

Phenolic acids of walnut (*Juglans regia* L.)¹

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S u m m a r y

The chromatographic separations showed the presence of eleven free phenolic acids (*trans*-cinnamic, *o*-coumaric, *p*-coumaric, chlorogenic, ferulic, gallic, caffeic, *p*-hydroxybenzoic, syringic, tannic and vanillic) in the walnut tissues. The *p*-hydroxybenzoic acid was a predominant compound in the green husks ($618.8 \mu\text{g}\cdot\text{g}^{-1}$), though, high content of it was found in leaves obtained in July ($478.3 \mu\text{g}\cdot\text{g}^{-1}$). The content of chlorogenic acid in green husks of walnut ($306.7 \mu\text{g}\cdot\text{g}^{-1}$) was twice higher than that in the leaves obtained in July. Similar level of coumaric acids was determined in the leaves and green husks (*o*-coumaric: $42.4\text{--}48.2 \mu\text{g}\cdot\text{g}^{-1}$; *p*-coumaric: $78.6\text{--}94.3 \mu\text{g}\cdot\text{g}^{-1}$), though, *o*-coumaric acid was not found in May-leaves. Moreover, leaves of walnut possessed high content of caffeic acid (leaves obtained in May – 456.9 ; leaves obtained in July – $262.1 \mu\text{g}\cdot\text{g}^{-1}$) and ferulic acid (leaves obtained in May – 93.5 ; leaves obtained in July – $292.5 \mu\text{g}\cdot\text{g}^{-1}$). The predominant compound of the male inflorescences was vanillic acid ($359.5 \mu\text{g}\cdot\text{g}^{-1}$). The green husks of walnut were rich in polyphenolic acids, syringic ($427.0 \mu\text{g}\cdot\text{g}^{-1}$) and tannic ($237.4 \mu\text{g}\cdot\text{g}^{-1}$). However, the content of tannic acid was higher in the leaves - especially in leaves collected in May ($350.7 \mu\text{g}\cdot\text{g}^{-1}$). In tested tissues of walnut, gallic acid was found in the lowest concentration (male inflorescences – $32.7 \mu\text{g}\cdot\text{g}^{-1}$ and leaves obtained in July – $12.7 \mu\text{g}\cdot\text{g}^{-1}$). In general, there was a great variation in a level of the phenolic acids between the studied organs of the walnut; nevertheless, walnut tissues are precious source of phenolic acids.

Key words: *hydroxycinnamic acids, hydroxybenzoic acids, walnut organs*

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INTRODUCTION

Phenolic acids are ubiquitous constituents of higher plants involved in defense against ultraviolet radiation and attack of pathogens and/or insects. Moreover, these compounds participate in antioxidative/oxidative reactions. Several hundreds of phenolic compounds have been described in plants and grouped into different classes according to their chemical structure. Among them are phenolic acids that have an aromatic ring hydroxylated and/or methoxylated to the different extend. Hydroxybenzoic acids have a general structure C6-C1, and they are able to polymerize to hydrolysable tannins and lignins. Chemical structure of hydroxycinnamic acids (C6-C3) possess an aromatic ring and three-carbon chain with one double bond. Phenolic acids have been also found within plant tissues as amino- and glycosyl-derivatives [1].

Walnut (*Juglans regia* L.) belongs to *Juglandaceae* family. It is a deciduous tree native to western and central Asia to south-eastern Europe, commercially cultivated all over the world. Antioxidant effects of polyphenols obtained from walnut have been reported previously [2]. In scavenging of hydroxyl and superoxide radicals for water and methanol extracts of *J. regia* are found [3]. Moreover, walnut leaves have been widely used in folk medicine, and earlier studies demonstrated their anti-inflammatory, antidiarrheic, antihelminthic, antiseptic and astringent properties [4, 5]. On the other hand, environmental interactions and allelopathic properties of the walnut have been suggested. One of the best known examples of allelopathy was vicinity of black walnut to other growing plants.

Composition of polyphenols within walnut is quite well known. Only phenolic acids in different parts of walnut during seasonal vegetation were studied for the less extension. In present paper the content of free phenolic acids within male inflorescences, leaves and green husks during various developmental stages of the growing season are reported.

MATERIAL AND METHODS

The *J. regia* inflorescences were collected in May, leaves were harvested in May and July, instead of green husks which were collected in July. Plant material was freeze-dried immediately after harvest and prepared for phenolics analysis. Extraction procedure was based on the method described by Kowalski and Wolski [6]. Phenolic acids were extracted from plant material (5 g) with 50 ml of 80% methanol. After centrifugation at 10000 g for 30 min the supernatant was defatted with petroleum ether. Solid sodium hydrogen carbonate and water was added to obtain 5% concentration of salt and 40% of methanol. Solution was left in darkness overnight and centrifuged. Free phenolic acids fraction was extracted with distilled ethyl acetate after acidification of the water phase to pH=2 with 6M hydrochloric acid.

Separation of phenolic acids was carried out using HPLC isocratic Varian system equipped with ProStar 210 pump, Photodiode Array Detector ProStar 335 and analytical column Microsorb – MV 100-5 C18 (250x4.6 mm ID, Varian) with ChromSep Guard Cartridge and 20 μL injection loop. Mobile phase was set as follows: methanol - water (25:75) with addition of 1% acetic acid. All separations were performed at the temperature of 27°C and the flow rate was 1 $\text{cm}^3 \cdot \text{min}^{-1}$. Identification of the phenolic acids was done with use of Varian software (Star Chromatography Workstation ver. 6.41, Aurora and PolyView 2000). Retention times and UV/VIS spectra of separated compounds were compared with standards obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Then, the quantity of phenolic acids present in each sample was then estimated by comparison of the peak area of separated phenolic acid with the reference standard. All experiments were repeated three times and results were expressed in $\mu\text{g} \cdot \text{g}^{-1}$ of dry matter (d.m.) as an average data \pm standard error.

RESULTS AND DISCUSSION

The chromatographic separations have shown the presence of eleven free phenolic acids: *trans*-cinnamic, *o*-coumaric, *p*-coumaric, chlorogenic, ferulic, gallic, caffeic, syringic, tannic, vanillic and *p*-hydroxybenzoic within the studied walnut organs. The content of *p*-hydroxybenzoic acid (fig. 1) was the highest in walnut husks ($618.8 \mu\text{g} \cdot \text{g}^{-1}$). Moreover, this acid was a predominant compound of leaves ($478.3 \mu\text{g} \cdot \text{g}^{-1}$). The amount of chlorogenic acid in green husks ($306.7 \mu\text{g} \cdot \text{g}^{-1}$) was twice higher than in leaves and four times higher than its content in male flowers. Coumaric acids (*p*-coumaric: $31.4\text{--}95.3 \mu\text{g} \cdot \text{g}^{-1}$ and *o*-coumaric: $31.2\text{--}48.24 \mu\text{g} \cdot \text{g}^{-1}$) in leaves and husks were found at a similar level, however, the *ortho*- form was not found in the leaves obtained in May. High levels of the di-substituted phenolic acids: caffeic and ferulic (fig. 2) were found in the leaves of the walnut. Other di-substituted derivative of benzoic acid (vanillic acid) was predominant compound of the male inflorescences ($359.5 \mu\text{g} \cdot \text{g}^{-1}$). Moreover, the polyphenolic acids containing three or higher number of oxygen substituents, such as gallic, syringic and tannic acids were also identified (fig. 3). Tannic acid was found in the leaves and green husks of walnut and the highest content of this compound occurred in leaves obtained in May ($350.7 \mu\text{g} \cdot \text{g}^{-1}$). Syringic acid was found only in leaves obtained in May and green husks, its content was considerably higher in green husks ($427.0 \mu\text{g} \cdot \text{g}^{-1}$). Gallic acid was determined in male flowers ($32.7 \mu\text{g} \cdot \text{g}^{-1}$) and leaves obtained in July ($12.6 \mu\text{g} \cdot \text{g}^{-1}$), and its amount was the lowest among identified phenolic acids in walnut.

Amaral et al. [7] have found a decrease in the content of total phenolic acids and flavonoids in the walnut from May to September, however, these authors did not analyse the free acids fraction. They identified 3-O-caffeoylquinic acid as well as derivatives of *p*-coumaric acid and quercetin. Wichtl and Anton [8] found caffeic, ferulic, *p*-coumaric, gallic, salicylic, chlorogenic and neochlorogenic acids in

walnut leaves. Jakopic et al. [9] have shown the presence of protocatechuic, gallic, chlorogenic acids and juglone as well as (+)-catechin in green walnut fruits extracted with methanol or ethanol. The content of analysed compounds was hooked on the solvent and tested cultivar during experiment. They assigned that methanol was a stronger solvent for extraction of the phenolic substances. Moreover, Jakopic et al. [10] found other phenolic acids, such as: syringic, sinapic, chlorogenic, vanillic and caffeic in ethanolic liqueur from green walnut fruits.

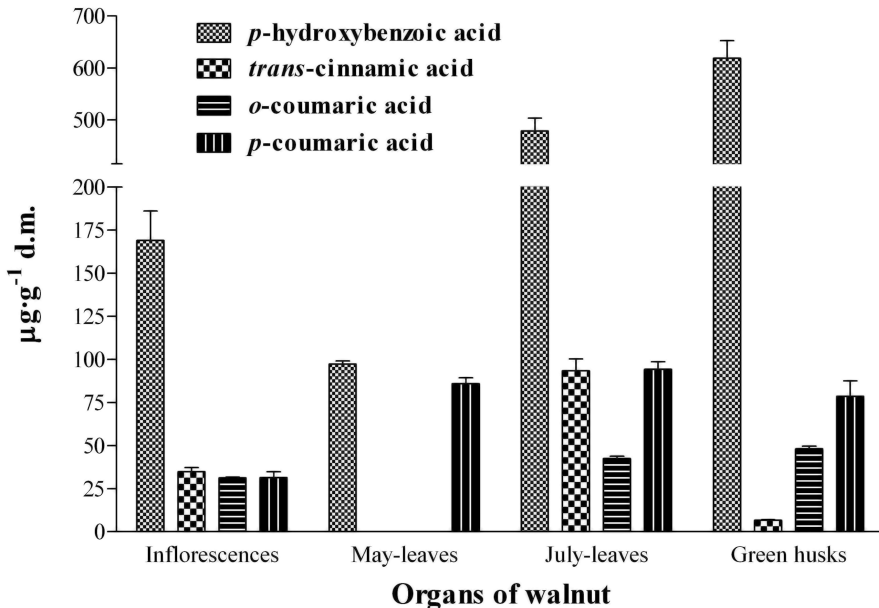


Figure 1.

The content of the mono-substituted phenolic acids in tissues of analysed organs of walnut

Herbs and spices contain natural antioxidants, and their activity has been attributed to the polar phenolic compounds [11]. The antioxidant activity of phenolics depends on the number of hydroxyl groups in the molecule and hydroxyl derivatives of cinnamates are more effective than benzoates counterparts [12]. It was also found that isomers of chlorogenic acid could scavenge superoxide anion radicals and inhibit oxidation of linoleate system [13]. Other activity of phenolic acids is connected with plant resistance to insects and antimicrobial properties. Santiago et al. [14] have found that level of free *p*-coumaric acid was correlated with the resistance of maize to stem borer (*Sesamia nonagrioides* Lefèbvre). Ferulic and *p*-coumaric acids were jointly able to provide resistance mechanisms through cell wall fortification and lignification. In addition, it has been reported that phenolic acids (ferulic, isovanillic, syringic, *p*-hydroxybenzoic and *p*-coumaric) were responsible for the antibacterial activity [15].

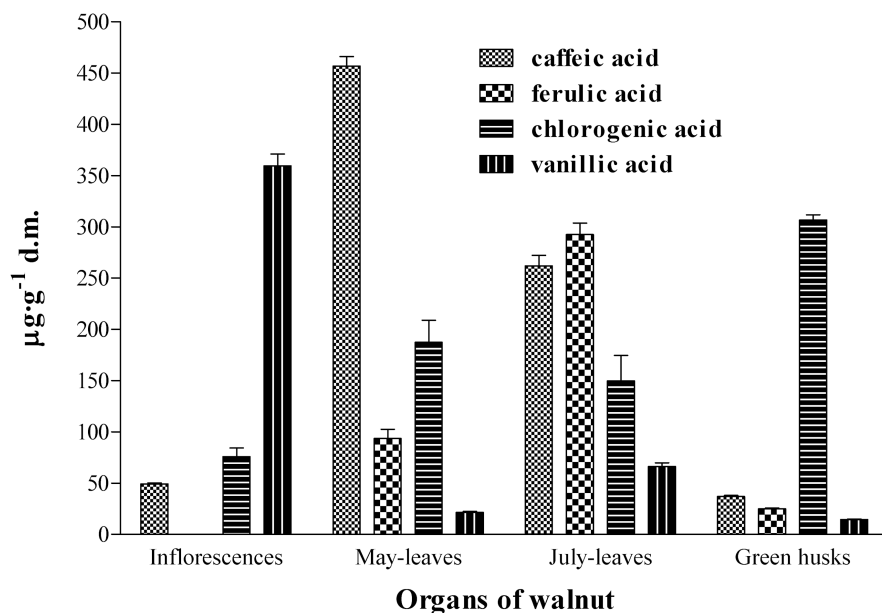


Figure 2.
The content of the di-oxygenated phenolic acids in tested tissues of the *J. regia*

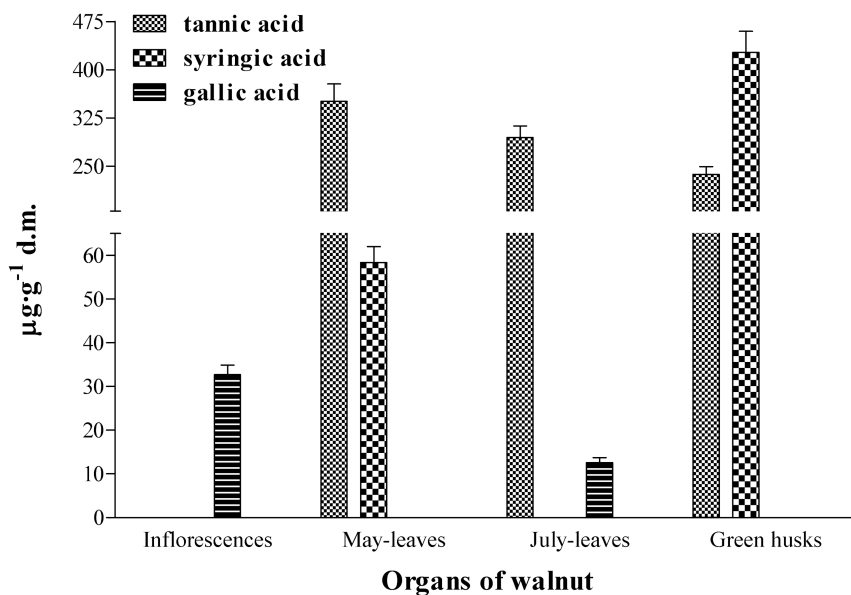


Figure 3.
The content of the poly-substituted phenolic acids in analysed tissues of walnut

Phenolic acids are very interesting from botanical and pharmaceutical points of view. Plants containing high level of flavonoids and phenolic acids were widely used as antioxidant agents [16]. Moreover, several studies demonstrated the antimicrobial activity of phenolics and/or phenolic extracts, making them a good alternative to antibiotics and chemical preservatives [5]. They also found that walnut can be used as a source of natural antimicrobial compounds.

Obtained results concerning the amount and variation in levels of the phenolic acids within the walnut tissues as well as the cited reference data suggest that walnut tissues are rich source of biologically active phenolic acids.

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KWASY FENOLOWE ORZECHA WŁOSKIEGO (*JUGLANS REGIA* L.)

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Streszczenie

Analiza chromatograficzna kwasów fenolowych ekstrahowanych z tkanek orzecha włoskiego wykazała obecność jedenastu kwasów: *trans*-cynamonowego, chlorogenowego, ferulowego, galusowego, kawowego, *o*- i *p*-kumarowego, *p*-hydroksybenzoesowego, syryngowego, tanninowego i wanilinowego. Dominującym związkiem zielonych owocni orzecha włoskiego ($618,8 \mu\text{g}\cdot\text{g}^{-1}$) oraz liści zbieranych w lipcu ($478,3 \mu\text{g}\cdot\text{g}^{-1}$) był kwas *p*-hydroksybenzoesowy. Najwyższą zawartość kwasu chlorogenowego ($306,7 \mu\text{g}\cdot\text{g}^{-1}$), dwukrotnie większą niż w liściach stwierdzono w zielonych owocniach. Stwierdzono, że zawartość kwasów kumarowych w liściach i zielonych owocniach była na podobnym poziomie (kwas *o*-kumarowy: $42,4\text{--}48,2 \mu\text{g}\cdot\text{g}^{-1}$, kwas *p*-kumarowy: $78,6\text{--}94,3 \mu\text{g}\cdot\text{g}^{-1}$), przy czym liście zbierane w maju nie zawierały kwasu *o*-kumarowego. Ponadto liście orzecha włoskiego bogate były w kwas kawowy (majowe – $456,9$, lipcowe – $262,1 \mu\text{g}\cdot\text{g}^{-1}$) oraz ferulowy (majowe – $93,5$, lipcowe – $292,5 \mu\text{g}\cdot\text{g}^{-1}$). Dominującym składnikiem kwiatostanów męskich był kwas wanilinowy ($359,5 \mu\text{g}\cdot\text{g}^{-1}$). Zielone owoce orzecha bogate były również w kwasy polifenolowe, syryngowy ($427,0 \mu\text{g}\cdot\text{g}^{-1}$) oraz tanninowy ($237,4 \mu\text{g}\cdot\text{g}^{-1}$), chociaż zawartość kwasu tanninowego wyższa była w liściach, zwłaszcza zbieranych w maju ($350,7 \mu\text{g}\cdot\text{g}^{-1}$). W badanych organach orzecha, najniższym stężeniem charakteryzował się kwas galusowy i jego obecność wykazano jedynie w męskich kwiatostanach ($32,7 \mu\text{g}\cdot\text{g}^{-1}$) oraz liściach zbieranych w lipcu ($12,7 \mu\text{g}\cdot\text{g}^{-1}$).

Podsumowując należy stwierdzić, że organy orzecha włoskiego charakteryzuje duża zmienność w zawartości kwasów fenolowych, niemniej orzech włoski stanowi cenne źródło kwasów fenolowych.

Słowa kluczowe: kwasy hydroksycynamonowe, kwasy hydroksybenzoesowe, organy orzecha włoskiego