

THE EFFICACY OF WALNUT (*JUGLANS REGIA* L.) BY-PRODUCT EXTRACT AS A FREE RADICAL SCAVENGER

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Abstract: Walnut leaves and green husk are agro-forest wastes generated in the walnut (*Juglans regia* L.) harvest that could be valued as a source of natural compounds with antioxidant properties. The free radical scavenging activity of studied extracts of walnut leaves and green husk was evaluated by employing antioxidant assay system. Phenolic compounds are the major contributors to the biological properties like antioxidant activities of walnut leaves and green husk. The total phenols content of the extracts was determined using the Folin-Ciocalteu method to assess their contribution to the antioxidant activity. Extract antioxidant activity as well as the kinetics of inhibition of free radicals were evaluated in terms of radical scavenging ability using the stable 2,2 diphenyl-1-picrylhydrazyl radical (DPPH[•]). The results of the present study suggest that walnut leaves and green husk extracts, a byproduct of walnut processing industry, can be used as an economical source of natural antioxidants for food, cosmetic and pharmaceutical industries.

Key words: walnut leaves, walnut green husk, solid-liquid extraction, total phenols content, DPPH antioxidant activity.

I. Introduction

Walnut is a crop of high economic interest to the food industry: the edible part of the fruit (the seed or kernel) is consumed, fresh or toasted, alone or in other edible products. It is globally popular and valued for its nutritional, health and sensory attributes. The fresh natural kernels are consumed mainly as whole nuts or used in various confectioneries. They are a nutrient-dense food mainly owing to their fat content and protein, vitamin and mineral profiles. Nowadays, there is an increasing interest in the study and processing of walnut by-products (leaves, green husks, membrane septum) [1].

Walnut leaves and green husks are considered a source of healthcare compounds, and have been widely used in traditional medicine. In some European countries, especially in rural areas, dry walnut leaves are frequently used to prepare infusions for their antiradical and antibacterial properties. The antioxidant compounds from walnut by-products could be used for protecting the oxidative damage in living systems by scavenging oxygen free radicals, and also for increasing the stability of foods by preventing lipid peroxidation [3, 4].

Special attention is focused on the extraction of bioactive compounds from walnut by-products, using different solvents. The extraction constitutes an important step in the manufacture of phytochemical-rich products. The application of this low-cost technology to obtain molecules to be used as food additives or nutraceutical products is an appropriate strategy for the exploitation of by-products such as the walnut leaves and green husks [5].

In this paper, the extraction of phenolic compounds from walnut leaves and green husks was optimized by applying Soxhlet installation. This method has been proven to be desirable for phenolics extraction and has many advantages such as increasing extraction yield, shortening extraction duration. Evaluation of the influence of solvent in the solid-liquid extraction process was measured by total polyphenol content and DPPH antioxidant activity. Finally, the optimized conditions were validated. Solid-liquid extraction was preceded by the selection of the best solvent for obtaining phenolics-enriched extracts of walnut leaves and green husks.

II. Materials and methods

2.1. Materials

Walnut (*Juglans regia* L.) leaves and green husks were collected during summer, July 2011, in Chisinau, Central Moldova. Fresh and healthy leaves and green husks were manually collected from the middle third of branches exposed to sunlight. The leaves and green husks were dried at room temperature and packed in paper bags in order to protect them from light. Voucher specimens were preserved in our laboratory for further reference.

2.2. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH[•]) as free radical form (95% purity), Folin-Ciocalteu's phenolic reagent (FCR), sodium carbonate were supplied by Sigma-Aldrich. 3,4,5-trihydroxybenzoic acid (gallic acid) were obtained from Alfa Aesar. Methanol (99,8%) and ethanol (99,9%) were provided by Eco-Chimie (Chisinau, Moldova). All the chemicals used were of HPLC or analytical grade.

2.3. Extraction procedure

Dried walnut leaves and green husks were grounded before extraction. The dried powder of leaves and green husks was extracted with water, ethanol and their mixtures at different concentrations from 0% to 100% EtOH for 2 h at 60°C and solid to liquid ratio 1 g per 10 ml of solvent. The extracts of walnut leaves and green husks were filtered with paper filter and after were used immediately in the experiments. Obtained extracts were analyzed for the total phenols content and antioxidant activity DPPH assays. For each extract sample analysis were carried out in triplicate.

2.4. Total phenols content measurement

For quantification of total phenols content, the Folin-Ciocalteu's method was used [8]. A volume of 0.5 ml of Folin-Ciocalteu's reagent was added to a dark flask, containing 0.5 ml of the each extract sample and 10 ml of distilled water. After 5 min, 8 ml of a 7.5% aqueous sodium carbonate solution was added to the mixture and the content was mixed thoroughly. The samples were kept in dark for 2h and then the absorbance was measured at 765 nm with HACH LANGE DR-5000 UV/vis spectrophotometer. Three parallel samples were analyzed. Gallic acid was used for constructing the standard curve. Concentration range of gallic acid was of 0.05-0.5 mg/ml. The results of total polyphenol content were expressed as mg of gallic acid equivalents per ml of extract (mg GAE/ml).

2.5. Determination of DPPH radical scavenging activity

The antioxidant activity of walnut leaves and green husks extracts as well as the kinetics of inhibition of free radicals were studied in terms of radical scavenging ability using the stable DPPH[•] method [2]. 0.1 ml of the extract sample was added to 3.9 ml of 60 μM solution of DPPH[•] in methanol. The reaction was carried in dark and the absorbance was recorded at 515 nm to determine the concentration of remaining DPPH[•]. Methanol as instead of DPPH[•] solution was used as blank solution. The values of [DPPH[•]]_t at each reaction time were calculated according to the calibration curve (in the concentration range of 0.38-38 μg/ml): $A_{515\text{ nm}} = 0.0293 [\text{DPPH}^{\bullet}]_t - 0.0072$, where the concentration [DPPH[•]]_t is expressed in μg/ml. The coefficient of linear correlation of the above relation is $R = 0.9999$. The radical scavenging activity (RSA) was calculated using the equation [6]:

$$\text{RSA} = 100\% \cdot ([\text{DPPH}^{\bullet}]_0 - [\text{DPPH}^{\bullet}]_{20}) / [\text{DPPH}^{\bullet}]_0$$

where [DPPH[•]]₀ is the concentration of the DPPH[•] solution (without sample) at t=0 min and [DPPH[•]]₂₀ is the remained DPPH[•] concentration at t=30 min. Lower [DPPH[•]]_t in the reaction mixture indicates higher free radical scavenging activity.

2.6. Statistical analysis

Variance analysis of the results was carried out by least square method with application of coefficient Student and Microsoft Office Excel program version 2007. Differences were considered statistically significant if probability was greater than 95% (p-value <0.05). All assays were performed by triplicate at room temperature 20 ± 1 °C. Experimental results are expressed as average \pm SD (standard deviation).

III. Results and discussion

Several studies on the extraction of phenolic compounds from different cultivars of walnut leaves and green husks have been published. Knowledge of the behavior of the factors influencing the process conditions is necessary to enhance the optimization extraction efficient for any bioactive compound. Previous findings have reported the influence of many independent variables, such as solvent composition, pH, temperature, extraction time, and solid to liquid ratio, on the yields of bioactive compounds which can be extracted from diverse natural products. The positive or negative role of each factor in the mass transfer of the process is not always clear; the chemical characteristics of the solvent and the diverse structures and compositions of the natural products mean that each material-solvent system has a different behavior, which cannot be predicted [1, 7].

Different solvents have been used for the extraction of phenols compounds from plant material. The extraction method must allow extraction of the principal compounds of interest, and it must avoid their chemical modification. The polarity of the solvent plays an important role in the selective extraction. Water and aqueous mixtures of ethanol, methanol, and acetone are commonly used in the plant extraction.

In a recent study was demonstrated the influence of solvent on the solid-liquid extraction of phenolic compounds from the leaves and green husks of *Juglans regia* L. However, to the best of our knowledge, there are small reports in literature on optimization of solvent extraction of these compounds from the walnut leaves and green husks. It was investigated the influence of water/ethanol solvent mixtures at different concentrations on the level of total phenols content of extracts from walnut leaves and green husks. Obtained experimental data are demonstrated in figure 1.

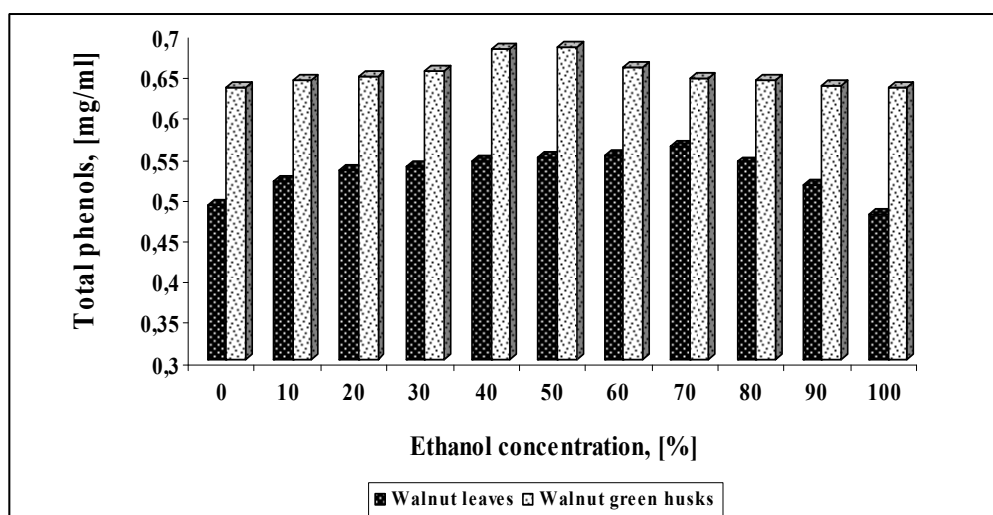


Figure 1. Dependence of solvent type on the level of phenols compounds extraction from walnut leaves and green husks.

During this study, there were also analyzed the UV/Vis spectra of the extracts from walnut leaves and green husks in the wavelength range 270 - 1100 nm. The spectrum of the extracts display strong peaks, typical for phenolic compounds at 400 and 450 nm. The walnut leaves contain chlorophyll, so this pigment was also extracted with ethanol containing solvent. The typical chlorophyll band at 670 nm is of different intensity depending of the ethanol concentration in a solvent.

The antioxidant activity analysis was performed with the same extracts from walnut leaves and green husks. The antioxidant activity value of tested extracts was expressed as radical scavenging activity and this parameter was in the range of 54.25 – 83.31 % for walnut leaves extracts. In this study the antioxidant capacity of the walnut leaves and green husks extracts were analyzed as the kinetics of inhibition of free radicals (the percentage of DPPH' remaining at steady state). Experimental results are shown in figures 2 and 3.

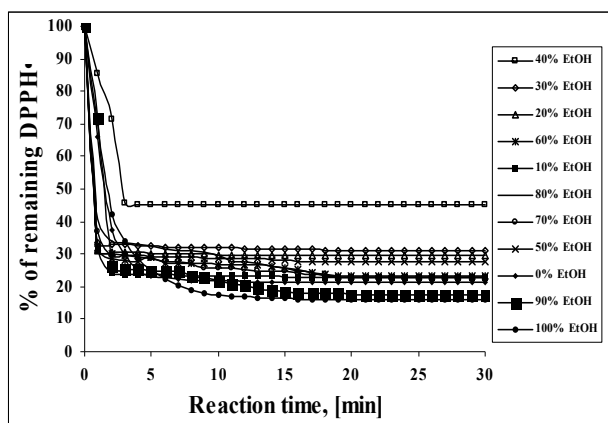


Figure 2. Reaction kinetics of DPPH[•] with extracts from walnut leaves

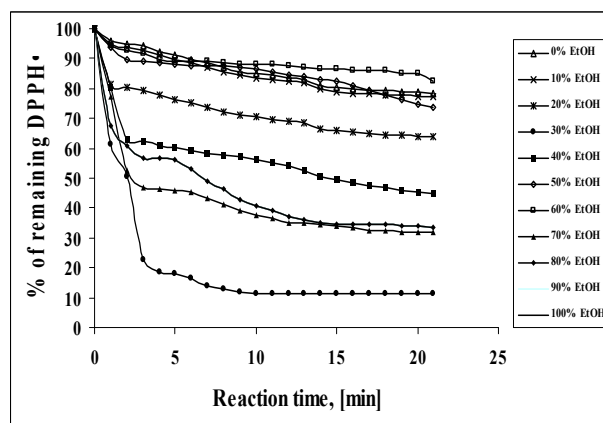


Figure 3. Reaction kinetics of DPPH[•] with extracts from walnut green husks

It is well known that the absorbance decreases as a result of a colour change from purple to yellow when the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H molecule. A more rapid decrease of the absorbance means more potent antiradical activity, expressed in terms of hydrogen donating ability of the compounds. The DPPH[•] scavenging capacity of biologically active compounds is mostly related to their phenolic hydroxyl groups.

IV. Conclusion

In this study water and ethanol, two environmentally and food safe solvents were used to optimize solid-liquid extraction of phenolic compounds from walnut leaves and green husks. Antioxidant activity of walnut leaves and green husks extracts was evaluated by measuring of the free radical scavenging capacities of the extracts using stable DPPH[•] and total phenols content using Folin-Ciocalteu reagent. It was established that optimal solvent for antioxidant extraction from walnut leaves is 70% and from walnut green husks is 50% mixture of water and ethanol, respectively. Walnut leaves and green husk extracts obtained by Soxhlete extraction possess considerable amounts of phenols compounds and a significant radical scavenging activity towards stable DPPH free radical.

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