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Antifungal effect of fresh and stored olive mill wastewater and its ethyl acetate extract against plant pathogenic fungi

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Abstract: Olive mill wastewater (OMW) has serious environmental implications due to its high organic matter content, particularly its phenolic compounds. Using OMW in crop protection has been suggested as an environmentally friendly alternative to reduce the impact of chemical pesticides on human health and the environment. This study aimed to investigate the antifungal activity of fresh and stored OMW, as well as its ethyl acetate extract, against several phytopathogenic fungi: *Syncephalastrum racemosum*, *Paramyrothecium roridum*, *Fusarium oxysporum*, and *Verticillium dahliae*. OMW was stored at 25 °C and 45 °C for three months, and both fresh and stored OMW were used in non-sterile, sterile, and centrifuged conditions. Phenolic and flavonoid compounds were extracted and identified by high-performance liquid chromatography (HPLC) analysis. Results showed that fresh OMW and its derivative compounds significantly inhibited the studied fungi. In contrast, OMW storage, sterilization, and centrifugation increased the mycelium growth of the fungi, particularly *S. racemosum*, which demonstrated relative resistance to stored OMW and its ethyl acetate extract. During storage, some phenolic and flavonoid compounds disappeared (resorcinol and vanillic acid), while the concentration of others increased (gallic acid, chlorogenic acid, and quercetin). This work highlights the potential use of fresh OMW as a bio-agent to protect plants from fungal diseases.

Keywords: crop protection; antifungal activity; phytopathogenic fungi; phenolic compounds; storage

Worldwide olive oil production is increasing annually by 5%, with olive mill wastewater (OMW) production estimated to be around 6 × 10^6 m^3, 98% of which is in the Mediterranean basin (FAOSTAT 2020). Since 2015, Saudi Arabia has witnessed a 58.5% increase in olive oil production each year, with an annual production of 80 000 t in the Kingdom of Saudi Arabia (KSA) in the northern regions of Al-Jouf, Al Qurayyat, Hail, and Tabuk cities, where climatic and geographical conditions are suitable for olive tree cultivation (Hemida et al. 2014; Alhajoj & Alowaiesh 2019). Therefore, OMW production in KSA is estimated at around 160 000 m^3 (Aly et al. 2014).

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OMW is characterized by high organic loads expressed in terms of chemical oxygen demand (COD), biological oxygen demand (BOD), total solids (TS), total suspended solids (TSS), total organic carbon (TOC), and phenolic compound concentrations. Phenolic compounds are responsible for OMW's phytotoxic and antimicrobial activities, making it one of the most polluting wastewater generated by the food industry (Jarboui et al. 2009; Dermeche et al. 2013; Senani-Oularbi et al. 2018). In addition, OMW contains soil fertilizing content due to its high mineral nutrient values. OMW's biological activity is also characterized by its suppressive effects on plant pathogens. Many microorganisms are associated with plant diseases, including phytopathogenic fungi, which are causal agents of some of the world's most serious plant diseases, capable of significantly reducing productivity on a large scale in agricultural fields. According to Jarboui et al. (2008) and Magdich et al. (2012), OMW contains phenolic compounds and free fatty acids that provide antimicrobial properties. In particular, flavonoid and phenolic compounds exhibit antimicrobial activity, making them useful as natural plant protectors against fungal species (*Fusarium oxysporum* f. sp. *lycopersici*, *Pythium* spp., *Sclerotinia sclerotiorum*, *Verticillium dahliae* (Vagelas et al. 2016; Cibelli et al. 2017), *Phytophthora capsici*, *Rhizoctonia solani, Diploceras hypericinum* (Cayuela et al. 2008; Cirak et al. 2014; Nawrocka et al. 2018), as well as phytopathogenic bacteria (*Pseudomonas putida*, *Pseudomonas syringaepv. tomato*, *Xanthomonas vesicatoria*, *Clavibacter michiganensis* subsp. *michiganensis*, *Ralstonia solanacearum* (Cirak et al. 2014; Arredondo-Valdés et al. 2020) weed species (*Amaranthus retroflexus*, *Solanum nigrum*, *Chenopodium album*, and *Sorghum halepense*), and nematodes (Cayuela et al. 2008).

Furthermore, OMW can be used as a phytoprotective compound during the growth, harvest, and storage of vegetables and fruits. It is a promising way to prevent fruit and vegetable losses following an attack by saprophytic and phytopathogenic fungi (Quaglia et al. 2016; Senani-Oularbi et al. 2018). OMW does not harm crop growth when used appropriately regarding dose and timing for crop protection (El-Abbassi et al. 2017). In this regard, using OMW or phenolic compound extracts as alternatives to pesticides during the growing season is an appropriate approach to protecting humans and the environment from the harmful effects of pesticides frequently applied to plants.

Therefore, the present study aimed to investigate the antifungal effect of fresh and stored OMW, stored at 25 and 45 °C for three months, and its ethyl acetate extract against phytopathogenic fungi.

MATERIAL AND METHODS

Sampling. The OMW was obtained from a three-phase continuous extraction factory in Al Jouf City, Saudi Arabia (29°52'40.8108"N, 40°6'15.5016"E) and underwent physicochemical characterization. The pH and electrical conductivity (EC) were measured directly using a pH meter (WTW inoLab 7110 model, Thermo Fisher, Sweden) and conductivity meter (CL 221 model, Lab Tech, Italy), respectively. The TS content was determined after drying the OMW overnight at 105 °C (JIS 1995). The volatile solids (VS) content was gravimetrically assessed after incinerating the dry OMW at 550 °C for 4 h (JIS 1995). The ash content was calculated as the total and volatile solid contents. The TSS was determined gravimetrically after centrifuging the crude OMW at 4 000 rpm for 15 min (JIS 1995). The COD was determined using the Knechtel method (Knechtel 1987). The BOD was measured using the manometric method with a pressure sensor using the OxiTop method. The TOC was measured with the Shimadzu TOC-500 analyzer (Shimadzu, Japan) after acidic hydrolysis of the sample with concentrated HCl (JIS 1995). The total phosphorus (TP) concentration was determined calorimetrically as a molybdovanadate phosphoric acid complex, while the total nitrogen (TN) concentration was determined by the Kjeldahl method (JIS 1995). The macro- and micro-elements were analyzed by an atomic absorption spectrophotometer (Hitachi Z-6100, Hitachi, Japan), and the polyphenol content was determined gravimetrically after extracting it with ethyl acetate. All OMW parameters were compared to the standard limits recommended by WHO (WHO 2004) and the presidency of meteorology and environment in KSA (PME 2001). All parameters were analyzed in triplicate.

Isolation and identification of phytopathogenic fungi. Phytopathogenic fungi were isolated from diseased plant organs (Table 1). To remove saprophyte microorganisms, surfaces of diseased plant organs were disinfected with 5% chlorite solution (NaClO) and washed with sterile distilled water for 30 min. Under sterile conditions, plant fragments were cut

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Table 1. Studied phytopathogenic fungi

Identified strain	GenBank Accession No.	ITS sequence length (bp)	CG (%)	Host	Disease symptoms
Syncephalastrum racemosum	MZ057792	602	34	*Ficus benjamina*	leaf blight
Paramyrothecium roridum	MZ057761	526	55.7	*Cucumis sativus*	leaf spot and root rot
Fusarium oxysporum	OL913805	527	48.8	*Solanum lycopersicum*	wilt
Verticilium dahliae	MW830379	536	54.8	*Olea europea*	wilt

ITS – internal transcribed spacer; GC% – guanine-cytosine percent; host – diseased plants from which fungi were isolated

with a sterile scalpel and then transferred onto Potato Dextrose Agar (PDA, Himedia, USA). The Petri dishes were incubated at 28 °C for 7 days. Isolated fungi were purified twice on PDA to confirm the purification of the selected isolates (Shaima et al. 2021). Isolated fungi were molecularly identified.

Molecular identification of isolated fungi was performed by sequencing the internal transcribed spacer (ITS). Therefore, the genomic DNA of isolated fungi was extracted from a seven-day-old fungal culture grown on PDA. The fungal mass was scraped out with a fine spatula and then grinded in liquid nitrogen. 100 mg of mycelium was used for genomic DNA extractions using the i-genomic™ Series DNA Extraction Mini Kit (iNtRON, Korean) specialized for extracting genomic DNA from the specific specimen.

The Internal Transcribed Spacer (ITS) gene amplification was performed using the ITS universal primer (Sigma Company, Germany). The universal primer, ITS1 (TCCGTAGGTGAACCTTGCGG) and ITS4 (TCCTCCGCTTATTGATGC), produced approximately 500 bp of amplicon products. PCR Master Mix (Promega, Netherlands) was used to amplify the ITS region. PCR mix without a DNA template was performed as a negative control. The PCR reaction conditions were 95 °C for 2 min (1 cycle), followed by 35 cycles of denaturation at 95 °C for 0.5 min, annealing at 52 °C for 30 min, and extension at 72 °C for 2 min, before a final extension at 72 °C for 15 min.

The resulting purified PCR products were subjected to individual Basic Local Alignment Search Tool (BLAST) searches to verify their authenticity in GenBank. The reported sequences of the ITS rDNA gene were submitted to the GenBank database and assigned accession numbers MZ057792, MZ057761, OL913805, and MW830379, respectively, according to the fungi. The guanine-cytosine (GC) ratio was studied for the ITS regions of the four identified fungi, and calculations were performed using ACUA software (Umashankar et al. 2007). Details regarding the identification, GC content, isolation source and disease symptoms of phytopathogenic fungi are presented in Table 1.

For the phylogenetic analyses, the sequences of the tested and the comparative isolates were aligned with highly similar sequences from the GenBank using the multiple sequence alignment (MUSCLE) software. The alignment of sequences was performed using Clustal X software (version 2.0) (Larkin et al. 2007). Phylogenetic analysis of ITS rDNA derived from Neighbour-Joining method using MEGA software (version 4.0).

OMW inhibition effect against phytopathogenic fungi. The OMW was used fresh and stored at 25 °C and 45 °C for three months. All samples were raw, centrifuged at 4 000 rpm for 15 min, and sterilized by autoclaving at 121 °C for 20 min. To evaluate their potential to inhibit the mycelium growth of the four studied phytopathogenic fungi, all OMW samples were diluted with PDA medium at ratios of 1/5 and 1/10. A 6-mm disk of mycelium from the periphery of seven-day-old fungal colonies was placed at the center of each plate, and then, plates were incubated at 28 °C. PDA medium was used as a negative control. The mycelial growth inhibition was assessed by observing the point at which the mycelium growth of the control reached the periphery of the Petri dishes (Onaran & Bayram 2018). The inhibition percentage of mycelium growth of studied fungi was determined according to the following formula:

$$\text{Inhibition } (\%) = \frac{D-d}{D} \times 100 \qquad (1)$$

where: D – the diameter of fungal growth mycelium in the control; d – the diameter of fungal growth mycelium in a Petri dish containing OMW

Microbial count and water content determination. For microbial count, 10 mL of raw, centri-

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fuged fresh, and stored OMW at 25 °C and 45 °C for three months were suspended in sterile physiological water (90 mL). The suspension was used for microbial count expressed as the total number of colonies forming units (cfu) according to ISO 7218 (ISO 1996). For each suspension, serial decimal dilutions were plated in triplicate on different agar media: Plate Count Agar (Pronadisa, Madrid, Spain), for the total count bacteria (TCB) incubated at 37 °C for 24 h, and PDA for yeasts and moulds enumeration, incubated at 28 °C for five days (AFNOR 1995).

Water content was gravimetrically determined before and after drying the studied OMW samples overnight at 105 °C (JIS 1995).

Ethyl acetate extract effect against phytopathogenic fungi. Phenolic compounds were extracted three times using ethyl acetate from raw fresh and stored OMW at 25 °C and 45 °C for three months. The pooled organic extracts were then filtered through a filter paper where sodium sulphate had been deposited, collected in a 250 mL pear-shaped flask, and the solvent removed in a rotavapor. The phenolic compounds were gravimetrically determined after weighing the pear-shaped flask initially and after solvent removal. The final concentration of phenolic compounds was estimated as 7.82 g ± 0.24, 8.54 g ± 0.36 and 9.66 g ± 0.82 of fresh and stored OMW at 25 °C and 45 °C respectively. After evaporation using a rotary evaporator, phenolic compounds were dissolved in 2 mL of methanol. The agar diffusion technique was used to determine the antifungal activity of OMW polyphenols against phytopathogenic fungi. 200 µL of each phenolic compound sample was poured onto the surface of the PDA medium, followed by incubation at 4 °C until complete adsorption in the medium. Then, a 6-mm disk of a 7-day-old fungal culture was transferred into the center of the PDA Petri dishes. PDA medium was used as a negative control. These were incubated at 28 °C until the mycelium growth of the control reached the Petri dishes periphery. The experiment was set with three replicates and repeated twice (Leontopoulos et al. 2015).

The inhibition percentage of tested fungi was determined according to the equation:

$$\text{Inhibition (\%)} = \frac{D-d}{D} \times 100 \qquad (2)$$

where: D – the diameter of fungal growth mycelium in the control; d – the diameter of fungal growth mycelium in a Petri dish containing phenolic compounds

Phenolic and flavonoid compounds analysis using the HPLC method. Phenolic and flavonoid compounds were quantified and identified using reversed-phase High Performance Liquid Chromatography (HPLC) (Agilent 1 200 series, Agilent, Santa Clara, CA, USA) equipped with a diode array detector and C18 column of 3 × 250 mm (Thermo Scientific, Waltham, USA). The mobile phase consisted of 0.05% trifluoroacetic acid in water (solvent A, Merck, Darmstadt, Germany) and acetonitrile (solvent B, HPLC grade ≥ 99.9%; Honeywell Seelze, Germany). The temperature was maintained at 40 °C, and the total running time was 20 min. The flow rate was 0.9 mL/min, and the injection volume was 10 µL. The mobile phase was consecutively programmed in a linear gradient as follows: 60–82% B over 20 min, followed by 5 min equilibration at 60% B. Detection was monitored at 280 nm, and the concentration of the identified compounds was determined based on the area of the relative peaks compared to the spectra of the commercial standards.

Statistical Analysis. The results were expressed as mean ± standard deviation using a one-way analysis of variance (ANOVA) test followed by a Student's *t*-test, employing IBM SPSS 20 statistical software (version 20). Differences were considered significant when $P \leq 0.05$. The results were expressed as means ± standard error (SE). All analyses were conducted in triplicate.

RESULTS

Molecular identification of phytopathogenic fungi. Four phytopathogenic fungi were isolated from different diseased plant organs (Table 1). The high similarity of the sequence analyses was comparable to those of the reference strains available in the GenBank database. The ITS rDNA sequence alignment revealed that the isolates were *S. racemosum*, *P. roridum*, *F. oxysporum*, and *V. dahliae*. *S. racemosum* showed the lowest GC content (34%), while *P. roridum* showed the highest content (55.7%), which provided a reasonable prediction. Figure 1 shows the constructed Neighbour-joining tree based on the sequences of the four tested and twenty-two comparative fungal strains obtained from GenBank belonging to four distinct species (*S. racemosum*, *P. roridum*, *F. oxysporum*,

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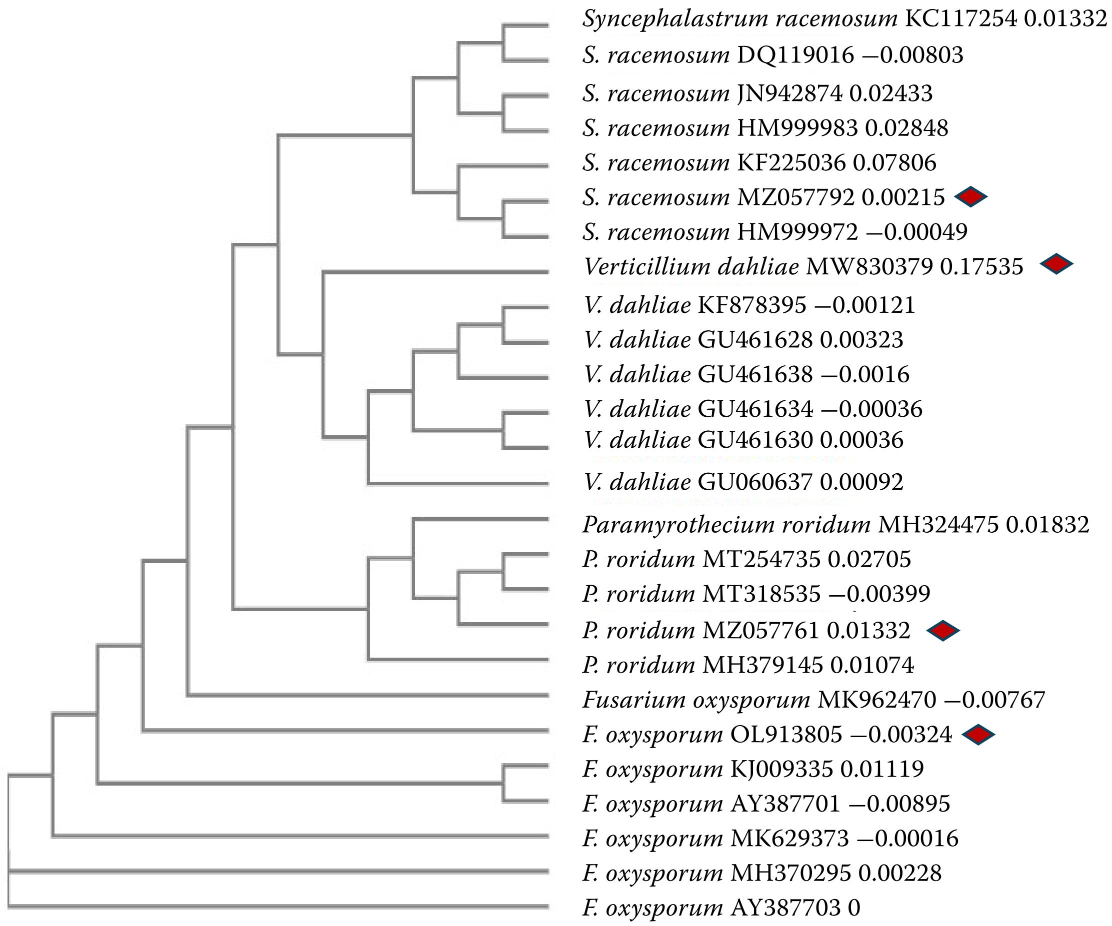

Figure 1. A phylogenetic analysis of ITS rDNA was derived from the neighbour-joining method using MEGA software (version 4.0); diamond shapes represent our isolates, and the remaining are reference sequences

and *V. dahliae*) (Table 2). The results showed four groups. The sequences of each isolated strain were closely related to those in a similar group. The sequence of *S. racemosum*, *P. roridum*, *F. oxysporum*, and *V. dahliae* exhibited a similarity of 100% with the accession strains MW407961, GQ853401, MT530243, and EF377335, respectively.

Olive mill wastewater physicochemical characterization. The physicochemical analysis of the OMW is presented in Table 3. The OMW pH was relatively acidic. The OMW had high organic loads (COD, BOD_5, TOC, TS, VS, TSS, and total N) and high mineral content, especially in fertilizing elements (ash, EC, total P, Ca, Mg, K, and Na). The CODs/BOD_5 rate (2.3) was lower than 2.5. Moreover, all the values of heavy metals in the OMW were within the standard limits recommended by WHO and the presidency of meteorology and environment in KSA.

Effect of OMW against phytopathogenic fungi. The suppressive effects of fresh and stored OMW at 25 °C and 45 °C against phytopathogenic fungi for three months were summarized in Table 4. The results indicated that all the studied OMW at 1/5 ilution showed the highest inhibition percentage for all the investigated phytopathogenic fungi compared to that at 1/10 dilution. Furthermore, non-sterile, raw, fresh OMW used at dilutions of 1/5 and 1/10 inhibited (100%) *P. roridum*, *F. oxysporum*, and *V. dahliae*. (Figure 2). A similar effect was obtained with raw, non-sterile OMW stored at 25 °C for three months on the mycelium growth of *P. roridum* at 1/5 and 1/10 and *F. oxysporum* at a dilution of 1/5. After storage at 45 °C for three months, raw, non-sterile OMW relatively decreased the inhibition percentage of all the studied fungi. The thermal treatment of OMW by autoclave strongly decreased the inhibition percentage of all the studied fungi, compared to centrifugation and non-sterilization processed OMW.

Furthermore, it was found that *S. racemosum* was significantly resistant ($P < 0.05$) to the effect of ster-

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Table 2. Comparative fungal strains from GenBank used for the phylogenetic analysis

Organisms	Origin	Aligned sequence length (base pairs)	Accession No.
Syncephalastrum racemosum	Saudi Arabia (Sakaka)	602	MZ057792
	USA (Bethesda)	589	JN942874
	France (Plouzane)	614	KF225036
	Egypt (Cairo)	569	KC117254
	France (Paris)	621	DQ119016
	Netherlands (Utrecht)	593	HM999983
	Netherlands (Utrecht)	603	HM999972
Paramyrothecium roridum	Saudi Arabia (Sakaka)	526	MZ057761
	China (Shandong)	556	MT318535
	Italy (Turin)	525	MH324475
	USA (Urbana)	577	MH379145
	Mexico (Sinaloa)	482	MT254735
Fusarium oxysporum	Saudi Arabia (Sakaka)	527	OL913805
	China (Hainan)	503	KJ009335
	Mexico (Oaxaca)	521	MH370295
	China (ChangChun)	545	MK962470
	China (Yunnan)	447	AY387701
	China (Yunnan)	441	AY387703
	Spain (Madrid)	460	MK629373
Verticillium dahliae	Saudi Arabia (Sakaka)	536	MW830379
	Egypt (Giza)	532	KF878395
	Germany (Lower Saxony)	565	GU060637
	USA (Salinas)	530	GU461638
	USA (Salinas)	544	GU461634
	USA (Salinas)	527	GU461630
	USA (Salinas)	494	GU461628

ile raw fresh OMW and OMW stored at 25 °C and 45 °C under all the studied conditions. However, the inhibition percentage was insignificant between *P. roridum*, *F. oxysporum*, and *V. dahliae* ($P > 0.05$).

In order to assess the effect of OMW humidity and microbial flora on fungal inhibition, water content, total count bacteria, and yeasts and moulds enumeration were determined (Table 4). The results showed a decrease in the water content of fresh raw non-sterile OMW after storage at 45 °C, which is more than 25 °C, for three months. Centrifugation increases the water content in all raw OMW samples, while sterilization decreases it. Fresh OMW showed the highest number of the total count bacteria, while the highest number of yeast and moulds was shown in OMW stored at 25 °C for three months. Centrifugation decreases the microbial flora count. After storage at 45 °C for three months, no microbial flora was detected in OMW.

Effect of ethyl acetate extract against phytopathogenic fungi. The inhibition percentages of the ethyl acetate extract on the mycelium growth of the tested phytopathogenic fungi are presented in Table 5. The fresh OMW ethyl acetate extract showed the highest inhibition growth [40% (*S. racemosum*) – 88% (*F. oxysporum*)] for all of the tested phytopathogenic fungi, while those extracted from OMW stored at 45 °C for 3 months exhibited the lowest inhibition percentages [0% (*S. racemosum*) – 55% (*F. oxysporum*)].

Phenolic compounds extracted significantly negatively affected *F. oxysporum*, while those extracted from stored OMW positively affected *S. racemosum* mycelium growth.

Identification and quantification of phenolic compounds and flavonoids in fresh and stored

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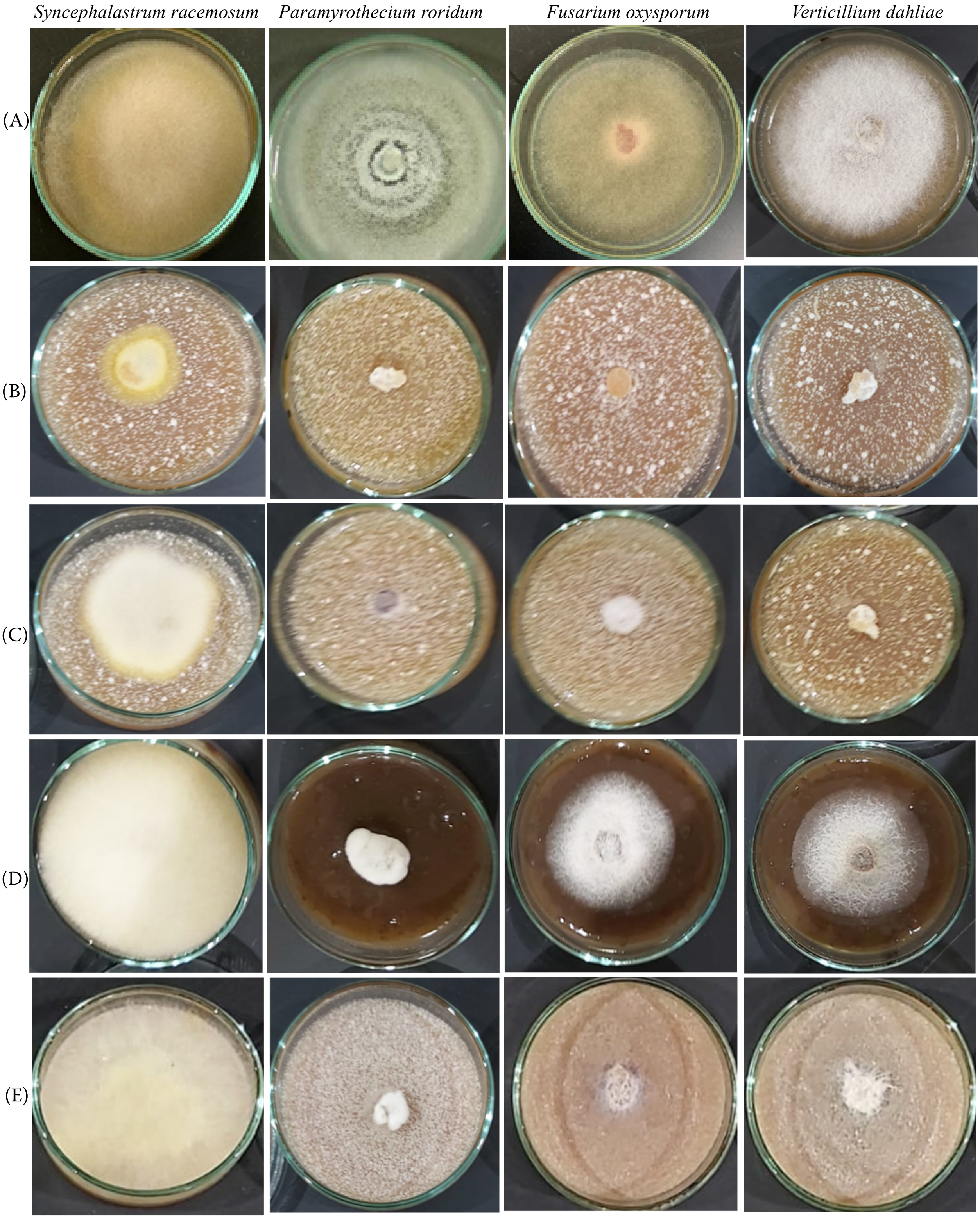

Figure 2. Inhibition effect of olive mill wastewater (OMW) used at dilution 1/5 against phytopathogenic fungi at different conditions: (A): Phytopathogenic Fungi on PDA (negative control); (B): Raw non-sterile fresh OMW; (C): Centrifuged non-sterile fresh OMW; (D): Raw sterile fresh OMW; (E): Raw non-sterile OMW stored at 25 °C for 3 months; (F): Centrifuged non-sterile OMW stored at 25 °C for 3 months; (G): Raw sterile OMW stored at 25 °C for 3 months; (H): Raw non-sterile OMW stored at 45 °C for 3 months; (I): Centrifuged non-sterile OMW stored at 45 °C for 3 months; (J): Raw sterile OMW stored at 45 °C for 3 months

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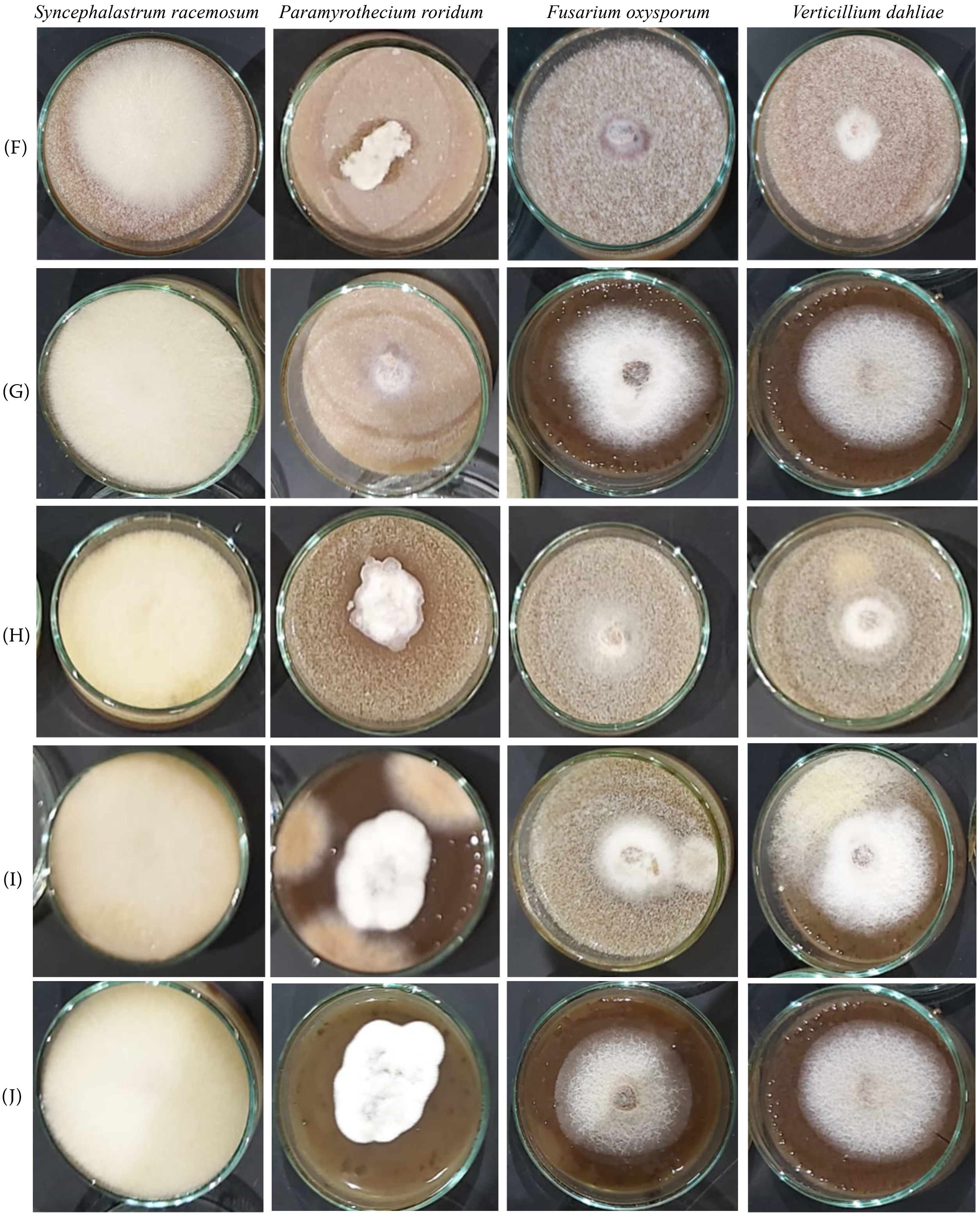

Figure 2 to be continued. Inhibition effect of olive mill wastewater (OMW) used at dilution 1/5 against phytopathogenic fungi at different conditions: (A): Phytopathogenic Fungi on PDA (negative control); (B): Raw non-sterile fresh OMW; (C): Centrifuged non-sterile fresh OMW; (D): Raw sterile fresh OMW; (E): Raw non-sterile OMW stored at 25 °C for 3 months; (F): Centrifuged non-sterile OMW stored at 25 °C for 3 months; (G): Raw sterile OMW stored at 25 °C for 3 months; (H): Raw non-sterile OMW stored at 45 °C for 3 months; (I): Centrifuged non-sterile OMW stored at 45 °C for 3 months; (J): Raw sterile OMW stored at 45 °C for 3 months

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Table 3. Physico-chemical characterization of olive mill wastewater (OMW)

Parameter	OMW	Maximum limit (WHO)	Maximum limit (KSA)
Basic index			
pH	5.02 ± 0.50	6.50–8.00	6.00–9.00
EC (mS/cm)	13.52 ± 0.58	3 000.00	nd
TS (g/L)	54.23 ± 1.20	nd	nd
TSS (g/L)	12.50 ± 0.85	500.00	15.00
COD(g/L)	76.00 ± 0.15	nd	150.00
CODs(g/L)	45.40 ± 0.10	nd	nd
BOD_5 (g/L)	19.70 ± 0.05	5.00	0.25
CODs/BOD_5	2.30	nd	nd
Organic content			
VS (g/L)	42.75 ± 1.25	nd	nd
TOC (g/L)	18.30 ± 1.20	nd	0.05
Total N (g/L)	1.50 ± 1.05	nd	0.05
Total phenols (g/L)	7.80 ± 0.08	nd	0.001
Inorganic content			
Ash (g/L)	11.48 ± 0.88	nd	nd
Total P (mg/L)	76.87 ± 2.30	< 5.00	1.00
Calcium (mg/L)	206.70 ± 1.24	10.00	
Potassium (mg/L)	5 500 ± 50	nd	nd
Sodium (mg/L)	250 ± 2.50	919.00	nd
Magnesium (mg/L)	128.60 ± 2.00	60.00	nd
Iron (mg/L)	26.85 ± 2.65	5.00	nd
Zinc (mg/L)	1.2 ± 0.10	3.00	1.00
Barium (mg/L)	0.29 ± 1.02	nd	nd
Boron (mg/L)	0.64± 0.40	nd	nd
Chromium (mg/L)	0.017 ± 0.56	0.05	0.10
Manganese (mg/L)	0.166 ± 0.24	0.50	nd
Copper (mg/L)	0.12 ± 0.06	0.20	0.20
Nickel (mg/L)	0.087 ± 0.04	0.20	0.20
Cadmium (mg/L)	0.012± 0.01	0.01	0.02
Molybdenum (mg/L)	0.012 ± 0.01	nd	nd
Lead (mg/L)	< 0.001	5.00	0.10
Cobalt (mg/L)	< 0.001	nd	nd
Vanadium (mg/L)	< 0.01	nd	nd

EC – electrical conductivity; TS – total solid; VS – volatile solids; TSS – total suspended solid; COD – chemical oxygen demand; CODs – soluble chemical oxygen demand; BOD_5 – biological oxygen demand; Total N – total nitrogen; Total P – total phosphorus; TOC – total organic carbon; nd – not determined; KSA – Kingdom of Saudi Arabia OMW physicochemical parameters were carried out in triplicate and compared to the standard limits recommended by WHO and the presidency of meteorology and environment in KSA

OMW. Representative chromatograms of the ethyl acetate extract compounds obtained from fresh and stored OMW are shown in Figure 3. The fresh OMW chromatogram showed peaks corresponding to monomeric phenolic compounds and flavonoids. It revealed six main phenolic compounds and flavonoids in fresh OMW (Table 6). Quinic acid was the primary representative compound in fresh OMW. Storage of OMW at 25 °C for three months increased the concentrations of quinic acid, chlorogenic acid (5-caffeoylquinic acid), vanillic acid, and quercetin. Moreover, the apparition of cinnamic acid with the disappearance of gallic acid and resorcinol was observed. However, the most abundant phenolic compound in OMW stored at 45 °C was gallic acid, with a concentration of 5 675 mg/L, while chlorogenic acid was the least detected phenolic compound, with a concentration of less than 2 mg/L.

DISCUSSION

OMW is one of the most polluting wastewater in olive oil-producing countries, and the environmental problems associated with it are primarily due to its complex composition, which makes it difficult to treat using economically sustainable methods, thereby limiting its management and disposal (Dermeche et al. 2013; Jarboui et al. 2023). In Al Jouf, KSA, the OMW was found to be highly polluted by organic and mineral matters, while the CODs/BOD_5 rate (2.3) was lower than 2.5, indicating that the OMW is biodegradable (Płuciennik-Koropczuk & Myszograj 2019). Therefore, OMW lacked heavy metal content within the standard limits recommended by WHO (2004) and the presidency of meteorology and environment in KSA (2001). These findings are consistent with those reported by several authors studying OMW treatment in the same region (Aly et al. 2014; Alhajoj & Alowaiesh 2019) or in other parts of the world (Cayuela et al. 2008; Jarboui et al. 2009; Caballero-Guerrero et al. 2022).

Managing OMW presents a challenging task for converting polluting wastewater into eco-friendly materials. A potential approach to achieving this is utilizing OMW's aromatic compounds to manage plant diseases caused by pathogenic fungi, including *Botrytis cinerea*, *Alternaria solani*, *Fusarium culmorum*, *Verticillium dahliae*, and *Sclerotinia sclerotiorum* (Vagelas et al. 2016; Drais et al. 2021).

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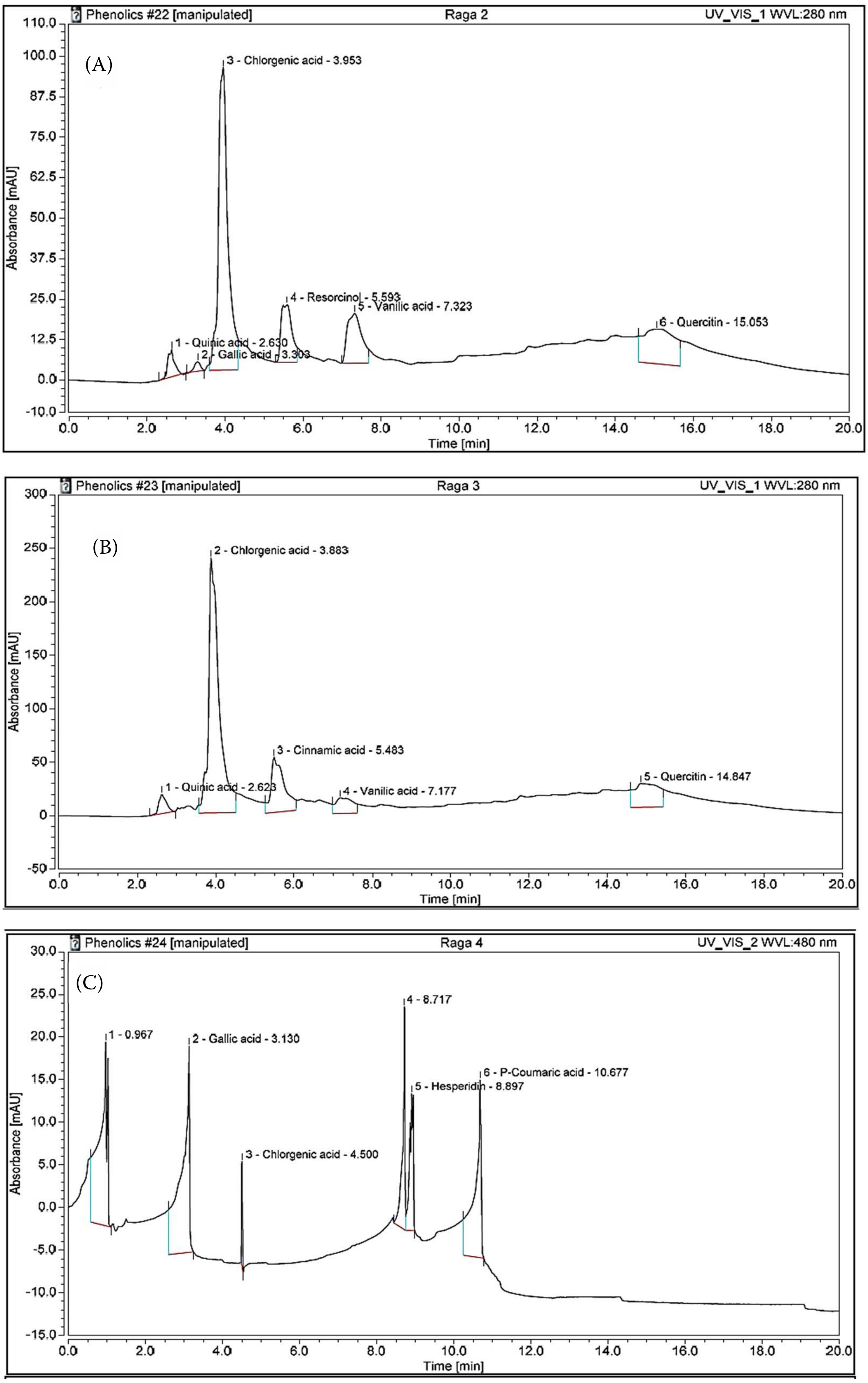

Figure 3. (A) HPLC chromatogram of phenolic and flavonoid compounds of fresh olive mill wastewater (OMW); (B) OMW stored at 25 °C for 3 months; (C) OMW stored at 45 °C for 3 months

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Table 4. Suppressive effect of fresh and stored olive mill wastewater (OMW) against phytopathogenic fungi

Olive mill wastewater (OMW)		dilution	Inhibition percentage of phytopathogenic fungi (%) *Syncephalastrum racemosum*	*Paramyrothecium roridum*	*Fusarium oxysporum*	*Verticillium dahliae*	water content (%)	microbial flora (log_{10} N (ufc/mL)
Fresh OMW	raw non-sterile	1/10	$80^{a} \pm 2$	100^{b}	100^{b}	100^{b}	94.16 ± 0.50	TCB: 4.48 ± 0.02 Y&M: 3.50 ± 0.07
		1/5	$90^{a} \pm 4$	100^{b}	100^{b}	100^{b}		
	centrifuged non-sterile	1/10	$40^{a} \pm 1$	$94^{b} \pm 2$	100^{b}	$90^{b} \pm 2$	97.25 ± 0.55	TCB: 3.35 ± 0.04 Y&M: 2.30 ± 0.02
		1/5	$60^{a} \pm 3$	100^{b}	100^{b}	100^{b}		
	raw sterile	1/10	0^{a}	$46^{b} \pm 4$	$40^{b} \pm 1$	$20^{b} \pm 2$	91.45 ± 0.26	TCB: nd Y&M: nd
		1/5	0^{a}	$70^{b} \pm 2$	$60^{b} \pm 2$	$50^{b} \pm 3$		
Stored at 25 °C for 3 months	raw non-sterile	1/10	0^{a}	100^{b}	$90^{ab} \pm 1$	$90^{b} \pm 2$	88.56 ± 0.25	TCB: 3.30 ± 0.20 Y&M: 4.80 ± 0.12
		1/5	0^{a}	100^{b}	100^{b}	$94^{b} \pm 4$		
	centrifuged non-sterile	1/10	0^{a}	$70^{b} \pm 4$	$80^{b} \pm 4$	$70^{b} \pm 2$	90.50 ± 0.50	TCB: 1.80 ± 0.20 Y&M: 2.80 ± 0.12
		1/5	0^{a}	$80^{b} \pm 3$	$90^{b} \pm 2$	$90^{b} \pm 4$		
	raw sterile	1/10	0^{a}	$60^{c} \pm 4$	$10^{b} \pm 1$	$20^{c} \pm 2$	86.35 ± 0.24	TCB: nd Y&M: nd
		1/5	0^{a}	$70^{c} \pm 2$	$36^{c} \pm 2$	$30^{c} \pm 2$		
Stored at 45 °C for 3 months	raw non-sterile	1/10	0^{a}	$70^{b} \pm 3$	$70^{b} \pm 2$	$50^{b} \pm 3$	70.24 ± 0.45	TCB: nd Y&M: nd
		1/5	0^{a}	$80^{b} \pm 2$	$80^{b} \pm 4$	$60^{b} \pm 2$		
	centrifuged non-sterile	1/10	0^{a}	$60^{b} \pm 4$	$40^{b} \pm 1$	$40^{b} \pm 1$	71.14 ± 0.24	TCB: nd Y&M: nd
		1/5	0^{a}	$66^{b} \pm 2$	$50^{b} \pm 2$	$54^{b} \pm 2$		
	raw sterile	1/10	0^{a}	$40^{c} \pm 2$	$16^{c} \pm 1$	$30^{c} \pm 2$	67.26 ± 0.85	TCB: nd Y&M: nd
		1/5	0^{a}	$50^{c} \pm 4$	$40^{c} \pm 1$	$50^{c} \pm 3$		

$^{a-c}$significant differences ($P < 0.05$) between different OMW types on studied fungi; ufc — unit forming colony; TCB — Total count bacteria; Y&M — Yeasts and molds; nd — not detected

In this study, fresh OMW exhibited the highest inhibition of the growth of the studied phytopathogenic fungi. In contrast, storage and thermal treatment of OMW by autoclaving positively affected the growth of fungi and mycelia. Interestingly, *S. racemosum* was significantly resistant ($P < 0.05$) to the effect of fresh sterile OMW and OMW stored at 25 °C and 45 °C under all studied conditions. Misra et al. (2016) showed that *S. racemosum* was highly resistant to liquid media added with 2% tannins. In 2002, Assas et al. mentioned that fresh OMW is characterized by a high amount of simple and highly toxic phenolic compounds, whereas, during storage, these compounds were auto-oxidized, leading to high molecular weight compounds such as tannin compounds.

Furthermore, the effect of storage on fungi was investigated by Al-Awadi and Al-Judaibi (2014), who observed high inhibition of *F. oxysporum* after treatment with fresh camel urine (rich in phenolic compounds), while the inhibition was decreased by 22% when camel urine was stored for six months.

Indeed, long-time storage of OMW decreased their water content and therefore a reduction in the

Table 5. Inhibition percentage (%) of ethyl acetate extract of fresh and stored olive mill wastewater (OMW) on mycelium growth of phytopathogenic fungi

OMW	*Syncephalastrum racemosum*	*Paramyrothecium roridum*	*Fusarium oxysporum*	*Verticillium dahliae*
Fresh	$40^{a} \pm 2$	$70^{a} \pm 4$	$88^{a} \pm 2$	$80^{a} \pm 1$
Stored at 25 °C for 3 months	0^{ab}	$50^{ab} \pm 2$	$62^{ab} \pm 1$	$56^{ab} \pm 2$
Stored at 45 °C for 3 months	0^{b}	$48^{b} \pm 2$	$55^{b} \pm 2$	$44^{b} \pm 1$

$^{a-b}$significant differences ($P < 0.05$) between different OMW types on studied fungi

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Table 6. Identification of phenolic and flavonoid compounds in fresh and stored OMW

Peak name	Retention time (min)	Phenolic and flavonoid compounds concentration (mg/L) ± SD		
		Fresh OMW	stored OMW at 25 °C	stored OMW at 45 °C
Quinic acid	2.630	141.14 ± 2.35	335.10 ± 4.25	nd
Gallic acid	3.303	0.28 ± 0.08	nd	5 675.54 ± 17.23
Chlorogenic acid	3.953	12.83 ± 0.56	35.88 ± 1.25	1.87 ± 0.20
Cinnamic acid	5.483	nd	18.14 ± 0.88	nd
Resorcinol	5.593	4.37 ± 0.84	nd	nd
Vanillic acid	7.323	6.04 ± 1.02	6.70 ± 0.52	nd
Hesperidin	8.897	nd	nd	1 118.00 ± 10.00
p-coumaric acid	10.677	nd	nd	3 784.24 ± 12.25
Quercetin	15.053	2.95 ± 0.30	4.77 ± 0.66	nd

Nd – not detected; SD – standard deviation; the HPLC analysis was carried out in duplicate

total count of bacteria was reported. The yeast and mold counts increased in OMW stored at 25 °C for three months, related to the OMW acidic pH and their ability to adapt at low humidity (Jarboui et al. 2009). After three months of storage at 45 °C, OMW becomes sterile. Furthermore, the thermal treatment by autoclaving sterilized the OMW, increasing the mycelium growth of phytopathogenic fungi compared to non-sterile OMW. This suggests that OMW's microbial flora is vital in inhibiting phytopathogenic fungi. Several authors have reported similar results (Bess 2000; El-Masry et al. 2002; Cibelli et al. 2017). El-Masry et al. (2002) and Bess (2000) showed that the primary cause of the antifungal activity of compost and compost extracts was their richness in microflora. Sterilization of compost or compost extract, either by autoclaving or microfiltration (0.22 µm), destroyed their active microflora and caused a loss of their suppressive power (El-Masry et al. 2002).

Furthermore, high temperatures may destroy some phenolic compounds, reducing their antifungal activity. More recently, Cibeli et al. (2017) reported that the decomposition of metabolites from OMW high-pressure homogenization strongly affected pathogenic fungi compared to the thermal treatment of OMW at 121 °C for 20 min. Foti et al. (2021) reported that OMW has been proposed for producing biopesticides and composts. When applied to soils and crops, OMW suppressed the growth of most phytopathogenic bacteria, fungi, and weed species without affecting crop production. Nevertheless, certain precautions should be taken when using OMW as a biopesticide, including dose and timing (El-Abbassi et al. 2017).

The characterization of OMW revealed a high abundance of phenolic and flavonoid compounds, which possess antimicrobial properties, as previously reported in previous studies (Jarboui et al. 2008; Dermeche et al. 2013; Caballero-Guerrero et al. 2022). After storage at 25 °C and 45 °C for three months, the phenolic compound content increased, possibly due to decreased water content during prolonged storage at these temperatures. The increase in phenolic content could also be attributed to the inactivation of the phenoloxidase degradation enzyme of phenolic compounds. However, Feki et al. (2006) observed a decrease in hydroxytyrosol derivative content and an increase in hydroxytyrosol concentration after four months of OMW storage. They suggested the addition of ethanol to stabilize OMW and prevent enzymatic and non-enzymatic oxidation reactions that could lead to the degradation or polymerization of phenolic compounds.

In our study, fresh OMW contained six major phenolic compounds, while OMW stored at 45 °C exhibited only three major phenolic compounds, with gallic acid being the dominant compound. Dermeche et al. (2013) reported that the OMW variability in phenolic compounds depends on the OMW intrinsic factors and the phenolic compounds extraction methods. Nevertheless, this variability significantly complicates their bioconversion treatments, which is why some consortia of microorganisms can effectively treat OMW, and others may be inhibited by phenolic content.

The high inhibition effect ovoid of ethyl acetate extract of fresh OMW against studied fungi could be due to the lethal concentration of phenolic and flavonoid constituents. The disappearance of some

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phenolic and flavonoid compounds (quinic acid, resorcinol, vanillic acid, and quercetin) at 45 °C might be due to their rapid degradation during the first time of storage or their polymerization into polyphenolic compounds that are not detectable by HPLC analysis. Similarly, during the fermentation of naturally black olives, Romero et al. (2004) reported the dominance of hydroxytyrosol in olive juice after 12 months of storage, despite the presence of other compounds such as hydroxytyrosol, oleuropein, and tyrosol at time zero.

The antifungal activity of phenolic and flavonoid compounds could be related to different mechanisms exhibited by these compounds against phytopathogenic fungi. Therefore, Oufensou et al. (2020) mentioned that this bioactivity depends on the ability of phenolic and flavonoid compounds to affect fungal cellular membranes, acidification of cytosolic pH and deterioration of cellular ionic homeostasis. Similarly, Hu et al. (2017) reported that the antifungal activity of phenolic compounds depends on their ability to disrupt the integrity of the plasma membrane and induce mitochondrial dysfunction, leading to metabolic stagnation. Martinez et al. (2017) mentioned that chlorogenic acid totally inhibited mycelium growth and spore germination of *S. sclerotium*, *F. solani*, *V. dahliae*, *B.cinerea* and *C. sojina*. More recently, Calheiros et al. (2023) reported that chlorogenic acid caused chitin, glucan and ergosterol reduction of the cell wall and the cell membrane of *C. paropsilosis*. Li et al. (2017) showed that gallic acid inhibits the ergosterol biosynthesis of *T. ruburum*. The efficacy of flavonoids as inhibitors of fungal growth has been referred to as their ability to react with nucleophilic amino acids in fungal proteins (Treutter 2005).

Several studies have reported the effect of temperature on the antioxidant activity and phenolic content of food products (Terefe et al. 2015; Rocha-Parra et al. 2016; Teles et al. 2018). Rocha-Parra et al. (2016) demonstrated that gallic acid was the most stable phenolic compound, and its content remained constant at all water activity levels while storing encapsulated red wine. Teles et al. (2018) observed that the phenolic compounds and antioxidant activity of grape pomace dried at 60 °C were slightly higher than those observed for samples dried at 50 °C and 40 °C. This increase in phenolic content was attributed to the inhibition of phenoloxidase activity, the enzyme responsible for degrading phenolic compounds. However, 40 °C and 50 °C drying temperatures were insufficient for inactivating the phenoloxidase enzymes. Terefe et al. (2015) reported that exposure of the peroxidase enzyme to temperatures of 60–70 °C for a prolonged time (more than 30 min) was sufficient for its inactivation.

The decrease in antifungal activity observed in the ethyl acetate fraction of OMW stored at 45 °C could be attributed to the sub-inhibitory concentration of gallic acid or the sterilization effect of OMW.

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CONCLUSION

The present study demonstrated that long-term storage of OMW at 25 °C and 45 °C negatively impacts its effect against phytopathogenic fungi. Moreover, the study highlighted an increase in the concentration of some phenolic compounds and the disappearance of others during the storage process. Additionally, storage of OMW led to a decrease in water activity, the microbial flora, the inactivation of phenoloxidase, and the initiation of a polymerization process. Notably, fresh OMW and its ethyl acetate extract were found to protect plants against phytopathogenic fungi. Consequently, these OMW extracts could reduce excessive chemical pesticides and prevent their impact on human health and the environment.

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