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Olea europaea L.'den elde edilen triterpenoid ve polifenol bileşiklerinin antimikrobiyal ve yaşlanma karşıtı etkilerinin değerlendirilmesi: Ekstraksiyon, Tanılama ve *in vitro* Testler

Assessment of antimicrobial and anti-aging effects of triterpenoid and polyphenol compounds from olea europaea L: extraction, identification and in vitro tests

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ABSTRACT

Aim: This study investigated the antimicrobial and anti-aging effects of bioactive compounds derived from Olea europaea L. leaves and flowers, widely used in traditional treatments in European and Mediterranean countries.

Materials and Methods: Following solid-liquid extraction, the control of purification processes was conducted using thin-layer chromatography. Identification of the obtained molecules was performed through high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analyses. For determining antimicrobial activity, Gram-positive and Gram-negative bacteria, *Bacillus cereus*, and *Escherichia coli* O15:H7, were respectively used for minimum inhibitory concentration (MIC) tests. In the assessment of Oleuropein's *in vitro* cytotoxicity on adult human dermal fibroblasts (HDFa), the MTT assay was employed using HDFa cell lines, while an ELISA Test kit was utilized to determine changes in collagen type-I levels.

Results: As a result of the study, oleanolic acid (L1), oleuropein (L2), and ursolic acid (L3) were isolated from olive leaves, whereas oleuropein aglycone (F1) molecule was isolated from olive flowers. According to the results of the MIC tests, compounds L1, L2, and L3 isolated from the leaves exhibited an inhibitory effect against *B.cereus* within the concentration range of 5-250 µg/mL, whereas Oleuropein aglycone (F1) did not demonstrate any activity. Furthermore, except for the Oleuropein (L2) molecule, no other compound was effective against *E.coli.* In the evaluation of Oleuropein's *in vitro* cytotoxicity, a dose-dependent effect on HDFa cell viability was observed, and collagen type-I levels were significantly higher than levels obtained with vitamin C.

Conclusion: Based on the results, it is believed that the active molecules derived from olive plant's leaves and flowers exhibit antimicrobial effects, potentially serving as natural preservatives in the cosmetics industry. Moreover, their contribution to cell regeneration suggests potential use in wound treatments.

Keywords: Oleuropein, antimicrobial effect, *olea europaea L.,* MIC, anti-aging.

ÖZ

Amaç: *Bu çalışmada geleneksel tedavilerde Avrupa ve Akdeniz ülkelerinde kullanılmakta olan Olea europaea L. yaprakları ve çiçeklerinden elde edilen biyoaktif bileşiklerin antimikrobiyal ve yaşlanma karşıtı etkileri araştırılmıştır.*

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Gereç ve Yöntem: *Katı-sıvı ekstraksiyonu ve saflaştırma adımlarını takiben, saflaştırma işlemlerinin kontrolü ince tabaka kromatografisi (ITK) ile yapılmıştır. Elde edilen moleküllerin tanımlanması yüksek basınçlı sıvı kromatografisi (HPLC) ve nükleer manyetik rezonans (NMR) analizleri ile gerçekleştirilmiştir. Antimikrobiyal aktivitenin belirlenmesinde minimum inhibisyon konsantrasyonu (MIC) testleri için gram pozitif ve gram negatif bakterilerin temsilcisi olarak sırasıyla Bacillus cereus ve Escherichia coli O15:H7 kullanılmıştır. Oleuropein'in insan dermal fibroblastları üzerindeki in vitro sitotoksisite değerlendirmesinde, HDFa hücre hatları kullanılarak MTT testi, kollajen tip-I seviyesindeki değişimin belirlenebilmesi için ise ELISA Test kiti kullanılmıştır.*

Bulgular: *Çalışma sonucunda zeytin yaprağından Oleanolik asit (L1), oleouropein (L2) ve Ursolik asit (L3) molekülleri, zeytin çiçeğinden ise oleuropein aglikon (F1) molekülü izole edilerek tanımlanmıştır. Minimal inhibisyon konsantrasyonu (MIK) testi sonuçlarına göre, yapraklardan izole edilen L1, L2 ve L3 bileşikleri, Bacillus cereus'a karşı 5-250 µg / mL konsantrasyon aralığında inhibisyon etkisi göstermiştir, ancak Oleuropein aglikon (F1) herhangi bir aktivite göstermemiştir. Ayrıca, Escherichia coli'ye karşı Oleuropein (L2) molekülünün dışında hiçbir bileşiğin etkili olmadığı bulunmuştur. Oleuropein'in insan dermal fibroblastları üzerindeki in vitro değerlendirmesinde, HDFa hücrelerinin hücre canlılığı üzerinde doza bağlı bir etki gözlenmiş ve kollajen tip-I seviyeleri vitamin C ile elde edilen seviyelerden önemli ölçüde yüksek bulunmuştur.*

Sonuç: *Sonuçlara dayanarak, zeytin bitkisinin yaprakları ve çiçeklerinden elde edilen aktif moleküllerin antimikrobiyal etkiler sergilediği ve kozmetik endüstrisinde doğal koruyucu olarak hizmet edebileceği düşünülmektedir. Dahası, hücre yenilenmesine olan katkıları, yara tedavilerinde potansiyel kullanımı önermektedir.*

Anahtar Sözcükler*: Oleuropein, antimikrobiyal etki, olea europaea L., MIC, yaşlanma karşıtı.*

INTRODUCTION

Olea europaea L. (olive) is known as one of the oldest cultivated trees and healthiest natural vegetable oil sources in the world (1). According to one theory, the homeland of the olive tree is Southwestern Asia and Upper Mesopotamia, which includes Syria and Southeast Anatolia (2). Encompassing economical, agricultural, nutritional, and environmental aspects, *Olea europaea L.* type is represented by two varieties in Türkiye; *Olea europaea* L. var. *europaea* Zhukovsky and *Olea europaea* L. var. *Sylvestris* (Miller) Lehr (3). In Mediterranean countries olive groves are spread over large areas and olive products have wide usage areas like table oils, cosmetic and medical ingredients.

As the number of studies on olives increases, it has been revealed that not only olive oils but also all parts of the olive plant (leaf, flower, seed) contain important bioactive compounds. For example; olive leaves and olive flowers are very rich in phenols (oleuropein), flavanols (rutin), catechin (flavan-3-ols), oleoside, flavones and secoiridoid glycoside (4-7) compounds that extensively valuable and widely used in pharmaceutical industry. Amounts and varieties of bioactive compounds of olives are depending to their species, the climate, and the geographical location (8). Literature findings showed that oleuropein is the main phenolic compound from the leaves and has an increasing interest in recent years due to its beneficial contributions in health such as antitumoral, blood pressure-lowering, hypertension, antimicrobial, cardioprotective, anti-inflammatory, antioxidant, anti-cancer, anti-angiogenic and neuroprotective functions (9-11).

Although various extraction methods such as maceration, percolation, solid-liquid, ultrasound, microwave and Soxhlet extraction can be used, there is no optimized method for all types of polyphenols because of their complex structures. Moreover, the recent studies have shown that the variation and quality of the phenolic contents can be affected by the extraction, purification and separation methods (12). For this purpose, in many studies Soxhlet extraction is preferred as an extraction method that because of higher purification efficiency than other methods (13).

Herein this study, we aimed to investigate the antimicrobial and anti-aging effects of triterpenoid and polyphenolic compounds that extracted from *Olea europaea L*. leaves and flowers collected from Türkiye and extracted with hexane, chloroform, ethyl acetate, methanol and distilled water by Soxhlet extraction method.

MATERIALS and METHODS

Plant material, extraction, separation and identification methods

Olea europaea L. leaves and flowers have been collected from Culhalar village (Aydın, Türkiye) and dried under shade and open-air conditions.

The dried samples were ground via industrial type grinder into a particle size of 0.5 mm.

In order to reach the maximum efficacy of liquid-solid extraction; hexane, chloroform, ethyl acetate, methanol and distilled water have been tested. Extraction procedure was carried out with an automatic Soxhlet apparatus at 80°C. According to the thin layer chromatography results (data not shown), methanol extract showed the highest band diversity and thus selected for further separation and purification steps. The extracted compounds stored in dry and dark conditions until use. Stock solutions were prepared in DMSO (dimethyl sulfoxide) and filtered with 0.45 µM filter (EMD Millipore, Bedford, MA, USA).

For purification 4.4 g methanol extract of flower was subjected to the silica-gel column using a mixture of chloroform-methanol (95:5, 80:20, 70:30 v/v) solvent system and 160 fractions were collected into flasks. A total of 7 combining processes were carried out and purification processes were continued. At the end of the purification procedure, one molecule was separated and identified as Oleuropein aglycon (F1).

On the other hand, 5,64 gr leaf extract was subjected to the silica-gel column using a mixture of hexane-ethyl acetate (50:50, 40:60, 30:70, 20:80 v/v) solvent system and 50 fractions collected into flasks. A total of 8 combining processes were carried out and purification processes were continued. At the end of the purification procedure, three molecules were identified as Oleanolic acid (L1), Oleuropein (L2) and Ursolic acid (L3).

Oleuropein molecules were identified with the standard molecules by High Pressure Liquid Chromatography (HPLC) method. For this aim, the standard calibration curve of pure oleuropein molecule was conducted and samples were analysed at the same method. Briefly; HPLC analysis was performed on a Thermo Scientific Ultimate 3000 (ThermoFisher Scientific, Massachusetts, USA) plus photo diode array apparatus using and Hypersil™ ODS C18 (Thermo Scientific™) column. Isocratic elution was performed, and the mobile phase comprised 0.01% trifluoroacetic acid in water (60%) and methanol (40%). 20µl samples were injected with a flow rate of 1.2 mL/min and detected at wavelength of 223nm.

For identification the other molecules, Nuclear magnetic resonance (NMR) method was used with ^{1}H (proton) analysis which performed and identified in the Ege University EBILTEM NMR Satellite Laboratory.

Antimicrobial activity

Minimum Inhibitory Concentration (MIC)

Antimicrobial effects of the compounds were tested against to gram negative *Escherichia coli* O157:H7 (RSKK 234) and gram-positive *Bacillus cereus ATCC 10876* obtained from the Microbiology Department Culture Collection of Ege University, Faculty of Science.

Broth microdilution method was carried on 96-well plate system according to the guideline of Clinical and Laboratory Standard Institute (14). For this purpose, bacteria strains were growth on Muller Hinton Broth (MHB) for 24h at 37° C. After incubation, 0.1 mL growth medium transferred on Muller Hinton Agar (MHA) plates and incubated for overnight at 37° C. Isolated colonies were picked by sterile pipette tips and suspended in 0.85% saline solutions. Optical density of the bacterial solution was adjusted to 0.3-0.5 optical density (approximately 10^6 cfu / mL) by spectrophotometer. 5 µL bacterial suspension was added to each well contains 195 µL MHB with a different final concentration of pure compounds listed in Table-1.

Table-1. Purified compounds from *Olea europaea* and final concentrations tested for antimicrobial activity (L1, L2 and L3; leaf, F; Flower).

Molecule Code	Final Concentrations
L1	5- 25- 50 µg/mL
L2	5- 25- 50 µg/mL
L3	50- 250- 500 µg/mL
F1	50- 250- 500 µg/mL

A set of wells containing only bacteria suspension served as positive control. Furthermore, 0.5%, 2.5% and 5% of DMSO plus bacteria containing wells checked for the potential inhibitory effects of DMSO. Plates were incubated for 18h at 37 °C under aerobic condition. At the end of the incubation period, 20 µL of 1% TTC (2,3,5-Triphenyltetrazolium chloride) solution was added to the wells for the determination of microbial activity.

In vitro cell viability testing and collagen type-I level assessment

Adult human dermal fibroblasts (HDFa, Gibco, C-013-5C) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) FBS, 1 % (v/v) L-glutamine and 0.5 % (v/v) penicillin/streptomycin (100U/100 mg/ml) at 37 °C and 5% CO_2. *In vitro* cytotoxicity and IC_{50} of oleuropein were evaluated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) assay. In brief, HDFa cells were seeded in 96-well plates ($5x10^5$ cells/well) and cultured overnight in the cell culture medium at 37 °C and 5% CO_2. Followed, cells were treated with 1- 1000 µg/ml of oleuropein for 7-days. At the end of the incubation, 100 µL of 10% (v/v) MTT solution was added to each well and incubated for 4 h at 37 °C. Finally, 100 µL DMSO added to each well, and absorbance measurement was performed to measure cell viability at 570 nm via microplate reader (Multiskan™ GO, Thermo Scientific). Vitamin C, of which effectiveness in collagen production and cell proliferation has been proven in the literature, and DMSO were used as a positive control and negative controls in the MTT test, respectively. To assess the effect of oleuropein on collagen type-I levels, ELISA Kit (E-EL-H0869, Elabscience) was used by following the manufacturer's instructions.

Statistical analysis

The statistical analyses were conducted using Two-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test using Prism 8.3 software (GraphPad, San Diego, CA, USA), with a confidence interval of ±95%.

RESULTS and DISCUSSION

Purification and identification of polyphenolic compounds

After the extraction process the extracted compounds stored in dry and dark conditions until use. Stock solutions were prepared in DMSO and filtered with 0.45 µM filter (EMD Millipore, Bedford, MA, USA).

Oleuropein aglycon (F1), Oleanolic acid (L1) and Ursolic acid (L3) were detected by NMR with ^1H (proton) analysis which performed and identified in the Ege University EBILTEM NMR Satellite Laboratory.

Oleuropein molecule (sample L2) was detected with standard molecule by HPLC method which described above. The standard calibration curve of oleuropein was established between 28.4-1000 µg/mL concentration (Figure-1). Isolated oleuropein molecule was prepared with a concentration of 1000 µg/mL dissolved in HPLC grade methanol (Figure-2).

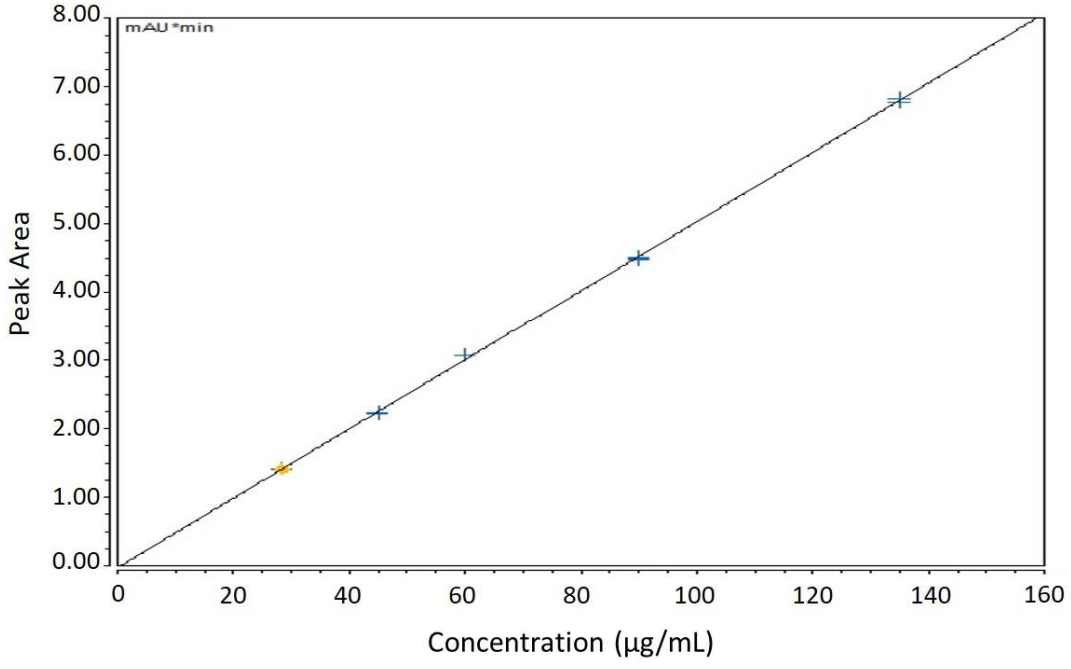
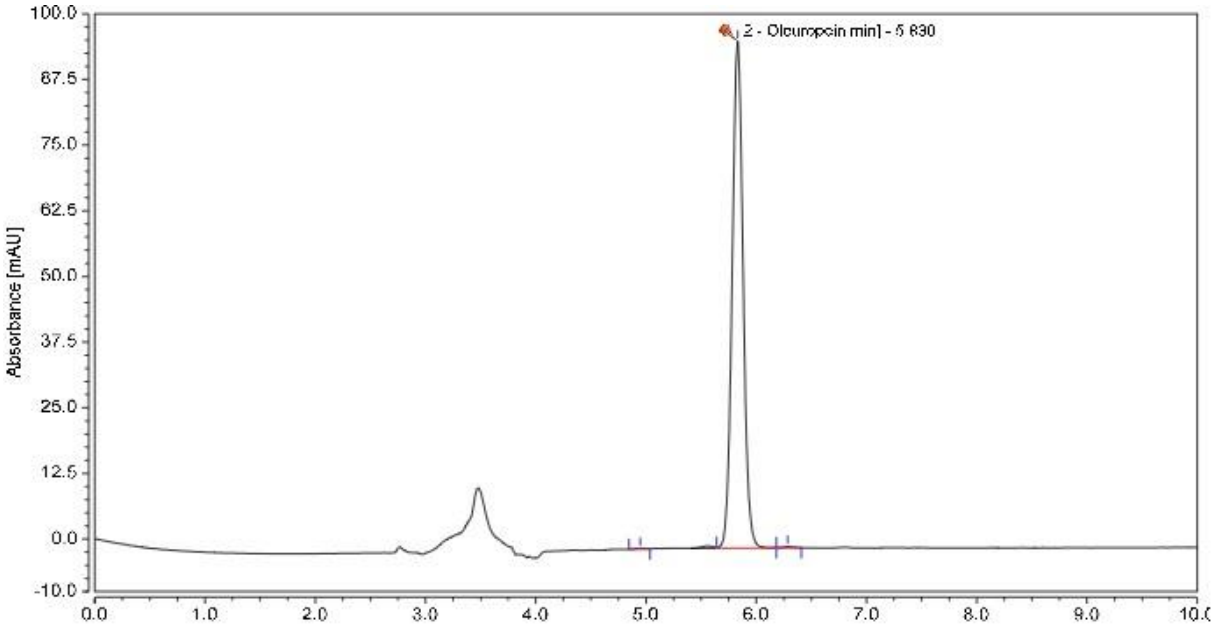


Figure-1. Standard calibration curve of pure oleuropein standard molecule. (R^2:0.9996)



No.	Peak Name	Retention Time	Area	Height	Relative Area
		min	mAU*min	mAU	%
UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1
1		4.947	0.01	0.113	0.09
2	Oleuropein	5.83	11.07	96.502	99.67

Figure-2. HPLC analysis results of the L1 sample with oleuropein standard calibration curve.

Table-2. The viability of the microorganisms after TTC solution addition. (nt: not tested) (+: positive results for formazan formation; -: negative results for formazan formation)

Molecule Names	***E.coli* O157:H7**					***B.cereus***				
Concentrations (µg/ml)	5	25	50	250	500	5	25	50	250	500
L1- Oleanolic acid	+	+	+	nt	nt	+	-	-	nt	nt
L2- Oleuropein	+	+	+	+	-	-	-	-	nt	nt
L3- Ursolic acid	nt	nt	+	+	+	nt	nt	+	-	-
F1- Oleuropein aglycon	nt	nt	+	+	+	nt	nt	+	+	+

Anti-Microbial Activity

According to the MIC results, none of molecules and concentrations showed antimicrobial activity against *E.coli* O157:H7 except Oleuropein (L2). As listed in Table-2, L2 sample showed an inhibition effect at 500 µg/mL concentration. In addition, Ursolic acid (L3) molecules reduced the viability and could show antimicrobial activity at concentrations higher than 500 µg/ml.

Topuz and Bayram (15) tested the antimicrobial activity of crude extract (CE), pure oleuropein (PO) and particularly purified oleuropein (PPO) molecules of olive leaves which collected from different location of Türkiye against *Escherichia coli* O157:H7, *Listeria monocytogenes, Salmonella typhimurium* and *Staphylococcus aureus* bacteria. According to their results, PO and PPO are more effective then CE samples. MIC values show differences between the microorganism groups and extraction methods. In summary, they identified the MIC value of PPO and PO as 12.5 mg/ml and 0.781 mg/ml against to *E.coli* and *S.aureus*, respectively. When compared with our results, L2 and L3 samples have a higher antimicrobial effect against *E. coli* O15:H7.

On the other hand, it has been determined that the Oleuropein (L2) molecule has an

antimicrobial effect on *B.cereus* even at a concentration of 5 µg/ml. While the Oleanolic acid (L1) molecule acts at 25 - 50 µg/ml, the L3 molecule appears to be effective at around 250 and 500 µg/ml. Moreover, it was observed that the molecules obtained from the olive flower did not show antimicrobial activity at the concentrations tested against both groups of microorganisms. Therefore, it was seen that the molecules obtained from the olive leaf are more effective against gr (+) *B.cereus*. In another study (16) olive leaf extract was added into pasteurized milk and tested for using as a potential natural preservative agent. They analyzed the extract with HPLC analyses and found out that oleuropein was the dominant compound. According to the agar well diffusion assay results, inhibition halos width of 6.75 ± 0.31 and 5.33 ± 0.17 mm were achieved with the concentrations of undiluted (at 1.44 mg/mL oleuropein) and diluted (1:2 v/v, at 0.72 mg/mL oleuropein) extracts against to *B.cereus*, respectively.

In order to control the inhibition effect of DMSO, pure DMSO was added to the wells with a final concentration of 0.5%-2.5% and 5% without active molecules. According to the results, it was observed that DMSO did not have an inhibitory effect against both organisms at the concentrations used.

Assessment of *in vitro* cytotoxicity and collagen level

Locating at the dermis layer of skin, fibroblasts are constantly exposed to various environmental insults. As they are responsible for the recovering and generating process of connective tissues, they were selected as main target for *in vitro* viability assessments in this study. Cytotoxicity assay was performed to evaluate the effect of various concentration of oleuropein on cell viability. Notably, although *in vitro* anticancer activity of oleuropein has been well characterized through various studies subjected breast cancer (17), hepatocarcinoma (18) or neuroblastoma (19) cancer cells, there are limited information about the effect of oleuropein on healthy cell lines. Herein this study, a dose-dependent effect on cell viability of HDFa was observed (Figure-3A) which was also in agreement with the 7-days of proliferation profile (Figure-3B).

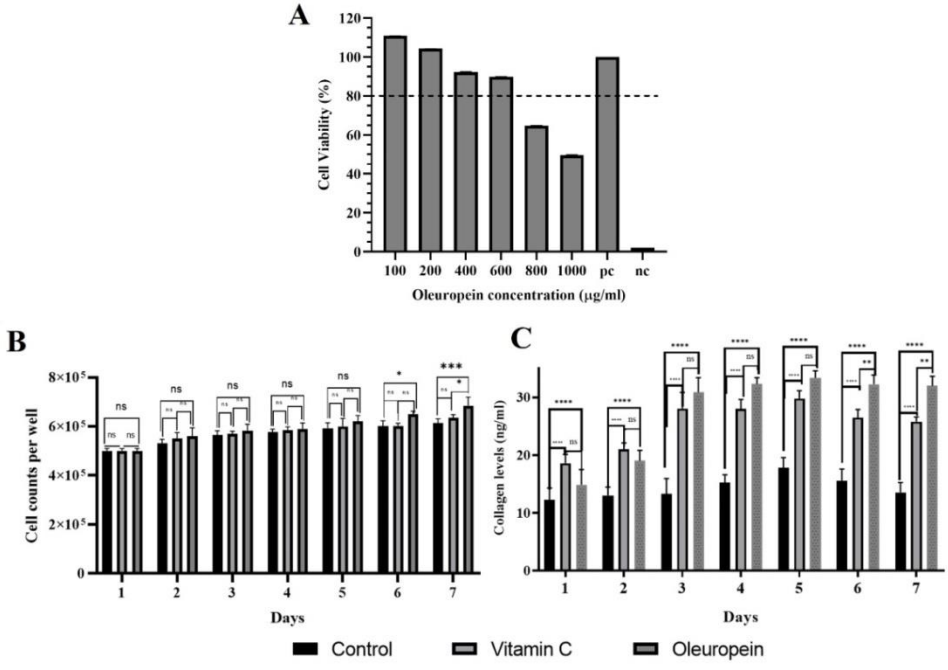


Figure-3. *In vitro* cytotoxicity and collagen type-I level assessment.

A) Cytotoxicity results of oleuropein (200 µg/ml).

B) Effects of oleuropein on proliferation of HDFa.

C) ELISA results representing the collagen type I levels in control (non-treated), vitamin C (200 µg/ml) and oleuropein (200 µg/ml) added groups. pc: positive control; nc: negative control; ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; Two-Way ANOVA, Multiple Comparison Test.

Bal et al. (20) also reported a dose-dependent cytotoxicity and proliferation phenomenon in healthy human bronchial epithelium cell lines, where they observed proliferation up to 1000 µg/ml. Moreover, on day 7, a higher rate of cell proliferation ($p<0.05$) was observed in the group treated with oleuropein (200 µg/ml) compared to the group treated with vitamin C (200 µg/ml), which is well-known for its function in stimulating collagen synthesis and cell proliferation (21, 22). Consisted with the cytotoxicity data, Goldsmith et al. (23), demonstrated that the application of oleuropein showed no effect on non-tumorigenic cells. Katsiki et al. (24), on the other hand, observed that the use of oleuropein resulted in a postponement of senescence-related characteristics, leading to an extension of the life span of human fibroblasts by around 15%.

Consistent with the cell proliferation and cytotoxicity data, ELISA results revealed that the collagen type-I levels were significantly higher than that of achieved with vitamin C treatment at day 6 ($p < 0.01$) and day 7 ($p < 0.01$**)** (Figure-3C). Overall, the increase in collagen type-I levels is thought to be associated with the tendency of oleuropeins to act as inhibitors of collagenase (25). Notably, besides the antioxidant, anti-inflammatory, and anticancer activities, the effectiveness of oleuropein has also been reported for wound healing studies considering such parameters like delaying senescence, reducing ROS levels, and showing increased proteasome activity (22). Moreover, the enhancement of wound healing through oleuropein treatment has been observed to entail a reduction in cell infiltration and improvement in the deposition of collagen fibers and re-epithelialization (26). Herein, the obtained data also indicates that oleuropein activates fibroblasts, leading to proliferation with a subsequent increase in collagen type-I expression. This achievement is attributed to the prevention of the deterioration of dermal skin due to aging and wrinkles, as tightly and well-organized collagen proteins support the mechanical interaction between fibroblasts. Thus, it has been concluded that the oleuropein molecule can be considered an effective stimulator for collagen expression and has great potential in anti-aging and tissue recovery studies. It has been emphasized that oleuropein, which exhibited a supportive effect on the proliferation of healthy fibroblasts and collagen synthesis in this study, also demonstrates inhibitory properties in studies conducted with cancer cells. This is shown to be associated with the excellent antioxidant and anti-inflammatory properties of oleuropein.

CONCLUSION

This work showed the antimicrobial effects of extracted molecules from *Olea europaea L.* leaves and flowers collected from Culhalar village (Aydin, Türkiye). In parallel with literature but at lower concentrations, especially for oleuropein molecule, showed inhibition effects on both gram positive and negative bacteria. Furthermore, oleuropein, which has been reported in the literature to selectively inhibit proliferation in cancer cell types, was found in the study to exhibit non-toxic properties on healthy dermal fibroblasts. Moreover, contrary to its effects on fibrotic tissues, it was demonstrated that oleuropein positively influences collagen type- I production in healthy cells. The observed contrasting mechanisms in cancer and fibrotic cells was attributed to oleuropein's sensitivity to ROS levels, selective toxicity on cell types and their collagenase inhibition capacities. Particularly, the presence of collagen type-I protein as a significant connective tissue fibril in the dermal layer of the skin, contributing to the structure of elastic fibrils and playing crucial roles in skin tightness and wrinkle formation, makes this finding noteworthy. The loss of elasticity in aging and wrinkled skin, parallel to the diminishing proliferative properties of fibroblasts and the decrease in the synthesis of collagen and other dermal matrix proteins, leads to adverse effects on the skin. In this context, it is possible to suggest that the application of the oleuropein molecule to human dermal fibroblasts under *in vitro* conditions has the potential to mitigate existing negative impacts, and this can be attributed to its ability to increase cell proliferation and stimulate collagen synthesis. Hence, these results support the usage of *Olea europaea L.* extracts as natural preservative agents in many application areas like cosmetic and by products through the selective activity of oleuropein molecules among healthy and damaged cells provide an excellent applicability property. The results of this study can be used for further studies to identify the mechanism of multifaceted effects of oleuropein and other phenolic compounds of *Olea europaea L.*

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