Chemistry of Natural Compounds, Vol. 44, No. 5, 2008

FATTY-ACID COMPOSITION AND SECONDARY METABOLITES FROM SLIGHTLY POLAR EXTRACTS OF THE AERIAL PART OF *Primula macrocalyx*

Yu. S. Kosenkova,[1*] M. P. Polovinka,[1] N. I. Komarova,[1] D. V. Korchagina,[1] S. V. Morozov,[1] A. I. Vyalkov,[1] N. Yu. Kurochkina,[2] V. A. Cheremushkina,[2] and N. F. Salakhutdinov[1]

UDC 665.3+547.972

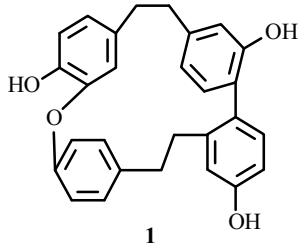
Flavones 2′,5′-dimethoxyflavone, 3′-methoxy-4′,5′-methylenedioxyflavone, 3′,4′-dimethoxyflavone, 5,6,2′,3′,6′-pentamethoxyflavone, and 5,6,2′,3′,5′,6′-hexamethoxyflavone; salicylates, methyl-4-methoxysalicylate and peonol; and bisbibenzyl polyphenol riccardin C were isolated for the first time from the acetone extract of the aerial part of Primula macrocalyx *Bge. The content of free and total fatty acids was determined by GC and GC—MS. Palmitic (16:0), octadecatetraenoic 18:4 (6,9,12,15), linoleic 18:2 (9,12), and α-linolenic 18:3 (9,12,15) were the principal acids from the aerial part of* Primula macrocalyx.

Key words: *Primula macrocalyx* Bge., Primulaceae (primrose), 2′,5′-dimethoxyflavone, 3′-methoxy-4′,5′-methylenedioxyflavone, 3′,4′-dimethoxyflavone, 5,6,2′,3′,6′-pentamethoxyflavone, 5,6,2′,3′,5′,6′-hexamethoxyflavone, methyl-4-methoxysalicylate, peonol, riccardin C, fatty acids, HPLC, GC, GC—MS.

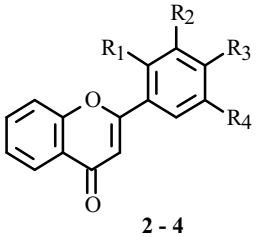
Primula macrocalyx Bge. is widely used in folk medicine. Tincture of roots is used as an expectorant for pneumonia and bronchitis and as a diuretic. The decoction possesses sedative, spasmolytic, and slight laxative action. The tea is a sudorific. The preparations Primulin and Primulat exhibit expectorant, sedative, spasmolytic, and diuretic activity and are used for vitamin deficiency. The tincture and powder of leaves are used for vitamin C and A deficiencies and as diuretics and laxatives. Tea of flowers is drunk for colds, headache, insomnia, paralysis, heart disease, rheumatism, and kidney diseases [1].

P. macrocalyx is similar to the European species *P. veris* L. that is endemic to Southern Europe, the Caucasus, and Iran; grows well in cultivation; and is promising for introduction [2].

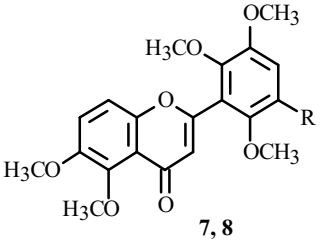
The chemical composition of *P. macrocalyx* is at present practically unstudied. Only the total amounts of saponins [3], provitamin A, ascorbic acid, carotene, carbohydrates [4], and a triterpene glycoside [5] in the plant have been determined.



1



2 - 4



7, 8

2: $R_1 = R_4 = OCH_3$, $R_2 = R_3 = H$; **3:** $R_1 = H$, $R_2 = OCH_3$, R_3-$R_4 = OCH_2O$
4: $R_1 = R_4 = H$, $R_2 = R_3 = OCH_3$; **7:** R = H; **8:** $R = OCH_3$

1) Novosibirsk Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences, 630090, Novosibirsk, prosp. Akad. Lavrent′eva, 9, e-mail: koesn@nioch.nsc.ru; 2) Central Siberian Botanical Garden, Siberian Branch, Russian Academy of Sciences, ul. Zolotodolinskaya, 101. Translated from Khimiya Prirodnykh Soedinenii, No. 5, pp. 457-460, September-October, 2008. Original article submitted May 2, 2008.

0009-3130/08/4405-0564

TABLE 1. Retention Time and Relative Content of Fatty-Acid Methyl Esters in Extract of the Aerial Part of *Primula macrocalyx* by GC—MS and GC

Ret. time, min		Acid	Acid fraction, mass %		Ret. time, min		Acid	Acid fraction, mass %	
GC—MS	GC		free	total	GC—MS	GC		free	total
6.13	5.86	6:0	-	0.3	20.66	19.89	18:3 (6,9,12)	0.8	4.8
7.75	7.42	7:0	-	0.3	20.76	19.99	18:4 (6,9,12,15)	1.8	10.7
7.87	7.63		-	0.2	20.86	20.06	18:2 (9,12)	4.3	14.8
9.30	8.92	8:0	-	0.1	20.87	20.10*	18:1 (9)	3.4	0.7
10.79	10.06	9:0	0.9	0.6	20.92	20.15*	18:3 (9,12,15)	1.8	6.6
12.19	11.72	10:0	0.1	0.1	21.10	20.23	18:0	0.6	1.9
13.51	13.10	11:0	0.1	0.1	20.64	21.09	19:0-*iso*	-	0.1
13.78	13.57		-	0.1	21.96	21.24	19:0	0.4	0.5
14.57	14.03	12:0-*iso*	-	0.1	22.62	21.92	20:1 (11)	-	1.2
14.69	14.08	12:0-*antiiso*	0.1	0.1	22.83	22.15	20:0	0.1	0.1
14.77	14.17	12:0	0.13	1.3	23.65	22.90	21:0	-	0.2
15.50	14.89	13:0-*iso*	-	0.1	24.44	23.65	22:0	0.3	1.0
15.94	14.97	13:0	0.1	0.2	25.20	24.43	23:0	0.1	0.1
16.23	15.82		-	0.3	26.03	25.14	24:0	0.3	0.6
16.88	16.13	14:0-*iso*	-	0.2	26.61	25.87	25:0-*iso*	-	0.1
16.92	16.25	14:0-*antiiso*	-	0.1	26.68	25.89	25:0-*antiiso*	-	0.1
17.09	16.38	14:0	0.1	2.7	26.98	25.93	25:0	0.1	0.1
17.32	17.03		-	0.1	28.10	26.70	26:0	0.4	0.1
17.75	17.14	15:0-*iso*	0.2	0.1	28.90	27.31	27:0-*iso*	-	0.1
17.83	17.40	15:0-*antiiso*	-	0.2	29.07	27.35	27:0-*antiiso*	-	0.1
18.14	17.50	15:0	0.7	0.3	29.43	27.44	27:0	-	0.1
18.97	18.19	16:0-*iso*	0.1	0.4	31.05	28.37	28:0	1.4	0.4
19.03	18.23	16:0-*antiiso*	0.2	0.1	32.30	28.72	29:0-*iso*	-	0.1
19.22	18.42	16:0	2.8	12.0	33.11	28.88	29:0	-	0.2
19.79	18.98	17:0-*iso*	0.1	0.1	35.60	30.02	30:0	0.1	0.1
19.87	19.00	17:0-*antiiso*	0.1	0.1			Total	20.6	63.4
20.14	19.32	17:0	0.2	0.2					

*Oleic and linoleic acids eluted in one GC peak. Their total content was determined by GC; the ratio, by mass spectrometry.

We studied slightly polar metabolites of *P. macrocalyx* using successive extraction of the aerial part by hexane and acetone [6]. Soaking in hexane removed chlorophyll and colored pigments. This facilitated isolation of the slightly polar metabolites. The major component of the acetone extract was riccardin C (**1**) [6].

The dried acetone extract was dissolved in a small amount of methanol. The insoluble part was separated (the precipitate had an HPLC profile of fatty acids). Solvent was removed. The viscous green residue was treated with hexane and mixed with a spatula. The mixture was separated into a green solution and a brownish residue. The green solution was treated successively with HCl solution (5%), water, $NaHCO_3$ solution (5%), and water again. The hexane solution was dried over $MgSO_4$. Solvent was removed. The residue was chromatographed and rechromatographed over columns of silica gel ($CHCl_3$ eluent with a gradient of ethanol from 0 to 10%) to isolate the following compounds in order of elution: 2′,5′-dimethoxyflavone (**2**), 3′-methoxy-4′,5′-methylenedioxyflavone (**3**), 3′,4′-dimethoxyflavone (**4**), methyl-4-methoxysalicylate (**5**), peonol (**6**), 5,6,2′,3′,6′-pentamethoxyflavone (**7**), 5,6,2′,3′,5′,6′-hexamethoxyflavone (**8**), and riccardin C (**1**). Flavones **2**, **3**, and **4** were isolated earlier from *P. veris* [7]. Peonol (**6**) was previously isolated from *P. veris* [8] and *P. auricula* [9]. Flavones **7** and **8** have not been previously isolated from plants of the genus *Primula* but have been isolated from the ethylacetate fraction of the leaf extract of *Casimiroa tetrameria* [10]. Compounds **2**, **3**, **7**, and **8** were also present (according to HPLC) in the hexane extract.

According to HPLC, both extracts of the aerial part of *P. macrocalyx*, hexane and acetone, contained a significant amount of free and bound fatty acids. Ground air-dried raw material was extracted several times by a mixture of hexane and diethylether in order to extract fatty acids. The combined extract was evaporated. Part of the resulting extract was preliminarily

chromatographed over a column to remove colored pigments in order to determine the content of free fatty acids (FFA). The other part of the extract was hydrolyzed in order to determine the content of total fatty acids (TFA), i.e., FFA and those acids that were esterified. Samples containing fatty acids were evaporated and treated with diazomethane. Fatty acids were identified as methyl esters using GC and GC—MS. The content of FFA methyl esters in the corresponding sample was 20.6%; of TFA in the hydrolyzed sample, 63.4%.

Table 1 shows that all monocarboxylic acids with 6 to 30 C atoms and a slight amount of dicarboxylic acids were present in the studied plant. The principal acids of the aerial part of *P. macrocalyx* were palmitic (16:0, rel. content 12%), octadecatetraenoic [18:4 (6,9,12,15), rel. content 10.7%], linoleic [18:2 (9,12), rel. content 14.8%], and α-linolenic [18:3 (9,12,15), rel. content 6.6%]. The contents of the other acids were in the range 0.2-4.8%. Some of the acids were present in trace quantities. The aerial part of *P. macrocalyx* contained also isomeric forms of unbranched saturated fatty acids (both iso- and antiiso-acids). The fraction of unsaturated acids was 38.8% of their total content. The presence in the plant of linolenic acid [18:3 (6,9,12), rel. content 4.8%] in combination with a significant amount of octadecatetraenoic [18:4 (6,9,12,15)] was a taxonomic marker of the genus *Primula* [11]. The fraction of FFA in the aerial part was 0.37% of the mass of dry raw material with 18.3% of the acids in the bound state.

Comparison of the total fatty-acid composition and the FFA fraction of the extracts revealed that practically all monocarboxylic acids were present in the plant in the free state whereas the dicarboxylic acids were only found in the bound state. All major acids were mainly esterified. The ratio of free to esterified forms was different for them. The different ratio of forms for 18:1 (9) and 18:3 (9,12,15) in the free and bound states was especially notable. The 18:1 (9) acid in the free form was one of the principal acids whereas 18:3 (9,12,15) acid predominated in the total fraction. It should be noted that the content of free and esterified forms was also different for 28:0 fatty acid, which was found more in the free state than in the bound.

Furthermore, α-tocopherol, phytol, and phytosterols were observed in the analyzed samples.

EXPERIMENTAL

HPLC was performed on a Milikhrom A-02 (ZAO EkoNova, Novosibirsk) microcolumn chromatograph using a standard chromatography column (2 × 75 mm) packed with a reversed-phase sorbent (ProntoSIL 120-5-C18, particle size 5 μm, Bischoff, Germany). We used gradient elution with simultaneous multiwavelength detection at six wavelengths (220, 240, 260, 280, 320, 360 nm) [12]. 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; methanol, 5 min. The temperature was 35°C; pressure 30-36 atm; flow rate, 150 μL/min.

Quantitative gas chromatography of methylated extracts was performed on an Agilent 6890 chromatograph with a flame-ionization detector and Agilent G1701 AA Chemstation data processing system. We used an HP-5 quartz capillary column (5% diphenyl- and 95% dimethylsiloxane copolymer); length, 30 m; inner diameter, 0.32 mm; stationary phase thickness, 0.25 μm. The carrier gas was He at flow rate 2 mL/min. The sample was introduced without dividing the flow (splitless) in order to increase the sensitivity and improve the reproducibility. The injector and detector temperatures were 280°C. The column temperature was varied according to the following program: 2 min at 50°C; increased to 280°C at 10°C/min; hold at that temperature for 20 min. The fatty acid composition was calculated using internal normalization. The content of separate fatty acids was expressed in percent of total mass of all substances.

GC—MS was carried out in an HP 6890 GC with an HP 5972 mass-selective detector. We used an HP-5 MS quartz caipllary column (5% diphenyl- and 95% dimethylsiloxane copolymer); length, 30 m; inner diameter, 0.25 mm; stationary phase thickness, 0.25 μm. The carrier gas was He at flow rate 0.8 mL/min. The sample was introduced without dividing the flow (splitless). The injector and detector interface temperatures were 280°C. The column temperature was varied according to a program analogous to that described above. Compounds were identified by comparison with retention times of several authentic samples and with mass spectra from the NIST 02 MS database (175,000 compounds) that was included in the Agilent G1701 AA Chemstation data processing system. Furthermore, selective detection according to the individual characteristic ions was used to increase the confidence level of the identification.

PMR and ^{13}C NMR spectra in $CDCl_3$ and DMSO-d_6 including LRJMD and 2D CH-COSY correlation spectra were recorded on Bruker AM-400 (operating frequencies 400.13 and 100.61 MHz) and AV-300 (300.13 and 75.47 MHz, respectively) instruments. The standards were solvent resonances (δ_H 7.24 ppm and δ_C 76.90 ppm for $CHCl_3$; δ_H 2.50 and δ_C 39.50 ppm

for DMSO). Column chromatography was performed over Merck 100-160 μm silica gel; TLC, on Silufol UV-254 plates using $CHCl_3$:C_2H_5OH (2-5% EtOH) with detection of spots in UV light.

Plant material was collected near Anos village of Chemal Region, Altai, in August 2004-2007. A voucher specimen is stored in the Herbarium of the CSBG, SB, RAS.

The first extraction method used ground raw material (413.03 g) that was treated with hexane (2 L) and soaked seven times for 12 h each until the bright green color of the solution disappeared. The combined hexane extract was evaporated to produce a residue (3.82 g). The extraction was continued by treatment with acetone (2 L) and soaking six times for 48 h each until riccardin C (HPLC monitoring) completely disappeared. The acetone extract was evaporated to produce a residue (5.29 g). The resulting acetone extract was treated with methanol (30 mL). The insoluble part weighed 0.79 g. The part that was soluble in methanol was evaporated to produce a residue (4.50 g) that was treated with hexane (30 mL) to give a green solution and a viscous brownish residue (2.30 g). The green hexane solution was separated from the brownish residue and treated successively with HCl solution (5%, 50 mL × 2), water (30 mL), $NaHCO_3$ solution (5%, 50 mL × 2), and water again. The organic layer was dried over calcined $MgSO_4$. The mass of the extract after processing and removal of solvent was 1.62 g.

Then this extract was separated over a column of silica gel (SiO_2:substance, ~20:1; eluent, $CHCl_3$ with an EtOH gradient from 0 to 10%). Elution by pure $CHCl_3$ produced fraction 1 (185.6 mg) that contained flavones **2**-**4** [7]. Addition to the eluent of ethanol (1-2%) produced fraction 2 (800 mg) that contained mainly **5** and **6**. Then, flavones **7** (7 mg, 0.0017% of the mass of dry raw material) and **8** (10 mg, 0.0024%) eluted from the column in that order [10]. The next fractions (totaling 405.7 mg, 0.098% of the dry raw material) contained riccardin C that was then purified over a reversed phase as described previously [6] to afford **1** (0.22 g).

Fraction 1 was chromatographed over a column of silica gel (SiO_2:substance, ~50:1; eluent $CHCl_3$ with an EtOH gradient up to 1%). The separation isolated three flavones, **2** (7 mg), **3** (5 mg), and **4** (8 mg) in yields of 0.0017, 0.0012, and 0.0019%, respectively, of the mass of dry raw material. The structures of the compounds were established using PMR and ^{13}C NMR spectra and comparison of them with the literature [7].

Fraction 2 was chromatographed over a column of silica gel (SiO_2:substance, ~40:1; eluent $CHCl_3$ with an EtOH gradient up to 3%) to isolate **5** (100 mg) and **6** (80 mg) in yields of 0.024 and 0.019%, respectively, of the mass of dry raw material.

PMR spectrum of **5** (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 3.80 (3H, s, OMe-C4), 3.89 (3H, s, OMe-C1′), 6.41 (1H, dd, $J_{5,6}$ = 8.4, $J_{5,3}$ = 2.3, H-5), 6.43 (1H, d, $J_{3,5}$ = 2.3, H-3), 7.71 (1H, d, $J_{6.5}$ = 8.4, H-6), 10.95 (OH) [13].

^{13}C NMR spectrum of **5** (75.47 MHz, $CDCl_3$, δ, ppm): 105.33 (s, C-1), 163.67 (s, C-2), 165.50 (s, C-4), 100.55 (d, C-3), 107.41 (d, C-5), 131.09 (d, C-6), 169.60 (s, C-1′), 51.83 (q, OMe-C1′), 55.33 (q, OMe-C4).

PMR spectrum of **6** (300 MHz, $CDCl_3$, δ, ppm, J/Hz): 2.53 (3H, s, Me-C1′), 3.81 (3H, s, OMe-C4), 6.39 (1H, d, $J_{3,5}$ = 2.5, H-3), 6.42 (1H, dd, $J_{5,6}$ = 8.4, $J_{5,3}$ = 2.5, H-5), 7.60 (1H, d, $J_{6,5}$ = 8.4, H-6), 12.73 (OH).

The ^{13}C spectrum of **6** agreed well with that in the literature [14].

The NMR spectra of **1**-**4**, **7**, and **8** agreed completely with those in the literature.

The second extraction method used raw material (20 g) that was treated with a hexane:Et_2O mixture (1:1, 100 mL each, 5 times, 8 h each) at room temperature with stirring. The mass of the resulting extract was 1.997 g.

A part (0.162 g) of the resulting extract was chromatographed over a column of neutral Al_2O_3 (extract:absorbent, 1:40). The eluents were hexane (50 mL, 1) and hexane:Et_2O (50 mL, 5:1, 2; 50 mL, 3:1, 3; 50 mL, 1:1, 4). Fractions 2, 3, and 4 were combined based on HPLC data (fatty-acid profile). The combined fractions, i.e., FFA, weighed 29.7 mg.

The content of total acids was determined by basic hydrolysis. Total extract (0.5 g, accurate weight) was treated with NaOH solution (15 mL, 1 M NaOH) in MeOH (80%) and stored in a thermostat at 50°C for 1 h. The resulting hydrolysate was cooled to room temperature, diluted with water to 100 mL, and extracted with Et_2O in 20-25 mL portions until the organic phase was colorless. The ether extracts were discarded. The aqueous layer was neutralized with HCl until the pH was 3-4 and again extracted with Et_2O (3-5 times). The ether extracts were combined, washed with water until the rinsings were neutral, and evaporated in vacuo. The mass of the acidic part after neutralization was 0.17 g.

The resulting fatty acids were dissolved in Et_2O (5-10 mL), treated with a catalytic amount [15] (1-2 drops) of methanol and freshly prepared diazomethane solution [16] in Et_2O until gas evolution ceased, and stored for 30-40 min. The solvent was removed in vacuo. The methyl esters were analyzed by GC and GC—MS.

ACKNOWLEDGMENT

The work was supported financially by the SB RAS (Lavrent′evskii Competition of Youth Projects, SB RAS 2006).

REFERENCES

1. J. Bruneton, *Pharmacognosy, Phytochemistry, Medical Plants*, Lavoisier, New York (1999).
2. A. K. Skvortsov, N. V. Trulevich, and Z. R. Alferova, *Introduction of Plants of the Natural Flora of the USSR* [in Russian], Nauka, Moscow (1979).
3. P. K. Mukhamedzyaeva and M. M. Alimbaeva, *Rastit. Resur.*, **5**, No. 3, 380 (1969).
4. O. V. Michnik, *Rastit. Resur.*, **12**, No. 3, 391 (1976).
5. A. M. Zakharov, O. I. Zakharova, D. A. Pakaln, and I. N. Sokol′skii, *Khim. Prir. Soedin.*, 672 (1971).
6. Yu. S. Kosenkova, M. P. Polovinka, N. I. Komarova, D. V. Korchagina, N. Yu. Kurochkina, V. A. Cheremushkina, and N. F. Salakhutdinov, *Khim. Prir. Soedin.*, 591 (2007).
7. J. Budzianowski, M. Morozowska, and M. Wesolowska, *Phytochemistry*, **66**, No. 9, 1033 (2005).
8. S. R. Jensen, B. J. Nielsen, and V. Norn, *Phytochemistry*, **16**, 2036 (1977).
9. *Bioactive Substances of Plant Origin* [in Russian], Nauka, Moscow (2001).
10. B. Heneka, H. Rimpler, A. Ankli, O. Sticher, S. Gibbons, and M. Heinrich, *Phytochemistry*, **66**, 649 (2005).
11. O. Sayanova, J. A. Napier, and P. R. Shewry, *Phytochemistry*, **52**, No. 5, 419 (1999).
12. G. I. Baram, M. A. Grachev, N. I. Komarova, M. P. Pelelroyzen, Yu. A. Bolvanov, S. V. Kuzmin, V. V. Kargaltsev, and E. A. Kuper, *J. Chromatogr.*, **264**, 69 (1983).
13. *Sadtler Standard Spectra: Nuclear Magnetic Resonance Spectra*, Vol. 30, Sadtler Research Lab., Philadelphia (1974), 19659 p.
14. A. K. Chakraborti, L. Sharma, and M. K. Nayak, *J. Org. Chem.*, **67**, No. 18, 6406 (2002).
15. N. S. Vul′fson, ed., *Preparative Organic Chemistry* [in Russian], Khimiya, Moscow (1964).
16. L. F. Fieser and M. Fieser, *Reagents for Organic Synthesis*, Wiley, New York (1968).