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Phenolic composition and antioxidant activity of different parts of *pistacia vera L.*

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Abstract: Phenolic compounds are widely distributed in almost all higher plants and are present in different tissues. The quality and quantity of phenolics are used as criteria for variety identification. The present study has been planned to determine the phenolic composition of *Pistacia vera*.

Total phenolic content and concentration of flavonoids of eight different extracts, from the floral buds and leaves of *Pistacia vera*. (*Anacardiaceae*) were determined using spectrophotometric methods. The total phenolic content ranged from 401.64 to 513.77 (mg GAE/g DW). The total flavonoid concentrations varied from 8.28 to 67.94 mg CE/g.

Antioxidant activity was assessed by three methods: DPPH°+, β-carotene and ABTS and determined respectively as IC50 (mg/mL), CAA and PI (%), all extract have shown to be endowed by a high antioxidant activity similar to that of BHT.

Ethanolic extract of male floral buds showed the highest phenolic and flavonoid concentration and the strongest antioxidant activity. The significant linear correlation was confirmed between the values for the total phenolic content and antioxidant activity of plant extracts.

Phenolic compounds were investigated by LTQ Orbitrap LC-MS analysis and revealed 17compounds split into 5 groups: Abscisic acids, phenolic acids and their derivatives, flavonols, flavanones, flavones, flavanols. The high contents of phenolic compounds indicated that these last contribute to the antioxidant activity. The *Pistacia vera* can be regarded as promising candidates for natural plant sources of antioxidants with high value.

Keywords: *Pistacia vera*; floral buds; leaves; total phenols; flavonoids; LC-MS; antioxidant activity.

Introduction

Phenolic compounds are one of the most widely occurring groups of phytochemicals with considerable physiological and morphological importance in plants.

The accumulation of phenolic compounds varies strongly with the growth state, development and responses to environmental stress and is a result of the balance between biosynthesis and further catabolism [1,2].
The pistachio of commerce is the only edible species among the 11 species in the genus *Pistacia*.

Its Latin name is *Pistacia vera* L. a member of the family *Anacardiaceae*. The tree has a pinnately compound leaf. Each leaf subtends a single axillary bud. Most of these lateral axillary buds differentiate into inflorescence primordia and produce a nut bearing rachis the following year; thus, pistachios bear laterally on one-year-old wood [3].

The pistachio tree is a dioecious plant with male and female flowers growing on separate trees [4].
Iran, Turkey, USA, Syria, Italy, Tunisia and Greece are the main producers of cultivated pistachio [5].
The chemical constituents of the *Pistacia* genus were studied and found to be: monoterpenes [6], tetracyclic triterpenoids [7], flavonoids [8], and other phenolics including gallic acid and essential oils [9]. Six gallotannins and seven flavonoid glycosides were isolated from *P. weinmannifolia* [10].

The main objective of the present study was to assess metabolites in flowers buds and leaves of male and female *Pistacia vera* L. CV "Mateur". The assessments were carried out by measuring total

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phenolics and flavonoids. Then by elucidating the compounds of each extract. Finally, the antioxidant activity was evaluated.

Material and Methods

Extract preparation

This work was carried out in *Pistacia vera* male and female cultivar "Mateur".

The plant parts used in extraction were classified as leaves and floral buds.

Pistacia vera L. buds were collected in April 2012 from plants grown in the North of Tunisia while the leaves were collected in June 2012 from the same site. The botanical identification was performed by Mr. Arbi Khouja. Voucher specimens were deposited in the herbarium of the High School of Food Industries for future reference.

Each of the above-cited plant parts was dried under shade and powdered to a fine grade using a laboratory scale mill. Ten grams of each plant part was weighed accurately and, two kinds of the extract were prepared with 96% ethanol and distilled water, separately, at room temperature macerating two times (×200 mL), every 24h the solvent is removed. Following the filtration, ethanol and aqueous extracts were concentrated to dryness under reduced pressure using a rotary evaporator at 40 °C to yield crude extracts.

Yield of each extract was calculated using the following formula:

$$Yield\ (\%) = \frac{m1}{\mathrm{m0}} \times 100$$

With m_0: mass of sample, m1: mass of crude extract

Spectrophotometric assays

The total phenolic content was estimated using the Folin–Ciocalteu reagent (Spanos & Wrolstad, 1990)[11], using 100 µl extract, 900 µl pure water and 5 mL reagent. For the preparation of a standard curve, 0.10–0.50 mg/mL gallic acid was used and data were expressed in mg gallic acid equivalents (GAE) per 100 g dry weight (DW). The absorbance of all samples was measured at 760 nm.

Total flavonoid content was determined according to Dewanto, Wu, Adom, and Liu (2002) [12], using aluminum
chloride and sodium nitrite as reagents. The absorbance of the samples was measured at 510 nm against a reagent blank. Catechin at concentrations of 0.01–0.25 mg/mL was used to create a calibration curve and data were expressed as mg catechin equivalents (CE) per 100 g DW.

Identification of phenolic profile

LTQ Orbitrap LC-MS analysis.

Structural characterization was assessed using a LTQ Orbitrap XL LC-MS (Thermo Fisher scientific, Bremen, Germany), fitted with an Accela 600 Pump, Accela PDA and Accela autosampler. The Orbitrap MS analyzed selected samples in the negative ion mode over the mass range *m/z* 120 - 1000.

The compounds were separated at 40°C on a Hypersil gold C18 (50 mm x 2.1 mm; 1.9 µm) column from
Thermo Fisher scientific (Bremen, Germany). The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A linear gradient program at a flow rate of 0.4 mL/min was used 0–5 min from 5% to 95% (B), 5–6 min 95 % (B) then 5% (B) for 3 min. the injection volume was 5 µL.

MS settings were as follows: capillary temperature (270 °C), vaporizer temperature (350 °C), sheath and
auxiliary gas pressures (35 and 10 arbitrary units). For negative ion measurements, the ion source voltage was set to –4.00 kV, the capillary voltage to –37 V and the tube lens voltage to –204.11 V. Data acquisitions and analysis were carried out with Xcalibur software (version 2.1).

Phenolic compounds were identified according to the corresponding spectral characteristics: mass spectra,
accurate mass, characteristic fragmentation, and characteristic retention time.

The quantification was performed by the external standard method as described previously (Oelschlaegel, Set *al.*, 2012) [13].

Evaluation of the antioxidant capacity

All experiments were done under subdued light. All samples were analyzed in triplicate at five different concentrations (10, 5, 1, 0.1, 0.01 mg/mL) using a UV/Vis spectrophotometer Jenway 6505.

DPPH assay.

DPPH is a free radical, being stable at room temperature and producing a purple solution in ethanol. In presence of antioxidant compounds, the DPPH is reduced, producing a colorless ethanol solution.

The radical-scavenging activity of extracts was evaluated using the test of the stable free radical DPPH (Chen, Y et *al.*, 1999) [14]. 2 mL of different concentrations of different extract in ethanol and 2 mL of ethanol for control sample were mixed with 2 mL of freshly prepared DPPH solution in ethanol (2.10^{-4} M) and allowed to stand for 30 min in the dark at room temperature. The absorbance of the solution was measured at 517 nm against ethanol as a blank. The radical scavenging activity was

expressed as IC50 (µg. mL^{-1}), the concentration providing 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated using the following formula:

$$\%inhibition = \frac{AC(0) - AS(t)}{AC(0)} \times 100$$

Where AC(0) is the absorbance of the control at t = 30 min and AS(t) is the absorbance of the tested sample at t = 30 min. BHT was used as a positive control. Tests were carried out in triplicate.

β-Carotene/linoleic acid bleaching method.

The β-carotene bleaching method is one of the most frequently applied methods for determining the total antioxidant property of the extracts. In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C and attacks the β-carotene molecules causing a reduction in the absorbance at 470 nm. Beta-carotene in the systems undergoes rapid discoloration in the absence of antioxidant and vice versa in its presence.

The presence of different antioxidants can delay the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system (Jayaprakash et al., 2003) [15]. Thus, the degradation rate of β-carotene–linoleate depends on the antioxidant activity of the extracts.

The antioxidant activity of extracts was estimated using β-carotene bleaching method as described by Suja, Jayalekshmy and Arumughan [16]. First, 0.2 mg of β-carotene was dissolved in 1 mL chloroform, then 20 mg linoleic acid and 200 mg Tween 40 were added. The chloroform was removed using a rotary evaporator at 40 °C for 5 min and 50 mL of oxygenated distilled water was added to the residue slowly with vigorous agitation, to form an emulsion. Aliquots (4 mL) of the β-carotene/linoleic acid emulsion were mixed with 0.2 mL of the sample solution in ethanol (0.2 mg. mL^{-1}), 0.2 mL of ethanol for negative control and 0.2 mL of BHT for positive control. The blank consisted on an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm until the color of β-carotene disappeared in the control (t = 120 min). Antioxidant activity percentages (%AA) were calculated using the following equation:

$$\%AA = \frac{AS(120) - AC(120)}{AC(0) - AC(120)} \times 100$$

Where AS (120) is the absorbance of the tested sample at 120 min, AC (120) is the absorbance of the control at 120 min and AC(0) is the absorbance of the control at 0 min. The tests were carried out in triplicate.

ABTS°+ radical scavenging activity assay.

Antiradical activity of extracts was assessed using the ABTS°+ free radical decolorization assay developed by Re et *al.*, (1999) [17] with some modification. Briefly, the performed radical monocation of ABTS°+ was generated by reacting ABTS°+ solution (7mM) with 2.45 mM $K_2S_2O_8$. The mixture was allowed to stand for 15 hours in the dark at room temperature. The solution was diluted with ethanol to obtain the absorption of 0.7 ± 0.2 units at 734 nm. Samples were separately dissolved in ethanol to yield the following concentrations (0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL). In order to measure the antioxidant activity of extracts, 10 µL of each one at various concentrations were added to 990 µL of diluted ABTS°+.

Results and Discussion

The yield of extraction is illustrated on the following Table1:

Table 1. Yields of different extracts.

Percent yields (%)	Floral Buds		Leaves	
	Male	**Female**	**Male**	**Female**
Ethanolic extracts	8.50	18.00	33.80	33.50
Aqueous extracts	28.10	36.79	26.40	32.70

Phenolics compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [18].

The accumulation of phenolic compounds is a carefully controlled process with both the levels of secondary metabolites and the composition of the phenolic pool varying considerably between organisms, tissues, developmental stages and, in relation to environmental conditions.[19].

The main function of phenolic is to maintain stable the concentration of free radical by producing and scavenging them and their physiological function may be shown by regulation of cell redox potential.[20].

The results showed significant differences in total phenolic compounds and total flavonoids contents in floral buds and leaves.

Ethanol and aqueous extracts of floral buds and leaves of *Pïstacia vera* (male and female) were studied for their contents of total phenols. Table 2 shows the total phenol contents measured by Folin Ciocalteu reagent in terms of gallic acid equivalent (GAE)

Table 2. Total polyphenol and flavonoids of Pistacia vera extracts.

Extracts	mg of GAE/g*	mg CE/g*
ML-EE	401,64±0.815	64,38±0.598
FL-EE	428,10±0.258	28,53±0.417
FL-WE	445,70±0.612	17,31±0.793
ML-WE	512,46±0.407	22,24±0.940
FFB-EE	418,87±0.556	47,28±0.386
MFB-EE	513,77±0.389	67,94±0.638
FFB-WE	428,25±0.432	12,93±0.317
MFB-WE	505,26±0.744	8,28±0.506

*Each value is the average of three analyses ± standard deviation.

M_{FB-EE}: ethanolic extract of male floral bud, **M_{FB-WE}**: water extract of male floral bud, **F_{FB-EE}**: ethanolic extract of female floral bud, **F_{FB-WE}**: water extract of female floral bud, **M_{L-EE}**: ethanolic extract of male leaves, **M_{L-WE}**: water extract of male leaves, **F_{L-EE}**: ethanolic extract of female leaves, **F_{L-WE}**: water extract of female leaves.

The total phenolic content of different extracts of leaves are ranging from 401.64 to 512.46 (mg GAE/g DW), however, those of floral buds range from 418.87 to 513.77(mg GAE/g DW).

The highest total phenolic content was observed in Ethanol extract of the Male Floral Buds Categories, and the lowest was observed in Ethanol extract of male leaves. The results showed that *Pistacia vera* tree leaves and floral buds fractions contained a mixture of phenolic compounds at different levels according to the polarity of solvent used in the extraction process.

As in the case of total phenolics, the amounts of flavonoids in the extracts were dependent on the employed solvent and the organ used. They ranged from 8.28 to 67.94 mg CE/g of extract. In this case, too we note that the highest amounts were observed on male floral buds ethanol extract.

Regardless of the organ studied, we showed that male ethanol extracts are the richest on flavonoids contents.

We note also that flavonoids are more extracted with ethanol than water either for male or female trees.

The investigation of chromatograms with ORBITRAP indicated some differences among *Pistacia vera* floral buds and leaves.

The amount (expressed as mg/l f.w) of each identified phenolic compound is reported in Table 3. In accordance with data reported above, it is evident that between pistachio floral bud and leaves, there are significant differences concerning not only the qualitative chemical profile, but also the quantitative one.

Ethanolic and aqueous extracts of male and female trees showed the presence of seventeen different compounds.
Gallic acid, protocatechuic_acid and chrysin were identical in all of the examined materials.
Rutin, caffeic acid and catechin were identified in all samples except on male and female floral buds extracted with ethanol.

Coumaric acid was not observed on male floral buds regardless of the type of solvent used.
In the case of gallic acid, the amount in Ethanol Extract of leaves is better than in Water extract while in floral buds Water is better. For protocatechic acid, Ethanol extract seems to give better yield.

Regardless the solvent used, we note that catechin is available on all leaves extracts. For p-Coumaric acid, the highest amount was observed on F_{FB-WE} and F_{FB-EE} with 7.81 and 14.27 mg/l respectively.

Ellagic acid, quercitin and rutin are marker for floral buds exceptionally for male floral buds aqueous extract.

In this way, two extracts appear to be of major interest which is male floral bud ethanol extract (**M_{FB-EE}**) and male leaves water extract (**M_{L-WE}**).

The wealth of floral buds in phenolic compounds can be explained by the fact that male flower buds rich in pollen will pollinate the female trees that produce pistachios which explain their high content of phenolic compounds.

For leaves, the variation of the composition between sex can be explained by the fact that phenolics compounds can be influenced by climatic and soil conditions.

The antioxidant capacity

The antioxidant activity of plants is mainly due to the active compounds presents in them.
The ethanol and aqueous extracts prepared from floral buds and leaves of *Pistacia vera* exhibited strong antioxidant activity assayed by the three different methods including DPPH, ABTS°+ free radicals scavenging, and β-carotene–linoleic acid system. The results of the investigation are shown in Table 4.

Table 3. Contents (mg/g) of phenolic compounds of Pistacia vera floral buds and leaves extracts.

Peak N°	Compound Name	Mass (mg/l)							
		M_L/EE	F_L/EE	M_L/WE	F_L/WE	F_{FB}/EE	F_{FB}/WE	M_{FB}/WE	M_{FB}/EE
Abscisic acids, phenolic acids and their derivatives									
1	**Gallicacid**	6,97	16,54	188,60	115,15	115,44	2,22	6,69	131,31
2	**Protocatechuic acid**	1,53	2,53	45,78	6,82	30,57	146,22	171,85	15,50
3	**Chlorogenic acid**	NF*	NF*	0,10	NF*	0,07	NF*	NF*	NF*
4	**Caffeic acid**	0,04	0,10	0,14	0,31	0,27	NF*	NF*	0,12
5	**p-Coumaric acid**	0,73	1,56	0,95	4,96	7,81	14,27	NF*	NF*
6	**Ellagic acid**	0,74	NF*	NF*	NF*	10,13	1,54	0,77	42,81
7	**cis,trans-Abscisic Acid**	NF*	NF*	NF*	NF*	NF*	NF*	NF*	0,32
Flavonols									
8	**Rutin**	1,40	1,70	0,05	3,15	3,10	NF*	NF*	9,84
9	**Mirycetin**	0,50	0,50	0,35	0,40	0,37	NF*	0,32	0,97
10	**Kaempferol**	NF*	NF*	NF*	NF*	0,19	NF*	NF*	0,43
11	**Quercetin**	0,31	0,20	NF*	0,15	2,16	NF*	NF*	3,54
Flavanones									
12	**Naringenin**	0,25	0,21	NF*	NF*	0,44	NF*	NF*	0,60
13	**Pinocembrin**	0,18	0,18	0,12	0,27	0,22	NF*	0,27	0,15
Flavones									
14	**Apigenin**	NF*	NF*	NF*	NF*	NF*	NF*	NF*	0,12
15	**Chrysin**	2,59	1,16	0,83	0,48	5,17	2,24	0,97	1,32
16	**Luteolin**	0,06	0,05	NF*	NF*	0,06	0,05	NF*	0,13
Flavanols									
17	**Catechin**	5,96	5,36	6,99	3,10	0,58	NF*	NF*	0,31

***NF**: **Not Found**

Table 4. Antioxidant activity of different extracts of floral buds and leaves *Pistacia vera**.

		DPPH assay IC_{50} (mg/mL)	**β-carotene method (CAA)**	**ABTS°+ assay PI (%)**
Floral buds	**FFB-WE**	0.95±1.21	557.49±2.07	84.04±1.43
	MFB-WE	0.10±0.64	547.038±0.36	89.45±0.02
	FFB-EE	0.34±1.48	545.29±1.17	68.80±1.12
	MFB-EE	0.009±0.001	716.02±1.24	87.46±1.66
Leaves	**FL-WE**	0.04±0.33	679.44±0.87	82.62±0.49
	ML-WE	0.01±0.001	667.24±3.02	78.20±1.17
	FL-EE	0.29±1.15	698.6±1.45	76.92±0.51
	ML-EE	0.01±0.32	686.91±0.92	80.76±0.22
BHT		0.02±0.23	641.11±0.17	87.30±0.20

*The values shown are the mean ± standard deviation of three replications

Little differences were observed between the three radical scavenging assays (DPPH•, β-caroten and ABTS°+).All the eight vegetal materials exhibited antioxidant properties due to the presence of phenolic compounds.

The relatively stable organic radical, DPPH, has been widely used in the determination of antioxidant activity of single compounds, as well as of different plant extracts (Katalinic et *al*., 2006) [21].

All tested extracts in the DPPH assay appeared to be endowed with good antioxidant properties. However, the antioxidant activity of the various extracts tested was basically dependent on their concentration.
The radical scavenging activity of extracts was measured by their IC_{50} values (amount of sample required for 50% scavenging of DPPH radical). The IC_{50} value is negatively related to the antioxidant activity. The higher the IC_{50}, the lower the antioxidant activity.

The values of IC50 calculated for *Pistacia vera* extracts from leaves and floral buds confirmed the reactivity of these samples against DPPH° free radicals.
The best results were obtained with the ethanol extract from male floral buds [IC50=(0.009±0.001) mg/mL] and the Ethanol and Water extracts from male leaves [IC50=(0.01±0.001) mg/mL].
Independently of the studied organ and the solvent used, male tree presents the highest antioxidant activity similar to that of BHT.

The ABTS°+ method gives a measure of the antioxidant activity of extract by determining the reduction of the radical cation as the percentage of inhibition (PI) of absorbance at 734 nm. Chakraborty reported that the decolorization of the ABTS°+cation reflects the capacity of an antioxidant to donate electrons or hydrogen atoms in order to inactivate this radical species [6].

The result of ABTS°+ radical scavenging effect of leaf and floral buds extracts is shown in Table 4. The extracts have shown the dose dependent scavenging of ABTS°+ radicals. The radical scavenging effect of floral buds extracts was higher than that of leaf extracts. Among solvent extracts, water extracts scavenged radicals more efficiently followed by ethanolic extracts.

A significant PI of $ABTS^{\bullet+}$ was observed due to the scavenging ability of the extracts. The higher percentage of inhibition was observed in the aqueous extract of male floral bud (89.45±0.02) % followed by the ethanolic extract of male floral bud (87.45±1.66) % and aqueous extract of female floral bud (84.04±1.43) % at 1mg/mL. However, at the same concentration, the ethanolic extract of female floral bud has only exhibited an inhibition percentage of 68.80±1.12 %.

For leaves extracts, a (82.62±0.49) % radical-scavenging activity was observed by aqueous extract of female leaves followed by ethanolic extract of male leaves, aqueous extract of male leaves and ethanolic extract of female leaves with an inhibition percentage of 80.76±0.22%, 78.20±1.17 and 76.92±0.51 respectively.
The scavenging of ABTS°+ radicals by extracts has tied in with the scavenging of DPPH radicals.

The comparable β-carotene bleaching rates of the control, BHT (standard) and water and aqueous extracts of different parts of *Pistacia vera* are shown in Table 4.

The result showed the control had a substantial and rapid oxidation of β-carotene. Accordingly, the sample extracts with the presence of antioxidant retained their color and also absorbance for a longer period.

Table 4 shows the mean antioxidant activity based on the β-carotene bleaching rate of the extracts of different parts of the pistachio plant (FFB-WE, MFB-WE, FFB-EE, MFB-EE, FL-WE, ML-WE, FL-EE, ML-EE). The extract with the lowest β-carotene degradation rate exhibits the highest antioxidant activity. Only three extracts had lower antioxidant activities than the standard (BHT).

For leaves, the highest antioxidant activity among the samples was observed in FL-EE whereas ML-WE had the lowest antioxidant activity. For the floral buds, the highest antioxidant activity among the samples was observed in MFB-EE whereas FFB-EE had the lowest antioxidant activity.

The result showed that there was considerable variation in the antioxidant activities, since it ranges from the lowest of 545.29 to the highest of 716.02 where the orders of the antioxidant activity are as follow: MFB-EE> FL-EE > ML-EE > FL-WE >ML-WE > FFB-WE > MBF-WE >FFB-EE.

Conclusion

Pistacia vera extracts contained high levels of total phenolic compounds and flavonoids. All the sample extracts from this species also exhibited high antioxidant and free radical scavenging activities and some even showed a higher potency than the standard synthetic antioxidants; for example, the aqueous extract of flowers had a higher activity in the DPPH assay than the BHT. The results of the present study suggest that the plant extracts provide a substantial source of secondary metabolites, which act as natural antioxidants.

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