

Investigation and quantification of the potential antioxidant, inflammatory, and antibacterial bioactive molecules of the extracts of Algerian black and green table olive brine

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Received: 9 December 2022; Accepted: 27 December 2022; Published: 12 January 2023

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ORIGINAL ARTICLE

Abstract

The table olive industry produces a large amount of wastewater that can be expensive to be treated and harmful to the environment. This study aimed to find a way to reuse brine water from the production of black and green table olive brines from Bejaia and Mascara of the Sigoise cultivar in order to create a valuable byproduct and contribute to sustainable development. In this context, the high-performance liquid chromatography–diode-array detection (HPLC-DAD) analysis revealed the highest concentration of hydroxytyrosol (4-(2-dihydroxy phenyl ethanol); 69.67 mg/100 mg) for green table olive brines of Mascara (EOGM) and tyrosol (Ty) (28.8 mg/100 mg) for black table olive brines of Bejaia (EOBB). Presence of polyphenols and ortho-diphenols could be responsible for their antioxidant, anti-inflammatory, and antibacterial properties. To assess antioxidant activity, the scavenging effects of DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-Azinobis[3-ethylbenzothiazoline-6-sulfonate]) radicals as well as hydrogen peroxide (H₂O₂) radicals were used. The antimicrobial activity showed that the black olive extract exhibited the best inhibitory effect, with a minimum inhibitory concentration (MIC) ranging from 0.625 mg/mL to 0.31 mg/mL. The anti-inflammatory activity of tested extracts of black olives of Bejaia (EOBB) and green of olives Mascara (EOGM) was 20.06 µg/mL and 20.21 µg/mL, respectively, which demonstrated the anti-inflammatory effect of these extracts on human beings.

Keywords: table olive; polyphenols; ortho-diphenols; HPLC-DAD; hydroxytyrosol; antioxidants; activity

Introduction

The olive tree (*Olea europaea* L.) is a spreading species native to the Mediterranean region. Even though the olive tree's fruits are mostly used to make high-quality olive oil, 11% of them are processed into table olives for immediate consumption. For the Mediterranean people, this food being of great nutritional value serves not only

as a source of calcium during times of scarcity but also as a means of survival. In Algeria, cultivation of olive occupies about 470,000 hectares of area and produces about 700,000 tons of olives (MARDE, 2017). The Sigoise variety olive of Tlemcen in Algeria, or olive of Tel, occupies 25% of the Algerian olive orchard. This variety is used mainly to produce excellent preserved green or black olives, with about 50 kg/tree production.

The fruit of olive tree, a bitter drupe, must be transformed into edible drupe and need to be processed in order to make it a tasty and palatable snack. The bitter oleuropein glucoside can be hydrolyzed to produce brine using a wide range of manufacturing techniques, depending on the variety and degree of ripeness. One way to do this is by breaking down the bitter compound oleuropein glucoside through hydrolysis, which creates brine. Different methods can be used to achieve this, depending on the type of olive and its level of ripeness. (Guan *et al.*, 2022; Huertas-Alonso *et al.*, 2021). Acid and progressive enzymatic or alkaline hydrolysis methods are the most-widespread systems. A fermentation process using yeast or lactic acid bacteria is often created to enhance palatability (Bonatsou and Panagou, 2022; Jardim-Botelho *et al.*, 2022; Yan *et al.*, 2022).

Olive fruit contains many polyphenols of various types, important for human health. Polyphenols regulate the balance of substances in the body that affect oxidative stress, including conditions such as neurodegeneration and cancer (Menezes *et al.*, 2020).

Polyphenols effectively reduce the severity and slow down the progression of various diseases, including cardiovascular, neurodegenerative, and cancerous diseases. Recent research has also identified specific biologically active compounds that are believed to contribute to these effects, such as a polymeric polyphenol derived from tea tree (*Camellia sinensis* L.) effectively blocks the primary protease of SARS-COV-2 (Hong *et al.*, 2022). The manner in which polyphenols work in the body is closely tied to their antioxidant effects. These compounds help to decrease the levels of harmful reactive oxygen species which harm cells. Along with their antioxidant properties, plants' polyphenols also offer other health benefits, such as anti-allergic, anti-atherogenic, anti-thrombotic, and anti-mutagenic properties (Hossain *et al.*, 2022; Liu *et al.*, 2022). Several studies have investigated the impact of polyphenols on the human immune system, including their ability to affect the proliferation and activity of blood cells and their potential to produce cytokines or other substances that support immune defense (Murgia *et al.*, 2021; Xu *et al.*, 2022).

In order to meet the increasing demands of healthier lifestyles and longer life expectancies, the food and pharmaceutical industries must find reliable and beneficial sources for medications and food additives that promote health and wellness (Achmon and Fishman, 2015). Table olive processing waters contain a high content of phenolic compounds, including hydroxytyrosol (4-(2-dihydroxy phenyl ethanol) [HT]; 3,4-dihydroxy phenyl), which is a very powerful natural antioxidant and the second-most abundant plant metabolite in olives (*Olea europaea*). Over the past decade, numerous research studies have

shown the health benefits of antioxidants in general and hydroxytyrosol in particular (Britton *et al.*, 2019). Indeed, hydroxytyrosol has been shown to neutralize free radicals during the oxidation process. It also promotes the production of new mitochondria in tissues, improving their function, and affects the pigment that gives skin its color, helping to lighten dark spots and give skin a brighter appearance. This polyphenol is easily absorbed by the digestive system. It rapidly enters the bloodstream, and works to counteract the harmful effects of accumulated free radicals in human tissues and helps to prevent bone loss.

A recent study has shown that a polyphenol extract called bonolive, derived from olive leaves, can effectively support rebuilding of bone tissue (Ciriminna *et al.*, 2016). On the other hand, the manufacturing processes of table olive brines particularly produce polluting wastewater, which is difficult to treat. Such wastewater is not allowed in municipal sewage systems because of its high organic load and high concentration of phenolic compounds known to have toxic effects on living organisms (Yakhlef *et al.*, 2018).

In general, many extraction techniques are used to extract phytochemicals from medicinal plants, including cold maceration, hot percolation, Soxhlet reflux, ultrasonic extraction, centrifugal extraction, supercritical fluid extraction, microwave-assisted extraction, and liquid-liquid extraction. However, these extraction techniques have their advantages and disadvantages, so selecting the best method for extraction of phytochemicals is important (Gopalasatheeskumar *et al.*, 2022; Jahromi, 2019). Extraction is characterized by its simplicity and low cost. It is based on using solvents of various polarities under different temperatures and pH conditions. A liquid-liquid extraction technique using ethyl acetate as a solvent was used to isolate and purify phenolic compounds from olive brine. Ethyl acetate is known to be the most suitable solvent for recovering phenolic monomers (Jahromi, 2019).

This study intended to determine the chemical composition and dosage of the key substance called hydroxytyrosol. It seeks to use *in vitro* techniques to investigate the antioxidant capabilities of extracts from brine waters of black and green table olive brines of the Sigoise cultivar of Bejaia and Mascara.

Materials and Method

Reagents and Standards

The majority of the chemicals used in this work are analytical in nature. Sigma-Aldrich (St. Louis, MO, USA)

provided the extraction solvent (ethyl acetate) and standards for high-performance liquid chromatography–diode-array detection (HPLC-DAD) and DPPH (2,2-diphenyl-1-picrylhydrazyl) analysis, including gallic acid, α -tocopherol, caffeic acid, ascorbic acid, antioxidant trolox, and ovalbumin. The reagents and culture media used to perform the biological activities were purchased from Merck (Darmstadt, Germany).

Samples and extraction

Brine water for table olives of the Sigoise variety (green and black) was obtained from the manufacturing industrial plants of different regions: Mascara (North-West) and Bejaia (North-East) of Algeria during the 2021–2022 season and stored at -80°C . The extraction of phenolic compounds was performed using ethyl acetate as a solvent (200 mL for 100 mL of sample), according to Barbieri *et al.* (2021)

HPLC–DAD analysis of phenolic compounds

The intracellular phenolic compounds were identified using HPLC and comparing the retention period of sample chromatographic peaks with those of authentic standards, as described by Maalej *et al.* (2022). The concentration of phenolic compounds in the ethyl acetate extracts was determined using an HPLC–DAD (1260-Agilent, Allemagne) system. Concerning the separation of compounds, it was achieved using a C18 column (Eclipse DB, particle size $5\text{ }\mu\text{m}$, $4.6\times 25.0\text{ mm}$) and following two mobile phases: solvent A (0.1% acetic acid in water) and solvent B (100% acetonitrile). Acetonitrile phase under the following gradient conditions: from 0 to 22 min (10% B), and from 22 to 32 min (50% B). Following conditions were used: 22 to 32 min (50% B), from 32 to 40 min (100% B), from 40 to 44 min (100% B), and from 44 to 50 min (10% acetonitrile). The analysis cycle lasted for 50 min. At 40°C , the flow rate and injection volume were 0.5 mL/min and 5 μL , respectively. The *ultraviolet*–visible (UV-VIS) spectra of phenolics were recorded from 190 to 400 nm, and the samples were detected at 254, 280, and 330 nm.

Total Phenolic Content (TPC)

Phenolic compounds were quantified by using the Folin–Ciocalteu method, as described by Rekik *et al.* (2021). Brine extract, 100 μL , was mixed with 250 μL of distilled water and 250 μL of Folin–Ciocalteu reagent. A UV-VIS spectrophotometer at 756 nm was used to take the reading against a blank sample. The results were reported as gallic acid equivalents (mg/g extract).

Ortho-diphenol content

Ortho-diphenol content was identified as described by Gargouri *et al.* (2013). Briefly, 2 mL of sample was added to 0.5 mL of sodium molybdate dihydrate solution (0.5 g L^{-1} in ethanol–water mixture, 1/1, v/v). The mixture was stirred, and the absorbance was measured after 15 min at 370 nm at room temperature. The caffeic acid standard was also formed following the same steps.

Antioxidant activity

The antioxidant potential of brine extracts was determined by the following standardized tests: DPPH, ABTS (2,20-Azinobis[3-ethylbenzothiazoline-6-sulfonate]), and hydrogen peroxide (H_2O_2). The results were expressed as $\mu\text{g}/\text{mg}$ of extract using the following references: ascorbic acid for the DPPH free radical scavenging test, water soluble antioxidant trolox for ABTS, and alpha tocopherol for H_2O_2 scavenging activity (Gargouri *et al.*, 2013).

DPPH radical scavenging activity

A volume of 0.1 mL of each extract was transferred into a test tube. The reaction was carried out for 30 min in the dark (Gargouri *et al.*, 2013). Then, absorbance was measured at 517 nm. An aliquot of 2 mL of DPPH solution (0.4%) in ethanol and 0.1 mL of phenolic extract at different concentrations ranging from 31.25 $\mu\text{g}/\text{mL}$ to 500 $\mu\text{g}/\text{mL}$ was mixed and shaken vigorously. After 30-min incubation at 30°C in the dark, the absorbance was measured at 517 nm against a blank sample.

ABTS radical scavenging activity

According to Gargouri *et al.* (2013), the ABTS radical scavenging activity was determined with slight modifications. The radical cation ABTS was prepared by reacting to an aqueous solution of ABTS (7 mM) with potassium persulfate (2.45 mM, final concentration), which was kept in the dark at 25°C for 12–16 h. The solution was diluted with ethanol to achieve an absorbance of $0.70 (\pm 0.02)$ at 734 nm. Aliquots of trolox or sample in water (20 μL) were added to 2.0 mL of this diluted solution, and the absorbance was determined at 734 nm at 30°C after 6 min of initial mixing. Blank samples for each assay were prepared using appropriate solvents. The antioxidant solution was tested by measuring its ability to reduce the concentration of radical cation ABTS, which was calculated based on the equivalent concentration of antioxidant trolox. The activity of the antioxidant was determined at three different concentrations.

Hydrogen peroxide scavenging test

The scavenging activity of the extracts against H_2O_2 was determined according to the method described by Remigante *et al.* (2022). Deionized hydrogen peroxide (dH_2O_2) solution, 40 mM, was prepared in a phosphate buffer at pH 7.4. Each extract was made of different concentrations, starting at a concentration of 1 mg/mL. To 0.6 mL of H_2O_2 solution, 1.4 mL of each extract was added. The final product was allowed to react at room temperature for 10 min. α -tocopherol (the reference) was prepared under the same conditions. The absorbance of H_2O_2 was measured at 230 nm against an extract-free control. The H_2O_2 inhibition rate (T%) was estimated according to the following formula:

$$T\% = \frac{Ac - Ae}{Ac} \times 100,$$

where Ac is the control absorbance, and Ae is the tested sample absorbance.

Evaluation of anti-inflammatory activity

The anti-inflammatory activity of the four extracts was evaluated according to the protein thermal denaturation inhibition assay described by Karthik *et al.* (2013). To this end, concentrations of different range, from 0.0625 to 1 g/mL, were prepared. A solution made up of 1 mL of each dilution and 1 mL of 0.2% ovalbumin (diluted in Du phosphate-buffered saline [PBS]) was incubated for 5 min at 72°C. Once vortexed and cooled, absorbance was measured at a wavelength of 660 nm against an extract-free blank sample. Diclofenac, the reference anti-inflammatory agent, was prepared following the same operating procedures as the extract. The inhibition rate (T%) of protein denaturation was calculated according to the following formula:

$$T\% = 100 - \frac{ODs}{ODc} \times 100,$$

where ODs: Optical Density of brine extract
ODc: Ovalbumine solution without extract

Antibacterial activity

Bacterial strains and culture conditions

The selected bacterial strains belong to the international culture collection (American Type Culture Collection [ATCC], Manassas, VA), consisting of Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and *Listeria monocytogenes* ATCC 19117 as well as Gram-negative

bacteria *Salmonella enterica* ATCC 14028 and *Escherichia coli* ATCC 8739.

Determination of minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC)

The liquid micro-dilution method was performed on a 96-well plate to evaluate the antibacterial potency of ethyl acetate extracts to determine minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) as described by Elhadef *et al.* (2021b).

The double-dilution method was used to create concentrations ranging from 20 to 0.031 mg/mL in 1% dimethyl sulfoxide (DMSO) to prepare a range of concentrations. The bacterial suspension was adjusted to a concentration of 106 colony forming unit (CFU)/mL, and 10 μ L of this inoculum was added to each well of a plate. The plates were incubated at 30°C, with continuous shaking overnight.

The MIC was defined as the lowest extract concentration that could inhibit the growth of any bacteria. The MIC was determined visually by examining the first well without turbidity. The MBC was determined by plating 10 μ L of the extracted germ solution and examined for bacterial growth. The MBC represents the lowest extract concentration that killed 99.9% of the bacterial strains present in the initial test solution.

Statistical analysis

The study of the differences within the samples of olive water is ensured by the application of one-way analysis of variance (ANOVA) with one factor after verification of normality by the Shapiro–Wilk test through all the following targeted parameters: contents of polyphenols, ortho-diphenols, hydroxytyrosol, tyrosol, caffeic acid, rutin, coumaric acid, IC50-ABTS, IC50-DPPH, IC50- H_2O_2 , IC50-anti-inflammatory activity, with a probability of 5% error. To determine if the variances of the parameter's polyphenols, ortho-diphenols, hydroxytyrosol, tyrosol, caffeic acid, rutin, and coumaric acid were homogeneous, Levene's test was performed. The mean values of these parameters were then compared using the Tukey's test, and any significant differences were identified at a significance level of $P \leq 0.05$. On the other hand, Dunnett test compares the mean values of IC50-ABTS, IC50-DPPH, IC50- H_2O_2 , and IC50 anti-inflammatory activity parameters with reference of each test to highlight the best-performing antioxidant power of different samples.

A series of linear correlations between the negative control concentrations of each test (ABTS, DPPH, H_2O_2 , anti-inflammatory activity, and extracts of four olive

water samples) was applied to investigate the intensity and significance of the existing relationships.

A linear correlation matrix was adopted to highlight all possible relationships between the following parameters: polyphenols, ortho-diphenols, hydroxytyrosol, tyrosol, caffeic acid, rutin, coumaric acid, IC50 ABTS, IC50 DPPH, IC50 H₂O₂, IC50 anti-inflammatory activity, MIC *Listeria monocytogenes*, MIC *Staphylococcus aureus*, MIC *Salmonella enterica*, and MIC *Escherichia coli*.

All statistical analyses and graphical representations were processed by SPSS version 26 (IBM SPSS, 2019).

Results

Contents of polyphenols, ortho-diphenols, and identification of phenolic compounds by HPLC–DAD

The one-factor ANOVA test showed a highly significant difference ($P < 0.001$) regarding the content of total polyphenols and ortho-diphenols within the samples (Figure 1). EOGM recorded the highest contents of polyphenols and ortho-diphenols (1884.66 mg GAE/mL and 271.39 mg CAE /mL, respectively). On the other hand, extract of green olive brines from Bejaia (EOGB) had the lowest content of polyphenols (1746.5 mg gallic acid equivalent [GAE]/mL). EOBB and extract of green

olive brines from Mascara (EOBM) were reported to have intermediate levels ranging from 1825.33 mg GAE/mL to 1862.33 mg GAE/mL, respectively. EOBB had the lowest ortho-diphenol content (237.55 mg CAE/mL). The EOBB and EOGB samples were found to have intermediate contents ranging from 246.84 to 262.8 mg CAE /mL (Table 1).

In addition, phenolic compounds were identified by HPLC–DAD. Table 2 and Figure 1 illustrate phenolic compounds of the four samples characterized by HPLC–DAD.

Study of phenolic compounds

Similarly, the one-factor ANOVA test reported a highly significant difference ($P < 0.001$) regarding contents of hydroxytyrosol, caffeic acid, rutin, and coumaric acid and a highly significant difference ($P < 0.01$) for tyrosol (Figure 2). EOGM had the highest hydroxytyrosol content (69.67 mg/100 mL) compared to other samples, which contain various contents from 50.36 mg/100 mL to 61.45 mg/100 mL. EOGM contained a low level of tyrosol (17.51 mg/100 mL). On the other hand, other samples had high contents of tyrosol, ranging from 17.48 mg/100 mL to 28.8 mg/100 mL. The EOGM and EOGB samples had high levels of caffeic acid (3.5 mg/100 and 4.17 mg/100 mL, respectively). Black samples from

Table 1. Polyphenol and ortho-diphenol contents in the four acetate extract samples (mean values $\pm$ SD).

Compounds	BB	BM	GB	GM	P-value
Polyphenols	1862.36 $\pm$ 2.75 ^b	1825.03 $\pm$ 2.56 ^c	1747.13 $\pm$ 1.50 ^d	1885.25 $\pm$ 5.10 ^a	0.000
Ortho-diphenols	262.8 $\pm$ 1.61 ^b	237.55 $\pm$ 0.93 ^d	246.84 $\pm$ 0.66 ^c	271.39 $\pm$ 0.31 ^a	0.000

Notes: Mean values followed by different superscript letters are significantly different according to 5% Tukey's test. *** $P < 0.001$. BB: Black Bejaia, BM: Black Mascara, GB: Green Bejaia, GM: Green Mascara.

Table 2. Identification of phenolic compounds by HPLC–DAD.

Compounds	TR (min)	λ (nm)	EOBB (mg/100 mg)	EOBM (mg/100 mg)	EOGB (mg/100 mg)	EOGM (mg/100 mg)
Hydroxytyrosol	9.98	280	58.81 $\pm$ 3.31	50.36 $\pm$ 1.74	61.45 $\pm$ 2.72	69.67 $\pm$ 2.16
Tyrosol	12.3	280	28.8 $\pm$ 2.46	17.48 $\pm$ 3.68	23.27 $\pm$ 2.20	17.51 $\pm$ 3.10
Caffeic acid	13.41	280	2.2 $\pm$ 0.53	0.77 $\pm$ 0.05	4.17 $\pm$ 0.64	3.5 $\pm$ 0.49
Rutin	14.95	254	0.92 $\pm$ 0.08	2.76 $\pm$ 0.3	0.44 $\pm$ 0.05	1.34 $\pm$ 0.07
Verbascosid	15.31	330	3.62 $\pm$ 0.41	8.27 $\pm$ 0.98	0.52 $\pm$ 0.08	0.78 $\pm$ 0.03
Coumaric acid	16.32	280	2.06 $\pm$ 0.26	0.61 $\pm$ 0.08	0.8 $\pm$ 0.08	1.1 $\pm$ 0.36
Oleuropein	20.29	280	2.43 $\pm$ 0.29	8.88 $\pm$ 0.27	8.52 $\pm$ 0.72	3.84 $\pm$ 0.52
Quercitin	23.25	254	0.36 $\pm$ 0.04	0.33 $\pm$ 0.07	0.1 $\pm$ 0.03	0.42 $\pm$ 0.04
Apiginin -7-Glu	17.62	330	0.76 $\pm$ 0.07	0.27 $\pm$ 0.04	0.11 $\pm$ 0.18	0.38 $\pm$ 0.03

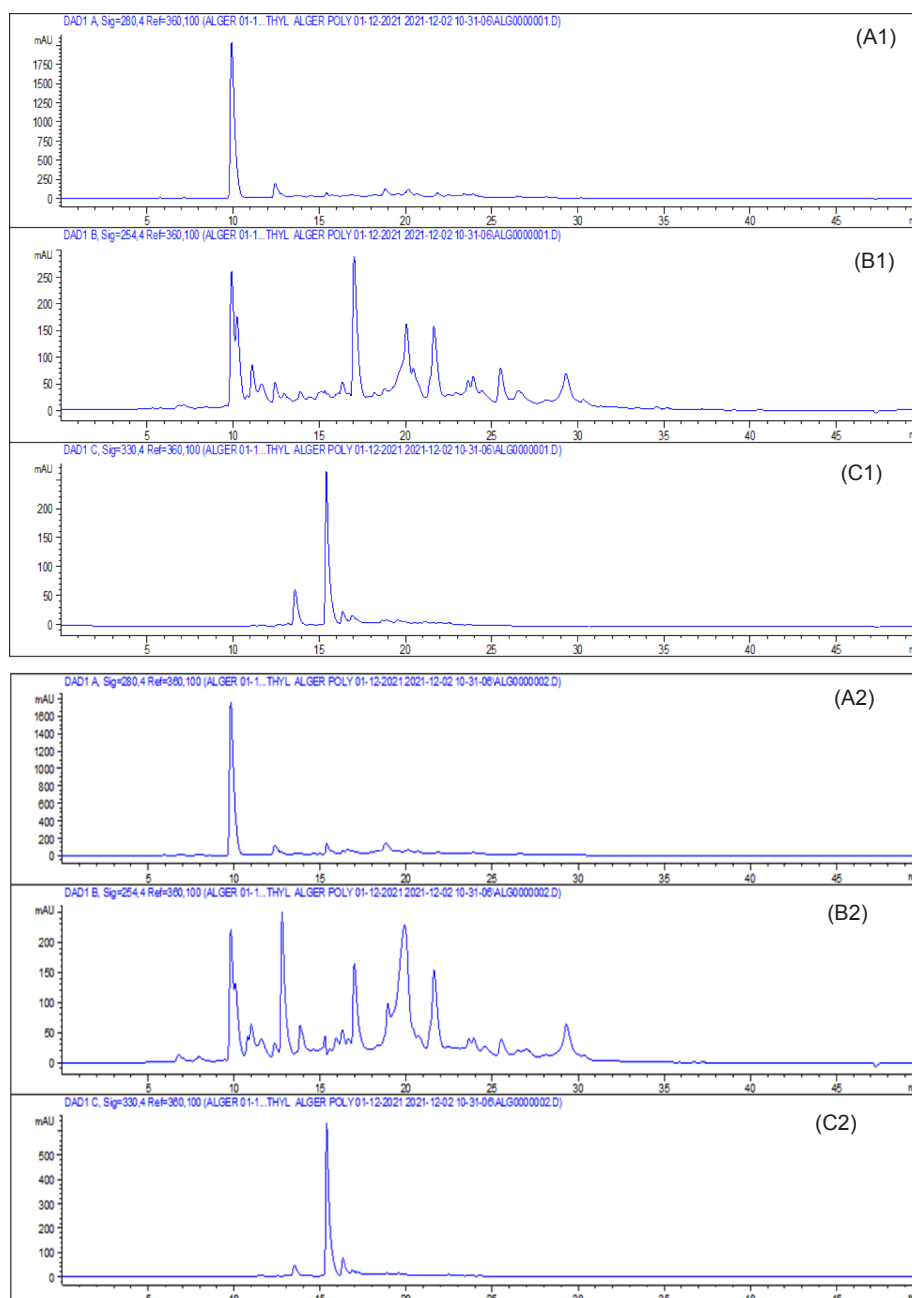


Figure 1. Chromatograms of ethyl acetate extract from brines at different wavelengths: 254 nm, 280 nm, and 330 nm. A1, B1, C1: EOBB; A2, B2, C2: EOBBM; A3, B3, C3: EOGB; A4, B4, C4 EOBB: Extract of the brines of black olive of Bejaia; EOBBM: extract of green olive brines from Mascara; EOGB: extract of green olive brines from Bejaia; EOGBM: extract of green olive brines of Mascara (Sig).

either Mascara or Bejaia contained low levels of caffeic acid (0.77 mg/100 mL and 2.2 mg/100 mL, respectively). EOBBM had the highest rutin content (2.72 mg/100 mL), compared to other samples, which had reliable contents of rutin, varying from 0.44 mg/100 mL in EOGB to 1.34 mg/100 mL recorded in EOGBM. EOBB recorded the highest level of coumaric acid (2.06 mg/100 mL). On the other hand, other samples had low contents that vary from 0.61 mg/100 mL in EOBBM to 1.1 mg/100 mL in EOGBM (Table 2).

Antioxidant activity

DPPH assay

At all the concentrations tested, the ethyl acetate extracts of olive water significantly inhibited DPPH radical in a dose-dependent manner ($P \leq 0.01$ against the negative control), and the Pearson correlation coefficient r varied from 0.92 to 0.99, which showed the existence of strong positive correlations in all relationships. EOGBM extract showed the highest activity compared to all extracts, even

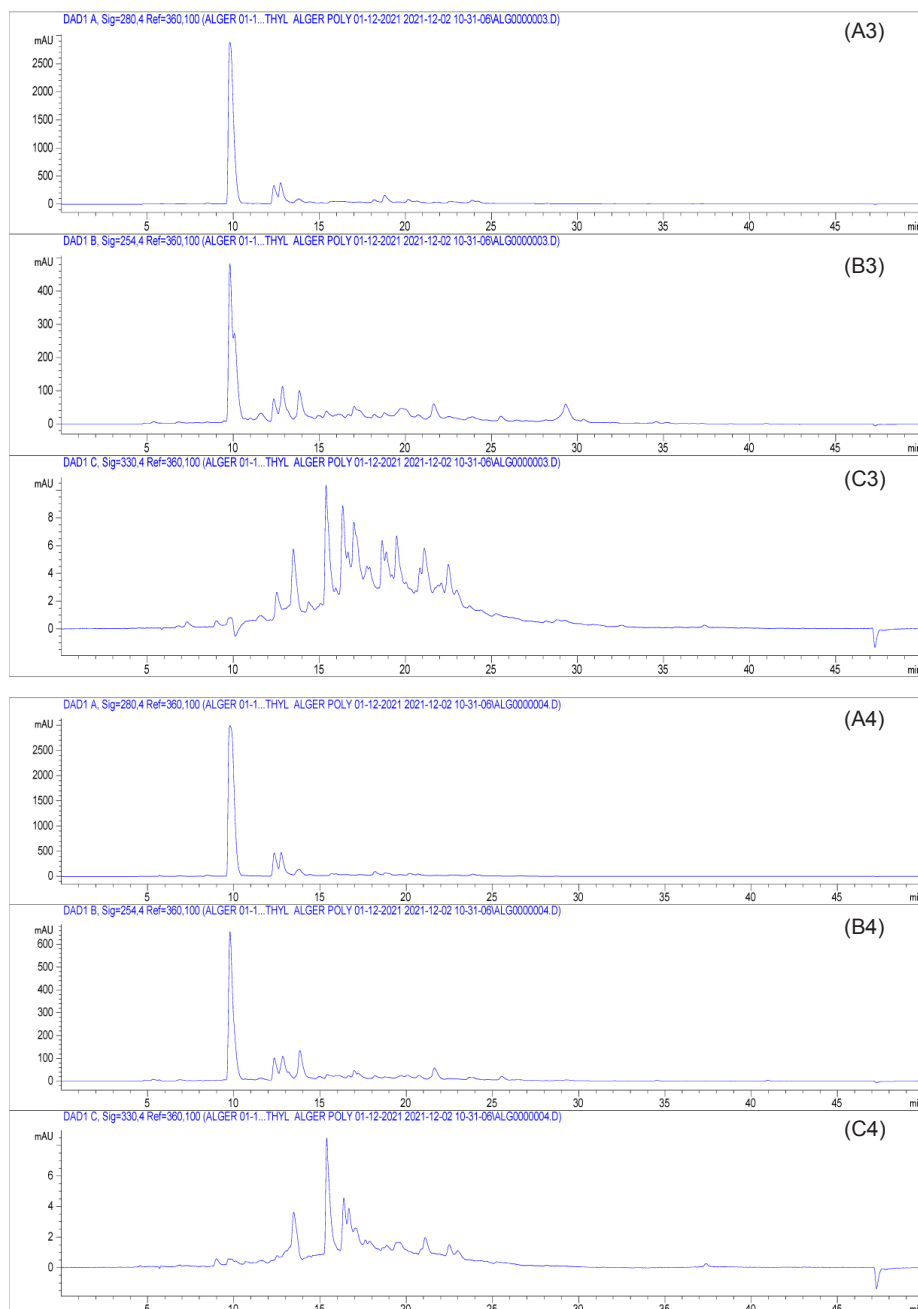


Figure 1. Continued.

ascorbic acid at the lowest concentration of 62.5 µg/mL. It inhibited 80.34% of DPPH radicals. As shown in Table 3, all extracts reached their maximum activity at the last concentration of 2,000 µg/mL. EOBM had the highest activity with an inhibition rate of 97.65 µg/mL, and EOGM reached an inhibition rate of 96.66% at the same concentration of 2,000 µg/mL.

According to the one-factor ANOVA test, the IC₅₀ values of all extracts differed statistically ($P < 0.001$) from the IC₅₀ value of the ascorbic acid reference (1.309 µg/mL); all four samples had very low values (ranging from 0.133 µg/mL to 0.649 µg/mL). The lowest value

characterized EOGM, indicating its significant antioxidant activity compared to other extracts tested (Table 3).

ABTS test

The cation radical ABTS⁺ was inhibited at all concentrations tested and dose dependent by ethyl acetate extracts of olive water. The correlation coefficient r varied from 0.91 to 0.96, which meant the existence of strong positive correlations among all the relations. Across all the extracts tested, at the lowest concentration of 62.5 µg/mL, EOGM extract showed the highest activity, with an inhibition rate of 77.05%. The inhibition proportions were progressively higher with the concentrations

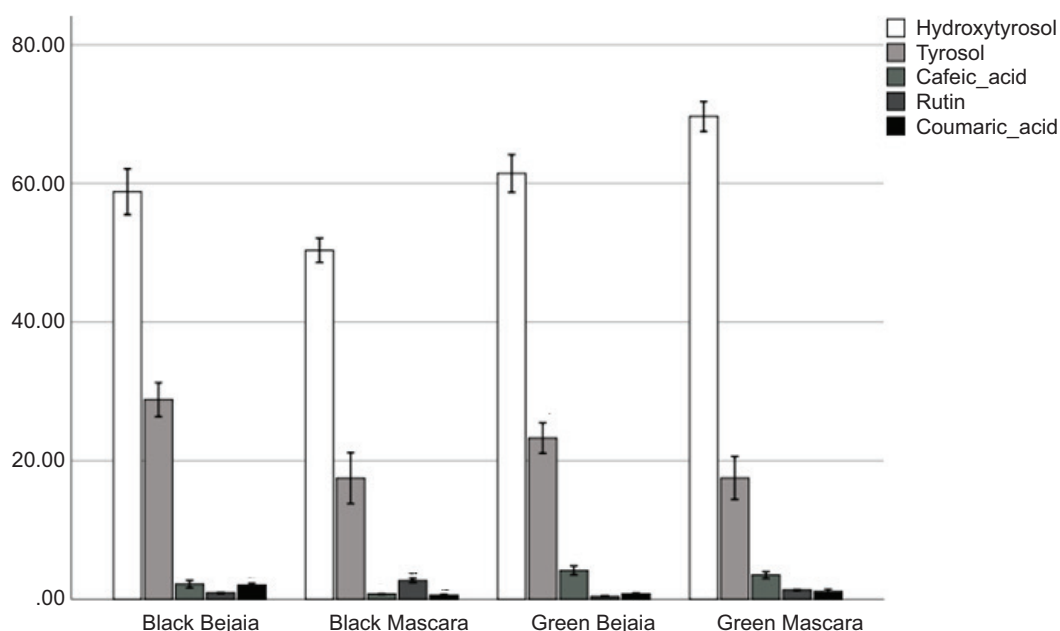


Figure 2. Phenolic compounds of four ethyl acetate extract samples.

Table 3. IC₅₀ values for DPPH, ABTS, H₂O₂, and protein denaturation (PD) tests (mean values $\pm$ SD).

Extrait	IC ₅₀ (μg/mL)	IC ₅₀ (μg/mL)	IC ₅₀ (μg/mL)	IC ₅₀ (μg/mL)
	ABTS	DPPH	H ₂ O ₂	PD
EOBB	1.312 $\pm$ 0.045	0.261 $\pm$ 0.041	11.33 $\pm$ 0.39	20.067 $\pm$ 0.117
<i>P</i> -value	0.000***	0.000***	0.001174**	0.000***
EOBM	1.488 $\pm$ 0.046	0.649 $\pm$ 0.052	15.31 $\pm$ 0.59	76.07 $\pm$ 0.421
<i>P</i> -value	0.000***	0.000***	0.000***	0.000***
EOGB	1.712 $\pm$ 0.023	0.314 $\pm$ 0.022	11.76 $\pm$ 0.45	24.49 $\pm$ 0.463
<i>P</i> -value	0.000***	0.000***	0.000***	0.000***
EOGM	1.015 $\pm$ 0.087	0.133 $\pm$ 0.014	5.215 $\pm$ 0.51	20.21 $\pm$ 0.295
<i>P</i> -value	0.000***	0.000***	0.000***	0.000***
References				
Trolox	0.63 $\pm$ 0.036			
Acide ascorbique		1.309 $\pm$ 0.012		
α -tocopherol			9.15 $\pm$ 0.55	
Diclofenac				11.55 $\pm$ 0.403

Notes: ** $P < 0.01$; *** $P < 0.001$ by Dunnett's test.

EOBB: Black olives of Bejaia, EOBM: Black olives of Mascara, EOGB: Green olives of Bejaia, EOGM: Green olives of Mascara.

used: 62.5 μ g/mL; 125 μ g/mL; 250 μ g/mL; 500 μ g/mL; 1,000 μ g/mL, and 2000 μ g/mL. All extracts reached their maximum activity at 2,000 μ g/mL. EOBM inhibited 98.84% at a concentration level of 2,000 μ g/mL. Trolox, used as a reference, inhibited 90.04% of ABTS⁺ cation at the highest concentration of 2,000 μ g/mL (Table 3).

H₂O₂ test

At all concentrations tested, ethyl acetate extracts of olive water significantly inhibited H₂O₂ in a dose-dependent

manner ($P < 0.01$), and the correlation coefficient r varied from 0.98 to 0.99. At the lowest concentration tested (62.5 μ g/mL), EOGM inhibited most of the cells (49.71%), outperforming even the reference α -tocopherol (47.68%). It was reported that all extracts recorded a progressive evolution of inhibition rates from one concentration to another to reach their maximum at the last concentration of 1,000 μ g/mL; at the later, the EOGM had the maximum rate of inhibition 89.03% (Figure 3).

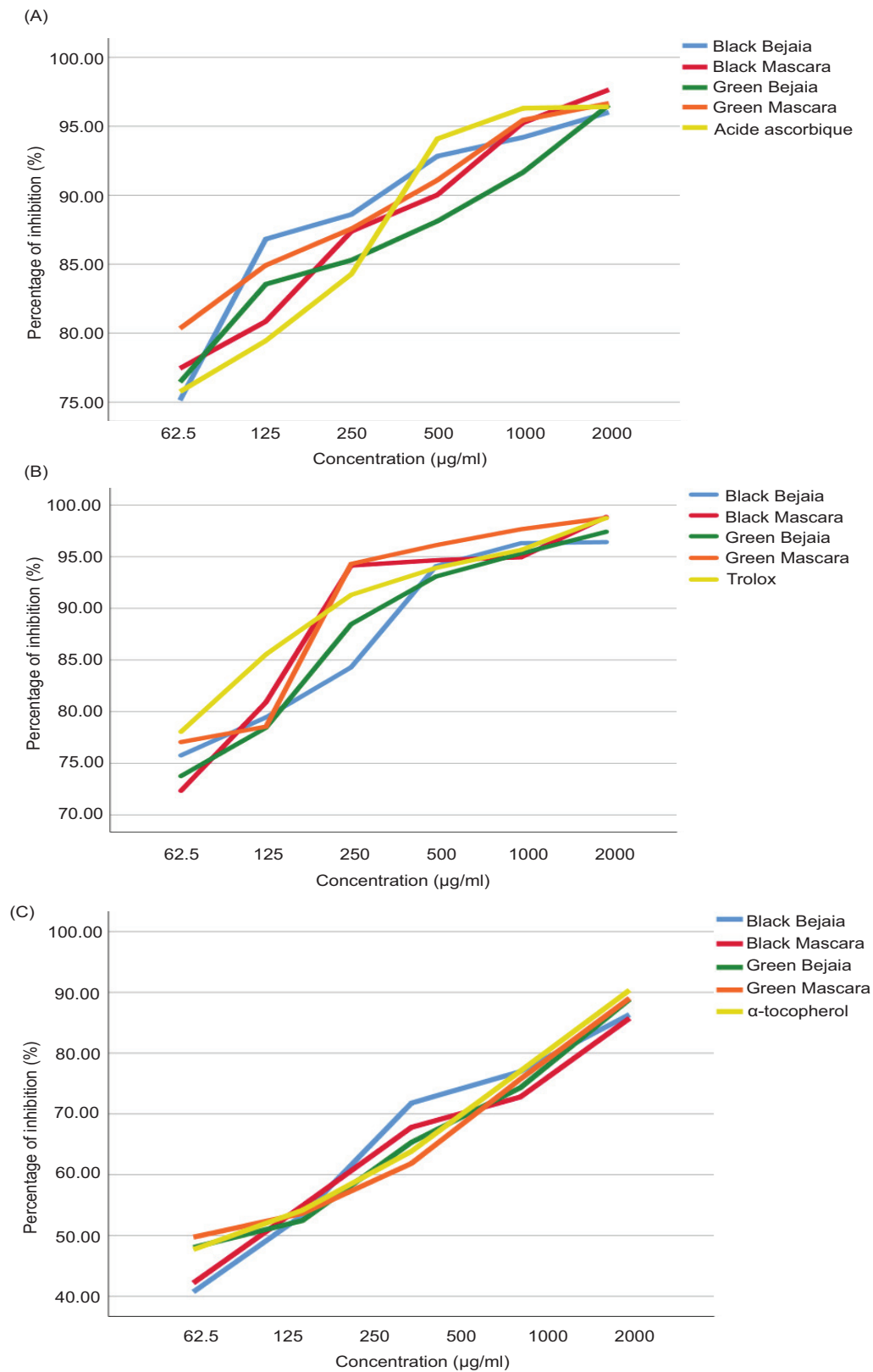


Figure 3. Percentage of inhibition of samples (a) Percentage of DPPH radical inhibition by ethyl acetate extracts of four samples and ascorbic acid. (b) Percentage of inhibition of ABTS+ cation by ethyl acetate extracts and trolox. (c) Percentage of H₂O₂ inhibition by four extracts of ethyl acetate as well as α-tocopherol.

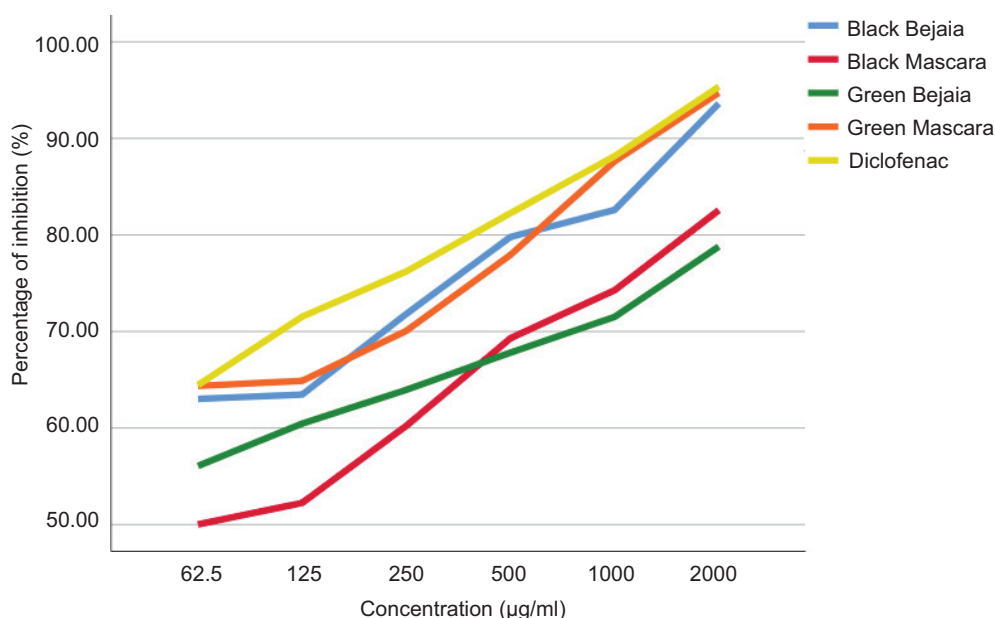


Figure 4. Rate of inhibition of ovalbumin protein denaturation or anti-inflammatory activity of four samples of ethyl acetate extracts together with diclofenac.

Statistical analysis by one-factor ANOVA showed a highly significant difference ($P < 0.001$) between the IC₅₀ value of reference α -tocopherol (9.15 µg/mL) and the IC₅₀ values of all tested extracts. Among the tested extracts, EOGM had the lowest IC₅₀ value of 5.215 µg/mL.

Anti-inflammatory activity

It was recorded across all the extracts tested that at the lowest concentration tested (62.5 µg/mL), EOGM showed the highest inhibition rate (64.36%), compared to other extracts tested, except the reference drug diclofenac, which had an inhibition rate of 64.44%. The inhibition proportions were progressively higher with the concentrations used. All extracts reached their maximum activity at 2,000 µg/mL; at the later concentration, EOGM had the maximum rate of inhibition (94.71%) but not higher than the Diclofenac reference (Table 3 and Figure 3).

The one-factor ANOVA showed a very highly significant difference ($P < 0.001$) between the IC₅₀ value of diclofenac reference (11.55 µg/mL) and the IC₅₀ values of all tested extracts. Across the extracts tested, EOBB and EOGM had the lowest values of 20.06 µg/mL and 20.21 µg/mL, respectively.

Antibacterial activity

According to the results shown in Tables 4 and 5, the recorded MIC values were between 0.31 mg/mL and 2.5 mg/mL; the lowest were those evaluated for EOBB extract. EOBB had a low Minimum bactericidal concentrations (MBC), compared to the four species tested. The value varied from 1.25 mg/mL to 5 mg/mL. In comparison to other species tested, EOGB has a MBC ranging from 5 mg/mL to 20 mg/mL. The MBC values of Mascara samples, either green or black, ranged from 10 mg/mL to 20 mg/mL.

Table 4. MIC values of four ethyl acetate extracts against four species of bacteria.

Ethyl acetate extract	CMI (mg/mL) <i>Listeria monocytogenes</i>	CMI (mg/mL) <i>Staphylococcus aureus</i>	CMI (mg/mL) <i>Salmonella enterica</i>	CMI (mg/mL) <i>Escherichia coli</i>
EOBB	0.625 ± 0.00 ^a	0.31 ± 0.00 ^a	0.31 ± 0.00 ^a	0.31 ± 0.00 ^a
EOBM	2.5 ± 0.00 ^c	2.5 ± 0.00 ^c	2.5 ± 0.00 ^b	1.25 ± 0.00 ^b
EOGB	1.25 ± 0.00 ^b	2.5 ± 0.00 ^c	2.5 ± 0.00 ^b	1.25 ± 0.00 ^b
EOGM	0.625 ± 0.00 ^a	1.25 ± 0.00 ^b	2.5 ± 0.00 ^b	1.25 ± 0.00 ^b

Table 5. MBC values of four ethyl acetate extracts against four bacterial species.

Ethyl acetate extract	MBC (mg/mL) <i>L. monocytogenes</i>	MBC/CMI	MBC (mg/mL) <i>S. aureus</i>	MBC/CMI	MBC (mg/mL) <i>S. enterica</i>	MBC/CMI	MBC (mg/mL) <i>E. coli</i>	MBC/CMI
EOBB	5 ± 0.00 ^a	8	2.5 ± 0.00 ^a	8	1.25 ± 0.00 ^a	4	5 ± 0.00 ^a	16
EOBM	20 ± 0.00 ^c	8	20 ± 0.00 ^d	8	10 ± 0.00 ^b	4	10 ± 0.00 ^b	8
EOGB	20 ± 0.00 ^c	16	5 ± 0.00 ^b	2	10 ± 0.00 ^b	4	5 ± 0.00 ^a	4
EOGM	10 ± 0.00 ^b	16	10 ± 0.00 ^c	8	20 ± 0.00 ^c	8	20 ± 0.00 ^c	16

Discussion

Phytochemical profile

The HPLC–DAD analysis of the phenolic phytochemical profile of brackish allowed the separation and quantification of phenolic compounds, among which nine compounds can be mentioned, namely hydroxytyrosol, tyrosol, caffeic acid, rutin, verbascoside, coumaric acid, oleuropein, quercetin, and apigenin. These compounds were identified based on their retention periods and their peaks' spectral characteristics relative to standards. Polyphenols were identified and quantified using 18 standards. The chromatogram showed that hydroxytyrosol and tyrosol were the most abundant products for all the samples analyzed, which varied from 58.81±3.31 mg/100 g to 69.67±2.16 mg/100 g for hydroxytyrosol, or the extracts of green olive brines highlighted the highest concentrations (Table 1, Figure 1). Most of the degradation of oleuropein into hydroxytyrosol occurred during fruit ripening or after plant growth. To release 100% of hydroxytyrosol content, hydrolysis measures such as heat or acid treatments were necessary (Johnson and Mitchell, 2018). This was significant because hydroxytyrosol had a high commercial value and was an important source of income from the processing of table olives into a valuable and highly sought-after substance because of the increasing demand of natural products with antioxidant and pharmaceutical properties (Belaqziz *et al.*, 2016). Tyrosol levels were significantly lower than hydroxytyrosol levels, ranging from 2 to 3 times lower than those quantified for the latter (Figure 2). This was expected because hydroxytyrosol was produced through the hydrolysis of oleuropein, the main secoiridoid glycoside present in table olives. In contrast, tyrosol was produced from legistroside, which was present in table olives to a lesser extent than oleuropein (Huertas-Alonso *et al.*, 2021).

Oleuropein, one of the most important constituents of olive phenolic fraction, was identified in the extracts analyzed and in the defatted olive pulp. Oleuropein can confer resistance to diseases and insect infestations on the tree. In addition, it is responsible for the bitter taste of olives and has antioxidant properties due to its catechol

fraction (Huertas-Alonso *et al.*, 2021). According to Benlarbi *et al.* (2018), the amount of oleuropein varies from 32.63 mg/100 g (Sigoise cultivar) to 1973.42 mg/100 g (Dahbia cultivar) for green olives and from 75.50 mg/100 g (Sigoise cultivar) to 373.01 mg/100 g (Dahbia cultivar) for black olives at two stages of ripening. Comparable oleuropein contents of 8.88 mg/100 g for EOBM and 8.52 mg/100 g for EOGB (Table 1) belonging to two different stages of ripening were observed. This could be explained by the effect of chemotype and transformation process of table olives during the debittering and fermentation phase under the effect of lactic acid bacteria (Bonatsou and Panagou, 2022; Jardim-Botelho *et al.*, 2022; Yan *et al.*, 2022).

Polyphenols and ortho-diphenols

The fruits of *Olea europaea* L. are an important source of nutrients and components with nutritional value because of their rich composition of unsaturated fatty acids, vitamin E, carotenoids, minerals, pentacyclic triterpenes, and polyphenols (Ghanbari *et al.*, 2012). Phenolic compounds in table olives are one of the sources of nutritional value (Huertas-Alonso *et al.*, 2021). EOGM had the highest concentrations of polyphenols (1884.66 mg GAE/kg) and ortho-diphenols (271.39 mg CAE/mL) whereas EOGB had the lowest concentration of polyphenols (1746.5 mg GAE/kg), and EOBM had the lowest concentration of ortho-diphenols (Table 1). The phenolic content of olives decreases during ripening (Soufi *et al.*, 2016). Diversity in phenolic content is due to the influence of cultivar, ripening, and processing method. Processing of table olives caused considerable changes in phenolic content, which showed a highly significant difference between total polyphenols and ortho-diphenols. This result could be due to cultivar characteristics, such as fruit diameter and/or moisture, since the decrease is related to the diffusion of these compounds. The value obtained for Sigoise cultivar could be explained by fruit diameter (Metouchi *et al.*, 2016). Phenolic compounds are secondary plant metabolites that are biosynthesized through the shikimic acid pathway. The polyphenolic profile differs even among plant varieties of the same species.

Antioxidant activity

The DPPH and ABTS tests, commonly used to measure the antioxidant properties of phenolic compounds, demonstrated a strong antiradical potential of ethyl acetate extracts through the free radical scavenging activity. The results of this activity, as shown in Table 3 and Figure 3, were compared to reference standards to provide more detailed and useful information. The extracts exhibited a significantly higher antiradical potential than the reference standards (ascorbic acid and tocopherol). The extract of olive brine water from Mascara evaluated the most potent antioxidant activity with IC₅₀ ranging between 0.133±0.014 µg/mL and 1.015±0.087 µg/mL (Table 3, Figure 3), which could be explained by the high levels of phenolic compounds (1885.25±5.10 mg CAE /mL) (Table 2), especially hydroxytyrosol (69.67±2.16 mg/100 g; Table 1 and Figure 2). These results followed Gueboudji *et al.* (2021), who showed that the extracts of margins water rich in phenolic compounds had a more remarkable antioxidant power than ascorbic acid and *butylated hydroxytoluene* (BHT) with IC₅₀s = 2.03±0.14 µg/mL and 4.27±0.38 µg/mL.

According to the considered results, all the tested extracts had a strong antioxidant activity, which neutralized H₂O₂ in a dose-dependent manner. All the concentrations used exhibited inhibition rates that exceeded 40% (Figure 3). The calculated IC₅₀s ranged from 5.215±0.51 to 15.31±0.59 µg/mL (Table 3). EOGM (IC₅₀ = 5.215±0.51 µg/mL) was found to be more potent compared to α-tocopherol (IC₅₀ = 9.15±0.55 µg/mL), which formed vitamin E. H₂O₂ itself is not very reactive. However, it can sometimes be toxic to cells, because it can produce hydroxyl radicals in cells. Thus, removing H₂O₂ is crucial for antioxidant defense in cellular or food systems (Charoenprasert and Mitchell, 2012). Several studies have shown that antioxidant activity is correlated with the content, nature, and structure of polyphenols abundant in the by-products of the olive industry (Moudden *et al.*, 2020). One of the most important natural antioxidant extracts is hydroxytyrosol, which has 10 times more antioxidants than green tea and twice as much as coenzyme Q10. In its chemical structure, this compound has an extra OH group in its benzene ring, compared to tyrosol. Therefore, it obtains a greater free radical scavenging function, increasing its antioxidant power and effectiveness under stressful conditions (Benincasa *et al.*, 2022). These bioactive molecules have been proposed to be beneficial in a multitude of diseases, including certain cancers, cardiovascular disease, passive smoke-induced oxidative stress, whole blood production of thromboxane B₂, and inflammatory diseases, such as osteoarthritis (Cardinali *et al.*, 2012).

Anti-inflammatory activity

Olive products are rich in natural antioxidants that inhibit oxidative stress, often involved in developing many diseases, such as neurodegenerative diseases, cancer, and coronary heart disease. The hydroxyl radical is known to react with all DNA molecules and damage purine, pyrimidine base, and deoxyribose (Juan *et al.*, 2021). The most studied DNA damage is the formation of 8-OH-G. Permanent changes in genetic material resulting from this oxidative damage represent the first step involved in mutagenesis, carcinogenesis, and aging (Juan *et al.*, 2021). However, ability of ethyl acetate extracts from brines to inhibit protein denaturation may signal their anti-inflammatory capacity.

EOBB and EOGM exhibited a remarkable dose-dependent inhibition of thermally induced ovalbumin denaturation with comparable IC₅₀s (20.067±0.117 µg/mL and 20.21±0.295 µg/mL), respectively (Table 3). The reference drug diclofenac, known for its anti-inflammatory properties, evaluated the best inhibitory effect with an IC₅₀ = 11.55±0.403 µg/mL (Table 3 and Figure 4). The observed anti-inflammatory effects of olive extract could be related to its phenolic composition, since it is rich in hydroxytyrosol. Olive phenolic compounds generally inhibit inflammatory enzymes and cyclooxygenase more effectively than ibuprofen (Laaboudi *et al.*, 2016). These promising results need to be complemented by further *in vivo* tests to extract bioactive molecules that could be used as natural alternatives with inflammatory properties to mitigate the side effects of synthetic drugs (Saleh *et al.*, 2020).

Antimicrobial activity

The antibacterial activity of brine's ethyl acetate extract was evaluated against a range of foodborne bacteria, covering both Gram-positive and Gram-negative bacteria. All extracts showed an inhibitory effect against the strains tested, where MICs ranged from 0.31 to 2.5 mg/mL. EOBB was the most effective in exerting inhibitory activity against the tested strains, with the lowest MICs located between 0.31 mg/L and 0.625 mg/L, compared to the other extracts analyzed. However, gentamine in positive control expressed significant activity against *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella enterica*, where the MIC = 0.097 mg/mL. On the other hand, the majority of concentrations used did not show any bactericidal effect because the recorded MBC values were higher than MICs or the calculated MBC–MIC ratio (4 < MBC–MIC < 16) reflected a bacteriostatic effect of most of the tested extracts, except EOGB, which was bactericidal toward *Staphylococcus aureus* and inhibited MBC = 5 mg/mL, which was two times the MIC. The

latter contained high levels of hydroxytyrosol and oleuropein. Indeed, the antimicrobial activity of hydroxytyrosol and its implicit application as a natural preservative have been proved by numerous studies. The studies have evaluated the *in vitro* sensitivity of hydroxytyrosol and oleuropein to many bacterial strains that occasionally caused respiratory and intestinal infections in humans. All brine ethyl acetate extracts were tested for antibacterial activity. EOBB highlighted a higher anti-bacterial activity than other extracts at concentrations ranging from 1.25 µg/mL to 5 µg/mL against all standard strains. It was emphasized that hydroxytyrosol was one of the bioactive molecules of olives with strong antibacterial potential; this has been confirmed also by previous studies (Brenes *et al.*, 2022). The study conducted by Medina *et al.* (2013) indicated that the addition of 400 µg/mL of hydroxytyrosol to different media significantly altered the parameters of the growth curve of *E. coli* strains, compared to the control group, proving that hydroxytyrosol had a bactericidal effect against a wide spectrum of bacteria. In our study, none of the tested extracts evaluated bactericidal activity, except for EOGB, which was found to be toxic to Gram-positive bacteria *S. aureus* (MBC–MIC = 2); these results were consistent with those of Akermi *et al.* (2020) and Allaoui *et al.* (2020), which showed that oil mill water rich in phenolic compounds was found to be more toxic against food-borne strains. It was observed that the bactericidal effect of hydroxytyrosol was stronger against Gram-positive bacteria than Gram-negative bacteria (Belaqziz *et al.*, 2016). On the other hand, other studies performed on hydroxytyrosol-rich water also confirmed the inhibitory properties of hydroxytyrosol toward phytopathogenic bacteria (Brenes *et al.*, 2022). Likewise, hydroxytyrosol demonstrated bactericidal activity against harmful (*E. coli* and *Clostridium perfringens*) and beneficial (*Bifidobacterium bifidum* and *Lactobacillus acidophilus*) bacteria of the gut microbiota (Huertas-Alonso *et al.*, 2021). The extract's antibacterial activity increased with decrease in the content of phenolic compounds. The diffusion of phenolic compounds in brines depended on several parameters, such as characteristics of the cultivar, permeability of the fruit skin, type of phenolic compounds presents in the olive flesh, and their ability to diffuse out of the fruit. Hydroxytyrosol and oleuropein were found to be cytotoxic to many strains of clinical bacteria. The study conducted by Medina *et al.* (2013) showed that the minimum inhibitory concentration of hydroxytyrosol and oleuropein against *S. aureus* was 3.5%.

Conclusions

Extracts of brine water from green olives showed substantial quantities of ortho-diphenols and polyphenols when the four samples under study were quantified. The

phytochemical profile showed that their main component, hydroxytyrosol, was present in large amounts. This investigation verified the extracts' strong antioxidant potential, directly tied to the chemical makeup of phenolic chemicals that made up those extracts. In numerous antioxidant activity tests, EOGM was discovered to be most effective in scavenging free radicals. Additionally, an intriguing antibacterial activity against bacterial strains with food origin was assessed for all the extracts. The black olive extract demonstrated the strongest inhibitory action, which had an MIC range of 0.625–0.31 mg/mL. The extracts were also found to have substantial anti-inflammatory properties. The findings from the extracts could be a useful source of chemicals for the diet and help to prevent diseases caused by free radicals. The extracts under study were a source of natural antioxidants, according to an *in vitro* testing, and could be used as basic materials to make functional food products. However, *in vivo* investigations are required in the future to evaluate more accurately the potential of table olives. Table olive extract-based screening has proven to be particularly effective for locating interesting therapeutic candidates, such as hydroxytyrosol.

Acknowledgments

The authors would like to acknowledge the support of the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (Contrat programme LR14ES08), Tunisia.

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