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Original article

Varietal and Time Dependent Differences in Juglone and Total Phenolic Contents of the Walnut (*Juglans regia* L.) Leaves

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Summary. From May to October, time dependent differences with two weeks intervals in juglone (5-Hydroxy-1,4-naphthoquinon) and total phenolic content of the leaves of five walnut (*Juglans regia* L.) cultural varieties grown in Turkey (cv. Şebin, cv. Yalova-2, cv. Yalova-3, cv. Yalova-4 and cv. 1974/7) were investigated. Juglone and phenolic contents were determined by spectrophotometric methods. Correlations between juglone-phenolic content, juglone-climatic factors and phenolic content-climatic factors were also established. In conclusion, there were significant differences between the varieties and the highest values of both juglone and phenolic content were recorded in Yalova-2 cultivar among the varieties. For example, maximum juglone content was determined in Yalova-2 (3,51 mg/g) but minimum in Yalova-4 (2,26 mg/g). Maximum phenolic content was also determined in Yalova-2 (51.8 mg/g), but minimum phenolic level was seen in 1974/7 cultivar (49,3 mg/g), as a mean. On the other hand, both juglone and phenolic content were generally higher from middle of August to middle of September and the lowest levels were seen in May. Further, while a significant positive correlation between juglone and phenolic contents of the leaves was found, there was almost no significant correlation between juglone and climatic factors except a significant negative correlation between phenolic content and wind speed in Yalova-2 cultivar.

Key words: Climatic factors, juglone, phenolic content, walnut cultivars, walnut leaf

Introduction

The walnut family (Juglandaceae) contains several species and is rather distributed on the world. The most common species among the species is *Juglans regia* (1). This species is grown commercially in several countries such as China, USA, France, India, Italy, Spain and Turkey for nut production (2). Several parts of the walnut mainly kernels, shells, bark and leaves are using for the pharmacological and cosmetic aims (3). Walnut leaves are easily available in abundant amounts and are considered a source of healthcare compounds and are intensively using in traditional medicine for treatment of several disorders (4).

Juglone (5-hydroxy-1,4-naphthoquinone) is a quinone group aromatic compound has been found in all parts of the walnut tree and it has also been reported that the amounts of juglone were in order of green peel>leaves>bark and it has phytotoxic and allelopathic properties (5). Juglone can be toxic for surrounding plant species, and is of great interest due to its chemical reactivity. Juglone is involved into the walnut pathogenic defense mechanism (6). Walnuts had the highest antioxidant activity among analyzed foods and drinks (7). A balance among competing source and sink mechanisms and rates will ultimately determine whether juglone is capable of attaining sufficient levels to be allelopathic to intercrops in a walnut

tree agroforestry system. Although the allelopathic effect of Juglone on plants is generally toxic (8), in some cases it may also be beneficial (9).

Detrimental impact of Juglone may be associated with suppressing the intensity of a wide range of physiological processes and biochemical reactions occurring in plant tissues. Some researchers reported that Juglone may inhibit the growth of both shoots and roots, photosynthesis, chlorophyll content, respiration, transpiration, stomatal conductance and disrupting root plasma membrane and decreasing of water uptake. Juglone influence the germination, growth, development, reproduction and distribution of a number of plant species (8). In spite of the large number of papers published on the biological activity of Juglone, little is known about the mechanism of its toxic effect on plant cells growth (10). Juglone has been found to have herbicidal effect on some weed species (11) and antioxidant activity on some enzymes (12). Its antiviral and antimicrobial activities have also been detected (1, 13). Recently its antifungal and antibacterial activities have been enhanced using PLGA nanoparticle system, as well (14, 15).

Phenolic compounds are secondary metabolites synthesized in plants by mainly mevalonic or shikimic acid pathways. Their synthesis depends on numerous enzymes involved in different metabolic pathways. They show pharmacological activities such as antioxidant, immune stimulating, antiseptic and spasmolytic so on (16). Phenolics are also involved in physiological processes such as fruit growth and they affect different aspects of fruit development (17). Phenols are active in defense against various types of stresses caused by pathogens or abnormal environmental conditions. Wounding in plants can also cause stress situations affecting the biosynthesis of phenolic compounds (18). Since phenolic compounds have been shown to have antioxidant, free radical-scavenging and metal-chelating activity in addition to their anticarcinogenic properties, they are considered beneficial for human health (19, 20).

Walnut is a rich source of phenolic compounds, including phenolic acids, naphtoquinones and flavonoids. In walnut leaves, naphtoquinones and flavonoids are considered as major phenolic compounds (19). The increasing interest in the powerful biological activities of plant phenolics has outlined the necessity of determining their content (21). There have been reports of seasonal variation in juglone level in leaves and soil beneath of *J. nigra* (22). But no reports regarding the varietal differences and time dependent variation of juglone and phenolic content in the leaves of Turkey's cultivars of *J. regia* have been encountered. For this reason, the present study was conducted to understand time dependent variations in juglone and total phenolic contents in the leaves of five walnut cultural varieties grown in Turkey, and relations between juglone-phenolic content, juglone-climatic factors and phenolic content-climatic factors. This information should allow for a better time dependent use of the leaves of walnut to extract juglone and phenolics, as well as for a better ecological definition of these compounds.

Materials and Methods

Leaf sampling

Walnut leaves were picked up from the five cultivars (cv. Şebin, cv. Yalova-2, cv. Yalova-3, cv. Yalova-4 and cv. 1974/7) of 20 years old walnut trees (*Juglans regia* L.) grown in the walnut orchard of Agricultural Faculty of Uludağ University in Bursa city of Turkey. Three trees per cultivar were chosen randomly and leaf collecting procedure was carried out during two following years with two weeks intervals from May to October. The collected leaves were put into the plastic bags and bring to the laboratory. Then the leaf samples were dried in an oven by keeping for 48 h at 70°C. The dried leaf samples were put into the plastic bags and labelled, and so they were kept in a cold room until to use. On the other hand, the meteorological values of Bursa city were obtained from General Directorate of Meteorology of Turkey (Table 1).

Determination of Juglone Content

Measurement of juglone content of the walnut leaves was carried out by spectrophotometric method (23). 2 g of leaf was homogenized in 50 ml petroleum ether and after filtration the filtrate was centrifuged at 18.000 rpm in refrigerated centrifuge for 15 min. The supernatant was diluted ten-fold with petroleum ether and its absorbance was recorded by spectrophotomet-

Table 1. Meteorological values of Bursa city where the walnut orchard from which the leaves were picked is exist. The values are mean of two following years of the research conducted from May to October.

Meteorological Parameters	May	June	July	August	Septemb.	October
Temperature (°C)	19.4	23.5	25.2	26.2	20.7	16.1
Rainfall (mm)[3]	10.7	55.0	7.7	2.2	47.2	60.5
Humidity (%)	59.0	57.0	53.5	55.5	63.5	74.5
Wind Speed (m/s)	1.9	1.8	2.0	1.7	1.7	1.3

ric measurement at 410 nm. Blank sample was only petroleum ether. In determining of juglone content of the leaves was used standart curve prepared by a series of pure juglone solutions in the range of 0.01, 0.02, 0.03, 0.04 and 0.05 mg juglone contents. The juglone content was expressed as "mg juglone/g dry leaf".

Determination of Total Phenolic Content

The amount of total phenolic compounds in the walnut leaves was determined spectrophotometrically (24). 2 g of leaf was homogenized in 50 ml methanol and after filtration the filtrate was centrifuged at 4.000 rpm in refrigerated centrifuge for 15 min. 10 ml of supernatant was taken and its methanol was evaporated in a rotary evaporator. Then the residue was dissolved in 2 ml of methanol, and 0.1 ml of this solution was mixed with 0.1 ml of Folin reagent, 0.5 ml of Na-carbonate solution (%20) and 9.3 ml of distilled water. After keeping at 25°C for 2 hours, absorbance of the mixture was measured in spectrophotometer at 765 nm. The mixture without 0.1 ml of leaf extract was used as blank sample. In determining of phenolic content of the leaves was used a standart curve prepared by a series of gallic acid standart solutions in the range of 0.02, 0.04, 0.06, 0.08 and 1 mg gallic acid contents. The phenolic content was expressed as "mg phenolic/g dry leaf".

Statistical Analyses

The mean values obtained from three replications were shown on the tables. LSD multiple comparison test was applied for comparing the significant differents among cultural varieties and sampling dates at 0.05 level for both juglone and phenolic content of the walnut leaves. Further, correlation test was applied to show significant correlations between juglone - phenolic content, as well as between juglone - meteorological parameters and phenolic content - meteorological parameters.

Results and Discussion

The increasing interest in the powerful biological activities of plant phenolics has outlined the necessity of determining their content in leaves of different walnut cultivars. In this study, walnut leaves from five walnut cultivars originating from the same orchard and from the same year of production were analyzed for their seasonal variations in juglone and phenolic content from May to October. The walnut cultivars were grown under the same agricultural, geographical and climatic conditions.

In this study, in all the cultivars, the lowest content of juglone in leaves was measured in May which the stage of vigorous development of spring growth unit. In a former study, the lowest content of juglone of walnut in annual shoots was also measured in May (18). It was followed an increase during the resting time between the spring and summer growth. That is, juglone contents increased from the spring growth cycle in May to the summer flush of growth in middle of September. The juglone pattern of seasonal variations was almost similar in all cultivars with some differences. In Yalova-3 and Yalova-4, it increased continuously from May until to October. The other varieties have showed some fluctuations in juglone content (Table 2). On the other hand, maximum juglone content was determined in Yalova-2 (3, 51 mg/g leaf) and minimum in Yalova-4 (2, 26 mg/g leaf), as average of the months. Some researchers proved that the content of juglone in leaves and nuts of pecan (*Carya illinoensis* K.) was low in early season and increased through July. In black walnut (*Juglans nigra* L.) great seasonal fluctuations in the concentrations of juglone were ascertained (25).

Formerly, it has been shown showed that measurements of seasonal change in juglone content among various tissues of pecan walnut revealed that

Table 2. Time dependent juglone contents of the leaves of the five walnut cultivars. The values in the table are mean of triplicate determinations of two following years. The last row values are mean of juglone contents for each cultivar.

Sampling Date	Şebin	Yalova-2	Yalova-3	Yalova-4	1974/7
May 15	1.03	0.61	0.68	0.21	0.65
June 1	1.08	2.11	1.20	0.72	0.98
June 15	1.71	2.73	2.00	1.75	2.32
July 1	2.92	3.45	2.71	2.27	3.06
July 15	2.92	4.23	2.96	2.31	2.83
Aug 1	2.37	4.86	3.23	2.86	3.70
Aug 15	3.45	5.31	3.03	2.92	4.73
Sept 1	3.57	4.21	3.36	3.18	4.61
Sept 15	3.55	4.46	3.87	3.02	3.81
Oct 1	3.20	3.67	3.53	3.65	3.23
Mean	2.53	3.51	2.66	2.26	3.01

A multiple comparison test LSD (%5) values for sampling dates and the cultivars were 1.07 and 0.81, respectively.

Table 3. Time dependent total phenolic contents of the leaves of the five walnut cultivars. The values in the table are mean of triplicate determinations of two following years. The last row values are mean of total phenolic contents for each cultivar.

Sampling Date	Şebin	Yalova-2	Yalova-3	Yalova-4	1974/7
May 15	18.7	29.3	19.3	25.0	24.3
June 1	20.6	32.5	17.5	15.0	24.3
June 15	23.7	31.8	25.0	19.3	27.5
July 1	34.3	38.7	42.5	25.6	35.6
July 15	43.1	36.5	51.8	59.3	48.7
Aug 1	58.1	63.1	56.8	66.2	56.2
Aug 15	65.0	68.7	66.2	58.7	63.7
Sept. 1	88.1	69.3	80.2	82.5	66.8
Sept. 15	85.0	72.5	71.2	76.8	66.8
Oct. 1	78.1	68.7	68.7	61.2	72.5
Mean	51.2	51.8	50.0	50.0	49.3

A multiple comparison test LSD (%5) values of sampling dates and the cultivars were 4.5 and 2.1, respectively.

the highest concentrations occurred in leaves in June and in nuts in September. On the other hand, at the end of vegetation period, low juglone was reported in the walnut *J. regia* (17). Also, regarding the seasonal changes, juglone showed a linear decrease over growing season in the leaves of green husks of *J. regia* **(26)**. This opposite result may be derived from different cultivars used or from different climatic conditions along the varied seasons. Other researchers showed that potential juglone abundance estimated in walnut leaves, hulls, and roots ranges from less than 0.1% to as much as 5% dry weight-basis, depending on the growing season and extraction techniques used **(26)**.

In our study, the total content of phenolic compounds was the lowest in the stage of vigorous spring growth in May (Table 3). After that, in the mid-time between the spring and summer growth (second sampling date) and at the beginning of the summer growth flush (third sampling date) in middle of September it strongly increased. As average of the months, maximum phenolic content was determined in Yalova-2 variety and minimum phenolic level was seen in 1974/7 variety (49, 3 mg/g leaf). Considering that the light may plays an essential role in phenol biosynthesis, some researchers attributed the increase in phenolics in July to the highest value of solar radiation in July.

Total content of phenols depends on sampling time (3). Our study is also consistent with this result. 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. However, no significant correlation between climatic parameters such as temperature, rain, humidity and wind, and phenolic or juglone contents was established (Table 4); variations in total phenols and juglone of walnut leaves reported in the present study (Table 2,3) may be explained by genetically programmed inner conditions of walnut species.

Most of the results show that contents of flavonoids and phenolic acids are lowest at the beginning of vegetative period and increase during summer to achieve the highest amounts at the end of phenological cycle (27). Our results suit to that pattern that cultivar variations in phenolic contents were estimated. The levels of phenols are influenced by environmental factors, soil composition, maturation level, cultivar and harvest year. Some researchers indicated that the average values of phenolic acids, flavonoids and total phe-

Table 4. Correlations between meteorological parameters and contents of juglon (J) and total phenolic (P). The values in the table are correlation coefficients.

Comparative Parameters	Şebin	Yalova-2	Yalova-3	Yalova-4	1974/7
J-P	0.869**	0.756*	0.922**	0.809**	0.848**
J-Temperature	-0.026	0.380	-0.017	-0.085	0.181
J-Rainfall	-0.046	-0.211	0.044	0.093	-0.125
J-Humidity	0.299	-0.006	0.350	0.443	0.150
J-Wind Speed	-0.331	-0.272	-0.450	-0.585	-0.347
P-Temperature	-0.305	-0.210	-0.152	-0.149	-0.241
P-Rainfall	0.195	0.111	0.018	-0.028	0.079
P-Humidity	0.585	0.530	0.443	0.391	0.550
P-Wind Speed	-0.628	-0.692*	-0.508	-0.438	-0.658

** (P<0.01), * (P<0.05)

nols seem to point out to a decrease until September, while others indicated that each phenolic group had its own curve of seasonal fluctuations (3).

On the other hand, in this study, a significant positive correlation between juglone and phenolic contents of the leaves was found (r: 0,893; P<0, 01). While there was no significant correlation between juglone and climatic factors, except a significant negative correlation between phenolic content and wind rate in Yalova-2 cultivar (r:-0,692 ; P<0,05) (Table 4).

The results obtained indicated that walnut leaves may become important in obtaining a noticeable source of the compounds with health protective potential and antimicrobial activity; therefore the walnut leaves should preferentially be collected from the middle of August to the early September, in that time juglone and phenolic contents are higher. It has been shown that cultivars and sampling date is important for the juglone and phenolic contents in the walnut leaves. Juglone was considered as a useful tool for distinguishing different genotypes, and may be a potential genetic marker to study variation. This approach may be more useful for achieving a quick and short-term objective of either increasing juglone content for juglone production or decreasing its content for nut production in horticulture and reducing allelopathy in agroforestry (2).

In conclusion; it may be concluded that there is a significant positive relation between juglone and phenolic content in walnut leaves depending on seasonal variation. Juglone and phenolic contents rather change according to walnut cultivars that the highest juglone content was determined in Yalova-2 cultivar. The results of the present study apparently indicated that the walnut (*J. regia* L.) leaves are constitute a suitable source of juglone and phenols and they could be used as alternative natural antioxidants. As juglone and phenolic contents are higher in that time, walnut leaves should preferentially be collected between late August and early September. The information obtained in the walnut leaves may be useful in planning the collecting time and cultivar selection for producing medicinal extracts.

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