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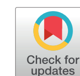
Phenolic profile (HPLC-UV) of olive leaves according to extraction procedure and assessment of antibacterial activity



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ABSTRACT

The aim of the present study is to firstly study the effect of the extraction solvents (ethanol, acetonitrile, distilled water), pH, temperature, and the extraction method (maceration, sonication, maceration in two steps) on the flavonoid and phenolic contents of olive leaves. Furthermore, qualitative and quantitative analyzes of phenolic compounds by (HPLC) were performed. Results showed that the extract macerated in two steps by ethanol followed by distilled water of dried leaves showed high contents of phenolic compounds and flavonoids compared to the extracts obtained by the other studied techniques and solvents. On the other hand, the macerated extracts were studied for their antibacterial activity against five pathogenic bacteria (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Listeria monocytogenes*). The results showed a strong antibacterial activity of the same macerated extract in two steps for dried leaves, which could be attributed to its richness in bioactive compounds such as oleuropein.

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1. Introduction

Recently, a number of medicinal plants have been used to treat different diseases in humans and animals [1], due to their richness in bioactive molecules among which we cite flavonoids and phenolic compounds [2]. These compounds are defined as secondary metabolites with strong biological activities such as antioxidant, anti-inflammatory, antimicrobial, hypoglycemic, antihypertensive, and antiviral activities [3].

In this context, *Olea europaea* is widly known as one of the most cultivated tree in the Mediterranean countries where it covers 8 million hectares, nearly 98% of the world's crop [4], which demonstrates the great economic and social importance of this crop in addition to the potential benefits of using its by-products [5,6]. Olive leaves are an important source of bioactive compounds in comparison to olive oil and fruit [7]. Among the phenolic compounds present in olive leaves, the essential ones are the hydroxytyrosol, tyrosol, catechin, caffeic acid, rutin and oleuropein [8].

The content of these leaves varies according to many factors such as climatic conditions, moisture content, age and variety of the plant, agricultural practices [9], and the extraction procedures used [10]. Thus, extraction is an important and determinative step in the analysis and the use of the cellular bioactive compounds contained in these leaves [7].Therefore, the identification of the appropriate extraction methods is a limiting step to increase the yield of these compounds.

In this context, we are interested in studying some parameters that influence the content of phenolic compounds and flavonoids of olive leaves likewise the nature of solvents (80% ethanol, 20% acetonitrile, distilled water), the extraction methods (maceration with a single step, sonication, maceration in two steps with different solvents), pH, temperature, and the state of these leaves (dry or fresh). Then the identification of these compounds such as: coumaric acid, oleuropein, protocatechuic acid, syringic acid, quercetin, gallic acid, ferulic acid, caffeic acid, luteolin, hydroxytyrosol, rutin and tyrosol in each extract by high performance liquid chromatography was carried out. Finally the evaluation of the antibacterial activity of different olive leaves extracts against a broad spectrum of pathogenic bacteria was performed in order to explore the relationship between this activity and the content of phenolic compounds and flavonoids of each extract.

2. Methods

2.1. Material

Methanol, acetonitrile, phosphoric acid, Folin's reagent, sodium carbonate (Na2CO3), dimethyl sulfoxide (DMSO), sodium nitrite

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($NaNO_2$), aluminum trichloride ($AlCl_3$), sodium hydroxide (NaOH), and standard compounds such as hydroxytyrosol, tyrosol, oleuropein, coumaric acid, gallic acid, ferulic acid, caffeic acid, luteolin, quercetin, syringic acid, protocatechuic acid and rutin were purchased from Sigma Aldrich. Stock solutions containing these standards were prepared in 80% methanol and stored at 4 °C until use.

2.2. Sample preparation

This study was carried out on *Olea europaea* leaves. Collection was done in early December (2016). Leaves were washed with distilled water, dried at 40 °C and crushed to obtain a fine powder which was subsequently kept in the dark until use.

2.3. Extracts preparation by maceration in a single step

Five grams of dried and crushed leaves were macerated in 50 mL of different solvents for four hours. The solvents used for this extraction are: 80% ethanol, 20% acetonitrile, distilled water, distilled water at 60 °C and distilled water at 60 °C with a pH = 3 (adjusted with a solution of HCl (0.2 N)). The extracts were then filtered using a filter paper. The extraction solvents were removed from the filtrate by evaporation under reduced pressure in a rotary evaporator. The concentrated extracts were stored at 4 °C until use.

2.4. Extracts preparation by sonication

For sonication, 5 g of dried and crushed leaves were mixed with 50 mL of different solvents. The mixtures were vortexed for 5 min and then extracted in an ultrasonic bath (Bransonic; 47 KHz) for 4 h at room temperature. The solvents used for this extraction are: 80% ethanol, 20% acetonitrile and distilled water. Then the extracts were filtered. The solvents were then removed from the filtrate by evaporation under reduced pressure in a rotary evaporator. The concentrated extracts were stored at 4 °C until use.

2.5. Extracts preparation by maceration in two steps with different solvents

Five grams of dried and crushed leaves were macerated in 50 mL of ethanol for four hours at room temperature. The filtrate was recovered and the solid residue was then extracted with methanol for 4 h. The filtrate was recovered once again then the two extraction solutions were combined and evaporated under reduced pressure. The concentrated extracts were stored at 4 °C until further use. The same procedure was followed as described above, methanol was replaced by distilled water in the second step of extraction.

2.6. Preparation of fresh leaves extract by maceration in ethanol followed by distilled water

Five grams of fresh and ground leaves were macerated in 50 mL of ethanol for four hours at room temperature. The filtrate was recovered and the solid residue was immersed again in distilled water during 4 h. Once again the filtrate was recovered, and these two extraction solutions were combined and then evaporated under vacum. The concentrated extracts were stored at 4 °C until they were used.

2.7. Determination of total phenolic compounds

The determination of total phenolic compounds of each extract was carried out by Folin-Ciocalteu method [11] using gallic acid as standard. 0.5 mL of Folin reagent was added to 0,1 mg/mL of extract. After 5 min, 2 mL of Na_2CO_3 (20%) were added. Then, the mixture was incubated for 30 min at room temperature in the dark. The absorbance was measured at 750 nm by spectrophotometer (Selecta). The total phenolic compound contents are expressed as milligram of gallic acid equivalent per gram of the dry vegetable matter (mg EAG/gMS) (R^2 = 0.999; N = 3).

2.8. Determination of total flavonoid compounds

Total flavonoid contents of each olive extract was determinated via the aluminum trichloride method [12]. 250 μL of the diluted extract (0.1 mg/mL) were mixed with 75 μL of a 7% $NaNO_2$ solution. After incubating for 6 min at room temperature, 150 μL of a freshly prepared solution of 10% $AlCl_3$ were added to the mixture. After standing for 5 min at room temperature, 500 μL of sodium hydroxide (NaOH.,1 M) were added to the mixture. The final volume was adjusted to 2.5 mL with distilled water. The absorbance of this preparation was measured at 510 nm. A calibration curve was carried out in parallel under the same operating conditions using quercetin. Flavonoid contents are expressed in milligram equivalent of quercetin per gram of dry vegetable matter (mg EQ/gMS) (R^2 = 0.989; N = 3).

2.9. Identification of phenolic compounds contained in each extract by HPLC

High performance liquid chromatography analysis was performed for the analytical qualification and quantification of phenolic compounds in each olive leaves extract according to the IOOC method [13]. The extracts (10 mg) were dissolved in 80% methanol (1 mL) and filtered with 0.45 μm filters before analysis by HPLC (UV–vis). Separation of phenolic compounds was performed on a Wakosil C18HG (5 μm, 4.6 × 150 mm) at a temperature of 40 °C. The elution was carried out in gradient mode using a binary solvent mixture composed of water acidified with 0.2% phosphoric acid (solvent A) and methanol / acetonitrile 50/50 (solvent B). A linear gradient was run from 96% (A) and 4% (B) to 50% (A) and 50% (B) during 40 min; it changed to 40% (A) and 60% (B) for 5 min; during 15 min it changed to 0% (A) and 100% (B), after reequilibration for 12 min to initial composition. The mobile phase flow rate was 1 mL/min and the injection volume of each sample was 20 μL. All phenolic compounds were identified by comparing their retention times with those of standards (coumaric acid, caffeic acid, protocatechuic acid, rutin, quercetin, luteolin, gallic acid, ferulic acid, hydroxytyrosol, oleuropein, syringic acid, tyrosol).

2.10. Assessement of antibacterial activity

2.10.1. Used strains

In order to evaluate the antibacterial activity of prepared extracts, five strains were used. They were all provided by the laboratory of biology and health of the faculty of sciences of Tetouan. *Pseudomonas aeruginosa* (ATCC27853), *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (CECT976), *Bacillus subtilis* (DSM6633) and *Listeria monocytogenes* (CECT4030). These strains were subcultured on Lauria Bertani medium (LB) and incubated at 37 °C for 24 h.

2.10.2. Determination of the antibacterial activity

Antibacterial activity was performed using agar diffusion method [14]. The LB medium (Luria-Bertani) was uniformly inoculated with a sterile swab using a saline suspension (NaCl 0.9%) of the studied strains, previously adjusted using the 0.5 Mc Farland standard with a turbidity of approximately 108 bacteria/mL. Sterile Whattman paper discs 6 mm of diameter were deposited on the culture media and then soaked with 10 μL of each tested extract. After 24 h of incubation at 37 °C, the diameter of the inhibition halos was

measured. Each extract was dissolved in dimethyl sulfoxide (DMSO) to prepare two different concentrations (30 mg/mL and 50 mg/mL). Discs soaked with Chloramphenicol were used as positive controls. The negative control was a disk containing 10 μL of DMSO. The experiments were repeated twice for each test.

2.11. Statistical analysis

Data were analyzed using One-way analysis in variance (ANOVA) in Excel, and presented as "mean ± standard deviation". Groups were compared against each others. F values with $P < 0.05$ were considered significant. All experimental measurements were carried out in triplicate (N = 3) exept for the antibacterial assay which was performed in duplicate.

3. Results and discussion

3.1. Determination of total phenolic compounds and flavonoids

In order to select the most efficient extraction method, several techniques and solvents were tested. The Fig. 1A shows that the extraction by maceration using ethanol 80% gave the highest contents of both total phenolic contents (101.5 ± 1.27 mg/g; $p < 0.01$) and total flavonoids (54.92 ± 0.73 mg/g; $p < 0.01$) in comparison to the other solvents tested. In the same context, the Fig. 1B shows that the highest contents of total phenolic contents (94.25 ± 1.76 mg/g; $p < 0.01$) and total flavonoids (36.4 ± 1.69 mg/g; $p < 0.01$) were shown by distilled water when using sonication for extraction. Based on these results, we aimed to assess the yield of the extraction when combining the two most performent solvents. As expected the obtained concentration of total phenolic contents (169.10 ± 0.57 mg/g; $p < 0.01$) and total flavonoids (98.15 ± 0.7 mg/g; $p < 0.01$) were significantly increased (Fig. 1C). In the other hand, knowing that the state of the leaves affect sthe contents on both total phenolic contents and total flavonoids (Fig. 1D), we hypothesize that the drying of the leaves is necessary for a high yield of phenolic compounds and flavonoids. Moreover, this step removes the water contained in leaves, which is responsible for the deterioration of the phenolic compounds, especially oleuropein, by enzymatic actions ([15]; [16]).

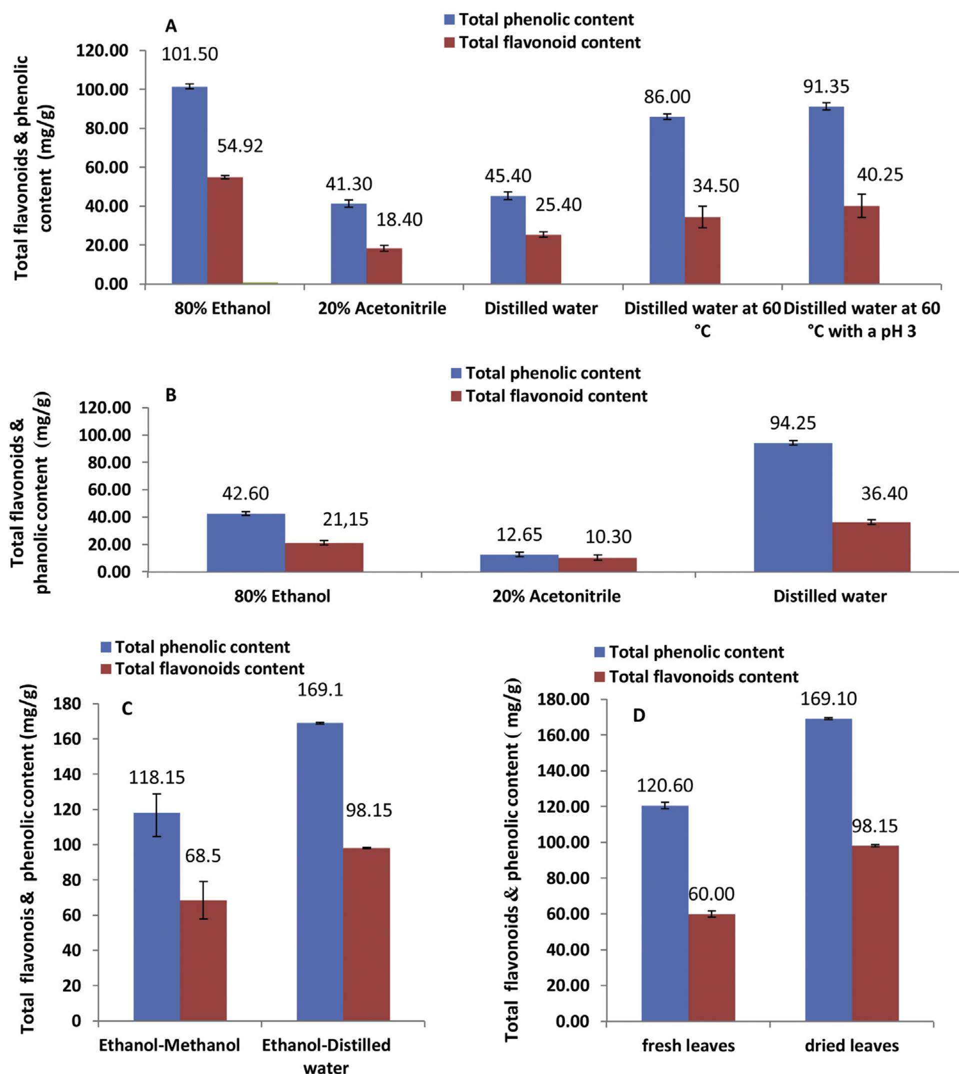

Fig. 1. Total flavonoid and phenolic contents of olive leaves extracts obtained by different methods and solvents. (A) Maceration (B) Sonication (C) Maceration in to steps with different solvents (D) Comparison between the total flavonoïds and phenolic contents in fresh and dried leaves. Data are expressed as (means ± standard deviation). n = 3; (*$^{*}p < 0.5$; $^{**}p < 0.01$; $^{***}p < 0.001$*).

Table 1
Concentration of identified phenolic compounds (mg/g dry weight) in olive leaves extracts obtained by maceration with different solvents.

Phenolic compounds	Retention time (min)	80% Ethanol	20% Acetonitrile	Distilled water
Coumaric acid	14.28	ND	ND	0.15 ± 0.06
Caffeic acid	12.13	0.34 ± 0.06	ND	ND
Protocatechuic acid	11.82	3.23 ± 0.26	ND	0.40 ± 0.00
Rutin	17.11	0.05 ± 0.01	ND	0.20 ± 0.00
Quercetin	21.74	0.02 ± 0.00	ND	ND
Luteolin	22.17	0.09 ± 0.01	ND	0.31 ± 0.13
Gallic acid	17.19	3.04 ± 0.06	ND	ND
Ferulic acid	09.61	ND	ND	ND
Hydroxytyrosol	07.66	0.02 ± 0.01	0.19± 0.01	0.25 ± 0.07
Oleuropein	18.42	15.17± 0.39	ND	27.20 ± 0.99
Syringic acid	08.10	ND	ND	ND
Tyrosol	10.68	ND	ND	0.20 ± 0.14

Values are mean ± standard error (n = 3).
○n.d. not detected.

3.2. Extracts analysis by HPLC

The identification of the phenolic compounds of each macerated extract is presented in Table 1. For the 80% ethanol extract, oleuropein (29.5 ± 0.10 mg/g) was the major compound followed by coumaric acid and gallic acid. Rutin, luteolin, hydroxytyrosol, tyrosol and quercetin were the minor phenolic compounds. However, caffeic acid, protocatechuic acid, ferulic acid, and syringic acid were not detectable. For the 20% acetonitrile extract, gallic acid (11.17 ± 1.65 mg/g) represented the major compound followed by oleuropein compared to rutin, luteolin, hydroxytyrosol, and tyrosol. Whereas coumaric acid, caffeic acid, protocatechuic acid, ferulic acid and acid syringic, were not detectable. Concerning the extraction by maceration in cold distilled water, the majority of phenolic compounds are undetectable except for rutin (0.5 ± 0.35 mg/g), oleuropein (0.1 ± 0.71 mg/g) and luteolin (0.03 ± 0.02 mg/g). These results were consistent with those found by Ortega-García et al. [17], who showed that the use of water as a co-solvent :with organic solvents increases the amount of oleuropein extracted and that the solvent mixtures are able to desactivate the enzymes responsible for the conversion of oleuropein into other molecules. In addition, the use of water in combination with alcohols leads to an increase in the swelling of plant materials and the contact area between the vegetable matrix and the solvent which ultimately improves the extraction yield [18]. While extraction in distilled water at 60 °C showed that oleuropein (19.3 ± 0.99 mg/g), coumaric acid (16.5 ± 1.77 mg/g), and caffeic acid (9.96 ± 1.36 mg/g), were major compounds and that protocatechuic acid, rutin, luteolin, and tyrosol were found to be minor compounds. Nevertheless, gallic acid, ferulic acid, hydroxytyrosol and syringic acid were not detectable. Besides, when this extraction was carried out in distilled water at 60 °C with a pH value of 3, it was found that the oleuropein content increases (23.36 ± 0.91 mg/g), and gallic acid, coumaric acid, rutin were major compounds while the other compounds were undetectable. These results were in agreement with Ansari et al. [19], who optimized a method of extracting oleuropein from olive leaves. They found that distilled water at 60 °C (pH = 3) for 4 h could allow the extraction of a large amount of oleuropein. Thus, all these results show that temperature, pH and the nature of solvent have an effect on the extraction of flavonoids and phenolic compounds including oleuropein. For sonication (Table 2) in 80% ethanol, oleuropein (15.17 ± 0.39 mg/g), gallic acid (3.04 ± 0.06 mg/g), and protocatechuic acid (3.23 ± 0.26 mg/g), were major compounds; whereas caffeic acid, rutin, quercetin, luteolin, and hydroxytyrosol were minor phenolic compounds. However the others were not detectable. In contrast, for acetonitrile 20% all phenolic compounds were undetectable except for hydroxytyrosol which was detected at a concentration of 0.19 ± 0.01 mg/g. For sonication in distilled water, oleuropein was the major compound representing 27.2 ± 0.99 mg/g compared to other compounds such as coumaric acid, protocatechuic acid, rutin, luteolin, hydroxytyrosol and tyrosol. Regarding the maceration technique with two steps (Table 3), it was found that in both extracts oleuropein was found to be the major compound with a concentration of 45.11 ± 1.25 mg/g, respecting the ethanol extraction followed by methanol and 80.67 ± 0.47 mg/g for extraction with ethanol followed by distilled water. Furthermore, it was found that in these two methods the majority of the phenolic compounds were extracted with different concentrations. In ethanol extraction followed by methanol, rutin, gallic

Table 2
Concentration of identified phenolic compounds (mg/g dry weight) in olive leaves extract obtained by sonication with different solvents.

Phenolic compounds	Retention time (min)	80% Ethanol	20% Acetonitrile	Distilled water
Coumaric acid	14.28	ND	ND	0.15 ± 0.06
Caffeic acid	12.13	0.34 ± 0.06	ND	ND
Protocatechuic	11.82	3.23 ± 0.26	ND	0.40 ± 0.00
acid	17.11	0.05 ± 0.01	ND	0.20 ± 0.00
Rutin	21.74	0.02 ± 0.00	ND	ND
Quercetin	22.17	0.09 ± 0.01	ND	0.31 ± 0.13
Luteolin	17.19	3.04 ± 0.06	ND	ND
Gallic acid	09.61	ND	ND	ND
Ferulic acid	07.66	0.02 ± 0.01	0.19 ± 0.01	0.25 ± 0.07
Hydroxytyrosol	18.42	15.17 ± 0.39	ND	27.20 ± 0.99
Oleuropein	08.10	ND	ND	ND
Syringic acid Tyrosol	10.68	ND	ND	0.20 ± 0.14

Values are mean ± standard error (n = 3).
○n.d. not detected.

Table 3
Concentration of phenolic compounds identified (mg/g dry weight) in olive leaves extracts obtained by maceration in two steps with different solvents.

Phenolic compounds	Retention time (min)	Ethanol-methanol	Ethanol -distilled water
Coumaric acid	14.28	1.58 ± 0.13	2.34 ± 0.16
Caffeic acid	12.13	1.18 ± 0.03	2.11 ± 0.01
Protocatechuic acid	11.82	0.08 ± 0.02	1.05 ± 0.07
Rutin	17.11	1.65 ± 0.07	1.15 ± 0.21
Quercetin	21.74	0.19 ± 0.01	0.37 ± 0.04
Luteolin	22.17	0.12 ± 0.00	0 .40 ± 0.28
Gallic acid	17.19	1.76 ± 0.06	2.44 ± 0.00
Ferulic acid	09.61	0.003 ± 0.00	0.10 ± 0.00
Hydroxytyrosol	07.66	0.25 ± 0.06	0.34 ± 0.00
Oleuropein	18.42	45.11 ± 1.25	80.67 ± 0.47
Syringic acid	08.10	0.02 ± 0.00	0.09 ± 0.01
Tyrosol	10.68	0.49 ± 0.06	1.95 ± 0.07

Values are mean ± standard error (n = 3).

acid, caffeic acid, luteolin, hydroxytyrosol, tyrosol, coumaric acid, syringic acid, ferulic acid, quercetin and protocatechuic acid, were found. Whereas in ethanol extraction followed by water rutin, gallic acid, caffeic acid, luteolin, hydroxytyrosol, tyrosol, coumaric acid, syringic acid, ferulic acid, quercetin and protocatechuic acid were detected (Fig. 2). These results are consistent with Benavente-Garcıa et al. [8], who showed that the phenolic compounds of olive leaves consist of oleurosides (oleuropein and verbascoside), flavones (luteolin, diosmetin, apigenin-7-glucose, luteolin-7-glucose and diosmetin-7-glucose), flavonols (rutin), flavan-3-ols (catechin) and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid). Moreover, they showed that the content of each compound could change according to the difference of solvents polarities [20].

Comparing the extraction of phenolic compounds from fresh leaves and dry leaves macerated in ethanol followed by water (Table 4), it was found that once again oleuropein was the major compound in both extracts, but its concentration in dry leaves was very high compared to that in fresh leaves (50.86 ± 0.20 mg/g; $p < 0.01$). These results were in agreement with Silva et al. [16], who showed that the low oleuropein content in fresh leaves was probably attributed to the conversion of this compound by the

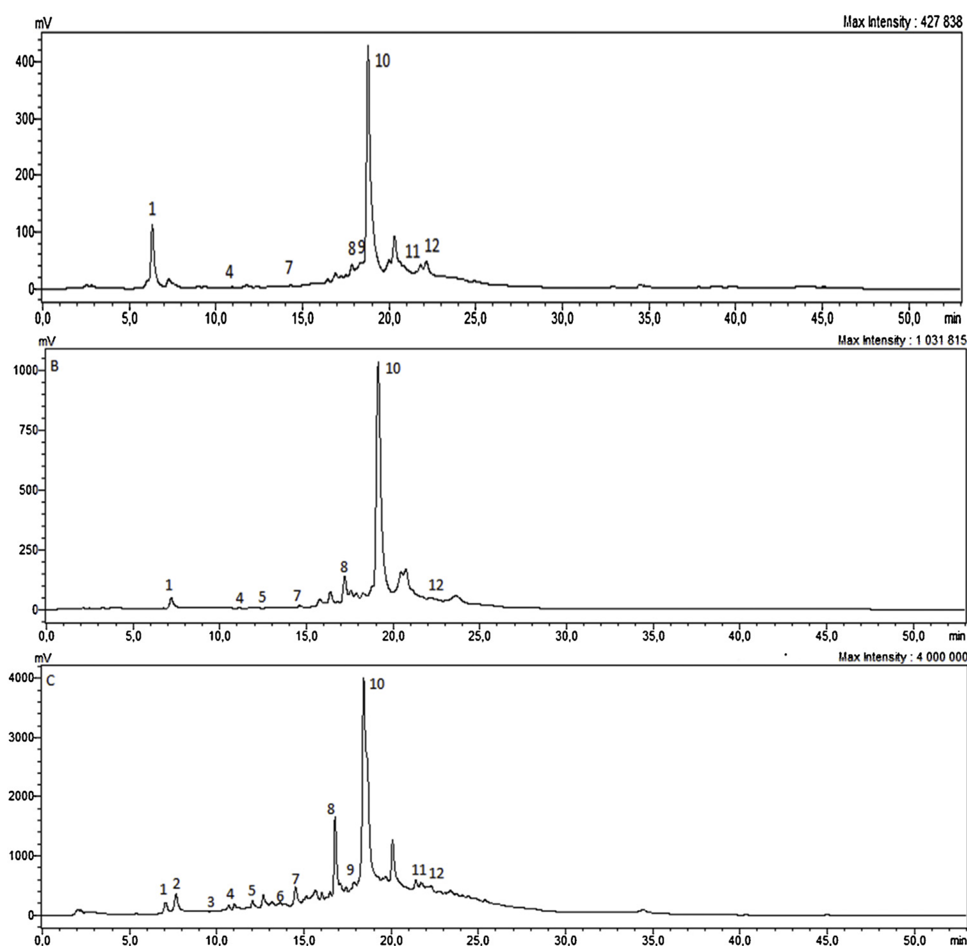

Fig. 2. HPLC chromatograms of the polyphenols of each olive leave extract. A) Maceration in 80% ethanol B) Sonication in distilled water C) Maceration in ethanol followed by water from dried leaves. 1: Hydroxytyrosol, 2: Syringic acid, 3: Ferulic acid 4: Tyrosol, 5: Protocatechuic acid, 6: Caffeic acid, 7: Coumaric acid, 8: Rutin, 9: Gallic acid, 10: Oleuropein, 11: Quercetin, 12: Luteolin. Data are expressed as (means ± standard deviation). n = 3.

Table 4
Concentration of identified phenolic compounds (mg/g dry weight) in fresh leaves macerated in ethanol followed by distilled water.

Phenolic compounds	Retention time (min)	Fresh leaves
Coumaric acid	14.28	1.04 ± 0.06
Caffeic acid	12.13	ND
Protocatechuic acid	11.82	0.65 ± 0.03
Rutin	17.11	0.96 ± 0.73
Quercetin	21.74	0.22 ± 0.06
Luteolin	22.17	0.01 ± 0.01
Gallic acid	17.19	1.15 ± 0.07
Ferulic acid	09.61	ND
Hydroxytyrosol	07.66	1.03 ± 0.01
Oleuropein	18.42	50.86 ± 0.20
Syringic acid	08.10	0.002 ± 0.03
Tyrosol	10.68	0.0007 ± 0.00

Values are mean ± standard error (n = 3).
○n.d. not detected.

β-glucosidase present in leaves. It was also found that in dried leaves the content of other phenolic compounds and flavonoids was very high compared to fresh leaves. Also no caffeic acid and ferulic acid were detected in fresh leaves.

3.3. Antibacterial activity

The results obtained for the antibacterial activity of each prepared extract (inhibition diameter is given by (mm)) are summarized in Table 5. The results of this activity showed different responses regarding the five bacteria tested reflected by different halos diameters. We also found that this antibacterial effect was more important for ethanol prepared extract followed by water for dried leaves (Fig. 3). Besides, this extract exhibited inhibitory activity against all the studied strains. Thus, these results showed that the high content of flavonoids and total phenolic compounds of these extracts canprobably explain their strong antibacterial activities compared to the other techniques used. It is also observed that these extracts have a high oleuropein content, this compound is a Secoiridoid contained in olive leaves, olives and olive oil. This last was responsible for a strong antibacterial activity that could be explained by its surfactant properties which could change the permeability of the cell membrane. [21]. The results of the antibiogram also showed that all strains tested are sensitive to Chloramphenicol with different inhibition diameters. These results are in agreement with the study of Pereira et al [22], who found that olive leaves have antibacterial activity in the following order against *Bacillus cereus* ˜ *Candida albicans>E. coli>S. aureus>P. aeruginosa*. Besides there wasn't any selectivity between Gram-positive and Gram-negative bacteria. This leaves extracthave been

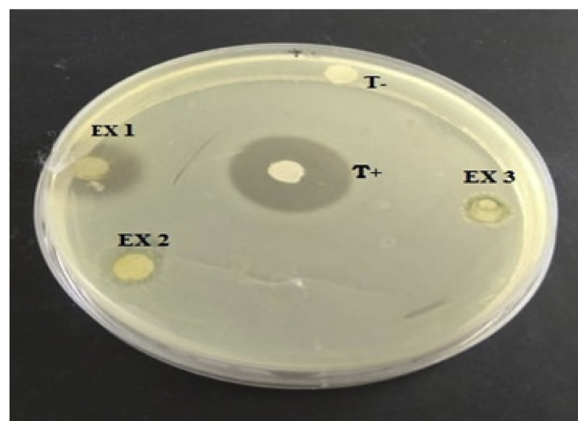

Fig. 3. Antibacterial activity of the best extract obtained by maceration in two steps with ethanol followed by water from dried leaves against *Escherichia coli* bacteria. T-: negative witness (DMSO); T+: positive witness (Chloramphenicol); EX 1: tested extract obtained by maceration with ethanol followed by water; EX 2: tested extract obtained by maceration distilled water; EX 3: tested extract obtained by maceration with 20% acetonitrile.

Table 5
Antibacterial activity of olives leaves extracts at different concentrations (Diameter of inhibition zone in mm).

Extracts	Conc. mg/ml	*Staph. aureus*	*Listeria monocytogene*	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Escherichia. coli*	*Escherichia. coli*
Maceration							
80% Ethanol	30	3.5 ± 0.0	03 ± 0.2	02 ± 0.5	01 ± 0.2	03 ± 0.0	03 ± 0.0
	50	8.5 ± 0.9	07 ± 0.6	5.5 ± 0.8	4.5 ± 0.6	07 ± 0.1	07 ± 0.1
20% Acetonitrile	30	–	–	–	–	–	–
	50	–	–	–	–	–	–
Distilled water	30	–	–	–	–	–	–
	50	–	–	–	–	–	–
Distilled water at 60°C	30	01 ± 0.3	01 ± 0.2	01 ± 0.2	–	02 ± 0.0	02 ± 0.0
	50	4.5 ± 1.0	04 ± 0.1	05 ± 0.2	01 ± 0.3	06 ± 0.4	06 ± 0.4
Distilled water at 60°C with a pH 3	30	01 ± 0.3	01 ± 0 0	02 ± 0.5	01± 0.0	01 ± 0.1	01 ± 0.1
	50	3.5 ± 1.0	3.2± 0.0	03 ± 0.2	02 ± 0.0	03 ± 0.3	03 ± 0.3
Sonication							
80% Ethanol	30	02 ± 0.0	02 ± 0.5	01 ± 0.3	0.9 ± 0.4	01 ± 0.0	01 ± 0.0
	50	03 ± 0.4	03 ± 0.1	01 ± 1.0	1.1 ± 0.4	02 ± 0.7	02 ± 0.7
20% Acetonitrile	30	–	–	–	–	–	–
	50	–	–	–	–	–	–
Distilled water	30	02 ± 0.9	03 ± 0.3	02 ± 0.5	03 ± 0.7	02 ± 1.1	02 ± 1.1
	50	03 ± 1.0	04 ± 0.4	05 ± 0.3	05 ± 1.4	03 ± 0.5	03 ± 0.5
Maceration in two steps							
Ethanol - Methanol	30	07 ± 0.4	06 ± 1.3	07 ± 2.5	2 ± 0.0	07 ± 2.1	07 ± 2.1
	50	10 ± 1.5	12 ± 1.0	09 ± 1.3	5 ± 1.2	10 ± 0.0	10 ± 0.0
Ethanol – water distilled	30	08.5 ± 1.0	09 ± 0.4	11 ± 1.3	09 ± 0.3	11 ± 0.3	11 ± 0.3
	50	10.5 ± 0.0	15.5 ± 0.4	15 ± 1.3	12.5 ± 1.3	15 ± 1.3	15 ± 1.3
Ethanol followed by water distilled	30	05 ± 1.2	08 ± 0.3	04 ± 0.5	03 ± 0.0	03 ± 0.3	03 ± 0.3
Fresh leaves	50	09 ± 2.0	10 ± 0.9	07 ± 0.3	07 ± 0.0	05 ± 0.3	05 ± 0.3
	30	16 ± 0.8	16 ± 2.3	16 ± 0.3	14 ± 2.4	19 ± 0.0	19 ± 0.0
Chloramphenicol	50	21 ± 2.9	20 ± 0.4	19 ± 2.3	20 ± 2.5	26 ± 0.9	26 ± 0.9

Values are mean ± standard error (n = 2).
-: No antibacterial activity.

able to denature proteins and affect the permeability of the cell membrane of the bacteria. Similarly, Lee and Lee [23] reported that the combined phenolic mixture prepared from the olive leaves extract exhibited inhibition effects against *B. cereus* and *S. enteritidis*. In addition, Owen et al. [24] reported that olive leaves showed antimicrobial activity against *E. coli*, *S. aureus*, *B. cereus*, *S. typhi* and *V. parahaemolyticus*.

4. Conclusion

In conclusion, the maceration of olive leaves in ethanol followed by water for dried leaves resulted in the higher yield of phenolic compounds and flavonoids in term of total contents of these compounds and also demonstrated by the variety of phenolics identified by HPLC method. This feature explains the inhibition effect towards the pathogenic bacteria tested.

The richness of olive leaves in valuable molecules could be exploited in recovery of phenolic compounds with various applications in food, cosmetic and pharmaceutical industries. In this way further studies are conducted to valorize oleuropein throughout bioconversion into hydroxytyrosol which is a high added value product.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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