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INFLUENCE OF PREPARING METHOD ON ANTIOXIDANT ACTIVITY AND POLYPHENOLS CONTENT OF GREEN WALNUTS COMFITURE

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ABSTRACT. *The present study reports on the proportion of endocarp, mesocarp and exocarp in green walnuts and on the total phenolic content and antioxidant activity of these fractions and of whole green walnuts (Juglans regia L.). The total phenolic content was determined by the Folin–Ciocalteu method and the antioxidant activity was assessed by the ability to quench the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH). The highest total phenolic content was recorded in endocarp, followed by exocarp and mesocarp, while the antioxidant activity of these fractions kept the same order. Traditionally the comfitures are made only from endocarp but this procedure removes mesocarp and exocarp which become wastes. In order to recover these valuable fractions, in this study, besides the comfiture made only from endocarp (V1), a comfiture from green walnuts without exocarp (V2) and another one from whole green walnuts were made and analysed in terms of sensorial properties, content of polyphenols and antioxidant activity. The comfiture made only from endocarp was superior to the other variants in terms of phenolics content and antioxidant activity. However the comfiture made from green walnuts without exocarp proved to be acceptable in terms of sensory features, had high phenolic content and antioxidant activity and allowed reducing waste from 48.84% to 16.52%.*

KEYWORDS: *green walnuts, endocarp, mesocarp, exocarp, comfiture, phenolics content, antioxidant activity*

INTRODUCTION

Walnut (*Juglans regia* L.) belongs to the *Juglandaceae* family and provides excellent antioxidant compounds and highly effective agents for disease prevention (Anderson et al. 2001; Bloomhoff et al. 2006; Samaranayaka et al. 2008; Carvalho et al. 2010; Cosmulescu et al. 2010). After testing many foods, Halvorsen et al. (2006) concluded that walnuts ranks second to blueberries in terms of antioxidant content. Different parts of walnut have been used as natural remedies in folk medicine for their antidiarrheal, antihelminthic, antiseptic and astringent properties. Numerous studies have demonstrated the antioxidant potential of walnut products, ie nuts (green or dry) and green walnuts mesocarp (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). According to studies conducted by Rahimipanah et al. (2010), green walnuts mesocarp is a valuable source of phenolic compounds and can be used as an alternative natural antioxidant in the food industry. Several studies suggest that regular consumption of walnuts may have beneficial effects against oxidative stress mediated diseases such as cardiovascular disease and cancer (Koren et al. 2001; Shimoda et al. 2009). Walnut fruits are rich in phenolic compounds (Prasad 2003). Thirty-seven compounds have been isolated from walnut extracts and their structures have been characterized (Fukuda et al. 2006). Stampar et al. (2006) identified thirteen phenolic compounds in green walnuts mesocarp: chlorogenic, caffeic, ferulic, sinapic, gallic, ellagic, protocatechuic, syringic, vanillic acids, catechin, epicatechin, myricetin and juglone. The content of phenolic compounds depends on environmental conditions, genotype and stage of development of the nuts (Solar et al. 2006) as well as geographical location or climatic conditions (Amaral et al. 2008). Regarding the seasonal variation in the content of phenolic compounds, the highest content was determined in May and July (Amaral et al., 2004). Oliveira et al. (2008) showed that green walnuts mesocarp is an important source for obtaining compounds with protective action for health, having high antimicrobial potential. Verardo et al. (2009) showed that walnuts have a total phenolic content greater than most foods.

Phenolic compounds have a major contribution to the antioxidant activity because they are effective hydrogen donors (Banerjee et al. 2005). Phenolic acids, flavonoids and naphtoquinones are the main phenolic compounds of walnuts and walnut leaves. A particular constituent of walnuts, unique, is juglone (5-hydroxy-1,4-napthoquinone), a chemical compound released by walnut which can be toxic at different levels to many plant species. Juglone is present in considerable amounts in all green parts of walnut (Cosmulescu et al. 2011) and in walnut mesocarp while the content of juglone in endocarp is very low or absent (Jakopic et al. 2008). Colarič et al. (2005) confirmed that green walnuts are rich in phenolic compounds and juglone is a well known component of the mesocarp, its content being significantly higher than the content of other phenolic compounds (Solar et al. 2006). In addition to making nocino, an Italian liqueur, fresh green walnuts can also be used to make delicious comfitures.

Preserved in syrup, green fresh walnuts have a delicious flavor and an exquisite firm texture. For preparing comfitures green walnuts with unformed kernels are peeled of mesocarp and exocarp that is until it begins to form the kernel which is white and soft. This procedure removes mesocarp and exocarp which become wastes. Therefore, the second objective of this work was to study the possibility of recovery of these fractions in preserves. To do this, there were proposed and carried three variants of manufacturing green walnuts comfiture which were analysed in terms of sensorial properties, content of phenolic compounds and antioxidant capacity.

MATERIALS AND METHODS

Walnuts of 'Sibisel 44' cultivar were harvested in green stage, before the hardening of the endocarp, in the second half of June 2013 from the experimental walnut orchard belonging to the Research Station of University of Craiova (44°20′N, 23°49′E). Green walnuts were randomly collected, put into paper bags and transported to the laboratory where they were evaluated by physicochemical analysis. Experiments were performed in triplicate and the results were expressed as means ± standard deviations.

Study regarding the proportion of endocarp, mesocarp and exocarp of green walnuts

60 green walnuts were taken into consideration, which were accurately weighed. A stainless steel knife was used to remove the exocarp, then the mesocarp, which were collected separately as well as the endocarp of the green walnuts. Each fraction was accurately weighed and the results were reported to the total mass of walnuts to get the corresponding proportion of endocarp, mesocarp and exocarp respectively. Each of these fractions has been characterized in terms of total phenolic content and antioxidant capacity.

Variants of manufacturing the comfiture from green walnuts

Green walnuts comfiture was made in three experimental variants according to Table 1.

Table 1. Experimental variants of manufacturing green walnuts comfiture.

Variants	Description
V1	Green walnuts comfiture, made only from endocarp
V2	Comfiture made from green walnuts which were peeled of exocarp (endocarp + mesocarp)
V3	Whole green walnuts comfiture (made from endocarp + mezocarp + exocarp)

Total phenolics content

Total phenolic content was assessed colorimetrically by using the Folin-Ciocalteu phenol reagent method (Singleton & Rossi 1965). Folin Ciocalteu reagent (2N, Merk), gallic acid (99% purity, Sigma), anhydrous sodium carbonate (99% purity, Sigma) and methanol were used in this experiment. Samples (3 g) were extracted with 5 mL methanol in an ultrasonic bath for 45 min at ambient temperature. After extraction, the samples were centrifuged for 5 min at 4200 rpm. Supernatants were filtered through polyamide membranes with pore diameter of 0.45 μm and stored at –20°C. 1 mL of each methanolic extract (diluted 1:20 with methanol) was mixed with 1 mL distilled water and 500 μL Folin-Ciocalteau reagent and stirred for one minute. After 2 min, 4 mL of 7.5% sodium carbonate aqueous solution were added and incubated for 2 hours at the room temperature (25 ^{o}C). The same procedure was also applied to the standard solutions of gallic acid. The absorbance of the mixture was measured at 765 nm using an Evolution 600 UV/VIS spectrophotometer (Thermo Scientific, USA). Gallic acid was used to prepare the standard curve (0-250 mg/L). Results were expressed as mg of gallic acid equivalents (GAE)/100 g FW.

Antioxidant activity

Relative antioxidant activity was measured in methanol extracts of whole walnuts, endocarp, mesocarp, exocarp and comfiture samples using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Methanol (Merck, Germany), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Merck, Germany) were employed. The extraction of samples was made according to the same protocol as described for total phenolic content. The free radical scavenging ability of the extracts against DPPH free radical was evaluated as described by Oliveira et al. (2008), with some modifications. Briefly, each methanol extract (50 µL) was mixed with 3 mL of a 0.004% (v/v) DPPH methanolic solution. The mixture was incubated for 30 min at room temperature in the dark and the absorbance was measured at 517 nm on Evolution 600 UV/VIS spectrophotometer (Thermo Scientific, USA). The DPPH free radical scavenging ability was subsequently calculated with respect to the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which was used as a standard reference to convert the inhibition capability of each extract solution to the mmol Trolox equivalent antioxidant activity (TEAC)/L. The radical was freshly prepared and protected from the light. A blank control of methanol/water mixture was run in each assay. Results were expressed in mmol Trolox/100 g FW.

Statistical Analysis

Experimental data were submitted to one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI software (StatPoint Technologies, Warrenton, VA, USA). All data were presented as mean values of three replicates ± standard deviation. Multiple sample comparison was also performed using LSD multiple range test at $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic content and antioxidant activity of endocarp, mesocarp, exocarp and whole green walnuts

Table 2 presents the results on the proportion of endocarp, mesocarp and exocarp in green walnuts, total phenolic content and antioxidant activity of green walnuts and their fractions. It can be noted that endocarp represents little over 50% by weight of the whole green walnuts, while the mesocarp and exocarp fractions, which usually constitute wastes, have a very high proportion, almost 50% of the mass of green walnuts.

Table 2. Total phenolic content and antioxidant activity of whole green walnuts and of their fractions.

No. crt.	Fraction	Proportion (%)	Total phenolic content (mg GAE/100 g)	Antioxidant activity (mmol Trolox/100 g)
1	Endocarp	51.16 ± 1.48	848.05 ± 35.61^{c}	5.48 ± 0.25^{d}
2	Mesocarp	32.32 ± 2.84	569.89 ± 21.65^{a}	2.64 ± 0.18^{a}
3	Exocarp	16.52 ± 3.47	783.19 ± 32.11^{b}	3.52 ± 0.21^{b}
4	Whole green walnut	100	744.23 ± 34.53^{b}	4.56 ± 0.30^{c}

∗Data expressed as average values ± SD of three samples analyzed separately. Different superscript letters within the same column indicate significant differences ($P < 0.05$) among samples

The results presented in Table 2 showed that the highest content of polyphenols was recorded in endocarp, followed by exocarp and mesocarp. Because of this, total phenolic content of whole green walnuts was lower than that of endocarp. The antioxidant activity of the fractions analyzed kept the same order. Besides, the antioxidant activity exhibited good correlation with the total phenolic content, the correlation coefficient between them being R^2 = 0.84. The high values of total phenolic content and antioxidant activity of mesocarp and exocarp show that these fractions are valuable in terms of functional health benefits, as numerous studies have shown actually. Based on these results, in this work we intended to try their capitalization in preserves, avoiding their establishment as wastes.

Preparing comfiture in the three experimental variants

The comfitures were made according to the following recipes and specific technologies: raw material corresponding to the three variants (500 g per batch) was initially submerged in water with slices of lemon and left overnight under refrigeration. The next day, the walnuts were scalded for 10 minutes, and then cooled by washing with cold water. 800 g of sugar and 500 ml of water were brought to boil in order to prepare the syrup in which 500 g of raw material were introduced. These were subjected to an intermittent boiling (15 min of boiling followed by 2 hours of break) until the samples reached the consistency of the comfiture and a soluble solid content of 70%. The total boiling time was 45 minutes (three rounds of boiling) for the comfiture made of exocarp and 75 minutes (five rounds of boiling) for the comfitures made in variants V2 and V3.

Figure 1. The appearance of the finished product in the three experimental variant: a - V1, b - V2, c - V3.

Figure 1 (a-c) presents the appearance of the finished product in the three experimental variants (a - V1, b - V2, c - V3). The comfiture made only from endocarp (V1) was superior in terms of overall appearance, color, transparency and sweetness, which was accompanied by a specific flavor of green walnuts. At the comfiture made from endocarp and mesocarp (V2), the colour of green walnuts and syrup turn in a dark mahogany brown (figure 1), the syrup had a lower transparency and the sweetness was accompanied by a slightly bitter taste and a more pronounced aroma of green walnuts that partially masked the sweet sensation. Its taste and aroma make it favorite to people who do not like intense sweetness.
The comfiture made from whole green walnuts (V3) was very dark, almost opaque, and fragments of exocarp detached from the fruit during boiling can be observed both in syrup and on the fruits surface (figure 1). The presence of these exocarp fragments could be considered a product deficiency. In terms of taste, the comfiture was quite bitter while the flavor of green walnuts was strong and predominant.

Total phenolic content and antioxidant activity of fruit and syrup from comfitures made in the three experimental variants

Data presented in Table 3 show that green walnuts comfiture made only from endocarp was superior to the other variants in terms of phenolics content and antioxidant activity. With slightly lower values of phenolic content in fruits but higher in syrup and with an antioxidant activity which is not significantly different from that of variant V1, it was noticed variant V3 made from whole green walnuts.

However, the presence of the exocarp fragments both in syrup and at the fruits surface downgrades this comfiture. To these are added the bitter taste overly pronounced and the stronger green walnut flavor. An interesting variant of manufacturing which deserves to be considered is V2, the comfiture made from green walnut endocarp with adherent mesocarp which presented high values of phenolic content and antioxidant activity. This had a slightly bitter taste and an intense green walnut flavor, which makes it acceptable in terms of sensory properties and even preferable for people who do not like intense sweetness. Note that the adoption of this

Table 3. Total phenolic content and antioxidant activity of the green walnuts comfiture made in the three experimental variants.

Experimental variants	Total phenolic content (mg GAE/100 g)	Antioxidant activity (mmol Trolox/100 g)
Fruits		
V1	1249.64 ± 43.71^{b}	7.88 ± 0.33^{b}
V2	1144.58 ± 46.92^{a}	7.16 ± 0.41^{a}
V3	1210.348 ± 54.46^{ab}	7.64 ± 0.31^{ab}
Syrup		
V1	380.056 ± 3.26^{b}	2.64 ± 0.18^{b}
V2	284.584 ± 13.09^{a}	2.06 ± 0.09^{a}
V3	428.488 ± 17.58^{c}	2.68 ± 0.13^{b}

∗Data expressed as average values ± SD of three samples analyzed separately. Different superscript letters within the same column indicate significant differences ($P < 0.05$) among variants

variant of manufacturing allows the capitalizing of green walnut in proportion of over 80% (83.75% in the case of the raw material considered for this study) compared with a recovery of about 50% of the raw material for the manufacturing of the comfiture made only from endocarp, which means a reduction of wastes from about 50% to 15-20%.

CONCLUSIONS

Green walnuts endocarp proved to be the richest in phenolic compounds and exhibited the highest antioxidant activity, followed by exocarp and mesocarp. In the manufacture of green walnuts comfiture only from endocarp, the mesocarp and exocarp fractions constitute waste and represent almost 50% of the mass of green walnuts.

Due to their high content of phenolic compounds we proposed their recovery by making comfiture assortments which could include these fractions.

The comfiture made only from endocarp was superior to that made from endocarp with mesocarp in terms of overall appearance, color, transparency and sweetness, the latter being darker in color, with lower transparency. However, the slightly bitter taste and the more pronounced aroma of green walnuts are features which make it favorite to people who do not like intense sweetness. Preparing comfiture in this manner allows the capitalizing of green walnut in proportion of over 80% compared with about 50% at the manufacturing of the comfiture made only from endocarp, and an important reduction of wastes.

The comfiture prepared from whole green walnuts was very dark, almost opaque, and presented fragments of exocarp detached from the fruits during boiling, both in syrup and on the surface of the fruits. Besides these, the comfiture was quite bitter and the flavor of green walnuts was predominant. Nevertheless, this product presented high phenolic content in fruits and syrup and an antioxidant activity not significantly different from that of the comfiture made only from endocarp.

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