COMPONENTS OF THE ETHYLACETATE EXTRACT OF *Hedysarum theinum* ROOTS

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Isoflavonoids (-)-medicarpin, (-)-vestitol, and formononetin and butylphenols raspberry ketone and rhododendrol were isolated for the first time from the ethylacetate extract of Hedysarum thienum *roots by column chromatography. GC—MS showed that the ethylacetate extract contained fatty acids, the principal ones being palmitic, linoleic, oleic, behenic, and lignocerinic.*

Key words: *Hedysarum theinum*, Fabaceae, isoflavonoids, medicarpin, vestitol, formononetin, raspberry ketone, rhododendrol, fatty acids, HPLC, GC—MS.

Hedysarum theinum is a perennial herbaceous plant with a thick reddish-brown root, because of which it is popularly called red root. This is a rare high-mountain subalpine species with a dispersed Central Asia—South Siberia distribution (Altai, Mongolia, Jugarskii Alatau). It is found most frequently in regions with a humid climate in the lower part of the high-mountain belt in subalpine and alpine meadows, rocky slopes, and forest glades in cedar—broadleaf woods [1]. In habitats with optimal ecological conditions that have not been greatly affected by civilization (collection, grazing), *H. theinum* is the dominant and subdominant species.

The decoction or tincture of red root, which has a whole range of unique biologically active properties, has been used since long ago in Altai as a systemic, tonic, and anti-inflammatory agent. Modern folk medicine considers red root to possess immunostimulant properties and to cleanse actively the vascular system, which facilitates restoration of all body functions [2].

The chemical composition of the root and aerial part of *H. theinum* has not previously been studied in detail. The yield of extracted substances has been reported for extraction of roots by various solvents [3]. The methanol extract reacts with vanillin and HCl to give a characteristic reaction for oligomeric proanthocyanidines [4].

We studied previously the composition of successive extracts (hexane, ether, ethylacetate) of *H. theinum* roots using GC—MS and HPLC [5]. They contained fatty acids, triterpenes, and phenols. However, compounds of the different classes were not well separated.

Herein we report results from the extraction of *H. theinum* roots by ethylacetate and the chemical composition of the extract. The yield of extract after six-fold extraction was 1.0%. The following results were obtained after separation of the extract by column chromatography over silica gel.

The nonpolar part of the ethylacetate extract (fraction 1, see Experimental) was methylated by diazomethane. The products were subsequently analyzed by GC—MS. The principal components of fraction 1 were the methyl esters of palmitic (16:0), linoleic (18:2), oleic (18:1), behenic (22:0), and lignocerinic (24:0) acids (Table 1). According to GC—MS, the ethylacetate extract contained only one triterpene, stigmast-4-en-3-one, whereas the neutral part of the hexane extract contained at least 12 compounds in addition to fatty acids, 8 of which were identified as triterpenes [5].

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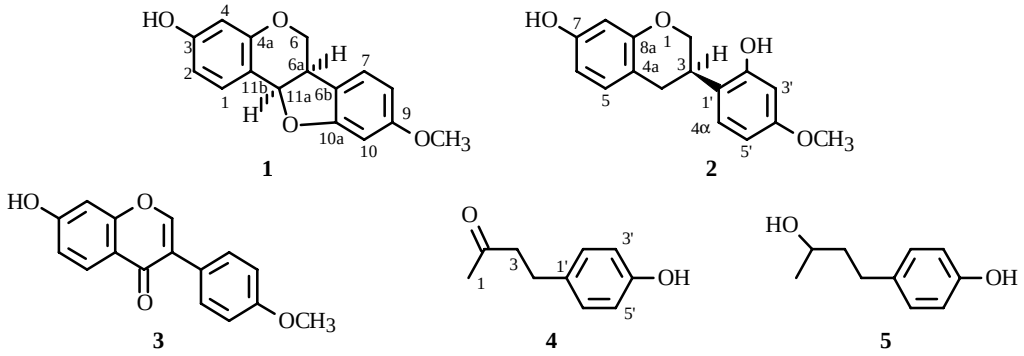
TABLE 1. Composition of Fraction 1 of the Ethylacetate Extract after Methylation by Diazomethane*

Compound	Content, %
Methyl hexadecanoate (16:0)	18
Methyl linoleate (18:2)	16
Methyl oleate (18:1)	13
Methyl linoleate (18:3)	1
Methyl octadecanoate (18:0)	4
Methyl eicosanoate (20:0)	1
Methyl docosanoate (22:0)	9
Methyl tricosanoate (23:0)	3
Methyl tetracosanoate (24:0)	14
Methyl hexacosanoate (26:0)	1
Stigmast-4-en-3-one**	3

*Components with a content >1% are listed

**Identified by comparison of mass spectra with Wiley library spectra without taking retention time into account.

Column chromatography over silica gel of the more polar fractions of the ethylacetate extract (fractions 3-5) isolated (-)-medicarpin (**1**), (-)-vestitol (**2**), formononetin (**3**), raspberry ketone (**4**), and rhododendrol (**5**) with HPLC monitoring of the separation.



The structures of **1-5** were proved using spectral data such as GC—MS, PMR, ^{13}C NMR, IR, and UV spectroscopy. Spectra of the isolated compounds were similar to those published (see Experimental). Compound **1** [(-)-medicarpin] has *cis*-fused six- and five-membered heterocyclic rings with SSCC $^{3}J_{H\text{-}6a,H\text{-}11a} = 6.5$ Hz. The CD spectrum for **1** has been reported [6] but the absolute configuration was not determined. For **2** [(-)-vestitol], signals for H-3′ (δ 6.34 ppm) and H-5′ (δ 6.46 ppm) were assigned in the PMR spectrum using the nuclear Overhauser effect (NOE) for the OCH_3 group. The NOE was 3% for H-3′ and 4% for H-5′. The signal for H-5 (δ 6.92 ppm, dd, 8 Hz, 1 Hz) had SSCC $^{4}J_{H\text{-}5,H\text{-}4} = 1$ Hz, which enabled H-5 and H-6′ (δ 6.99 ppm, d, 8 Hz) to be distinguished. (-)-Vestitol was assigned the (3*R*)-configuration [7, 8].

Compounds **1-5** possessed various types of biological activity including antimicrobial [9, 10], antibacterial [11, 12], anticancer [13, 14], and anticholesterolemic [15].

All these compounds were isolated from *H. theinum* for the first time. Compounds **1-3** were isolated from *H. polybotrus* Hand.-Mazz growing in China [16]. Also, isolation of **4** and **5** from the *Hedysarum* genus has not been previously described [17].

EXPERIMENTAL

GC—MS was performed in a Hewlett—Packard instrument with an HP 5890 Series II gas chromatograph and HP 5971 (EI, 70 eV) mass-selective detector using an HP5MS capillary column [diphenyl (5%)—dimethylsiloxane (95%), 30 m × 0.25 mm × 0.25 μm]; He carrier gas at 1 mL/min; programmed column temperature at 50°C for 2 min, from 50°C to 300°C at 4°C/min, and 300°C for 30 min; vaporizer temperature 280°C; ion-source temperature 175°C, and scan rate 1.2 scans/s in mass range 30-650 amu. Mixture components were determined using the LPMR NIOCh library of mass spectra of natural compounds (1000 compounds) and the Wiley/NBS Registry of Mass Spectral Data (400,000 compounds). The contents (%) of the compounds were determined from peak areas in chromatograms without using correction coefficients.

HPLC was performed in a Milichrom A-02 microcolumn chromatograph (ZAO EkoNova Novosibirsk) using a standard chromatography column (2 × 75 mm) packed with reversed-phase sorbent (ProntoSIL 120-5-C18, 5 μm, Bischoff, Germany). Gradient elution with simultaneous multiwavelength detection at six wavelengths (220, 240, 260, 280, 320, 360 nm) was used [18]. The eluent was methanol with trifluoroacetic acid (TFA, 0.1%). The gradient was 0-30% methanol, 0.1% TFA, 5 min; 30-50% methanol, 0.1% TFA, 5 min; 50-70, 70%, 0.1% TFA, 10 min; 70-90, 90%, 0.1% TFA, 10 min; and 5 min to methanol. The temperature was 35°C; pressure, 30-36 atm; flow rate, 150 μL/min.

IR spectra were recorded on a Vector 22 instrument in KBr; UV spectra, on an HP 8453 UV Vis instrument in EtOH (c 10^{-4} M). PMR spectra were recorded on Bruker AM-400 (400.13 MHz) and DRX-500 (500.13 MHz) instruments; ^{13}C NMR spectra, on Bruker AM-400 (100.61 MHz) using JMOD regime and DRX-500 (125.76 MHz) using JMOD and NOESY regimes for 5-10% solutions in $CDCl_3$ or $(CD_3)_2SO$. The standard was the solvent signal. Column chromatography was performed over Merck 60-200 μm silica gel; TLC, on Silufol UV-254 plates using hexane:diethylether (1:3). Spots were observed in UV light or developed using HCl and *p*-nitroaniline and sodium nitrite (yellow, brown color with phenolic compounds) [19].

Plant Raw Material. *H. theinum* Krasnob. was collected in 2003 on Korgonsk ridge of Altai Krai at 2000 m above sea level in a subalpine grassy glade. The density of the cenopopulation was 3.2 specimens per square meter. The underground part of mature specimens in g2 and g3 states was collected. The mass of fresh root of one specimen was 1-3 kg. The plant specimen agreed with a specimen of *H. theinum* preserved in the CSBG SD RAS herbarium.

Successive Extraction by Ethylacetate:Ethanol. Ground air-dried roots of *H. theinum* (2812 g) were extracted with ethylacetate (6 × 9 L) with boiling for 5.5 h, yield 29.46 g (1.0%), and by ethanol (7 × 9 L) with boiling for 7 h, yield 139.07 g (4.9%).

The ethylacetate extract (5.01 g) was separated by column chromatography over silica gel (50 g) with elution by hexane with a diethylether gradient. The compositions of fractions were analyzed by TLC. Similar fractions were combined to afford fraction 1, 1.615 g (hexane:diethylether, 25%); fraction 2, 217 mg; fraction 3, 407 mg; fraction 4, 190 mg; fraction 5, 216 mg (hexane:diethylether, 50%); fraction 6, 579 mg (hexane:diethylether, 75%). Further elution by diethylether produced fraction 7, 111 mg; elution by ethylacetate, fraction 8, 501 mg; elution by methanol, fraction 9, 630 mg.

Fraction 1 (32 mass % of extract) did not contain phenolic compounds according to TLC and gave color with HCl and *p*-nitroaniline and sodium nitrite. Fraction 1 (53 mg) was dissolved in hexane (2 mL). Diazomethane in diethylether (0.8 mL) was added dropwise [20] at room temperature. The mixture was stirred and analyzed by GC—MS. Table 1 gives the results.

Fraction 3 was separated by column chromatography over silica gel with elution by hexane with a diethylether gradient to afford fraction 3-1, 90 mg (hexane:diethylether, 30%); fraction 3-2, 93 mg (hexane:diethylether, 35%); and fraction 3-3, 90 mg (hexane:diethylether, 45 and 50%).

Fractions 2 and 3-1 were combined according to HPLC data. Column chromatography over silica gel with elution by $CHCl_3$:CH_3OH (100:1) afforded **1** (289 mg, 5.8 mass % of extract) in 79% purity according to HPLC. Chromatography of the sample (21 mg) twice on Silufol plates (hexane:diethylether, 25%) afforded **1** (7 mg) in 95% purity according to HPLC.

Fraction 3-2 was chromatographed on Silufol plates using hexane:diethylether (50%) to afford **4** (18 mg, 0.4 mass % of extract) in 85% purity according to GC—MS.

Fractions 3-3 and 4 were combined according to HPLC. Column chromatography twice on silica gel (hexane:diethylether, 35%; $CHCl_3$:CH_3OH, 100:1) afforded **2** (74 mg, 1.5 mass % of extract) in 80% purity according to HPLC. Chromatography of the sample (27 mg) twice on Silufol plates (hexane:diethylether, 35%) afforded **2** (6 mg) in 94% purity according to HPLC.

Recrystallization of fraction 5 three times from diethylether isolated **3** (55 mg, 1.0 mass % of extract) in 94% purity according to HPLC. The mother liquor was evaporated and chromatographed successively over silica gel (hexane:diethylether,

45%) and twice on Silufol plates (hexane:diethylether, 35%; $CHCl_3$:CH_3OH, 100:8) to afford **5** (28 mg, 0.6 mass % of extract) in 84% purity according to HPLC.

(-)-Medicarpin (1) (Demethylhomopterocarpan, 9-methoxy-6α,11α-dihydro-6*H*-benzo[4,5]furo[3,2-*c*]chromen-3-ol). $C_{16}H_{14}O_4$. $[\alpha]_{580}$ -257° (*c* 0.47, $CHCl_3$), lit. [21] $[\alpha]_{589}$ -220.6° (*c* 0.75, $CHCl_3$). IR spectrum (cm^{-1}): 3593, 1712, 1622, 1600, 1497. UV spectrum (λ_{max}, nm, log ε): 225 (3.98), 287 (3.75). $[M]^+$: 270, 255, 161, 148, 135. Found $[M]^+$: 270.08960; Calc. $[M]^+$: 270.08920.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 3.52 (1H, ddd, J = 11, 6.5, 4, H-6a), 3.61 (1H, dd, J = 11, 11, H-6β), 3.74 (3H, s, OCH_3), 4.22 (1H, dd, J = 11, 4, H-6α), 4.95 (1H, br.s, OH), 5.48 (1H, d, J = 6.5, H-11a), 6.39 (1H, d, J = 2, H-10), 6.43 (1H, d, J = 2, H-4), 6.44 (1H, dd, J = 8, 2, H-8), 6.53 (1H, dd, J = 8, 2, H-2), 7.11 (1H, d, J = 8, H-7), 7.37 (1H, d, J = 8, H-4).

^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 39.46 (C-6a), 55.42 (OCH_3), 66.48 (C-6), 78.44 (C-11a), 96.87 (C-8), 103.61 (C-4), 106.36 (C-10), 109.66 (C-2), 112.67 (C-11b), 119.04 (C-6b), 124.64 (C-7), 132.14 (C-1), 156.64 (C-4a), 156.91 (C-3), 160.63 (C-10a), 161.11 (C-9). PMR [22] and ^{13}C NMR [23] data have been published.

(-)-Vestitol (2) [(3*R*)-(2-hydroxy-4-methoxyphenyl)-chroman-7-ol]. $C_{16}H_{16}O_4$. $[\alpha]_{580}$ -5° (*c* 0.40, $CHCl_3$), lit. [7] $[\alpha]_{580}$ -18.9° (*c* 0.5, CH_3OH). IR spectrum (ν, cm^{-1}): 3597, 1706, 1621, 1596, 1508. UV spectrum (λ_{max}, nm, log ε): 223 (3.98), 282 (3.56). $[M]^+$: 272, 150, 137.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 2.87 (1H, ddd, J = 15, 5, 1.5, H-4α), 2.97 (1H, dd, J = 15, 10, H-4β), 3.48 (1H, dddd, J = 10, 10, 5, 3.5, H-3), 3.74 (3H, s, OCH_3), 4.01 (1H, dd, J = 10, 10, H-2β), 4.31 (1H, ddd, J = 10, 3.5, 1.5, H-2α), 5.05 (1H, br.s, OH), 6.33 (1H, d, J = 2, H-8), 6.34 (1H, d, J = 2, H-3′), 6.37 (1H, dd, J = 8, 2, H-6), 6.46 (1H, dd, J = 8, 2, H-5′), 6.92 (1H, dd, J = 8, 1, H-5), 6.99 (1H, d, J = 8, H-6′). NOE of OCH_3 group: δ 6.34 (H-3′), 3%; 6.46 (H-5′), 4%.

^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 30.21 (C-4), 31.59 (C-3), 55.22 (OCH_3), 69.79 (C-2), 102.05 (C-3′), 103.12 (C-8), 105.89 (C-5′), 107.82 (C-6), 114.57 (C-4a), 119.75 (C-1′), 128.09 (C-6′), 130.31 (C-5), 154.14 (C-7), 154.72 (C-8a), 155.04 (C-2′), 159.25 (C-4′). PMR [6] and ^{13}C NMR [24] data have been published.

Formononetin (3) [7-hydroxy-3-(4-methoxyphenyl)-chromen-4-one]. $C_{16}H_{12}O_4$. mp 260-264°C, lit. [25] mp 262-264°C. IR spectrum (ν, cm^{-1}): 3430, 1638, 1596, 1513, 1453, 1248. UV spectrum (λ_{max}, nm, log ε): 249 (4.40), 301 (4.03). $[M]^+$: 268, 132.

PMR spectrum (DMSO-d_6, δ, ppm, J/Hz): 3.78 (3H, s, OCH_3), 6.87 (1H, d, J = 2, H-8), 6.94 (1H, dd, J = 8, 2, H-6), 6.80 (2H, d, J = 8, H-3′,5′), 7.50 (2H, d, J = 8, H-2′,6′), 7.96 (1H, d, J = 8, H-5), 8.33 (1H, s, H-2), 10.86 (1H, s, OH).

^{13}C NMR spectrum (DMSO-d_6, δ, ppm): 55.14 (OCH_3), 102.14 (C-8), 113.60 (C-3′,5′), 115.21 (C-6), 116.61 (C-4a), 123.16 (C-1′), 124.24 (C-3), 127.29 (C-5), 130.08 (C-2′,6′), 153.15 (C-2), 157.45 (C-8a), 158.95 (C-7), 162.60 (C-4′), 174.63 (C-4). PMR [26] and ^{13}C NMR [27] data have been published.

Raspberry Ketone (4) [4-(4-hydroxyphenyl)-butan-2-one]. UV spectrum (λ_{max}, nm): 224, 278. $[M]^+$: 164, 107.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 2.11 (3H, CH_3), 2.75 (4H, m, H-3,4), 6.72 (2H, d, J = 8, H-3′,5′), 7.02 (2H, d, J = 8, H-2′,6′). PMR [28] data have been published.

Rhododendrol (5) [4-(4-hydroxyphenyl)-butan-2-ol]. $[M]^+$: 166, 148, 133, 107.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 1.21 (3H, d, J = 6, CH_3), 1.72 (2H, m, H-3), 2.61 (2H, m, H-4), 3.81 (1H, q, J = 6, H-2), 6.73 (2H, d, J = 8, H-3′,5′), 7.05 (2H, d, J = 8, H-2′,6′), 13.40 (1H, s, OH).

^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 23.39 (C-1), 31.04 (C-4), 40.81 (C-3), 67.57 (C-2), 115.11 (C-3′,5′), 133.83 (C-1′), 129.27 (C-2′,6′), 153.60 (C-4′). PMR [29] and ^{13}C NMR [30] data have been published.

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