbal therapies, which have been used for many centuries, are used as alternative treatments for individuals with cancer [1]. Emerging evidence has demonstrated that many natural products isolated from plant sources possess antitumor properties [2-4]. *Brachyglottis monroi* (Hook. f.) B. Nord. (Asteraceae), previously *Senecio monroi*, is a shrub endemic to New Zealand [5]. The use of *B. monroi* by indigenous Maori Polynesians, has been documented widely in relation to the treatment of sores and wounds [6].

Apoptosis, which is also known as programmed cell death, occurs in several pathological situations in multicellular organisms, and constitutes a component of a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells [4]. Apoptotic cell death is characterized by cell shrinkage, chromatin condensation, and DNA fragmentation [7]. The cytotoxicities of most classical antitumor drugs are thought to be mediated by their abilities to induce apoptosis [8].

5-Fluorouracil (5-FU), a water-soluble fluorinated pyrimidine analog, is a popular anti-neoplastic agent and used to treat various types of solid cancers, such as cancer of the liver, stomach, colon, pancreas, and breast, alone or in combination with leucovorin (LV) [9]. The application of its high doses causes adverse side effects that include bone marrow depression and thombocytopenia [10]. Therefore, new agents possessing chemotherapeutic and chemopreventive activities should be developed.

In our experiments, 13(*E*)-labd-13-ene-8α,15-diol [13(*E*)] isolated from *Brachyglottis monroi* showed anticancer

activity carcinomic human alveolar basal epithelial (A549) and human larynx carcinoma (Hep2) cells in a dose-dependent manner [11]. In this study, we examined the effects of 13(*E*) on the inhibition of cell proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the induction of apoptosis in human breast cancer (MDA-MB-231) cells by 4',6-diamidino-2-phenylindole (DAPI) staining and DNA fragmentation assay. We also were assessed anti-tumor activity of 13(*E*) and effect of 13(*E*) on biochemical markers (LPO, lipid peroxidation; GSH, glutathione; ALT, alanine aminotransferase; AST, Aspartate aminotransferase) in MDA-MB-231 cells xenografted mice.

EXPERIMENTAL SECTION

Cell line, reagents and chemicals

Human breast cancer (MDA-MB-231) cells were purchased from the Korea Cell Line Bank (KCLB, Seoul, South Korea), and were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) and 0.01% antibiotic-antimycotic solution (Gibco BRL; Grand Island, NY, USA) at 37□ in 5% CO_2 and 95% O_2 in a humidified cell incubator. The tissue culture plates were purchased from Falcon (BD Biosciences, NJ, USA). 3-(4,5-dimethylthiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 4',6' diamino-2-phenylindole (DAPI) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 13(*E*) was isolated from *Brachyglottis monroi* using a previously described method [11].

Cell viability assay

For the measurement of cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously [12]. Briefly, MDA-MB-231 cells were seeded at 1×10^5 cells/well in a 24-well plate, and were treated with various concentrations of 13(*E*) for 24 h and 48 h. At the end of culturing, the cells were washed, and 50 μL of MTT (2 mg/mL) was then added to each well. After incubation of the plate for 4 h in the dark, the formed formazan was dissolved in DMSO solution, and absorbance was measured at 570 nm using a microtiter plate reader (Bio-TEK Instruments, USA). For determination of cell viability, percent viability was calculated as (absorbance of sample/control absorbance × 100).

Apoptosis

DAPI staining was performed according to the previously described protocol [13]. MDA-MB-231 cells were first cultured on 4-chamber slides at a density of 2×10^4 cells/chamber. After treatment with 13(*E*) for 48 h, cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde for 30 min. After washing in PBS, the cells were incubated in 1 μg/mL DAPI solution for 30 min in the dark. The cells were then observed with a fluorescence microscope (Leica DM IRB; Leica, Wetzlar, Germany).

In vivo anti-tumor property

Female Balb/c mice (6 weeks old) were purchased from the Central Laboratory Animal (Seoul, Korea). Experiments were performed in accordance with regulatory standards and guidelines and were approved by the institutional Animal Care and Use Committee.

To establish a xenograft carcinoma animal model, 5×10^6 MDA-MB-231 cells were injected into the flanks of female Balb/c mice. Tumor cells were allowed to grow into visible masses for 14 days, the mice were randomly divided into five groups (10 mice/group), and treated with variable strategies. 13(*E*) was intraperitoneally injected with 20 mg/kg three times per week for 2 weeks and 5-FU was intraperitoneally injected with 20 mg/kg three times per week for 2 weeks. Tumor volume for xenografts was determined by a caliper and was calculated as volume = length × width^2 × 0.5, where the width is the smallest measurement and the length is the longest measurement [14]. The weight of body during animal test monitored twice each week.

Biochemical parameters

After collection of blood samples, the mice were sacrificed. Then the liver was excised, rinsed in ice cold normal saline followed by ice-cold 10% KCl solution, blotted, dried and weighed. A 10% (w/v) homogenate was prepared in ice-cold KCl solution and centrifuged at 1500 rpm for 15 min at 4℃. The supernatant thus obtained was used for the estimation of LPO by thio-barbituric acid reaction [15], GSH by enzymatic recycling [16], ALT and AST by the method described by Reitman and Frankel in 1957 year [17].

Statistical analyses

The results are presented as mean ± S.D. of thee replicates from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Student's t test. The values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

To measure cytotoxic effect of 13(E) on MDA-MB-231 cells, MDA-MB-231 cells incubated with 13(*E*) at a concentration of 4, 8, 16, and 32 μg/mL for 24 h showed 92.87% 89.34%, 78.67%, and 44.37% viability compared to the control value, respectively (Fig. 1). In addition, MDA-MB-231 cells incubated with 13(*E*) at a concentration of 4, 8, 16, and 32 μg/mL for 48 h showed 69.69% 67.07%, 55.23%, and 0.92% viability compared to the control value, respectively (Fig. 1). These results showed that 13(*E*) inhibited the proliferation of MDA-MB-231, and this cytotoxic effect was increased in a time- and dose-dependent manner.

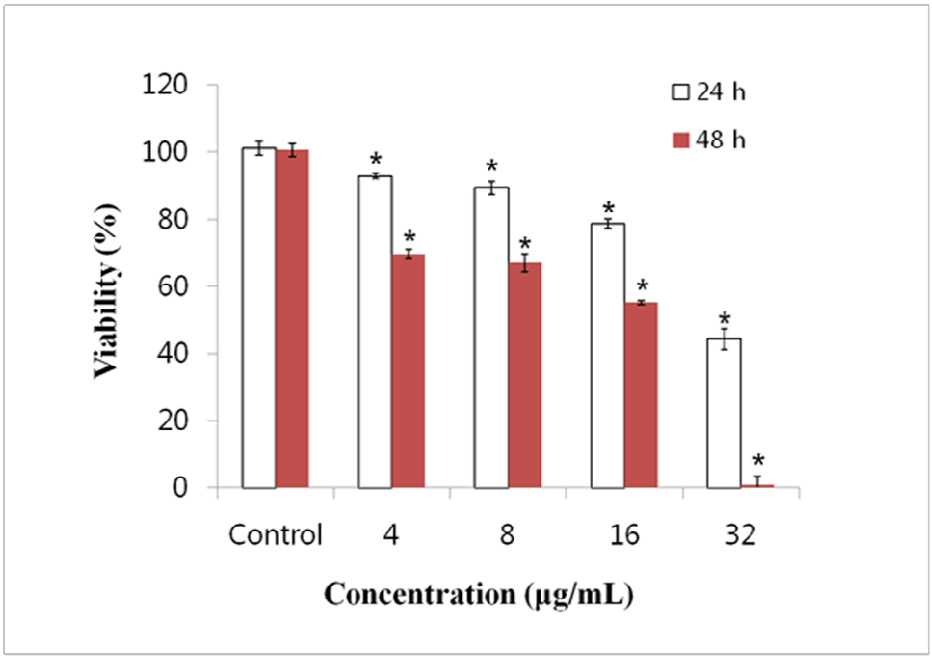

Fig.1: Cytotoxic effects and 13(*E*)-induced cell death in MDA-MB-231 cells

MDA-MB-231 human breast cancer cells were incubated with 13(*E*) at various concentrations (4, 8, 16, and 32 μg/mL) for 24 and 48 h, and the viability was determined by the MTT assay. Results are represented as the mean ± standard error for three independent experiments, each with a minimum of three cultures. 13(*E*), 13(*E*)-labd-13-ene-8α,15-diol. *represents $p<0.05$ compared to the control after 24 h and **represents $p<0.05$ compared to the control after 48 h.

To confirm that the cytotoxic effect of 13(*E*) was induced by apoptosis, 13(*E*) was treated in MDA-MB-231 cells. The changes in cell morphology induced by 13(*E*) were examined by phase-contrast microscopy. In Fig. 2A, 13(*E*)-treated cells at a concentration of 16 and 32 μg/mL for 48 h were detached from the culture dish, underwent cell rounding and cytoplasmic blebbing, and became irregular in shape. To confirm the induction of apoptosis in MDA-MB-231 cells, 13(*E*) was analyzed by the DAPI assay, which specifically stains the nuclei. The assay revealed that some nuclei of the cells that had been treated with 13(*E*) at a concentration of 16 and 32 μg/mL for 48 h exhibited typical features of apoptosis such as nuclear condensation and nuclear fragmentation (Fig. 2B).

After DAPI staining, the images recorded by fluorescence microscope. Photomicrograph of a phase-contrast microscope (A), photomicrograph of a fluorescence microscope after MDA-MB-231 cells were stained with DAPI (B).

We also investigated the anti-tumor activity of 13(*E*) in a MDA-MB-231 carcinoma animal model using Balb/c mice. The MDA-MB-231 tumor-xenograft mice displayed increased tumor volume during test period (Fig. 3A). Treatment with 13(*E*) or 5-FU reduced the tumor volume significantly when compared to that of cancer control group (Fig. 3, $p<0.05$). Treatment with 13(*E*) produced tumors similar in size to those produced using 5-FU (Fig. 3A). Furthermore, 13(*E*) didn't show acute side effects, including body weight loss and mortality (Fig. 3B and 3C). This is, to the best of our knowledge, the first study demonstrating that 13(*E*) inhibits tumor growth in a MDA-MB-231 carcinoma animal model.

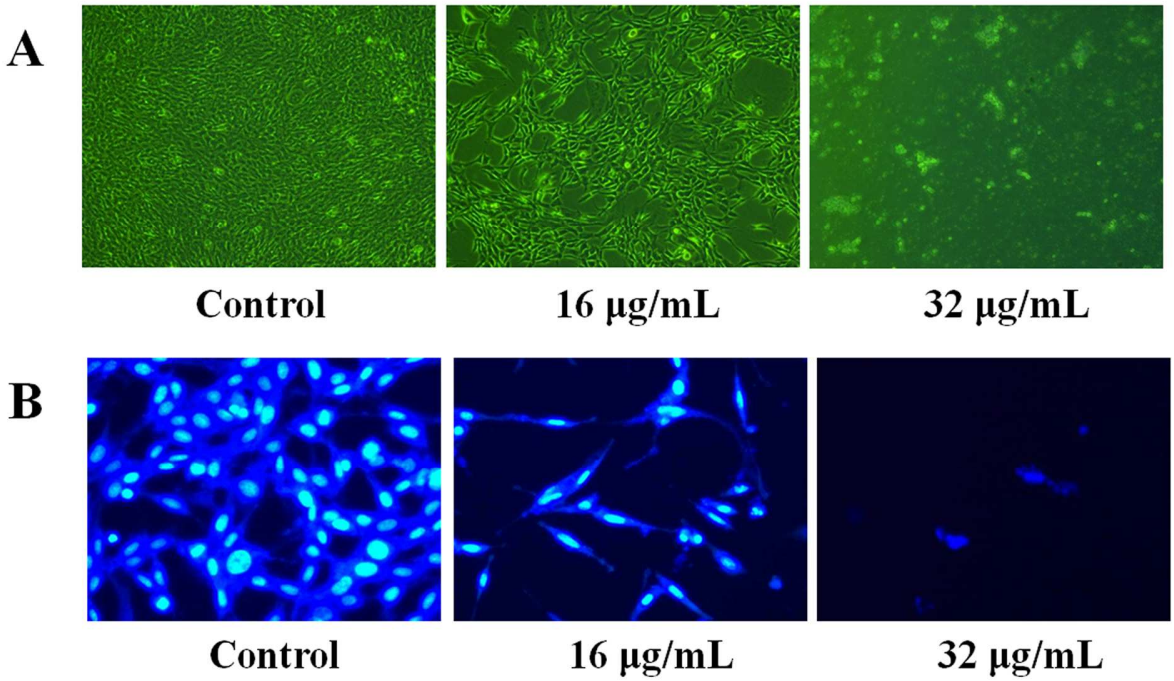

Fig. 2: MDA-MB-231 human breast cancer cells were incubated with 13(*E*) at concentrations of 16 μg/mL and 32 μg/mL for 48 h

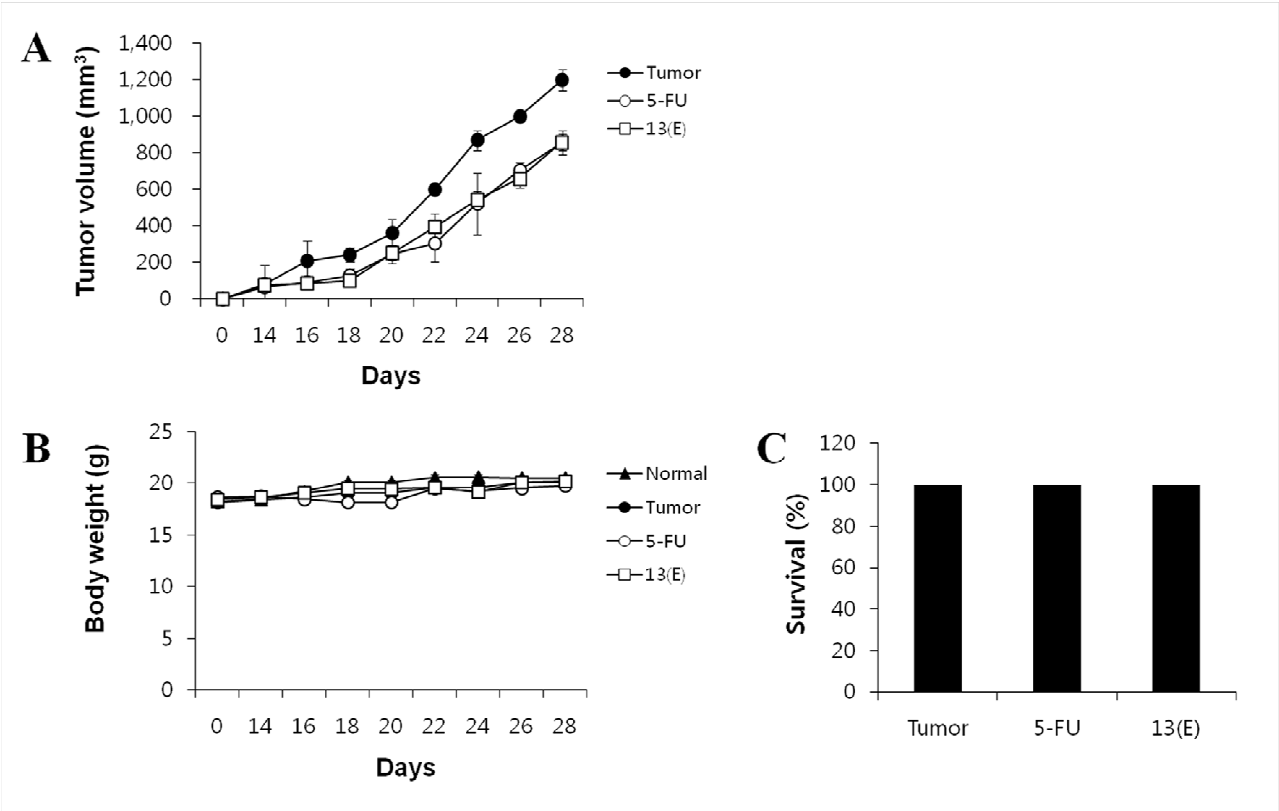

Fig. 3: Effect of 13(*E*) on tumor volume (A), body weight (B) and survival (C) in tumor bearing Balb/C mice

MDA-MB-231 cells were inoculated in Balb/C mice and then after 2 weeks the mice intraperitoneally administered with 20 mg/kg of 13(*E*) three times per week for 2 weeks. Tumor volume was determined once per 2 days during tested period. The weight of body during animal test monitored twice each week. The survival measured after tested period. 13(*E*), 20 mg/kg; 5-FU, 20 mg/kg 5-fluorouracil. 13(*E*), 13(*E*)-labd-13-ene-8α,15-diol. *Significantly different from normal group ($p<0.05$). **Significantly different from tumor group ($p<0.05$).

The inoculation of MDA-MB-231 cells caused significant decreases in the levels of the antioxidant enzymes GSH while significantly increasing the levels of LPO, ALT and AST in liver of MDA-MB-231 animals, when compared to the normal group (Table 1). Treatment with 13(*E*) reversed these changes towards the normal levels. Almost similar results were observed with 5-FU treatment.

Table 1: Effect of 13(*E*) on LPO, GSH, ALT and AST levels in liver of mice

Group	LPO (nmol/g)	GSH (μmol/g)	ALT (Unit/mL)	AST (Unit/mL)
Normal	45.4 ± 1.4	6.0 ± 0.3	205.2 ± 4.5	160.3 ± 4.1
Tumor	82.5 ± 2.2*	3.0 ± 0.6*	402.4 ± 6.2*	378.1 ± 4.3*
5-FU	64.2 ± 5.8**	3.7 ± 0.5	311.3 ± 3.1**	271.4 ± 5.6**
13(*E*)	51.3 ± 4.1**	5.8 ± 1.3**	214.9 ± 5.3**	199.3 ± 2.0**

*Each value represents the mean ± S.D. (n=10). *Significantly different from normal group (p<0.05). **Significantly different from tumor group (p<0.05). LPO, lipid peroxidation; GSH, glutathione; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; 13(E), 20 mg/kg 13(E)-labd-13-ene-8α,15-diol; 5-FU, 20 mg/kg 5-fluorouracil.*

Labdane diterpenes, which are the predominant compounds in *C. creticus* as well as in the resin "Labdano", are known for their cytotoxicities [18]. Labdane-type diterpenes exhibit cytotoxic activity against human leukemic cell lines *in vitro*. The structures of the labdane-type diterpenes may have strong correlation with their cytotoxic activities. Specifically, the absence of the double bond between C-7 and C-8 in 13(*E*) is essential for its cytotoxic activity [19]. In a previous report, 13(*E*) was reported to be effective against murine leukemia cell lines [20]. Also, our previous study showed that 13(*E*) exhibited dose-dependent inhibitory effects on A549 and Hep2 cells [11]. In this report, 13(*E*) showed cytotoxicity in MDA-MB-231 cells.

Rotundifuran, a labdane-type diterpene isolated from the fruit of *Vitex rotundifolia*, has been shown to inhibit the proliferation of HL-60 leukemia cells by activating the apoptotic pathway [21]. Dimas et al. (1999) reported that sclareol, a labdane-type diterpene from the leaves of *Cistus incanus* sub. sp creticus (L.), kills tumor cells through a cell cycle-specific mechanism and induces apoptosis. Our results showed that 13(*E*) induces apoptosis in MDA-MB-231 cells.

There is no doubt that reactive oxygen species (ROS) play an important role in pathological changes in the liver, particularly in toxic liver diseases [22]. Oxidative stress induced by an increase in free radicals and/or decrease in antioxidant defenses is well documented in animal model [23, 24].

Liver is the largest gland and major site for drug metabolism [25]. Liver injury induced by chemicals, drugs, and viruses is a well-recognized toxicological problem [26]. Serum enzymes including ALT and AST are used in the evaluation of hepatic disorders and a increase in these enzyme activities reflects active liver damage [27]. The serum levels of GSH also can be used as indices of oxidative stress [28]. Our studies revealed that 13(*E*) is able to decrease human breast tumor with decreasing in the levels of the antioxidant enzymes GSH and significantly increasing the levels of LPO, ALT and AST in liver of MDA-MB-231 animals.

The results from the present study have demonstrated that 13(*E*) induces apoptosis and inhibits tumor growth in mouse carcinoma MDA-MB-231 in vivo. Moreover, it reversed LPO, GSH, ALT and AST levels towards the normal levels. Therefore, these findings suggest that 13(*E*) is a good source of chemotherapeutic agents involved in the inhibition of tumor growth.

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