Phenolic Content, Antioxidant and Antimicrobial Activities of “Chemlali” Olive Leaf (*Olea europaea* L.) Extracts

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Abstract. In last decade, there is an increasing interest in researches for production of biologically active compounds from natural sources. *Olea europaea* L. is used in traditional medicine in the Mediterranean areas. The aim of the current study was to investigate the content of phenol compounds and flavonoids extracted from “Chemlali” olive leaf collected from Mascara region (west of Algeria) followed by the assessment of *in vitro* antioxidant and antimicrobial activities of the phenolic extracts against a wide spectrum of resistant human pathogens. Extraction was conducted at room temperature using four solvents: deionised water (ddH₂O), 80% methanol (MeOH), and petroleum ether. Total phenols and total flavonoids were measured using the Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. The antioxidant properties have been determined by DPPH test. Results showed that the total phenol and flavonoid contents of the olive leaf extracts ranged from 3.64 in petroleum ether extract to 21.47 ± 0,05 mg gallic acid equivalents (GAE)/ g dried matter and from 3.33mg ± 0.07 to 17.64mg ± 0.07mg catechin equivalents (CE)/ g dried matter, respectively. Furthermore, our results revealed that extracting solvents have a significant influence on the antioxidant and antimicrobial properties. The three extracts possessed antibacterial activity against tested Gram-positive and Gram-negative bacteria particularly petroleum ether extract. The extract antimicrobial activity may be due to the presence of secoiridoid class. In conclusion, the data obtained in this study confirming the traditional use of this plant in treatment of infectious diseases. Phenolic compounds in olive leaves are major contributors to the antioxidant and antimicrobial effects of olive leaves. However, further detailed studies are required to determine the active ingredients responsible for these effects and to determine the mechanism of action of these compounds in the anti-microbial activity.

1. Introduction

The success story of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms. The investigation of some indigenous plants for their antimicrobial properties may yield useful results. A large number of plants indeed were used to combat different diseases and known to possess antimicrobial activity [1].

Olive tree (*Olea europaea* L.) (Figure 1) is one of the most important fruit trees in Mediterranean countries; they cover 207.822 ha in Algeria [2]. It represents a great economic and social importance, and it may be a potential source of the possible benefits to be derived from utilization of any of its byproducts. *Olea europaea* preparations have been used widely in folk medicine in European Mediterranean area, Arabia peninsula, India and other tropical and subtropical regions as diuretic, hypotensive, emollient and for urinary and bladder infections [3]. Olive leaves contain high quantities of phenol substances very similar to those present in olives and their derived products. Methanolic extracts of olive leaves contain secoiridoids such as oleuropein, ligostroside, dimethyleuropein, and oleoside [4] flavonoids, including apigenin, kaempferol, and

luteolin; as well as phenolic compounds such as caffeic acid, tyrosol, and hydroxytyrosol [5]. Oleuropein, the major phytochemical in olive leaf, is a complex phenol present in large quantities in olive leaves but in lower quantities in olive oil [6]. Oleuropein can be hydrolyzed to hydroxytyrosol, elenolic acid, oleuropein aglycone, and glucose [7, 8].

Figure 1. Illustration of Olive aerial parts (*Olea europaea*).

In vivo, olive leaf extract (OLE) lowered blood cholesterol [9] and lipid [10] concentrations in cholesterol-fed rats and lowered blood pressure in nitro-L-arginine methyl ester-induced hypertensive rats [11] as well as in normotensive rats [12]. In streptozotocin-induced diabetic rats, OLE decreased serum concentrations of glucose, lipids, uric acid, creatinine and liver enzymes [13]. To the best of our knowledge, no study has been done on the antioxidant and antimicrobial activities and phenolic compounds of the Algerian *Chemlali* olive leaves variety. Hence, the aim of this study was to investigate the antioxidant and antimicrobial activities as well as total phenolics, total flavonoids of the aqueous, methanolic and petroleum ether *Chemlali* olive leaves extracts.

2. Material and Methods

2.1. Plant material

Plant material used in present research (Algerian *Chemlali* olive leaves), were collected from the region of Mascara (Hachemet Zalamta) in the west of Algeria. Olive leaves variety was confirmed by ITAF (Technical Institute for Fruit Trees) experts. The plant material was air dried in shade at room temperature. The leaf petioles were carefully manually separated and dry leaves were pulverized (3x1 min in high speed grinder) into powder and stored at 4°C protected from light until further use.

2.2. Sample preparation

For the extraction method optimization, a fine dried powder (50 g) of sample were added to 500 mL distilled water, methanol or petroleum ether, homogenized and shaking at 150 rpm for 24 h, at room temperature. The extracts were filtered through Whatman No.1 filter. The residue was then extracted with two additional 100 mL portions of solvent. The combined extracts were evaporating by using a rotary evaporator or freeze dryer to give the crude dried extract. The dried extracts were stored at -20°C until used.

2.3. Phytochemical screening

The screening of chemical constituents was carried out with the methanolic, petroleum ether and aqueous extracts using chemical methods and thin-layer chromatography (TLC) according to the methodology given in [14, 15].

2.4. Determination of total phenol content

Phenolic compounds concentration in the extracts was estimated by a colorimetric assay based on procedures described by Singleton *et al.* [16] with some modifications. Briefly, 1 mL of sample was mixed with 1 mL of Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of saturated Na₂CO₃ solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Values of total phenol content (TPC) were estimated by comparing the absorbance of each sample with a standard response curve generated using gallic acid (0, 12.5, 25, 50, 100 and 200 µg/mL). The results were expressed as mg gallic acid equivalents (GAE)/ g dried matter. All the measurements were taken in triplicate and the mean values were calculated.

2.5. Total flavonoids content

The total flavonoid content was determined by using the colorimetric assay according to Lamaison and Carnet 1990 [17] and Huang *et al.* 2004 [18]. A calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CE)/ g dried matter. Briefly, an aliquot of 1 mL of each sample or standard solution was added individually to equal volumes of solution of 2 % AlCl₃, 6 H₂O, mixed evenly and allowed to stand at room temperature for 10 minutes. The absorbance was then read at 367 nm.

2.6. Determination of antioxidant activity using the DPPH method

In order to measure antioxidant activity, DPPH (2,2-diphenyl-2-picrylhydrazyl) free radical scavenging assay was used. The method was carried out as described by Mansouri *et al.*, 2005 [19]. DPPH solution was prepared by solubilization of DPPH (2.4 mg) in methanol (100 mL). Next, (50 µL) of each extract was removed and mixed with the DPPH solution (1.95 mL) in a test tube. After 30 minutes, the absorbance of these solutions was read at 517 nm. A duplicate reading was performed for each sample. The positive control in this assay was ascorbic acid and BHT used at the same concentrations of extracts, and subjected to the same aforementioned procedures for the quantification of antioxidant activity.

The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH Scavenging Effect (\%)} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \cdot 100$$

2.7. Determination of the antimicrobial activity

- Test microorganisms

The following microorganisms were used as test organisms in the screening: two Gram-positive bacteria namely, *Staphylococcus aureus* (Clinical isolated) and *Bacillus subtilis* subsp. *spizizenii* (ATCC 6633), four Gram-negative bacteria, *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Salmonella enterica* subsp. *heidelberg* (ATCC 8326), *Klebsiella pneumoniae* (clinical isolated) and one yeast *Candida albicans* (Clinical isolated). The microorganisms were supplied from three origins, Algerian Pasteur Institute, the laboratory of microbiology (Yessad Khaled hospital of Mascara) and the laboratory of microbiology in Mascara University (Algeria).

- Antimicrobial activity assay

Antimicrobial activity was determined by the agar disc diffusion assay [20]. The extracts were dissolved in Dimethyl Sulfoxide (DMSO) or distilled water. Petri plates were prepared with 20 ml of sterile Mueller Hinton Agar (MHA) (Sigma, France) inoculate by suspension of cell (1 ml) adjusted by McFarland 0.5 method (10^6 CFU /ml). Sterile filter paper discs of 6 mm diameter were impregnated with 20 μ L of the extract solution. The plates were incubated at 37°C for 24 h. Gentamycin (15 μ g), Amoxicilline (25 μ g), Erythromycin (15UI) and Amphotericin (10%) were used as positive controls. Negative controls were performed using paper discs loaded with 20 μ L of the solvent used DMSO. The antimicrobial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. After that, the inhibition zones were measured in millimeters by Vernier Calipers. All tests were repeated three times to minimize test error. An inhibition zone of 12 mm or greater (including diameter of the disc) was considered as high antibacterial activity.

2.8. Statistical analysis

Values are given as arithmetic means $\pm$ standard error of the mean (S.E.M.). Data were analyzed using one-way analysis of variance (ANOVA), *P* values <0.05 were considered as significant.

3. Results and Discussion

Understanding of the chemical composition and potential biological properties of plant extracts is of essential importance for their further use in the food industry or alternative therapy. A range of spectrophotometric assays was applied to create a data base which could be used to evaluate the potential of olive leaves as an inexpensive and accessible natural resource for the production of polyphenol-rich extracts with strong antimicrobial and antioxidant activity.

3.1. Phytochemical analysis

The preliminary phytochemical qualitative screening of the aqueous, methanolic and petroleum ether extracts of the leaves of *Olea europaea* revealed the presence of iridois, flavonoids and tannins in methanolic and aqueous extracts as shown in **Table 1**.

Table 1. Preliminary phytochemical screening of the investigated extracts of *Olea europaea*.

Extracts	Flavonoids	Condensing tannins	Hydrolysable tannins	Coumarins	Iridoids
Pet. ether	+/-	-	-	+/-	+/-
Methanol	++	+++	-	-	+
Aqueous	+++	+++	-	-	+

(+) Presence ; (-) Absence.

Water and methanol are the most polar solvents that elute polar substances, but petroleum ether has a medium polarity, so the substances that have the most important presence in our extracts were polar ones.

3.2. Extract Yields, Total Phenolic and Total Flavonoid Contents

The results of extract yields, total phenolic and flavonoid contents are shown in Table 2. The significant differences in the extraction yield between metabolic, aqueous and petroleum ether extract of same sample might be ascribed to the varying availability of extractable components. A higher extraction yield was obtained in the methanolic extract.

Table 2. Results of extract yields, total phenolic and flavonoid contents of *Chemlali* Olive Leaf (*Olea europaea* L.) Extracts.

Extract	Yields	Total phenolic contents	Total flavonoid contents
Pet. ether	2.1	3.64 ± 0.91	3.33 ± 0.07
Methanol	30	21.47 ± 0.05	17.64 ± 0.17
Aqueous	7.9	10.5 ± 1.23	6.45 ± 0.03

The quantitative estimate of the total phenolic compounds *Olea europaea* leave extracts estimated by the Folin-Ciocalteu method revealed that the highest total phenolic content was found in methanolic extract (21.47 ± 0.05 mg GAE/g dried matter) followed by aqueous extract (10.5 ± 1.23 mg GAE/g dried matter), the lowest content was registered in petroleum ether extract (3.64 ± 0.91 mg GAE/g dried matter). In general, the results indicate that the solvent of extraction influenced the phenolic content and the extract yields.

Generally, polyphenols values of the methanolic extract are higher than those reported by Boudhioua N. et al. 2008 [21] in a Tunisian *Chemlali* variety ($13.80 \text{ mg} \pm 0.05$ mg GAE/g dried matter).

The flavonoids results expressed as mg catechin equivalents (CE)/ g dried matter with reference to a standard curve ($Y = 0.0067x + 0.0132$, $r^2 = 0.999$), established their presence in all olive leave extracts with a highest value in methanolic extract ($17.64 \text{ mg} \pm 0.07$) and minimum value in petroleum ether ($3.33 \text{ mg} \pm 0.07$).

3.3. DPPH Radical Scavenging Activity

The characteristic feature of antioxidants to scavenge DPPH free radical is well accepted and is therefore most often selected as a reliable tool to evaluate the free radical scavenging capacity of different plant extracts. Interestingly, the DPPH radical assay is incredibly sensitive towards active ingredients even at lower concentrations. Another beneficial aspect is that this test is time saving and can be used to analyze a batch of samples in a shorter time. This procedure has been often used for the assessment of free radical scavenging ability of plant-based antioxidant extracts [22]. It is generally predicted that DPPH radical scavenging activity, and with it antioxidant activity, is strongly affected by the amount of phenolic compounds as well as the degree of hydroxylation of the phenolic compounds [23].

The DPPH radical scavenging activity of olive leaves extracts was tested and compared with Butylated Hydroxytoluene (BHT) and ascorbic acid (Figure 2). The scavenging activity of Ascorbic acid was found to be higher than those of the three extracts. The antioxidant activity of methanolic and aqueous extract were significantly ($p < 0.05$) higher than petroleum ether extract antioxidant activity.

The correlation coefficient (r) between the free radical (DPPH) scavenging activity (%) and the total phenolic and total flavonoids content was determined. The DPPH scavenging activity and the total phenolic content displayed a better correlation ($r^2 = 0.60$, $p < 0.01$). However, no significant correlation was obtained between flavonoids content and free radical (DPPH) scavenging ability ($r^2 = 0.39$).

It had been reported that Olive leaf has antioxidant properties associated with oleuropein [24]. Caffeic acid was also reported to have antioxidant activity through the scavenging of superoxide anion [24]. On the other hand, olive leave flavonoids, phenols and oleuropeosides have been shown to possess important antioxidant activity towards free radicals, which is principally based on the redox properties of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure [25, 26].

Figure 2. Free radical scavenging activity of different phenolic extracts of olive leaves.

3.3. Antimicrobial activity

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. The World Health Organization estimates that plant extract or their active constituents are used as folk medicine in traditional therapies of 80% of the world's population. In the present work, the extracts obtained from “*Chemlali*” olive leaves show strong activity against most of the tested bacterial and fungal strains. The results were compared with standard antibiotic and fungicide drugs.

Antimicrobial activity as summarized in Table 3, revealed that the *Chemlali* olive leaf (*Olea europaea*) extracts showed antimicrobial activity against a wide spectrum of micro-organisms.

The Petroleum ether extract exhibited strong activities against *E. coli* with Diameter of Inhibitory Zone (DIZ) measured at 13 mm, *S. enterica* (13 ± 0.50 mm) (Figure 3), while moderate antibacterial activity was found against other bacterial strains. On the other hand, the methanolic extract was found to be less active against the most tested micro-organisms with an exception of *S. enteric* which registered strong effect ($DIZ = 15 \pm 1.1$ mm). Further, *K. pneumoniae* was the most resistant microorganism.

Table 3. Antimicrobial activity of the crude extracts of olive leaves in agar diffusion assay.

Extracts	Microbial strains						
	<i>E. co.</i>	<i>E. cl.</i>	<i>K. p.</i>	<i>S. e.</i>	<i>S. a.</i>	<i>B.s.</i>	<i>C. a.</i>
Pet. ether	13±0.4	8.16±0.15	-	10.86±0.15	13±0.5	8.33±2.3	10.86±0.15
Methanol	-	11±0.20	-	8±0.09	15	10.5±0.43	8±0.09
Aqueous	-	-	-	9±0.22	14±0.09	11±0.07	9±0.22
Amoxicillin	25	18	15	18	-	-	nt
Gentamycin	28	-	23	25	26	20	nt
Erythromycin	-	-	-	-	27	12	nt
Amphotericin	nt	nt	nt	nt	nt	nt	-
DMSO	-	-	-	-	-	-	-

E.co.: *Escherichia coli* ATCC 25922; *E.cl.*: *Enterobacter cloacae* ATCC 13047; *K.p.*: *Klebsiella pneumoniae* (Clinical isolated); *S.e.*: *Salmonella enterica* subsp. *heidelberg* (ATCC 8326); *S. a.*: *Staphylococcus aureus* (Clinical isolated); *B.c.*: *Bacillus subtilis* subsp. *spizizenii* ATCC 6633; *C.a.*: *Candida albicans* (Clinical isolated); (-): no activity; nt: not tested; Inhibition zones including the diameter of the paper disc (6 mm); each value is represented as mean $\pm$ DS, (n=3).

Aqueous extract showed the maximum DIZ against *S. aureus* and *B. subtilis* (14 ± 0.09 mm and 11 ± 0.07 , respectively), while control discs possessed significantly higher antimicrobial activity compared with the extracts.

As previously described, individual phenolic compounds present in the olive leaves extracts were identified and quantified, but we choose to submit the entire extracts to antimicrobial activity studies for several reasons. First, in a general way, the antimicrobial capacity of phenolic compounds is well-known [27]. In addition, extracts may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extracts [28]. According to Liu [29] additive and synergistic effects of phytochemicals are responsible for their potent bioactive properties.

The high content of phenolic compounds identified in the extract might contribute for its antimicrobial properties. Hydroxytyrosol and oleuropein are other phenolic compounds that have been proven to inhibit or delay the rate of growth of several human intestinal or respiratory tract pathogens, namely *Haemophilus influenzae*, *Moraxella catarrhalis*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Vibrio cholerae* and *Vibrio alginolyticus* [30].

Figure 3. Antimicrobial activity of “Chemlali” *Olea europaea* leaves extracts against assessed by disc diffusion method.

The unusual combined antibacterial and antifungal action obtained in this study for olive leaf extracts is in agreement with that reported by Nychas et al. [31]. Olive leaves may be useful in cases where prolonged use of antibiotics encourage development of opportunistic infections [32], being especially effective against *S. aureus* and *S. enterica*, two bacterial genera which pose the major resistance problems. Furthermore, the *Olea europaea* leaves extracts have the potential use as dietary ingredients or nutraceutical agents [34, 35].

Conclusion

Polyphenol-rich fractions from “Chemlali” *Olea europaea* leaves were found to possess promising antioxidant and antimicrobial activities to treat infectious diseases due to multi-resistant bacterial strains. However, further detailed studies are required to determine the active ingredients responsible for these effects and to determine the mechanism of action of these compounds in the anti-microbial activity.

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