www.czasopisma.pan.pl PAN www.journals.pan.pl
POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 58/2: 45–55, 2016
DOI: 10.1515/abcsb-2016-0016

PAN POLSKA AKADEMIA NAUK ODDZIAŁ W KRAKOWIE

CHEMICAL COMPOSITION AND UVA-PROTECTING ACTIVITY OF EXTRACTS FROM *LIGUSTRUM VULGARE* AND *OLEA EUROPAEA* LEAVES

MONIKA E. CZERWIŃSKA*, KATARZYNA DUSZAK, ANDRZEJ PARZONKO AND ANNA K. KISS

Department of Pharmacognosy and Molecular Basis of Phytotherapy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

Received April 21, 2016; revision accepted September 14, 2016

Plants belonging to the family Oleaceae have been traditionally used in treatment of various inflammatory skin disorders. One of the most well-known species is *Olea europaea* L. (olive), cultivated in the Mediterranean countries. Another species is *Ligustrum vulgare* L. (common privet), occurring particularly in Northern Europe and Asia.
The aim of the study was a comparison of the composition of aqueous and ethanolic extracts from leaves of *O. europaea* and *L. vulgare* (HPLC-DAD-MS), and determination of the total content of phenolics and flavonoids, as well as the content of the major compound, oleuropein. Secondly, we aimed to study the protective effect of extracts on reactive oxygen species (ROS) production by human fibroblast cells (NHDF), cell viability (MTT assay), and apoptosis rate (Annexin V/propidium iodide staining) after UVA-irradiation.
The phytochemical analysis allowed us to identify compounds belonging to the groups of flavonoids, phenylpropanoids and secoiridoids in the extracts. The compounds from the group of lignans (olivil) were identified as being unique to *O. europaea* extracts. Echinacoside, ligustroflavone and ligustaloside A were identified in *L. vulgare* extracts in contrast to olive preparations. It was established that the aqueous and ethanolic extracts from leaves of both species, except the privet aqueous extract at a concentration of 5 μg/ml, did not show any significant inhibition of ROS production after UVA-irradiation in the model of NHDF cell line. The aqueous extracts of both species at concentrations of 5 and 25 μg/ml had a protective effect on the viability of UVA-treated cells in contrast to the ethanolic extract.
In conclusion, no significant difference in the activity of olive and privet leaf extracts has been observed, which suggests that both plant materials' extracts, particularly aqueous ones, are effective herbal medicines and photoprotectors, which – to some extent – confirms the use of their preparations in skin disorders.

Keywords: *Ligustrum vulgare*, *Olea europaea*, secoiridoids, Oleaceae, fibroblasts

INTRODUCTION

Plants belonging to the family Oleaceae have traditionally been used in treatment of various inflammatory disorders (Koca et al., 2011). In particular, *Olea europaea* L. (olive) has, by and large, been mostly cultivated in the Mediterranean countries for production of olive oil and medicinal preparations since ancient times. Decoctions from olive leaves have been indicated to treat skin nodules, inflammatory wounds and burns (Koca et al., 2011). Therefore, the composition and activity of olive have been widely studied to date. The monographs of *Oleae folium* and *Oleae folii extractum siccum* are published in the European Pharmacopoeia 8.0 (2014). On the other hand, *Ligustrum vulgare* L. (common privet) is a decorative shrub occurring particularly in Northern Europe and Asia, where it has been used in folk medicine, but the knowledge of its activity is still limited.

The Oleaceae plants are characterized by the presence of iridoids accompanied by phenylethanoid derivatives in the form of esters and glycosides of tyrosol (3-HPEA) and hydroxytyrosol (3,4-DHPEA). In the genera *Olea* and *Ligustrum* most of the identified iridoids formally belong to secoiridoids, as well as oleoside-type glycosides (Jensen et al., 2002). However, the chemical composition of these two species has not been compared up until now. It was previously shown that the most

* Corresponding author, email: mczerwinska@wum.edu.pl

PL ISSN 0001-5296

abundant secoiridoid glucoside of both olive and common privet leaves is oleuropein (Romani et al., 2000; Savournin et al., 2001). Both extracts and oleuropein have been shown to exhibit antioxidant and anti-inflammatory activity, including inhibition of some proinflammatory enzymes, such as cyclooxygenase type 2 (COX-2) and lipoxygenase (Czerwińska et al., 2013; De La Puerta et al., 1999; Macková et al., 2013). Despite some reports on external usage of *O. europaea* and *L. vulgare* leaves and their antioxidant and anti-inflammatory effects, their influence on wound healing has not been fully elucidated. Based on the available data, it is considered that the aqueous extract of olive leaves enhances the development of fibroblasts and epithelialization, as well as reduces congestion and infiltration of neutrophils, while demonstrating a significant healing effect in rats and mice (Koca et al., 2011). Furthermore, the inhibitory activity of olive extracts on skin thickness and elasticity, along with inhibition of the expression of metalloproteinases and COX-2 level, has been proved in UV-irradiated mice (Kimura and Sumiyoshi, 2009).

It is considered that symptoms of UV-induced cutaneous damage and skin dysfunction are linked with photoaging process, immunosuppression, photocarcinogenesis and exacerbation of photodermatoses. This can lead to a significant decrease in skin antioxidants, leading to the skin being less able to protect itself against free radicals generated after sunlight exposure (Svobodova et al., 2006). In particular, UVA radiation (315-400 nm) may enhance this process. It penetrates into the deep epidermis and dermis of the skin affecting the connective tissues. As a result the loss of elasticity, wrinkling and consequently premature aging proceeds. For this reason, we used human fibroblasts – as the most abundant cells of connective tissue – as a wound therapy model to assess the activity of privet and olive leaf extracts. Human fibroblasts might be involved in the process of photoaging via biochemical and morphological changes induced by oxidative agents, such as UV-radiation. It is considered that they play an important role in the skin protection process by initiating the proliferative phase of repair, as well as secreting collagens and glycosaminoglycans (Sharifi et al., 2013).

Taking into consideration some references regarding the anti-inflammatory and UV-protecting activity of olive extracts, as well as lack of scientific data on the potential wound healing activity of privet extracts, we have studied for the first time the biological effect of privet extracts in human fibroblasts with reference to olive ones. The phytochemical differences between the species have not been indicated either. Therefore, the first aim of our study was the comparison of the phytochemical composition of aqueous and ethanolic extracts from leaves of *O. europaea* and *L. vulgare* using a HPLC-DAD-MS/MS method.

Additionally, we determined the total content of phenolics and flavonoids, as well as the content of the major compound, oleuropein, in the tested extracts. In the second part of the study, we compared the influence of extracts on UVA-induced ROS generation in normal human skin fibroblasts (NHDF), and then evaluated their potential cytoprotective and anti-apoptotic effect against damage caused by UVA irradiation.

MATERIALS AND METHODS

CHEMICALS

Dichlorofluorescein diacetate ($DCFH_2$-DA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypsin and echinacoside were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Annexin V Apoptosis Detection Kit and accutase were purchased from BD Biosciences (San Diego, USA). Phosphate buffered saline (PBS) and Penicillin/Streptomycin were purchased from PAA Laboratories (Pasching, Austria). DMEM medium (Dulbecco's Modified Essential Medium) and HEPES buffer were purchased from Lonza (Verwiers, Belgium). Fetal Bovine Serum (FBS) was purchased from Thermo Scientific (Logan, USA). The standard of verbascoside was purchased from HWI Analytik GmbhH (Reulzheim, Germany). Solvents used for the HPLC analysis were HPLC grade. Water was obtained using water purification system MILLIPORE Simfilter Simplicity UV (Molsheim, France).

PLANT MATERIAL AND EXTRACTS PREPARATION

Ligustrum vulgare L. leaves were collected in July 2013 in Warsaw. *Olea europaea* L. leaves were collected in August 2012 on the island of Čiovo in Croatia. Plant material of both species was collected in nature. Leaves were dried in the shade at room temperature. The specimens (No LV062012, OE082012) of leaves are available in the herbarium of the Department of Pharmacognosy and Molecular Basis of Phytotherapy, the Medical University of Warsaw, Poland. The plant material was identified by Anna Kiss (Medical University of Warsaw).

Aqueous extract: 5 g of powdered plant material was macerated in water (1:10) for 1 hour and then extracted four times with water (1:10) in an ultrasonic bath Sonorex Super RK 106 (Bandelin, Germany) at a temperature of 30°C for 30 minutes.

Ethanolic extract: 5 g of powdered plant material was heated under reflux four times with aqueous ethanol (60%, v/v) in a ratio of 1:10 for 30 min-

utes. The collected extracts were concentrated at a low pressure and lyophilized.

Lyophilized extracts were dissolved in PBS (2.5 mg/ml) and added to DMEM to obtain the required concentration of extracts in experiments. The extracts were tested at concentrations of 5 and 25 μg/ml.

HPLC-DAD-MS/MS ANALYSIS

HPLC-DAD-MS/MS analysis was performed on a UHPLC-3000 RS system (Dionex, Germany) with DAD detection and an AmaZon SL ion trap mass spectrometer with ESI interface (Bruker Daltonik GmbH, Germany). Separation was performed on a Zorbax SB C18 column (150 x 2.1 mm, 1.9 μm) (Agilent, USA). The column temperature was set at 25°C. The mobile phase (A) was water/acetonitrile/formic acid (95:5:0.1, v/v/v) and the mobile phase (B) was methanol. A linear gradient system was used: 0–60 minutes 1-60% B. The flow rate was 0.18 ml/min. The column was equilibrated for 10 minutes between injections. UV spectra were recorded over a range of 200–450 nm, chromatograms were acquired at 240 nm, 280 nm, 325 nm and 350 nm. The LC eluate was introduced directly into the ESI interface without splitting. The nebuliser pressure was 40 psi; dry gas flow 9 L/min; dry temperature 300°C; and capillary voltage 4.5 kV. Analysis was carried out using a scan from m/z 200 to 2,200. Compounds were analyzed in a negative ion mode. The MS^2 fragmentation was obtained for the most abundant ion at the time.

TOTAL PHENOLS AND FLAVONOIDS CONTENT

The sum of phenols was determined using a modified spectrophotometric method with Folin-Ciocalteu's reagent (Singleton et al., 1999). The assay was performed in 96-well plates: 10 μl of extracts' solution (5 mg/ml in 50% methanol), 105 μl of 10% Folin-Ciocalteu reagent (diluted in distilled water) and 85 μl Na_2CO_3 (1M) were mixed and incubated for 15 minutes at room temperature in the darkness. The absorbance was measured at 765 nm in a microplate reader (SYNERGY 4, BioTek, Winooski, USA) and the results were expressed as gallic acid equivalent (mg of GAE/g of extract).

The sum of flavonoids was determined using a spectrophotometric method with 2% $AlCl_3$ solution in methanol preceded by hydrolysis with hydrochloric acid (25%) according to the monographs of Polish Pharmacopoeia X (2014). The assay was performed as follows: 10 ml of extracts' solutions (0.2 mg/ml in 50% ethanol) and 2 ml of 2% methanolic $AlCl_3$ solution were mixed, and diluted with the mixture of acetic acid and methanol (95:5, v/v) to the volume of 25 ml. After 45 minutes incubation in the darkness, the absorbance was measured at 425 nm in a spectrophotometer (Evolution 60S, Thermo Scientific, Madison, USA) and the results were expressed as hyperoside equivalent (mg of HPE)/g of extract), as well as quercetin equivalent (mg of QE/g of extract) (Quettier-Deleu et al., 2000).

The content of oleuropein was determined using a HPTLC-photodensitometry method (Czerwińska et al., 2015).

HUMAN SKIN FIBROBLASTS MODEL

The NHDF human skin fibroblast cells were obtained from Lonza (Verwiers, Belgium). The cells (3–6 passage) were cultured in the DMEM supplemented with 10% FBS, penicillin and streptomycin, in 5% CO_2 and at 37°C. The cells were cultured until they formed a confluent monolayer. The medium was changed every two days. The cells were then detached using accutase or trypsin (0.25%). For each experiment, the cells were seeded on 12-well plates (4×10^4 cells per well) or 96-well plates (5×10^3 cells per well) in DMEM with FBS (1%). All determinations were made using 96-well plates and were measured in a microplate reader SYNERGY 4 (BioTek, Winooski, USA).

UV IRRADIATION

Before UV irradiation, the cells were washed once with PBS and exposed to UV radiation in a thin layer of PBS. The cells were irradiated with UVA in a dose of 25 J/cm^2 using a CL-1000 L crosslinker (UVP, USA) emitting UVA peaking at 365 nm. After irradiation, PBS was replaced with serum-free DMEM (with tested extracts in some experiments) and incubated at 37°C in humidified atmosphere of 5% CO_2.

MITOCHONDRIAL FUNCTION ASSESSMENT

Mitochondrial function was assessed using MTT assay. In brief, the cells were incubated with extracts for 24 h before and after UVA irradiation and then MTT (0.5 mg/ml) solution was added for 2 h. The converted dye was then solubilized with acidic isopropanol (0.04 M HCl in absolute isopropanol), and absorbance was measured at 570 nm with background subtraction at 650 nm, using a microplate reader. The cell viability was calculated according to the formula:

$$\text{Cell viability [\%]} = A_{sample}/A_{control} \times 100$$

INTRACELLULAR ROS GENERATION AFTER UV IRRADIATION

The dichlorofluorescein diacetate ($DCFH_2$-DA) assay was used to assess the extent of ROS generation following UVA exposure. The cells were seeded in 12-well plates and incubated with tested extracts for 24 h. Following UV exposure, the cells were washed twice with PBS and incubated with various concentrations of the extracts for 24 h. Following incubation the cells were washed with PBS and incubated with dihydrodichlorofluorescein diacetate ($DCFH_2$-DA, 10 μM) for 30 minutes at 37°C. The cells were then detached using accutase, washed and fluorescence was determined using a FACS Calibur flow cytometer (Becton Dickinson, USA).

STAINING WITH ANNEXIN V-FITC/PI

To detect apoptosis/necrosis, after UV irradiation the cells were incubated in the presence or absence of the tested extracts for 24 h. The cells were detached using accutase and annexin staining was determined using an Annexin V-FITC Apoptosis Detection Kit, following manufacturer guidelines. The samples were analyzed by flow cytometry, using a FACS Calibur (Becton Dickinson, USA) flow cytometer and CellQuest software. The results were presented as rates of apoptotic and necrotic cells.

STATISTICAL ANALYSIS

The results were expressed as a mean ± SEM. Statistical significance of differences between means was established by ANOVA with Tukey's or Duncan post hoc test. *P* values below 0.05 were considered statistically significant. All analyses were performed using Statistica 10 (StatSoft, Poland).

RESULTS AND DISCUSSION

In the present study, the phytochemical composition of aqueous and ethanolic extracts from leaves of *Olea europaea* and *Ligustrum vulgare* has been compared for the first time.

The HPLC-DAD-MS/MS analysis allowed us to compare the phytochemical composition of olive and privet leaf extracts in groups of compounds, such as flavonoids, phenylpropanoids and secoiridoids (Table 1). The most abundant ones for both species were secoiridoids, such as oleoside (*m/z* 389; R_t=10.4), oleoside-11-methylester (*m/z* 403; R_t=24.8), 10-hydroxyoleuropein (*m/z* 555; R_t=36.7), oleacein (*m/z* 319; R_t=43.8), oleuropein (*m/z* 539; R_t=47.8) and ligstroside (*m/z* 523; R_t=52.4). It is worth noting that the aglycone of ligstroside, oleocanthal, was present only in privet leaf aqueous extract. The flavonoids, luteolin 7-*O*-glucoside (*m/z* 447; R_t=41.6) and quercetin 3-*O*-rutinoside (*m/z* 609; R_t=42.9), and phenylpropanoid verbascoside (*m/z* 623; R_t=40.6) were identified in both olive and privet leaf extracts. Based on their molecular weight the compounds from the group of lignans, such as olivil glucoside (*m/z* 537; R_t=5.6), olivil (*m/z* 375; R_t=6.5) and flavonoid chrysoeriol glucoside (*m/z* 461; R_t=47.1) were identified only in olive extracts. Although, the presence of pentacyclic triterpens, such as oleanolic and maslinic acids, has been previously established (Guinda et al., 2015), we did not detected them in the olive leaf ethanolic extract. The composition of aqueous (Fig. 1a) and ethanolic (Fig. 1b) extracts from privet leaves was characterized by the presence of more diverse phytochemicals than olive extracts. In contrast to olive preparations, a phenylpropanoid, echinacoside (*m/z* 785; R_t=34.6), as well as flavonoid, ligustroflavone (*m/z* 723; R_t=45.5) were identified in common privet extracts. The unique secoiridoid for privet extracts turned out to be ligustaloside A (*m/z* 555; R_t=39.3). Moreover, based on UV-Vis spectra and MS/MS we were able to identify the rare glucarates of *p*-coumaric acid, as well as *p*-coumaroyl and feruloyl quinic acid derivatives in common privet extracts, which were not present in olive extracts.

Analyzing the quantity of the total phenolic and flavonoids content in extracts, it was established that the ethanolic extracts from olive and common privet leaves were characterized by a higher content of total phenols and flavonoids than aqueous extracts (Table 2). However, significant differences in the quantities of compounds between olive and common privet were observed in the case of aqueous extracts. A significantly higher content of phenolic compounds (84.3 ± 5.2 mg GAE/g of dry extract) was determined in the olive leaf aqueous extract rather than in common privet aqueous extract (75.8 ± 4.2 mg GAE/g of dry extract). Depending on the olive cultivars the total phenolic content in aqueous extracts from leaves of *O. europaea* ranges from 78.52 ± 2.18 to 102.69 ± 1.63 μg GAE/mg of dry extract (Orak et al., 2012). Thus, our results stay in agreement with the previously established range of phenolic content in olive leaf aqueous extracts. On the other hand, a higher quantity of flavonoids was noted for common privet aqueous extract (3.7 ± 0.1 mg HPE/g of extract; 2.6 ± 0.1 mg QE/g of dry extract) in comparison with olive aqueous extract (1.7 ± 0.1 mg of HPE/g of dry extract; 1.2 ± 0.1 mg QE/g of dry extract) (Table 2). The total flavonoid content in olive aqueous extract was lower than in the studies of Orak et al. (2012), where flavonoid content ranged from 5.46 ± 0.06 to 12.47 ± 0.12 mg QE/g of dry extract (Orak et al., 2012). Additionally, in our study, it has been indicated with the HPTLC-

TABLE 1. Retention times, UV-Vis, and MS/MS data in the negative ion mode for compounds present in *Ligustrum vulgare* and *Olea europaea* leaves extracts

	Compounds	Retention time [min]	UV [nm]	$[M-H]^-$ m/z	MS^2ions
1	olivil glucoside[a,c]	5.6	235	537.4	493.4, **375.4**, 331.2, 179.0
2	olivil[a,c]	6.5	235	375.5	213.0, 169.1, 107.2
3	hydroxytyrosol glucoside	9.7	220,280	315.3	153.0, **135.0**
4	oleoside	10.4	235	389.3	**227.0**, 183.0, 165.0
5	*p*-coumaroyl glucarate	11.3	230, 310	355.3	337.2, **209.0**, 190.9
6	*p*-coumaroyl glucarate	12.5	230, 310	355.3	337.2, **209.0**, 191.0
7	*p*-coumaroyl glucarate	13.8	230, 310	355.2	337.1, **209.0**, 191.0
8	*p*-coumaroyl glucarate	15.0	230, 310	355.2	337.1, **209.0**, 191.0
9	*p*-coumaroyl glucarate	16.3	230, 310	355.2	337.1, **209.0**, 191.0
10	secoiridoid derivative	20.2	230	393.3	375.1
11	secoiridoid derivative	20.9	230	611.4*	565.4, **403.3**
12	secoiridoid derivative	23.1	235	389.2	**345.2**, 209.0
13	oleoside-11-methylester	24.8	240	403.4	371.1, **223.0**, 179.0
14	unidentified	26.0	240, 325	519.5	325.2, 235.0, **193.0**
15	secoiridoid derivative	27.7	235	571.4	553.4, **419.5**, 410.0
16	*p*-coumaroyl quinic acid	28.3	230,310	337.5	**173.0**
17	secoiridoid derivative	28.7	235	571.4	553.4, **419.5**, 410.0
18	feruloyl quinic acid	30.6	230, 320	367.8	**173.0**
19	secoiridoid derivative	32.7	230	393.6	375.2, 307.1, **273.1**
20	oleoside-11-methylester isomer	34.2	240	403.7	**371.2**, 223.0, 179.0
21	echinacoside[b]	34.6	240, 290, 330	785.6	**623.8**, 461.4
22	10-hydroxyoleuropein	36.7	240	555.4	**537.4**, 403.5, 393.6, 323.3, 291.3
23	secoiridoid derivative	38.8	230	393.5	375.2, 273.1
24	ligustaloside A	39.3	240, 280	555.4	523.4, **393.5**, 273.4
25	verbascoside[b]	40.6	240, 290, 330	623.4	**461.6**, 315.2
26	luteolin 7-*O*-glucoside	41.6	260, 350	447.4	**285.3**
27	quercetin 3-*O*-rutinoside	42.9	260, 355	609.4	343.1, 301.0
28	oleacein[b]	43.8	230, 280	639.4**	**319.2**, 195.0
29	oleuropein isomer	44.7	240	539.4	377.3
30	ligustroflavone	45.5	270, 340	723.5	577.8
31	apigenin 7-*O*-rutinoside	45.9	270, 340	577.4	269,1
32	apigenin 7-*O*-glucoside[c]	46.5	230	431.8	269.0
33	chrysoeriol 7-*O*-glucoside[a,c]	47.1	240,340	461.5	446.2, 299.2
34	oleuropein[b]	47.8	240, 280	539.4	377.4, **307.2**, 275.3
35	oleocanthal	50.0	230	607.7*	303.1, 285.3, 165.0
36	ligstroside	52.4	230	523.4	**361.5**, 291.2, 259.3
37	oleuropein isomer	53.5	230	539.4	**377.4**, 307.2

* [M-H-HCOOH], ** [2M-H]; ions in **bold** – most abundant ion peak.
[a] The possible compounds identified based on molecular weight.
[b] Identity confirmed with chemical standard.
[c] Compounds identified in olive extracts.

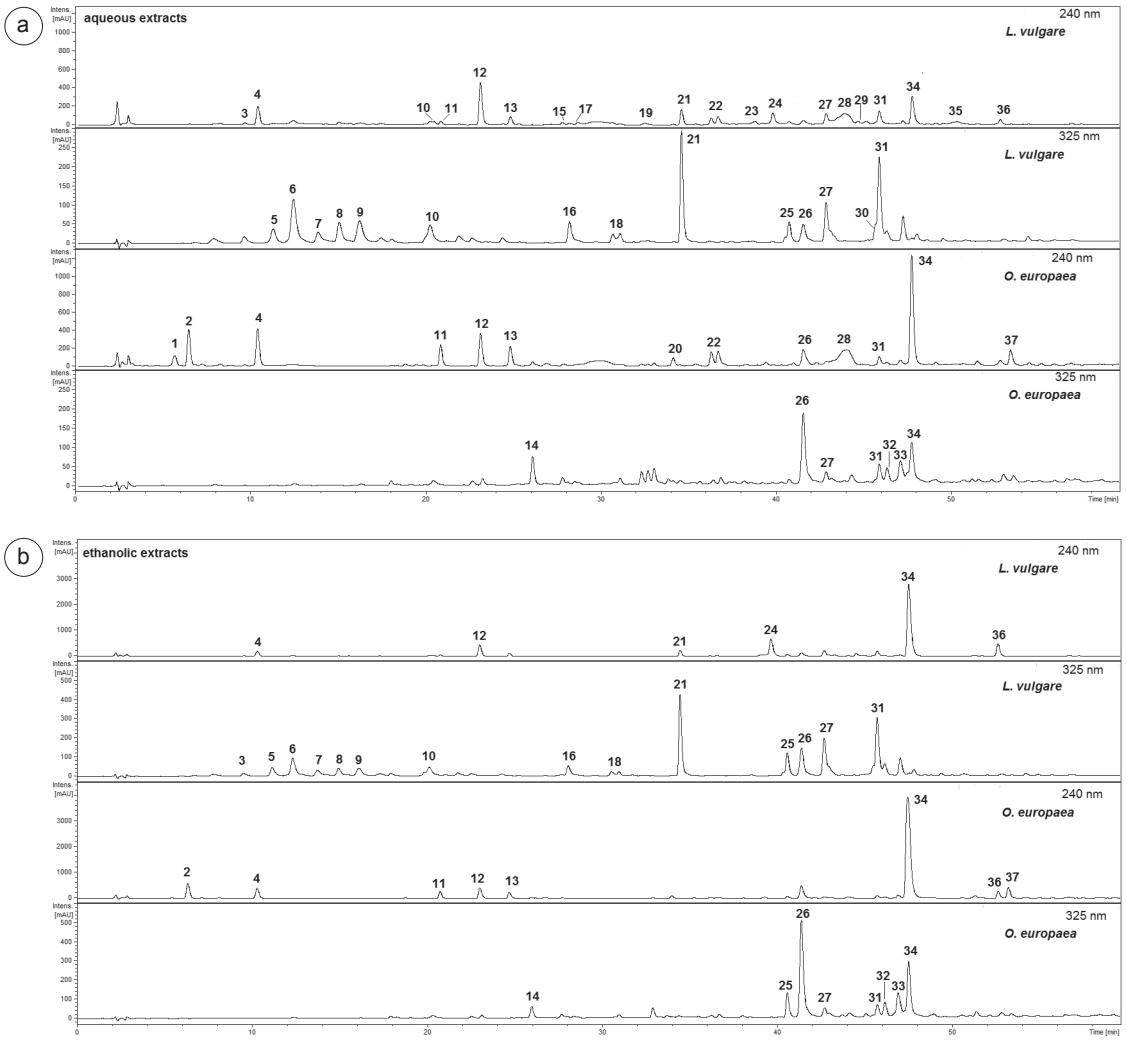

Fig. 1. HPLC UV-Vis chromatograms of *Ligustrum vulgare* and *Olea europaea* aqueous extracts (**a**) and ethanolic extracts (**b**) acquired at 240 nm and 325 nm.

photodensitometry method that olive ethanolic extract is characterized by a higher content of oleuropein (23.4 ± 2.7%) compared to privet extract (13.4 ± 1.4%) (Table 2). According to the monograph of *Oleae folii extractum siccum* (European Pharmacopoeia 8.0, 2014; Polish Pharmacopoeia X, 2014), a concentration of oleuropein in the dried olive extract of not less than 16% fulfills the pharmacopoeia requirements. The quantity of oleuropein in the aqueous extract from olive leaves was 6.3 ± 1.7% (Table 2). However, in the case of privet aqueous extract the oleuropein content was not established due to the limitations of quantification.

Although leaves of olive and privet are considered as sources of antioxidants and have been traditionally used as anti-inflammatory agents, in particular olive preparations, their biological activity has not been widely studied. Taking into consideration the available data on the potential involvement of olive leaf extracts in the wound healing process, as well as protection against UV-induced skin damage, we decided to assess the antioxidant and photoprotective activity of both species extracts in the NHDF cell model. First, considering the previous data on antioxidant activity of preparations of olive and common privet, assigned to the presence of phenolic compounds, we compared the potential protective activity of the tested extracts against ROS generation by UVA-irradiated NHDF (Fig. 2). It was established that only aqueous extract from privet leaves at a concentration of 5 μg/ml showed significant inhibition of ROS production in this model. In the previous studies of plant-derived extracts containing

TABLE 2. The comparison of total phenolic, flavonoids and oleuropein content in extracts from leaves of *Olea europaea* and *Ligustrum vulgare*.

leaves extracts	*Olea europaea*	*Ligustrum vulgare*
total phenolic content [mg of GAE/g of extract]*		
aqueous extract	84.3 ± 5.2[b]	75.8 ± 4.2
ethanolic extract	96.1 ± 5.2[a]	91.5 ± 3.1[a]
flavonoids content [mg of HPE/g of extract]**		
aqueous extract	1.7 ± 0.1	3.7 ± 0.1[c]
ethanolic extract	5.8 ± 0.3[a]	6.8 ± 0.5[a]
flavonoids content [mg of QE/g of extract]**		
aqueous extract	1.2 ± 0.1	2.6 ± 0.1[c]
ethanolic extract	4.1 ± 0.2[a]	4.8 ± 0.4[a]
oleuropein quantity [%]***		
aqueous extract	6.3 ± 1.7	-
ethanolic extract	23.4 ± 2.7[a,b]	13.4 ± 1.4

* The total phenolic content expressed as gallic acid equivalent [mg of GAE/g of extract].
** The flavonoids content expressed as hyperoside equivalent [mg of HPE/g of extract]; quercetin equivalent [mg of QE/g of extract].
*** The quantity of oleuropein [%] determined with HPTLC-photodensitometry method.
[a] $P < 0.05$ vs. aqueous extracts; [b] $P < 0.05$ vs. common privet leave aqueous extract; [c] $P < 0.05$ vs. olive leave aqueous extract.

phenolic compounds, the inhibition of ROS production in UVA-irradiated fibroblasts by extracts from *Galinsoga* herb, as well as by *Oenothera paradoxa* defatted seeds extract was observed (Bazylko et al., 2015; Jaszewska et al., 2013). However, in the case of olive and privet extracts, it is likely that their constituents, such as secoiridoids, are highly unstable compounds in the presence of the destructive effects of UV radiation (Bruneton, 1999). Additionally, flavonoids are also distinguished by a greater sensitivity to UV radiation (Cvetković et al., 2011). The antioxidant activity of olive leaf extracts has never been investigated in UV-irradiated fibroblasts. For this reason, the effect of UV-radiation on some phenolics, in particular secoiridoids, structures and their weak antioxidant effect in this model need to be further elucidated.

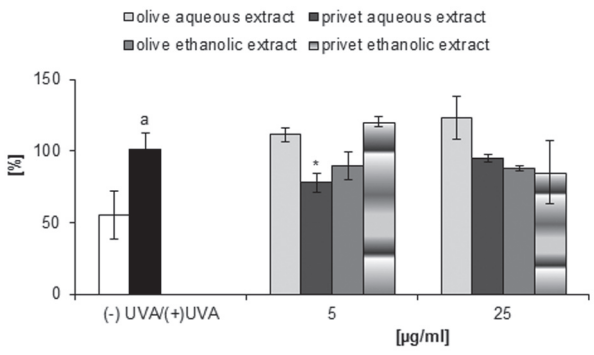

Fig. 2. The effect of extracts from leaves of *Olea europaea* (olive) and *Ligustrum vulgare* (privet) on ROS production in UVA-irradiated NHDF [%]. [a] $P < 0.05$ vs. control (-) UVA; * $P < 0.05$ vs. control (+) UVA.

It is believed that ROS can induce apoptosis in many different cell systems under physiologic and pathologic conditions (Simon et al., 2000). Moreover, the crucial factors involved in the wound healing process are cell viability and proliferation. In this respect, we evaluated the proliferative activity of living cells assessing the mitochondrial function with MTT assay. Initially, we determined the effect of extracts on the viability of non-UV-treated NHDF cells. None of the extracts have decreased cell viability (Fig. 3). On the other hand, the fibroblasts exposed to UVA-irradiation showed a reduction in viability (48.6 ± 4.9% viable cells) compared with the non-treated cells (Fig. 4). The incubation of fibroblasts with aqueous extracts from leaves of olive and privet prevented the decrease of viability activity after UVA-irradiation at concentrations of 5 and 25 µg/ml (Fig. 4). For the olive leaf aqueous extract the cell viability ranged from 85.8 ± 8.9% (25 µg/ml) to 136.9 ± 29.6% (5 µg/ml). The cell viability after privet leaf aqueous extract treatment ranged from 104.6 ± 13.9% (25 µg/ml) to 122.8 ± 30.6% (5 µg/ml). In the case of ethanolic extracts, the photoprotective effect

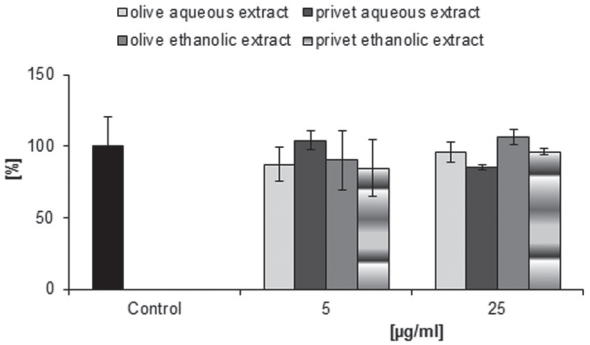

Fig. 3. The effect of extracts from leaves of *Olea europaea* (olive) and *Ligustrum vulgare* (privet) on NHDF cell viability [%].

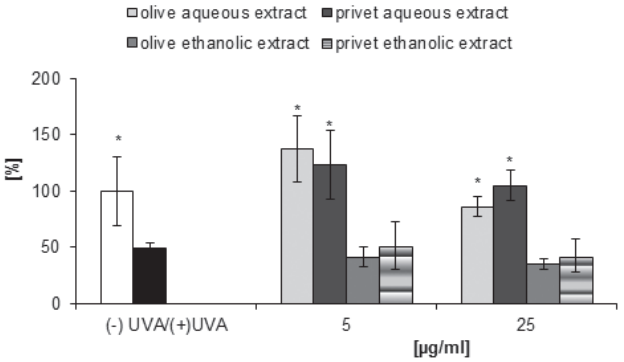

Fig. 4. The effect of extracts from leaves of *Olea europaea* (olive) and *Ligustrum vulgare* (privet) on cell viability in UVA-irradiated NHDF [%]. * $P < 0.05$ vs. control (+) UVA.

was not observed. Similarly, the inhibition of cell proliferation was recently observed when UVA-irradiated human fibroblasts were treated with ethanolic extracts form *Galinsoga* herb (Bazylko et al., 2015). The authors suggested the presence of some lipophilic compounds, including sterols, fatty alcohols or diterpene alcohols, in ethanolic extracts. In the previous studies on the mouse embryonic fibroblasts it was shown that ethanolic extracts from olive leaves suppressed their cytotoxicity. However, in the study the cytotoxicity, as well as apoptosis, were induced by hydrogen peroxide (H_2O_2) as a model substance simulating UV damage, and the cells were co-treated with ethanolic extract. Thus, it seems that cells protection against oxidative stress and apoptotic effect of olive extract resulted from its free radicals scavenging activity (Ha et al., 2009). On the other hand, the different observations were found for flavonolignans from *Silybum marianum*, which protected human fibroblasts against oxidative stress only when the cells were pretreated with silymarin before H_2O_2 induction. It may suggest that the protection is not due to the direct interaction of silymarin and H_2O_2. Silymarin is more likely to alter the cell membrane (Sharifi et al., 2013). Taking into consideration these data, the mechanism of cytotoxicity of ethanolic extracts from Oleaceae plants in UVA-irradiated fibroblasts seems to be more complex and there is a necessity for further investigation. However, there are some reports on photocytotoxicity of natural compounds, such as flavonoids of *Hypericum perforatum* (Onoue et al., 2011; Wilhelm et al., 2001). It is believed that some phototoxic chemicals are able to generate ROS, including singlet oxygen and superoxide under light exposure leading to peroxidation of fatty acids, which may additionally justify the weak antioxidant effect of the extracts tested in this study, in particular ethanolic ones. Since Onoue et al. (2011) observed the significant differences in photoreactivity of glycoside and aglycone moieties, the discrepancies between aqueous and ethanolic extracts' activity might be the result of their different composition, particularly due to the presence of aglycones in aqueous extracts. Another potential mechanism of photocytotoxicity might be genotoxic effect caused by interaction between DNA and UV-excitated photosensitizers (Onoue et al., 2011).

For further investigation of the protective activity of the tested extracts, we measured apoptosis and necrosis of the cells. The population of living cells decreased from 90.32% to 54.14% after UVA-irradiation (Fig. 5a). The cell viability results were confirmed in annexin V/propidium iodide staining experiments. It was shown that the aqueous extracts prevented the apoptosis of fibroblasts after UVA-irradiation (Fig. 5b). The population of living cells was 75.30% and 67.47% when fibroblasts were incubated with aqueous extract (25 µg/ml) from privet and olive leaves, respectively. Surprisingly, in the case of aqueous extracts from common privet leaves we did not observe any significant dose-dependent anti-apoptotic effect, and we are unable to explain it. Due to the fact that the extracts did not significantly inhibit ROS production in the NHDF cell system, it is likely that the anti-apoptotic effect of aqueous extracts is not the result of the antioxidant activity of the extracts. On the other hand, a completely different effect for ethanolic extract was observed, just as it was previously proved in MTT test. The privet and olive leaf ethanolic extracts at a concentration of 25 µg/ml decreased the population of live cells to 62.53% and 35.97%, respectively (Fig. 5c). The cytotoxic effect of ethanolic extracts may be associated with the presence of some lipophilic compounds compared with the aqueous extracts.

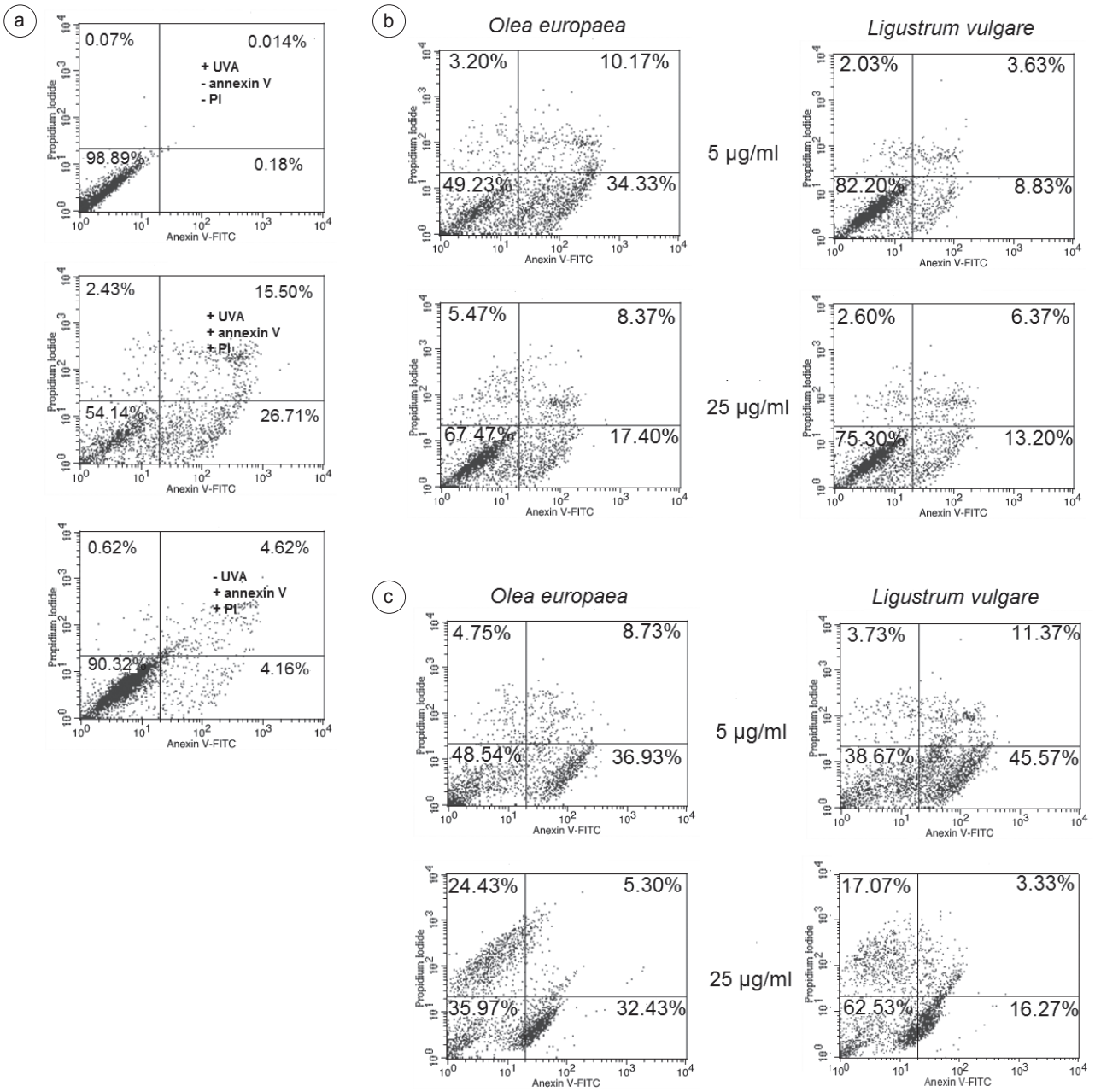

Fig. 5. The results of Annexin-FITC/PI staining test in NHDF control cells before and after UVA-irradiation (**a**). The effect of aqueous (**b**) and ethanolic (**c**) extracts from leaves of *Olea europaea* (olive) and *Ligustrum vulgare* (privet) on cell viability in UVA-irradiated NHDF [%].

CONCLUSION

To the best of our knowledge, this is the first study describing the photoprotective effect of *L. vulgare* leaf extracts in human fibroblasts referring to the activity of another Oleaceae species, *O. europaea* leaf extracts. In the current study, we have not observed any significant differences in the potency of both species extracts. However, the substantial difference between aqueous and ethanolic extracts of both species in the UVA-induced cytotoxicity was demonstrated. Both in MTT test and in annexinV/propidium iodide staining, ethanolic extracts had a cytotoxic effect, whereas aqueous extracts exert-

ed a protective influence on human fibroblasts. It seems that the presence of more lipophilic compounds in ethanolic extracts may be responsible for the differences in activity of both types of extracts.

In conclusion, the aqueous extract from leaves of *L. vulgare* and *O. europaea* are likely to be effective photoprotectors, which partially confirms the use of their preparations in skin disorders.

AUTHORS' CONTRIBUTIONS

The following declarations about authors' contributions have been made: MC: study conception, supervision of the research design, monitoring of the data collection tools, manuscript preparation, KD: biological experiments and data analysis, AP: draft of the biological methods, AK: participation in HPLC-MS analysis, critical revision of the manuscript. The authors declare that they have no conflict of interest to disclose.

ACKNOWLEDGEMENTS

This project was carried out with the use of CePT infrastructure financed by the European Regional Development Found within the Operational Programme 'Innovative economy' for 2007–2013.

REFERENCES

European Pharmacopoeia 8.0. 2014. 1-1456. https://www.edqm.eu/en/european-pharmacopoeia-8th-edition-1563.html [13 April 2016].

Polish Pharmacopoeia X. 2014. Volume 1, 1-2040. President of the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products, Warsaw.

Bazylko A, Borzym J, and Parzonko A. 2015. Determination of *in vitro* antioxidant and UV-protecting activity of aqueous and ethanolic extracts from *Galinsoga parviflora* and *Galinsoga quadriradiata* herb. *Journal of Photochemistry and Photobiology B: Biology* 149: 189–195.

Bruneton J. 1999. Monoterpenes. Iridoids. In: *Pharmacognosy, phytochemistry, medicinal plants*, 590-611. Techniques and Documentation, Intercept, Lavoisier Publishing Inc., Paris.

Cvetković D, Marković D, Cvetković D, and Radovanović B. 2011. Effects of continuous UV-irradiation on the antioxidant activities of quercetin and rutin in solution in the presence of lecithin as the protective target. *Journal of the Serbian Chemical Society* 76: 973–985.

Czerwińska ME, Granica S, and Kiss AK. 2013. Effects of an aqueous extract from leaves of *Ligustrum vulgare* on mediators of inflammation in a human neutrophils model. *Planta Medica* 79: 924–932.

Czerwińska ME, Ziarek M, Bazylko A, Osińska E, and Kiss AK. 2015. Quantitative determination of secoiridoids and phenylpropanoids in different extracts of *Ligustrum vulgare* L. leaves by a validated HPTLC-photodensitometry method. *Phytochemical Analysis* 26: 253–260.

De la Puerta R, Ruiz Gutierrez VR, and Hoult JRS. 1999. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochemical Pharmacology* 57: 445–449.

Guinda A, Castellano JM, Santos-Lozano JM, Delgado-Hervás T, Gutiérrez-Adánez P, and Rada M. 2015. Determination of major bioactive compounds from olive leaf. *LWT – Food Science and Technology* 64: 431–438.

Ha JY, Goo SY, Sung JS, and Shin HS. 2009. Antioxidant and cytoprotective activity of the olive leaf (*Olea europaea* L. var. Kalamata) extracts on the mouse embryonic fibroblast cell. *Food Science and Biotechnology* 18: 965–970.

Jaszewska E, Soin M, Filipek A, and Naruszewicz M. 2013. UVA-induced ROS generation inhibition by *Oenothera paradoxa* defatted seeds extract and subsequent cell death in human dermal fibroblasts. *Journal of Photochemistry and Photobiology B: Biology* 126: 42–46.

Jensen SR, Franzyk H, and Wallander E. 2002. Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers. *Phytochemistry* 60: 213–231.

Kimura Y, and Sumiyoshi M. 2009. Olive leaf extract and its main component oleuropein prevent chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice. *Journal of Nutrition* 139: 2079–2086.

Koca U, Süntar I, Akkol EK, Yilmazer D, and Alper M. 2011. Wound repair potential of *Olea europaea* L. leaf extracts revealed by *in vivo* experimental models and comparative evaluation of the extracts' antioxidant activity. *Journal of Medicinal Food* 14: 140–146.

Macková A, Mučaji P, Widowitz U, and Bauer R. 2013. In vitro anti-inflammatory activity of *Ligustrum vulgare* extracts and their analytical characterization. *Natural Product Communications* 8: 1509–1512.

Onoue S, Seto Y, Ochi M, Inoue R, Ito H, Hatano T, and Yamada S. 2011. *In vitro* photochemical and phototoxicological characterization of major constituents in St. John's Wort (*Hypericum perforatum*) extracts. *Phytochemistry* 72: 1814–1820.

Orak HH, Isbilir SS, and Yagar H. 2012. Determination of antioxidant properties of lyophilized olive leaf water extracts obtained from 21 different cultivars. *Food Science and Biotechnology* 21: 1065–1074.

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin JC, Bailleul F, and Trotin F. 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology* 72: 35–42.

Romani A, Pinelli P, Mulinacci N, Vincieri FF, Gravano E, and Tattini M. 2000. HPLC analysis of flavonoids and secoiridoids in leaves of *Ligustrum vulgare* L. (Oleaceae). *Journal of Agricultural and Food Chemistry* 48: 4091–4096.

Savournin C, Baghdikian B, Elias R, Dargouth-Kesraoui F, Boukef K, and Balansard G. 2001. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *Journal of Agricultural and Food Chemistry* 49: 618–621.

Sharifi R, Pasalar P, Kamalinejad M, Dehpour AR, Tavangar SM, Paknejad M, Mehrabani Natanzi M, Nourbakhsh M, Ahmadi Ashtiani HR, Akbari M, and Rastegar H. 2013. The effect of silymarin (*Silybum marianum*) on human skin fibroblasts in an *in vitro* wound healing model. *Pharmaceutical Biology* 51: 298–303.

Simon HU, Haj-Yehia A, and Levi-Schaffer F. 2000. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 5: 415–418.

Singleton VL, Orthofer R, and Lamuela-Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology* 299: 152–178.

Svobodova A, Walterova D, and Vostalova J. 2006. Ultraviolet light induced alteration to the skin. *Biomedical papers of the Medical Faculty of Palacký University, Olomouc, Czech Republic* 150: 25–38.

Wilhelm KP, Biel S, and Siegers CP. 2001. Role of flavonoids in controlling the phototoxicity of *Hypericum perforatum* extracts. *Phytomedicine* 8: 306–309.