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Screening of Chemical Composition, Antimicrobial and Antioxidant Activities of Essential Oil and Volatile Fraction from Olive Mill Wastewater

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Abstract: Oil mill wastewater is the main by-product of the olive oil industry resulting mainly from the treatment and pressing of olives in mills. It is a rich source of nutrients and phytochemicals with a wide spectrum of biological properties. The present study focuses on the chemical analysis and evaluation of the antimicrobial and antioxidant activity of the essential oil (EO) and the volatile fraction (VF) obtained, respectively, by hydrodistillation. Chemical analysis by gas chromatography coupled to mass spectrometry (GC/MS) and a flame ionisation detector (GC/FID) revealed the predominance of phenolic compounds (25.71%, 60.36%) and fatty acids (62.37%, 38.25%) for the VF and EO, respectively. It was also shown that the main compounds were oleic acid (24.9%) for the VF and 4-ethylphenol (28.5%) for the EO. The results of the antimicrobial activity in terms of MIC values against twelve microorganisms showed that, overall, the VF was more active than the EO. The antioxidant activity of the VF and EO was evaluated using the DPPH assay and expressed as half-maximal inhibitory concentration (IC₅₀), where the EO (218 µg/mL) showed better antioxidant activity than the VF (244 µg/mL). The results also revealed that the antimicrobial activity and antioxidant activity values for both oils were significantly lower than the standards used.

Keywords: olive mill wastewater; essential oil; volatile fraction; antimicrobial activity; antioxidant activity

1. Introduction

The Mediterranean region constitutes the best environment for the growth and the development of olive trees and the largest producer of olive oil. More than 98% of the world's olive oil is produced there, with Spain, Italy, Greece, Turkey and Portugal being the main producing countries [1]. The International Olive Council (IOC) reported that for the 2020–2021 harvest periods, world olive oil production was estimated at 3,034,000 tons. In addition, Algeria is the ninth-largest olive oil producer in the world, with about 35 million trees whose production of olives and olive oil is estimated at about one million and 80,000 tons per year, respectively [2,3]. Nowadays, numerous olive-oil-extraction processes are used, including the traditional extraction, press system, two-phase and three-phase extraction. The two-phase centrifugation process generates effluents in smaller quantities as compared to the former process and the three-phase centrifugation system [4]. This later produces from 10 to more than 30 million cubic meters annually of olive mill wastewater (OMW) [5], which includes 83 to 92% water coming from the olives themselves and the water used in the olive-oil-extraction process [6]. It is characterised by a dark colour (red to

brown), an acidic pH (between 3.0 and 6.0), high conductivity, and a solid content ranging from 4.1 to 16.4% [7]. It is well recognised that many factors (origin and location, type of species and their cultivation, maturity of olives, storage time, climatic conditions and extraction procedure) have a direct impact on the characteristics and chemical composition of olive oil and OMW [8]. The chemical analysis of the OMW has revealed the presence of a high content of organic matter (2–8 g/100 g) [9] including nitrogen compounds, sugars, organic acids, and phenolic compounds, which leads to an increase in organic load, high chemical oxygen demand (COD) (between 40 and 220 g/L) and biochemical oxygen demand (BOD) (between 35 and 170 g/L) [10]. In addition, pectins, mucilages, lignins and tannins (1.0–1.5%), lipids (0.03–1.1%) and inorganic substances (0.4–2.5%) have also been described in OMW [11]. It should be noted that during the extraction of olive oil, more than 99% of the important phenolic compounds present in the olive fruit are completely transported in the wastewater of the oil mill. Currently, more than 50 and 40 phenolic compounds have been isolated and characterised in OMW and olive oil, respectively [11]. These phenolic compounds constitute the major chemical class with concentrations ranging from 0.5 to 24 g/L [12], where flavonoids are the main components of this effluent, representing 45–65% of the total phenolic content [13]. However, they are phytotoxic [14] and cause environmental problems such as water pollution for aquatic organisms and soil contamination for microorganisms [15,16].

Phenolic compounds are characterised by their high bioactivity and great therapeutic potential, including antioxidant activity, antimicrobial activity [17,18], as well as anti-inflammatory [18,19], antibiotic/antiviral [20], antiproliferative and anti-atherogenic activities [21]. Recent studies performed on pharmacological activities of OMW revealed interesting antioxidant [17,22], antimicrobial [17,23], anticoagulant [24], anti-haemolytic and anti-inflammatory properties [25].

The smell of olive oil is associated with volatile components such as aldehydes, alcohols, ketones, ethers and esters. More than 120 volatile compounds have been identified and different oils can be classified based on their organoleptic properties [26]. This may be related to characteristic compounds in the volatile fraction of olive oil that are responsible for specific aromas [27]. These compounds are biogenerated through the lipoxygenase pathway (LOX) from polyunsaturated fatty acids when the cells of olive fruits are disrupted by crushing and oil extraction [28].

As a follow-up to our review [29], the present study focuses on the chemical analysis of the EO and the VF obtained by hydrodistillation from the crude OMW and the crude extract, prepared by liquid–liquid extraction of OMW, respectively. Both oils were first assessed for their antimicrobial activity against twelve microorganisms and then for their antioxidant activity by the DPPH assay. Based on the literature review [30] and to the extent of our knowledge, this is the first time that a study of the chemical analysis of OMW's VF and EO and their biological activities has been carried out.

2. Materials and Methods

2.1. Sampling and Origin of Olive Mill Wastewater

Crude OMW was obtained from an olive oil mill located in Larbaa (Lat. 36°33'54.20" N—Long. 3°9'5.63" E)—Blida (Algeria) in November, during the 2019/2020 olive season. The recovered OMW was the by-product of a three-phase olive-oil-extraction process. The samples were subjected to filtration followed by centrifugation to remove suspended solids. Finally, storage was carried out at room temperature in a dry and ventilated area.

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α -tocopherol, ethyl acetate, hexane, methanol, hydrochloric acid and sodium sulphate anhydrous (99%) were purchased from Prochima-Sigma (Algeria). All reagents and solvents used were pure and of analytical grade.

2.3. Preparation of Crude Extract

First, 300 g of crude OMW (pH = 5) were centrifuged for 10 min at 4500 rpm and 5 °C, then the pH was adjusted to 2 using HCl (0.5 N). The obtained solution was treated with 100 mL of hexane twice to remove the lipid fraction. The aqueous phase was collected for further liquid–liquid extraction using 100 mL of ethyl acetate. The organic phase obtained after five repeated extractions was dried over anhydrous sodium sulphate and the solvent was evaporated to give 1.5 g of a crude extract, which corresponds to 0.5% (*w/w*) yield.

2.4. Extraction of Volatile Fraction

The VF of the OMW was prepared using a modified Dean-Stark apparatus. First, 300 mg of crude extract were steam-distilled with 500 mL of distilled water for 1 h.

The VF was separated from the water by liquid–liquid extraction with ethyl acetate. The organic phase was dried over anhydrous sodium sulphate and the solvent was removed using a rotary evaporator resulting in 20 mg of VF, corresponding to a yield of 6.6% (*w/w*).

2.5. Extraction of Essential Oil

First, 400 g of the centrifuged OMW were mixed with 1 L of distilled water. The hydrodistillation process was carried out for 3 h. The separation of the EO from the aqueous phase was carried out in a similar way to that of the VF. The extraction yield of the EO was 0.0075% (*w/w*). The obtained EO was weighed and stored in brown vials at 4 °C. It should be noted that the extraction time for the preparation of the VF and EO by hydrodistillation was chosen on the basis of preliminary experiments and in reference to our previous work [31].

2.6. Chromatographic Analyses of VF and EO

The coupled gas chromatography/mass spectrometry (GC/MS) and gas chromatography/flame ionisation detection (GC/FID) analyses were performed for qualitative and quantitative purposes, respectively. The analysis was performed using a GC-QP2010 system (Shimadzu, Kyoto, Japan) coupled to an FID detector. The analysis system was equipped with an auto-sampler injector and a separation capillary column HP-5 fused silica column (30 m × 0.25 mm i.d., df 0.25 µm). The oven temperature was held at 60 °C for 5 min, and then increased to 250 °C at a rate of 3 °C·min⁻¹. The injector temperature was set at 250 °C. Helium was used as a carrier gas at a constant rate of 28.8 mL/min and a linear velocity of 33.3 cm/s corresponding to an inlet pressure of 43.6 kPa. The interface and ion source temperatures were 250 °C and 200 °C, respectively. The acquisition was made in full scan mode in the mass range of 40–500 *m/z*, with a scanning rate interval of 0.2 s. Samples were dissolved in ethyl acetate (10% *w/w*) and then, 1 µL was injected with a split ratio 1:90. The mass range scanned was 35–550 *m/z*. The delay time was set at 5 min. The GC/FID analysis was also performed under the same conditions. To identify the chemical composition, the following databases were used: W11N17 (DB1) (Wiley11-Nist17, Wiley, Hoboken, NJ, USA; and FFNSC 3.0 (DB2) (Shimadzu, Kyoto, Japan). The identification was performed by applying two filters, namely spectral similarity match over 85% and linear retention index (LRI) match calculated using a C₇ and C₄₀ saturated n-alkane homologue series (1000 g/mL, 49452-U) supplied by Merck KGaA (Darmstadt, Germany) with a filter window of ±10 LRI units. Further identification was achieved based on mass spectra reported by specialized [32].

2.7. ATR-FTIR Spectroscopy Analyses of VF and EO

An FTIR spectrometer, model Nicolet iS10, was used for ATR dry film measurements. It was equipped with a deuterated triglycine sulphate (DTGS) detector, 0.5 µL liquid samples (EO or VF) were dried over the diamond ATR crystal of mono-reflection Dura Sample IR II accessory for liquids, from Smiths Detection Inc. (Andover, MA, USA). The OMNIC 9.8 software, from Nicolet iS10, was used for instrument control and data treatment.

The absorbance was recorded over the range 4000–525 cm^{-1} at a resolution of 4 cm^{-1} with an average of 32 scans per spectrum.

2.8. Antimicrobial Assay of VF and EO

2.8.1. Test Microorganisms

Ten bacterial strains, including the Gram-positive bacteria *Staphylococcus aureus* (ATCC 44300), *Bacillus cereus* (ATCC 14975), *Listeria monocytogenes* (ATCC 13932), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 14110), and the Gram-negative *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Agrobacterium tumefaciens* (ATCC 23308), *Enterococcus faecium* (ATCC 51559) and *Enterobacter cloacae* (ATCC 13047) were used to assess the anti-bacterial properties and the anti-fungal activity against *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) of the VF and EO. These strains have been provided by Pasteur Institute of Algiers (Algeria).

2.8.2. Determination of the Minimum Inhibitory Concentration of VF and EO

The minimum inhibitory concentration (MIC) values were determined according to the CLSI guidelines. Inoculums were prepared by transferring one colony of each strain from the agar plate into flasks containing the nutrient broth. They were then incubated for 18 h at 37 °C. The microbial suspensions were adjusted to 1×10^8 CFU/mL for bacteria and 1×10^5 CFU/mL for *C. albicans* (ATCC 10231) and *S. cerevisiae* (ATCC 9763) using a UV-visible spectrometer at a wavelength of 625 nm. The 96-well plates were prepared by adding 100 μL of the VF and EO into the first well. Then, 50 μL of nutrient broth were added to each well, from the second to the twelfth well. A series of two-fold dilutions of the EO was performed in a concentration range from 0.0488 to 50 mg/mL by successively transferring 50 μL from the first to the eleventh well. The last well containing 50 μL of nutrient broth without the VF and EO was used as a negative control, and then 50 μL of the suspensions were added to the wells of each strip leading to a final volume of 100 μL . The plates were incubated at 37 °C for 18 to 24 h for bacteria and 36 to 48 h for the antifungal test. Kanamycin and chloramphenicol were used as references; their highest concentration was 10 mg/mL. All tests were performed in triplicate under sterile conditions.

2.9. Antioxidant Activity of VF and EO

The DPPH free-radical-scavenging test was performed to determine antioxidant activity [33]. A 0.4 mL volume of the methanolic solution of the VF and EO at different concentrations (from 7.81 to 4000 $\mu\text{g}/\text{mL}$) was mixed with 1.6 mL of DPPH methanolic solution (0.1 mM). The mixture was incubated at 37 °C and left in the dark for 30 min, and then the absorbance was measured at 517 nm, using a UV-visible reader. Butylhydroxytoluene (BHT), butylhydroxyanisole (BHA) and α -tocopherol were used as antioxidant standards. Results were expressed as IC_{50} . The scavenging activity was calculated using Equation (1):

$$\% \text{DPPH scavenging} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where A_{control} is the absorbance of the negative control and A_{sample} is the absorbance of the sample oils at 517 nm.

3. Results and Discussions

3.1. ATR-FTIR Analyses of VF and EO

The analysis of the infrared spectrum of the EO and VF (Figure 1) revealed the presence of strong absorption bands at 1463 cm^{-1} , 2923 cm^{-1} , 2853 cm^{-1} , most likely corresponding to methyne, methylene and methyl groups. The IR spectrum also showed a strong absorption band between 1600 and 1800 cm^{-1} centred around 1700 cm^{-1} . This broad band could regroup the elongation movements of the $\text{C}=\text{C}$ at 1600 cm^{-1} , indicating the presence of aromatic compounds and those of the carbonyl group $\text{C}=\text{O}$ at 1720–1740 cm^{-1} , in relation to the presence of compounds bearing a carbonyl function. The presence of

aromatic compounds is most likely associated with the presence of phenolic compounds. This hypothesis is most plausibly confirmed by the presence of OH groups of phenolic compounds, but also of alcohols, at 3358 cm^{-1} [34]. The correlation between the VF and EO spectra was estimated at 71.69%.

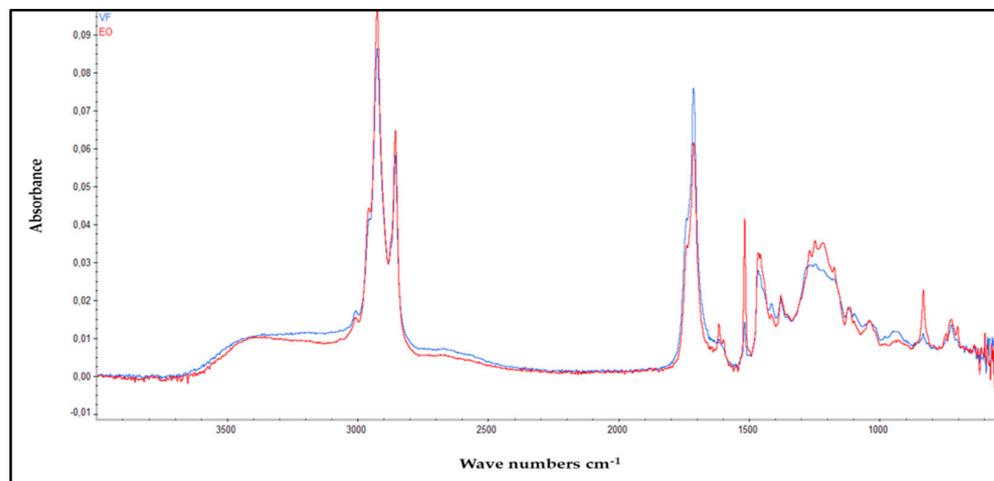


Figure 1. ATR—FTIR spectra of the VF (Blue) and EO (Red) extracted from OMW.

3.2. GC/MS and GC/FID Analyses of VF and EO

First, it should be noted that VF and EO displayed different chromatographic profiles. Forty-one and thirty-nine compounds accounting for 90.17 and 99.26% of the total compositions were identified in the VF and EO, respectively (Table 1). As described above, GC/MS allowed the identification of the different compounds on the basis of the MS database. The GC/FID analysis has a double objective including the calculation of retention indices and the determination of the percentage content of different compounds.

Table 1. Chemical composition of VF and EO.

N°	Compound	Database	Chem. Class	LRI _{lit}	LRI _{cal}	VF (%)	EO (%)
1	Ethanol, 2,2'-oxybis-	DB1, DB2	O	967	962	0.5	t
2	Hexanoic Acid	DB1, DB2	FA	984	980	0.23	0.3
3	Phenol	DB1, DB2	PC	986	982	0.4	0.5
4	Benzyl alcohol	DB1, DB2	PC	1032	1033	2	4.9
5	cis-furan Linalool oxide	DB1, DB2	T	1070	1072	–	0.12
6	p-Cresol	DB1, DB2	PC	1077	1077	0.32	–
7	o. Guaiacol	DB1, DB2	PC	1088	1088	0.11	0.5
8	Nonanal	DB1, DB2	O	1104	1104	0.17	–
9	Benzene ethanol	DB1, DB2	PC	1116	1112	3.61	7
10	Cyclohexanecarboxylic acid	DB1, DB2	O	1127	1120	–	0.12
11	cis-Limonene oxide	DB1, DB2	T	1134	1134	–	t
12	Camphor	DB1, DB2	T	1143	1141	t	–
13	4-Ethylphenol	DB1, DB2	PC	1170	1170	11.7	28.5
14	cis-pyran Linalool oxide	DB1, DB2	T	1173	1174	–	0.41
15	Octanoic acid	DB1, DB2	FA	1178	1175	0.8	–
16	Isomenthol	DB1, DB2	T	1182	1182	0.65	–
17	Catechol	DB1, DB2	PC	1197	1205	1.1	0.2
18	4-vinylphenol	DB1, DB2	PC	1226	1221	0.3	0.7
19	cis-p-Propenylanisole	DB1, DB2	PC	1269	1265	–	0.3
20	Nonanoic acid	DB1, DB2	FA	1272	1272	0.8	t
21	4-Ethylguaiacol	DB1, DB2	PC	1282	1279	1.31	3.8
22	Cinnamic acid, methyl ester	DB1, DB2	PC	1350	1352	0.20	1.4
23	Neric acid	DB1, DB2	FA	1347	1358	–	0.2
24	o-Toluic acid, methyl ester	DB1, DB2	FA	1362	1365	0.2	0.4

Table 1. Cont.

N°	Compound	Database	Chem. Class	LRI _{lit}	LRI _{cal}	VF (%)	EO (%)
25	Eugenol	DB1, DB2	PC	1361	1368	–	11
26	Capric acid	DB1, DB2	FA	1380	1370	–	0.17
27	4-Ethylcatechol	DB1, DB2	PC	1392	1388	2.11	0.8
28	Tyrosol	DB1, DB2	PC	1427	1427	2	0.36
29	9-Oxononanoic acid	DB1, DB2	FA	1483	1480	0.26	0.12
30	α-Farnesene	DB1, DB2	T	1508	1509	–	t
31	2,4-di-tert-Butylphenol	DB1, DB2	PC	1519	1514	0.15	–
32	5-tert-Butylpyrogallol	DB1, DB2	PC	1526	1527	0.2	0.7
33	Tyrosol, acetate	DB1, DB2	PC	1567	1566	0.20	–
34	Tetradecanoic acid	DB1, DB2	FA	1768	1761	0.1	0.16
35	Palmitoleic acid, methyl ester	DB1, DB2	FA	1890	1905	–	0.2
36	Palmitic acid, ethyl ester	DB1, DB2	FA	1926	1927	0.14	1.5
37	Palmitoleic acid	DB1, DB2	FA	1944	1943	0.55	1.7
38	Palmitic acid	DB1, DB2	FA	1969	1967	3.3	2.1
39	Ethyl (E)-9-palmitoleate	DB1, DB2	FA	1978	1974	1.04	1.1
40	Palmitic acid, ethyl ester	DB1, DB2	FA	1990	1995	4.4	7.4
41	cis-Linoleic acid, methyl ester	DB1, DB2	FA	2096	2095	0.7	0.7
42	Elaidic acid, methyl ester	DB1, DB2	FA	2110	2101	2.01	2
43	Oleic Acid	DB1, DB2	FA	2146	2145	24.9	6
44	Linoleic acid, ethyl ester	DB1, DB2	FA	2164	2164	5.7	3
45	Elaidic acid, ethyl ester	DB1, DB2	FA	2174	2170	16.42	11
46	Stearic acid, ethyl ester	DB1, DB2	FA	2199	2195	0.3	0.2
47	Isopentyl palmitate	DB1, DB2	FA	2246	2246	0.21	–
48	Tributyl acetyl citrate	DB1, DB2	O	2254	2269	0.4	–
49	Tetracosne	DB1, DB2	O	2400	2400	0.2	–
50	2-Octyldodecyl propionate	DB1, DB2	O	2411	2411	0.17	–
51	Oleic acid, pentyl ester	DB1, DB2	FA	2421	2421	0.31	–

T, trace (<0.1%); T, terpenes; FA, fatty acids; PC, phenolic compounds; O, others; LRI_{lit}, linear retention index reported in the literature; LRI_{cal}, calculated linear retention index. Database used for LRI match.

The chemical composition of the VF and EO can be divided into four chemical classes (Table 1 and Figure 2): terpenes (VF—0.65%; EO—0.53%), phenolic compounds (VF—25.71%; EO—60.36%), fatty acids (VF—62.37%; EO—38.25%) and others (variously functionalised compounds; VF—1.44%; EO—0.12%). Previous studies have already reported the presence of fatty acids [35], phenols [36], and terpenes [37,38] as the main chemical composition of olive oil and OMW.

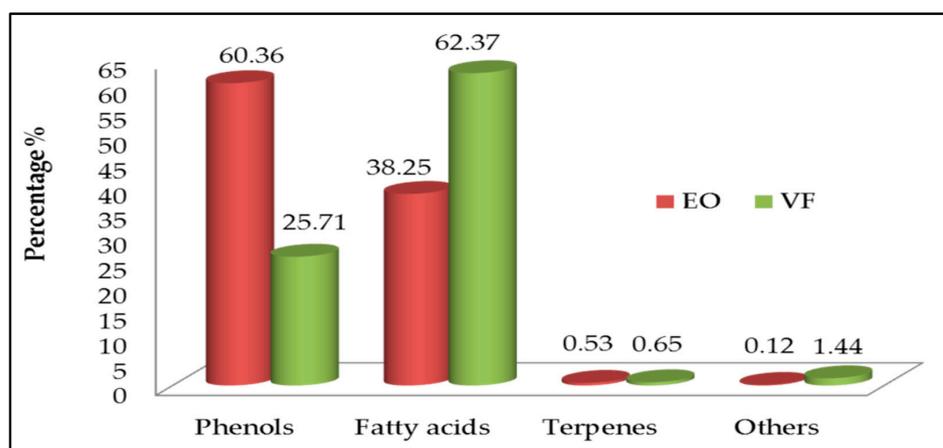


Figure 2. Percentage composition of the four chemical classes in VF and EO.

A closer look at Figure 2 shows that the sum of the percentages of phenolic compounds and fatty acids in the VF and EO are 88.08% and 98.61%, respectively. Thus, it can be seen that almost the entire chemical composition of both oils is phenolic compounds and/or

fatty acids at approximately similar percentages. The findings are most probably related to a bioconversion/degradation process of phenols into fatty acids and vice versa. As mentioned above and according to Figure 2, phenolics and fatty acids are the predominant chemical classes in the VF and EO, respectively. This result is rather surprising given that the two oils, obtained from OMW, should, in all likelihood, have a relatively similar chemical composition, especially with regard to the chemical nature of the main class. It seems, therefore, that the hypothesis of the intervention of a degradation process is more than likely. Thus, as the EO was directly prepared from the OMW while the VF was obtained following two steps including the preparation of the extract and then its hydrodistillation, it is plausible that the phenols, which are very sensitive to heat, within the VF underwent degradation reactions into fatty acids. It is possible that the degradation process followed the same chemical mechanism as the bioconversion of phenols to fatty acids.

Thus, the simultaneous coexistence of the four main classes in the chemical composition of the VF and EO could most likely be related to biosynthetic considerations, as illustrated in Figure 3 [39], which describes the biosynthetic pathways of the different chemical classes and some plausible pathways suggested based on the chemical composition of the VF and EO. The results also revealed that the main chemical class in the EO was made up of phenolic compounds (60.36%), of which 4-ethylphenol (28.5%) was the predominant one. On the other hand, and unexpectedly, the volatile fraction was characterised by the presence of fatty acids as the predominant class (62.37%), with oleic acid being the main compound at 24.9%. The low content of terpenes in the VF and EO is probably related either to their loss during the preparation process of the OMW extract by liquid extraction, or to their degradation under the effect of heat, although this last hypothesis remains less probable. In the present study, we noticed the presence of cis-limonene oxide, cis-pyran linalool oxide, isomenthol and camphor as monoterpenes and farnesene as a sesquiterpene.

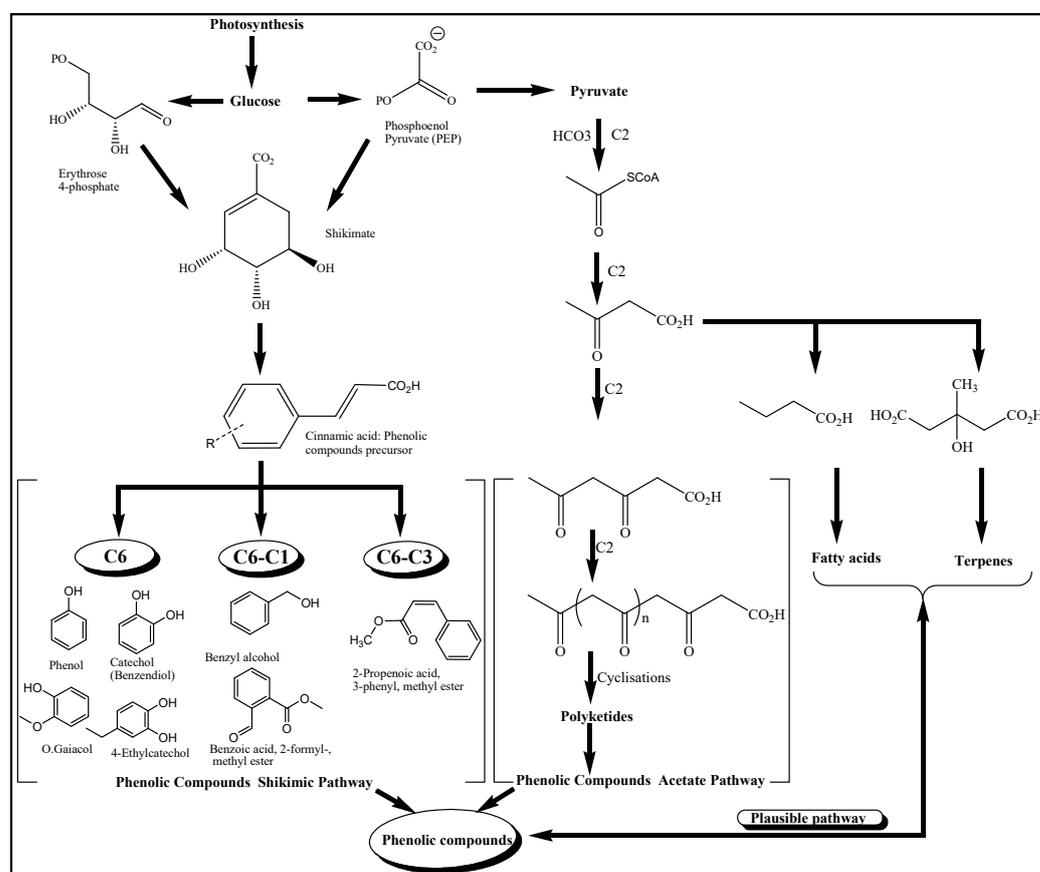


Figure 3. Interconnection of the biosynthetic pathways of the different chemical classes present in VF and EO.

As illustrated in Figure 3, shikimic acid is the biosynthetic precursor of the majority of phenolic compounds, whereas acetyl Coenzyme A is the precursor of fatty acids, which is also the precursor of terpenes via mevalonic acid and of phenolic compounds via malonyl Coenzyme A. It is quite permissible that the contents of the four chemical classes are most likely inter-related.

Thus, it is worth noting the presence of linalool and limonene in the volatile fraction of olive oil [40,41], while, in the present study, they were described in the EO as limonene oxide and linalool oxide (furan and pyran). This finding is most likely related to their chemical transformation under the effect of steam or their bioconversion by specific microorganisms present in the medium. In this context, the biotransformation of linalool to limonene oxide via limonene, as well as to furanoid and pyranoid linalool oxide was reported [42] using different strains of *A. niger*, a fungus found and isolated in OMW [43]. In the same order, plausible pathways (PPs) have been proposed for obtaining camphor and isomenthol from limonene. Additionally, the biosynthesis of neomenthol (isomer of isomenthol) has been described from limonene in *Mentha arvensis* L. essential oils [44]. The previous several reactions are illustrated by Figure 4, which clearly explains the presence of limonene oxide pyran and furan linalool oxide in the EO.

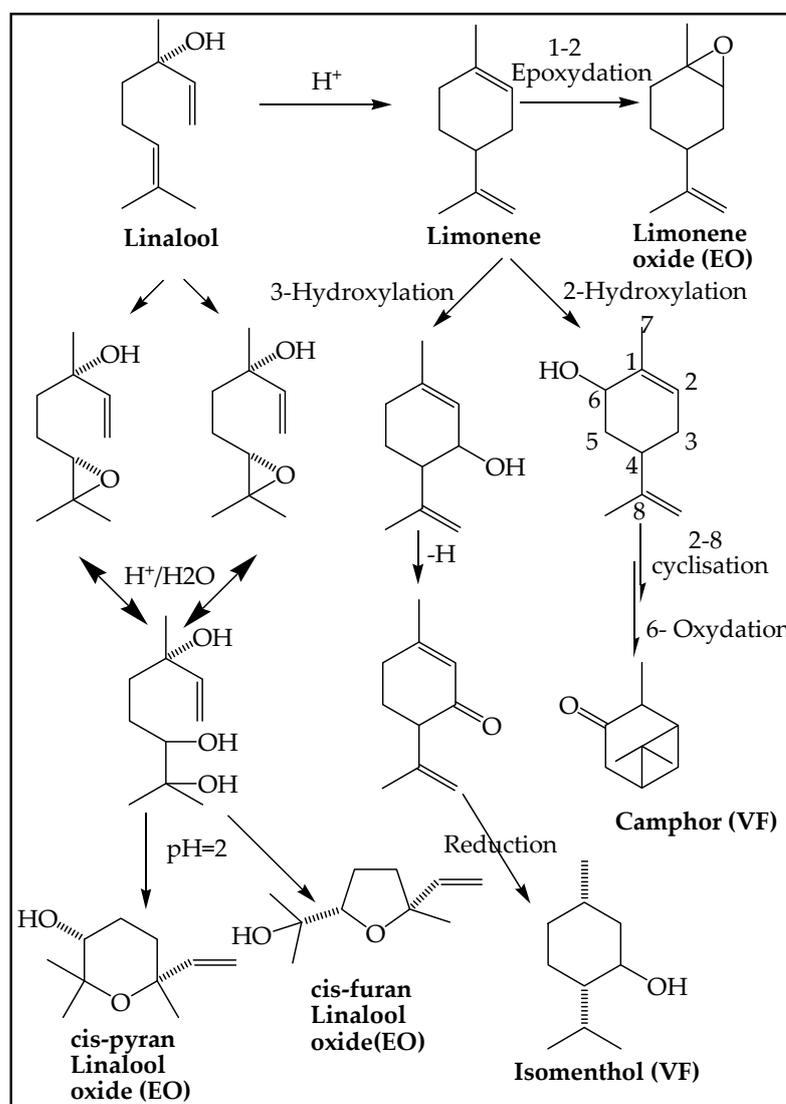


Figure 4. Bioconversion pathways of limonene and linalool using *Aspergillus niger* and proposed pathways (PPs) leading towards camphor and isomenthol.

Furthermore, the presence of farnesene, even in trace amounts, in the EO, and not in the VF, is linked to the relatively long hydrodistillation time (3 h) which favours its entrainment since it is a compound with a high molecular weight and low vapor pressure. The presence of farnesene and limonene has previously been proven in olive oil treated by hydrodistillation and solid-phase microextraction (SPME) [38]. It should be pointed out that monoterpenes, sesquiterpenes and benzenoids have been described as being more abundant in the volatile fraction of virgin olive oil obtained by hydrodistillation than by SPME [45], and vice versa for the abundance of their fatty acids.

A further examination of the chemical composition (Table 1) revealed the presence of oleic acid and its isomer elaidic acid (identified as an ethyl ester), tyrosol and its derivative tyrosol acetate (considered a secoiridoid); both compounds have previously been reported in virgin olive oil [46].

3.3. Antimicrobial Activity

According to Table 2, the VF exhibited strong antimicrobial activity compared to the EO. Thus, both the VF and EO inhibited the growth of all the tested microorganisms at different concentrations. The MIC values were in the range of 195.30–1562.5 µg/mL and 390.62–3125 µg/mL for the VF and EO, respectively. Based on the above data, it is evident that the VF exhibited a stronger inhibitory effect with respect to *B. subtilis*, *M. luteus*, *E. coli*, *A. tumefaciens*, *E. faecium*, *C. albicans* and *S. cerevisiae*. Furthermore, both oils showed the same activity against *B. cereus*, *S. aureus*, *L. monocytogenes* and *P. aeruginosa*. However, the EO was ahead of the VF only once against *E. cloacae*. Kanamycin and chloramphenicol showed the lowest MIC values, i.e., a very strong inhibitory effect against all the microorganisms tested in comparison with the VF and EO. It must be noted that *B. subtilis*, *B. cereus*, *L. monocytogenes* and *P. aeruginosa* were the most resistant microorganisms. Conversely, *E. coli* and *S. cerevisiae* were the most sensitive ones.

Table 2. Minimum inhibition concentrations (MIC) values of VF and EO.

Microorganisms	Gram	MIC (µg/mL)			
		EO	VF	Kanamycin	Chloramphenicol
<i>B. subtilis</i>	+	3125.0	3125.0	62.50	3.90
<i>B. cereus</i>	+	1562.5	1562.5	7.81	1.95
<i>S. aureus</i>	+	781.25	781.25	15.62	15.62
<i>M. luteus</i>	+	1562.5	781.25	15.62	7.81
<i>L. monocytogenes</i>	+	1562.5	1562.5	15.62	7.81
<i>E. cloacae</i>	-	781.25	1562.5	7.81	3.90
<i>E. faecium</i>	-	1562.5	781.25	62.50	3.90
<i>A. tumefaciens</i>	-	1562.5	781.25	1.95	15.62
<i>E. coli</i>	-	390.625	195.30	–	3.90
<i>P. aeruginosa</i>	-	1562.5	1562.5	1.95	3.90
<i>C. albicans</i>	Yeast	1562.5	781.25	3.90	3.90
<i>S. cerevisiae</i>	Yeast	781.25	390.625	–	3.90

The high antibacterial activity of the VF could be related to its chemical composition, which is particularly rich in fatty acids, especially unsaturated ones. The latter are also famous for their high biological activity and are involved in various biosynthetic processes. In this regard, long-chain unsaturated fatty acids are endowed with antibacterial activity [47]. In addition, it has been reported that fatty acids inhibit bacterial growth by disrupting bacterial membranes or by inhibiting fatty-acid synthesis [48]. According to the GC/MS analysis of the VF, the fatty acid fraction consisted of oleic acid and its isomer elaidic acid, linoleic acid, their ethyl ester and stearic acid ethyl ester, which are known for their antibacterial activity against several microorganisms such as *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, among others [49].

Furthermore, synergism between these fatty acids could take place and boost the antimicrobial activity of the VF. In this regard, it was found that oleic and linoleic acid

exhibited a synergistic antimicrobial effect when they were mixed [50]. In addition, the presence of tyrosol in the VF (2%) composition at a higher amount as compared to the EO (0.3%), and tyrosol acetate (0.2%), which was only found in the VF could also be reasons for its high activity. In this scenario, it has been reported that these two components are endowed with good antimicrobial activities [51].

3.4. Antioxidant Capacity

The capacity of scavenging the DPPH free radicals by the VF, EO and standards was obtained graphically using a linear curve by plotting radical-scavenging percentages with respect to the sample concentrations. The antioxidant activity of both the oils and standards was expressed in terms of IC₅₀ (µg/mL). The results in Table 3 show that the antioxidant activity of the VF and EO was significantly lower than all the controls used. The recorded IC₅₀ for the EO was relatively better than for VF. It has previously been reported that OMW alkyl aromatic alcohols show significant antioxidant properties and that the best result was associated with the presence of polyphenols [51,52]. This result is mainly due to the high phenolic content in the EO as they represent the main chemical class. The difference in antioxidant activity was most probably related to the presence of gaiacol, phenol and 5-tertbutylpyrogallol, which were only present in the EO. In addition, benzyl alcohol (4.9%), benzene ethanol (7%) and 4-ethylphenol (28.5%) were comparatively much more present in the EO than in the VF.

Table 3. Antioxidant activity of VF and EO expressed as IP% and IC₅₀.

Samples	DPPH Assay	
	IP (%)	IC ₅₀ (µg/mL)
VF	73.9	244
EO	79.8	218
BHA	90.14	6.14
BHT	95.02	12.99
α-Tocopherol	89.52	13.02

IP: Inhibition percentage at the first concentration of: 4000 µg/mL for EO and VF; 800 µg/mL for BHA, BHT and α-Tocopherol.

4. Conclusions

In the present work, the chemical analysis of the VF and the EO of OMW revealed the presence of four chemical classes including phenolics, fatty acids, terpenes and a group of variously functionalised compounds. The antimicrobial test revealed that the VF showed a strong inhibitory effect compared to the EO against most of the microorganisms tested, including *B. subtilis*, *M. luteus*, *E. coli*, *A. tumefaciens*, *E. faecium*, and especially the yeasts, *C. albicans* and *S. cerevisiae*. This high antimicrobial activity is most likely related to its richness in fatty acids, especially unsaturated fatty acids, which are known to have strong antimicrobial properties. The antioxidant capacity of the two oils by the DPPH method, through the free-radical-scavenging capacity, showed that the EO had a relatively higher antioxidant activity than the VF in terms of IC₅₀.

In general, the in vitro assays carried out showed that the essential oil and the volatile fraction of OMW provide an important source of natural antimicrobial and antioxidant agents that could be used in the food, cosmetic or pharmaceutical industries, while reducing their hazardous impact on the environment.

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