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Effect of Growth Stage and Solvent Extract on the Antioxidant Potential of Olive Leaves

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Abstract: The effect of growth stage on the physicochemical composition and antioxydant activities of olive leaves extracted with solvents of increasing polarity was investigated at two different stages. The total phenolic, total *o*-diphenols and total flavonoid were measured. Therefore, the levels of chlorophyll and carotenoids were also measured. The content of phenols varied significantly with two different growth stages October and February. The lowest level of phenols and *o*-diphenols was detected in the first vegetative stage whereas the highest content was enregistered during the second vegetative stage. The results showed that methanol extract contains the maximum phenolic content. Furthermore, the differences in total flavonoid contents of leaves between two different stages and between solvent extract were highly significant. Thus, the AA exhibited asignificant ($p<0.05$) increase during leaves developpement. In fact, methanol and ethyl acetate extract was a considerably more effective on DPPH radical scavenger than other solvents extracts.

Keywords: *Olea europaea* L., Growth Stage, Antioxidant Activity, Chlorophylls, Phytochemical Composition

1. Introduction

Plants of importance in modern agriculture and trade are not restricted to traditional food, forage, and fiber crops, but increasingly include species with secondary metabolites having desired aromatic or therapeutic qualities, or providing source material for the perfume and chemical industries [1].There is an increasing interest in finding naturally and biologically produced antioxidants capable of inhibiting free radical reactions, retarding oxidative rancidity of lipids, protecting the human body from diseases, and preserving foods from spoiling [2-3]. The plant kingdom produces a wide range of natural antioxidants. However, there is still not enough knowledge aboutthe practical usefulness of most of them. In the group of secondary plant metabolites, antioxidant phenolics are commonly found invarious fruits, vegetables and herbs and they have been shown toprovide a defense against oxidative stress from oxidizing agents and free radicals [4]. Many herbal infusions, frequently used as home medicines have antioxidative and pharmacological properties related to the presence of phenolic compounds, especially phenolic acids and flavonoids. Polyphenolsare also known for their ability to prevent fatty acids from oxidativedecay, and provide an additional value to plants used as foodingredients, rich for example in rosmarinic acid [5]. Antioxidant properties of polyphenols arise from their high reactivity as hydrogen orelectron donors, and from the ability of the polyphenol derived radical to stabilise and delocalise the unpaired electron (chain-breaking function), and from their ability tochelate transition metal ions (termination of the Fenton reaction) [6].

The health promoting effect of antioxidantsfrom plants is thought to arise from their potential effects on thereactive oxygen/nitrogen species. In addition, antioxidants havebeen widely used in food industry to prolong the shelf life.However, there is widespread agreement that some synthetic antioxidantssuch as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) need to be replaced with natural antioxidants due to their potential health risks and toxicity [7]. Therefore, it is very important to find out new sources ofsafe and inexpensive antioxidants of natural origin.In addition to this, the presence of phenolic

compounds (phenolic acids, polyphenols and flavonoids) in plants, herbs and spices, isgaining increasing attention because of their various functions,such as antioxidant activity and flavouring properties [8].

Despite all the reported investigations on phenol composition of *Olea europaea* L. from different origins, there is no bibliographic data reporting the variation of total phenol during the growth of *Olea europaea* L. The objective of this study was to investigate variation of the phenol, *o*-diphenols and flavonoïd contents and the antioxidative properties of hexane, chloroform, ethyl acetate and methanol extracts from olive leaves cultivar during two vegetative growth stages in order to determine the optimal harvest time characterized by the highest content of bioactive compounds.

2. Materials and Methods

2.1. Plant and Sample Material

Fresh leaves of the cultivar *chemlali* of *O. europaea* L. were collected from the coastal region -Mahdia –in the centre of Tunisia at two different harvesting stages. The sample collection was done according to the biological cycle of olive leaves [9], when the leaves are still growing (October) and when the leaves have completed their growth (February). The leaves were separated from the other parts. Voucher specimens have been deposited in the Herbarium of the Laboratory of Biochemistry, Faculty of Medicine of Monastir, Tunisia.

2.2. Determination of Pigment Content

The procedure was carried out at 4°C and dark. Aleaf sample (0.25 g) was mashed in a mortar andpestle with 80% acetone (v/v), the extract wasfiltered through two layers of nylon and centrifugedin sealed tubes at 15000g for 5 min. The supernatantwas collected and the absorbance wasread at 663 and 647nmfor chlorophyll a and chlorophyll b, respectively,and at 470 nm for carotenoid content. The concentrations for chlorophyll a, chlorophyll b, and the sum of leaf carotenoids (xanthophylls and carotenes) were given in µg ml $^{-1}$ extract solution according to the equations of Lichtenthaler and Buschmann (2001) [10]:

$$\text{Chlorophyll a} = 12.25A663 - 2.79\ A647;$$

$$\text{Chlorophyll b} = 21.50A647 - 5.10\ A663;$$

$$\text{Carotenoid} = (1000\ A470 - 1.82\text{Chl a} - 95.15\text{Chl b})/225.$$

2.3. Preparation of Leaf Extracts

Fresh leaves were air-dried in an oven at 40°C for three days and then were pulverized to 0.2–0.4 mm particle in a cutting mill. For extraction, four different solvents in ascending polarity (hexane, chloroform, ethyl acetate and methanol) were used to fractionate the soluble compounds from the *Olea europaea* L. leaves. The dried leaves sample (5 g) was extracted with 100 ml of each solvent in a shaker (Eyela Model MMS-300, Tokyo Rikakikai Co., Ltd., Japan), at room temperature and then the solvents from the combined extracts were evaporated by vacuum rotary evaporator (EYELA N1000, Japan). After filtration, all extracts obtained were then transferred to vials and kept in the dark at –20 ° C prior to use.

2.4. Total Phenols and o-diphenols

Total phenolic contents and *o*-diphenols of fractions were determined according to the method of Montedoro et al.(1992) [11] with minor modifications. For total phenols, 0.4 milliliter of each fraction and 10 ml of diluted Folin–Ciocalteu reagent were mixed. After 1-min incubation, 8 ml of sodium carbonate (75g/L) was added and the mixture was incubated for 1 h. The absorbance was measured at 765 nm. The same extract was used to determine total *o*-diphenols. Then, 1 ml of a solution of HCl (0.5 N), 1 ml of a solution of a mixture of $NaNO_2$ (10 g) and $NaMoO_4.2H_2O$ (10 g) in 100 ml H_2O and finally 1 ml of a solution of NaOH (1 N) were added to 100 µl of the leaves extract. After 30 min, *o*-diphenols were read at 500nm. The total phenols and o-diphenols were expressed on a dry weight basis as mg hydroxytyrosol equivalents / 100 g of sample.

2.5. Determination of Total Flavonoïds

Total flavonoïd contents (TF) of the leaves extracts were determined according to the colorimetric assay developed by Zhishen et al.(1999) [12]. One ml of properly diluted leaves extract was mixed with 4 ml of distilled water. At zero time, 0.3 ml of (5% w/v) $NaNO_2$ was added. After 5 min, 0.3 ml of (10% w/v) $AlCl_3$ was added. At 6 min, 2 ml of 1 M solution of NaOH were added. Finally, the volume was made up to 10 ml, immediately by the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the absorbance was read at 510 nm. The results were also expressed on a dry weight basis as mg catechin equivalents (CEQ) / 100 g of sample.

2.6. Antioxidant Activity (DPPH Radical Scavenging Assay)

The DPPH method [13] was used to determine antioxidant activity of olive leaf extracts. 20-µL from the stock solution of the sample were dissolved in absolute ethanol to a final volume of 1 mL and then added to 1 mL DPPH (0.1 mM, in absolute ethanol). The reaction mixture was kept at roomtemperature. The optical density (OD) of the solution was measured at 517 nm, after 60 min. The optical densities of the samples in the absence of DPPH weresubtracted from the corresponding OD with DPPH. The % reduction values were determined and compared toappropriate standards.Inhibition of the free radical DPPH, in percent (I %) was calculated using the following equation:

$$I\% = (A_{blank} - A_{sample}) / A_{blank} \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the tested compound), and A_{sample} is the absorbance of the tested compound.

2.7. Statistical Analyses

All assays were run in triplicate. The results are reported as mean values of three analysis and standard deviation. Data were subjected to statistical analysis using the SPSS programme, release 11.0 for Windows (SPSS, Chicago, IL, USA). The one-way analysis of variance (ANOVA) followed by Duncan multiple range test were employed to study the effect of solvent and the differences between individual means were deemed to be significant at $p < 0.05$. Differences between the two harvested dates were carried out using Student-*t* test. Simple associations between variables were calculated as the Pearson correlation. Simple associations between variables were calculated as the Pearson correlation. Hierarchical cluster analysis (HCA) was carried out using XLStat-Pro 7.5 (2007) for Windows (Addinsoft, New York, NY, (USA).

3. Results and Discussion

3.1. Chlorophyll Content

Figure 1 reports the amount of chlorophylls a and b, their sum and total carotenoids, respectively. The highest concentration of chlorophylls a and b, total chlorophylls and total carotenoids was observed in leaves of October, followed by leaves of February. These results show that the highest chlorophyll and carotenoids values are in the leaves of the first stage of growth (Figure 1). Statistical analysis of this parameter showed significant differences between leaves of the two times of harvest ($p \leq 0.01$). Such a comparison was very difficult for leaves in two stage of growth with olive leaves from other cultivar.

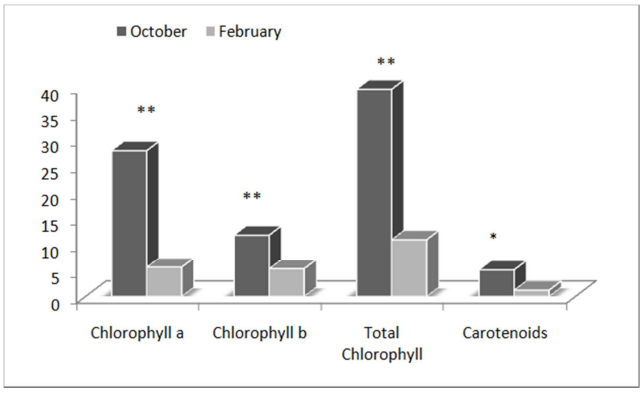

Fig. 1. *Total pigment content of the olive leaves sampled harvested in two different stages. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of p < 0.05.*

3.2. Phytochemical Composition

Total phenolic assay was carried out based on the absorbance values of the various extract solutions, reacted with Folin–Ciocalteu reagent and compared with the standard solutions of hydroxytyrosol equivalents as described above. Data obtained from the total phenolic assay supports the key role of phenolic compounds in free radical scavenging and/or reducing systems. Results of quantitative estimation of phenols in the aerial parts of *O. europaea* L. during the different growth stages are given in Fig. 2. The content of phenols varied significantly with two different growth stages October and February. The amount of total phenolic contents of the leaf extracts (hexane, chloroform, ethyl acetate and methanol) of *O. europaea* L.was tested, and occurred in the range of 0.86–587.42 mg /100 g DW (Fig. 2). The lowest level of phenols was detected in the first vegetative stage whereas the highest content was enregistered during the second vegetative stage.The total phenolic contents of the leaves extract of the second growth stage of methanol, ethyl acetate, chloroform and hexane were noted to be 587.42, 62.25, 17.042 and 1.62 mg/100 g DW, respectively. A consistent decrease in the amounts of phenols was observed in the leaves extract during the first growth stages with 0.86, 4.01, 25.71 and 294.65mg /100 g DW, in hexane, chloroform, ethyl acetate and methanol, respectively. In fact, the results showed that methanol extract contains the maximum phenolic content, followed by ethyl acetate, chloroform and hexane (Fig. 2). The extraction of phenolic compounds from the plant materials has been achieved mainly with methanol or aqueous methanol [14]. Thus, the results showed significant differences ($p \leq 0.05$), among solvents extracts.In fact, these results suggest that the second growth stage could be characterized by the maximum polyphenols totaux.

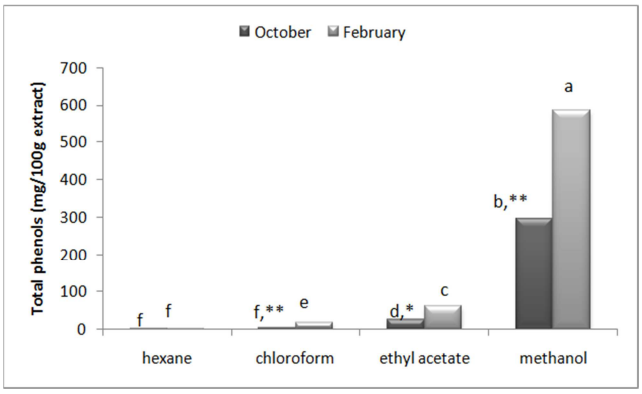

Fig. 2. *Total phenolic content of extracts of olive leaves harvested in October and February obtained by different solvent extraction. Results are expressed as hydroxytyrosol equivalents. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of p < 0.05.*

The content of extractable *o*-diphenols in extracts, expressed in hydroxytyrosol equivalents, varied between 5.43 mg and 171.08 mg /100 g DW (Fig. 3). As expected, amount of the total *o*-diphenols was very high in methanol extracts from leaves of October and February, respectively (68.11 and 171.08 mg /100 g DW). It was followed by chloroform extract of October and February with a value of 12.52 mg and 48.09mg /100 g DW, respectively (Fig. 3). In our recent studies, it has been reported that the yield of extractable compounds was highest in methanol extract from the olive leaves in comparison with the solvents such as ethyl acetate and chloroform [15]. Although the extraction yield of total extractable compounds with hexane was the smallest, it was

comparable with the proportion of total *o*-diphenols compounds found for extracts obtained with chloroform and ethyl acetate (Fig. 3).Statistically, the content of total *o*-diphenols in leaves extracts of cv. *chemlali* was significant when collected at two growth stages (Fig. 3). In fact, the content of *o*-diphenols varied significantly ($p \leq 0.05$), among hexane, chloroform, ethyl acetate and methanol extracts of October and February. Variation of phenolic concentration during the growth of olive leaves affirm the influence of both phonological stages and climate factors on production and release of these metabolites.

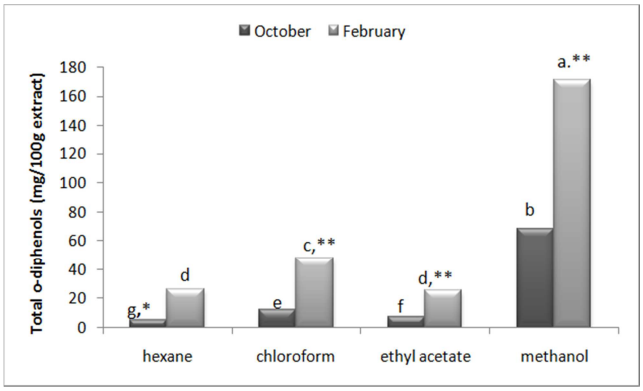

Fig. 3. *Total o-diphenols content of extracts of olive leaves harvested in October and February obtained by different solvent extraction. Results are expressed as hydroxytyrosol equivalents. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of p < 0.05.*

Results of total flavonoids, expressed as catechin equivalents, in the leaves extracts of October were 27.94, 133.68, 122.00 and 154.94 mg CEQ/100 g DW, with hexane, chloroform, ethyl acetate and methanol, respectively (Fig. 4). The content of flavonoids in the leaves extracts of February decreased in the same order of methanolic fraction > chloroform fraction >ethyl acetate fraction > hexane fraction. (Fig.4). The results given in Fig.4 showed that the highest content of total flavonoids compounds was found in extracts obtained with methanol and chloroform, whereas the contents obtained with hexane were much smaller. This data showed, then, that methanol and chloroform are good solvent systems for the extraction of total flavonoids. Studying seasonal changes in contents of phenolic compounds in Rhus, Euonymus and Acer leaves, Ishikura (1976) [16] have found that the total flavonoid per leaf increased rapidly at the second growth stages. In the other hand, the accumulation of flavonoids in olive leaves during the second vegetative stage maybe due to the fact that during this stage, the plant protection is mainly secured by phenolics which are highly accumulated during this stage. Indeed, it is well known that an important function of flavonoids and phenolic acids is their action in plant defence mechanisms [17].The accumulation of phenols duringthe second growth stage could be related to ecological roles such as intensifying antifungal defences and attracting pollinators [18]. Furthermore, the differences in total flavonoid contents of leaves harvested at two different stages and among solvent extract were highly significant ($p < 0.001$).

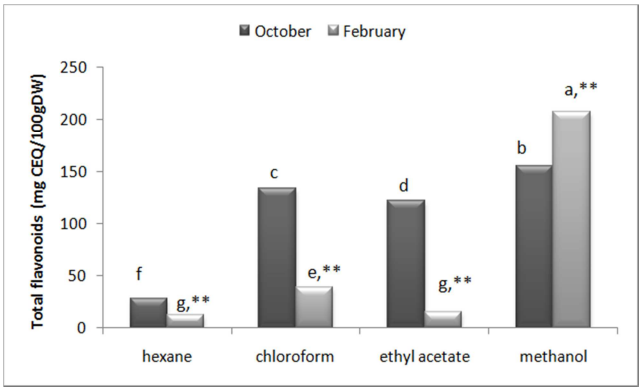

Fig. 4. *Total flavonoids content (as catechin equivalents) in extracts from olive leaves sampled in October and February. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of p < 0.05.*

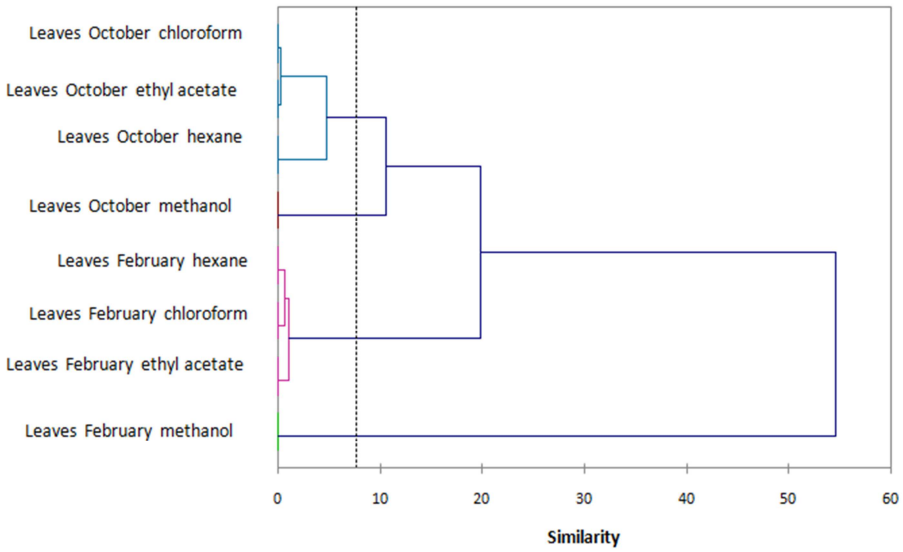

Fig. 5. *Hierarchical classification the olive leaves sampled from cultivar chemlali harvested in two different stages based on the levels of phenol composition.*

The hierarchical cluster analysis based on the contents of phytochemical composition was performed on the leaves from cultivar *chemlali* treated with solvents and collected at two different stages of the vegetative cycle of the olive. The results obtained following HCA are shown as a dendogram (Figure 5) in which three well-defined clusters are observed. Samples will be grouped in clusters in terms of their nearness or similarity.The first group of samples is clearly noticeable which is composed of leaves harvested in October and treated with chloroform, ethyl acetate, hexane and methanol. The second cluster consists of leaves harvested in February and treated with chloroform, hexane and ethyl acetate, while the third cluster includes leaves of February extracted with methanol. The results showed that that the leaves collected in October and treated with hexane, chloroform and ethyl acetate have the levels in phenol composition relatively similar with the leaves collected in February and treated with hexane, chloroform and ethyl acetate. Therefore, the leaves of first stage of growth (October) have an intermediate composition. In fact, the hierarchical cluster analysis presented a clear separation of leaves *chemlali* collected in the second stage of growth and treated with methanol, which show different composition from that of other extracts leaves studied.

3.3. Antioxidant Activity

3.3.1. DPPH Radical Scavenging Activity

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants [19].Vegetables are the main sources of antioxidant vitamins (vitamin E, vitamin C, precursor of vitamin A i.e., ß-carotene), which act as free radical scavengers. However, more than 80% of the total antioxidant capacity in vegetables comes from the ingredients other than antioxidant vitamins, indicating the presence of phenolic compounds are the dominant antioxidants that exhibit scavenging efficiency on free radicals and reactive oxygen species are numerous and widely distributed in the plant kingdom [20]. As can be seen from the Fig.6, free radical scavenging effect of the extract of leaves from October was found to decrease in the order, ethyl acetate extract >methanol extract>chloroform extract>hexane extract. In fact, the free radical scavenging activities of methanol and ethyl acetate extracts (56.99% and 53.01%, respectively) were found superior to all other extracts. Therefore, low polar extracts exhibited low DPPH scavenging activity. The scavenging effect of hexane, chloroform, ethyl acetate and methanol extracts from the leaves of February on DPPH radicals increased from 49.94 to 60.5% and were 49.94 %, 52.23%, 59.41% and 60.5%, respectively, indicating that the ethyl acetate and methanol extract was a considerably more effective DPPH radicals scavenger ($p < 0.05$). The antioxidant activity of phenolic compounds in methanol and ethyl acetate extracts is reported to be mainly due to their redox properties [21], which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Organic extracts may be more beneficial than isolated constituents, because other compounds present in the extracts can change the chemical or biological properties of bioactive individual component [22].Significant differences ($p > 0.05$) in antioxidant activities were found between solvents extracts, Besides, phenolics were also found to be one of the most plentiful classes of constituents in ethyl acetate extract of olive leaves. This is due to the presence of high bioactive compounds in ethyl acetate extract as compared to other organic extracts. The key role of phenolic compounds to scavenge free radicals has been emphasized in several reports [23].Thus, the AA exhibited a significant ($p<0.05$) increase during leaves developpement (except for the chloroform extract) in leaves extracts. In fact, regarding theses variations in the accumulation of secondary metabolites and antioxidant activities in olive leaves, it could be concluded that the physiological stage of the plant affects the choice of best harvesting time.

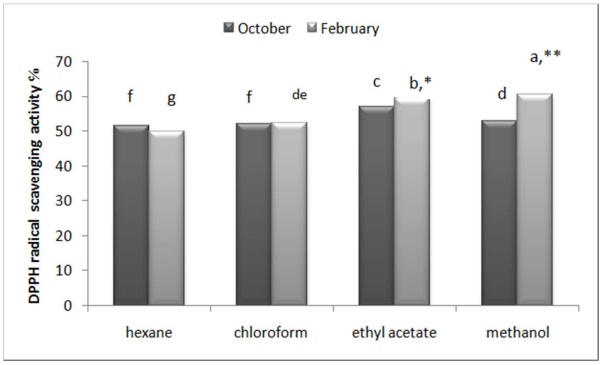

Fig. 6. *Free radical-scavenging capacities of the extracts measured in DPPH assay. Values are expressed as means ± standard deviation (n = 3). Means with different letters were significantly different at the level of p < 0.05.*

3.3.2. Correlation Between Phytochemical Compounds and Antioxidant Activities

In order to assess the contribution of total polyphenols, total *o*-diphenols and total flavonoids to the total antioxidant activity ofthe olive leaves, the Pearson's correlation coefficient was calculated.Highly positive relationship was found between antioxidant capacity values and contents of antioxidant components (total phenols, r = 0.585 and total total*o*-diphenols,r = 0.532),A significant correlation ($p<0.01$) was also observed between total flavonoids and total polyphenols (r = 0.759) and total *o*-diphenols (r = 0.653).No significant correlation was observed between flavonoids content and DPPH radical cation scavenging activity in the olive leaves (r = 0.414). However, important correlation was observed between polyphenols content and total *o*-diphenols (r = 0.957). The strong correlation found between the antioxidant capacity and total phenolsand *o*-diphenols contents suggests a strong contribution of these compounds in the total antioxidant capacity and healthiness of olive leaves.

4. Conclusion

Plant secondary metabolites generally display remarkable biological activities such as antioxidant properties which are useful for preserving foods from decay and contamination and/or preventing living tissues from various diseases. Accordingly in this study, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. To explore the suitability of different extracting solvents with different polarity, we have compared the total phenolic content and antioxidant properties of hexane, chloroform, ethyl acetate and methanol extracts. Methanol and ethyl acetate extracts showed the highest scavenging activities against DPPH. In the other hand, phenolic content showed marked variation with plant growth stage and the maximum phenolic amounts were detected during the second vegetative stage. The presence of these phenolic compounds in appreciable amounts makes olive leaves in the second vegetative stage an excellent source of these compounds for agro alimentary industry and for the formulation of functional foods.

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