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Olive Oil Wastewaters from Northern Morocco: Physicochemical Characterization and Antibacterial activity of Polyphenols

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Abstract

This work examines the influence of extraction system, growing season and production site on some physicochemical parameters of olive oil mill wastewaters (OMW) samples generated in Taza province (northern Morocco). Antibacterial activity of OMW polyphenols extracts was tested against certain strains known for their pathogenicity. ANOVA analysis showed that pH, electrical conductivity and total phenols content were mainly under the influence of the extraction system, while chlorides content variability was largely due to the effect of production site and its interaction with extraction system. Moreover, mean comparison statistics revealed that the highest value of total phenols content was recorded in OMW obtained using triple phase centrifugation decanter , whereas, OMW released by super pressure revealed the lowest pH. Among sites, BniFrassen exhibited higher values for electrical conductivity and chlorides content. Significant difference was observed, between the two growing seasons, only for total phenols content. The two principal component (PC) axes accounted for 80% of total variance. PC1 was better explained by production site, while system extraction and growing season fitted PC2 variability. For the in vitro bioassays, OMW polyphenols extracts showed a differential antibacterial activity against the four bacteria studied. This variability depends mainly on the tested microorganism and the concentration of polyphenols extract. In fact, a complete inhibition of growth forall the strains was observed at a concentration of 0.205 mg/ml of polyphenols.

1. Introduction

Taza province (northern Morocco) is one of the most important olive-growing regions in Morocco with an acreage of about 78800 ha. Olives produced by the province account for 7% of national production, standing at 90000 tons in a normal year, 70% of this production is reserved for the crushing and extraction of olive oils. Olive oil industry in the province of Taza is made up of traditional mills 'Maasra' (1000 units) and modern and semi-modern units (49 units including 19 in super pressure, 23 with triple phase decanter and only 7 units with dual phase decanter) [1].

Despite the economic importance of olive oil extraction industry, great amounts of olive oil mill wastewatersare produced. This black liquid wastewater comes from the olive fruit vegetation water, the water used for washing and treatment and a portion of the olive pulp and residual oil [2]. The volume of OMW varies from 40 to 60 L for pressing method, but it ranged from 80 to 100 L for triple phase centrifugation process per 100 kg of olives [3]. Whereas, OMW released by dual phase decanter are of small amounts compared to the other systems, mainly due to the addition of very low quantities of water during the olives crushing.

Several studies conducted on the composition of OMW indicated that this effluent contains 83-92 % of water as a major part [4], large amounts of organic molecules, particularly polyphenolic mixtures with different

molecular weights [5], and other organic molecules, including nitrogen compounds, sugars, organic acids, and pectins that increase their organic load [6-12].
Furthermore, the physicochemical characteristics of OMW are rather variable, depending on several factors including soil conditions, yearly climatic fluctuations, olive cultivars, agronomic and cultivation practices, irrigation management, degree of fruit maturation, harvesting period, storage time, and extraction procedure [13-19].
OMW claimed to be one of the most contaminating effluents among those produced by the agrofood industries, owing to its high mineral salt content, low pH and the presence of phytotoxic compounds, especially phenols [20]. OMW are highly toxic to plants and to some microorganisms [21]. Negative effects have also been recorded on soil properties, including immobilization of available nitrogen [22, 23], displacement of the exchange complex calcium by potassium in an anfisol, increased salinity [24] and decreased plant-available magnesium, probablybecause of the antagonistic effect of potassium [2].
Treatment of OMW requires high capital and operating cost units with limited efficiency due to high polluting loads. Recently, several research carried out on OMW had focused on the valorization of its valuable elements. In fact, phenols as the main pollutant elements of OMW have been the subject of various studies that revealed their high antibacterial potential [26-29].
The objectives of this work were: (i) to examine the composition of fresh OMW for some polluting physicochemical parameters (pH, electrical conductivity, chlorides content and total phenols) produced by different extraction systems in three sites of northern Morocco during two consecutive growing seasons, and (ii) to evaluate the antibacterial activity of OMW phenols against some bacterial strains known for their pathogenicity.

2. Materials and Methods

2.1. *Samples*

Fresh olive oil mill wastewaters, produced by three different extraction systems: dual phase (C2) and triple phase (C3) centrifugation decanters, and super-pressure system (SP), were sampled from three different sites (Figure 1) in the province of Taza (northern Morocco), namely Taza city ‘T-C’ (34 ° 12'36 "N, 3 ° 52'0" W), Bouchfaa ‘BC’ (34 ° 5'17 "N, 4 ° 17'6" W) and BniFrassen ‘B-F’(34 ° 21'35 "N, 4 ° 22'57" W).
OMW samples were taken in a closed plastic container during November and December of two consecutive growing seasons (2013-2014 and 2014-2015) and were maintained at 4 °C to prevent biodegradation.

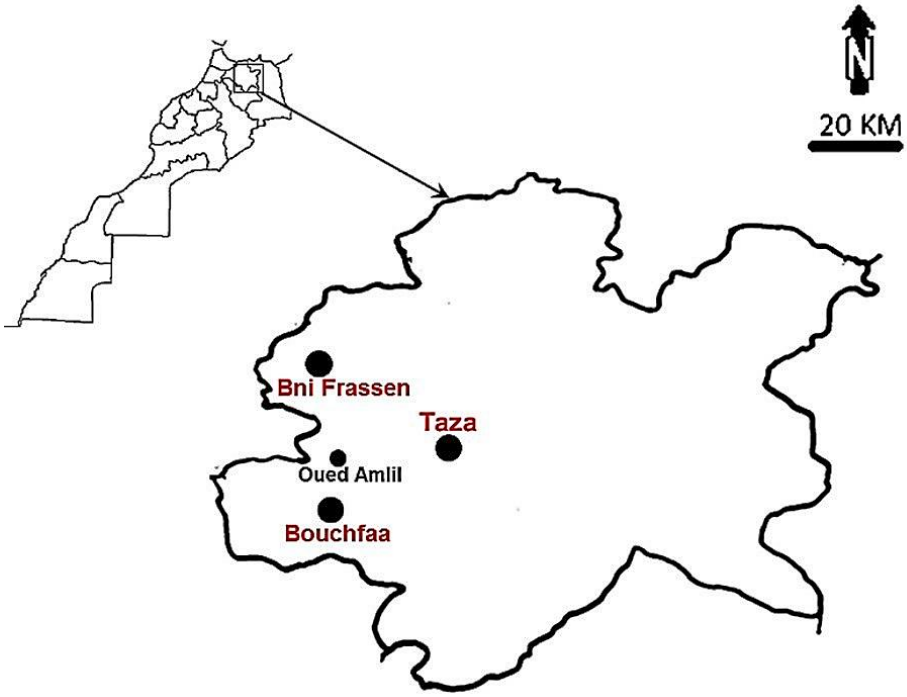

Figure 1: Geographical localization of sampling sites in Taza province (northern Morocco)

2.2. *Analytical methods*
2.2.1. *Physicochemical analyses*

pH:
Hydrogen potential (pH) was measured*in Situ*, immediately after sampling, using a multi-parameter analyser (CONSORT C535, Turnhout, Belgium) previously calibrated with buffer solutions pH 4, 7 and 10, according to the manufacturer's instructions.

Electrical conductivity:
A calibrated multi-parameter analyser (CONSORT C535, Turnhout, Belgium) was used to measure the electrical conductivity of the OMW samples, expressed in mS.cm-1.

Total phenols content:
Extraction of phenolic compounds was carried out using the analytical methodology described by De Marco *et al.* [30] with some modifications. OMW samples were washed with hexane [1:1, (v/v)] in order to remove the lipid fraction: 10 ml of OMW were mixed with 15 mlof hexane; the mixture was shaken and then centrifuged during 5 min at 3000 rpm. The phases were separated and the washing was repeated successively two times. Extraction of phenolic compounds was then carried out with ethyl acetate: OMW samples, preventively washed, were mixed with 10 mlof ethyl acetate; the mixture was vigorously shaken and centrifuged for 10 min at 3000 rpm. The phases were separated and the extraction was repeated successively four times. The ethyl acetate was evaporated and the dry residue was dissolved in methanol. .
Total phenols content was determined by spectrophotometry (Spectrophotometer JENWAY 6100, Dunmow, Essex, U.K) using Folin-Ciocalteau reagent and the extinction was measured at 750 nm according to the Folin-Cicalteau method [31] using caffeic acid (Sigma-Aldrich, St. Louis, MO, USA) as standard. Values for total phenols content are given as g caffeic/l.

Chlorides content:
Chlorides content was determined according to the standard [32]; it was measured using the Mohr method. Chromate ions are used as an indicator in the titration of chloride ions with a silver nitrate standard solution. After the precipitation of all chlorides as white silver chloride, the first excess of titrant results in the formation of a silver chromate precipitate, which signals the end point.

2.2.2. *Antibacterial activity assays*
Biological material:
The four bacteria strains used in this study were supplied by the Spanish Type Culture Collection (CECT), including Gram-negative: *Pseudomonas aeruginosa* CECT118, *Escherichia coli* K12 (CECT433), and Gram-positive: *Staphylococcus aureus* CECT976, and *Bacillus subtilis* DSM6633.
The bacterial strains were cultivated twice in Muller–Hinton agar (MH) and incubated overnight at 37°C except for *Bacillus* species, which were incubated at 30 °C. Then, each strain of cultures was cultivated in 3 ml of Mueller-Hinton broth (MH) and was incubated at 37 °C for 18 h. Each culture of used bacteria was diluted in MH broth to obtain a final concentration of about 10^8 CFU/ml.

Determination of inhibition zones:
Antibacterial activity of polyphenols extract was tested by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). This method is presented as a consensus standard by the CLSI [33]. Extracts of polyphenols were diluted with DMSO (Dimethylsulfoxide) 10 %. Antimicrobial tests were carried out by the disc diffusion method using 100 µl of suspension containing 10^8 CFU/ml of bacteria spread on Mueller-Hinton agar (MH) in sterilized Petri dishes (90 mm diameter). The discs (6 mm in diameter) were impregnated with 10 µl of different DMSO polyphenol extracts dilution and placed onto the inoculated agar. Tetracycline (10 µg/well) was used as a positive control in antibacterial tests. Negative control consisted of 10% DMSO that is used to dissolve the solid polyphenol extracts. The plates were incubated for 24 h at 37°C and antibacterial activity was evaluated by measuring the inhibition zone diameter (mm) against the tested organism. Experiments were run in triplicate, and the developing inhibition zones were compared with those of reference discs.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):
Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the CLSI [33]. The MIC was defined as the lowest concentration of polyphenols at which microorganisms show no visible growth. Referring to the results of the MIC assay, the tubes showing complete absence of growth were identified and 5µl solutions from each tube was transferred to agar plates and incubated at 37 °C, for 24 hours. The MBC is defined as the lowest concentration of polyphenols at which inoculated microorganisms were 99.9% killed.

2.3. *Statistical analysis*
All determinations were performed in triplicate. Combined analyses of variance (ANOVA) were carried out over extraction systems, production sites and growing seasons. Least significant difference (LSD) values were calculated at the 5% probability level. The relationships between the studied traits were established. Principal component analyses (PCA) were performed on the correlation matrix, calculated on the mean data of all the replicates. The STATGRAPHICS Centurion XVII package was used for all the calculations.

3. Results

3.1. *Physicochemical analysis*
Results of the combined analyses of variance of OMW samples (Table 1) revealed that pH was highly influenced by the extraction system, which explained about 70% of total observed variance. Electrical conductivity was affected in equal proportion by the extraction system and its interaction with production site. Total phenols content, the most indicator of OMW toxicity, was mainly under the effect of extraction system (61%) and to a lesser extent the growing season (29%). Chlorides content variability was mainly due to the effect of production site and its interaction with extraction system, which explained 25% and 31%, respectively.

Table 1: Mean squares of the combined analyses of variance of OMW samples produced in different sites of northern Morocco (Taza city, BniFrassen and Bouchfaa), using three extraction systems (dual and triple phase centrifugation decanters and super-pressure system) during two growing seasons (2013-2014 and 2014-2015).

Source of variation	Df	pH	Conductivity	Total Phenols	Chlorides
Site	2	0.083**	26.073***	0.0163**	0.604***
System	2	1.294***	6.817**	0.8130***	0.065
Season	1	0.022	0.145	0.3900***	0.336**
Site × System	4	0.235***	30.050***	0.1015***	0.748***
Site × Season	2	0.010	4.575	0.0012	0.021
System × Season	2	0.014	3.935	0.0004	0.196**
Site × System × Season	4	0.174***	8.908***	0.0051	0.333***
Replicate	1	0.007	0.027	0.0014	0.032
Residual	35	0.012	0.925	0.0019	0.030
Total	53				

** Significant at 0.05 probability level; ** Significant at 0.01 probability level; *** Significant at 0.001 probability level*

Mean comparison among production sites (Table 2) showed that BniFrassen exhibited higher values for electrical conductivity and chlorides content (11.70 mS/cm and 1.16 g/l, respectively). On the contrary, Taza city gave OMW with the lowest conductivity and chlorides content values (9.48 mS/cm and 0.83 g/l, respectively). No statistical differences among sites were recorded for the other parameters.
Among extraction systems, significant differences were observed in total phenols content and pH values (Table 2). Higher content of total phenols (1.78 g/l caffeic) was found in OMW samples from triple phase decanter, whereas, OMW released by super-pressure system were much more acidic (pH = 4.86) than those from other systems.
When comparing the two growing seasons (Table 2), significant differences were observed only for total phenols content. The highest value (1.67 g/l caffeic) was measured for the 2014-2015season. No statistical differences were registered for the other parameters.

Table 2: Means and range values of analyzed physicochemical parameters of OMW samples produced in three different sites of northern Morocco (Taza city [T-C], BniFrassen [B-F] and Bouchfaa [BC]), using three extraction systems (dual [C2] and triple [C3] phase centrifugation decanters and super-pressure [SP] system) during two growing seasons (2013-2014 and 2014-2015).

	pH		Conductivity (mS/cm)		Total Phenols *(g/lcaffeic)*		Chlorides (g/l)	
	Mean	*Range*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Sites								
B-F	5.03 a	4.97-5.08	11.70 a	11.24-12.16	1.62 a	1.60-1.64	1.16 a	1.08-1.25
BC	5.07 a	5.02-5.13	9.78 b	9.32-10.24	1.56 a	1.54-1.58	1.12 a	1.04-1.21
T-C	5.16 a	5.11-5.21	9.48 b	9.02- 9.94	1.58 a	1.56-1.60	0.83 b	0.75-0.91
Extraction system								
C2	5.38 a	5.33-5.44	10.60 a	10.14-11.06	1.31 c	1.34-1.38	0.99 a	0.90-1.07
C3	5.02 b	4.96-5.07	10.74 a	10.28-11.10	1.78a	1.76-1.81	1.03 a	0.94-1.11
SP	4.86 c	4.81-4.91	9.61 a	9.15-10.07	1.61b	1.59-1.63	1.10 a	1.02-1.19
Growingseason								
13-14	5.11 a	5.06-5.15	10.27 a	9.89-10.64	1.50 b	1.48-1.52	1.12 a	1.05-1.19
14-15	5.07 a	5.02-5.11	10.37 a	9.99-10.74	1.67 a	1.65-1.69	0.96 a	0.89-1.03

Means for each character followed by the same letter are not significantly different according to LSD test at P < 0.05

To detect the combination of variables that best explained the existing variability, principal component analysis (PCA) was performed on the correlation matrix based on data mean values as shown in figures 2, 3 and 4. The first two PC axes accounted for 80% of total variance: 43% and 37% for axes 1 (PC1) and 2 (PC2), respectively. Axis 1 measures the variability of all tested parameters, with the exception of pH. Towards its positive direction, there was a joint increase of chlorides content and electrical conductivity. Toward its negative direction, there was an increase in total phenols content. On the second axis, the observed variation was caused mainly by pH. Production site means (Figure 2) plotted on the same plan determined by the two axes are grouped in clusters. PC1 discriminated clearly between samples from BniFrassen with high values of chlorides and conductivity on the positive side and those from Taza city and Bouchfaa, which are partially superimposed on the left side.

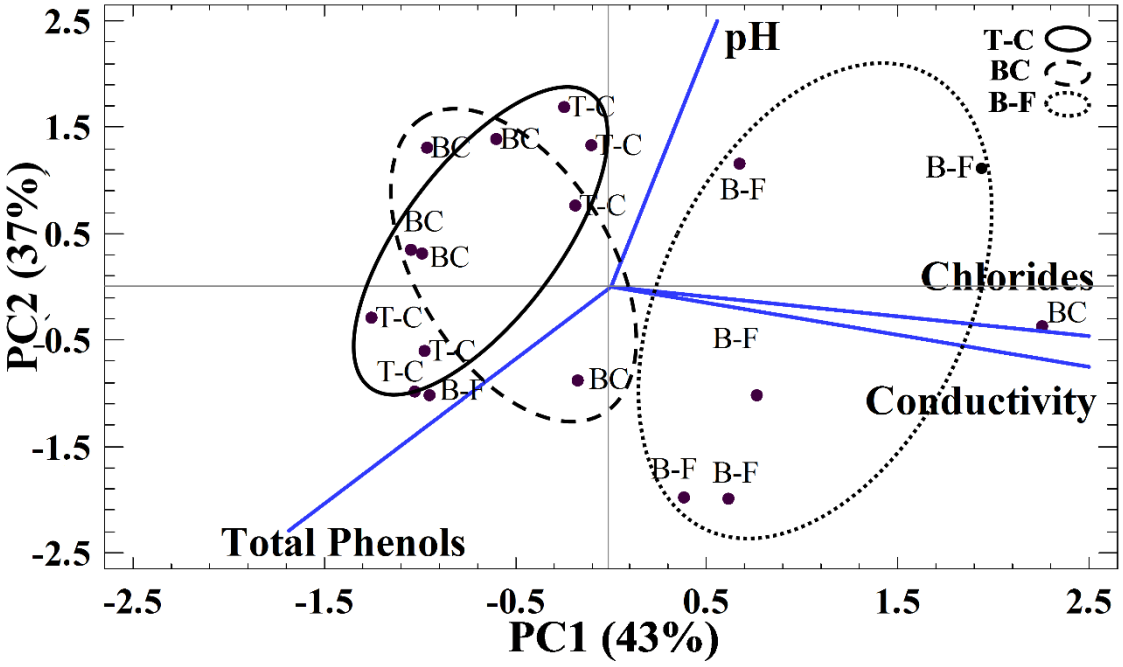

Figure 2: PCA projections on axes 1 and 2 accounting for 80% of total variance. Eigenvalues of the correlation matrix are symbolized as vectors representing traits that most influence each axis. The 18 points representing OMW samples means for each site (*T-C*: Taza City, *BC*: Bouchfaa, and *B-F*: BniFrassen) are plotted on the plane determined by axes 1 and 2.

On other hand, as it can be seen on figures 3 and 4, PC2 separated properly between extraction systems with C2 on the positive side and the two others systems (C3 and SP) towards the negative one. In addition, OMW samples from C2 had higher values of pH, and low amounts of total phenols in contrast to C3 and SP. Thus confirmed what was previously demonstrated by mean comparisons (Table 2). Similarly, PC2 separated the two growing seasons; 2013-2014 interacted with high amounts of chlorides content on its positive side, whereas 2014-2015 was associated with higher total phenols content on the negative direction.

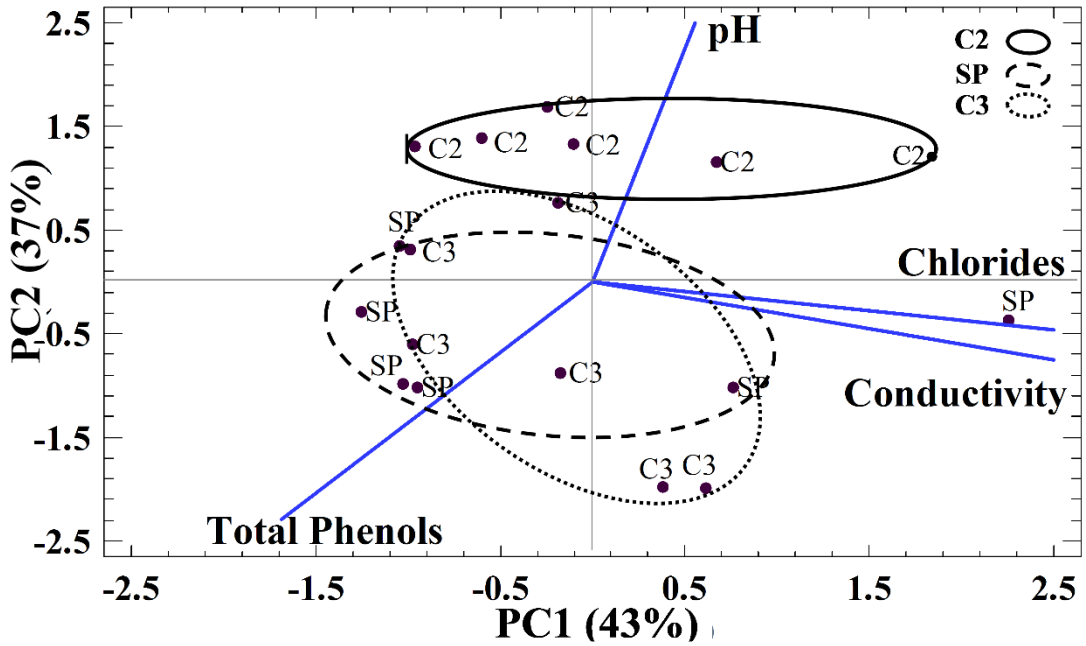

Figure 3: PCA projections on axes 1 and 2 accounting for 80% of total variance. Eigenvalues of the correlation matrix are symbolized as vectors representing traits that most influence each axis. The 18 points representing OMW samples means for each extraction system (*C2*: dual phase decanter, *C3*: triple phase decanter, and *SP*: super-pressure) are plotted on the plane determined by axes 1 and 2.

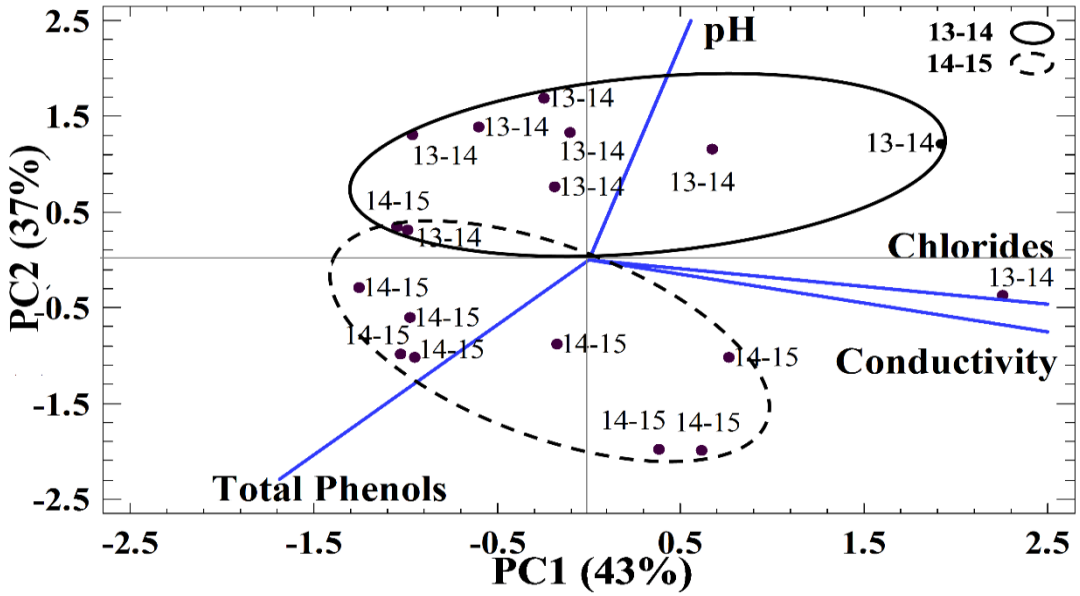

Figure 4: PCA projections on axes 1 and 2 accounting for 80% of total variance. Eigenvalues of the correlation matrix are symbolized as vectors representing traits that most influence each axis. The 18 points representing OMW samples means for each growing season (*2013-2014* and *2014-2015*)are plotted on the plane determined by axes 1

The study of relationships among the studied parameters (Table 3) revealed that electrical conductivity was positively associated to chlorides content, while pH values were negatively correlated to total phenols content. The rest of correlations were not significant and of minor importance.

Table 3: Correlations between pH, electrical conductivity, total phenols and chlorides for data from OMW samples produced in three different sites of northern Morocco (Taza city, BniFrassen and Bouchfaa), using three extraction systems (C2, C3 and SP) during two growing seasons (2013-2014 and 2014-2015).

	pH	**Conductivity**	**Total Phenols**	**Chlorides**
pH		-0.0784	-0.4785**	-0.0758
Conductivity			-0.0795	0.6584**
Total Phenols				-0.2087
Chlorides				

3.2. *Antibacterial activity of polyphenols*

The antibacterial properties of three different polyphenol residues, extracted from OMW produced using super-pressure, dual and triple phase centrifugation decanter systems, were tested against four pathogenic bacteria (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis*), with a negative control (DMSO 10%) and a positive one (Tetracycline). Mean comparisons following the analysis of variance (data not shown) are presented intable4.

No antibacterial activity was observed for the negative control (DMSO 10%), while the zone of inhibition for positive control was as follows: 19 mm, 16 mm, 32 mm, 30 mm against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus subtilis*, respectively (Table 4, Figure 5A).

The results showed that the three DMSO polyphenols extracts exhibited growth inhibition of all the tested microorganisms with various degrees. The antibacterial activity was not the same by using identical concentration of polyphenols applied to the four bacteria and gave inhibition zones diameters between 8 and 13 mm. Furthermore, inhibition zone was positively correlated to the applied concentration of polyphenols, which depends on the extraction system (Table 4).

When comparing inhibition zones between tested strains, we noted that the polyphenols had a higher inhibitory effect against *Staphylococcus aureus* (Gram-positive) with an inhibition diameter of 13 mm. However, the minimum zone of inhibition was against *Escherichia coli* and *Pseudomonas aeruginosa* (8 mm), both are Gram-negative bacteria (Table 4).

Table 4: Antibacterial activity of OMW polyphenols by the disk method on studied bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis*)

	Inhibition zones (mm)			
	Gram (-)		Gram (+)	
	Escherichia coli	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
C2	8 c	8 c	11 c	9 c
C3	10 b	11 b	13 b	12 b
SP	10 b	11 b	11bc	10 c
Control (+)	16 a	19 a	32 a	30 a
Control (DMSO10%)	–	–	–	–

Means for each strain followed by the same letter are not significantly different according to LSD test at P < 0.05

The MIC is the lowest concentration of a product that prevents visible growth of a bacterium after 18 or 24 h of incubation at 37 °C. MIC results of OMW polyphenols from the three systems are presented in tables 5, 6 and 7. The studied microorganisms did not exhibit the same sensitivity towards polyphenols extracts. In fact, polyphenols from SP caused inhibition to *Escherichia coli* at 0.205 mg/ml, while growth of *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*were inhibited at 0.102 mg/ml. The MICs of polyphenols from C3 were 0.226 mg/ml for *Escherichia coli*, and 0.113 mg/ml for all other bacteria. For

polyphenols from C2 (Figure 5B), the highest MIC (0.160 mg/ml) was also for *Escherichia coli*, whereas, the lowest one was recorded for *Bacillus subtilis* (0.320 mg/ml).
On the basis of MIC results, the minimum bactericidal concentration (MBC) was determined. Results are listed in table 5, 6 and 7. Bactericidal effect of OMW polyphenols was at the concentration of 0.205 mg/ml for *Escherichia coli* (Gram-negative), the strain that showed more resistance compared to other bacteria (Figure 5C). The MBC for *Staphylococcus aureus* was 0.113 mg/l,while the less resistant strains were *Pseudomonas aeruginosa* and *Bacillus subtilis* with a bactericidal effect at 0.102 mg/l.

Table 5: Antibacterial activity of OMW polyphenols from super- pressure (SP) on studied bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and*Bacillus subtilis*)

Germs	Minimum Inhibitory Concentration (**MIC)**					Minimum Bactericidal Concentration(**MBC)**				
	1 (0.819 mg/ml)	1/2 (0.409 mg/ml)	1/4 (0.205 mg/ml)	1/8 (0.102 mg/ml)	1/16 (0.051 mg/ml)	1 (0.819 mg/ml)	1/2 (0.409 mg/ml)	1/4 (0.205 mg/ml)	1/8 (0.102 mg/ml)	1/16 (0.051 mg/ml)
Escherichia coli	–	–	–	+	+	–	–	–	+	+
Pseudomonas aeruginosa	–	–	–	–	+	–	–	–	–	+
Staphylococcus aureus	–	–	–	–	+	–	–	–	+	+
Bacillus subtilis	–	–	–	–	+	–	–	–	–	+

Table 6: Antibacterial activity of OMW polyphenols from triple phase centrifugation decanter (C3) on studied bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*)

Germs	Minimum Inhibitory Concentration (**MIC)**					Minimum Bactericidal Concentration(**MBC)**				
	1 (0.903 mg/ml)	1/2 (0.451 mg/ml)	1/4 (0.226 mg/ml)	1/8 (0.113 mg/ml)	1/16 (0.056 mg/ml)	1 (0.903 mg/ml)	1/2 (0.451 mg/ml)	1/4 (0.226 mg/ml)	1/8 (0.113 mg/ml)	1/16 (0.056 mg/ml)
Escherichia coli	–	–	–	+	+	–	–	–	+	+
Pseudomonas aeruginosa	–	–	–	–	+	–	–	–	–	+
Staphylococcus aureus	–	–	–	–	+	–	–	–	–	+
Bacillus subtilis	–	–	–	–	+	–	–	–	–	+

Table 7: Antibacterial activity of OMW polyphenols from dual phase centrifugation decanter (C2) on studied bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Bacillus subtilis*)

Germs	Minimum Inhibitory Concentration (**MIC)**					Minimum Bactericidal Concentration(**MBC)**				
	1 (0.639 mg/ml)	**1/2** (0.320 mg/ml)	**1/4** (0.160 mg/ml)	**1/8** (0.080 mg/ml)	**1/16** (0,040 mg/ml)	**1** (0.639 mg/ml)	**1/2** (0.320 mg/ml)	**1/4** (0.160 mg/ml)	**1/8** (0.080 mg/ml)	**1/16** (0.040 mg/ml)
Escherichia coli	–	–	+	+	+	–	–	+	+	+
Pseudomonas aeruginosa	–	–	–	+	+	–	–	–	+	+
Staphylococcus aureus	–	–	–	+	+	–	–	–	+	+
Bacillus subtilis	–	–	–	–	+	–	–	–	+	+

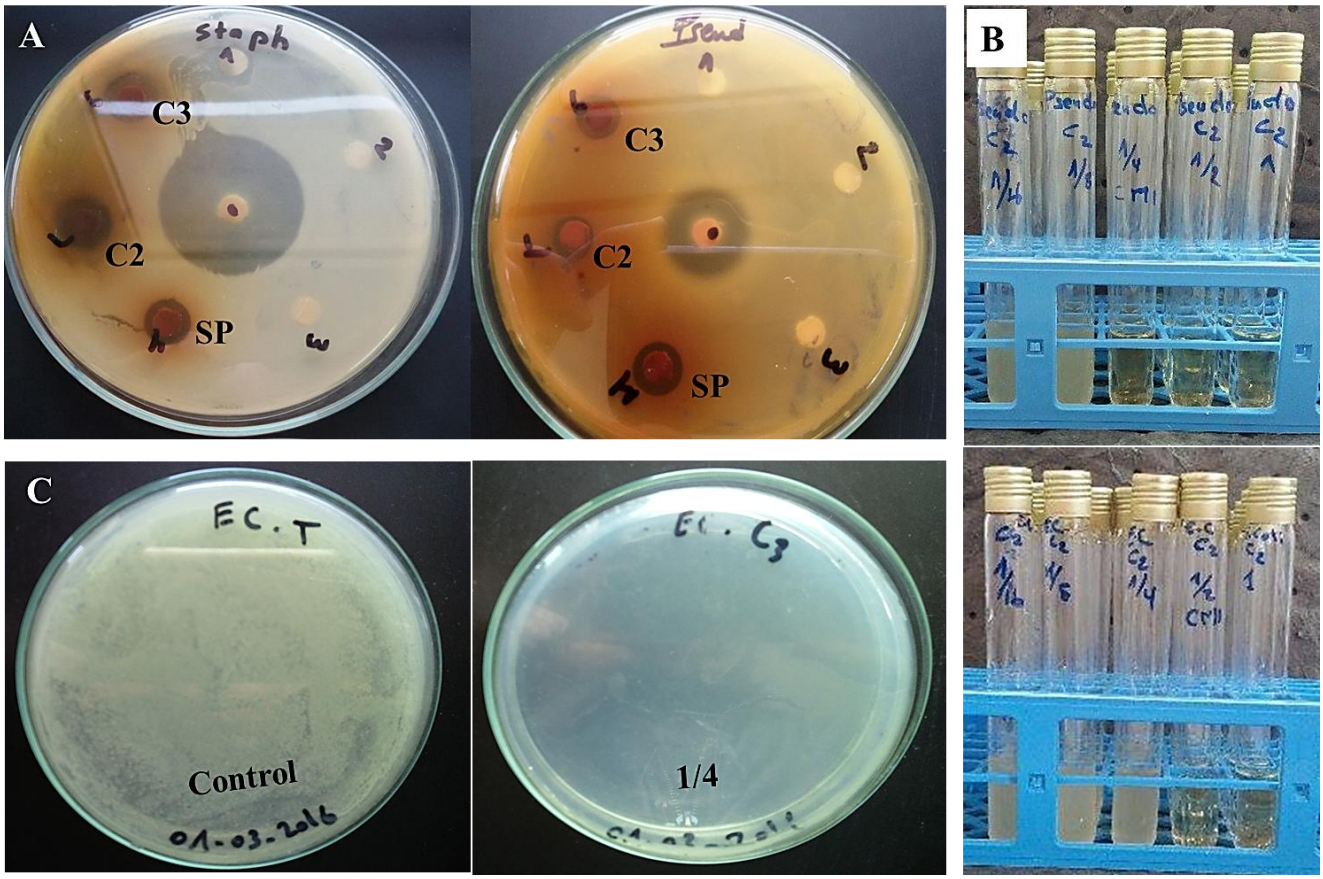

Figure 5: Illustration of some *in vitro* bioassay results. **A** =Inhibition zones of polyphenols extracts on *Staphylococcus aureus* (left) and *Pseudomonas aeruginosa* (right). **B** = Minimum inhibitory concentration (MIC) of C2 polyphenols extracts on *Pseudomonas aeruginosa* (top) and *Escherichia coli* (bottom). **C**= Minimum bactericidal concentration (MBC) of C3 polyphenols extracts on *Escherichia coli:* control (left) and at a concentration of 0.205 mg/ml (right).

3.1. *Physicochemical analyses*

Results from the present work confirmed the OMW toxicity on some pathogenic bacteria due mainly to their low pH and richness in total phenols [21]. Generally and for all analyzed samples, low pH values, ranging from 4.81 to 5.26, were in agreement with those obtained in other studies [17, 34, 35], more much acidic values were reported by other authors [14, 36, 37]. For total phenols content, the mean value was around 1.59 g caffeic/l, in agreement with concentrations measured by Bouknana*etal.* [12] and Achak*etal.* [35]. Different concentrations were encountered in many researches [14, 17, 34, 36, 37]. This difference was probably due to the fresh OMW analyzed in our work, while most studies were conducted on OMW collected from storage basins. It could also be justified by variability between olive cultivars and ripening stages [38].

Degree of OMW mineralization was assessed by measuring the electrical conductivity; values observed were comparable to those found by Adhoum and Mounser [34] and Achak *et al.* [35]. The salt content was evaluated by chlorides concentration closely related to salting practices for conservation of olives before the extraction process. Analyzed OMW samples appeared to be rich in chlorides (1.04 g/l) in comparison to lowest contents encountered by Hanafi *et al.* [37] in OMW from Marrakech (southern Morocco). Significant variability among production sites was found for chlorides content and consequently for electrical conductivity, which depends closely on the amount of total dissolved salts, or the total amount of dissolved ions in the water. Values scored for both parameters were slightly lower than those reported in other studies [10, 14, 39]. In addition, farmers in BniFrassen have the habit to use large amounts of salts in order to conserve olives before crushing, because of the limited availability of extraction units as compared to the other sites of our study.

Regarding total phenols content, OMW samples collected from C3 were the richest in total phenols, while those from C2 had the lowest content. These findings are in agreement with those obtained by Bouknana *et al.* [12].Total phenols differences could be explained by the amount of added water and phenols solubility. C3 process requires addition of large amount of warm water, which increases the losses of phenols by solubilization. These differences could be attributed to other variables involved in the process of extraction such as the olive crushing and malaxation machinery, temperature applied and the duration of contact with water [40-42]. Another important difference between extraction systems was the pH values. C2 gave the less acidic wastewaters (pH=5.38). This might be due to less content of total phenols characterizing this system in our case. In fact, Hanafi *et al.* [37] attributed the high acidity of OMW to their richness in organic acids (phenolic acids, fatty acids, etc.).

Total phenols content was the only dependent parameter on the growing season. In fact, the prevailing climatic conditions in each season could explain this difference. Therefore, 2014-2015 season was marked by a reduced amount of rainfall compared to the previous season. This water shortage might be the cause of variability in phenols content. Water shortage tends to generate a stress situation in the olive tree that induces phenol production in the olive fruit [43]. In the same context, Tura *et al.*[44] reported that, at similar olive ripening stage, polyphenols were higher in the years with the highest heat summation.

3.2. Antibacterial activity of polyphenols

The results obtained in our study are in agreement with those reported by other authors [27, 28, 45] who founded that OMW polyphenol extracts were active against all the challenge bacteria.

Differences in inhibitory effect of polyphenols found in our work between Gram-positive and Gram-negative bacteria were also observed in other studies [28, 46, 47]. Nevertheless, GuesmiandBoudabous [48] reported no selective antimicrobial activity against both Gram-positive and Gram-negative bacteria.

These differences could be attributed partially to the great complexity of the double membrane-containing cell envelope in Gram-negative bacteria compared to the single membrane structure of positive ones [49]. In the same trend, Leouifoudi*et al.* [29] explained the different activities against Gram-negative and Gram-positive bacteria by the differences in cell wall composition. Gram-negative bacteria have a lipopolysaccharide component in their outer membrane that makes them more resistant to antibacterial compounds.

Antibacterial activities of polyphenols extracted from OMW were confirmed by several researches [27-29]. In fact, the stronger inhibitory effect of polyphenols extracted from OMW were attributed to their acidic side chain, which makes phenolic acids much less polar and might, therefore, facilitate the transport of these molecules across the cell membrane[28]. Polyphenols effect could also be explained by their interaction with membrane lipids by a neutralization of the membrane's electric potential following penetration of the molecule [28]. Morever, antibacterial activities were related to well-denominated phenolic acid especially caffeic acid, vanillic acid, p-coumaric acid, and 4-hydroxybenzoic acid [50-51], verbascoside [52], and oleuropein and hydroxytyrosol [27].

Conclusions

Physicochemical characterization, by evaluating some parameters of olive oil wastewaters produced in north of Morocco (Taza province), confirmed the polluting load and toxicity of this type of effluent due to their acidity and high content in salts and total phenols. Moreover, differences were observed regarding extraction system, production sites and growing season effects. In addition, our result showed effectiveness antibacterial activities of polyphenol residues extracted from OMW against some pathogenic bacteria. This could make the way for a possible alternative valuation of OMW. In perspective, optimization of phenolic acids compounds extraction needs more study in order to be used and applied as antibiotics or food additives against pathogenic microorganisms.

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