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Research Article

Physicochemical Characterisation of Two-Phase Olive Mill Wastewater (*Olea Europaea* L. Cv. Ayvalık) Collected from Ayvalık Geographical Region

Ayvalık Coğrafi Bölgesinden Toplanan İki Fazlı Zeytin Değirmeni Atık Suyunun (*Olea Europaea* L. Cv. Ayvalık) Fizikokimyasal Karakterizasyonu

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ABSTRACT

Olive mill wastewater (OMWW) is the liquid by-product generated during the olive oil production process. When discharged into the soil, it poses harmful effects on soil microorganisms, aquatic ecosystems, and the atmosphere due to sulfur dioxide emissions. OMWW contains a high amount of bioactive compound, namely phenols which are highly powerful antioxidants. Besides their antioxidant activity, these compounds also demonstrate other biological properties such as antimicrobial, anticancer, anti-inflammatory, immunomodulatory, and anti-hyperglycemic owing to their ability to reduce oxidative stress. During production, only a very small portion of these phenolic compounds transfers into the olive oil, while the majority remains in the wastewater. This study aimed to characterize polyphenolic profile of two-phase OMWW (Olea europaea L. cv. Ayvalık) collected from Ayvalık region. The physicochemical characterization study was assessed using TPC (Total Phenolic Content), DPPH analysis (2,2-diphenyl-1-picrylhydrazyl), HPLC (High Pressure Liquid Chromatography), ICP-MS (Inductively Coupled Plasma Mass Spectrometry) and FT-IR (Fourier Transform Infrared Spectroscopy) measurements. The qualitative and quantitative HPLC analyses of the extracts revealed that hydroxytyrosol (75.253±2.6 mg/g), and tyrosol (38.213±1.8 mg/g) were the most abundant phenolic compounds while p-coumaric, caffeic, ferulic and vanillic acids were also present. Major mineral compounds are potassium, with a concentration of 24.37±0.39 g/kg, followed by calcium at 5.84±0.05 g/kg, phosphorus at 1.92±0.03 g/kg, sodium at 1.63±0.02 g/kg, and magnesium at 1.42±0.01 g/kg while iron, manganese, zinc and copper were also found as primary micronutrients. OMWW exhibited a TPC of 139±3.6 mg GAE/g and an antioxidant activity EC_{50} value of 9.37±0.12 mg/mL, which confirmed that OMWW is a cheap source of natural phenolic source that shows promise for use in fortified foods and pharmaceutical products after additional processing steps such as isolation and purification.

Keywords: Olive mill wastewater, Phenolic compounds, By-product, Chemical composition, HPLC

ÖZ

Zeytin atık suyu (OMWW), zeytinyağı üretim sürecinde ortaya çıkan sıvı bir yan üründür. Toprağa boşaltıldığında, kükürt dioksit emisyonu nedeniyle toprak mikroorganizmaları, su ekosistemleri ve atmosfer üzerinde zararlı etkilere neden olur. OMWW, güçlü antioksidanlar olan fenoller gibi pek çok biyoaktif bileşik içerir. Antioksidan aktivitesinin yanı sıra, bu bileşikler oksidatif stresi azaltma özellikleri sayesinde antimikrobiyal, antikanser, antienflamatuar, immünomodülatör ve antihiperglisemik gibi diğer biyolojik özellikler de sahiptir. Üretim sırasında, bu fenolik bileşiklerin sadece çok küçük bir kısmı zeytinyağına geçerken, çoğunluğu atık suda kalır. Bu çalışma, Ayvalık bölgesinden toplanan iki fazlı OMWW'nin (Olea europaea L. cv. Ayvalık) polifenolik profilini karakterize etmeyi amaçlamıştır. Fizikokimyasal karakterizasyon çalışması, TPC, DPPH analizi, HPLC, ICP-MS ve FT-IR ölçümleri kullanılarak yapılmıştır. Ekstraktların kalitatif ve kantitatif HPLC analizleri, hidroksitirosol (75,253±2,6 mg/g) ve tirosol (38,213±1,8 mg/g) en bol bulunan fenolik bileşikler olduğunu, p-kumarik, kafeik, ferulik ve vanilik asitlerin de mevcut olduğunu ortaya koymuştur. Başlıca mineral bileşikler, 24,37±0,39 g/kg potasyum, 5,84±0,05 g/kg kalsiyum, 1,92±0,03 g/kg fosfor, 1,63±0,02 g/kg sodyum ve 1,42±0,01 g/kg magnezyumdur. Demir, manganez, çinko ve bakır da birincil mikro besinler olarak bulunmuştur. OMWW, 139±3.6 mg GAE/g TPC ve 9.37±0.12 mg/mL EC_{50} antioksidan aktivitesi sergilemiştir, bu da OMWW'nin, biyoaktif bileşenlerinin izolasyonu ve saflaştırılması gibi ilave işlemlerden sonra, zenginleştirilmiş gıdalarda ve farmasötik ürünlerde kullanım için umut vaat eden, ucuz bir doğal fenolik antioksidan kaynağı olduğunu göstermektedir.

Anahtar kelimeler: Zeytin değirmeni atık suyu, Fenolik bileşikler, Yan ürün, Kimyasal bileşim, HPLC

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INTRODUCTION

Olive mill wastewater (OMWW) is the dark liquid effluent generated as a by-product during the olive oil extraction process. Olive cultivation has a high economic importance in Mediterranean basin, where approximately 95% of the world's olive trees are located due to the specific climatic requirements [1, 2, 3]. According to the International Olive Council (IOC, 2024), annual olive and olive oil production in Europe reaches approximately 10 and 2.57 million tonnes, respectively. Among the major olive oil producer countries such as Spain, Italy, Greece, Tunisia, Syria, Morocco, and Portugal, Türkiye ranked second in globally with a 14.8% production share, yielding approximately 380,000 tonnes in the 2022/23 season [4]. OMWW generation in the Mediterranean basin reached an estimated 30 million tonnes annually in 2022 [5]. In Türkiye alone, approximately 923,000 m^3 of OMWW is produced each year, of which around 600,000 tonnes are released into the environment without any treatment [6, 7, 8]. This leads to significant risks for soil microorganisms, aquatic ecosystems, and the atmosphere due to sulphur dioxide emissions and high phytotoxicity. The chemical oxygen demand (COD) of OMWW typically ranges from 50 to 200 g/L, and biochemical oxygen demand (BOD) can reach up to 100 g/L [1, 3, 9, 10].

Modern olive oil extraction technologies mainly involve two systems: two-phase and three-phase centrifugation [11, 12]. The three-phase process requires approximately 880-950 m^3 of water per 1000 tonnes of olives and generates around 1200 m^3 of wastewater [1] while the two-phase system uses only 250-350 m^3 of water for the same throughput. In this way, it reduces effluent volume by approximately 80% and energy consumption by up to 20% [13]. Nevertheless, it still produces considerable amounts of OMWW containing high concentrations of organic matter. Therefore, the characterization and management of OMWW remain important research topics. In the present study, two-phase OMWW was selected as the subject of investigation owing to its widespread adoption in industrial olive oil production [1, 14]. Although its chemical composition depends on many factors such as olive variety, climatic conditions, cultivation type, fruit maturity, storage time, and extraction technique, OMWW is primarily composed of water (83–92%), sugars, organic acids, and minerals [14]. The usage of chemicals, solvents, or high temperatures in conventional extraction methods may may alter the complex physicochemical properties of target compounds, whereas membrane-based technologies used in the present study provide a non-destructive approach for the separation and concentration of bioactive compounds [2, 15].

OMWW contains a diverse range of organic substances including sugars, proteins, carotenoids, tocopherols, and phenolic compounds such as hydroxytyrosol, tyrosol, verbascoside, and oleuropein [16, 17]. These phenolics possess strong antioxidant activity and exhibit a range of biological properties including antimicrobial, anticancer, anti-inflammatory, immunomodulatory, antihypertensive, and anti-hyperglycaemic effects, as well as inhibition of blood platelet aggregation and phospholipid oxidation [3, 9]. Despite the distinctive polyphenol composition of olive fruit, only 1–2% of total phenols are transferred into the final olive oil, whereas the vast majority, approximately 98%, remains in the by-products [18, 19]. Consequently, OMWW can be considered a phenolic-rich source up to 100-fold more concentrated than olive oil itself [1]. Because of its unique characteristics, many processes have been developed to treatment and valorization of these bioactive constituents, including enzymatic preparation, solvent extraction [20], natural deep eutectic solvent extraction [21], membrane separation, and chromatographic procedures [3, 14].

Previous physicochemical characterisation studies on OMWW have been conducted in southern Italy (*Olea europaea cv. Coratina*) [22], the Kabylia region of Algeria (*Olea europaea*) [23], Akkar in North Lebanon [24], the Loukkos region of Morocco [25], Jerash city in Jordan [26], Touta in North-East Tunisia [27], the Sousse region of Tunisia [28], Kalamata in Greece [29], and the Andalusia and Cordoba regions of Spain [30, 31]. Existing researchs on OMWW from the Ayvalık region have largely focused on the purification of effluents derived from three-phase extraction processes [32, 33, 34]. However, there is no comprehensive physicochemical characterization of two-phase OMWW, specifically associated with the Ayvalık cultivar (*Olea europaea L. cv. Ayvalık)* in this region. This study therefore aims to address this knowledge gap by providing a detailed characterisation of polyphenol content (HPLC), total phenolic content (TPC), radical-scavenging activity (DPPH), mineral composition (ICP-MS), physicochemical parameters, and structural analysis (FT-IR) of two-phase OMWW from this region. The findings are intended to contribute to potential applications in food preservation, nutraceutical formulation, and cosmetic development.

MATERIALS and METHODS

Materials

Olive mill wastewater (OMWW) obtained from *Olea europaea* L. cv. Ayvalık (39° 19′ 8″ N, 26° 41′ 43″ E) which is the most common olive tree variety in the mediterranean region was supplied from an olive oil company using two-phase continuous system (Haus Olive Pro 31, 4742, Türkiye) with the process condition that malaxation temperature: 27°C, malaxation time: 45 min, centrifugation speed: 3000 rpm (Selme Olive Oil Co., Ayvalık, Balıkesir, Türkiye)

between November and March during the olive period of the year 2021-2022. The total sample volume was specified as 3×2 L, collected from different production days. To prevent the oxidation of phenolic compounds, pH was adjusted 5 by adding acetic acid [22]. After arriving at the laboratory, the OMWW was immediately processed and kept at −20°C until used (Raw OMWW).

Chemicals

Solvents used for extraction procedures and high-performance liquid chromatography (HPLC) were HPLC-grade water, methanol, acetonitrile, phosphoric acid, acetic acid were all purchased from Sigma Aldrich-Merck (Darmstadt, Germany). The commercial analytical standards of Hydroxytyrosol (purity around 90%), (HT), tyrosol (>95%), (Tyr), vanillic acid (>98%), oleuropein (>98%), p-coumaric acid (>98%), gallic acid (>98%), syringic acid, luteolin-7-glucoside and rutin were purchased from Sigma- Aldrich Chemical Co. (Darmstadt, Germany). Folin–Ciocalteu reagent, 2,2- diphenyl-1-picrylhydrazyl, trolox (%97), were purchased from Sigma-Aldrich (Darmstadt, Germany).

Preparation of Sample

The extraction procedure used for the olive pulp was adapted from D'Antuono et al. [22] and Sygouni et al. [35]. Just before the filtration, OMWW was sieved using Whatman glass microfiber filters (1.6 µm pore diameter) to eliminate solid particles and colloids (OMWW-F1). Afterwards, the sample which was separated from large suspended particles, was filtered through a 0.1 µm pore diameter filter (1000 Da, Sepra, Italy) to remove all high molecular weight proteins, fat, lipids, carbohydrates and solids, while the phenolic compounds were concentrated (OMWW-F2). All the filters employed acted as molecular sieves without any chemical interactions with the matrix. Concentrated sample was frozen at −20°C before lyophilization using a freeze-dryer (Christ Alpha 2-4 LSCPlus, VWR GmbH, Darmstadt, Germany) and was stored at -20°C for analysis (OMWW).

Methods

The physicochemical properties of the materials were evaluated by measuring pH (Mettler Toledo-S220K, USA) and electrical conductivity (Hanna-HI-99300, UK) in a 1:10 (w/v) aqueous extract. The volatile solids, which reflect the organic matter content was determined by loss on ignition at 430°C for 24 h [36]. Total carbohydrate content of olive mill wastewater (OMWW) samples was determined using the phenol-sulfuric acid colorimetric method described by Alburquerque et al. [37]. Prior to analysis, samples were centrifuged at 10,000 x g for 10 min and filtered through a 0.45 µm membrane, followed by appropriate dilution with distilled water. Briefly, 1.0 mL of sample was mixed with 1.0 mL of 5% (w/v) phenol solution, and 3.0 mL of concentrated sulfuric acid was rapidly added. The mixture was incubated at room temperature for 20-30 min for color development, and absorbance was measured at 490 nm using a UV-Vis spectrophotometer. A calibration curve was prepared using D-glucose as standard, and all analyses were performed in triplicate with appropriate blanks [37, 38].

Total solids (TS) were determined using method 2540 B, and total suspended solids (TSS) were determined using method 2540 D of Standard Methods. This involved evaporating a 10 ml sample in a porcelain cup, previously dried and weighed, to 105°C until constant weight [39].

Concentrations of lignin and cellulose were determined according to the American National Standards Institute and American Society for Testing and Materials [40, 41] and holocellulose content was determined by the sodium chlorite ($NaClO_2$) delignification [42]. Prior to analysis, the sample was ground to pass through a 0.5 mm sieve and extracted with an ethanol-toluene mixture (1:2, v/v) for 6 h using a soxhlet apparatus to remove extractives. Exactly 5 g of the extractive-free, oven-dried sample was transferred into a 250 mL erlenmeyer flask containing 150 mL of distilled water pre-heated to 75°C, and the suspension was mechanically stirred throughout the reaction. Ten drops of glacial acetic acid and 1.5 g of sodium chlorite ($NaClO_2$, 80%) were added to initiate the delignification reaction. After 1 h of vigorous stirring at 75°C, the same amounts of glacial acetic acid and sodium chlorite (1.5 g) were added again. This addition cycle was repeated every hour for a total reaction time of 4 h, until complete delignification was achieved, as visually confirmed by the whitening of the residue. The reaction mixture was then cooled in an ice bath to terminate the oxidation. The holocellulose residue was vacuum-filtered through a pre-weighed crucible, washed successively with cold distilled water until the filtrate was acid-free (neutral pH), and then rinsed with acetone to facilitate drying. The crucible and residue were dried at 105°C to constant weight, cooled in a desiccator, and weighed gravimetrically. Holocellulose content was expressed as g/kg on a dry matter basis. Hemicellulose content was calculated as the difference between holocellulose and cellulose concentrations.

Total nitrogen analysis was performed according to AOAC Official Method 990.03 [43]. The lipid content was determined using AOAC method 920.39, employing the traditional method of extraction using a Soxhlet apparatus with diethyl ether (boiling point 40–60°C) as the solvent, followed by weighing [44].

Total Polyphenol Content

Total phenolic content was determined by the Folin–Ciocalteu method, as described by Lesage-Meessen et al. [1]. Briefly, 0.5 g of freeze dried OMWW and 0.5 mL of Folin–

Ciocalteu reagent were added to a 25 mL volumetric flask. Afterwards, 3 min, 1 mL of Na2CO3 (35%, w/w) was added. Deionized water was used to fill the flasks to the necessary volume level, and they were then kept in the dark for 60 min. A Shimadzu UV-1700 UV/Vis spectrophotometer (Kyoto, Japan) was used to measure absorption at 750 nm. Gallic acid (0-100 ppm) was used to create a standard curve. TPC was expressed as mg gallic acid equivalents (GAE) per g OMWW.

Determination of Antioxidant Activity by DPPH

The antioxidant activity of freeze dried OMWW was measured based on hydrogen-donating or radical-scavenging ability using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), following the method reported by Chanioti and Tzia [21], with some modifications. Initially, DPPH radical solution of DPPH was prepared (0.0025 g/100 mL methanol) prepared prior to measurements. 0.5 mL of the diluted sample was added with 3 mL of 0.1 mM of DPPH solution and the mixture was vortexed. After 20 min remaining in darkness at ambient temperature the absorbance of the mixture was recorded at against blank at 517 nm. Equation (1) was utilized to calculate the percentage of inhibition of the DPPH radical:

$$\text{Inhibition}(\%) = 100 \times (\text{Acontrol} - \text{Asample})/\text{Acontrol} \quad (1)$$

where Acontrol is absorbance of the control and Asample is absorbance of the sample. Caffeic acid (R^2=0.9937) and trolox (R^2=0.9955) were used as the reference antioxidant compound. Results were expressed as EC_{50} (mg/mL). EC_{50} is the concentration of extract that declines 50% of the initial concentration of DPPH radical. Low EC_{50} value indicates strong ability of the extract to act as DPPH scavenger.

Heavy Metals Analysis

For each element to be determined, standards between 0 and 500 mg/L (depending on the field of the linear calibration curve of each element) were prepared. Na, Mg, P, K, Ca, Mn, Fe, Cu, Zn and Se were detected by employing inductively coupled plasma/mass spectrometry (ICP-MS) (7850 Agillent ICP-MS, USA) after microwave digestion.

0.5 g of the OMWW samples weighed into Teflon PFA container vessels, 5 ml of nitric acid (65%) solution and 2 mL of hydrogen peroxide (30%) added and digested for 40 minutes at 210°C in the microwave unit. After digestion procedures, samples diluted with distilled water and filtrated using 0.45 um filter and analyzed using ICP-MS device. Results expressed g/kg and mg/kg level according to calibration curves on the basis of dry matter [45, 46].

HPLC Analysis

The phenolic composition of OMWW was performed with HPLC-DAD (Shimadzu, Kyoto, Japan) liquid chromatography equipped with a binary gradient pump LC 20AD. A photodiode array detector (PDA, 200-600 nm) and a CTO-10 ASVp column thermostat set at 40°C. LG solution software was used for spectra and data processing. An analytical Phenomenex Luna C18 (5 µm) column (4.6×250 mm) was used throughout this experiment.

The OMWW samples are dissolved in adequate amount of MeOH were filtered through a 0.45 µm pore size PTFE filter and an aliquot (20 µL) was injected into the chromatograph. The solvent system consisted of (A) Acetonitril and (B) water (pH 2.5 adjusted with 0.15% phosphoric acid), % 0.1 phosphoric acid in ultrapure water (w/v). The elution profile of the linear gradient was: 0–10 min, 7% A(isocratic); 10–57 min, 7–17% A; 57–80 min, 17–22% A; 80–90 min, 22–7% A. The total run time was 90 min. The flow rate was 1 mL/min [22].

The only phenolic compounds detected in appreciable amounts in our samples as identified by comparison of their retention time and spectra with those obtained from the corresponding standards. The concentration measurement for each identified compound was performed using regression curve obtained using the available standards and results expressed mg/g OMWW on the basis of dry matter.

Fourier Transform Infrared (FT-IR) Spectra

Fourier transform infrared (FT-IR) spectra of the samples were recorded using an FT-IR spectrometer (Agilent Cary 630, Germany) equipped with an attenuated total reflection (ATR) accessory and a diamond crystal. Spectral measurements were carried out over the wavenumber range of 4000–500 cm^{-1}, with a resolution of 4 cm^{-1}. Each spectrum was obtained by averaging 50 scans at room temperature.

Statistical Analysis

All replicated experimental results (n=3) were expressed as mean values and standard deviations (SD). One-way analysis of variance (ANOVA) was performed using the MINITAB software package (Version 16, State College, PA, USA). Differences between means were evaluated using the Tukey test (α=0.05).

RESULTS and DISCUSSION

Physicochemical Characteristics of Raw OMWW

To determine the physical and chemical parameters; pH, conductivity, total solid, lignin, cellulose, hemicellulose, fat, total N, carbohdrate analysis were performed. The analysis revealed that the pH of the raw OMWW sample was 5.1±0.254, its electrical conductivity measured 0.79±0.02 $S{\cdot}m^{-1}$, and the moisture content was 63.92±1.22%. The acidic nature of olive mill wastewater aligns with previous studies in the literature [46, 47]. pH appeared to be a little lower than that reported in the study conducted by Lesage-Meessen et al. [1], they found pH close to 5.7 for the olive oil residue which is extracted using a classical three-phase centrifugation system. Electrical conductivity of raw OMWW sample was measured as 0.79±0.02 S m^{-1}. According to some studies this value was variyed between 0.55 and 1.0 S/m [48, 49]. The OMWW from two-phase extraction systems has different characteristics compared to the olive pomace from traditional press and three-phase system [14].

The moisture content of the sample was found to be 63.92%. According to previous studies, the moisture content of OMWW obtained from the two-phase extraction system ranges between 65–75%, compared to 22–25% in traditionally pressed olive pomace and 40–45% in three-phase systems [14, 37].

The characterization of the raw OMWW revealed that it is primarily composed of carbohydrates, organic acids, and mineral nutrients, with their distribution varying according to the extraction process and agronomic practices. In the present study, total suspended solids (TSS) were measured at 885.71±3.41 g/kg in raw OMWW, and decreased significantly to 17.41±0.09 g/kg and 0.11±0.02 g/kg after treatment with Filter 1 and Filter 2, respectively. Correspondingly, total dissolved solids (TDS) were found to be 115.32±1.52 g/kg, 104.83±0.69 g/kg, and 59.73±0.72 g/kg (Table 1). The observed reduction in TSS and TDS can be attributed directly to the filtration process with the removal of main components such as cellulose, hemicellulose, and lignin. In addition, fat and total nitrogen are also present in significant quantities [14, 50]. As shown in Table 1, the concentrations of cellulose, hemicellulose, and lignin in raw OMWW were found to be 184.11±0.57 g/kg, 285.43±1.56 g/kg, and 327.23±2.82 g/kg, respectively. After first filtration, large particles and colloids such as lignin, cellulase and hemicellulose have been removed. Following the second filtration, high molecular weight proteins, lipids and carbohydrate have been separated from the wastewater.

Total Polyphenol Content

Total phenolic content (TPC) analysis is a crucial step in evaluating OMWW due to its high concentration of phenolic compounds, which have a significant impact on industrial applications. Therefore, assessing the TPC of OMWW provides valuable insights into its potential for valorization as a source of bioactive compounds. In the present study, TPC analysis was performed to quantify the The total phenolic content, estimated as gallic acid equivalents, was found to be 139±3.6 mg GAE/g confirming its richness in bioactive molecules.

According to the study investigated a multistage recovery process for phenolic antioxidants from OMWW concentrates, TPC concentration have been found between 32.50±0.70 and 40.10±0.80 mg GAE/g depending on the applied process including acidification, delipidization, solvent extraction, and solid-phase extraction of two-phase system OMWW [51]. In the study on operational parameters of a full-scale anaerobic digestion process powered by

Table 1. Physicochemical composition of the raw olive mill wastewater before and after filtration

	OMWW (Raw)	OMWW-F1 (After fiter 1)	OMWW-F2 (After filter 2)
	(g/kg)*	(g/kg)*	(g/kg)*
Total Suspended Solid	885.71±3.41	17.41±0.09	0.11±0.02
Total Dissolved Solid	115.32±1.52	104.83±0.69	59.73±0.72
Cellulose	184.11±0.57	-	-
Hemicellulose	285.43±1.56	-	-
Lignin	327.23±2.82	-	-
Fat	79.32±0.37	76.12±0.14	1.18±0.08
Total N	9.72±0.03	2.41±0.03	0.83±0.04
Carbohydrate	63.35±0.11	54.77±0.81	11.37±0.11

*dry weight basis

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olive oil by-products, Tamborrino et al. [52] reported that TPC of the biomass (olive pulp and pomace) ranged from 1840 to 3040 mg GAE/kg. Elayeb et al. [53] investigated the valorization of phenolic compounds from olive mill wastewater (OMWW) using a separation hydrocyclone process. They measured the TPC up to 13.8 mg GAE/g in the extracted polyphenol-rich fractions. An assessment of phenolic compound variability in OMWW subjected to different processing times and milling techniques revealed that OMWW contained up to 19.61 mg GAE/g of total phenolic compounds [54]. An investigation into the antioxidant properties and protective effects of phenolic extracts from two OMWW varieties revealed that TPC in polyphenol extracts was 26.32±0.12 mg GAE/g for the Sigoise variety and 43.53±0.56 mg GAE/g for the Chemlal variety highlighting the presence of bioactive compounds with potential health benefits [55].

The TPC results of OMWW given with these studies are lower than the present study. This can be explained by the fact that the samples of olive oil mill wastewaters are filtered by employing membran technology before the determination of polyphenols concentrations, while other studies used another extraction technology. Besides extraction methodologies, the phenolic profile of OMWW is highly dependent on olive variety and cultivation practices which affect the phenolic biosynthesis in olives. Furthermore, the processing conditions employed during olive oil production such as traditional pressing, two-phase, or three-phase centrifugation have a significant impact on both the concentration and composition of phenolic compounds in OMWW. As documented in previous researches, storage conditions prior to the extraction of OMWW can also lead to the degradation or transformation of phenolic compounds through oxidation, microbial activity, and polymerization reactions [14, 50, 56, 57].

Antioxidant Activity

This study investigated the compounds' antioxidant activity using the DPPH test.The antioxidant capacity is commonly expressed as the efficient concentration (EC_{50}), which represents the quantity of the sample needed to decrease the initial concentration of the DPPH free radical by 50%. Caffeic acid (3,4-dihydroxycinnamic acid) which possess three hydroxyl moieties in their structures and trolox were used as the reference antioxidant compounds.

DPPH radical scavenging ability of OMWW sample was found to be EC_{50} 9.37±0.12 mg/L while EC_{50} 1.241±0.01 mg/L for pure caffeic acid and 3.85±0.20 for trolox. Belaqziz et al. [58] reported the antioxidant activity as EC_{50} 32.32±4.7 mg/L after solvent-solvent extraction of OMWW obtained from an olive oil company using three phase milling techniques located in the Tensift region of Morocco. In the study in which the samples obtained from a commercial two-phase olive oil mill located at Correggiola region in Italy, crude OMWW showed EC50 activity ranged between 14.49 and 38.50 mg/L depending on the used solvent [59]. Azaizeh et al. [60] reported the best with EC_{50} of 20 mg/L after ethyl acetate extraction of the OMWW sample. OMWW extract showed considerably higher antioxidant activity.

Analysis of Phenolic Compouns Profile by HPLC

The analysis of individual phenolic compounds in OMWW using HPLC is fundamental for identify and quantify specific phenolics to provide detailed insights into their distribution and concentration. Although two-phase centrifugal decanters yield virgin olive oils with a greater phenolic compound concentration than those produced using three-phase systems, the vast majority of them (approximately 98%) still remains in the OMWW [1, 14].

OMWW contains a diverse range of phenolic compounds, including hydroxytyrosol, tyrosol which contribute to its antioxidant potential and bioactive properties. Hydroxytyrosol which is hydrolysis products of verbascoside and the hydroxytyrosol-secoiridoid. They have a powerfull antioxidant activities [61], inhibitory effect on human LDL oxidation, and exhibit anti-inflammatory and anticancer properties [16].

According to the results presented in **Table 2**, the most representative compounds identified in OMWW, include gallic acid, hydroxytyrosol, tyrosol, vanillic acid, syringic acid, epicatechin, p-coumaric acid, Luteolin 7-glucoside, oleuropein and rutin. Among the identified phenolic compounds in OMWW, hydroxytyrosol, tyrosol, and gallic acid were found to be the most abundant, with concentrations of 75.253±2.6 mg/g, 38.213±1.8 mg/g, and 19.144±0.5 mg/g, respectively. These results indicate that hydroxytyrosol is the predominant compound, accounting for approximately 55.6353% of the total identified phenolics, followed by tyrosol at 28.2512% and gallic acid at 14.1533% (**Table 2**). These results are comparable with the study on the recovery and profiling of phenolic compounds from OMWW [14, 12, 57, 62]. In the study on the phenolic profile and antioxidant activities of olive mill wastewater obtained from modern and semi-modern three-phase systems, hydroxytyrosol was also found the most abundant phenolic compound, representing about 55–70% of the total phenolic concentration [16]. In the study carried out by Lesage-Meessen et al. [1] p-coumaric acid and vanilic acid was also found major phenolic compounds. Minor compounds such as verbascoside, rutin, caffeoylquinic acid, luteolin-4-glucoside, 11-methyloleoside, hydroxytyrosol-10-β-glucoside, luteolin-7-rutinoside, and oleoside identified in the study conducted by Dermeche

et al. [14]. According to the study developed a multistage recovery process combining ethanol-based aqueous extraction from two-phase olive mill wastewater, maximum recovery of hydroxytyrosol was reported as 4.7 mg/g when ethanol/$(NH_4)2SO_4$ extraction was applied [51]. In another study focusing on the recovery of valuable bioactive compounds from OMWW derived from two-phase olive mills, hydroxytyrosol and tyrosol levels were stated as 6.9 mg/g and 5.1 mg/g, respectively, demonstrating the potential for high-value compound recovery from OMWW [63].

Table 2. Polyphenol composition and % distribution of OMWW

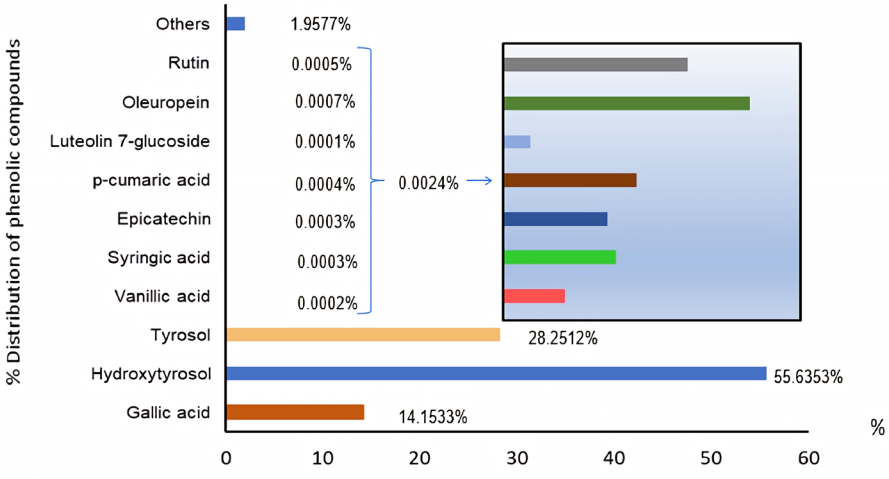
Phenolic compounds	(mg/g)	Phenolic compounds	(mg/g)
Gallic acid	19.144±0.5	Epicatechin	0.394±0.02
Hydroxytyrosol	75.253±2.6	p-cumaric acid	0.506±0.02
Tyrosol	38.213±1.8	Luteolin 7-glucoside	0.1±0,005
Vanillic acid	0.231±0.03	Oleuropein	0.941±0.002
Syringic acid	0.428±0.01	Rutin	0.7±0.01

Tyrosol, detected at a concentration of 38.213±1.8 mg/g (**Table 2**), has been reported to be effective in preserving cellular antioxidant defenses [64]. Gallic acid, which constitutes approximately 14.1533% of the total phenolic compounds in OMWW samples, was quantified at 19.144±0.5 mg/g (**Table 2**). Gallic acid has been reported to possess strong antioxidant activity in lipid systems and to exhibit both antihyperglycaemic and antioxidant properties [65]. Owing to these bioactive properties, gallic acid is widely utilized in processed foods, cosmetics, and food packaging materials to prevent lipid peroxidation-induced rancidity and spoilage [66].

Oleuropein has been reported to be beneficial in the treatment of various inflammatory diseases [15] was identified at relatively low concentration of 0.941±0.002 mg/g with representing approximately 0.0007% of the total phenolic components (**Table 2**). Visioli et al. [67] indicated that oleuropein, an ester of elenolic acid and hydroxytyrosol, is one of the major phenolic compounds in OMWW, whereas Allouche et al. [20] failed to identify this compound in their analysis. One possible explanation for the variation in oleuropein amounts in OMWW is related to the maturity stage of the olives at harvest. During mechanical olive oil extraction, oleuropein can be enzymatically degraded into elenolic acid and hydroxytyrosol by an esterase [67]. This enzymatic activity may account for the high levels of hydroxytyrosol quantified in OMWW, as also reported by Dermeche et al. [14], Bianco et al. [68] and De Marco et al. [69]. More remarkably, even if the major phenolic compounds are generally the same, the phenolic composition of OMWW varies significantly between studies. The treatments applied to extract the oil from the olives and to treat the olive mill wastes, as well as the olive variety and growth environment, have a significant impact on the quantitative and qualitative phenolic content of OMWW [14].

Table 3. Amounts and % distribution minerals in OMWW

Mineral compounds	(g/kg)	Mineral compounds	(mg/kg)
P	1.92±0.03	Fe	122.02±0.91
K	24.37±0.39	Cu	13.26±0.09
Ca	5.84±0.05	Mn	24.19±0.07
Mg	1.42±0.01	Zn	14.08±0.09
Na	1.63±0.02	Se	nd



https://doi.org/10.24323/akademik-gida.1975900

Mineral Composition of OMWW

Olive mill wastewater contains a complex mixture of organic and inorganic compounds, including valuable minerals. In the context of the food industry and sustainable waste management, mineral analysis of OMWW is necessary for assessing its potential for valorization.

Mineral analysis has shown that the major elements in OMWW are potassium, with a concentration of 24.37±0.39 g/kg, followed by calcium at 5.84±0.05 g/kg, phosphorus at 1.92±0.03 g/kg, sodium at 1.63±0.02 g/kg, and magnesium at 1.42±0.01 g/kg. This elemental composition is a common characteristic of olive mill wastes. The primary micronutrient identified was iron, with a concentration of 122.02±0.91 mg/kg. In addition, manganese was measured at 24.19±0.07 mg/kg, zinc at 14.08±0.09 mg/kg, and copper at 13.26±0.09 mg/kg, while selenium was not detected in the sample, as shown in **Table 3**.

These results are consistent with those reported in previous studies by Dermeche et al. [14], Rajib et al. [46] and Hanafi et al. [70]. In two-phase OMWW samples, OMWW, Cegarra et al. [38], reported a potassium content of 25.6 g/kg, whereas Alburquerque et al. [37], recorded a slightly lower value of 19.8 g/kg. Regarding sodium, their findings showed concentrations of 8.3 g/kg and 0.8 g/kg, respectively. The observed variability in mineral concentrations can be attributed to factors such as soil characteristics, type of irrigation, and the cultivation practices used for olive trees, as noted by Paredes et al. [36].

Measurement of Fourier Transform Infrared (FT-IR)

FT-IR is widely used for analyzing the complex organic content of OMWW. This technique allows the determination of major functional groups such as phenolic compounds, aliphatic compounds, carboxylic acids, aromatics, and polysaccharides present in OMWW.

The FT-IR spectra of OMWW illustrated in **Figure 1**. displays characteristic peaks of carbonyl groups in the range of 3650-3000 cm^{-1} which corresponds to the O–H elongation vibration of alcohols, phenolic groups, carboxylic groups and the N–H hydrogen vibration of amide groups.

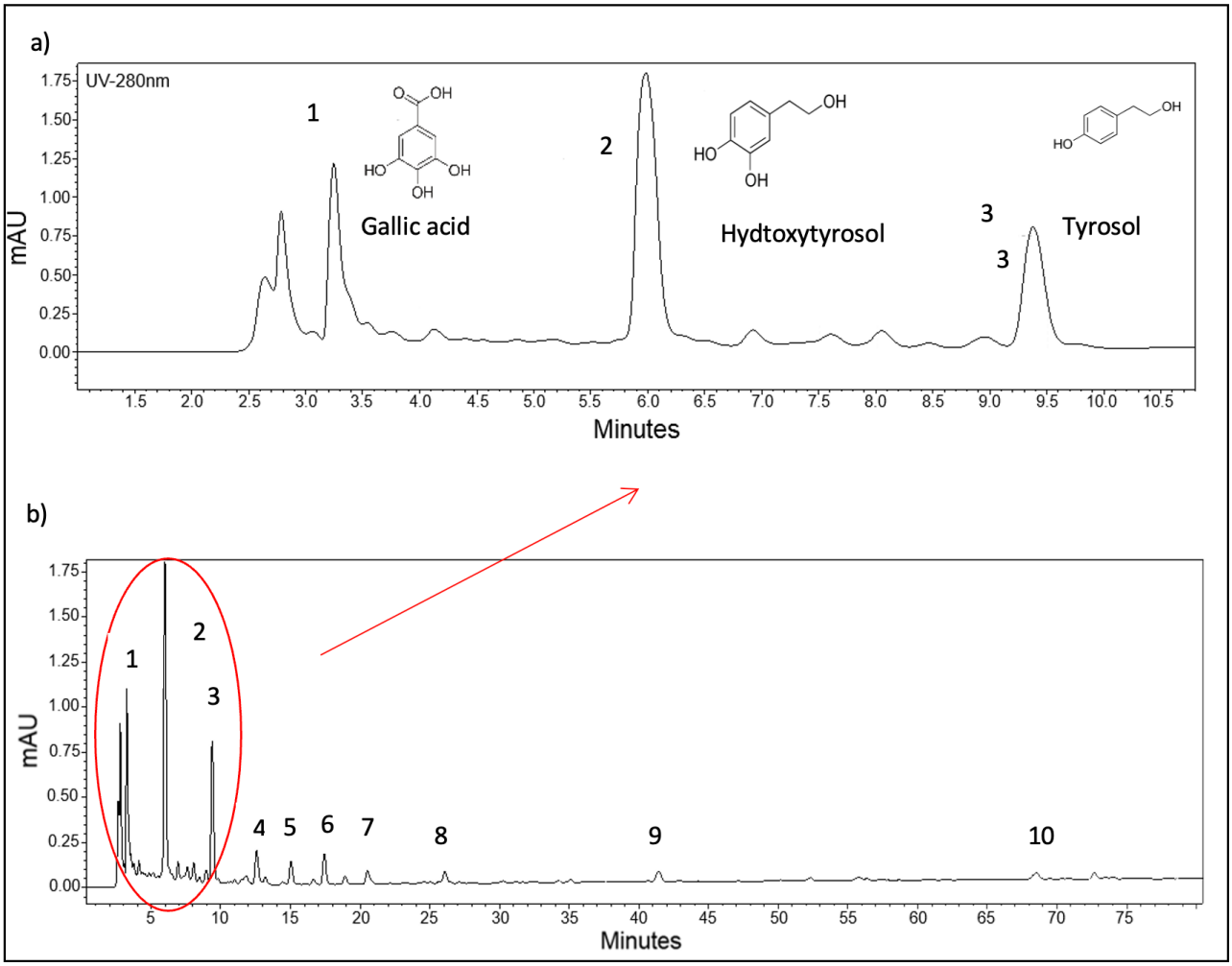


Figure 1.a) Major phenolics compound chromatogram of OMWW b) Phenolics present in the OMWW 1. Gallic acid, 2. Hydroxytyrosol, 3. Tyrosol, 4. Vanillic acid, 5. Syringic acid, 6. Epicaatechin, 7. p-coumaric acid, 8. Luteolin 7-glucoside, 9. Oleuropein, 10. Rutin

Aliphatic methylene molecules containing CH, CH_2, and CH_3 groups are found in the two peaks at 2900 and 2800 cm^{-1}, representing the lipids. The band at 1650 cm^{-1} indicates the aromatic compounds due to the C=C stretching of the aromatic ring of quinone. Additionally, the band at 1400 cm^{-1} is mainly attributed to the O–H stretching vibration of the phenolic compounds and aromatic ethers. The absorbance at 1100 cm^{-1} is associated with C–O stretching, corresponding to the structure of glucidic compounds (**Figure 2**). These findings are in agreement with earlier studies, which confirm that olive mill wastewater is primarily composed of aliphatic fatty acids and a diverse range of phenolic compounds [14, 57, 71].

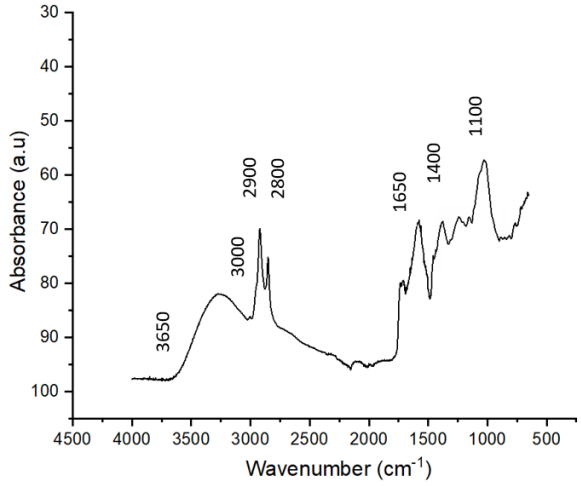


Figure 2. FT-IR spectra of OMWW

CONCLUSION

Olive mill wastewater is the major waste produced by the olive industry. The waste causes large-scale environmental problems because of its high polluting power due to a high organic load and a high antimicrobial activity. This by-product has also demostrated useful biological properties such as antimicrobial, anticancer, or anti-inflammatory thanks to their antioxidant activity exerted mainly by various phenolic compounds. Olive mill wastewater generated during the production of olive oil from the *Olea europaea* L. cv. Ayvalık olive cultivar which is among the most widely cultivated olive in Aegean Region evaluated comprehensivelly.

The characterization of OMWW showed that it mainly composed of, carbohydrates, phenolic compounds and mineral nutrients. This work confirmed the potential feature of olive mill wastewaters as a source of natural antioxidant with the 139±3.6 mg/g TPC and EC_{50} 9.37±0.12 mg/mL antioxidant activity. Hydroxytyrosol, tyrosol and gallic acid are the most abundant phenolic compounds present in olive mill wastewater. The high antioxidant potential of the OMWW phenolic extracts was related to their high contents of hydroxytyrosol (75.253±2.6 mg/g) tyrosol (38.213±1.8 mg/g) and gallic acid (19.144±0.5 mg/g). On the basis of these findings, it was concluded that OMWW-derived phenolics can be concidered as a promising phenolic source for fortified food application, food preservation and formulation of pharmaceutical products. This added value by product will pave the way for provide new strategy to valorization of OMWW. This approach aligns with circular economy principles by transforming a challenging agro-industrial waste into a valuable secondary resource.

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