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Polyphenolic-polysaccharide compounds from selected medicinal plants of *Asteraceae* and *Rosaceae* families: Chemical characterization and blood anticoagulant activity

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ABSTRACT

The preparations from selected traditional medicinal plants in Poland (*Asteraceae* and *Rosaceae* families), were prepared in the multi-step process of isolation and their anticoagulant activity was measured by APTT and PT tests. The most promising effect was observed for the substances extracted from *Fragaria vesca* (*Rosaceae*) and *Echinacea purpurea* (*Asteraceae*). They showed interesting activity with respect to the activity of 5th International Standard for Unfractionated Heparin in APTT method. The structure characterization by IR, HPLC, GLC-MS and colorimetric methods revealed that these preparations are macromolecular polysaccharide–polyphenolic conjugates, similar to cell wall acidic macromolecular fragments which are common in the higher plants. The high content of hexuronic acids, as well as phenolic glycoconjugates seems to be responsible for the observed anticoagulant activity.

1. Introduction

The pharmaceutical industry uses on a large scale animal polysaccharides, especially for treatment of cardiovascular system diseases. The most popular of them with the widest practical application is heparin. Heparin is one of the glycosaminoglycans, an acidic mucopolysaccharide containing sulphonic groups (BeMiller, 2008; Hirsh, 1991). Since its discovery by McLean in 1915 (McLean, 1959), heparin has become a widely used anticoagulant for the treatment and prevention of thrombotic diseases and for maintaining blood fluidity in extracorporeal devices (Freedman, 1992; Johnell et al., 2002). Blood coagulation is an enzymatic event initiated in response to tissue damage. Binding of circulating factor VII (FVII) to exposed tissue factor (TF) starts a cascade of reactions that ultimately leads to the formation of thrombin, which clots blood. However, dysfunctions of this process contribute to development and progression of many cardiovascular diseases, which are a major epidemiological problem all over the world. Anticoagulants inhibit thrombin generation and fibrin formation. The anticoagulant effect of heparin is mediated primarily by its binding to antithrombin (AT), thereby accelerating the latter's inhibitory function of factor Xa (FXa) and thrombin in plasma (Bisacchi, 2003). Unfortunately, the main complication with heparin therapy is that it occasionally causes life-threatening bleeding. Heparin causes also other problems including heparin-induced thrombocytopenia, and poor bioavailability (Blaisdell, 1996; Greinacher & Warkentin, 2006). There is a clinical need for new parenteral anticoagulants that are effective and safe when used in conjunction with either fibrynolytic therapy or antiplatelet agents in patients with cardiovascular diseases (Hirsh, 2001; Stone & Shore-Lesserson, 2006).

Polysaccharides with anticoagulant activity have been found not only in mammals (BeMiller, 2008; Bisacchi, 2003), but also in marine alga (Chuch, Meade, Treanor, & Whinna, 1989; Farias, Valente, Pereira, & Mourão, 2000; Mayer & Hamann, 2002, 2004, 2005, 2007; Pereira, Mulloy, & Mourão, 1999), and invertebrates (Mourão & Pereira, 1999). They all are highly anionic compounds mainly due to the presence of sulfate groups and, in some cases, hexuronic acids (Côte & Hahn, 1994). In contrast, polysaccharides isolated from higher plants do not contain sulfate groups and their anticoagulant activity is due to the presence of hexuronic acids residues, like GlcA or GalA, and its derivatives (Yoon et al., 2002). The higher plants rich in these saccharides belong to *Magnoliiceae*, *Hamameliceae*, *Rosaceae*, *Dilleniceae* and *Asteraceae* families. The higher plants cell walls components like hemiceluloses, pectins and pectin-like substances are the polysaccharides containing hexuronic acids (Côte & Hahn, 1994). Such substances showing antithrombotic and thrombolytic effects were isolated from root of *Paeonia suffructicosa* (Liapina et al., 1995), *Paeonia anomala* (Liapina, Kondashevskaia, Ziadetdinova, & Uspenskaia, 2000) and *Paeonia lutea* (Liapina, Kondashevskaia, & Smolina, 1997; Pastorova, Liapin-

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a, Uspenskaia, & Ziadetdinova, 1999). Polysaccharides rich in GalA, which inhibit blood clotting were also isolated from medicinal plants like *Porana volubilis, Listea cerbeba, Parameria laevigate* and *Piper betle*, collected in Asian countries: Korea, China, Indonesia and Malaysia (Yoon et al., 2002). All described plants grow however in Central Asia. They are often very demanding in cultivation in Europe, and require special climatic conditions. The extracts from flowers and seeds of European medicinal plant *Filipendula ulmaria* is also known to have high anticoagulant and fibrinolytic activity *in vitro* and *in vivo* tests (Liapina & Kovalchuk, 1993). Our research group has been working on isolation, structure characterization and biological activity of polysaccharides and their conjugates with polyphenols, from European and Polish folk medicine plants. We have found that macromolecular polyphenolic-polysaccharide conjugates isolated from several different plants of *Asteraceae* and *Rosaceae* families, have interesting anticoagulant activity *in vitro* tests, and except *Filipendula ulmaria* were not described previously.

2. Experimental

2.1. Materials

The plant materials were obtained from 17 popular Polish plants belonging to *Asteraceae* and *Rosaceae* families, purchased from local markets or collected from the natural environment. The list of studied plants is given in Table 1. They were identified by Professor Krystyna D. Kromer and by MSc Jolanta Kochanowska from Botanical Garden of Wrocław, Wrocław University, Wrocław, Poland and herbarium voucher specimens were deposited in Botanical Garden of Wrocław, Wrocław University, Wrocław, Poland. All reagents including monosaccharides and quercetin standards, were purchased from Sigma–Aldrich Co. The solvents were of analytical grade.

2.2. Extraction and purification

Dried parts of plant (200 g) were minced, suspended in 2 l of hexane and refluxed for 5 h at 69 °C, then filtered and the hexane fraction was removed. The procedure was repeated with the new portion of hexane. Then the plant residue was suspended in 2 l of 0.1 M NaOH, refluxed for 5 h at 97 °C, and a clear supernatant was obtained after centrifugation at 8000 rpm for 15 min at room temperature. The alkaline fraction was neutralized with 1 M HCl and concentrated on a rotary evaporator under reduced pressure to obtain the dry crude plant extract (it corresponds to 11.58 ± 4.85% of dried plant material). The extract was dissolved in 1 l of water, then 1 l of diethyl ether was added, and the mixture was stirred for 6 h at 34 °C. After that time the water-soluble fraction was collected by separating from the organic one. The procedure was repeated with the new portion of diethyl ether. Then 1 l of chloroform was added to the combined water extract. The mixture was refluxed for 6 h at 61 °C. Extraction process with chloroform was repeated, and then the water-soluble fraction was refluxed twice with the mixture of chloroform and ethanol (3:2) for 6 h at 70 °C. The water-soluble fraction after the multi-step extraction process with organic solvents was evaporated to dryness on a rotary evaporator under reduced pressure. Then it was suspended in 500 ml of methanol, stirred for 24 h at room temperature, and finally filtered off. This procedure was repeated 5 times. The precipitate was dried at room temperature, dissolved in distilled water, and dialyzed against water for 6 days (cut-off of dialysis tubing membrane was 12.5 kDa). The retentate was evaporated under reduced pressure to give macromolecular substance (1.58 ± 0.83% of weight of dried plant material) (Gancarz, Pawlaczyk, & Czerchawski, 2006).

2.3. General methods

The contents of hexoses and hexuronic acids were determined based on Dubois, Gilles, Hamilton, Rebers, & Smith, 1956 and

Table 1
Plants tested for anticoagulant activity, used in Polish phytotherapy with their therapeutic activities* (Stone & Shore-Lesserson, 2006; Strzelecka & Kowalski, 2000; Yoon et al., 2002).

No	Family name	Scientific name	English name	Parts used in article	Voucher number	Traditional use in Poland*
1	*Asteraceae*	*Arctium lappa* L.	Great burdock/	Flowers	007376	Diuretic, diaphoretic, antidiabetic, antiplatelet, bacteriostatic, anti-balding, antiseborrheic
2		*Arnica montana* L.	Mountain arnica	Flowers	002355	Antiseptic, on bruises, anti-inflammatory, circulatory stimulant, diastolique, anti-arthritis
3		*Artemisia vulgaris* L.	Common wormwood	Herb	004942	Bacteriostatic, cholagogue, carminatif, diastolique, on menstrual problems
4		*Calendula officinalis* L.	Garden marigold	Flowers	006689	Anti-inflammatory, bacteriostatic, on bruises, hemostatic, reduces cholesterol in blood, on menstrual problems
5		*Centaurea cyanus* L.	Cornflower	Flowers	008189	Diuretic, anti-inflammatory in eyes diseases
6		*Echinacea purpurea* (L.) Moench.	purple Coneflower	Flowers	011493	Immunostimulator, anti-inflammatory, anti-flu, on wounds healing, anti-eczemic
7		*Helichrysum arenarium* (L.) Moench.	Sandy everlasting daisy	Flowers	006305	Stomach tonic, cholagogue, diastolique, in atonic gallbladder, in gouty arthritis
8		*Hieracium pilosella* L.	Mouse-ear hawkweed	Flowers	002652	Diuretic, cholagogue, bacteriostatic
9		*Inula helenium* L.	Elecampane	Roots	002670	Cholagogue, diuretic, antihelminthic, expectorant, on wounds healing
10		*Solidago virgaurea* L.	Golden rod	Flowers	003501	Cholagogue, diuretic, anti-inflammatory on kidneys, immunostimulator
11		*Taraxacum officinale* Web.	Common dandelion	Roots	004998	Cholagogue, diuretic, arthritis, antihepatitic, antidiabetic, on upper airways infection, immunostimulator
12	*Rosaceae*	*Alchemilla vulgaris* L.	Common lady's mantle	Flowers	002274	Anti-inflammatory, gastritis, antidiarrheal, carminative, in burns
13		*Filipendula ulmaria* (L.) Maxim.	Meadowsweet	Flowers	005184	Anti-inflammatory, analgesic, antipyretic, anti-flu, bacteriostatic, cholagogue, on wounds healing
14		*Fragaria vesca* L.	Wild strawberry	Herb	004954	Diuretic, antidiarrheal, in vitamins deficiency
15		*Potentilla anserina* L.	Silverweed	Flowers	004984	Antidiarrheal, diastolique, on wounds healing, on menstrual problems
16		*Rosa canina* L.	Dog rose	Fruits	004009	In vitamin-C deficiency diseases, anti-flu, diuretic, cardiotonic
17		*Rubus plicatus* W. et N.	Blackberry	Leaves	013914	Antidiarrheic, bacteriostatic, anti-inflammatory, antidiabetic

carbazole (Dische, 1947) reactions, respectively. Hexoses and hexuronic acids were assayed using Glc and GalA, respectively, as standards. After acid hydrolysis of the plant substance (2 M HCl for 8 h at 100 °C) the monosaccharides were converted to the 1-phenyl-3-methyl-5-pyrazolone derivatives (PMP-derivatives), by the method described by Honda et al. (1989). The contents of monosaccharides in the acid hydrolyzate were estimated by the reversed-phase liquid chromatography (Macherey-Nagel C_{18} column, Shimadzu SLC-6B, LC-6A, SPD-6A with the UV detector). Independently the percentages of the different monosaccharides in the acid hydrolyzate were estimated in the form of their acetylated, borohydrate-reduced alditol acetates (Bierrnann & McGinnis, 1989) by gas–liquid chromatography (Hewlett-Packard Model 5891A Series II chromatograph equipped with a HP-1 column (0.22 mm × 25 m), the temperature program of 150–250 °C (8 °C/min) to 270 °C). Phenolic compounds were assayed, according to the Folin-Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventós, 1999; Singleton & Rossi, 1965) after acid hydrolysis. Samples were diluted in 0.1 M NaOH to final concentration 62.5 μg/ml, and the absorbance was measured at 765 nm. The amount of phenolic groups were evaluated based on the calibration curve with quercetin. Infrared spectra were recorded on KBr pellets with Perkin Elmer System 2000 FT-IR infrared spectrometer. All colorimetric assays were measured using UV–vis HP, 8452 A spectrophotometer.

2.4. Clotting assays

Clotting assays of activated partial thromboplastin time (APTT) and of prothrombin time (PT) were performed using standardized human plasma – MDA Reference Plasma® (purchased from BioMérieux S.A., Warsaw, Poland). Two separate assays measuring APTT and PT were carried out to investigate the stage, at which blood clotting pathways were inhibited. Each of the measured substances, plant extracts, plant purified substances and monosaccharide standards, were diluted in 0.1 M phosphate buffered saline (PBS) to give the following concentrations: 12.50, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20 and 0.10 mg/ml, in standardized human blood plasma, respectively.

The method described by Brown (1988) was used for the determination of PT. Thromboplastin–calcium reagent – Simplastin HTF® (BioMérieux S.A., Warsaw, Poland) was reconstituted with distilled water according to the manufacturer's instructions. It was then incubated in a water bath at 37 °C for at least 10 min before the test was commenced. MDA Reference Plasma ® was also reconstituted with distilled water according to the manufacturer's instructions. Plasma (100 μl) was placed in a test tube and incubated in the water bath for 3 min. For the controls, 100 μl of prewarmed PBS, followed by 200 μl of the prewarmed thromboplastin–calcium reagent was rapidly added to the plasma while simultaneously starting a timer. The test tube was then gently tilted back and forth, until a clot formed, and the clotting time was recorded. For the tests, 100 μl of the prewarmed sample solution in PBS was mixed with plasma, just before adding the thromboplastin–calcium reagent. All experiments were carried out in, at least duplicates.

The method described by Brown (1988) was used for the APTT tests. The partial tromboplastin with activator – Automated APTT® (BioMérieux S.A., Warsaw, Poland) was reconstituted with distilled water according to the manufacturer's instructions, and then was prewarmed in a water bath to 37 °C. The 0.02 M solution of calcium chloride was also incubated in the same conditions. Then 50 μl of plasma solution was placed in a test tube. After incubating for 3 min in the water bath, 50 μl of the partial tromboplastin solution with activator was added, and the contents were mixed rapidly. The mixture was then incubated for another 3 min, after which 50 μl of the prewarmed calcium chloride solution was added while simultaneously starting a timer. The tube was then allowed to remain in the water bath while, gently tilting the test tube every 5 s. At the end of 20 s, the test tube was removed, wiped clean with dry gauze, and gently tiled back and forth until a clot was seen and the time recorded. For the samples, 50 μl of the material was added to the contents of the test tube just prior to the addition of calcium chloride solution, and readings taken as before.

The anticoagulant activity of the test samples was expressed as clotting time measured in APTT test or IU/mg using a parallel standard curve based on the 5th International Standard for Unfractionated Heparin (ISUH) (229.5 IU/mg), established in 1998 (NIBSC Code: 97/578).

3. Results and discussion

3.1. Medicinal plants containing substances with anticoagulant activity

We have prepared the crude plant extracts, consisting of 12 ± 5% of mass of the dried plant material, from 17 plants, popular

Table 2
Activated thromboplastin time (APTT) of crude extracts isolated from different species of *Asteraceae* and *Rosaceae* families.

Plant extracts	APTT TEST							
	Control time[s]	Concentrations of extracts [mg/ml]						
		12.50	6.25	3.13	1.56	0.78	0.39	0.20	0.10
Arctium lappa L.	48.0	**>600.0**	**>600.0**	389.0 ± 12.5	201.0 ± 8.7	74.2 ± 3.3	31.2 ± 1.3	34.0 ± 1.5	29.8 ± 1.2
Arnica motana L.	42.4	**>600.0**	**>600.0**	**>600.0**	366.8 ± 18.2	205.8 ± 9.4	92.6 ± 7.4	53.1 ± 2.1	41.5 ± 1.7
Artemisia vulgaris L.	49.9	**>600.0**	130.4 ± 5.4	29.0 ± 1.1	26.1 ± 1.0	36.8 ± 1.1	47.0 ± 2.2	48.6 ± 2.5	48.9 ± 2.1
Calendula officinalis L.	46.3	423.0 ± 18.1	209.4 ± 9.3	129.0 ± 6.1	60.0 ± 2.8	49.4 ± 2.2	44.9 ± 2.0	42.6 ± 1.8	44.0 ± 2.2
Centaurea cyanus L.	45.8	130.1 ± 6.5	89.6 ± 4.4	58.0 ± 2.7	48.1 ± 2.2	44.0 ± 1.8	43.5 ± 2.0	42.3 ± 2.3	32.5 ± 2.4
Echinacea purpurea (L.) Moench.	46.1	**>600.0**	**>600.0**	**>600.0**	**>600.0**	544.7 ± 21.5	234.8 ± 11.6	96.7 ± 4.9	53.0 ± 2.6
Helichrysum arenarium (L.) Moench.	45.8	**>600.0**	479.4 ± 21.3	202.5 ± 9.7	87.6 ± 4.3	59.9 ± 3.1	50.9 ± 2.1	46.4 ± 2.1	46.0 ± 2.2
Hieracium pilosella L.	49.9	**>600.0**	**>600.0**	351.4 ± 14.2	150.0 ± 6.7	86.9 ± 4.4	60.7 ± 2.9	56.0 ± 2.3	51.2 ± 2.2
Inula helenium L.	54.8	225.0 ± 10.1	145.1 ± 6.9	83.0 ± 3.8	65.4 ± 3.2	52.2 ± 2.2	49.0 ± 1.8	53.6 ± 2.0	51.0 ± 2.5
Solidago virgaurea L.	46.5	**>600.0**	**>600.0**	**>600.0**	480.0 ± 19.6	199.3 ± 8.5	80.6 ± 3.8	50.4 ± 2.5	37.2 ± 1.6
Taraxacum officinale Web.	46.1	**>600.0**	281.2 ± 12.9	161.8 ± 7.7	78.2 ± 3.8	51.6 ± 2.4	40.0 ± 1.9	39.8 ± 2.0	41.0 ± 2.1
Alchemilla vulgaris L.	54.8	**>600.0**	**>600.0**	591.0 26.6	187.2 ± 8.7	137.7 ± 6.9	58.0 ± 2.8	52.7 ± 2.2	47.4 ± 1.9
Filipendula ulmaria (L.) Maxim.	54.8	**>600.0**	**>600.0**	**>600.0**	242.0 ± 11.5	89.8 ± 4.3	48.0 ± 2.1	46.3 ± 1.7	54.8 ± 2.2
Fragaria vesca L.	48.0	**>600.0**	**>600.0**	**>600.0**	**>600.0**	349.0 ± 15.7	204.0 ± 10.1	84.0 ± 4.1	50.8 ± 2.4
Potentilla anserina L.	55.8	**>600.0**	**>600.0**	**>600.0**	231.0 ± 11.7	97.4 ± 5.1	55.7 ± 2.2	48.0 ± 1.9	52.4 ± 2.3
Rosa canninia L.	49.9	**>600.0**	**>600.0**	383.2 ± 15.9	205.5 ± 9.7	83.0 ± 4.1	55.0 ± 2.6	48.0 ± 2.0	50.8 ± 2.4
Rubus plicatus W. et N.	39.5	**>600.0**	**>600.0**	**>600.0**	**>600.0**	243.6 ± 11.5	189.3 ± 8.8	79.4 ± 4.1	54.6 ± 2.3

The bold values indicates the clot was not observed in measured samples.
Values are expressed as mean of 5 measurements ± *SD*.

Table 3
Prothrombin time (PT) of crude extracts isolated from different species of *Asteraceae* and *Rosaceae* families.

Plant extracts	PT TEST							
	Control time[s]	Concentrations of extracts [mg/ml]						
		12.50	6.25	3.13	1.56	0.78	0.39	0.20	0.10
Arctium lappa L.	13.0	**>300.0**	51.4 ± 2.3	27.0 ± 1.3	18.0 ± 0.8	16.8 ± 0.7	14.2 ± 0.6	13.9 ± 0.6	14.4 ± 0.5
Arnica motana L.	13.7	**>300.0**	130.8 ± 6.3	39.5 ± 1.8	26.3 ± 1.2	18.4 ± 0.8	15.6 ± 0.7	14.1 ± 0.5	14.4 ± 0.6
Artemisia vulgaris L.	14.7	75.8 ± 3.7	29.9 ± 1.4	14.8 ± 0.7	13.6 ± 0.6	13.4 ± 0.5	14.0 ± 0.5	15.1 ± 0.7	15.1 ± 0.5
Calendula officinalis L.	13.8	32.1 ± 1.5	22.1 ± 10.2	16.5 ± 0.7	14.0 ± 0.5	13.3 ± 0.5	13.6 ± 0.6	13.6 ± 0.5	12.9 ± 0.4
Centaurea cyanus L.	13.4	17.8 ± 0.8	15.4 ± 0.7	14.2 ± 0.6	14.0 ± 0.5	14.0 ± 0.4	13.5 ± 0.5	14.0 ± 0.7	13.7 ± 0.4
Echinacea purpura (L.) Moench.	13.0	**>300.0**	**>300.0**	56.0 ± 2.6	25.0 ± 1.1	17.6 ± 0.7	15.3 ± 0.7	14.1 ± 0.6	13.5 ± 0.5
Helichrysum arenarium (L.) Moench.	13.4	81.7 ± 3.9	29.9 ± 1.4	20.1 ± 0.9	17.0 ± 0.7	14.3 ± 0.6	14.3 ± 0.5	13.8 ± 0.5	13.7 ± 0.3
Hieracium pilosella L.	14.7	177.0 ± 8.8	66.2 ± 3.0	29.5 ± 1.3	19.7 ± 0.9	15.6 ± 0.6	14.1 ± 0.7	13.4 ± 0.5	14.8 ± 0.4
Inula helenium L.	14.0	19.9 ± 1.0	17.1 ± 0.8	16.6 ± 0.8	14.4 ± 0.7	14.6 ± 0.5	15.2 ± 0.3	16.5 ± 0.7	15.7 ± 0.7
Solidago virgaurea L.	14.5	**>300.0**	**>300.0**	64.6 ± 3.1	38.2 ± 1.8	30.0 ± 1.3	20.3 ± 1.0	18.0 ± 0.7	13.6 ± 0.4
Taraxacum officinale Web.	13.0	25.9 ± 1.1	18.3 ± 0.8	15.8 ± 0.8	14.1 ± 0.6	13.4 ± 0.5	13.2 ± 0.6	13.0 ± 0.5	12.7 ± 0.5
Alchemilla vulgaris L.	14.0	**>300.0**	170.0 ± 8.7	30.4 ± 1.3	17.4 ± 0.8	14.8 ± 0.7	14.3 ± 0.6	15.0 ± 0.6	15.6 ± 0.7
Filipendula ulmaria (L.) Maxim.	14.0	**>300.0**	**>300.0**	59.8 ± 3.0	19.1 ± 0.8	15.0 ± 0.6	13.0 ± 0.7	13.5 ± 0.7	13.9 ± 0.6
Fragaria vesca L.	13.0	**>300.0**	**>300.0**	67.8 ± 3.3	25.0 ± 1.1	18.0 ± 1.0	15.5 ± 0.8	14.2 ± 0.7	13.4 ± 0.7
Potentilla anserina L.	15.6	**>300.0**	82.0 ± 3.9	29.9 ± 1.5	18.2 ± 0.8	15.3 ± 0.7	13.9 ± 0.5	13.8 ± 0.6	14.8 ± 0.7
Rosa canninia L.	14.7	**>300.0**	66.1 ± 3.0	29.1 ± 1.4	18.6 ± 1.0	14.9 ± 0.6	14.7 ± 0.7	14.4 ± 0.8	15.8 ± 0.6
Rubus plicatus W. et N.	12.8	**>300.0**	**>300.0**	75.2 ± 3.6	31.0 ± 1.4	18.4 ± 0.8	15.4 ± 0.6	14.2 ± 0.7	13.1 ± 0.5

The bold values indicates the clot was not observed in measured samples.
Values are expressed as mean of 5 measurements ± *SD*.

in Poland, belonging to *Asteraceae* and *Rosaceae* families (Table 1). We studied alkaline extracts, filtrated from precipitates and finally neutralized. Their anticoagulant activity was tested using activated partial thromboplastin time (APTT) and prothrombin time (PT). The results are shown in Tables 2 and 3. The most interesting activity was demonstrated by extracts isolated from *Arnica montana, Echinacea purpurea*, *Solidago virgaurea*, *Filipendula ulmaria*, *Fragaria vesca* and *Rubus plicatus*. They inhibited completely the plasma clot formation in APTT test, at the concentration as low as 3.13 mg/ml, whereas the extracts from *Echinacea purpurea*, *Fragaria vesca* as well as *Rubus plicatus* – still completely inhibited clotting process, even at the concentration of 1.56 mg/ml of plasma. The crude extracts from these plants completely inhibited clot formation in PT test, at the concentration of 6.25 mg/ml (Tables 2 and 3).

The strong anticoagulant activity of some crude extracts was the reason for further isolation and purification of the active products from selected plants. The last process was based on the multistep, two-phases extraction, the extraction from the solid state, and finally dialysis against water (Gancarz et al., 2006). The yield achieved value of 1–2.5% of mass of dried plant material (Table 6). The separated products were chemically characterized and their anticoagulant activity was measured by APTT test and PT test. The results are shown in Tables 4 and 5. The purified plant products demonstrated enhanced anticoagulant activity in comparison to the crude extracts. These purified products completely inhibited the clot formation, even at the concentration of 0.78 mg/ml of standardized human blood plasma, in APTT test, and at the concentration of 3.13 mg/ml in PT test. From all of the products, which demonstrated the highest anticoagulant activity in both clotting assays, two were outstanding – from *Echinacea purpurea* and from *Fragaria vesca*. These products in APTT test prolonged clotting time, even at the concentration of 100 μg/ml of human plasma. The purified product from *Echinacea purpurea* inhibited the clotting time in PT test still at the concentration of 0.78 mg/ml, and the product from *Fragaria vesca* – even at 0.39 mg/ml of human plasma.

The results of APTT test were compared with the 5th International Standard of Unfractionated Heparin (ISUH) (229.5 IU/mg) (Fig. 2A). The activity of plant products was compared with the standard calibration curve based on 5th ISUH (NIBSC Code: 97/578) and expressed in international units (IU). The activity of the product isolated from *Fragaria vesca* was the highest – almost 2 IU/mg. The activity of the product isolated form *Echinacea purpurea* was also high – 1.4 IU/mg (Fig. 2B). It is worth to point out that the activity of these extracts are greater than that of *Filipendula ulmaria* – 0.5 IU/mg, reported previously as promising anticoagulant plant extract (Liapina & Kovalchuk, 1993).

Since in the carbohydrate parts of the plant products we have found Ara – the monosaccharide typical for the pectin-like polysac-

Table 4
Activated thromboplastin time (APTT) of purified plant substances and selected monosaccharides.

Plant substances/monosaccharides	APTT TEST						
	Control time[s]	Concentrations of substances [mg/ml]					
		3.13	1.56	0.78	0.39	0.20	0.10
Arnica motana L.	53.0	**>600.0**	**>600.0**	**>600.0**	258.0 ± 11.2	102.2 ± 4.8	65.4 ± 3.3
Echinacea purpurea (L.) Moench.	53.0	**>600.0**	**>600.0**	**>600.0**	**>600.0**	419.0 ± 18.7	124.2 ± 5.5
Solidago virgaurea L.	53.0	**>600.0**	**>600.0**	**>600.0**	569.8 ± 26.2	210.6 ± 8.8	112.0 ± 5.1
Filipendula ulmaria (L.) Maxim.	57.3	**>600.0**	**>600.0**	**>600.0**	274.0 ± 10.2	113.9 ± 5.1	56.1 ± 2.1
Fragaria vesca L.	57.8	**>600.0**	**>600.0**	**>600.0**	**>600.0**	**>600.0**	240.9 ± 10.4
Rubus plicatus W.et N.	57.8	**>600.0**	**>600.0**	**>600.0**	435.5 ± 21.5	208.7 ± 8.9	111.1 ± 4.8
Arabinose (Ara)	49.0	50.6 ± 2.3	58.2 ± 2.7	62.0 ± 3.5	50.6 ± 2.7	51.4 ± 2.3	54.4 ± 2.5
Rhamnose (Rha)	49.0	50.4 ± 2.2	55.4 ± 2.4	54.1 ± 2.5	49.8 ± 2.2	55.2 ± 2.5	55.4 ± 2.6
Glucuronic acid (GlcA)	41.6	**>600.0**	50.8 ± 2.2	46.1 ± 2.3	41.0 ± 2.0	44.0 ± 2.3	44.3 ± 2.2
Galacturonic acid (GalA)	49.0	**>600.0**	56.4 ± 2.7	54.4 ± 2.5	45.8 ± 2.2	42.0 ± 1.8	41.2 ± 2.2

The bold values indicates the clot was not observed in measured samples.
Values are expressed as mean of 5 measurements ± *SD*.

Table 5
Prothrombin time (PT) of purified plant substances and selected monosaccharides.

Plant substances/monosaccharides	PT TEST					
	Control time[s]	Concentrations of substances [mg/ml]				
		3.13	1.56	0.78	0.39	0.20	0.10
Arnica motana L.	15.9	**>300.0**	85.0 ± 4.4	42.7 ± 2.2	28.1 ± 1.1	16.3 ± 0.9	13.8 ± 0.6
Echinacea purpurea (L.) Moench.	15.9	**>300.0**	**>300.0**	**>300.0**	58.0 ± 2.8	27.1 ± 1.4	15.8 ± 0.8
Solidago virgaurea L.	15.9	**>300.0**	**>300.0**	84.8 ± 4.4	38.4 ± 1.9	20.6 ± 1.2	15.9 ± 0.7
Filipendula ulmaria (L.) Maxim.	14.5	**>300.0**	50.0 ± 2.2	28.0 ± 1.1	17.5 ± 0.7	16.0 ± 0.7	14.7 ± 0.5
Fragaria vesca L.	14.6	**>300.0**	**>300.0**	**>300.0**	**>300.0**	25.5 ± 1.1	17.2 ± 0.7
Rubus plicatus W.et N.	14.6	**>300.0**	138.6 ± 6.6	38.6 ± 1.8	20.6 ± 0.8	16.4 ± 0.7	14.0 ± 0.6
Arabinose (Ara)	13.1	13.2 ± 0.2	13.7 ± 0.3	13.0 ± 0.2	13.0 ± 0.3	13.4 ± 0.4	13.6 ± 0.4
Rhamnose (Rha)	13.1	13.3 ± 0.3	12.5 ± 0.2	12.6 ± 0.3	13.2 ± 0.2	12.4 ± 0.4	12.9 ± 0.3
Glucuronic acid (GlcA)	13.0	**>300.0**	18.4 ± 0.7	14.0 ± 0.5	13.4 ± 0.3	13.6 ± 0.3	13.4 ± 0.2
Galacturonic acid (GalA)	13.1	**>300.0**	21.7 ± 0.8	15.8 ± 0.6	14.6 ± 0.4	13.2 ± 0.4	13.0 ± 0.3

The bold values indicates the clot was not observed in measured samples.
Values are expressed as mean of 5 measurements ± *SD*.

charides of the higher plants, Rha – deoxysaccharide and uronic acids (UA), the anticoagulant activity of these monosaccharides was also examined. The mono-hexuronic acids – GlcA and GalA showed much weaker anticoagulant activity in both tests, than the purified plant products. Both hexuronic acids completely inhibited clot formation in the APTT test, at the concentration as high as 3.13 mg/ml of plasma (Tables 4 and 5).

The results indicate that not only the presence of the sulfate groups like in heparin, determines the anticoagulant activity. It can also be due to carboxylic groups, though the effect of the acidic monosaccharides is not very strong. The effect is undoubtedly strengthened by the polymeric composition of the acidic polysaccharides (Yoon et al., 2002) and the polyphenols. This is the case of the products isolated by us from the selected plants.

3.2. Chemical analysis

All the substances isolated from the selected six different plant materials were found to be macromolecular compounds. They do not penetrate through the dialysis membrane with the cut-off 12.5 kDa. These substances constituted only about 1–2.5% of mass of the dried plant materials, from which they were isolated (Table 6). All of them were dark brown water-soluble products. They were also very good soluble in alkali solutions, and non-soluble in organic solvents like methanol, ethanol, acetone, chloroform, diethyl ether, hexane, with the exception of DMSO. All of these products were analysed by FT-IR spectroscopy. The all spectra FT-IR were very similar (Fig. 1), and typical for polyphenolic pigments isolated from higher plants, for example from tea (Bilinska, 1996; Paim, Linhares, Magrich, & Martin, 1990; Sava, Yang, Hong, Yang, & Huang, 2001). A group of signals observed at 3410–3383 cm^{-1} corresponds to the stretching vibrations, and at 1074–1033 cm^{-1} to bonding vibrations of O—H groups. Signals between 2950 and 2850 cm^{-1} are from stretching vibrations of aliphatic C—H groups, especially from methyl (CH_3) group of the methyl esters. The strong bands at 1650 and 1614 cm^{-1} are due to vibrations of carbonyl (C=O) and aromatic (C=C) groups. The carbonyl absorption band shown at 1750 cm^{-1} comes from esterified (COO-R) carbonyl groups. Signals from stretching vibrations of carboxylic (COO^-) group: asymmetric at 1531–1519 cm^{-1}, and symmetric at 1415–1403 cm^{-1} are also present. A strong band at about 1263–1228 cm^{-1} is due to stretching asymmetric vibrations of C—O bonds. The absence of strong signals at 1350–1300 cm^{-1} and at 1160–1040 cm^{-1} indicates the absence of sulfate esters in analysed substances. The region at 1200–1000 cm^{-1} is also dominated by ring vibrations overlapped with stretching vibrations of (C—OH) side groups and the glycosidic (C—O—C) bond vibrations between monosaccharides in polysaccharide chains (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The absorption bands typical for anomeric regions of polysaccharides are also present, at 892, 868 and 856 cm^{-1} for β-anomers, and signals at 816, 812 and 804 cm^{-1} suggest the α-type of glycosidic linkages (Kačuráková et al., 2000).

The analysed substances composed of saccharide part and phenolic part were also characterized after acidic hydrolysis. The presence of neutral carbohydrates was detected by the phenol-sulfuric method (Dubois et al., 1956), which is non-specific method indicating aldoses, ketoses and alduronic acids. Neutral saccharides were found in all six plant substances. The presence of hexuronic acids was detected by the carbazole assay (Dische, 1947). The analysed preparations contained UA in the concentration range 25–55.9% of weight of the total saccharide compositions. The highest amounts of UA were found in the substances isolated from *Filipendula ulmaria* (55.9%), *Fragaria vesca* (53.0%) and from *Rubus plicatus* (41.9%). The FT-IR spectrum of the substance isolated from *Filipendula ulmaria* suggests the presence of the carboxylic groups in the esterified (COO-R) forms in greater concentration than in other analysed plant extracts. Chemical analysis showed that besides hexoses like Glc, Gal and Man, the other monosaccharides typical

Table 6
Plant substances after isolation and purification process. Content of monosaccharides in carbohydrate parts. Phenol content in the phenolic parts of the analysed plant substances, expressed as quercetin equivalents. For details see text.

Plant substance isolated from	Yield after puriffication process (wt%)	Yield of the polysaccharide part (wt%)	Content of monosaccharides HPLC and GLC-MS analysis (wt%)						UA	Total phenols mean ± *SD* [μM]
			Rha	Ara	Xyl	Man	Glc	Gal		
Arnica motana L.	0.9	11.3	3.5	21.1	0.8	0.9	21.0	22.8	29.9	4831.56 ± 5.04
Echinacea purpurea (L.) Moench.	1.8	16.4	4.2	28.6	2.4	0.6	8.0	23.5	32.7	4792.19 ± 4.28
Solidago virgaurea L.	1.0	13.4	6.0	17.2	1.6	1.6	17.8	30.8	25.0	5084.67 ± 4.11
Filipendula ulmaria (L.) Maxim.	2.5	18.5	5.5	13.6	4.2	0.9	4.2	15.7	55.9	4957.83 ± 3.56
Fragaria vesca L.	1.4	15.2	10.0	6.8	1.4	0.9	8.6	19.3	53.0	6704.60 ± 5.95
Rubus plicatus W.et N.	2.0	8.0	2.4	9.8	1.1	2.9	25.8	16.1	41.9	6260.24 ± 6.76

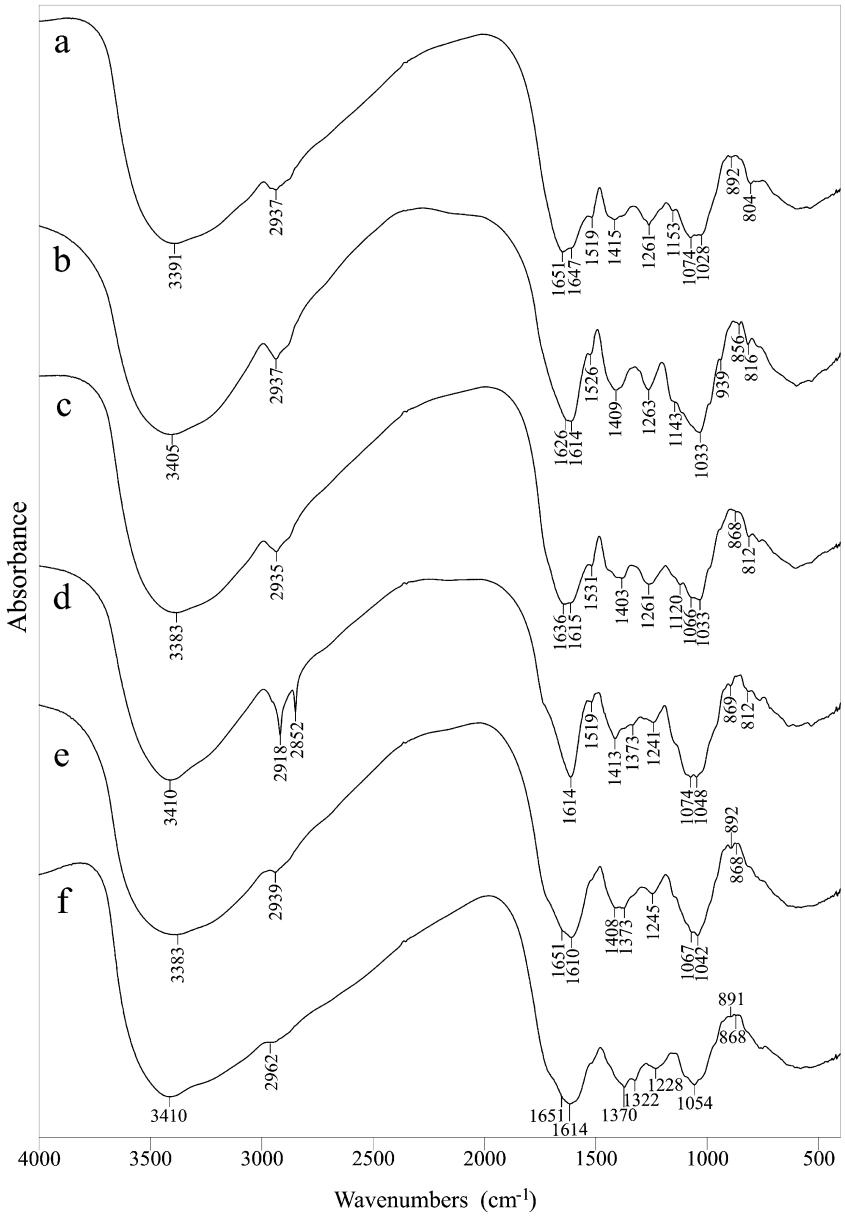

Fig. 1. FT-IR spectra of plant substances isolated from *Echinacea purpurea* (a), *Arnica montana* (b), *Solidago virga aurea* (c), *Filipendula ulmaria* (d), *Fragaria vesca* (e), *Rubus plicatus* (f).

for cell wall acidic polysaccharides of the higher plants like Ara, Rha, and Xyl are present (Côte & Hahn, 1994). The composition of saccharide parts of the plant substances was determined by high pressure liquid chromatography (HPLC) and by gas–liquid chromatography–mass spectrometry (GLC–MS). The results are shown in Table 6. HPLC and GLC–MS analyses indicated the presence of Gal (15.7–30.8 wt.%), Ara (6.8–28.6 wt.%) and Rha (2.4–10.0 wt.%), and much smaller amounts of Xyl (0.8–4.2 wt.%) and Man (0.6–1.6 wt.%).

After acidic hydrolysis of each of the six plant products, the dark brown precipitates were received. They were non-soluble in water as well as in organic solvents except DMSO. They were however well soluble in alkaline solutions, at pH 8.9–9.5. These behavior and conclusions from FT-IR spectra suggested that they are phenolic substances. This hypothesis was examined by Folin–Ciocaltelu assay (Singleton & Rossi, 1965; Singleton et al., 1999). To determine the quantity of phenolic substances in the precipitates quercetin as a phenolic standard was used for calibration (Singleton & Rossi, 1965; Singleton et al., 1999). The precipitates isolated by us from all these plant materials possessed very high amounts of phenolic groups (Table 6). The highest amounts of phenolic fragments were observed in the preparations isolated from *Fragaria vesca* and from *Rubus plicatus* (Ivanova, Gerova, Chervenkov, & Yankova, 2005).

4. Conclusion

Plants, which belong to *Asteraceae* and *Rosaceae* families, are very popular in folk medicine in Poland (Kohlmünzer, 2003; Ożarowski & Jaroniewski, 1987; Strzelecka & Kowalski, 2000). These plants are also known as being rich in acidic polysaccharides, due to presence of hexuronic acids or its derivatives (Côte & Hahn, 1994). The aim of the presented work was to obtain heparin-like acidic polysaccharides. Our procedure was not directed for isolation of the polyphenolic substances, however, the preparations we have obtained appeared to contain polyphenols of macromolec-

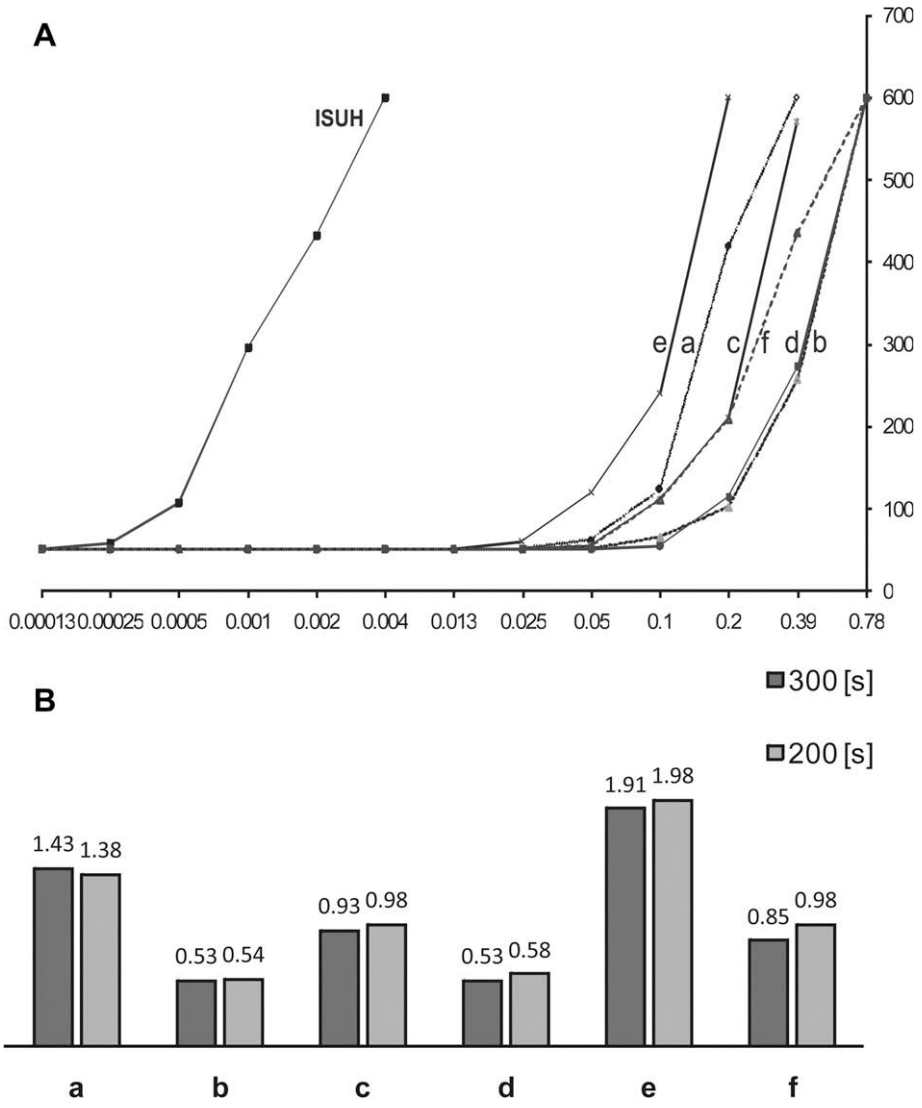

Fig. 2. (A) The anticoagulant activity of the purified plant substances isolated from *Echinacea purpurea* (a), *Arnica montana* (b), *Solidago virga aurea* (c), *Filipendula ulmaria* (d), *Fragaria vesca* (e), *Rubus plicatus* (f), determined based on APTT assays, and expressed as IU/mg using a parallel standard curve, based on the 5th International Standard for Unfractionated Heparin (ISUH) (229.5 IU/mg). (B) The anticoagulant activity of the purified plant substances expressed as IU/mg.

ular nature in addition to polysaccharides. The presence of acidic polysaccharides and polyphenols could be the result of polysaccharide chains functionalization by polyphenols, either by covalent bonds (Renard, Guyot, & Drilleau, 2001) or by complex formation. The presented polyphenolic glycoconjugates were isolated using the procedure similar to that described by Yoon et al. (2002), but the anticoagulant activity of the preparations from the plant materials we have studied had not been reported earlier, except of *Filipendula ulmaria* (Liapina & Kovalchuk, 1993). The obtained extracts are mixtures of high molecular weight polymers and further separation of the plant preparations by gel filtration could lead to better purified compounds with higher biological activity (Yoon et al., 2002).

Our studies, as well as the literature data on the plant polysaccharides and their therapeutic properties, permit us to judge, that after fractionation of the presented plant preparations and receiving more active compounds, they may become a new source of anticoagulant compounds. Their advantage is that they do not bring some threats of enzootic, bacterial or virus diseases. The low costs of manufacturing of plant medicines are additional advantages of usage the plant materials.

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