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Original Article

ANTIBACTERIAL, ANTIFUNGAL AND ANTIOXIDANT ACTIVITIES OF TUNISIAN *OLEA EUROPAEA SSP. OLEASTER* FRUIT PULP AND ITS ESSENTIAL FATTY ACIDS

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ABSTRACT

Objective: This study was conceived to evaluate the essential fatty acids, secondary metabolite, antimicrobial and antioxidant activities of *Olea europaea ssp. oleaster* fruits pulp methanolic extract.

Methods: Analysis of the lipid content from unexploited *Olea europaea ssp. oleaster* pulp was carried out using gas chromatography. The antioxidant activity was evaluated by DPPH radical scavenging. The antimicrobial activity was also tested against seven pathogenic bacteria, two fungal species and one yeast strain using two methods.

Results: The obtained results showed that the major components of fatty acids were oleic acid (77.4%) and elaidic acid (17.58%). Moreover, the tested extract was rich in phenol (84.04±0.01 mg GAE/g DW) than in flavonoids (60.41±0.02 mg RE/g DW). In addition, it showed puissant antioxidant (IC_{50} = 28±0.01 µg/mL), antibacterial and antifungal activities. The inhibition zones diameters and the minimum inhibition concentration values for tested microorganisms were in the range of 13-18 mm and 3.125-25 mg/mL, respectively.

Conclusion: This study shows that *Olea europaea ssp. oleaster* fruit pulp could be developed into ingredients for use in foods as the natural antioxidant and antimicrobial agent.

Keywords: Essential fatty acids, Antiradical activity, Antimicrobial activity, *Olea europaea ssp. Oleaster*, Secondary metabolite.

INTRODUCTION

Secondary metabolites are present in all higher plants. They play an important role in the plants protection against bacteria, virus, fungi and insects. For example, phenolic compounds are plant secondary metabolites which play important roles in disease resistance [1, 2]. In fact, *Olaecea* is one of the large plant families used as a framework to evaluate the occurrence of some typical secondary metabolites [3]. The olive (*Olea europaea* L.) is a small tree, which belongs to the family *Oleaceae* and is native to tropical and warm temperate regions of the world. *Olea europea* L. is the most widespread and important plant in the Mediterranean countries. Indeed, 98% of olive production worldwide is concentrated in the Mediterranean basin. A considerable part of olive production is processed. The olive is the cultivated form, whereas the oleaster is the wild form of *Olea europaea* subsp. *Europaea* [4]. They are called var. *europaea* and var. *sylvestris*, respectively [5]. The crop is propagated either by cuttings or grafts and therefore cultivars are clones. The transition between the oleaster and the olive is based on the size of the pit remains in artifact records. Nevertheless, they are more than 2000 cultivars in the Mediterranean basin that displays huge diversity based on fruit morphology, pit size and morphology. Several modern cultivars display as small pits as the oleaster, making the distinction criteria doubtful [6].

The tree, famous for its fruit, also called the olive, is commercially important in the Mediterranean region as a prime source of olive oil [7]. This plant can be used in crude from without refining. Thus conserving the essential vitamins, fatty acids and other natural elements of dietic importance. *Olea europaea* L. is widely studied for its alimentary use (the fruits and the oil are important components in the daily diet of a large part of the world's population), whereas the leaves are important for their secondary metabolites such as the secoiridoid compounds, oleacein and oleuropein. Previous investigations carried out on olive leaf extracts have demonstrated their hypotensive, hypoglycaemic, hypouricaemic, antimicrobial and antioxidant activities [8-10]. Even though they are a number of studies about the secondary metabolite and biological activities of *O. Oleaster*,the information about the characteristic of oleaster has been scarcely investigated. The aims of the current work were to evaluate the essential fatty acid composition, secondary metabolite as well as antimicrobial and antiradical activities of *O. Oleaster* pulp harvested in the north-western part of Tunisia (Béja).

MATERIALS AND METHODS

Plant material and preparation of the methanol extract

The plant material (fruits pulp of *Olea europaea ssp. oleaster*) used in this study was collected from the north-western part of Tunisia (Béja) in March 2012. The plant was botanically confirmed using available literature [11]. The air-dried fruits pulp was finely ground with a blade-carbide gringing (IKA-WERK Type: A: 10). Triplicate sub-samples of 1 g were separately extracted by stirring with 10 ml of pure methanol for 30 min. The extract was then kept for 24 h at 4°C, filtered through a Whatman No. 4 filter papers, evaporated under vacuum to dryness and stored at 4°C until analyzes [12].

Fatty acids extraction and analysis conditions

Fatty acids (FAs) of *O. oleaster* fruit pulp was extracted with cylohexane in a soxhlet apparatus for 6 h. The extract was concentrated under reduced pressure using a rotary evaporator at 60 °C. The extract was kept in obscurity at 4 °C until analyzes. For FAs analyses, 20 mg of oil was stabilized with 1 mL TBME (Tert Butyl Metyl Ether). Then, FAs were derivatized into their corresponding methyl esters (FAMEs) by adding 50 µL of trimethyl sulfonium hydroxide (TMSH, 0.5M) in methanol. The analyses were done on a capillary gas chromatography Varian (CPG). The injection was split 1:100 at 250 °C. The column used was Select CB for FAME fused silica WCOT (50 m x 0.25 mm; film thickness 0.25 mm). The temperature gradient was 185 °C for 40 min, then 15 °C/min to 250 °C, and 250 °C for 10 min. The analysis time was 55 min. The detector FID was set up 250 °C. The helium was the carrier gas at 1.2 mL/min.

Determination of total phenolic contents

Total phenolic contents of the tested extract were determined using Folin–Ciocalteu reagent according to the method previously reported by Slinkard and Singleton [13]. Total phenolic content of plants was expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW) through the calibration curve with gallic acid.

Determination of total flavonoids contents

Total flavonoid contents were determined according to that previously described by Popova et al. [14], with some modifications. 1 mL of 2% aluminium trichloride ($AlCl_3$) in methanol was mixed with the same volume of 1 mL of plant extract and the volume was made up to 25 mL with methanol. The mixture was left for 40 min and the absorbance at 420 nm was measured in a Shimadzu 160-UV (Tokyo, Japan) spectrophotometer. The results are given as rutin equivalent per gram dry weight (mg RE/g DW). All samples were analyzed in triplicate.

Condensed tannin content

In presence of concentrated H_2SO_4, condensed tannins were transformed by the reaction with vanillin to anthocyanidols [15]. 50 µL of the methanolic organ extract - appropriately dilute was mixed with 3 mL of 4% methanol vanillin solution and 1.5 ml of H_2SO_4. After 15 min, the absorbance was measured at 500 nm. Condensed tannin content of tested extract (three replicates per treatment) was expressed as mg catechin equivalents per gram dry weight (mg CE/ g DW) through the calibration curve with catechin.

Antioxidant activity evaluation using free radical scavenging activity (DPPH)

The effect of methanolic extract of *Olea europaea ssp. oleaster* fruit pulp on DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging was estimated according to Hatano et al. [16] Tests were carried out in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation: Scavenging effect (%) = $[(A_0\text{-}A_1)/A_0] \times 100$, where A_0 and A_1 are the absorbance of the control and the sample, respectively, after 30 min at 517 nm. Sample concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against sample (extract solution) concentrations.

Antimicrobial activity

Microbial strains

The methanol extracts of *O. Oleaster* fruit pulp was tested for their antibacterial and antifungal activities against ten indicators microorganisms including seven bacteria reference pathogenic (*Escherichia coli* ATCC 8739, *Salmonella typhimurium* NCTC 6017, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Aeromonas hydrophila* EI, *Listeria monocytogenes* ATCC 7644, and *Bacillus cereus* ATCC 1247), two fungi (*Aspergillus niger* and *Aspergillus flavus*) and one yeast (*Candida albicans* ATCC 2091) species [17].

Bacterial strains were grown in trypto-caseine soja agar (TSA) and incubated at 37°C for 24h. Fungal species were grown on potato dextrose agar (PDA) plate at 28ºC for 72h. *Candida albicans* was grown on sabouraud dextrose agar (SDA) plate at 30°C for 48h.

Disc diffusion method

The antibacterial and antifungal activities of the methanol extract of *O. Oleaster* fruit pulp were determined, firstly, by disc diffusion assay [17]. Briefly, 100 µL of suspension containing 10^8 CFU/mL of bacteria cells, 10^6CFU/mL of yeast, and 10^4spore/mL of fungi were spread on Petri plates containing TSA, SDA, and PDA medium, respectively. The paper discs (6 mm in diameter) were separately impregnated with 15 µL of the evaluated extract and placed on the agar which had previously been inoculated with the selected test microorganisms. Gentamicin (10 µg/disc) and amphotericin B (20 µg/disc) were used as a positive reference for bacteria and fungi, respectively. Discs without samples were used as a negative control. Plates were kept at 4 ºC for 1 h. The inoculated plates were incubated for 24 h for pathogenic bacterial strains, 48 h for yeast and 72 h for fungi isolates at 37°C, 30°C and 27°C, respectively.

Antimicrobial activity was assessed by measuring the diameter of the growth-inhibition zone in millimetres (including disc diameter of 6 mm) for the tested micro-organisms comparing to the controls. The measurements of inhibition zones were carried out three times and values were the average of three replicates.

Determination of MIC and MBC

The minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were also determined using a broth dilution method [17]. All antibacterial and antifungal tests were performed in TCS and Sabouraud broth, respectively. Overnight broth cultures were diluted in peptone water (0.1% (v/v)) to obtain working culture (10^5 CFU/mL). Serial dilutions, ranging from 0.07 to 50 mg/mL of the studied extract, were used, including one growth control (TCS for bacterial strains and Sabouraud for fungal ones) and one sterility control (appropriate medium + tested extract). Tubes were incubated for 24 h for pathogenic bacterial strains, 48 h for yeast and 72 h for fungi at 37°C, 30°C and 27°C, respectively and the MICs and MBCs were determined. Microbial growth was indicated by the presence of turbidity and a 'pellet' on the tube bottom. MICs were determined presumptively as the first tube, in ascending order, which did not produce a tube bottom. To confirm MICs and to establish MBCs, 10 µL of broth was removed from each well and inoculated on TCS plates for bacterial strains, on PDA plates for fungal and on SDA for yeast. After incubation, the number of surviving organisms was determined. The MIC was the lowest concentration which resulted in a significant decrease in inoculum viability (> 90%), while the MBC was the concentration where 99.9% or more of the initial inoculum was killed. Each experiment was repeated at least three times and the modal MIC and MBC values were selected.

RESULTS AND DISCUSSION

Fatty acid composition

As shown in table 1, twenty fatty acids were detected in the *O. oleaster* oil. Oleic acid (77.4%) and elaidic acid (17.58%) were considered as major fatty acids. It is clearly shown that this oil is an important source of essential fatty acids. This gives a nutritional and medicinal importance for these plant seeds. Moreover, the monounsaturated fatty acid has an important physiological role [18]. Oleic acid was found in larger amounts in pulp of *O. oleaster* indicates that the oil obtained from these varieties are of good quality and beneficial for health, because this un-saturated fatty acid constitutes structural component of membranes. Fatty acids have important functions such as being used as a source of energy invertebrate and invertebrate animals and forming structure of biological membranes [19]. It is possible to generalize fatty acids found in high organized plants and animals.

Table 1: Fatty acid composition of *Olea europaea ssp. oleaster* fruit pulp

Nomenclature	Percent (%)	Name
C10:0	0.39 ±0.01	Capric acid
C12:0	0.07 ±0.001	Lauric acid
C13:0	0.30 ±0.005	Tridécyclic acid
C14:0	0.11 ±0.005	Myristic acid
C15:1	0.08 ±0.005	Pentadecyclic
C16:0	0.06 ±0.006	Palmitic acid
C17:0	0.07 ±0.005	Marguaric acid
C17:1	0.07 ±0.0005	Heptaguaric acid
C18:0	0.05 ±0.002	Stearic acid
C18:1 cis	**77.40 ±0.45**	**Oleic acid (omega- 9)**
C18:1 trans	**17.58 ±0.28**	**Elaidic acid**
C18:2	1.30 ±0.05	Linoleic acid
C18:3	0.82 ±0.01	α-linolenic
C20:0	0.10 ±0.01	Arachidic acid
C20:3	0.17 ±0.005	Homo-gamma-linolenic acid
C20:4	0.12 ±0.005	Eicosatetraenoic acid
C22:0	0.83 ±0.005	Behenic acid
C22:1	0.24 ±0.005	Erucic acid
C24:0	0.18 ±0.005	Lignoceric acid

Total polyphenol and flavonoids contents

The results of the quantitative determination of the polyphenols, tannins and flavonoids of the tested extract are summarized in table 2. This extract was rich in phenol than in flavonoids. In fact, total phenol content was 84.04±0.01 mg GAE/g DW, whereas total flavonoid content was 60.41±0.02 mg ER/g DW. The condensed tannin content was 40.8±0.01 mg CE/g DW. Generally, plant materials rich in phenolics are increasingly being used in the food industry because they retard oxidative degradation of lipids and improve the quality and nutritional value of food [20]. Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified [21].

Phenolic compounds of plants are also very important because their hydroxyl groups confer scavenging ability. The high contents of total phenolic compounds in *O. oleaster* fruit pulp methanolic extract can contribute to important antiradical and antimicrobial activities of this extract. Those findings justify the use of different plant organ of the studied species overall its distribution area in the world by ancient and actual local human populations.

Table 2: Total phenol, flavonoid, condensed tannin contents and antioxidant activities of *Olea europea ssp. oleaster* extract

Total phenol content (mg GAE/g DW)	Flavonoides (mg ER/g DW)	Condensed tannin (mg CE/g DW)	DPPH IC_{50} (µg/mL)	Positive control (BHT) (µg/mL)
84.04±0.01	60.41±0.05	40.08±0.01	28±0.01	27±0.01

Antioxidant activity

The antioxidant activity of the methanol extract of *O. oleaster* fruit pulp was evaluated by DPPH. The extract improved 50% inhibition at higher concentrations, indicating lesser antioxidant capacity. Lower IC_{50} value indicated higher antioxidant activity. The evaluated extract showed important antioxidant activity being close to control positive (Table 2). In fact, the obtained result was not significantly different from Butylated Hydroxytoluene (BHT), with its IC_{50} values were 28 µg/mLand 27 µg/mL, respectively. This could be assigned to the presence of some phenolic compounds (Table 2). It is well known that phenolic substances such as flavonoids, phenolic acids and tannins contribute directly to the antioxidant capacity of plants [22]. Indeed, various studies highlighted the correlation between the phenolic content and the antioxidant capacity of plant extracts [23, 24], indicating that extracts with highest polyphenol contents show higher antioxidant activity. Phenolic compounds exhibit considerable free radical-scavenging activities and metal ion-chelating properties, preventing metal-induced free radical formation [25]. In addition, phenols are organic compounds that contain a hydroxyl group bound directly to the aromatic ring, and the H-atom of the hydroxyl group can trap peroxyl radicals, preventing other compounds to be oxidized [26].

Antifungal and antibacterial activities

The *in vitro* antimicrobial potential of *O. oleaster* fruit pulp extract against the employed micro-organisms was assessed by the presence or absence of inhibition zones, MIC and MBC values. The obtained results showed that the tested extract had great potential for antibacterial, antifungal and anticandidal activities against all evaluated microorganisms (Table 3). The maximal inhibition zones diameters, MIC and MBC values for tested microorganisms, which were sensitive to the extract, were in the range of 13-18 mm, 3.125-25 mg/mL and 6.25-50 mg/mL, respectively. In addition, the extract appeared to be more efficient against fungus and yeast species than against bacterial strains. In fact, *O. oleaster* fruit pulp extract was most efficient against both Gram-positive (*B. cereus*, *L. monocytogens*, *S. aureus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *A. hydrophila*, *S. typhimurium*). There is evidence in the literature that Gram-positive bacteria are more sensitive to plant oil and extracts than Gram-negative bacteria ones because of hydrophobic lipopolysaccharide in the outer membrane which provides protection against different agents [27, 28]; however, the results in this study showed that the methanolic extract of *O. oleaster* fruit pulp did not have selective antibacterial activity on the basis of the cell wall differences of bacterial microorganisms.

The antimicrobial effect of phenols has long been known. In literature, it has been indicated that the antibacterial and antifungal activities depends on the nature of the phenolic compound. There are several phenolic acids, such as chlorogenic, caffeic, vanillic and syringic, as well as some other phenolic compounds like quercetin, hydroxytyrosol and resveratrol identified to have antimicrobial activity [29, 30]. In fact, [31]. showed that oleuropein and other phenolic compounds (p-hydroxybenzoic, vanillic and p-coumaric acids) completely inhibit the growth of both Gram-negative and Gram-positive bacteria (*B. cereus, E. coli, Kl. pneumoniae*) and fungi (*Asp. flavus*). Phenolic structures similar to oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans [32, 33]. All these found can explain the growth inhibition power of *Olea europaea ssp. oleaster* fruit pulp against the tested strains.

Table 3: Zones of growth inhibition (mm), the MIC and MBC values (mg/ml) of *Olea europaea ssp. oleaster* fruit pulp methanol extract against ten microorganisms.

Micro-organisms[a]		Inhibition zone diameters (mm)[b]		MIC (mg/mL)	MBC (mg/mL)
		Extract	Antibiotics		
Gram negatif bacteria	*E. coli* ATCC 8739	17±0.5	24[1]	12.5	25
	S. typhimurium NCTC 6017	16±1	23[1]	12.5	25
	A. hydrophila EI	15±0.5	23[1]	25	50
	P. aeruginosa ATCC 27853	18±2	21[1]	12.5	25
Gram positif bacteria	*S. aureus* ATCC 29213	17±1	20[1]	12.5	25
	L. monocytogenes ATCC 7644	14±0.5	18[1]	25	50
	B. cereus ATCC1247	13±0.5	21[1]	25	50
Fungus	*Asp. flavus*	17±1	11[2]	3.125	6.25
	Asp. niger	18±0.9	12[2]	3.125	6.25
Yeast	*C. albicans*	16±0.5	17[2]	6.25	12.5

[a]Final bacterial density was around 10^5 cfu/mL. [b]Inhibition zone diameters (mm) produced around the wells by adding 15 µL of methanol extract.[1]Gentamicin. [2]Amphotricin. ±: Standard deviation.

CONCLUSION

The essential fatty acids, secondary metabolite and biological activities of *O. oleaster* fruit pulp methanolic extract have been studied here for the first time.

The fatty acids composition revealed that this oil contained twenty acids with the major components were oleic acid (77.4%) and elaidic acid (17.58%). Moreover, the tested extract was rich in phenol

(84.04±0.01 mg GAE/g DW) than in flavonoids (60.41±0.02 mg RE/g DW). In addition, it showed strong antiradical, antibacterial, antifungal and anticandidal activities. In this manner, the methanolic extract of *O. oleaster* fruit pulp may be suggested as potential sources of natural antioxidants as well as pharmaceuticals and natural therapies of infectious diseases in the human as antimicrobial agents.

CONFLICT OF INTERESTS

Declared None.

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ABBREVIATIONS

ATCC: American Type Culture Collection

DPPH: 2,2-diphenyl-1-picrylhydrazyl

GC: Gas Chromatography

IZ: Inhibition Zones

MIC: Minimum inhibitory concentration

MBC: Minimum bactericidal concentration

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