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Full Length Research Paper

Research update: Lectin enriched fractions of herb and dry extract of *Urtica dioica* L.

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***Urtica dioica* L is a plant rich in flavonoids, carotenoids, caffeoylmalic acid and has an established medical value. Although content of mineral and organic substances of *U. dioica* L. herb is well characterized, presence of bioactive polypeptides is much less appreciated. Seeds and roots of nettle, have been established as a common source for isolation of lectins. Therefore data on the presence of lectins in herb of nettle is ambiguous. Lectin-enriched protein fractions were isolated from herb (fresh and dry) and dry extract of *U. dioica* L. by using homogenisation with fluid nitrogen, extraction in 0.01 M phosphate-buffer saline (PBS), concentrating, salting and precipitation. The amount of protein was measured using photometric Bradford method. A proteomic analysis using 2D gel electrophoresis was performed for lectin – enriched protein fractions isolation and analysis. We estimated quantity of protein and lectins, assessed their blood cell agglutinating activity using tests employing rabbit erythrocytes. The highest concentration of protein and specific hemagglutination activity was observed for protein fractions isolated from fresh herb. The highest lectins content was presented in protein fractions isolated from the dry extract.**

Key words: *Urtica dioica* L., lectins, hemaglutination, electrophoresis.

INTRODUCTION

Plant lectins are a class of carbohydrate-binding nonimmune origin protein (Goldstein et al., 1980; Sharon, 1989; Peumans et al., 2001; de Meija et al., 2003). In past few decades a lot of lectins were purified and identified. They have attracted great interest because of their various biological activities, such as cell agglutination, antiproliferative, antitumor, immuno-modulatory, antifungal and antiviral (Broekaert et al.,1989; Wang et al.,1996; Does et al., 1999; Wong et al., 2003; Singh et al., 2004). *Urtica dioica* L. is widely used herb in medicine. Stinging nettle is well known for its diuretic, anti-inflammatory effects. It is also used for joint and muscle rheumatic disease, bile system disease and arthritis (Bauer et al., 2009). Roots of the nettle have been established as a common source for isolation of lectins. Stinging nettle rhizomes contain considerable amounts of a lectin which exhibits carbohydrate binding specificity for N-acetylglucosamine oligomers (Chapot et al., 1986).

An unusual lectin has been isolated from stinging nettle (*U. dioica* L.) rhizomes. It is a small (8.5 kDa) monomeric protein with high contents of glycine, cysteine and tryptophan. The *U. dioica* agglutinin (UDA) is not blood group-specific and is specifically inhibited by N-acetylglucosamine oligomers. As compared to other plant lectins. UDA has a very low specific agglutination activity. Nevertheless, it induces HuIFN-y in human lymphocytes at concentrations comparable to those of other inducers (Peumans et al., 1984). Our research novelty is purificating experiments of lectins with well known herb of

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nettle (*U. dioica* L.). Therefore data on the presence of lectins in the herb of stinging nettle is ambiguous. In this study, we prepared lectin-enriched protein fractions from extracts of fresh and dry herb and dry extract of *U. dioica* L. and estimated protein and lectin quantity. We assessed lectin blood cell agglutinating activity using tests employing rabbit erythrocytes.

MATERIALS AND METHODS

The dry herb (humidity – 11.8%) and dry extract of *U. dioica* L. herb (DER: (5-10:1), extraction solvent: water) was imported from Poland. The fresh herb (humidity – 85%) of stinging nettle was collected in Lithuania, Kaunas district, Cekiskes.

Homogenisation

40 g of plant material (for choice - dry herb, fresh herb or dry extract of *U. dioica* L.) was homogenized with fluid nitrogen in mortar.

Extraction

Homogenised material was extracted in 1:5 (w/w) 0.01 M phosphate-buffer saline (PBS), pH 7.2, with protheasis inhibitor and 2% PVPP (Polyvinyl prolidone) for 2 to 16 h at 4 to 5°C. PBS consists of 10.9 g NaH_2PO4 (anhydrous), 3.2 g NaCl, 90 g H_2O in1 liter. Protheasis inhibitorconsists of 5 mM (0.8 mg/ml) Benzamidyne 5 mM (0.66 mg/ml) ε-amino-capronic acid.

Concentrating and salting

The extract was filtered through filter and centrifuged at 1500 rpm for 30 min at 4 to 5°C. The filtrate was purified followed by 40% saturation with ammonium sulfate (w/v) and constant stirring for 15 min. Then the solution with precipitate was centrifugated again at 15000 rpm for 30 min. The extract was then subjected to a “salting out” step with 40 to 80% ammonium sulfate (w/v) and stirred about 24 h. Forthcomming precipitate (first protein fraction) was dissolved in the little amount of PBS solution and left at 4°C. The 80% saturated solution was centrifuged at 15000 rpm for 30 min.The filtrate was purified followed by 80 to 100% saturation with ammonium sulfateand stirred again for 24 h. Forthcomming precipitate (second protein fraction) was dissolved in the little amount of PBS solution and left at 4°C. The 100% saturated solution was centrifuged at 15000 rpm for 30 min. Final filtrate was eliminated and precipitate (third protein fraction) was dissolved in the little amount of PBS solution. The precipitate (of three fractions) was resuspended and dialyzed against three changes of PBS for 48 to 72 h. The amount of ammonium sulfate was: 0 to 40%-233,38 g/L; 40 to 80% - 266,41 g/L, 80 to 100% -143,35 g.L/ at 4°C. Peptide solutions were concentrated using Centiprep YM-3 filters. Methods prepared according to Dhuna (2005, 2010), Tulasi (2002) and Gupta (1997).

Protein precipitation

Protein precipitation accomplished with trichloracetic acid (TCA), 0.2 M TCA (0,4 g/2 ml) mixed with 2 ml of peptide solution. Coagulation of protein can be prevented at reduced temperature (0 to 4°C). Peptide solution were kept on ice for 30 min to avoid protein denaturation. Solutions centrifugated at 20000 rpm 30 min at 4°C supernatant was poured. TCA residual was washed with acetone (at temperature -20°C) and incubated on ice for 10 min. Protein solution was centrifugated at 2000 rpm for 10 min. Acetone was removed and washing repeated once again. Solution dissolved in sodium dodecyl sulfate (SDS) buffer and kept for 30 min at 60°C (Bell et al., 1983).

Measurement of protein amount

The amount of protein was measured using photometric Bradford method. Standarts were prepared in 0.5 ml test-tubes: 0.125; 0.25; 0.5 and 1.0 mg/ml Bull Serume Albumine (BSA) solutions. Standarts were devided in to small volumes and kept at 20°C. Negative control was prepared in 0.5 ml test-tube–5 µl buffer. Examples were prepared in 0.5 ml test-tubes -5 µl protein extract. 5 volumes of Roti-Quant (Carl Roth GmbH, Karlsruhe, Germany) reagent diluted with 4 volumes of deionisated water. 250 µl of Rothi-Quant reagent was poured on standarts, negative standarts and mixed. Solutions were kept for 5 to 30 min at 20°C. Light absorbtion was measured with Eppendorf BioPhotometerin 595 nm wavelength (Lowry, 1951).

Gel electrophoresis

Electrophoresis is widely used in trials for protein isolation and analysis. Most of protein trials are made in 2D polyacrylamide gel electrophoresis (PAGE) method. Protein are separated by their charge with isoelectric focusing method and fractionated by their size in polyacrylamide gel. Protein are visible like spots after staining. PAGE was carried at pH 8.8 using 12% (w/v) acrylamide slab gel with running conditions 12 mA per mini-gel for 2 h (Ausubel, 2003). Coomasie Blue G-250 (Fackelmayer, 1998) and silver nitrate (Møller et al., 2003) were used to visualize the protein bands.

Preparation of erythrocytes

Rabbit blood was used during experiments, got from Lithuanian Univercity of Health Sciences, Veterinary academy. 2% erythrocyte solution was prepared, where amount of erythrocytes was $5x10^9$ ml. 1 ml of rabbit blood mixed with 14 ml PBS solution. Erythrocytes were washed 4 times: 2% erythrocyte solution was centrifugated at 800 rpm for 10 min at 4°C, the supernatant was eliminated, precipitate resuspended in 15 ml PBS solution and procedure with centrifugation was repeated. After washing procedure, erythrocytes were treated with 7.5 ml trypsin (Biochrom AG, L2103, 1310 USP U/mg) solution (1 mg/ml PBS), incubated for 1 h at 37°C. Trypsintreated erythrocytes were washed 4 times: it was provided centrifugation at 800 rpm for 10 min at 4°C, Supernatant was poured, precipitate resuspended with 15 ml of PBS solutions and centrifugated at 800 rpm for 10 minat 4°C. After washing procedure erythrocytes resuspended with 15 ml of PBS solution (Ove et al., 2003).

Hemagglutionation test

50 µl 0.01 M PBS solution (7.0 mM Na_2HPO_4, 2.7 mM NaH_2PO_4, 154.0 mM NaCl, pH 7.2) was added to U shaped microplate wells. To the first well was poured 50 µl protein extract and mixed. 50 µl of extract was moved to the next well. Dilution repeated in 1 to 15 wells. 50 µl of sample from 15‘th well was eliminated. To the 16‘th well added 50 µl PBS solution, mixed and 50 µl eliminated. In each well 50 µl 2% trypsine-treated erythrocytes solution were added.

Microplate was softly mixed and covered. Titre was measured on

Table 1. Characteristics of protein fractions from *Urtica dioica*L. herb (fresh and dry) and dry extract.

Source / isolation step	Protein quantity mg /g weight	Specific hemoagglutinating activity[1]	Lectin content, %[2]
Fresh herb			
Precipitate of 40% saturated AS	1.18 ± 0.0253	2.95 ± 0.0131	0.01 ± 0.0014
Precipitate of 80% saturated AS	2.85 ± 0.0376	2.23 ± 0.0856	0.01 ± 0.0007
Dry herb			
Precipitate of 40% saturated AS	0.09±0.0141	0.44±0.0468	0.05±0.0051
Precipitate of 80% saturated AS	0.41±0.0225	0.2±0.0215	0.1±0.0013
Supernatant of 80% saturated AS	0.64±0.0481	1.7±0.0653	0.01±0.0064
Dry extract			
Precipitate of 40% saturated AS	0.40±0.0139	0.83±0.0782	0.02±0.0011
Precipitate of 80% saturated AS	0.31±0.0154	0.95±0.0241	0.02±0.0085
Supernatant of 80% saturated AS	0.24±0.0181	0.06±0.0046	0.33±0.0045

1- Specific hemagglutinating activity was defined as the ratio of the titer/ml and protein concentration (mg/ml); Titer per ml was defined as the reciprocal of the highest dilution giving visible agglutination of the rabbit erythrocytes. 2 - Lectin content is based on cell agglutinating activity as compared to specific hemagglutinating activity of lectin from *S. tuberosum.* Tables include representative results from at least three separate experiments. AS,ammonium sulphate.

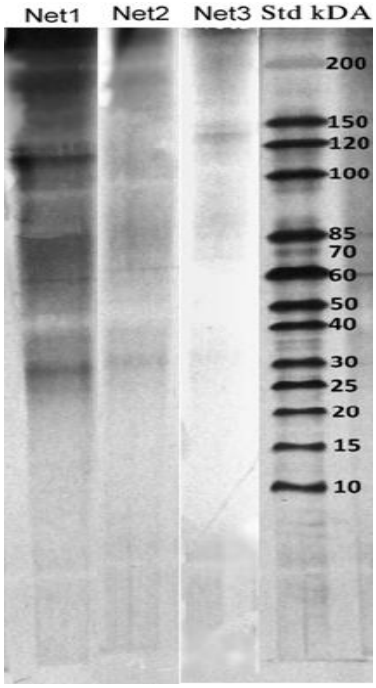

Figure 1. Gel electrophoresis of dry *Urtica dioica* L. herb, pH 8.8 using 12% (w/v) acrylamide slab gel. Flow direction fom top (+) to the bottom (-), 12 mA per minigel, duaration 2 h.Std - PageRuler™ Protein Ladder. Total amount of proteins in each well : Net1 – 0.3 µg in 40 % AS saturated precipitate; Net2 – 0.6125 µg in 80% AS saturated precipitate; Net3 – 1.25 µg in 80 % AS saturated supernatant. Sample was stained with silver nitrate.

the light table after 30 min of incubation at 22°C. Hemagglutination titre (per 1 ml of sample) is the maximum dilution in which total aglutination was observed. Specific activity of hemagglutination was calculated by dividing the value of titre from protein concentration in the sample. Specifical hemagglutination activity was counted by dividing hemagglutination titre meaning from protein concentration in example (Laija et al., 2010).

Statistical analysis

Analysis of standart deviation of means were carried out on all data using statistical analysis system (SAS 76, SAS Institute Inc).

RESULTS

Protein concentration (mg/g), specific hemagglutinating activity and lectins content (%) of *U. dioica* L. herb (fresh and dry) and dry extract presented in Table 1. Protein quantity of fresh and dry herb increased in precipitate from 40 to 80% saturated ammonium sulfate (AS) samples. Meanwhile protein quantity in dry extract decreased in precipitate from 40 to 80% saturated AS samples. Fresh *U. dioica* L. herb protein extract contained relatively high level of hemagglutinating activity against trypsin-treated rabbit erythrocytes. Maximum of specific hemagglutinating activity of dry *U. dioica* L. herb was in 80% AS saturated precipitate. Maximum of specific hemagglutinating activity of dry *U. Dioica* L. extract reached in 80% saturated AS supernatant. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis test was made with protein fractions of *Urtica dioica* L. herb dry (Figure 1). In protein precipitate fraction (40 % saturated AS) (Net1) was found particles sized 200 kDa, 120 kDA

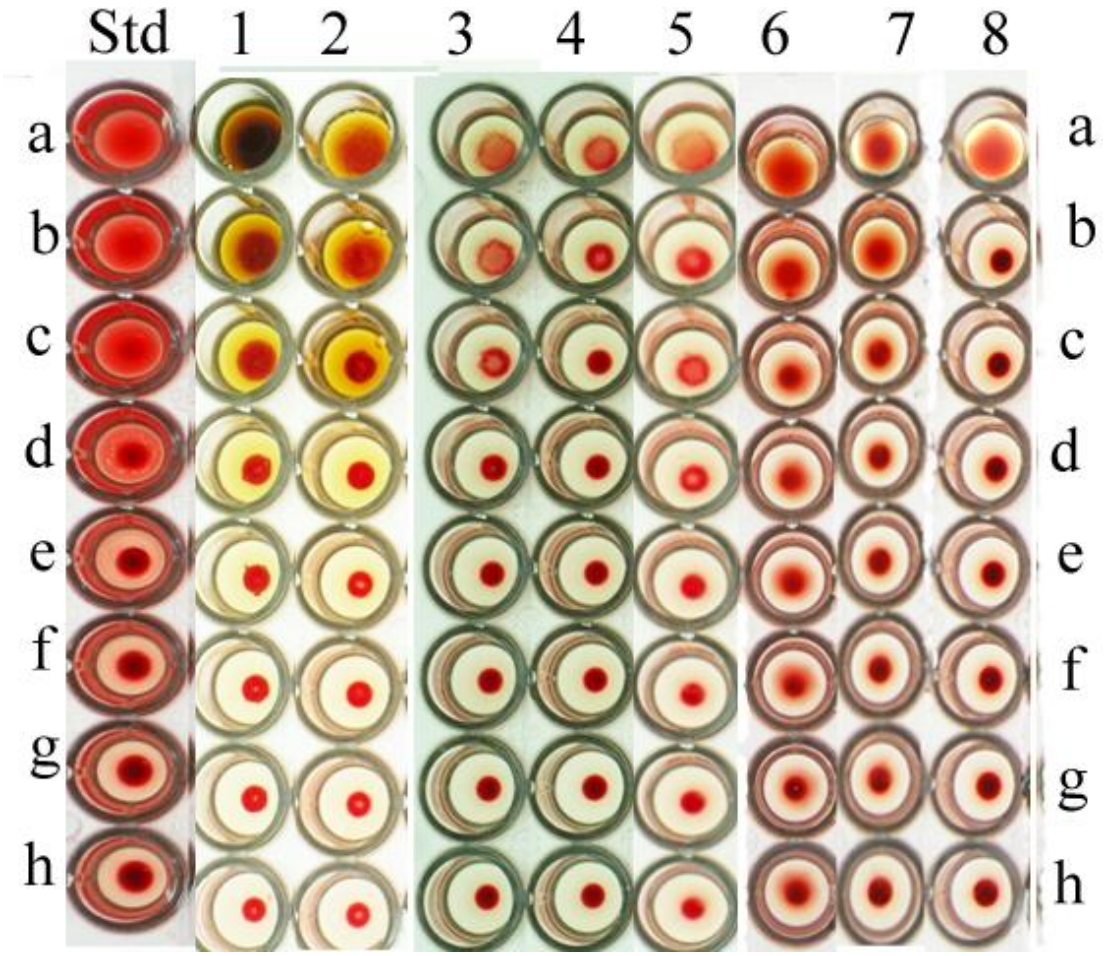

Figure 2. Hemagglutination test results of fresh, dry *U. dioica* L. herb and dry extract lectin enriched fractions. Std - Soy bean lectin standart, 1 – fresh *U. dioica* L. herb 40 % AS saturated precipitate, 2 - fresh *U. dioica* L. herb 80 % AS saturated extract precipitate, 3 Dry *U. dioica* L. herb 40 % AS saturated precipitate. 4 – dry *U. dioica* L. 80 % AS saturated precipitate, 5 – Dry *U.dioica* L. 80 % AS saturated supernatant, 6 – Dry *U. dioica* L. extract 40 % AS saturated precipitate, 7 - Dry *U. dioica* L. extract 80 % AS saturated precipitate, 8 Dry *U. dioica* L. extract 80 % AS saturated supernatant.

and 30 kDA. In protein precipitate fraction (80% saturated AS) (Net2) particles size was 120 kDA and 30 kDA. In protein supernatant fraction (80% saturated AS) (Net3) particles size was 120 kDA. According to literature lectin size is from 29 kDA to 34 kDa (Hänsel et al., 2007). Hemaglutination activity titre was measured by full agglutination of rabbit erythrocytes, diluted in serial way (ratio 1:1) lectin extracts. Std –Soy bean lectin standart (starting concentration (conc.) 0.5 mg/ml). Standart control agglutination founded in Std-a 0.5 mg/ml conc., Std-b 0.25 mg/ml conc. and Std-c 0.125 mg/ml conc. Hemagglutination was found in 1a, 1b, 1 c, 2a, 2b, 3a, 4a, 5a, 6a, 6b, 7a, 7b and 8a wells, Lectin content in each column : 1 to 0.01, 2 to 0.01, 3 to 0.05, 4 to 0.1, 5 to 0.01, 6 to 0.02, 7 to 0.02, 8 to 0.33% based on cell agglutinating activity as compared to specific hemagglutinating activity of lectin from *Solanum tuberosum*. (Figure 2).

DISCUSSION

It was investigated several *U. dioica* L. rhizomes samples and was found the general lectin –UDA in previous experiments. UDA is actualy a mixture of up to 11 different isolectins, which are monomeric proteins formed by 80 to 90 amino acids. With a molecular weight between 8300 and 9500 Dalton these lectins are among the smallest so far identified (Peumans et al.,1984).The quantitative results obtained by capillary electrophoresis (the total lectin content varied from 0 to 0.42% in the samples) were accurate. SDS-PAGE electrophoresis test showed that protein fractions include lectins particles. According to literature lectin size is from 29 kDA, to 34 kDa (Hänsel et al., 2007). In this study protein fractions of *U. dioica* L. herb and dry extract were isolated. The total concentration of protein in herb and dry extract of *U. dioica* L. revealed that lectin-like glycoprotein enriched

fractions contained specific hemagglutination activity up to 1.3 and 3.0 mg protein/ml, respectively and protein concentration was 0.09 to 2.25 mg /g weight. We established particles sized of 200, 120 and 30 kDA. The finding of separated lectins requires more detailed investigation.

Conclusions

Presence of hemagglutination activity was observed for all of the protein fractions isolated by salting out with ammonium sulphate from fresh and dry herb of *U. dioica* L. and dry extract of stinging nettle. The highest concentration of protein and specific hemagglutination activity were observed for protein fractions isolated from fresh herb. Estimated lectins content in fresh *U. dioica* L. herb based on functional activity using lectin from *S. tuberosum* as a standard 0.01%, in dry *U. dioica* L. herb varied from 0.01 to 0.1% and in dry *U. dioica* L. extract varied from 0.02 to 0.33%. The highest lectins content was presented in protein fractions isolated from the dry extract. However, hemagglutination activity was not detected in 80% saturated ammonium sulphate fraction.

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