

Research Article

Journal of Food Quality Evaluation of Effect of Extraction Solvent on Selected Properties of Olive Leaf Extract

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The quest for natural preservatives and functional foods with health benefits has seen an increasing demand for natural products having therapeutic value. Herein, we investigated the influence of ethanol, methanol, acetone (50%, 70%, and 90% v/v), and distilled water on selected properties of olive leaf extract and determined the yield, total phenolic content (TPC), antioxidant activity, and antimicrobial activity. Extracts were analyzed for their oleuropein, hydroxytyrosol, and tyrosol contents by high-performance liquid chromatography (HPLC). The highest extraction yield of 20.41% was obtained when using 90 vol% methanol, while the highest total polyphenol contents of 232 and 231 mg_{gallic-acid-equivalent}/100 g were obtained for 90 vol% methanol and 90 vol% ethanol, respectively. Antioxidant activity was determined using the α,α -diphenyl- β -picrylhydrazyl (DPPH) radical scavenging assay, by determining the ferric reducing antioxidant power (FRAP), and using the Fe²⁺-chelating activity assay, which provided the highest values when 90 vol% methanol was used (33.84%, 0.75, and 12.91%, respectively). HPLC analysis showed that the highest oleuropein contents corresponded to the extracts obtained using 90 and 70 vol% methanol (26.10 ± 0.20 and 24.92 ± 1.22 g/L, respectively), and the highest antimicrobial activity was observed for 90 vol% methanol and distilled water. Olive leaf extracts using 90 vol% methanol had high levels of polyphenols and were highly antioxidant and antimicrobial. The results of this study facilitate the commercial applications of natural extracts with antioxidant and antibacterial activities and are expected to establish a foundation for further optimization studies.

1. Introduction

The increasing demand for natural preservatives and new functional foods with health benefits has inspired numerous studies on biologically active compounds found in plant extracts and the by-products of plant processing [1–3]. Among these compounds, phenol derivatives (phenolics) exhibit a wide range of physiological effects, including anti-allergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, anticancer, cardioprotective, and vasodilatory activities [4]. Since phenolics are typically extracted from natural matrices or food industry by-products that are usually discarded or used for animal feed production [5], the influence of solvent on the extraction of phenolics from vegetable substrates has been extensively researched [6]. For example, solvent polarity is known to strongly affect extraction

efficiency and other parameters [7–9]. The differences in the structures of phenolic compounds determine the solubilizing abilities of solvents whose polarities are different. Therefore, the type of extraction solvent and separation procedure can have an important influence on the amounts of polyphenols extracted from plant substances. Although the phenolic contents of food have been widely investigated and extraction conditions optimized for antioxidant activity, some studies have shown that the optimum separation procedure normally depends on the characteristics of the plant [10, 11].

Olive (*Olea europaea*) fruit, oil, and leaves have a long-standing history of medicinal and nutritional use [12]. Olive leaves are a by-product of olive processing, accounting for up to 10% of the total olive weight, and are considered to be an inexpensive raw material source of antioxidant compounds [13]. Olive leaves have traditionally been used in

animal feed, but because they contain high-value compounds with antioxidant and antibacterial properties, they have recently been used as food additives, in functional foods and in pharmaceuticals. [14, 15]. Olive leaves, in particular, exhibit antioxidant, antihypertensive, and anti-inflammatory activities and are effective against hypoglycemia and hypocholesterolemia [16, 17]. The antioxidant activities of olive leaf extracts have been ascribed to the presence of phenolics such as oleuropein, luteolin, and hydroxytyrosol [18]. For example, oleuropein, the main component of olive leaf extract, exhibits antihypotensive, anti-inflammatory, and strong antioxidant activities [19–21]. Consequently, there is a growing interest in recovering phenolic compounds from olive leaves [22]. However, the conditions currently used for the extraction of biologically active compounds need to be improved in order to increase extraction efficiency, decrease extraction costs, and preserve functional activity in a better way [23]. In view of the above, in this study, we investigated the effect of the solvent (water, aqueous methanol, aqueous ethanol, and aqueous acetone) used to extract olive leaves on yield, as well as the antioxidant and antimicrobial activities of the extract.

2. Materials and Methods

Olive leaves were imported from Spain (Teetraum, Wollenhaupt Co., Ltd., Germany) and purchased through CJ mall in Korea. Oleuropein, hydroxytyrosol, and tyrosol standards, and α,α -diphenyl- β -picrylhydrazyl (DPPH) were obtained from the Sigma-Aldrich Chemical Co., Korea.

2.1. Preparing the Olive Leaf Extract. Distilled water (DW), aqueous ethanol (50, 70, and 90 vol%), aqueous methanol (50, 70, and 90 vol%), and aqueous acetone (50, 70, and 90 vol%) were used as extraction solvents. Typically, a mixture of dried olive leaf powder (5.0 g) and the solvent of choice (100 mL) were agitated in a shaking incubator at room temperature (25°C) and 250 rpm for 1 h and then centrifuged at 10000 rpm for 10 min. The supernatant was concentrated in vacuo at 50°C using a rotary evaporator, and the residue was freeze-dried.

2.2. Determining Extraction Yield. Extraction yield (%) was calculated as follows: $100\% \times m_{\text{Extract}}/m_{\text{Powder}}$, where m_{Extract} and m_{Powder} are the masses of the extract and olive leaf powder (g), respectively.

2.3. Determining Total Polyphenol Content (TPC). TPC was determined using a slight modification of the method reported by Wei et al. [24]. In brief, the test solution (100 μ L) was treated with the Folin–Ciocalteu reagent (100 μ L, 1 N) and incubated at room temperature for 3 min. The mixture was then treated with aqueous Na_2CO_3 (300 μ L, 1 N), incubated at room temperature for 90 min, and diluted with DW (1 mL). The absorbance of the resulting solution was measured at 725 nm using an OPTIZEN 2120 UV spectrophotometer (Mecasys Co., Ltd., Korea). A standard curve was prepared using 100,

250, 500, and 1000 ppm gallic acid, the results of which were used to calculate the TPC, which is expressed as milligrams of gallic acid equivalents (GAEs) per 100 g ($\text{mg}_{\text{GAE}}/100 \text{ g}$) of the sample. Gallic acid was used as the standard in these experiments. The linear equation for the gallic acid calibration curve can be written as follows: $y = 0.0057x - 0.2484$ ($R^2 = 0.991$), where y is the TPC and x is the absorbance value.

2.4. α,α -Diphenyl- β -Picrylhydrazyl Radical Scavenging Activity (DPPH), Ferric Reducing Antioxidant Power (FRAP), and Fe^{2+} -Chelating Activity. DPPH radical scavenging activity was determined using the method of Blois [25]. In brief, a solution of DPPH in MeOH (1 mL, 1.5×10^{-4} M) was added to the test solution (4 mL) with stirring, and the resulting mixture was incubated at room temperature for 30 min after which absorbance was measured at 517 nm using the abovementioned spectrophotometer.

FRAP was determined using a slightly modified method reported by Oyaizu [26]. In brief, samples were mixed with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $\text{K}_3\text{Fe}(\text{CN})_6$ (2.5 mL, 1% (w/v)). The obtained mixture was incubated at 50°C for 20 min and treated with trichloroacetic acid (2.5 mL, 10% (w/v)). The upper layer of the mixture (2.5 mL) was diluted with DW (2.5 mL) and FeCl_3 (0.5 mL, 0.1% (w/v)), after which absorbance was measured at 700 nm using the abovementioned spectrophotometer.

Fe^{2+} -chelating activity was determined using the method of Dinis et al. [27]. Briefly, 0.5 mL of the sample was mixed with a solution of FeCl_2 (2 mL, 1 mM) in 95 vol% ethanol. The reaction was initiated by the addition of aqueous ferrozine (2.5 mL, 2 mM), and the mixture was vortexed for 10 min, filtered through a nylon syringe filter (0.45 μ m), after which absorbance was measured at 562 nm using the abovementioned spectrophotometer. Fe^{2+} -chelating activity (%) was calculated as follows:

$$100\% \times (\text{absorbance control} - \text{absorbance sample}) / (\text{absorbance control}). \quad (1)$$

2.5. Quantitating Oleuropein by High-Performance Liquid Chromatography (HPLC). Oleuropein, hydroxytyrosol, and tyrosol were quantified by HPLC (Agilent 1100 series, USA) after sample filtration through a 0.45 μ m PVDF membrane filter (Pall Life Science). The mobile phase contained 5% formic acid (A) and methanol (B), and the following gradient was used: 5% B, then 15% B after 3 min, 25% B after 13 min, 35% B after 25 min, 45% B after 35 min, 50% B after 40 min, 100% B after 45 min, 5% B after 46 min, and re-equilibrate to the initial composition for 4 min. The flow rate was 0.9 mL/min, and elution was performed at room temperature. The injection volume was 10 μ L. A Supelcosil LC-ABZ column (250 mm \times 4.6 mm, 5 μ m) was used, and the absorbance detector was operated at 280 nm.

2.6. Determining Antimicrobial Activity. Total viable counts (TVC) were determined on 3M™ Petrifilm™ aerobic count plates (3M, Seoul, Korea) incubated at 35°C for 24 h.

Coliforms and *Escherichia coli* (*E. coli*) were determined on 3M™ Petrifilm™ *E. coli*/coliform count plates (3M, Seoul, Korea) after incubation for 24 h at 35°C. Colonies were identified and counted as per manufacturer's instructions.

2.7. Statistical Analysis. All experiments were performed in triplicate. Experimental data were analyzed by one-way analysis of variance using SPSS/PC Statistics 23.0 software (SPSS Inc., Chicago, IL, USA). The obtained results are presented as means with corresponding standard deviations. Tukey's multiple range tests were used to determine significant differences between mean values, and $P < 0.05$ was taken as an indicator of statistical significance.

3. Results and Discussion

3.1. Extraction Yields. Table 1 lists extraction yields and selected properties of the extracts obtained using each solvent. The highest extraction yields of 20.41 and 18.88% were observed for 90 vol% methanol and DW, respectively, while the lowest yield of 10.83% was observed for 90 vol% acetone, which is similar to the trend reported by Butsat and Siriamornpun; when olive leaves were extracted for 6 h using 80% methanol, 80% ethanol, 80% acetone, and DW, the highest extraction yield was observed for 80% methanol and the lowest for 80% acetone [28]. These yields were ascribed to the effect of solvent polarity on the solubilities of the extract components, i.e., proteins and carbohydrates are more soluble in water and methanol than in ethanol or acetone [29].

3.2. Total Polyphenol Content (TPC). Figure 1 shows the effect of solvent on TPC and reveals that the highest values of 231.98 and 230.61 $\text{mg}_{\text{gac}}/100 \text{ g}$ were obtained for 90 vol% methanol and 90 vol% ethanol, respectively. The lowest TPC of 192.03 $\text{mg}_{\text{gac}}/100 \text{ g}$ was observed for DW and was significantly different to the values obtained using the other solvents ($p < 0.05$). TPC was observed to decrease with increasing water content for each solvent, in agreement with previously reported results. Thus, when compared to the other extraction solvents, water results in a higher non-phenolic compound content (e.g., carbohydrates and terpenes) because some phenolic compounds soluble in methanol, ethanol, and acetone can be extracted through complex formation. Hence, compounds that contain more phenol groups or have higher molecular weights than simple phenols are found in the water extract [30]. Moreover, compounds extracted with methanol have been reported to exhibit higher antioxidant activities and phenolic contents than those prepared using other solvents [29, 31], which is consistent with the results of this study. Some researchers have revealed that methanol is typically preferred for the effective extraction of phenolic compounds from plants [32, 33] and that methanol decreases the degeneration of phenols in plant extracts by controlling polyphenol oxidative enzyme activity [34]. Moreover, Moudache et al. showed that the TPC content of an olive leaf extract increases with increasing organic content in the extraction solvent [15].

3.3. Antioxidant Activity. The effects of the various solvents on the DPPH radical scavenging activity, FRAP, and Fe^{2+} -chelating activity of the extracts are summarized in Table 1. The first of these parameters is primarily used to quantify the FRAP of natural antioxidants. The original violet color of the DPPH radically changes to yellow when reduced to the corresponding stable diamagnetic molecule. Consequently, determining DPPH radical scavenging activity by observing this color change allows one to characterize numerous samples within a short period, and the method is sensitive enough to detect active ingredients at low concentrations [35]. The highest DPPH radical scavenging activity of 33.84% was observed for 90 vol% methanol; however, the values of 32.97 and 33.27% obtained for 50 and 70 vol% methanol, respectively, were not significantly different ($P > 0.05$). For aqueous ethanol and aqueous acetone, statistically similar ($P > 0.05$) values of ~31% were observed, irrespective of water content. The lowest DPPH scavenging activity of 26.75% ($P < 0.05$) was observed for DW.

FRAP is a parameter that quantifies antioxidant activity related to the electron-donating capability of a molecule. The Fe^{3+} in $\text{K}_3\text{Fe}(\text{CN})_6$ is reduced to Fe^{2+} in the presence of an antioxidant, which results in the initial yellow test solution turning green or blue [36]. High FRAP values were obtained for all solvents in this study, with the exception of DW, and decreased in the order: aqueous methanol > aqueous acetone > aqueous ethanol >> pure water. The olive leaf methanol extract was determined to have strong antioxidant properties; hence, the compounds in this methanol extract are outstanding electron donors capable of terminating oxidation chain reactions by reducing oxidized intermediates to stable forms [37].

Determining Fe^{2+} -chelating activity relies on the ability of the extract to complex Fe^{2+} ions, thereby inhibiting the formation of the Fe^{2+} -ferrozine complex. The highest Fe^{2+} -chelating activity was obtained for 90 vol% methanol, while the lowest value was obtained for DW ($P < 0.05$). These findings show that the radical scavenging activity of the olive leaf extract depends on the polarity of the solvent used, in agreement with previous results [38]. Sepúlveda-Jimenez et al. demonstrated that extracts of the same plant origin obtained using methanol exhibited higher antioxidant activities than those extracted with water [39]. Franco et al. showed that the polarity of the extraction solvent strongly influences the extraction efficiency and the antioxidant activities of *Rosa rubiginosa* and *Gevuina avellana* extracts [40]. Fractions with different antioxidant activities could be separated on the basis of the polarity of the extracting solvent, with oxygenated compounds selectively extracted in accordance with their chemical structures, polarities, and solubilities [41].

3.4. Analyzing Olive Leaf Extract by HPLC. The oleuropein, hydroxytyrosol, and tyrosol contents of the olive leaf extracts were quantified by HPLC (Table 2, Figure 2), which revealed that 90 vol% methanol was best able to extract these phenolic compounds. Oleuropein has previously been identified as an important component of olive leaf extract [42, 43]. The

TABLE 1: Extraction yields and antioxidant activities of olive leaf extracts obtained using various solvents.

| Solvent | Extraction yield (%) | DPPH radical scavenging activity (%) | FRAP | Fe ²⁺ -chelating activity |
|----------|----------------------------|--------------------------------------|---------------------------|--------------------------------------|
| Ethanol | | | | |
| 50% | 17.55 ± 0.88 ^{bc} | 31.17 ± 0.10 ^b | 0.71 ± 0.01 ^{bc} | 9.78 ± 0.12 ^{bc} |
| 70% | 17.08 ± 0.77 ^{bc} | 31.68 ± 0.40 ^b | 0.69 ± 0.01 ^c | 10.93 ± 0.24 ^b |
| 90% | 17.44 ± 1.13 ^{bc} | 31.56 ± 0.19 ^b | 0.59 ± 0.02 ^d | 10.06 ± 0.07 ^{bc} |
| Methanol | | | | |
| 50% | 17.17 ± 0.25 ^{bc} | 32.97 ± 0.23 ^a | 0.75 ± 0.01 ^a | 8.81 ± 0.67 ^{cd} |
| 70% | 16.45 ± 0.64 ^{bc} | 33.27 ± 0.41 ^a | 0.76 ± 0.00 ^a | 9.68 ± 0.81 ^{bcd} |
| 90% | 20.41 ± 0.63 ^a | 33.84 ± 0.47 ^a | 0.75 ± 0.01 ^a | 12.91 ± 0.37 ^a |
| Acetone | | | | |
| 50% | 16.47 ± 0.84 ^{bc} | 31.83 ± 0.13 ^b | 0.72 ± 0.00 ^b | 9.12 ± 0.30 ^{cd} |
| 70% | 16.03 ± 1.29 ^c | 31.67 ± 0.35 ^b | 0.70 ± 0.00 ^{bc} | 8.38 ± 0.77 ^{de} |
| 90% | 10.83 ± 0.13 ^d | 31.90 ± 0.22 ^b | 0.72 ± 0.01 ^b | 9.19 ± 0.37 ^{cd} |
| 100% DW | 18.88 ± 1.83 ^{ab} | 26.75 ± 0.46 ^c | 0.64 ± 0.00 ^e | 7.22 ± 0.17 ^e |

Means with different superscripts (^{a-e} in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations from three replicates.

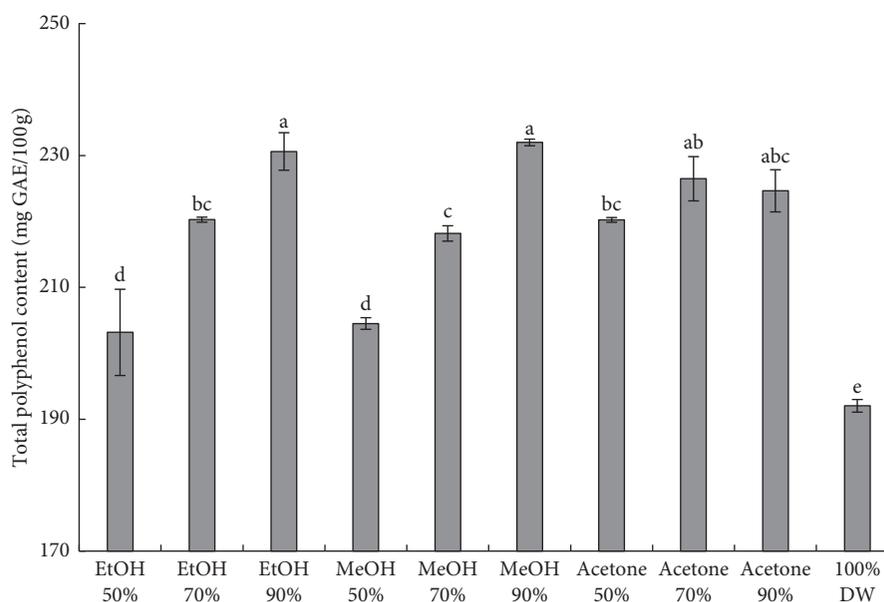


FIGURE 1: Effect of solvent on the TPC of olive leaf extract. Means with different superscripts (^{a-e} in the same column) differ significantly ($P < 0.05$). All values represent means ± standard deviations for three replicates.

highest oleuropein content of 26.10 g/L was obtained when extracted with 90 vol% methanol ($P < 0.05$), while the lowest content of 5.36 g/L was observed for DW, which is the same as the TPC trend. Hydroxytyrosol and tyrosol were detected in considerably smaller amounts, which is in agreement with previous results [44]. Thus, among the tested solvents, methanol was found to be most favorable for extracting oleuropein from olive leaves, which is in agreement with the findings of Bouaziz and Sayadi [18].

3.5. Antimicrobial Activity. Table 3 shows the antimicrobial activities of fractions extracted with various solvents, which reveals that antimicrobial activity decreases in the order: DW > 90 vol% methanol > 70 vol% methanol > 90 vol% ethanol > 90 vol% acetone. The above extracts were examined for their effectiveness against experimental microorganisms. All extract did not detect in coliform count plate and *E. coli*

count plate (data not shown). These findings are in agreement with the previously reported abilities of olive leaf extract to inhibit the growth of certain pathogenic bacteria [45, 46]. The observed antimicrobial activities are attributable to the phenolic contents of the extracts [47, 48]; the high contents of oleuropein and other phenolic compounds identified in the extracts contribute to the observed antibacterial properties. In this study, the total viable counts of the 90 vol% methanol, ethanol, and acetone extracts are low because of the high phenol contents of these extracts. Oleuropein has been reported to improve the production of nitric oxide in a dose-dependent manner (it is known to be cytotoxic to various pathogenic bacteria) in endotoxin-challenged mouse macrophages [49]. The effects of oleuropein and its derivatives contribute to the in vivo defense system against bacterial infection. The total phenolic content and the amount of oleuropein determined by HPLC in the DW extract were the lowest; however, this extract exhibited the highest antimicrobial

TABLE 2: Major phenolic compounds in olive leaf extracts obtained using various solvents.

| Solvent | Compound (g/L) | | |
|----------|----------------------------|---------------------------|----------------------------|
| | Hydroxytyrosol | Tyrosol | Oleuropein |
| Ethanol | | | |
| 50% | 0.61 ± 0.09 ^{abc} | 0.10 ± 0.00 ^{ab} | 19.72 ± 1.27 ^{bc} |
| 70% | 0.63 ± 0.12 ^{abc} | 0.11 ± 0.02 ^a | 21.96 ± 1.96 ^b |
| 90% | 0.62 ± 0.09 ^{abc} | 0.07 ± 0.01 ^c | 21.89 ± 1.13 ^b |
| Methanol | | | |
| 50% | 0.72 ± 0.05 ^{abc} | 0.07 ± 0.00 ^c | 19.47 ± 0.85 ^{bc} |
| 70% | 0.77 ± 0.03 ^a | 0.08 ± 0.01 ^{bc} | 24.92 ± 1.22 ^a |
| 90% | 0.74 ± 0.02 ^{ab} | 0.07 ± 0.00 ^c | 26.10 ± 0.20 ^a |
| Acetone | | | |
| 50% | 0.54 ± 0.07 ^{bc} | 0.08 ± 0.00 ^c | 17.70 ± 0.47 ^c |
| 70% | 0.52 ± 0.06 ^c | 0.03 ± 0.01 ^d | 17.66 ± 0.64 ^c |
| 90% | 0.61 ± 0.10 ^{abc} | 0.02 ± 0.00 ^d | 17.79 ± 0.48 ^c |
| 100% DW | 0.25 ± 0.02 ^d | 0.03 ± 0.00 ^d | 5.36 ± 0.78 ^d |

Means with different superscripts (^{a-d} in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations for three replicates.

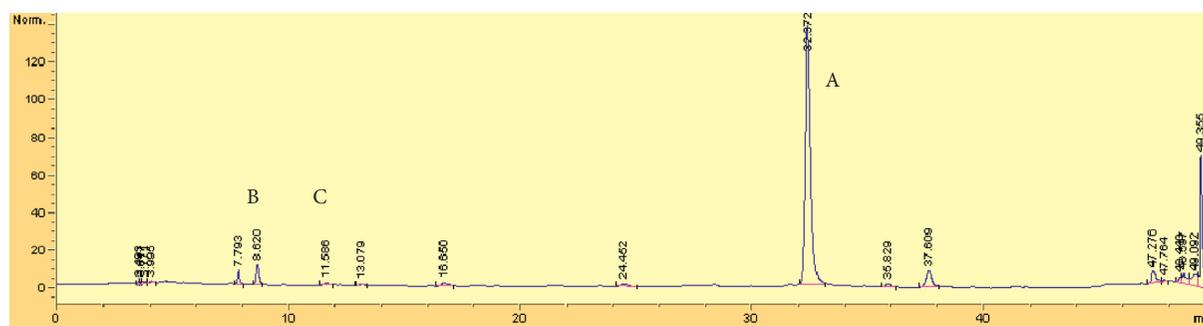


FIGURE 2: HPLC trace of phenolic compounds extracted from olive leaves with 90 vol% methanol: (A) hydroxytyrosol, (B) tyrosol, and (C) oleuropein.

TABLE 3: Total viable counts (TVCs) in olive leaf extracts obtained using various solvents.

| Solvent | Antimicrobial (CFU/mL) |
|----------|-------------------------------|
| Ethanol | |
| 50% | 771.67 ± 34.03 ^b |
| 70% | 756.67 ± 91.70 ^b |
| 90% | 145.00 ± 56.35 ^c |
| Methanol | |
| 50% | 598.33 ± 117.30 ^b |
| 70% | 116.67 ± 2.89 ^c |
| 90% | 36.67 ± 18.93 ^c |
| Acetone | |
| 50% | 1070.00 ± 105.00 ^a |
| 70% | 780.00 ± 173.86 ^b |
| 90% | 165.00 ± 17.32 ^c |
| 100% DW | 33.33 ± 16.07 ^c |

Means with different superscripts (^{a-c} in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations for three replicates.

activity of 33.33 CFU/mL. According to previous reports, antimicrobial activity is not only related to the total phenol content, but also to the types and relative distributions of the phenolic components, which are important for biological activity. Bacterial resistance is also related to the structure of the polyphenol. Therefore, the compounds in the DW extract need to be identified through further studies.

4. Conclusions

We determined optimal conditions for olive leaf extraction by examining the effect of extraction solvent on selected extract properties. The highest extraction efficiency of 20.41% was obtained using 90 vol% methanol, while the lowest value of 18.88% was obtained using DW, and the highest total polyphenol contents were obtained with 90 vol% methanol and 90 vol% ethanol, while the lowest was obtained using DW ($P < 0.05$). The highest antioxidant activity was observed for the extract obtained using 90 vol% methanol, and the oleuropein content was highest when 90 and 70 vol% methanol were used as the extraction solvents. Finally, extracts with the highest antimicrobial activities were obtained using 90 vol% methanol and DW. Thus, we conclude that 90 vol% methanol is the optimal extraction solvent, affording extracts with high antioxidant and antibacterial activities in high yields. The results of this study are expected to be of importance for the development of a wide range of products based on olive leaf extract.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

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