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Effects of extraction condition on structural features and anticoagulant activity of *F. vesca* L. conjugates

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ABSTRACT

From the air-dried Wild strawberry (*Fragaria vesca* L., family Rosaceae) leaves five water-soluble glycoconjugates **Fv I–V** by different extraction conditions have been isolated. Effects of extraction steps/agents on chemical composition and anticoagulant activity of **Fv I–V** were examined. Dark brown *F. vesca* conjugates **Fv I–V** were recovered in 4.5–8.4% yields, based on dry herb. Isolates were composed of carbohydrate, phenolic and protein components. **Fv I–V** displayed on HPLC broad molecule-mass distribution patterns with dominance of low molecule-masses 9–14 kDa. Their carbohydrate parts revealed high hexuronic acids content (35–60%) while the dominant neutral sugars – galactose, arabinose and rhamnose were found in lower amounts and indicated the presence of rhamnogalacturonans associated with arabinogalactans in all *F. vesca* preparations. In all **Fv I–V** isolates high polyphenolic contents were determined, whereas proteins were found in low amounts only. In *in vitro* experiments on human pooled plasma **Fv I–V** showed at higher concentrations complete inhibition of plasma clot formation and the most active conjugates in aPTT, PT and TT tests were shown to be **Fv I** and **Fv III**, containing the highest amounts of phenolics.

1. Introduction

The medicinal plant *Fragaria vesca* L. (Rosaceae) is commonly known as the Woodland strawberry or Wild strawberry. It naturally occurs throughout the Northern hemisphere. Woodland strawberry fruit is strongly flavored, and is cultivated and collected for domestic use as well as commercially as an ingredient into jams, sauces, liqueurs, cosmetics and components of natural medicines (Strzelecka & Kowalski, 2000). Various beneficial biological effects of strawberry fruits consumption have been documented, such as an increase of the serum antioxidant capacity in humans (Cao, Russell, Lischner, & Prior, 1998), anti-carcinogenic activity (Carlton et al., 2001; Wedge et al., 2001) and antithrombotic effects (Naemura et al., 2005). Moreover, the water extract of leaves is a direct, endothelium-dependent vasodilator (Mudnic et al., 2009). These beneficial effects have been mostly attributed to the polyphenolic compounds found in large quantities in strawberry fruits (Hannum, 2004). However, strawberry leaves, as a source of bioactive compounds with potentially beneficial biological effects, have been largely overlooked by researchers. Apart from the reports on the use of Wild strawberry leaves in traditional medicine as an aqueous extract for the treatment of several diseases (Strzelecka & Kowalski, 2000), scientific reports of its effects on biological systems are lacking. Furthermore, dried and milled leaves are regularly distributed by herbal pharmacies and are commonly used in the general population for the preparation of a strawberry tea.

The antithrombotic effect of strawberry fruits (Naemura et al., 2005) motivated our attention to look into vegetative parts of this medicinal plant in term of bioactive compound contents as well as their possible anticoagulant activities. It has been found that cardiovascular diseases are the most spread illnesses nowadays all over the world. Between years 2006 and 2015, deaths due to non-communicable diseases, half of which will be due to cardiovascular disease, are expected to increase by 17%. The clinical manifestations of these diseases include angina, myocardial infarction, transient cerebral ischemic attacks and strokes (World Health Organization, 2007). Their treatment requires mainly anticoagulant and/or antiplatelet agents. These pharmaceuticals inhibit the enzymes of the coagulation pathway or the activation and aggregation of platelets (Freson, Thys, Wittevrongel, & Geet, 2006; Levine, Hirsh, & Kelton, 1988). In the world there is a continuous interest for searching

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of novel, therapeutically better, more effective anticoagulant and anti-platelet agents, with multiple targets, without unfavorable side effects. Medicinal plants were found to be a good source for searching of new anticoagulant agents of plant origin (Chua & Koh, 2006). Recently, plant glycoconjugates from Asteraceae and Rosaceae families showed interesting anticoagulant and/or pro-coagulant activities (Pawlaczyk, Capek, et al., 2011; Pawlaczyk et al., 2010; Pawlaczyk, Czerchawski, et al., 2011; Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska, & Gancarz, 2009).

The aim of this work was to isolate polysaccharide–polyphenolic conjugates from leaves of *F. vesca* L. using different extraction steps/agents, to characterize and to compare their chemical compositions, *i.e.* carbohydrate, phenolic and protein contents, monosaccharide composition, uronic acids content and to verify their possible anticoagulant or pro-coagulant effects on human blood plasma using aPTT, PT and TT *in vitro* tests.

2. Materials and methods

2.1. Plant material

Dry leaves of the medicinal plant *F. vesca* L. were purchased from the local market. The identity of the plant was certified by Prof. Krystyna D. Kromer and MSc Jolanta Kochanowska from Botanical Garden of Wrocław University, Wrocław, Poland and a voucher specimen (No. 004954) has been deposited in the Botanical Garden of Wrocław University, Wrocław, Poland.

2.2. Isolation of Fragaria vesca glycoconjugates

F. vesca glycoconjugates **Fv I–V** were isolated according described procedure with some modifications (Gancarz, Pawlaczyk, & Czerchawski, 2006). Air-dried leaves were minced and divided into five equal parts. One part was suspended in 0.1 M NaOH at room temperature for 24 h and refluxed for 6 h at 97 °C. The solid part was removed by centrifugation (1850 × *g*; 20 min) and the supernatant was neutralized (1 M HCl). The supernatant was further concentrated to smaller volume and extracted twice with hexane (water:hexane 1:1, v/v) for 6 h at 69 °C, then with diethyl ether (1:1, v/v) for 6 h at 34 °C, then with chloroform (1:1, v/v) for 6 h at 61 °C, and finally with same proportions of chloroform and ethanol mixture (chloroform:ethanol 3:2, v/v) for 6 h, at 70 °C. After every extraction process the organic part was removed. The water-soluble material was evaporated to a paste like form and treated with methanol at room temperature. The soluble part was removed by filtration while residue was dissolved in distilled water and exhaustively dialyzed (MWCO 12–14 kDa) against distilled water, and freeze-dried to give the dark brown *F. vesca* conjugate **Fv I** (Fig. 1).

The second part of the plant material was defatted with methanol in Soxhlet apparatus (4 days) followed by acetone (4 days). The insoluble part was air-dried and suspended in 0.65 M NaOH for 24 h at room temperature and refluxed for 6 h at 97 °C. The solid part was removed by centrifugation (1850 × *g*; 20 min) and the supernatant was neutralized (1 M HCl). The next purification steps of the supernatant were similar as that of **Fv I** and gave after freeze-drying the dark brown *F. vesca* conjugate **Fv II** (Fig. 1).

The third part of the plant material was defatted by methanol and acetone, as that of **Fv II** sample. The plant material was air-dried and suspended in 0.1 M NaOH for 24 h at room temperature, and refluxed for 6 h at 97 °C. The solid part was removed by centrifugation (1850 × *g*; 20 min) and the supernatant was neutralized (1 M HCl). The next purification steps of the supernatant by organic solvents were similar, as that of **Fv I**. The final product after dialysis was freeze-dried to give the dark brown *F. vesca* glycoconjugate **Fv III** (Fig. 1).

The next part of the plant material was defatted by methanol and acetone as those of **Fv I** and **Fv II** samples. The plant material was air-dried and suspended in sodium acetate buffer (pH 5.0) for 24 h at room temperature, and refluxed for 6 h at about 100 °C. The solid part was removed by centrifugation (1850 × *g*; 20 min) and the supernatant was neutralized (0.1 M NaOH). The next purification steps of the supernatant by organic solvents were similar as those of **Fv I–III** conjugates. The final product was freeze-dried to give the dark brown *F. vesca* glycoconjugate **Fv IV** (Fig. 1).

The last *F. vesca* glycoconjugate **Fv V** was received after next steps of procedure made on glycoconjugate **Fv IV**. Glycoconjugate **Fv IV** was dissolved in 0.1 M NaOH at room temperature for 24 h and refluxed for 6 h at 97 °C. Thereafter, the solution was centrifugated eventually to remove some precipitation (1850 × *g*; 20 min) and the supernatant was neutralized (1 M HCl), and concentrated under the reduced pressure to lower volume. The concentrated solution was dialyzed (MWCO 12–14 kDa) against distilled water, and freeze-dried to give the dark brown *F. vesca* conjugate **Fv V** (Fig. 1).

2.3. General methods

Glycoconjugates were hydrolyzed with 2 M TFA for 1 h at 120 °C and the quantitative determination of the neutral monosaccharides was carried out in the form of their borohydrate-reduced alditol acetates (Bierrmann & McGinnis, 1989) by GLC–MS method on a Focus ITQ 700 chromatograph coupled with ion trap detector, Thermo Scientific, equipped with a Rtx-225 column (0.25 mm × 30 m). The oven temperature program in the range of 170–180 °C (1 °C/min heating rate) followed by heating in the range of 180–235 °C (3 °C/min heating rate) and the flow rate of carrier gas – helium 1 mL/min (Shapira, 1969). The total content of carbohydrates in the samples was estimated by the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The hexuronic acids content was determined with *m*-hydroxybiphenyl reagent (Blumenkrantz & Asboe-Hansen, 1973). The content of phenolics was measured by Folin–Ciocalteu assay, using gallic acid as a standard, and the result was expressed as gallic acid equivalent (GAE) (Singleton, Orthofer, & Lamuela-Raventós, 1999). The protein content was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). All colorimetric assays were measured using Cecil CE 2021 spectrophotometer.

2.4. FT-IR, UV–vis and NMR spectroscopies

Fourier-transform infrared (FT-IR) spectra were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra of KBr pellets (2 mg of a sample/200 mg KBr) were collected in the middle region from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. The UV–vis spectra of water solutions of glycoconjugates (c = 0.2 mg/mL) were recorded on the UV-1800 spectrophotometer (Shimadzu, Japan). ^{1}H NMR spectra of samples dissolved in D_2O were measured at 25 °C on 600 equipped with HCN ^{13}C enhanced salt tolerant cold probe and HSQC spectra at 400 MHz VNMRS Varian in ^{1}H–$^{19}F/^{15}N$–^{31}P 5 mm PFG AutoX DB NB probe. gHSQCAD Sequence from Varian pulse sequence library was used for measurement of ^{1}H–^{13}C heterocorrelated spectra.

2.5. In vitro clotting assays

The substrates for aPTT and PT times measurements including standardized human plasma – MDA Reference Plasma®, TriniCLOT aPTT HS, and TriniCLOT PT HTF, produced by Trinity Biotech Ireland, were purchased from Horiba ABX Sp. z o.o. (Warsaw, Poland).

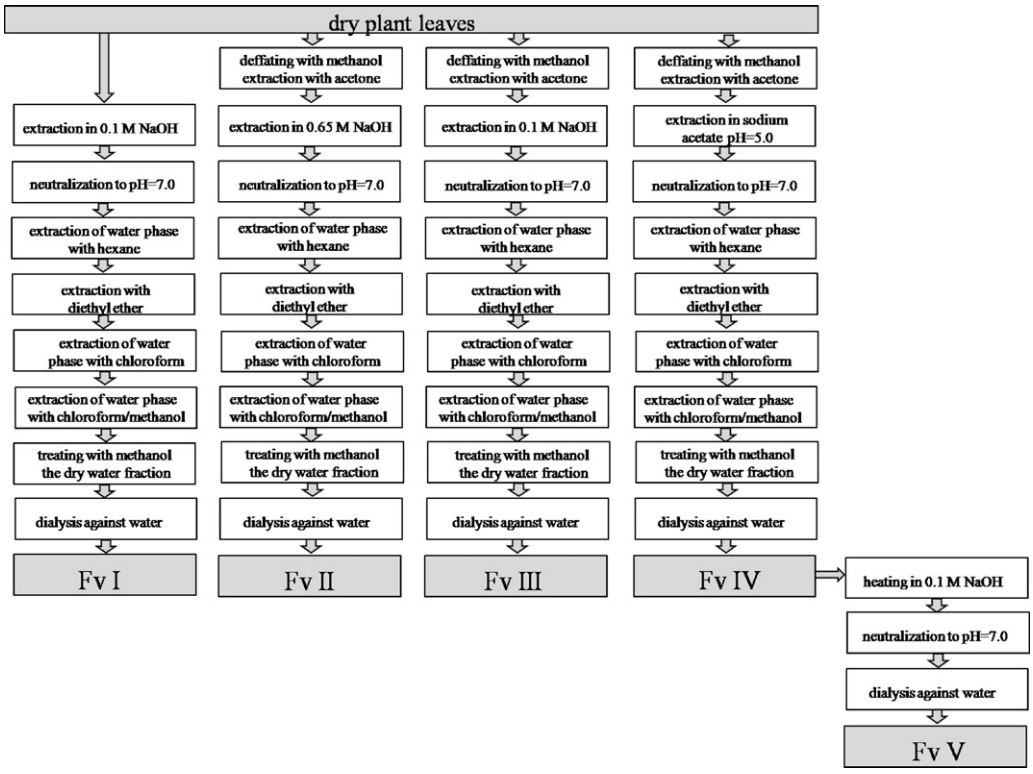

Fig. 1. Isolation procedure of *F. vesca* (**Fv I–V)** conjugates.

All experiments were carried out three times, and were measured automatically using Option 2 Plus coagulometer (BioMérieux, France). Three separate assays measuring aPTT, PT and TT (Brown, 1988) were carried out to investigate the stage, at which extrinsic, intrinsic blood clotting pathways, and the final stage of conversion of fibrinogen to fibrin, were prolonged. The anticoagulant activity of the series of the tested *F. vesca* glycoconjugates in the different concentrations was expressed in seconds, as clotting time measured in aPTT, PT and TT tests. The following concentrations of glycoconjugates were used in the clotting mixtures: 4000, 2000, 1000, 500, 250, 125, 62.50, 31.25, 15.63 and 7.81 μg/mL. The anticoagulant activity of samples measured in aPTT test was compared to unfractionated heparin (144 IU/mg). The determination of aPTT (Brown, 1988) was made using the partial thromboplastin with activator –TriniClot aPTT HS which was at first pre-warmed in a dry bath to 37 °C. The solution of calcium chloride (0.025 M) was also incubated in the same conditions. Then 50 μL of plasma solution was placed in a test tube with 25 μL of water, for control test. After incubating for 3 min in the dry bath, 50 μL of the partial thromboplastin solution with activator was added, and the contents were mixed rapidly. The mixture was then incubated for another 3 min in 37 °C, and then 50 μL of the pre-warmed calcium chloride solution was added 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, instead of water 25 μL of the pre-warmed sample solution was mixed with 50 μL of plasma. The experiment was done for unfractionated heparin in different concentrations.

To determine PT (Brown, 1988) thromboplastin-calcium reagent – TriniClot PT HTF was reconstituted with distilled water according to the manufacturer's instructions. It was then incubated in a dry bath at 37 °C for at least 30 min before the test was commenced. Human plasma was also reconstituted with distilled water according to the manufacturer's instructions. 50 μL of plasma was placed in a test tube and 25 μL of water, for control test. This mixture was incubated in the dry bath for 3 min. Thereafter, 100 μL of pre-warmed 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, instead of water 25 μL of the pre-warmed sample solution was mixed with 50 μL of plasma.

2.6. Statistical analysis

Statistical evaluation was carried out with Microsoft Office Excel 2010. Data are expressed as the mean ± S.D. Significant differences between the treated groups and the control were determined by the Student's *t*-test, at a level of $P < 0.05$.

3. Results and discussion

3.1. Isolation and analyses of F. vesca glycoconjugates

From the leaves of medicinal plant *F. vesca* by different extraction condition (defatted or not with methanol and then acetone, 0.1 and 0.65 M sodium hydroxide, and sodium acetate buffer of pH 5.0) followed by multi-steps two-phases organic extractions, dialysis and freeze-drying five dark-brown glycoconjugates **Fv I–V** have been isolated (Fig. 1). Their characteristics, *i.e.* yield, carbohydrates, proteins and phenolics contents, compositional analyzes and molecular mass are given in Table 1. The **Fv I–V** conjugates were recovered in 3.9–8.4% yields of starting the dry plant material (w/w). The highest yield 8.4% was recovered from not defatted leaves by 0.1 M NaOH (**Fv I**) and lowest one 3.9% by acetate buffer (**Fv IV**). However, the high yield in **Fv I** probably was due to elimination of the first step, *i.e.* methanol and acetone extractions, where significant amounts of extractive compounds may be removed. There were no significant differences in yields of **Fv II–V** conjugates

Table 1
Characterization of *F. vesca* **Fv I–V** glycoconjugates.

Plant conjugate	Yield (wt%)	[a]Total phenols [mM]	[b]Total sugar content (wt%)	Protein content (wt%)	[c]UA content (wt%)	Monosaccharide composition of carbohydrate part (wt%)							
						Rha	Fuc	Ara	Xyl	Man	Gal	Glc	[d]UA
Fv I	8.4	3.29	21.1	1.1	12.8	13.9	0.4	9.4	4.4	0.6	7.9	2.7	60.7
Fv II	5.3	1.17	28.5	0.5	8.8	13.2	n.d.	22.8	6.7	n.d.	15.4	11.0	30.9
Fv III	4.5	3.54	31.7	1.3	11.2	25.1	0.6	16.5	5.0	0.7	12.1	4.7	35.3
Fv IV	3.9	2.79	28.6	1.0	15.3	9.2	0.5	15.4	4.6	1.5	12.2	3.1	53.5
Fv V	3.7	0.81	29.0	0.8	12.9	3.9	n.d.	21.0	4.5	1.5	17.2	7.4	44.5

n.d. – not detected.
[a] Phenolic content expressed in mM of gallic acid equivalent (GAE) per 1 g of the plant glycoconjugate.
[b] Total sugar content determined by phenol-sulfuric assay.
[c] UA – total uronic acids content (wt%) in **Fv I–V** conjugates estimated by *m*-hydroxybiphenyl reagent.
[d] UA – uronic acid contents (wt%) calculated on carbohydrate parts in **Fv I–V** conjugates.

in which methanol and acetone purification steps were involved. The highest carbohydrate content 31.7% was determined in **Fv III** and the lowest one 21.1% in **Fv I** conjugates. From the Table 1 it is evident that there is no significant difference in carbohydrate contents in **Fv II–V**, although various extraction agents were used. Protein content was relatively low in all conjugates and varied from 0.5 to 1.3%. However, phenolic components were present in significant amounts in all **Fv I–IV** conjugates. The highest content was found in **Fv III** (3.5 mM of GAE) and **Fv I** (3.3 mM of GAE), both extracted by 0.1 M NaOH. Surprisingly, the conjugate extracted stronger basic condition, *i.e.* 0.65 M NaOH (**Fv II**) contained much lower amounts of phenolics rich in free —OH groups (1.2 mM of GAE). More surprisingly looks the result of this analysis for **Fv V** glycoconjugate, received after heating in 0.1 M NaOH solution, where phenolic content of these compounds with free —OH groups (0.8 mM of GAE) was the lowest. From Table 1 it is evident that all preparations are composed of carbohydrates and phenolics as their dominant structural elements, and proteins, however, they occur in small amounts only.

HPLC analyses of **Fv I–V** conjugates showed similar broad molecular mass distribution patterns. They contained one to five not completely separated molecular peaks, what is typical for such conjugates. Usually these natural polymers are mixtures of chains with similar or the same building fragments. Besides, from the chromatogram (Fig. 2) the dominance of lower molecule-mass components 9–14 kDa is evident.

The **Fv I** glycoconjugate was recovered in 8.4% yield of the dry plant material. HPLC analysis showed a broad molecular mass distribution pattern (M_p 4–982 kDa) containing five peaks, with a dominance of low molecular mass peak centered at about 11 kDa (Fig. 2). It was rich in phenolics (3.3 mM of GAE) and carbohydrates (21%) with low protein content (1.1%). The carbohydrate part was rich mainly in hexuronic acids (61%) while from neutral sugars (~40%) Rha, Ara and Gal residues dominated. It is evident that rhamnogalacturonan (~75%) with arabinogalactan (~17%) are the main components of **Fv I** carbohydrate part (Table 1).

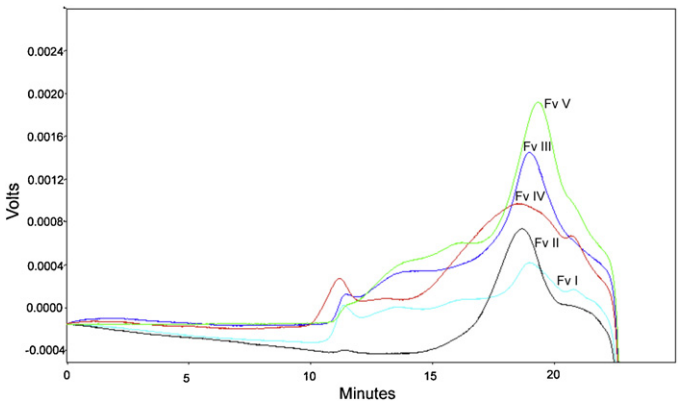

Fig. 2. Molecular-mass distribution patterns of *F. vesca* (**Fv I–V**) conjugates.

The next **Fv II** glycoconjugate was recovered in 5.3% yield and on HPLC revealed one peak of low molecular mass (M_p ~13.5 kDa). It contained about 29% of carbohydrates, however, much lower phenolics (1.16 mM of GAE) and proteins (0.5%) contents of all isolates were determined in this conjugate. Relatively lower hexuronic acids content (~31%) indicates the prevalence of neutral polysaccharide types in **Fv II**, *i.e.* arabinogalactan (38%) and xyloglucan (18%), as indicates the highest glucose content of all isolates (Table 1).

The glycoconjugate **Fv III** was isolated in 4.5% yield and displayed three peaks in HPLC analysis, two of low intensity, and centered at M_p 253 and 944 kDa, and a sharp dominant one, centered at M_p 11.4 kDa. Phenolics (3.54 mM of GAE), carbohydrates (32%) and proteins (1.3%) contents were the highest of all preparations. In the carbohydrate part of **Fv III** over 35% of hexuronic acids were determined, indicating thus the predominance of an acidic polymer, *i.e.* rhamnogalacturonan (60%) associated with arabinogalactan (29%), similarly as in that of **Fv I** (Table 1).

The glycoconjugate extracted by acetate buffer **(Fv IV)** revealed the lowest yield (~4%) of all isolates, however, its carbohydrates content (~29%) was similar as those of **Fv II** and **V** isolates. It showed in HPLC analysis three peaks, two of them with low intensity, at M_p ~4 and ~1100 kDa, and the very broad dominant peak of M_p ~14 kDa, weakly tailing toward the lower molecular mass. **Fv IV** showed quite high phenolic content 2.79 mM of GAE), and the abundance of proteins (1.0%) was relatively low, similarly to all of analyzed conjugates. The sugar analysis of carbohydrate part revealed high hexuronic acids content (~54%) and relatively low rhamnose amount (4%), indicating thus not only rhamnogalacturonan type of polymer, but as well the presence of homogalacturonan segments occurring in this pectin material. The arabinose (15%) and galactose (12%) residues are probably integral parts of the hairy regions of this pectin material (Table 1).

The **Fv V** conjugate was recovered in the yield ~4%, contained 29% of carbohydrates, similarly to **Fv IV**, which was the starting material to prepare **Fv V**. The carbohydrate part was rich in hexuronic acids (~45%), arabinose (~21%) and galactose (~17%). Relatively low rhamnose content (~4%) indicates the presence of homogalacturonan fragments in **Fv V** conjugate. It showed in HPLC analysis four peaks, three of them with low intensity at M_p 942, 229 and 58 kDa, and one dominant with M_p ~9 kDa, weakly tailing toward the higher molecular mass.

3.2. FT-IR spectroscopy of *F. vesca* glycoconjugates

FT-IR spectra of *F. vesca* glycoconjugates **Fv I–V** isolated by different extraction steps/agents are presented in Fig. 3A. Individual

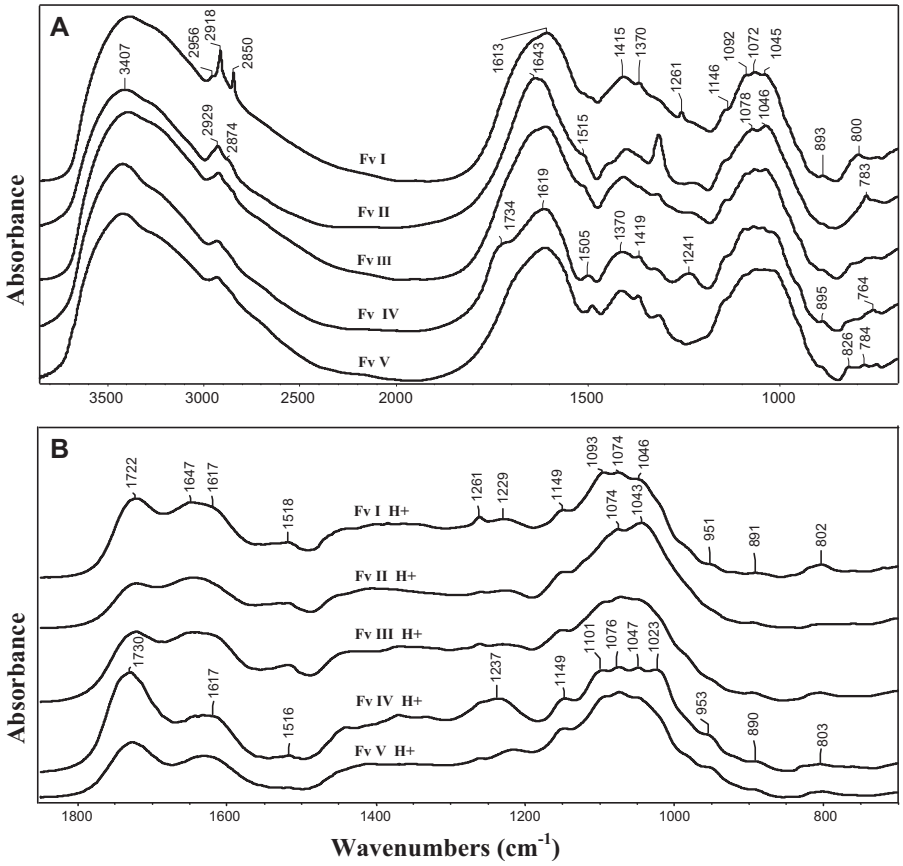

Fig. 3. FT-IR spectra of *F. vesca* conjugates **Fv I–V** (A, uronic acids are in sodium salt) and **Fv I–VH⁺** (B, uronic acids are in acidic form).

FT-IR spectra of **Fv I–V** conjugates (uronic acids were present in the form of sodium salts) showed similar spectral pattern. Bands in the region of 1200–900 cm^{-1} are characteristic for polysaccharide backbones and in the regions 1259–1367 cm^{-1} (phenyl—OH) and 1515 cm^{-1} (C=C) for phenolics (Günzler & Gremlich, 2002; Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The bands found at about 1150, 1100, 1073, 1045 and 1023 cm^{-1} [ν(C—OH), ν(C—O—C), ν(C—C) and ring vibrations] derive from two dominant polysaccharide moieties, arabinogalactan and galacturonan/rhamnogalacturonan. But their spectra are overlaped and no conclusions concerning types of polymers could be expressly deduced from the new shape of spectra. However, bands at about 1613 and 1415 cm^{-1}, assigned to the antisymmetric and symmetric vibration mode of $\nu(COO^-)$ groups of hexuronic acids, indicate the presence of acidic polymers (galacturonan/rhamnogalacturonan) in **Fv I–V**. Moreover, several bands of low intensity in the region of 2962–2848 cm^{-1}, derived from stretching vibrations of ν(C—H) bonds and the broad band with maximum at about 3400 cm^{-1}, originate from stretching vibrations of ν(O—H) bonds (Fig. 3A). As can be seen from Fig. 3A, the presence of phenolic rests connected with the presence of bands at 1516 or 1505 cm^{-1} can be clearly recognized in **Fv IV** and **Fv V** conjugates, while in **Fv I–III** ones, at 1645 cm^{-1}, mentioned bands were fused with bands derived from vibrations of $\nu(COO^-)$ groups and water ν(H—O—H).

To get the evidence in FT-IR technique about the presence of phenolics (identified by colorimetric assay) in all **Fv I–V**, the, conjugates were passed through cation exchanger in acidic form and freeze-dried. FT-IR spectra of *F. vesca* glycoconjugates in acidic form **Fv I–V H⁺** (—COONa groups of hexuronic acids were transformed to —COOH rests) are shown in Fig. 4. New absorption bands appeared in the region of 1730–1722 cm^{-1} [instead of bands at 1615 and 1415 cm^{-1}, due to $\nu(-COO^-)$ groups], assigned to the ν(C=O)

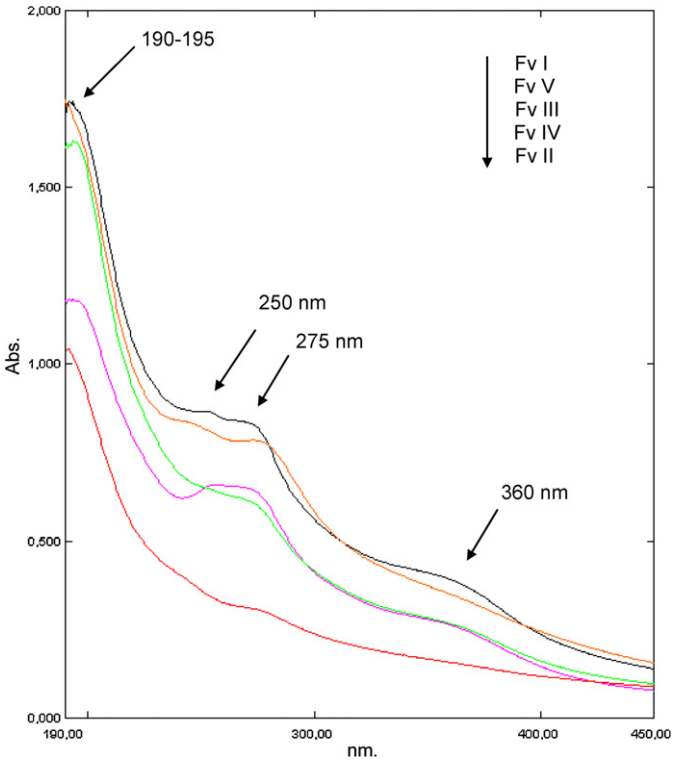

Fig. 4. UV–vis spectra of *F. vesca* (**Fv I–V**) conjugates.

stretching vibrations of —COOH groups, are clearly visible in case of all conjugates. From the intensity of bands at 1730–1722 cm^{-1}, and those at 1644–1649 cm^{-1}, it is evident that the highest hexuronic acids content is in **Fv IV** and **Fv-V** glycoconjugates, while in **Fv I–III** conjugates lower contents of carboxyl groups were observed (Fig. 3B). This finding is in agreement with the chemical analysis of hexuronic acids analysis results received in colorimetric measurements (Table 1). Moreover, **Fv I–V H^+** forms complex bands in the region of 1261–1518 cm^{-1} typical for phenolics appeared in all plant preparations, although they are less pronounced as carbohydrate ones (Fig. 3B). As it can be seen in Fig. 3B, distinguishable bands at 1518 and 1261 cm^{-1} could be recognized and confirm thus the presence of phenolic compounds in *F. vesca* glycoconjugates (Günzler & Gremlich, 2002).

3.3. UV–vis spectroscopy of F. vesca glycoconjugates

The UV–vis spectroscopy was used to characterize individual *F. vesca* glycoconjugates, in order to see some differences in their spectral patterns. It is a non-destructive technique, applicable to study such natural macromolecules as are polysaccharide–phenolic complexes. The UV–vis spectra of *F. vesca* **Fv I–V** glycoconjugates are shown in Fig. 4. In the wavelength range of 190–450 nm, four absorption maxima at about 190–195, 250, 275 and 360 nm were observed. The maximum in 190–195 nm range indicates the possible presence of carboxyl groups derived from pectin material, found in all glycoconjugates in the relatively high amount, or carboxyl and carbonyl groups derived from phenolic compounds. The height of these bands was dominant for all conjugates. The spectra of **Fv I** and **Fv IV** conjugates showed four absorption peaks while in **Fv II, Fv III** and **Fv V** conjugates two or three peaks were observed. The band at about 250 nm can be assigned to hexuronic acid groups, which can originate during isolation process (β-elimination reaction of esterified GalA residues in rhamnogalacturonan or galacturonan core), which comprised strong alkaline conditions and high temperature (Ragnar, 2001). Absorbance maximum around 270–275 nm may derive from phenolic or polyphenolic compounds (C=C bonds of aromatic rings), found in the case of spectra of all **Fv I–V** glycoconjugates, in high quantities. Besides, proteins can absorb irradiation in this wavelength as well, however, their content is very low in all conjugates (0.5–1.3%). As can be seen from Fig. 4, the less intensive maxima, found in some conjugates at 360 nm may indicate the presence of conjugated phenolic units (conjugated to the C=C bonds of aromatic rings).

3.4. NMR spectroscopy of F. vesca glycoconjugates

Differences in the composition of individual fractions caused by different extraction procedures could be observed also in NMR spectra after sample dissolutions in deuterated water. ^{1}H NMR spectra of **Fv I–V** samples (Fig. 5) show signals due to protein as broad lines in the region of δ 3.0–0.5 ppm, while phenolic components are present as very broad signals at δ 7.7–6.0 ppm. Overlapped signals due to carbohydrate components were present in the region δ 5.4–4.44 for anomeric and δ 4.68–3.22 for skeletal protons. HSQC spectra of investigated **Fv I–V** samples are shown in the Fig. 6. Detailed assignment of signals was not possible due to a complexity of mixtures. Attribution of signals was performed on the basis of chemical shift comparison with literature data. Intensities of signals in both, ^{1}H NMR spectra as well as of cross peaks in HSQC spectra varied reflecting different compositions of samples. In α-anomeric region three types of α-arabinofuranose (α-Ara*f*) residues could be identified. In the HSQC spectra of all fractions (**Fv I–V)** the H1/C1 cross peak at δ 5.22/110 was present. Very good accordance with data published for pectic polysaccharides (arabinorhamnogalacturonan) (Habibi, Heraud, Mahrouz, &

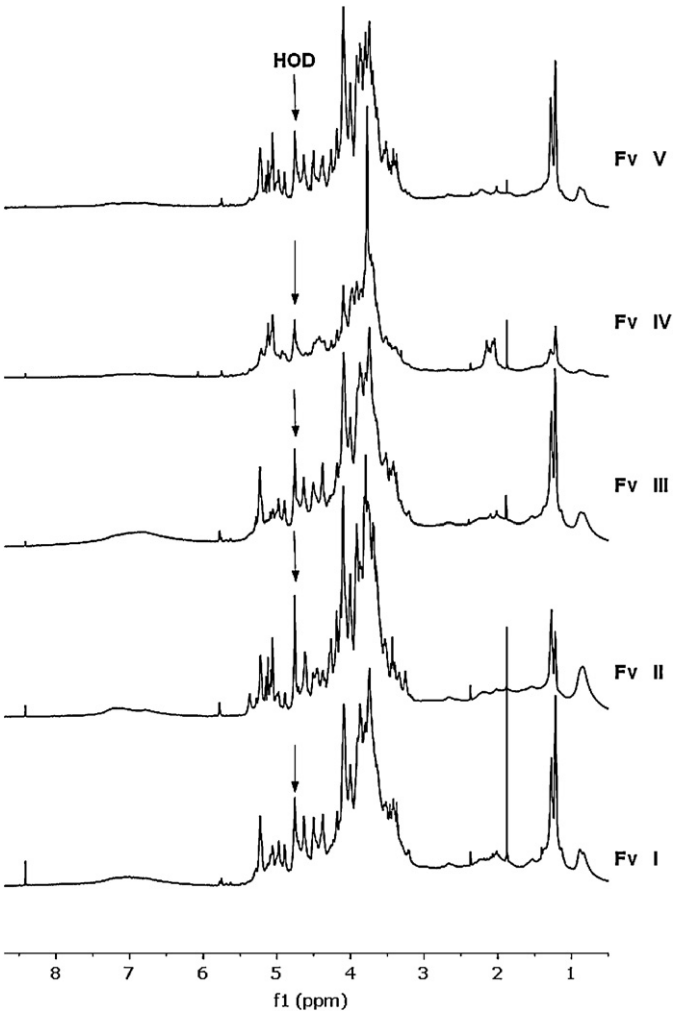

Fig. 5. ^{1}H NMR spectra of *F. vesca* (**Fv I–V**) conjugates.

Vignon, 2004) suggested that this signal is due to terminal α-Ara*f* linked O2 or O3 of some other sugar. The presence/absence of internal 1,5-linked α-Ara*f* in the sample could be revealed on the basis of characteristic chemical shift of α-Ara*f* C5 signal downfield shifted to ~67 ppm. In the HSQC spectrum of **Fv I** this characteristic signal was missing. Other cross peaks were present there: that one due to α-Ara*f* at δ 5.07/107.98, α-rhamnose (α-Rha) at δ 5.24/98.37, α-galacturonic acid (α-GalA*p*) at 5.10/98.8 and β-galactose (β-Gal) at δ 4.52–4.47/103.3–102.2. Due to missing characteristic H5, H5′/C5 signals the H1/C1 at δ 5.07/107.98 of the second α-Ara*f* unit could not be attributed to 1,5-linked α-Ara*f* unit as the literature data might suggest (Habibi et al., 2004; Habibi, Mahrouz, & Vignon, 2005). Comparison with the literature data showed that this signals might be due to some other linkage as a terminal unit. Comparison with literature NMR data has shown a very good accordance with chemical shifts of terminal α-Ara*f* unit linked to O6 of β-Gal*p* residue in arabinogalactan oligosaccharides isolated from instant coffee (Matulová, Capek, Kaneko, Navarini, & Suggi-Liverani, 2011). Obtained data for **Fv I** are consistent with arabinorhamnogalacturonan structure of polymers.

Downfield shifted α-Ara*f* C5 signals was observed in **Fv II–V** HSQC spectra (H5, H5′/C5 at 3.98–3.72/66.8 ppm) suggesting the presence of 1,5-linked α-Ara*f* in these fractions. Corresponding H1/C1 cross peak for this unit might be that one at δ 5.09–5.06/107.2.

Chemical shifts of 1,5-linked arabinofuranose units are sensible to further branching at O2 and/or O3. H1/C1 chemical shift of

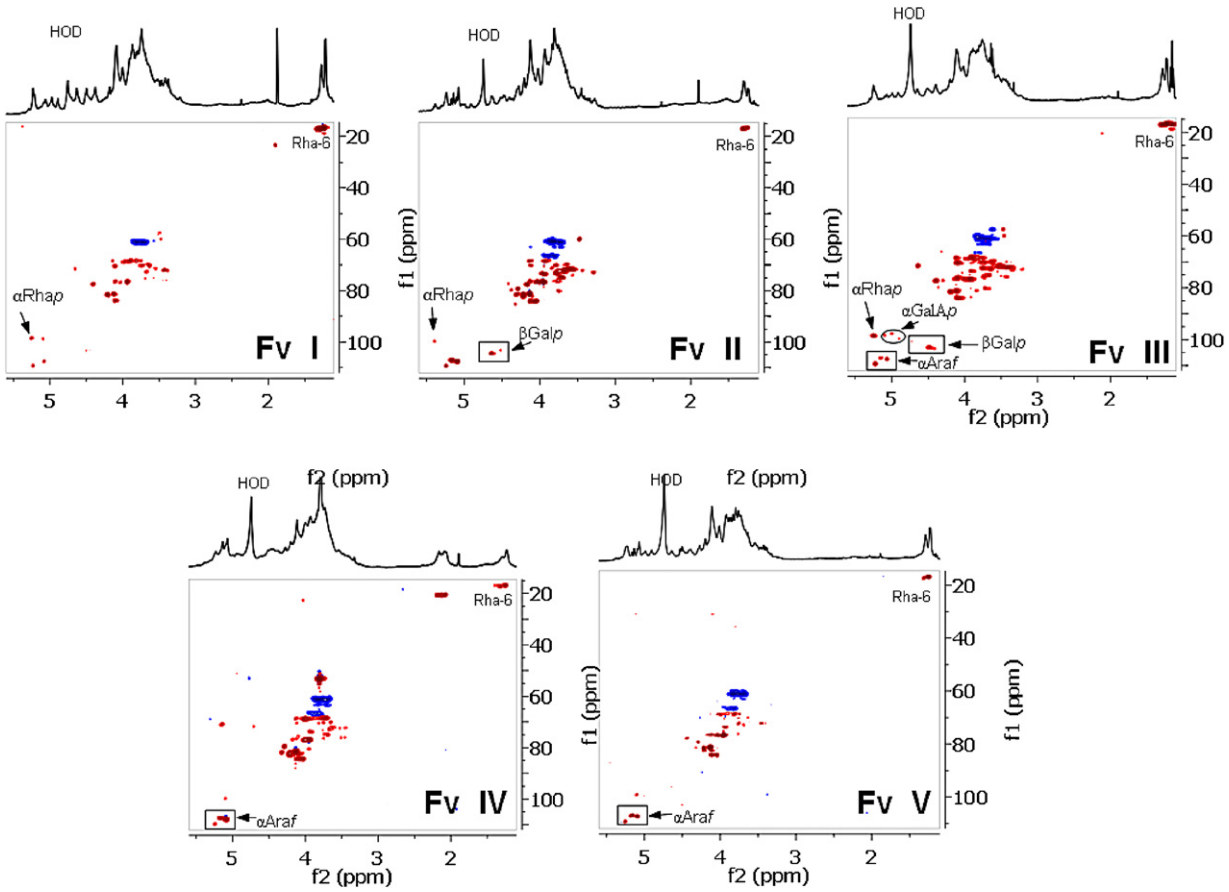

Fig. 6. HSQC NMR spectra of *F. vesca* (**Fv I–V**) conjugates.

branched 1,5-linked Ara*f* residue may vary according the structure (Habibi et al., 2004, 2005). In HSQC spectra due to fractions **Fv II** and **Fv III** characteristic signals due to β-galactose appeared at δ 4.45/102.75 (terminal βGal), 4.69/104.2 (internal 1,4-linked βGal and/or internal 1,3-linked βGal) (Habibi et al., 2005; Hinz, Verhoef, Schols, Vincken, & Voragen, 2005; Matulová et al., 2011; Sengkhamparan et al., 2009). In these fractions new αAra*f* cross peak appeared at δ 5.16/107.3 indicating its origin in 1,2,5- or 1,3,5-linked αAra*f*, the most probably due to its branching by βGal and/or its oligosaccharides. This conclusion was supported by the fact that in the HSQC spectra of **Fv IV** and **Fv V** the δ 5.16/107.3 cross peak was missing and no cross peaks due to βGal were observed.

In all fractions a doublet signals ($^3J_{H5,H6}$) typical for H6/C6 signals due to rhamnose (Rha) was located at δ 1.24/17.2. Corresponding H1/C1 signal to this Rha unit appeared at 5.24/98.37. The H1/C1 and H6/C6 chemical shifts values are characteristic of internal α-1,2-linked Rha residues in →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→ repeating unit of rhamnogalacturonans (Habibi et al., 2005; Renard, Lahaye, Mutter, Voragen, & Thibault, 1998; Sengkhamparan et al., 2009). Cross peaks H1/C1 due to α-galactopyranuronic acid (α-Gal*p*A) in HSQC spectra due to studied fractions were observed at δ 5.12/98.09 and 5.00/97.52. Obtained data indicate the presence of branched rhamnoarabinogalacturonans.

Fractions **Fv IV** and **Fv V** showed αAraf signals as dominant in HSQC spectra. This fact suggests that their origin is in arabinans. Chemical shift H1/C1 at δ 5.08/107.8 is in accordance with those published for internal 1,5-linked αAraf residues while that one at δ 5.17/107.4 for terminal Ara*f* in arabinans (Dourado, Cardoso, Silva, Gama, & Ciombra, 2006).

3.5. Anticoagulant activity of F. vesca glyconjugates

The *in vitro* anticoagulant activity of *F. vesca* preparations **Fv I–V** on human plasma was studied. It has been found that all glycoconjugates are rich in hexuronic acids and phenolics, which give them polyelectrolyte properties similar to polysaccharide anticoagulants like glycosaminoglycans, *i.e.* heparin – the most know anticoagulant (rich in sulfated esters) of animal origin (Middeldrop, 2008). The anticoagulant activity of **Fv I–V** glycoconjugates was measured by activated partial thromboplastin time test (aPTT), prothrombin time test (PT), and thrombin time test (TT) (Tables 2–4). The human plasma, pooled from many healthy donors, was used as a reservoir of coagulation cascade enzymes. All plant glycoconjugates were measured in the range of concentration from 4000 to 7.81 μg/mL, in order to evaluate the strength of the biological activity. In our previous study (Pawlaczyk et al., 2009) we have found that *F. vesca* conjugate, isolated by the similar procedure as was used for the **Fv I** glycoconjugate, showed complete inhibition of plasma clot formation in *in vitro* experiments, *i.e.* in aPTT in the range of concentration 3000–780 μg/mL, and in PT test in the range of concentration 3000–390 μg/mL.

It was very important to check the influence of different isolation condition on the anticoagulant activity of the received product, from the same plant material. As the first method aPTT assay was used. This test is a universally accepted screening procedure used to detect abnormalities in the intrinsic coagulation system. It can be used to detect deficiencies of Factors II, V, VIII, IX, X, XI, and XII, but is insensitive to platelet Factor III (Brown, 1988). In aPTT test the strongest anticoagulant activity showed **Fv I** and **Fv III** conjugates (Table 2), but the second one was the most active. Both of

Table 2
Activated thromboplastin time (aPTT) measurements of the *F. vesca* glycoconjugates made *in vitro* experiments in human pooled plasma. The bold value indicates that the clot was not observed in measured samples. Values are expressed as mean of 5 measurements ± S.D.

Concentrations of a sample in the clotting mixture [μg/mL]	*In vitro* aPTT measurements [s]				
	Fv I	**Fv II**	**Fv III**	**Fv IV**	**Fv V**
1000.00	**>600.0**	**>600.0**	**>600.0**	**>600.0**	**>600.0**
500.00	**>600.0**	528.9 ± 6.1	**>600.0**	356.8 ± 4.2	185.4 ± 3.1
250.00	455.0 ± 5.7	192.8 ± 3.4	520.0 ± 6.0	143.6 ± 3.7	112.7 ± 2.2
125.00	199.5 ± 2.3	100.6 ± 2.1	222.2 ± 4.8	86.9 ± 2.1	67.3 ± 1.9
62.50	114.2 ± 2.0	54.9 ± 1.0	84.2 ± 2.4	50.3 ± 1.0	36.6 ± 0.7
31.25	61.5 ± 1.5	61.5 ± 1.5	45.4 ± 1.7	36.2 ± 0.8	34.8 ± 0.6
15.63	37.8 ± 0.8	39.2 ± 0.6	34.2 ± 0.8	38.9 ± 0.7	35.7 ± 0.6
7.81	32.7 ± 0.5	34.7 ± 0.6	35.1 ± 0.5	35.7 ± 0.5	36.8 ± 0.5
Control – 0	36.8 ± 0.6	36.8 ± 0.6	36.8 ± 0.6	36.8 ± 0.6	36.8 ± 0.6

Table 3
Prothrombin time (PT) measurements of the *F. vesca* glycoconjugates made *in vitro* experiments in human pooled plasma. The bold value indicates that the clot was not observed in measured samples. Values are expressed as mean of 5 measurements ± S.D.

Concentrations of a sample in the clotting mixture [μg/mL]	*In vitro* PT measurements [s]				
	Fv I	**Fv II**	**Fv III**	**Fv IV**	**Fv V**
4000.00	**>300.0**	**>300.0**	**>300.0**	**>300.0**	43.6 ± 1.8
2000.00	**>300.0**	144.7 ± 6.2	**>300.0**	105.5 ± 1.7	21.8 ± 0.9
1000.00	92.8 ± 3.9	48.0 ± 2.8	**>300.0**	34.5 ± 0.8	14.5 ± 0.7
500.00	28.5 ± 1.1	20.1 ± 1.0	46.2 ± 1.5	16.3 ± 0.6	11.2 ± 0.5
250.00	12.6 ± 0.5	14.1 ± 0.7	36.2 ± 1.3	13.6 ± 0.5	10.0 ± 0.4
125.00	11.5 ± 0.4	10.7 ± 0.4	12.2 ± 0.6	9.5 ± 0.4	9.7 ± 0.4
62.50	10.9 ± 0.4	10.2 ± 0.3	9.9 ± 0.5	10.5 ± 0.5	9.1 ± 0.3
31.25	9.4 ± 0.3	9.9 ± 0.3	10.5 ± 0.4	10.8 ± 0.4	9.6 ± 0.4
15.63	9.8 ± 0.4	10.1 ± 0.3	11.9 ± 0.5	10.6 ± 0.4	11.7 ± 0.4
7.81	10.5 ± 0.3	10.2 ± 0.2	10.3 ± 0.4	11.0 ± 0.3	11.2 ± 0.2
Control – 0	11.0 ± 0.3	11.0 ± 0.3	11.0 ± 0.3	11.0 ± 0.3	11.0 ± 0.3

Table 4
Thrombin time (TT) measurements, of the *F. vesca* glycoconjugates made *in vitro* experiments in human pooled plasma. The bold value indicates that the clot was not observed in measured samples. Values are expressed as mean of 3 measurements ± S.D.

Concentrations of a sample in the clotting mixture [μg/mL]	*In vitro* TT measurements [s]				
	Fv I	**Fv II**	**Fv III**	**Fv IV**	**Fv V**
4000.00	**>300.0**	**>300.0**	**>300.0**	**>300.0**	**>300.0**
2000.00	**>300.0**	78.5 ± 3.4	**>300.0**	71.6 ± 3.3	38.1 ± 1.7
1000.00	67.4 ± 3.1	38.9 ± 1.8	**>300.0**	22.4 ± 1.0	27.6 ± 1.2
500.00	22.1 ± 0.9	34.5 ± 1.1	36.4 ± 1.0	18.7 ± 0.8	22.1 ± 0.9
250.00	20.8 ± 0.8	28.6 ± 0.9	24.6 ± 0.8	17.0 ± 0.7	22.6 ± 0.9
125.00	17.7 ± 0.6	17.7 ± 0.7	20.1 ± 0.8	13.5 ± 0.6	18.9 ± 0.7
62.50	12.5 ± 0.4	14.6 ± 0.6	14.9 ± 0.6	11.7 ± 0.5	14.9 ± 0.6
31.25	12.6 ± 0.5	12.1 ± 0.4	12.8 ± 0.5	12.0 ± 0.4	13.2 ± 0.5
15.63	12.1 ± 0.4	12.3 ± 0.5	11.9 ± 0.5	12.3 ± 0.5	12.0 ± 0.4
7.81	12.7 ± 0.6	11.5 ± 0.4	12.1 ± 0.4	12.0 ± 0.4	12.5 ± 0.4
Control – 0	11.5 ± 0.4	11.5 ± 0.4	11.5 ± 0.4	11.5 ± 0.4	11.5 ± 0.4

them completely inhibited the plasma clot formation in the concentration of 500 μg/mL in the clotting mixtures, and both strongly prolonged time of clotting still at the concentration of 62.5 μg/mL. The conjugates **Fv II**, **Fv IV** and **Fv V** were also inhibitors of clotting process, but with lower activity. They completely stopped clot formation in the concentration of 1.0 mg/mL in the clotting mixtures, and still prolonged time of clotting at the concentration of 125 μg/mL, but **Fv V** conjugate showed lower activity. It was weaker anticoagulant than that of **Fv IV** isolate (Table 2).

Anticoagulant activity of the *F. vesca* **Fv I–V** conjugates, measured in aPTT test, was compared with the activity of the unfractionated heparin (144 IU/mg). As it can be seen from Fig. 7, the anticoagulant activity of **Fv III** isolate was 0.96 IU/mg while conjugates **Fv I**, **Fv II**, **Fv IV** and **Fv V** reached 0.90, 0.83, 0.68 and 0.45 IU/mg, respectively. From these results it is evident that studied plant macromolecular compounds are weak inhibitors of the intrinsic coagulation pathway, but with still interesting activity, *i.e.* when compared to the activity of *Lythrum salicaria* glycoconjugate activity – 0.7 IU/mg (Pawlaczyk, Capek, et al., 2011).

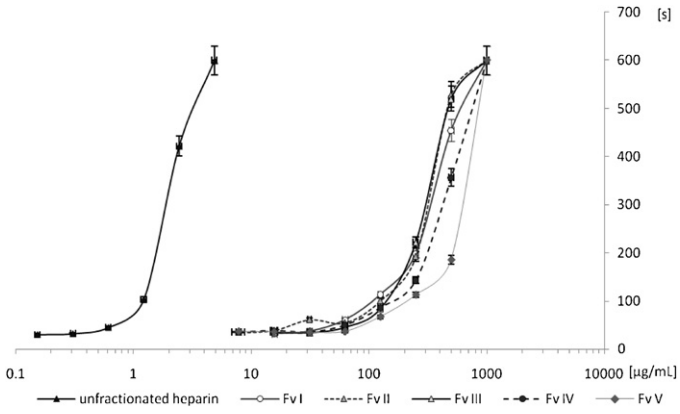

Fig. 7. The *in vitro* activity of *F. vesca* **Fv I–V** conjugates, determined in aPTT test on human blood plasma, compared with the activity of unfractionated heparin (144 IU/mg).

The PT test is used to detect bleeding disorders. An abnormal or extended PT test usually indicates a deficiency in one or more of the factors in the extrinsic or common pathway of blood coagulation. This deficiency can be caused by hereditary coagulation disorders, vitamin K deficiency, liver disease or drug administration. The PT test is commonly used to monitor oral anticoagulant therapy as it is sensitive to deficiency in Factors II, VII and X1,2 and for general pre-operative screening. The PT also is used to perform factor assays for the extrinsic system (*i.e.* Factors II, V, VII and X). The prothrombin time is not sensitive to deficiencies in the intrinsic coagulation system (*i.e.* Factors VIII, IX, XI and XII), or to platelet dysfunctions (Brown, 1988). In PT test **Fv III** was found as the most active anticoagulant (Table 3). This glycoconjugate completely inhibited clotting process in the concentration of 1.0 mg/mL in the clotting mixtures, and strongly prolonged the process even at the concentration of 250 μg/mL. The conjugate **Fv I** was less active, about one row weaker than that of **Fv III** and **Fv V** was almost not active in this test (Table 3).

The TT test is based on the ability of thrombin to catalyze the polymerization of fibrinogen to form fibrin. The mechanism involves the splitting of fibrinogen by the proteolytic action of thrombin. Polymerization of the fibrin monomer ultimately results in the production of a fibrin gel clot. Abnormalities affecting this stage of coagulation include quantitative and qualitative alterations in fibrinogen, increased fibrinolytic activity causing variations in fibrin degradation products (FDP), and interferences with fibrinogen polymerization. The TT test is sensitive to heparin and other circulating antithrombins (Brown, 1988). In TT test, **Fv III** was the most active inhibitor of clotting process, and the results were very similar to that of PT test (Table 4). This glycoconjugate completely inhibited clot formation in the concentration of 1.0 mg/mL in the clotting mixtures, and strongly prolonged this process even at the concentration of 250 μg/mL. Less active was shown to be **Fv I** conjugate, about one row weaker than that of **Fv III** isolate (Table 4).

4. Conclusion

It can be summarized that all five plant glycoconjugates **Fv I–V** isolated from the medicinal plant *F. vesca* L. by different steps/agents were composed of polyphenolic and polysaccharide components, with low protein contents. Their carbohydrate parts were composed of hexuronic acids (αGalA), rhamnose, arabinose and galactose residues. Chemical analyses of carbohydrate parts of **Fv I–V** conjugates revealed the presence of galacturonan/rhamnogalacturonans associated with arabinan/arabinogalactans. High contents of pectic polysaccharides and polyphenolics (containing carboxylic groups) make from these conjugates potentially active compounds, similarly as a heparin. The preliminary anticoagulant *in vitro* tests showed that all *F. vesca* isolates displayed the anticoagulant activity, similarly to those of isolates from strawberry fruits (Naemura et al., 2005) and *L. salicaria* flowers (Pawlaczyk, Capek, et al., 2011; Pawlaczyk, Czerchawski, et al., 2011). Their anticoagulant activities were determined in the order **Fv I > Fv III > Fv II > Fv IV > Fv V**. However, their anticoagulant activities were lower than that of unfractionated heparin, the strongest anticoagulant agent widely used in a clinical practice. Nevertheless, it is evident that only two glycoconjugates, *i.e.* **Fv III** and **Fv I** have been shown to have a significant biological activity, promising to be studied in more advanced experiments. As it was shown, both most active conjugates revealed the similar amounts of galacturonic acid and the highest phenolics contents.

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