LOW-MOLECULAR-WEIGHT PHENOLIC COMPOUNDS FROM *Hedysarum theinum* ROOTS

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*Isoflavonoids (-)-medicarpin, (-)-vestitol, formononetin, 8-hydroxydaidzein, and 6″-*O*-acetylononin were isolated for the first time in addition to (-)-catechin, (-)-epicatechin, protocatechoic acid, raspberry ketone, and rhododendrol from the alcohol extract of* Hedysarum theinum *roots.*

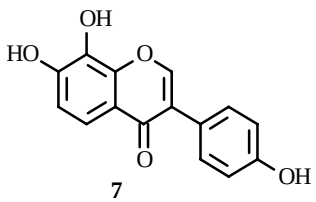
Key words: *Hedysarum theinum* Krasnob., Fabaceae, 8-hydroxydiadzein, 6″-*O*-acetylononin, (-)-catechin, (-)-epicatechin, protocatechoic acid.

In continuation of research on the chemical composition of *Hedysarum theinum* Krasnob. [1], which has valuable medicinal properties, we studied the chemical composition of the low-molecular-weight fractions of the alcohol extract of roots of this plant.

Roots of *H. theinum* were extracted successively multiple times with EtOAc and ethanol. The chemical composition of the EtOAc extract has been reported by us [1]. Alcohol extracts have previously been separated into fractions of monomeric and oligomeric compounds by dissolving the dried extract in water and then extracting successively with organic solvents, e.g., diethylether, EtOAc, and BuOH [2]. However, in our instance the use of this method led to formation of emulsions and a precipitate during the extraction by diethylether or $CHCl_3$. This complicated the separation process. In order to avoid this step, which caused losses, we separated the dried ethanol extract by extraction in a Soxhlet apparatus successively by $CHCl_3$, EtOAc, acetone, and ethanol. HPLC analysis showed the presence of low-molecular-weight compounds in the $CHCl_3$ and EtOAc fractions of the alcohol extract and practically none in the acetone and ethanol fractions.

Column chromatography of the $CHCl_3$ fraction over silica gel and preparative TLC on silica gel isolated medicarpin (**1**), raspberry ketone (**2**), vestitol (**3**), and rhododendrol (**4**).

The EtOAc fraction was separated by chromatography over polyamide into water-soluble compounds (water eluent) and aglycons (MeOH eluent). Preparative separation of the aglycon fraction using column adsorption chromatography over silica gel with subsequent rechromatography over reversed-phase sorbent (Diasorb C16T) isolated vestitol (**3**), formononetin (**5**), protocatechoic acid (**6**), the isoflavonoids 8-hydroxydiadzein (**7**) and 6″-*O*-acetylononin (**8**), and (-)-catechin (**9**) and (-)-epicatechin (**10**). The structures of **1**-**10** were proved by spectral analysis including PMR, ^{13}C NMR, IR, and UV spectroscopy in addition to GC—MS. The resulting spectra were similar to those reported in the literature.



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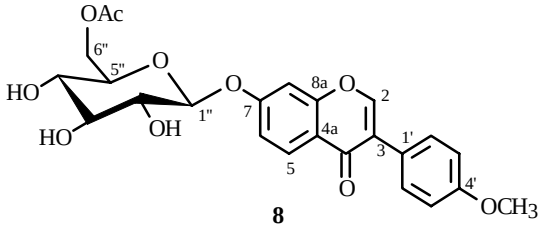

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Certain differences were observed in comparing the PMR spectra of **8** with the literature data. This was most likely explained by the effect of solvents, DMSO-d_6 in this study and acetone-d_6 in the literature [3]. This hypothesis was supported by the fact that differences in the chemical shifts were observed for protons directly bound to O atoms (OH groups) or through a C atom. Just these protons should be the most sensitive to a change of solvent. Chemical shifts of C atoms in the ^{13}C NMR spectrum of **8** recorded in DMSO-d_6 were similar to those of the spectrum recorded in acetone-d_6.

The configuration of the sugar unit in **8** was established by analyzing the double-resonance PMR spectra. SSCC of glycoside ring protons were consistent with axial—axial coupling ($J_{1'',2''}$ = 7.5 Hz, $J_{3'',4''}$ = 9 Hz, $J_{4'',5''}$ = 10 Hz) and corresponded to SSCC for a β-glucoside. The presence of an acetyl in the 6″-position of the glucoside unit was confirmed by analyzing spectra of ononin (**7**-*O*-β-glucosylformononetin) in DMSO-d_6, which is unsubstituted in this position. The PMR spectrum of acetylated **8** had resonances for protons H-6″a and H-6″b at 4.08 and 4.34 ppm that were shifted to weak field relative to those of the corresponding protons of ononin (3.54 and 3.77 ppm) [4]. The ^{13}C NMR spectrum showed a resonance for C-6″ that was also shifted to weak field, 63.3 ppm instead of 60.2 ppm for the corresponding C atom in ononin.

The results show that the low-molecular-weight compounds from *H. theinum* consist of a wide range of compounds, among which there is no major component. Apparently the biological activity of this plant is due to the action of a complex of minor components. Half of the compounds isolated by us were isoflavonoids, **1**, **3**, **5**, **7**, and **8**. Compounds **1**-**5** were observed by us previously in the EtOAc extract of *H. theinum* [1]. Compounds **6**-**10** were isolated from *H. theinum* for the first time. Compounds **7** and **8** have not previously been isolated from the *Hedysarum* genus. Formononetin (**5**) and analogs of **8**, ononin and malonylononin, were found in *in vitro* cultivated roots of *H. theinum* [5].

The presence in *H. theinum* of isoflavonoids enables it to be recommended as a potential plant raw material for developing medicinal preparations.

EXPERIMENTAL

General Comments. IR, UV, MS, and HPLC chromatograms were recorded using instruments and conditions as before [1]. PMR and ^{13}C NMR spectra were recorded on Bruker AV-300 (300.13 and 75.47 MHz) and AM-400 (400.13 and 100.61 MHz) instruments using 5-10% solutions in DMSO-d_6. Optical rotation angles were measured on a Polamat A instrument. Mass spectra were recorded in a Reflex III (Bruker, Germany) MALDI-TOF mass spectrometer using a N_2 laser (VSL-337 ND, Laser Science Inc., USA). The matrix was a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile (50%).

Column chromatography was performed on Merck silica gel (60-200 μm) using eluents $CHCl_3$:CH_3OH (100:0, 100:1, 100:2, 100:5, 100:10, 100:20, v/v, 1) and hexane:diethylether (6:4, 1:1, 2:8, 1:9, v/v, 2). Preparative TLC over LSL 5-40 silica gel used eluent 1 or 2 (4:1). Sorbent TU 6-09-10-822-73, particle size 0.25-0.50 mm, was used for column chromatography over polyamide. TLC was carried out on Sorbfil PTLC AF-V-UF plates using eluent 2 (1:3). Spots were developed in UV light or by *p*-nitroanilinium hydrochloride and sodium nitrite (yellow or brown color with phenolic compounds) [6].

Preparative reversed-phase (RP) HPLC was performed over Diasorb-130-C16T sorbent (7 μm, ZAO BioKhimMakST) using a column (0.7 × 25 cm) and isocratic elution by MeOH:TFA (0.1%) (40:60 or 70:30, 3). HPLC analysis of the products used a "Millichrome A-02" microcolumn liquid chromatograph.

We used *H. theinum* roots collected by an expedition of the LLiPBAC NIOKh SD RAS that were analogous to those described earlier [1].

Successive Extraction by EtOAc:EtOH. Ground air-dried roots (2812 g) were extracted with EtOAc (6 × 9 L) by boiling for 5.5 h, yield 29.46 g (1.0%); with EtOH (7 × 9 L), by boiling for 7 h, yield 139.07 g (5.0%).

Extraction in a Soxhlet Apparatus. Dried EtOH extract (49.84 g) was extracted with $CHCl_3$ for 5 h, yield 1.60 g (3% of the EtOH extract mass) and extracted successively with EtOAc twice for 7 h (yield 7.07 g, 14%) and acetone five times for 7 h (yield 16.39 g, 33%). The solid after extraction was dissolved in EtOH and filtered. Solvent was vacuum distilled to afford the EtOH extract (20.51 g, 41%).

Separation of the $CHCl_3$ Fraction. The $CHCl_3$ fraction (1.50 g) was separated by column chromatography over silica gel (50 g) using eluent 1 (from 100:0 to 100:10).

The fraction eluted by the 100:1 system (395 mg) was separated again over a silica-gel column (eluent 1, 200:1) and by preparative TLC (eluent 2, 4:1) to afford medicarpin (**1**, 24 mg) and raspberry ketone (**2**, 19 mg).

Preparative TLC (eluent 1, 100:4) of the fraction eluted by the 100:2 system (109 mg) afforded (-)-vestitol (**3**, 33 mg).

The fraction eluted by the 100:5 system (231 mg) was separated again over a silica-gel column (eluent 1, 100:3.5) and by preparative TLC (eluent 1, 100:4) to afford rhododendrol (**4**, 5 mg).

Separation of the EtOAc Fraction. The EtOAc fraction (5.86 g) was separated by column chromatography over polyamide (12 g) into a fraction of water-soluble compounds (2.50 g) using water as eluent; a fraction of aglycons (2.22 g); MeOH. The aglycon fraction (2.14 g) was separated by column chromatography over silica gel (22 g) using eluent 1 (from 100:0 to 100:10).

Fraction A, which was eluted by the 100:2 system (118 mg) afforded after recrystallization formononetin (**5**, 51 mg).

Fraction B, which was eluted by the 100:2 system (152 mg), was rechromatographed over a silica-gel column using eluent 2 (6:4) to afford vestitol (**3**, 13 mg).

Fraction C, which was eluted by the 100:10 system (178 mg), was separated by column chromatography over silica gel (eluents 2 and then 1) and by preparative RP HPLC (eluent 3, 40:60 and 70:30) to afford **6** (15 mg), **7** (13 mg), and **8** (4 mg).

Fraction D (66 mg), which was eluted by the 100:10 system (263 mg total) was separated by preparative RP HPLC (eluent 3, 40:60) to afford catechin (**9**, 23 mg) and epicatechin (**10**, 39 mg).

The physicochemical analytical results for **1**-**5** were analogous to those reported previously by us [1].

Protocatechoic Acid (6) (3,4-dihydroxybenzoic acid). IR spectrum (KBr, ν, cm^{-1}): 3370, 1680, 1603, 1443, 1293, 1202, 1140. UV spectrum (EtOH, λ_{max}, nm, log ε): 260 (4.44), 295 (4.24). $[M]^+$ 154.02670, $C_7H_6O_4$, calc. $[M]^+$ 154.02660.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.78 (1H, d, J = 8, H-5), 7.28 (1H, br.d, J = 8, H-6), 7.33 (1H, br.s, H-2), 9.30, 9.70 (2H, s, OH), 12.24 (1H, s, COOH).

^{13}C NMR spectrum (75 MHz, DMSO-d_6, δ, ppm): 115.12 (C-5), 116.53 (C-2), 121.62 (C-1), 121.85 (C-6), 144.88 (C-3), 150.01 (C-4), 167.30 (COOH). PMR and ^{13}C NMR spectra have been published [7].

8-Hydroxydiadzein (7) [7,8-dihydroxy-3-(4-methoxyphenyl)chromen-4-one]. UV spectrum (EtOH, λ_{max}, nm): 258. Mass spectrum (MALDI): $[M + H]^+$ 271.68, $C_{15}H_{10}O_5$, calc. $[M]^+$ 270.23.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.80 (2H, d, J = 8.5, H-3′,5′), 6.95 (1H, d, J = 8.6, H-6), 7.39 (2H, d, J = 8.5, H-2′,6′), 7.47 (1H, d, J = 8.6, H-5), 8.31 (1H, s, H-2).

^{13}C NMR spectrum (75 MHz, DMSO-d_6, δ, ppm): 114.50 (C-5), 115.27 (C-3′, C-5′), 115.3 (C-6), 117.80 (C-4a), 123.03* (C-1′), 123.26* (C-3), 130.46 (C-2′, C-6′), 133.22 (C-8), 147.07 (C-8a), 150.29 (C-7), 152.97 (C-2), 157.47 (C-4′), 175.49 (C-4). PMR and ^{13}C NMR spectra have been published [8].

6″-*O*-Acetylononin (8) [6″-*O*-acetyl-7-(*β*-D-glucopyranosyloxy)-3-(4-methoxyphenyl)chromen-4-one]. UV spectrum (EtOH, λ_{max}, nm): 256. Mass spectrum (MALDI): $[M + H]^+$ 473.69, $C_{24}H_{24}O_{10}$, calc. $[M]^+$ 472.43.

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.01 (3H, s, CH_3CO), 3.21 (1H, ddd, $J_{4″,5″}$ = 10.0, $J_{4″,3″}$ = 9.0, $J_{4″,OH\text{-}4″}$ = 5.0, H-4″), 3.26-3.36 (2H, m, H-2″,3″), 3.75 (1H, ddd, $J_{5″,4″}$ = 10.0, $J_{5″,6″a}$ = 7.0, $J_{5″,6″b}$ = 2.0, H-5″), 3.80 (3H, s, OCH_3), 4.08 (1H, dd, $J_{6″a,6″b}$ = 12.0, $J_{6″a,5″}$ = 7.0, H-6″a), 4.34 (1H, dd, $J_{6″b,6″a}$ = 12.0, $J_{6″b,5″}$ = 2.0, H-6″b), 5.16 (1H, d, $J_{1″,2″}$ = 7.5, H-1″), 5.19 (1H, d, $J_{OH\text{-}3″,3″}$ = 4.5,, OH-3″), 5.29 (1H, d, $J_{OH\text{-}4″,4″}$ = 5.0, OH-4″), 5.46 (1H, d, $J_{OH\text{-}2″,2″}$ = 4.5, OH-2″), 7.00 (2H, d, J = 8.8 H-3′,5′), 7.15 (1H, dd, $J_{6,5}$ = 8.8, $J_{6,8}$ = 2.2, H-6), 7.23 (1H, d, $J_{8,6}$ = 2.2, H-8), 7.54 (2H, d, J = 8.8, H-2′,6′), 8.07 (1H, d, $J_{5,6}$ = 8.8, H-5), 8.42 (1H, s, H-2).

^{13}C NMR spectrum (100 MHz, DMSO-d_6, δ, ppm): 20.64 ($\underline{CH_3}CO$), 55.15 (OCH_3), 63.32 (C-6″), 69.76 (C-4″), 73.03 (C-2″), 73.78 (C-5″), 76.22 (C-3″), 99.62 (C-1″), 103.44 (C-8), 113.62 (C-3′, C-5′), 115.51 (C-6), 118.52 (C-4a), 123.39 (C-1′), 123.97 (C-3), 126.98 (C-5), 130.08 (C-2′, C-6′), 153.73 (C-2), 157.01 (C-8a), 159.02 (C-4′), 161.17 (C-7), 170.22 ($CH_3\underline{COO}$), 174.66 (C-4). PMR and ^{13}C NMR spectra have been published [3].

(-)-Catechin (9), $[\alpha]_{580}$ −4.76° (*c* 0.41, MeOH) {lit. [9] $[\alpha]_{589}$ −4.1° (*c* 0.41, acetone)}, mp 178-180°C (lit. [9] mp 175-178°C). IR spectrum (KBr, ν, cm^{-1}): 3396, 1778, 1608, 1519, 1468, 1143. UV spectrum (EtOH, λ_{max}, nm, log ε): 280 (3.43). $[M]^+$ 290.07910, $C_{15}H_{14}O_6$, calc. $[M]^+$ 290.07903.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 2.35 (1H, dd, J = 16, 8, H-4_{ax}), 2.66 (1H, dd, J = 16, J = 5, H-4_{eq}), 3.81 (1H, m, H-3), 4.47 (1H, d, J = 7, H-2), 4.88 (1H, br.s, OH), 5.88 (1H, br.s, H-6), 5.68 (1H, br.s, H-8), 6.59 (1H, d, J = 8, H-6′), 6.68 (1H, d, J = 8, H-5′), 6.72 (1H, br.s, H-2′), 8.84, 8.88, 8.96, 9.20 (4H, br.s, OH).

^{13}C NMR spectrum (75 MHz, DMSO-d_6, δ, ppm): 27.73 (C-4), 66.24 (C-3), 80.91 (C-2), 93.80 (C-8), 95.09 (C-6), 98.99 (C-4a), 114.46, 115.00 (C-2′, C-5′), 118.30 (C-6′), 130.55 (C-1′), 144.74 (C-3′, C-4′), 155.26 (C-8a), 156.06* (C-5), 156.36* (C-7). PMR and ^{13}C NMR spectra have been published [10].

(-)-Epicatechin (10), $[\alpha]_{580}$ −27.75° (*c* 0.21, MeOH) {lit [11] $[\alpha]_{580}$ −34° (*c* 0.08, MeOH)}, mp 244-246°C (lit. [12] mp 242-243°C). IR spectrum (KBr, ν, cm^{-1}): 3418, 1680, 1630, 1521, 1468, 1204, 1146. UV spectrum (EtOH, λ_{max}, nm, log ε): 280 (3.32). $[M - 18]^+$ 272.06819, $C_{15}H_{12}O_5$, calc. $[M - 18]^+$ 272.06847.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm): 2.46, 2.67 (2H, m, H-4), 3.99 (1H, m, H-3), 4.72 (1H, br.s, H-2), 5.88 (1H, br.s, H-6), 5.71 (1H, br.s, H-8), 6.65 (2H, s, H-5′,6′), 6.88 (1H, br.s, H-2′), 8.82, 9.16 (4H, br.s, OH).

^{13}C NMR spectrum (75 MHz, DMSO-d_6, δ, ppm): 28.20 (C-4), 64.90 (C-3), 78.04 (C-2), 94.06 (C-8), 95.07 (C-6), 98.47 (C-4a), 114.75, 114.87 (C-2′, C-5′), 117.94 (C-6′), 130.60 (C-1′), 144.42, 144.48 (C-3′, C-4′), 155.75 (C-8a), 156.53* (C-5), 156.22* (C-7). PMR and ^{13}C NMR spectra have been published [10].

Assignments of signals marked with * in ^{13}C NMR spectra of **7**, **9**, and **10** may be interchanged.

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