Journal of Medicinal Plants Research Vol. 5(19), pp. 4938-4942, 23 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Seasonal variation of total phenols in leaves of walnut (*Juglans regia* L.)

Sina COSMULESCU[1*] and Ion TRANDAFIR[2]

[1]Faculty of Horticulture, University of Craiova, 13, A.I.Cuza Str., Craiova 200585, Romania.
[2]Faculty of Chemistry, University of Craiova, 107, Calea Bucuresti Str., Craiova 200529, Romania.

Accepted 26 July, 2011

The total phenols content was studied in walnut (*Juglans regia* L.) leaves of twelve different cultivars, over eight distinct harvest times (June 1st to September 15th). Total phenols content was determined by colorimetric assay and their amount ranged from 15.87 (cv. Jupanesti) to 33.67 mg GAE/100 g (cv. Valrex). Total phenols increase over the months of June and July, then decrease over the month of August, then a new increase is recorded by the beginning of September. These differences in terms of total phenols at different sampling time are supposed to be the effect of change in ecological parameters. The results obtained indicated that walnut leaves may become important in obtaining a noticeable source of compounds with health protective potential and antimicrobial activity; therefore the walnut leaves should preferentially be collected in July and early September, when phenolic content is higher.

Key words: Walnut leaves, total phenols, seasonal changes.

INTRODUCTION

Walnut (*Juglans regia* L.) has important amounts of phenolic compounds (Labuckas et al., 2008). The fact is known that the higher the phenols content the stronger is the antioxidant activity. Walnut leaves have been used in both cosmetic and pharmaceutical industries. Leaves have been widely used in folk medicine for treatment of venous insufficiency and haemorrhoidal symptomatology, and also for their antidiarrheic, antihelmintic, depurative and astringent properties (Valnet, 1992). Different scientific papers demonstrated the antioxidant potential of walnut products, kernel (Labuckas et al., 2008; Samaranayaka et al., 2008), green husk (Oliveira et al., 2008) and leaves (Pereira et al., 2008; Rahimipanah et al., 2010; Stampar et al., 2006). The content of phenols in walnut is variable. The concentrations of phenols depend on time period (Amaral et al., 2004), on time period and geographic origin (Cosmulescu et al., 2010), on ontogenetic stage of the shoots (Solar et al., 2006), on climatic conditions and farming practices (Amaral et al., 2008), on cultivar choice and picking date (Jakopic et al., 2007) and on some agricultural factors (Areias et al., 2000). The levels of phenols compounds in nuts are influenced by environmental factors (Ghasemi, 2011), soil composition, maturation level (Wakeling et al., 2001), cultivar and harvest year (Bolling et al., 2010). As regards the connection between crop period and polyphenols content, Amaral et al. (2004) indicated that the average values of phenolic acids, flavonoids and total phenols contents seem to point out to a decrease until September, while Solar et al. (2006) indicated that each phenolic group had its own curve of seasonal fluctuations.

Kornsteiner et al. (2006) reported that the mean content of total phenolics, in 10 different nut types, varied between 32 mg gallic acid equivalents/100 g (pines) and 1625 mg (walnuts). A large variation in phenols and antioxidant capacity was found in the nuts (eleven different kinds of nuts) by Abe et al. (2010). Oliveira et al. (2008), reported that total phenols content amount ranged from 32.61 (cv. Mellanaise) to 74.08 mg/g of GAE (cv. Franquette). Other authors have also established that phenols content extracts are depending on the type of solvent (Jakopič et al., 2009). Previous researches showed that green husk and leaves of Romanian walnut cultivars proved to be an important source of phenolic compounds (Cosmulescu et al., 2010, 2011); and walnut fruits are important sources of nutritive elements (Cosmulescu et al., 2009).

*Corresponding author. E-mail: sinacosmulescu@hotmail.com.

Dry walnut leaves are still largely used as an infusion. Because antioxidant capacity and phenols content is correlated and total phenol presented seasonal fluctuation, in the present paper, the total phenols of walnut leaves have been studied in twelve different cultivars growing under the same agricultural, geographical and climatic conditions. Evolution of phenolic compounds from June to September was monitored.

MATERIALS AND METHODS

Plant material and extraction

Extraction, identification and quantification of total phenols have been carried out on walnut leaves in twelve different cultivars that are grown under the same experimental area. Cultivars of different origins were analyzed; 6 of Romanian origin ('Orastie', 'Valcor', 'Jupanesti', 'Valmit', 'Valrex', 'Muscelean'), 4 from France ('Franquette', 'Lara', 'Fernor', 'Fernette') and 2 from U.S.A. ('Vina', 'Hartley'). The samples were taken out from an experimental walnut orchard belonging to the University of Craiova and located at Vâlcea research station (45°07' lat. N and 24°22 long. E). Samples for the experiment were collected over 2 consecutive years, from June the 1st until September the 15th and they were preserved by freezing them at -40°C temperature. The walnut leaves were cut into small pieces, in the amount of 1000 mg and weighed with a precision of 0.0001, then placed in conical vessels together with 20 mL methanol with 1% BHT (2,6-di-*tert*-butyl-4-methylphenol), covered with parafilm and aluminium foil and they were kept at the temperature of 25°C on an ultrasonic bath for 40 min. Extracts were separated by centrifugation at 1200 g. Supernatants were filtered through polyamide membranes with pore diameter of 0.22 µm and stored at a temperature of -20°C.

Chemicals and reagent

Folin-Ciocalteu 2N reactive (Sigma-Aldrich), gallic acid (Sigma), anhydrous sodium carbonate (Sigma-Aldrich).

Total phenol determination

The amount of total phenolic compounds in the walnut leaves extract was determined colorimetrically with Folin-Ciocalteu reagent by using the method described by Singleton and Rossi (1965) with some modifications. To 1 ml extracts (diluted 1:10 with ultrapure water), 1 ml bidistilled water (blank), 1 ml of each standard solution were introduced in laboratory flasks of 25 ml and added every 5 ml reactive Folin-Ciocalteu (diluted 1:10 with ultrapure water). After 2 min, 4 ml of sodium carbonate solution 7.5% was added and they were kept in the incubator during 2 h at the room's temperature. The absorbance was measured at 765 nm by using a model evolution 600, double beam scanning UV-visible spectrophotometer, PC control with VISIONpro software. A standard curve was prepared by using 50, 100, 150, 200 and 250 mg/L solutions of gallic acid in methanol and water (60:40, v/v). Gallic acid was used as the reference standard and the results (total phenolic content) were expressed as gallic acid equivalents (GAE) in milligrams per gram.

Statistical analysis

All analyses were run in triplicate and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by using the Statistic software (STAT2011) and XLSTAT 7.5.2 – principal component analysis (PCA). Differences between means were first analyzed by ANOVA test and then by least significant difference (LSD) test ($p < 0.05$). Data were subjected to Pearson correlations.

RESULTS AND DISCUSSION

Total phenolic content has been analyzed in twelve cultivars, over 8 sampling dates (from June 1st to September 15th). Total phenolic content of leaves in 12 walnut cultivars is shown in Table 1. The statistically important differences on total phenols were obtained in different harvest times in walnut cultivars ($p<0.05$).

Amount of total phenolic content was continuously increased from the 1st sampling to the 4th sampling in all cultivars (Figure 1). Seasonal changes, as regards the sum of total polyphenols (in all twelve cultivars under study), are developing by following a polynomial curve of the 2nd degree (r=0,894***), with a continuous increase until the beginning of August; then they decrease over the month of August, and they increase again by the beginning of September (Figure 1).

Over the eight time sampling, the mean total phenol content in twelve walnut cultivars ranged from 15.87 to 33.67 mg GAE/100 g (Table 1). This represents a 2.12 fold difference between 'Jupanesti' and 'Valrex' cultivars. 'Lara' cultivars had the second highest total phenol content at 29.95 mg GAE/100g, 11% less than that of 'Valrex' cultivars. Closer values, as regards average content, were recorded in three cultivars: 'Fernette' (27.5 mg GAE/100 g), 'Fernor' (27.83 mg GAE/100 g) and 'Vina' (27.52 mg GAE/100 g), which represented about 18.3% less than that of 'Valrex' cultivar. 'Franquette', 'Hartley', Muscelean', 'Orastie', 'Valcor' and 'Valmit' cultivars had similar levels of total phenols, ranging from 18.76 to 22.78 mg GAE/100 g. The study revealed a considerable amount of variation among the cultivars tested; this was not surprising when considering that cultivar dependent phenolic content variations have previously been observed in walnut (Oliveira et al., 2008) and many other horticultural crops, including blueberry (Connor et al., 2002), apple (Lata et al., 2009) and plum (Vasantha Rupasinghe et al., 2006).

Differences in the cultivar, depending on time of sampling, were found to be: 12.77 to 30.77 mg GAE/100 g in 'Muscelean' cultivar; 12.54 to 27.57 mg GAE/100 g in 'Valmit' cultivar; 10.67 to 17.66 mg GAE/100 g in 'Jupanesti' cultivar; 25.4 to 45.66 mg GAE/100 g in 'Valrex' cultivar; 16.33 to 47.65 mg GAE/100 g 'Lara' cultivar; 11.97 to 43.54 mg GAE/100 g in 'Fernette' cultivar; 19.33 to 39.55 mg GAE/100 g in 'Fernor' cultivar; 15.38 to 37.5 mg GAE/100 g in 'Vina' cultivar; 9.26 to 30.61 mg GAE/100 g in 'Franquette' cultivar; 10.55 to 31.87 mg GAE/100 g in 'Hartley' cultivar; 8.46 to 27.14 mg GAE/100 g in 'Valcor' cultivar, and 11.36 to 28.65 mg GAE/100 g in 'Orastie' cultivar, respectively (Table 1).

Table 1. Mean total phenolic content of walnut leaves (mg GAE/100 g)*

Sampling date / cultivar	June 1st	June 15th	July 1st	July 15th	August 1st	August 15th	September 1st	September 15th	Average (SD)
Fernette	6.47 (0.20)	11.97 (0.02)	23.74 (0.15)	27.27 (0.08)	32.43 (0.19)	22.74 (0.01)	43.54 (0.05)	30.87 (0.02)	27.50 (3.68)
Franquette	7.21 (0.04)	9.26 (0.19)	18.43 (0.07)	30.61 (0.11)	25.00 (0.01)	21.74 (0.15)	29.02 (0.16)	25.40 (0.14)	22.78(2.73)
Vina	10.49 (0.16)	24.85 (0.17)	15.38 (0.01)	31.36 (0.03)	37.5 (0.02)	26.34 (0.23)	28.14 (0.07)	29.11 (0.15)	27.52 (2.55)
Hartley	6.02 (0.27)	10.55 (0.04)	31.87 (0.03)	25.79 (0.07)	14.84 (0.14)	24.11 (0.06)	21.95 (0.13)	22.38(0.04)	21.64 (2.66)
Muscelean	13.47 (0.33)	12.77 (0.14)	19.68 (0.13)	30.77 (0.01)	20.75 (0.03)	21.44 (0.13)	24.00 (0.04)	24.21(0.02)	21.95 (2.06)
Valmit	10.99 (0.51)	22.64 (0.02)	16.68 (0.17)	12.54 (0.13)	16.97 (0.28)	13.66 (0.11)	27.57 (0.07)	20.08(0.01)	18.59 (1.99)
Fernor	10.15 (0.08)	22.13 (0.08)	19.93 (0.10)	39.55 (0.10)	23.52 (0.14)	28.31 (0.04)	28.30 (0.29)	33.06(0.09)	27.83 (2.57)
Valcor	5.48 (0.12)	11.52 (0.10)	25.41 (0.09)	27.14 (0.07)	16.06 (0.25)	16.12 (0.01)	22.87 (0.61)	8.46(0.14)	18.22 (2.68)
Jupinesti	5.51 (0.10)	10.96 (0.28)	13.69 (0.16)	17.66 (0.05)	18.90 (0.13)	10.67 (0.07)	17.45 (0.76)	21.8(0.23)	15.87 (1.58)
Lara	11.94 (0.07)	16.38 (0.09)	16.33 (0.01)	31.21 (0.14)	34.86 (0.08)	34.43 (0.06)	47.65 (0.09)	28.78(0.08)	29.95 (4.17)
Valrex	21.91 (0.42)	26.52 (0.12)	45.66 (0.28)	28.78 (0.03)	29.32 (0.15)	25.40 (0.03)	41.75 (0.17)	38.28(0.21)	33.67 (3.06)
Orastie	9.75 (0.26)	18.99 (0.04)	18.76 (0.02)	19.53 (0.01)	12.51 (0.04)	11.36 (0.02)	28.65 (0.04)	21.52(0.04)	18.76 (2.18)
Sum	119.39	213.6	266.63	337.28	283.74	271.4	361.98	319.04	
Average (SD)	9.94 (1.72)	16.96 (1.91)	21.98 (2.82)	26.81(2.29)	22.74 (2.48)	21.23 (2.27)	28.85 (2.62)	24.82 (2.33)	

*Values are expresssed as mean (standard deviation) of two years.

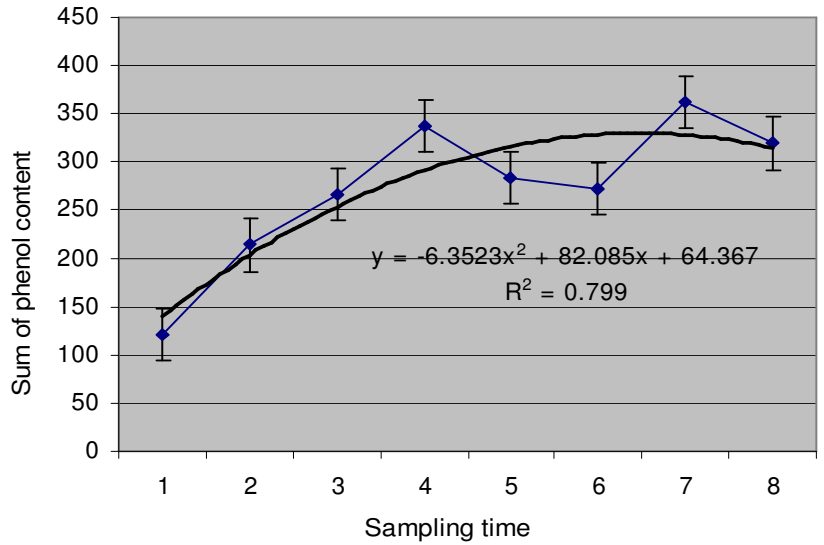

Figure 1. Evolution of total phenol amount (sum of twelve cultivars) between June and September.

Table 2. Correlation matrix and eigenvalues of harvest date and total phenol content. Correlation matrix (Pearson (n)).

Variables	June 1st	June 15th	July 1st	July 15th	August 1st	August 15th	September 1st	September 15th
June 1st	1							
June 15th	0.692**	1						
July 1st	0.561**	0.228	1					
July 15th	0.197	0.075***	0.153	1				
August 1st	0.442*	0.430	-0.027	0.546**	1			
August 15th	0.372	0.239	0.204	0.793***	0.750***	1		
September 1st	0.674**	0.472*	0.281	0.315	0.641**	0.664**	1	
September 15th	0.714***	0.592**	0.319	0.483*	0.643**	0.626**	0.592**	1
Principal component analysis: Eigenvalues								
	F1	F2	F3	F4	F5	F6	F7	F8
Eigenvalue	4.31	1.50	0.94	0.51	0.27	0.21	0.17	0.07
Variability (%)	53.88	18.78	11.82	6.42	3.44	2.64	2.08	0.91
Cumulative (%)	53.88	72.67	84.49	90.92	94.36	97.01	99.08	100.000

*,**,***Significant at P<0.05, 0.01 and 0.001, respectively.

Total phenols increase over the months of June and July, then they decrease over the month of August, then a new increase is recorded by the beginning of September; the values are varying between 9.94 mgGAE/100 g (June 1st) and 30.07 mgGAE/100g (September 1st). Amaral et al. (2004) reported that the amounts of total phenols in walnut leaves in six walnut cultivars, have varied from 12.4 to 34.5 g/kg and all cultivars have presented slightly higher values of total phenolic compounds over the months of May and July. This fact shows that the total content of phenols depends on sampling time and this is supported by other authors (Solar et al., 2006; Jakopic et al., 2007; Cosmulescu et al., 2010). These differences in terms of total phenols at different sampling times are supposed to be the effect of change in ecological parameters. Biosynthesis of phenolic compounds can be induced by stronger sunlight and length of daytime, therefore the phenols content is increasing until the beginning of August. High temperature stress promotes production of phenolic compounds; the increases observed in phenolic content of walnut leaves collected in July may be attributed to higher values of temperatures.

The lower concentration during August is probably caused by vegetation stage (fruits' growth). A likely factor that causes the increasing of phenols content is the stress condition (differences between day and night temperature, irregular rainfall, drought), that explains the increasing of content over the month of Septem ber. Plants can accumulate phenolic compounds in their different tissues in response to challenge by various stress factors (Pasqualini et al., 2003). The influence of environmental conditions on quantity of active constituents in various plants has been reported. Variations in total phenols of walnut leaves reported in the present study can be explained by qualitative and quantitative changes of environmental circumstances over the year. It is difficult to determine which environ-mental factor is mainly responsible for the variations observed, because the changes are small and irregular. As a result, further studies are required to be carried out in order to elucidate the induction of biosynthesis of total phenols by ecological parameters. Analysis of PCA (Principal component analysis) has shown that the first two data collection (F1- the 1st of June and F2- the15th of June) have represented 53.88 and 18.78%, respectively, of the variant's total (Table 2). The rest of data collection (F3…F8) represented 27.33% of the variant's total. As regards correlation (Pearson correlation) between sampling date and accumulation of total phenols, all correlations were positive, except for negative correlation between the 1st of July and the 1st of August.

Conclusion

It can be concluded that there is a relationship between phenolic content in walnut leaves and seasonal, genetic and ecological factors. The results of the present study apparently indicated that Persian walnut leaves may constitute a suitable source of phenols and they could be used as alternative natural antioxidants. Bearing in mind that total phenolic acids have been the subject of several studies owing to their antioxidant potential (Samaranayaka et al., 2008; Oliveira et al., 2008; Pereira et al., 2007). The results obtained suggest that, for this purpose, walnut leaves should preferentially be collected in July and early September, when phenolic content is higher. Information obtained through analysis of total phenols in walnut leaves could be useful in planning the collecting time of walnut products for medicinal extracts.

ACKNOWLEDGEMENT

This work was supported by CNCSIS-UEFISCSU, project number PNII-IDEI code 430 /2008.

REFERENCES

Abe LT, Lajolo FM, Genovese MI (2010). Comparison of phenol content and antioxidant capacity of nuts. Ciênc. Tecnol. Aliment, 30(1): 254-259.

Amaral JS, Seabra RM, Andrade PB, Valentao P, Pereira JA, Ferreres F (2004). Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves. Food Chem. 88: 373-379.

Amaral JS, Valentão P, Andrade PB, Martins RC, Seabra RM (2008). Do cultivar, geographical location and crop season influence phenolic profile of walnut leaves? Molecules,13: 1321-1332.

Areias F, Valentão P, Andrade PB, Ferreres F, Seabra RM (2000). Flavonoids and phenolic acids of sage: influence of some agricultural factors. J. Agric. Food Chem., 48(12): 6081-6084.

Bolling BW, Dolnikowski G, Blumberg JB, Chen CYO (2010). Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chem., 122: 819-825.

Connor AM, Luby JJ, Tong CBS, Finn CE, Hancock JF (2002). Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. J. Am. Soc. Hort. Sci., 127: 89-97.

Cosmulescu S, Baciu A, Achim G, Botu M, Trandafir I (2009). Mineral composition of fruits in different walnut (*Juglans regia* L.) cultivars. Not. Bot. Horti. Agrobo., 37(2): 56-160.

Cosmulescu S, Trandafir I, Achim G, Botu M, Baciu A, Gruia M (2010). Phenolics of green husk in mature walnut fruits. Not. Bot. Hort. Agrobo., 38(1): 53-56.

Cosmulescu SN, Trandafir I, Achim G, Baciu A (2011). Juglone Content in Leaf and Green Husk of Five Walnut (*Juglans regia* L.) Cultivars. Not. Bot. Hort. Agrobo., 39(1): 237-240.

Ghasemi K, Ghasemi Y, Ehteshamnia A, Mohammad Nabavi S, Fazel Nabavi S, Ali Ebrahimzadeh M, Pourmorad F (2011). Influence of environmental factors on antioxidant activity, phenol and flavonoids contents of walnut (*Juglans regia* L.) green husks**.** J. Med. Plants Res., 5(7): 1128-1133.

Jakopic J, Colaric M, Veberic R, Hudina M, Solar A, Stampar F (2007). How much do cultivar and preparation time influence on phenolics content in walnut liqueur? Food Chem., 104(1): 100-105.

Jakopič J, Veberič R, Štampar F (2009). Extraction of phenolic compounds from green walnut fruits in different solvents. Acta Agric. Slov., 93(1): 11-15.

Kornsteiner M, Wagner KH, Elmadfa I (2006). Tocopherols and total phenolics in 10 different nut types. Food Chem., 98: 381-387.

Labuckas DO, Maestri DM, Perelló M, Martínez ML, Lamarque AL (2008). Phenolics from walnut (*Juglans regia* L.) kernels: Antioxidant activity and interactions with proteins. Food Chem., 107(2): 607-612.

Lata B, Trampczynska A, Paczesna J (2009). Cultivar variation in apple peel and whole fruit phenolic composition. Sci. Hort., 121(2): 176-181.

Oliveira I, Sousa A, Ferreira I, Bento A, Estevinho L, Pereira JA (2008). Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. Food Chem. Toxicol., 46(7): 2326-2331.

Pasqualini V, Robles C, Garcino S, Greff S, Bousquet-Melou A, Bonin G (2003). Phenolic compounds content in *Pinus halepensis* Mill. needles: a bioindicator of air pollution. Chemosphere, 52: 239-248.

Pereira JA, Oliveira I, Sousa A, Ferreira I, Bento A, Estevinho L (2008). Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. Food Chem. Toxicol., 46(6): 2103-2111.

Pereira JA, Oliveira I, Sousa A, Valentão P, Andrade PB, Ferreira IC, Ferreres F, Bento A, Seabra R, Estevinho L (2007). Walnut (*Juglans regia* L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. Food Chem. Toxicol., 45(11): 2287-2295.

Rahimipanah M, Manoochehr H, Mojgan M (2010). Antioxidant activity and phenolic contents of Persan walnut (*Juglans regia L.*) green husk extract. Afr. J. Food Sci. Technol., 1(4): 105-111.

Samaranayaka AGP, John JA, Shahidi F (2008). Antioxidant activity of english walnut (*Juglans regia* L.). J. Food Lipids, 15(3): 384-397.

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic - phosphotungstic acid reagents. Am. J. Enol. Vitic., 16: 144-158.

Solar A, Colarič M, Usenik V, Stampar F (2006). Seasonal variations of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.). Plant Sci., 170(3): 453-461.

Stampar F, Solar A, Hudina M, Veberic R, Colaric M (2006). Traditional walnut liqueur - cocktail of phenolics. Food Chem., 95: 627-631.

Valnet J (1992). Phytotherapie Traitement des maladies par les plantes. Paris: Maloine, pp. 476-478.

Vasantha Rupasinghe HP, Jayasankar S, Lay W (2006). Variation in total phenolics and antioxidant capacity among European plum genotypes. Sci. Hort., 108(3): 243-246.

Wakeling LT, Mason RL, D'arcy BR, Caffin NA (2001). Composition of pecan cultivars Wichita and Western Schley (*Carya illinoinensis* (Wangenh.) K. Koch) grown in Australia. J. Agric. Food Chem., 49(3): 1277-1281.