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TANNINS OF *Geranium sanguineum*

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A study has been made of the phenolic compounds of Geranium sanguineum. *One tannin of the hydrolyzable series has been isolated from the epigeal part, and for this the structure of 1,3-bis-O-digalloyl-2-O-galloyl-4,6-(hexahydroxydiphenoyl)-β-D-glucose is proposed. Three new proanthocyanidins have been isolated from the roots, and most probable structures have been put forward for these.*

Plant tannins possess a broad spectrum of pharmacological action [1-10], and a number of effective drugs have been created from them. Investigations carried out in many countries in recent years have confirmed their high antimicrobial [11], antioxidant [12], hypotensive [13], and other types of activity [14-23]. All this accounts for the great interest in the study of the structure of this class of natural compounds.

We have reported previously that flavanols, phenolic acids, and hydrolyzable tannins have been isolated from the epigeal part of *Geranium sanguineum* – blood-red geranium – and have been characterized; two of them – bis(hexahydroxydiphenoyl)trigalloylglucose and 1,2,3-tri-O-galloyl-4,6-(hexahydroxydiphenoyl)-β-*D*-glucose – proved to be new compounds, not previously described in the literature [24, 25].

We have continued the study of the polyphenols of this plant. Using the procedure described in [24], the polyphenolic complex of the epigeal part of the plant was subjected to fractionation on a column of rawhide powder. The total polyphenols isolated from an aqueous acetone fraction were chromatographed on a column of silica gel using eluent systems 3 and 4. In the case of system 4, fractions were obtained containing a substance with R_f 0.43, contaminated with a substance having R_f 0.40 (system 1). After rechromatography on a column of silica gel, a substance was isolated that consisted of a finely disperse powder, light yellow with a greenish tinge, having mp 261°C. UV spectrum of the substance (MeOH, λ_{max}, nm): 226, 276 (log ε 4.80, 4.34).

The products of the complete acid hydrolysis of the substance obtained were gallic acid, glucose, and ellagic acid, in a ratio of 5:1:1, respectively. The gallic acid was determined colorimetrically [26], the ellagic acid gravimetrically, and the glucose by the semimicromethod of determining sugars [27]. Together with other compounds, 2-O-galloylglucose was detected among the products of mild acid hydrolysis. The rate of hydrolytic splitting out of the hexahydroxydiphenic acid showed that it esterified the hydroxy groups of the fourth and sixth atoms of glucose.

In the IR spectrum of the substance we observed an absorption band in the 3500-3100 cm^{-1} region, showing the presence of free and bound hydroxy groups in the molecule. In the PMR spectrum (acetone-d_6) there were overlapping signals of the protons of galloyl and hexahydroxydiphenoyl groups in the 7.20-6.65 ppm region. The signal of one of the glucose protons appeared at 6.60 ppm (1H) and those of the other six protons in the 5.60-4.21 ppm region. On the basis of the only slight change in the chemical shifts of the glucose protons and the considerable displacement of the signals of the aromatic protons of the acetylated substance into the weak-field region, it could be concluded that all the hydroxy groups of the glucose were substituted and only the phenolic hydroxyls were acetylated.

A comparison of the results of chemical transformations and of spectral investigations of this compound with literature information [28-30] permitted us to propose for it as the most probable structure 1,3-bis-O-digalloyl-2-O-galloyl-4,6-(hexahydroxydiphenoyl)-β-*D*-glucose (**1**).

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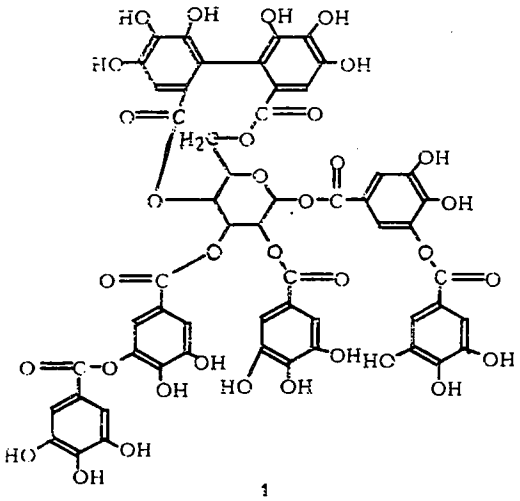

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For a more complete and deeper study of this plant, we investigated the polyphenol complex of the roots. The total phenols were isolated from the roots and, unlike the polyphenols of the epigeal part, they proved to be tannins of the condensed series (qualitative reaction with the vanillin reagent). With the aid of paper chromatography, five compounds were detected in the polyphenol complex of the roots. When it was subjected to column chromatography on silica gel, an ethyl acetate fraction yielded compounds having R_f 0.64 and 0.49 (system 1) which were identified by their physicochemical constants as (+)-catechin and (±)-gallocatechin. The other three compounds were isolated from a butanol fraction after chromatography on cellulose columns and gel filtration on Sephadexes G-50 and G-100 [31]. From their qualitative reactions and chromatographic behavior they were assigned to the proanthocyanidins. (The first had R_f 0.32-0.34 in system 1 and 0.33-0.36 in system 2; the second had R_f 0.15-0.25 and 0.22-0.28; and the third 0.00-0.10 and 0.00-0.12, respectively).

In the products of the alkaline cleavage of the proanthocyanidins we detected phloroglucinol and protocatechuic and gallic acids, and in the products of acid hydrolysis under severe conditions (2 N HCl, heating) anthocyanidins (cyanidin and delphinidin). Acid hydrolysis under mild conditions (0.1 N HCl, room temperature) led to the formation of (+)-catechin, (±)-gallocatechin, and glucose.

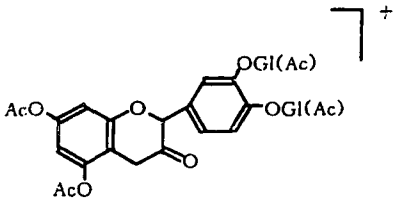
The ratio of (+)-catechin and (±)-gallocatechin in proanthocyanidins (**1**) and (**3**) was 1:1, and in (**2**) 2:1. The ratios of the phenolic and sugar parts in the proanthocyanidins were: for (**1**), 1:1.5; for (**2**), 1:1; and for (**3**), 1:2. The amounts of catechins in the decomposition products of the proanthocyanidins were determined by high-pressure liquid chromatography [32], and the amounts of glucose by the semimicro method for determining sugars.

In order to identify the terminal fragments of the proanthocyanidins we used thioglycolic acid [33]. In the products of the reactions of all three proanthocyanidins after 15 min we detected (+)-catechin and thioethers. After reduction of the thioethers over Raney nickel, the reaction products of proanthocyanidins (**1**) and (**2**) gave (+)-catechin, while (**3**) gave (+)-gallocatechin. Furthermore, the results of thiolytic cleavage showed the absence of glucose from the lower parts of the proanthocyanidins.

In the IR spectrum of proanthocyanidin (**1**) there was an intense band in the 3450-3150 cm^{-1} region relating to the stretching vibrations of hydroxy groups. Absorption bands at 1680, 1580, 1510, and 1435 cm^{-1} related to the stretching vibrations of aromatic rings. In addition, absorption bands were observed at 2930 and 1490 (CH−, $-CH_2-$), 1340, 1220, and 1050 (O−H, C−O), and 1280 cm^{-1} (C−O−C). Analogous patterns were observed in the IR spectra of proanthocyanidins (**2**) and (**3**).

In the mass spectrum of the acetylated derivative of proanthocyanidin (**1**) at a temperature of the ionization chamber of 170°C the peak of a high-mass ion was observed at 1002 a.m.u., together with a number of other ion peaks. Of these, the maximum and the most characteristic peak was that of an ion with *m/z* 331 of an acylated glucopyranose residue. Analysis of the key ions of the mass spectrum showed that the ion with *m/z* 1002 corresponded in mass to the ion of an acylated derivative of catechin split out from the oligomeric structure at the fairly high temperature.

The fragmentation of $M^{+\cdot}$ 1002 took place mainly by the elimination of catechin and acetic acid molecules and the formation of the ion with *m/z* 331. In addition, there was breakdown of the $M^{+\cdot}$ ion with the ejection of fragments of the acylated glucopyranose moiety – an ion with *m/z* 774. The latter, in its turn, decomposed with the ejection of fragments having 42 and 60 a.m.u.



Analogous breakdown was observed in the case of acetyl derivatives of proanthocyanidins (**2**) and (**3**).

On the basis of the mass-spectral results it could be assumed that the proanthocyanidins under investigation were glycosides of tri- and tetramers.

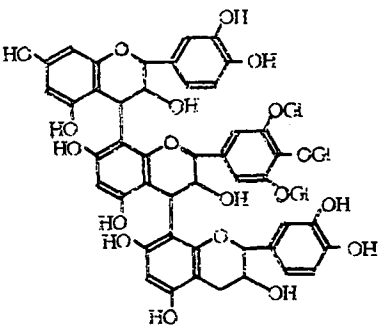
We also recorded the PMR spectra of the proanthocyanidins and their acetyl derivatives. In the PMR spectrum of proanthocyanidin (**1**), signals of the protons of the phenolic moiety of the molecule were observed at 7.3-6.4 ppm, and signals of the protons of the aglycon part of the molecule and also of the protons of the sugar moiety in the 6.2-3.4 ppm region. The large number of overlapping signals showed that the number of units in (**1**) was more than three, i.e., the compound was an oligomer.

In the PMR spectra of the acetylated proanthocyanidins (**1-3**) the signals of the protons of the sugar moiety were uniformly shifted into the weak-field region. Slight changes in the chemical shifts of the protons of the aromatic parts of the molecule in the direction of the weak-field region were also observed. This showed that some of the phenolic hydroxyls were blocked by sugar residues, the ratio of aromatic and "sugar" acetyl groups in (**1**) being 1:1.5.

Analysis of the PMR spectra of proanthocyanidin (**3**) and its acetyl derivative permitted the assumption that the structure of (**3**) was more complicated than that of (**1**), the number of sugar residues relative to the aglycon being higher.

The complex nature of PMR spectra of proanthocyanidins (**1-3**) was due not only to an overlapping of the signals of the monomeric units but also to the presence of a large number of rotational isomers with respect to the interflavan bonds [31].

On the basis of an analysis of the results obtained, the following may be put forward as the most probable structures for proanthocyanidins (**1-3**):



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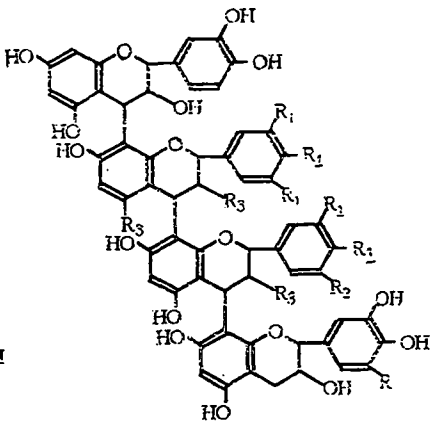

1. R=H
   R_1=R_2=OGl
   R_3=OH

3. R=OH: R_2=H
   R_1=R_3=OGl

EXPERIMENTAL

To separate the polyphenol complex of *G. sanguineum* we used chromatographic columns of type L 40/100 silica gel, rawhide powder, cellulose, and Sephadexes G-50 and G-100. The qualitative composition of the polyphenols was determined by the PC method.

The following solvent systems were used: 1) butanol–acetic acid–water (40:12:28); 2) 2% acetic acid; 3) diethyl ether–ethyl acetate (1:1); 4) ethyl acetate; 5) water–acetone (1:1); 6) water–ethanol (1:1); 7) butan-1-ol-85% formic acid–water (95:10:20); 8) butan-1-ol-benzene-acetic acid–water (2:10:2:1); 9) 2 N solution of HCl in ethanol–water (2:1); 10) acetic acid–HCl (conc.)–water (5:1:6); 11) 2 N solution of HCl in *n*-butanol; and 12) acetonitrile–acetic acid–water (17:5:2).

As revealing agents we used: 1) 1% alcoholic solution of $FeCl_3$; 2) mixture of 1% aqueous solutions of $FeCl_3$ and $K_3Fe(CN)_6$ (1:1); and 3) 1% solution of vanillin in HCl (conc.).

The melting points of the compounds isolated were determined in a Boëtius instrument with a PHHK-9.5 visual apparatus (Germany). UV spectra were taken on a SF-26 spectrophotometer; IR spectra on a Specord-71 instrument (Carl Zeiss); PMR spectra on XL-200 (Varian) and XL-100 (Varian) instruments; and mass spectra on a MKh-1300 instrument (St. Petersburg) at an ionizing potential of 50 V and a temperature of the ionization chamber of 170-180°C, using a system for the direct injection of the sample. The catechins in the products from the proanthocyanidins were determined quantitatively in a Milikhrom-1A high-pressure liquid chromatograph.

Isolation of a Tannin from the Polyphenol Complex of the Epigeal Part. By a method described previously [24], 10 g of total polyphenols was subjected to fractionation on rawhide powder. A solution in 15 ml of acetone of the powder isolated from the aqueous acetone fraction (1.3 g) was mixed with silica gel and was dried in a vacuum desiccator until the solvent had been completely eliminated. The silica gel with the adsorbed substance (50 g) was transferred to a column (3.0 × 40 cm), and the column was then washed successively with systems 3 and 4, 60-ml fractions being collected. The course of separation was monitored by TLC in system 1. Fractions 3-8 contained mainly a substance having R_f 0.43, with a small amount of an impurity having R_f 0.40. The fractions were combined, concentrated, and rechromatographed on a column of silica gel (2.0 × 30 cm, 30 g of silica gel) using system 4. The fractions containing only the substance having R_f 0.43 were combined, dried with anhydrous Na_2SO_4, concentrated, and precipitated with a fivefold volume of petroleum ether. The precipitate was filtered off through a Schott No. 3 funnel and was dried in a vacuum desiccator. This gave 0.27 g of a tannin having R_f 0.43.

Acid Hydrolysis of the Tannin. A solution of 0.1 g of the compound obtained in 30 ml of 5% H_2SO_4 was heated at 100°C for 1.5 h in a flask fitted with a reflux condenser while nitrogen was passed through the reaction mixture. The ellagic acid that precipitated was filtered off on a Schott No. 4 funnel, washed, and dried in a vacuum desiccator over freshly calcined $CaCl_2$. The yield was 0.021 g, corresponding to 87% of the ellagic acid in the $C_{55}H_{38}O_{34}$. The amount of gallic acid, determined photocolorimetrically, was 0.062 g, corresponding to 90.5% of the gallic acid content of the $C_{55}H_{38}O_{34}$. The glucose content was found by a micro method for determining sugars. When 3 ml of the solution being analyzed was treated with potassium permanganate, 0.620 mg of cuprous oxide was formed, which, according to a conversion table, is equivalent to 0.13 mg of glucose. Consequently, 30 ml of the solution contained 1.3 mg of glucose, which corresponds to 91.5% of the glucose in $C_{55}H_{38}O_{34}$.

Isolation of the Total Polyphenols from the Roots. The dried and comminuted roots of the plant (1.3 kg) were covered with 10 liters of chloroform–benzene (2:1) and were steeped for a day. The extract was poured off, and the residue was dried in the air until the smell of the solvent had disappeared. Then the roots were extracted with 80% aqueous methanol (3 × 5 liters), and the concentrate was treated successively with ethyl acetate and *n*-butanol. The ethyl acetate extracts were combined (7 liters), dried over anhydrous Na_2SO_4, and concentrated (to 0.9 liter) under reduced pressure in a current of nitrogen, and the total polyphenols were precipitated by the addition of a fivefold volume of petroleum ether. The precipitate was filtered off on a Schott No. 3 funnel and was dried in a vacuum-drying chest at 35°C. The yield amounted to 39.3 g (3.2%). The butanol extract (8 liters) was also concentrated (to 0.7 liter) under reduced pressure in a current of nitrogen, and the total polyphenols were precipitated by the addition of a threefold volume of chloroform. After filtration and drying, 83.85 g (6.45%) of substance was obtained.

Separation of the Polyphenols. The total polyphenols from the ethyl acetate extract (2 g) were repeatedly triturated with moist diethyl ether in a mortar. Only the monomeric catechins, completely free from condensation products, passed into the ether. The ethereal solution was chromatographed on a column of silica gel (3.0 × 60 cm, 50 g of silica gel). Moist ether was used as eluent, 50-ml fractions being collected. Fractions 12-18 and 23-26 contained substances with R_f 0.64 and 0.49 (system 1), respectively. These fractions were combined, dried with freshly calcined Na_2SO_4, and evaporated to dryness under reduced pressure in a current of nitrogen. The dry residues were dissolved in small amounts of hot water. On standing in the cold, the solutions deposited crystalline substances.

Substance 1 (0.30 g), mp 174-175°C (water), UV spectrum (EtOH, max) 279, $[\alpha]_D^{20}$ +17.0° (system 5); identified as (+)-catechin.

Substance 2 (0.34 g), mp 172°C (decomp.), UV spectrum (EtOH, max) 270, optically inactive; identified as (±)-gallocatechin.

Isolation of the Proanthocyanidins. A solution of 10 g of the total polyphenols from the butanol fraction in 10 ml of solvent system 1 was chromatographed on a column of cellulose (4 × 60 cm, 60 g of cellulose), using system 1 as eluent, with the collection of 80-ml fractions. Fractions 13-20, 23-28, and 34-40 contained substances having R_f 0.32-0.34, 0.15-0.25, and 0.00-0.10, respectively. The fractions containing mainly the substance with R_f 0.32-0.34 were combined, concentrated under vacuum to small volume (100-120 ml), and rechromatographed on a column of cellulose (2.0 × 35 cm, 25 g of cellulose), followed by gel filtration on Sephadex G-100 (1.0 × 20 cm, 10 g) in system 6. Eluents containing only one substance were concentrated in vacuum to small volume and were precipitated with a fivefold volume of petroleum ether, the precipitate being filtered off and dried in a vacuum-drying chest at 30-35°C. This gave 2.1 g of proanthocyanidin (**1**).

Analogously, fractions 23-28 and 34-40 yielded proanthocyanidins (**2**) and (**3**), respectively. They were purified by gel filtration – on Sephadex G-50 for proanthocyanidin (**2**) and Sephadex G-100 for proanthocyanidin (**3**).

Proanthocyanidin (1) – light yellow amorphous powder, mp 208°C (decomp.); soluble in water, in aqueous solutions of acetone and of ethyl alcohol, and in ethanol; insoluble in diethyl ether, benzene, and chloroform.

Proanthocyanidin (2) – light brown amorphous powder, mp 202°C (decomp.); soluble in water, methanol, and ethanol; insoluble in petroleum ether, diethyl ether, and chloroform. Its yield was 1.2 g.

Proanthocyanidin (3) – dark brown amorphous powder, mp 231°C (decomp.). Soluble in methanol, ethanol, and aqueous acetone; insoluble in ethyl acetate and chloroform. Its yield was 1.4 g.

Alkaline Hydrolysis of the Proanthocyanidins. The alkaline cleavage of 50 mg of each proanthocyanidin was carried out by the procedure of [34]. After concentration, the ethyl acetate extracts of the cleavage products were chromatographed on paper in systems 7 and 8. Phloroglucinol (R_f 0.64 and 0.16), gallic acid (R_f 0.56 and 0.09), and protocatechuic acid (R_f 0.73 and 0.58) were detected in the cleavage product from all the proanthocyanidins.

Acid Hydrolysis of the Proanthocyanidins under Severe Conditions. Solutions of 50 mg of each of the proanthocyanidins in 25 ml of system 9 were boiled in the water bath with a reflux condenser for 40 min. The contents of the flask became quite red. After cooling, the hydrolyzates were treated with *n*-butanol, whereupon the crimson color passed into the butanol layer. The butanol extracts of the products of acid hydrolysis were chromatographed in systems 10 and 11 in the presence of cyanidin and delphinidin. We detected delphinidin (R_f 0.32 and 0.70) and cyanidin (R_f 0.22 and 0.35) in the hydrolyis products of all three proanthocyanidins.

Acid Hydrolyis of the Proanthocyanidins under Mild Conditions. Solutions of 100 mg of each proanthocyanidin in 45 ml of 0.1 N HCl in methanol were heated in the water bath in flasks fitted with reflux condensers for 1 h. By PC in system 1, (+)-catechin, (±)-gallocatechin, and glucose were detected in the hydrolysis products from all the proanthocyanidins. It was established that the amounts of (+)-catechin, (±)-gallocatechin, and glucose were, respectively: in proanthocyanidin (**1**) – 21.6, 25.2, and 37.2 mg; (**2**) – 35.1, 20.8, and 34.1 mg; (**3**) – 18.3, 21.8, and 40.2 mg. The quantitative determination of the catechins was carried out by means of high-pressure liquid chromatography [32] in a column (10 cm long) with the reversed-phase adsorbent Separon-C_{18} in system 12. The amount of glucose was determined by the semimicro method of determining sugars.

Identification of the Terminal Fragments of the Proanthocyanidins. Solutions of 30 mg of each proanthocyanidin in 5 ml of ethyl alcohol were each treated with 4 ml of thioglycolic acid. Samples were taken after 15 and 30 min and then every hour for 12 h and were analyzed by PC in system 1. The reaction products from all the proanthocyanidins were (+)-catechin and thioethers. A saturated solution of sodium bicarbonate was added to each reaction mixture and it was extracted with ethyl acetate. The (+)-catechin passed into the ethyl acetate layers. The aqueous residues were acidified and were then extracted with ethyl acetate, and the extracts were concentrated under vacuum, giving the oily thioethers. Then to 10 mg of each thioether was added 12 ml of a suspension of catalyst – Raney nickel – in ethanol. After 3 h, the reaction mixtures were filtered and were analyzed by PC in system 1 in the presence of the markers (+)-catechin and (±)-gallocatechin. (+)-Catechin was found in the products of the reduction of the thioethers from proanthocyanidins (**1**) and (**2**), and (±)-gallocatechin in those from proanthocyanidin (**3**).

Preparation of Acetyl Derivatives of the Proanthocyanidins. In each case, 100 mg of a proanthocyanidin was dissolved in 5 ml of absolute pyridine, 6 ml of acetic anhydride was added, and the mixture was kept at room temperature for 48 h. Then the solution was poured into ice water and left for several hours. The precipitate that deposited was filtered off on a Schott No. 4 funnel, washed with water, and dried in a vacuum desiccator over P_2O_5. For purification, each acetyl derivative

was dissolved in 15-20 ml of dry acetone and precipitated from solution by the addition of a fivefold volume of petroleum ether. The precipitate was filtered off and dried in a vacuum desiccator.

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