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# Comparison of biochemical and antioxidant activities of ultrasonic-assisted extraction with different solvents in olive leaf

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## Abstract

Olive leaves are considered to have great potential as natural sources of antioxidants and phenolic compounds. In this study, dried olive leaves were extracted using four different solvents (water, methanol, ethanol, and 80:20(v/v) methanol-water) with ultrasonic-assisted extraction. The biochemical (total phenolics and flavonoids, total protein, free amino acids, total soluble, and reducing sugars) and antioxidant activities (CUPRAC, DPPH, FRAP, and FIC) of these extracts were evaluated. Total phenolics content was significantly affected by the different solvents and the highest total phenolics content was obtained in methanol-water (234 mg $g^{-1}$) extraction. The highest total flavonoid (47 mg $g^{-1}$) and total protein (5,1 mg $g^{-1}$) content were obtained in methanol extraction. Yield of the free amino acids was lowest in ethanol (1,5 mg $g^{-1}$), while it was highest in water (2,3 mg $g^{-1}$) and methanol-water (2,2 mg $g^{-1}$) extractions. The highest total soluble sugars were obtained from methanol-water (70,4 mg $g^{-1}$) and ethanol (65,4 mg $g^{-1}$) extractions, while the highest total reducing sugar contents were obtained from methanol (112,2 mg $g^{-1}$) and methanol-water (111,6 mg $g^{-1}$). While methanol-water extraction showed the highest antioxidant capacity with 0,63 mmol TR $g^{-1}$ CUPRAC value, it also showed the strongest radical scavenging activity with 1,09 mmol TR $g^{-1}$ DPPH radicals value and 0,065 mmol TR $g^{-1}$ FRAP potential value. FIC capacity was higher in water than in other solvent extraction methods. Methanol and methanol-water solvents were the most effective solvents for measuring phenolic and antioxidant activities in olive leaves.

## Introduction

Olive (*Olea europaea* L.), belonging to the Oleaceae family, is an evergreen tree with hard branches, grayish bark, and high-value oil, used in the food, medicine, and cosmetic industries. Since olive leaves are cheap, edible and considered a source of various biologically active compounds (Şahin & Şamlı, 2013), they can be used as a by-product after harvesting, and oil extraction of olive fruits. Leaves are lanceolate, positioned opposite to each other with short-stalked mucronate, green top, and hoary bottom surfaces (Castejón et al., 2020). Olive leaves have been used in traditional medicine for the treatment of illnesses. The dried leaf decoction is used to treat diabetes (Alarcon-Aguilara et al., 1998), and the leaf extracts are used to treat stomach and intestinal conditions as well as to clean the mouth (Bellakhdar et al., 1991). They also have been used to treat hypertension and induce diuresis from fresh leaf extracts (Pieroni et al., 1996; Ribeiro et al., 1986). Due to the phenolic and flavonoid compounds they contain, olive leaves have demonstrated that they exhibit antimicrobial activity, including antiviral, antifungal,

and antibacterial effects against various pathogenic microorganisms, as well as containing a number of biologically active compounds that support health-promoting effects (Sánchez-Gutiérrez et al., 2021; Topuz & Bayram, 2022).

Metabolites in the leaves have crucial roles in fulfilling the vital functions of the olive tree (Papoti & Tsimidou, 2009), and olive leaves contain many pharmacologically active compounds, especially antioxidants. Olive leaves have gained increasing attention from the scientific and industrial community due to their rich oleuropein content. Secoiridoids and flavonoids other than oleuropein may contribute to antioxidant activity in leaf extracts (Goulas et al., 2010). Antioxidants typically have the ability to delay or prevent the initiation and spread of oxidative chain reactions. Phenolic and flavonoid compounds from plants are an antioxidant class that acts with free radical scavenging ability because the formation of free radical derivatives during the oxidation process and the propagation chain of stable derivatives during this reaction are inhibited by antioxidant activities (Castejón et al., 2020). Additionally, these compounds function as metal chelators to inhibit the production of hydroxyl radicals (Gouvinhas et al., 2017).

The yields of many beneficial biological components in plant samples are largely dependent on the extraction method and period. The effectiveness of extraction is affected by changes in the extraction parameters (type of extraction solvent, solvent concentration, extraction time, and extraction temperature) (Xu et al., 2017). The biochemical content and antioxidant capacity of plant extracts can be used with maximum efficiency when the extraction parameters are optimized (Chew et al., 2021). Plant extracts consist of different active biological component structures, physicochemical properties, and polarities, and these properties affect extraction efficiency. Therefore, biochemical contents and antioxidant capacities cannot be determined by using a single universal method. Many methods are used for the extraction of biochemical and antioxidant components from leaves, but solvent extraction is the most widely method (Wissam et al., 2016). Several solvents have been used to extract the polyphenols from the samples effectively. It has been reported in many studies that the most efficient extraction of polyphenols is usually obtained from polar solvents rather than non-polar solvents (Liu et al., 2007; Wissam et al., 2016). Therefore, water and organic solvents (methanol and ethanol) are commonly used in the extraction of samples. Consequently, the specification of optimum extraction parameters for each sample is required for the correct determination and evaluation of biochemical contents and antioxidant capacities. While determining the extraction yield, the cost, safety, and environmental effects of the process should also be considered. The use of conventional extraction methods has begun to decline because of the following reasons: i) time-consuming extraction protocols of plant samples; ii) the loss of some phenolic and flavonoid compounds due to oxidation caused a decrease in antioxidant capacity and ionization (Şahin & Şamlı, 2013). The ultrasonic-assisted extraction technique can be used as an alternative, which has proven to be more effective than other techniques in recent years (Chew et al., 2021).

Although studies on the antioxidant capacities of olive leaves and fruits are plentiful (Dobrinčić et al., 2020; Goulas et al., 2010; Topuz & Bayram, 2022), and have received increasing attention, there are few studies on the effect of ultrasonic-assisted extraction with different solvents on the biochemical content and antioxidant capacity (Ahmad-Qasem et al., 2013; Şahin & Şamlı, 2013). Therefore, the aim of the study was to investigate to what extent the biochemical content and antioxidant capacity are affected by ultrasonic-assisted extraction with different solvents of dried olive leaves, which are sources of bioactive components and can be used for commercial purposes throughout the year.

## Materials and Methods

### Plant material

The leaves of Memecik variety were used in the study. Olive leaves were collected from an orchard in Karatepe village (37° 55´ N, 28° 05´ E), Köşk, Aydın in March 2021. The olive trees in the orchard were irrigated with flood irrigation in summer. Leaf samples were collected between 9 and 10 AM and branches containing mature leaves of 1-year old shoots were selected from the entire perimeter of the trees to minimize environmental and orientation variability in the samples. After sampling, the leaves were immediately cleaned of dust and then dried at room temperature (RT) for 14 days. Finally, the dried samples were placed in airtight opaque glass jars and stored in a dry, dark, and cool place until analysis.

### Chemicals

Methanol (99%), ethanol (99%), and glycerol (99%) were purchased from ISOLAB Laborgeräte GmbH (Isolab, Germany). (±)-Catechin hydrate, phenol, sulfuric acid (%99), sodium borate, 2-cyanoacetamide, copper(II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), neocuproine (Nc-$C_{14}H_{12}N_2$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron(II) chloride and ferrozine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium carbonate, sodium nitrate, aluminum chloride, sodium hydroxide, ninhydrin, sodium citrate, *L*-valine, potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), gallic acid, trichloroacetic acid (TCA) were purchased from Merck (Merck Company, Darmstadt, Germany). All other chemicals used were analytical grade and obtained from either

Sigma-Aldrich or Merck.

**Preparation of leaf extracts**

Ultrasonic-assisted extraction of olive leaves with four different solvents (water, methanol, ethanol, and methanol-water (80:20, v/v) was carried out in accordance with a method described by Zhang et al. (2009) with minor modifications. Prior to extraction of the dried leaves, the samples were powdered with an electric grinder (SCM 2934, Sinbo, Türkiye) and the obtained powder was immediately used for extraction. The samples (1 g) were extracted in an ultrasonic bath system (LAB.ULT.4045, interior dimensions: 300 mm × 150 mm × 100 mm) using 40 mL of solvent. General extraction parameters were: temperature: 70 °C; time: 10 min; solid/solvent ratio: 1:40 (w/v), maximal nominal output power: 150 W, and ultrasonic frequency: 40 kHz. The extracts were then filtered through a 0,45 µm PTFE filter (Isolab, Germany) and filtered aliquots were stored in the dark at -20 °C until further analyses.

**Determination of total phenolic and flavonoid contents**

The total phenolics of the samples, extracted with different solvents, were determined using the modified Folin-Ciocalteau method (1927). The extract solution (0,1 mL) was mixed with 2,5 mL of deionized water and 0,1 mL of Folin-Ciocalteu reagent (Merck Company, Darmstadt, Germany), and the reaction was terminated using 0,5 mL of 20% sodium carbonate. The reaction mixture was incubated at RT for 30 min in the dark and the absorbance was measured at 760 nm with a UV–vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The standard curve was prepared using different concentrations of gallic acid (GA).

Total flavonoids were measured by the aluminum chloride reaction (Sakanaka et al., 2005). The extract solution (0,25 mL) was mixed with 1,25 mL of deionized water, and 75 µL of 5% sodium nitrate. After 6 min, 0,15 mL of 10% aluminum chloride was added; after 5 min, 0,5 mL of 1 M sodium hydroxide was added. The absorbance of all the sample solutions against a blank was measured at 510 nm and (±)-catechin concentrations were used to construct the standard curve.

**Determination of protein and free amino acids contents**

The protein content in the different solvent extracts was measured by the Bradford method (Bradford, 1976). The extract solution (0,1 mL) was mixed with 3 mL of Bradford reagent and gently vortexed. The reaction mixture was incubated for 5 min at RT for the color formation and the absorbance was measured at 595 nm with a UV–vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The standard curve was prepared using bovine serum albumin (BSA).

Total free amino acids were measured by the ninhydrin method (Lee & Takahashi, 1966). The extract solution (0,1 mL) was mixed with 1,9 mL of reaction solution (0,5 mL 1% ninhydrin + 1,2 mL glycerol + 0,2 mL 0,5 M sodium citrate). The reaction mixture was boiled in a water bath for 12 min, and cooled in an ice bath and absorbance was measured at 570 nm. Total free amino acids content was calculated using a standard curve prepared with *L*-valine.

**Determination of total soluble and reducing sugar contents**

Total soluble sugar was measured by the phenol-sulphuric method (Dubois et al., 1956). The extract solution (1 mL) was mixed with 0,5 mL of 5% phenol and 2,5 mL of concentrated sulfuric acid. The reaction mixture was incubated for 10 min at RT for the color formation and the absorbance of all the sample solutions against a blank was measured at 490 nm. Total soluble sugar content was calculated using a standard curve prepared with glucose.

Total reducing sugar was measured using the Somogy-Nelson method (Somogyi, 1952). An aliquot of each extract (1 mL) was mixed with 2,5 mL of sodium borate (100 mM, pH 9.0) and 0,5 mL of 1% 2-cyanoacetamide and vortexed well. The reaction mixture was boiled in a water bath for 12 min, and cooled in an ice bath and absorbance was measured at 280 nm. A standard curve prepared with glucose.

**Cupric Reducing Antioxidant Capacity (CUPRAC) method**

The CUPRAC test was performed according to the method of Apak et al. (2006). CUPRAC reactions were set up as follows: 1 mL of 0,01 M copper(II) chloride, 1 mL of 0,0075 M neocuproine solution, and 1,0 mL of 1 M ammonium acetate buffer solution (pH 7.0) were added successively into a glass tube. Subsequently, X mL of extract solution and "1,1 – X" mL deionized water were added to obtain a total volume of 4,1 mL and mixed well. Absorbance against a reagent solution without a sample was measured at 450 nm after 30 min. The antioxidant activity was calculated as Trolox equivalents per g of dry weight (mmol TR $g^{-1}$ DW), through a calibration curve with Trolox standard.

$$CUPRAC\ (mmol\ TR\ g^{-1}) = \frac{A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

Where; *A*: Sample absorbance measured at 450 nm; $\varepsilon_{TR}$: molar absorption coefficient of TR compound in the CUPRAC method (1,67 × $10^4$ L $mol^{-1}$ $cm^{-1}$); *Vm*: Total volume of CUPRAC method measuring solution; *Vs*: Sample volume (mL); *Df*: Dilution factor (if needed); $V_E$: Volume of the prepared extract (mL); *m*: The amount of sample taken in the extraction process (g).

**2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging method**

DPPH radical-scavenging activity was measured according to Bener et al. (2022). DPPH method was applied as follows: X mL of extract solution, "2 – X" mL

99% ethanol, and 2 mL of 0,2 mM of DPPH• solution were added to a glass tube and mixed well. The reaction mixture was incubated at RT in the dark for 30 min. The absorbance of the samples was measured at 515 nm against ethanol with a UV-vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan). The free radical-scavenging activity was expressed as Trolox equivalents per g of dry weight (mmol TR $g^{-1}$ DW) and calculated according to the following equation:

$$DPPH\ (mmol\ TR\ g^{-1}) = \frac{\Delta_A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

where $\varepsilon_{TR}$: molar absorption coefficient of TR compound in the DPPH method (2,16 × $10^4$ L $mol^{-1}$ $cm^{-1}$), $V_s$: sample volume, $V_m$: total volume of method, *Df*: dilution factor (if needed), $V_E$: extract volume and, *m* is the mass of dry extract.

**Ferric reducing antioxidant power (FRAP) method**

The ferric reducing antioxidant power was measured according to the method of Berker et al. (2007). The following reactions were set up to measure FRAP activity: X mL of extract solution, "1 – X" mL 96% ethanol, 2,5 mL of 0,2 M phosphate buffer (pH 6.6), and 2,5 mL of 1% potassium ferricyanide solution were added to a glass tube and incubated for 20 min in a water bath at 50 °C. After incubation, 2,5 mL of 10% trichloroacetic acid (TCA) was added, and thoroughly mixed. An aliquot of 2,5 mL was mixed with 2,5 mL of distilled water and 0,5 mL of 0,1% ferric chloride solution; then the absorbance of the resulting Prussian blue solution was measured at 700 nm after 2 min against a reagent blank. Ferric reducing antioxidant power activity was expressed as Trolox equivalents per g of dry weight (mmol TR $g^{-1}$ DW) and calculated according to the following equation:

$$FRAP\ (mmol\ TR\ g^{-1}) = \frac{A}{\varepsilon_{TR}} x\ \frac{V_m}{V_s}\ x\ D_f\ x\ \frac{V_E}{m}$$

Where; *A*: Sample absorbance measured at 700 nm; $\varepsilon_{TR}$: molar absorption coefficient of TR compound in the FRAP method (1,77 × $10^4$ L $mol^{-1}$ $cm^{-1}$); *Vm*: Total volume of FRAP method measuring solution; *Vs*: Sample volume (mL); *Df*: Dilution factor (if needed); $V_E$: Volume of the prepared extract (mL); *m*: The amount of sample taken in the extraction process (g).

**Ferrous ion-chelating (FIC) method**

The ferrous ion-chelating ability was determined in accordance with a method described by Decker & Welch (1990) with minor modifications. FIC reactions contained 1 mL of extract solution, 3,7 mL of distilled water, and 100 µL of 2 mM iron(II) chloride. The reaction was initiated by the addition of 200 µL of 5 mM ferrozine. The reaction was well mixed and incubated for 20 min at RT. After incubation, absorbance was determined at 562 nm against a blank. Distilled water (1 mL) was used as a blank instead of the ferrozine solution, which helped with error correction due to the uneven color of the sample solutions. FIC was calculated as follows:

$$FIC\ (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

where $A_0$ was the absorbance without samples and $A_1$ was the absorbance of the sample.

**Statistical analysis**

All analyzes were performed with three replicates. Results were subjected to analysis of variance (ANOVA) using SPSS Statistics 22.0 software (IBM, Armonk, NY, USA). Duncan's multiple range test (Duncan, $P \leq 0.05$) was used to separate significant differences between the means. To show the relationship between the biochemical contents and antioxidant capacities, Pearson's linear correlation analysis (Heatmap correlation) was calculated using OriginPro software (version 2021, OriginLab, Northampton, MA). All values were expressed as mean ± standard deviation and the results were based on dry weights (DW) of samples.

## Results and Discussion

The mechanical activity of ultrasound accelerates the distribution of the solvent toward the tissues, and thus the bioactive substances in the plant tissue are easily transferred to the solvent. There is no single or standard extraction method for bioactive compounds in plants. Methods and solvents differ according to the active substance desired to be obtained (Ignat et al., 2011). The solubility of compounds found in plants is affected by the polarity of the solvent in which they are dissolved. It is therefore very difficult to have a single procedure for all plant components (Garcia-Salas et al., 2010). In the ultrasonic-assisted extraction method, the biochemical and antioxidant capacities of the samples can be extracted most effectively by using hot or cold solvents. In the present study, the components of the olive leaf dried by ultrasonic-assisted extraction using four solvents of different polarities were investigated in detail.

Organic solvents (such as ethanol, methanol, and acetone), distilled water, and aqueous mixtures are commonly used for solvent extraction. The results of the extractions from olive leaves with different solvents obtained through the ultrasonic-assisted extraction are listed in Figure 1. In this study, total phenolic and total flavonoid contents in the ultrasonic-assisted extraction of olive leaves with water, methanol, ethanol, and methanol-water solvents were determined. The highest total phenolic content was obtained in the order of methanol-water > methanol > ethanol > water (Figure 1a) and the highest total flavonoid content in the order of methanol > ethanol > methanol-water > water (Figure 1b). The highest total phenolic content was obtained from methanol-water (234 mg $g^{-1}$) and methanol (192 mg $g^{-1}$) solvent extracts, and the highest total flavonoid content was obtained from methanol (47 mg $g^{-1}$) and ethanol (46 mg $g^{-1}$) solvent extracts. Olive leaves are rich in antioxidants, such as phenolics

and flavonoids (Ahmad-Qasem et al., 2013; Dobrinčić et al., 2020; Hannachi et al., 2019; Talhaoui et al., 2014), and values in the current study were higher than the total polyphenol content (61,09 to 92,49 mg $g^{-1}$) in olive leaves in ultrasonic-assisted extraction with ethanol solvent (50%, v/v) reported by Dobrinčić et al. (2020). Phenolic and flavonoid compounds are potent chain-breaking antioxidants, and recent studies have focused specifically on determining total phenolic and total flavonoid contents (Chew et al., 2021; Dobrinčić et al., 2020; Guo et al., 2011). Flavonoids and polyphenols are located in the cell wall, while free phenolic compounds are found in the cell vacuoles and phenolic and flavonoid compounds can be extracted with suitable solvents (Chew et al., 2021). The total phenolic content of leaves of different olive cultivars was between 52,1-60,4 mg $g^{-1}$ in the extracts obtained from methanol-water solvent (80:20) (Talhaoui et al., 2014), and the total phenolic content was 66 mg $g^{-1}$ in the ultrasonic-assisted extraction of olive leaves with ethanol-water solvent (80:20) by Ahmad-Qasem et al. (2013). Kenaf (*Hibiscus cannabinus*) leaves extracted with 95% ethanol yielded significantly higher total flavonoid content (48,195 mg CHE $g^{-1}$), while water extract yielded the lowest total flavonoid content (3,843 mg CHE $g^{-1}$), which is consistent with the results of the current study (Chew et al., 2021). Regarding the use of water in ultrasonic-assisted extraction, it was observed that ultrasounds were effective in extracting phenolic compounds even when applied for a short time (Ahmad-Qasem et al., 2013). As a result of different solid/liquid ratios and different times at ultrasonic-assisted extraction of olive leaves, the total polyphenol content ranged from 0,897 to 5,309 g GAE (gallic acid equivalent) 100 $g^{-1}$, and the total flavonoid content ranged from 0,042 to 0,239 g RE (rutin equivalent) 100 $g^{-1}$ (Hannachi et al., 2019). Therefore, the solubility of various phenolic and flavonoid components in olive leaves was significantly affected by differences in solvent polarities (Lafka et al., 2013).

Flavonoids can interact with and bind proteins, affecting the antioxidant capacity of sample extracts (Arts et al., 2002; Packer et al., 1999). Therefore, the effect of ultrasonic-assisted extraction with different solvents on protein content was also investigated. While the total protein content in olive leaves was not significantly affected by extraction with methanol and ethanol solvents, the total protein content was significantly reduced in methanol-water, and water extracts (Figure 1c). Total protein content was found to be 5,1 mg $g^{-1}$, 4,9 mg $g^{-1}$, 3,5 mg $g^{-1}$, and 1,9 mg $g^{-1}$ in the leaf extracts obtained with methanol, ethanol, methanol-water, and water solvents, respectively. The total protein content of creosote bush (*Larrea tridentata*) leaves in different organic solvents (methanol, ethanol, or acetone, in a concentration of 90%, 70%, 50%, or 30% v/v) and water extracts changed between 5,79-131,84 mg $g^{-1}$ (Martins et al., 2012). The lowest protein content in the extracts of creosote bush was observed in water extracts, while the highest protein content was observed in methanol (90%, v/v) extracts. The results support the opinion that flavonoids in samples extracted with different solvents interact by binding proteins in the extracts, which may affect their antioxidant capacity.

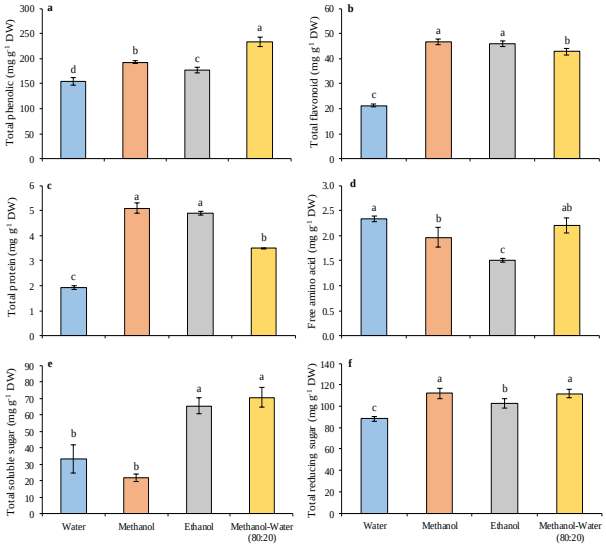


**Figure 1.** Total phenolic (a), total flavonoid (b), total protein (c), free amino acid (d), total soluble sugars (e) and total reducing sugars (f) in olive leaf extractions with four different solvents. Results represent means ± standard deviation. Different letters indicate significant differences at $P \leq 0.05$ level.

Free amino acids, an important group of polar nitrogen metabolites, are the precursors of various cell components, such as proteins, nucleotides, phenylpropanoids, and alkaloids (Zhang et al., 2017). The total free amino acids content was highest in extracts obtained from water (2,3 mg $g^{-1}$) and methanol-water (2,2 mg $g^{-1}$), but the yields were not statistically different ($P \leq 0.05$) (Fig 1d). However, free amino acids content was significantly lower in olive leaves extracted with methanol (2,0 mg $g^{-1}$) and ethanol (1,5 mg $g^{-1}$). The total free amino acid content in the ultrasonic-assisted extraction of olive leaves with methanol solvent was reported to be 0,09% (Luo et al., 2019). The free amino acid content of tea flowers (*Camellia sinensis*) extracted with distilled water at 90 °C was 8089 µg $g^{-1}$ (Wang et al., 2010).

Total soluble sugars are metabolic substrates that play a fundamental role in structure and metabolism at the cellular and whole plant levels (Afzal et al., 2021). Ultrasonic extraction with different solvents of olive leaves changed the total soluble sugar content (Figure 1e). The soluble sugar content was highest in methanol-water (70 mg $g^{-1}$) and ethanol (65 mg $g^{-1}$) extractions. However, extractions with water (33 mg $g^{-1}$) and methanol (22 mg $g^{-1}$) yielded significantly lower soluble sugar contents compared to the methanol-water and ethanol extractions. This probably changed depending on the polarity of each solvent and the solubility of soluble sugars in them. The total soluble

sugar content in the ultrasonic-assisted extraction of olive leaves with methanol solvent was reported to be 14,14% (Luo et al., 2019). The total soluble sugar content of olive leaves extracted with 80:20 (v/v) ethanol-water at 85 °C was 18,20-17,71 mg $g^{-1}$ (Eris et al., 2007).

Reducing sugars play an important role in metabolic pathways and assist in the production of secondary metabolites (Khatri & Chhetri, 2020). The values obtained from different extraction solvents for the reducing sugar content of olive leaves are given in Figure 1f. The highest reducing sugars contents were obtained from methanol (112,2 mg $g^{-1}$) and methanol-water (111,6 mg $g^{-1}$) extracts, and the lowest from ethanol (102,8 mg $g^{-1}$) and water (88,2 mg $g^{-1}$) extracts. The reducing sugar content in olive leaves varied considerably with respect to the solvent used, possibly depending on the polarity of each solvent and the solubility of the reducing sugars in them.

Four different techniques (CUPRAC, DPPH, FRAP, and FIC) were used to determine the antioxidant capacities of the extracts which are highly sensitive methods with reproducible results. It is recommended that at least two methods should be included to evaluate antioxidant capacity in extracts (Boeing et al., 2014). The CUPRAC assay uses copper(II)–neocuproine reagent as the oxidizing agent and is a very fast way to evaluate the antioxidant activity of extracts in a short time. In this method, the increase in absorbance was measured at 450 nm based on the reduction of copper(II)-neocuproin to the highly colored copper(I)-neocuproin chelate as a result of the color change from light blue to orange-yellow. Figure 2a shows that the copper(II) ion-reducing ability changes significantly depending on the extraction with different solvents ($P \leq 0.05$). The CUPRAC activity in methanol-water and methanol extracts was 0,63 and 0,50 mmol TR $g^{-1}$, respectively, while the activity in ethanol and water extracts was 0,43 and 0,25 mmol TR $g^{-1}$, respectively. CUPRAC activity in 12 different solvent extractions (water, ethanol (50, 80, and 100 %), methanol (50, 80, and 100 %), acetone (50, 80, and 100 %), methanol-DMSO, and ethanol-DMSO of yuzu (*Citrus junos*) ranged from 702.4 to 2195,2 mg TE 100 $g^{-1}$ in peels, from 348,7 to 1067,1 mg TE 100 $g^{-1}$ in pulp, from 338,9 to 785,1 mg TE 100 $g^{-1}$ in seeds (Assefa & Keum, 2017). In addition, aqueous forms of organic solvents gave better results for CUPRAC assay in yuzu. The CUPRAC activity in leaves of Chondrolia Halkidiki, Kalamon, and Koroneiki olive cultivars extracted with 70:30 (v/v) methanol-water at 70 °C changed between 271-398 mM TE $g^{-1}$ (Yancheva et al., 2016). The alteration in CUPRAC capacity when olive leaves are extracted with different solvents might be the difference in the polarity of the solvents, changing the extraction efficiency of certain antioxidant compound groups and affecting the antioxidant properties of the samples.

The DPPH assay measures the radical scavenging ability of extracts. DPPH radical scavenging activity was higher than CUPRAC and FRAP capacities, and DPPH radical scavenging activity was found to be in the order of methanol-water (1,09 mmol TR $g^{-1}$) > methanol (1,01 mmol TR $g^{-1}$) > ethanol (0,99 mmol TR $g^{-1}$) > water (0,46 mmol TR $g^{-1}$) (Figure 2b). Regarding solvent extracts, the DPPH radical scavenging activity in the ethanolic extract of Chinese truffle (*Tuber indicum*) was 1,61 mg $mL^{-1}$ for the $EC_{50}$ value (Guo et al., 2011). Hon-shimeji (*Hypizigus marmoreus*) scavenged DPPH radical by 59,7% and 34,0% at 5 mg $mL^{-1}$ ethanolic and cold-water extracts (Lee et al., 2007), whereas scavenging abilities in the ethanol extract of bracket fungus (*Laetiporus sulphureus*) was 14%, 26%, 55% and 86% inhibition at 100, 200, 400 and at 800 µg $mL^{-1}$ concentrations, respectively (Turkoglu et al., 2007). As a result of the extraction of olive leaves with methanol-water solvent (80:20), DPPH radical scavenging activity Arbequina leaves had the highest scavenging activity with 7,2 µg $mL^{-1}$ for $EC_{50}$, followed by Sikitita and Picual cultivars with 11,3 µg $mL^{-1}$ for $EC_{50}$ (Talhaoui et al., 2014). This indicates that cultivars differ in their antioxidant capacity.

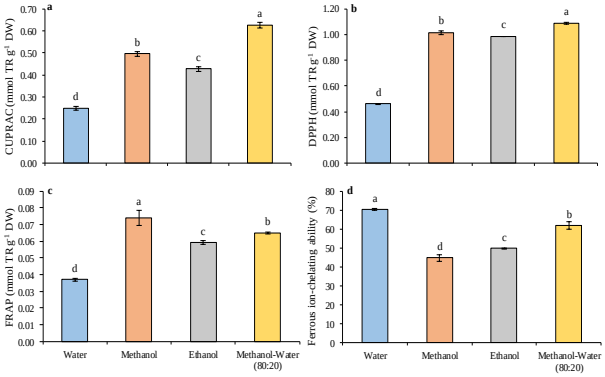


**Figure 2.** Results of CUPRAC (a), DPPH (b), FRAP (c) and FIC (d) assays from olive leaf extracts with four different solvents. Results represent means ± standard deviation. Different letters indicate significant differences at $P \leq 0.05$ level.

FRAP test was used to determine the reducing ability of antioxidant compounds in olive leaf extracts. FRAP functioned as a reducing agent with the ability to donate a single electron or hydrogen atom for the reduction of antioxidants (Rabeta & Nur Faraniza, 2013). The lowest FRAP capacity was observed in water extract with 0,037 mmol TR $g^{-1}$ and was determined as 0,059, 0,065 and 0,074 mmol TR $g^{-1}$ in ethanol, methanol-water and methanol extracts, respectively (Figure 2c). The FRAP capability of ultrasonic extraction of kenaf leaves with ethanol solvent was 18,39 mg TEAC $g^{-1}$ (Chew et al., 2021). Ahmad-Qasem et al. (2013) reported that the FRAP capacity was between 41,1-89,2 mg TR $g^{-1}$ in olive leaves extracted with ultrasonic-assisted extraction with ethanol-water (80:20) solvent. The FRAP capacity in olive leaves was due to the presence of antioxidant compounds involved in electron transfer. These antioxidant compounds have the ability to neutralize free radicals,

transform them into stable compounds and terminate the reactions initiated by free radicals (Muddathir et al., 2017).

Excess iron in the body causes the production of hydroxyl radicals (OH•) by stimulating lipid peroxidation (Guo et al., 2011). Moreover, the OH• is very toxic even at low concentrations and damages macromolecules (e.g. DNA, lipids, and proteins), causing their structural disorders (Özyürek et al., 2008). Metal ions chelation may be important to prevent the production of hydroxyl radicals that can damage biomolecules. In addition, natural metal chelating compounds, including phenolics and flavonoids, are desirable instead of synthetic chelating agents, which are associated with the problem of toxicity (Gulcin & Alwasel, 2022). Ferrous ions are also considered the most effective pro-oxidants widely used in the food industry. Ferrozine generates complexes with the $Fe^{2+}$ ion, but the chelating agents in the reaction disrupt the complex formation, causing a decrease in the color intensity in the reaction. Therefore, the decrease in color intensity provides an estimate of the ion-chelating activity for the chelator in the reaction. FIC activity was expressed as percent inhibition of ferrozine-$Fe^{2+}$ complex formation in olive leaves with different solvents. As shown in Figure 2d, all solvent extractions interfered with the formation of the ferrous and ferrozine complex and thus olive leaves have ferrous ion-chelating ability. The highest ferrous ion-chelating ability in olive leaves was observed in water (70%) and methanol-water (62%) extractions, while the lowest was observed in ethanol (50%) and methanol (45%) extractions. The ethanolic extracts of Chinese truffle had ferrous ion-chelating abilities of 73,4% at 6 mg $mL^{-1}$ and 77,4% at 12 mg $mL^{-1}$, and the FIC ability of the samples increased as the concentration increased (Guo et al., 2011). Lee (2007) observed that the FIC abilities of Hon-shimeji were 3,30% and 62,9% in the 0,5 mg $mL^{-1}$ ethanolic and cold-water extracts and 79,2% and 94,1% in the 5 mg $mL^{-1}$ ethanolic and cold-water extracts, respectively. The metal chelating activity was 14,24%, 3,71%, and 18,53% in eggplant (*Solanum melongena*) extracts with 70% methanol, 70% ethanol, and 70% acetone, respectively (Boulekbache-Makhlouf et al., 2013).

Overall, the hydromethanol mixture (methanol-water (80:20)) was the most effective solvent for the ultrasonic-assisted extraction of biochemical content and antioxidant capacity from olive leaves. Other studies have shown that hydroalcoholic mixtures (aqueous mixtures of methanol and ethanol) were more effective than pure solvents in biochemical and antioxidant capacity analyzes (Wang et al., 2009; Waterman & Mole, 1994). The effects of CUPRAC, DPPH radical-scavenging, and FRAP had different antioxidant and radical scavenging activities in solvent extracts, and the lowest antioxidant activity was observed in water extracts regardless of the assay used. The olive leaves extracted with methanol and methanol-water solvents showed higher antioxidant activity than those extracted with the other solvents. In addition, total phenolic content is associated with antioxidant capacities, because the structural properties of phenolic compounds are responsible for antioxidant activity (Katalinic et al., 2004). In the study, antioxidant activities calculated against reactive oxygen species were higher in methanol and methanol-water solvents, consistent with the total phenolic content observed in different solvent extracts. The differences between the findings of the current study and the other studies may come from varietal differences, cultivation practices, environmental and geographical factors, and different plant parts used in the studies.

Based on the results of ultrasonic-assisted extraction with four different solvents, heat-mapped correlation analysis was conducted to examine the relationship between biochemical contents and antioxidant capacity. The result of the correlation analysis based on the extraction of olive leaves with different solvents is provided in Figure 3. Of the 45 coefficients, five were significant at the $P \leq 0.05$ level. Of these five significant correlations, four were positively correlated and one was negatively correlated with each other. The significant positive correlation ($r$ = 0,97) between total phenolic content and CUPRAC may indicate that phenolic compounds mainly contribute to CUPRAC capacity. The significant positive correlation ($r$ = 0,96) between total flavonoid content and DPPH may indicate that flavonoids mainly contribute to DPPH capacity. In addition, the positive correlation of total reducing sugars with DPPH ($r$ = 0,95) and FRAP ($r$ = 0,97) indicates that they are significantly correlated. The significant negative correlation ($r$ = -0,98) between total protein content and FIC assay indicates that proteins have an effect on ferrous ion-chelating abilities. Prolonged ultrasonic-assisted extraction of kenaf leaves with ethanol revealed a weak positive correlation between total flavonoid content and DPPH capacity, as it could cause more losses of flavonoid compounds (Chew et al., 2021). The observation of a linear correlation ($r$ = 0,90) between total protein content and total flavonoid in the extraction of creosote bush leaves with different organic solvents (methanol, ethanol, or acetone, in a concentration of 90%, 70%, 50%, or 30% v/v) and distilled water (Martins et al., 2012) was consistent with the linear correlation ($r$ = 0,93) between total protein and total flavonoid in current results.

We have evaluated the biochemical content and antioxidant capacity of the extracts based on the ultrasonic-assisted extraction of olive leaves with water, methanol, ethanol, and methanol-water solvents. Methanol and ethanol were the best solvents for extracting total flavonoids and total protein; methanol-water extraction gave better results for total phenolics, soluble sugars, CUPRAC and DPPH capacity. The total free amino acids were better extracted with water and methanol-water, and the reducible sugars

with methanol and methanol-water solvents. FRAP assay was better when samples were extracted with methanol and FIC assay with water. Therefore, no single solvent can simultaneously extract all the molecules and antioxidant capacity of a sample.

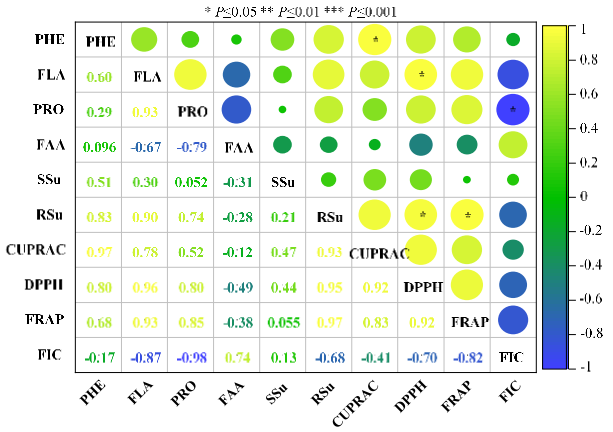


**Figure 3.** Relationships and correlations between examined parameters generated by a heat map using mean values. The color scale indicates the intensity of the normalized mean values of the different parameters [PHE, total phenolic; FLA, total flavonoid; PRO, total protein; FAA, free amino acid; SSu, total soluble sugars; RSu, total reducing sugars; CUPRAC, cupric reducing antioxidant power; DPPH, DPPH radical-scavenging; FRAP, ferric reducing antioxidant power; FIC, Ferrous ion-chelating].

## Conclusion

In this work, the effect of ultrasonic-assisted extraction with different solvents of dried olive leaves on biochemical content and antioxidant capacity was investigated. The results showed that methanol and methanol-water solvents were better extraction solvents for measuring the biochemical content and antioxidant capacity of olive leaves. The extracts obtained from the ultrasonic-assisted extraction of olive leaves with methanol or methanol-water are also a valuable natural product sources with antioxidant capacity and can be used in industrial applications, such as medicine and food. However, due to the toxicity of methanol, the next step in research efforts should focus on finding lesser or non-toxic solvents that can provide high extraction results, such as methanol.

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