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Isolation of oleuropein from olive leaf by effective method and investigation of its antimicrobial properties

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Natural products play an important role in the production and development of food and medicine. In olive tree cultivation and olive processing industry, vast amounts of by-products are yielded. Olive leaves constitute the major by-product of olive industry. Olive leaf is potential source of phenolic compounds. Plant polyphenols have attracted great interest due to their antimicrobial activities. It was aimed to isolate the oleuropein compound from olive leaf and to identify its antimicrobial effects in this research. In the first stage, methanol extract was obtained from the olive leaf. After then, ethyl acetate extract (including oleuropein with 93.5% purity) procured from methanol extract. Ethyl acetate extract was subjected to chromatographic technique to obtain oleuropein in high purity (97.6%). The structure of oleuropein was determined by spectroscopic method including ^{1}H and ^{13}C NMR besides comparing isolated oleuropein with the standard commercially obtained. Antimicrobial activity of methanol extract, ethyl acetate extract and oleuropein was investigated on *Bacillus subtilis, Candida tropicalis, Lactobacillus brevis, Lactobacillus plantarum, Proteus vulgaris, Saccharomyces cerevisiae, Salmonella enteritidis* microorganisms by 96-well microtiter plate method. It was determined that MIC (Minimum Inhibitory Concentration) values of olive leaf products ranged between 1:1 (50 mg/mL) and 1:8 (6.25 mg/mL) for tested microorganisms.

Keywords: Antimicrobial, Minimum Inhibitory Concentration (MIC), Oleuropein, Olive leaf

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Olive trees are indigenous to tropics and temperate zones. They belong to *Olea europaea sativa* sub-species of *Olea europaea* of Olea genus of Oleaceae family with 29 genus and about 600 species[1,2]. Olive fruits are processed into table olives and olive oil worldwide. The rest of the olive trees and the waste materials of processing industry are considered as by-products of olive[3]. Majority of olive by-products are not used through technological processes. Disposal of them most of the time constitutes a cost item for growers. Olive leaves are accumulated during the truncature of olive trees, collection of olive fruits and processing olives into olive oil process (cleaning-blending). The quantity of leaves generated through pruning varies with the type of pruning and age of tree and such values vary between 12-30 kg/tree[4]. Olive leaves constitute about 10% of olive weight collected for oil extraction[5]. In some regions with intensive olive culture, olive leaves are used to feed livestock or used together with branches as fuel wood[4]. Olive leaves have many traditional uses. Olive leaves are used orally for stomach, intestinal diseases and bronchial asthma and chewed as a mouth cleanser. In addition to these, decoctions of leaves are taken for diarrhea and to treat urinary tract infections[6]. Studies conducted on humans and animals revealed hypoglycemic[7], antihypertensive[8], anticarcinogen[9], antioxidant[10], antimicrobial[11], anti-inflammatory[12] effects of olive leaves.

As it was in other phenolic compounds, oleuropein demonstrates its antimicrobial effect through destruction of bacterial membranes or peptidoglycan layer of the cells. Several researches have been carried out elucidate interactions between oleuropein and membrane lipids. The ortho-diphenol constitution of oleuropein has been indicated as the active factor

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for antimicrobial mechanism. However, this is not certain and the mechanism has not been fully elucidated, yet[13]. Since oleuropein scavenges free radicals, it has quite strong antimicrobial activity[14]. Such attributes of oleuropein were proved through *in vitro* and *in vivo* tests. Antioxidant mechanism of oleuropein is primarily attributed to existence of hydroxyl groups (particularly 1,2-dihydroxybenzen section). Hydroxyl groups donate their hydrogens to inhibit oxidation and neutralize free radicals. Oleuropein has metal chelating activity as well as free radical scavenging activity. Therefore, it can protect membranes against metal-induced lipid oxidation[15]. Majority of these activities of olive leaf come from oleuropein compound of the leaf. Therefore, recent studies mostly focused on production of supreme value-added compounds from olive leaves[16].

This study was conducted for extraction, purification and structural analysis of high value-added oleuropein compound from olive leaves and to determine antimicrobial activity of oleuropein against microorganism inducing intoxication and infections in humans and used as starter culture in foodstuffs.

Materials and Methods

Collection of samples

Olive leaves (Trilye cultivar) were collected from Manisa (Turkey) in December 2016. Collected leaves were dried, placed into polypropylene bags and brought to Plant Research Laboratory, Department of Chemistry in Tokat Gaziosmanpaşa University.

Bacterial culture conditions

Pathogen tests, test cultures of *Bacillus subtilis, Salmonella enteritidis, Proteus vulgaris* and *Candida tropicalis* strains were supplied from Department of Genetics and Bioengineering in Tokat Gaziosmanpaşa University. Starter cultures of *Lactobacillus brevis, Lactobacillus plantarum* isolated from pickles and commercial *Saccharomyces cerevisiae* were supplied from Department of Food Engineering in Tokat Gaziosmanpaşa University. Before the analyses, *S. enteritidis* (at 37±2°C), *B. subtilis, P. vulgaris* (at 30±2°C) and *C. tropicalis* (at 25±2°C) were developed in triptic soy broth (TSB) medium (Lab M, Lancashire, UK), *S. cerevisiae* (at 25±2°C) was developed in 2% glucose-containing nutrient broth (NB) medium (Merck, Germany), *L. brevis* and *L. plantarum* (at 30±2°C) were developed in de man rogosa sharpe broth (MRS) medium (Lab M, Lancashire, UK) for 18-24 h. A second activation process was applied to the cultures by applying the same conditions.

Oleuropein purificiation

Firstly, olive leaves were ground and 200 g were weighed into a vessel. It was extracted with methanol (3x1.0 L) for 15 h. After filtration, the solvent was removed under reduced pressure to yield the methanol extract. It was named as methanol extract. The extraction efficiency of the obtained methanol extract was calculated by specified Equation 1:

$$\frac{(S2-S1)}{S}\times 100 = \text{Equation 1}$$

Where;

S_2: Flask tare (g) + Methanol extract (g)
S_1: Flask tare (g)
S: Dried and ground olive leaf weight (g)

A sample of methanol extract (25 g) dissolved in distilled water (250 mL, 60°C) filtered to remove the insoluble particle and extracted with ethyl acetate. The ethyl acetate phase was separated with the help of seperatory funnel. After then, ethyl acetate solvent was removed by reduced pressure to yield ethyl acetate extract. This extract was named as ethyl acetate extract.

Ethyl acetate extract (3.0 g) was subjected to Sephadex LH-20 column chromatography. Methanol was used as the mobile phase. The flow rate of the methanol (mobile phase) was adjusted to 0.25 mL/min using a peristaltic pump (Longer pump, China). The fractions containing oleuropein were combined with the observation of thin layer chromatography (TLC) (Merck, Germany). After, solvent removed under reduced pressure. The resulting extract was named as purified oleuropein. Isolated oleuropein was determined as 97.6% purity with LC-MS/MS (Shimadzu, LCMS-8050, Japan) analysis.

Quantitative and structural analysis of oleuropein

Oleuropein quantity of the extracts were identified with the help of LC-MS/MS device. With methanol, 1 ppm methanol extract, ethyl acetate extract and purified oleuropein solutions were prepared. Obtained extract solutions were filtered by 0.22 μm (Millex-HV) membrane filter. Filtered extracts were taken into auto-sampler viols of LC-MS/MS. Shimadzu-brand LC-MS-8050 is equipped with SIL-30 AC auto-sampler, DGU-20A_{3R} degasser, LC-30 AD pump,

CTO-10AS colon furnace (40°C). Mobile phase A (5 mM ammonium acetate) and mobile phase B (methanol) were used in this study. Flow rate of LC was 0.4 mL/min. LC-MS/MS gradient system solvent concentration is provided in Table 1.

The structure of oleuropein isolated from olive leaf was elucidated by ^{1}H and ^{13}C NMR spectral analyses as well as comparing with the standard in LC-MS/MS analysis. Nuclear Magnetic Resonance (NMR) spectra were recorded on a spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR (Bruker Avence II NMR Spectrometer, USA). The spectral values of ^{1}H and ^{13}C were given in Table 4. Oleuropein (7 mg) was dissolved in deuterated dimethyl sulfoxide in special tube (20.3 cm) then, the tube was inserted to the NMR instrument for NMR analysis.

Minimum inhibitory concentrations (MIC) of the extracts and oleuropein

Antimicrobial activity of 10% solutions (prepared with distilled water) of methanol extract, ethyl acetate extract and purified oleuropein against pathogenic microorganisms (*B. subtilis, C. tropicalis, P. vulgaris, S. enteritidis*) and starter microorganisms (*L. brevis, L. plantarum and S. cerevisiae)* were determined with the use of "96-well MIC Plate" method. All MIC conditions were implemented and all analyzes were carried out according to the method of Topuz and Bayram[17].

Statistical analysis

Statistical analyses of data obtained during the research were carried out using the Duncan test by SPSS (version 20.0) program.

Results and Discussion

Extraction efficiency

The amount of olive leaf phenolic extract can be affected by many factors. Geographical region, type of olive tree, harvest time, environmental and climatic conditions are some of these factors[18].

Methanol extract efficiency of olive leaf was found as 54.42% in this study. Bilgin and Şahin[19] investigated the effects of geographical origin and extraction method on extraction efficiency. Leaves were collected from three olive trees of the same cultivar grown in the same region. Samples were collected from 6 different locations of Anatolia. It was reported that extraction efficiencies varied between 102.27-443.16 mg/g dry leaf (10.23-44.32%) in homogenizator-assisted extraction method and between 88.75-350.82 mg/g dry leaf (8.88-35.08%) in ultrasound-assisted extraction method.

Zeitoun *et al.*[20] applied 4 different pre-treatments (fresh, sun-drying, oven-drying, oven-drying before decoloring) to olive leaves and obtained leaf extracts with the use of three different solvents (70% ethanol, 70% methanol, water). It was reported that extraction efficiencies of olive leaves subjected to different pre-treatments varied between 22.36-32.03% in ethanol-extracted samples, between 20.03-32.91% in methanol extracted samples and between 17.96-23.17% in water-extracted samples. The greatest extraction efficiency (32.03%) was observed in methanol-extracted sun-dried samples.

Oleuropein quantity

The data for standard oleuropein quantification with LC-MS/MS device was provided in Table 2. Chromatogram for oleuropein standard was presented in Figure 1 and a sample chromatogram for olive leaf product was provided in Figure 2. In addition, Oleuropein amounts of olive leaf and extracts were given in Table 3. Oleuropein quantity of olive leaf was determined as 197.67 mg/g dry leaf. Such a value

Table 1 — LC-MS/MS gradient system solvent concentration

Time (min)	Ammonium acetate (%)	Methanol (%)
0	95	5
8	5	95
8-10.3	5	95
10.3	95	5
10.3-14	95	5

Table 2 — LC-MS/MS data for oleuropein standard

Data	Values
R^2	0.9956
Retention time (min)	6.4
MS, m/z M^-	538.90
MS/MS ion m/z	139.10, 149.10, 275.20

Table 3 — Oleuropein quantities of olive leaves and extracts

	Oleuropein quantity (mg/g)
Dry leaf	$197.67\pm6.89^{d*}$
Methanol extract	363.23 ± 12.65^{c}
Ethyl acetate extract	934.69 ± 27.40^{b}
Purified oleuropein	976.21 ± 12.44^{a}
	Oleuropein quantity (%)
Dry leaf	19.77 ± 0.69^{d}
Methanol extract	36.32 ± 1.27^{c}
Ethyl acetate extract	93.47 ± 2.74^{b}
Purified oleuropein	97.62 ± 1.24^{a}

*Means indicated with different small letters in the same column indicate significant differences ($p<0.05$).

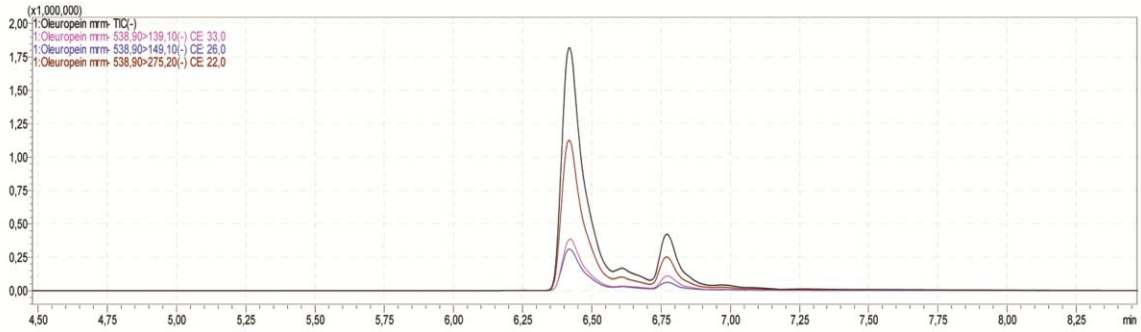

Fig. 1 — Chromatogram for oleuropein standard

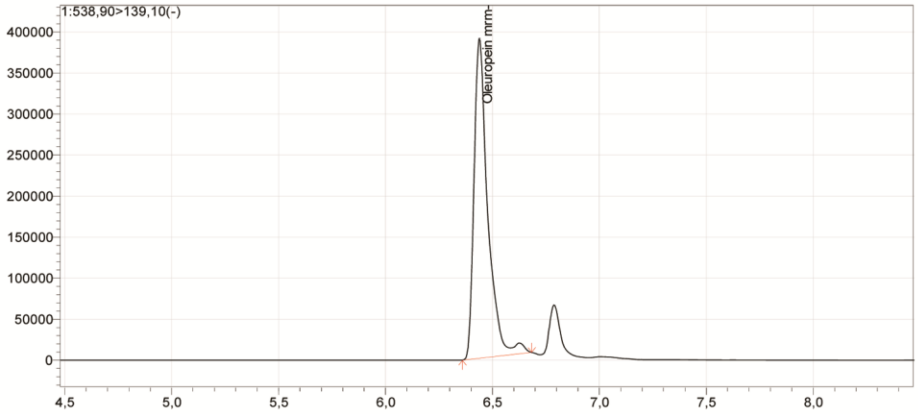

Fig. 2 — A sample chromatogram for olive leaf product

corresponded to 19.77% oleuropein content of olive leaf. Oleuropein quantity of methanol extract, ethyl acetate extract and purified oleuropein was respectively identified as 363.23 mg/g, 934.69 mg/g and 976.21 mg/g. Such values corresponded to 36.32%, 93.47% and 97.62% oleuropein contents, respectively. The differences in oleuropein quantities of purification stages were found to be significant ($p<0.05$). It was found that the amount of oleuropein increased due to the purification treatment.

Savournin *et al.*[21] reported oleuropein contents of dry leaves of 14 olive trees as between 9.0-14.3%. Similarly, Bouaziz and Sayadi[22] indicated varying oleuropein contents based on harvest time and reported oleuropein contents of dry olive leaves as between 12.4-14.2%. Jemai *et al.*[23] reported oleuropein content of dry leaves of Chemlali cultivar as 4.32%. Japón-Luján *et al.*[24] used dynamic ultrasound extraction method and reported oleuropein quantity of dry olive leaves as 22.61 mg/g (2.3%). Ansari *et al.*[25] reported oleuropein quantities of Iran-originated 7 olive trees as between 6.1-13.0 mg/g dry leaf. Mohamed *et al.*[26] determined phenolics of olive leaves obtained from 21 different olive trees of Southern Sudan with LC-MS and reported leaf oleuropein quantities as between 0.11-4.74 mg/g dry leaf. Şahin[27] extracted phenolics of olive leaves with Soxhlet method and used ethanol, methanol and water as solvent and reported ethanol, methanol and water-extracted oleuropein quantities respectively as 2.90 mg/g, 37.55 mg/g and 3.83 mg/g dry leaf. Şahin[27] also extracted olive leaf phenolics with the aid of supercritical CO_2, used ethanol, methanol and water again as the solvent and reported ethanol, methanol and water-extracted oleuropein quantities respectively as 2.90 mg/g, 14.24 mg/g and 10.91 mg/g dry leaf.

Elucidation of oleuropein

In ^{1}H NMR spectrum of oleuropein compound, the singlet peak observed at δ 7.52 belonged to H-5 proton. The doublet peak observed at δ 6.64 belonged to H-7' proton and had 8.0 Hz *ortho* coupling with H-8'. The singlet signal observed at δ 6.61 belonged to H-4' proton. The doublet appeared at δ 6.48 belonged to H-8 proton and had coupling with H-7' (J=8.0 Hz). One of the signals observed in downfield was H-8 proton. It splited into quartet with the neighboring methyl and resonated at δ 5.97 ppm (1H, q, J=8.0 Hz, J=2.0 Hz). Since the electrons current effect along π

bond of carbon-carbon double bond, alkene hydrogen was exposured to shielding effect and resonated at downfield. H-6 proton signal appeared as a singlet at δ 5.87 ppm. Since the protons connected to C-1' were diastereotopic, they had different chemical shift. While H-1'a proton yielded a signal at δ 4.12 (1H, m, Ha) ppm, H-1'b proton yielded a signal at δ 4.07 (1H, m, Hb) ppm. H-11 methoxy protons signal appeared as a singlet at δ 3.65. The signal of H-2' was observed as multiplet at δ 2.69 ppm (2H, m). The doublet observed at δ 2.65 ppm belonged to H-2a proton. H-2b proton gave the signal at δ 2.40 ppm. H-9 (methyl protons) protons resonated as a singlet δ 1.65 ppm. Anomeric proton peak appeared at δ 4.49 ppm (1H, d, J=8.0 Hz, H-1"). Glucose protons resonated between 3.09-3.71 ppm (Table 4). In ^{13}C NMR spectrum, observation of twentyfive signals accorded with the literature[28]. The chemical structure of the oleuropein molecule isolated from olive leaf is shown in Figure 3.

Antimicrobial effects of extracts and oleuropein on pathogen microorganisms

MIC values of methanol extract, ethyl acetate extract, purified oleuropein against the microorganisms causing intoxications and infections in humans and used as starter culture in foodstuffs are provided in Table 5.

Antimicrobials of plant origin are very important against pathogens and especially antibiotic resistant strains[29]. It was aimed to determine antimicrobial activity of methanol extract, ethyl acetate extract and purified oleuropein against *B. subtilis*, *C. tropicalis*, *P. vulgaris* and *S. enteritidis*.

MIC values of methanol extract, ethyl acetate extract and purified oleuropein for *B. subtilis* were determined respectively as 1:4 (12.5 mg/mL), 1:4 (12.5 mg/mL), 1:8 (6.25 mg/mL). Markin *et al.*[30] investigated inhibitory effect of water-extracted phenolics of olive leaves on *B. subtilis* (10^6 cfu/mL) and reported that 20% phenolic extract provided inhibition of *B. subtilis*. Zehra *et al.*[31] obtained extracts of some plant leaves against *B. subtilis.* It was determined that *Olea europaea* leaf extract showed the highest antimicrobial activity. In a study investigating the antimicrobial activity of olive leaf extract against *B. subtilis*, the MIC value of extract against microorganism was determined as 3.125 mg/mL[32]. In another study, the MIC values of B. *subtilis* ATCC 14579 against 4 different Tunisian olive leaf methanol extracts were determined as 64,

Table 4 — ^{1}H NMR (400 MHz, DMSO-d6) and ^{13}C NMR (100 MHz) spectral values of oleuropein compound

	^{1}H NMR values	^{13}C NMR values
1		171.1
2	2.65 (1H, dd, J=16.0 Hz, J=4 Hz, Ha), 2.40 (1H, dd, J=16 Hz, J=4.0 Hz, Hb)	34.2
3		30.6
4		108.2
5	7.52 (1H, s)	153.9
6	5.87 (1H, s)	93.5
7		129.7
8	5.97 (1H, q, J=8.0 Hz, J=2.0 Hz)	123.5
9	1.65 (3H, d, J=8.0 Hz)	13.5
10		166.7
11	3.65 (3H, s)	51.7
1'	4.12 (1H, m, Ha), 4.07 (1H, m, Hb)	65.5
2'	2.69 (2H, m)	34.2
3'		128.9
4'	6.61 (1H, s)	116.7
5'		145.6
6'		144.2
7'	6.64 (1H, d, J=8.0 Hz)	116.0
8'	6.48 (1H, d, J=8.0 Hz)	120.0
1"	4.49 (1H, d, J=8.0 Hz)	99.51
2"	3.09-3.11(m)	73.8
3"	3.17-3.21 (m)	77.0
4"	3.09-3.11 (m)	70.4
5"	3.17-3.21 (m)	77.8
6"	3.60-3.71 (m) 3.45-3.48 (m)	61.6

Table 5 — MIC values of extracts and purified oleuropein

Microorganisms (6 log cfu/mL)	Antimicrobials (mg/mL)		
	Methanol extract	Ethyl acetate extract	Purified oleuropein
B. subtilis	12.5	12.5	6.25
C. tropicalis	-	12.5	25
L. brevis	-	50	50
L. plantarum	-	50	50
P. vulgaris	12.5	12.5	12.5
S. cerevisiae	-	50	50
S. enteritidis	25	6.25	25

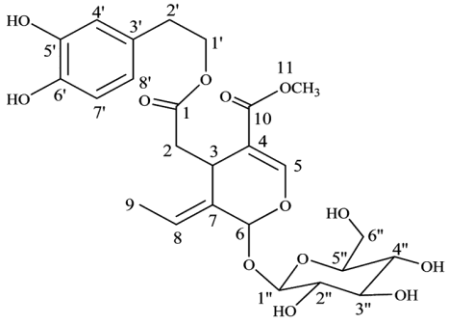

Fig. 3 — Oleuropein molecule isolated from *Olea europaea* leaves

32, 128, 128 μg/mL[33].

Antimicrobial effect of methanol extract on *C. tropicalis* was not detected. MIC values of ethyl acetate extract, and purified oleuropein for *C. tropicalis* were respectively, identified as 1:4 (12.5 mg/mL), 1:2 (25 mg/mL). Shialy *et al.*[34] investigated antimicrobial effect of olive leaf extract on *Candida* and *Aspergillus* species and indicated that olive leaf extract did not have any antimicrobial effects on *C. tropicalis*.

MIC values of methanol extract, ethyl acetate extract, and purified oleuropein for *P. vulgaris* were respectively, identified as 1:4 (12.5 mg/mL), 1:4 (12.5 mg/mL), and 1:4 (12.5 mg/mL). Gokmen *et al.*[35] reported MIC value of olive leaf extract on *P. vulgaris* as ≥16 mg/mL. On the contrary, Babker *et al.*[36] found that olive leaf extract (at 1-1.5-5 mg/mL levels) does not have any antimicrobial activity against *P. vulgaris.*

MIC values of methanol extract, ethyl acetate extract, and purified oleuropein for *S. enteritidis* were respectively, identified as 1:2 (25 mg/mL), 1:8 (6.25 mg/mL), and 1:2 (25 mg/mL). Liu *et al.*[11] indicated that 62.5 mg/mL olive leaf extract yielded inhibition of *S. enteritidis*. It was also determined that olive leaf extract inhibited biofilm formation of *S. enteritidis*. Lee and Lee[37] investigated antimicrobial effect of ethanol-extract of olive leaf and commercial oleuropein. In the same study, antimicrobial effect was not detected on *B. cereus*, *S. aureus* and *E. coli*, but olive leaf extract had antimicrobial effect on *B. cereus* besides *S. enteritidis*.

As it was in other phenolic compounds, oleuropein demonstrates its antimicrobial effect through destruction of bacterial membranes or peptidoglycan layer of the cells. Antimicrobial effect is characterized by functionality of exocyclic 8,9-olefinic[38] and mostly attributed to oleosins able to damage cell membranes due to surface-active characteristics[39]. Destruction of bacterial membrane functions is a common characteristic of phenolic compounds. Membrane destruction alters membrane permeability and generates loss of function in membrane-related enzymes. Loss of low-molecular weight metabolites from the cells is accompanied by protein and nucleic acids degraded by otolithic enzymes[40]. The mechanism of the antimicrobial activity of oleuropein has not been fully clarified, yet[41].

Antimicrobial effects of extracts and oleuropein on starter microorganisms

L. brevis, *L. plantarum* and *S. cerevisiae* are microorganisms used as starter in the fermentation process[42]. For example, it is known that microorganisms responsible for fermentation in olives, which are a product derived from vegetable raw materials, are initially lactic acid bacteria and in advanced stage, yeasts[43]. It was aimed to determine antimicrobial activity of methanol extract, ethyl acetate extract and purified oleuropein against *L. brevis*, *L. plantarum*, *S. cerevisia*e microorganisms involved in olive fermentation.

Methanol extract did not have antimicrobial effect on *S. cerevisiae*. MIC values of ethyl acetate extract, purified oleuropein for *S. cerevisiae* were respectively identified as 1:1 (50 mg/mL), 1:1 (50 mg/mL). Korukluoglu *et al.*[44] obtained the extracts of fresh olive leaves with the use of different solvents and investigated the antimicrobial effects of these extracts on some yeasts. Water-extracts did not have antimicrobial effects on test microorganisms. Test yeasts were sensitive to acetone and ethyl acetate-extracts and *S. cerevisiae* ATCC 9763 were identified as the most resistant yeasts. Schaide *et al.*[45] indicated that olive leaf extract promoted the development of *S. cerevisiae* and this strain could be used in fermentation of table olives with olive leaves.

Methanol extract did not have antimicrobial effect on *L. brevis*. MIC values of ethyl acetate extract, purified oleuropein for *L. brevis* were respectively identified as 1:1 (50 mg/mL), 1:1 (50 mg/mL). Olive leaf methanol extract did not have any antimicrobial effects on *L. plantarum*. MIC values of ethyl acetate extract, purified oleuropein for *L. plantarum* were respectively, identified as 1:1 (50 mg/mL), 1:1 (50 mg/mL). Ruiz Barba *et al.*[46] obtained extracts of alkaline-treated, heat-treated and untreated brined green olives and investigated the antimicrobial effects of water-extract solutions (4 mg/mL) on *L. plantarum* (10^6 cfu/mL). While 121°C 15 min heat-treatment yielded inhibition of bacteria, alkaline-treated oleuropein promoted the development of *L. plantarum*. Another study, the growth inhibitory activity of oleuropein compound against four *L. plantarum* strains isolated from different sources was investigated. It was determined that oleuropein at concentration level found in olive products does not inhibit the growth of *L. plantarum*. It was determined that oleuropein concentrations higher than 100 mM are needed to inhibit *L. plantarum* growth[47].

Conclusions

In this study, a fast, cheap and efficient method to isolate the oleuropein is provided. The ethyl acetate extract included the oleuropein of the same purity with the standard. After isolation of oleuropein by chromatographic technique such as Sephadex LH-20, it yielded the oleuropein which has the same purity with ethyl acetate extract. Hence, the oleuropein was yielded by extraction without column chromatography in a high yield (93.5%).

This study was conducted for extraction, purification and structural analysis of high value-added oleuropein compound from olive leaves and to determine antimicrobial activity of oleuropein against microorganisms inducing intoxication and infections in humans and used as starter culture in foodstuffs. In previous studies conducted in Turkey, only the oleuropein quantities of crude olive leaf extracts were determined and purification was not studied at all. In present study, crude extract (methanol extract) was obtained from olive leaves, then oleuropein of that extract was purified and physical and spectral characteristics of oleuropein were determined with the aid of NMR spectroscopy.

Olive leaf crude extract quantities of the present study were greater than the values of earlier studies. Considering the oleuropein quantities of the extracts, the greatest quantity was observed in purified oleuropein and it was followed by ethyl acetate extract and methanol extract. Increasing oleuropein quantities with the process of purification proved the accuracy of selected purification method. Such a case can also be proved through ^{1}H NMR and ^{13}C NMR analyses of purified oleuropein. Antimicrobial analyses revealed that methanol extract, ethyl acetate extract and purified oleuropein had antimicrobial effects on *B. subtilis*, *P. vulgaris* and *S. enteritidis* bacteria. For all tested microorganisms, ethyl acetate extract and purified oleuropein had greater antimicrobial activity than the methanol extract of olive leaves. It can be stated based on present findings that there were not much differences between antimicrobial effects of olive leaf extracts on gram-positive and gram-negative bacteria. While antimicrobial activity of methanol extract was not detected on *C. tropicalis*, antimicrobial activity of ethyl acetate extract and purified oleuropein were determined. Besides, antimicrobial activity of extracts and purified oleuropein against starter cultures used in food industry (*L. brevis*, *L. plantarum* and *S. cerevisiae)* were identified at 50 mg/mL upper most concentration. It was concluded based on present findings that methanol extract or oleuropein could be used in foodstuffs to increase their shelf lives and to improve functionality.

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Conflict of Interest

All authors declared that there is no conflict of interest.

Authors' Contributions

MB is the coordinating researcher. MB made significant contributions to the design and execution of the study. MB, ST, ME, CK, ŞK and AO contributed in designing and conduction of research. MB, ST, ME, CK, ŞK performed the experiments. MB, ST, RE, ŞK calculated, analysed and interpreted data. MB and ST attented in designing and writing the manuscript. RE and ŞK revised the manuscript for significant intellectual scope. MB gave final confirmation for the submission of revised version. All authors read, approved final manuscript.

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