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# Chemical Characterization and Anti-inflammatory Effect of Rauvolfian, a Pectic Polysaccharide of *Rauvolfia* Callus

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**Abstract**—The pectic polysaccharide named rauvolfian RS was obtained from the dried callus of *Rauvolfia serpentina* L. by extraction with 0.7% aqueous ammonium oxalate. Crude rauvolfian RS was purified using membrane ultrafiltration to yield the purified rauvolfian RSP in addition to glucan as admixture from the callus, with molecular weights 300 and 100-300 kD, respectively. A peroral pretreatment of mice with the crude and purified samples of rauvolfian (RS and RSP) was found to decrease colonic macroscopic scores, the total area of damage, and tissue myeloperoxidase activity in colons as compared with a colitis group. RS and RSP were shown to stimulate production of mucus by colons of the colitis mice. RSP appeared to be an active constituent of the parent RS. The glucan failed to possess anti-inflammatory activity.

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*Key words*: callus, *Rauvolfia serpentina*, pectin, anti-inflammatory effect, colitis

Pectic polysaccharides have been earlier shown to possess an anti-inflammatory effect that depends on the structural features of their macromolecules [1]. Some pectins have been demonstrated to protect intestinal wall on administration for 6-21 days. Different physiological effects of pectin appeared to interfere during prolonged exposition, thereby obstructing the elucidation of structure–activity relationships [2-5]. Therefore, the search for and chemical characterization of pectins with anti-inflammatory capacity on single administration is of great interest. Pectins have been earlier obtained from the aerial part of the marsh cinquefoil *Comarum palustre* L. (comaruman) and berries of common cranberry *Vaccinium oxycoccus* L. (oxycoccusan) and were found to inhibit colitis in mice on single administration before induction of colonic inflammation [6, 7].

The chemical composition and biological activity of compounds isolated from native plants are well known to be variable due to differences in area, climate, soil composition, and so on. Callus culture has been developed for standardizing and stabilizing chemical compositions and biological activities of substances isolated from native plants. The callus culture of *Rauvolfia serpentina* L. has been developed for production of anti-arrhythmic alkaloids [8]. The structural features and physiological activity of the *Rauvolfia* callus polysaccharides have not been previously studied.

The present work is devoted to isolation, chemical characterization, and elucidation of anti-inflammatory effect of the pectic polysaccharides of the callus of *R. serpentina*.

## MATERIALS AND METHODS

**General methods.** The samples were preliminarily dried in vacuum under $P_2O_5$ till constant weight, and the content of glycuronic acids was determined using interaction with 3,5-dimethyl phenol in the presence of concentrated sulfuric acid [9] (calibration curve was obtained for D-galacturonic acid). Total protein contents were deter-

*Abbreviations*: LPS) lipopolysaccharides; MPO) myeloperoxidase; RS) initial rauvolfian; RSH) galacturonan; RSP) low methyl esterified pectin; SCFAs) short chain fatty acids.

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mined according to the Lowry method using a calibration curve for BSA [10], and methoxyl contents were estimated as described earlier [11] using a calibration curve for methanol. Spectrophotometric measurements were run on an Ultrospec 3000 instrument (Pharmacia Biotech, England). Optical rotations were measured on a Polartronic MHZ (Schmidt Haensch, Germany) polarimeter at 20°C in water. Descending paper chromatography was run on Filtrak FN-12 and FN-13 papers in the solvent system of *n*-butanol–pyridine–water (6 : 4 : 3 v/v) followed by detection of sugars with aniline hydrogen phthalate at 105°C. The neutral sugars were quantified by gas–liquid chromatography (GLC) as the corresponding alditol acetates using *myo*-inositol as the internal standard. The molar ratios were calculated from the peak areas. GLC was performed with a Hewlett-Packard 4890A chromatograph (USA) fitted with an RTX-1 (0.25 mm × 30 m) capillary column (Restek, USA) with argon as a carrier gas using a flame-ionization detector and HP 3395A integrator [12].

The molecular weights of polysaccharides were analyzed by HPLC [13]. Sample (3 mg) was dissolved in 1 ml of 0.15 M NaCl in bidistilled water followed by filtration. The following chromatographic system was used for analysis: a SD-200 pump (Dynamax, USA), a Shodex Asahipak GS-620 HQ column (7.6 mm × 30 cm; Shimadzu, Japan) with a Shodex GS-26 7B pre-column (7.6 mm × 5 cm; Shimadzu), CTO-10AS thermostat, and RID G136A refractometer (Shimadzu). Elution was carried out with 0.15 M NaCl at 40°C with an effluent rate of 0.5 ml/min. Dextran sulfates with molecular masses in the ranges of 36-50 and 400-600 kD (Sigma, USA) were employed for column calibration.

NMR spectra were recorded with a Bruker DRX-500 spectrometer (Germany) using 3-5% carbohydrate solution in $D_2O$ at 55 and 70°C; the internal standard was acetone, $\delta_H$ 2.225 ppm, $\delta_C$ 31.45 ppm. Two-dimensional COSY, TOCSY, ROESY, and HSQC spectra were recorded using the standard Bruker procedures as described earlier [14]. The ROESY spectra were performed using 200 msec mixing time. A 60 msec duration of the MLEV17 spin-lock was used for TOCSY experiments.

**Isolation of polysaccharide from callus culture.** The callus of *R. serpentina* (L.) Benth. ex Kurz (Apocynaceae) was obtained as described earlier [8]. The callus cells were subcultured for 60 days at 26°C in darkness.

The callus (200 g) dried at 40°C was treated with 0.5% aqueous formalin solution for binding polyphenols and inhibition of enzymes. The residual material was treated with diluted HCl (up to pH 4) at 50°C for 3 h, and the material obtained was extracted with 0.7% aqueous ammonium oxalate as described earlier [15]. The solution obtained was concentrated and dialyzed using ultrafiltration membranes with molecular weight cutoff of 100 kD (apparatus for membrane ultrafiltration; Vladisart, Russia). A polysaccharide fraction was precipitated with four volumes of 96% ethanol. The precipitate was dissolved in distilled water followed by lyophilization to afford the parent polysaccharide named rauvolfian (RS).

All aqueous solutions were concentrated in vacuum using a rotary evaporator at 40-45°C and centrifuged at 7000-8000*g* for 10-20 min. The samples obtained were lyophilized.

**Purification of RS using membrane ultrafiltration.** Rauvolfian RS (400 mg) was dissolved in distilled water (200 ml) and was fractionated using polysulfonic membranes (Millipore, USA) with a molecular weight cutoff of 300 kD. The purified rauvolfian RSP (220 mg) and a contaminating glucan of the callus (153 mg) with molecular weight more than 300 and 100-300 kD, respectively, were isolated after concentration and lyophilization.

**Complete acidic hydrolysis.** Polysaccharides (2-5 mg of each) were hydrolyzed with 2 M trifluoroacetic acid (TFA, 0.5-1 ml) at 100°C for 4-6 h in sealed tubes. The acid was removed by the repeated co-evaporation with methanol. The neutral sugars were quantified by GLC.

**Partial acidic hydrolysis.** The parent rauvolfian RS (45 mg) was dissolved in of 0.05 M TFA (80 ml) and the mixture was incubated at 80°C for 4 h followed by centrifugation. The supernatant was evaporated with methanol, the solution obtained was concentrated, and ethanol was added for precipitation. The precipitate was dissolved in water and subjected to ultrafiltration using Millipore membranes with molecular weight cutoff of 100 kD to afford galacturonan RSH (17 mg): complete hydrolysis of the material obtained with 2 M TFA was found to furnish galacturonic acid as a single sugar constituent.

**Enzymatic hydrolysis.** Rauvolfian (100 mg) was dissolved in water (10 ml), pH was adjusted to 4.1, and 2.9 mg of pectinase (EC 3.2.1.15, 500 U/mg; Fluka, Germany) was added and the resulting mixture was incubated at 50°C. Digestion was monitored by the Somogyi test [16] to estimate the reducing sugar content. The mixture obtained was heated on a boiling water bath for 5 min. The coagulated protein was removed by centrifugation, the supernatant obtained was concentrated, and ethanol (four volumes) was added to precipitate polysaccharides. The precipitate was separated by centrifugation, dissolved in water, dialyzed, and lyophilized. The presence of GalA was determined in the supernatant using paper chromatography.

**Determination of lipopolysaccharide (LPS).** The presence of LPS in the samples of polysaccharides was determined by the QCL-1000 chromogenic test based on the lysate of amebocytes of *Limulus polyphemus* (LAL-test; Cambrex, USA) in accord with the manufacture's instructions. An LPS specimen from *Escherichia coli* 0111:B4 (Sigma, USA) was used as a reference standard.

**Application of polysaccharides and induction of experimental colitis.** Male A/HeJ mice weighing 20-25 g were used. They were housed in standard environmental

conditions. Animals were singly treated orally with the polysaccharide samples (0.2 ml) dissolved in water using flexible rubber catheter two days before induction of colitis. Control mice received the same amount (0.2 ml) of water. Positive control and reference groups received prednisolone (5 mg/kg) and apple pectin (100 mg/kg; MP Biomedicals, USA), respectively.

The mice were fasted for a 15 h before induction of colitis. The mice were lightly anesthetized with ether. Acetic acid (5%, pH 2.5, 0.15 ml) was instilled into the colon lumen through the plastic catheter (2 cm long, external diameter 1 mm) [17].

**Assessment of colitis severity.** The mice were killed by cervical dislocation 24 h after rectal administration of acetic acid. The colonic fragments (5 cm) were isolated for visual evaluation. Macroscopic scoring of colon damage was performed using the following criteria: 0, no inflammatory changes; 1, hyperemia; 2, facial ulcers without mucosal damage; 3, ulcers with mucosal damage. The total area of damage was expressed in relative percent (%) of the total surface area of a colonic fragment [18].

**Determination of colonic myeloperoxidase (MPO) activity.** The colonic samples (100-150 mg) were homogenized in PBS (pH 7.4) and centrifuged at 10,000*g* for 20 min at 4°C. MPO activity in supernatants was assayed by mixing the supernatant with citric phosphate buffer (pH 5.0) containing 0.4 mg/ml of *o*-phenylenediamine and 0.002% hydrogen peroxide. The change in absorbance (OD) at 492 nm was measured spectrophotometrically, and MPO activity was expressed as units/mg of tissue [19].

**Evaluation of adherent colonic mucus.** The mucus contents of the colon were determined spectrophotometrically by Alcian blue dye binding method. The colon was excised and immersed for 2 h in 0.1% Alcian blue in a 0.16 M sucrose solution buffered with 0.05 M sodium acetate. The unbound dye was then removed by two subsequent washings for 15 and 45 min in 0.25 M sucrose solution, and the mucus-bound dye was eluted by immersing the colon in a 0.5 M $MgCl_2$ solution for 2 h. The solution obtained was centrifuged at 2000*g* and OD of supernatant was read at 605 nm. Amounts of Alcian blue corresponded per gram of wet colonic sample were then calculated from standard curves [20].

**Statistics.** The data were processed to obtain the arithmetic mean and the standard root-square-deviation. The reliability of differences was evaluated using Student's *t*-test.

**Table 1.** Yield and monosaccharide composition of rauvolfian and its fractions

| Rauvolfian and its fractions | Yield, % | $[\alpha]_D^{20}$ | Content, %*** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | GalA | neutral monosaccharides | | | | | |
| | | | | Gal | Ara | Rha | Glc | Xyl | Man |
| RS | 23* | 174 | 51.0 | 6.8 | 7.1 | 2.6 | 31.3 | – | 0.7 |
| RSP | 55** | 210 | 82.0 | 5.0 | 4.1 | 2.1 | 5.0 | 1.8 | – |
| RSH | 38** | 298 | 99.7 | – | – | tr. | – | – | – |

Note: tr., trace; Rha, rhamnose; Gal, galactose; Ara, arabinose; Xyl, xylose; Glc, glucose; Man, mannose.
* Of air-dried raw.
** Of parent RS.
*** Contents of D-galacturonic acid and neutral monosaccharides were determined as molar %.

**Table 2.** $^1$H- and $^{13}$C-NMR-spectroscopy of glucan of *Rauvolfia* callus and galacturonan RSH

| Residue | Chemical shifts, $\delta_H$ acetone 2.225 ppm and $\delta_C$ acetone 31.45 ppm | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | H-1 | C-1 | H-2 | C-2 | H-3 | C-3 | H-4 | C-4 | H-5 | C-5 | H-6,6' | C-6 |
| →4)-α-Glc*p*-(1→ | 5.39 | 101.1 | 3.62 | 72.7 | 3.95 | 74.8 | 3.64 | 78.4 | 3.83 | 73.0 | 3.84, 3.78 | 61.9 |
| →4)-α-Gal*p*A-(1→ | 5.10 | 100.8 | 3.74 | 69.6 | 3.99 | 70.0 | 4.44 | 79.5 | 4.86 | 72.4 | – | 175.6 |

## RESULTS

**Isolation and characterization of polysaccharides of *Rauvolfia* callus.** The parent rauvolfian RS was obtained from the dried *Rauvolfia* callus by extraction with aqueous ammonium oxalate (yield 23%). RS was shown as a result of acidic hydrolysis to consist of galacturonic acid residues (51%) as a main constituent. The polysaccharide fraction of storage glucan was found to be extracted during isolation of the parent rauvolfian as proved by high contents of glucose residues (Table 1). Pectinase digestion of RS was found to give D-galacturonic acid only in the hydrolyzate. These data indicated that galacturonan appeared to be a constituent of RS, which is a pectin. The feature of rauvolfian was shown to be low degree of esterification of carboxylic groups in the residues of D-galacturonic acid (OMe-group contents, ca. 0.3%).

Rauvolfian RS was separated using membrane ultrafiltration to yield the purified RSP, and the second fraction appeared to be glucan, as proved using NMR spectroscopy (Table 2). HPLC indicated that RSP and the glucan possess molecular weights of 300 and 100 kD, respectively (Fig. 1).

Galacturonan RSH was obtained using acidic hydrolysis followed by ultrafiltration in order to carry out further structural analysis (Table 1). NMR spectra confirmed that RSP represented a segment of galacturonan as the backbone of rauvolfian (Table 2). A similar galacturonan was obtained earlier for other pectic substances

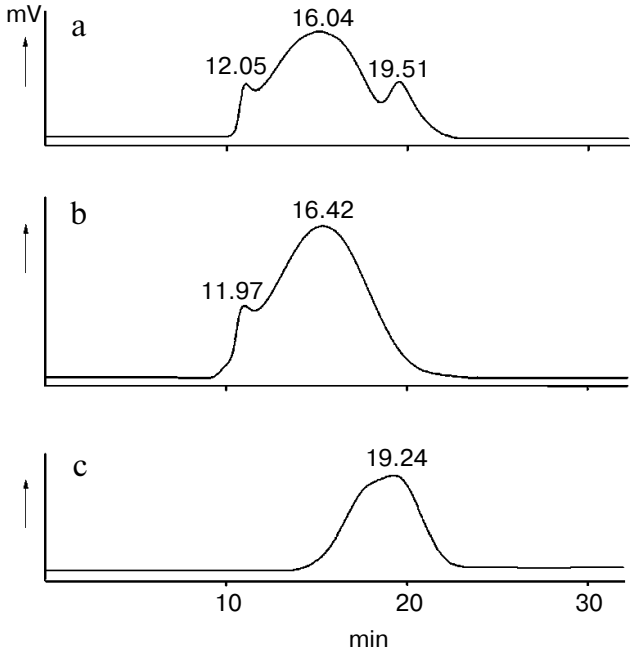


**Fig. 1.** HPLC elution patterns of RS (a), RSP (b), and glucan (c). Dextran sulfate standards with molecular weights of 36-50 (22.23-22.88 min), 100 (18.44-19.49 min), and 300 kD (15.22-16.24 min) were employed for column calibration.

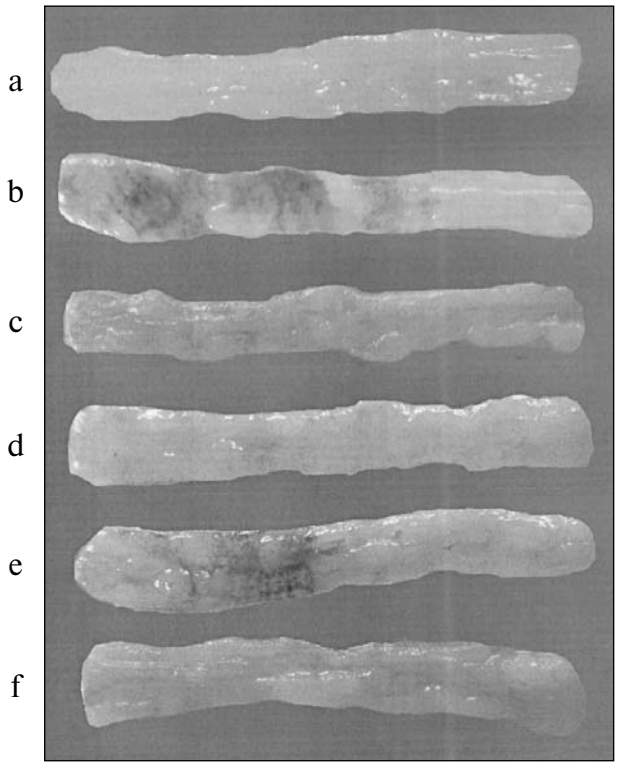


**Fig. 2.** Samples of colonic wall of mice before (a) and 24 h after acetic acid infusion (b-f). Mice were treated two days before with $H_2O$, rauvolfian (100 mg/kg), RSP (100 mg/kg), glucan (100 mg/kg), and prednisolone (5 mg/kg) (b-f, respectively).

[21, 22]. The presence of the rhamnopyranose residues in the composition of fragment RSH as a minor constituent confirmed by the NMR spectra (the signal at 1.24 ppm showed correlation with C-atom at 17.9 ppm in the $^{1}H/^{13}C$ HSQC spectrum) is additional confirmation that rauvolfian represented a typical pectin with rhamnogalacturonan as the backbone.

As may be seen, polysaccharide RS isolated from the callus culture represented a mixture of low methyl esterified pectin (RSP) and glucan. The samples were found to be contaminated with endotoxin. LPS content was 15, 9, and 1 ng/mg in RS, RSP, and glucan, respectively, as tested by the LAL-test. A sample of apple pectin used for a comparison was determined to consist of the galacturonic acid residues (85%) altogether with residues of galactose, arabinose, glucose, rhamnose, and xylose in ratios 7.6 : 2.7 : 2.0 : 1.4 : 1.0.

**Protective effect of rauvolfian on intestinal inflammation.** The anti-inflammatory effect of rauvolfian was studied in mice using the model of ulcerative colitis induced by the infusion of acetic acid rectally. Induction of colitis resulted in mucosal damage and inflammation in the distal colonic wall after 24 h (Fig. 2b). Contents of colonic MPO were shown to increase twofold and mucus was found to decrease.

**Table 3.** Protective effect of rauvolfians (RS and RSP) and glucan on the colon of mice

| Peroral administration 48 h before induction of colitis, mg/kg | Degree of injury | |
|---|---|---|
| | score | area, % |
| $H_2O$ (control) | 3.0 ± 1.0 | 24 ± 14 |
| Rauvolfian RS | | |
| 25 | 3.0 ± 0.8 | 21 ± 7 |
| 50 | 2.0 ± 1.0 | 18 ± 9 |
| 100 | 1.6 ± 0.5* | 11 ± 4** |
| Prednisolone, 5 | 1.1 ± 0.4* | 11 ± 6** |
| Apple pectin, 100 | 2.6 ± 0.5 | 19 ± 7 |
| $H_2O$ (control) | 2.6 ± 1.0 | 22 ± 6 |
| Rauvolfian RS | | |
| 100 | 1.4 ± 0.5** | 12 ± 8** |
| 200 | 0.8 ± 0.7* | 5 ± 3* |
| Glucan | | |
| 100 | 2.0 ± 0.8 | 14 ± 6 |
| 200 | 1.7 ± 1.1** | 18 ± 3 |

Note: The data represent the arithmetic mean ± standard deviation; $n = 7$.

* and **, differences are significant versus control at $p < 0.01$ and $p < 0.05$, respectively.

Oral administration of rauvolfian RS two days before induction of colitis was found to diminish intestinal inflammation (Fig. 2c). In the mice treated with RS, the degree of colonic injury was significantly reduced as determined microscopically (Table 3). The preventive effect of RS (dose 100 mg/kg) was comparable with that of prednisolone administered perorally in dose of 5 mg/kg. Apple pectin used as the reference pectic substance failed to influence the colitis. A peroral pretreatment of mice with RS was shown to decrease the MPO activity in the intestinal wall (Fig. 3) and to enhance the amounts of mucus (Fig. 4).

The purified rauvolfian RSP was found to inhibit development of colitis. Macroscopic (Table 3) and biochemical indexes of injury (Fig. 5) were reduced in mice receiving 100 mg/kg of RSP orally. At the same time, glucan obtained from RS using ultrafiltration failed to prevent colitis progression as there were ulcers on the mucus surface (Fig. 2e); the area of damage failed to decrease and mucus amounts failed to recover after administration of glucan.

## DISCUSSION

In the present study, rauvolfian RS was demonstrated to prevent inflammation when given orally at doses of 50-100 mg/kg. The doses indicated coincide with intake by humans of pectin as a constituent of plant-based food [23]. Apple pectin used widely as a food ingredient did not possess anti-inflammatory effect. Therefore, the effect of RS is assumed to depend on the structural features of the pectin.

RS isolated from the *Rauvolfia* callus using extraction with aqueous ammonium oxalate was shown to contain polysaccharide chains of two types as follows: pectin RSP and glucan, as determined using membrane ultrafiltration. The abnormal yield of polysaccharide (23%) from callus is to be accounted for by the presence of glucan in RS. The callus cells are suggested to contain the high contents of glucans of starch type, which are necessary for the growth of cultured tissue.

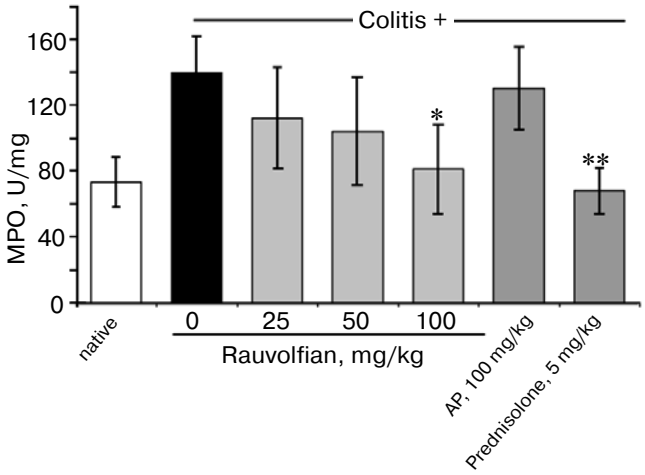


**Fig. 3.** Myeloperoxidase (MPO) activity in the colonic wall of mice before (native) and 24 h after rectal infusion of acetic acid. Mice were treated two days (48 h) before with rauvolfian (0-100 mg/kg), apple pectin (AP, 100 mg/kg), and prednisolone (5 mg/kg). Here and in Figs. 4 and 5 the data represent the arithmetic mean ± standard deviation ($n = 7$); * and **, differences are significant versus control at $p < 0.05$ and $p < 0.01$, respectively.

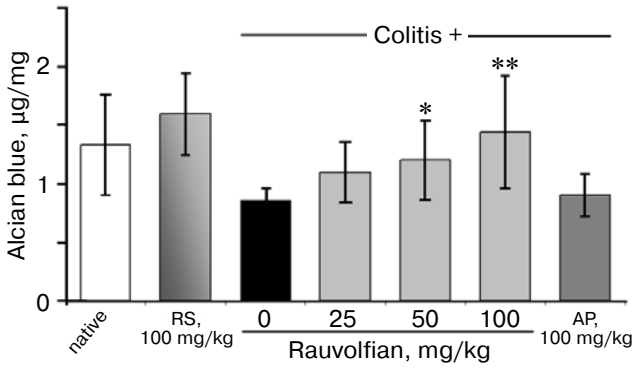


**Fig. 4.** Amounts of mucus in the colonic samples of mice before (native) and 24 h after rectal infusion of acetic acid. Mice were treated two days (48 h) before with rauvolfian (0-100 mg/kg) and apple pectin (AP, 100 mg/kg).

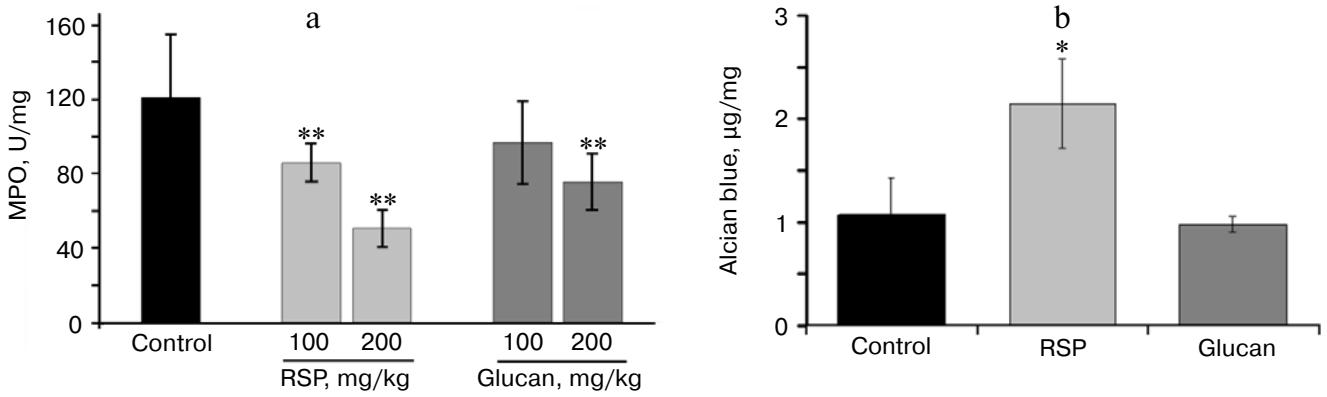


**Fig. 5.** Effect of RSP and glucan on the MPO activity (a) and amounts of mucus (b) in the colonic wall of mice with experimental colitis.

Rauvolfian RSP purified free from glucan proved to possess the main characteristics of all the pectic macromolecules. The yield of pectin RSP calculated per weight of the callus biomass (13%) is higher than that of pectins from other calluses or native plants [1, 24].

Pectin RSP was found to exert a protective function in colitis, whereas glucan failed to influence colonic inflammation. The mechanism of anti-inflammatory effect of RSP remains unclear and appeared to involve a reduction of neutrophil activity. Since MPO is component of azurophilic granules of neutrophils, the MPO activity has been earlier shown to be proportional to number of neutrophils in the colitis intestinal wall [25]. Decrease in the MPO activity by rauvolfian may indicate that pectin inhibits infiltration of intestinal wall by neutrophils. Pectins have been earlier shown to reduce adhesion capacity of neutrophils *in vitro* [26]. However, direct interaction of RSP ingested and circulating neutrophils appeared to be unlikely due to low leakage of macromolecular compounds through the intestinal wall. The anti-inflammatory effect of RSP seemed to be associated with short chain fatty acids (SCFAs) produced during digestion of pectins by colonic microflora. SCFAs are well known to inhibit activity of neutrophils [27] and to stimulate mucus production in the colon [28].

RSP appeared to interact with intestinal mucosa resulting in enhanced protection of the epithelial layer against injury induced by acetic acid. The low esterified rhamnogalacturonans and the linear oligogalacturonides have been earlier shown to possess significant bioadhesion to the colonic mucus, thereby increasing its resistance against toxic factors [29].

LPS contamination (15 ng/mg) of rauvolfian seemed to be low in comparison with that of other plant polysaccharides. Pectins (lemnan, silenan, potamogetonan, and others) isolated earlier in our laboratory have been shown to contain LPS in the range of 50-1000 ng per mg as measured using the same LAL-test. Pectins of *Angelica sinensis*, *Glycyrrhiza glabra*, and *Bupleurum falcatum* have been found to contain more than 1000, 80, and 45 ng LPS per mg, respectively [30]. The LPS contaminations appeared to be more associated with the polysaccharide chains of RSP than those of glucan since the main part of LPS (9 ng/mg) was determined in the pectin. The data are consistent with those obtained earlier [31] demonstrating that pectin of high molecular weigh (1000 kD) contains more LPS (640 ng/mg) than that with molecular weigh 40 kD (4 ng/mg). The origin of LPS contaminations in pectic polysaccharides is unknown. Both plant raw materials and materials used during extraction of pectin may be contaminated by LPS. At the same time, false positive LAL test might have originated from the ability of pectins to interact with factor C of hemolysate from amebocytes of horseshoe crab [32]. According to [30], the false positive reaction to LPS presence is assumed to be mediated by the galactose residues in the pectin. The galactose residues as constituents of the side chains of RS and RSP are suggested to interact with factor C of the test system used in the present study. Administration of RSP at doses 50-100 mg/kg is calculated to be equal of LPS administration at a dose of 450-900 ng/kg. Ability of LPS in these doses to exert anti-inflammatory effect is unknown.

Thus, the pectin rauvolfian inhibiting colonic inflammation induced by rectal infusion of acetic acid in mice was isolated from the *Rauvolfia* callus. The pectin fraction with mol. weight of 300 kD appeared to mediate anti-inflammatory effect of rauvolfian contained non-active glucan with molecular weight of 100 kD.

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