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CHEMICAL STUDY OF *Caragana spinosa* SEEDS

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Seeds of *Caragana* Fabr. (thal ka rdo rje; Fabaceae) species are used in Tibetan medicine as immunostimulating agents. These are most often seeds of *C. spinosa* (L.) Vahl. Ex Hornem. [1]. Information on the chemical composition of this plant raw material has not been published. The goal of the present work was to study the chemical composition of the fatty oil, phenolic compounds, and water-soluble polysaccharides (WSPS) from *C. spinosa* seeds.

Seeds of *C. spinosa* were collected near Nizhnii Ubukun (Selengin Region, Republic of Buryatiya, 25 Aug., 2010, 51°52′54″ N, 106°89′35″ E). The species was determined by Cand. Pharm. Sci. G. V. Chekhirova (IGEB, SB, RAS). Specimens of *C. spinosa* seeds are preserved in the seed bank at IGEB, SB, RAS (No. Fb/s-31/10-03/1008).

Fatty oil was isolated by extracting ground seeds (150 g) in a Soxhlet apparatus with hexane. This produced a hexane fraction (13.11 g). Then, raw material was extracted with EtOH (70%, 1:20, ×3) on a boiling water bath. The alcohol extract was concentrated to a watery residue that was worked up with EtOAc to afford an EtOAc fraction (3.64 g). After lipophilic and alcohol-soluble compounds were removed, raw material was extracted with H_2O on a boiling water bath (1:25, ×3). The aqueous extract was concentrated to one fourth the volume and dialyzed against distilled H_2O (72 h). The undialyzed residual was deproteinated by the Sevag method [2] and then protease from *Streptomyces griseus* [3]. Polysaccharides were precipitated by acetone (1:6). The resulting precipitate was centrifuged (6000 g, 20 min) and dried to afford the WSPS fraction (3.091 g).

Fatty oil (FO) of *C. spinosa* seeds (8.74% yield of air-dried seed mass) was a viscous transparent bright-yellow liquid with a pleasant nutty aroma. The physicochemical parameters of the FO were ρ 0.8862 g/cm^3; n_D^{20} 1.4773; freezing temperature (–18)-(–25)°C; UV spectrum (λ_{max}, nm): 207, 271, 427, 447, 475, 669. IR spectrum (ν, cm^{-1}): 3009, 2956, 2924, 2854, 1745, 1457, 1418, 1377, 1234, 1160, 1098, 913, 845, 722; acid number 5.51 ± 0.22 mg KOH/g; saponification number 184.04 ± 7.36 mg KOH/g; iodine number 154.70 ± 4.55 g I_2/100 g. Fatty acids of FO contained 20 components, which were dominated by fatty acids with C18 chain length including 18:2ω6 (linoleic, 41.24%), 18:1ω9 (oleic, 21.49%), and 18:3ω3 (linolenic, 17.19%) (Table 1). The unsaturation coefficient of fatty acids with C18 chain length was 1.84. The total content of saturated/unsaturated fatty acids was 13.74/86.25%. Polyunsaturated fatty acids dominated over monounsaturated. Chemical analysis showed that FO from *C. spinosa* seeds contained 34.51 ± 1.04 mg% carotinoids; 67.24 ± 2.01 mg% tocopherols; and 10.73 ± 0.16% unsaponified substances including 23.85 ± 0.50 mg/g *β*-sitosterol.

The EtOAc fraction (3 g) was separated over a column of Sephadex LH-20 (4 × 40 cm) that was eluted by an EtOH:H_2O gradient (96:4→0:100). Subfractions were chromatographed over polyamide (5 × 4 cm, EtOH:H_2O eluent, 96:4→0:100), silica gel (2 × 50 cm, $CHCl_3$:EtOH eluent, 100:0→70:30), and TLC (silica gel, EtOAc:CH_2Cl_2:AcOH:HCOOH:H_2O, 10:2.5:1:1:1). Five compounds were isolated and identified using melting points, mass spectrometry, IR and UV spectroscopy, and PMR and ^{13}C NMR spectra as umbelliferone (**1**, 3 mg) [4], quercetin (**2**, 4 mg) [5], eriodictyol (**3**, 5,7,3′,4′-tetrahydroxyflavanone, 8 mg) [6], pyracanthoside (**4**, eriodictyol-7-*O*-glucoside, 24 mg) [7], and rutin (**5**, quercetin-3-*O*-rutinoside, 7 mg) [8].

Eriodictyol (**3**), $C_{15}H_{12}O_6$, mp 270°C. UV spectrum (EtOH, λ_{max}, nm): 230sh, 289, 325sh; +$AlCl_3$: 310, 368. ESI-MS *m/z* 289 $[M + H]^+$. IR spectrum (ν, cm^{-1}): 3364, 1634, 1601, 1518, 1450, 1158, 1088. PMR spectrum (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.70 (1H, dd, J = 17.1, 3.0, H_b-3), 3.14 (1H, dd, J = 17.1, 12.6, H_a-3), 5.42 (1H, dd, J = 12.6, 3.0, H-2), 5.96 (1H, m, J = 2.2, H-6, 8), 6.87 (2H, s, H-5′, 6′), 7.01 (1H, s, H-2′), 9.10 (2H, s, 3′,4′-O<u>H</u>), 12.20 (1H, br.s, 5-O<u>H</u>). ^{13}C NMR spectrum (125 MHz, DMSO-d_6, δ, ppm): 42.88 (C-3), 79.31 (C-2), 95.22 (C-6), 96.14 (C-8), 101.97 (C-10), 114.11 (C-5′), 115.38 (C-2′), 116.60 (C-6′), 129.70 (C-1′), 145.42 (C-4′), 145.74 (C-3′), 163.73 (C-9), 164.57 (C-5), 166.62 (C-7), 196.46 (C-4).

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TABLE 1. Fatty-Acid Composition of Fatty Oil from *C. spinosa* Seeds, % of Fatty-Acid Mass

Acid	%	Acid	%	Acid	%	Acid	%
14:0	0.11	22:0	0.19	20:1 ω9	0.18	MUFA[a]	27.22
15:0	0.05	23:0	0.04	20:1 ω11	0.57	PUFA[a]	59.03
16:0	8.42	24:0	0.27	18:2 ω6	41.24	UFA[a]	86.25
17:0	0.54	16:1 ω7	0.87	18:3 ω3	17.19	PUFA/MUFA	2.17
18:0	2.81	16:1 ω9	0.42	20:3 ω6	0.49	UFA/SFA	6.28
20:0	0.88	18:1 ω7	3.69	21:5 ω3	0.11	USC18[b]	83.61
21:0	0.43	18:1 ω9	21.49	SFA[a]	13.74	$K_{C18\ US}$[c]	1.84

[a]Total content of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), and unsaturated fatty acids (UFA); [b]total content of unsaturated fatty acids with C18 chain length; [c]unsaturation coefficient of fatty acids with C18 chain length $\{K_{C18\ US} = [(1 \times C18:1) + (2 \times C18:2) + (3 \times C18:3)]/\Sigma C18\}$.

Pyracanthoside (**4**), $C_{21}H_{22}O_{11}$, mp >300°C. UV spectrum (EtOH, λ_{max}, nm): 228sh, 286, 335sh. ESI-MS (*m*/*z*, %, +-ion mode): 451 (53) $[M + H]^+$, 289 (100) $[aglycon + H]^+$. IR spectrum (ν, cm^{-1}): 3450, 1690, 1600, 1515, 1454, 1149, 1084. PMR spectrum (500 MHz, DMSO-d_6, δ, ppm, J/Hz): aglycon: 2.75 (1H, dd, J = 17.1, 3.0, H_b-3), 3.28 (1H, dd, J = 17.1, 12.6, H_a-3), 5.44 (1H, dd, J = 12.6, 3.0, H-2), 6.08 (1H, m, J = 2.2, H-6), 6.11 (1H, d, J = 2.2, H-8), 6.81 (2H, s, H-5′, 6′), 6.92 (1H, s, H-2′), 9.04 (2H, s, 3′,4′-O<u>H</u>), 12.17 (1H, br.s, 5-O<u>H</u>); glucopyranose: 3.18 (1H, t, J = 9.0, H-4″), 3.21 (1H, dd, J = 7.6, 9.2, H-2″), 3.27 (1H, dd, J = 9.2, 9.0, H-3″), 3.37 (1H, m, H-5″), 3.43 (1H, dd, J = 11.5, 5.5, H_a-6″), 3.66 (1H, dd, J = 11.5, 3.2, H_b-6″), 5.00 (1H, d, J = 7.6, H-1″). ^{13}C NMR spectrum (125 MHz, DMSO-d_6, δ, ppm): aglycon: 42.24 (C-3), 78.83 (C-2), 95.57 (C-8), 96.31 (C-6), 102.31 (C-10), 115.04 (C-5′), 115.39 (C-2′), 117.44 (C-6′), 129.63 (C-1′), 145.30 (C-4′), 145.79 (C-3′), 162.79 (C-9), 162.94 (C-5), 165.24 (C-7), 197.04 (C-4); glucopyranose: 60.47 (C-6″), 69.12 (C-4″), 72.84 (C-2″), 76.29 (C-3″), 77.10 (C-5″), 99.64 (C-1″).

All compounds were isolated from *C. spinosa* seeds for the first time; **3** and **4**, for the first time from the genus *Caragana*. The presence of **3** and **4** in seeds of other representatives of the Fabaceae family was observed before. Compound **3** was found in *Dipteryx odorata* (Aubl.) Willd. [9] and *Tamarindus indica* L. [10]; **4**, in *Gleditsia caspia* Desf. [7]. HPLC was used (conditions 1) to establish that **4** was the dominant phenolic compound in *C. spinosa* seeds (19.37 mg/g of air-dried raw material mass). Compound **4** was also present in seed pericarp (380.88 μg/g) and in trace quantities in flowers of *C. spinosa*. This compound was not found in leaves and stems.

The yield of WSPS from *C. spinosa* seeds was 2.06% of the air-dried raw material mass. An aqueous solution of WSPS gave a positive reaction with I_2 solution and had a positive specific rotation. The physicochemical parameters of the WSPS were $[\alpha]_D$ +98.1° (*c* 0.5, 1% NaOH). IR spectrum (ν, cm^{-1}): 3419, 2936, 1732 [$\nu(C{=}O)_{eth}$], 1651 [$\nu_{as}(COO^-)$], 1437 [$\delta_{as}(CH_3)_{eth}$], 1410 [$\nu_s(COO^-)$], 1362, 1340, 1230, 1150, 1101, 1079, 1044, 1023, 927 [ρ $(CH_3)_{eth}$, $\nu(C_1{-}H_\alpha)$ – type 1], 847 [$\nu(C_1{-}H_\alpha)$ – type 2a], 831, 786, 760 [$\rho(CH_3)_{ring}$, $\nu(C_1{-}H_\alpha)$ – type 3]; carbohydrate content 91.47%; uronic acids 13.21%; protein, phenols, ash <0.5%. Total hydrolysis of WSPS produced glucose, galacturonic acid, galactose, arabinose, and rhamnose in a 32.6:5.5:3.3:2.6:1 ratio and traces of mannose and xylose (Table 2). The preliminary data suggested that WSPS from *C. spinosa* seeds were a mixture of α-glucans and pectinic substances.

The dominant components of the WSPS (2.857 g) were isolated by separation over a column of DEAE-cellulose (OH^--form, 3 × 60 cm) and were eluted successively by H_2O, NaCl (0.1–1 M), and NaOH (0.1 M) to afford six fractions: CSSW-1 (H_2O, 1.892 g), CSSW-2 (0.1 M NaCl, 0.154 g), CSSW-3 (0.3 M NaCl, 0.084 g), CSSW-4 (0.5 M NaCl, 0.024 g), CSSW-5 (1 M NaCl, 0.211 g), and CSSW-6 (0.1 M NaOH, 0.312 g). The dominant fraction CSSW-1 contained 92.4% glucose, arabinose and galactose in a 1:1.3 ratio, and traces of mannose and galacturonic acid (Table 2). Fractions eluted by NaCl and NaOH solutions typically had a higher content of galacturonic acid (7.4–53.7 mol%) and did not give a reaction with I_2 solution. This indicated that these fractions were pectinic substances.

Fraction CSSW-1 contained according to HPLC three polymers with molecular weights 147 (CSSW-1-1), 94 (CSSW-1-2), and 54 kDa (CSSW-1-3). These were isolated using preparative gel chromatography on Sephacryl 300-HR (4 × 80 cm, 0.1 M NaCl eluent). Separation of CSSW-1 (1.5 g) afforded three homogeneous (HPLC, conditions 2) components CSSW-1-1 (1.076 g), CSSW-1-2 (0.141 g), and CSSW-1-3 (0.047 g). Only glucose was observed in CSSW-1-1 and CSSW-1-2. Arabinose and galactose in a 1:1.3 ratio and traces of mannose were found in CSSW-1-3.

TABLE 2. Characteristics of WSPS from *C. spinosa* Seeds

Fraction	Yield, %	$[\alpha]_D$, °[d]	Reaction with I_2	Monosaccharide composition, mol%						
				Ara	Gal	Glc	Man	Rha	Xyl	GalUA
WSPS	2.06[a]	+98.1	+	5.8	7.2	71.8	0.2	2.2	0.5	12.2
CSSW-1	66.2[b]	+101.7	+	3.3	4.2	92.4	Tr.			Tr.
CSSW-1-1	71.7[c]	+107.4	+			99.9				
CSSW-1-2	9.4[c]	+104.2	+			99.9				
CSSW-1-3	3.1[c]	n.d.	–	43.7	56.2		Tr.			
CSSW-2	5.4[b]	n.d.	–	31.4	32.7	24.4	0.9	2.7	0.4	7.4
CSSW-3	2.9[b]	n.d.	–	40.4	39.2	14.7	0.7	2.1	0.2	10.6
CSSW-4	0.8[b]	n.d.	–	16.9	38.9	12.0	0.3	2.4	Tr.	29.4
CSSW-5	7.4[b]	+152.7	–	31.7	21.4	7.3	3.1	5.2	Tr.	31.2
CSSW-6	10.9[b]	+164.4	–	13.9	19.8	4.8	0.3	7.4		53.7

[a]Of air-dried raw material mass; [b]of WSPS mass; [c]of WSPS mass; [d](*c* 0.5, 1% NaOH); n. d., not determined; Tr. < 0.05.

The two dominant glucans were investigated further. The polysaccharides were fully methylated and hydrolyzed. The decomposition products were analyzed as alditolacetates using GC/MS. This showed the presence of 1,5-di-*O*-Ac-2,3,4,6-tetra-*O*-Me-Glc*p*; 1,4,5-tri-*O*-Ac-2,3,6-tri-*O*-Me-Glc*p*; 1,4,5,6-tetra-*O*-Ac-2,3-di-*O*-Me-Glc*p*; and 1,5,6-tri-*O*-Ac-2,3,4-tri-*O*-Me-Glc*p* in 1.54:5.07:1.55:1 and 1.84:15.41:1.82:1 ratios for CSSW-1-1 and CSSW-1-2, respectively. The presence of the first three derivatives indicated that glucopyranoses were (1→4)-bonded in the main chain, which contained C-6 side branches consisting of single glucopyranose units and longer chains of (1→6)-bonded glucopyranoses. This was evident from the detection of 1,5,6-tri-*O*-Ac-2,3,4-tri-*O*-Me-Glc*p*. The ratio of (Glc*p*-1→) and (→6-Glc*p*-1→) units was 1.54:1 and 1.84:1 for CSSW-1-1 and CSSW-1-2, respectively. The degrees of substitution of the main chains in CSSW-1-1 and CSSW-1-2 were 23.4 and 10.6%, respectively.

Oxidation of CSSW-1-1 and CSSW-1-2 acetates by CrO_3 with subsequent hydrolysis of the oxidation products formed glucose, indicating that it had the α-configuration.

Thus, the dominant components of WSPS from *C. spinosa* seeds were branched α-(1→4)-glucans containing in C-6 side chains single units and (1→6)-bonded chains of α-glucopyranose. Arabinogalactans and pectinic substances were minor constituents of the WSPS complex.

TLC was performed on Sorbfil PTSKh-AF silica gel plates (Imid Ltd.); column chromatography, over silica gel (100/400, Woelm); ion-exchange chromatography, over DEAE-cellulose (Whatman); gel chromatography, over Sephacryl 300-HR (Sigma Aldrich). Spectrophotometric studies were carried out on an SF-2000 spectrophotometer (OKB Spektr). Optical rotation was measured on an SM-3 polarimeter (Zagorsk Optical Mechanical Plant). IR spectra were recorded as films on ZnSe substrate windows on an FT-801 IR-Fourier spectrometer (Simeks) in the range 4000–600 cm^{-1}. GC/MS analysis was carried out in a 6890N GC/MS connected to a 5973N quadrupole mass detector (Agilent Technologies, electron-impact ionization, ionization energy 70 eV, total ion current detection, scan range 41–450 amu) using an HP-Innowax capillary column (Agilent Technologies, 30 m × 0.25 mm × 0.50 μm; stationary phase polyethyleneglycol). The mobile phase was He at flow rate 1 mL/min. The sample volume (1% solution in hexane) was 0.2 μL with 20:1 flow division, column temperature 150–250°C (rate 2°C/min), vaporizer 250°C, ion source 230°C, detector 150°C, lines connecting the chromatograph with the mass spectrometer 280°C. MS analysis was carried out in a MAT 8200 high-resolution mass spectrometer (Finnigan). PMR and ^{13}C NMR spectra were recorded on a VXR 500S NMR spectrometer (Varian). HPLC used a Summit liquid chromatograph (Dionex) with conditions 1 (Prodigy ODS 3 column; Phenomenex, 250 × 4.6 mm, 5 μm; H_2O:MeOH:AcOH 14:6:1 mobile phase at flow rate 1 mL/min; 20°C; UVD 170S UV detector at 330 nm) and conditions 2 (TSK gel GMP×1 column; Supelco, 300 × 7.8 mm, 5 μm; H_2O mobile phase at flow rate 1 mL/min; 20°C, UVD 170S UV detector at 190 nm). Chemical analyses of FO included determination of acid and iodine numbers; titration for saponification number; spectrophotometry for carotinoid and tocopherol contents [11]; gravimetry for saponified substances; and HPTLC-densitometry for β-sitosterol [12]. Total contents of carbohydrates were determined by the phenol–H_2SO_4 method [13]; uronic acids, by reaction with 3,5-dimethylphenol [14]; protein, by the Bradford method [15]; ash content, gravimetrically after dry ashing at 550°C. Hydrolysis and methylation were performed as before [16]; oxidation by CrO_3, by the literature method [17].

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