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Influence of enzymatic and ultrasonic extraction on phenolics content and antioxidant activity of *Hibiscus Sabdariffa* L. flowers

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The scientific support of the manifold benefits of phyto-molecules to human health has stimulated an increased consumption of natural products globally. Among phyto-molecules, polyphenols of intermediate and high molecular weights have been intensively studied because of their biological activities and uses. Flowers of roselle (*Hibiscus sabdariffa* L.) – a medicinal and culinary herb, contain compounds of polyphenolic structure, mainly anthocyanins, known for their antioxidant and anti-inflammatory activities. The extraction efficiency of biomolecules from various sources is tested through applying various technologies, conventional and non-conventional, in order to recover high amounts of such compounds and preserve their bioactivities. Consequently, an efficient approach is required to enhance the extractability of polyphenolic-based compounds from roselle flowers. The present study describes a combined technique of cellulase- and ultrasound-assisted extraction of *Hibiscus sabdariffa* phenolic compounds with enhanced yields. High amount of total anthocyanins (676.03±8.34 mg $100g^{-1}$ DM) and strong antioxidant activity of the crude acidified hydro-ethanolic extracts as measured by FRAP and DPPH assays were obtained in ultrasonic extracts pre-treated with cellulases. The optimum incubation time with the enzyme was 60 min, at which flavonoids and tannins were recovered in the highest amounts. The obtained results support the use of non-conventional environment-friendly extracting technologies to obtain an extract rich in antioxidants with potential uses in food, cosmetics and pharmaceutical industry

Keywords: *Hibiscus sabdariffa*, enzyme-assisted extraction, ultrasonication, phenolics, antioxidant activity.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a wild-grown or cultivated plant of the Malvaceae family, genus *Hibiscus*, widely used in tea production. Due to the particular content of micronutrients and antioxidant compounds (anthocyanins), the flowers of *Hibiscus sabdariffa* L. have been also used for medicinal and food purposes, as shown by Da-Costa-Rocha *et al.* [1] and Camelo-Méndez *et al.* [2]. Several health benefits of roselle have been reported, such as analgesic, anti-inflammatory, sedative, antimicrobial, immunomodulatory, as well as cardiovascular disease protection [3]. Although there are vast studies investigating the chromatic attributes of fresh petals of *Hibiscus sabdariffa* L., and their pharmacological properties [2], not the same could be noticed regarding the optimization of the extraction technologies, conventional and non-conventional. Testing the optimal extraction conditions using single or combined technologies is essential for high recovery of bioactive compounds, in particular polyphenols. Such biomolecules are efficiently extracted using polar organic solvents or acidified solvent mixtures which usually increase the extraction of anthocyanins due to the presence of the red flavylium cation as described by Giusti and Wrolstad [4]. The use of large quantities of organic solvents may generate a negative environmental impact and a few impurities in the final extract, leading to a time-consuming extraction. Ultrasound-assisted extraction (UAE) has been successfully used as a non-conventional technique for effective extraction of phenolic compounds from various plant materials through optimization of different experimental parameters [5]. The UAE of bioactive compounds is usually performed at frequencies ranging from 20 to 100 kHz and various ultrasonic amplitudes, applying direct and indirect irradiation through various devices. Other non-conventional extraction methods have been investigated with various yields, *e.g.* microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction [6-8]. In recent years, mild "green" extraction of bioactive compounds with the aid of hydrolytic enzymes has emerged as a promising tool [9].

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Enzymes such as cellulases, pectinases or hemicellulases have been used for the disruption of plant cell walls and consequent liberation of increased amounts of targeted compounds. The extraction yield highly depends on the plant material, type of compounds/enzymes and process parameters. As the UAE reduces extraction time and increases extractability of phenolic compounds, it may provide efficiency in combination with the enzyme-assisted extraction.

This work aimed at evaluation of the optimal parameters of the combined enzyme- and pulsed ultrasound-assisted extraction of polyphenolic compounds from *Hibiscus sabdariffa* L. red petals. The following antioxidant compounds were targeted: anthocyanins, flavonoids, flavones and flavonols, phenolics and condensed tannins. The total antioxidant activities of the crude roselle extracts were determined as well.

EXPERIMENTAL

Plant material and chemical reagents

Commercially available *Hibiscus sabdariffa* L. petals were purchased from a local producer (Sibiu, Romania). Samples were ground and mixed using the Grindomix Retsch GM 200 mill and stored at -80 °C until analysis. The moisture content was determined using the moisture analyzer (MAC 210/NP Radwag, Poland).

All chemical reagents used for analysis were of analytical grade. Cellulase from *Aspergillus niger* with activity > 60,000 U/g (MP Biomedicals) was used.

Extraction procedure

The extraction approach consisted of the enzymatic technique combined with ultrasonication. The optimal UAE conditions previously described were applied [10]. Three incubation times of roselle samples with cellulase solution in acetate buffer of pH 4.8 were used (60 min, 120 min, 180 min) at 40 °C. An enzyme/substrate ratio of 0.166 (w/w) was used. After incubation, enzymes were inactivated at 100 °C for 5 min. Extraction was further performed by UAE using 70% ethanol acidified with 1% acetic acid. Control samples (without cellulase pre-treatment) were also investigated. UAE was performed using an ultrasonic device (Sonifier SLPe-150, Branson, USA) of 150 W power and 40 kHz frequency, equipped with a transducer.

The obtained extracts were centrifuged at 8000 rpm, at 4 °C for 10 min. The NF800R refrigerated centrifuge (Universal 320, Hettich, Germany) was used.

Determination of the total content of anthocyanins

The content of total anthocyanins was determined spectrophotometrically by the pH differential method [4]. The Specord 200Plus UV–Vis spectrophotometer (Analytik Jena, Germany) was used. The results were expressed as mg cyanidin-3-O-glucoside 100 g^{-1} DM.

Determination of the total content of phenolics

The phenolics content was determined according to Folin-Ciocalteu method [11]. The results were expressed as mg gallic acid equivalents GAE 100 g^{-1} DM.

Determination of the total content of flavonoids

The flavonoids content was determined spectrophotometrically as described in [12]. The results were expressed as mg quercetin 100 g^{-1} DM.

Determination of the total content of flavones and flavonols

The content of flavones and flavonols was determined spectrophotometrically as described in [13]. The results were expressed as mg rutin 100 g^{-1} DM.

Determination of the total content of tannins

The tannins content was determined spectrophotometrically as described in [14]. The results were expressed as mg catechin 100 g^{-1} DM.

Antioxidant activity assays

Ferric reducing antioxidant power (FRAP). The total antioxidant activity of enzyme ultrasonic extracts was determined by the ferric reducing ability assay [15]. The results were expressed as mg ascorbic acid 100 g^{-1} DM.

Radical scavenging activity (RSA) using 1, 1-diphenyl-2-picrylhydrazyl (DPPH). The RSA activity of enzyme ultrasonic extracts was determined by DPPH assay [16]. The results were expressed as inhibition percentage calculated according to the formula:

$$inhibition\ (\%) = 100 \times \frac{A_0 - A}{A_0}$$

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

Statistical analysis

The experimental parameters were analyzed in duplicate. Data were expressed as mean values±SD of duplicate experiments. The statistical analysis of the experimental data was carried out using the Systat v.10 software.

RESULTS AND DISCUSSION

Hybrid approach of enzyme- and ultrasound-assisted extraction of phenolic-based compounds from roselle

The UAE conditions that proved efficiency in extracting polyphenolic compounds from *Hibiscus sabdariffa* L. petals using 70% ethanol acidified with 1% acetic acid in a solvent/solid ratio of 40/1 were: 30 min extraction time at ultrasonic amplitude of 70%, as previously described by our group [10]. In order to further develop an efficient "green" extraction under mild conditions, we hereby report the development of a hybrid approach of enzymatic and ultrasonication technology. Three incubation times (60, 120 and 180 min) of samples with cellulase solution in acetate buffer of pH 4.8 were tested at 40 °C. The measured temperature of the mixtures after ultrasonication varied between 26.6 and 31.6 °C, depending on the experiment run.

The cellulolytic multi-enzyme complex used for extraction contains exo- and endo-β-1,4-D-glucanases which hydrolyze glucosidic bonds, and β-glucosidase which degrades small molecular weight cellulose hydrolysates.

The results regarding the content of total anthocyanins as a variation of incubation time are presented in Figure 1. The mean values of total anthocyanins contents were 676.03±8.34 mg 100 g^{-1} DM for enzyme ultrasonic extracts and 668.11±9.89 mg 100 g^{-1} DM for control, respectively. No statistically significant differences were found between combined method and control (single UAE). By increasing incubation time with cellulase, a decrease in total anthocyanins content was noticed, probably due to the time-dependent influence of acidic conditions given by the sample/solvent mixture on the enzyme denaturation. Based on these findings, evaluation of the other phenolic compounds was done after 60 min incubation of samples with cellulase followed by UAE. The results are shown in Table 1.

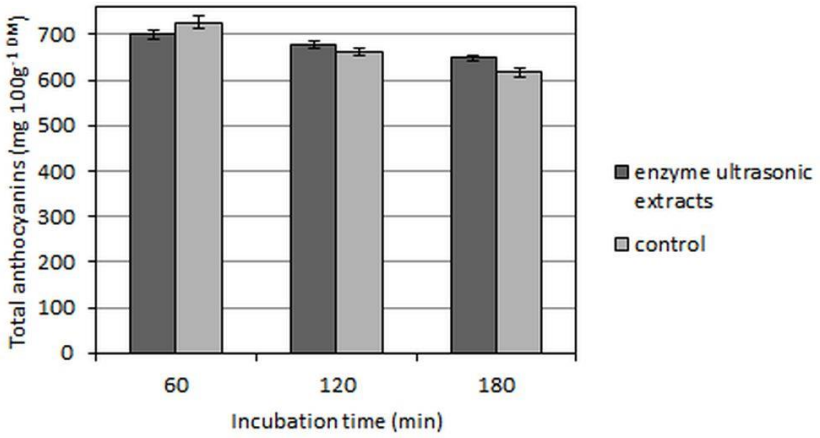

Fig. 1. The effect of incubation time on total anthocyanins content of enzyme ultrasonic extracts of roselle petals.

Table 1. The content of bioactive compounds of roselle extracts by enzymatic and ultrasonication combined technique.

Type of extraction / Bioactive compounds	Enzyme + Ultrasonication	Ultrasonication (Control)
Phenolics (mg GAE 100 g^{-1} DM)	1336.743±18.371	1338.169±21.584
Flavonoids (mg quercetin 100 g^{-1} DM)	2715.513±42.243	2533.792±33.104
Flavones and flavonols (mg rutin 100 g^{-1} DM)	1673.792±46.895	1671.569±40.784
Tannins (mg catechin 100 g^{-1} DM)	6561.118±80.559	6271.147±85.573

In experimental runs involving enzymatic pre-treatment, an increase in total content of flavonoids by 7% and tannins by about 5% was found. This might be explained by the enzymatic release of high-molecular weight tannins and pro-anthocyanidins from roselle samples, because such

compounds are usually associated with cell wall polysaccharides [17]. Our results showed that of total flavonoids, the contribution to their overall yield increase was not related to flavones and flavonols, but to other representatives of the class, mainly anthocyanins.

The effects of the improved material transfer determined by cavitation process during UAE as previously described by other authors [18] were further positively influenced by the cellulase pre-treatment of roselle samples for 60 min, resulting in high recovery of flavonoids/anthocyanins and tannins.

To our knowledge, no studies have been published on enzyme-assisted extraction, single or combined technology, of polyphenolic compounds from roselle. However, there are studies reporting a high recovery of anthocyanins from red and purple *Hibiscus sabdariffa* L. calyces under single UAE [19]. Regarding the use of enzymes to improve extraction of bioactive compounds, our findings are consistent with previous reports on other plant materials, mainly fruits, when various types of commercial hydrolytic enzymes have been involved [20].

In vitro antioxidant activities of enzyme ultrasonic extracts

The total antioxidant activity of the roselle enzyme ultrasonic extract was measured *in vitro* using different analytical assays, based on the measurement of ferric ion reducing capacity and on single electron transfer by DPPH. The results are presented in Figures 2 and 3.

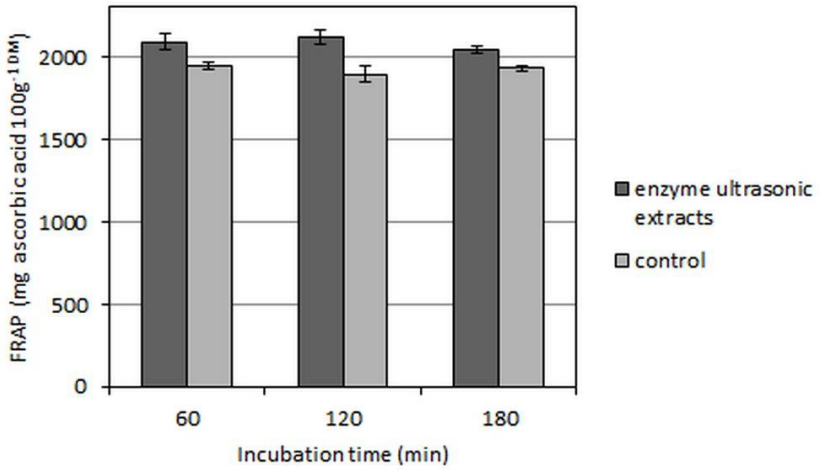

Fig. 2. The total antioxidant activity by FRAP of roselle enzyme ultrasonic extracts in relation to incubation time.

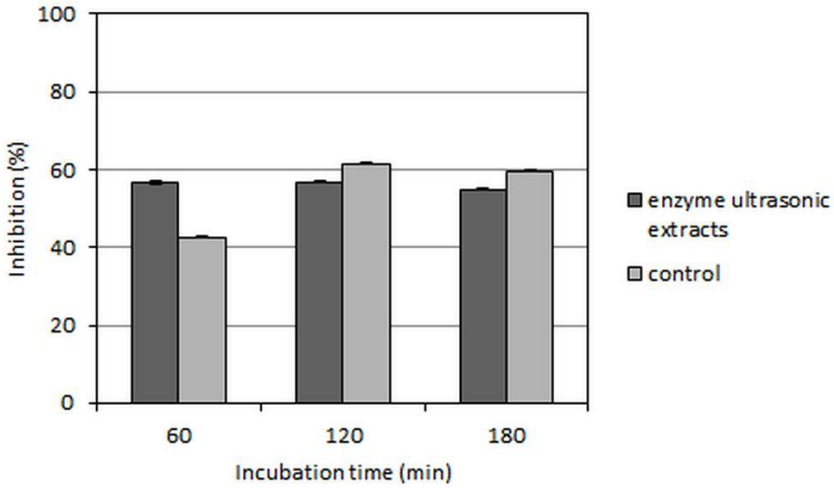

Fig. 3. Radical scavenging activity by DPPH of roselle enzyme ultrasonic extracts in relation to incubation time.

Regarding the antioxidant activity as measured by FRAP technique, the values obtained for the enzyme ultrasonic extracts were higher than those of the ultrasonic ones which did not undergo enzymatic pre-treatment (control). The mean FRAP values increased by 8.3% when cellulase pre-treatment was involved, compared to control samples.

The roselle crude extracts reduced the purple coloration of DPPH giving inhibition percentages > 50%, which confirm a good radical scavenging activity. The increase in RSA values was of about 4% when cellulase pre-treatment was involved, compared to control.

A study published in literature described the high antioxidant activity exhibited by extracts obtained through maceration of *Hibiscus sabdariffa* L. calyces in methanol, which was correlated to their high content of flavonoids [21].

CONCLUSIONS

Flowers of roselle (*Hibiscus sabdariffa* L.) contain high amounts of antioxidant compounds of polyphenolic structure.

An efficient enzyme-assisted extraction using cellulase combined with ultrasonication was developed. Lower incubation time (60 min) favored tannins and flavonoids extraction, improving their extractability by 5-7%.

The high antioxidant activity of acidified ethanol extracts, in terms of FRAP and radical scavenging activity by DPPH, was found in enzyme ultrasonic extracts.

The obtained results confirmed the efficacy of non-conventional extraction technologies with the development of "green" ones by using enzymes to formulate an extract rich in antioxidants with potential uses in food, cosmetics, textiles and pharmaceutical industry.

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