Chemistry of Natural Compounds, Vol. 48, No. 2, May, 2012 [Russian original No. 2, March-April, 2012]

POLYPRENOLS AND TRITERPENOIDS FROM LEAVES OF *Alcea nudiflora*

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UDC 547.315

Polyprenols and triterpenoids from leaves of Alcea nudiflora *were studied for the first time. It was shown that the principal components of the unsaponified fraction were polyprenols, sterols, a phytol, and tocopherols. The composition of the polyprenols from* Alcea nudiflora *was established. Minor components of polyprenols with chain lengths 8, 9, and 14 isoprene units were observed for the first time in plants of the genus* Alcea*. A total of 28 terpene components of the unsaponified fraction, 26 of which were not previously observed in this species, were determined by GC–MS.*

Keywords: *Alcea nudiflora*, Malvaceae, unsaponified fraction, polyprenols, triterpenoids, hydrocarbons.

Plants of the family Malvaceae are exceptional among leafy plants because of their high content of polyprenols (PPs), which are chemotaxonomic markers [1–6]. Such plants also have a characteristically high content of cyclopropane acids, which are practically not observed in plants of other families [7].

Alcea nudiflora belongs to this family, is widely distributed, and is common in the plant cover of the whole Tian-Shan (Chatkal, Kuramin, Ugam, Pskom, etc. ranges) and Pamir-Alai (Alai, Turkestan, Nuratau, Zarafshan, etc. ranges) [8–10]. However, neutral triterpenoids and bioactive PPs from this plant are insufficiently studied [11].

Various qualitative and quantitative analytical methods for PPs and dolichols from plant material have been reported. However, each of them has certain shortcomings. Thus, comparison of HPLC analyses of an extract and a chromatographic standard concentrate of PPs does not consider seasonal cycles of the component ratio in plant material according to vegetation periods. Furthermore, PPs and dolichols in certain plant species are present as esters of aliphatic acids that are unsuitable for HPLC analysis [12, 13]. HPLC with refractive-index detection also has shortcomings. Normalization to the total peak area in the chromatogram does not consider differences in the extinction or refraction.

PMR analysis of polyisoprenoid concentrates also has several shortcomings. A PMR spectrum of a PP sample is a superposition of resonances of similar structural fragments. The regular structure of the PPs produces total resonances that are stronger than those of impurities. This leads to a substantial overestimation of the quantitative characteristics of the studied PP fraction. Solanesol can be cited as an example [14].

Use of densitometry of thin-layer chromatograms compared with a standard [15] also usually causes substantial overestimation of quantitative results because accompanying mono-, sesqui-, and diterpene alcohols overlap the spot for the PP fraction. Thus, borneol, *cis*-abienol, isoabienol, dehydroabietinol, and other alcohols that enhanced the intensity of the PP spot were present as impurities in the total fraction during a study of PPs from conifers [16, 17]. Bisabolol was isolated from essential oil of cotton buds [18]. The chromatographic mobility of total extracted compounds depends on the chain length of the PPs in them and their form (as alcohols or esters). Therefore it is practically impossible to choose an adequate standard for an unstudied raw material.

HPLC with a reversed-phase sorbent that uses an external standard, tocopherol palmitate, with spectral and chromatographic properties corresponding best to the studied sample is practically devoid of these shortcomings for the analysis of extracts and fractions for PP content [19]. Furthermore, the standard is typically stable to external factors because its chemical and spectral properties do not change over several years both in the pure state and in solution.

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Translated from *Khimiya Prirodnykh Soedinenii*, No. 2, March–April, 2012, pp. 169–173. Original article submitted July 8, 2011.

 0009-3130/12/4802-0180

TABLE 1. Qualitative and Quantitative Composition of Polyprenols of Various Purities from *Alcea nudiflora* Leaves (% of Total Polyprenols)

Sample	P_8*	P_9	P_{10}	P_{11}	P_{12}	P_{13}	P_{14}	Total polyprenol content in sample, wt% of unfractionated sample (US)
Alcea nudiflora UF	Tr.	Tr.	9.5	39.0	42.7	7.1	1.7	18.7
Alcea nudiflora PP	0.9	1.9	3.0	39.0	44.2	10.3	0.6	94.6

*Polyprenol with eight isoprene units; Tr = traces.

The optical density and retention time are important parameters for the method. It should be considered that an external standard can be used to study any PP-containing mixtures or mixtures of PP derivatives regardless of the composition and source and the chemotaxonomic and seasonal features of the raw material. An advantage of the proposed method over semi-quantitative estimation using TLC [20] is the greater differentiation of PPs from accompanying impurities and the ability to distinguish these compounds that are present in the plant material as esters of fatty aliphatic acids in addition to free and acetylated PPs.

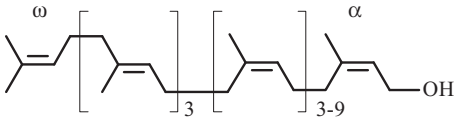
We used just this method in our investigations. Extracts from leaves of *A. nudiflora* and fractions were analyzed for PP content in a Milikhrom chromatograph with UV detection at 210 nm using a column (6.3 × 0.2 cm) packed with Lichrosorb RP-18 sorbent (5 μm) and MeOH:$(CH_3)_2CO$ (1:3, v/v) eluent. PPs of basidial fungi and extracts of the aerial part of *Hypericum perforatum* were previously studied by this method [21, 22].

Primary analysis of PPs was carried out without further purification of the fraction of unsaponified substances. The component composition and PP content were determined on enriched PP fractions obtained using column chromatography over silica gel that produced 12 fractions. Fraction 4 contained PPs of 94.6% purity (HPLC). Their PMR spectrum corresponded to that of sea-buckthorn PPs [12].

Table 1 presents the component ratio of PPs in the total unsaponified fraction (UF) of the extract from leaves of *A. nudiflora* and in the purified fraction of PPs in addition to the content of the polyisoprenoid fraction in each sample.

Table 1 shows that HPLC of the unpurified UF can rather accurately determine the qualitative and quantitative compositions of polyisoprenoids. However, minor components with a short chain have the same retention time as the impurities. Therefore, the sample must be purified to 60% and greater in order to determine accurately the components with 8–9 isoprene units in the chain. The prenologs P_8, P_9, and P_{14} in addition to previously identified components were detected [23].

Thus, it was proven that *A. nudiflora* PPs are ficaprenols with 3–9 *cis*-units and 3 *trans*-units.



The principal components of the UF from leaves of *A. nudiflora* that were identified by direct GC–MS of the unsaponified solid without further fractionation are listed below (wt% of UF):

Component	*Amt., %*	*Component*	*Amt., %*
Hexahydrofarnesylacetone	1.3	Stigmasterol	4.6
Phytol	26.9	β-Sitosterol	27.9
Squalene	2.6	Fucosterol	1.2
γ-Tocopherol	1.7	β-Amyrin	7.1
Cholesterol	1.5	α-Amyrin	2.8
α-Tocopherol	3.1	Lupeol	2.6
Campesterol	2.1	Uvaol	1.6.

These data show that the principal components of the unfractionated sample (US) were β-sitosterol and phytol. Furthermore, another 12 compounds that, with the exception of α- and β-amyrin, were not reported for raw material of this species were identified [20, 24–26].

The content of minor components in the mixture was 13% of the total US. Additional fractionation revealed the composition of the minor components. Thus, the fraction of aliphatic hydrocarbons (fraction 1, F-1) that was obtained upon purification of the PPs contained greater than 20 components, the principal ones of which were tricosane, pentacosane,

heptacosane, and nonacosane. GC–MS established the composition of unbranched aliphatic hydrocarbons as given below (wt% of fraction F-1):

Component	*Amt., %*	*Component*	*Amt., %*
Hexadecane	0.5	Tricosane	5.9
Heptadecane	0.5	Tetracosane	1.5
Octadecene	0.3	Pentacosane	10.0
Octadecane	0.7	Heptacosane	20.8
Nonadecane	0.7	Nonacosane	13.3
Heneicosane	1.4	Unident. branched hydrocarbons	44.1.

Furthermore, the fraction contained 0.3% caryophyllene oxide.

Branched aliphatic hydrocarbons (fraction 2) could not be identified because the database did not contain the corresponding spectra.

The component composition of the UF of *A. nudiflora* after chromatographic fractionation was summed and is given below (wt% of UF, fractions 3 and 5–12). Another 14 triterpenoid compounds in addition to the analytical results for the US could be identified:

Component	*Amt., %*	*Component*	*Amt., %*
Hexahydrofarnesylacetone	1.2	Stigmasterol	4.5
Phytol	24.6	β-Sitosterol	24.8
Squalene	2.4	Fucosterol	1.3
γ-Tocopherol	1.7	β-Amyrin	6.8
Cholesterol	1.3	α-Amyrin	2.8
Cholestanol	0.4	β-Amyren-3-one	0.6
Cholest-Δ^7-en-3β-ol	0.6	α-Amyren-3-one	0.2
Stigmast-Δ^7-en-3β-ol	2.8	Obtusifoliol	3.6
Stigmast-4-en-3-one	0.5	Citrostadienol	0.4
α-Tocopherol	3.1	Lupeol	2.5
Campesterol	2.1	Uvaol	1.6
Stigmastanol	3.0	Erythrodiol	1.5
Stigmast-7,16-dien-3β-ol	2.9	Cycloartenol	0.9
Stigmast-4,22-dien-3-one	0.3	24-Methylenecycloartenol	0.2.

Thus, the composition of PPs from *A. nudiflora* was established. Minor components of PPs with 8, 9, and 14 isoprene units in the chain were observed for the first time in plants of this genus. GC–MS determined 28 terpene components of the UF, 26 of which were not previously observed in this raw material. The principal components of the aliphatic hydrocarbon fraction were found.

EXPERIMENTAL

Plant material was collected in Namangan Oblast (Yangikurgan Region, Khazratishokh village) in July 25, 2010. The UF of the leaf extract from *A. nudiflora* was obtained by the literature method [2] in 4.6% yield of the air-dried plant mass. Decaprenol and undecaprenol from leaves of sea buckthorn that were isolated as before [12] and pentadecaprenol and hexadecaprenol that were supplied by A. M. Moiseenkov were used as standards for HPLC analysis. The tocopherol palmitate internal standard was synthesized from tocopherol (PAO Altaivitaminy) [12, 27].

GC–MS. GC–MS spectra were recorded on a Hewlett–Packard G 1800 A instrument consisting of an HP 5890 Series II GC and an HP 5971 mass-selective detector using a column (30 m × 0.25 mm × 0.25 μm with HP-5MS sorbent, 5% diphenyl, 95% dimethylsiloxane) and He carrier gas (1 mL/min). The column temperature was 2 min at 50°C, 4 min to 300°C, and 30 min at 300°C. The vaporizer temperature was 280°C; ion source, 170°C.

Quantitative Determination of PPs in the Extract. The method was based on HPLC over a reversed-phase sorbent using as an external standard tocopherol palmitate with spectral and chromatographic properties corresponding best to those of the studied sample. The analytical instrument was a Milikhrom LC with UV detection at 210 nm, a column (6.3 × 0.2 cm) packed with Lichrosorb RP-18 sorbent (5 μm), and MeOH:$(CH_3)_2CO$ (1:3, v/v) eluent.

The analysis was carried out under conditions analogous to those used for PPs of sea buckthorn, fungi, and *H. perforatum* [12, 19-21].

The quantitative content of PPs in the studied fraction was calculated using the formula:

$$C = \frac{S_s^{pr}}{S_{es}} \times \frac{M_{es}}{M_s^{pr}} \times \frac{100}{C_1} \times 100 \times K,$$

where C is the percent PPs in the analyzed sample; S_s^{pr}, the peak area of the principal component in the chromatogram of the analyzed sample; S_{es}, the peak area of the external standard (peak areas were determined as the product of the peak height and width at half-height); M_{es}, the weight of standard (μg); M_s, the weight of sample (μg) (sample and standard weights were determined as the product of the concentration of the corresponding solution and the sample volume injected into the chromatograph); K, a calculation coefficient reflecting the nature of the external standard, which is equal to 0.667 for tocopherol palmitate (calculated from a chromatogram of an artificial mixture of pure PP and tocopherol palmitate). Decaprenol and undecaprenol from leaves of sea buckthorn that were isolated as before [12] and pentadecaprenol and hexadecaprenol supplied by A. M. Moiseenkov were used to prepare the artificial mixtures. The quantity C_1 is the content of principal component in the PP mixture in the analyzed sample that was determined by analyzing the chromatogram of the fraction of enriched PPs.

The method developed for sea buckthorn PPs was used with certain modifications. The method was adapted to the unstudied raw material for which the component ratio was unknown by using the total peak area of the PP components instead of S_s^{pr}, setting coefficient $100/C_1$ equal to unity, and calculating the component ratio using peak areas.

The studied sample (4–5 mg, ±0.0001 g, weighed on an analytical balance) was dissolved in a penicillin vial in working eluent in order to prepare a solution of concentration 5 mg/mL. Freshly prepared solution was analyzed. The solution of external standard was prepared at concentration 0.5 mg/mL.

The chromatograph pump was primed with working eluent from which air was removed beforehand by purging for 20–30 s with a gentle stream of He from a cylinder. Working eluent (4 μL) and/or external-standard solution (4 μL) was injected into the sample port using a needle.

Samples were taken at 50 μL/min; elution, 100 μL/min. Detection was performed at 210 nm with measurement time 0.3 s and sensitivity 0.8–3.2. The recorder speed was 12 mm/min. The analysis time of a single sample was 12–15 min.

Primary analysis of PPs was performed without additional purification. The component composition was measured on enriched PP fractions isolated by column chromatography over silica gel and by preparative TLC.

Chromatographic Purification of PPs. A weighed portion of UF (100 mg) was dissolved in Et_2O and placed on a silica gel column (2.0 g) in dry form. For this, silica gel was added to a solution (0.15 g). The solvent was removed in a rotary evaporator with heating to 30°C and residual pressure 400 mm Hg. The dried silica gel with the deposited extract sample was carefully poured through a funnel onto a prepared column so that a solvent layer less than 0.3 cm covered the silica-gel surface. The column was eluted by hexane with Et_2O increasing from 0 to 30%. Fractions (1.5 mL) were collected in tubes (10 mL). Fractions were combined according to TLC results on Silufol and Armsorb plates. This produced 12 fractions, each of which was analyzed for PP content using HPLC as described above. Fraction 1 (2.3 mg) was a mixture of aliphatic hydrocarbons; fraction 2 (2.0 mg), a mixture of unidentified components; fraction 3 (3.7 mg), a mixture of tocopherols and triterpene ketones; fraction 4 (19.2 mg), a concentrate of PPs; fraction 5 (16.0 mg), a concentrate of aliphatic and triterpene alcohols with phytol dominating; fractions 6–11 (47.3 mg), a concentrate of sterols; fraction 12 (8.0 mg), a concentration of sterols with an impurity of triterpene diols and unidentified polar compounds.

Fraction 4 was studied using HPLC with markers of pure PPs from sea-buckthorn leaves. Mixed samples with fir PPs (n = 14–20) were used to identify components with longer chain lengths.

Decaprenol from Sea-buckthorn Leaves (l = 3, m = 5, n = 0). Colorless oil. MS *m/z*: 698 $[M]^+$, 680 $[M - H_2O]^+$. PMR spectrum (δ, ppm, J/Hz): 1.58 (12H, 4 Me), 1.66 (18H, 6 Me), 1.73 (3H, d, J = 1.5, Me-3), 1.9-2.2 (36H, m, allyl Me), 4.07 (2H, d, J = 7, 2H-1), 5.1 (9H, m, olefinic protons), 5.43 (1H, tq, J = 7, 1.5, H-2).

Undecaprenol from Sea-buckthorn Leaves (l = 3, m = 5, n = 0). Colorless oil. MS *m/z*: 766 $[M]^+$, 748 $[M - H_2O]^+$.

Fractions 1, 3, and 5–12 were identified by comparing retention times with authentic samples and correlating complete experimental mass spectra with those from the NIST 02 MS database (175,000 compounds) that was incorporated into the data processing system of an Agilent G 1710AA Chemstation that is constantly updated with new data obtained by staff members of the NIOC, SB, RAS. Furthermore, selective detection of pure characteristic ions was used to increase the reliability level of the identification.

Tocopherol palmitate was synthesized from tocopherol (PAO Altaivitaminy). For this, tocopherol (4.5 g) was dissolved in anhydrous Py (7.5 mL), treated with palmitic acid chloride (5 mL), and stirred on a magnetic stirrer for 3 h at 70°C. When

the reaction was finished, the mixture was diluted with H_2O and extracted with Et_2O (4 × 50 mL). The Et_2O extracts were washed by HCl solution (10%, 2 × 50 mL) to remove Py and by H_2O until neutral and dried over anhydrous Na_2SO_4. The yield of product of 85% purity (according to HPLC) was 85% of theoretical. The product was purified by chromatography over silica gel impregnated with $AgNO_3$ (5%) and by subsequent crystallization from EtOH. The HPLC-pure product had mp 42–43°C; lit. [27] mp 42.4°C.

ACKNOWLEDGMENT

We thank staff members of LPMR, Vorozhtsov NIOC, SB, RAS for GC–MS analyses of the samples. The work was supported by Grant No. FA-A12-T117 RUzb "Creation of New Technologies of Highly Effective Drugs Based on Polyprenols and the Study of Immunomodulator Activity."

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