Antibacterial and antioxidant potentials of phenolic extracts from olive mill wastewater and their use to enhance the stability of olive oil

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Fresh olive oil mill wastewaters (OMW) were collected in northern Morocco from industrial mills operating with three extraction systems (two [C2] and three-phase [C3] centrifugation decanters and super-pressure system [SP]) during the 2017 growing season. Physicochemical and microbiological characteristics of these effluents were examined. OMW phenolic extracts were prepared and their antibacterial and antioxidant activities were evaluated. Moreover, the improvement of virgin olive oil stability with the addition of OMW phenolic extracts (at 100, 300 and 500 ppm) was investigated. Results from this work confirmed the pollutant load of these effluents characterised by an acidic pH, strong organic content, and considerable amounts of mineral matter. Concerning the microbiological counts, yeasts and moulds were the most abundant. Among the extraction system, C2 displayed OMW with the high levels for all measured parameters, except for the total phenols that were more abundant in C3. For the in vitro bioassays, OMW phenolic extracts showed a promising antibacterial activity against the two studied bacteria. In fact, C2 extract was the most active for inhibiting the bacteria growth, while *Escherichia coli* (Gram-) was more resistant to bactericidal phenols than *Listeria innocua* (Gram+). Findings from DPPH and FRAP tests showed a good antiradical potential and an important reducing power capacity and were both concentration-dependent. For the two tests, antioxidant activity of OMW phenolic extracts from C2 was also the most important. The addition of phenolic extracts to virgin olive oil, stocked at 60°C, resulted in lower records for peroxide value, K232 and K270 compared to the control, indicating a good protective effect against oil oxidation. The great antioxidant effect of OMW extracts was at 500 ppm, like that of ascorbic acid.

Keywords: OMW, phenolic extract, antibacterial activity, antioxidant potential, olive oil stability

1. INTRODUCTION

The increase in demand for olive oil has made its production more expensive because of its beneficial health properties including antioxidant, anti-atherogenic, anti-inflammatory, anti-aging, anti-tumour, anti-viral, anti-cancer and immune modulator activities [1 - 4]. About 97% of worldwide production is provided by Mediterranean countries [5]. In spite of the socio-economic contribution of the olive oil industry for these countries, huge amounts of olive oil mill wastewaters (OMW) are produced annually, estimated to reach 30 million per m^3 [6]. Uncontrolled disposal of these effluents creates a substantial environmental problem. In addition, dispersed distribution of the olive mills and high financial requirement of OMW treatments complicate their management and therefore hinder the sustainable development of the olive oil industry. Therefore, OMW management is becoming nowadays a big challenge for producers.

OMW contains 83-92% of water as a major component and large amounts of

organic molecules, especially polyphenols, nitrogen compounds, sugars, organic acids and pectins [7 - 11]. The studies carried out on the OMW effect on soil properties, ecosystems and plant growth have all confirmed that their toxicity is mainly due to the phenolic content [12 - 15].
OMW, known by their highly polluting and complexing organic load, are also a promising source of bioactive compounds and substances of high value and great interest. As olive oils, they could have some health benefits to be exploited. Phenolic compounds are one of the most valuable substances that OMW might provide as an interesting alternative to attenuate the negative impact of these effluents [16].
Recently, several studies have focused on the functional properties of OMW phenolic compounds and their possible uses. The antioxidant activity of OMW phenolic extracts was widely studied and the results were encouraging compared to synthetic antioxidants [17 - 20]. Visioli et al. [21] have evaluated and confirmed the antioxidant and anti-inflammatory activities of OMW phenols extracts. Other excellent biological properties such as antimicrobial and anti-carcinogenic activities of the OMW biophenols are documented [16]. El-Abbassi et al. [20] reported that natural phenols from olives and its by-products are recognised as potential targets for the food, cosmetic and pharmaceutical industries. The production of functional foods, especially olive oils, enriched by phenolic compounds extracted from OMW has also been developed [22 - 24]. Indeed, phenolic compounds are known to function as preventive agents against oxidative damages (formation of flavour and toxic compounds), because of their ability to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation [19, 25, 26].
The objectives of this study were: i) to investigate physicochemical and microbiological characteristics of fresh OMW as affected by extraction systems, ii) to evaluate antioxidant potentials of OMW phenolic extracts, iii) to try improving the stability of virgin olive oil by adding OMW phenolic extracts.

2. EXPERIMENTAL PART

2.1 SAMPLING

Fresh olive mill wastewaters (OMW) for this study were collected from three industrial units of olive oil extraction (using two phase [C2] and three phase [C3] decanter centrifugation and super-pressure system [SP]), located in Taza province (34°12'36"N, 3°52'0"W) (northern Morocco).
OMW samples were taken at the end of December 2017, in a closed plastic container, and stored at 4°C until analysis.

2.2 PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION

Hydrogen potential (pH) and the electrical conductivity of OMW samples were measured in situ using a multiparameter type (CONSORT C535, Turnhout, Belgium). Suspended solids were determined by centrifuging an OMW aliquot during 20 min at 8,000 rpm and drying the pellet at 105°C for 24 h. Total solids, consisting on all organic and mineral substances in solution or in suspension, were assessed by heating a volume sample until reaching a constant weight, then the organic matter was determined after combustion at 550°C for 4 h. The difference between the total solids and organic matter was defined as a mineral matter. Chemical Oxygen Demand was performed using the open reflux method based on a boiling oxidation (150°C for 2 hours) of the reducing matter with an excess of potassium dichromate in an acidic medium. The remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to determine the consumed potassium dichromate and the oxidizable matter were calculated in terms of oxygen equivalent. Chlorides content was determined according to the standard using the Mohr method. Total kjeldahl nitrogen was evaluated using the Kjeldahl method: a warm mineralisation of the OMW samples was carried out in an acidic medium using a catalyst. Ammonia nitrogen (mineralisation product) was then moved to ammonia after the addition of sodium hydroxide. The ammonia is driven by steam and trapped in boric acid and titrated with hydrochloric acid. All traits mentioned above were analysed according to Rodier et al. [27]. Protein content was calculated by multiplying the total nitrogen by 6.25 [28].
Total phenols were extracted following the method of De Marco et al. [29]. The obtained dry residue was dissolved in methanol and total phenols content was determined by spectrophotometry (Spectrophotometer JENWAY 6100, Dunmow, Essex, UK) using Folin-Ciocalteu reagent [30] using caffeic acid (Sigma-Aldrich, St. Louis, MO, USA) as the standard. Total sugars content was determined spectrophotometrically following the method described by Dubois et al. [31]. Total organic carbon was calculated by dividing organic matter by 1.724 [32].
For microbial count, 1 mL of each OMW sample was diluted tenfold in 10 ml of sterile buffered peptone water (0.1% w/v, pH 7.4) followed by preparation of serial decimal dilutions, using the peptone solution, in order to obtain CFU counts in the range of 30-300 per plate. 100 µL of the corresponding decimal dilutions were inoculated in four different media:
- PCA (Plate Count Agar, Difco), at 30°C for count of Total Aerobic and Mesophilic Flora.
- DPA (Potato Dextrose Agar, Difco), at 30°C for count of Yeasts and Molds.
- MRS agar (Man Rogosa Sharpe, Difco,), at 30°C for

count of Lactic Acid Bacteria.
- Macconkey agar (Difco), at 37°C for count of Total Coliform Bacteria.

2.3 ANTIBACTERIAL ACTIVITY OF OMW PHENOLIC EXTRACTS

To evaluate the antibacterial activity of the OMW phenolic exacts, we used two reference bacteria strains obtained from the Spanish Type Culture Collection (CECT): *Escherichia coli* K12 (CECT433), and *Listeria innocua* CECT4030. The used strains were cultivated twice in Muller–Hinton agar (MH) and incubated overnight at 37°C. Then, each strain of cultures was cultivated in 3 mL of Mueller-Hinton broth (MH) and incubated at 37°C for 18 h. Each culture of the used bacteria was diluted in MH broth to obtain a final concentration of about 10^8 CFU/mL.
The susceptibility test of the used microorganisms to the phenolic extracts was performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute [33]. The extracts were diluted with 10% DMSO (Dimethylsulfoxide). The discs (6 mm Ø) were impregnated with 20 µL of different DMSO phenolic extracts dilution and placed onto the inoculated agar. Disks impregnated with 10% DMSO served as negative controls and a disk with gentamicin (15 µg) served as a positive control. Replicas at each concentration were performed. Plates were incubated at 37°C for 24 h, and antibacterial activities were evaluated by measuring inhibition zone diameters (mm).
The two bacterial strains were then used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the MH broth microdilution in 96 well-plates [34]. 100 µL of sterile MH broth were dispensed into all the wells and 100 µL of phenolic extract solution were added on the first column, then serially dilutions were made to obtain final concentrations ranging from 9.77 µg/mL to 25 mg/mL. All the wells, except the negative control column, were inoculated with 10 µL of *E. coli* and *L. innocua* standardized inoculum (adjusted to approximately 10^8 CFU/mL using 0.5 McFarland standard). The microplates were incubated at 37°C for 24 h. The MIC was defined as the lowest phenolic concentration demonstrating complete inhibition of microorganisms' growth. Referring to the results of the MIC assay, the wells demonstrating no visible growth were identified and 20 µL solutions from each well was removed to agar plates and incubated at 37°C for 24 hours. MBC is recorded as the lowest MIC concentration of at which inoculated microorganisms were 99.9% killed.

2.4 ANTIOXIDANT ACTIVITY OF OMW PHENOLIC EXTRACTS

2.4.1 DPPH radical scavenging activity

The antioxidant activity of the OMW phenol extracts was evaluated by using the stable 2,2-diphenyl-2-picrylhydrazyl radical (DPPH*) according to the method described by El-Abbassi et al. [20]. The hydrogen atom donating ability of OMW phenolic extracts was determined by the decolourisation of the methanolic solution of DPPH. DPPH produces violet/purple color in methanolic solution and fades to shades of yellow colour in the presence of antioxidants [35].
The DPPH* solution was prepared by dissolving 4 mg of DPPH in 100 ml of methanol. A 100 µl of each methanolic solution of OMW phenol extracts at different concentrations (0-400 mg/mL), was added to 3 ml of DPPH working solution in a cuvette. All cuvettes were covered, well shaken, and kept in the dark and the decrease in absorbance was measured at 517 nm after 60 min, using a spectrophotometer (SPECUVIS, UV-Visible) against methanol as a blank.
The radical scavenging activities were calculated according to the following formula:

$$\% \text{ Radical scavenging activity} = \\ = \{1 - (A_{sample} / A_{control}\} \times 100$$

Where A_{sample} is the absorbance of the test sample and $A_{control}$ is the absorbance of DPPH in methanol (3 mL) plus methanol (100 µL) instead of samples.
Synthetic antioxidant reagents (ascorbic and caffeic acids) were used as positive controls for comparison. Measurements were repeated three times.
Test sample (or control) concentration providing 50% inhibition (IC50, expressed in µg/mL) was calculated from the graph plotted radical scavenging activities (%) against concentration.

2.4.2 Ferric reducing antioxidant power (FRAP) Assay

FRAP assay is based on the ability of the tested antioxidants (OMW phenols extracts) to reduce ferric iron (Fe^{+3}) present in the $[K_3Fe(CN)_6]$ complex to the ferrous form (Fe^{+2}).
The reducing power of OMW phenolic extracts was evaluated according to the method reported by Yen and Chen [36]. A 1 mL of sample solutions at different concentrations (0-100 mg/mL) was mixed with 2.5 ml of phosphate buffer solution (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide $[K_3Fe(CN)_6]$ at 1%. The mixtures were incubated at 50°C for 30 min, then, 2.5 ml of trichloroacetic acid (10%) were added. The whole was centrifuged at 3,000 rpm for 10 min. The upper layer of each solution (2.5 mL) was then mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm using a spectrophotometer (SPECUVIS1, UV-Visible) against blank. A typical blank solution contained the same solution mixture without OMW phenolic extracts (or controls) was incubated under the identical conditions. Higher absorbance of the reaction mixture indicated

higher reducing power.
Ascorbic and caffeic acids were used as positive controls. Measurements were repeated three times.

2.5 OLIVE OIL ENRICHMENT WITH OMW PHENOLIC EXTRACTS

2.5.1. Virgin olive oils (VOO) characterization

VOO were extracted from olives belonging to 'Moroccan Picholine' and collected in an industrial mill unit using a continuous process (three phase decanter) located in the Taza province (northern Morocco) during the 2017 extraction period. Quality characteristics (free fatty acids, peroxide value and extinction coefficients at 232 and 270 nm) of these oils were determined according to the European Union Commission Regulation EEC/2568/91 [37]. Total phenols were extracted according to the method described by Zunin et al. [38], and the content was determined by spectrophotometry (Spectrophotometer JENWAY 6100, Dunmow, Essex, UK) following the Folin-Cicalteu method [30] using the caffeic acid as standard. Content of chlorophyll and carotenoid compounds were determined from the absorption of the olive oil dissolved in cyclohexane, at 670 and 470 nm respectively following the method of Minguez-Mosquera et al. [39].

2.5.2 Total phenols extraction from OMW

OMW were drawn from the same three phase decanter unit simultaneously with the VOO sampling. Total phenols (TP) were extracted using the analytical methodology described by De Marco et al. [29]. OMW samples were acidified and washed with hexane [1:1, (v/v)] in order to remove the lipid fraction: 10 ml of OMW were mixed with 15 ml of hexane; the mixture was shaken and then centrifuged during 5 min at 3,000 rpm. The phases were separated, and the washing was successively repeated twice. TP extraction was then carried out with ethyl acetate: OMW samples, preventively washed, were mixed with an equal volume of ethyl acetate; the mixture was vigorously shaken and centrifuged for 10 min at 3,000 rpm. The phases were separated, and the extraction was repeated successively four times. The ethyl acetate was evaporated, and the dry residue was recovered.

2.5.3 Enriched virgin olive oil (VOO) preparation and oxidation system

Weighed quantities of OMW phenolic extracts were dissolved in an appropriate volume of ethanol/water (50/50; v/v) and added at different concentrations (100 ppm, 300 ppm and 500 ppm) to VOO to obtain an enriched-virgin olive oil. OMW phenolic extracts were mixed with virgin olive oil by stirring for 30 min and ethanol traces were evaporated at 37°C [40]. The same procedure was applied to ascorbic acid (AA) as standard. VOO enriched with OMW phenolic extract and ascorbic acid and VOO without enrichment (control) were stored in the dark at 60°C for 22 days, during which the stability of oils was evaluated at the initial time and every 7 days by the measurement of peroxide value and conjugated dienes and trienes formation [22].

2.6 STATISTICAL ANALYSIS

All analytical determinations were performed in triplicate. For OMW characterisation, the least significant differences (LSD) test was applied at the 5% probability level using the STATGRAPHICS Centurion XVII package. For the antioxidant activity and enrichment of virgin olive oil with OMW phenolic extracts, data are expressed as means ± standard deviation and curves were fitted using Microsoft Excel software.

3. RESULTS AND DISCUSSION

3.1 OMW CHARACTERISATION

The microbiological and physicochemical characteristics of the fresh OMW are reported in Table I. Results confirmed the pollutant load of these effluents and were similar to several previous researches, although some differences were observed for certain characteristics, due to many factors such as variety, maturity of olives, period of production, climatic conditions, farming methods, region of origin, and the oil extraction technology [15, 41, 42, 43]. The pH was acidic (4.89) as a result of high amount recorded for total phenols (1.75 g/l caffeic acid) that was consistent with previous findings [9, 11, 44, 45]. High values of chlorides content (0.91 g/l) and consequently an important electrical conductivity (10.18 mS/cm) were found, mainly related to salt adding for olive conservation before oil extraction [46]. The investigated OMW also showed a strong organic load, expressed by the high values of chemical oxygen demand (164.09 g O_2/l), sugar amount (5.19 g/l) and organic matter (73.13 g/l). These findings are usually associated to cultivation practices, irrigation management, olive cultivar and fruit maturity [11, 47]. OMW in our case were heavily loaded by total solids (91.25 g/l) and suspended solids (42.97 g/l) compared to other studies [48 - 50]. The separation efficiency of the used centrifuge or decanter could explain differences in solid contents. Considerable amounts of mineral matter (18.12 g/l), total kjeldahl nitrogen (0.49 g/l) and proteins (3.04 g/l) were recorded in line with those detected by Ayoub et al. [51] and De Leonardis et al. [28]. Calculated C/N ratios were similar to values reported by Amaral et al. [7]. For microbial counts, total aerobic and mesophilic flora was around 21.89 10^4 CFU/ml, including yeasts and moulds that were the most abundant (17.76 10^4 CFU/ml), while the counted lactic acid bacteria were approximately 5.04 10^4

Table I - Microbiological and physicochemical characteristics of fresh OMW collected in northern Morocco from industrial mills operating using three extraction systems (two [C2] and three [C3] phase centrifugation decanters and super-pressure [SP] system) during the 2017 growing season.

	C2	C3	SP
pH	5.07[a]	4.77[c]	4.84[b]
Electrical conductivity *(mS/cm)*	11.1[a]	9.7[b]	9.7[b]
Suspended solids *(g/l)*	64.9[a]	31.5[b]	32.5[b]
Total solids *(g/l)*	129.7[a]	71.7[b]	72.2[b]
Mineral matter *(g/l)*	29.6[a]	9.5[c]	15.2[b]
Organic matter *(g/l)*	100.0[a]	56.6[c]	62.7[b]
Chemical oxygen demand *(g O_2/l)*	226.7[a]	116.0[b]	149.53[b]
Total phenols *(g/l caffeic acid)*	1.5[b]	1.9[a]	1.8[a]
Chlorides *(g/l)*	1.1[a]	0.9[ab]	0.8[b]
Sugar *(g/l)*	8.0[a]	2.7[c]	4.5[b]
Total kjeldahl nitrogen *(g/l)*	0.5[a]	0.4[c]	0.5[b]
Total organic carbon *(g/l)*	58.0[a]	32.9[c]	36.4[b]
C/N ratio	109.3[a]	75.3[b]	74.5[b]
Proteins *(g/l)*	3.3[a]	2.7[c]	3.1[b]
Total Aerobic Mesophilic Flora *(10^4 CFU/ml)*	23.9[a]	19.9[c]	21.8[b]
Lactic Acid Bacteria *(10^4 CFU/ml)*	6.3[a]	4.1[c]	4.7[b]
Total Coliform Bacteria *(10^4 CFU/ml)*	-	-	-
Yeasts & Molds *(10^4 CFU/ml)*	19.4[a]	16.8[b]	17.2[b]

[abc] Means for each trait followed by the same letter are not significantly different at $P < 0.05$.

CFU/ml. No total coliform bacteria were observed. Our results were close to those revealed by El Yamani et al. [52] and Esmail et al. [53], but lower than those disclosed by Ben Sassi et al. [41] and Amaral et al. [7].

When comparing among extraction systems for physicochemical characteristics, significant statistically differences were found between C2 on one hand and both C3 & SP on the other hand. C2 displayed OMW with the high levels of all traits, except for total phenols which the great amount was for C3. The same findings were documented previously [42]. C3 and SP presented relatively similar characteristics. In fact, the large quantities of added water during olive oil extraction using C3 and SP allowed a dilution of OMW produced by these two systems. Moreover, C3 requires addition of warm water that increases the loss of phenols by solubilisation through the OMW [11].

For microbiological characteristics, wide variations were observed between the three systems, especially for total aerobic and mesophilic flora, and lactic acid bacteria, with C2 exhibiting the highest counts and C3 the lowest ones. Yeasts and moulds were more abundant in OMW from C2, whereas, no significant difference was found between C3 and SP. The richness of OMW from C3 in total phenols makes them more acidic that could explain the low microbiological counts in these effluents [54].

3.2 ANTIBACTERIAL ACTIVITY OF OMW PHENOLIC EXTRACTS

The phenolic extracts (from C2, C3 and SP systems) were screened for their antibacterial properties against two pathogenic bacteria (*Escherichia coli*, and *Listeria innocua*), with a negative control (DMSO 10%) and a positive one (Gentamicin). The results of the disc diffusion test are presented in Table II. No antibacterial activity was noted for the negative control, while among phenolic extracts, C2 exhibited more antibacterial potential showing the highest inhibition zones (8.5 mm, 10 mm against *E. coli* and *L. innocua*) compared to the other extracts (C3 and SP). The inhibition zones for gentamicin were the largest (14 mm, 17 mm against *E. coli* and *L. innocua*). In addition, gram positive bacteria (*L. innocua*) was more sensitive than the negative one (*E. coli*) for all extracts.

The MIC and MBC values for the two studied trains are also indicated in Table II. Different sensibility was revealed towards the three phenolic extracts. In fact, the C2 extract caused the complete inhibition of *E. coli* and *L. innocua*, respectively, at 6.3 and 3.2 µg/mL. The MICs for C3 were 25.0 µg/mL for *E. coli*, and 6.3 µg/ml for *L. innocua*. For the phenolic extract from SP, the highest MIC (12.5 µg/mL) was also for *E. coli*, whereas, the lowest one was recorded for *L. innocua* (6.3 µg/mL). The bactericidal effect of the C2 extract was at 12.5 and 6.3 µg/mL, respectively, for *E. coli* and *L. innocua*, while the MBCs of C3 and CP were respectively, 25.0 and 12.5 µg/mL for the two bacteria strains.

Our findings are in harmony with those disclosed in several investigations [55 - 57] indicating a good antibacterial potential of OMW phenolic extracts mainly related to their low polarity, due to the acidic side chain, which facilitate their transport over the cell membrane [58]. The phenolic effect could be also associated to their interaction with membrane lipids by a neu-

Table II – Antibacterial activities of OMW phenolic extracts (from two [C2] and three [C3] phase centrifugation decanters and super-pressure [SP] system) against *Escherichia coli* and *Listeria innocua*, expressed as inhibition zones, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Germs	Inhibition zone (mm)			MIC (µg/mL)			MBC (µg/mL)		
	C2	C3	SP	C2	C3	SP	C2	C3	SP
Escherichia coli	8.5[a]	7.0[c]	8.0[b]	6.3[c]	25.0[a]	12.5[b]	12.5[b]	25.0[a]	12.5[b]
Listeria innocua	10.0[a]	7.0[c]	9.0[b]	3.2[b]	06.3[a]	06.3[a]	06.3[c]	25.0[a]	12.5[b]

[abc] Means within a line followed by the same letter are not significantly different at $P < 0.05$.

tralisation of the membrane's electric potential following the penetration of the molecule [58]. Difference in effectiveness among the three extracts could be attributed to the chemical composition of phenolic compounds that depended mainly on the extraction process [42]. In fact, antibacterial activities were predominantly linked to caffeic acid, vanillic acid, p-coumaric acid, and 4-hydroxybenzoic acid [59, 60], verbascoside [61], and oleuropein and hydroxytyrosol [55]. The antibacterial test indicated that *E. coli* (Gram negative) was more resistant to the phenolic effect than *L. innocua* (Gram positive), probably due to differences in the cell wall composition [62, 63]. Gram-negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds [56].

3.3 ANTIOXIDANT ACTIVITY OF OMW PHENOLIC EXTRACTS

Although the environmental problem created by OMW because of their polluting load, these effluents seem to be a promising source for valuable compounds such as phenols. In fact, Bouaziz et al. [64] previously reported that olive oil and olive by-products provide a rich source of natural antioxidants with phenols as one of the most important groups [65]. In this work, we focused on the antioxidant activity of OMW phenolic extracts from three extraction systems (C2, C3 and SP), using two different assays: DPPH and FRAP.

3.3.1 DPPH radical scavenging activity

The DPPH radical-scavenging activity by hydrogen donation is a well-known antioxidation mechanism. Results represented in Figure 1 showed an antiradical potential of all OMW phenolic extracts and standards that is related to antioxidant concentration (concentration-dependent). The percentage of DPPH scavenging activity increase with increasing concentration until the stationary phase is reached, corresponding to the almost complete DPPH scavenging. OMW phenolic extracts exhibited an antiradical activity greater than 90% at concentrations around 320 µg/mL for C2 and 390 µg/ml for both C3 and SP, while the same score is reached at a relatively low concentration (about 156 µg/ml) for CA and AA.

IC 50, the concentration providing 50% of inhibition, was calculated to further compare among phenolic extracts as well as to standards. Results are shown in Figure 2. The comparison of IC50 values showed significant differences between all tests. Among phenolic extracts, C2 displayed the highest antiradical activity (the lowest IC50 equal to 32.5 µg/mL), contrariwise, the lowest one was observed for C3 extract (42.5 µg/mL). Although low concentrations of phenolic extracts allowed significant antiradical activity, those recorded for both standards remained stronger. IC50 values for CA and AA were 7.5 and 15 µg/mL, respectively.

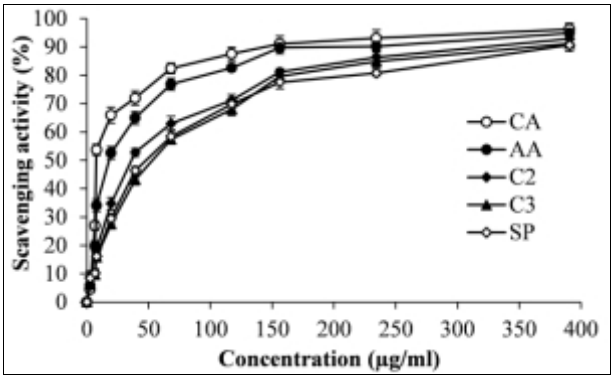

Figure 1 - DPPH radical scavenging activities (%) of OMW phenolic extracts from three extraction systems (C2: two phase decanter, C3: three phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards. Data presented as means ± SD.

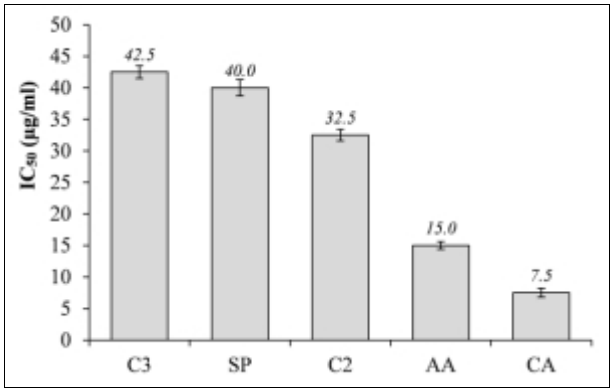

Figure 2 - Concentration providing 50% DPPH radical-scavenging (IC50) of OMW phenolic extracts from three extraction systems (C2: two phase decanter, C3: three phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards. Data presented as means ± SD.

3.3.2 Ferric reducing antioxidant power (FRAP) assay

In addition to the DPPH test, antioxidant potential of OMW phenolic extracts, from C2, C3 and SP systems and the standards caffeic acid (CA) and ascorbic acid (AC), were also assessed from their ability to reduce ferric iron (Fe^{+3}) in the [$K_3Fe(CN)_6$] complex to the ferrous form (Fe^{+2}), which was monitored by measuring the formation of Perl's Prussian blue at 700 nm. Results for this FRAP test are displayed graphically in Figure 3. OMW phenolic extracts exhibited a good reducing power capacity, increasing in a concentration-dependent way that was higher for C2 phenolic extract. Nonetheless, the reducing powers of CA and AA (standards) were more pronounced. Indeed, the order of FRAP activity of all samples and standards were as follows: CA > AA > C2 > SP > C3. At a concentration of 100µg/ml, the reducing power (absorbance at 700 nm) were found to be 1.393 ± 0.025, 1.234 ± 0.026, 0.957 ± 0.027, 0.815 ± 0.023 and 0.931 ± 0.021 for CA, AA, C2, C3 and SP, respectively.

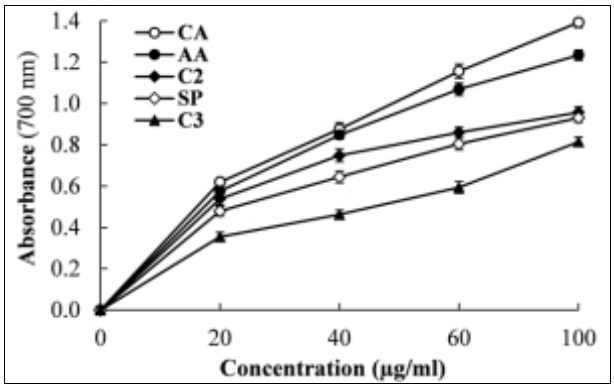

Figure 3 - Reducing power of OMW phenolic extracts from three extraction systems (C2:two phase decanter, C3: three phase decanter and SP: super-pressure) compared to ascorbic acid [AA] and caffeic acid [AC]) as standards by using the FRAP assay: increase in absorbance indicates increasing reducing power. Data presented as means ± SD.

Our results concerning the antioxidant activity of OMW phenolic extracts were very encouraging and similar to several previously published researches [19, 20, 22, 42, 55]. For both tests, the antioxidant activity of phenolic extracts was found to be concentration-dependent. This observation agrees with that reported in various studies [19, 22, 66]. The correlation of antioxidant activity with phenolic extract content was also reported for olive leaves [67, 68] and tea extracts [36]. At equal concentrations, phenolic extract from C2 exhibited the strong antioxidant activity, probably due to the phenolic compound composition characterising each extract. Similarly, Lesage-Meessen et al. [42] announced that the C2 extract displayed the highest antiradical activity, with a two-fold value greater than that of the C3 extract and attributed the difference to the highest concentrations of antioxidant phenolic compounds in the C2 extract such as hydroxytyrosol. Farag et al. [40] had also noticed a close relationship between the antioxidant effectiveness and the chemical composition of phenolic compounds. In another study conducted on olive leaf extracts, it was suggested that the effect of the phenolic profile on the antioxidant activity was greater rather than the total phenolic content [68]. Rice-Evans et al. [69] attributed the antioxidant activity of an extract to both content and structure of phenolic compounds, especially, the number of hydroxyl groups in the phenolic ring.

3.4 VOO ENRICHMENT WITH OMW PHENOLIC EXTRACTS

The improvement of virgin olive oil with OMW phenolic extracts was also investigated here in the light of the importance of antioxidants in protecting the quality of foods and considering the abundant source of phenols in olive oils and OMW as the main antioxidant and stabilising agents [70]. Initial olive oil quality was determined before enrichment with phenolic extracts and the results revealed low mean values for free fatty acids (0.78% oleic acid), peroxide value (4.13 meqO_2/kg) and extinction coefficients (K232 = 1.87 and K270 = 0.17) falling into the extra virgin olive oil (VOO) category according to the European Union Commission requirements [37], which indicated a good oil quality with low oxidation status. Chlorophyll and carotenoid amounts were 1.52 mg/kg and 0.85 mg/kg, respectively, while the total phenols content was 398 mg/kg caffeic acid.

The same concentrations (100, 300 and 500 ppm) of C2 phenolic extracts (PE) and ascorbic acid (AA) were added to VOO samples. Enriched VOO along with the control (VOO without additives) were stored in the dark at 60°C for 22 days. Olive oil stability was monitored by measuring PV, K232 and K270 and the results are displayed in Figures 4 and 5.

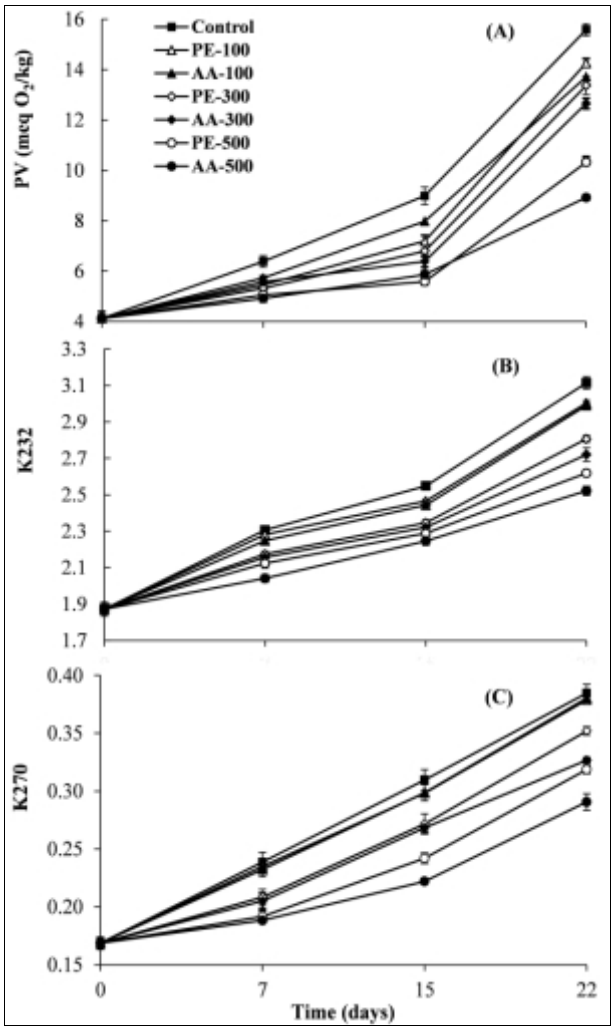

Figure 4 - Evolution of peroxide value [PV] (A), K232 (B) and K270 (C) of virgin olive oils (VOO) enriched with different concentrations of OMW phenolic extracts [PE] and ascorbic acid [AA] as standards during the storage period at 60°C. Data presented as means ± SD. AA-100, AA-300, AA-500 = VOO enriched with 100, 300 and 500 ppm of acid ascorbic, respectively. PE-100, PE-300, PE-500 = VOO enriched with 100, 300 and 500 ppm of OMW phenolic extract, respectively.

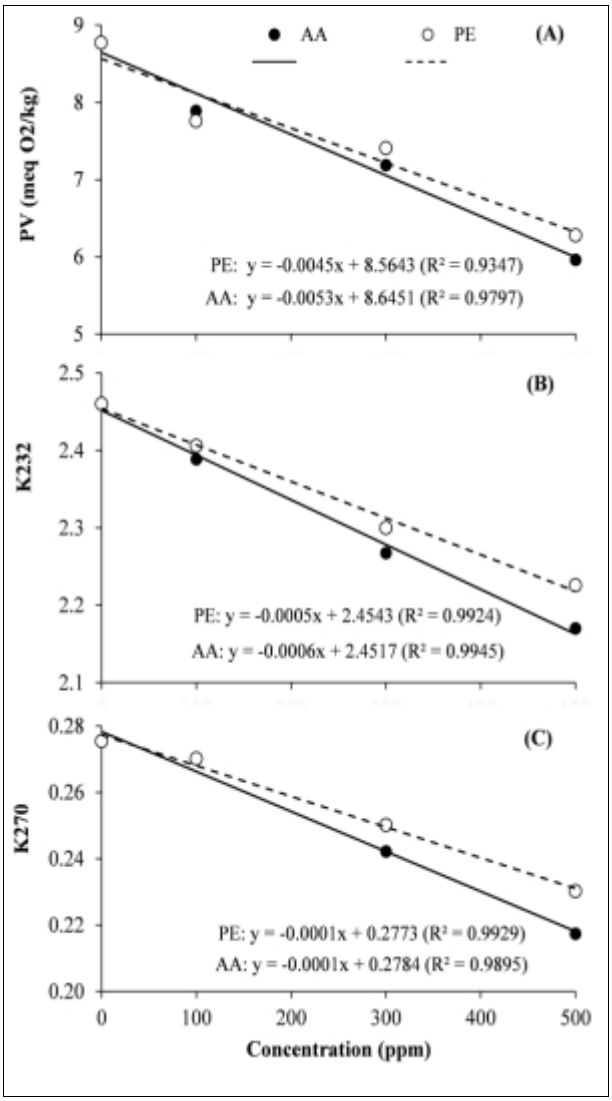

Figure 5 - Regressions of peroxide value [PV] (A), K232 (B) and K270 (C) of enriched virgin olive oils against different concentrations of additives (OMW phenol extract [PE] and ascorbic acid [AA]).

Figure 4 shows significant increases of PV, K232 and K270 values in relation to the storage time, indicating a significant rise in both lipid hydroperoxides and conjugated dienes and trienes formation, for all samples. However, the increase was more important in the control compared to samples enriched with OMW phenolic extracts (PE) and ascorbic acid (AA). Indeed, PE-500 and AA-500 gave a slower increase for the three traits, with a more antioxidant effectiveness of AA. Moreover, the effect of PE and AA against lipid oxidation increased with an increasing concentration as shown in Figure 5. In fact, when PV, K232 and K270 values were plotted against PE and AA concentrations (Figure 5A, B, C, respectively), we observed a decrease in the values of the three oxidation parameters with the increase of the additive concentration.

Our results were encouraging and showed a significant oxidation delay in PE-enriched virgin olive oil as demonstrated by low increases in PV, K232 and K270 values, compared to the control. Moreover, this oxidation delay was concentration-dependent. Similarly, Lafka et al. [20] evaluated the oxidative stability of commercial virgin olive oil and sunflower oil, enriched with OMW phenol extracts, by the measurement of peroxide value and induction time, and they reported a reduction of the peroxides formation and an increase in induction time in samples enriched at a concentration of 150 ppm compared to control samples. Fki et al. [22] reported that the addition of OMW extract, 3,4-dihydroxyphenylacetic acid and hydroxytyrosol in refined oils displayed a significant delay in the oxidation rate, which was dose-dependent. The same authors also indicated that PV values were lower in samples enriched with OMW extracts at 500 ppm than those enriched with butylated hydroxyanisole (BHA), p-hydroxyphenylacetic acid or tyrosol. The effect of the total and free phenolic compounds from Picual and Koronaki leaves and fruits on sunflower oil stability was also demonstrated by Farag et al. [40] indicating that total and free phenolic compounds produced a significant antioxidant activity and protected the stability of sunflower oil. Bouaziz et al. [64] have disclosed a decrease in the formation of both lipid hydroperoxides and conjugated dienes and trienes formation as well as an improvement of the oxidative resistance (assessed by Rancimat test) in refined olive oil and refined olive husk oil enriched with phenolics recovered from olives. De Leonardis et al. [71] examined the influence of natural antioxidants extracted from OMW on the oxidative stabilisation of lard and reported that the oxidative resistance, measured using PV and Rancimat, was greatly enhanced by the addition of OMW extracts. Similarly, olive phenols recovered from OMW were added to raw and cooked fresh pork sausages during aerobic storage and resulted in a decrease in pH and peroxide value [72, 73].

4. CONCLUSIONS

This study confirmed the pollution load of OMW. Their direct disposal into the environment might be a serious problem. However, phenols, the main harmful elements in these effluents, could constitute a promising source of natural antioxidants. OMW phenolic extracts showed great antibacterial and antioxidant potentials either by the *in vitro* tests or by their effect on the oxidation delay of the olive oil. The low cost of this source of bioactive compounds encourages their use as alternatives to synthetic products for other purposes.

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