ANTIOXIDANT ACTIVITY AND POLYPHENOLIC PROFILE OF WALNUT (*JUGLANS REGIA* L.) GREEN HUSKS AND LIQUEURS

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SUMMARY

The beneficial effects of walnuts and walnut products on human health, due primarily to their rich polyphenolic content, have been appreciated as an empirical fact for centuries. The purpose of this study is to determine the polyphenolic contents of liqueurs made from the walnut selections 'Rasna' and 'Sava' and the walnut cultivar 'Šampion' (all harvested at three different times), as well as the polyphenolic contents of their green husks. The walnut liqueurs were prepared according to two traditional recipes using young walnut fruits. The highest antioxidant capacity was recorded in the liqueur made from the 'Rasna' walnut selection (89.94%), whereas the highest contents of phenols (83.28 mg GAE/g FW), flavonoids (0.83 mg QE/g FW) and proanthocyanidins were found in the liqueur made from the 'Šampion' cultivar (14.75 mg CE/g FW). The youngest 'Rasna' walnuts, harvested at the first experimental time point, exhibited the highest phenolic and tannin contents, whereas the highest flavonoid content was observed in the 'Šampion' cultivar. The biochemical results obtained indicate a decrease in the polyphenolic content of walnut fruits with their growth and development. Moreover, the polyphenolic profiles of the walnut liqueurs considered were found to be greatly affected by the method of preparation and the cultivar/selection of walnuts.

Key words: flavonoids, phenols, liqueur, proanthocyanidin, green husks

Abbreviations: TP - total phenols; GAE - gallic acid equivalents; FW - fresh weight; PVPP - polyvinylpyrrolidone; QE - quercetin equivalent; PA - proanthocyanidins; CE - catechin equivalents; DPPH radicals - 2,2-diphenyl-1-picrylhydrazyl radicals.

INTRODUCTION

Over the course of centuries, walnut liqueurs have been found to exert beneficial effects on human health. As little or no pesticides are used in walnut production, walnut liqueurs can be considered healthy for containing little or no pesticide residues. Compared to other fruit crops, the walnut is highly susceptible to oxidation reactions, which is indicative of the increased presence of antioxidant compounds inhibiting lipid autoxidation (Čadenović et al., 2012). Walnut fruits, bark, roots, leaves, cotyledons, meats and oil are used for medicinal purposes (Stampar et al., 2005; Upadhyay et al., 2010). The *Junglandaceae* family, *Juglans regia*, is referred to as the royal walnut family for its oxidative stress-relieving properties (Shah et al., 2015). Norouzi et al. (2013) argue that consuming walnuts is the easiest way to improve health. A number of studies have suggested that nutrition is of crucial importance to the

prevention of serious diseases such as congenital defects, coronary diseases, cancer and cataracts (Taha & Al-Wadaan, 2011; Čadenović et al., 2012; Noumi et al., 2012; Shah et al., 2015). *Juglans regia* fruits are used in traditional medicine for treating respiratory diseases, skin and bone disorders, anorexia, diarrhoea, stomach-ache, scrofula, various endocrine diseases, infectious diseases, and cancer. Walnuts also have wide applications in the cosmetic and pharmaceutical industries (Stampar et al., 2005, Upadhyay et al., 2010., Taha & Al-Wadaan, 2011., Shah et al., 2015). *J. regia* bark and leaves are highly therapeutic (Noumi et al., 2012). In some European countries, especially in Portugal, dry walnut leaves are used for infusion therapy (Amaral et al., 2004). In the Middle East, walnuts are added, individually or in combination with other fruits, to traditional dainties such as Ma'moul (Taha & Al-Wadaan, 2011). In Modena, Italy, the liqueur made from unripe walnuts (namely the *nocino* liqueur) is used as an after-dinner drink (Alamprese et al. 2005). Phenolic compounds are one of the most active antioxidant compounds (Saeed et al., 2015). Phenols actively partake in the physiological processes of walnut fruit growth and development, exerting various impacts on walnut fruits before and after harvest. Catanzaro et al. (2018) suggest that the phenolic content of walnuts is conditioned by both genetic and environmental factors, whereas Wianowska et al. (2016) report that the flavonoid and phenolic contents of walnut extracts depend on the *J. regia* cultivars used. Cosmulescu et al. (2010) conclude that the phenolic content of walnut green husks decreases significantly with walnut maturation. Solar et al. (2006) assert that the phenolic content of walnuts depends on their developmental stage. Mikulic-Petkovsek et al. (2011) argue that total phenols essentially contribute to disease resistance. Phenolic contents vary in different parts and growth phases of the Persian walnut: total phenols increase in early and mid summer, followed by a decrease in late summer and a new increase before the harvest period (Cosmulescu & Trandafir, 2011). Stampar et al. (2005) identified a total of 13 phenolic compounds in walnut husks. Walnut green husks are rich in phenolic compounds such as juglone (Colarič et al., 2005). Juglone (5-Hydroxy 1,4-naphthoquinone) is a specific phenolic compound that exists only in walnuts, especially in green walnut parts such as leaves, husks, stems and roots. Juglandin has exhibited cytotoxicity to different types of breast, lung and skin cancer cells (Catanzaro et al., 2018). Phenols impart a bitter yet pleasant taste to walnut liquors (Stampar et al., 2005). The purpose of this study is to determine the polyphenolic contents of liqueurs made from the walnut selections 'Rasna' and 'Sava' and the walnut cultivar 'Šampion' (all harvested at three different times), as well as the polyphenolic contents of their green husks.

MATERIAL AND METHODS

Plant material

The walnut cultivar 'Šampion' and selections 'Rasna' and 'Sava' were enrolled in this study. The walnuts considered were harvested at three different times in 2017: 5th May, 25th May and 5th September.

Preparation of walnut ethanol extract

Green husks were removed from the fruits and chopped in small pieces. A plant mass of 0.5 g was extracted using 40% ethanol. After 24 h, the filtered sample was prepared for analysis. The dehushing method employed was the same for all the three samples examined.

Preparation of walnut liqueur

The walnut liqueur recipe consists of a small number of ingredients: 140 g of young walnut fruits, 250 g of sugar, 50 g of honey, and 1L of grape brandy per glass jar.

Fruits of all the walnut selections and cultivars considered were first harvested on 25th May 2017. The fruits were then carefully washed with cold water and all the impurities were removed. Two different methods of liqueur preparation were used with each of the walnut cultivar and selections considered, thus resulting in a total of six different walnut liqueurs. The first method involved washing the walnut fruits, cutting off the peduncle and placing the whole fruits into a previously well-washed glass jar. However, the second method involved cutting the fruits into halves with a sharp knife. The prepared fruits (140 g) were placed into six jars with a capacity of 1.5 kg, after which 250 g of sugar was added to each. Cellophane covers were placed over the jar tops to avoid contact with the metal lid during the process. The jars were sealed and left in a sunny place over a period of 40 days (with regular shaking every two days). After 40 days, a litre of grape brandy and 50 g of honey were added to each jar, after which new cellophane covers were placed over the jar tops and the jars were tightly sealed with metal lids. The liqueurs thus prepared were left in a dark room at room temperature. The jars were occasionally shaken to avoid honey settling. Following another 40 days, the liqueurs were ready for consumption and analysis.

Determination of the phenolic content of walnut extracts

The concentration of total phenols (TPs) in the walnut extracts considered was determined spectrophotometrically using the "Folin-Ciocalteu" reagent (Hagerman et al., 2000). This method is based on measuring the reducing capacity of phenols and other compounds. The amount of TPs was calculated as the gallic acid equivalent, relative to the calibration curve of gallic acid standard solutions (covering the concentration range between 0.1 and 1.0 mg/ml), and expressed as mg gallic acid equivalents/g fresh weight (mg GAE/g FW).

Determination of the tannin content of walnut extracts

In order to determine the tannin content of the walnut extracts considered, the method for determining the phenolic content was employed, but the samples used were obtained by the centrifugation (10.000 x g, 10 min) of the reaction mixture consisting of 0,1 g of polyvinylpolypyrrolidone (PVPP), 1 ml of water and 1 ml of the extract. After the removal of tannins by adsorption on an insoluble matrix (PVPP), the non-tannin phenolic content in the supernatant was determined. The tannin content of the samples was calculated from the difference between the content of total phenols and non-tannin phenolic compounds, and expressed as mg gallic acid equivalents/g fresh weight (mg GAE/g FW).

Determination of the flavonoid content of walnut extracts

The concentration of flavonoids was calculated as the quercetin equivalent (QE), relative to the calibration curve of quercetin standard solutions, and expressed as mg quercetin equivalents/g fresh weight (mg QE/g FW) (Markham, 1989).

Determination of the proanthocyanidin content of walnut extracts

The concentration of proanthocyanidins (PA) in the walnut extracts considered was determined using a butanol-HCl assay (Sun et al., 1998). The aliquots of the prepared extracts (0.5 ml) were placed into test tubes. After the addition of butanol-HCl reagent (3 ml) and 2% ferric reagent (0.1 ml), the test tubes were vortexed and placed in a boiling water bath for 60 min. After cooling, the absorbance was recorded at 550 nm against a blank containing solvent (0.5 ml) instead of the extract. The amount of proanthocyanidins was expressed as mg catechin equivalents/g fresh weight (mg CE/g FW).

Determination of DPPH radical neutralization

The spectrophotometric determination of the "scavenging" activity of these extracts was based on monitoring the transformation of DPPH radicals (2,2-diphenyl-1-picrylhydrazyl radicals) in a reduced (DPPH-H) form. The DPPH radical is purple and it has a maximum absorption at 517 nm. Through reactions with antioxidants, it binds a hydrogen atom to its unpaired electron and thereby turns yellow. The DPPH assay was done according to the method of Lee et al. (1998).

Statistical analysis

The results obtained were statistically analysed using StatSoft (2017) and the analysis of variance (ANOVA). The parameter mean difference was set at a significance level of 0.01% using the Duncan's multiple range test. The results obtained are shown in Tables 1 and 2.

RESULTS AND DISCUSSION

The biochemical composition of walnut green husks and the phenolic content of the walnut cultivar and selections considered are given in Table 1. The highest phenolic content of green husks was found in the selection 'Rasna' harvested at the 1st experimental time point (68.08 mg GAE/g FW), whereas the lowest phenolic content of green husks was recorded in the selection 'Sava' harvested at the 3rd experimental time point (17.50 mg GAE/g FW). The results obtained indicate that the concentration of polyphenols and their antioxidant activity decrease with walnut fruit ripening, with the exception of proanthocyanidins which increase with fruit ripening during the second harvest period (25th May) and then decrease in the 3rd harvested period. Moreover, it can be argued that the concentration of polyphenols depend on the walnut cultivar/selection. Wianowska et al. (2016) investigated the phenolic content of walnut green husks from different cultivars and seedlings. The concentration of phenols at harvest was in the range of 90.64- 117.707 mg GAE/g FW. Ghasemi et al. (2011) report that the phenolic content of the 11 walnut cultivars examined ranged between 15.15 and 108.11 mg GAE/g FW, whereas Jalili et al. (2012) report that the average phenolic content of the seven walnut extract considered ranged 23.40 ± 0.89 mg GAE/g FW. Oliveira et al. (2008)

found that the total concentration of phenols in walnut extracts ranged from 32.61 to 74.04 mg GAE/g FW, depending on the walnut cultivars used.

Table 1. Average concentrations of phenols, tannins, flavonoids and proanthocyanidins in walnut fruits harvested at three experimental time points (1st, 2nd and 3rd) and the DPPH-assay results

Sample	Cultivar/Selection	Phenols (mg GAE/g FW)	Tannins (mg GAE/g FW)	Flavonoids (mg QE/g FW)	Proanthocyanidins (mg CE/g FW)	DPPH (%)
1.	‘Rasna’ 1 st time point	68.08^a	63.18^a	2.92 ^b	32.40 ^d	88.25^a
2.	‘Sava’ 1 st time point	62.64 ^b	59.00 ^b	3.70 ^{ab}	58.02 ^b	83.55 ^b
3.	‘Šampion’ 1 st time point	66.74 ^a	58.73^b	4.15^a	48.96 ^c	88.14^a
4.	‘Rasna’ 2 nd time point	22.02 ^{de}	18.65 ^e	0.96 ^{cd}	53.52 ^b	24.41 ^{de}
5.	‘Sava’ 2 nd time point	25.86 ^d	22.74 ^d	1.32 ^c	74.38 ^a	32.26 ^d
6.	‘Šampion’ 2 nd time point	33.75 ^c	29.54 ^c	1.21 ^c	76.38^a	49.11 ^c
7.	‘Rasna’ 3 rd time point	24.23 ^d	19.86 ^{de}	0.47 ^e	23.39 ^e	15.34 ^e
8.	‘Sava’ 3 rd time point	17.50 ^f	13.84 ^f	0.52 ^e	15.41 ^f	9.50 ^f
9.	‘Šampion’ 3 rd time point	24.13 ^e	18.75 ^e	0.72 ^d	22.88 ^e	13.25 ^{ef}

The highest concentration of tannins was found in the ‘Rasna’ green husks harvested at the first experimental time point (63.18 mg GAE/g FW), whereas the lowest tannin concentration was recorded in the ‘Sava’ green husks harvested at the 3rd experimental time point (13.75 mg GAE/g FW). Chrzanowski et al. (2010) report a total tannin content of 237.4 mg GAE/g FW in walnut green husks, which is much higher than our results probably due to different harvest timings.

According to Wianowska et al. (2016), the flavonoid content of walnuts ranges from 0.280 mg QE/g FW to 2.570 mg QE/g FW depending on the cultivar/seedling. The highest concentration of flavonoids was found in the ‘Šampion’ green husks harvested at the 1st experimental time point (4.15 mg QE/g FW), whereas the ‘Rasna’ green husks harvested at the 3rd experimental time point were found to have the lowest concentration of flavonoids (0.47 mg QE/g FW).

The highest concentration of proanthocyanidins was recorded in the ‘Šampion’ green husks harvested at the 2nd experimental time point (76.38 mg of CE/g FW), i.e. in late May, and the lowest concentration of proanthocyanidins was observed in the ‘Sava’ green husks harvested at the 3rd time point (15.51 mg CE/g FW). The antioxidant activity is directly proportional to the concentration of polyphenols (Malenčić et al., 2012).

The DPPH scavenging activity was the highest in the ‘Rasna’ green husks harvested at the 1st experimental time point (88.25%) and the ‘Šampion’ green husks harvested at the 1st experimental time point (88.14%). However, the lowest DPPH scavenging activity was recorded in the ‘Sava’ green husks harvested at the 3rd experimental time point (9.50%), suggesting that walnut fruits exhibit higher DPPH activity when harvested at the beginning of ripening.

Different aqueous extracts of walnut (*J. regia* L.) green husks show antimicrobial activity and can be used as an easily affordable source of health-protective compounds (Pereira et al., 2007) and a protective system against pathogenic fungi (Ebrahimi et al., 2018). Alamprese et al. (2005) report that the antioxidant potential of walnut liqueur is directly correlated with the total content and characteristics of walnut phenols. The biochemical composition of the walnut liqueurs examined and the DPPH scavenging activity of the extracts are presented in Table 2.

Table 2. Average concentrations of total phenols, tannins, flavonoids and proanthocyanidins in the walnut liqueurs examined and the DPPH scavenging activity of the extracts

Sample	Cultivar/Selection	Phenols (mg GAE/g FW)	Tannins (mg GAE/g FW)	Flavonoids (mg QE/g FW)	Proanthocyanidins (mg CE/g FW)	DPPH (%)
1.	‘Rasna’ whole fruits	63.36 ^c	55.46 ^c	0.27 ^c	9.86 ^c	82.30 ^{ab}
2.	‘Šampion’ whole fruits	74.24 ^b	64.80 ^b	0.83^a	14.75^a	68.17 ^c
3.	‘Sava’ whole fruits	65.55 ^c	58.00 ^{bc}	0.41 ^d	11.26 ^b	79.77 ^b
4.	‘Rasna’ cut fruits	73.45 ^b	64.62 ^b	0.64 ^b	9.64 ^c	89.94^a
5.	‘Šampion’ cut fruits	83.28^a	71.54^a	0.51 ^c	11.58 ^b	82.52 ^{ab}
6.	‘Sava’ cut fruits	72.46 ^b	64.34 ^b	0.88^a	9.82 ^c	77.86 ^b

The highest phenolic content was found in the liqueur made from the cut ‘Šampion’ walnuts (83.28 mg GAE/g FW), whereas the lowest phenolic content was recorded in the liqueur made from the whole ‘Rasna’ walnuts (63.36 mg GAE/g FW). The decisive effect of walnut cultivars on the phenolic content of walnut liqueurs was also argued by Jakopic et al. (2007), who determined higher phenolic concentrations in the liqueur prepared from the ‘Franquette’ cultivar than in that made from the ‘Elit’ cultivar. Jakopič et al. (2009) recorded changes in the phenolic content of walnuts in different solvents. In methanol, a phenolic content of 161.07 mg GAE/g FW was recorded in the ‘Franquette’ cultivar, compared to 148.98 mg GAE/g FW recorded in the ‘Elit’ cultivar. In ethanol, a phenolic content of 135.27 mg GAE/g FW was recorded in the ‘Franquette’ cultivar, compared to 126.20 mg GAE/g FW observed in the ‘Elit’ cultivar.

In the present research, the highest tannin content was found in the liqueur made from the whole ‘Šampion’ walnuts (71.54 mg GAE/100 g of dry material), whereas the lowest tannin content was recorded in the liqueur made from the whole ‘Rasna’ fruits (55.46 mg GAE/100 g of dry material). The highest flavonoid content was found in the liqueur made from the cut ‘Sava’ walnuts (0.88 mg QE/g FW) and whole ‘Šampion’ walnuts (0.83 mg QE/g FW), whereas the liqueur made from the whole ‘Rasna’ walnuts had the lowest flavonoid content (0.27 mg QE/g FW). The concentration of proanthocyanidins was the highest in the liqueur made from the whole ‘Šampion’ walnuts (14.75 mg CE/g FW), whereas the lowest proanthocyanidin concentration was found in the liqueur made from the whole ‘Rasna’ walnuts (9.86 mg CE/g FW), the cut ‘Rasna’ walnuts (9.64 mg CE/g FW) and the whole ‘Sava’ walnuts (9.82 mg CE/g FW).

The DPPH scavenging activity was the highest in the ‘Rasna’ selection (89.94%) and the lowest in the ‘Šampion’ cultivar (68.17%). Shah et al. (2015) argue that methanolic walnut extracts contain higher phenolic contents (94.39 ± 5.63 mg GAE/gFW) than aqueous walnut extracts (27.92 ± 1.40 mg GAE/gFW). Furthermore, methanolic walnut extracts exhibit the highest antioxidant activity (EC₅₀ of 0.250 mg/ml), followed by water extracts (EC₅₀ of 0.325 mg/ml) in the Fenton’s reaction (0.199 ± 0.023 and 2.991 ± 0.740 , respectively).

CONCLUSION

The results obtained indicate that young walnut fruits are an important source of polyphenols (namely proanthocyanidine, tannin and flavonoid) and thus can be considered a functional food, especially in the form of liqueurs and other beverages. Concentrations of tannins, flavonoids, proanthocyanidines and phenols were found to be the highest in walnut liqueurs, particularly in the one made from walnut halves. However, the antioxidant activity and polyphenolic content of walnuts were found to decrease with their growth and development.

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