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FLAVONOIDS AND SILICON IN CERTAIN PLANT POLLEN

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The flavonoid composition of certain plant pollens is studied. Pollen from representatives of various families differs in the quantitative and qualitative composition of the flavonoid complex. A specially developed method is used to estimate the content of three Si species that differ in the strength of bonding to organic substances in the pollen samples. The coexistence in plant pollen of the bioflavonoids and Si increases the pharmacological value of this natural product.

Plant pollen has been used since antiquity in fold medicine and pharmacology. The natural compounds responsible for the pharmacological value of pollen are bioflavonoids [1-3], which possess of vitamin P activity [4]. The flavonoid complex of plants used for food and medicine is well known [1-5]. However, most attention has been paid to the leaves and racemes whereas pollen has been little studied.

The Si content of plants has recently been of interest in addition to the flavonoids [6-9]. Silicon is no longer considered an inert (ballast) component of plant tissues. It is completely justifiably assigned to a group of elements that are necessary for the normal growth and development of animal and human organisms [6]. Silicon plays an important role in functioning of connective tissues, facilitates the biosynthesis of collagen and the formation and calcification of bone, and participates in the metabolism of lipids and phosphorus and the maintenance of Ca equilibrium, which is involved with the aging process [6].

We proposed a method for determining organogenic (chemically bound to an organic substance in plant tissues) Si and developed an analysis procedure based on it for three [7] and five [9] Si species in plants (in the presence of amounts of P typical of plant tissues). The method can determine Si in plants that is contained in phospholipids (where it partialy replaces P) and Si that is chemically bound to protein, lignin, cellulose, and pectins [7]. This excludes Si from plant detritus, soil particles, and dust that contaminate the plant material from the analysis. Plant pollen, which contains pectin and cellulose, turned out to be a good sample for applying our method. The present work presents analytical results on the Si species in plant pollen and certain new data on the flavonoid composition of pollen.

Pollen samples were collected from plants in the Moscow region (MGU Biostation, Zvenigorod) in the summer of 1998. Edible (vegetable) chrysanthemum was grown on the starting plot of the All-Russia Scientific Research Institute of Selection and Vegetable Seed Culture of the Russian Academy of Agricultural Sciences (Odintsovo). Flower pollen was collected using a paint brush into a plastic container taking all precautions to exclude dust from air. It was stored at 0°C before analysis.

All pollen samples examined by us had a high polyphenol content (photometric method with vanillin and conc. HCl) and contained bioflavonoids. Edible (vegetable) chrysanthemum pollen contained 4.33% polyphenols including quercetin (1.93%), luteoline, and luteolin-7-O-glucoside (1.72%). The data are presented per absolute dry weight. For comparison, its leaves contained only quercetin and its glycosides (isoquercitrin and rutin); the racemes (heads), 3,5,7,3′,4′-pentamethoxyflavone, which was converted into quercetin after saponification. Polymethoxyflavones were not observed in vegetable chrysanthemum pollen (like other studied plant species).

Pollen of timothy (*Phleum pratense*) and black sedge (*Carex nigra*) contains compounds giving a positive reaction for anthocyans, a specific flavone with properties of tricine (5,7,4′-trioxy-3′,5′-dimethoxyflavone), in addition to isorhamnetin. The total polyphenol content was 1.44 and 0.97%, respectively. Cyanidine glucoside (probably chrysanthemin) was identified in the anthocyan fraction of timothy pollen. Isorhamnetin was present at 0.31%. Pollen of *Carex nigra* contains almost two times less isorhamnetin (0.17%).

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TABLE 1. Content of Silicon Species in Certain Plant Pollen, % (±0.005) of Absolutely Dry Mass

Plant	Si, sum of three species	Free Si	Readily hydrolyzed Si	Tightly bound Si
Chrysanthemum coronarium	0.47	0.13	0.29	0.05
Phleum pratense	0.73	0.25	0.41	0.07
Achillea millefolium	0.87	0.25	0.51	0.11
Tanacetum vulgare	0.84	0.22	0.52	0.10
Hypericum maculatum	0.78	0.26	0.43	0.09
Carex nigra	0.59	0.21	0.31	0.07
Crataegus sanguinea	0.51	0.19	0.27	0.05

Pollen of hawthorn contains apigenin and its rhamnoside, in addition to hyperoside (1.14%), quercitrin (0.95%), and rutin (0.53%). The total flavonoid content in hawthorn pollen was 3.97%. Qualitatively the flavonoid composition of the flavonoid complex of hawthorn pollen was similar to amaranth leaves (*Amaranthus cruentus*, green variety), where rutin is present in addition to the glycosides mentioned above.

Pollen of common tansy contained 4.51% polyphenols. The flavonoid complex included quercetin (1.71%), quercitrin (1.34%), isorhamnetin (0.77%), and luteolin and rutin (<0.1%).

Yarrow pollen (with a total polyphenol content of 4.77%) contained quercetin (2.12%), isoquercitrin (1.43%), isohramnetin (0.51%), and luteolin. St. John's wort pollen contained 3.84% polyphenols including quercetin (1.44%), quercitrin (1.31%), hyperoside (0.97%), and traces of luteolin. Pollen of St. John's wort also yielded hypericin, which is also present in the its leaves. The amount of this multinuclear hydroxyquinone was 0.17% of the absolutely dried pollen.

The analytical results for the three Si species are given in Table 1.

The total content of the three Si species in the studied pollen samples was from 0.47 to 0.87% (per absolutely dry weight). The amount of readily hydrolyzed Si was 52.5-61.9% of the total Si content in pollen; of strongly bound, 9.6-12.6% of the total for the three Si species; and free Si, 27.6-37.2% of the total. Pollen from yarrow and common tansy typically had the highest Si content. The outer coating of pollen grains (exine) is known to be very strong [10]. The flavonoids are localized on its surface [3]. The exine strength is probably due to Si in polysaccharide complexes. Silicon that is extracted by acid hydrolysis is chemically bound to not only cellulose but also pollen pectins (mainly as orthosilicic esters) and can (as a microelement) be assimilated by the organism in order to satisfy the physiological requirement for this element [6-8].

Flavonoid glycosides exhibit the ability to expand arteries and are capable (in conjunction with ascorbic acid) of reducing the permeability and hardness of capillaries [4]. Silicon imparts strength to cardiovascular vessel walls and prevents the entry of lipids into blood plasma [6]. The fact that Si in addition to flavonoids are present in plant pollen increases the value of this natural product

EXPERIMENTAL

Fluorescence spectra of pigments were recorded on a Hitachi MPF 4 (Japan) spectrofluorimeter. IR spectra were taken on a IKS-14 (Russia) spectrometer in KBr pellets. Absorption spectra were recorded using a Hitachi 557 (Japan) spectrophotometer equipped with a computer. Quantitative photometric analysis was carried out on an SF-16 (Russia) spectrophotometer.

Flavonoid Investigation. Flavonoids were extracted by methanol or 96% ethanol from pollen previously treated with *n*-hexane. They were separated using partition column chromatrography on silica gel (Woelm, ICN Pharm. GmbH and Co., Germany) containing 20% water as the stationary phase. The mobile phase was a mixture of *n*-butanol, CH_3COOH, and water (4:1:5, organic phase) or $CHCl_3$, methanol, and water (7:3:0.5). The resulting fractions were further separated by paper chromatography (Whatman 3MM, USA) using a mixture of *n*-butanol, CH_3COOH, and water (4:1:5) or 15% CH_3COOH. The positions of the spots were visualized by fluorescence in UV light (3M chromatoscope).

Glycosides were hydrolyzed by a mixture of CH_3OH and HCl (12 N) (1:1) with subsequent extraction of the aglycone

by isoamyl alcohol and purification of it on LH-20 Sephadex. Hydrolysates of sugars were analyzed (together with standards) by paper chromatography using mixtures of acetone, *n*-butanol, and water (7:2:1) or ethylacetate, isopropanol, and water (65:25:11). The chromatographs were visualized using $AgNO_3$ and 3,5-dinitrosalicylic acid.

Flavonols were determined using a specially developed method that compared the fluorescence and excitation fluorescence spectra of ethanol solutions of the pigments with the spectra of their complexes with $AlCl_3$, H_3BO_3, and citric acid. Emission maxima in the range 470-520 nm (for ethanol solutions), 480-500 nm (for $AlCl_3$ complexes), and 520-540 nm (for H_3BO_3 and citrate complexes) were used to identify the pigments. The excitation fluorescence spectra contained bands at 365-375, 420-430, and 440-470 nm, respectively. Flavones and their glycosides in addition to flavonol glycosides were identified by their color reactions (with $ZrOCl_2$, CH_3COONa, $FeCl_3$, $AlCl_3$, H_3BO_3, and citrate), estimating the bathochromic shifts of band 1 in their absorption spectra [11-13]. The flavonoids were determined quantitatively by the literature method [14] and by fluorescence using the fluorescence intensity of ethanol solutions of flavonols colored by 10% $AlCl_3$ (a calibration curve was constructed using the corresponding quercetin complex).

Isorhamnetin. The following tests showed the presence of 3′-methylquercetin in the pollen. Band 1 in the absorption spectrum experienced a bathochromic shift of 100 nm with zirconyl chloride (hydroxyls are present at positions 3, 5, and 4′). Fluorescence properties (complex with $AlCl_3$) confirmed the presence of a free 3-OH group. A positive test with boric and citric acids indicated the presence of a 5-OH group in addition to 3-OH. A test with CH_3COONa in ethanol was positive (7-OH group). The reaction with CH_3COONa and H_3BO_3 was negative. Mild hydrolysis (demethylation) using HI converted the pigment into quercetin. The IR spectrum contained a band at 2945 cm^{-1} (CH_3O group).

Tricine. The methanol extracts of timothy and black sedge pollen formed a tight band during column chromatography on silica gel. The band contained quercetin and a colorless flavone with absorption maxima at 274 and 345 nm. The pigments were separated by paper chromatography using *n*-butanol:CH_3COOH:water (3:2:95). The flavone remained on the start. Quercetin migrated as a pale yellow band. The flavone was purified by rechromatography (*n*-butanol:CH_3COOH:water, 4:1:5), forming a spot with green fluorescence (in NH_3 vapor under UV light) with R_f 0.74. The pigment turned rose-colored with metallic Mg in the presence of HCl, yellow with Benedict solution, and brown with $FeCl_3$. Alkaline hydrolysis of the pigment (2 N NaOH, 2 h) gave syringic acid and phloroglucinol. Quercetin and phloroglucinol are known under these conditions to form protocatechuic acid [11, 12]. The pigment contained no sugar residue. The properties taken together suggest that it is 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone. IR spectroscopy (a band at 2947 cm^{-1}) confirmed that a methoxy group was present.

Anthocyanin Analysis. Pollen was extracted with 1% HCl in methanol. The extract was dried under vacuum and chromatographed on a silica-gel column (as above). A rose-colored band was isolated on the column. The material in the band had a characteristic absorption maximum at 535-540 nm (in acidic methanol). The eluate from this band was evaporated under vacuum. The dried solid was dissolved in 1% HCl in methanol. The solution was saturated with $(CH_3COO)_2Pb$. The resulting precipitate was treated with absolute methanol saturated with gaseous HCl. Anthocyanins were precipitated from the methanol solution by an excess of ethyl ether. The material from this was hydrolyzed by a mixture of methanol and conc. HCl (1:1) for 5 min with boiling. The aglycone was extracted by isoamyl alcohol. The aglycone had R_f = 0.51 (paper chromatography using CH_3COOH:HCl:water, 30:3:10). The hydrolysate contained glucose. The aglycone gave color reactions characteristic of a cyanidine: violet with CH_3COONa, blue with $FeCl_3$, and red upon dissolution in "cyanidine reagent" (cyclohexanol:toluene, 1:5). The absorption spectrum of the aglycone in 1% HCl in ethanol exhibited a maximum at 550 nm. The isolated anthocyan was identified from these properties as chrysanthemin (cyanidin-3-glucoside).

Hypercin Isolation. St. John's wort pollen was extracted with acetone. The extract was diluted with water. The pigment was transfered into diethyl ether. The ether extract was evaporated to dryness. The solid was dissolved in methanol and chromatographed on a column filled with $MgCO_3$ and supersil in a 3:2 ratio [15]. The eluents were CH_3OH and a mixture of CH_3OH and HCOOH (9:1, 3:1, and 1:1). The material from a rose-colored band was purified by paper chromatography using CH_3OH:diethyl ether (1:9). The resulting pigment had absorption bands at 330, 480, 535, and 575 nm (in ethanol, pH 7.0) and a fluorescence maximum at 600 nm. The red color changed to green on heating in acetic anhydride in the presence of H_3BO_3 (with appearance of an absorption maximum at 620-630 nm). The IR spectrum of the pigment exhibited bands of quinoid carbonyl (doublet at 1712 and 1727 cm^{-1}). Taken together, the properties are indicative of hypericin [16].

Silicon Determination. Silicon in the pollen was analyzed by a micromethod developed by us to analyze Si in protein, lipids, and pectin isolated from plant tissues [7]. The presence of three Si species differing in binding strength to the organic substance was established using stepwise hydrolysis by CF_3COOH (TFA). Free (molybdate-active) Si was determined directly in a 2N H_2SO_4 extract, which is used for colorimetric determination of Si by the Si-Mo blue method [17]. The total free and

readily hydrolyzed Si was extracted by 1 M TFA (10 min treatment at 100°C). By subtracting from this amount the quantity of free Si, the content of readily hydrolyzed Si was found. The fraction of strongly bound Si was found by difference between its content in the hydrolysate (2 M TFA, 10 min, 120°C) and the total readily hydrolyzed and free Si.

A portion of the preparation (25-50 mg) was treated with TFA (5.0 ml, acid concentration and extraction conditions as given above) in closed Teflon tubes. Then the TFA was removed in a vacuum desiccator. The remainder of the acid was neutralized with ammonia. The solution was held for 5 min at 80°C (pH 8.0). The ammonia was carefully neutralized. Sulfuric acid (1.53 ml, 96%) was added. Water was added to bring the volume to 15.0 ml. The resulting solution was treated with ammonium molybdate (1.0 ml, 10%). The pH was adjusted to 1.5. The solution was poured into a separatory funnel after 5 min. Concentrated H_2SO_4 (10 ml) was added. The Si-Mo acid was extracted with two portions of isoamyl alcohol (15.0 ml each). The combined organic extracts were washed with 1 N H_2SO_4 (10 ml) and evaporated under vacuum to a volume of 15.0 ml. Two drops of tin chloride (50% solution of $SnCl_2 \cdot H_2O$ in 6 N HCl) and ethyl ether (2 ml) were added. The total volume was brought to 20.0 ml using isoamyl alcohol. The absorbance of the blue solution of Si-Mo blue was determined at 750 nm after 5 min using a spectral chromatographic detector (SKB AMN) in a microcuvette of 15 µl volume and 1.0 cm optical length. The accuracy of the determination was ±0.005%.

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