Chemical and biological evaluation of olive leaves as a waste by-product of olive oil industry

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Background and objectives
Finding new uses for by-products of cultivated plants is of great value economically and to the environment. Leaves represent about 10% of the total weight of olives yield. It is worth to obtain value-added products from this material. In this article, the leaves were evaluated chemically and biologically for phenolics and flavonoids as well as for microelements and macroelements and fatty acids. Also, antioxidant and antimicrobial activities were carried out.
Materials and methods
Air-dried powdered olive leaves were defatted with hexane and the marc was then soaked in 80% methanol and successively extracted with CH_2Cl_2, EtOAc, and n-BuOH. Total phenolic and flavonoid contents were determined as chlorogenic acid and rutin equivalents, respectively. Microelements and macroelements were detected in addition to fatty acids. The antioxidant effect was determined *in vitro* using 1,1-diphenyl-2-picrylhydrazyl and antimicrobial activity was carried out using in-vitro agar well diffusion method.
Results and conclusion
Total phenolics were found to be highest in the 80% methanolic extract and the lowest in water and ethyl acetate fractions. 1,1-diphenyl-2-picrylhydrazyl-free radical scavenging activity of olive leaf extracts were in this order: 80% methanolic extract, water extract, ethyl acetate fraction and butanol fraction. Also, the calcium : potassium value was 15: 1. Fatty acid profile revealed that linolenic acid was the major fatty acid in terms of percent (49.45%).
Ethyl acetate fraction showed positive antibacterial activity and negative antifungal activity whereas water, 80% methanol, and butanol fractions have positive antifungal and negative antibacterial activity.
Conclusion
Olive leaves could be considered as a potential inexpensive source for food supplements for human health.

Keywords:
antimicrobial, antioxidant, by-product, fatty acids, flavonoids, olive leaves, phenolics, waste

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Introduction

Olea europaea (subsp. *europaea* L.) is a member of the *Oleaceae* family which comprises 600 species in 25 genera distributed on all continents [1]. The olive fruit, oil, and the leaves have a rich history of nutritional and medicinal uses and are considered as an important crop in the Mediterranean region and North Africa [2]. Plants waste is currently an important issue both in developing and developed countries. The majority of the wastes is underutilized and may cause environmental problems if not properly handled [3]. Olive crops, fruits, and their by-products represent valuable sources for nutritional products with health benefits [4]. In ancient Egypt, olive leaves were first used to treat several diseases such as fever, cough, and cystitis [5].

Worldwide O. *europaea* are used to treat various ailments as they have ethnomedical properties. The plant materials and its isolated components have shown a wide spectrum of in-vitro and in-vivo pharmacological activities [6].

The oil extract from leaves which is considered as an agricultural waste by-product has many potential health benefits such as antioxidant activity [7–12], anti-HIV properties [13], anti-proliferative and apoptotic effects [14], protective effect against

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human leukemia [15], and has lipid-lowering activity [10].

Also, using olive leaf in the sheep diet led to an increase in omega 3 fatty acids and conjugated linoleic acid (C18:2) amounts in Awassi sheep milk [16] as well as improved the meat quality [17].

Chemically, leaves of olive contain considerable biophenols such as the other parts of the olive tree. Oleuropein and its metabolites, including tyrosol and hydroxytyrosol, are the most abundant phenolic compounds known in the olive leaf. Oleuropein has antibacterial, antiviral, antitumor, blood pressure, and blood lipid-reducing factor, anticancer, and cardioprotective activities [18–20].

Few studies have been carried out on the leaves of olive plants cultivated in Egypt [21,22]. As part of our study to evaluate the by-products of economically important cultivated plants for their value, olive leaves were subjected to intensive investigation chemically and biologically. The fatty acid composition, total phenolic, and flavonoid contents of different leaf extracts were carried out as well as the determination of microelements and macroelements, antimicrobial, and antioxidant activities.

Materials and methods

Plant material

Olive leaves (*O. europaea* L.) were collected from an olive farm located at Ein-Helwan, Cairo, Egypt in August 2016. The plant was authenticated by Prof. Dr Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University. Olive leaves were washed with tap water (three times), dried in oven at 40°C, finely ground and kept in a dark and dry bottle for phytochemical and biological studies.

Experimental

GC/MS conditions

GC/MS analyses were carried out using a trace GC ultra Gas chromatographs (Thermo scientific corp., USA) coupled with a thermo mass spectrometer detector (ISQ single Quadrupole Mass spectrometer). The injected volume was 0.2 µl where helium was used as a carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1 : 10 using the following temperature program: 80°C for 1 min, rising at 4.0°C/min to 300°C, and held for 1 min. The injector and detector were held at 240°C. Mass spectra were obtained by electron ionization at 70 eV, using a spectral range of m/z 40–450.

Extraction

Six hundred grams of the dried powdered leaves were sonicated with hexane (3×1 l). The extracts were combined and evaporated under reduced pressure at 40°C to yield a dark greenish black residue (~12.26 g).

The dried marc was extracted with 80% methanol (3×1 l) and the extracts were combined and evaporated under reduced pressure at 45°C to yield a dark brown residue (~154 g), which was dissolved in 200 ml distilled water and successively extracted with methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and n-butanol (n-BuOH) (3×500 ml for each). The extracts were evaporated under reduced pressure and examined by thin-layer chromatography using ethyl acetate : hexane (3 : 1) and (1 : 1) as solvent systems. Also water extract was prepared from 50 g of leaves for biological tests.

Determination of total phenolic and flavonoid contents

The total phenolic content of the leaf extracts was determined by the Folin–Ciocalteu method [23]. The amount of phenolics was calculated as chlorogenic acid equivalents/1 g plant as described by Meda *et al.* [23]. The total flavonoid content was also determined by the same method and expressed as mg of rutin equivalent/g of extract.

Free radical scavenging activity on 1,1-diphenyl-2-picrylhydrazyl

The 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of different leaf extracts was measured according to Nenadis and Tsimidou [24]. IC_{50} values of the extracts (concentration of extract necessary to decrease the initial concentration of 1,1-diphenyl-2-picrylhydrazyl by 50%) were calculated.

Determination of macronutrients and micronutrients

Dry ashing technique was performed for digesting of plant sample according to Akinyele and Shokunbi [25]. One gram of olive leaves was washed with water and then heated in oven at 500°C for 12 h until complete ashing. The plant residue was dissolved in 1 M nitric acid and filtered. The minerals of olive leaves were analyzed by atomic absorption spectrophotometer.

Preparation of lipid constituents

Five grams of hexane extract from olive leaves were dissolved in hot acetone and left in the refrigerator overnight, and then filtered.

Saponification of acetone-soluble fraction

The acetone-soluble fraction was saponified by refluxing with 100 ml N/2 alcoholic KOH for 8 h [26]. The alcoholic solution was concentrated to

about 25 ml and diluted with cold, distilled water. The unsaponifiable matter was extracted by shaking with successive portions of ether and then collected and evaporated *in vacuo* till dryness and finally analyzed by GC/MS.

Preparation of total fatty acids

Mother liquor produced after saponification was rendered acidic (pH=2) with sulfuric acid. The liberated fatty acids were extracted several times with ether and then washed with distilled water and dehydrated over anhydrous Na_2SO_4 and finally evaporated *in vacuo* at 40°C till dryness.

Preparation of fatty acid methyl esters

The fatty acid fraction was dissolved in 30 ml dry methanol containing 4–5% dry HCl and refluxed on a boiling water bath for 3 h. The reaction mixture was diluted with distilled water and extracted with successive portions of ether (3×25 ml) and then washed with distilled water, dried over sodium sulfate anhydrous, and filtered and evaporated *in vacuo* at 40°C. Fatty acid methyl esters were analyzed by GC/MS.

Antimicrobial activity

The examination of antimicrobial activity was based on the in-vitro agar well diffusion method according to Hindler *et al.* [27]. The prepared extracts were tested against standard microorganisms of Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram negative bacteria (*Salmonella spp.* and *Escherichia coli*) at a concentration of 5 mg/ml. For antifungal activity, two types of fungi were used; *Aspergillus fumigatus* and *Candida albicans*. The diameter of the inhibition zones were measured (mm) at 37°C after 24 h. Ampicillin, gentamicin, and amphotericin B are used as positive controls.

Result and discussion

Total phenolic and total flavonoid contents

The total phenolics and flavonoid content of extracts obtained from olive leaves are shown in Table 1.

The extractable total phenolics were found to be highest in the 80% methanolic extract and the lowest concentration was observed in ethyl acetate and water extracts. Besides, the highest flavonoids content is reported in the n-butanol extract, while the lowest value is determined in the water extract. Actually, the plant phenolics have received considerable attention due to their biological activity including anti-inflammatory, anticarcinogenic, and anti-atherosclerotic activities [28].

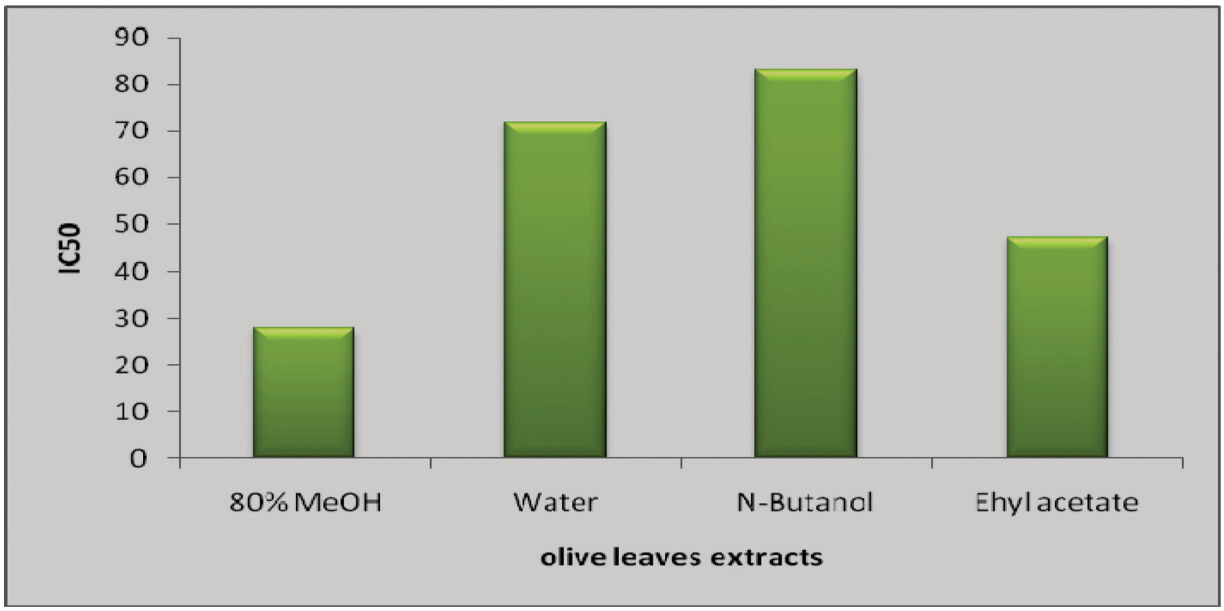
Free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl)

Radical scavenging activity (expressed as absorbance percentage) and IC_{50} of olive leaf is shown in Fig. 1.

Table 1 Quantitative estimation of total phenolics and flavonoids of olive leaves

Extracts	Total polyphenols (mg/g) extract	Total flavonoids (mg/g) extract
80% MeOH	253.23±0.75	121.40±0.23
EtOAc	152.83±0.68	111.47±0.55
n-Butanol	211.98±0.43	138.62±0.45
Water	148.7±0.88	86.47±0.41

Figure 1



IC50 of different olive leaves extracts.

1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity of olive leaf extracts was in this order: 80% methanolic extract, water extract, ethyl acetate fraction and butanol fraction.

Evaluation of macronutrients and micronutrients

Olive leaves contain essential macroelements such as K, Ca, Mg, and Na as well as microelements such as Fe, Zn, and Cu (Table 2). Calcium is the predominant element with Mg being the most dominant element in relation to all detected elements. These results reveal a considerable amount of minerals that exist in Egyptian olive leaves compared with previous literatures. It can be useful in improving the mineral content of bread and cake products when added to them. It could be also observed that the Ca : P ratio in our olive leaves is 15.1 [22]. The ratio of Ca : P should not be less than 1.0 in foods as recommended by the FAO/WHO [29].

Table 2 Evaluation of macroelements and microelements of olive leaves

Elements	Olive leaves sample (dry method) mg/100 g	Ibrahim *et al*. 2016 [22] (wet method) mg/100 g
Ca	2730	1570
P	180	115
Na	360	140
K	2000	656
Mg	550	193
Fe	24.5	19.1
Mn	1.91	4.3
Zn	7.1	2.5
Cu	0.5	0.9

Fatty acid profile

The amount of total lipids in *O. europaea* cultivated in Egypt was considered high (2%) and the main fatty acid composition is determined and listed in Table 3. The result showed high concentrations of saturated fatty acids (35.74%) mainly palmitic acid. High content of linolenic acid (49.45%) turns this by-product into a valuable source of n-3 polyunsaturated fatty acid. It is important to mention that the kind of unsaturated fatty acids found in the leaves is different from those found in the oil [30–32]. For olive oil, oleic acid is the main unsaturated fatty acid whereas for the leaves, linolenic acid is the major one which is around 49.45%. This is very important and gives the leaves (by-product) a nutritional and pharmaceutical importance.

Antimicrobial activity

Olive leaf extracts were screened for their antibacterial and antifungal activity against four different bacterial human pathogens and two fungi, respectively, and compared with those of Ampicillin, Gentamicin, and Amphotericin B which are positive controls (Table 4). The activity was determined based on the capacity to inhibit the growth of pathogenic bacteria and fungi measured as the zone of inhibition in MM. All tested bacteria and fungi were sensible against standards, while olive leaf extracts varied from one species to other. Methylene chloride and ethyl acetate extracts appeared to be active against *S. aureus*, *B. subtilis*, and *Salmonella spp.* but has no activity of the fungi species used. Butanol, 80% methanol, and water

Table 3 GC/MS analysis of fatty acids of olive leaves

Number	R_{RT}	Area %	Chemical structure	Compounds
1	0.602	1.05	$C_{15}H_{24}O$	Butyl hydroxy toluene
2	0.763	0.93	$C_{15}H_{30}O_2$	Myristic acid methyl ester
3	0.819	0.42	$C_{19}H_{36}O_2$	Oleic acid methyl ester
4	0.879	0.69	$C_{17}H_{32}O_2$	Palmitoleic acid methyl ester
5	0.893	24.10	$C_{17}H_{34}O_2$	Palmitic acid methyl ester
6	0.940	0.12	$C_{18}H_{34}O_2$	Cis-10-heptadecenoic acid methyl ester
7	0.954	0.45	$C_{18}H_{36}O_2$	Methyl margarate
8	0.994	10.87	$C_{19}H_{34}O_2$	Linoleic acid methyl ester
9	1.000	49.45	$C_{19}H_{32}O_2$	α-Linolenic acid methyl ester
10	1.012	5.02	$C_{19}H_{38}O_2$	Stearic acid methyl ester
11	1.110	0.42	$C_{21}H_{40}O_2$	Cis-11-eicosenoic acid methyl ester
12	1.123	2.34	$C_{21}H_{42}O_2$	Eicosanoic acid methyl ester
13	1.176	0.37	$C_{22}H_{44}O_2$	Heneicosanoic acid methyl ester
14	1.226	0.81	$C_{23}H_{46}O_2$	Behenic acid methyl ester
15	1.275	0.24	$C_{24}H_{48}O_2$	Tricosanoic acid methyl ester
16	1.322	0.43	$C_{25}H_{50}O_2$	Lignoceric acid methyl ester
17	1.396	0.33	$C_{45}H_{74}O$	Solanesol
18		Total saturated fatty acids		35.74
19		Total unsaturated fatty acids		62.30
		Unidentified compound		1.96

R_{Rt}, relative to α-linolenic acid methyl ester retention time=40.70 min.

Table 4 Mean of inhibition zone in mean±SD produced on a range of environmental and clinically pathogenic microorganisms for successive extracts of olive leaves growing in Egypt (5 mg/ml)

Items	Antibacterial activity zone of inhibition (mm)				Antifungal activity zone of inhibition (mm)	
	Gram positive		Gram negative			
	Staphylococcus aureus (RCMB010010)	*Bacillissubtilis* (RCMB 010067)	*Salmonella spp.* (RCMB010043)	*Escherichia coli* (RCMB 010053)	*Aspergillus fumigatus* (RCMB 02568)	*Candida albicans* (RCMB 05036)
80% methanol	NA	NA	NA	NA	8.1±2.1	NA
Methylene chloride	10.1±0.63	15.5±0.44	8.2±0.37	NA	NA	NA
Ethyl acetate	8.3±1.2	12.4±1.5	9±0.46	NA	NA	NA
Butanol	NA	NA	NA	NA	7.1±2.1	NA
Water	NA	NA	NA	NA	7.8±0.5	NA
Ampicillin	23.8±1.2	32.4±0.63	NA	NA	NA	NA
Gentamicin	NA	NA	17.3±0.58	19±1.2	NA	NA
Amphotericin B	NA	NA	NA	NA	23.7±0.58	25.4±1.5

Data are expressed in mean±SD. NA, no activity.

extracts were only active against *A. fumigatus*. None of the prepared extracts showed activity against *C. albicans*.

The results obtained in this study encourage the researchers to carry out more studies on olive leaves and consider this waste as a potential inexpensive source for food supplements for human health.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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