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Carbohydrate Polymers 47 (2002) 27–33

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Structural characterization and antitussive activity of a glucuronoxylan from *Mahonia aquifolium* (Pursh) Nutt.

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Received 31 July 2000; revised 23 October 2000; accepted 20 November 2000

Abstract

From the stems of *Mahonia aquifolium* (Pursh) Nutt. a water-soluble (4-*O*-methyl-α-D-glucurono)-D-xylan was isolated by alkaline extraction and fractionation of the crude hemicellulose, employing ion-exchange chromatography and gel filtration. The results of compositional and linkage analysis, supported by NMR spectral data of the polysaccharide, showed the (1 → 4)-linked β-D-xylopyranosyl backbone with about 15% of 4-*O*-methyl-D-glucuronic acid attached to *O*-2 of the xylose residues. The distribution pattern of uronic acid units along the xylan chain was determined by the method based on interpretation of the activity coefficient of the calcium counter-ions, estimated in a molecular-disperse solution of calcium salt of the polysaccharide. The results evidenced that the branching of the xylan molecule is not regular. When tested for antitussive activity on mechanically induced coughing in cats, the glucuronoxylan exhibited a much greater effect in comparison to the drugs used in clinical practice to treat coughing.

Keywords: *Mahonia aquifolium*; Glucuronoxylan; Structure; Antitussive activity

1. Introduction

Mahonia aquifolium (Pursh) Nutt. from the Berberidacea family is a shrub widespread in the forests of the North American Pacific coast and is known also as a cultivated plant in gardens and parks called mahonia. It has been used for a long time in homoeopathy as an organotropic drug for treatment of inflammatory scaling dermatoses and, now, is available also as a topical antipsoriatic drug. The active principle of the mahonia extract in the treatment of psoriasis is thought to be the alkaloids (Hänsel, 1992). However, there are opinions in the literature that other, not yet determined compounds also may be responsible for the above-mentioned effect of the tincture. Since our ongoing program is directed towards the isolation of new, active polysaccharides of plant origin, we subjected the mahonia tincture, for the first time, to analysis for the presence of carbohydrates and found that in addition to sugars it contained a mixture of low-molecular-weight polysaccharides (Kardošová & Košt'álová, 1999). The finding in our preliminary experiments that these polysaccharides showed immunomodulating activity induced our interest in mahonia as another plant source of potentially active polysaccharides. In the present work, we have studied the hemicellulosic material obtained from the mahonia stems and provide the results on chemical structure and antitussive activity of its dominant component, a glucuronoxylan.

2. Experimental

2.1. Materials

The plant mahonia was gathered in the garden of the Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic and the voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Comenius University. DEAE-Sephadex A-50 was obtained from Pharmacia (Sweden), Bio-Gel P-4 from Bio-Rad (USA), and Sep-pak C_{18} cartridges from Waters Associates (USA). All chemicals used were of analytical grade.

2.2. General methods

Concentrations were performed under diminished pressure at a bath temperature not exceeding 45°C. Free-boundary electrophoresis of polysaccharide solution (10 mg ml^{-1}) was performed in 0.05 M sodium tetraborate with a Zeiss 35 apparatus at 150 V and 8 mA for 30 min. Optical rotation (1-ml cell) was measured at 20 ± 1°C with

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0144-8617/02/$ - see front matter
PII: S0144-8617(00)00341-6

a Perkin–Elmer Model 141 polarimeter. The number-average molecular mass $\bar{M}_n$ was determined osmometrically at 30°C using a Knauer pressure osmometer.

High-pressure gel-permeation chromatography (HPGPC) was performed using a commercial instrument (Laboratorní prístroje, Prague, Czech Republic) equipped with two Labio Prague Biospher GM 300 and 1000 exclusion columns (8×250 mm^2) and using aqueous 0.1 M $NaNO_3$ as solvent (0.4 ml min^{-1}). The eluate was monitored by RI and UV detectors. The infrared spectrum of the methylated polysaccharide was recorded with a Nicolet Magna 750 spectrometer.

Descending paper chromatography was performed on Whatman No. 1 paper in the following solvent systems: S1, 8:2:1 EtOAc–pyridine–water; and S2, 18:3:1:4 EtOAc–AcOH–formic acid–water, the sugars being detected with anilinium hydrogen phthalate. Carbohydrates were determined by the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) and the uronic acid content was determined potentiometrically and by the method of Blumenkrantz and Asboe (1973). The total hydrolysis of polysaccharides was effected with 2 M CF_3CO_2H (TFA) at 120°C for 2 h. The sugars in the hydrolyzate were converted to alditol trifluoroacetates and analyzed by gas chromatography on a Hewlett-Packard 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m) at a temperature program of 110–125°C (2°C min^{-1}) to 165°C (20°C min^{-1}) and a flow rate of hydrogen 20 ml min^{-1}. GC–MS analysis of partially methylated alditol acetates of saccharides was carried out on a Finnigan Mat SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80–240°C (6°C min^{-1}), 70 eV, 200 μA, and ion-source temperature 150°C. Potentiometric titrations were performed using a Radiometer PHM 64 (Denmark) equipped with a GK 2401 combined electrode. Spectrophotometric measurements were carried out on a Specol 11 (Zeiss, Jena, Germany) spectrometer. Reagents used in determination of activity coefficients of Ca^{2+} were: 0.084 mol l^{-1} KOH, 0.020 mol l^{-1} $Ca(OH)_2$, 0.005 mol l^{-1} $CaCl_2$, redistilled water freed from carbon(IV) oxide, and tetramethylmurexide. Ultracentrifugation of the solution of calcium salt of glucuronoxylan was performed on a Beckman Type L8-50M/E ultracentrifuge with a Ti-50 rotor at 45 000 rpm for 90 min. NMR spectra were recorded at 25°C on an FT NMR Bruker DPX 300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.46 MHz) in D_2O and chemical shifts of signals were referenced to internal acetone (2.225 and 31.07 ppm for ^{1}H and ^{13}C, respectively).

2.3. Isolation of (4-O-methyl-α-D-glucurono)-D-xylan

The methanol-pretreated, cold- and hot-water extracted, air-dried mahonia stems (400 g) were extracted with 0.25 M NaOH (8 l) for 24 h at room temperature. The plant residue was separated from the supernatant by filtration. The supernatant after centrifugation (5000 g) was precipitated with 4 volumes of ethanol and the precipitate was collected by centrifugation, suspended in water, exhaustively dialyzed, and freeze-dried. The brownish product was further purified by washing with 80% aqueous ethanol acidified with HCl (1 vol.%) to give 11.2 g of crude hemicellulose (CHC). CHC (200 mg) was loaded onto a column (5×100 cm^2) of DEAE-Sephadex A-50 (carbonate form) and irrigated successively with water (W) and 0.25 M (C1) and 0.5 M (C2) carbonate solutions. The largest, water-eluted fraction W was further purified by gel filtration on a column (2.5×130 cm^2) of Bio-Gel P-4 by water elution to give a homogeneous polysaccharide composed of D-xylose and 4-*O*-methyl-D-glucuronic acid.

2.4. Methylation analysis

The polysaccharide (50 mg), pretreated with $NaBH_4$, was methylated according to the method of Ciucanu and Kerek (1984) to give a fully methylated product, as evidenced by the absence of IR absorption for hydroxyl. The methylated sample was recovered using a Sep-pak C_{18} cartridge by the procedure of Waeghe, Darvill, McNeil and Albersheim (1983). The product was then converted into partially methylated alditol acetates by hydrolysis, reduction with $NaBD_4$, and acetylation, and was subjected to linkage analysis by GC–MS (Jannson, Kenne, Liedgren, Lindberg & Lönngren, 1976).

2.5. Determination of activity of calcium ions in the solution of (4-O-methyl-α-D-glucurono)-D-xylan and distribution of the uronic acid units in the polysaccharide

The polysaccharide solution was percolated through a Dowex 50Wx2 (H^+) cation-exchanger and then neutralized to the point of equivalence with saturated $Ca(OH)_2$ solution. The activity of Ca^{2+} ions was determined in the solution of the calcium salt of the polysaccharide (3.00 mmol $COOCa_{0.5}$ l^{-1}) by a spetrophotometric method using tetramethylmurexide ($c = 4 \times 10^{-5}$ mol l^{-1}) as the metallochromic indicator (Kohn, 1974; Kohn & Furda, 1967). The solution did not contain any additional inert electrolyte. The calibration curve was constructed from the data obtained with $CaCl_2$ solution. The single-ion activity coefficient γ_{Ca}^{2+} was calculated from the activity of Ca^{2+} in the solution ($c_{Ca}^{2+} = 1.5$ mmol l^{-1}) of calcium salt of the polysaccharide.

The mean distance b (nm) between two adjacent carboxyl groups in their perpendicular projection on the axis of the xylan chain was estimated from the analytical curve $\gamma_{Ca}^{2+} = f(b)$. This function was introduced by Kohn and Furda (1967) using the activity coefficient values determined in model solutions (Kohn, 1974).

2.6. Antitussive activity tests

The experiments were performed on adult non-anaesthetized

Table 1
Characterization of the crude hemicellulose (CHC) and the hemicellulose fractions (W, C1, C2)

Characteristics	CHC	W	C1	C2
Yield (%)	2.8[a]	60.27[b]	36.43[b]	3.28[b]
Ash (%)	5.6	nd[c]	nd[c]	nd[c]
M_n	nd[c]	36 700	17 024	19 324
$[D]_{22}^{D}$ ($c = 0.5$, water)	nd[c]	−52.7°	+29.35°	+58.6°
Uronic acid (%)	15.5[d]	13.8[d,e,f]	42.3[d,g]	50.3[d,g]
Composition of neutral sugar components (mol%)				
Rhamnose	3.57	1.75	9.93	9.78
Arabinose	5.41	1.99	5.87	12.35
Xylose	83.57	92.85	70.92	53.90
Mannose	1.53	0.59	1.87	3.90
Glucose	4.04	1.98	3.46	9.86
Galactose	1.85	0.81	7.92	10.18

[a] Related to dry plant.
[b] Related to CHC.
[c] Not determined.
[d] Potentiometric titration was used for determination of uronic acid.
[e] The *m*-hydroxydiphenyl method was used for determination of uronic acid.
[f] The uronic acid was proved to be 4-*O*-methyl-D-glucuronic acid.
[g] The uronic acid was proved to be D-galacturonic acid.

cats of both sexes weighing 1500–2500 g (10 in each set). After several days' quarantine, the animals were surgically implanted with a tracheal cannula. This enabled the mechanical stimulation of airways and the recording of the side tracheal pressure. The mucous membranes of the laryngopharyngeal (LP) and tracheobronchial (TB) areas were stimulated consecutively five times with a 0.35-mm-diameter nylon fibre. The cough parameters, i.e. the number of efforts (NE), intensity of cough attacks in expirium (IA^+) and inspirium (IA^-), cough frequency ($NE\ min^{-1}$), and intensity of maximal cough effort in expirium (IME^+) and inspirium (IME^-), were evaluated on the basis of the pressure values recorded on a Biograph 12-03 electromanometer. The water solution of the tested compound was administered perorally in a dose of 50 mg kg^{-1} b.w. The values of cough parameters obtained prior to the administration of the tested compound represented the normal value (*N*). For comparative purposes commercial products generally used in clinical practice to treat coughing, i.e. prenoxdiazine (P), dropropizine (D), and codeine (C), were tested along with the polysaccharide. The effect of drugs was monitored in time intervals 0.5, 1, 2, and 5 h. Statistical evaluation of the results was carried out by the method of Wilcoxon and Wilcox (1964). The doses of the individual comparative drugs used herein, i.e. P = 30 mg kg^{-1} b.w., D = 100 mg kg^{-1} b.w., C = 10 mg kg^{-1} b.w., represented the amounts which, in earlier experiments, exhibited the highest antitussive effect.

Table 2
Characterization of (4-*O*-methyl-D-glucurono)-D-xylan (X)

D-Xylose (%)	85.25
4-*O*-Methyl-D-glucuronic acid (%)	14.75
$-OCH_3$ (%)	1.24
$\bar{M}_n$	37 273
$[\alpha]_{22}^{D}$ ($c = 0.5$, water)	−54.7°
Xylose: 4-*O*-Methyl-D-glucuronic acid	8.4:1.0

3. Results and discussion

3.1. Isolation and structural characterization of the glucuronoxylan

From the stems of *Mahonia aquifolium* a CHC was obtained in 2.8% yield (on dry weight basis) by alkaline extraction, followed by ethanol precipitation. In order to decolourize and eliminate most of the accompanying polyphenolic compounds, the precipitate was washed with acidified aqueous ethanol. The undialyzable portion, the sugar composition of which suggested heterogeneity (Table 1), was subjected to ion-exchange chromatography to give three fractions (W, C1, C2) of different sugar compositions (Table 1). The largest fraction W represented 60% of CHC and contained besides the prevalent D-xylose component, only trace amounts of other neutral sugars. It was further purified by gel filtration to give a polysaccharide (X) homogeneous by free-boundary electrophoresis and HPGPC. As determined by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe, 1973) and potentiometric titration, the polysaccharide contained 14.75% uronic acid, which was proved in the hydrolyzate to be 4-*O*-methyl-D-glucuronic acid, confirmed also by the methoxyl content (Table 2) and ^{13}C NMR spectral data (Table 3).

The main product of methylation of the polysaccharide was 2,3-di-*O*-methyl-D-xylopyranose (Table 4), arising from the (1 → 4)-linked xylose chain units. The amount of the 3-*O*-methyl derivative 13.79 mol% (12.81 from single substitution + 0.98 from double substitution), fitting well the amount of uronic acid found by previous methods (Table 2), evidenced that the uronic acid is attached to *O*-2 of D-xylose units. The detected small amount of non-reducing xylose units 1.82 mol% should then be attached to *O*-3 of the chain xylose residues, as indicated by the amount of the 2-*O*-methyl derivative 1.64 mol% (0.66 from single substitution + 0.98 from double substitution).

The chemical shifts of the carbon signals in the ^{13}C NMR spectrum of the glucuronoxylan (Table 3) confirmed the results of the linkage analysis. The C-4 signal of the internal unsubstituted xylose units was downfield shifted to 77.21 ppm, thus confirming the involvement of this carbon in a (1 → 4) linkage. For substituted internal xylose units, besides the C-4 signal at 76.90 ppm, the C-2 signal also was downfield shifted to 77.67 ppm owing to *O*-2 substitution with 4-*O*-methyl-D-glucuronic acid. The signals generated by the resonance of anomeric carbons of xylose units appeared at 102.58–103.01 ppm and reflected, together with the vicinal coupling constant $^3J_{1,2} = 7.3\ Hz$, the β-type of the xylose interunit bonds. The α-linkage of 4-

Table 3
^{13}C NMR data for sugar residues of the glucuronoxylan

Sugar residue	Chemical shift (ppm)						
	C-1	C-2	C-3	C-4	C-5	C-6	OMe
Xyl-int[a]	102.58	73.57	74.53	77.21	63.38		
Xyl-int-s[b]	102.17	77.67	72.95	76.90	63.83		
Xyl-nr[c]	103.01	na[d]	76.50	70.06	66.67		
4-*O*-Me-D-GlcA	98.58	73.13	73.13	82.98	71.95	176.49	60.82

[a] Refers to the internal unit.
[b] Refers to the internal substituted unit.
[c] Refers to the non-reducing unit.
[d] The signal was not assigned.

O-methyl-D-glucuronic acid was evidenced by the vicinal coupling constant $^3J_{1,2} = 3.4\ Hz$.

The above-mentioned results prove that X has a (1 → 4)-linked β-D-xylopyranosyl backbone with a monomeric 4-*O*-methyl-D-glucuronic acid attached to each eighth chain unit, on the average. A negligible amount of non-reducing xylose units (below 2%) was found to be linked at the *O*-3 position.

3.2. Distribution of uronic acid in the X molecule

The distribution pattern of uronic acid in the xylan molecule was elucidated on the basis of the single-ion activity coefficient of calcium counter-ions determined in the solution of calcium salt of the polysaccharide. The prerequisite for applying this method is the electrostatic bond between the calcium ions and carboxyl groups of the polysaccharide. The activity coefficients of counter-ions bound by an electrostatic bond to the carboxyl groups of the polysaccharide are a function of linear charge density of the macromolecule characterized by the mean distance (b, nm) between two adjacent carboxyl groups (Kohn & Luknár, 1975). The function $\gamma_{Ca}^{2+} = f(b)$ has a general validity for linear acid polysaccharides, regardless of the kind of uronic acid unit in the macromolecule. Though this relationship was proved to be valid for linear acidic polysaccharides, we consider this function applicable to the present glucuronoxylan since the uronic acid is attached to the linear chain exclusively as a single terminal unit, similarly as in the case of the glucuronoxylan from *Rudbeckia fulgida* where this method was applied and where the results were confirmed by another method (Kardošová, Matulová & Malovíková, 1998).

Table 4
Partially methylated alditol acetates obtained from the methylated glucuronoxylan

Derivative[a]	Mol%	Linkage indicated
2,3,4-Me_3-Xyl	1.82	Xyl*p*-(1 →
2,3-Me_2-Xyl	83.70	→ 4)-Xyl*p*-(1 →
2-Me-Xyl	0.66	→ 3,4)-Xyl*p*-(1 →
3-Me-Xyl	12.81	→ 2,4)-Xyl*p*-(1 →
Xylose	0.98	→ 2,3,4)-Xyl*p*-(1 →

[a] The derivative 2,3,4-Me_3-Xyl refers to 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-D-xylitol, etc.

To ascertain the electrostatic bond between calcium ions and the carboxyl groups, i.e. that the solution of the calcium salt of the polysaccharide was molecular-disperse and did not contain any aggregates or microgel particles which would indicate intermolecular bonds of chelate nature, the solution was subjected to ultracentrifugation and the calcium ion activity was determined also in the supernatant. It was found that the value of the activity coefficient of calcium ions in the supernatant was identical with that determined in the solution of the polysaccharide before ultracentrifugation, i.e. $\gamma_{Ca}^{2+} = 0.52$. Moreover, the HPGPC chromatogram profile of the original polysaccharide was identical to that of the compound in the supernatant.

The distance between two vicinal carboxyl groups in the studied glucuronoxylan, obtained from the linearized form of the function $\gamma_{Ca}^{2+} = f(b)$, is $b = 1.95$ nm. When considering the analogy with the cellulose or mannuronan molecules which have the same type of glycosidic bonds (diequatorial transglycosidic β-(1 → 4) bonds of saccharide units in $^4C_1$ conformation), the length of one xylose unit in the glucuronoxylan chain is $b_0 = 0.515$ nm (Atkins, 1977). Then from the above-obtained value of 1.95 nm it follows that approximately each fourth chain xylose residue carries one terminal uronic acid unit. However, the polysaccharide was found to contain on average one uronic acid unit per 8.4 xylose units (Table 2). If the distribution of uronic acid in the molecule is regular, at such a ratio of the component sugars, the mean distance between the neighbouring carboxyl groups should be $b_1 = 4.32$ nm. As the value found was much lower (1.95 nm), it is apparent that the glucuronoxylan molecule is irregularly branched. We assume that it contains "acidic blocks" (uronic acid attached to each fourth xylose unit) alternating with "neutral blocks" composed of exclusively unsubstituted xylose units or containing only a small number of uronic acid units.

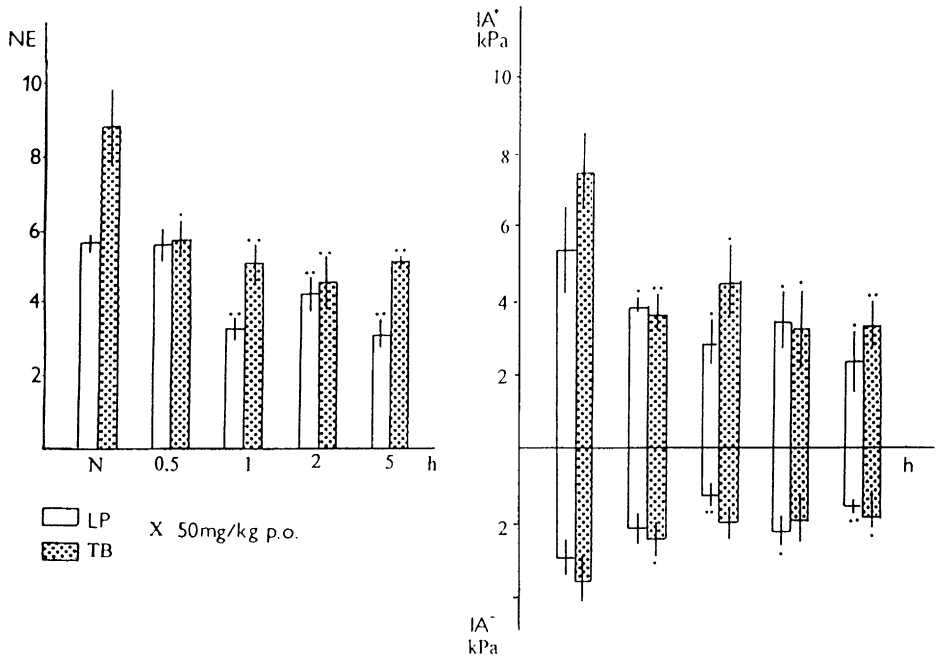

Fig. 1. Changes in the number of cough efforts (NE) and intensity of cough attack in expirium (IA^+) and inspirium (IA^-) from laryngopharyngeal (LP) and tracheobronchial (TB) areas after peroral administration of glucuronoxylan (X) in the dose 50 mg kg^{-1} b.w. N = normal value of cough parameters. The columns represent the mean values of cough parameters, the range denotes standard error of means, 5% significance is marked with one dot, 1% with two dots.

3.3. Antitussive activity of the glucuronoxylan (X)

The results of tests showed that peroral administration of X brought about a statistically significant decrease in the number of cough efforts (NE) and intensity of cough attacks in expirium and inspirium (IA^+ and IA^-) from both stimulated regions (LP and TB) of the airways (Fig. 1). This significant inhibition of cough attacks contributes to positive assessment of the antitussive effect of the tested compound. It is important from the therapeutic point of view because an increase of intrathoracic pressure may have negative consequences for the patient.

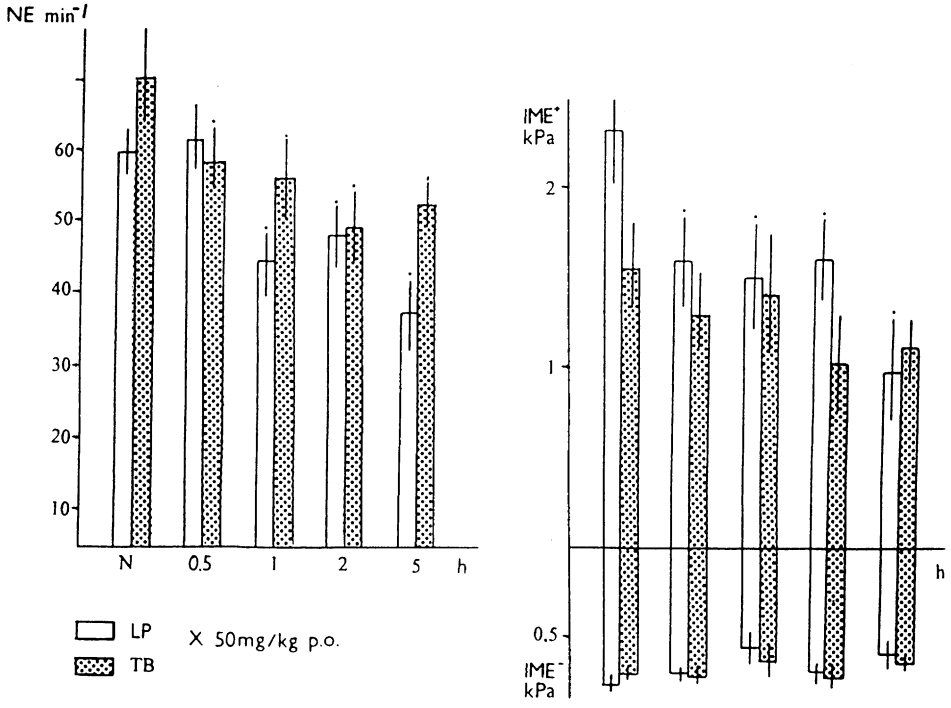

Fig. 2. Changes of cough frequency (NE min^{-1}) and intensity of maximum expiratory (IME^+) and inpiratory (IME^-) cough efforts after administration of X.

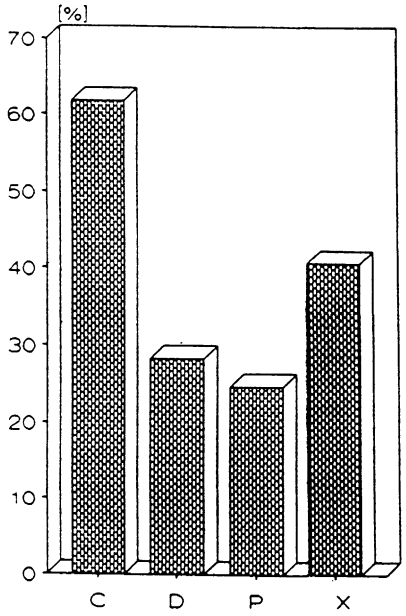

Fig. 3. Effect of X on cough parameters (NE + IA) in comparison to those of commercial drugs C (codeine), D (dropropizine), and P (prenoxdiazine); 100% is equivalent to *N* in Figs. 1 and 2.

Likewise, the frequency of coughing (Fig. 2) was reduced significantly from both LP and TB regions. It is noticeable that the number of efforts and frequency of coughing from the TB region decreased 30 min after administration of the compound and lasted throughout the whole experiment, while from the LP region the decrease was registered 1 h after administration. This means that the onset of the effect of glucuronoxylan was prompt, important for clinical practice.

The intensity of maximum cough efforts (IME^+, IME^-) from the TB region (Fig. 2) was not affected significantly, indicating that expectoration was not suppressed. This is important because the mucus does not cumulate in the airways. Comparison of cough parameters from the TB and LP regions revealed different abilities to influence the mechanism regulating the quality and quantity of coughing. The tests confirmed our earlier finding (Korpáš & Nosál'ová, 1991) that compounds with dominant peripheral mechanisms reduce the frequency of coughing but have much less influence on its amplitude. The frequency of coughing depends probably on the condition of the cough receptors, while the amplitude is determined by the condition of the cough centre. We assume that in the mechanism of action of the glucuronoxylan anti-inflammatory, antioxidant, and antiproliferative activities take part. In this way the protective effect on the cough receptors (Müller & Ziereis, 1994; Müller, Ziereis & Gawlik, 1995; Wiesenauer & Ludtke, 1996) is achieved. Comparative tests performed under the same conditions with some drugs used in clinical practice revealed that the glucuronoxylan was less active (X = 40.1%) than the most frequently used opioid antitussive drug codeine (C = 61.8%), but much more active than the two non-narcotic synthetic drugs dropropizine (D = 28.3%) and prenoxdiazine (P = 24.7%) (Fig. 3).

A glucuronoxylan of similar primary structure but different distribution pattern of uronic acid in the molecule, isolated from the herbaceous plant *Rudbeckia fulgida* (Kardošová et al., 1998), was found to possess a similar cough-reflex suppressing activity (Nosál'ová, Kardošová & Franová, 2000). This suggests that the uronic acid distribution has no effect. It may be concluded that owing to the high antitussive activity, the defined structure of the natural polymer and the possibility of obtaining this compound in large amounts from plant source, this polymer should be considered among prospective antitussive agents.

Acknowledgements

This investigation was supported by the Slovak Scientific Grant Agency VEGA (Grant No. 2/7138).

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