ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 19:5 (2025) 596-610

records of natural products

Promising Natural Polyphenols from Olive (*Olea europaea*) Leaves and Seeds: Dual Benefits in the Prevention of Ultraviolet B-induced Fibroblast Skin Damage and Anti Skin Hyperpigmentation

Asih Gayatri[1], Fahd M. Abdelkarem[2], Yhiya Amen[1,3], Masako Matsumoto[1], Maki Nagata[1] and Kuniyoshi Shimizu[1*]

[1]*Department of Agro-Environmental Sciences, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 819-0395, Japan*

[2]*Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt*

[3]*Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt*

(Received June 18, 2025; Revised July 25, 2025; Accepted August 08, 2025)

Abstract: The development of phytochemicals from olive leaves and seeds for cosmeceutical applications is still scarce. This study aimed to perform comprehensive investigations of the bioactive compounds and biological activities of olive leaves and seeds for antioxidant, skin protection from ultraviolet B (UVB) irradiation in human dermal fibroblast (NHDF) cells from adult donors, and anti-melanogenesis in B16 melanoma cells. Two bioactive compounds were isolated from olive leaves, identified as oleuropein (**1**) and oleuroside (**2**). Additionally, one bioactive compound, pinoresinol (**3**) was elucidated for the first time from olive seeds. Among these compounds, pinoresinol demonstrated the most potent melanin inhibitory in B16 melanoma cells at the low concentration of 1.25 µg/mL with no cytotoxicity, decreasing the melanin content to 54 ± 5.48 %. In addition, oleuropein at 100 µg/mL inhibited melanin production to 35 ± 2.15 %, without cytotoxicity. Furthermore, oleuropein at concentrations of 100-200 µg/mL promoted cell proliferation above 120 % and exhibited skin protective activity at 200 µg/mL against UVB irradiation (250 mJ/cm^2), maintaining cell viability to 76.54 ± 5.05 %. This study highlights the isolated compounds from olive leaves and seeds, which are agricultural by-products, offer potential natural sunscreen products in the future with fewer side effects.

Keywords: Natural polyphenols; cytotoxicity assay; skin protectant; anti melanogenesis; cosmetic.

1. Introduction

The olive oil sector plays a significant role in the Mediterranean area from a social economy to a cultural perspective due to its beneficial effects. However, olive tree cultivation and oil extraction produce numerous by-products. The by-product during olive tree cultivation is estimated to generate 6.23 kg of pruning waste (branches and leaves) [1]. The largest contributor to waste generation are the olive leaves, an abundant and unavoidable byproduct. Olive (*Olea europea* L.) leaves represent approximately 4-7% of the total weight of olives arriving at any given olive mill, which are generally discarded, causing economic and environmental issues [2]. Leaves are also accumulated in large

*Corresponding author: E-Mail: shimizu.kuniyoshi.381@m.kyushu-u.ac.jp ; Phone:+81-92-8024946; *Fax:*+81-92-8024946

The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products September-October 2025 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.544.2506.3557
Available online: August 22, 2025

volumes around 300–750 kg/ha or 25 kg per olive tree on farms during the pruning of the olive trees, although the quantities may vary depending on culture conditions, tree age, production and/or local pruning practice [3]. Currently, the process of reducing leaf wastes is to burn them which has environmental impacts [4]. Therefore, olive leaves represent one of the main by-products derived from both olive tree cultivation and the olive-processing industry.

In addition to olive leaf wastes, a by-product from olive oil extraction (olive stone) is also becoming a concern in decreasing the waste. The olive stone is composed of two main parts:the endocarp (outer shell) and the seed inside. The endocarp (outer shell), which constitutes about 9–27 % of the olive fruit, serves as a protective layer surrounding the seed and is particularly rich in lignocellulosic materials, primarily lignin, cellulose, and hemicellulose [5]. Olive stones, particularly olive seeds, are rich in dietary fiber (47.6 %), lipids (30.4 %), proteins (13.5 %), and phenolic compounds (8.10 %) [6]. During the process of olive oil extraction, 100 kg of fresh olive fruits produce 22 kg of olive stone containing approximately 4 kg of seed wastes [7]. Until now, olive stones are used in small boilers in the own olive mills and in buildings around the world, and the reduction process of olive seed wastes is only to edible fungi production [8]. Olive stones present valuable opportunities in food and cosmetic industries, enhancing both nutritional value in bakery products and providing green cosmetic products. Meanwhile, olive leaf and seed wastes contain promising secondary metabolites that can be developed for pharmaceutical or cosmeceutical industries.

Rich in polyphenols, the main chemical compounds in olive leaves and seeds, it is a promising valorization system of valuable compounds [9]. Olive leaf extract has been utilized medicinally and marketed as an antioxidant, cardioprotective, immune stimulator, antibiotic, anti-inflammatory, and blood sugar-regulating agent [10]. In addition, olive seed extract from *Olea europaea* L., cultivated in Kyushu Island, Japan, possessed potential antioxidant properties [11]. Furthermore, olive seed extract shows a significant inhibitory effect on acetylcholinesterase, butyrylcholinesterase, and tyrosinase enzymes involved in neurodegenerative diseases [12]. At present, the use of bioactive compounds from olive seeds and leaves as cosmetics is not comparable to pharmaceuticals. Meantime, the demand for natural ingredients for cosmetics in sunscreen products has increased due to safety reasons. Therefore, the recovery of biophenolic components in the seeds and leaves from olive oil by-products for antioxidants, anti melanogenesis, and skin protectant from UVB are an innovative strategy to promote natural sunscreen product with fewer adverse reactions

Currently, skin cell damage mainly caused by overexposure to UVB irradiation which contains high-energy photons is a critical problem [13]. The harmful wavelength of UVB light can be absorbed by skin chromophores in the dermis layer [14]. The absorption of photons by skin chromophores in dermal fibroblast layer can upregulate ROS production and structural rearrangement of nucleotides, causing skin cancer [15]. Therefore, antioxidants as free radical scavengers and skin protection from UVB are the best treatment. Furthermore, anti melagonesis activity should be considered in commercial sunscreens. This is because the stimulated melanocytes (pheomelanin) by UVB will produce a high level of ROS ($O_2^{\cdot-}$, $^{\cdot}OH$, and H_2O_2), triggering melanin content production which causes the risk of pigmentation disorder [16-18].

In the present study, bioassay-guided isolation of ethyl acetate-soluble fraction from olive leaves led to the isolation of two bio-phenolic secoiridoid glycosides. Moreover, one phenolic lignan from the ethyl acetate fraction of olive seeds was successfully purified for the first time. The chemical structure of all isolated compounds was elucidated by 1D and 2D nuclear magnetic resonance (NMR) spectroscopic data and high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) and then compared with previously reported data. Furthermore, all isolated compounds were evaluated for antioxidant, cytotoxic activity, protective effect on UVB-exposed human dermal fibroblast (HDFs), and melanogenesis inhibitory on B16 melanoma cells for the development of natural and safe sunscreen products. In addition, the structure-activity relationship for secoiridoid glycosides and lignan compounds from olive leaves and seeds for the anti melanogenesis and skin protectant activities were discussed in this study for the first time.

2. Materials and Methods

2.1. Plant Material

This study used olive leaves and seeds of the Mission (*Olea europaea* 'Mission') cultivar, collected from Nagayo Olive Farm in Nagasaki (Latitude 32° 49' 30.68"N and Longitude, 129° 52' 30.16"E) Japan, in October 2021. The olive leaves were obtained during the routine tree pruning process, while the seeds were collected during the separation of olive fruit (used for olive oil extraction). Fresh samples voucher no. SFFPS0182023 were deposited at the Systematic Forest and Forest Products Sciences Laboratory, Faculty of Agriculture, Kyushu University, Japan. These samples were promptly frozen to preserve their integrity until further use.

2.2. Extraction and Isolation of Compounds

Fresh olive leaves were dried at room temperature for 10 days and then ground into a fine powder using a mixer. The resulting olive leaves powder (7 kg) was subjected to extraction four times with 100% methanol (MeOH, 20 L each) at room temperature, yielding a crude extract weighing 1359 g. Subsequently, 330 g of the crude extract was partitioned sequentially using *n*-hexane, dichloromethane (DCM), and ethyl acetate (EtOAc). This partitioning process yielded four main fractions *n*-hexane-soluble fraction (65.3 g), the DCM-soluble fraction (80.3 g), the EtOAc-soluble fraction (100.5 g), and the aqueous-soluble fraction (80.7 g).

These fractions were then further analyzed for their antioxidant activity. The EtOAc-soluble fraction (4 g) from olive leaf with the most potent antioxidant activity and skin photo protection was fractionated over Biotage® Sfär Silica HC 25 g column, equipped with a UV detector (254 and 280) nm. The fraction was eluted with a gradient system of *n*-hexane:dichloromethane:methanol (10:0:0, 5:5:0, 0:9:1, 0:8:2, 0:6:4, 0:4:6, 0:2:8, 0:0:10) *v/v/v* to obtain 3 fractions evaluated on HPLC analysis based on the similarity of chemical fingerprinting. Fraction 1 with the potent antioxidant activity were further fractionated by a pure C-850 medium pressure liquid chromatography (MPLC, Buchi, Switzerland). The MPLC was set with preparative mode and UV detection (220, 254, 280, and 320) nm, connected with reversed flash column (Inertsil ODS-P column 5 µm, 20 ×250 mm, GL Sciences, Tokyo, Japan). The flow rate was (10 mL/min) during the elution. The fraction was eluted with the same mobile phase water (solvent A) and methanol (solvent B) with a different gradient system. Fraction 1 (46.0 mg) was separated with the gradient elution of 6 min 10% B, 3 min 45% B, 4 min 55% B, 4 min 65% B, 4 min 70% B, 4 min 70% B, 3 min 75%B, 5 min 80%B, and 15 min 100%B to obtain compound **1** (18.6 mg) from sub-fraction 1-5 and compound **2** (7.1 mg) from sub-fraction 1-6.

Furthermore, the EtOAc-soluble fraction (9 g) from olive seeds with the great antioxidant activity, was fractionated over Biotage® Sfär Silica HC 100 g column, equipped with a UV detector (254 and 280) nm and eluted with a gradient system of dichloromethane : ethyl acetate (7:3 to 0:10) *v/v* to obtain 5 fractions. Fraction 1 (91.8 mg) with the most active antioxidant activity was separated with the gradient elution of H_2O: MeOH (80:20) to (0.0:100) to obtain compound **3** (20 mg) from sub-fraction 1-3.

The chemical structure of isolated compounds was elucidated by 1D, 2D NMR, and HR-ESI-MS. HR-ESI-MS was measured using a quadrupole time-of-flight (QTOF) mass spectrometry (Agilent (Technologies, Santa Clara, CA, USA) equipped with a dual ESI source for the simultaneous mass reference spraying to calibrate the observed *m/z* values). 1D (^{1}H and ^{13}C NMR) and 2D NMR (HSQC, HMBC, COSY) experiments used A JNM-ECS 400 and 600 spectrometer (JEOL, Tokyo, Japan) and CD_3OD was used for the solvent. The purity of isolated compounds was checked using HPLC (Agilent 1260-LC system (Agilent Technologies)) equipped with a vacuum degasser, an autosampler, binary pump and DAD detector (Agilent Technologies) using a Inertsil ODS-3 column (150 × 4.6 mm, 5 µm). Mobile phase A consisted of H_2O and mobile phase B contained MeOH. The gradient program was as follows: 0 %-100 % B (0.00-22.00 min), 100 % B (22.00-27.00 min), % B was decreased into 5 % in 3 min. Elution was carried out with flow rate of 0.75 mL/min.

2.3. Antioxidant Activity Test

The scavenging activity of free radical DPPH· in extracts, fractions, and isolated compounds was determined using a microplate reader (Biotek, Winooski, VT, USA). In a 96-well plate, 50 µL of samples (12.5-200 µg/mL) solution was mixed with 80 µL of DPPH (0.05 mM) radical solution in methanol. Ascorbic acid (3.2-50 µg/mL) was used as the positive control or reference to compare the antioxidant activity. The mixtures were incubated for 30 min in a dark room. Furthermore, the absorbance was measured at a wavelength of 570 nm.

2.4. MTT Assay on Normal Human Dermal Fibroblast Cells

Normal human dermal fibroblast (NHDF)-Adult cells (PromoCell, C-12302, North America) with the passage number 5-10 were seeded into a 96-well plate at a density of 1 x 10^5 cells/mL and incubated for 24 h at 37 °C with 5% CO_2. The EtOAc-soluble fraction, DCM-soluble fraction, the isolated compounds from olive leaves, galic acid (as positive control) at various concentrations, and DMSO (as negative control) were added into a serum-free medium, and incubated for a further 24 h. The cells were then washed with PBS (200 µL) and added to the fresh serum-free medium (100 µL). After that, 20 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent was added to each well and incubated for 4 h. Finally, the supernatant was replaced by 100 µL of isoprophyl alcohol (containing 40 mM HCl) to dissolve formazan crystals. Measurement was taken at 570 nm using a microplate reader (Biotek, Winooski, VT, USA)

2.5. Skin Protection Assay from UVB Irradiation

The skin protection evaluation from UVB of EtOAc, DCM-soluble fractions and the isolated compounds was assessed in NHDF-AD cells. The cells 1 x 10^5 cells/mL with the passage number 5-10 were seeded in 96-well and incubated for 24 h at 37°C under 5% CO_2. After incubation, the tested DCM-soluble fraction, EtOAc-soluble fraction, isolated compounds from olive leaves at various concentrations and DMSO (as the negative control) were added to the fresh serum-free medium in 96-well plates and incubated for a further 5 h under 5% CO_2. The cells were then washed with 200 µL of PBS. Subsequently, 100 µL of PBS was added to each well and the cells were irradiated with UVB light at a dose of 250 mJ/cm^2 for 1 min. The UVB dose given is based on the optimization result of previously conducted experiments with slight modification [19]. UVB irradiation was performed with Bio-Link (Vilber Lourmat, Torcy, France). The supernatant was removed and fresh serum-free medium (100 µL) was added to each well incubated for an additional 24 h as well. After the incubation, an MTT assay was performed as above to assess cell viability.

2.6. Cell Viability Assay and Determination of Melanin Content in B16 Melanoma Cells

The B16 melanoma cells (RIKEN BRC, RCB0557, Ibaraki, Japan) with the cell density of (1 x 10^5 cells/mL) and passage number of 5-10 were placed in a 96-well plate and adhered at 37 °C under 5% CO_2 for 24 h. Samples were added to the cells followed by incubation for 72 h. The measurement of cellular melanin content was conducted according to [20] with slight modification. The cells were washed with PBS following lysis in 200 µL of 1 M NaOH by heating at 100 °C for 30 min to solubilize the melanin. The resulting lysate (intracellular melanin content) was read in the absorbance at 405 nm using a microplate reader. The melanin production activity is expressed as a percentage of the control cells treated with DMSO. After that, the cell viability assay was performed using MTT, same as above.

2.7. Statistical Analysis

All biological assays were performed in three independent biological and technical replication. The data are presented as mean ± SD. Student's *t-test* was used for statistical analysis. Values of $p < 0.05$ were considered significant at a 95% confidence interval.

3. Results and Discussion

3.1. Assessment of Antioxidant Activity of Olive Leaf Extracts

The olive leaves were extracted with 100% MeOH and partitioned into *n*-hexane, DCM, and EtOAc, respectively. Then, each extract was assessed for its antioxidant activity by measuring its potential to inhibit free radical DPPH· (Table 1). This assay proposed to screen the promising extract for isolating the secondary metabolites in olive leaves as skin protectant from UVB irradiation in NHDF cells and anti melanogenesis in B16 melanoma cells. According to the result, the EtOAc-soluble fraction exhibited the most potent radical scavenging against DPPH with the IC_{50} value of 63.50 ± 1.70 µg/mL. The DCM-soluble fraction showed moderate antioxidant activity with the IC_{50} value of 159.92 ± 2.00 µg/mL. Meanwhile, crude extract, *n*-hexane, and aqueous-soluble fractions, compared with EtOAc-soluble fraction, did not exhibit antioxidant activity with the IC_{50} > 200 µg/mL (Table 1). Therefore, the EtOAc and DCM-soluble fractions from olive leaves were further evaluated against UVB-induced fibroblast skin damage and melanogenesis inhibitory activity.

Table 1. Antioxidant activity of the crude extracts and fractions of olive leaves and seeds in comparison with ascorbic acid

Samples	DPPH (IC_{50}, µg/mL)
Olive Leaves	
Crude Extract	354.68 ± 3.70
n-hexane-soluble fraction	333.29 ± 6.80
DCM-soluble fraction	159.92 ± 1.90
EtOAc-soluble fraction	63.50 ± 1.70
Aqueous-soluble fraction	284.39 ± 4.90
Fraction 1	68.37 ± 0.93
Fraction 2	164.38 ± 12.99
Fraction 3	Not active
Olive Seeds	
EtOAc-soluble fraction	137.70 ± 3.50
Fraction 1	30.75 ± 1.60
Ascorbic acid	2.70 ± 1.51

The results are shown as mean ± SD (n=3).

3.2. Evaluation of Ethyl Acetate and Dichloromethane-Soluble Fractions for Skin Protectant from UVB Irradiation and Anti Melanogenesis

The EtOAc and DCM-soluble fractions from olive leaves with promising antioxidant activity were examined against skin protectant on NHDF cells and anti melanogenesis on B16 melanoma cells. The EtOAc-soluble fraction was confirmed to possess cell growth stimulating activity above 120 % at 50-400 µg/mL on NHDF cells before UVB irradiation and skin protection after exposure to UVB at a given concentration of 400 µg/mL (Figure 1). The treatment of EtOAc-soluble fraction from olive leaves at 400 µg/mL on UVB-irradiated fibroblast cells with the dose of 250 mJ/cm^2 was capable of maintaining the cell viability until 94.28 ± 14.79 %, compared to UVB control. In another research, the capability of olive leaf extract to inhibit UVB-induced skin damage on HaCaT cells was reported [21]. In the study, the effect of ethanol (EtOH) extract from olive leaves indicated that the given treatment of EtOH extract at 500 µg/mL after UVB irradiation (687.6 mJ/cm^2) with the cell density of 1 × 10^4 cells/well maintain cell viability more than 80% [21]. Meanwhile, in our study, the protection of fibroblast skin damage from UVB irradiation (250 mJ/cm^2) on NHDF cells with the cell density of 1 × 10^5 cells/well using EtOAc-soluble fraction at 400 µg/mL maintained the cell viability up to 94.28 ±

14.79 %. Based on the result of our study, the treatment with EtOAc-soluble fraction was able to cover the deeper layer of skin (dermis) effectively.

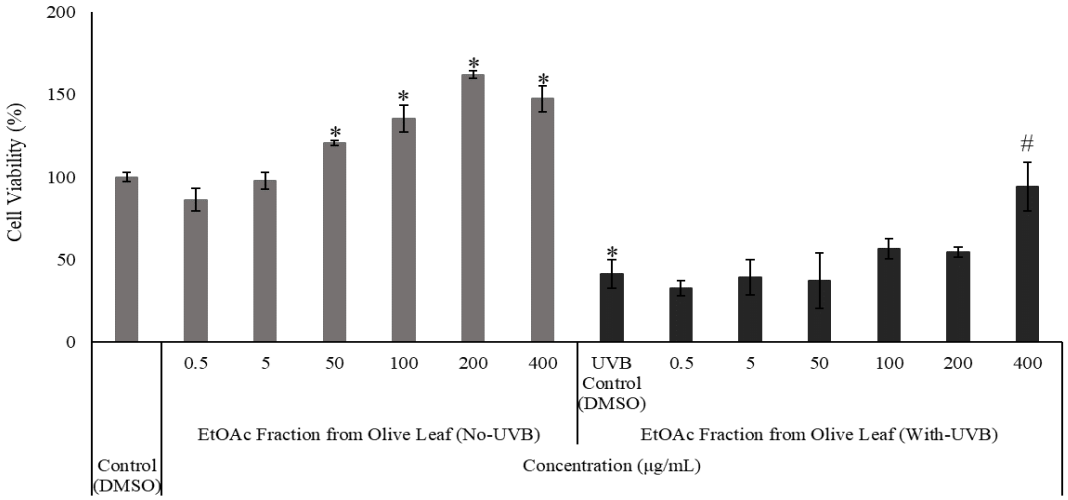

Figure 1. Cell viability evaluation of NHDF cells treated with EtOAc fraction (olive leaf) with no-UVB irradiation and irradiated with UVB. EtOAc fraction (olive leaf); $*p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with UVB control. The results are shown as mean ± SD (n=3)

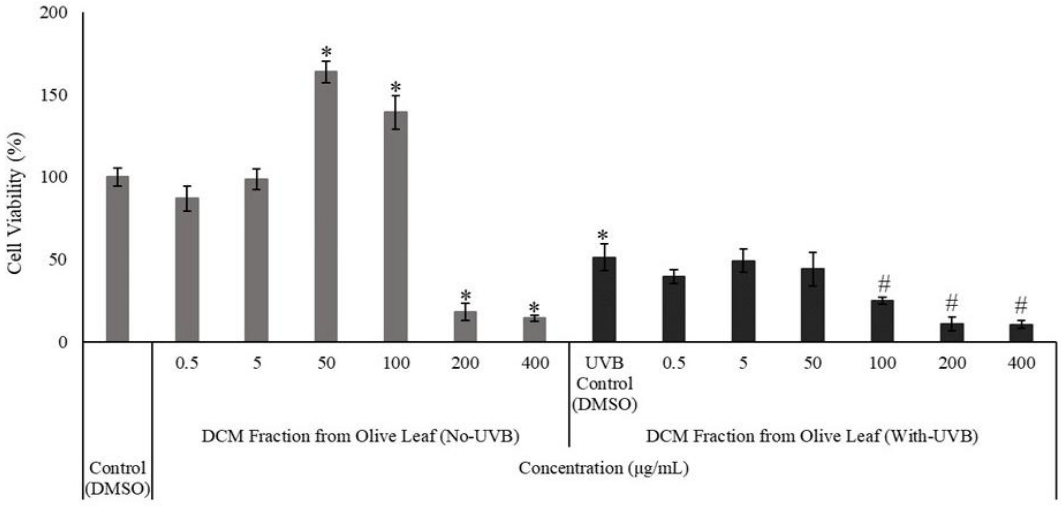

Figure 2. Cell viability evaluation of NHDF cells treated with DCM fraction with no-UVB irradiation and irradiated with UVB. DCM; $*p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with UVB control. The results are shown as mean ± SD (n=3)

The DCM-soluble fraction showed the stimulation of cell proliferation above 130% at the given treatments of 50-100 µg/mL. However, the DCM-soluble fraction did not prevent UVB-induced fibroblast cell death (below 50 %) in a dose-dependent manner up to 400 µg/mL (Figure 2). Therefore, the EtOAc-soluble fraction from olive leaves was selected to assay anti hyperpigmentation activity on B16 melanoma cells. The EtOAc-soluble fraction was applied to these cells at the concentrations of 1.25-100 µg/mL to determine cytotoxic effect and inhibition of melanin formation **(**Figure 3A & B). The intracellular melanin production decreased significantly to 44.18 ± 2.05 % against control in the presence of concentration at 100 µg/mL without cytotoxicity. In conclusion, at the same time, the given concentration (100 µg/mL) of EtOAc-soluble fraction from olive leaves increased the cell viability and

decreased the melanin content **(**Figure 3A & B). According to the antioxidant, skin protectant from UVB, and anti melanogenesis evaluations, the EtOAc-soluble fraction from olive leaves was selected to isolate the secondary metabolites for developing sunscreen products.

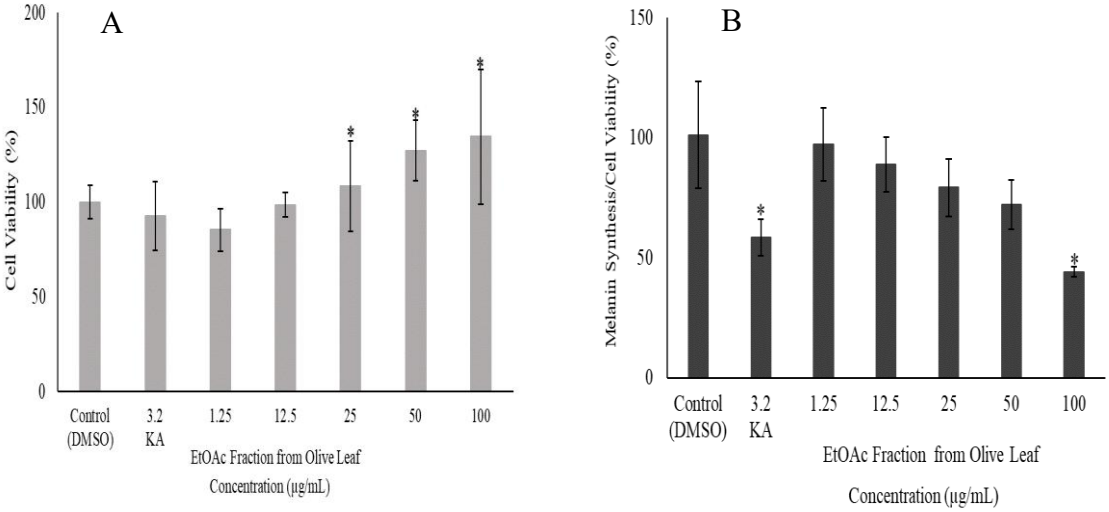

Figure 3. Cell viability evaluation in B16 melanoma cells treated with EtOAc fraction (olive leaf) (A). Melanogenesis inhibitory (B). EtOAc fraction (olive leaf); *$p < 0.05$ compared with control. The results are shown as mean ± SD (n=3). KA: Kojic acid (positive control)

3.3. Structural Elucidation of the Isolated Compounds from Ethyl Acetate-Soluble Fraction of Olive Leaves

Bioassay-guided purification of the EtOAc-soluble fraction from olive leaves led to isolation of two phenolic secoiridoid glycosides, namely oleuropein (**1**) and oleuroside (**2**) (Figure 4). Furthermore, one phenolic lignan, pinoresinol (**3**), was successfully isolated for the first time from olive seeds. Then, their structures were elucidated based on 1D, 2D NMR, HR-ESI-MS (Table S1 and Figures S1-S14).

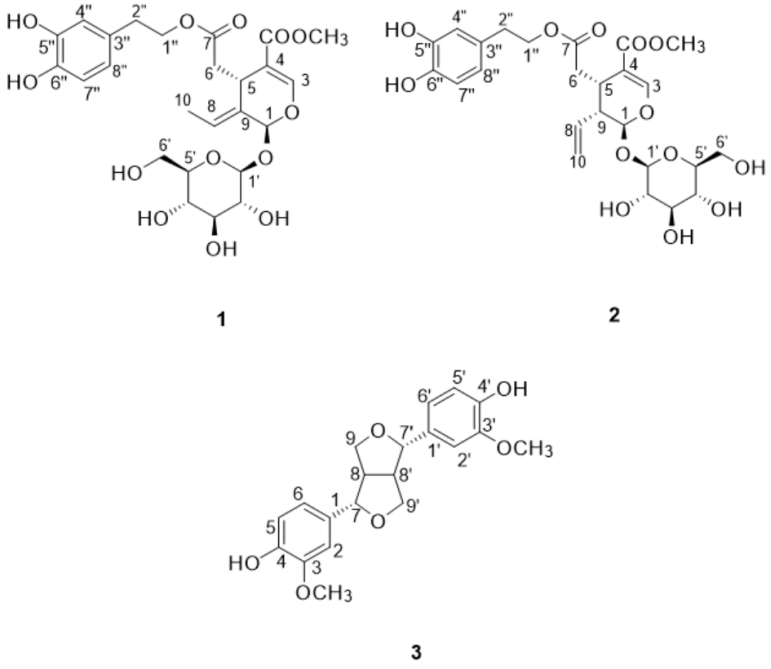

Figure 4. Chemical structure of isolated compounds oleuropein (**1**), oleuroside (**2**), and pinoresinol (**3**) from EtOAc-soluble fraction of olive leaves and seeds

The other constituents of olive seed that have been elucidated are class of secoiridoid glycosides, including nuzhenide, nuzhenide 11-methyl oleoside, and methoxynuzhenide [22]. Moreover, nuzhenide is the most concentrated phenolic in the seeds and not distributed in other parts of the olive [23]. Meanwhile, tyrosol, hydroxytyrosol, catechol, rutin, oleuropein, luteolin, quercetin, kaempferol, apigenin, ligstroside, dimethyloleuropein, oleuroside, contain in olive leaves [24]. Vomifoliol has been also isolated for the first time from olive leaves in the *oleaceae* family [25]. The most abundant compound in olive leaves is oleuropein, followed by hydroxytyrosol, the flavone-7-glucosides of luteolin and apigenin and verbascoside [26,27]. Olive leaves also contain the triterpenic compounds of erythrodiol, uvaol, oleanolic acid, betulinic acid, ursolic acid, maslinic acid, and 18β-glycyrrhetinic acid with the oleanolic acid being the prevailing component [28]. In addition, olive leaves contain bioactive compound of alpha-tocopherols [29], and rich in carotenoids and chlorophyll [30].

3.4. The Bioactivity of Isolated Compounds Against Skin Protection from UVB Irradiation

The isolated compounds **1** and **2** from olive leaves with promising antioxidant activity against free radical scavengers were evaluated for the protection on UVB-induced fibroblast cell death. The UVB intensity of 250 mJ/cm^2 was selected for this assay because it can maintain cell viability at 52.30 ± 10.66 % as the UVB control. Meanwhile, gallic acid (GA) was used as a positive control for this assay. Gallic acid protected the UVB-irradiated fibroblast cell damage at a dose of 25 µg/mL, without notable cytotoxicity. In addition, GA at the concentration of 25 µg/mL maintained the cell viability up to 73.98 ± 4.17 % after UVB irradiation (Figure 5).

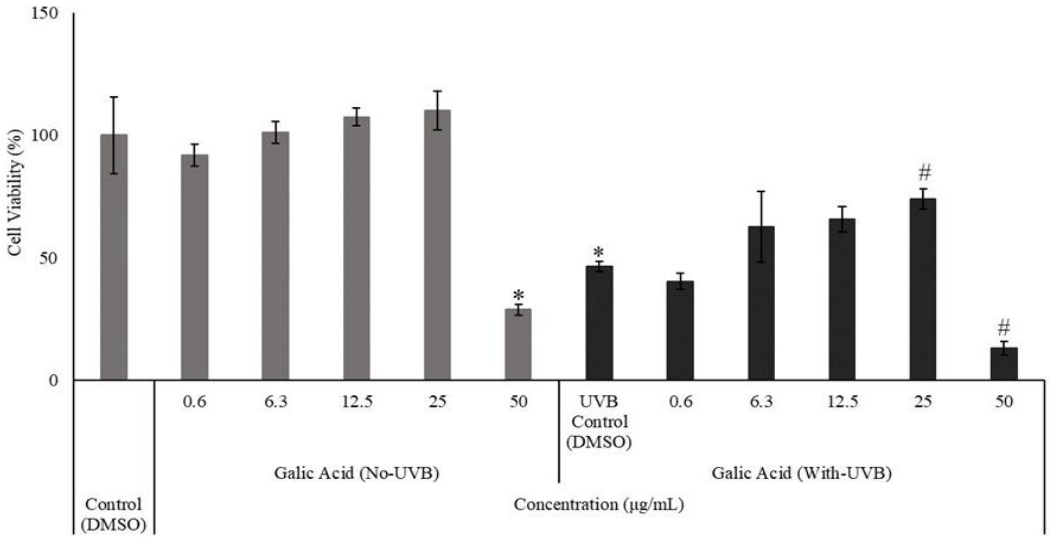

Figure 5. Cell viability evaluation of NHDF cells treated with galic acid with no-UVB irradiation and irradiated with UVB. Galic acid; $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with UVB control. The results are shown as mean ± SD (n=3)

Subsequently, the previous study noted that oleuropein has been able to prevent human fibroblast (Hs68) cell damage more than 50% compared to control under UVA exposure with concentrations of 5-25 µg/mL [31]. In our study, oleuropein was evaluated in response to UVB irradiation, which is more energetic ultraviolet and little penetrate to dermal layer. This study should bring a good ability to prevent fibroblast skin damage. The isolated compound **1** (oleuropein) at the treatment condition of 100-200 µg/mL exhibited cell growth stimulating activity above 120 % and was able to significantly maintain the cell viability of 76.54 ± 5.05 % at 200 µg/mL in UVB-irradiated fibroblast skin, compared with UVB control (Figure 6). The capability of compound **1** as protection against UVB irradiation in fibroblast cells was confirmed with the antioxidant properties against DDPH free radical (IC_{50} 35.48 ± 2.68 µg/mL). Furthermore, compared with gallic acid as a positive control for skin photo protection

(Figure 5), oleuropein (**1**) at high concentrations generated cell proliferation ability which can be applied for wound healing activities in the future. The presence of a sugar moiety in oleuropein at high concentrations (100-200 µg/mL) suggested its capability to enter NHDF cells. The faster passage of oleuropein through the membrane cells should indicate the more rapid activation of specific gene related to the growth of cells especially in transforming growth factor-beta (TGF-β). Therefore, the oleuropein could accelerate the regulation of proliferative cells at high concentrations. This statement is supported by another study targeting the TGF-β pathway using the polyphenols of *Echinacea purpurea* (L.) Moench to enhance cell proliferation for wound healing in a rat model [32]. It was discussed that the existence of phenylethanoid glycosides in *Echinacea purpurea* (L.) Moench flower enhances the wound healing via expression of the TGF-β gene with the use of aqueous extract (AE) at 5% and 10% w/w. The given treatments of 5% and 10% w/w from AE succeeded in increasing the rate of wound-healing over 9 days of the treatment [32].

Moreover, compound **1** suggested to have potent antioxidant activity and skin protection from UVB exposure due to the presence of two hydroxyl substituents in the hydroxytyrosol ring. The hydrogen atom of hydroxyl groups in the hydroxytyrosol moiety would donate to a free radical through homolytic O-H bond cleavage and form the phenoxy radical. The presence of phenoxy radical will be stabilized through electron delocalization on the aromatic structure (through π-delocalization) [33]. Furthermore, in another study, the protective ability of oleuropein (**1**) was analyzed using UVB absorption mechanisms. The existence of a hydroxytyrosol ring in oleuropein confirms the assignment of electron transitions from π → π*, involving HOMO → LUMO and HOMO-1 → LUMO [34]. Therefore, the potential of oleuropein for skin photoprotective against UVB was confirmed by its radical scavenger activity and UVB absorption. Furthermore, the promising oleuropein for skin photoprotection against UVB irradiation has been further evaluated at the molecular study level. In another study, olive leaf extract (300 and 1000 mg/kg) and oleuropein (10 and 25 mg/kg) were administered orally twice daily every day for 30 weeks in male hairless mice skin (5 week old) [35]. After the treatment, both olive leaf extract and oleuropein downregulate gene expressions (MMP-2, MMP-9, and MMP-13) related to collagen breakdown in the skin of hairless mice [35].

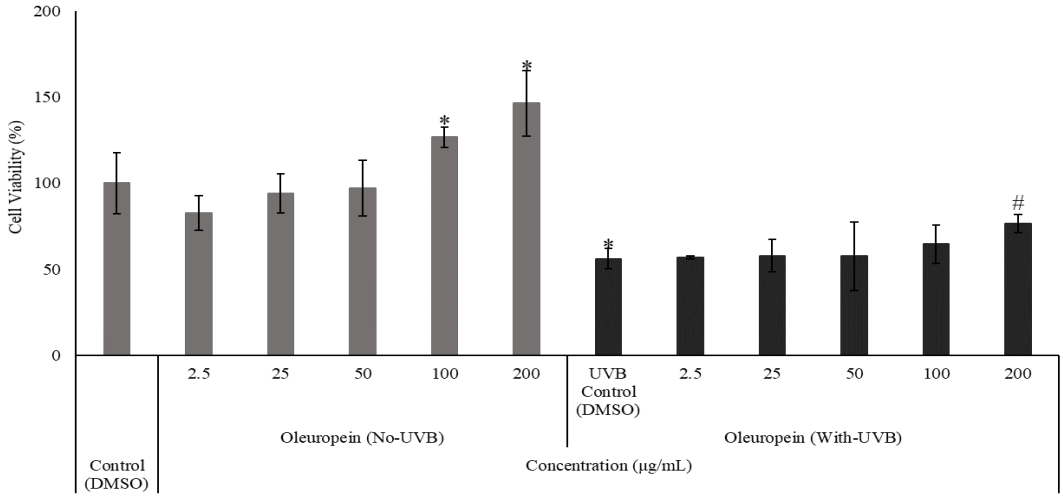

Figure 6. Cell viability evaluation of NHDF cells treated with oleuropein with no-UVB irradiation and irradiated with UVB. Oleuropein; $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with UVB control. The results are shown as mean ± SD (n=3)

Compound **2** (oleuroside) showed a significant cytotoxic effect at the concentrations of 0.6-50 µg/mL and no skin protection activity against UVB irradiation (Figure 7). At these tested concentrations, it was observed that the cell viability decreased to below 80%. Though the compound **2** could scavenge DPPH free radical with the IC_{50} value of 41.28 ± 1.17 µg/mL, it did not protect fibroblast cells-damage against UVB irradiation due to the slight cytotoxic effect. This phenomenon suggests that the shifting position of the double bond in C-8/C-10 of the compound **2** affected the cell viability (Figure 4).

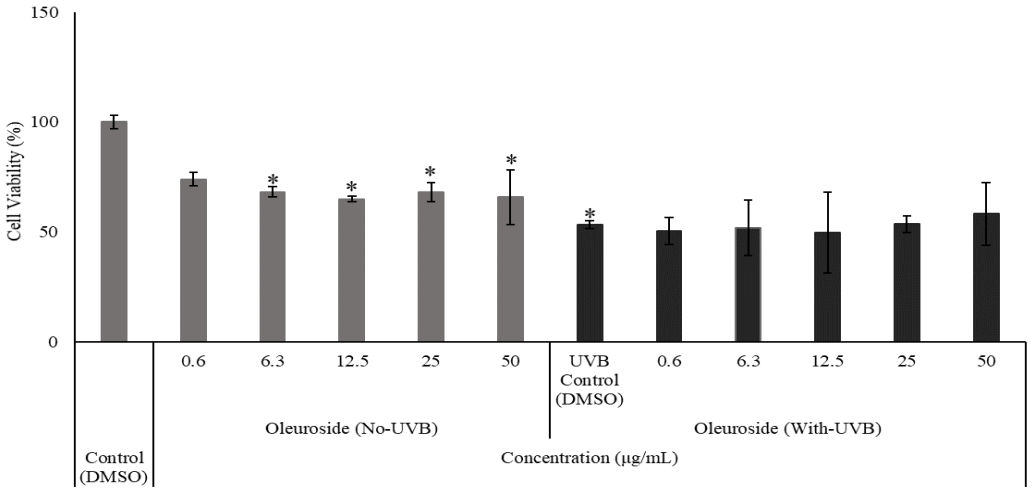

Figure 7. Cell viability evaluation of NHDF cells treated with oleuroside with no-UVB irradiation and irradiated with UVB. Oleuroside; $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with UVB control. The results are shown as mean ± SD (n=3)

3.5. Anti Melanogenesis Activity of Isolated Compounds from Olive Leaves and Seeds

The isolated compounds **1** and **2** from olive leaves with promising antioxidant and skin protectant as well as one purified compound from olive seeds were examined for melanin inhibitory effect in B16 cells. Among the tested compounds, the compound **3** (pinoresinol) isolated from the EtOAc-soluble fraction of olive seeds was the most potent antimelanogenic activity. The potential anti skin hyperpigmentation of the pinoresinol was also confirmed by its EtOAc-soluble fraction from olive seeds. The EtOAc-soluble fraction from olive seeds at given treatments of 25-50 µg/mL in B16 melanoma cells diminished the melanin content production below 75% against the negative control, without cytotoxicity (Figure 8A & B).

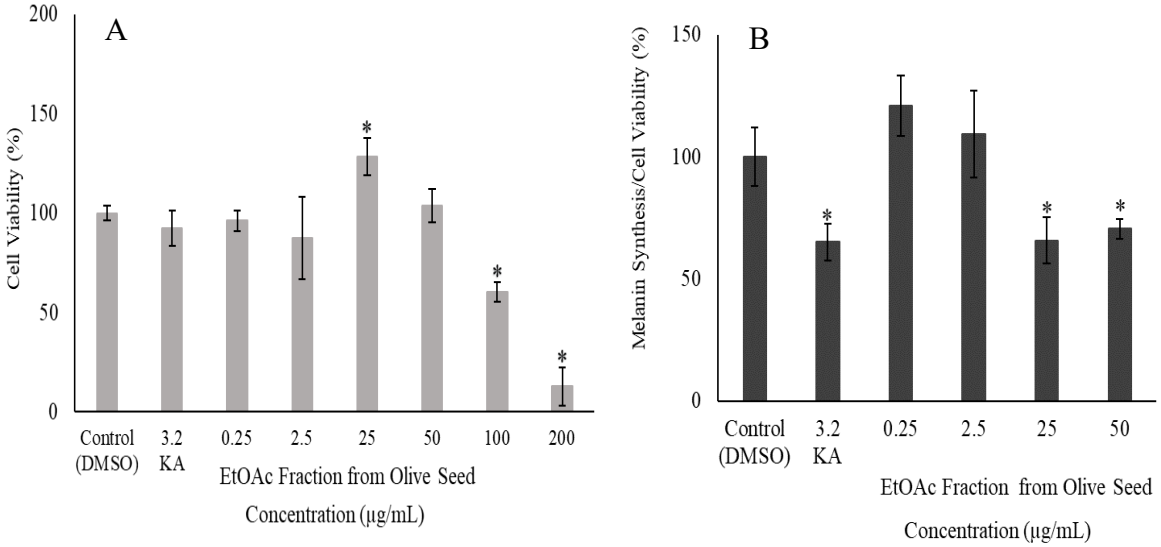

Figure 8. Cell viability evaluation in B16 melanoma cells treated with EtOAc fraction (olive seed) (A). Melanogenesis inhibitory (B). EtOAc fraction (olive seed); $^{*}p < 0.05$ compared with control. The results are shown as mean ± SD (n=3). KA: Kojic acid (positive control)

In addition, pinoresinol at a low concentration of 1.25 µg/mL reduced significantly the intracellular melanin formation to 54.01 ± 5.48 % against the negative control, without cytotoxic effect (Figure 9A & B). Compared to kojic acid as the positive control, pinoresinol was a greater melanogenesis inhibitor. The intracellular melanin formation downregulated to 70.41 ± 6.57 % against the negative control when treated with kojic acid at 3.2 µg/mL. The key factor of pinoresinol exhibited potent anti melanogeneisis activity should be due to the presence of methoxyl and hydroxyl groups in phenyl rings. This explanation was coherence with a reported study [36]. The 4-hydroxy-3-methoxyl group on the β-phenyl ring of cinnamamide derivatives shows more potent inhibitory activity of tyrosinase enzyme (19.33 ± 1.36) than kojic acid (20.57 ± 2.02 %).

However, the treatment of EtOAc-soluble fraction (200 µg/mL) in B16 cells showed a significant cytotoxic effect, decreasing the cell viability up to 12.81 ± 9.65 % (Figure 8A). In addition, the given dose of 100 µg/mL of the compound **3** in B16 cells resulted in a noticeable reduction of cell viability to 14.00 ± 2.63 % (Figure 9A).

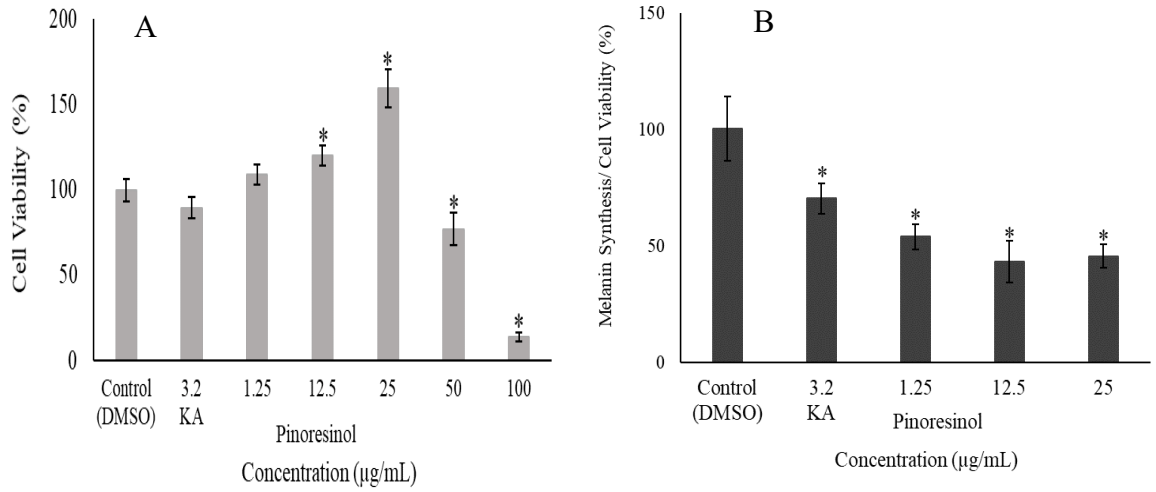

Figure 9. Cell viability evaluation in B16 melanoma cells treated with pinoresinol (A). Melanogenesis inhibitory (B). Pinoresinol; *$p < 0.05$ compared with control. The results are shown as mean ± SD (n=3). KA: Kojic acid (positive control)

Subsequently, oleuropein (**1**) with 100 µg/mL, purified from olive leaves, exhibited significantly active proliferation, and the melanin production process was also inhibited. This compound at the 100 µg/mL inhibited strongly the melanin formation in B16 cells to 35.16 ± 2.15 %, without cytotoxicity (Figure 10A & B). In previous study [37], oleuropein is confirmed to be melanogenesis inhibitory by the downregulation of MITF expression. Microphthalmia-associated transcription factor (MITF) is a gene related to the inducer of melanin enzymes (tyrosinase, TRP-1, and TRP-2), promoting melanogenesis. The study found that the given oleuropein concentrations of 100-200 µM inhibites MITF secretion [37]. Furthermore, the presence of two hydroxyl groups in phenyl rings of oleuropein contributed to the decrease of melanin formation in B16 melanoma cells. This phenomena confirms with another study on the structure-activity relationship from (2-[2-methyl-5-(propane-2-yl)phenoxy]-2-oxoethyl(2E)-3-(2,4dihydroxyphenyl)prop-2-enoate) [38]. The structure inhibits enzyme related melanin production (tyrosinase) due to substitution pattern of hydroxyl groups at phenyl ring [38]. Meanwhile, compound **2** (oleuroside) at the highest concentration (50 µg/mL), compared to compound **1** (oleuropein), did not show melanin inhibition in B16 cells (Figure 11A & B). This result suggested that methyl did not appear in the C-10 due to the shifting of double position C-8/C-10 in the compound **2** (Figure 4), causing no melanogenesis inhibitory in the B16 cells.

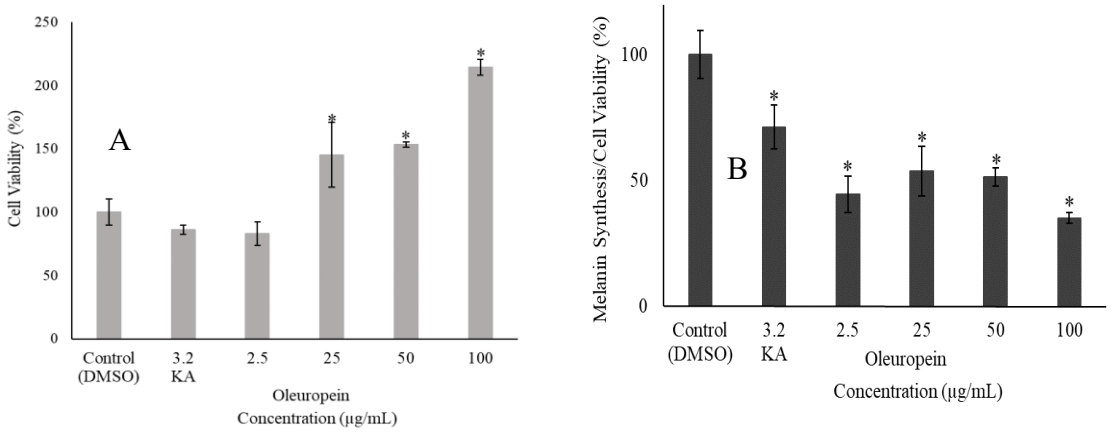

Figure 10. Cell viability evaluation in B16 melanoma cells treated with oleuropein (A). Melanogenesis inhibitory (B). Oleuropein; **p* < 0.05 compared with control. The results are shown as mean ± SD (n=3). KA: Kojic acid (positive control)

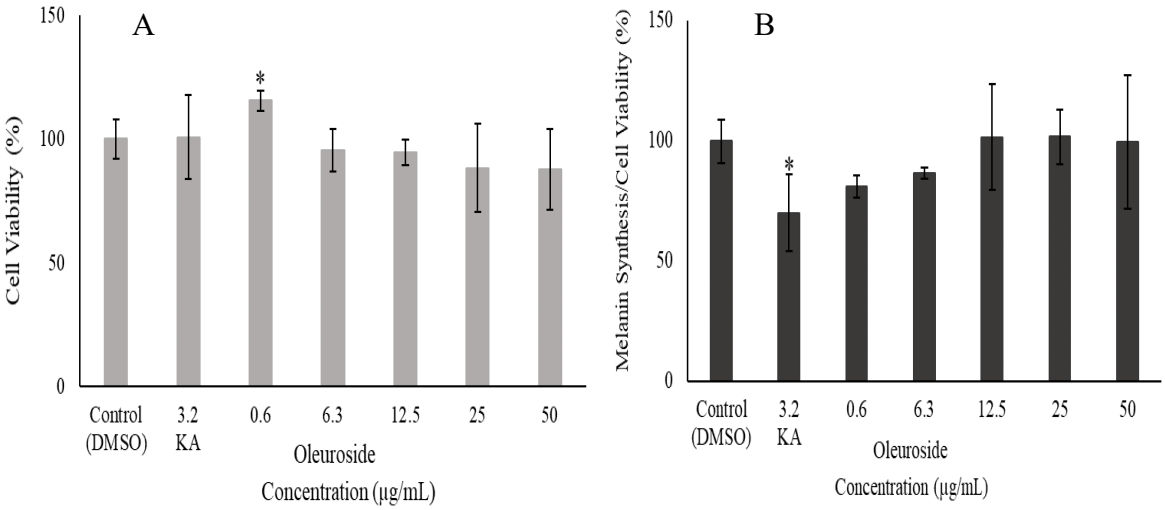

Figure 11. Cell viability evaluation in B16 melanoma cells treated with oleuroside (A). Melanogenesis inhibitory (B). Oleuroside; **p* < 0.05 compared with control. The results are shown as mean ± SD (n=3). KA: Kojic acid (positive control)

4. Conclusion

In conclusion, antioxidant-guided isolation of the EtOAc-soluble fraction from the olive leaves resulted in two phenolic secoiridoid glycosides. They were identified as oleuropein (**1**) and oleuroside (**2**). The purification of the EtOAc-soluble fraction from the olive seeds successfully reported pinoresinol (**3**) for the first time, highlighting olive seeds as a previously unrecognized source. Pinoresinol was the most effective in diminishing the melanin content formation in B16 melanoma cells, compared to kojic acid as positive control. Moreover, the isolated compounds of oleuropein from olive leaves demonstrated cell growth-stimulating activity and skin protection against UVB irradiation in NHDF cells. The identified bioactive compounds in olive leaves and seeds, which are agricultural by-products, can be utilized to produce natural sunscreen products in the cosmeceutical industry with higher activity and less side effects for the human and environment.

Acknowledgments

The Faculty of Agriculture scholarship, Kyushu University, is acknowledged for the scholarship to Asih Gayatri. Our gratitude to the Institute for Materials Chemistry and Engineering, Kyushu University, Japan, for carrying out the NMR measurements. The authors would like to express their gratitude to Mrs. Hidaka Ayako to carry out HR-MS measurements. Our gratitude to Ms. Michelle Anastasha Tomara to prepare the crude extract of olive leaves and Mr. Mitsusaki, Mr. Kawaguchi, and Mr. Takeda to provide olive seed and leaf powders to support the research.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

Conflict of Interest

The authors declare that there is no conflict of interest

ORCID
Asih Gayatri: 0009-0001-4289-0131
Fahd M. Abdelkarem: 0000-0002-6388-0821
Yhiya Amen: 0000-0003-2552-4567
Masako Matsumoto: 0000-0002-8470-0471
Maki Nagata: 0000-0001-5157-562X
Kuniyoshi Shimizu: 0000-0001-5960-1503

References

[1] M. E. Martínez-Navarro, J. Escobar-Talavera, C. Cebrián-Tarancón, S. Llorens, G. L. Alonso and M. R. Salinas (2024). Gastrointestinal digestion of olive leaf waste from olive mill production chain in a simulator of the gastrointestinal tract, *J. Sci. Food. Agric.* **104**, 7550–7556.

[2] E. Ruiz, J. M. Romero-García, I. Romero, P. Manzanares, M. José, J. M. Negro and E. Castro (2017). Olive-derived biomass as a source of energy and chemicals, *Biofuel. Bioprod. Biorefining-Biofpr*. **11**, 1077-1094.

[3] M. L. Clodoveo, P. Crupi, A. Annunziato and F. Corbo (2022). Innovative extraction technologies for development of functional ingredients based on polyphenols from olive leaves, *Foods* **11**, 103.

[4] M. Krzywonos, G. Difonzo and A. Pasqualone (2025). Challenges and technological requirements in agri-food waste upcycling : The case study of olive leaf extract, *Future Foods*. **11**, 100547

[5] J. F. García Martín, M. Cuevas, C. H. Feng, P. Alvarez-Mateos, M. Torres-García and S. Sánchez (2020). Energetic valorisation of olive biomass: Olive-tree pruning, olive stones and pomaces, *Processes* **8**, 511.

[6] M. E. Fessikh, H. Elhrech, A. Yahyaoui, E. Idrissi, L. H. Lee, W. A. Abdulmonem, N. E. Omari and A. Bouyahya (2025). Sustainable valorization of olive stone by-products: Opportunities and challenges, *J. Food Comp. Anal.* **142**, 107495.

[7] G. Rodríguez, A. Lama, R. Rodríguez, A. Jiménez, R. Guillén and J. Fernández-Bolaños (2008). Olive stone an attractive source of bioactive and valuable compounds, *Bioresour. Technol.* **99**, 5261–5269.

[8] G. Koutrotsios, M. Patsou, E. K. Mitsou, G. Bekiaris, M. Kotsou, P. A. Tarantilis, V. Pletsa, A. Kyriacou and G. Zervakis (2019). Valorization of olive by-products as substrates for the cultivation of ganoderma lucidum and pleurotus ostreatus mushrooms with enhanced functional and prebiotic properties, *Catalysts* **9**, 537.

[9] E. M. Kabbash, Z. T. Abdel-Shakour, S. H. El-Ahmady, M. Wink and I. M. Ayoub (2023). Comparative metabolic profiling of olive leaf extracts from twelve different cultivars collected in both fruiting and flowering seasons, *Sci. Rep.* **13**, 612.

[10] S. Selim, M. Albqmi, M. M. Al-Sanea, T. S. Alnusaire, M. S. Almuhayawi, H. A. Elgawad, S. K. Al Jaouni, A. Elkelish, S. Hussein, M. Warrad and M. T. El-Saadony (2022). Valorizing the usage of olive leaves, bioactive compounds, biological activities, and food applications: A comprehensive review, *Front. Nutr*. **9**, 1008349

[11] A. Kishikawa, A. Ashour, Q. Zhu, M. Yasuda, H. Ishikawa and K. Shimizu (2015). Multiple biological effects of olive oil by-products such as leaves, stems, flowers, olive milled waste, fruit pulp, and seeds of the olive plant on skin, *Phytother. Res.* **29**, 877–88

[12] M. F. A. Rahman, E. Elhawary, A. M. Hafez, E. Capanoglu, Y. Fang and M. A. Farag (2024). How does olive seed chemistry, health benefits and action mechanisms compare to its fruit oil? A comprehensive review for valorization purposes and maximizing its health benefits, *Food Biosci*. **59**, 104017

[13] R. R. Sharma, A. Deep and S. T. Abdullah (2022). Herbal products as skincare therapeutic agents against ultraviolet irradiation-induced skin disorders, *J. Ayurveda Integr. Med.* **13**, 100500.

[14] R. Daré, R. G., C. V. Nakamura, V. F. Ximenes and S. O. S Lautenschlager (2020). Tannic acid, a promising anti-photoaging agent: Evidences of its antioxidant and anti-wrinkle potentials, and its ability to prevent photodamage and MMP-1 expression in L929 fibroblasts exposed to UVB, *Free Radic. Biol. Med*. **160**, 342–355.

[15] M. Cavinato and P. Jansen-Dürr (2017). Molecular mechanisms of UVB-induced senescence of dermal fibroblasts and its relevance for photoaging of the human skin, *Exp. Gerontol*. **94**, 78–82.

[16] A. Mariano, I. Bigioni, A. Scotto d'Abusco, A. Baseggio Conrado, S. Maina, A. Francioso, L. Mosca and M. Fontana (2021). Pheomelanin effect on UVB irradiation-induced oxidation/nitration of L-tyrosine, *Int. J. Mol. Sci.* **23**, 267

[17] P. R. Upadhyay, R. J. Starner, V. B. Swope, K. Wakamatsu, S. Ito and Z. A. Abdel-Malek (2022). Differential induction of reactive oxygen species and expression of antioxidant enzymes in human melanocytes correlate with melanin content: implications on the response to solar UV and melanoma susceptibility, *Antioxidants* **11**, 1204.

[18] D. Feng, Z. Fang and P. Zhang(2020). The melanin inhibitory effect of plants and phytochemicals: A systematic review, *Phytomedicine* **107**, 154449.

[19] A. Gayatri, F. M. Abdelkarem, M. Matsumoto and K. Shimizu (2025). In vitro evaluation on the antioxidant and skin photo protection effects of lignans from the olive (*Olea europaea*) seed, *Nat. Prod. Res*. **1–8** (online first), doi:10.1080/14786419.2025.2491824

[20] K. Yamauchi, T. Mitsunaga, M. Inagaki and T. Suzuki (2014). Synthesized quercetin derivatives stimulate melanogenesis in B16 melanoma cells by influencing the expression of melanin biosynthesis proteins MITF and p38 MAPK, *Bioorg. Med. Chem.* **22**, 3331–3340.

[21] F. Xu, X. Yi, X. Zhang, D. Pei, J. Yuan, N. Wang, D. Di, W. Zeng, Y. Liu and H. Wang (2023). Identification of anti-photoaging components of Olea europaea leaves based on spectrum-effect relationship, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci*. **1226**, 123807

[22] S. Silva, L. Gomes, F. Leitão, M. Bronze, A. V. Coelho and L. V. Boas (2010). Secoiridoids in olive seed: Characterization of nüzhenide and 11-methyl oleosides by liquid chromatography with diode array and mass spectrometry, *Grasas y Aceites*. **61**, 157–164.

[23] M. Servili, M. Baldioli, R. Selvaggini, A. Macchioni and G. F. Montedoro (1999). Phenolic compounds of olive fruit: One- and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit, *J. Agric. Food Chem*. **47**, 12–18.

[24] I. Khelouf, Karoui, A. Lakoud, M. Hammami and M. Abderrabba (2023). Comparative chemical composition and antioxidant activity of olive leaves *Olea europaea* L. of Tunisian and Algerian varieties, *Heliyon*. **9**, e22217.

[25] A. Elkattan, A. Gohar, M. Amer, Z. M. Naeem, A. Ashour and K. Shimizu (2019). Melanin synthesis inhibitors from olea europeae, *Rec. Nat. Prod*. **14**, 139–143.

[26] O. Benavente-García, J. Castillo, J. Lorente, A. D. R. J. Ortuño and J. A. Del Rio (2000). Antioxidant activity of phenolics extracted from *Olea europaea* L. Leaves, *Food Chem*. **68**, 457–462.

[27] A. Guinda, J. M. Castellano, J. M. Santos-Lozano, T. Delgado-Hervás, P. Gutiérrez-Adánez and M. Rada (2015). Determination of major bioactive compounds from olive leaf. LWT, *Food Sci. Technol.* **64**, 431–438.

[28] A. Guinda, M. Rada, T. Delgado, P. Gutiérrez-Adánez, and J. M. Castellano (2010). Pentacyclic triterpenoids from olive fruit and leaf, *J. Agric. Food Chem.* **58**, 9685–969

[29] E. Botsoglou, A. Govaris, I. Ambrosiadis and D. Fletouris (2012). Lipid and protein oxidation of α-linolenic acid-enriched pork during refrigerated storage as influenced by diet supplementation with olive leaves (*Olea europea* L.) or α-tocopheryl acetate, *Meat Sci*. **92**, 525–532.

[30] I. Tarchoune, C. Sgherri, J. Eddouzi, A. Zinnai, M. F. Quartacci and M. Zarrouk (2019). Olive leaf addition increases olive oil nutraceutical properties, *Molecules* **24**, 545.

[31] P. Machała, O. Liudvytska, A. Kicel, A. Dziedzic, M. A. Olszewska and H. M. Żbikowska (2022). Valorization of the photo-protective potential of the phytochemically standardized olive (*Olea europaea* L.) leaf Extract in UVA-irradiated human skin fibroblasts, *Molecules* **27**, 5144.

[32] M. I. Ezzat, M. M. Abdelhafez, A. K. Al-Mokaddem and S. M. Ezzat (2025). Targeting TGF-β/VEGF/NF-κB inflammatory pathway using the polyphenols of *Echinacea purpurea* L. Moench to enhance wound healing in a rat model, *Inflammopharmacology* **33**, 2151.

[33] A. Moazzen, N. Öztinen, E. Ak-Sakalli and M. Koşar (2022). Structure-antiradical activity relationships of 25 natural antioxidant phenolic compounds from different classes, *Heliyon* **8**, 10467

[34] A. C. P. da Silva, J. P. Paiva, R. R. Diniz, V. M. Dos Anjos, A. B. S. M. Silva, A. V. Pinto, E. P. Dos Santos, A. C. Leitão, L. M. Cabral, C. R. Rodrigues and et. al (2019). Photoprotection assessment of olive (*Olea europaea* L.) leaves extract standardized to oleuropein: In vitro and in silico approach for improved sunscreens, *J. Photochem. Photobiol. B.* **193**, 162–171.

[35] Y. Kimura and M. Sumiyoshi (2009). Olive Leaf extract and its main component oleuropein prevent chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice, *J. Nutr.* **139**, 2079–2086

[36] S. Ullah, C. Park, M. Ikram, D. Kang, S. Lee, J. Yang, Y. Park, S. Yoon, P. Chun and H. R. Moon (2019). Tyrosinase inhibition and anti-melanin generation effect of cinnamamide analogues, *Bioorg. Chem.* **87**, 43–55.

[37] H. Wang, J. Chen, J. Hu, J. Si, Y. Xie, J. Wei, Y. Liu and D. Pei (2024). Tyrosinase inhibitor screened from Olea europaea L. leaves: Identification, molecular docking analysis and molecular mechanisms, *Ind. Crops. Prod.* **210**,118112.

[38] M. N. Masum, K. Yamauchi and T. Mitsunaga (2019). Tyrosinase inhibitors from natural and synthetic sources as skin-lightening agents*, Rev. in Agric. Sci*. **7**, 41–58.