

Research Article

Antioxidant and Antimicrobial Activity of Polyphenols Extracted after Adsorption onto Natural Clay “Ghassoul”

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Natural polyphenols contained in olive mill wastewaters (OMW) have been usually associated with great bioactive properties as “antioxidants”. In this work, we recovered the polyphenols after adsorption onto natural clay “ghassoul” by different solvents: water, ethyl acetate, and methanol (PPW, PPA, and PPM, respectively) to avoid environmental pollution. Also, we tested the antioxidant activity of the extracted polyphenols by two methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC). Then, we analyzed antimicrobial activity by the microdilution technique to determine at the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The OMW of the Fez-Meknes region has a very acidic pH, considerable amounts of mineral matter, and a high concentration of polyphenols and organic content. The results of the test from DPPH showed good antiradical potential for polyphenols extracted with water, but the TAC showed an important capacity for all extracts unless PPA. The antibacterial activity is not the same on the four bacteria studied (*Escherichia coli*, *Salmonella sp*, *Staphylococcus aureus*, and *Enterococcus faecalis*), and all extracts inhibit most tested germs that do not have the same MIC and the same sensitivity. Only the PPW showed the minimum bactericidal concentration (MBC) that is equal to 0.290 mg/mL for *Salmonella sp* and *Staphylococcus aureus*, which confirms that the extraction by water of the adsorbed polyphenols is an original solution to recover the polyphenols and also to obtain a natural phenolic antioxidant which can be used in the pharmaceutical, nourishment, and cosmetic industry.

1. Introduction

The olive oil production in the Fez-Meknes region of Morocco generates considerable volumes of olive mill wastewaters (OMW) that are directly discharged in soils without any treatment [1–3]. This has become a big environmental problem in the cities of this region. However, the residue of

olive oil contains a rich source of polyphenols 100 times more concentrated than in olive oil [4, 5]. OMW is a gently acidic liquid of high conductivity, particularly rich in organic matter, such as fatty acids [3] and polyphenols. Those constituents give OMW a brownish-black color. Furthermore, the high pollution of this effluent is generally attributed to its excessive phenolic content, toxic for the flora

and fauna [6]. The composition of OMW varies qualitatively and quantitatively with the olive variety, climate conditions, cultivation practices, storage time, and olive oil production method [7–11]. Polyphenols play an important role in preventing chronic human diseases such as cardiovascular diseases and inflammatory diseases [12, 13] and also can be used as natural antioxidants in food and pharmaceutical industries [12, 14].

In this way, numerous techniques have been used to recover polyphenols from olive-derived products, which includes enzymatic treatment [15], solvent extraction [4, 16–18], membrane separation [19–21], and centrifugation and chromatographic procedures [22]. Solvent extraction is the most common technique employed to extract polyphenols [1]. The objective of this work is to extract polyphenols previously adsorbed onto natural clay (ghassoul) and to evaluate their biological effectiveness, by studying their antioxidant and antibacterial effects. The study of the antioxidant activity is realized by two methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) and total antioxidant capacity (TAC). The antibacterial activity of the extracts is evaluated by determining their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) on Gram-positive and Gram-negative bacteria.

2. Material and Method

2.1. Olive Mill Wastewater Samples. OMW samples of the Fez-Meknes region (Morocco) were obtained from two phases during the period of November 2018 to February 2019.

All samples were stored in the vials protected from light and conserved at 4°C.

2.2. Material. Ghassoul is a natural clay abundant in Morocco (Fez-Meknes region). The material was previously characterized by different techniques (X-ray diffraction, FTIR spectroscopy, SEM/EDX, DTA/TGA, and BET) [23], and it was sieved using a sieve. The fraction below 63 µm was retained for the experiments and denoted Gh-B.

2.3. Extraction of Polyphenols after Adsorption onto Natural Clay “Ghassoul”. Adsorption tests were done in the non-transparent vials to avoid the degradation of polyphenols. 50 mg of Gh-B was immersed in 50 mL of OMW diluted in water ($C_0 = 30$ mg/L). After 3 hours of agitation, the solution is separated by centrifugation. This protocol is repeated 4 times to recover the maximum amount of polyphenols. The pellet containing the polyphenols adsorbed onto Gh-B was extracted with methanol (PPM), water (PPW), and ethyl acetate (PPA). The mixture was stirred for 30 min and then kept in the dark for two hours to avoid auto-oxidation and subsequent polymerization of the phenolic compounds. The mixture was centrifuged at 8000 rpm for 20 min. The supernatant of all extracts (PPM, PPW, and PPA) was evaporated. After that, the polyphenols concentrations were determined. Then, the antibacterial and antioxidant tests were performed.

2.4. Determination of Polyphenols Concentration (PPC). The polyphenol concentration of all the extracts after its recovery onto Gh-B was determined by the Folin–Ciocalteu spectrophotometric method [24]. 0.4 mL of each extract was introduced into test tubes, 2 mL of Folin reagent was diluted 10 times, and 1.6 mL of 7.5% of sodium carbonate was added into tubes. After, the mixture was stirred and incubated for two hours. The absorbance was measured at 760 nm using a UV-visible spectrometer (Shimadzu).

The PPC was calculated using the linear equation from calibration curve (1):

$$\begin{aligned} A &= 4.8153X; \\ R^2 &= 0.99, \end{aligned} \quad (1)$$

where A is the absorbance and X is the concentration of polyphenols (PPC) in g/L.

2.5. Antioxidant Tests

2.5.1. Antioxidant Activity Measurement by DPPH Method. The free radical of 1,1-diphenyl-2-picrylhydrazyl “DPPH” (Figure 1) scavenging capability of each extract solution was determined as described previously by Brand-Williams et al. [25]. The samples prepared at different concentrations of polyphenols (0.890 g/L, 0.810 g/L, and 0.240 g/L) were mixed with 1.2 mL of 0.020% DPPH and 200 µL of ethanol solution. The mixture was incubated for 30 min in the dark at ambient temperature, and the absorbance was measured at 517 nm according to Shariffar et al. [26] using a UV-visible spectrometer (Shimadzu), with ascorbic acid (AA) as a positive control.

The percentages of inhibition (I%) were determined using the following equation:

$$\% \text{ scavenging} = \frac{A_0 - A_s}{A_0}, \quad (2)$$

where A_0 is the absorbance of the control and A_s is the absorbance of the sample at 517 nm.

The 50% inhibitory concentration of the DPPH (IC_{50}) activity of each extract was determined graphically by inhibition percentages as a function of different concentrations of the extracts [27].

2.5.2. Determination of Total Antioxidant Capacity (TAC). The total antioxidant capacity test (TAC) of all extracts was determined by the phosphomolybdenum method as described by Prieto et al. [28]. Each sample (0.6 mL) is blended with 6 mL reagent solution (sodium phosphate 28 mM), sulfuric acid (0.6 M), and ammonium molybdate (4 mM). The tubes were incubated for 90 min at 95°C. After cooling, the absorbance was measured at 695 nm and the same way for the control sample (6 mL reagent solution was mixed with 0.6 mL of methanol) which was incubated within the same conditions as the samples. The calculation of TAC was performed from the linear equation for calibration curve (3) and presented in terms of ascorbic acid equivalents (AA) in µg/mg of crude extract:

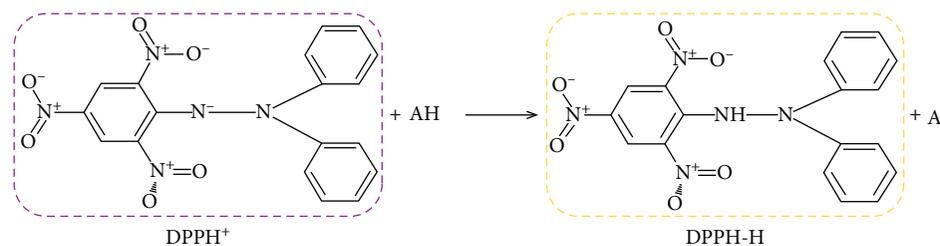


FIGURE 1: Reaction mechanism involved in the DPPH test between the free radical species DPPH^{\bullet} and an antioxidant (AH).

TABLE 1: Concentration providing 50% DPPH radical scavenging (IC_{50}) and TAC of polyphenol extracts after adsorption onto ghashoul.

	PPW	PPM	PPA	AA
Concentration of polyphenols (g/L)	0.89 ± 0.00	0.81 ± 0.00	0.24 ± 0.00	—
IC_{50} ($\mu\text{g}/\text{mL}$)	55.01 ± 0.00	47.00 ± 0.66	62.87 ± 1.11	44.00 ± 0.00
TAC ($\mu\text{g}/\text{mg}$)	75.00 ± 0.44	83.00 ± 0.44	33.00 ± 0.66	—

$$\begin{aligned}
 A &= 0.027B + 0.0117; \\
 R^2 &= 0.99,
 \end{aligned}
 \tag{3}$$

where A is the absorbance and B is expressed in $\mu\text{g}/\text{mg}$.

2.5.3. Antibacterial Activity. The antibacterial activity of extracts was tested against Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella sp.*). The determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was performed in 96-well flat-bottom microplates [29,30]. Briefly, a decreasing concentration of extracts (PPM, PPW, and PPA) was prepared in sterilized distilled water, and then $50 \mu\text{L}$ of Mueller Hinton broth and $50 \mu\text{L}$ of a bacterial suspension at 10^6 cfu/mL were added to each well [31]. A well containing only bacterial suspension with Mueller Hinton broth was served as a positive control. However, a well containing sterile water and extract was served as a negative control. After microplate incubation at 37°C for 24 h, $40 \mu\text{L}$ of TTC (2, 3, 5-triphenyl tetrazolium chloride) was added to each well and reincubated at 37°C for 30 min. MIC was determined at the lowest concentration at which the bacterial growth was not observed [32, 33].

The MBC was confirmed by plating $2 \mu\text{L}$ of samples from the wells in which no growth was observed on the Mueller Hinton agar medium. The plates were incubated at 37°C for 24 h. The lowest concentration that did not produce any bacterial colony was taken as MBC [34].

This experiment was repeated three times for each concentration, and the MBC/MIC ratio allows us to determine the bactericidal efficacy of the extract studied. If the MBC/MIC ratio is below 4, the effect is bactericidal, and if MBC/MIC is greater than 4, the effect is bacteriostatic [35].

3. Results and Discussion

3.1. Determination of the Free Radical Scavenging Activity (DPPH) and Total Antioxidant Capacity (TAC). Table 1 shows that the PPW and PPM extracts present a very remarkable IC_{50} , which is equal to $55.01 \mu\text{g}/\text{mL}$ and $47.00 \mu\text{g}/$

mL , respectively, and these values are very near to IC_{50} of the standard ascorbic acid ($44.00 \mu\text{g}/\text{mL}$), followed by the PPA. According to several studies [12,36], we found that the antioxidant capacity of different polyphenols extracts was directly correlated to the percentage of free hydroxytyrosol which belongs to the family of polyphenols and that their antioxidant activity depends on the type of phenolic compounds and their concentration. Hydroxytyrosol is found in the form of its ester of oleanolic acid: oleuropein or in the form of hydroxytyrosol acetate capable of trapping free radicals easily.

Therefore, the extraction process of polyphenols after adsorption with water is very important than that of methanol. This is an original, easy method, which is efficient to recuperate polyphenols, reduce pollution, and probably the polyphenols are used as natural antioxidants in various pharmacologic applications. The IC_{50} of PPW and PPM extracts after adsorption are better compared with the works of other authors. Leouifoudi et al. [2] have obtained the values $30.70 \mu\text{g}/\text{mL}$ and $11.70 \mu\text{g}/\text{mL}$ of IC_{50} for the polyphenols extracted from olive mill wastewater from plain area and mountainous area, respectively. These values are higher than the standard (IC_{50} acid ascorbic = $3.20 \mu\text{g}/\text{mL}$) [2]. Also, Belaqqiz et al. used different milling techniques and found the following values 15.83, 32.32, 173.00, 126.30, and $261.30 \mu\text{g}/\text{mL}$ of the flavonoid family, polyphenols for table olive wastewater, green olive brine (GTOW), black olive brine (BTOW), and purple olive brine (PTOW), respectively [36].

The aqueous extracts of PPW and PPM present the values of TAC very near compared with the PPA. These values of TAC are very important compared to the value (near 0.12) obtained in the study of the phenolic profile and antioxidant activities of olive mill wastewater [12].

However, the TAC of PPA is slightly lower than that of PPW and very important compared to PPM. The extraction of polyphenols by water after adsorption is an original, efficient method and respectful for the environment, compared to the extraction of polyphenols from olive mill wastewater with chemical treatment such as methanol and ethyl acetate. Therefore, the PPW shows good antiradical potential, which is able to extend the shelf life of food and

TABLE 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the studied extracts.

Bacteria	Gram	PPM			PPA			PPW		
		MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>Escherichia coli</i>	-	0.270 ± 0.006	—	0.125 ± 0.000	0.125 ± 0.000	—	—	0.018 ± 0.006	-	-
<i>Salmonella</i> sp	-	0.070 ± 0.000	—	0.015 ± 0.000	0.125 ± 0.000	8.333	8.333	0.075 ± 0.000	0.290 ± 0.000	3.866
<i>Staphylococcus aureus</i>	+	0.070 ± 0.000	0.270 ± 0.000	0.062 ± 0.004	3.857	—	—	0.075 ± 0.004	0.290 ± 0.004	3.866
<i>Enterococcus faecalis</i>	+	0.017 ± 0.004	—	0.125 ± 0.000	—	—	—	0.300 ± 0.000	-	-

Note. "—" indicates no effect.

pharmaceutical products by decreasing the oxidation rate of the products.

3.2. *Determination of the Minimum Inhibitory Concentration (MIC) of Pathogenic Strains and the Minimum Bactericidal Concentration (MBC)*. The test of antibacterial activity was repeated 3 times for the determination of MIC and MBC which is expressed by mg/mL (Table 2).

The results presented in Table 2 show that all polyphenols extracts inhibit the tested bacteria with a different degree. *Escherichia coli* is more sensitive to PPW (0.018 mg/mL) than PPA (0.125 mg/mL) and PPM (0.270 mg/mL), while *Enterococcus faecalis* is more sensitive to PPM (0.017 mg/mL) than PPA (0.125 mg/mL) and PPW (0.300 mg/mL). However, the extracts present a great activity against *Salmonella sp* and *Staphylococcus aureus* (MIC between 0.015 and 0.075 mg/mL).

In this study, the MBC/MIC ratios of PPM and PPW extracts are less than 4 for the two microbial strains *Staphylococcus aureus* and *Salmonella sp*. These two extracts seem to have a bactericidal action against these two strains; on the other hand, the PPA has a bacteriostatic effect against *Salmonella sp*.

The different extracts show that water extract has a high level of antibacterial activity mostly for the value of MBC which is equal to 0.290 mg/mL. This explains that the PPW contains flavonoids in particular quercetin [37] and luteolin [38] that could be considered as antibacterial compounds against *Staphylococcus aureus* and *Salmonella sp* bacteria.

4. Conclusion

In the context of discovering new antioxidants from natural sources, this work represents the extraction of polyphenols from olive mill wastewater after adsorption onto natural clay “ghassoul” by different solvents such as water, methanol, and ethyl acetate.

The antioxidant activity tests were evaluated using two different tests: 1,1-diphenyl-2-picrylhydrazyl (DPPH), total antioxidant capacity (TAC), and the antibacterial activity test was determined by the microdilution technique. The results showed that the aqueous extracts of PPW and PPM had the highest antioxidant activity with the IC₅₀ of 55.01 µg/mL and 47.00 µg/mL, respectively, which are near to ascorbic acid standard (44.00 µg/mL) following the PPA which is equal to 62.87 µg/mL, unlike the TAC values which are close to each other for all extracts.

For antibacterial activity, all polyphenol extracts inhibit the tested bacteria with a different degree. The exception of the PPW is a high MBC that is equal to 0.290 mg/mL following PPA (0.270 mg/mL) and PPM (0.125 mg/mL) for *Salmonella sp* and *Staphylococcus aureus*. The values of the MBC/MIC ratio show that the PPM and PPW extracts had bacteriostatic activity contrariwise PPA.

Furthermore, the extracts of polyphenols by water were effective in inhibiting bacterial growth. Indeed, it is an original, economical, and environmentally friendly method.

It allows decontamination of the material “ghassoul” after adsorption and is a most promising antioxidant source which can contribute to further potential biological applications in the biomedical domains, especially as natural anticancer agents.

Data Availability

The authors confirm that all data underlying the findings of this study are fully available without restriction.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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