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Original article

Ethanol Extract of *Oldenlandia diffusa* – an Effective Chemotherapeutic for the Treatment of Colorectal Cancer in Humans

-Anti-Cancer Effects of *Oldenlandia diffusa*-

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Key Words

anti-cancer activity, HT-29 human colorectal adenocarcinoma cells, *Oldenlandia diffusa*

Abstract

Objectives: *Oldenlandia diffusa* is traditionally used to relieve the symptoms of and to treat various diseases, but its anti-cancer activity has not been well studied. In the present study, the authors investigated the anti-cancer effects of an ethanol extract of *Oldenlandia diffusa* (EOD) on HT-29 human adenocarcinoma cells.

Methods: Cells were treated with different concentrations of an EOD, and cell death was assessed by using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Analyses of the sub G1 peak, the caspase-3 and -9 activities, and the mitochondrial membrane depolarizations were conducted to confirm cell death by apoptosis. Also, intracellular reactive oxygen species (ROS) generation was determined using carboxy-H2DCFDA (5-(and-6)-carboxy-20,70-dichlorodihydrofluorescein diacetate).

Results: EOD inhibited the proliferation of HT-29 cells for 24 hours by 78.6% ± 8.1% at 50 μg/mL, 74.4% ± 4.6% at 100 μg/mL, 65.9% ± 5.2% at 200 μg/mL, 51.4% ± 6.2% at 300 μg/mL, and by 41.7% ± 8.9% at 400 μg/mL, and treatment for 72 hours reduced the proliferation at the corresponding concentrations by 43.3% ± 8.8%, 24.3 ± 5.1 mV, 13.5 ± 3.2 mV, 6.5 ± 2.3 mV, and by 2.6 ± 2.3 mV. EOD increased the number of cells in the sub-G1 peak in a dose-dependent manner. The mitochondrial membrane depolarization was elevated by EOD. Also, caspase activities were dose-dependently elevated in the presence of EOD, and these activities were repressed by a pan-caspase inhibitor (zVAD-fmk). The ROS generation was significantly increased by EOD and N-acetyl-L-cysteine (NAC; a ROS scavenger) remarkably abolished EOD-induced cell death. In addition, a combination of sub-optimal doses of EOD and chemotherapeutic agents noticeably suppressed the growth of HT-29 cancer cells.

Conclusion: These results indicate that EOD might be an effective chemotherapeutic for the treatment of human colorectal cancer.

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1. Introduction

Traditional herbal medicines are being increasingly studied on a scientific basis [1], and the benefits of a number of these medicines are being appreciated in the West. Given the worldwide increases in cancer rates, the anti-tumor effects of some traditional herbal medicine have been attracting research attention [2], and some have been shown to possess unique advantages for the treatment of certain tumors; in addition, some light has been shed on the natures of the anti-

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tumor mechanisms [3-5]. *Oldenlandia diffusa* is a well known medicinal plant that is used in Asia to treat hepatitis, tonsillitis, and malignant tumors of the liver, lung and stomach. Several studies indicate that *Oldenlandia diffusa* has multiple biological activities, which include antitumor, chemopreventive, anti-angiogenic, anti-inflammatory, anti-oxidant, and proapoptotic effects [6, 7].

Apoptosis is a programmed, physiological mode of cell death and is characterized by morphological changes, such as chromatin condensation and nuclear fragmentation. The apoptotic process is triggered by signals involving mitochondria (the intrinsic pathway) or death receptors (the extrinsic pathway). The mitochondrial pathway involves the release of cytochrome c and other pro-apoptotic factors into the cytoplasm through pores in the mitochondrial membrane, and these releases lead to the activation of caspase-9. These pores are produced by a process that increases mitochondrial membrane permeability (MMP) and leads to loss of mitochondrial membrane integrity. The signal that triggers the apoptosis process is the product of a delicate balance between apoptotic and anti-apoptotic proteins [8-10]. However, the underlying apoptotic mechanisms of an ethanol extract of *Oldenlandia diffusa* (EOD) in HT-29 human colorectal adenocarcinoma cells are not clearly understood.

In the present study, we investigated the anti-cancer effects of an EOD on HT-29 cells (a human colorectal adenocarcinoma cell-line). EOD was found to trigger the apoptosis of HT-29 cells via caspase activation and mitochondrial dysfunction. In addition, in combination with other chemotherapeutic agents, EOD was found to suppress markedly the growth of HT-29 cells.

2. Materials and Methods

The powder form of an EOD (Catalog number: CA04-019) was obtained from the plant extract bank at the Korean Research Institute of Bioscience and Biotechnology (KRIBB) in Daejeon, Korea. The powder was then immersed in ethanol, sonicated for 15 minutes, and extracted for 72 hours. The extract was filtered through non-fluorescent cotton and evaporated under reduced pressure by using a rotary evaporator (N-1000SWD, Eyela, Japan) at 45°C. The condensed extract was then lyophilized using a Modul Spin 40 dryer (Biotron Corporation, Calgary, Canada) for 24 hours. The final yield of lyophilized powder (EOD) was 12.3%. The EOD was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/mL and stored at 4°C as a stock solution, which was later diluted with medium to the desired concentration prior to use. The HT-29 human colorectal adenocarcinoma cells were obtained from the American type culture collection (Rockville, MD) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2-mM glutamine, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin in a 5% CO_2/95% relative humidity (RH) atmosphere at 37°C.

To investigate cell viability, we used a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HT-29 cells were seeded into the wells of 12-well plates and cultured in RPMI 1640 for 72 hours. The MTT solution [100 μL, 5 mg/mL in phosphate buffer solution (PBS)] was then added to each well, and the plates were incubated for 4 hours at 37°C. After the supernatants had been removed, they were gently shaken with 200 μL of DMSO (Jersey Lab Supply, Livingston, USA) for 30 minutes, and the absorbance was measured at 570 nm by using a microtiter plate reader (SpectraMax 190; molecular Devices Corporation, Sunnyvale, USA).

Propidium iodine (PI) staining was used to investigate cell cycle disruption, as previously described [11, 12]. About 1 $\times 10^6$ cells were placed in an e-tube, and 700 μL of ice-cold fixation buffer (ethyl alcohol) was slowly added with vortexing. Tubes were sealed with parafilm, incubated at 4°C overnight, and spun for 3 minutes at 106 g and 4°C, after which the supernatants were aspirated and discarded. Cell pellets were resuspended in 200 μL of PI staining solution (2 μL of PI @ 5 mg/mL and 2 μL of RNase in 196 μL of PBS), spun at 20817 g for 5 s, and left for 30 minutes in the dark at room temperature. Samples were analyzed using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Mountain View, USA) at λ = 488 nm and Cell-Quest software (Becton-Dickinson, Franklin Lakes, USA). The deoxyribonucleic acid (DNA) content distributions of normally growing cells are characterized by two peaks, that is, the G1/G0 and G2/M phases, where the former represents normal functioning and the resting state of the cell cycle and has a high diploid DNA content, whereas the latter G2/M phase has a low diploid content and also contains tetraploid DNA. On the other hand, cells in the sub-G1 phase, which have the least DNA content, are termed hypodiploid, and represent DNA fragmentation [12].

Mitochondrial membrane depolarization was evaluated by using a 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescence probe, according to the manufacturer's instructions (Molecular Probes, Eugene, USA). HT-29 cells were labeled with 2 μM JC-1 for 30 minutes at 37°C and were then analyzed at an excitation wavelength 488 nm by using flow cytometry with 530/30 or 585/42 nm bypass emission filters. Cells not exhibiting red fluorescence were regarded to manifest mitochondrial membrane depolarization.

Caspase-3 and -9 assay kits (Cellular Activity Assay Kit Plus) were purchased from BioMol (Plymouth, USA). After experimental treatments, cells were centrifuged (1000 g, 4°C, 10 minutes), washed with PBS, resuspended in ice cold cell lysis buffer, and incubated on ice for 10 minutes. Samples were centrifuged at 10000 g (4°C, 10 minutes), and supernatants were removed. Supernatant samples (10 μL) were then incubated with 50 μL of substrate (400-lM Ac-DEVD-pNA) in 40 μL of assay buffer at 37°C. The absorbance at 405 nm was read at several time-points. The pNA concentrations in the samples were determined using a standard plot of absorbance versus pNA concentration. The pan-caspase inhibitor (zVAD-fmk, Calbiochem, Millipore Corporation, Billerica, USA) was used to validate the assay method.

Intracellular reactive oxygen species (ROS) generation was determined using carboxy-H2DCFDA [5-(and-6)-carboxy-20,70-dichlorodihydrofluorescein diacetate; Molecular Probes, Eugene, USA]. Briefly, after treatments, cells

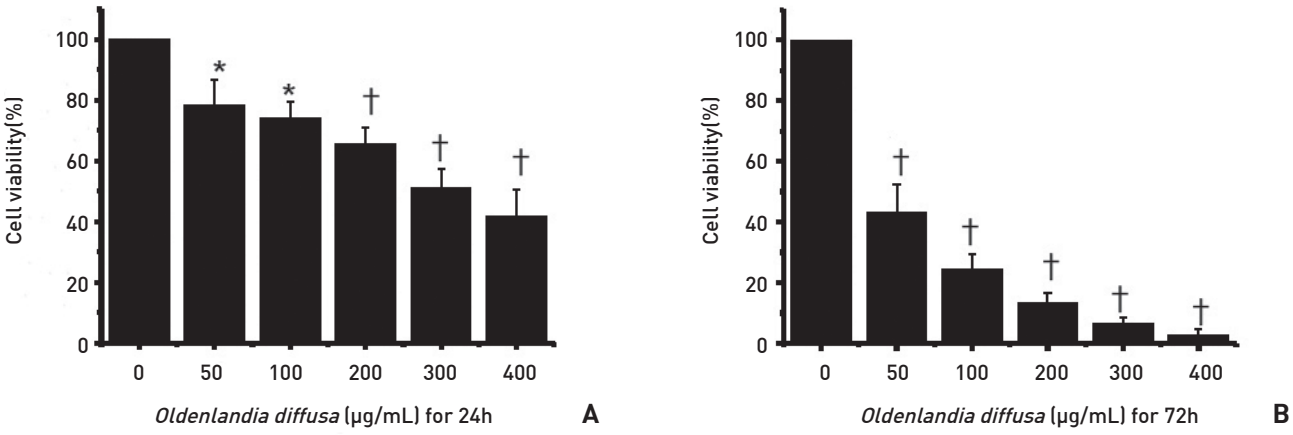

Figure 1 Cytotoxic effects of the EOD.

HT-29 cells were incubated with EOD at the indicated concentrations (μg/mL), and after (A) 24 hours or (B) 72 hours of treatment, cell viabilities were assessed by using an MTT assay as described in Materials and Methods. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.
EOD, ethanolic extract *of Oldenlandia diffusa*; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide ; SD, standard deviations.

were treated with 100 μM carboxy-H2DCFDA in culture medium, incubated at 37°C for 30 minutes, and washed with PBS. Fluorescence was measured using a FACScan (Becton-Dickinson, Mountain View, California, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Unless otherwise indicated, results are expressed as means ± standard deviations (SDs) of experiments performed in triplicate. Statistical analysis was performed using a paired Student's *t*-test. Statistical significance was accepted for *P* values < 0.05.

3. Results

Treatment with EOD (50 — 400 μg/mL) for 24 hours reduced the viability of HT-29 human colorectal cancer cells by 78.6% ± 8.1% at 50 μg/mL, 74.4% ± 4.6% at 100 μg/mL, 65.9% ± 5.2% at 200 μg/mL, 51.4% ± 6.2% at 300 μg/mL, and 41.7% ± 8.9% at 400 μg/mL (Fig. 1A), and treatment for 72 hours reduced cell viabilities at the corresponding concentrations by 43.3% ± 8.8%, 24.3 ± 5.1 mV, 13.5 ± 3.2 mV, 6.5 ± 2.3 mV, and by 2.6 ± 2.3 mV (Fig. 1B). These findings show that EOD has a cytotoxic effect on HT-29 human colorectal cancer cells.

After treatment of the HT-29 cells with EOD for 24 hours, cells were stained with PI, and cell cycle progression was assessed by using flow cytometry. EOD increased the number of cells in the sub-G1 peak in a dose-dependent manner, indicating the induction of cell death (Fig. 2). After 24 hours of treatment with different concentrations of EOD, the following cell percentages were seen in the sub-G1 phase; 5.3% at 100 μg/mL, 7.4% at 200 μg/mL, 8.6% at 300 μg/mL, and 24.3% at 400 μg/mL; however, only 2.6% of untreated cells were in this phase. At the same time, the following percentages were in the G1 phase; 60.6% at 100 μg/mL, 65.2% at 200 μg/mL, 75.5% at 300 μg/mL, and 57.4% at 400 μg/mL; however, 19.4% at 100 μg/mL, 13.5% at 200 μg/mL, 10.1% at 300 μg/mL, and 13.2% at 400 μg/mL were in the G2/M phase, which were lower than the percentages for untreated cells in this phase (Fig. 2). These results suggest that the anti-cancer effect of EOD is closely associated with the induction of apoptosis.

In addition, mitochondrial membrane depolarization (an early event during intrinsic apoptosis signaling) was elevated after treatment with EOD. The mitochondrial membrane depolarizations caused by treatment with EOD were markedly increased by 12.2% ± 2.1% at 50 μg/mL, 16.3% ± 2.2% at 100 μg/mL, 21.1% ± 4.1% at 200 μg/mL, 23.2% ± 5.3% at 300 μg/mL, and 52.4% ± 6.2% at 400 μg/mL as determined by using flow cytometry (n = 5; Fig. 3A). Also, because caspase activation is required for apoptotic cell death, caspase activity assays were performed to assess the activities of caspase-3 and -9. The caspase activities were found to be dose-dependently elevated in the presence of EOD (at concentrations from 50 to 400 μg/mL) for 24 hours, and these activities were found to be repressed by zVAD-fmk (Fig. 3B). These observations suggest that EOD-induced apoptosis is mediated by a mitochondrial/caspase-dependent pathway in HT-29 cells.

Because intracellular ROS play important roles in apoptosis, we examined whether EOD generates ROS in HT-29 cells. Cells were treated with various concentrations of EOD from 50 to 400 μg/mL for 24 hours, after which the levels of ROS generation were measured by using flow cytometry. As shown in Fig. 4A, ROS generation was significantly and dose-dependently increased by up to almost 1.5 fold in EOD-treated cells versus untreated controls. In addition, cell viabilities were measured after the cells had been treated for 24 hours with EOD at concentrations of from 50 to 400 μg/mL plus N-acetyl-L-cysteine (NAC; 1 mM), a ROS scavenger. As shown in Fig. 4B, with NAC ad-

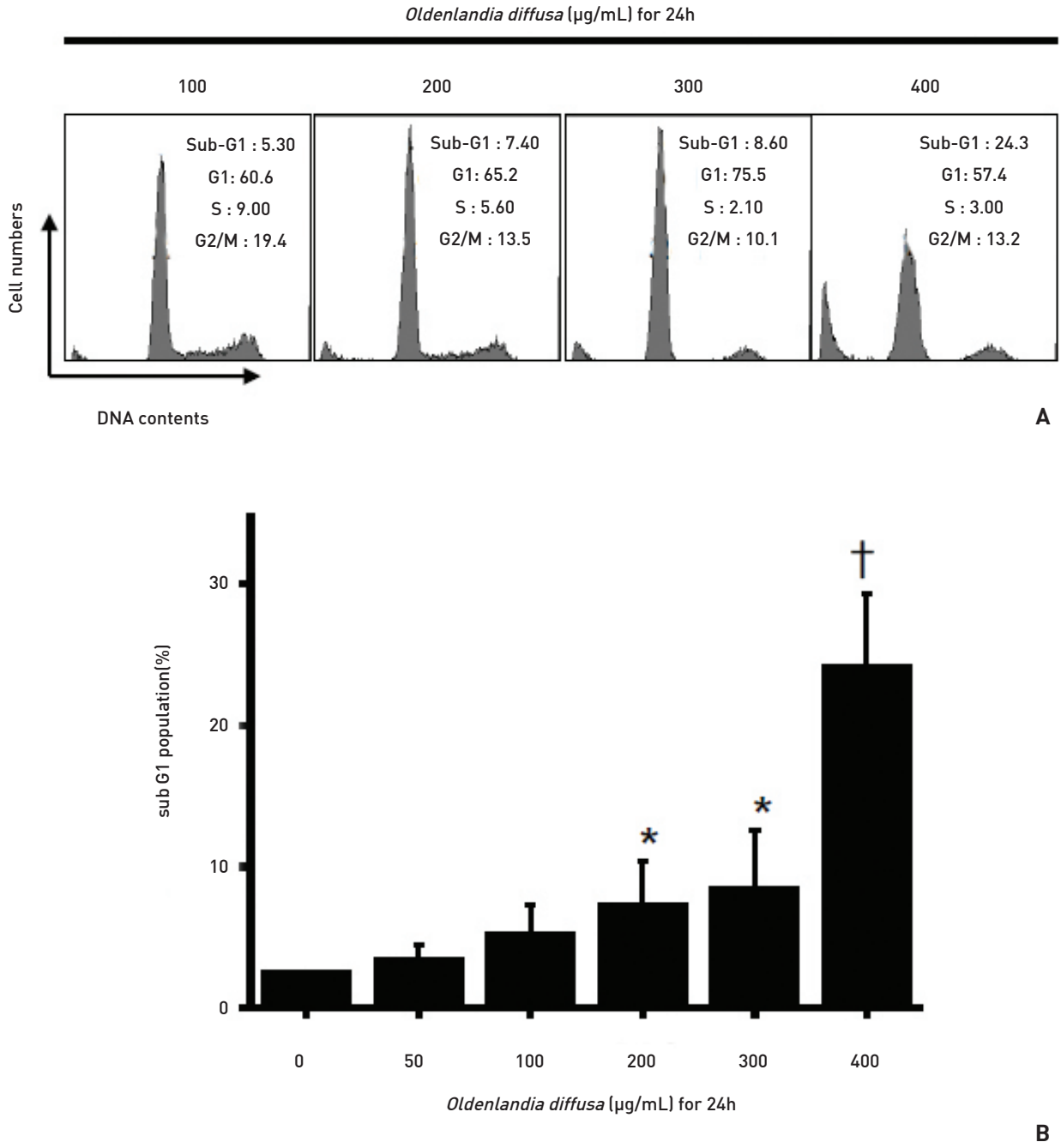

Figure 2 EOD increased HT-29 cell apoptosis.

Cells were incubated with EOD at the indicated concentrations (μg/mL). (A) After 24 hours, sub-G1 peaks were measured using a FACS can as described in Materials and Methods. (B) Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.
EOD, ethanolic extract of *Oldenlandia diffusa*; FACS, fluorescence-activated cell sorter; SDs, standard deviations.

ministration, EOD-induced cell death had vanished. These results show that EOD enhanced ROS generation.

We also investigated whether EOD enhanced the sensitivity of HT-29 cells to chemotherapeutic agents, such as paclitaxel, 5-fluorouracil, cisplatin, ectoposide, doxorubicin, and docetaxel. Combinations of EOD with these chemotherapeutic agents were found to suppress cell growth more than these agents alone (Fig. 5). In particular, paclitaxel and doxorubicin plus EOD markedly suppressed cell growth. These results suggest that EOD increases cell chemosensitivity.

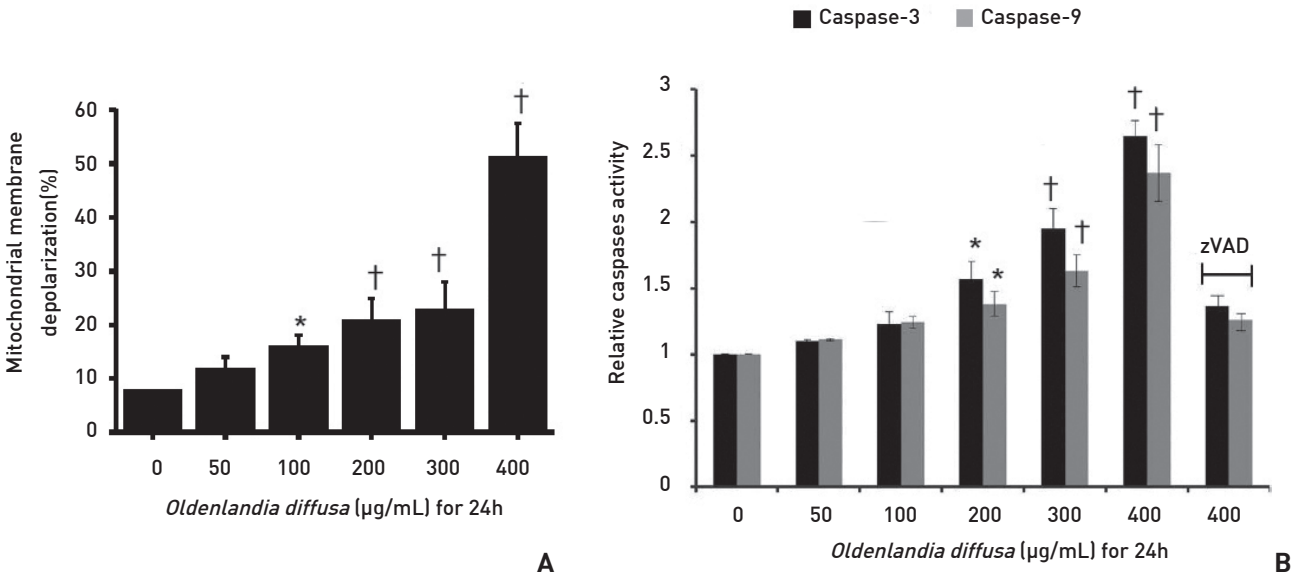

Figure 3 EOD increased mitochondrial membrane depolarization and the activities of caspase-3 and -9.

HT-29 cells were incubated with EOD at the indicated concentrations (μg/mL) for 24 hours. (A) Mitochondrial membrane depolarization was measured by using a FACScan as described in Materials and Methods. (B) The activities of caspase-3 and -9 were assessed by using enzyme assays. Specific activities were determined by using four samples per group. The caspase activity of untreated cells was taken to be 100%. The pan-caspase inhibitor, zVAD-fmk, (zVAD) was used at 20 μM to confirm the validity of the analytical method. Column represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.
EOD, ethanolic extract of *Oldenlandia diffusa*; FACS, fluorescence-activated cell sorter; SDs, standard deviations.

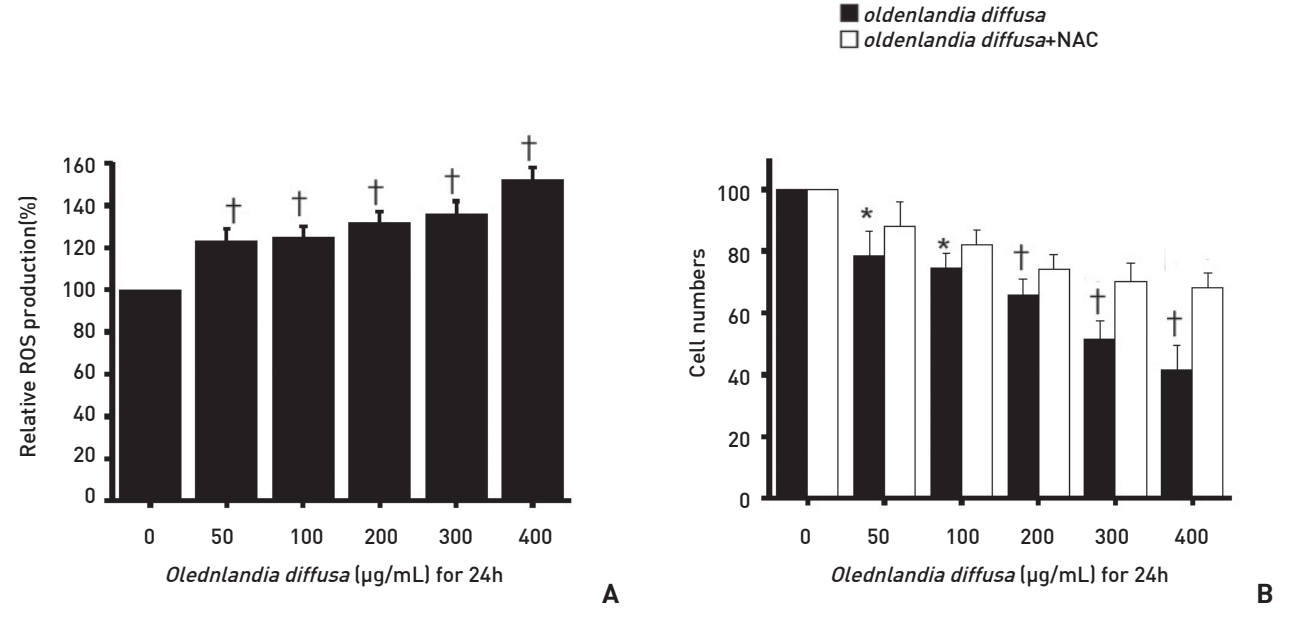

Figure 4 EOD enhanced ROS accumulation in HT-29 cells.

(A) Intracellular ROS was detected in HT-29 cells treated with the indicated concentrations of EOD for 24 hours. ROS productions are presented as percentages of untreated controls. (B) Cells were treated with EOD plus NAC for 24 hours, and cell viabilities were determined by using MTT assay. Columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.
EOD, ethanolic extract of *Oldenlandia diffusa*; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDs, standard deviations.

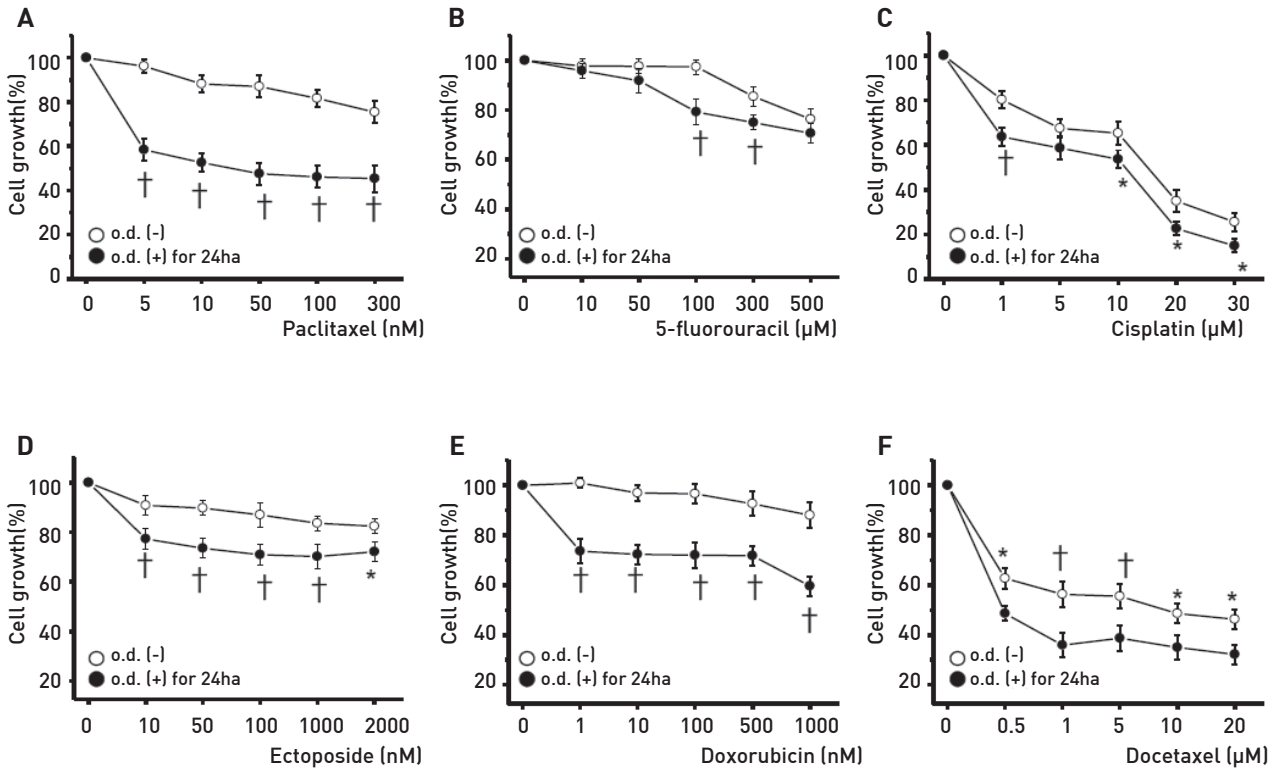

Figure 5 EOD increased cellular chemosensitivity.

HT-29 cells were co-treated with EOD and chemotherapeutic agents, that is, paclitaxel, 5-fluorouracil, cisplatin, ectoposide, doxorubicin, or docetaxel at the indicated concentrations prior to the MTT assay. Results are expressed as percentages (%) of non-treated controls and columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.
O.d., *Oldenlandia diffusa*; EOD, ethanolic extract of *Oldenlandia diffusa*; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDs, standard deviations.

4. Discussion

Oldenlandia diffusa is commonly used in traditional medicine to treat various diseases, including cancer. *Oldenlandia diffusa* contains oleanolic acid (3b-hydroxyolean-12-en-28-oic acid, OA), ursolic acid (3b-3-hydroxyurs-12-ene-28-oic acid, UA), asperuloside (IG1), E-6-O-p-coumaroyl scandoside methyl ester (IG2), and E-6-O-p-coumaroyl scandoside methyl ester-10-methyl ether (IG3) [13, 14]. Of these, OA and UA have been reported to exhibit significant antitumor effects on and to have significant cytotoxic activities in many cancer cell lines, such as, liver cancer cells, gastric cancer cells, colon carcinoma cells, and fibrosarcoma cells [15-20]. UA was reported to inhibit significantly the growth of R-HepG2 cells in nude mice via the apoptosis-inducing factor (AIF) signaling pathway [15], and UA and OA were found to cause apoptosis of HepG2, Hep3B, Huh7, and HA22T cell lines [16]. Both UA and OA were reported to inhibit HCT15 (a human colon carcinoma cell line) proliferation through cell-cycle arrest, although UA had the greater effect [17]. OA was also reported to induce the apoptosis of MKN28 cells (a gastric cancer cell line) via the mitochondrial pathway in an AKT and c-Jun N-terminal kinase (JNK) dependent manner, and interestingly, OA increased JNK phosphorylation, and decreased AKT phosphorylation, but did not affect the phosphorylations of p38 and extracellular signal-regulated kinase (ERK). Furthermore, OA also enhanced the messenger RNA (mRNA) expressions of caspase 3, caspase 9 and Apaf-1 in MKN28 cells significantly [18]. In HepG2 cells (a hepatocellular carcinoma cell-line), OA inhibited hepatocellular carcinoma via ERK-p53 induced cell cycle arrest and mitochondrial-dependent apoptosis [19]. In another study, OA or UA induced HuH7 (a human hepatocellular carcinoma cell-line) apoptosis via a mitochondrial-dependent pathway and the downregulation of X-linked inhibitor of apoptotic protein (XIAP) at the transcriptional level [20]. However, no study, to the best of our knowledge, has investigated the effects of *Oldenlandia diffusa* on the growth of human colorectal cancer cells.

Our results indicate that EOD reduced, in a concentration-dependent manner, HT-29 cell viability by inducing apoptosis (Fig. 1), which was confirmed by increases in sub-G1 populations (Fig. 2) and mitochondrial dysfunction (Fig. 3A). Furthermore, EOD activated caspase-3 and -9 (Fig. 3B), and increased intracellular ROS generation (Fig. 4). In addition, a combination of sub-optimal doses of EOD and chemotherapeutic agents synergistically sup-

pressed HT-29 cancer growth (Fig. 5).

The extrinsic apoptotic pathway can be triggered by interactions between transmembrane death receptors and their cognate ligands [21, 22] whereas the intrinsic apoptotic pathway involves non receptor-mediated stimuli that produce mitochondrial-mediated signals, resulting in an opening of mitochondrial permeability transition (MPT) pores, the loss of mitochondrial membrane potential, and the release of pro-apoptotic proteins like cytochrome c. Our results show that EOD-induced HT-29 apoptosis is associated with caspase cascade induction and loss of mitochondrial membrane potential (Figs. 2, 3, 4), and indicate that EOD-induced mitochondrial dysfunction leads to apoptosis. They also suggest that EOD enhances the anti-cancer effects of chemotherapeutic agents (Fig. 5). However, no *in vivo* experiments on the effects of EOD in colorectal adenocarcinoma animal models, to the best of our knowledge, have been reported, so whether or not EOD really has anti-tumor effects on animals or humans is still not known. Therefore, further investigation is required to understand the detailed mechanism by which EOD reduces proliferation and induces the apoptosis of human colorectal adenocarcinoma cancer cells both *in vivo* and *in vitro*.

5. Conclusion

This study shows that EOD reduces proliferation and induces apoptosis (as confirmed by an increase in the sub-G1 phase) of HT-29 cells. Furthermore, EOD-induced apoptosis was found to be associated with caspase activation and mitochondrial dysfunction. EOD also activated caspase-9 and -3 and increased the generation of intracellular ROS, and combined treatment with sub-optimal doses of EOD and chemotherapeutic agents noticeably suppressed cancer growth. These results indicate that EOD offers a potential alternative adjuvant herbal treatment for colorectal cancer.

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Conflict of interest

The authors declare that there are no conflict of interest.

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