Chemistry of Natural Compounds, Vol. 39, No. 4, 2003

POLYPHENOLS FROM *Vitis vinifera* SEEDS

A. Zh. Pirniyazov, N. G. Abdulladzhanova, S. M. Mavlyanov, F. G. Kamaev, and D. N. Dalimov

UDC 547.982/83/84

The chemical composition of polyphenols from grape seeds was studied. It has been found that they contain four catechins and two proanthocyanidins. The probable structure of the proanthocyanidins was proposed on the basis of physicochemical investigations.

Key words: flavan-3-ols, (-)-epicatechin gallate, (+)-catechin, (-)-epicatechin, (±)-gallocatechin, (-)-epigallocatechin gallate, proanthocyanidins.

About 80% of medicines used worldwide in medical practice are derived from plant sources. Phenolic compounds have a broad spectrum of pharmacologic activity and are used to treat several diseases, in particular, preparations based on them are used for lowering blood chloesterol [1, 2]. They also have antibacterial [3, 4], antihepatoxic [5], antioxidant [6-9], and other activities.

We investigated phenolic compounds from seeds of grapes (*Vitis vinifera* L.), which are a multi-ton waste from viticulture.

Grape seeds were treated with $CHCl_3$ and aqueous ethanol (90%) to isolate the total polyphenols. The aqueous-ethanol extract was evaporated in vacuum. The aqueous residue was treated with ethylacetate and then *n*-butanol. The total polyphenols were isolated by adding $CHCl_3$ and petroleum ether. The yield of total polyphenols from the ethylacetate fraction was 5.1 mass %; from the butanol fraction, 3.8 mass % of the air-dried raw material.

Chromatography has shown that the polyphenols of the ethylacetate fraction are mainly monomeric catechins with a small impurity of proanthocyanidins. The total polyphenols were separated. Pure catechins were isolated by column chromatography over silica gel to afford four catechins: (-)-epicatechingallate, (+)-catechin, (-)-epicatechin, and (±)-gallocatechin.

Paper chromatography (PC) of the butanol fraction detected weak spots of (+)-catechin, (-)-epicatechin, (±)-gallocatechin, and a brightly colored band beginning at the origin until R_f 0.40 (proanthocyanidin-1, PA-1). A compound that precipitated upon concentrating the butanol fraction also appeared as a band beginning at the origin until R_f 0.05 (proanthocyanidin-2, PA-2). A significant quantity of it was concentrated at the origin. This is indicative of a high molecular weight.

Pure proanthocyanidins were isolated and purified using column chromatography over cellulose and Sephadex G-50.

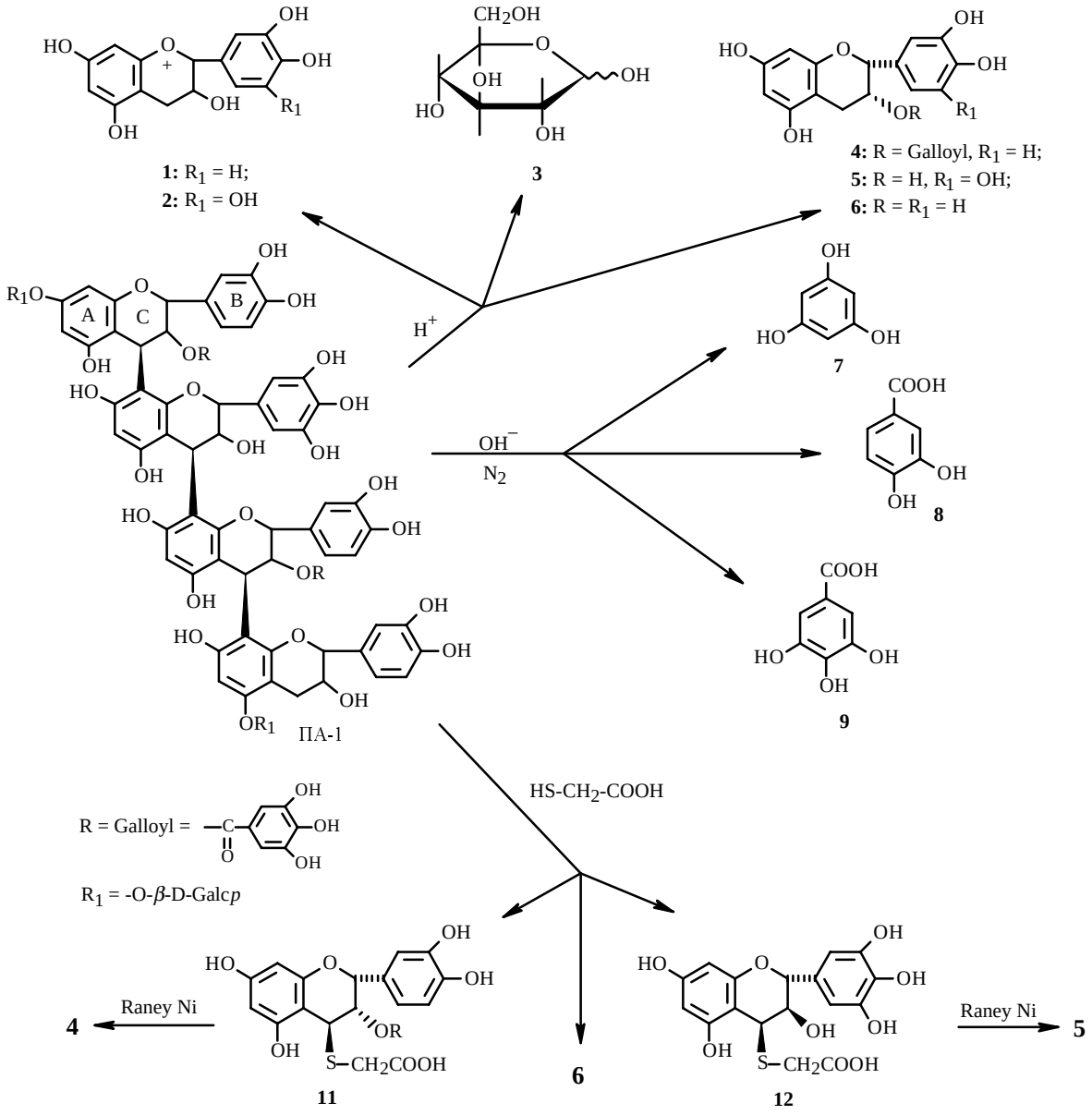
The masses of the proanthocyanidins were determined by ultracentrifugation [10]. Results of two determinations gave approximate molecular weights of PA-1 = 1800; PA-2 = 2140.

The flavane fragments, of which the isolated proanthocyanidins are constructed, were identified using chemical transformations (Schemes 1 and 2).

Acid hydrolysis of PA-1 and PA-2 by HCl (2 N) with heating (forcing conditions) produced cyanidin (**1**) and delphinidin (**2**). Acid hydrolysis of PA-1 under mild conditions (0.1 N HCl) produced glucose (**3**), (-)-epicatechingallate (**4**), (±)-gallocatechin (**5**), and (+)-catechin (**6**); of PA-2, (-)-epigallocatechingallate (**4′**), (±)-gallocatechin (**5**), and glucose (**3**). The ratio **4**:**5**:**6** in PA-1 was 2:1:1; of **4′**:**5** in PA-2, 2:2; of the phenolic and sugar parts in PA-1, 4:2; in PA-2, 5:1.

A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (371) 162 70 71. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 281-285, July-August, 2003. Original article submitted July 8, 2003.

0009-3130/03/3904-0349$25.00



Scheme 1

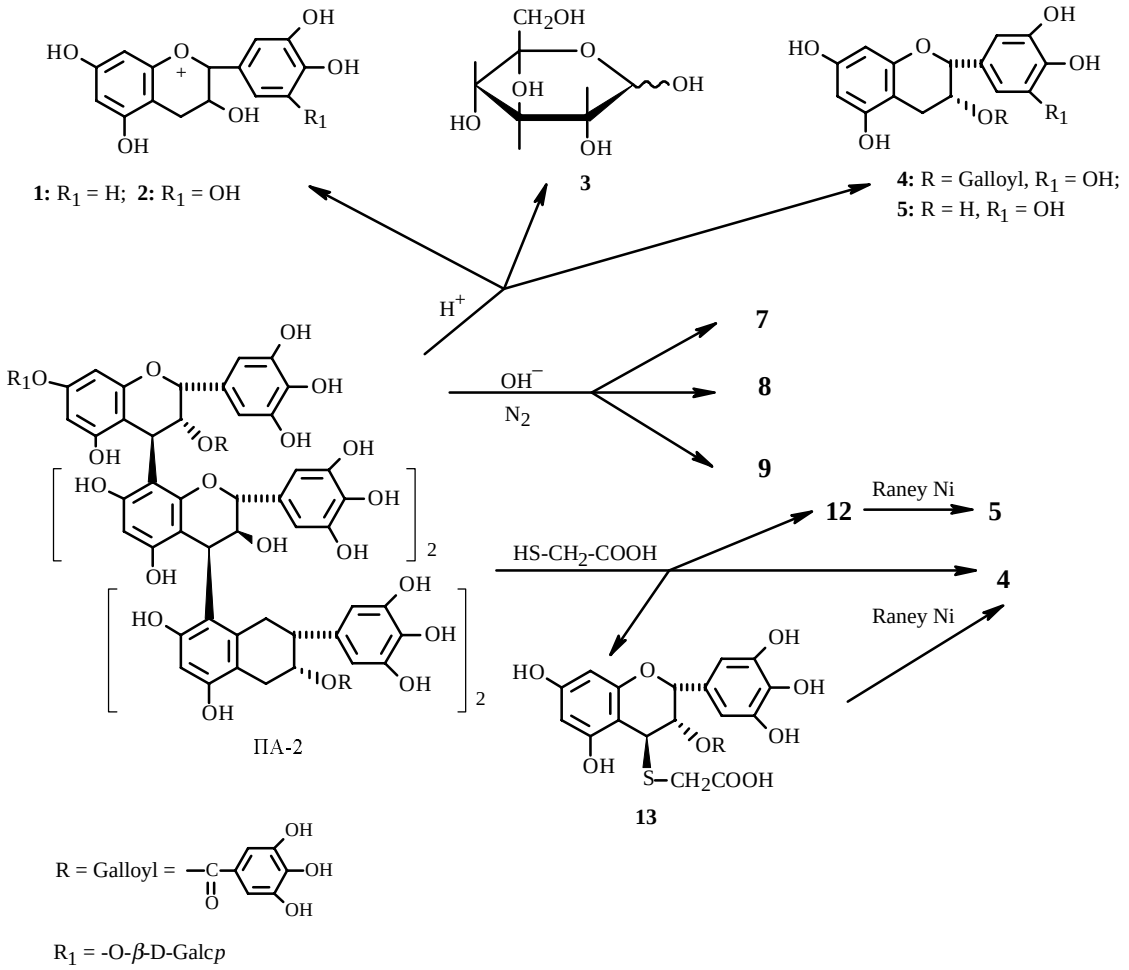
The catechin content in the hydrolysis products of PA-1 and PA-2 was determined by a combination of PC and colorimetry [11]; of glucose, by a semimicro sugar-determination method [12].

Base cleavage of PA-1 and PA-2 under N_2 formed three compounds each. These were identified by their physicochemical properties as fluoroglucinol (**7**) and protocatechuic (**8**) and gallic (**9**) acids.

Reductive hydrolysis of PA-1 by SO_3 produced three catechins: (-)-epicatechingallate, (-)-gallocatechin, and (+)-catechin; of PA-2, (-)-epigallocatechingallate and (±)-gallocatechin.

Thioglycolic acid was used to determine the terminal groups of PA.

The "lower" sections of PA-1 and PA-2 afforded **6** and **4′**, respectively; the "upper" sections, a mixture of two thioesters **11**/**12** and **11**/**13**. Reduction of the thioesters over Raney nickel produced a mixture of the corresponding catechins, which were separated by gel chromatography over Sephadex LH-20 with elution by ethanol. The compounds obtained from PA-1 were identified as **4** and **5**; from PA-2, **4′** and **5**.



Scheme 2

Mild thiolytic hydrolysis of PA-1 showed that the upper and lower sections contain a sugar unit. Considering this and the steric hindrance in the "middle" sections of the proanthocyanidins, the most probable binding site of the sugars to the carbohydrates is thought to be the C-5 position of the lower and the C-7 position of the upper catechin sections.

The IR spectrum of PA-1 exhibits absorption bands at 3550-3250 cm^{-1} (OH); 1640, 1570, and 1430 (aromatic ring), 2780 and 1330 (CH_1–CH_2–), 1350 (=C–OH, C–H), and 1260 (C–O–C).

The IR spectrum of PA-2 is analogous.

PMR spectra of PA-1 and PA-2 were recorded. They contain several unique signals overlapping themselves and OH signals. Therefore, the hydroxides were deuterated to simplify somewhat the interpretation of the spectra.

The PMR spectrum of PA-1 was recorded in CH_3OH-d_4 and is consistent with the structure proposed on the basis of the chemical transformations. The spectra exhibit signals centered at 2.70 ppm for the two H-4 protons from the lower catechin sections. A set of singlets centered at 6.85 ppm was assigned to aromatic protons H-2′ and H-6′ of the gallic acids. Comparison with the H-4 protons indicates that there are two of these protons. Nine H-2′, H-3′, and H-6′ protons of rings B resonate at 6.4-6.6 ppm. The sugar protons (without the angular protons) were found at 3.40-4.00 ppm. A quantitative comparison with the H-4 protons confirms that PA-1 does in fact contain two sugar units. Unfortunately, the most informative part of the spectrum at 6.2-4.4 ppm could not be interpreted owing to superposition and overlap of signals.

The PMR spectrum of PA-2 has a multiplet for the two H-4 protons of the lower catechin section at 2.97 ppm. A set of singlets for the H-2′ and H-6′ protons of three gallic acids is observed at 6.85 ppm. A set of singlets centered at 6.05 ppm belongs to the ten protons of rings B. The glucose protons (without the angular proton) appear at 3.9-3.4 ppm. Their quantity indicates that one sugar unit is present.

Unfortunately, like for PA-1, the most informative signals at 5.7-4.3 ppm could not be intrepreted owing to superpositioning.

Nevertheless, we are confident that the PMR spectra are consistent with the proposed structures for PA-1 and PA-2.

Thus, PA-1 is apparently the oligomeric glycosylated proanthocyanidin (-)-epicatechingallate-7-O-(β-D-Glc*p*)-(4β→8)-(-)-gallocatechin-(4β→8)-(-)-epicatechingallate-(4β→8)-(+)-catechin-5-O-(β-D-Glc*p*); PA-2, (-)-epigallocatechin-3-O-gallate-7-O-(β-D-Glc*p*)-(4β→8)-[(-)-gallocatechin]$_2$-(4β→8)-[(-)-epigallocatechin-3-O-gallate]$_2$.

EXPERIMENTAL

Paper for chromatography (Filtrak), the solvent systems *n*-butanol:acetic acid:water (40:12:28, system 1), acetic acid:HCl (conc.):water (5:1:6, system 2), and HCl (2 N) in *n*-butanol (system 3), and developer vanillin (1%) in HCl (conc.) were used to study the polyphenol composition.

Melting points of pure compounds were determined on a Boetius block with a RNNK-9.5 (Germany) viewing system; optical density, on a CM circular polarimeter. The tube length was 2 cm. UV spectra were recorded on an SF-26 spectrophotometer; PMR spectra, on an XL-100 NMR spectrophotometer; IR spectra, on a Specord UR-75 spectrophotometer.

Isolation of Total Polyphenols from Grape Seeds. Grape seeds (1 kg) were ground (to 0.1-0.3 mm) and extracted with $CHCl_3$ (4 × 5 L) to remove lipophilic substances. The raw material was dried and extracted three times with ethanol (90%, 1:8). The extracts were combined and concentrated in vacuum under N_2. The aqueous concentrate was treated with $CHCl_3$ (three times) and then repeatedly in a separatory funnel with ethylacetate (aqueous:ethylacetate, 2:1) and *n*-butanol. The ethylacetate extracts were combined, dried over anhydrous Na_2SO_4, and concenetrated in vacuum. The polyphenols were precipitated by hexane (ethylacetate concentrate:hexane, 1:5). The solid was filtered off, redissolved in ethanol, concentrated, and precipitated. The solid was filtered off through a Schott funnel and dried in a vacuum desiccator. Yield of light-brown amorphous powder of total polyphenols, 51 g.

The aqueous solution remaining after ethylacetate extraction was treated repeatedly with *n*-butanol. The butanol extracts were combined and concentrated under vacuum. The polyphenols were precipitated by $CHCl_3$. Yield of amorphous brown power of total polyphenols, 38 g.

Polyphenol Separation. Total polyphenols (5 g) from the ethylacetate fraction were repeatedly ground in a mortar with moist diethylether. The ether solution was chromatographed over a silica-gel column with elution by diethylether:ethylacetate in various ratios to afford pure flavan-3-ols, which were identified by physicochemical properties as (+)-catechin (R_f 0.64), (-)-epicatechin (R_f 0.56), (-)-epicatechingallate (R_f 0.72), and (±)-gallocatechin (R_f 0.49) (system 1).

Isolation of Proanthocyanidins. The butanol fraction (10 g) was chromatographed over cellulose (system 1) and then Sephadex G-50 (5 × 100 cm) columns (acetone:water, 1:1).

The eluate fractions appearing on the chromatogram as a band from the origin to R_f 0.40 were combined. The acetone was distilled off. The aqueous condensate was lyophilized to yield a brown powder (PA-1, 1.03 g). The eluate fractions appearing as a stationary spot at the origin were also combined. The acetone was distilled off. The aqueous condensate was lyophilized to yield a dark-brown powder (PA-2, 0.9 g).

Proanthocyanidin-1 (PA-1), $C_{86}H_{78}O_{43}$, amorphous brown powder, M = 1800, R_f 0.40 (system 1), $[\alpha]_D^{22}$ +88.5 (*c* 1.0, acetone:water 1:1). UV spectrum (MeOH, λ_{max}, nm): 224, 282. IR spectrum (KBr, cm^{-1}): 3550-3250 (OH); 1640, 1570, 1430 (aromatic ring); 2780, 1330 (CH_1–CH_2–); 1350 (=C–OH); 1260 (C–O–C).

PMR spectrum (MeOH-d_4, ppm): 6.85 (H-2, H-6, galloyl), 6.6-6.4 (H-2′, H-3′, H-6′, galloyl), 2.70 (H-4 catechin), 3.40-4.00 (H glucose).

Proanthocyanidin-2 (PA-2), $C_{102}H_{85}O_{52}$, amorphous brown powder, M = 2140, R_f 0.05 (system 1), $[\alpha]_D^{22}$ +93 (*c* 1.0, acetone:water 1:1). UV spectrum (MeOH, λ_{max}, nm): 218, 276. IR spectrum (KBr, cm^{-1}): 3500-3210 (OH); 1620, 1540, 1445 (aromatic ring); 2930, 1490 (CH_1–CH_2–); 1320 (=C–O–H); 1200 (C–O–C).

PMR spectrum (MeOH-d_4, ppm): 6.85 (H-2′, H-6′, galloyl), 6.05 (nine H, ring B), 3.9-3.4 (H glucose), 2.97 (H-4 catechin).

Acid Hydrolysis of Proanthocyanidins Under Forcing Conditions. Proanthocyanidins (30 mg) were dissolved in HCl (15 mL, 2 N) in aqueous ethanol (50%) and boiled for 30 min. The contents of the flask became noticeably red. The hydrolysate was cooled and treated with *n*-butanol. The butanol extract was separated by PC using systems 2 and 3 and

authentic specimens of cyanidin and delphinidin. The hydrolysates of both proanthocyanidins contained cyanidin (R_f 0.33, 0.69) and delphinidin (R_f 0.21, 0.35) using systems 2 and 3, respectively.

Acid Hydrolysis of Proanthocyanidins under Mild Conditions. Proanthocyanidins (100 mg) were hydrolyzed by the literature method [13]. PC of the hydrolysis products of PA-1 detected (+)-catechin, (-)-epicatechingallate, (+)-gallocatechin, and glucose; of PA-2, (-)-epigallocatechingallate, (+)-gallocatechin, and glucose.

Samples (3 mL) of the hydrolysates were taken for analysis of sugars using a semimicro determination method. It was found that the starting hydrolysate of PA-1 contained 16.4 mg of glucose; of PA-2, 6.6 mg. This is 84 and 80% of the calculated quantity of sugars in the studied proanthocyanidins.

Quantitative Determination of Catechins. Catechins were isolated by exhaustively treating the hydrolysates with ethylacetate in a separatory funnel. Then, the ethylacetate extract of the hydrolysate (1 mL) was placed on chromatography paper using a micropipette. The width of the band was 20 cm. Two spots of the same solution were placed along its edges and chromatographed using system 1 as a control. The chromatogram was dried. The control bands were cut out and developed with vanillin reagent. The catechin bands were marked with a pencil. The control bands were dried and attached to the main chromatogram. The catechin bands were carefully cut out, placed in small flasks (25 mL) with ground-glass stoppers, and saturated with diethylether (10 mL). The flasks were sealed with stoppers and placed for 12 h in a thermostat at 35°C. The flasks were shaken from time to time. A sample (1 mL) of each catechin band was treated with vanillin reagent (5 mL). The intensity of the resulting color was measured after 5 min on a FEK 56M (0.5-cm cuvette with a blue light-filter). Calculations were performed using calibration curves constructed from pure catechins from authentic samples).

We found that the whole hydrolysate of PA-1 contained (-)-epicatechingallate (40.8 mg), (±)-gallocatechin (13.8 mg), and (+)-catechin (13.2 mg), which are 85.0, 83.2, and 83.7%, respectively, of the theoretical content of these compounds in this proanthocyanidin. It was also found that the whole hydrolysate of PA-2 contained (-)-epigallocatechingallate (52.0 mg) and (±)-gallocatechin (23.1 mg) or 82.0 and 81.9% of the theoretical content of these compounds in this proanthocyanidin.

Base Cleavage of PA-1 and PA-2. The compounds (30 mg) were cleaved by the literature method [14]. Fluoroglucinol (R_f 0.64) and protocatechuic (R_f 0.73) and gallic (R_f 0.56) acids were isolated and identified in the reaction mixture.

Reductive Hydrolysis by SO_3. PA-1 and PA-2 (100 mg) were dissolved in aqueous alcohol (20 mL, 50%) and heated on a boiling-water bath with constant passage through the solution of SO_3 for 1 h. The cooled hydrolysates were treated with ethylacetate (3 × 5 mL). The ethylacetate extracts were evaporated in a ceramic cup. The solid was thoroughly treated with diethylether. The ether solutions were evaporated to 1/3 the volume and chromatographed on paper using system 1 and (-)-epicatechingallate, (-)-gallocatechin, (+)-catechin, and (-)-epigallocatechingallate. The chromatograms were developed by vanillin reagent and showed that the products of reductive hydrolysis of PA-1 were (-)-epicatechingallate (R_f 0.72), (-)-gallocatechin (R_f 0.49), and (+)-catechin (R_f 0.64); of PA-2, (-)-epigallocatechin-3-O-gallate (R_f 0.64) and (-)-gallocatechin.

Thiolytic Hydrolysis of PA-1 and PA-2. The compounds (100 mg) were hydrolyzed by the literature method [15]. PA-1 produced (+)-catechin (**10**) and a mixture of two thioesters (**8**, **9**); PA-2, (-)-epigallocatechingallate (**12**) and a mixture of two thioesters (**13**, **9**). Catalytic cleavage of the PA-1 thioesters and separation by chromatography over Sephadex LH-20 afforded (-)-epicatechingallate (**12**) and (-)-gallocatechin (**11**); of the PA-2 thioesters, (-)-epigallocatechingallate and (-)-gallocatechin.

REFERENCES

1. K. Tebib, L. Bitri, P. Besamcon, and J. Ronanet, *Food Chem.*, **49**, 403 (1994).
2. G. A. Jones, T. A. Allister, A. D. Muir, and K. L. Cheng, *Environ. Microbiol.*, **60**, 1374 (1994).
3. W. G. Li, X. Y. Zhang, Y. J. Wu, and X. Tian, *Acta Pharmacol. Sin.*, **22**, 1117 (2001).
4. K. K. Chakraborti and S. S. Nanda, *Indian Drugs*, **27**, 161 (1989).
5. A. Luximon-Ramma, T. Bahorun, M. A. Soobrattee, and O. I. Aruoma, *J. Agric. Food Chem.*, **50**, 5042 (2002).
6. A. A. Shahat, P. Cos, T. De Bruyne, S. Apers, F. M. Hammouda, S. I. Ismail, S. Azzam, and M. Claeys, *Planta Med*, **68**, 539 (2002).
7. T. Hatano, H. Miyatake, M. Natsume, N. Osakabe, T. Takizawa, H. Ito, and T. Yoshida, *Phytochemistry*, **59**, 749 (2002).

8. C. K. Sen, S. Khanna, G. Gordillo, D. Bagchi, M. Bagchi, and S. Roy, *Ann. N. Y. Acad. Sci.*, **957**, 239 (2002).
9. M. Sato, N. Maulik, and D. K. Das, *Ann. N. Y. Acad. Sci.*, **957**, 122 (2002).
10. A. B. Makhamatkulov, Z. A. Kuliev, A. D. Vdovin, M. R. Yagudaev, and V. M. Malikov, *Khim. Prir. Soedin.*, 59 (1992).
11. A. S. Sadykov, A. I. Ismailov, A. K. Karimdzhanov, and Sh. I. Islambekov, Pat. No. 492,791; *Byull. Izobret.*, No. 43 (1975).
12. A. I. Ermakov, *Methods of Biochemical Investigation of Plants* [in Russian], Agropromizdat, Leningrad (1987).
13. S. M. Mavlyanov, Sh. Yu. Islambekov, F. G. Kamaev, U. A. Abdullaev, A. K. Karimdzhanov, and A. I. Ismailov, *Khim. Prir. Soedin.*, 238 (1997).
14. F. Hasimoto, G. I. Nonaka, and I. Nishioka, *Chem. Pharm. Bull.*, **37**, 3255 (1989).
15. R. S. Thompson, D. Jacques, E. Haslam, and R. J. N. Tanner, *J. Chem. Soc., Perkin Trans. 1*, No. 11, 1387 (1972).