Chemistry of Natural Compounds, Vol. 43, No. 6, 2007

LAMIACEAE CARBOHYDRATES. II. WATER-SOLUBLE POLYSACCHARIDES FROM *Mentha x piperita*

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UDC 582.949.27+547.917

The composition of water-soluble carbohydrates from the aerial part of Mentha x piperita *variety Krasnodar 2 was studied. Fructose, glucose, saccharose, and raffinose were observed in the free carbohydrates. Water-soluble polysaccharides were represented by* α*-(1→4)-glucans branched at C-6 (MPW$_C$1, MPW$_C$4, MPW$_H$1) and* β*-(1→4)-galactans (MPW$_C$2, MPW$_C$3, MPW$_C$5, MPW$_H$2, MPW$_H$3). The isolated compounds were found to have membrane-stabilizing and antiatherogenic activities.*

Key words: *Mentha x piperita*, Lamiaceae, free carbohydrates, water-soluble polysaccharides, glucans, galactans, biological activity.

Pectinic substances and hemicelluloses from the aerial part of *Mentha x piperita* L. (Lamiaceae) variety Krasnodar 2 were previously isolated and characterized [1]. The goal of the present work was to isolate and study free carbohydrates and water-soluble polysaccharides (WSPS) from *M. piperita*.

WSPS were isolated from raw material that was freed beforehand of lipophilic and alcohol-soluble substances and successively treated with cold and hot water. Precipitation with ethanol produced polysaccharide complexes MPW_C and MPW_H, respectively.

The alcohol extract contained free carbohydrates (8.98-9.59% of the absolute dry mass) such as fructose (2.9-3.1%), glucose (2.4-2.8%), saccharose (1.2-1.3%), and raffinose (0.9-1.2%).

WSPS from *M. piperita* (MPW_C and MPW_H) are colored substances with a significant amount of phenols and ash components. The carbohydrate component makes up 16-30% of the mass of the whole complex (Table 1). They typically have a positive specific rotation and give a reaction with iodine. They also lack uronic acids in the hydrolysate. The principal monosaccharides for MPW_C and MPW_H are Glc and Gal (1:1.2) and Glc, Ara, and Gal (2:1.3:1), respectively.

WSPS were heterogeneous according to gel chromatography. Treatment of MPW_C and MPW_H with KU-2-8 ion-exchange resin (H^+-form) produced carbohydrate components $MPW_{C'}$ and $MPW_{H'}$ that did not contain ash elements, protein, and phenolic compounds (carbohydrates 93-95%, ash content <1%, nitrogen, not detected) and fractions of phenolic compounds Ph_1 and Ph_2 [positive reactions with $FeCl_3$, $NH_4Fe(SO_4)_2$, Folin reagent].

The monosaccharide and component compositions of $MPW_{C'}$ and $MPW_{H'}$ were identical to those of MPW_C and MPW_H. Preparative gel chromatography of $MPW_{C'}$ isolated the five substances MPW_C1, MPW_C2, MPW_C3, MPW_C4, and MPW_C5 in the ratio 2:1:1:2:7; of MPW_H, the three substances MPW_H1, MPW_H2, and MPW_H3 in a 3:2:9 ratio. Table 2 gives the principal properties of the isolated substances.

Polysaccharides MPW_C1, MPW_C4, and MPW_H1 contained a significant amount of Glc (86.4-96.3%). Cleavage of the substances by α-amylase gave Glc, maltose, and substance X as the main hydrolysis products. Lactose was also found for MPW_C4. Preparative isolation (paper chromatography, PC) of substance X and quantitative hydrolysis of it established that it contained Glc and Ara in a 1:1 ratio. The ability to be cleaved by α-amylase, the presence of maltose after enzymatic hydrolysis, and a positive $[\alpha]_D^{20}$ suggested that it contained α-1→4-bound Glc. The periodate consumption (~1 mol/anhydrous unit), the presence of erythritol after Smith degradation of polyols, and results from CrO_3 oxidation (Glc in the hydrolysate) confirmed this.

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 0009-3130/07/4306-0648

TABLE 1. Physicochemical Properties of MPW_C and MPW_H

Component	Content in raw matl., %	Content, %				$[\alpha]_D^{20}$,° (*c* 0.1, water)	pH (*c* 0.1, water)	Monosaccharide composition, mol %				
		carbohydrates	phenols	ash	nitrogen			Ara	Gal	Glc	Rha	Xyl
MPW_C	1.55-2.93	16.23±0.45	28.56±1.57	29.37±0.86	13.21±0.52	+10	7.0-7.2	8.3	46.7	39.9	1.4	3.7
MPW_H	1.32-1.66	30.43±1.21	24.11±1.15	28.11±1.63	9.56±0.18	+18	6.8-7.1	25.6	41.7	20.2	2.4	12.1

TABLE 2. Properties of MPW_C' and MPW_H' Components

Component	Content in total fraction, %	$M \cdot 10^4$, Da	$[\alpha]_D^{20}$, ° (*c* 1.0, water)	Cleavage by α-amylase	$NaIO_4$ consumption*	HCOOH release	K^{br}**	Monosaccharide composition				
								Ara	Gal	Glc	Rha	Xyl
					MPWC′							
MPW_C1	14.72	5.50	+70	+	0.98	0.099	9-10	6.6	-	93.4	-	-
MPW_C2	8.70	3.20	-	-	-	-	-	14.7	78.6	-	3.5	3.2
MPW_C3	7.30	1.60	-	-	-	-	-	16.2	75.8	-	2.4	5.6
MPW_C4	16.73	0.65	+58	+	0.94	0.284	3-4	3.2	9.6	86.4	-	0.8
MPW_C5	52.55	0.35	-	-	1.05	0.270	4	8.2	62.2	22.2	1.7	5.7
					MPW_H'							
MPW_H1	20.56	5.00	+64	+	0.94	0.074	15	2.6	-	96.3	-	1.1
MPW_H2	14.15	2.80	-	-	0.88	0.142	5	18.6	74.8	3.1	-	3.5
MPW_H3	65.29	1.50	-	-	0.94	0.224	4	33.3	46.3	-	3.6	16.8

* mol/anhydrous unit, ** K^{br} indicates the number of main-chain units used for one branch.

Exhaustive Hakomori methylation of MPW_C1, MPW_C4, and MPW_H1 produced the permethylates, which were analyzed by TLC after formolysis and hydrolysis. Comparison of mobilities with those of authentic samples of methylated glucopyranoses and subsequent densitometry of the chromatograms identified 2,3,6-tri-*O*-Me-Glc and 2,3-di-*O*-Me-Glc in 8.5:1.0, 3.0:1.0, and 15.0:1.0 ratios for MPW_C1, MPW_C4, and MPW_H1, respectively. Trace amounts of 2,3,4,6-tetra-*O*-Me-Glc were also observed. The presence of 2,3,6-tri-*O*-Me-Glc confirmed that (1→4)-bound glucose was present in the main polymer chains. The observance of 2,3-di-*O*-Me-Glc indicated that branching occurred at the glucose C-6. The tetrasubstituted glucopyranose was generated from terminal glucose units.

The principal monosaccharides for MPW_C2, MPW_C3, MPW_H2, and MPW_H3 were Ara and Gal; for MPW_C5, Glc and Gal (Table 2). Periodate oxidation consumed ~1 mol/anhydrous unit (Table 2). Erythritol was the main hydrolysis product of the reduced polyols.

Gal was not observed in the hydrolysate after CrO_3 oxidation. This is possible if the main chain had β-bonds. Maxima in the IR spectra at 890-891 cm^{-1} were characteristic of all substances. These facts suggest that β-1→4-bound Gal was probably present.

Chromatography (TLC) of the hydrolysate of MPW_C5, MPW_H2, and MPW_H3 permethylates detected 2,3,6-tri-*O*-Me-Gal and 2,3-di-*O*-Me-Gal in 3.0:1.0, 4.0:1.0, and 3.0:1.0 ratios, respectively. Traces of 2,3,4,6-tetra-*O*-Me-Gal were also found. The methylation results indicate that the studied polymers contained (1→4)-bound Gal in the main chain with branching at C-6.

Analysis of Ph_1 and Ph_2 showed that they consisted of phenol (76.24 and 81.04%) and carbohydrate (21.82 and 18.28%) parts. The chemical nature of Ph_1 and Ph_2 was established using acid hydrolysis, which produced glucose and gallic acid. Gel chromatography of Ph_1 and Ph_2 indicated that they were homogeneous. The elution maxima of the phenolic compounds (Folin method) corresponded with components of molecular weights ~$1.0 \cdot 10^3$ (Ph_1) and $1.5 \cdot 10^3$ Da (Ph_2). The results indicate that Ph_1 and Ph_2 are probably tanning agents of the hydrolyzed group.

TABLE 3. Membrane-stabilizing and Antiatherogenic Activities of WSPS Components

Component	IC_{50}, mg/mL		Atherogenic lipid binding, %
	Peroxide hemolysis	Osmotic hemolysis	
MPW_C	0.2667	0.1078	24.32±0.12
MPW_C'	0.0709	0.4582	48.24±0.94
Ph_1	0.0043	0.7532	8.55±0.06
MPW_H	0.3489	0.2113	20.73±0.18
MPW_H'	0.0284	0.4270	37.52±0.71
Ph_2	0.0099	0.7674	8.72±0.07
Caffeic acid	0.0054	0.7621	5.12±0.03
Heparin	-	-	100

It is noteworthy that WSPS from the aerial part of *M. piperita* are a heterogeneous complex consisting of branched α-(1→4)-glucans and β-(1→4)-galactans.

Membrane-stabilizing and antiatherogenic activities were found for MPW_C, MPW_H, $MPW_{C'}$, $MPW_{H'}$, Ph_1, and Ph_2.

MPW_C and MPW_H were less effective in the peroxide hemolysis model than their components (Table 3). $MPW_{H'}$ was more active than $MPW_{C'}$. Ph_1 had the highest membrane-stabilizing activity and was 1.3 times more active than caffeic acid.

The picture was reversed for osmotic hemolysis. MPW_C and MPW_H were more active than their component parts. The polysaccharides were more capable of stabilizing membranes than the polyphenols.

The antiatherogenic activity was studied using the binding of atherogenic lipoproteides of blood. The experiments showed that the polysaccharide components were more active than Ph_1 and Ph_2. It is noteworthy that $MPW_{C'}$ was the most active and was 48.24% as active as heparin.

EXPERIMENTAL

The aerial part of *M. x piperita* was purchased from a drugstore chain (Herbs of Bashkiriya, batch No. 070604, Krasnodar 2 variety).

Isolation of WSPS was carried out after removal of lipophilic ($CHCl_3$:EtOH, 9:1) and alcohol-soluble substances (80% EtOH) by water extraction (1:30 ratio) at 20 and 100°C. The extract was concentrated and precipitated (95% EtOH, 1:5). The resulting precipitates were purified by reprecipitation and dried by changing solvents to produce fractions of WSPS MPW_C and MPW_H for extraction at 20 and 100°C, respectively.

The carbohydrate content was determined by the anthrone method [2]; phenols, by the Folin method [3] calculated as gallic acid; protein, by the ninhydrin method after hydrolysis calculated as alanine [4]; ash content, gravimetrically after ashing at 500°C and subsequent treatment with conc. HNO_3 and H_2O_2 (25%); optical rotation, on a Coers polarimeter with l = 10 cm at 20°C; pH, using a Checker®1 (Hanna Instruments) pH-meter. Spectrophotometric studies were performed on a Cecil CE-2011 spectrophotometer in 10-mm quartz cuvettes; PC, on Filtrak FN-2 paper; TLC, on Silufol (Kavalier) plates; HETLC, on Armsorb (Reakhrom) plates impregnated with phosphate buffer (0.07 M Na_2HPO_4, 0.07 M KH_2PO_4; 96.7:3.3, pH 8.0).

The solvent systems were *n*-PrOH:$CHCl_3$:DMSO:phosphate buffer (1, 51:29:11:9), *i*-PrOH:H_2O (2, 80:20), $CHCl_3$:CH_3OH (3, 9:1), and acetic acid (4, 15%).

The developers were diphenylamine:phosphate (1), aniline:oxalate (2), $KMnO_4$:$NaIO_4$:benzidine (3), and $FeCl_3$ (4, 5%).

Total acid hydrolysis was carried out in H_2SO_4 (10%, 4 h, 100°C); partial hydrolysis, using KU-2-8 (H^+-form, 1:10, 15 min, 100°C).

Enzymatic hydrolysis used α-amylase [5]; hydrolysis of Ph_1 and Ph_2, HCl (5%, 2 h, 100°C).

Quantitative composition of fractions and components was determined by HETLC and densitometry (system 1, developer 1) using a Mustek 2000 planchette scanner and TLC-Manager 3.1 (@PinSoft 2005) scanning densitometry programs. The standard carbohydrates were glucose (Roquette), arabinose, galactose, raffinose, fructose (Acros Organics), xylose, saccharose (Reakhim), and rhamnose (Dia M).

Gel chromatography was performed over Sephadex G-100 (Pharmacia, Uppsala, 1.2 × 50 cm) with elution by NaCl solution (0.3%) flowing at 1 mL/5 min. The volume of effluents was 0.5 mL; column temperature, 20°C. The column was calibrated using Dextran 100,000 (25 mL), 70,000 (32 mL), 50,000 (38 mL), 20,000 (53 mL), and 10,000 (65 mL) (Bio Chemica for GPC, Fluka) and calcion indicator (1109 Da, 106 mL). The external volume of the column was determined using "blue" dextran (2,000,000 Da, Pharmacia, Uppsala, 22 mL). The elution volumes of polysaccharides were detected using anthrone—H_2SO_4 reagent at wavelengths 570 and 625 nm; phenols, Folin reagent at 725 nm. The concentrations of polysaccharides were 7-8 mg/mL; of standard dextrans, 1 mg/mL. The sample volume was 2 mL.

Preparative gel chromatography was carried out under the same conditions using the substance (30 mg). Fractions of a single composition were combined, concentrated, precipitated by acetone, and dried.

Preparative PC used system 2 and developer 2.

Treatment of MPW_C with KU-2. MPW_C (500 mg) was dissolved in water (100 mL), treated with dry KU-2 cation-exchanger (10 g, H^+-form), stirred for 1 h, and filtered to remove the cation exchanger. The solution was concentrated and treated with dry acetone (100 mL). The precipitate was filtered off after 1 h, washed with acetone, and dried ($MPW_{C'}$, 70 mg, 14%). The supernatant and rinsings after isolation of $MPW_{C'}$ were combined, concentrated, and dried in vacuo at 40°C (Ph_1, 124 mg, 25%).

MPW_H was treated analogously to afford $MPW_{H'}$ and Ph_2 in yields of 169 mg (28%) and 128 mg (21%), respectively.

Periodate oxidation and Smith degradation were carried out by the literature method [6]. Periodate consumption was calculated from the decrease of absorbance at 223 nm [7]; quantitative determination of HCOOH, by titration with NaOH (0.01 M). The hydrolysate after $NaBH_4$ reduction was analyzed by HETLC (system 1, developer 3) and PC (system 2, developer 3).

CrO_3 oxidation was performed as before [8].

IR spectra in KBr disks were recorded on a Vector 22 spectrometer.

Methylation of polysaccharides was performed using the Hakomori method [9] with demethylation by the Ciucanu—Kerek method [10]; formolysis and hydrolysis of the permethylates, as described previously [1]. The hydrolysates were analyzed by TLC (system 3, developers 1 and 2) with subsequent densitometry of the chromatograms.

The Ph_1 and Ph_2 hydrolysates were analyzed using PC (system 4, developer 4) and HETLC (system 1, developer 1).

Membrane-stabilizing and antiatherogenic activities were evaluated as described earlier [1].

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