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Original scientific paper

Influence of enzyme-aided extraction and ultrasonication on the phenolics content and antioxidant activity of *Paeonia officinalis* L. petals

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Abstract: Peony is a less utilized herbaceous plant of ornamental and medicinal relevance. The petals are rich in bioactive phenolics and hence, the development of efficient extraction is required for maximum recovery and bioactivity. In this study, the optimal conditions for the ultrasound-assisted extraction of peony phenolics were first investigated by comparing to conventional extraction and testing the data fit by mathematical models (linear, two factor interactions, quadratic) followed by investigation of cellulase pre-treatment combined with ultrasonication. High amounts of flavonoids, phenolics and tannins were extracted in 30 min under ultrasound conditions of 150 W, 40 kHz, 50 % amplitude, in 70 % ethanol with 50/1 solvent/solid ratio. A further cellulase pre-treatment at 40 °C for different incubation times combined with ultrasonication facilitated the extraction of anthocyanins (720.48 mg/100 g DW), phenolics (3985.50 mg/100 g DW), flavonoids (2369.28 mg/100 g DW) and tannins (8917.81 mg/100 g DW). Good antioxidant activities by FRAP (350.03 mg ascorbic acid/100 g DW) and DPPH (inhibition values >80 %) were registered showing great potential for developing high-value bioingredients. The FTIR analysis revealed the presence of characteristic functional groups. The hereby-combined extraction methods could be extended to other peony species.

Keywords: cellulose; DPPH; FRAP; peony; polyphenols; ultrasounds-assisted extraction.

INTRODUCTION

Scientific evidence regarding the health benefits of countless phytochemicals has stimulated globally an increased consumption of natural products. Among phytochemicals, polyphenols of intermediate molecular weight (flavonoids, anthocyanins) and high molecular weight (hydrolysable or condensed tannins) have

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been intensively studied because of their remarkable biological activities and uses.[1–3] Medicinal, aromatic and culinary herbs are rich sources of such compounds, but flowers of ornamental use may contain higher amounts of bioactive compounds, thus becoming potential sustainable sources of bioingredients.

Among the most wide-spread herbaceous peony species (*Paeonia officinalis* L., *Paeonia lactiflora* Pall. and *Paeonia tenuifolia* L.), *P. officinalis* L. has been less studied from the standpoint of content of antioxidant compounds in the petals, extracted through different strategies, and of their bioactivities. Common peony (*P. officinalis* L.) is native to south-eastern Europe, being widely cultivated as a garden plant, but also used for medicinal purposes.[4] In particular, the root extracts have been used in Indian and Chinese medicine for a long time based on their pharmacological properties, such as neuroprotection, antihypertensive and anti-ulcer.[4] Ethanol extracts of *P. officinalis* L. petals showed antioxidant and antibacterial activities on *Staphylococcus aureus* and *Escherichia coli*.[5–6] There are reports on some food uses of peony petals of the red varieties, mainly as a tea infusion containing less than 33 % peonies in its composition, acting as adjuvant for several disorders (arthritis, asthmatic cough, chest pain) and as a textile dye.[7] The main anthocyanins detected in herbaceous peony petals are peonidin-3,5-di-*O*-glucoside, peonidin-3-*O*-glucoside-5-*O*-arabinoside, peonidin-3-*O*-glucoside, pelargonidin-3,5-di-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside and cyanidin-3-*O*-glucoside.[8]

Although there are vast studies on chromatic attributes of fresh *Paeonia* petals,[8] there are few reports on extraction optimization and quantitative analysis of phenolics from *P. officinalis* red petals. Efficient extraction technologies are required to maximize the recovery of such phyto-molecules. Hydrosoluble antioxidants of polyphenolic structure are efficiently extracted using polar solvents, under conventional or non-conventional procedures. Among the latter, ultrasound-assisted extraction (UAE) has been successfully applied to various materials, evolving as an energy saving method through optimization of different parameters.[9] Recently, enzyme-aided extraction has emerged as a promising tool that provides enhanced recovery of bioactive compounds by facilitating their release through disruption of plant cell walls.[10] Enzymes such as cellulases, pectinases or hemicellulases have frequently been investigated. The extraction yield strongly depends on the starting plant material, the compound of interest, type of enzyme and its preparation, and the subsequent extraction method that followed the enzymatic pre-treatment. Considering these, testing of the efficiency of several extraction technologies for bioactive compounds from peony petals is required as the literature is scarce on the optimization of such procedures for *Paeonia* spp.

This work was performed to develop a combined enzyme pulsed UAE procedure for high recovery of the phenolics from common peony red petals. The following antioxidant compounds flavonoids, anthocyanins, phenolics and con-

densed tannins were targeted. Different extraction solvents, matrix and process parameters were evaluated and mathematical models were elaborated. The total antioxidant activities of the hydroethanolic crude extracts were also evaluated.

EXPERIMENTAL

Plant material and chemical reagents

Petals of cultivated herbaceous red peony (*Paeonia officinalis* L.) were collected from the Sibiu region (Romania) and air-dried at 25 °C. The sample size was reduced by grinding using a knife mill (Grindomix GM 200, Retsch, Germany). The moisture content was determined using a moisture analyzer (MAC 210/NP Radwag, Poland). The samples were stored at −70 °C until analysis. All chemical reagents were of analytical grade.

Cellulase from *Aspergillus niger* as a powder readily soluble in water with activity >60,000 U g^{-1} (MP Biomedicals, CAS 150583) was used.

Extraction procedures

Three series of extraction experiments were performed, using conventional and non-conventional methods (UAE and enzyme-aided, Table S-1 of the Supplementary material to this paper). The first series of experiments were performed to evaluate the effect of two solvents, water and 70 % ethanol solution, which are generally recognized as safe (GRAS) according to https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices. Three ratios of solvent/solid were investigated for each type of solvent, according to several published data that reported values ranging from 16/1 to 40/1.[11,12] Three extraction temperatures were tested for each solvent and for each solvent/solid ratio. Based on the results obtained from the first experimental series, the second series were performed under UAE using the ethanol solution. The optimal conditions were tested for the following parameters: solvent/solid ratio, extraction time and ultrasonic amplitude. Starting from the UAE results that gave the highest recovery of investigated bioactive compounds, the last series of experiments were performed in order to maximize further the yield through a cellulolytic enzyme pre-treatment, previously reported as favoring compounds extraction[10] at different incubation times.

UAE was performed using an ultrasonic device (Sonifier SLPe-150, Branson, USA) of 150 W power and 40 kHz frequency, equipped with a transducer.

Regarding the enzyme-aided extraction, the pre-treatment with cellulase solution in acetate buffer pH 4.8 was applied at 40 °C. An enzyme/substrate mass ratio of 0.166 was used. The enzyme concentration was chosen according to published reports on the influence of Cellubrix (mixture of cellulase and cellobiase) pre-treatment on anthocyanins recovery from other flowers.[13] After incubation for different times, the enzymes were inactivated at 100 °C for 5 min and extraction was further performed by the optimal UAE. Control samples (without enzymatic pre-treatment) were maintained in buffer solution under similar conditions (time, *t*) and further processed by ultrasonication. The mixtures were filtered and centrifuged at 8000 rpm, at 4 °C for 10 min. An NF800R refrigerated centrifuge (Universal 320, Hettich, Germany) was used.

Determination of the total content of compounds of polyphenolic structure

The total phenolics content was determined using the Folin–Ciocalteu method.[14] A Specord 200Plus UV–Vis spectrophotometer (Analytik Jena, Germany) was used. The results are expressed in mg gallic acid equivalents GAE/100 g DW.

The total flavonoids content was determined as described.[15] The results are expressed as mg quercetin equivalents/100 g DW.

The content of condensed tannins was determined as described in the literature.[16] The results are expressed as mg catechin equivalents/100 g DW.

The total anthocyanins content was determined spectrophotometrically by the pH differential method.[17] The results are expressed as mg cyanidin 3-*O*-glucoside/100 g DW.

*Determination of ferric reducing antioxidant power (*FRAP*)*

The total antioxidant activity of the peony enzyme ultrasonic extracts was determined by the ferric reducing ability assay.[18] The results are expressed as mg ascorbic acid/100 g DW.

Determination of the radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The radical scavenging activity (*RSA*) of the peony enzyme ultrasonic extracts was determined by the DPPH assay.[19] The results are expressed as inhibition percentage calculated according to the following equation:

$$\text{Radical scavenging activity, \%} = \frac{A_0 - A}{A_0} \quad (1)$$

where A_0 is the absorbance at 515 nm of control and A is the absorbance at 515 nm of the sample.

ATR-FTIR analysis

Fourier Transform-infrared (FTIR) measurements were performed using an Alpha FTIR spectrometer (Bruker, Germany) with combined QuickSnapTM sampling modules and a ZnSe attenuated total reflection (ATR), with a resolution of 4 cm^{-1}. An average of 32 scans was recorded in the ATR mode.

Statistical analysis and mathematical modeling

Data are expressed as mean values ± *SD* of duplicate experiments. Differences between extractions variables were statistically compared using the Kruskall–Wallis one-way analysis of variance by Systat, v. 12.0.[20] Mathematical models were obtained on process variables and bioactivity content.

RESULTS AND DISCUSSION

The influence of extraction methodology and experimental parameters on the content of peony phenolic compounds

Conventional extraction of phenolic-based compounds from *Paeonia officinalis* L. red petals was performed using water and 70 % ethanol by varying matrix and the process parameters, such as solvent/solid ratio (48/1, 24/1, 12/1) and extraction temperature (30, 40, 50 °C). The extraction time was set at 120 min. The adequacy of several mathematical models was tested in support of correlations between content of compounds and the variables. According to the sequential model sum of squares, the suggested models that best fit the data were as follow: linear for total phenolics, two factor interactions (2FI) for total flavonoids and quadratic for total tannins. The 3D response plots for both investigated extraction solvents are shown in Figs. 1–3.

According to the summary statistics of the model, close matches between experimental and predicted values of the suggested models were obtained for phenolics (R^2 values of 0.9902 and 0.9845) and tannins (R^2 values of 0.9835 and 0.9382).

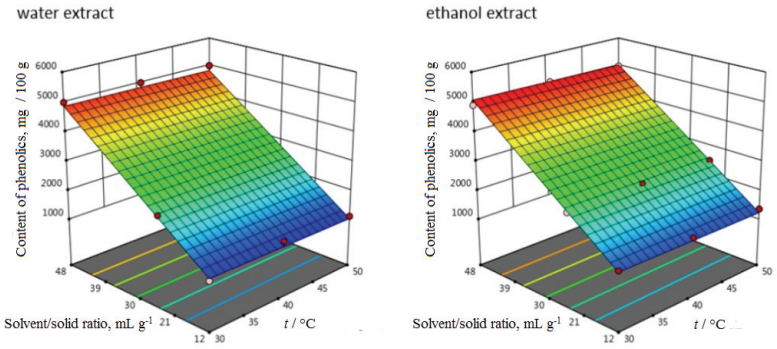

Fig. 1. Response plots of linear model for extraction efficiency of phenolics from red peony petals as a function of temperature and solvent/solid ratio.

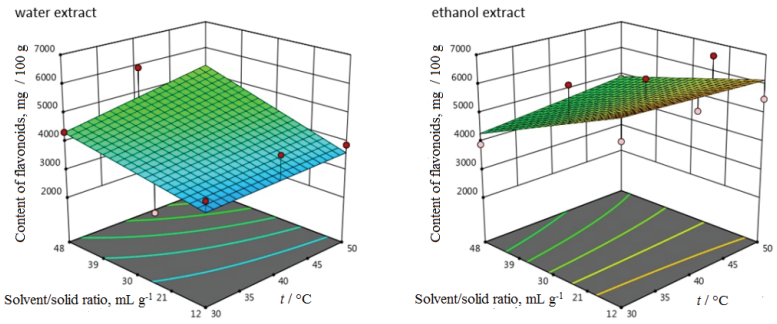

Fig. 2. Response plots of the 2FI model for the extraction efficiency of flavonoids from red peony petals as a function of temperature and solvent/solid ratio.

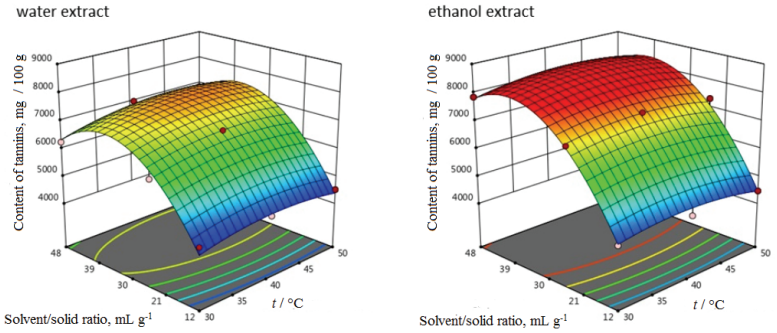

Fig. 3. Response plots of the quadratic model for the extraction efficiency of tannins from red peony petals as a function of temperature and solvent/solid ratio.

The maximum contents of phenolics, flavonoids and tannins were achieved for the highest solvent/solid ratio (48/1) at 40 °C. A high ratio provides better contact of the sample with the solvent. Despite increasing the temperature usually enhances the extraction due to high diffusion and solubility coefficients, a slight

decrease of phenolics content by 3 % was noticed at 50 °C. Such findings are consistent with previously reported ones showing degradation of polyphenols and pro-anthocyanidins occurs at temperatures above 50 °C.[21] Regarding the solvent efficiency, the results showed that 70 % aqueous ethanol improved the conventional extraction by 7.5–34 % depending on the type of compounds. Ethanol, which is less polar than water, provided a more satisfactory extraction of phenolic compounds for tested temperatures compared to water. The mixture of water and ethanol improves the extraction of compounds soluble in water and organic solvent also contributing to a weakening of the H bonds of polyphenols with proteins. The low extractability in water might be also explained by the fact that there are phenolic compounds associated with other molecules, in particular polysaccharides from the cell wall.[21] Correlation analysis by calculating the Pearson correlation coefficients showed significant positive correlations between phenolics and tannins extracted using either ethanol (r = 0.8374, P < 0.01) or water (r = 0.7263, P < 0.05).

There are few literature studies reporting the contents of total phenolics and flavonoids of common peony petals, while no studies have been identified dealing with their tannin composition. However, there are papers reporting the content of total phenolics and flavonoids of other herbaceous peony species, such as *Paeonia lactiflora* Pall. different cultivars.[22–24] Syrup prepared from the Romanian cultivated *Paeonia peregrina* Mill. var. romanica showed high amounts of phenolics (642.03 mg GAE g^{-1} DW).[25]

The release of cell wall-bound phenolics cannot be achieved through conventional extraction methods[26] and hence it was decided to increase the yield by applying non-conventional methods, UAE and enzyme-aided extraction. Using the solvent selected in the first experimental series (70 % ethanol), the second experimental series were performed using UAE under different conditions of solvent/solid ratio (40/1, 50/1), extraction time (10, 20, 30 min) and ultrasonic amplitudes (50, 70 %). A low solvent ratio is economic but may lead to decreased yield, and hence, two values situated within the interval of the previous optimum determined value were selected. The other parameters (time, amplitude) were selected based on economic efficiency – thus lower extraction times than those used in the initial experiments and medium and high operating amplitudes, which are directly related to the intensity of the cavitation created in liquids, were tested. The solvent/sample mixtures were irradiated using the pulsed mode operation that prevents a very fast temperature increase resulting degradation of heat-sensitive phyto-molecules. According to the sequential model sum of squares, the fitted models were linear for phenolics and tannins, 2FI for flavonoids. The 3D response plots for both investigated ratios are shown in FigS. S-1–S-3 of the Supplementary material.

The optimal UAE conditions for the extraction of high amounts of phenolic compounds from *P. officinalis* L. petals were determined by testing the significant differences using the Kruskall–Wallis one-way analysis of variance. Thus, significant differences resulting in high yield of phenolics were found for the 50/1 solvent/solid ratio, 30 min extraction time and 50 % ultrasonic amplitude. UAE is preferred because it shortens extraction times and uses moderate energy. To best of our knowledge, there are no reports on the use of UAE technology on polyphenolic compounds from cultivated *Paeonia officinalis* red petals.

The effect of the hybrid approach of enzyme pre-treatment and UAE on the content of peony phenolic compounds

The study was performed using a pre-treatment of petals with cellulolytic enzymes combined with direct ultrasonication at parameters that provided the highest extraction of phenolics. The cellulolytic enzymes consisted of a multi--enzyme complex of so-called C_1 enzyme that disrupts the structure of native cellulose rendering it more susceptible to the action of the other enzymes, exo-β--1,4-D-glucanases/endo-β-1,4-D-glucanases, which hydrolyze the glucosidic bonds, and β-glucosidase, which degrades small MW cellulose hydrolysates. As previously described, such treatments with complex cellulase enzymes enhance the extraction of insoluble-bound phenolics through disruption of glycosidic or –OH bonds.[27]

Three incubation times of samples with cellulase solution at pH 4.8 (60, 120, 180 min) were tested at 40 °C. Control samples without cellulase pre-treatment were also investigated. The results are presented in Fig. 4.

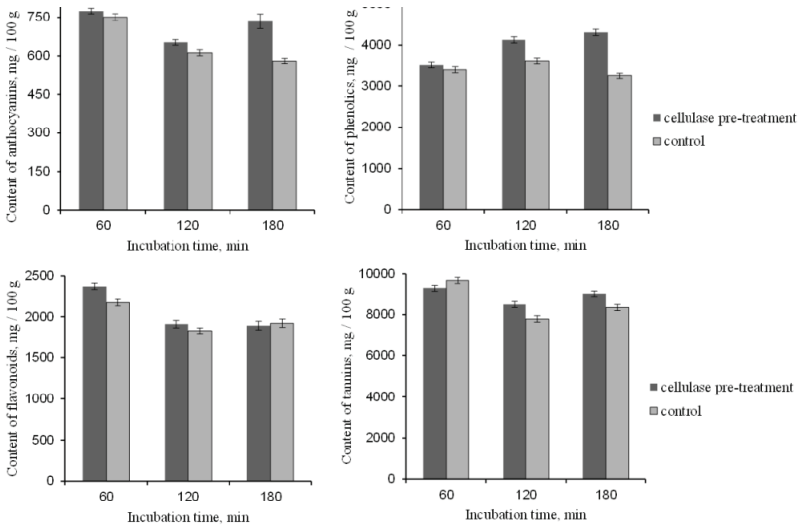

Fig. 4. The effect of incubation time on total content of bioactive compounds in enzyme pre-treated ultrasonic extracts of peony petals.

A significant release of phenolic compounds from their matrix was found in samples time-dependently incubated with cellulases, the increase in their content being of 3.6–16 %. The shortest incubation time (60 min) favored the extraction of flavonoids and tannins, while the longest incubation time (180 min) improved the extraction of phenolics. Particularly, the content of phenolics and anthocyanins increased by 16 and 11 %, respectively, due to the enzymatic release of high MW pro-anthocyanidins, which are usually associated with cell wall polysaccharides.[28] By applying this hybrid approach, the ability of the enzymes to release phenolics from peony petals was additionally accelerated by the applied low-frequency ultrasonication, known to favor the development and collapse of cavitation bubbles, and to improve the transport of enzymes to the surface of the substrate.[29] The maximum recovery of phenolics (3985.5±73.9 mg GAE/100 g DW), flavonoids (2369.3±51.6 mg quercetin/100 g DW), tannins (8917.8±167.8 mg catechin/100 g DW) and anthocyanins (720.5±20.7 mg cyanidin-3-*O*-glucoside/100 g DW) were obtained.

To best of our knowledge, no studies have been published on enzyme-assisted extraction of polyphenolic compounds from *P. officinalis* red petals. Nevertheless, the present findings confirmed previous reports on the efficient extraction of phenolics from other plant materials by the use of enzymes.[30]

Changes in the antioxidant activities of peony extracts with enzyme-aided extraction

The antioxidant activities of ultrasonic extracts of peony with and without cellulolytic enzyme pre-treatment were evaluated *in vitro* using two assays based on the measurement of ferric ion reducing capacity of antioxidants as *FRAP* and on electron transfer by DPPH. The results on antioxidant activities as *FRAP* (mg ascorbic acid/100 g DW) and DPPH (radical scavenging activity, *RSA* / %) are presented in Table I.

TABLE I. Antioxidant activities of peony extracts, according to different incubation time with cellulase at 40 °C

Type of extract	Incubation time, min					
	60	120	180	60	120	180
	FRAP / mg ascorbic acid (100 g DW)$^{-1}$			DPPH, *RSA* / %		
Enzyme ultrasonic	361.6±6.7	486.4±13.7	202.1±9.4	80.2±0.9	81.0±0.5	82.0±0.5
Control	257.2±8.9	228.5±4.2	187.8±5.6	83.6±0.8	83.6±0.4	83.5±0.8

The mean FRAP values increased by 56 % when cellulase pre-treatment was applied (350.03±12.44 mg ascorbic acid/100 g DW). A decrease in the *FRAP* values at the prolonged incubation time was registered, probably due to flavonoids/anthocyanins hydrolysis at the glucosidic bonds. In untreated samples, the correlation analysis revealed a positive good correlation between *FRAP* and

anthocyanins (R^2 = 0.9018). The correlation coefficient R^2 of *FRAP* with phenolics was 0.5198. No significant correlation was found in the treated samples. The increased *FRAP* value in the treated samples might be explained by the release of bound-phenolics and other antioxidant compounds not extracted in the untreated samples. There is no information regarding the ability of enzyme-aided extraction of phenolics from *P. officinalis* L. red petals to enhance the antioxidant activity, which was confirmed in this study, but other authors reported increased *FRAP* activity of rice bran when complex enzymatic pre-treatment was performed.[27] An extract obtained by the classic method from red petals of cultivated *P. officinalis* L. using ethanol revealed an antioxidant activity of 555.2±11.4 μmol Trolox/g FW.[6] Strong *FRAP* antioxidant activity expressed as Trolox equivalents (836.6±5.5 μmol Trolox/g DW) of aqueous extracts classically obtained from other herbaceous peony (*P. lactiflora* Pall.) has been reported by other authors.[22]

The peony crude extracts highly reduced the purple coloration of DPPH giving inhibition percentages > 80 % irrespective of the applied enzyme pre-treatment, showing significant *RSA* (Table I). No significant differences were found between control and enzyme-treated samples. The correlation analysis revealed a positive high correlation of DPPH with phenolics in both treated (R^2 = = 0.9317) and untreated samples (R^2 = 0.8248), which demonstrates the contribution of soluble phenolics to the radical scavenging activity. Similar inhibition values (87.13 %) were reported by other authors for syrup prepared from *P. peregrina* Mill. var. romanica petals,[25] while no studies have been identified dealing with enzyme-aided extracts of *P. officinalis*.

ATR-FTIR analysis

As shown in Fig. 5, the extract exhibited characteristic peaks attributed to stretching and bending vibrations in different areas, as follows.

–OH stretching (broad band of phenol –OH at 3354 cm^{-1} and C–O stretching at 1274 cm^{-1}), –CH stretching (–CH_3 at 2974 and 2928 cm^{-1}, –CH_2/ /methoxy –CH_3 at 2894 cm^{-1}), C=O stretching of aromatic compounds/flavones/hydroxyarylketones of flavonols (strong band at 1649 cm^{-1}), –CH_3 bending (1453–1327 cm^{-1}), C–O–C stretching attributed to carbohydrate residues (strong bands at 1086 and 1044 cm^{-1}), and aromatics –CH bending (strong band at 878 cm^{-1}).

The FTIR characterization of red peony petals extract, coupled with other analytical methods, may become useful for the identification of peony varieties. Similar major spectral characteristics were shown by other authors using an extract of peony petals (species not described).[31]

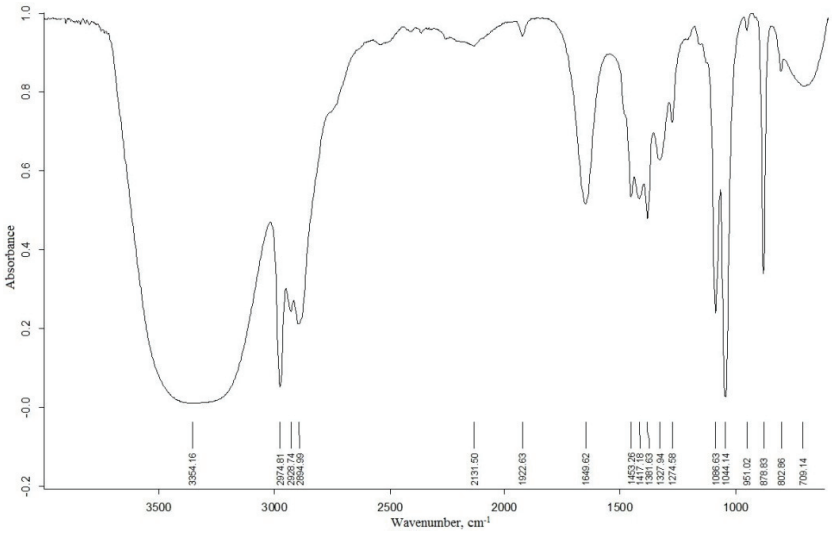

Fig. 5. ATR-FTIR spectrum of the crude peony (*Paeonia officinalis* L.) ethanol extract.

CONCLUSIONS

UAE of polyphenolic compounds from peony petals improved the efficacy and reduced the time compared to conventional solvent extraction. Optimal ultrasonic conditions for maximum recovery of phenolics, flavonoids and tannins were as follow: 50/1 solvent/solid ratio, 30 min extraction time, 50 % ultrasonic amplitude, at 150 W power and 40 kHz frequency. Cellulases pre-treatment of the raw materials followed by optimized ultrasonication further improved the extractability of phenolics.

Strong antioxidant activity in terms of ferric reducing power and radical scavenging activity was found in the enzyme ultrasonic extracts. The ATR-FTIR analysis confirmed the structural characteristics of polyphenols.

The results are encouraging, showing that enzyme ultrasonic extracts of peony petals are rich in antioxidant compounds, exhibiting great potential for the development of high-value bio-ingredients for food, textiles, cosmetics or pharmaceuticals industries.

SUPPLEMENTARY METERIAL

Additional data are available at http://www.shd.org.rs/JSCS/, or from corresponding author on request.

ИЗВОД

УТИЦАЈ ЕНЗИМА И УЛТРАЗВУКА ТОКОМ ЕКСТРАКЦИЈЕ НА САДРЖАЈ ФЕНОЛНИХ ЈЕДИЊЕЊА И АНТИОКСИДАТИВНУ АКТИВНОСТ ЛАТИЦА *Paeonia officinalis* L.

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Божур је биљка која се користи због својих украсних и медицинских својстава. Латице су богате биоактивним фенолним једињењима, те је за њихово успешно изоловање потребно развити ефикасан метод екстракције. У овом раду су оптимизовани услови екстракције фенолних једињења из латица божура применом ултразвука, упоређена је ефикасност са стандардном методом изоловања и подаци су тестирани применом математичких модела (линеарни, интеракције два фактора, квадратни). Додатно је испитан ефекат претретмана целулазом. Велике количине флавоноида, фенолних једињења и танина су изоловане након 30 min екстракције у 70 % етанолу, користећи ултразвук (150 W, 40 kHz, 50 % амплитуда), при масеном односу растварача и чврсте супстанце 50:1. Претретман целулазом на 40 °C у различитим временским периодима повећао је екстракцију антоцијанина (720,48 mg/100 g суве масе), фенолних једињења (3985,50 mg/100 g), флавоноида (2369,28 mg/100 g) и танина (8917,81 mg/100 g). Велика антиоксидативна активност екстраката је измерена применом FRAP (350,03 mg аскорбинске киселине/100 g суве масе) и DPPH метода (инхибиција > 80 %) указујући на значајан потенцијал описане методе изоловања у добијању драгоцених биосастојака. FTIR анализа је потврдила присуство карактеристичних функционалних група. Комбиновани метод екстракције би могао да се примени и на другим врстама божура.

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