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Anti-oxidant activities and total phenolics contents of leaf extracts from 14 cultivars of walnut (*Juglans regia* L.)

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SUMMARY
Walnut (*Juglans regia* L.) leaves provide a source of healthy compounds, phenolics, which could be useful for the prevention of diseases in which free radicals are involved. In this study, walnut leaves from 14 different cultivars were studied for their total phenolics contents and anti-oxidant activities. Anti-oxidant activities were evaluated by measuring the scavenging activities of leaf extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The total phenolics contents of the cultivars ranged from 17.7 mg gallic acid equivalents (GAE) g^{-1} FW to 39.6 mg GAE g^{-1} FW. The highest scavenging activities were found in methanol extracts of walnut leaves. Total phenolics contents were highly correlated with anti-oxidant activity (R^2= 0.94 – 0.92).

Anti-oxidants help protect against certain chronic diseases of ageing, including cardiovascular and neurological disease, and carcinogenic food products due to their ability to control those free radicals known to have a negative influence on human health. Natural anti-oxidants such as phenolic compounds are important for their benefit to human health. Many fruits and vegetables have the status of 'functional foods' by being able to promote good health and/or to prevent or alleviate disease (Kaur and Kapoor, 2001). Phenolic compounds are major contributors to the anti-oxidant activity of fruits and vegetables as they are effective hydrogen donors (Banerjee *et al*., 2005).

Walnut (*Juglans regia* L.) belongs to the family Juglandaceae and provides excellent and effective natural anti-oxidants and chemo-preventive agents (Anderson *et al*., 2001; Bloomhoff *et al*., 2006; Samaranayaka *et al*., 2008; Carvalho *et al*., 2010; Cosmulescu *et al*., 2010). Many different foods were tested by Halvorsen *et al*. (2006), and the result was that walnut ranked second only to blackberry in terms of their anti-oxidant contents. Several parts of walnut trees have been used in folk medicine as natural remedies based on their anti-diarrheic, anti-helmintic, antiseptic and astringent properties. Dried leaves are often used as a herbal tea or infusion. Many reports have demonstrated the anti-oxidant potential of walnut products such as nuts and green husks (Ghasemi *et al*., 2011; Oliveira *et al*., 2008; Rahimipanah *et al*., 2010;), leaves (Pereira *et al*., 2007), bark (Noumi *et al*., 2011), or flowers (Nabavi *et al*., 2011). Walnut leaves may be used as an easily accessible source of natural bio-active compounds to inhibit the growth of various Gram-positive bacteria responsible for dental plaque and oral hygiene problems (Sharafati-Chaleshtori *et al*., 2011). According to Rahimipanah *et al*. (2010), green walnut husks constitute a suitable source of phenolics and could be used as an alternative natural anti-oxidant in the food industry. The allelopathic influence of juglone was demonstrated by Sytykiewicz (2011).

Interest in natural resources prompted a new investigation into the anti-oxidant activities of walnut. In this study, leaves from 14 walnut cultivars grown in Romania ('Orastie', 'Idaho', 'Valcor', 'Jupanesti', 'Valmit', 'Valrex', 'Muscelean', 'Franquette', 'Lara', 'Fernor', 'Fernette', 'Ferjan', 'Vina', and 'Hartley') were studied for their total phenolics contents and anti-oxidant activities. Determinations were carried out on 15 July, when previous literature showed that total phenolics contents were high. Data on the anti-oxidant activity of walnut leaves do not exist. Therefore, anti-oxidant potentials were assessed using a reducing power assay, or by the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.

MATERIALS AND METHODS

Plant material

The extraction, identification, and quantification of total phenolic compounds were carried out on leaves of 14 different walnut cultivars ('Orastie', 'Idaho', 'Valcor', 'Jupanesti', 'Valmit', 'Valrex', 'Muscelean', 'Franquette', 'Lara', 'Fernor', 'Fernette', 'Ferjan', 'Vina', and 'Hartley'), all grown on the same experimental site. Samples were from the experimental walnut orchard belonging to the University of Craiova, located at the Valcea Research Station (45°07' N; 24°22' E). Leaf samples were collected at random from the orchard, using ten trees for each cultivar (sampling 10 g per tree). Samples were collected on 15 July in two consecutive years (2010 and 2011) and were preserved by freezing at –40°C.

Previous research on seasonal variations in total

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phenolic contents in walnut leaves had shown that concentrations were usually higher in July (Cosmulescu and Trandafir, 2011).

Determination of total phenolics contents

Chemicals: Gallic acid (Sigma-Aldrich, Munich, Germany), Folin-Ciocalteu reagent (Sigma-Aldrich), anhydrous sodium carbonate, methanol (Merck, Darmstadt, Germany), and 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) were used. All chemicals were of analytical grade according to ISO 3696:1987 (Barnstead).

Preparation of leaf extracts: Walnut leaf tissue (1.0 g) from each cultivar was cut into small pieces, then placed in a conical flask with 20 ml methanol containing 1% (v/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT). Each flask was covered with Parafilm™ and aluminium foil and kept at 25°C in an ultrasonic bath for 40 min. Each leaf extract was separated by centrifugation at 1,200 × *g*. The supernatant was filtered through a 0.22 µm polyamide membrane and stored at –20°C.

Method: The total phenolics content of each leaf extract was measured according to the Folin-Ciocalteu method, as described by Singleton and Rossi (1965), with some modifications. A 1.0 ml sample of each leaf extract, or 1.0 ml double-distilled water (blank), or 1.0 ml of each standard gallic acid solution (see below) was placed in a 25 ml flask and 5 ml of Folin-Ciocalteu reagent was added (diluted 1:10 with ultrapure water). After 2 min, 4 ml of 7.5% (w/v) sodium carbonate was added and the flasks were kept at room temperature (24º – 26ºC) for 2 h. The absorbance was measured at 765 nm using an Evolution 600, UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) computer control led by VISION Pro-software (Thermo Scientific). A standard curve was prepared using 50, 100, 150, 200, or 250 mg l^{-1} gallic acid in 60:40 (v/v) methanol and water. Gallic acid was used as a reference standard, and the results (total phenolics contents) were expressed as gallic acid equivalents (GAE) in mg GAE g^{-1} FW of leaf material.

Anti-oxidant activity

Chemicals and reagents: Methanol (Merck), 2,2-diphenyl-1-picrylhydrazyl (DPPH) ascorbic acid, ascorbic acid, gallic acid (all from Sigma-Aldrich) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Merck) were used to determine anti-oxidant activities.

Method: The scavenging activities of methanolic extracts of *J. regia* leaves against DPPH radicals were measured according to Hatano *et al*. (1988) and Oliveira *et al*. (2008), with some modifications. An aliquot of each methanolic leaf extract (50 µl) was mixed with 3 ml of 0.004% (v/v) DPPH in methanol. Each reaction mixture was incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm. Standards of various concentrations were used: ascorbic acid (0, 25, 50,100, 150, and 200 mg l^{-1}), gallic acid (0, 25, 50, 75, 100, and 125 mg l^{-1}), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; 0, 0.5, 1, 1.5, 2, 2.5, and 3.5 mM). Ultrapure water was used as a blank. The radical scavenging activity (RSA) against DPPH radicals was calculated according to the following equation:

$$\mathrm{RSA} = \frac{A_{517}^{blank} - A_{517}^{sample}}{A_{517}^{blank}} \times 100$$

where A_{517}^{blank} was the absorbance of the control, and A_{517}^{sample} was the absorbance of the leaf extract or standard solution.

All assays were conducted in triplicate. Anti-oxidant capacity was expressed in mg ascorbic acid g^{-1} FW of plant material, or in mg g^{-1} FW plant material, or in mg Trolox g^{-1} FW plant material.

Statistical analysis

Phenolics contents data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between means was compared by the LSD test at a significance level of $P \leq 0.05$. All data were expressed as means ± standard deviations. Correlation analyses of anti-oxidant activity *vs.* total phenolics contents were carried out using the correlation and regression programme (NCSS Statistical System, Kaysville, USA).

RESULTS AND DISCUSSION

Total phenolics contents (TPC)

Total phenolics concentrations in leaf extracts of 14 cultivars of *J. regia* are shown in Table I. Total phenolics contents of walnut leaves ranged from 39.6 mg GAE g^{-1} FW in 'Fernor' to 17.7 mg GAE g^{-1} FW in 'Jupanesti' (Table I). This represents a 2.23-fold difference between 'Fernor' and 'Jupanesti'. The average total phenolics content was 28.1 mg GAE g^{-1} FW.

Many studies have been carried out on the total phenolics contents of walnut products. In previous work, Cosmulescu and Trandafir (2011) studied the evolution of phenolics compounds in the leaves of different walnut varieties from June to September. Contents amount ranged from 15.8 – 33.6 mg GAE 100 g^{-1} plant material. According to Jalili and Sadeghzade (2012), the average total phenolics content of walnut leaf extracts was 22.16 mg g^{-1}. Higher phenolics contents were found in green walnut husks by Oliveira *et al*. (2008), ranging from 32.6

TABLE I

Total phenolics contents, anti-oxidant activities, and the significance of the differences in phenolics contents in 14 walnut cultivars

Cultivar	Total phenolics content (TPC; mg GAE g^{-1} FW)	Anti-oxidant activity (% inhibition of DPPH activity)
'Fernette'	27.27 ± 0.98†	91.37 ± 0.02
'Idaho'	28.08 ± 0.78	91.87 ± 0.04
'Vina'	31.36 ± 0.69*	92.09 ± 0.03
'Muscelean'	30.77 ± 1.12	90.67 ± 0.05
'Fernor'	39.55 ± 0.79***	91.36 ± 0.01
'Pedro'	33.71 ± 0.46**	92.07 ± 0.02
'Lara'	31.21 ± 0.57	92.49 ± 0.03*
'Valrex'	28.78 ± 0.68	93.52 ± 0.06**
'Orastie'	19.53 ± 0.49	91.52 ± 0.05
'Franquette'	30.61 ± 0.67	91.73 ± 0.06
'Hartley'	25.79 ± 0.73	91.24 ± 0.07
'Valcor'	27.14 ± 0.52	91.21 ± 0.04
'Jupanesti'	17.66 ± 0.91	81.91 ± 0.12
'Ferjan'	21.85 ± 0.84	92.05 ± 0.07
Mean	28.09 ± 5.71	91.07 ± 2.72

†Values are means ± SD (n = 2). Significant differences from the mean value in each column are indicated as *, **, *** at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

mg GAE g^{-1} FW (in 'Mellanaise') to 74.1 mg GAE g^{-1} FW (in 'Franquette'). According to Rahimipanah *et al.* (2010), the total phenolics and flavonoids contents of walnut green husk amounted to 3.43 g GAE 100 g^{-1} FW and 144.6 mg quercetin equivalents 100 g^{-1} DW respectively, in dried samples.

The phenolics contents data in the present study were subject to one-way analysis of variance (ANOVA) and the significance of the differences between means was determined by Student's *t*-test at a significance level of $P \leq 0.05$. The results indicated significant differences in phenolics contentsbetween the 14 walnut cultivars analysed (Table I). These differences may be due to genetic factors. Cultivar-dependent differences in phenolics contents have been observed in walnut (Oliveira *et al.*, 2008) and in many other horticultural crops such as honeysuckle (Rop *et al.*, 2011), *Hypericum* (Ghasemi Pirbalouti *et al.*, 2011), ginger (Ghasemzadeh *et al.*, 2011) and black chokeberry (Rop *et al.*, 2010).

Anti-oxidant capacity

Methanolic extracts of walnut leaves were evaluated for their anti-oxidant capacity using the DPPH radical-scavenging method. Ascorbic acid, gallic acid, and Trolox were used as reference standards. The system used for scavenging DPPH free radicals is a simple and acceptable method to evaluate anti-oxidative activity. Extracts of walnut leaves exhibited high DPPH free-radicals scavenging ability at different concentrations, and percentage inhibition values were calculated (Table I). The anti-oxidant activity of walnut leaf extracts over 30 min varied from 81.9% inhibition of DPPH radical activity in 'Jupanesti', to 93.5% inhibition of DPPH radical activity in 'Valrex'. Thus, anti-oxidant activity varied with walnut variety grown under the same orchard culture conditions.

Trolox equivalent (TE) anti-oxidant capacity (TEAC), GAE anti-oxidant capacity, and ascorbic acid equivalent (AAE) anti-oxidant capacity values are presented in Table II. Anti-oxidant capacities varied among the different walnut genotypes and in the different methods of measurement used. For example, from 16.4 mg Trolox g^{-1} FW in 'Jupanesti' to 50.7 mg Trolox g^{-1} FW in 'Fernor', 2.7 mg GAE g^{-1} FW in 'Jupanesti' to 10.6 mg GAE g^{-1} FW in 'Fernor' and 10.9 mg AAE g^{-1} FW in 'Jupanesti' to 32.5 mg AAE g^{-1} FW in 'Fernor' (Table II). A wide range of anti-oxidant capacities were measured among the 14 cultivars. Average values of anti-oxidant capacities were 31.7 mg TE g^{-1} FW, 6.2 mg GAE g^{-1} FW, and 21.5 mg AAE g^{-1} FW (Table II). Above average values were recorded in > 50% of the genotypes studied by each method. 'Fernor', 'Fernette', 'Vina', 'Muscelean', 'Lara', and 'Franquette' showed the highest anti-oxidant activities, regardless of the assay method used (Table II). It has been shown that walnut polyphenols are effective inhibitors of *in vitro* human plasma low-density lipoprotein (LDL) and LDL oxidation (Anderson *et al.*, 2001). The kernel pellicles of walnut are rich in ellagitannins with anti-oxidative activity (Shimoda *et al.*, 2009). Ethanol extracts of phenolic compounds from walnut skin displayed the highest anti-oxidative capacity, evaluated by TEAC assays (Samaranayaka *et al.*, 2008). Pereira *et al.* (2007) reported a high anti-oxidant activity in walnut leaves from trees grown in Portugal.

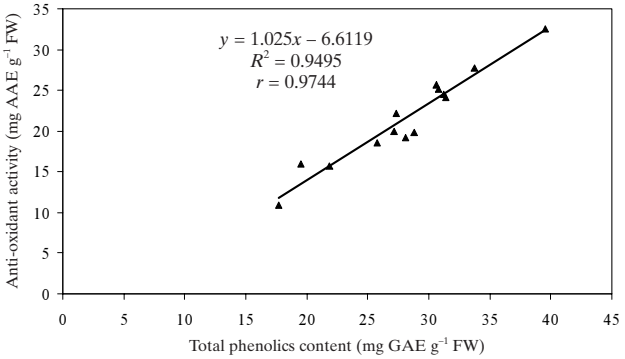

FIG. 1
Linear correlation between anti-oxidant activity (mg AAE g^{-1} FW) and total phenolics contents (mg GAE g^{-1} FW) of methanolic extracts of 14 cultivars of walnut (solid triangles).

The highest correlation coefficients were obtained between anti-oxidant activity and total phenolics contents. A strong positive correlation (R^2 = 0.94; y = 1.025x – 6.6119) was found between total phenolics content and AAE anti-oxidant capacity (Figure 1). Similar observations (R^2 = 0.92; y = 1.4353x – 7.6731) were made for the correlation between TEAC and total phenolics content (Figure 2), and between GAE anti-oxidant capacity and total phenolics content (R^2 = 0.92; y

TABLE II
Anti-oxidant activities and total phenolics contents of 14 cultivars of walnut

Cultivar	Total phenolics content (mg GAE g^{-1} FW)	Anti-oxidant activity		
		TEAC (mg Trolox g^{-1} FW)	GAE (mg GAE g^{-1} FW)	AAE (mg ascorbic acid g^{-1} FW)
'Fernette'	27.27 ± 0.98†	33.46 ± 0.29	5.98 ± 0.01	22.22 ± 0.32
'Idaho'	28.08 ± 0.78	32.33 ± 0.34	5.57 ± 0.12	19.22 ± 0.24
'Vina'	31.36 ± 0.69	39.47 ± 0.30**	7.18 ± 0.15	24.16 ± 0.12
'Muscelean'	30.77 ± 1.12	32.81 ± 0.38	6.88 ± 0.16	25.16 ± 0.25*
'Fernor'	39.55 ± 0.79	50.72 ± 0.44***	10.56 ± 0.17***	32.48 ± 0.18***
'Pedro'	33.71 ± 0.46	39.14 ± 0.37**	8.07 ± 0.18**	27.70 ± 0.19**
'Lara'	31.21 ± 0.57	36.79 ± 0.54*	6.49 ± 0.01	24.49 ± 0.21
'Valrex'	28.78 ± 0.68	27.56 ±.062	4.77 ± 0.13	19.87 ± 0.16
'Orastie'	19.53 ± 0.49	24.10 ± 0.47	4.65 ± 0.2	15.97 ± 0.28
'Franquette'	30.61 ± 0.67	32.28 ± 0.36	7.51 ± 0.16*	25.67 ± 0.32*
'Hartley'	25.79 ± 0.73	28.26 ± 0.36	5.50 ± 0.18	18.56 ± 0.41
'Valcor'	27.14 ± 0.52	24.70 ± 0.28	6.34 ± 0.21	20.01 ± 0.26
'Jupanesti'	17.66 ± 0.91	16.40 ± 0.41	2.65±0.17	10.87 ± 0.27
'Ferjan'	21.85 ± 0.84	26.71 ± 0.53	4.73 ± 0.16	15.69 ± 0.19
Mean	28.09 ± 5.71	31.76 ± 8.33	6.20 ± 1.87	21.57 ± 5.56

†Values are means ± SD (n = 2). Significant differences from the mean value in each column are indicated as *, **, *** at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

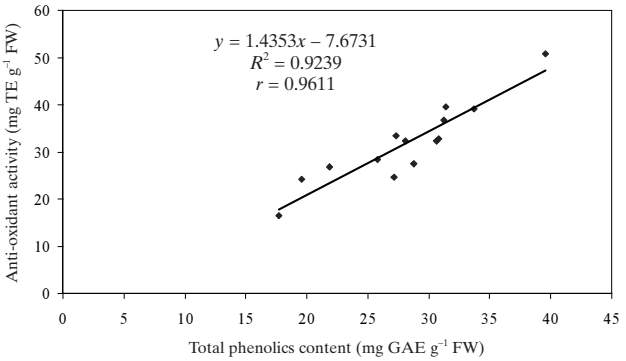

FIG. 2
Linear correlation between anti-oxidant activity (mg Trolox equivalents g^{-1} FW) and total phenolics contents (mg GAE g^{-1} FW) of methanolic extracts of 14 cultivars of walnut (solid diamonds).

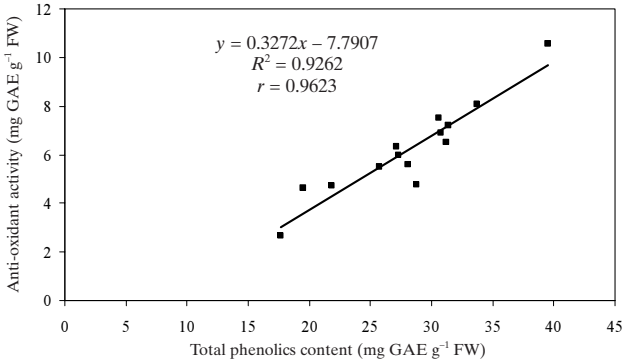

FIG. 3
Linear correlation between anti-oxidant activity (mg GAE g^{-1} FW) and total phenolics contents (mg GAE g^{-1} FW) of methanolic extracts of 14 cultivars of walnut (solid squares).

= 0.3272x – 2.7907; Figure 3). There was an excellent linear relationship (R^2 = 0.94 – 0.92) between the values for total phenolics contents and anti-oxidant activities which suggests that phenolics compounds largely account for the anti-oxidant capacity of walnut. Other authors have reported a correlation between anti-oxidant activity and the total phenolics contents of different extracts of *Melissa officinalis* (Koksal *et al*., 2011), *Clematis flammula* (Atmani *et al*., 2011), *Lonicera caerulea* (Rop *et al*., 2011), and *Camellia sinensis* (Chaturvedula and Prakash, 2011) leaves.

The relationship between the content of phenolic compounds and anti-oxidant capacity is not simple. The structure of phenolic compounds is a key determinant of their radical-scavenging activity. Anti-oxidant activity depends on the numbers and positions of hydroxyl groups in relation to functional carboxyl groups (Balasundram *et al*., 2006; Cai *et al*., 2006). High phenolics contents do not necessarily result in high anti-oxidant capacity (Conforti *et al*., 2009). Further studies are required to assess the potential of particular components of walnut leaves as effective natural remedies.

CONCLUSIONS

The results obtained here indicate that methanol extracts of *J. regia* leaves have significant ($P \leq 0.05$) total phenolics contents, DPPH-scavenging abilities, and anti-oxidant activities. The anti-oxidant activities of methanol extracts correlated well with their contents of phenolics compounds. In conclusion, this study suggests that *J. regia* leaves represent a potential source of natural anti-oxidants for use in the food, cosmetics, and pharmaceutical industries. However, further investigations on *in vivo* anti-oxidant activities are recommended.

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