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Fatty acid composition, phytochemical constituents and antioxidant activity of olive (*Olea europea* L.) leaves extract

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Abstract - In this study, the total lipid content, fatty acid profile, phytochemical constituents and the antioxidant activity of olive leaves of the variety Chétoui from different provenances were investigated. Preliminary results indicate the presence of lipids with lower proportions (≈ 4 g/100 g). The lipid profile was highly unsaturated (74.49-77.52%) with linolenic (31.77-35.85%) and linoleic (17.32+21.32%) acids as main compounds. The saturated fatty acids fraction (22.48-25.51%) was dominated by palmitic (18.64-21.01%). Regarding phenolic compounds, all samples showed elevated contents on total phenol content (38.49-50.30 mg GAE/g extract) and total flavonoid contents (53.84-113.13 mg CE/g extract) and exhibited good DPPH and ABTS radical scavenging activity, and have an excellent ferric reducing ability. Olive leaves from the variety Chetoui could be considered as potential source of essential fatty acids and natural antioxidants.

Key words: Olive leaves, Lipids, Essential fatty acids, Phytochemicals, Radical scavenging activity; Reducing power

1. Introduction

Olive oil is the highest prized and the most consumed oil in Mediterranean region owing to its unique taste, nutritional benefits and functional properties (Boskou, 2006). Its health claims were mainly attributed to its peculiar chemical composition including high content of monounsaturated fatty acids (oleic acid), and other minor compounds such as phenolics, tocopherols (vitamin E) and carotenoids (Visioli and Galli, 1998). In recent years, interest in the phenolic compounds of olive oil has increased because of their potential biological activities like antioxidant, lipid lowering, hypocholesterolemic, anti-diabetic, and anti-obseity, properties among others (Laribi, 2015). The phenolic fraction of olive oil (commonly known as biophenols) contained phenyl alkohols (tyrosol and hydroxytyrosol), secoiridoids (oleuropein, oleuropein aglycone, demethyleuropein, ligstroside, ligstroside aglycone, and elenoic acid), phenolic acids (caffeic, vanillic, sinapic, coumaric and cinnamic), flavonoids (luteolin and apigenine) and lignin (pinoresinol) (Bravo, 1998; Ryan et al., 2002a). Their direct contribution to the flavor, stability and nutritional value of the oil has also been reported (Gigon and Le Jeune, 2010; Perrin, 1992). However, the profitability of phenolic compounds derived from olive oil remains a difficult task due to their low daily intake (Rubió et al., 2012), and their quantitative and qualitative variations which in turn depend on genetic factors (cultivar), physiological (maturation stage at harvest), environmental (climate and soil), agronomic and technological factors (Valls et al., 2015). Therefore, the search for new source of these phenolic compounds is of great interest.

Olive leaves, considered as a by-product of olive oil production have recently emerged as potential source of biophenols. They include oleuropein end its derivatives, hdroxytyrosol, tyrosol, rutin, caffeic acid, luteoline and its glycosylated derivatives, diosmine, pinoresinol, apigenine, and diometine to which the antioxidant, antimicrobial, hypoglycemic, antidiabetic and anti-carcinogenic properties were attributed (Taamalli et al., 2012). Given these biological activities, olive leaves have been used for the development of novel functional foods/beverages (olive tea) preventing lipid peroxidation and extending the shelf life of foods (Talhaoui et al., 2014). However, as for olive oil, the content of leaf phenolic compounds depends greatly on the growing region, variety, and extraction methods. The variety "Chetoui" is the second important olive cultivar commonly planted in the northern (north-eastern and north-western) regions of Tunisia. It is known for its

high quality of olive oil owing to its richness on phenolic compounds and antioxidant pigments (Taamalli et al., 2012). Given these data, the present contribution aimed to study the phytochemical profile of olive leaves from the Chetoui variety, and to evaluate their in vitro antioxidant activity.

2. Materials and Methods

2.1. Plant material

Olive leaves of the variety "Chetoui" were collected in autumn 2018 from the regions of Kairouan, Zaghouan, Houemed and Sidi Saâd. After washing with tap water to remove dust, leaves were dried at 40°C in a convection oven until constant weight, ground to a fine powder, and subsequently essayed for their lipid content, fatty acid composition, phenolic and flavonoid determination and their antioxidants activity.

2.2. Lipid content and fatty acid composition

Total lipids were extracted manually from olive leaves powder (1g) using Chloroform/methanol (2:1 v/v) (Bligh and Dyer 1959). The chloroform fraction was evaporated to dryness to remove solvent, and converted into fatty acid methyl esters (FAMEs) using solution of sodium methoxide (3% in methanol). After neutralization of the reaction mixture with 0.1 mL of H_2SO_4 and phase separation, the organic phase containing FAMEs was recovered and analyzed by gas phase chromatography with flame ionization detection (GC-FID).

The chromatographic analysis of FAMEs was carried out according to the IUPAC 2.301 (1987) standard method. FAMEs analysis was performed on a Hewlett-Packard HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a TR-FAMEs polar capillary column (Thermo Fisher Scientific Inc., Bordeaux, France) (60 m × 0.25 mm, film 0.25 m thickness). The column temperature was initially held at 100°C for 5 min, increased to 240°C (4°C/min), then held isothermal for 15 min. The temperature of the injector and the FID detector were maintained at 240°C and 260°C, respectively. The FAMEs were identified by comparing their retention times with those of commercial standards (Sigma-Aldrich, Steinheim, Germany). Individual FAME content was expressed as a percentage of total peaks calculated from electronic integration and adjusted for internal standard (Habibi et al., 2021).

2.3. Preparation of olive leaves extract

The olive leaves powder was extracted using 80% methanol for 24 h. After centrifugation for 15 min at 3800 rpm, the Hydro-alcoholic extract was filtered using a 0.45 µm filter paper and concentrated in a rotary evaporator to obtain the olive leaves extract, which was stored in light protected glass vials at −20°C until analysis and use (Ryan et al., 2002b).

2.4. Determination of total phenol content (TPC)

The TPC of olive leaf extract was determined by the method of Skerget et al. (2005) using Folin-Ciocalteu (FC) as reagent and gallic acid as standard with some modifications. Briefly, a 0.5 mL of the diluted extract is mixed with 2.5 mL of 10-fold diluted FC reagent. After 3 min, 2 mL of sodium carbonate (Na_2CO_3) (75 g/L) was added and the mixture was left to stand at room temperature in the dark for 1 h. The absorbance of the solution was then measured at 760 nm against a blank using a spectrophotometer (Jasco Corp., Tokyo, Japan). The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g) through the calibration curve of gallic acid ($R^2 = 0.99$).

2.5. Determination of total flavonoid content (TFC)

The TFC was determined using the colorimetric method of aluminum trichloride ($AlCl_3$) using catechin as standard (Dewanto et al., 2002). Briefly, 250 µL of diluted sample extract was mixed with 75 µl of 5% of sodium nitrite ($NaNO_2$) solution and left to stand for 6 min at room temperature. Thereafter, 150 µL of a 10% $AlCl_3$solution and 500 µL of NaOH (1M) were added, and the final volume was adjusted to 2.5 ml with distilled water. The mixture was then stirred and then incubated in the dark for 30 min before reading the optical density at 510 nm against a blank. The TFC were expressed as mg catechin equivalents per gram of dry weight (mg CE/g) through the calibration curve with catechin ($R^2 = 0.99$).

2.6. Determination of antioxidant activities *in vitro*

2.6.1. DPPH radical scavenging activity

The DPPH-free radical scavenging activity was evaluated according to the slightly modified method of Kim et al. (2005). For this, 100 µL of the diluted olive leaf extract was added to 1.9 mL of a freshly prepared methanolic solution of DPPH (0.1 mM). After incubation for 1 h in the dark, the absorbance of the reaction

mixture was measured at a 515 nm and compared with the absorbance of the blank control (methanol). The percentage inhibition (I%) of $DPPH^{\cdot}$ radicals is calculated according to the following equation:

$I\ (\%) = [(A_0-A_s)\ /A_0] \times 100$

With A_0, A_s were the Absorbance of the blank and sample, respectively.

The anti-radical activity of the leaf extracts was expressed in µmole trolox equivalents per Kg of dry extract (µmole TE/kg of dry extract) through the calibration curve made with the positive antioxidant standard trolox ($R^2 = 0.99$).

3.6.2. ABTS radical scavenging activity

In the present work, the method of Yuan et al. (1999) is adopted for the evaluation of the ABTS radical scavenging activity. A test portion of 1 ml of the standard ABTS solution (7 mM) prepared in ultra-pure water was oxidized with 2 mL of $K_2S_2O_8$ (2.45 mM). The mixture was left to stand in the dark and at room temperature for 12 to 16 h. The $ABTS^+$ solution obtained is blue-green in color (stable in this form for more than two days). Before use, the solution formed is diluted in methanol until an absorbance of 0.7 at the 734 nm wavelength was obtained. In this study, 100 µL of diluted olive leaf extract was mixed with 2900 µL of the prepared solution of $ABTS^+$ (0.7 at 734 nm) and the mixture was incubated in the dark at room temperature for 15 minutes, then the absorbance was read at 734 nm. As for the DPPH assay, the ABTS radical scavenging activity was expressed as µmol equivalents Trolox per kg of dry extract (µmol TE/kg dry extract).

2.6.3 Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed of olive leaves extracts was performed according to the method described by Benzie and strain (1996). The FRAP reagent is freshly prepared at 37°C by mixing 25 mL of sodium acetate buffer (300 mM/L, pH 3.6), 2.5 mL tripyridyltriazine (TPTZ) (10 mM/L to 40 mM/L HCl) and 25 mL FeCl3, 6 H2O (20mM) using the ratio 10:1:1 (Wang et al. 2014). An aliquot of each diluted olive leaf extract (100 µL) is mixed with 1900 µL of the FRAP solution. After incubation for 30 min at 37 °C, the absorbance wass read at 593 nm. Trolox was used as a positive standard and the iron-reducing antioxidant power of the leaf extracts was expressed as µmol Trolox equivalent per kg of extract (µmol TE/kg extract).

2.7. Statistical analysis

All experiments were performed in triplicate and the results are expressed as mean value ± standard error of the mean (SEM). The comparison between means was carried out using a one-way analysis of variance (ANOVA) followed by the Tukey's Post hoc test at the significance level of $p < 0.05$. All analyzes were performed using Statistical Package for the Social Sciences (SPSS) software (version 22.0 for Windows, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Total lipid content and fatty acids composition

The total lipid content and the average of FAMEs composition expressed in g/100 g of total fatty acids are given in Table 1. As shown, total lipid content ranged from 2.84 to 3.67% (w/w) with the sample from kairouan Being the richest one. These results were similarto those reported for some Tunisian varieties including Chemlali (3.11%), Chemchali (3.58%), Chetoui (3.88%) and Zarrazi (3.21%) (Bahloul et al., 2014). A high lipid content (>9%) has been described for Arbosana, Ascolano, Grappolo, Koroneiki, and Negrinha do frexio (Cavalheiro et al. 2015) suggesting the deep influence of the genetic and the origin factors. As expected, the FAMEs profiles showed high proportions of polyunsaturated fatty acids (PUFA) with linolenic (31.77-35.85%) and linoleic (17.32-21.32%) acids being the most abundant FAMEs. The monounsaturated fatty acids (MUFA) included oleic acid (19.64-23.48%) as the main compounds. Saturated fatty acids (SFA) were mainly composed of palmitic (18.64-21.01%) and stearic (3.26-4.79%) acids.

Compared with the scarce literature data, the FAMEs composition reported herein was qualitatively quite similar to that observed for other variety such as Chemlali, Chemchali, and Zarrazi. The oil from these varieties was predominantly unsaturated with linolenic (30-42.16%), linoleic (14.48-15.84%) and oleic acid (18.28-26.36%) as major compounds, while palmitic 18.22-22.42) and stearic acid (2.59-3.88%) were the main saturated fatty acids (Bahloul et al., 2014). Similar FAMEs profiles (linolenic (34.4-41.3%), linoleic (6.84-8.26%), oleic (19.8-21.5%) and palmitic acid (23-26.9%)) have also been described for Arbosana, Ascolano, Grappolo, Koroneiki, and Negrinha do frexio (Cavalheiro et al. 2015).

From these results, it can be inferred that the observed discrepancies might presumably be attributed to genetic, pedo-climatic, cultural practices, extraction and analytical procedures.

Table 1. Total Lipid content (g/100 g) and fatty acid composition (% of total area) of olive leaves variety Chetoui from different provenances

	Zaghouan	Houemed	Sidi Saâd	Kairouan
Total lipid content (% w/w)	2.84 ±0.24^{c}	3.18 ±0.12^{b}	3.43 ±0.14^{b}	3.67 ± 0.08^{a}
Palmitic acid (C16 :0)	18.64±0.68^{a}	21.01±2.41^{a}	19.67±1.22^{a}	19.22±0.44^{a}
Palmitoleic acid (C16 :1)	0.88±0.04^{c}	2.01±0.12^{a}	1.40±0.04^{b}	1.34±0.06^{b}
Stearic acid (C18 :0)	3.91±0.14^{b}	4.50±0.22^{a}	4.79±0.18^{a}	3.26±0.44^{c}
Oleic acid (C18 :1)	19.64±1.26^{b}	21.16±1.02^{b}	23.48±1.46^{a}	20.47±2.28^{b}
Linoleic acid (C18 :2)	21.32±1.74^{a}	19.54±1.38^{a}	17.32±0.74^{b}	19.86±1.52^{a}
Linolenic acid (C18 :3)	35.61±1.66^{a}	31.77±2.32^{b}	33.34±2.12^{b}	35.85±2.44^{a}
SFA	22.55	25.51	24.46	22.48
MUFA	20.52	23.17	23.88	21.81
PUFA	56.93	51.32	51.66	55.71

*Values represent the mean + SEM of triplicate; Values in the same raw with different superscript letters are significantly different at $p<0.05$. SFA: Saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

3.2. TPC, TFC and antioxidant activity

Table 2 shows the TPC, TFC, the radical scavenging activity and the ferric reducing power of the leaf extract. Samples from Zaghouan showed the highest TPC and TFC with average values of 50.3 mg GAE/g and 113.13 mg EC/g dry weight. This sample was also distinguished by its high DPPH-, and ABTS-radical scavenging activity. In contrast, samples from Kairouan exhibited the anti-radical lowest activity which could be associated to its low TPC (35.42 mg GAE/g dry weight).

Table 2. Phytochemical and antioxidant activity of hydroalcoholic extract of olive leaves variety Chetoui from different provenances

	Zaghouan	**Houemed**	**Sidi Saâd**	**Kairouan**
Total phenolic content (mg GAE/g)	50.30 ± 0.17^{b}	38,94±2,60^{a}	38.49 ± 2.76^{a}	35.42 ± 2.07^{a}
Total flavonoid content (mg CE/ g)	113.13 ± 4.37^{c}	65.37 ± 0.86^{b}	53.84 ± 0.80^{a}	58.90 ± 3.31^{ab}
DPPH (µmole TE/g)	316.98 ± 18.88^{b}	240.58 ± 1.80^{a}	253.04 ± 19.43^{a}	226.81 ± 0.75^{a}
ABTS (µmole TE/g)	176.78 ± 2.26^{b}	159.74 ± 10.90^{b}	167.40 ± 11.29^{b}	103.02 ± 4.30^{a}
FRAP (µmole TE/g)	4.71 ± 0.14^{a}	7.06 ± 0.42^{bc}	6.50 ± 0.05^{b}	7.52 0.07^{c}

*Values represent the mean + SEM of triplicate; Values in the same raw with different superscript letters are significantly different at $p<0.05$.

Regarding the FRAP assay, the reciprocal trends has been observed since the sample from Kairouan has shown the greatest ability to reduce the Fe^{3+} to Fe^{2+} (7.52 µmol TE/g). The lowest ferric reducing power was observed for the samples from Zaghouan with an average value of 4.71µmol TE/g.

From these results, it can be concluded that the ferric reducing ability was not correlated to the presence of phenolic compounds, but it involves other antioxidant compounds such as pigments, vitamins, or terpenic compounds.

Previous phytochemical investigations on olive leaves revealed their richness of the phenolic compounds which are responsible for their antioxidant activity. In this context, an average value of 47.47 mg GAE/g extract dry weight has been reported for TPC in Chetoui variety from Beja (Edziri et al., 2019). This variety also showed the highest TFC (7.29 mg EC/g extract), exhibiting consequently the highest DPPH and ABTS-

radical scavenging activities in comparison to the standards BHT and trolox, and the other varieties including Meski, Oueslati and Jarboui (Edziri et al., 2019).

4. Conclusion

The results of the present study, together with a review of literature data clearly indicate that olive leaves are a rich source of essential fatty acids, phenolic compounds with a manifest antioxidant activity.

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