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An anticoagulant effect and chemical characterization of *Lythrum salicaria* L. glycoconjugates

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ABSTRACT

An ion-exchange chromatography of *Lythrum salicaria* glycoconjugate afforded twelve fractions (LsF1–F12) by stepwise elution with water, sodium chloride and sodium hydroxide solutions. Carbohydrate, phenolic and protein moieties have been determined in all fractions. Individual glycoconjugates LsF1–F12 varied in carbohydrate, phenolic and protein contents, molecular mass and monosaccharide composition. Rhamnogalacturonans and/or homogalacturonans associated with arabinogalactans, arabinan and hexosan types of polysaccharides were identified in ion-exchange fractions. *In vitro* anticoagulant activity tests showed complete inhibition of plasma clot formation by LsF2, LsF7 and LsF12 fractions and a slight procoagulant effect of LsF3 and LsF4 ones while other ion-exchange fractions were not active.

1. Introduction

Lythrum salicaria L. (Lythraceae) is a herb, shrub or tree, native to Europe, Asia and Africa, however, nowadays is widespread all over the world. It is an invasive and competitive plant, easily adapted to the environment. Flowering parts, leaves and roots of this plant were used as drugs for many centuries for their astringent and styptic properties. *L. salicaria* contains many biologically active compounds of flavonoids and phenolics origin which justify its use in the folk medicine for treatment of bleeding, moderating menstrual flow, healing of wounds, ulcers, dysentery, diarrhea, chronic intestinal catarrh, hemorrhoid, eczema, varicose veins and venous insufficiency (Mantle, Eddeb, & Pickering, 2000; Rauha et al., 2000; Thompson, Stuckey, & Thompson, 1987). Moreover, antimicrobial, anti-inflammatory, antioxidant, anti-nociceptive, hypo- and hyperglycemic activities of *L. salicaria* extracts have been reported in a last decade (Kahkönen et al., 1999; Lamela, Cadavid, & Calleja, 1986; Tunalier, Koşar, Küpeli, Çaliş, & Başer, 2007). Recently, we have isolated and characterized high-molecule mass compounds, polyphenolic–polysaccharide conjugates, which showed interesting anticoagulant and procoagulant activities *in vitro* as well *in vivo* experiments (Pawlaczyk, Czerchawski, Pilecki, Lamer-Zarawska, & Gancarz, 2009; Pawlaczyk, Czerchawski, & Kańska, 2010; Pawlaczyk, Czerchawski, & Kuliczkowski, 2010).

The aim of our present paper was to investigate the complex character of the glycoconjugate isolated from flowering parts of *L. salicaria* in term of type and molecular mass of components as well as to determine the content and composition of carbohydrate, phenolic and protein contents, and to verify the influence of each glycoconjugate fraction in the *in vitro* coagulation process of human plasma.

2. Materials and methods

2.1. Plant material and chemicals

Flowering parts of medicinal plant *L. salicaria* L. were collected from Proboszczów, Lower Silesia, Poland. A voucher specimen has been deposited in the Botanical Garden of Wrocław University, Wrocław, Poland (No. 005291). The substrates for aPTT and PT times measurements including standardized human plasma – MDA Reference Plasma®, TriniCLOT aPTT HS, and TriniCLOT PT HTF,

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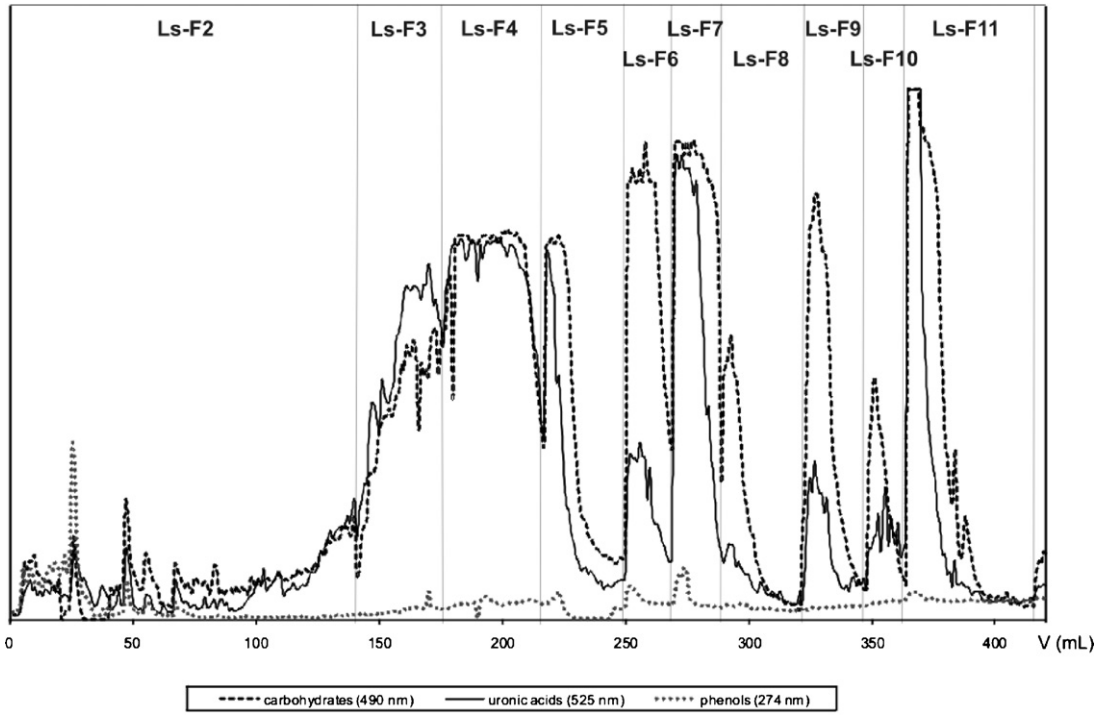

Fig. 1. DEAE-Sephadex A-50 chromatography of L. salicaria glycoconjugate (Ls). Fractions (LsF2-F11) were eluted with different concentrations of sodium chloride solutions (0.05–1.3 M). The collected fractions were assayed for the presence of carbohydrates (- - -), hexuronic acids (—) and phenolics (···).

produced by Trinity Biotech Ireland, were purchased from Horiba ABX Sp. z o. o. (Warsaw, Poland). Monosaccharide standards and solvents of analytical grade were purchased from Sigma – Aldrich Co.

2.2. Isolation of Lythrum glycoconjugate

The isolation of *L. salicaria* glycoconjugate (Ls) was already described (Gancarz, Pawlaczyk, & Czerchawski, 2006; Pawlaczyk, Czerchawski, & Kańska, 2010). Shortly, air-dried flowering parts of *L. salicaria* were minced and then suspended in 0.1 M NaOH at room temperature for 24 h, and refluxed for 6 h at 97 °C. The solid-state parts of plant were separated by centrifugation (1850 × *g*; 20 min) and the supernatant was neutralized using 1 M HCl. The water fraction was concentrated and extracted gradually twice with hexane (water:hexane – 1:1 v/v) for 6 h at 69 °C, diethyl ether (1:1) for 6 h at 34 °C, chloroform (water:chloroform – 1:1 v/v) for 6 h at 61 °C, and with similar proportions of chloroform and ethanol mixture (chloroform:ethanol – 3:2 v/v) for 6 h, at 70 °C. 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 a crude *Lythrum* glycoconjugate (Ls).

2.3. Ion-exchange chromatography of Lythrum glycoconjugate

A water solution (20 mL) of *Lythrum* glycoconjugate (Ls, 500 mg) was applied to a column (4 cm × 50 cm) of DEAE-Sephadex A-50 in chloride form. The column was successively eluted with water, 0.05 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 0.6 M, 0.7 M, 0.8 M, 0.9 M, 1.0 M, 1.1 M, 1.2 M and 1.3 M NaCl solutions, followed by 1.5 M NaOH solution. Fractions of 10 mL were collected and analyzed for carbohydrate content by phenol–sulfuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), uronic acid content by *m*-hydroxybiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) and for phenolics by absorbance measurement at 274 nm which is typical for phenolic pigments (Sava, Yang, Hong, Yang, & Huang, 2001). According to the elution solvent, Ls glycoconjugate was separated into twelve fractions, i.e. non-retained water fraction LsF1, sodium chloride fractions LsF2 (0.05–0.3 M NaCl), LsF3 (0.4 M NaCl), LsF4 (0.5 M NaCl), LsF5 and F6 (0.6 M NaCl), LsF7 and LsF8 (0.7 M NaCl), LsF9 (0.8 M NaCl), LsF10 (0.9 M NaCl), LsF11 (1.0–1.3 M NaCl) and LsF12 (1.5 M NaOH). Individual tubes were pooled, dialyzed against deionized water and freeze-dried to give brown colour fluffy materials. The fraction eluted by sodium hydroxide solution was neutralized with 4 M HCl to pH 7, dialyzed and freeze-dried to recover a dark brown fluffy material (Fig. 1).

2.4. Determination of molecular mass

Molecular mass determination of ion-exchange fractions was performed with HPLC Shimadzu apparatus (Vienna, Austria) using a tandem of two columns HEMA-BIO 300 followed HEMA-BIO 1000 column, Tessek (Prague, Czech Republic) of dimensions 8 mm × 250 mm. As a mobile phase 0.02 M phosphate buffer, pH 7.2 containing 0.1 M NaCl was used at a flow rate 0.4 mL/min. Differential refractometer was used as a detector. A set of dextran standards was used for calibration of the columns (Gearing Scientific, Polymer Lab. Ltd., UK).

2.5. General methods

Polysaccharide fractions of *Lythrum* were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120 °C and the quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates (Englmaier, 1990) by gas chromatography on a Hewlett-Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m), the temperature program of 110–125 (2 °C/min) −165 °C (20 °C/min) and flow rate of hydrogen 20 mL/min (Shapira, 1969). Carbohydrate content in the samples was estimated by the phenol–sulfuric acid assay (Dubois

et al., 1956). The uronic acid 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 (Singleton, Orthofer, & Lamuela-Raventós, 1999). The protein content was determined by Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The colorimetric assays were measured using Specol 11 spectrophotometer, Carl Zeiss Jena. Infrared spectra were obtained on a NICOLET Magna 750 spectrometer with DTGS detector and OMNIC 3.2 software, where 128 scans were recorded with 4 cm^{-1} resolution. The ^1H NMR spectra were recorded in D_2O on AMX-300 spectrometer, operating at 300.13 MHz using a standard Bruker software package.

2.6. In vitro clotting assays

Clotting assays of activated partial thromboplastin time (aPTT) and prothrombin time (PT) were performed using standardized human plasma (Brown, 1988). The following final concentrations of *Lythrum* glycoconjugate (Ls) and obtained ion-exchange fractions were used: 465.0, 233.5, 116.25, 58.13, 29.06, 14.53, 7.27 and 3.63 μg/mL in both tests.

Determination of aPTT activity (Brown, 1988) was done using partial thromboplastin with activator (TriniClot aPTT HS) prewarmed in a water bath to 37 °C. Similarly, the solution of 0.025 M calcium chloride was also incubated in the same conditions. For a control test, 50 μl of human plasma (MDA Reference Plasma®) was placed in a test tube containing 33 μl of distilled water. After incubating for 3 min at 37 °C, 50 μl of the partial thromboplastin solution with activator was added and the contents were rapidly mixed. The mixture was incubated for another 3 min at 37 °C and 50 μl of the prewarmed 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, 33 μl of the prewarmed sample solution was mixed with 50 μl of plasma. The experiment was done for unfractionated heparin (144 IU/mg) in its different concentrations. All experiments were carried out threefold, using Coag Chrom 3003 coagulometer (Bio-Ksel Sp. z o. o., Grudziądz, Poland). The anticoagulant activity of samples was expressed as IU/mg using a parallel standard curve based on unfractionated heparin (144 IU/mg).

Determination of prothrombin time (PT) was done using thromboplastin–calcium reagent (TriniClot PT HTF), which was diluted with distilled water according to the manufacturer's instructions (Brown, 1988). It was then incubated in a dry bath at 37 °C for at least 30 min before the test was started. Thereafter 50 μl of human plasma (MDA Reference Plasma®) was placed in a test tube and 33 μl of water, for control test. This mixture was incubated in the dry bath for 3 min. Thereafter, 100 μl of prewarmed thromboplastin-calcium reagent was rapidly added to the plasma mixture 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, 33 μl of the prewarmed sample solution was mixed with 50 μl of plasma. All experiments were carried out threefold, using Coag Chrom 3003 coagulometer (Bio-Ksel Sp. z o. o., Grudziądz, Poland).

3. Results and discussion

3.1. Ion-exchange chromatography of L. salicaria glycoconjugate and characterization of its components

In our previous study (Pawlaczyk, Czerchawski, & Kuliczkowski, 2010) a water-soluble *Lythrum* glycoconjugate (Ls) composed of carbohydrate, phenolic and protein moieties has been isolated from air-dried flowering parts of medicinal plant *L. salicaria.* The yield was 8% based on the dry plant material (w/w). It has been shown in "*in vitro*" and in "*ex vivo*" experiments that it completely inhibits plasma clot formation, irrespectively of the plasma type (human or rat). Further, in "*in vivo*" application of Ls into the Wistar rat blood circulation system, it showed pro-coagulant activity which is controversial effect when compared with in *in vitro* test. This controversial effect of Ls on blood coagulation process prompted us to investigate it in more detail, mainly in term of polysaccharide composition.

To get more information about the nature of *Lythrum* glycoconjugate, the crude Ls was fractionated into twelve fractions (LsF1–F12) using ion-exchange chromatography by step-wise elution with water (LsF1), sodium chloride solutions of increasing concentrations (LsF2–F11) and finally with 1.5 M sodium hydroxide solution (LsF12). The yield was 90% (from 5 g of crude Ls extract 4 g of total mass of fractions LsF1 to LsF12 were obtained). Their carbohydrates, proteins and phenolics contents, monosaccharide composition, uronic acids content and molecular mass of each fraction are given in Table 1. As it can be seen from Table 1 the highest masses were in sodium hydroxide (LsF12 ~ 25%), 0.7 M sodium chloride (LsF7 ~ 14% and LsF8 ~ 13%) and 0.5 M sodium chloride fraction (LsF4 ~ 11%) while the mass of other fractions fluctuated from 0.4 to 7.4%. In fractions LsF7 (60%), LsF11 (46%), LsF6 (43%), LsF5 (42%) and LsF4 (40%) a significant increase of carbohydrates has been noticed in comparison with the native Ls glycoconjugate (30%) whereas a marked decrease of carbohydrate content in LsF8 (~6%) and LsF10 (~9%) fractions was determined.

Phenolics were present in all fractions. The highest contents expressed as gallic acid equivalent (GAE) per 1 g of the analyzed material, were found in LsF12 (2.8 mM/g), LsF2 (0.93 mM/g) and LsF1 (0.85 mM/g) fractions, i.e. eluted with sodium hydroxide solution, 0.05–0.3 M sodium chloride solutions and water, respectively. Protein content was relatively low in all eluted fractions and varied from 0.1 to 1.2%. From Table 1 it is evident that all fractions are composed of complex phenolic and carbohydrate structures with small protein content. The HPLC analysis showed significant differences in molecular masses of fractions and indicated relatively high degree of polydispersity of Ls. It showed a wide range of molecular masses with two distinguishable, however, not complete separated regions centered at ~1.1×10^6 and ~6400 (Table 1).

The ion-exchange chromatography of Ls revealed the presence of glycoconjugates differing in molecular mass, neutral sugars composition, uronic acids, phenolic and protein contents. As it can be seen from Table 1, the water fraction LsF1 represents 0.4% of total Ls only. It was composed of neutral sugars, high phenolic and low protein contents.

The first sodium chloride fraction LsF2 (0.05–0.3 M NaCl) represents 4.4% of Ls and HPLC analysis showed two peaks, one with small intensity centered at mass ~1.47×10^6 and the second, dominant peak, with average molecule mass ~8500. LsF2 was rich in phenolics and protein content was the highest from all obtained fractions. It contained also high amount of uronic acids, arabinose and rhamnose residues while other sugars were present at very low quantities. The rhamnogalacturonan (~60%) and arabinan (25%) are the main components of this fraction.

The LsF3 fraction (0.4 M NaCl) contained 5.2% of total mass and on HPLC showed one peak of average molecule mass ~10,300. It showed low protein, phenolic and carbohydrate contents. Polysaccharide part was rich in uronic acids (over 99%) and indicated the presence of homogalacturonan type of polysaccharide.

The LsF4 fraction (0.5 M NaCl) contained 11.1% of total elution mass and displayed two peaks on HPLC, one broad with low intensity and centered at mass ~545×10^3 and sharp intensive centered at molecular mass ~9400. Both, phenolic and protein content were

Table 1
Yields and characteristics of DEAE-Sephadex fractions of *Lythrum salicaria* (Ls) glycoconjugate.

Fraction	Eluent	Yield (wt%)	Carbohydrate content (wt%)	Protein content (wt%)	Monosaccharide composition (wt%)							Uronic acid (wt%)	Total phenols mean (mM)	$M_p \times 10^3$
					Rha	Fuc	Ara	Xyl	Man	Glc	Gal			
Ls	–	**100.00**	**29.7**	**0.8**	**10.2**	**0.6**	**8.7**	**0.5**	**0.5**	**1.9**	**11.6**	**66.0**	**1.19**	**1100; 6.4**
Ls-F1	H_2O	0.4	29.5	0.3	63.4	Nf[a]	25.1	Nf[a]	Nf[a]	Nf[a]	11.5	Nf[a]	0.85	Nd[c]
Ls-F2	**0.05–0.3 M NaCl**	**4.4**	**22.4**	**1.2**	**23.7**	**3.3**	**25.1**	**2.2**	**1.7**	**2.4**	**3.2**	**38.4**	**0.93**	**1470; 8.5**
Ls-F3	0.4 M NaCl	5.2	27.7	0.2	0.3	Nf[a]	0.1	Nf[a]	Nf[a]	Nf[a]	Tr[b]	99.5	0.12	10.3
Ls-F4	0.5 M NaCl	11.1	39.5	0.2	0.7	Tr[b]	0.2	Tr[b]	Tr[b]	0.1	0.4	98.5	0.10	545; 9.4
Ls-F5	0.6 M NaCl	4.4	42.1	0.3	22.1	0.6	4.4	0.6	0.5	19.0	13.6	39.3	0.20	790; 4.8
Ls-F6	0.6 M NaCl	3.8	42.7	0.3	16.5	1.1	6.9	2.4	3.6	54.8	10.7	4.1	0.14	2.4
Ls-F7	**0.7 M NaCl**	**14.3**	**60.1**	**0.3**	**20.8**	**Nf[a]**	**7.0**	**2.4**	**2.6**	**59.4**	**4.9**	**2.8**	**0.11**	**3.6**
Ls-F8	0.7 M NaCl	13.1	6.3	0.1	10.8	Nf[a]	33.3	Nf[a]	Nf[a]	41.7	Nf[a]	14.2	0.07	2.9
Ls-F9	0.8 M NaCl	5.2	17.2	0.2	10.2	Nf[a]	38.3	8.5	3.6	33.7	Nf[a]	6.2	0.10	3.6
Ls-F10	0.9 M NaCl	5.3	8.5	0.1	16.9	Nf[a]	72.6	Nf[a]	Nf[a]	Nf[a]	Nf[a]	10.5	0.07	3.6
Ls-F11	1.0–1.3 M NaCl	7.4	45.8	0.4	3.9	Nf[a]	5.4	1.4	1.3	77.3	6.3	4.4	0.22	4.8
Ls-F12	**1.5 M NaOH**	**25.4**	**13.5**	**1.2**	**Tr[b]**	**Nf[a]**	**Tr[b]**	**Tr[b]**	**Tr[b]**	**Tr[b]**	**Tr[b]**	**99.5**	**2.80**	**4.9**

Bold letters indicate anticoagulant active conjugates.
[a] Not found.
[b] Traces.
[c] Not determined.

relatively low in comparison with the native Ls, however, carbohydrate content was relatively high (~40%). LsF4 was rich in uronic acids (over 98%) indicating the presence of homogalacturonan type of polymer, similarly as in LsF3.

The LsF5 fraction, eluted with 0.6 M NaCl, contained 4.4% of total mass and on HPLC revealed a broad molecular mass distribution pattern with two distinguishable peaks with area ratio about 1:1, one of average molecule mass ~790×10^3, weakly tailing towards the lower molecule masses and the second one of average molecule mass ~4800. Both, phenolic and protein contents were relatively low, however, the carbohydrate content (~42%) was higher as that of Ls (~30%). LsF5 showed lower uronic acids content (~40%) in comparison with those of LsF3 and LsF4 fractions, however, hexose content, i.e. rhamnose, glucose and galactose increased significantly (~55%). Sugar analysis evidently showed, that LsF5 glycoconjugate is composed of rhamnogalacturonan and hexosan type of polymers.

The fraction eluted with 0.6 M NaCl (LsF6), accounted for 3.8% of total Ls material recovered from the ion-exchange column, showed on HPLC analysis a sharp peak of low molecular mass ~2400. It contained 43% of carbohydrates with very low uronic acids content (~4%) and low phenolic and protein contents. Carbohydrate analysis showed a high glucose (55%) content and also rhamnose (17%) and galactose (11%) residues, indicating the presence of hexosan type of polysaccharides in LsF6.

Elution with 0.7 M NaCl afforded two glycoconjugate fractions LsF7 and LsF8 accounting for 14.3% and ~13% total mass, respectively. Both fractions showed molecular homogeneity on HPLC with single peaks of molecular mass ~3600 (LsF7) and ~2900 (LsF8). Besides, low protein and phenolic contents were detected in both fractions. It should be stressed that the LsF7 fraction has the highest carbohydrate content (~60%) of all obtained fractions. Its sugar analysis showed ~97% of neutral sugars and ~3% uronic acids only. High glucose (59%) and rhamnose (21%) contents indicated the hexosan type of polysaccharide in LsF7. However, the LsF8 fraction contained the lowest carbohydrate content (6.3%) of all fractions. Its carbohydrate part was rich in glucose (42%), arabinose (33%), uronic acids (14%) and rhamnose (11%) residues, thus indicating the presence of hexosan, rhamnogalacturonan and arabinan types of polymers in LsF8.

The glycoconjugate fractions LsF9 and LsF10, eluted with 0.8 M and 0.9 M NaCl, respectively, were obtained in small yields (~5%) and in low protein, phenolic and carbohydrate contents. Besides, both conjugates LsF9 and LsF10 showed low molecule mass ~3600. Sugar analyses indicated the presence of hexosan, pentosan and rhamnogalacturonan types of polymers in LsF9 and rhamnogalacturonan and arabinan in LsF10. Surprisingly, in both glycoconjugates very high contents of arabinose residues (the highest of all fractions) have been determined.

The LsF11 fraction (1.0–1.3 M NaCl) represented ~7% of total mass. It had average molecule mass ~4800. LsF11 showed higher amount of phenolic and protein contents in comparison with those of LsF3-LsF10 fractions, and the very high carbohydrate content (46%) (second with respect to all fractions). Composition analysis showed high amount of neutral sugars (~96%) and low uronic acids (~4%) content. The dominant part of this fraction was glucose residues (~77%) indicating hexosan type of polysaccharide in LsF11.

The last LsF12 glycoconjugate, eluted with 1.5 M NaOH solution, contained ~25% of total mass. The carbohydrate content was relatively low ~14% in this fraction. Its average molecule mass was estimated to be ~4900. Moreover, LsF12 had the highest phenolic and protein contents of all obtained fractions. Sugar analysis revealed, similarly to LsF3 and LsF4 fractions, the high uronic acid content and indicated almost exclusively the presence of homogalacturonan (Table 1).

FT-IR spectra of *Lythrum* glycoconjugate (Ls) and its fractions which showed significant biological activity are presented in Fig. 2. In the native Ls the bands in the region 900–1200 cm^{-1} characteristic for carbohydrates and at 1515 and 1367–1259 cm^{-1} for phenolics were identified (Himmelsbach, Hartley, Borneman, Poppe, & Van Halbeek, 1994; Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The spectral shape of the dominant group of bands at 1016, 1071, 1091 and 1144 cm^{-1} as well as the intensive bands at 1593 and 1408 cm^{-1} regions, typical for resonances of COO^- groups of galacturonic acids, confirmed the prevalence of galacturonan and/or rhamnogalacturonan type of polymers in Ls. Besides, the band of lower intensity at 1728 cm^{-1} suggests the presence of acidic form (COOH) of carboxylic groups in uronic acids. The bands at 2924–2850 cm^{-1} derived from stretching resonances of CH, CH_2 or CH_3 groups, and the strong band at 3400–3300 cm^{-1} originates from stretching vibrations of OH groups in sugar moieties.

The FT-IR spectrum of LsF2 fraction, eluted with the lowest concentrations of NaCl, showed simpler polysaccharide spectral pattern (1200–900 cm^{-1}) in comparison with that of Ls.

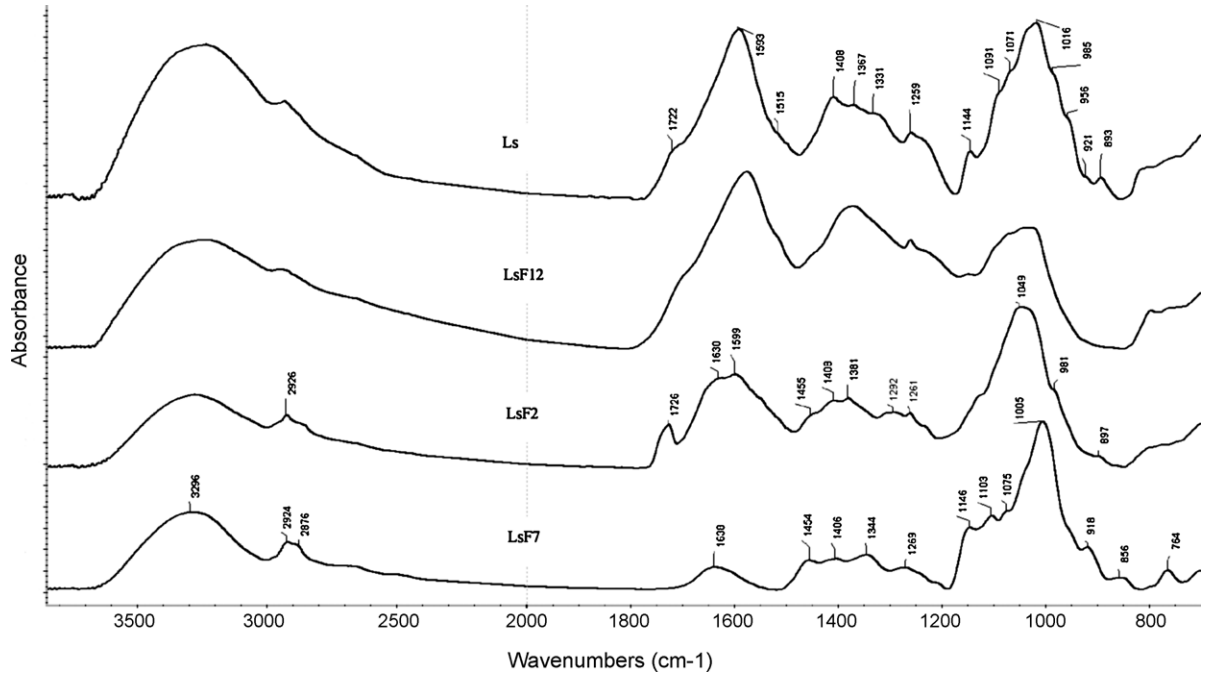

Fig. 2. FT-IR spectra of *L. salicaria* glycoconjugate (Ls) and its anticoagulant active ion-exchange fractions LsF2, LsF7 and LsF12.

Similarly, characteristic bands at 1559, 1408 and 1726 cm^{-1} due to resonances of carboxylic groups and bands in the region 1381–1261 cm^{-1} due to phenolics could be recognized. Moreover, clearly distinguishable IR band at 1630 cm^{-1} derived from the ν(C=O) stretch vibrations of the peptide bonds (amide I) was observed.

FT-IR spectra of LsF3–F5 glycoconjugates (not shown) revealed the carbohydrate spectral pattern typical for homogalacturonan and/or rhamnogalacturonan (Kačuráková et al., 2000) with strong signals at 1611 and 1414 cm^{-1}, characteristic for resonances of COO^{-} groups of uronic acids. However, spectral patterns of LsF6–F11 showed different shape in comparison with those of LsF2–F5. FT-IR spectra of LsF6–F11 showed similar spectral patterns, due to very low uronic acids contents and high content of hexosan type of polysaccharides. As it can be seen from Fig. 2, the FT-IR spectrum of LsF7 (typical for all LsF6–F11 fractions) showed the shape of carbohydrate pattern similar to that of α-glucans (Kačuráková et al., 2000), i.e. one main and three less intensive resonances at 1005, 1075, 1103 and 1146 cm^{-1}, respectively. In fractions LsF6–F11, exclusive of LsF10, a high content of glucose residues has been determined. Sodium hydroxide fraction LsF12 showed different spectral pattern in comparison with all other fractions, due to high phenolic contents and relatively low abundance of carbohydrates (Fig. 2).

In the ^{1}H NMR spectrum of *Lythrum* glycoconjugate (Ls), signals due to carbohydrates, proteins and phenolics could be observed. As it can be seen from Fig. 3, the Ls glycoconjugate shows very complex ^{1}H NMR spectrum due to its complex nature. At least 13 not very well resolved signals attributable to anomeric protons of galacturonic acid, galactose, rhamnose, arabinose etc. were distinguishable in the spectrum of Ls. The presence of anomeric signals at δ 5.30–4.90 and δ 4.80–4.60 showed that the sugar residues are both α- and β-linked (Gorin, 1981). In the spectrum resonances due to the methyl protons of 6-deoxysugars (rhamnose and fucose) at δ 1.2 as well as signals due to ring protons of saccharides at δ 3.5–4.5 were recognised.

The ^{1}H NMR spectrum of LsF2 showed a similar spectral pattern as that of the native Ls (Fig. 3). In addition, in the anomeric region two broad humps of signals in the region δ 5.5–4.90 were observed and thus indicating a complex character of LsF2. Its spectral complexity could be due to a large variability of constituent sugars and a high polydispersity of its macromolecules.

The ^{1}H NMR spectrum of LsF7 has been shown to be less complex in comparison with the native Ls glycoconjugate. As it can be seen from Fig. 3, the spectrum of LsF7 is very well resolved. In the anomeric region one dominant signal at δ 4.90 and four small intensity signals at δ 5.25, 5.15, 5.10 and 4.70 were found. Sugar analysis

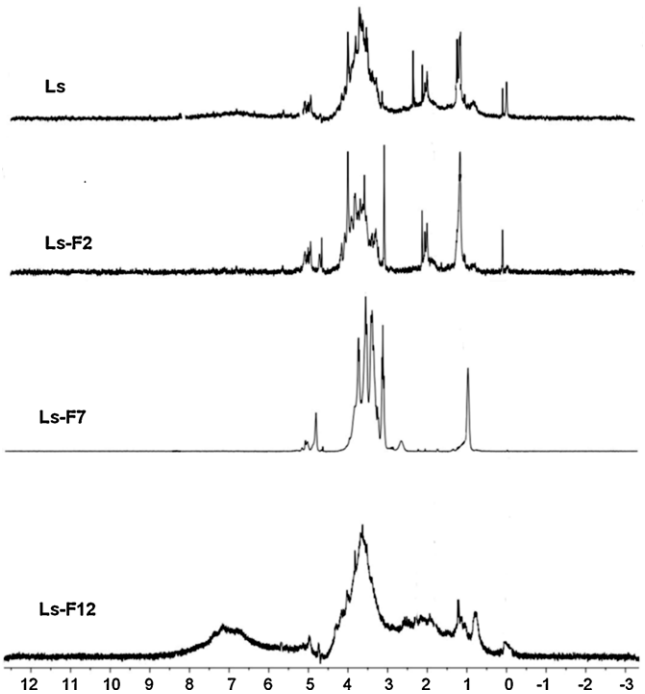

Fig. 3. ^{1}H NMR spectra of *L. salicaria* glycoconjugate (Ls) and its anticoagulant active ion-exchange fractions LsF2, LsF7 and LsF12.

Table 2
Activated thromboplastin time (aPTT) of *L. salicaria* glycoconjugate (Ls) and its ion-exchange fractions (LsF1–F12). The bold values indicate the clot was not observed in measured samples. Values are expressed as mean of 5 measurements ±SD.

	Control time (s)	Concentrations (μg/mL) in the clotting mixture							
		465.00	232.50	116.25	58.13	29.06	14.53	7.27	3.63
Ls	31.0 ± 1.1	**>300.0**	85.9 ± 3.2	43.1 ± 2.1	40.4 ± 2.0	36.5 ± 1.0	31.6 ± 0.8	31.6 ± 1.0	32.0 ± 1.0
Ls-F1	31.0 ± 1.1	101.4 ± 5.0	47.1 ± 3.2	35.9 ± 2.3	31.9 ± 1.2	31.5 ± 0.8	31.7 ± 0.9	32.4 ± 0.9	32.1 ± 1.1
Ls-F2	31.0 ± 1.1	**>300.0**	**>300.0**	123.0 ± 5.9	75.7 ± 3.4	49.1 ± 2.4	37.9 ± 1.7	34.6 ± 1.5	33.0 ± 1.1
Ls-F3	31.0 ± 1.1	22.7 ± 1.0	23.6 ± 1.1	27.3 ± 1.2	33.6 ± 1.5	35.7 ± 1.7	37.9 ± 1.7	36.2 ± 1.8	37.9 ± 1.8
Ls-F4	31.0 ± 1.1	33.2 ± 1.5	36.7 ± 1.6	37.8 ± 1.8	39.2 ± 1.8	38.2 ± 1.7	37.2 ± 1.8	36.5 ± 1.6	36.4 ± 1.6
Ls-F5	31.0 ± 1.1	75.0 ± 3.6	72.3 ± 3.5	58.2 ± 2.8	55.7 ± 2.6	37.9 ± 1.7	35.3 ± 1.6	35.2 ± 1.6	35.6 ± 1.7
Ls-F6	31.0 ± 1.1	72.1 ± 3.4	75.0 ± 3.6	70.7 ± 3.5	70.6 ± 3.3	30.7 ± 1.4	41.3 ± 1.9	37.8 ± 1.8	38.4 ± 1.7
Ls-F7	31.0 ± 1.1	**>300.0**	**>300.0**	132.2 ± 3.9	84.7 ± 3.9	74.6 ± 3.8	47.7 ± 2.4	37.5 ± 1.8	37.2 ± 1.6
Ls-F8	31.0 ± 1.1	63.1 ± 3.0	44.2 ± 2.1	37.6 ± 1.7	36.6 ± 1.6	37.4 ± 1.6	37.8 ± 1.7	38.6 ± 1.8	36.3 ± 1.6
Ls-F9	31.0 ± 1.1	81.7 ± 3.9	78.1 ± 3.7	69.0 ± 3.4	50.2 ± 2.4	37.9 ± 1.7	36.5 ± 1.6	36.7 ± 1.7	37.7 ± 1.7
Ls-F10	31.0 ± 1.1	64.3 ± 3.1	54.6 ± 2.4	44.5 ± 2.2	40.8 ± 2.0	43.3 ± 2.1	41.8 ± 2.0	40.8 ± 2.0	40.0 ± 1.8
Ls-F11	31.0 ± 1.1	70.7 ± 3.4	70.0 ± 3.5	63.3 ± 3.0	60.5 ± 2.9	52.1 ± 2.5	36.8 ± 1.7	38.1 ± 1.8	40.8 ± 1.9
Ls-F12	31.0 ± 1.1	**>300.0**	**>300.0**	144.6 ± 5.3	92.5 ± 4.7	63.5 ± 3.0	46.4 ± 2.2	41.1 ± 2.0	38.8 ± 1.8

revealed very high glucose (~60%) and rhamnose (~21%) contents in LsF7 while other sugars were found in small quantities only. The dominant H1 signal at δ 4.90 could derive from α-1,6-linked glucose residues (Maina, Tenkanen, Maaheimo, Juvonen, & Virkki, 2008) and thus indicates the prevalence of glucan type of polymer in LsF7. Moreover, the resonances due to the methyl protons of rhamnose residues at δ 1.2 were found as well.

^{1}H NMR spectrum of sodium hydroxide fraction LsF12 was not very well resolved. Besides signals due to carbohydrates, also those due to a protein part in the region δ 3.0–0.8 and phenolics (a broad hump of signals at δ 8.0–6.5) could be observed. This dark brown material contained the highest phenolic content of all fractions and was higher even than that of the native Ls glycoconjugate. In the anomeric region only signals of low intensities at δ 4.95–5.00 could be observed due to uronic acids, which represent the core of the carbohydrate part in LsF12.

3.2. Anticoagulant and procoagulant properties of *L. salicaria* ion-exchange glycoconjugates

The *in vitro* anticoagulant activity of *L. salicaria* preparation (Ls) and fractions obtained by separation on ion-exchange column (LsF1–F12) were studied. It has been found that some fractions are rich in uronic acids and phenolics, which give them polyelectrolyte properties similar to heparin – the most known anticoagulant polysaccharide (rich in sulfated esters) of animal origin (Middeldrop, 2008). The anticoagulant activity of Ls and its fractions were measured by activated partial thromboplastin time test (aPTT) and prothrombin time test (PT) (Tables 2 and 3). The standardized human plasma from health donors was used as a reservoir of coagulation cascade enzymes. All fractions were measured in different concentrations, in order to evaluate the strength of the biological activity. In our previous work (Pawlaczyk, Czerchawski, & Kuliczkowski, 2010) we have found that Ls showed complete inhibition of plasma clot formation in "*in vitro*" experiments, i.e. in aPTT as well as in PT tests. In aPTT test Ls clotting inhibition was observed at the concentration 465 μg/mL of human plasma, whereas its anticoagulant activity measured in PT test was very low. However, in "*in vivo*" tests the controversial effect (i.e. the pro-coagulant activity) of Ls on animal blood system was noticed. This controversial effect of Ls on blood coagulation process prompted us to separate Ls extract according to its negative charge by an ion-exchange chromatography. We have investigated the effect of individual ion-exchange fractions on coagulation process of human plasma in "*in vitro*" tests in order to find the most active component of Ls.

In vitro anticoagulant activities of Ls and its fractions are summarized in Tables 2 and 3. From the tables it is evident that the native Ls and only three fractions, i.e. LsF2, LsF7 and LsF12 have been shown to have a significant biological activity. Their activity increased in the order Ls < LsF2 < LsF7 < LsF12. The native Ls showed the complete inhibition of clotting process measured as a aPTT test at 465 μg/mL while other fractions, i.e. LsF2, LsF7 and LsF12 were more active and the complete inhibition of clotting process was observed as well at 232.5 μg/mL. Moreover, these fractions extended clotting process at 116.25 μg/mL 4–5 times and still even at 58.13 μg/mL 2–3 times (Table 2). Of all fractions tested, LsF12 has the highest anticoagulant activity. However, Ls-F2 fraction showed complete inhibition of clotting process at 465 and 230 μg/mL in PT test while Ls-F7 was not active at all whereas LsF12 extended clot-

Table 3
Prothrombin time (PT) of *L. salicaria* glycoconjugate (Ls) and its ion-exchange fractions (LsF1–F12). The bold values indicate the clot was not observed in measured samples. Values are expressed as mean of 5 measurements ±SD.

	Control time (s)	Concentrations (μg/mL) in the clotting mixture							
		465.00	232.50	116.25	58.13	29.06	14.53	7.27	3.63
Ls	13.2 ± 0.6	46.5 ± 2.2	24.3 ± 1.2	18.5 ± 0.9	14.7 ± 0.6	12.1 ± 0.6	11.6 ± 0.5	12.0 ± 0.5	13.2 ± 0.6
Ls-F1	13.2 ± 0.6	10.1 ± 0.3	9.7 ± 0.3	10.0 ± 0.4	10.3 ± 0.5	11.5 ± 0.6	11.4 ± 0.6	12.0 ± 0.6	13.0 ± 0.6
Ls-F2	13.2 ± 0.6	**>300.0**	**>300.0**	20.4 ± 1.0	15.8 ± 0.6	11.6 ± 0.5	9.9 ± 0.4	9.8 ± 0.4	10.6 ± 0.5
Ls-F3	13.2 ± 0.6	7.9 ± 0.3	9.0 ± 0.4	10.4 ± 0.5	11.4 ± 0.5	12.5 ± 0.6	13.3 ± 0.6	13.0 ± 0.6	13.5 ± 0.6
Ls-F4	13.2 ± 0.6	9.9 ± 0.5	11.2 ± 0.5	12.2 ± 0.6	13.0 ± 0.6	13.0 ± 0.6	13.3 ± 0.6	13.6 ± 0.5	13.5 ± 0.5
Ls-F5	13.2 ± 0.6	11.9 ± 0.6	12.2 ± 0.6	12.5 ± 0.6	12.8 ± 0.6	12.8 ± 0.6	13.0 ± 0.6	12.9 ± 0.6	13.1 ± 0.6
Ls-F6	13.2 ± 0.6	12.2 ± 0.5	12.4 ± 0.5	13.8 ± 0.7	12.9 ± 0.6	13.2 ± 0.6	13.4 ± 0.6	13.2 ± 0.6	13.6 ± 0.6
Ls-F7	13.2 ± 0.6	12.3 ± 0.5	12.0 ± 0.6	11.9 ± 0.6	12.0 ± 0.5	12.1 ± 0.6	12.5 ± 0.6	12.8 ± 0.6	13.0 ± 0.6
Ls-F8	13.2 ± 0.6	12.2 ± 0.5	12.7 ± 0.6	13.1 ± 0.6	13.0 ± 0.6	13.3 ± 0.6	13.3 ± 0.6	13.2 ± 0.6	13.2 ± 0.6
Ls-F9	13.2 ± 0.6	11.8 ± 0.5	11.7 ± 0.5	12.2 ± 0.5	12.6 ± 0.6	12.9 ± 0.6	13.1 ± 0.6	13.3 ± 0.6	13.5 ± 0.6
Ls-F10	13.2 ± 0.6	15.1 ± 0.7	15.9 ± 0.7	14.9 ± 0.7	15.6 ± 0.7	15.6 ± 0.7	15.1 ± 0.6	15.4 ± 0.6	14.2 ± 0.6
Ls-F11	13.2 ± 0.6	11.7 ± 0.5	11.4 ± 0.6	11.4 ± 0.5	12.0 ± 0.6	12.7 ± 0.6	12.9 ± 0.6	13.1 ± 0.6	13.2 ± 0.6
Ls-F12	13.2 ± 0.6	57.3 ± 2.8	46.0 ± 2.2	26.9 ± 1.2	18.4 ± 0.9	13.5 ± 0.6	12.5 ± 0.6	12.1 ± 0.6	12.3 ± 0.6

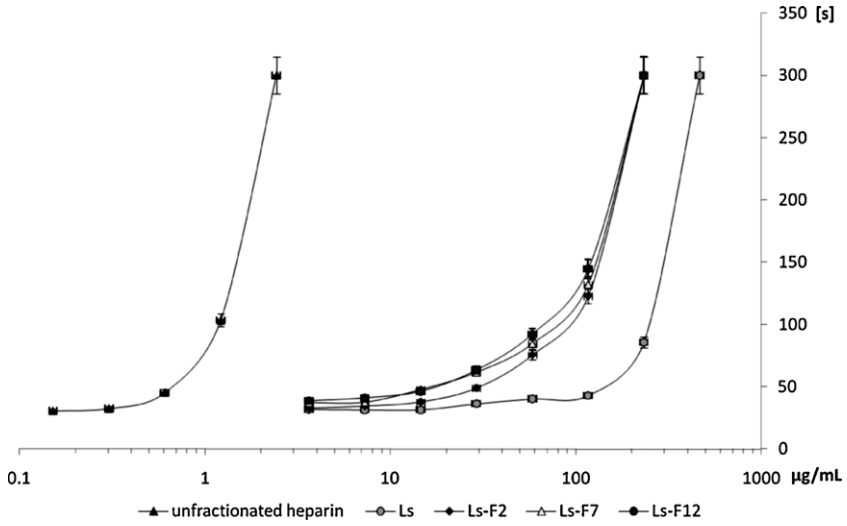

Fig. 4. The *in vitro* anticoagulant activity of the *L. salicaria* glycoconjugate (Ls), measured in aPTT test on human blood plasma, in comparison with the activity of unfractionated heparin anticoagulant (144 IU/mg).

ting process 4.3 and 3.5 times, respectively, being more active than LsF2 at concentration 116.25 μg/mL (Table 3).

Anticoagulant activities of the native Ls extract and its active fractions LsF2, LsF7 and LsF12, measured in aPTT test, were compared with the activity of the unfractionated heparin (144 IU/mg). As it can be seen from Fig. 4, the anticoagulant activity of the native Ls glycoconjugate was 0.7 IU/mg while some of its fractions, LsF2, LsF7 and LsF12 reached 2.4, 2.9 and 3.2 IU/mg, respectively. From these results it is evident that some of the Ls components show 3.4–4.6 times higher anticoagulant activity than that of the native Ls glycoconjugate.

From the results of *in vitro* aPTT and PT tests (Tables 2 and 3) it can be seen that besides fractions with significant or moderate anticoagulant activity, there are fractions with procoagulant effect in Ls. LsF3 fraction showed a weak procoagulant activity in both tests at the concentrations of 465 and 232.5 μg/mL, while LsF4 at this concentrations was effective in PT test only. Although pro-coagulant activities of LsF3 and LsF4 fractions were not very significant, they could partially explain *in vivo* procoagulant effect of the native *Lythrum* extract. It is known that the coagulation mechanisms in the blood circulation system are connected not only with enzymes of coagulation cascade, but also with blood cells, mainly with platelets. The mechanisms affecting the balance between coagulant and anticoagulant processes in the blood circulation system – the normal *in vivo* haemostasis, are complex, with many interrelated pathways and component factors, affected by a variety of cellular elements and the endothelium itself (Stone & Shore-Lesserson, 2006). It would be also possible that the procoagulant activity of *Lythrum* extract could be probably connected not only with the presence of glycoconjugates in LsF3 and LsF4, but also with the interaction with other factors in blood circulation system, like platelets. However, it will require additional more advanced biological studies of these complex plant macromolecules. To be able to answer such question, it is necessary to find if some of the components may induce platelet aggregation.

In conclusion, the ion-exchange chromatography of *L. salicaria* extract afforded twelve fractions which were characterized from chemical and biological point of view. *In vitro* anticoagulant tests revealed three fractions with higher anticoagulant activity as that of the native Ls and their effect increased in the order Ls < LsF2 < LsF7 < LsF12. The most active LsF12 showed the highest phenolic content of all fractions and a low occurrence of carbohydrates rich in uronic acids. Although carbohydrates content in LsF12 is low, their presence is important due to the water solubility of phenolic being probably in glycoside form. This strongest bound to the ion-exchange resin conjugate had low molecular mass ~4900. The second most active LsF7 was rich in neutral carbohydrates, and it had a negligible uronic acids content and about 25 times lower phenolic content as that of LsF12. Moreover, it showed low molecular mass ~3600. LsF2 conjugate was rich in phenolics while the carbohydrate part was relatively low and rich in uronic acids. Besides, it showed molecular heterogeneity. It has been found that the polyanionic character of heparin macromolecules is responsible for its high anticoagulant activity. Chemical analyses of active fractions revealed high phenolic and uronic acids contents in Ls, LsF2 and LsF12 and thus confirmed polyanionic character of these macromolecules. It has been found that this polyanionic character is important for anticoagulant activity (Pawlaczyk et al., 2009; Yoon, Pereira, Pavão, Hwang, Pyun, & Mourão, 2002). However, LsF7 fraction with low phenolics content and negligible amounts of uronic acids in revealed a high anticoagulant activity. To enlighten this fact found for LsF7, additional biological studies are required.

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