Food Sci. Biotechnol. Vol. 18, No. 5, pp. 1193 ~ 1198 (2009)

Food Science and Biotechnology

Photo-protective and Anti-melanogenic Effect from Phenolic Compound of Olive Leaf (*Olea europaea* L. var. Kalamata) Extracts on the Immortalized Human Keratinocytes and B16F1 Melanoma Cells

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Abstract Ethylacetate and butanol fractions of leaf extracts (OLE) showed the higher contents of total phenolic compounds than hexane and water fractions. 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. Treatment of ultraviolet-B (UVB) irradiated cells with 3 OLEs prepared by using ethylacetate and butanol at concentrations 0.001, 0.005, and 0.01% respectively showed significant recovery of cell viabilities. Treatment of dexametason 1 mM reduced tumor necrotic factor (TNF)-α secretion by about 40%. UVB irradiated immortalized human keratinocytes (HaCaT) cells were treated with 3 different OLEs at the same concentrations. Ethylacetate fraction showed the strongest inhibition activity with respect of reduction of the elevated TNF-α. Cytotoxicity of OLEs on the B16-F1 cells was evaluated through thiazolyl blue tetrazolium bromide (MTT) assay. Ethylacetate fraction has no cytotoxicity in the range of 0.005-0.01%. A slight cytotoxicity was observed at the concentration of 0.1% butanol fraction of OLE that caused 10% decrease in cell viability.

Keywords: olive leaf extract, oleuropein, phenolic compound, photo-protective, anti-melanogenic effect

Introduction

Olive crop (*Olea europaea* L. var. Kalamata) has an important place in the Mediterranean countries because of its physiologically active polyphenols. Olive tree demonstrates the great economic and social importance of this crop and possible health benefits to be derived from any of its byproducts. Ultraviolet (UV) irradiated skin cells produce reactive oxygen species (ROS) that induced the skin inflammatory response (1). UV is responsible for cellular oxidative damages and collagen degradation initiated by the generation of ROS (2,3). Keratinocytes are the primary targets of UV irradiation and the UV irradiated keratinocytes produce a variety of pro-inflammatory cytokines such as tumor necrotic factor (TNF)-α, interleukin (IL)-1α, IL-6, and IL-8 (4-6). TNF-α plays a key role in early responses to UV irradiation on keratinocytes since the expression of TNF-α is markedly increased within 3 days after UV-B (UVB) irradiation to cultured keratinocytes, which eventually leading to initiation of inflammation (5,7). Skin inflammation and ROS are thought to be the major cause of natural and photoaging of human skin (6,8).

In addition, UV irradiation stimulates melanocytes to induce melanin synthesis by enhancing the expression of tyrosinase that catalyzes the first two steps in melanogeneses in human skin (9). Melanin synthesized from melanocytes is composed of insoluble brown pigment and metallic protein and located inside of melanosomes. Skin pigmentation, however is developed only after the melanosomes are transferred from melanocytes to the neighboring keratinocytes via dendrites of melanocytes (10). There are many other factors that affect melanogenesis beside UV irradiation such as inflammatory diseases or genetic backgrounds (11). The first biochemical reaction in a melanogenesis is oxidation of tyrosin by tyrosinase to make dopaquinone or dopachrome and further oxidization processes results in production of eumelanin or pheomelanin respectively (12). Depigmenting agents that can prevent formation of melanin in melanocytes have been widely used for 'whitening' cosmetics as cosmeceutical. Kojic acid, arbutin, and hydroquinone are well known direct inhibitor of tyrosinase activity *in vitro* (13). Some tyrosinase inhibitors however do not inhibit tyrosinase directly. Instead, they rather negatively regulate the expression of tyrosinase or genes involved in the melanogenesis. In addition, many phytochemicals from diverse natural sources have been screened. In addition, suppression of melanosome transfers from melanocytes to keratinocytes is now known to be a new mechanism behind the depigmenting activities of many novel whitening cosmeceutical ingredients (14).

The objectives of this study were to evaluate the phenolic compounds from olive leaf extract with respect of photo-protective and anti-melanogenic effect by using a series of assays including thiazolyl blue tetrazolium bromide (MTT) assay, protection efficacy assay on UV damage, and melanin assay on immortalize human keratinocytes and melanoma cells.

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Received April 13, 2009; Revised June 3, 2009;
Accepted June 5, 2009

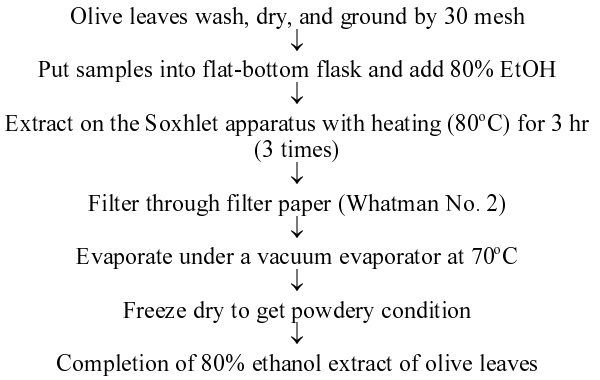

Fig. 1. Preparation of 80% ethanol of *O. europaea* L. leaves extracts (OLE).

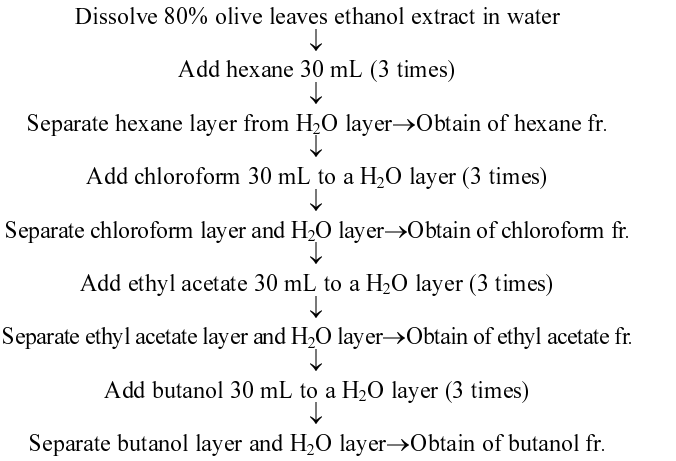

Fig. 2. Preparation of *O. europaea* L. leaves fractions.

Materials and Methods

Olive leaf Olive leaves originated from Greece were purchased from Asia Pharm. Ind. Co., Ltd. (Seoul, Korea). The olive leaves of foreign substances were eliminated by washing and drying at room temperature. After dried, olive leaves were around 30 mesh milled and used.

Materials B16F1 melanoma cells were purchased from KCLB (Seoul, Korea), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), α-melanocyte stimulating hormone (α-MSH), kojic acid, dexamethason, ascorbic acid, dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO, USA) and Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics from Welgene (Daegu, Korea). TNF-α enzymelinked immunosorbent assay (ELISA) kits were purchased from Biosource International (Camarillo, CA, USA).

Sample preparation A 80% ethanol extract and 5 different fractions were made used the method of Lee *et al.* (15). Twenty-five g of olive leaves were approximately 30 mesh grounded and extracted with 10 times of 80% 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°C and 80% ethanol olive leaf extract (OLE) was freeze-dried (Freeze dryer 3; Labconco, Kansas City, MO, USA). Preparation steps of 80% ethanol OLE were shown in Fig. 1.

Different fractions of OLE that was fractionated by adding different solvents based on different polarity were obtained. The 80% 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, we obtained chloroform, ethylacetate, and butanol fractions by adding solvent of chloroform, ethyl acetate, and butanol (Dae Jung Chemicals and Metals Co.), respectively. Final residue solution was called water fraction. Fractions that were evaporated with rotary vacuum evaporator, and freeze-dried to remove solvent were obtained (Fig. 2).

Analysis of total phenol content of olive leaf fractions The total phenol contents of samples were determined to use Folin-Dennis method that phenol compound and phosphomolybdic react to indicate a blue color (16). The sample of 1 mL was dissolved in a certain quantity of DMSO (Sigma-Aldrich) and added distilled water of 7 mL and Folin-Dennis reagent (Sigma-Aldrich, Buchs, Switzerland) of 0.5 mL. And then, after accurately 3 min, added saturated solution of sodium carbonate anhydrous 1 mL (Samchum Pure Chemical Co., Seoul, Korea), distilled water of 0.5 mL and determined absorbance using UV/VIS spectrophotometer (Mecasys Co., Ltd., Daejeon, Korea) at 725 nm at that time. Standard curve was made by using tannic acid (Sigma-Aldrich, Switzerland) Folin-Dennis reagent was made by adding sodium tungstate (Shinyo Pure Chemical Co., Osaka, Japan) of 10 g, phosphomolybdic acid 2 g, phosphoric acid 5 mL that using a reflux condenser in water bath at 80°C.

High performance liquiid chromatography (HPLC) analysis of the phenol compounds Analysis of the phenol compounds were used HPLC (Dionex Co., Sunnyvale, CA, USA). The sample solution was prepared by dissolving 80% ethanol OLE and each of different solvent fraction in methanol and the sample was filtered by using 0.45-μL membrane filter (Omnipore, Milipore, Tullagreen, Ireland) and we used. The column was a μ-Bondapak C_{18} (300×3.9 mm, Waters Co., Milford, MA, USA). Solvent is used 0.2% KH_2PO_4 (pH 3.0)/acetonitrile (85:15), flow rate was 1.5 mL/min, detector was UV detector (wavelength of 280 nm), injection volume was 20 μL. Standard sample was used catechin (Epicatechin; Tokye Kasei Kogyo Co., Ltd., Tokyo, Japan), vanillin, rutin, and oleuropein. But, in case of analysis condition of oleuropein, it was different from the condition as stated above. Solvent used was water (pH 2.5, H_3PO_4)/acetonitrile (77.5:22.5), detector was UV detector (wavelength of 240 nm). Flow rate and injection volume was under same analysis condition above mentioned.

Cell culture Immortalize human keratinocytes (HaCaT) and B16-F1 melanoma cells were maintained in DMEM containing 10% FBS and 1% antibiotics and incubated at

37°C in a humidified atmosphere of 5% CO_2.

MTT assay After 48 hr culture, medium was removed and incubated with MTT solution at 37°C for 90 min and 0.04 N HCl-isopropyl alcohol solution at room temperature for 30 min. Harvested solution was centrifuged at 11,000×g, 5 min. Absorbance was measured at 570 nm by microplate reader (Perkin Elmer, Waltham, MA, USA).

UV irradiation and treatment HaCaT cells were seeded at a density of 1×10^5 cells/well in 120 mm culture dishes and cultured in DMEM for 24 hr. Prior to UVB irradiation, cell culture media were removed and cells were rinsed with phosphate buffered saline (PBS). And then, cells were irradiated to UVB at 312 nm, 25 mJ/cm^2 as measured with an SX-312 research radiometer (Uvitec, Cambridge, UK). After UVB irradiation, cells were maintained in serum free DMEM with or without OLE for 24 hr.

α-MSH treatment B16-F1 melanoma cells seeded at a density of 1.5×10^5 cells/well at 6-well plates and cultured in DMEM for 24 hr. After 24 hr, medium changed to fresh one supplemented with α-MSH 5 nM and proper concentration of olive fraction samples and additional culture for 48 hr was added. Untreated α-MSH was used as a negative control and 200 and 400 μM of kojic acid were used as a positive control.

Measurement of protection efficacy on UV damage To measure protection efficacy on cell damage induced by UVB, we were performed by MTT assay. After UVB irradiation, HaCaT cells were cultured in serum free DMEM with or without OLE. Then, cell culture media was removed and replaced with MTT solution (0.33 g/L) for 90 min at 37°C. The supernatants were discarded and added isoprophanol to dissolve the formazan materials. The intensity was measured at 570 nm.

TNF-α analysis To evaluate TNF-α secretion, HaCaT cells were grown in serum free DMEM with or without OLE after UVB exposure. Then cultured HaCaT cells culture media was obtained. Secretion of human TNF-α in cultured media were determined using a commercial ELISA kits (BioSource International).

Melanin assay Secreted melanin was harvested from cultured medium. After 48 hr culture, medium was harvested and centrifuged at 9,000×g, 10 min. Absorbance was measured at 405 nm by microplate reader (Perkin Elmer).

Statistical analyses The results were analyzed by Sigma Stat 2.0 (Jandel Corp., San Rafael, CA, USA). One-way analysis of variance (ANOVA) was performed. Appropriate comparisons were made using the Student-Newman-Keuls test for one-way ANOVA analysis.

Results and Discussion

Analysis of total phenolic compound in olive leaf fractions Olive leaf has been reported as physiological activity materials. Some phenolics have an effect on olive plant growth while others protect the more vulnerable cell constituents against photooxidation by UV light by virtue of their strong UV absorption. Phenoilc compounds in olive leaf have been reported possible health benefits including antioxidant activity and antimicrobial activity (17).

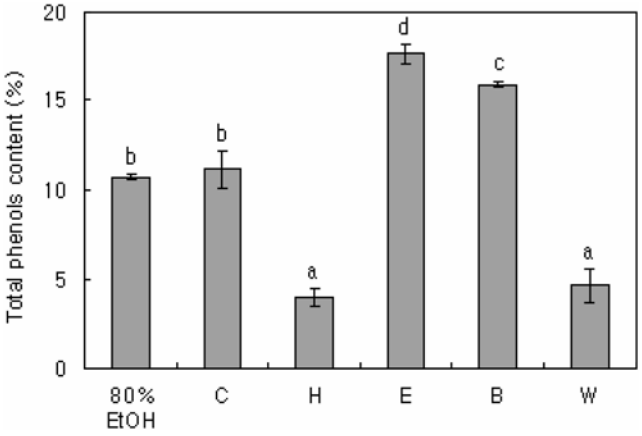

Fig. 3. Total phenol contents of olive leaf extract and fractions raised in Greece. 80% EtOH, 80% ethanol extract; C, chloroform fraction; H, hexane fraction; E, ethyl acetate fraction; B, butanol fraction; and W, water fraction; [a-c]Means in the same column not sharing a common letter are significantly different ($p<0.05$) by Student-Newman-Keuls method. All values are expressed as mean±SD of triplicate determinations.

The result of total phenolic contents of 80% ethanol extract, hexane, chloroform, ethyl acetate, butanol, and water fractions were compared in Fig. 3. Ethyl acetate and butanol fractions show the higher contents of total phenolic compounds than other fractions. Total phenolic compounds in hexane and water fraction were lower than other fractions. Olive leaf raised from Australia (*Olea europaea* L. var. Picual) and Spain (*Olea europaea* L. var. Hojiblanca) in butanol fraction showed the highest total phenolic content (18%) (18). Lee *et al.* (15) reported that the result of total phenolic content of OLE and fractions were ranged from 3.4 to 17.5% that was slightly lower content than our results. But among other samples, buthanol fraction, ethyl acetate fraction and 80% ethanol fraction shows the relatively higher content of total phenolic content, and water fraction and hexane fraction were shown lower content (15,18). This tendency was similar to our result of total phenol contents. Silva *et al.* (19) reported that the values of total phenolic content measured in the fresh and dry olive leaves were ranged from 11.6 to 40.1 g/kg, respectively. Skerget *et al.* (20) reported that total phenol content of methanol extract of olive leaves were showed 144 g galic acid/kg.

HPLC analysis of the phenol compounds The HPLC analysis of phenol compounds present in olive leaf extract and fractions are shown in Table 1. Among phenolic compounds, the highest abundant compound in OLE is oleuropein. Other compounds were catechin, caffeic acid, vanillin, and rutin which existed in very small quantities in olive leaves.

Oleuropein contents from 80% ethanol extract, hexane fraction, chloroform fraction, ethyl acetate fraction, and

Table 1. Phenolic compound contents of olive leaf fractions raised in Greece (mg/100 g)[2,3)]

Sample	Catechin	Caffeic acid	Vanillin	Rutin	Oleuropein
80% EtOH	0.07±0.02$^{a1)}$	ND$^{2)}$	ND	0.13±0.03^{c}	4.21±0.57^{b}
Hexane fr.	ND	0.03±0.04^{a}	ND	0.02±0^{a}	3.92±0.43^{b}
Chloroform fr.	ND	0.03±0.04^{a}	ND	0.06±0^{c}	0.32±0.03^{a}
Ethyl acetate fr.	ND	0.10±0^{b}	ND	0.17±0^{c}	5.76±0.32^{c}
Butanol fr.	0.14±0.01^{b}	0.02±0.01^{a}	0.02±0.0^{a}	ND	32.47±0.25^{d}
Water fr.	ND	ND$^{1)}$	ND	0.04±0.02^{b,c}	ND$^{1)}$

[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.

butanol fraction 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, respectively. Butanol fraction showed the highest oleuropein content compared to other fractions including ethyl acetate and water fraction, and ethanol extract. The result of butanol fraction is comparable to previous reports for same condition of oleuropein contents analysis that are similar tendency (15,18). 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 (15,18). Benavente-Garcia *et al.* (21) quantified various polyphenols found in olive leaves and reported that oleuropein was found to be the major phenolic compound. Soler-Rivas *et al.* (22) reported that oleuropein presented in high amounts (60±90 mg/g dry weight) in the leaves of the olive tree. Bouaziz *et al.* (23) demonstrated that oleuropein concentration in leaves were 12.4 to 14.2%(w/w) during all harvesting periods, and Luque de Castro *et al.* (24) reported that up to 14%(w/w) of oleuropein has been extracted in varieties of olive leaves. Pereira *et al.* (25) showed that aqueous extract exhibited a profile in which oleuropein was the compound present in highest amount, representing 73% of total identified compounds.

Although other compounds are very small amount, butanol fraction contains high amount catechin (0.14±0.01 mg/100 g) and ethyl acetate fracion showed an high content of rutin (0.17±0.009 mg/100 g) and caffeic acid (0.10± 0.001 mg/100 g), comparatively. Antioxidant activity of rutin, catechin, and caffeic acid as measured by Trolox equivalent antioxidant activity (TEAC) were 2.4, 1.3, and 2.4 mM, repectively and vitamin C and E contents were 1.0 mM (26). Butanol and ethyl acetate fractions showed high contents of oleuropein, rutin, catechin, and caffeic acid which are classified as strong natural antioxidants.

Photo-protective effect UVB irradiation plays an harmful role in skin damage, including skin cancer, inflammation, pigmentation, and skin wrinkles (5,27). We investigated whether treatment of olive extract protects UVB induced cell damages such as cell death and initiation of inflammation. Irradiation of HaCaT cells with UVB at 312 nm, 25 mJ/cm^2 decreased cell viability to 60% but treatment of ascorbic acid 25 μM recovered the cell viability to around 80%. Treatment of UVB irradiated cells with 3 different olive extracts prepared by using 80% ethanol ethylacetate and butanol at concentrations 0.001, 0.005, and 0.01%, respectively, showed significant recovery of cell viabilities

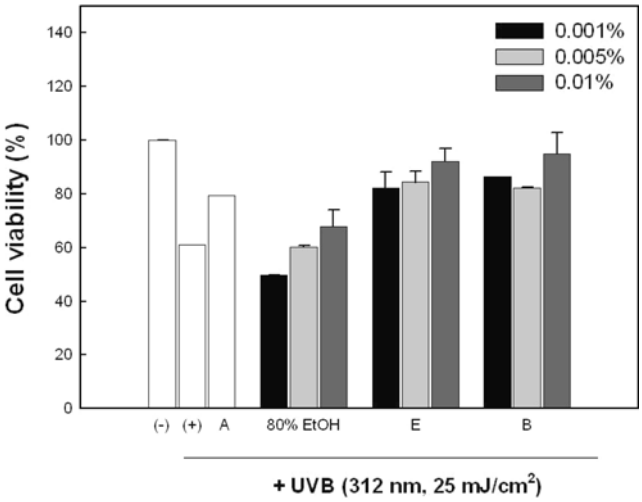

Fig. 4. Olive extract protected HaCaT keratinocytes from UVB damage. (-), non treated UVB; (+), treated UVB; A, ascorbic acid 25 μM; 80% EtOH, 80% ethanol extract; E, ethyl acetate fraction; and B, butanol fraction. All values are expressed as mean±SEM of duplicate determinations.

(Fig. 4). Ethylacetate and butanol fractions seemed to have similar protection activity but 80% ethanol extract did not show any significant recovering effect from the cell damages caused by UVB exposure. Further experiments will be performed to analyze if the olive leaf fraction protects cellular DNA degradation induced by UV.

Inhibitions of pro-inflammatory cytokines such as TNF-α, IL-1α, and IL-6 are of the first option to prevent inflammation. Especially, searching for the new phytochemicals that can inhibit TNF-α production in cells is a standard screening method for development of anti-aging cosmeceuticals since TNF-α is a primary responsive inflammatory cytokine in skin (4). These pro-inflammatory cytokines are involved in diverse inflammatory skin conditions and the progression of photoaging as well. Treatment HaCaT cells with 25 mJ/cm^2 UVB sharply increased TNF-α within 48 hr after UVB irradiation. Dexamethason is a potent anti-inflammatory agent and was used as a positive control. Treatment of dexametason 1 mM reduced TNF-α secretion by about 40%. UVB irradiated HaCaT cells were treated with 80% ethanol extract and 2 different fractions of OLE at the same concentrations with the previous cell viability assays for 24 hr. Among 3 fractions of olive leaf, the ethylacetate fraction had the strongest inhibition activity

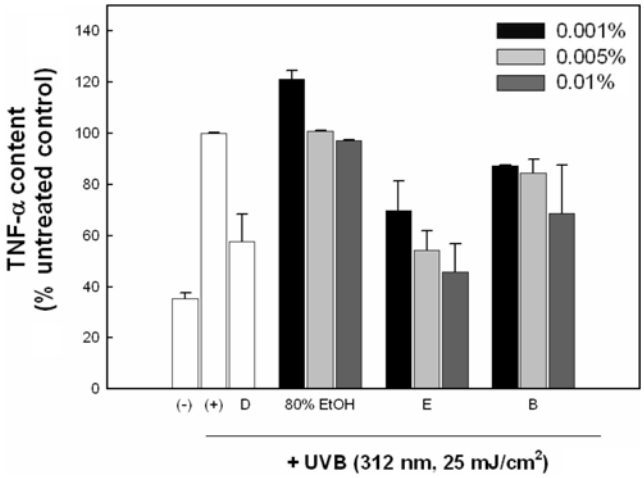

Fig. 5. Olive extract inhibited the UVB induced TNF-α secretion. (-), non treated UVB; (+), treated UVB; D, dexamethason 1 μM; 80% EtOH, 80% ethanol extract; E, ethyl acetate fraction; and B, butanol fraction. All values are expressed as mean±SEM of duplicate determinations.

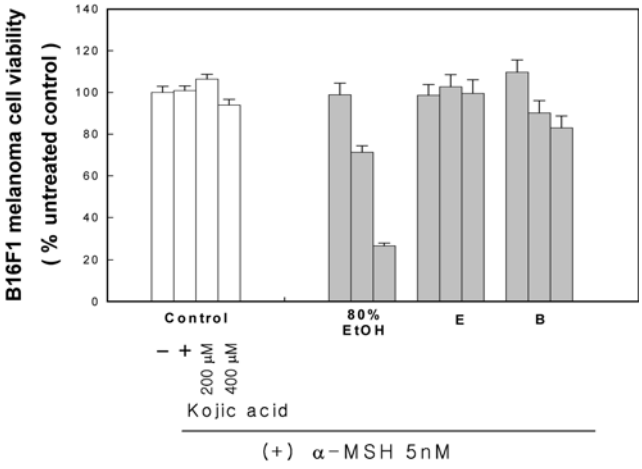

Fig. 6. Cytotoxicity of olive fraction samples on B16F1 melanoma cells by MTT assay. 80% EtOH, 80% ethanol extract (0.01, 0.03, and 0.05%); E, ethyl acetate fraction (0.005, 0.008, and 0.01%); B, butanol fraction (0.01, 0.05, and 0.1%).

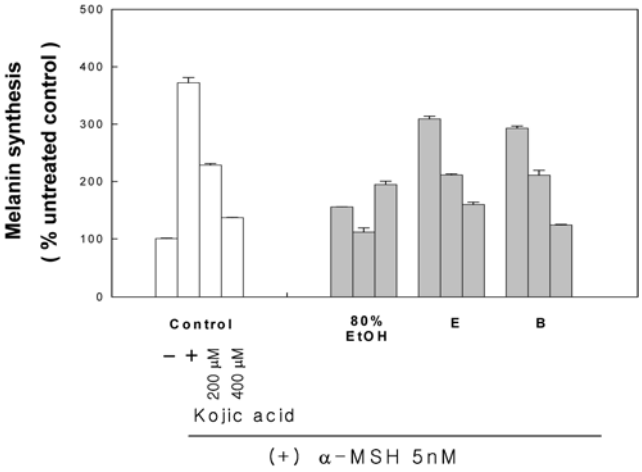

Fig. 7. Effect of reduced melanin secretion of olive fraction samples by melanin assay. 80% EtOH, 80% ethanol extract (0.01, 0.03, and 0.05%); E, ethyl acetate fraction (0.005, 0.008, and 0.01%); B, butanol fraction (0.01, 0.05, and 0.1%).

with respect of reduction of the elevated TNF-α. The amount of TNF-α secreted from the cells was decreased by more than 55% when 0.01% of ethylacetate fraction was treated. The butanol fraction similarly inhibited the TNF-α secretion but the inhibition activity was less than that of ethylacetate fraction. However, 80% ethanol extract did not have any suppression activity against TNF-α secretion (Fig. 5). These result shows that ethylacetate fraction and butanol fraction of olive leaf extract could be used for anti-inflammatory agent for alleviating skin inflammatory conditions and for anti-aging cosmeceuticals.

Anti-melanogenic effect Searching for the new phyto-chemicals from the natural sources that can effectively prevent the formation of pigment in skin become popular activities (28). Inflammation is one of the key factors that induce melanogenesis. Since anti-inflammatory effect of some fractions of olive leaf was seen, effects of olive leaf fractions on the melanin production from a melanoma cell line, B16-F1 was analyzed.

Firstly, cytotoxicity of each sample fraction on the B16-F1 cells was evaluated through MTT assay. Ethylacetate fraction of OLE has no cytotoxicity in the range of 0.005-0.01%. A slight cytotoxicity was observed at the concentration of 0.1% butanol fraction of OLE that caused 10% decrease in cell viability. 80% EtOH extract of olive leaf showed the significant cytotoxicity at the concentration over 0.03% (Fig. 6). In order to induce melanogenesis, 5 nM α-MSH was treated and the treatment stimulated melanin synthesis more than 3.5 folds. Koijic acid that has been used as a standard direct inhibitor of tyrosinase reduced melanin synthesis dose dependently (13). Both ethylacetate and butanol fractions showed significant inhibitory activities with dose dependent manner. The ethylacetate fraction had the strongest anti-melanogenic efficacy considering relative concentrations treated to cells. At 0.01% of ethylacetate fraction, more than 2.3 folds of reduction were observed (Fig. 7). In order to elucidate mechanisms behind the inhibition of melanin secretion by ethylacetate and butanol fraction of olive leaf, further studies are required. For example, *in vitro* tyrosinase inhibition assay, intracellular tyrosinase assay, tyrosinase zymography, and assays for other regulatory factors involved in melanogenesis.

Taken together, ethylacetate fraction of olive leaf seems to contain certain components responsible for anti-inflammatory efficacy and depigmenting activity as well. It seems that ethylacetate fraction has strong enough anti-inflammatory and whitening efficacies to be used as a new cosmeceutical.

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