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Cent. Eur. J. Biol. • 7(1) • 2012 • 126-131
DOI: 10.2478/s11535-011-0091-z

Central European Journal of **Biology**

Optimization and use of a spectrophotometric method for determining polysaccharides in *Echinacea purpurea*

Research Article

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Received 01 June 2011; Accepted 29 September 2011

Abstract: *Echinacea purpurea* is one of the most widely used immunostimulant plants. Its main active compounds are polysaccharides, glycoproteins, caffeic acid derivatives, alkamides, and melanins. The article describes an optimized extraction procedure that enables spectrophotometric quantification of polysaccharides from *Echinacea purpurea*. The extraction procedure can be widely applied as it demonstrated to be useful for determining polysaccharide content in flowers and leaves, in summer and autumn plants, in plants with green and red stem, and in plants from two different plantations. A significantly higher content of polysaccharides in flowers in comparison to leaves, as well as in plants with green stems in comparison to plants with red stems was determined. Statistical differences were absent in plants collected in different seasons and growing at different plantations.

Keywords: *Echinacea purpurea* • *Extraction* • *Polysaccharides* • *Spectrophotometry*

1. Introduction

Echinacea purpurea is one of the most widely used immunostimulant plants for treating flu and common cold. For medicinal treatment, root and herb extract as well as pressed juice of fresh aerial plant parts are used. The main active compounds are polysaccharides, glycoproteins, caffeic acid derivatives (cichoric acid), alkamides, and melanins [1-3]. Protein-free polysaccharide preparations called EPS (Echinacea polisacharide fraction), PSI (35 kDa 4-*O*-methylglucuronoarabinoxylan), PSII (50 kDa acidic arabinorhamnogalactan), and arabinogalactan have been studied [4-6]. These polysaccharides affect phagocytosis, chemotaxis, and production of cytokines in granulocytes and macrophages *in vitro* [4,7]. In animal studies, it was shown that EPS enhances phagocytosis [7] and arabinogalactan enhances resistance against systemic infections with *Listeria monocytogenes* and *Candida albicans* in mice [8,9]. Polysaccharides purified from *Echinacea purpurea* tissue culture injected intravenously into patients undergoing chemotherapy for gastric cancer showed lower leucopoenia [10].

Distribution of alkamides, polyenes/ynes, and phenolics within the same plant is well documented [11-13]. Isolation, separation and analytical determination of these compounds has also been demonstrated using high-performance liquid chromatography [14-16], capillary electrophoresis [17-19] and liquid chromatography with mass spectrometry (LC-MS) [20]. In contrast, only limited analytical data exist on polysaccharides in plant tissue. General procedure of polysaccharide isolation was described by Wagner [7] with only a few compounds being characterized, such as purified polysaccharides [7], methylglucuronoarabinoxylan, and arabinorhamnogalactan [5].

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The aim of our work was to optimize a method for isolation and quantification of polysaccharides from *Echinacea purpurea*, and to determine content in flowers and leaves, in summer and autumn plants, in plants with green and red stem, and in plants from two different plantations.

2. Experimental Procedures

2.1 Plant material

Aerial plant parts of *Echinacea purpurea* Moench were collected during flowering time (at the beginning of July and October). First group of 20 samples was obtained from The Institute for Hop Research and Brewing (Žalec, Slovenia): 5 plants with red stem and 5 plants with green stem collected in summer and autumn harvest, respectively. Second group of samples was obtained from two different plantations and collected in summer and autumn period, respectively. Flowers and leaves were collected separately (5 samples of each of two organs from each of two plantations and each of two harvests = 40 samples). Plant material was dried at room temperature, cut into 1cm particles, and powdered in the mortar.

2.2 Polysaccharide isolation

Various isolation protocols were tested. The basic method of polysaccharide isolation was adopted from literature [7] and optimized. All extraction steps were performed by sonication procedure as the studies have shown that ultrasound-assisted extraction indicates more efficient extraction in shorter time and at lower temperatures owing to disruption of cell walls and enhanced mass transfer of cell contents such as polysaccharides [21]. Influence of solvent volume, time of extraction, and ethanol precipitation were then studied. The optimal isolation method was: 50 mg of powdered herbal drug was extracted with 10 mL of petroleum ether for 10 min at room temperature in an ultrasonic bath and centrifuged at 3400x*g* 10 min. After removal of supernatant, the plant material in the sediment was extracted with methanol (10 mL for 10 min at room temperature). Petroleum ether and methanol extracts contained colored substances (chlorophyll, carotene and other pigments). Removal of these compounds was important, to prevent interaction with the colorimetric assay for polysaccharides. Supernatant was discarded and the pellet extracted with 10 mL of 0.5 M sodium hydroxide first for 10 min in an ultrasonic bath and then at room temperature for 24 h. For comparison, some samples were analyzed by using a method that contained a precipitation step. Precipitation was carried out by adding 8 mL of ethanol to 2 mL of supernatant incubating for 1 h at −20°C, and then centrifuging for 10 min at 3400x*g*. The precipitate was dissolved in 2 mL of distilled water, sonicated for 5 min, and centrifuged for 10 min at 3400x*g*. The supernatant was analyzed spectrophotometrically. Precipitation with 80% ethanol selects for polysaccharides but the oligosaccharides, disaccharides and monosaccharides remain soluble [22].

2.3 Determination of polysaccharide concentration

Various protocols for color reaction were tested as described in the results section. The optimal method was: 200 µL of polysaccharide solution (containing 5−50 µg of polysaccharides) were diluted with 300 µL of distilled water and 500 µL of 5% (w/w) phenol solution was added. Then, 2.5 mL of 96.5% sulfuric acid was added rapidly and the mixture was shaken. After 60 min incubation at 70°C, absorbance was measured at 490 nm (spectrtophotometer: Perkin Elmer Lambda Bio+). A negative control sample was prepared with distilled water.

2.4 Thin layer chromatography (TLC) analysis of sugars in polysaccharide hydrolysate

A sample of 10 mL of polysaccharide extract was dried on a rotary evaporator. The sample was treated with 2 mL of 2 M trifluoroacetic acid and heated under reflux for 1 h. Following incubation, 10 mL of water was added, the mixture was dried on a rotary evaporator and this procedure was repeated twice. The residue was dissolved in 0.5 mL of 80% ethanol and 1 µL was applied on a Silicagel 60 TLC plate (Merck). The plate was developed with acetonitrile:water (85:15) and derivatized by spraying with 0.5% thymol and 5% sulphuric acid in ethanol and heating for 5 minutes at 120°C.

2.5 Statistical evaluation

ANOVA, t-test, and linear regression were calculated using Microsoft Office Excel 2003.

3. Results and Discussion

3.1 Optimization of polysaccharide extraction

When performing the polysaccharide isolation process with 10 mL of solvents (petroleum ether, methanol and sodium hydroxide solution) per 50 mg of herbal drug in comparison to 1 mL per 50 mg of herbal drug, the amount of extracted polysaccharides was 9% higher. The increased volumes of petroleum ether and methanol resulted in more effective removal of pigments, while the increased volume of sodium hydroxide and sonication

resulted in more efficient extraction of polysaccharides from the plant matrix. Efficacy of extractions performed for 1, 2, 3, 4, and 24 hours with 0.5 and 2 M sodium hydroxide solution is shown in Table 1. Extraction efficiency reaches the maximum after 4 hours, but 24 hours were selected for routine experiments to assure complete extraction in all cases and more practical organization of work.

After extraction of polysaccharides, precipitation with 80% ethanol was performed in two ways, 1 hour at −20°C and 24 hours at 4°C. There were only slight differences in quantity of precipitated polysaccharides as determined by the spectrophotometric analysis (data not shown).

We performed additional tests to see the composition of sugars in the polysaccharide. Composition of precipitated polysaccharides as well as polysaccharides that remain soluble in 80% ethanol was evaluated by hydrolysis and thin-layer chromatography using xylose, galactose, and glucose as standards. In both samples, all of the three sugars and one additional unidentified sugar were detected, and among them xylose was prevailing (semiquantitatively determed as >50%). The amount of glucose was low (semiquantitatively determed as <10%). To further elucidate, if the glucose was a part of heteroglycane or if it originated from the starch contamination, we incubated the polysaccharide solution with alpha-amylase. After precipitation, the amount of polysaccharides determined in colorimetric assay was equal with and without amylase. This demonstrated that the isolated polysaccharide did not contain significant amount of starch.

3.2 Optimization of determination of polysaccharide concentration

Polysaccharide concentration was determined by the phenol-sulfuric method [23], which was modified and optimized to improve repeatability. We found out that poor repeatability of the method originates (besides in incomplete reaction) also in heterogeneous solution, which is formed after addition of sulfuric acid. In the solution, two phases can be seen, which cause diffuse scattering of a light beam during absorbance measurement, moreover, additional problem is formation of bubbles as a consequence of heating of the solution caused by sulfuric acid. Good repeatability (relative standard deviation, RSD=2.1%) of spectrophotometric analysis was reached by prolonged incubation at higher temperature (Table 2). The repeatability of overall method (extraction and spectrophotometric analysis) was RSD=3.8%.

Linearity of optimized phenol-sulfuric method was good (Pearson correlation R^2=0.991 for glucose standard and R^2=0.9899, and for Echinacea purpurea polysaccharide extract) in a tested range (see Table 3).

3.3 Quantification of Polysaccharides in *Echinacea purpurea* Plants

Total content of polysaccharides was significantly higher in green stem plants (2-way ANOVA: P=0.04227; t-test P=0.029) (Figure 1). It is obvious, that the same factor, that increases the content of red pigment in the stem, decreases the content of polysaccharides. This might be due to genetic or environmental (light) factor. On the other hand, both statistical tests showed no significant difference in polysaccharide content between plants collected in summer or autumn (2-way ANOVA: P=0.516772). Total content of polysaccharides was significantly higher in flowers as compared to leaves

Extraction time	0.5 M NaOH	2 M NaOH
1 h	1.09	1.57
2 h	1.24	1.69
3 h	1.21	1.70
4 h	1.42	1.78
24 h	1.42	1.76

Table 1. Influence of solvent molarity and extraction time on efficacy of polysaccharide extraction determined spectrophotometrically by measuring absorbance at 490 nm. All experiments were repeated three times. Efficacy of extraction is expressed as absorbancy units.

Incubation	Absorbance			Relative standard deviation (RSD) [%]
	1	2	3	
30 min, room temperature	0.869	0.976	0.970	6.4
60 min, room temperature	1.077	0.947	0.953	7.4
30 min, 50°C, shaking	1.044	0.982	0.954	4.6
60 min, 50°C, shaking	0.903	0.970	0.939	3.6
30 min, 70°C, shaking	0.889	0.847	0.862	2.5
60 min, 70°C, shaking	0.895	0.867	0.860	2.1

Table 2. Influence of incubation time, temperature, and shaking on repeatability of the phenol-sulfuric method. All experiments were repeated three times.

	Glucose	Echinacea
Concentration range	5−50 μg	20−90 mg
Regression equation	y=0.0199x + 0.0118	y=0.0236x − 0.0127
R^2	0.991	0.9899

Table 3. Linearity of the phenol-sulfuric acid method.

(2-way ANOVA: P=0.00002; t-test P=0.001) (Figure 2). This implies that it is favorable to include high proportion of flowers in the herbal material for producing medicinal products. There was a slightly higher concentration of polysaccharides in autumn flowers and leaves in comparison to plant parts collected in summer, but the difference was not statistically significant (2-way ANOVA: P=0.103; t-test P=0.110). There were also no differences found between plants from different plantations. Regarding polysaccharide content determined without or with precipitation with ethanol, it has to be emphasized that values measured without precipitation were approximately 2-fold higher. This can be attributed to the loss of smaller polysaccharides and oligosaccharides due to inefficient precipitation or to higher stringency in selectivity of the precipitation step. These two variations of analytical method will enable that the products which will be tested in future for their (*in vitro* or *in vivo*) activity, will be characterized on the content of the total polysaccharides and the precipitable polysaccharides to determine which fraction of the polysaccharides is more relevant for efficacy.

Using the modified and optimized method for extraction and quantification of total polysaccharides in *Echinacea purpurea*, we demonstrated that there is a significantly higher content of polysaccharides in flowers in comparison to leaves, and higher content in plants with green stems in comparison to plants with red stems. On the other hand no statistical differences were found between plants collected in different seasons or different plantations. In our previous research, we similarly found no difference in content of caffeic acid derivatives in plants harvested in summer or in autumn [13]. A limitation to the isolation and quantification method that we describe is that it does not show the qualitative composition of polysaccharides. However, since absorbance response varies for different sugars, the high heterogeneity of monosaccharide composition within a polysaccharide makes metabolomic approaches for quantification difficult and highly complex.

Here we demonstrated a simple and reliable method that offers useful and important improvements of polysaccharide determination in plant material suitable for routine work.

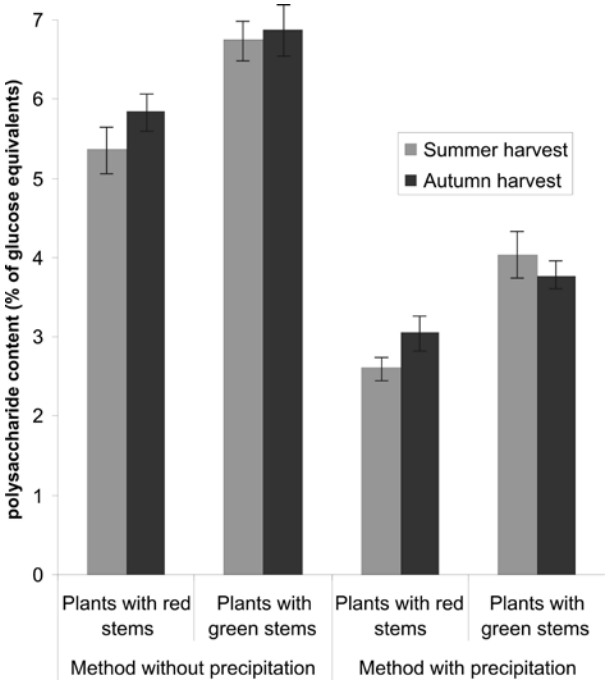

Figure 1. Polysaccharide content in % of glucose equivalents between plants with red and green stems collected in summer and autumn period, respectively, determined without or with precipitation with ethanol. Each column represents the average of analyses of five individual plants. Error bars represent standard errors.

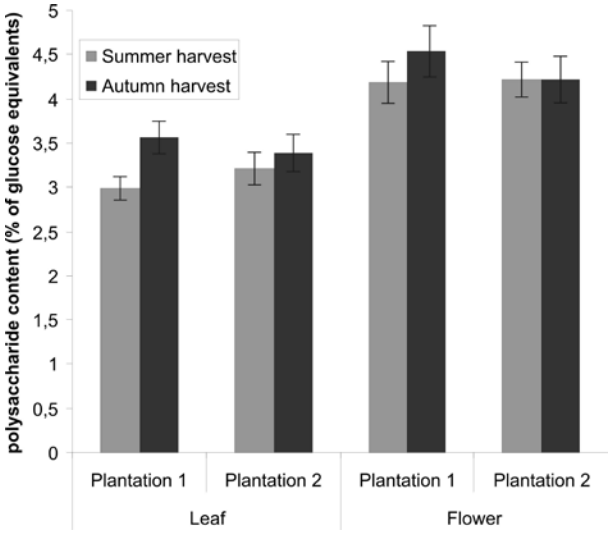

Figure 2. Polysaccharide content in % of glucose equivalents in flower and leaf plant parts from two different plantations collected in summer and autumn period, respectively. Each column represents the average for five individual plants. Error bars represent standard errors.

Acknowledgements

The authors are grateful to Ms. Nina Kroflič and Ms. Katja Delić for their skilful laboratory assistance.

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