Antioxidant Properties and Protective Effect of Olive Mill Wastewater Against Lipid Peroxidantion

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Abstract

Olive mill wastewater generated from olive oil industry can indeed be a significant environmental concern due to its pollution potential. It contains various pollutants, including organic matter, phenolic compounds, which can be harmful to the environment if not properly managed. Despite its pollution olive mill wastewater is a rich source of natural antioxidant (polyphenols). The objective of this study is to evaluate the antioxidant activity and the protective effect of the phenolic extracts of olive mill wastewater against lipid peroxidation. The antioxidant activity of the phenolic extracts of olive mill wastewater (OMWW) was evaluated using three tests, DPPH radical scavenging test, ferric reducing antioxidant power (FRAP) assay and the neutralization of hydrogen peroxide. The protective effect of the tested extracts against lipid peroxidation was evaluated in an *in vitro* model of rat liver homogenates through the production of thiobarbituric acid reactive substances (TBARS). The results showed that the phenolic extracts of olive mill wastewater are rich in phenolic compounds mainly flavonoids, with the highest content found in Chamlal OMWW (43.53±0.56 mg GAE/g of dried extract for polyphenols and 14.12± 0.014 mg QE/g of dried extract for flavonoids). Moreover, the studied extracts have demonstrated an important anti-free radical activity and reducing power. Indeed, these extracts are also capable to neutralize hydrogen peroxide (H_2O_2). Chamlal OMWW showed the highest antioxidant activity. The studied extracts exhibited a protective effect against lipid peroxidation and decrease malonic dialdehyde (MDA) level. Chamlal OMWW showing the highest protective effect. In the light of the obtained results, olive mill wastewater has an important antioxidant potential and it can be used as natural agents in food and pharmaceutical industries further its negative environmental impact can be reduced.

Keywords: Olive mill wastewater, Phenolic compounds, Lipid peroxidation, Antioxidant properties, Protective effect.

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1. Introduction

Olive oil production is a significant industry in Mediterranean countries. It generates olive mill wastewater (OMWW) as byproduct of this process. OMWW is a complex and variable mixture, it is primarily composed of water (83-94%), organic compounds (4-16%) including polyphenols and 0.4-2.5% mineral salts. The chemical composition of OMWW can vary depending on factors such as the olive variety, ripeness, extraction method, and processing conditions [1] [2]. Uncontrolled disposal of OMWW can indeed create significant environmental problems. Olive mill wastewater contains various organic compounds, polyphenols and other substances which can be highly polluting. It can contaminate soil and water sources, leading to damage to ecosystems and harm to aquatic life. The phenolic compounds in OMWW are toxic to some microorganisms which can disrupt local ecosystems by reducing biodiversity and affecting the microbial balance in the soil and water. Despite their polluting power, OMWW is a rich source of natural phenolic compounds that can be extracted and used in food, pharmaceutical, and cosmetic industries [3]. These compounds have been the subject of several scientific researchers due to their potential functional proprieties such as antioxidant, antimicrobial and other beneficial effects [4] [5]. In recent times, researchers have been focusing on the treatment and the exploitation of OMWW, on the one hand to limit their pollution and minimize their environmental impact, and on the other hand to use their polyphenols in food production, cosmetic industry and pharmacology. To this end the objective of the present study is to evaluate the antioxidant activity and the protective effect of olive mill wastewater against lipid peroxidation. In the aim to investigate the possible use of OMWW as a natural antioxidant to fight disorders caused by oxidative stress and preventing lipid peroxidation.

Materials And Methods

Biological Material

Olive mill wastewaters used in this study were obtained from modern oil mills located in two different regions of Algeria: Sig Region (35° 32’ 00” north, 0° 11’ 00” west. Altitude, 56 m) producing Sigoise variety and region of Tizi Ouzou (Latitude: 36°42’42” North Longitude: 4°02’45” Est) processing Chamlal variety.

Extraction of phenolic compounds from olive mille wastewater

Delipidation of olive mill wastewater: Twenty ml of olive mill wastewater (OMWW) are centrifuged for 10 min at 3200 g. Two phases are obtained: an aqueous phase and a precipitated pellet. Ten ml of the aqueous part are added to 20 ml of hexane. The solution is mixed using a vortex for 3 min at a speed of 13,500 rpm. After 15 min, the OMWW is collected after complete separation into two phases: hexane (supernatant) and the delipidated OMWW (pellet) ready for liquid-liquid extraction.

Extraction of phenolic compounds: A test portion of 100 ml of delipidated OMWW is mixed with 100 ml of solvent (ethanol 75%)). The mixture is then stirred for 4 hours followed by evaporation to dryness of the solvent at 60°C. The obtained dry extract was stored at -20°C [6].

Determination of Total Phenolic Content (TPC)

The level of total phenolic content of OMWW extracts was determined using Folin-Ciocalteu reagent based on a method described by Singleton et al [7]. 500 µl of each extract at increasing concentrations ranging from 0.125 to 4 mg/ml was mixed with 500 µl of Folin-ciocalteau reagent (1/10 with distilled water). After 5min of incubation in the dark, 1.5 ml of 7.5% Na_2CO_3 was added. The resulting mixture was again incubated for 30min in the dark at room temperature. The absorbance was measured at a 760 nm wavelength. A standard curve of gallic acid was drawn with a concentration range of 3.125×10^{-3} to 5×10^{-2} mg/ml. The content of the total phenolics was expressed as mg of gallic acid equivalents per gram of dried extract (mg GAE/g). All determinations were carried out in triplicate.

Total Flavonoid Content (TFC)

The flavonoid content of the tested extracts was measured using a colorimetric method, which based on the formation of a complex between the aluminum ion and the carbonyl and hydroxyl groups of flavonoids that produce a yellow color [8]. One milliliter (1 ml) of studied extracts solutions (0.5 mg/ml to 8 mg/ml) was mixed with 1 ml of a 2% aluminum chloride solution. Following incubation for 30 min, the absorbance of the reaction mixture was measured at 430 nm against a distilled water blank. A standard curve of quercetin was drawn with a concentration range of 3.0×10^{-4} to 4.0×10^{-3} mg/ml, and the results were expressed as mg quercetin equivalents per gram of dried extract (mg QE/100g).

Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power of the studied extracts was determined by the method of Yen & Duh [9] with slight modifications. 2.5 ml of the tested extract solutions at various concentrations (0.125 mg/ml to 4 mg/ml) were mixed with 2.5 ml of potassium ferricyanide (1%) and phosphate buffer (2.5 ml, 0.2 M, pH 6.6). The mixtures were incubated for 20 min at 50 °C. After incubation, 2.5 ml of trichloroacetic acid (10%) was added to the mixtures, followed by centrifugation at 3000 rpm for 10 min. 1 ml of the upper layer was mixed with 1 ml of distilled water and 0.5 ml of ferric chloride (0.1%). Vitamin C and gallic acid were used as reference standards. The increase in absorbance provided an indication of higher reducing power of the samples being analyzed. The reducing potential of the studied extracts and antioxidant standards (gallic acid and vitamin C) is expressed by the values of the effective concentrations 50% (EC_{50}) that correspond to the concentration of sample needed to give an absorbance equal to 0.5 at 700 nm. The lowest EC_{50} corresponds to the most important antioxidant activity.

Free radical scavenging activity (DPPH test)

The antioxidant scavenging activity was studied using 1,1-diphenyl- 2-picrylhydrazyl free radical (DPPH) as described by Tien et al. [10] with some modifications; 1.5 ml of various solution of the tested extracts at different concentrations (0.125 mg/ml to 1 mg/ml) were mixed with 1.5 ml of a 0.2 mM ethanolic DPPH solution. After incubation period of 30 min at 25 °C, the absorbance at 517 nm, the wavelength of maximum absorbance of DPPH, were recorded as a (sample). A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as A (blank). The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\%\ \text{inhibition} = 100 \times \frac{\text{A (blank)} - \text{A (sample)}}{\text{A (blank)}}.$$

The antioxidant activity of the tested extracts was expressed as IC_{50} defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Vitamin C and gallic acid were used as a standard.

Test of hydrogen peroxide scavenging capacity

The ability of the olive mill wastewater phenolic extracts to scavenge hydrogen peroxide was determined according to the method of Kumar et al. [11]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (0.1 M, pH7.4). 2 ml of solution of each extract at different concentrations (0.125 mg/ml to 2 mg/ml) in ethanol (70%) was added to a hydrogen peroxide solution (1.2 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of phenolic extracts and standard antioxidants were calculated according to the following formula:

$$\%\ \text{Scavenged}\ [H_2O_2] = 100 \times \frac{AC - AS}{AC}$$

Where:

AC: the absorbance of the control

AS: the absorbance in the presence of the phenolic extracts or standards

Evaluation of protective effect of olive mill wastewater extracts against lipid peroxidation
Animals

To evaluate the protective effect of the tested extracts against lipid peroxidation, six healthy male Wistar rats (252 ± 1.71 g) aged 3 months were used. The animals were obtained from the Pasteur Institute of Algiers (Algeria) and kept in individual polystyrene cages under animal house conditions (temperature 22 ± 1 °C, 12/12 h light-dark cycle, and relative humidity 60 ± 10%) for

two weeks at the Veterinary Sciences Institute of Ibn khaldoun University, Tiaret. Animals were sacrificed and specific organs (liver) were obtained for further analysis. The authors are members of the Algerian Association of Sciences in Animal Experimentation (AASEA) (Agreement Number: 45/DGLPAG/DVA.SDA.14).

Preparation of liver homogenate

The liver was isolated from 6 normal albino Wistar rats. The organs were weighed and 10% (w/v) homogenate was prepared in phosphate buffer (0.1 M, pH 7.4 having 0.15 M KCl) using the homogenizer at 4°C. The homogenate was centrifuged at 3000 rpm for 15 min and the clear cell free supernatant obtained was used for the study [12].

In vitro evaluation of lipid peroxidation assay

The protective effect of the tested extract against lipid peroxidation was evaluated according to the method described by Gupta and Sharma [13] for this 580 µL of Phosphate buffer (0.1 M; PH 7.4) was mixed with 200 µl of extract or standard and 200 µl liver homogenate then 20 µl of ferric chloride (100 mM, H_2O_2 0.50 % prepared in phosphate buffer 0.1 M, pH 7.4) were added to the mixture that was placed in a shaking water bath for 1 h at 37°C. The measurement of malonic dialdehyde (MDA) content was determined according to the method described by Yagi [14] a volume of 800 µl of TBA (0.375% w/v) was added to 200 µl of the previously prepared solution. After shaking for 2 min, the mixture was incubated in a water bath at 100°C for 10 min. During this step, the aldehyde functions of MDA were released by acid hydrolysis at 100°C. They react with TBA forming a pink colored complex (MDA-TBA). To stop the reaction, the tubes were placed in ice the complex thus formed is extracted with 2 ml of 1-butanol for 2 minutes. After centrifugation at 4000 rpm for 10 minutes at 4°C, the supernatant was collected and the absorbance of the pink chromogen obtained was measured at 532 nm. The tissue concentration of malondialdehyde (MDA) was calculated using a linear PET curve (y = 0.0151x - 0.0457).

The percentage of MDA inhibition was determined according to the following formula:

$$\text{MDA (\%)} = \frac{C0 - C1}{C0} \times 100$$

Where:

C0: MDA concentration without protection

C1: MDA concentration with protection

Statistical analysis

All assays were performed in triplicate and the results represent the means ± standard deviation. The data analysis was performed using the Statistica StatSoft software (version 6.1, Statsoft, Tulsa,

UK). The one-factor ANOVA was used to compare the means, followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$.

2. Result and discussion

Extraction yield, total phenolic and flavonoid content

The extraction yield, total phenolic and flavonoid content of the tested extracts are illustrated in the following table:

Table 1: Extraction yield, total phenolic and flavonoid content of the studied extract.

Extract	Extraction yield (%)	Phenolic content (mg GAE/g dried extract)	Flavonoid content (mg QE/g dried extract)
Chamlal OMWW	3.08±0.91a	43.53±0.56a	14.12±0.014a
Sigoise OMWW	4.53±0.16b	26.32±0.12b	5.47±0.12b

The values followed with different letters indicate significant differences by ANOVA post hoc LSD Tukey test $p < 0.05$.

Extraction yield

Our finding reported that Sigoise OMWW had the highest extraction yield (4.53±0.16%) compared to Chamlal OMWW (3.08±0.91%). The yield of polyphenol extraction from olive mill wastewater can vary depending on several factors, including the extraction method used, the quality and the variety of olives, solvent type, extraction time, temperature, and solvent-to-sample ratio. The extraction efficiency can differ based on the solubility and stability of phenolic compounds.

Total polyphenol and flavonoid content

The obtained results indicated that the phenolic extract of Chamlal OMWW had exhibited the higher polyphenols (43.53±0.56 mg GAE/g dried extract) and flavonoids (14.12±0.014 mg QE/g of dried extract) contents compared to Sigoise OMWW which has a phenolic content in the order of 26.32±0.12 mg GAE/g dried extract and flavonoids content estimated at 5.47±0.12 mg QE/g of dried extract. The variation in the

concentration of phenolic compounds in olive mill wastewater depends on climate cultivation area, soil composition, the time of collection. In addition to these variability factors, there are the effects of storage conditions prior to extraction, and the processing techniques [15] [16].

Antioxidant activity of olive products

Ferric Reducing Antioxidant Power (FRAP test)

The results of the ferric reducing antioxidant power (FRAP) test of the studied extracts and the standards antioxidant (gallic acid vitamin C) are presented in Figure 1.

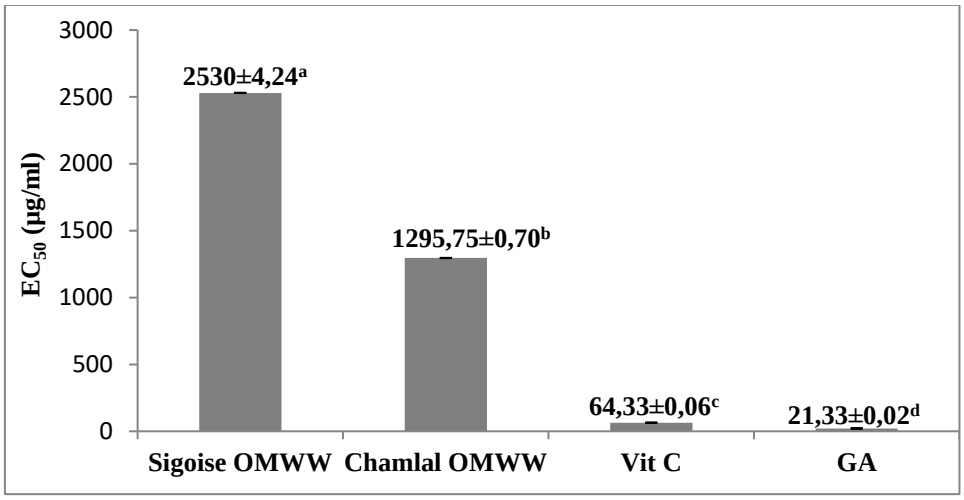

Figure 1. Reducing power of standards antioxidant and tested extracts (the values followed with different letters indicate significant differences by ANOVA post hoc LSD Tukey test p < 0.05).

The result of the antioxidant activity of the phenolic extracts of OMWW evaluated by the ferric reducing antioxidant power (FRAP) assay reported that Chamlal OMWW and Sigoise OMWW have an important reducing powers with EC_{50} values in the order of 1295.75±0.70 µg /ml and 2530±4.24µg /ml respectively. These reducing powers are much lower than those of gallic acid (EC_{50} = 21.33±0.02µg/ml) and vitamin C (EC_{50}= 64.33±0.06 µg/ml). The reducing power of Chamlal OMWW was significantly higher than that of Sigoise variety, maybe due to Chamlal 's higher phenolic content. The classification of the reducing power in the decreasing order of strength is as follows: Gallic acid > Vitamin C > Chamlal OMWW> Sigoise OMWW. We found a positive correlation between the total polyphenol content and the reducing power for the two studied extracts with a high coefficient of correlation (r) for both Chamlal (r = 0.994) and Sigoise (r =0.996) olive mill wastewater. The reducing power of olive mill wastewater refers to its ability to reduce certain chemical compounds, often including ferric (Fe^{3+}) ions to their reduced form typically ferrous (Fe^{2+}) ions. This is a measure of the antioxidant capacity of the OMWW indicating its ability to counteract oxidative stress by donating electrons to unstable molecules. The reducing power of olive mill wastewater is primarily attributed to its high polyphenol content. Polyphenols are known for their antioxidant properties, and they can help protect cells and tissues from damage caused by oxidative stress. The exact reducing power of olive mill wastewater can vary depending on factors such as the olive variety, processing methods, and storage conditions.

Free radical scavenging activity (DPPH test)

The findings of the DPPH free radical scavenging activity of the tested extracts and standard antioxidants are summarized in Figure 2.

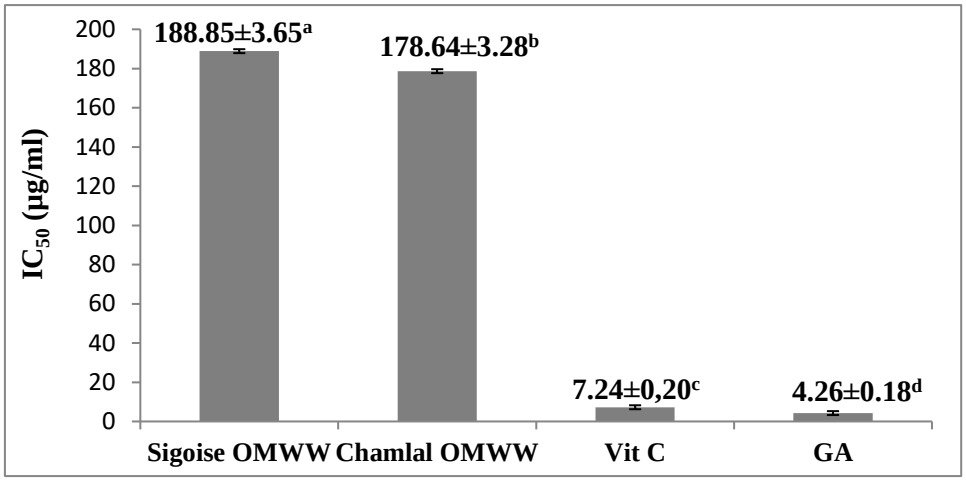

Figure 2. Results of DPPH test of standard antioxidants and tested extracts (The values followed by different letters indicate significant differences by ANOVA post hoc LSD Tukey testp < 0.05).

According to the obtained results it was found that the studied extracts possess a strong antiradical activity against DPPH free radical. As for the reducing power, it clearly appears that the extract of Chamlal OMWW showed a high antiradical activity (IC_{50}= 178.64±3.28µg/ml) in comparison of the extract of Sigoise OMWW (IC_{50}= 188.85±3.65 µg/ml). The antiradical activity of the tested extracts is much lower than that of the standard antioxidants vitamin C and gallic acid, which have a significant antiradical activity with IC_{50} values of 7.24± 0.20 µg/ml and 4.26±0.18 µg/ml, respectively. Therefore the antiradical activity was classified in the following descending order: Gallic acid > Vitamin C > Chamlal OMWW > Sigoise OMWW. A strong correlation was noted between the phenolic content and the DPPH radical scavenging activity with high coefficients of correlation (r) (r=0.954 for Chamlal OMWW and r=0.94 for Sigoise OMWW). The antiradical activity of the phenolic extract of olive mill wastewater has showed by several studies. El Moudden et al. [17] reported that the phenolic extract of the Moroccan OMWW exhibited an important antiradical activity, evaluated by the DPPH and ABTS essay. This activity is higher than that of the standard Trolox. El yamani et al. [18] showed that the phenolic extract of their studied OMWW had an antiradical activity against the DPPH free radical. Pérez-Bonilla et al. [19] have showed that hydroxytyrosol, oleuropein, and tyrosol, three predominant polyphenols in OMWW have a scavenging activity against the DPPH• free radical.

Hydrogen Peroxide scavenging capacity

The hydrogen peroxide scavenging capacity of the tested extracts and the standards antioxidant are represented in the following Figure.

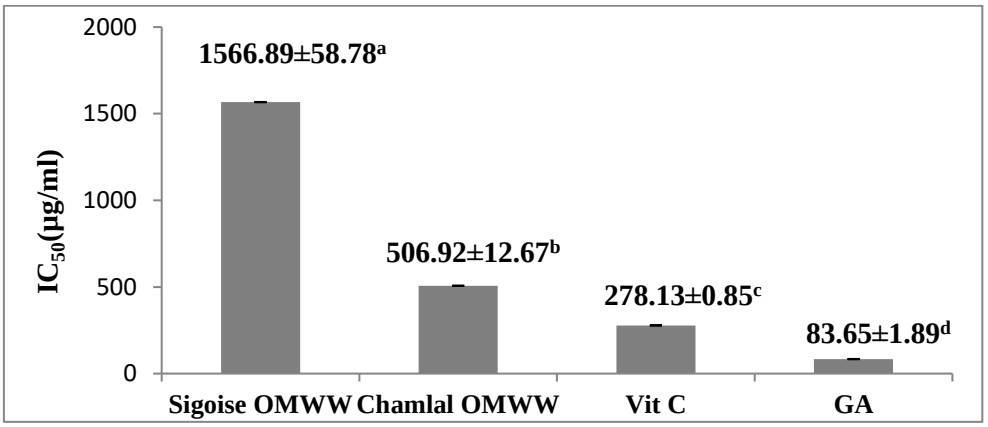

Figure 3. Hydrogen peroxide scavenging activity (%) of standards and extracts(The values followed with different letters indicate significant differences by ANOVA post hoc LSD Tukey test $p < 0.05$).

It's important to note that human body produces and uses H_2O_2 as a signaling molecule for various physiological processes. However, excessive exposure to H_2O_2 can have harmful effects on human cells if it present in high concentrations can generate oxidative stress in cells by producing reactive oxygen species (ROS). This can damage cellular components like DNA, proteins and lipids leading to cell dysfunction. High levels of H_2O_2 can cause cell death through apoptosis or necrosis disrupting tissue function. Proper control and regulation of H_2O_2 levels are essential for maintaining cellular health. Our finding reported that the studied phenolic extracts have an important hydrogen peroxide scavenging capacity. Chamlal OMWW showed a better scavenger activity (CI_{50}=102.99±0.88 µg/ml) then that of Sigoise OMWW (IC_{50}=176.26±3.44 µg/ml). The tested extracts showed a lower scavenging capacity than that of standards antioxidant gallic acid and vitamin C which exhibited an important scavenging activity with an IC_{50} of 83.65±1.89 µg/ml and 250.48±0.74 µg/ml respectively. The classification of the scavenging capacity in the decreasing order of strength is as follows:

Gallic acid >Vitamin C > Chamlal OMWW > Sigoise OMWW. The scavenging capacity of OMWW was affected by the phenolic content with high coefficient of correlation (r= 0.996 for Chamlal OMWW and r = 0.999 for Sigoise OMWW). The antioxidant activity of olive mill wastewater may be due to the presence of potential bioactive compounds such as oleuropein, hydroxyterisol and tyrosol, which have an important antioxidant and radical scavenging activities [20]. Hydroxytyrosol is a natural phenolic compound found in olives and olive mill wastewater. It is known for its potent antioxidant properties and protects the body's cells from oxidative damage caused by free radicals. Hydroxytyrosol can neutralize and scavenge free radicals, which are unstable molecules that can damage cells and DNA. It has anti-inflammatory effects, which can reduce oxidative stress and inflammation in the body. Hydroxytyrosol has been associated with cardiovascular health benefits due to its ability to improve blood vessel function and reduce oxidative stress in the cardiovascular system. Some studies suggest that hydroxytyrosol may protect the brain from oxidative damage and potentially reduce the risk of degenerative diseases [21] [22].

The benefits effect of hydroxytyrosol can vary depending on factors such as dosage and individual health. Oleuropein another natural phenolic compound found in olive mill wastewater, it has an antioxidant properties and protect cells from damage caused by free radicals. Oleuropein's antioxidant activity is attributed to its ability to scavenge free radicals and reduce oxidative stress, which may have various health benefits, such as reducing the risk of chronic diseases and promoting overall well being.

Correlation between the antioxidant tests

Our results showed a positive correlation between the antioxidant activity obtained from the DPPH test and those obtained from the FRAP assay for the two tested extracts (r= 0.993 for Chamlal OMWW and r= 0.903 for Sigoise OMWW). This indicated that the antioxidants substances present in OMWW extracts are capable to scavenge free radicals (DPPH.) and reduce oxidants such as ferric ions. Moreover a positive correlation has been found between FRAP assay and H_2O_2 scavenging capacity with high coefficients of correlation (r= 0.999 for Chamlal OMWW and r= 0.976 for Sigoise OMWW). Furthermore the result revealed a strong positive correlation between the DPPH test and H_2O_2 scavenging capacity (r= 0.994 for Chamlal OMWW and r= 0.971 for Sigoise OMWW).

Protective effect of olive mill wastewater extracts against lipid peroxidation

The results of the protective effect of the phenolic extracts of OMWW and standards antioxidants against lipid peroxidation are illustrated in Figure 4.

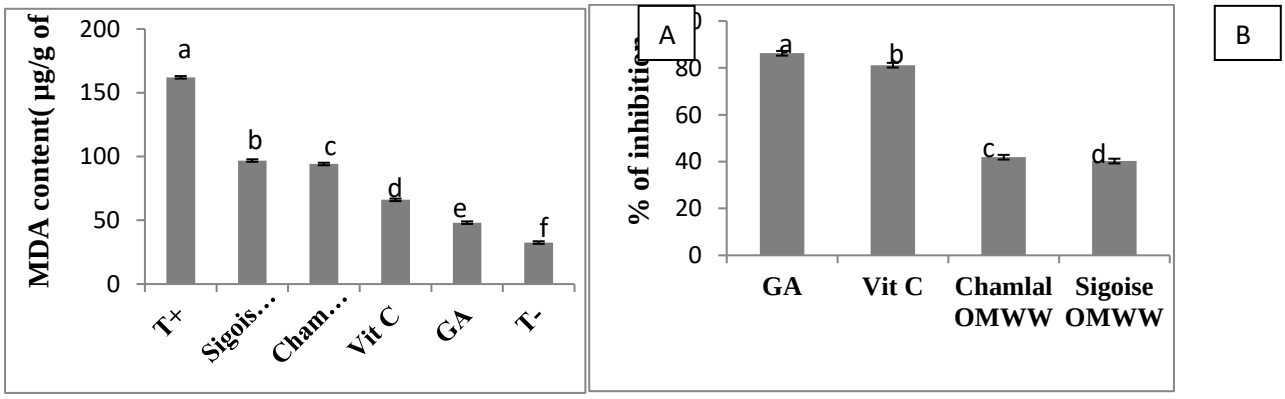

Figure 4: Protective effect of OMWW extracts, gallic acid and vitamin C against lipid peroxidation. expressed in MDA µmol/g of tissue (A) and the percentage of peroxidation inhibition (B). (The values followed with different letters indicate significant differences by ANOVA post hoc LSD Tukey test $p < 0.05$).

The obtained results revealed that the tested extracts have a protective effect against lipid peroxidation confirmed by the significant decrease in MDA level in comparison to the positive control (without protection against induced stress) which has exhibited the higher MDA level (162.05±0.94 µmol/g of tissue). The negative control (without stress) has the lower level of MDA

(32.55±0.21µmol/g of tissue). The extract of Chamlal OMWW was the most efficient against lipid peroxidation with a lowest MDA content (94.17±0.46µmol/g of tissue) and the better inhibitory effect (41.88±0.28%) this is can be attributed to its high phenolic and flavonoid contents compared to the Sigois extract. However, the extract of Sigoise OMWW showed the lowest protective effect with a higher MDA level (96.82± 0.46µmol/g of tissue) and the lowest inhibitory effect (40.24±0.28%). This can be due to its lower phenolic and flavonoid contents. The protective effect of the studied extracts was lower than that of the standards gallic acid and vitamin C which have significantly ($p < 0.05$) decreased the MDA level (48.14±1.87µmol/g of tissue for gallic acid and 66.02±0.93µmol/g of tissue for vitamin C) and demonstrated the highest inhibitory effect of lipid peroxidation (86.26±0.53% for gallic acid C and 81.16±0.26% for vitamin C). Our results are similar to those obtained by El-Abbassi et al. [23] which reported that OMWW has an inhibitory effect against lipid peroxidation. The protective effect of the phenolic extracts of OMWW against lipid peroxidation can be due to polyphenol found in this extracts like oleuropein, hydroxytyrosol, and tyrosol. It was indicated that oleuropein, one of the major phenolic compounds in the extracts of OMWW protects cell membrane from lipid oxidation and therefore prevents heart disease [15]. Manna et al. [24] demonstrated that hydroxytyrosol protected human erythrocytes against lipid peroxidation induced by H_2O_2. Our findings suggest that OMWW can prevent the initiation of lipid peroxidation and protect the human body against oxidative stress.

Conclusion

The results of the present study showed that the phenolic extract of the studied OMWW are rich in natural bioactive compounds which present an important antioxidant power and a high protective effect against lipid peroxidation thereby envisaging their use as potential source of natural antioxidants substances which can be used in food and pharmaceutical industry. Moreover *in vivo* studies are needed to better understand the mechanism of action of phenolic compounds of olive mill wastewater. Furthermore, we recommend isolation, purification and characterization of these compounds to full knowledge the chemical composition of olive mill wastewater.

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