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Antioxidant and Cytoprotective Activity of the Olive Leaf (*Olea europaea* L. var. Kalamata) Extracts on the Mouse Embryonic Fibroblast Cell

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Abstract Oleuropein content of olive leaf extracts (OLE; ethanol extract) was evaluated by high performance liquid chromatography analysis. Oleuropein contents were 4.21±0.57, 3.92±0.43, 0.32±0.03, 5.76±0.32, and 32.47±0.25 mg/100 g for ethanol extract, and hexane, chloroform, ethyl acetate, and butanol fraction, respectively. The removal of DPPH free radical increased in OLE and all 5 fractions of OLE in a concentration dependent manner. In order to investigate the antioxidant effect of OLE *in vitro*, 80%(v/v) ethanol OLE, H_2O_2, or combined treatment of 80%(v/v) ethanol OLE and H_2O_2 were applied on mouse embryonic fibroblast (MEF) cells. Cells were damaged by oxidative stress decreased their viability followed by increasing concentration of H_2O_2, but co-treatment of OLE and H_2O_2 showed an increase in cell growth about 20% compare to the cells treated with H_2O_2. OLE suppresses cytotoxicity induced by H_2O_2 in dose dependent manner. OLE treatment on MEF cells was also examined by analyzing cell cycle and apoptotic rate using flow cytometry. Apoptotic and necrotic cell accumulation was decreased in addition of OLE to H_2O_2 compare to the oxidative damaged cells. Taken together, these results demonstrated that OLE suppresses cytotoxicity induced by H_2O_2 and protect cells against oxidative stress on MEF cells.

Keywords: olive leaf, oleuropein, antioxidant activity, apoptosis, cell viability

Introduction

Olive crop (*Olea europaea* L. var. Kalamata) has an important place in the Mediterranean countries because of its physiologically active polyphenols (1). Olive tree demonstrates the great economic and social importance of this crop and possible health benefits to be derived from any of its byproducts (2). By products and wastes from plant, which represent a major problem for the industry, are sources of value added ingredients, with emphasis on the retrieval of bioactive compounds. Olive leaf is rich in polyphenols, especially in oleuropein (Fig. 1) and also contains rutin, verbacoside, apigenin-7-glucoside, and luteolin-7-glucoside (3). Polyphenols are responsible for the particular resistence to oxidative peroxidation. Many studies have been speculated on the possible antioxidant activity of natural compounds to avoiding lipid oxidation in the arteries and accumulation of low density lipoprotein (LDL) cholesterol in the arterial walls, which is a major cause of coronary heart diseases (4). Antioxidants are substances, which play a preventive role against human diseases by removing reactive oxygen species (ROS) from biological system (5). Several synthetic antioxidants including butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) have high antioxidant activity but the use of common synthetic antioxidants has become controversial issue because of adverse effect report (5). Therefore, there has been a growing interest in replacing them with natural ingredients which have a various biochemical activities, such as ROS generation inhibition, scavenging of free radicals and changes in cell cycle. Considerable interest has been directed to identification of plants with antioxidant activity that may be used for disease prevention in human. In this study, we used mouse embryonic fibroblasts (MEF) to assess antioxidant activity of olive leaf extracts. Fibroblasts are the most common cells of connective tissue in mammals and are generally carried out for ROS-induced cell death signaling pathway associated studies (6-8). Studies have demonstrated the ability of several constituents of plant such as genistein (9), genistein-8-C-glucoside (G8CG) (10), or polyphenols (11) to protect from oxidative stress in mouse fibroblasts.

The objectives of this study were to evaluate the oleuropein contents and DPPH free radical scavenging activity from OLE and various fractions of OLE and to determine antioxidant properties of OLE in a series of assays including cell viability, flow cytometry analysis of cell cycle, apoptosis assay on MEF cells.

Materials and Methods

Materials Olive (*Olea europaea* L. var. Kalamata) leaf was obtained from Asia Pharm. Ind. Co., Ltd. (Seoul, Korea), which was originated from Greece. The olive leaf of foreign substances was removed by washing and drying. After dried, olive leaf was approximately 30 mesh grounded and used.

Sample preparation A 80%(v/v) ethanol extract and 5 different fractions were made used the method of Lee *et al.* (12). Tewnty-five g of olive leaves were approximately 30

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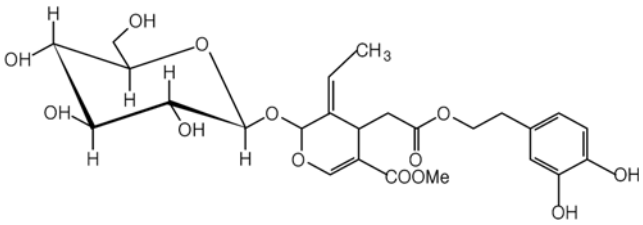

Fig. 1. Structure of oleuropein.

Olive leaf wash, dry, and ground by 30 mesh
↓
Put samples into flat bottom flask and add 80%(v/v) EtOH
↓
Extract on the Soxhlet apparatus with heating (80°C) for 3 hr (3 times)
↓
Filter through filter paper (Whatman No. 2)
↓
Evaporate under a vacuum evaporator at 70°C
↓
Freeze dry to get powdery condition
↓
Completion of 80%(v/v) ethanol extract of olive leaf

Fig. 2. Preparation of olive leaf extract by 80%(v/v) ethanol (OLE).

mesh grounded and extracted with 10 times of 80%(v/v) ethanol using a Soxhlet apparatus for 9 hr at 80°C. After vacuum filtration, sample was fixed for 24 hr at 4°C and filtered again. This solution was evaporated with rotary vacuum evaporator (R-124; Buchi Co., Zurich, Switzerland) at 70 and 80%(v/v) ethanol olive leaf extract (OLE) was freeze-dried (Labconco, Kansas City, MO, USA). Preparation steps of 80%(v/v) ethanol OLE are shown in Fig. 2.

Different fractions of OLE were fractionated by adding different solvents based on different polarity. The 80%(v/v) ethanol OLE was dissolved in water and added 30 mL of hexane (Dae Jung Chemicals and Metals Co., Siheung, Korea) and separated of hexane layer and water layer. After it was evaporated with rotary vacuum evaporator, we obtained a hexane fraction. Using same process, chloroform, ethyl acetate, and butanol fractions by adding solvent of chloroform, ethyl acetate, and butanol (Dae Jung Chemicals and Metals Co.) are obtained, respectively. Final residue solution was called water fraction. Obtained different fractions were evaporated with rotary vacuum evaporator, and freeze-dried to remove solvent.

For cell culture, cell viability, flow cytometry and apoptosis assay, Each dry samples for 80%(v/v) ethanol fraction (52 mg), hexane fraction (30.6 mg), butanol fraction (30 mg), water fraction (30 mg), chloroform fraction (20 mg), and ethyl acetate fraction (5.2 mg) were liquefied in 10 mL of phosphate buffered saline (PBS; WelGENE Inc., Daegu, Korea) and adjusted pH to between 6 and 7. Then, the extract was sterilized through 0.25-μm filter (Sartorius, Goettingen, Germany). Each extract was stored at –20°C until further use (Fig. 3).

Dissolve 80%(v/v) olive leaf ethanol extract in water
↓
Add hexane 30 mL (3 times)
↓
Separate hexane layer from H_2O layer→Obtain of hexane fr.
↓
Add chloroform 30 mL to a H_2O layer (3 times)
↓
Separate chloroform layer and H_2O layer→Obtain of chloroform fr.
↓
Add ethyl acetate 30 mL to a H_2O layer (3 times)
↓
Separate ethyl acetate layer and H_2O layer→Obtain of ethyl acetate fr.
↓
Add butanol 30 mL to a H_2O layer (3 times)
↓
Separate butanol layer and H_2O layer→Obtain of butanol fr.
↓
Liquefy to powdery fractions in PBS
↓
Adjust pH to between 6 and 7
↓
Filter through 0.25-μm filter
↓
Extracts stored at –20°C until use

Fig. 3. Preparation of olive leaf fractions and cell culture sample.

High performance liquid chromatography (HPLC) analysis of oleuropein Analysis of the oleuropein was used HPLC (Dionex Co., Sunnyvale, CA, USA). The sample solution was prepared by dissolving 80%(v/v) ethanol OLE and each of different solvent fraction in methanol and the sample was filtered by using 0.45-μL membrane filter (Omnipore; Millipore, Tullagreen, Ireland) and we used.

The column was a μ-Bondapak C_{18} (300×3.9 mm, Waters Co., Milford, MA, USA). Solvent used was water (pH 2.5, H_3PO_4)/acetonitrile (77.5:22.5), flow rate was 1.5 mL/min, detector was UV detector (wave length of 240 nm) (Optizen 2120UV; Mecasys, Daejeon, Korea), injection volume was 20 μL.

DPPH free radical scavenging activity 2,2-Diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 80%(v/v) ethanol to 200 μM and sonicated for 5 min to obtain the stable free radical DPPH. Each 100 μL of 80%(v/v) ethanol extract and 5 different fractions containing of OLE and 100 μL of freshly prepared 200 μM DPPH ethanolic solution was thoroughly mixed in a 96-well plate (SPL Inc., Pocheon, Korea). The plate was incubated at the room temperature in the dark for 30 min. A 200 μL of extract, PBS, 200 μM DPPH, and 80%(v/v) ethanol were used as standard, standard control, control, and blank, respectively. All tests were performed in triplicate. The absorbance of the mixture was measured by enzyme-linked immunosorbent assay (ELISA, Molecular Devices, Sunnyvale, CA, USA) at wavelength of 517 nm, and the percentage of free radical scavenging activity was calculated according to the following formula:

$$\text{Scavenging activity (\%)} = [1 - \{(Abs_{\text{sample 517 nm}} - Abs_{\text{blank 517 nm}} - Abs_{\text{standard 517 nm}})/Abs_{\text{control 517 nm}}\}] \times 100$$

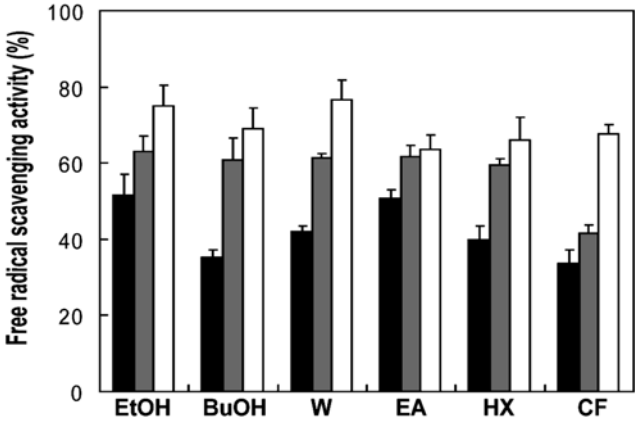

Fig. 4. DPPH radical scavenging activity of olive leaf extract (OLE) and its solvent-partitioning fractions. All extracts were added at 3 different dilutions of 1/1,000 (■), 1/100 (■), and 1/10 (□). EtOH, 80%(v/v) ethanol extract; BuOH, butanol fraction; W, water fraction; EA, ethyl acetate fraction; HX, hexane fraction; CF, chloroform fraction. All test values are mean±SEM (n=3).

Cell culture Mouse embryonic fibroblast (MEF) wild type cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc.) supplemented with 10%(v/v) fetal bovine serum (FBS; WelGENE Inc.) and 1%(v/v) penicillin (100 IU/mL)/streptomycin (100 μg/mL; WelGENE Inc.), and incubated at 37°C in a 5%(v/v) CO_2 atmosphere. The medium was replaced every 2 days after checking the cell growth under a microscope.

Measurements of cell viability MEF cells were harvested at approximately 70-80% confluence and seeded onto 96-well plate (SPL Inc., Pocheon, Korea) at 3.0×10^3 or 1.25×10^3 cells/well in 100 μL of medium. The culture plates were incubated in a CO_2 incubator at 5% CO_2 and 37°C for 24 hr to make the cells adhere to the inner wall of the plates. Cells were washed with PBS. Cells were treated with various concentrations of 80%(v/v) ethanol OLE in triplicates for each treatment group, and incubated for another 24 hr. Cells were then washed twice with PBS and incubated with 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) (5 mg/mL) for 3 hr at 37°C. The medium was discarded, and then, 200 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added. After 10 min incubation, the absorbance was measured by ELISA (Molecular Devices) at wavelength of 570 nm.

Flow cytometry analysis of cell cycle Cells were seeded in 100-mm dishes in 10 mL medium and incubated for 24 hr. At 60% confluence, the dishes were treated with control, 26 μg/mL of 80%(v/v) ethanol OLE, 450 μM hydrogen peroxide (H_2O_2; Sigma-Aldrich), and co-treated with 26 μg/mL of 80%(v/v) ethanol OLE and 400 μM H_2O_2 and then incubated for another 24 hr. Cells were harvested by trypsinization, and the cells were washed with PBS, and then fixed with ice-cold 100%(v/v) ethanol at 4°C for 4 hr. Cells were washed again with PBS, and

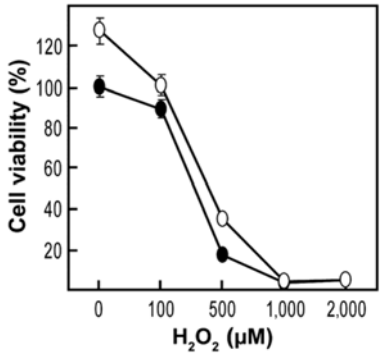

Fig. 5. Effect of olive leaf extract (OLE) on H_2O_2 induced cytotoxicity in MEF cells. Cells (3.0×10^3 cells/well) were treated for 24 hr with (○) or without (●) 80%(v/v) ethanol fraction of OLE (104 μg/mL) in the presence of H_2O_2 (100-2,000 μM). Values are mean±SEM (n=3).

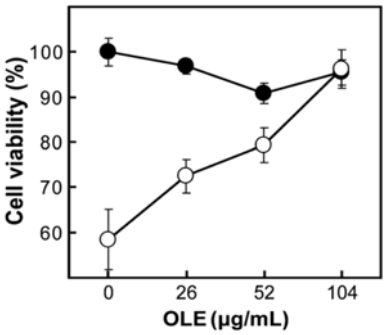

Fig. 6. Dose-dependent antioxidative effect of olive leaf extract (OLE) in MEF cells. Cells (1.25×10^3 cells/well) were treated for 24 hr with (○) or without (●) 700 μM H_2O_2 in the presence of different concentrations of 80%(v/v) ethanol fraction of OLE (26, 52, and 104 μg/mL). Values are mean±SEM (n=3).

incubated with propidium iodide (PI; BD Biosciences Pharmingen, San Diego, CA, USA) solution containing Triton X-100 (0.1%, v/v) (Sigma-Aldrich), 0.1 mM ethylenediamine tetraacetic acid (EDTA; Sigma-Aldrich), RNase A (50 μg/mL) (Amresco, Solon, OH, USA) and PI (50 μg/mL). The fluorescence was measured with a FAC scan laser flow cytometer (Becton Dickinson, San Jose, CA, USA), and analyzed by using Becton Dickinson software (Lysis Cellfit).

Apoptosis assay Cells were seeded in 100-mm dishes in 10 mL medium and incubated for 24 hr. At 60% confluence, the dishes were treated with control, 104 μg/mL of 80%(v/v) ethanol OLE, 1,000 μM H_2O_2, co-treated with 104 μg/mL of 80%(v/v) ethanol OLE and 1,000 μM H_2O_2, and then incubated for another 24 hr. Cells were harvested by trypsinization, and both of adherent and floating cells were included. The rest of steps were followed by manufacturer's instruction using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences Pharmingen). Apoptosis was quantified by FACScan flow cytometry of Annexin V-FITC/PI double staining.

Statistical analyses The results were analyzed by Sigma Stat 2.0 (Jandel Corp., San Rafael, CA, USA).

Appropriate comparisons were made using the Student-Newman-Keuls test for one-way analysis of variance (ANOVA) analysis.

Results and Discussion

HPLC analysis of oleuropein Oleuropein is a major compound of phenolic compound in olive (*Olea europaea* L. var. Kalamata) leaf (12). Oleuropein contents of the 80%(v/v) ethanol OLE and different 5 fractions from HPLC analysis are shown in Table 1. Oleuropein contents of different fractions were 4.21±0.57, 3.92±0.43, 0.32±0.03, 5.76±0.32, and 32.47±0.25 mg/100 g for 80%(v/v) ethanol extract, hexane fraction, chloroform fraction, ethyl acetate fraction, and butanol fraction, respectively. Butanol fraction showed the highest oleuropein content compared to other fractions including ethyl acetate and water fraction, and ethanol extract. Olive leaf has been widely used in Mediterranean diet, and several studies suggest its biological benefits including retard progression of atherosclerosis and hypertension, and anti-cancer (13-15). Oleuropein is one of the representative phenolic compounds in olive leaf and reported the anti-ischemic, antioxidative, and hypolipidemic effects (16,17).

The result of butanol fraction is comparable to previous reports for same condition of oleuropein contents analysis that are similar tendency (12,18). It has been known that oleuropein contents for butanol fraction of olive leaves raised in Australia (*Olea europaea* L. var. Picual) and Spain (*Olea europaea* L. var. Hojiblanca) were higher than other fractions (12,18). Benavente-Garcia *et al.* (16) quantified various polyphenols found in olive leaf and reported that oleuropein was found to be the major phenolic compound. Soler-Rivas *et al.* (4) reported that oleuropein presented in high amounts (60±90 mg/g dry weight) in the leaves of the olive tree. Bouaziz *et al.* (3) demonstrated that oleuropein concentration in leaves were 12.4 to 14.2%(w/w) during all harvesting periods, and Luque de Castro and Japon Lujan (19) reported that up to 14%(w/w) of oleuropein has been extracted in varieties of olive leaves. Pereira *et al.* (20) showed that aqueous extract exhibited a profile in which oleuropein was the compound present in highest amount, representing 73% of total identified compounds. It has been reported that catechin, caffeic acid, vanillin, rutin, vanillic acid, hydroxytyrosol, and tyrosol exist in very small quantities in olive leaves (3,16,19,20).

Antioxidative effect of olive leaf by scavenging free radical Oxidative stress is mainly caused by reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radicals, and superoxide anions. Oxidative stress mediated by ROS plays an important role in regulating various physiological processes such as aging, inflammation, and cancer (21,22).

In order to measure free radical scavenging activity of 80%(v/v) ethanol extract and 5 different fractions containing of OLE, cell-free DPPH assay was carried out. DPPH is stable radical, and if the free radical reacts with hydrogen donors, the radical is reduced to the corresponding hydrazine (23). The DPPH free radical scavenging activity of varying amounts of OLE and 5 fractions [80%(v/v) ethanol, butanol, water, ethyl acetate, hexane, and chloroform] of olive leaves is shown in Fig. 4. The removal of DPPH free radical increased in OLE and 5 fractions of OLE in a concentration dependent manner. Of those fractions, 80%(v/v) ethanol OLE was found to be more effective than other 5 fractions of OLE.

Table 1. Oleuropein contents of olive leaf (*Olea europaea* L. var. Kalamata) fractions

Sample	Oleuropein (mg/100 g)
80%(v/v) EtOH	4.21±0.57[b1)]
Hexane fraction	3.92±0.43[b]
Chloroform fraction	0.32±0.03[a]
Ethyl acetate fraction	5.76±0.32[c]
Butanol fraction	32.47±0.25[d]
Water fraction	ND[2)]

[1)]All values are expressed as mean±SD of triplicate determinations; Means in the same column not sharing a common letter are significantly different ($p<0.05$) by Student-Newman-Keuls method.
[2)]Not detected.

The content of oleuropein in olive leaf extract was showed the highest in butanol fraction, but the highest capacity of DPPH free radical scavenging activity was 80%(v/v) ethanol extract which was less oleuropein content than butanol fraction. These results are comparable to previously reports for same condition of DPPH free radical activity that was similar tendency. (18) The highest fraction was also not equal to result of oleuropein content and DPPH free radical activity. It seems that polyphenol compounds which are catechin, caffeic acid, vanillin, rutin, and hydroxytyrosol in olive leaf act on antioxidant. The 80%(v/v) ethanol OLE is also reported to possess the highest superoxide dismutase (SOD)-like activity among other fractions of OLE, where SOD is an antioxidant enzyme plays a key role in cells exposed to oxidative stress, among other fractions of OLE (24).

Olive leaves suppresses cytotoxicity induced by oxidative stress on MEFs To evaluate antioxidant capacity of olive leaf as functional food ingredient, our experiment was conducted to use 80%(v/v) ethanol extracted sample because ethanol is used with edible solvent. In order to investigate the antioxidant effect of OLE *in vitro*, 80%(v/v) ethanol OLE, H_2O_2, or combined treatment 80%(v/v) ethanol OLE and H_2O_2 were applied on MEF cells. Cells which were damaged by oxidative stress decreased their viability followed by increasing concentration of H_2O_2, but co-treatment 80%(v/v) ethanol OLE and H_2O_2 showed increase on cell growth slightly up to about 20% compare to the cells treated with H_2O_2 (Fig. 5). The 80%(v/v) ethanol OLE treatment on MEF cells did not show any cytotoxcicity in different concentrations as shown in Fig. 6. However, cells exposed to the same concentration of H_2O_2 (700 μM) increased their cell survival with 80%(v/v) ethanol OLE treatment in dose dependent manner. At the highest concentration of 80%(v/v) ethanol OLE, the damaged cells recovered their viability almost up to control cells. These results strongly suggest that 80%(v/v) ethanol OLE suppresses cytotoxicity induced by H_2O_2 in dose dependent manner.

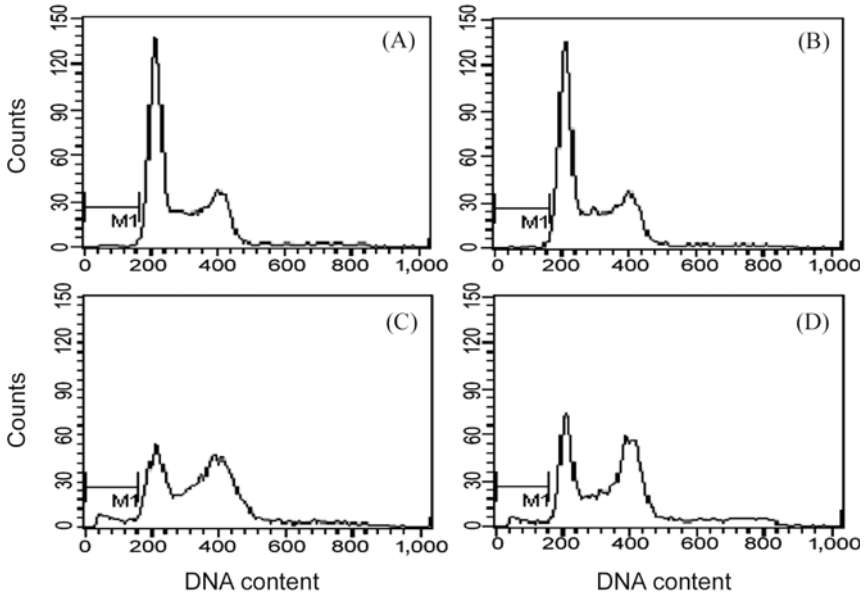

Fig. 7. Effect of olive leaf extract (OLE) treatment on cell cycle progression in oxidative damaged MEF cells. Cells were treated with PBS as control (A), 26 μg/mL of 80%(v/v) ethanol fraction of OLE (B), 450 μM H_2O_2 (C), and 26 μg/mL of 80%(v/v) ethanol fraction of OLE with 400 μM H_2O_2 (D) for 24 hr. The cell cycle was analyzed using flow cytometry with PI staining.

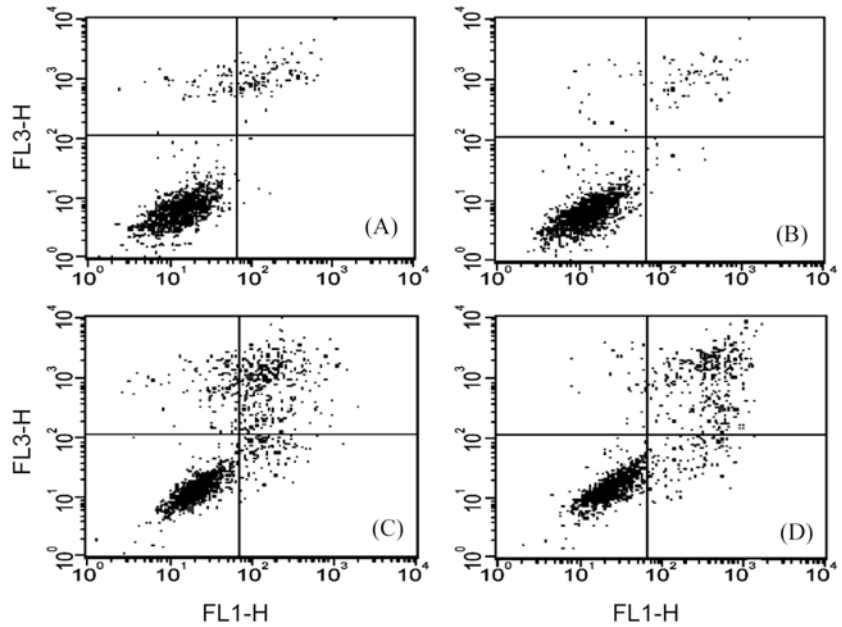

Fig. 8. Effect of olive leaf extract (OLE) treatment on the rate of apoptosis in MEF cells damaged by H_2O_2. Cells were treated with PBS as control (A), 104 μg/mL of 80%(v/v) ethanol fraction of OLE (B), 1,000 μM H_2O_2 (C), and 104 μg/mL of 80%(v/v) ethanol fraction of OLE with 1,000 μM H_2O_2 (D) for 24 hr. The rate of apoptosis was measured using flow cytometry with Annexin V-FITC and PI double staining.

Cell cycle analysis and apoptosis quantification using flow cytometry Effect of 80%(v/v) ethanol OLE treatment on MEF cells was also examined by analyzing cell cycle and apoptosis rate using flow cytometry. In the MEF cells treated with 80%(v/v) ethanol OLE, cell cycle showed normal pattern with significant peaks for both G_0/G_1 and G_2/M (Fig. 7B). Co-treatment of 80%(v/v) ethanol OLE and H_2O_2 still showed crumbled peaks on both G_0/G_1 and G_2/M, which destroyed by H_2O_2, but the cell cycle was restored to the normal state compare to the H_2O_2 treatment group and the portion of cells in sub-G_0 decreased from 4.86 to 2.56% (Fig. 7D). Also, apoptotic and necrotic cell accumulation was decreased in addition of 80%(v/v) ethanol OLE to H_2O_2 compare to the oxidative damaged cells (Fig. 8D). These results can imply possible protecting effects for 80%(v/v) ethanol OLE against oxidative damaged cells via cell cycle pathways.

Our data show the antioxidant potency of olive leaves on MEF cells, and these suggest that olive leaves suppresses

cytotoxicity induced by H_2O_2 and protect cells against oxidative stress.

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