

Study of Phenolic Composition and Biological Activities Assessment of Olive Leaves from different Varieties Grown in Tunisia

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Abstract

In the present study, olive leaves of eight varieties were investigated for the total phenol and flavonoid content, for the major compound and for the *in vitro* antioxidant properties. The different varieties showed a high content of polyphenol and flavonoid. Oleuropein was the major compound of the leaf extract for all varieties. Also, olive leaves extract exhibited a good antioxidant activity and a reduced power, each variety showed its own feature. A significant negative correlation between anti-radical activities and oleuropein content was observed only for four varieties ($r = -0.94$). The high oleuropein content and the important antioxidant activities of olive leaves extract could be useful sources for industrial extraction and pharmacological application in the promotion of health and prevention of damages caused by radicals.

Keywords: Olive leaves; Polyphenol; Flavonoid; Oleuropein; Antiradical activity; Reduced power; Antioxidant activity

Introduction

The high levels of free radicals in living systems can oxidize biomolecules, leading to tissue damage, cell death, or various diseases such as skin irritations, degenerative processes associated with ageing, cardiovascular diseases, arteriosclerosis, diabetes, neural disorders, and cancer [1-3]. Antioxidant compound can deactivate and scavenge free radical by donating hydrogen atom or chelating metals. Therefore, commercial antioxidants are in high demand, and most of them are synthesized, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG). However, these synthetic antioxidants are reported to be toxic and carcinogenic in animal models [4,5]. Thus, there is a growing request and interest on natural and safer antioxidants found in various kinds of land plants, such as cereals, vegetables, fruits and herbs; in which tocopherol, vitamin C, carotenoid and polyphenols are good sources of antioxidant [6].

In the Mediterranean area, olive leaves are one of the by-products of farming of the olive grove; they can be found in high amounts in the olive oil industries (10% of the total weight of the olives) and they accumulate during the pruning of the olive trees [7]. Olive leaves are considered as a cheap raw material which can be used as a useful source of high-added value products (phenolic compounds) [8]. Interestingly, the interest in the olive leaf and its chemical composition has recently been increasing. In fact, the olive leaves extract have been found to have anti-oxidative, anti-inflammatory and antimicrobial activities against bacteria and fungi [9-11]. Moreover, the olive leaf extract exhibits anti-viral activities against several virus like haemorrhagic septicaemia rhabdovirus [12]. The main phenolic compound in olive leaves are oleuropein, verbascoside, ligstroside, tyrosol and hydroxytyrosol [13]. Somova et al. reported that oleuropein and its derivatives had various biochemical roles including hypotensive, coronary dilating antiarrhythmic action [14]. Furthermore, it was reported that these polyphenols could prevent low-density lipoprotein oxidation and platelet aggregation [15,16].

The objective of the present study is to assess the amount of total phenolic and flavonoid content of ethanol extracts of olive leaves and then to evaluate their antioxidant properties.

Material and Methods

Chemicals

Ethanol (HPLC grade) and acetonitril (HPLC grade) was

purchased from Alpha chimie, sodium carbonate was obtained from Prolabo. Sodium nitrite, sodium hydroxide, peroxodisulphate and Folin-Ciocalteu were purchased from Fluka (Buchs, Switzerland). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was from Sigma Co. (St. Louis, MO, USA). Oleuropein, Gallic acid, (+)-catechin, and aluminium chloride were obtained from Sigma-Aldrich (Steinheim, Germany). Butylated hydroxytoluene (BHT) was purchased from Sigma.

Plant Material

Experiments were carried out on olive leaves of 8 varieties from different origin; Sevillane (Spain), Rosicola and Meski (Italia), Lucques (France), Gerboua, Chetoui, Limouni and Chemlali (Tunisia). Leaves were collected on october 2010 (maturation period) in the National Agronomic Institute (Tunisia). The different cultivars from different origins were submitted to the same cultural conditions and the same geographical, geological, and climatic conditions. Leaves were dried on site in a microwave oven (2500 MHz), three times for 2 min at maximum power. Dried leaves were powdered and stored in a dry place in the dark.

Preparation of olive leaves extract.

A mixture of ethanol and water (20 ml, 70 :30 (v/v)) was added to the olive leaves powdered (1 g). The mixture was left to stand for at least one week at room temperature in the dark. Subsequently, the solution was filtered using a 0.45 μ m filter paper. Each extract was stored at +4°C until analysis [17].

Determination of total polyphenol content.

Total phenolics of olive leaves were determined using the Folin-Ciocalteu (F-C) reagent, according to the method described by Singleton et al. [18]. Folin-ciocalteu reagent contains metals like

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polytungston. Phenolic content from the sample reduces the metal and changes the colour from yellow to Prussian blue. The intensity of the colour is directly proportional to the phenolic content. In the experiment, each extract was diluted with the same solvent used for extraction to an appropriate concentration for analysis. An aliquot of 1 ml of diluted extract was added to 5 ml of deionized water and 1 ml of 20% Na₂CO₃. After shaking, the mixture was incubated for 3 min at room temperature. Then, 1 ml of the diluted (F-C) reagent solution was added, and held for 30 min at 40°C. The absorbance of the solution was then measured at 760 nm against a blank. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g) through the calibration curve of gallic acid (R² = 0.99). The sample was analyzed in three replications [18].

Total flavonoid

Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants. Also, they are commonly known for their antioxidant activities [19]. In our study, total flavonoid content was measured according to Dewanto et al. [20]. 250 µl ethalonic leaves extracts appropriately diluted was mixed with 75 µl NaNO₂ (5%). After 6 min, 150 µl of 10% AlCl₃ and 500 µl of NaOH (1 M) were added to the mixture. Finally, the mixture was adjusted to 2.50 ml with distilled water. The absorbance versus prepared blank was read at 510 nm. Total flavonoid contents of leaves (three replicates per treatment) were expressed as mg catechin equivalents per gram of dry weight (mg CE/g) through the calibration curve with catechin. The calibration curve range was 25–500 µg/ml (R² = 0.99) [20].

HPLC analysis of the olive leaves extracts

The HPLC analysis given in the literature was used for identification and quantification of polyphenol compound [21]. HPLC analysis was performed on a KNAUER apparatus, manual injection valve, UV detector model 2500 using a column 250 x 4.60 mm with percolumn, packing: Eurospher 100-5 C18. Data acquisition and quantitation were performed with Eurochrom software (KNAUER). The mobile phase was 79% distilled water acidified (pH=3) with 0.10 M orthophosphoric acid (v/v, 1000 : 2.30) and 21% acetonitrile (Carlo Erba, HPLC grade) acidified with 0.10 M orthophosphoric acid (v/v, 1000 : 2.30). The flow rate was 1 ml/min, and the injection volume was 20 µl. Routine quantitation of oleuropein was assessed at 280 nm. Run time was 37 min.

To calculate the oleuropein amount in the olive leaf extracts, a five-point calibration curve (R² = 0.99) prepared using the standard solutions (Tyrosol) with increasing concentrations.

DPPH assay

The electron donation ability of the obtained ethanol extracts was measured by bleaching of the purple-coloured solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato et al. [22]. Extracts (1 ml, 5–200 µg.ml⁻¹) were added to 0.25 ml of 0.20 mM DPPH methanolic solution. After an incubation period of 30 min at room temperature, the absorbance was determined against a blank at 517 nm [22].

Percentage inhibition of free radical DPPH (PI%) was calculated as follow:

$$PI\% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

Where A_{blank} was the absorbance of the control reaction and A_{sample} was the absorbance in the presence of plant extract. Extract

concentration providing 50% inhibition (IC₅₀ µg/ml) was calculated from the log-dose inhibition curve regression equation prepared from the concentration of the extracts and the inhibition percentage. BHT was used as a positive control. Samples were analyzed in triplicate.

β-carotene bleaching test

The antioxidant activity of olive leaves extracts was evaluated according to a slightly modified version of the β-carotene bleaching method of Pratt [23]. β-Carotene (5 mg), 25 µl of linoleic acid and 200 mg of Tween 40 (polyoxyethylene sorbitan monopalmitate) were mixed with 10 ml of chloroform. Chloroform was removed at 50°C, under vacuum, using a rotary evaporator (Heidolph, Germany). The resulting mixture was immediately added to 100 ml oxygenated distilled water and was mixed well for 1–2 min. Aliquots (2.50 ml) of this emulsion were transferred into different test tubes containing 0.35 ml of test samples. BHT was used for comparative purposes. The tubes were placed, at 50°C, in a water bath. Absorbance of all the samples at 490 nm were taken at zero time (t = 0), the measurement of the absorbance was continued, until the colour of the β-carotene disappeared in the control reaction (t = 180 min), at 15 min intervals. A mixture prepared as above, without β-carotene, served as blank. All determinations were performed in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula:

$$AA = \% \text{ inhibition} = 100[1 - (A_0 - A_t) / A_0 - A^*t]$$

Where A₀ and A⁰ were the absorbance values measured at zero time of the incubation for test sample and control, respectively. A_t and A^t were the absorbances measured in the test sample and control, respectively, after incubation for 180 min [23].

Reducing power

The ferric-reducing power (FRAP) of the extracts and reference compound was tested using the assay of Oyaizu [24]. Ethanol extracts (1 ml) as well as ascorbic acid as reference (all at concentration 500 µg/mL) for comparative purposes was mixed with 2.50 ml of a 0.2 M sodium phosphate buffer (pH = 6.6) and 2.50 ml of 1% potassium ferricyanide (K₃Fe(CN)₆), and incubated in a water bath at 50°C for 20 min. Then, 2.50 ml of 10% trichloroacetic acid were added to the mixture that was centrifuged at 3000 rpm for 10 min. The supernatant (2.50 ml) was then mixed with 2.50 ml distilled water and 0.50 ml of 0.1% ferric chloride solution. The intensity of the blue–green colour was measured at 700 nm. The increase in the absorbance of the reaction indicated the increase in reducing power of the extracts [24].

Statistical analysis

All tests were carried out in triplicate and the results were presented as the mean ± standard deviation. Analysis of variance (ANOVA) was carried out using Origin 6.1. Differences at *P < 0.05 and **P < 0.01 were considered statistically significant. Pearson's correlation (R²) was carried out to study the relationships between different analytical parameter.

Results and Discussion

The evaluation of yields of extracts reveals the following order : chetoui > Gerboua > Lucques > Meski > Sevillane > Limouni > Rosicola > Chemlali (Table 1). This difference was probably related to genetic feature of each variety since its growth is in the same climatic and geographical conditions and the extraction was carried out with the same solvent. The total phenol (TP) and flavonoid (TF) content of olive leaves, obtained from different varieties were shown in Table 1.

| Varieties | Extraction yields | Total phenols | | Total flavonoïdes | |
|----------------------|--------------------------|------------------------------|------------------|------------------------------------|------------------|
| | | Our study | Previous studies | Our study | Previous studies |
| Gerboua | 40.00 ^a | 142.21 ± 3.53 ^a | | 125.64 ± 3.36 ^a | |
| Limouni | 33.66 ^{a,e,f} | 144.19 ± 10.27 ^b | | 120.88 ± 0.78 ^{b,c,d,e,f} | |
| Chetoui | 46.00 ^{c,d,f} | 102.32 ± 1.18 ^{a,b} | 24.36 [28] | 94.03 ± 4.19 ^{a,c,d,f} | 15.83 [28] |
| Chemlali | 28.33 ^{a,b} | 99.71 ± 11.45 | | 82.74 ± 10.73 ^{a,b} | |
| Sevillane | 35.00 | 73.05 ± 15.52 ^a | | 56.75 ± 6.72 ^{a,b} | |
| Lucques | 39.00 ^{b,c,e} | 106.80 ± 9.24 ^{a,b} | | 97.74 ± 12.67 ^{a,c,d,e} | |
| Rosicola | 33.00 ^a | 91.90 ± 11.04 ^a | | 76.01 ± 8.72 ^{a,c} | |
| Meski | 36.33 ^{a,b,c,d} | 110.03 ± 20.04 | | 91.32 ± 1.13 ^{a,c,d} | |
| <i>Olea europaea</i> | | | 144 [25] | | 1.47 [25] |
| | | | 40.27 [26] | | 16.78 [26] |
| | | | 1.60 [27] | | |

Results are expressed ± SD of the three replicates
For each variety, values in the same column bearing different letters are significantly different (a, b, c, d, e and f) at p < 0.05 and p < 0.01

Table 1: Yield of extraction (%), total phenols (mgGAE/g) and total flavonoïdes(mgCE/g)) in olive leaves extracts.

The means of TP in olive leaves extracts in terms of mg GAE/g of dry material ranged from 73.05 ± 15.52 to 144.19 ± 10.27 (p < 0.05). The highest amount was observed in the order Limouni > Gerboua > Meski > Chetoui compared to Sevillane content which had the lowest amount. Our study showed a high level of TP compared to other investigations reported by Skerget et al., Makris et al., Hayes et al., and Abaza et al. (Table 1). Moreover, Mylonaki et al. found the maximum theoretical yield of total polyphenol content to be 2.50 ± 0.76 using optimal recovery techniques of polyphenol from olive while our results were higher in spite of the use of a classic technique of extraction [25-29]. Our data revealed a difference in TP content between most varieties such as Lucques variety with 106.80 ± 9.24 and Limouni variety with 144.19 ± 10.27 (p < 0.01). Tunisian varieties contained relatively higher levels of total phenolics than the Spanish and Italian varieties. This was probably due to the natural climatic conditions of Tunisian varieties.

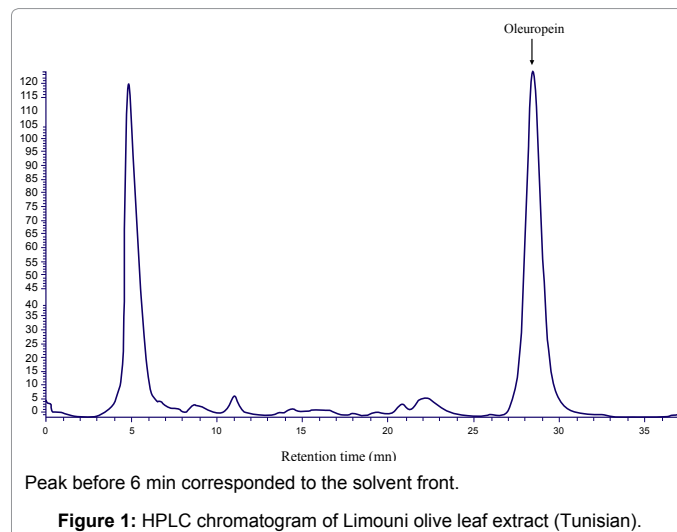
The TF content was also expressed as mg CE/g of dry material and ranged from 56.57 ± 6.0 to 125.64 ± 3.36 (p<0.05). Varieties of Gerboua exhibited an important amount of TF followed by Limouni and Lucques. Sevillane still had the lowest level of TF compared to the other varieties, whereas it was superior to those reported in previous studies [25,26,28] (Table 1). Drying method could influence the amount of obtained polyphenol. Another previous study showed that microwave drying avoided ester hydrolysis of saponins, which occurred with air-drying [30]. The drying technique using microwave energy was based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation which took place simultaneously [31]. Moreover, the variation found in the phenolic and flavonoid content of the olive leaves extract reported by different studies could be due to the different methods applied for extraction (extraction technique, solvent nature and time of extraction) and also the influence of the sample origin.

Chromatographic profiles of the different cultivars studied showed no difference in qualitative composition (Figure 1). Oleuropein was the major compound of the leaf extract. The identification of oleuropein was based on the comparison of the chromatographic retention time, UV absorbance spectra comparison with the authentic standard and confirmed by results of studies carried out by Savournin et al. and Lee-Huang Zhang et al. using the same HPLC conditions [21,32]. The obtained analytical data concerning oleuropein contents in *Olea*

europaea leaves were shown in Table 2. A high amount of oleuropein was obtained using aqueous ethanol (70%, v/v) as an extraction solvent [17,33]. In fact, we have chosen ethanol as a more preferable solvent because of its nontoxic effect, environmentally safe role and inexpensive feature. Besides, the study of Altiok et al. showed that water had an important role in extraction processes by increasing the diffusion of extractable polyphenol through the plant tissues [33]. In our study, Oleuropein content reached 57.24 mg/g of dry weight and was far in excess in comparison with the other phenolics. These data confirm how olive leaves are a source of oleuropein [5,34].

The isocratic HPLC method described in our study allowed a rapid separation and an identification of the major compounds of olive tree leaves. In fact, the analysis by HPLC using solvent gradient gave a no stable line base and no good separation of peaks. Indeed, the isocratic method could be used for the routine analysis of leaves extracts and industrial products [21,32]. The peak areas from a high-performance liquid chromatography analysis of biophenols extracted from olive leaf, especially oleuropein, can be used as chemotaxonomic markers to construct chemometric models in order to discriminate and classify the varieties of *Olea europaea* olive trees growth in same geographical zone. In our investigation, Oleuropein was present in high amount in extracts from different varieties. This chemical study showed intrinsic variations among sampled cultivars though they were collected from several varieties exposed to the same environmental (geographical, geological, and climate) and cultural (pruning, watering, and harvesting) conditions. Therefore, it could lead to characterization of varieties and evaluation of their homogeneity [34,35].

In order to evaluate the *in vitro* anti-oxidant activities of olive leaves extract, the DPPH radical scavenging activity of the leaf extracts were measured and compared to that of BHT (IC₅₀ = 17.02 µg/ml) (Table 3). The olive leaves extracts were able to scavenge the free radical. They exhibited antioxidant potential comparable to BHT. In fact, many varieties displayed a higher activity than BHT as Chemlali (IC₅₀ = 7.90 µg/ml), Rosicola (IC₅₀ = 10.88 µg/ml), Limouni (IC₅₀ = 12.25 µg/ml), Chetoui (IC₅₀ = 15.09 µg/ml), Meski d'Italie (IC₅₀ = 15.24 µg/ml) and Lucques (IC₅₀ = 16.10 µg/ml). The strong antioxidant activity of olive leaves could be attributed to their high total polyphenolic content. In fact, it was reported that polyphenols were one of the most effective antioxidative constituents in the plant [36]. Furthermore, a positive correlation between phenolic composition and antioxidant activity was proved [5,37]. Moreover, antioxidant activities could be attributed to



the higher oleuropein amount in olive leaves from different varieties. We did not observe a correlation between oleuropein content and anti-radical activity, though high oleuropein content corresponded to high anti-radical activity for some varieties. We observed a negative correlation between IC₅₀ values and oleuropein content only for four varieties; Gerboua, Lucques, Meski and Chetoui (R² = - 0.94). The good anti-radical activity of oleuropein could be due to the presence of the hydroxyl groups in its chemical structure (Figure 2). According to these results, the olive leaves extracts could prevent oxidative damage in system as a biological result of its ability to scavenge oxygen species such as hydroxyl radicals [27].

Beta-carotene bleaching was measured by the decrease in the initial absorbance at 490 nm and was slowed down in the presence of the extract. According to the results shown in figure 3, we found that Chemlali and Chetoui varieties had the higher inhibition percentage of beta-carotene. Its profiles showed 70% inhibition during 100 min, which were as strong as the synthetic BHT. However, Lucques, Meski, Sevilleane, Gerboua and Limouni varieties showed the same activities and were less effective in minimizing the oxidation of lipids. Rosicola varieties had the lowest activity.

The Fe³⁺-Fe²⁺ transformation in the presence of sample extract obtained from different varieties (0.5 mg/ml) was investigated to measure the reducing power of each extract. As shown in Table 3, the olive leaves extracts of different varieties demonstrated powerful Fe³⁺ reducing ability with statistically differences. The higher reduced power was observed in olive leaves extract of Chemlali variety followed by Gerboua and Sevilleane. The results demonstrate that olive leaves extract from different varieties had marked ferric ions (Fe³⁺) reducing ability and electron donor properties for neutralizing free radicals by forming stable products. However, this reducing power was lower than the standard antioxidant used. The reducing power reflects the

| | Oleuropein (mg/g of dry leaves) |
|----------------|--|
| Gerboua | 45.02 1.59 ^a |
| Limouni | 52.20 5.60 ^{a, b, c, d, e, f} |
| Che'toui | 57.24 ± 2.05 ^{a, b, c, d, e, f} |
| Chemlali | 47.42 1.41 ^b |
| Sevilleane | 30.76 2.64 ^{a, b, c, e} |
| Lucques | 52.12 1.96 ^{a, b, c, d} |
| Rosicola | 48.48 1.10 ^{a, c, e} |
| Meski d'Italie | 52.63 1.95 ^{a, b, d} |

Results are expressed ± SD of three replicates

For each variety, values in the same column bearing different letters (a, b, c, d, e, and f) are significantly different at p <0.05 and p <0.01

Table 2: Contents (as mg/g dry weight) of oleuropein in different olive leaves varieties determined by HPLC.

| | IC50 (ug/ml) | FRAP (absorbance at 700) |
|----------------|--------------|--------------------------|
| Gerboua | 18.64 ± 7.43 | 0.83 ± 0.04 |
| Limouni | 12.25 ± 0.47 | 0.68 ± 0.01 |
| Che'toui | 15.09 ± 1.76 | 0.66 ± 0.09 |
| Chemlali | 7.90 ± 0.56 | 0.84 ± 0.03 |
| Sevilleane | 17.02 ± 1.54 | 0.75 ± 0.07 |
| Lucques | 16.10 ± 0.06 | 0.67 ± 0.02 |
| Rosicola | 10.88 ± 1.78 | 0.54 ± 0.00 |
| Meski d'Italie | 15.24 ± 0.20 | 0.56 ± 0.03 |
| Ascorbic acid | n.d | 2.10 ± 0.11 |
| BHT | 17.02 ± 3.55 | n.d |

Means ± SD (n=3)

n.d: not determined

Table 3: Antiradical activity and reducing ability of each olive leaves variety.

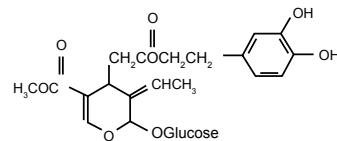


Figure 2: Chemical structure of oleuropein [38].

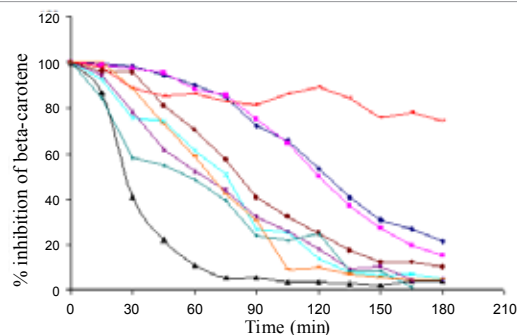


Figure 3: The antioxidant activities of olive leaves extracts of different varieties as determined by the beta-carotene bleaching test.

electron donating capacity of bioactive compound and was associated with antioxidant activity. The reducing capacity of compound might be served as an indicator of its potential antioxidant activity [38].

Conclusion

There are a wide number of systems available in order to evaluate the antioxidant properties of the extracts. The results of a single method can give only a reductive idea about the antioxidant activity of the plant. For that reason, in our study, we combined different techniques with the aim of giving a global idea about properties for each cultivar. Each olive variety had its own feature, however we could confirm that olive tree was a wide source of polyphenols, especially a source of oleuropein and it possesses a good antioxidant activity and strong reduced power. These results could be due to the solvent nature used for extraction. In fact, aqueous ethanol (70%, v/v) was the best solvent of extraction since it has exhibited a good antioxidant activity. Also, it yielded a high polyphenol and oleuropein content. It is important to reveal that the antioxidant assays carried out in this research are strictly based on chemical reaction *in vitro* and do not necessarily correlate with their activity in biological system. However, they can be useful indicators on the potency of some compounds in order to valorize plant species and use them in biological models.

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