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Antimicrobial Activities of Olive Oil Mill Wastewater Extracts against Selected Microorganisms

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Abstract: Discovering eco-friendly alternatives to synthetic chemicals has become an increasingly popular area of research. Natural products are now in the spotlight for their potential use as replacements for synthetic chemicals. To maximize the benefits of these natural products, it is important to use efficient extraction methods, especially from agroindustrial waste. Olive oil mill wastewater (OOMW) is a byproduct of the olive oil production process and is considered a pollutant; however, OOMW contains a wide range of phenolic compounds that have proven antimicrobial properties. This study investigates the extraction of these compounds from OOMW, with the aim of determining their potential antimicrobial activities against several bacterial strains and fungi, including *Bacillus spizizenii*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella aerogenes*, *Streptococcus uberis*, *Enterococcus faecalis*, and *Candida albicans*. The OOMW extracts (OEs) were prepared by using three different solvents: ethyl acetate, ethanol, and methanol. The highest total phenolic contents (4.03 g, GAE/L) and the strongest antibacterial activity were obtained with methanol extraction. All OEs showed no antifungal activity against *C. albicans*. OEs, particularly methanol extracts of OOMW, can be used as bioactive substances in various industries as nutraceuticals and food ingredients, respectively.

Keywords: olive oil processing; antibacterial; biorefinery; extraction; food waste

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1. Introduction

Countries in the Mediterranean Basin have highly developed olive and olive oil production processes; however, the methods used for olive oil production vary depending on the type of olive being processed. This leads to the generation of different types of waste products, such as olive oil mill wastewater (OOMW) and olive pomace, either separately or combined (as shown in Figure 1), which are released into the environment. In the traditional pressing method, olives are pressed to extract the oil, resulting in the formation of olive pomace and oily water. A small amount of water is used in this process, which generates a significant amount of OOMW (40–60 L/100 kg olives) with a high chemical oxygen demand (COD). The continuous centrifugation method utilizes decanters that can operate as either two-phase or three-phase systems. In the three-phase system, a large amount of OOMW (80–120 L/100 kg olives) and pomace are released due to the excessive use of hot water. On the other hand, the two-phase system involves using a small amount of water to generate a mixture of pomace and OOMW (10 L/100 kg olives) in an aqueous form as waste [1].

Pomace can be used as a raw material to produce biodegradable food packaging material or as an additive in food and animal feeds since it is rich in polyunsaturated fatty acids and phenolic compounds [2]. Furthermore, pomace can also be used as a fertilizer as it provides macro and micro elements that stimulate plant growth [3], and its antimicrobial actions make it effective against plant pathogens due to its antimicrobial actions [4]. On the other hand, the liquid waste rich in phenolic compounds have low pH and plant toxicity

with its high organic load and high COD contents [5,6]. In addition, the liquid waste causes environmental pollution and threatens natural aquatic life due to its characteristic odor and dark black-brown color [7].

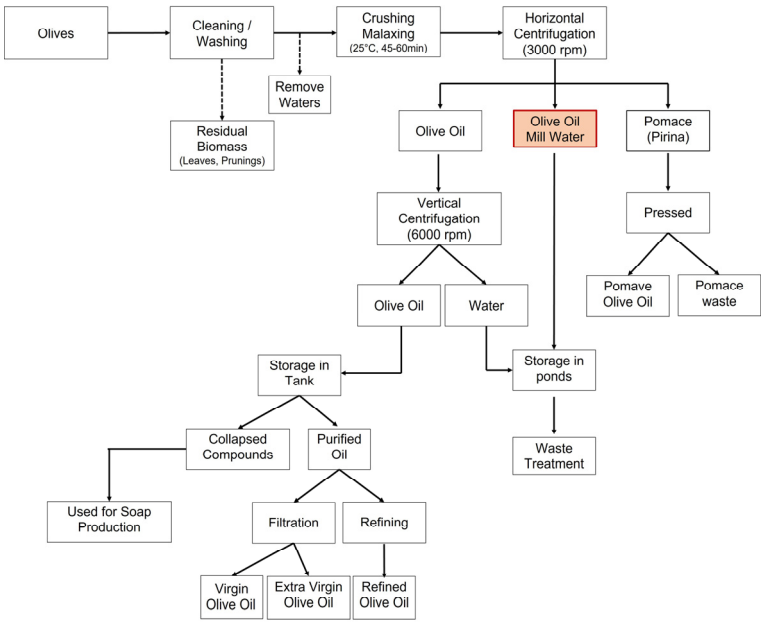

Figure 1. Schematic representation of the continuous centrifugation processing of olive oil industry. The OOMW generated during the process, which was also used in this study, has been highlighted in red.

Various studies have been conducted to reduce the toxicity of OOMW and convert it into valuable products, such as microbial biomass, biosurfactants, enzymes, hydrogen, biogas and bacterial cellulose, through microbial processes [8–11]. These studies have successfully demonstrated the potential applications of OOMW for various purposes, including COD removal. However, due to its nitrogen deficiency, extra nitrogen needs to be added to microbial production processes, which can increase production costs [5,10]. Alternatively, phenolic compounds can be extracted from OOMW using effective extraction methods [12,13]. Bioactive phenolic compounds recovered from olive wastes (e.g., OOMW, olive pomace and olive leaves) typically include hydroxytyrosol (3,4-DHPE), tyrosol (4-HPE), oleuropein (3,4-DHPE-EA-glu), quercetin-3-O-rutinoside (rutin), luteolin-7-O-glucoside, and 3,4-Dihydroxyphenylglycol (3,4-DHPG) [14]. The concentrations of phenolic compounds recovered from OOMW may differ depending on the olive variety and olive oil processing method [15]. Recovered phenolic compounds, mainly hydroxytyrosol, tyrosol, oleuropein and verbascoside, have been investigated for their antimicrobial, anticancer, antioxidant, cardiovascular, immunomodulatory, gastrointestinal, respiratory and anti-inflammatory activities to be evaluated in pharmacology [14,16–18]. Recovering polyphenols from olive oil wastes and reusing them in the process can enrich the quality of olive oil, resulting in economic benefits by reducing waste disposal costs and improving the quality of food products [19].

In the food industry, chemical disinfectants are commonly used to eliminate microorganisms on food contacted surfaces. However, the use of these chemicals can lead to the production of by-products that may pose a risk to human health [20]. As an alternative, natural disinfectants, such as organic acids, phenolic compounds, fruit/vegetable extracts, and essential oils, have been investigated for their antimicrobial and antibiofilm activities against microbial pathogens [21–24]. Extracts from olive by-products, particularly olive leaves, have been found to exhibit antibacterial activity against a range of bacteria such as *E. coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *S. aureus*, *Yersinia enterocolitica*, *E. faecalis*, *Pseudomonas fluorescens*, *P. savastanoi* pv. *savastanoi*, and *Agrobacterium tumefaciens* [25–29].

The extracts of olive oil mill wastewater (OEs) have various applications in industries, such as food, pharmacology and natural colorant, depending on their intended use [30,31]. OEs rich in polyphenols can be used to enhance the phenolic compounds of virgin olive oil to obtain extra virgin olive oil [19,32]. OEs can also be considered as food preservatives. For example, when OEs are mixed with red meat, they preserve the color of red meat, cause retardation of both fat degradation and microbial growth, thus extending the shelf life of meat [33]. In addition, OEs can be considered as natural disinfectants or natural biofilm control agents in food production processes due to their natural antimicrobial properties [21,22,24]. Natural phenolics from olive by-products, such as OOMW, are also recognized as potential sources in the cosmetic and pharmaceutical industries [34]. Furthermore, the natural color of OEs allows them to be used as a natural colorant in the food, textile or packaging industries [35,36].

Although various methods have been evaluated for the recovery of bioactive components, liquid–liquid extraction with ethyl acetate is a widely applied and practical method to recover bioactive phenolic compounds from OOMW [1]. Meanwhile, ethyl acetate is suitable for substrates with high oil content, i.e., OOMW, due to its ability to dissolve oil [37]. Alternatively, other solvents such as ethanol, isopropanol, n-propanol, methanol and acetone are also commonly used for extracting phenolics from food processing by-products [22,24,38]. However, the recovery yield of phenolic compounds differs due to the different polarities of solvents [39].

In this study, the use of OOMW as a potential antimicrobial substrate in pharmacology and food industry was evaluated. For this purpose, the effects of three different solvents with different polarities were investigated comparatively to obtain phenolic rich extracts from OOMW. In addition, antimicrobial activities of these extracts were tested against ten different microorganisms (*B. spizizenii*, *B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa*, *K. aerogenes*, *S. uberis*, *E. faecalis*, and *C. albicans*) by using agar well diffusion method.

2. Materials and Methods

2.1. Substrate

Olive oil mill wastewater (OOMW) was gifted by an olive oil manufacturer (Dizem Yapı Gıda, Çanakkale, Turkey) to be used in this study. The OOMW used in the study is the aqueous waste generated in the first stage of the three-phase oil extraction process (Figure 1) of *Olea europaea* L. fruits (Edremit yaglik cultivar). OOMW collected in November 2019 was stored at 4 °C for a maximum of one month.

2.2. Extraction

OOMW extracts (OEs) were prepared according to Papazi et al. [8] with some modifications. OOMW was extracted by liquid–liquid extraction by using ethyl acetate (EtOAc, 99.8%), ethanol (EtOH, 99%) and methanol (MeOH, 99.8%), separately (Figure 2).

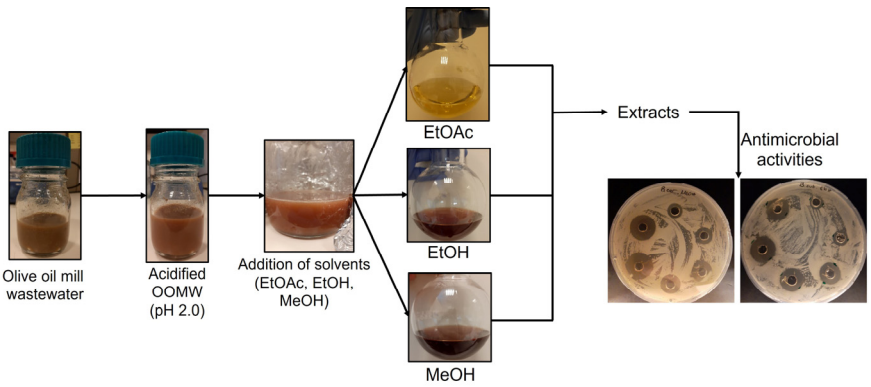

Figure 2. Schematic representation for preparation of OEs and determination of their antimicrobial activities.

- *Ethyl acetate extractions*

Initially, 25 mL of OOMW was acidified to pH 2.0 with 2 N HCl. The acidified OOMW was mixed with 25 mL of ethyl acetate and shaken vigorously using a magnetic stirrer at 350 rpm for 5 min. The ethyl acetate phase was separated and collected. The OOMW phase was remixed with 25 mL of ethyl acetate, and all the supernatants (ethyl acetate phases, total 50 mL) were gathered. The supernatant ethyl acetate liquid phase was evaporated under a vacuum (Heidolph, Schwabach, Germany) at 70 rpm at 45–47 °C for 60 min. Additionally, a different extraction was performed by mixing OOMW and ethyl acetate phases. For this, 50 mL of ethyl acetate phases and 25 mL of OOMW phase were mixed, and then solid substances were removed by centrifugation at 4 °C for 15 min. This mixture was evaporated at 70 rpm at 45–47 °C for 60 min.

- *Ethanol and Methanol extractions*

OOMW was also extracted by using ethanol and methanol (1:1, v/v), separately. OOMW (25 mL) was acidified and then mixed with ethanol (25 mL) or methanol (25 mL), similar to ethyl acetate extraction protocol. The extracts were shaken using a magnetic stirrer for 5 min, then the solid substances of OOMW were removed by centrifugation at 4 °C for 15 min. The liquid fraction (mixture of OOMW with solvent) was re-extracted with ethanol or methanol (25 mL). The ethanol and methanol extracts (50 mL of solvent and 25 mL of OOMW) were evaporated under a vacuum (Heidolph, Schwabach, Germany) at 60 °C and at 57 °C, for 60 min and 80 min, respectively.

- *Control extractions*

The extraction was repeated using water instead of OOMW to determine the possible effects of the solvents (ethyl acetate, ethanol and methanol) used in the extraction on the antimicrobial effects. The OOMW was also used to determine its antimicrobial activities as a negative control. For this, OOMW was centrifuged to remove solid substances and then sterilized by using 0.22 µm sterile syringe filter.

- *Analysis of the extracts*

The extracts of free solvents were stored at −20 °C and used for analysis of total phenolic compounds and their antimicrobial activities. The residual solvents of the extracts were analyzed by using NH_2 column (250 mm × 4.6 mm, 5 µm, GL Sciences Inc., Shinjuku, Tokyo, Japan) in the HPLC system equipped with a refractive index (RI) detector and autosampler. The HPLC analysis was operated at 25 °C with 1 mL min^{-1} of acetonitrile (60%, v/v) as the eluent [40].

2.3. Total Phenolic Contents

Total phenolic compounds of OEs were determined according to the Folin–Ciocalteu method using a spectrophotometer. To determine phenolic compounds, 100 µL of undiluted samples (OOMW and different types of OEs) and 250 µL of Folin–Ciocalteu reagent (0.2 N) were mixed and then 8 mL of Milli-Q water was added. This reaction mixture was incubated for 3 min under dark conditions. The mixture was mixed with 1 mL of Na_2CO_3 (35%, w/v) and 10 mL of Milli-Q water and incubated for 60 min under dark condition [38]. The absorbance at a wavelength of 725 nm was measured using a spectrophotometer. Results were expressed as gallic acid equivalents (GAE), and gallic acid was purchased from Fisher Scientific UK (Leicestershire, UK).

2.4. Antimicrobial Analysis

Antimicrobial activities of OEs were screened on ten different microorganisms (*B. spizizenii* (formerly known as *B. subtilis* subsp. *spizizenii*, ATCC® 6633), *B. cereus* (ATCC® 11778), *S. aureus* subsp. *aureus* (ATCC® 25923 and ATCC® 29213), *E. coli* (ATCC® 25922), *P. aeruginosa* (ATCC® 27853), *K. aerogenes* (basionym *Enterobacter aerogenes*, ATCC® 13048), *S. uberis* (ATCC® 700407), *E. faecalis* (ATCC® 29212), and *C. albicans* (ATCC® 10231)) by using the agar well diffusion method. Each strain was grown in Mueller–Hinton Broth

(MHB, Merck, Darmstadt, Germany) for 16 h. The OD_{600} values of the pre-grown cultures were adjusted to 0.1 using saline water (0.9%, *w*/*v*). 100 µL of each diluted cultures was spread into Petri dishes containing Mueller-Hinton Agar (17 mL). Six wells (7 mm diameter) were punched on each inoculated Petri dish using a sterile Pasteur pipette. Then, different dilutions (no dilution, 1/2 and 1/4) of each extract (40 µL) was transferred into the each well [22,24]. The agar plates were incubated at 37 °C for *E. coli*, *E. faecalis*, *P. aeruginosa*, *S. uberis*, *S. aureus* and *K. aerogenes*, and 30 °C for *Bacillus spizizenii*, *B. cereus* and *C. albicans*, for 24 h. After incubations, inhibition zones of microorganisms by the extracts were measured using a ruler. Each treatment was applied at least three times to determine antimicrobial activities of the extracts.

2.5. Statistical Analysis

The data obtained at the end of the study were analyzed using the MINITAB® software (Minitab Inc., State College, PA, USA) with ANOVA (analysis of variance). Significant differences were considered when the *p*-value was less than 0.05 within a 95% confidence interval.

3. Results and Discussion

The olive oil industry is a leading commercial product, particularly in the Mediterranean region. Despite its economic significance, the production of olive oil generates environmentally polluting by-products. These by-products, while requiring appropriate waste management, are potential biological sources. Various technologies exist for assessing olive oil mill wastewater; however, the recovery and evaluation of its phenolic compounds for potential antimicrobial applications are crucial for ensuring sustainability. In this study, the recovery of phenolic compounds from OOMW was studied by using three different solvents, comparatively. Moreover, antimicrobial activities of the extracts were investigated against various microorganisms by agar well diffusion method.

3.1. Phenolics

The OOMW used in this study is a by-product product of the Edremit yaglik (Ayvalık) olive variety, which constitutes approximately 25% of the olive trees in the Aegean Region of Turkey and has economic value with its high oil content (around 24%) [41]. In addition, the concentration of phenolic compounds in olive oil from the Edremit yaglik cultivar is 21,326.12 mg/kg, of which the three predominant phenolic compounds are oleuropein (3965 mg/kg), trans-cinnamic acid (1399 mg/kg) and luteolin-7-glucoside (1071 mg/kg) [42]. Edremit yaglik is widely used in Turkey's olive oil industry because of its high phenolic content and economic benefits. It is therefore important to evaluate olive industry wastes in circular biotechnology applications.

Total phenolic compound levels of OOMW and OEs are given in Figure 3. The OOMW obtained in this study was derived from three-stage olive oil processing, and the phenolic content of OOMW varies depending on the variety type, season, and olive oil processing type [43]. Although the total phenolic compounds of OOMW have been reported in a wide range (0.68–17.15 g/L) in the literature, it was determined as 2.41 g(GAE)/L in this study [8,44]. Similar to the results obtained in this study, Papazi et al. [8] determined that the OOMWs obtained from 14 different olive oil mills in Greece were between 0.68 and 2.22 g(GAE)/L. The lower phenolic content of the OOMW used in this study may be related to the use of more water for olive oil extraction in the olive oil processing. Since the olive waste generated in the traditional method processes is in a concentrated form, it naturally contains more phenolic compounds [44,45].

To recovery of phenolic compounds from OOMW, ethyl acetate (both solvent phase and mixtures), ethanol and methanol were evaluated as solvents. The total phenolic contents of OOMW and OEs were calculated as equivalent to the gallic acid value. There was no significant difference between the total phenolic contents of OOMW and ethyl acetate extracts (2.16 and 2.44 g(GAE)/L) (p = 0.124). Although ethanol extract (2.97 g(GAE)/L) is a promising solvent compared to ethyl acetate extracts regarding their total phenolic con-

tents ($p < 0.05$), the highest total phenolic level (4.03 g(GAE)/L) was detected in methanol extract ($p = 0.028$). Ethanol and methanol extracts contained 23% and 67% more phenolic compounds, respectively, compared to OOMW (Figure 3).

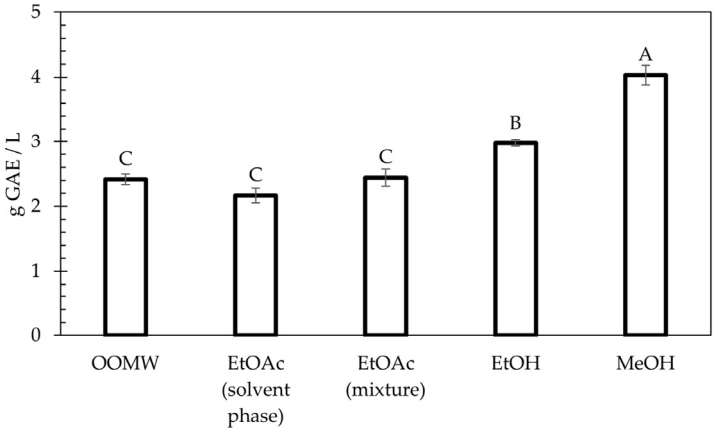

Figure 3. Total phenolic compounds of OOMW and OEs. Values are the averages of three determinations; error bars represent standard deviations. Different letters indicate significant ($p < 0.05$) differences.

Polar solvents are often used to recover phenolic compounds from fruit and plant materials [46]. Ethyl acetate has lower polarity (relative polarity, 0.228), while ethanol (relative polarity, 0.654) and methanol (relative polarity, 0.762) have higher polarities. Although ethyl acetate is often used for the extraction of phenolics from OOMW, it tends to extract mainly low- and medium-molecular-weight phenols [47]. As a result, ethyl acetate may not be able to extract some phenolics found in OOMW, such as protocatechuic acid, veratric acid, syringic acid, cinnamic acid, or p-hydroxyphenylacetic acid [48]. Methanol can be used as an alternative solvent for the extraction of low-molecular-weight polyphenols [49]. Lafka et al. [38] investigated the effects of different solvents (ethanol, methanol, n-propanol, isopropanol and ethyl acetate) for the recovery of phenolic compounds from OOMW and obtained the highest phenolic content with methanol extraction. Therefore, the effectiveness of the solvent on the extraction will change the phenolic contents of the extracts.

3.2. Antimicrobial Activity

All extracts were analyzed by HPLC to detect possible residues of the solvents that interfere with the antimicrobial activities of the OEs. According to HPLC analysis, there was no ethanol, methanol and ethyl acetate (only in mixture phases) detected in OEs. Therefore, it was confirmed that all antimicrobial activities were determined by solvent-free extracts, and they were not affected by possible antimicrobial effects of solvents. In addition, ethyl acetate was mixed with distilled water instead of OOMW, and the extraction method was performed in a similar way. The antimicrobial activities of the residue were also tested, and no zones were detected against microorganisms.

The antimicrobial effects of undiluted OEs and their dilutions (1/2 and 1/4) were screened against selected food-related microorganisms using the agar well diffusion method (Tables 1 and 2). Initially, the antimicrobial effect of OOMW was used as a negative control, but no activity was measured. The OOMW was extracted with ethyl acetate (using solvent phase) and found that the antimicrobial activities of the extract were low on *Pseudomonas*, *Bacillus* and *Staphylococcus* strains. The inhibition zones are 9.5 ± 0.7 mm, 11.5 ± 0.7 mm, 12.0 ± 0.1 and 11.5 ± 0.7 mm for *P. aeruginosa*, *B. spizizenii*, *B. cereus* and *S. aureus*, respectively. There was no antibacterial activity observed against other strains (Table 1). The mixture of the extract of ethyl acetate and OOMW phase gave relatively higher antimicrobial activities (Table 1) and were generally more effective against *S. uberis*, *E. faecalis*, *B. spizizenii* and *B. cereus* strains ($p = 0.004$), with 18.0 ± 1.8 mm, 18.8 ± 3.2 mm, 16.5 ± 0.6 mm, and 16.8 ± 1.0 mm inhibition zones, respectively. The higher antimicrobial

activities could be due to the higher phenolic contents or other components, such as fatty acids, found in the mixture [50].

Table 1. Inhibition zones (mm) of OEs (solvent phase and mixture) obtained by use of ethyl acetate with different dilutions (D: no dilution; 1/2; and 1/4) on selected microorganisms. Values are the averages of three determinations, and results are given as mean values ± standard deviations (n = 3). The well diameter (7 mm) is included in the results.

Microorganisms	Dilutions	Inhibition Zones (mm)	
		Ethyl Acetate (Solvent Phase)	Ethyl Acetate (Mixture)
Bacillus spizizenii ATCC 6633	D	11.5 ± 0.7 ^{f}	16.5 ± 0.6 ^{bc}
	1/2	10.0 ±0.1 ^{g}	12.8 ± 1.0 ^{e}
	1/4	7.0 ± 0.0	9.0 ± 0.8 ^{h}
Bacillus cereus ATCC 11778	D	12.0 ± 0.1 ^{ef}	16.8 ± 1 ^{bc}
	1/2	10.0 ± 0.1 ^{f}	13.0 ± 0.8 ^{de}
	1/4	7.0 ± 0.0	11.0 ± 0.8 ^{ef}
Staphylococcus aureus ATCC 25923	D	11.5 ± 0.7 ^{f}	15.5 ± 1.0 ^{bc}
	1/2	8.5 ± 0.7 ^{g}	12.0 ± 0.8 ^{de}
	1/4	7.0 ± 0.0	9.3 ± 0.5 ^{ef}
Staphylococcus aureus ATCC 29213	D	11.5 ± 0.7 ^{de}	13.8 ± 1.0 ^{cd}
	1/2	8.5 ± 0.7 ^{f}	11.5 ± 0.6 ^{de}
	1/4	7.0 ± 0.0	9.8 ± 1.0 ^{ef}
Escherichia coli ATCC 25922	D	7.0 ± 0.0	13.3 ± 1.0 ^{de}
	1/2	7.0 ± 0.0	11.5 ± 1.0 ^{de}
	1/4	7.0 ± 0.0	8.3 ± 0.5 ^{f}
Klebsiella aerogenes ATCC 13048	D	7.0 ± 0.0	12.8 ± 0.5 ^{ab}
	1/2	7.0 ± 0.0	10.8 ± 1.0 ^{bc}
	1/4	7.0 ± 0.0	8.0 ± 0.0 ^{d}
Pseudomonas aeruginosa ATCC 27853	D	9.5 ± 0.7 ^{d}	12.8 ± 1.0 ^{abc}
	1/2	8.0 ± 0.1 ^{e}	10.8 ± 0.5 ^{bcd}
	1/4	7.0 ± 0.0	7.0 ± 0.0
Streptococcus uberis ATCC 700407	D	7.0 ± 0.0	18.0 ± 1.8 ^{a}
	1/2	7.0 ± 0.0	13.8 ± 1.5 ^{bc}
	1/4	7.0 ± 0.0	7.0 ± 0.0
Enterococcus faecalis ATCC 29212	D	7.0 ± 0.0	18.8 ± 3.2 ^{a}
	1/2	7.0 ± 0.0	13.5 ± 1.3 ^{abc}
	1/4	7.0 ± 0.0	10.5 ± 0.7 ^{c}
Candida albicans ATCC 10231	D	7.0 ± 0.0	7.0 ± 0.0
	1/2	7.0 ± 0.0	7.0 ± 0.0
	1/4	7.0 ± 0.0	7.0 ± 0.0

The letters indicate significant ($p < 0.05$) differences in all extracts with different dilutions for each microorganism.

The methanol extract exhibited the highest antimicrobial activities on all bacterial strains (13.5–22.5 mm, Table 2), and they gave similar results with ethyl acetate on *S. uberis* (inhibition zone of 17.5 ± 0.7 mm) and *E. faecalis* (inhibition zone of 18.5 ± 0.7 mm) ($p > 0.05$). As expected, the antibacterial activities of the OEs decreased with increasing dilution rates. Additionally, all extracts were tested on *C. albicans* but no inhibition was observed. Similarly, Nunes et al. [51] investigated the antimicrobial effectiveness of a patented processed olive pomace as a by-product of olive industry on Gram-negative *E. coli* and Gram-positive *S. aurues* bacteria and *C. albicans.* Although they could measure antibacterial activities of the pomace against both bacteria, they found no activity on *C. albicans* [51].

Table 2. Inhibition zones (mm) of OEs obtained by use of ethanol and methanol with different dilutions (D: no dilution; 1/2; and 1/4) on selected microorganisms. Values are the averages of three determinations, and results are given as mean values ± standard deviations (n = 3). Different letters indicate significant ($p < 0.05$) differences. The well diameter (7 mm) is included in the results.

Microorganisms	Dilutions	Inhibition Zones (mm)	
		Ethanol	Methanol
Bacillus spizizenii ATCC 6633	D	17.5 ± 0.7 ^{ab}	18.5 ± 0.7 ^{a}
	1/2	15.5 ± 0.7 ^{cd}	15.0 ± 0.0 ^{d}
	1/4	11.0 ± 0.1 ^{fg}	11.5 ± 0.7 ^{f}
Bacillus cereus ATCC 11778	D	18.5 ± 0.7 ^{ab}	20.0 ± 0.0 ^{a}
	1/2	15.5 ± 0.7 ^{cd}	15.5 ± 0.7 ^{cd}
	1/4	12.0 ± 0.1 ^{ef}	12.5 ± 0.7 ^{ef}
Staphylococcus aureus ATCC 25923	D	16.5 ± 0.7 ^{ab}	18.5 ± 0.7 ^{a}
	1/2	13.5 ± 0.7 ^{cd}	14.0 ± 0.1 ^{bcd}
	1/4	9.5 ± 0.7 ^{ef}	10.5 ± 0.7 ^{ef}
Staphylococcus aureus ATCC 29213	D	16.0 ± 0.1 ^{bc}	22.5 ± 0.7 ^{a}
	1/2	14.0 ± 0.1 ^{cd}	18.5 ± 0.7 ^{b}
	1/4	10.5 ± 0.7 ^{ef}	14.5 ± 0.7 ^{c}
Escherichia coli ATCC 25922	D	14.0 ± 1.4 ^{bc}	15.5 ± 0.7 ^{fa}
	1/2	12.0 ± 0.1 ^{cd}	12.0 ± 0.1 ^{b}
	1/4	9.5 ± 0.7 ^{ef}	10.0 ± 0.1 ^{cd}
Klebsiella aerogenes ATCC 13048	D	13.0 ± 0.1 ^{ab}	13.5 ± 0.7 ^{a}
	1/2	11.0 ± 0.1 ^{bc}	11.0 ± 0.1 ^{bc}
	1/4	8.5 ± 0.7 ^{d}	9.0 ± 0.1 ^{cd}
Pseudomonas aeruginosa ATCC 27853	D	14.0 ± 1.4 ^{ab}	16.0 ± 1.4 ^{a}
	1/2	11.0 ± 1.4 ^{bcd}	10.5 ± 0.7 ^{bcd}
	1/4	9.0 ± 0.1 ^{d}	8.5 ± 0.7 ^{d}
Streptococcus uberis ATCC 700407	D	13.0 ± 0.1 ^{bc}	17.5 ± 0.7 ^{a}
	1/2	10.0 ± 0.1 ^{c}	15.0 ± 0.1 ^{ab}
	1/4	7.0 ± 0.0	7.0 ± 0.0
Enterococcus faecalis ATCC 29212	D	16.5 ± 0.7 ^{ab}	18.5 ± 0.7 ^{a}
	1/2	13.0 ± 1.4 ^{bc}	13.0 ± 1.4 ^{bc}
	1/4	7.0 ± 0.0	7.0 ± 0.0
Candida albicans ATCC 10231	D	7.0 ± 0.0	7.0 ± 0.0
	1/2	7.0 ± 0.0	7.0 ± 0.0
	1/4	7.0 ± 0.0	7.0 ± 0.0

The letters indicate significant ($p < 0.05$) differences in all extracts with different dilutions for each microorganism.

All three OEs exhibited broad-spectrum antibacterial activities against nine different microorganisms. Although antibacterial effects of three OEs were determined, the extract with the highest total phenolic content, the methanol extract, exhibited the best antimicrobial effect. This finding is consistent with the study conducted by Yakhlef et al. [29], who reported that the extract of OOMW with the highest phenolic content had the best antibacterial activities against *S. aureus*, *P. fluorescens*, *E. coli* and *E. faecalis*. Moreover, Bisignano et al. [52] reported that hydroxytyrosol and oleuropein in olives have cytotoxic activity against clinical bacterial strains, including ATCC reference strains. Pannucci et al. [27] determined in-vitro antimicrobial activities of hydroxytyrosol-rich extracts obtained from OOMW against two olive tree pathogens, *P. savastanoi* pv. *savastanoi* and *A. tumefaciens*. Extracts rich in these phenolic compounds with antimicrobial activities can also be obtained from olive leaves similar to OOMW [26,53]. On the other hand, in addition to olive-based products, Macaúbas-Silva et al. [54] extracted a tyrosol derivative, araçain, from *Psidium guineense* Sw. and reported that this extract showed moderate to high antibacterial activity against *Klebsiella pneumoniae* strains. Gram-negative bacteria (e.g., *E. coli* and

K. aerogenes) were found to be more resistant than Gram-positive bacteria (e.g., *Bacillus* and *Staphylococcus* strains) against polyphenolic compounds [55]. Similarly, Casadey et al. [56] found that tyrosol and its derivatives had a stronger antibacterial effect on *S. aureus* and weak antibacterial effect on *E. coli*. Furthermore, Topuz and Bayram [28] also stated that the most sensitive microorganism was *S. aureus*, while the most resistant microorganism was *E. coli* against oleuropein extracted from olive leaves. Balaban et al. [24] also reported that pomegranate peel extracts had more antibacterial activities against Gram-positive bacteria than Gram-negative bacteria. This is probably due to the action of the lipopolysaccharide membranes of Gram-negative bacteria acting as a permeability barrier, which is very effective at the passage of antimicrobial substrates [14,56]. On the other hand, no antifungal activity was found against *C. albicans* with OEs used in this study, which is consistent with the findings of a study conducted by Obied et al. [57]. In addition to the solvent type, the extraction method is also effective in the antimicrobial activities of the extracts. Sánchez-Gutiérrez et al. [26] compared the Soxhlet and microwave-assisted extraction (MAE) methods and determined that MAE had the strongest antimicrobial activity.

Olive fruit is rich in various phenolic compounds such as tyrosol, hydroxytyrosol, oleuropein, luteolin and apigenin [58]. The action antimicrobial mechanism of olive polyphenols targets the cell membranes of bacteria [52]. Canal et al. [58] investigated the potential antimicrobials of these phenolic compounds on *Aureobasidium pullulans* and *Saccharomyces cerevisiae* and observed high antimicrobial activities at concentrations of 200 ppm and above. It has been found that tyrosol and luteolin treatments affect the amide and fatty acid structures of microorganisms, while other phenolics, except tyrosol, affect carbohydrates in the cell structure [58]. Although phenolic compounds have been reported to exhibit antimicrobial activities by different mechanisms, they mainly exhibit antibacterial effects by damaging cell peptidoglycans and/or cell [21,59].

4. Conclusions and Future Perspectives

The study evaluated the potential of natural products to replace synthetic chemicals in industry. OEs extracted from OOMW were tested against ten microorganisms, revealing significant antibacterial activities, but no antifungal activity against *C. albicans*. The highest total phenolic compounds and the strongest antibacterial effects were observed in the methanol extract of OOMW. These findings indicate that OEs, particularly methanol extracts, have potential applications in the food and pharmaceutical industries, as nutraceuticals and food ingredients. The study highlights the importance of recovering phenolic compounds from OOMW, contributing to both waste treatment and the bioeconomy in a sustainable way to be used in pharmacology and food industries. Future research should investigate OEs' minimum inhibition concentrations and minimum bactericidal concentrations on foodborne pathogenic bacteria and their antibiofilm activities. OEs' natural disinfectant and food preservative activities also need to be explored. Additionally, the potential use of the OEs can be compared with the production of biotechnological products from OOMW through modeling studies regarding their potential economic benefits to the industry.

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