Plant Polyphenols

2. THE COUMARINS OF *SOLANUM PINNATISECTUM*

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The South American and Mexican species of the tuberous *Solanum* plants are important as a source of breeding material for improving the domestic potato. The polyphenols are being studied in connexion with genetical work (see, for example, Dodds & Long, 1955; Harborne, 1957), but they are also of interest because they may play a part in disease resistance in the domestic potato (Valle, 1957; Kuc, Henze, Ullstrup & Quackenbush, 1956; Kuc, 1957; Lee & Le Tourneau, 1958). In a preliminary survey of the polyphenols present in the petals of the tuberous *Solanum* plants, it was observed that the Mexican diploid *S. pinnatisectum* and two related species contained some polyphenols which were absent from some 70 other species investigated. When examined in ultraviolet light, chromatograms of extracts of these particular plants contained three distinctive brightly fluorescent spots in addition to the usual spots of the anthocyanins, flavonol glycosides and caffeic acid esters. This paper describes the identification of these three compounds. The other phenolic components of *Solanum pinnatisectum* have also been identified.

MATERIALS AND METHODS

Plant material. The Commonwealth Potato Collection (Hawkes, 1944) is maintained at this Institution under glasshouse conditions. Plants from four tuber lines (C.P.C. 2291, 2298, 2300 and 2301) of *Solanum pinnatisectum* and hybrids between two of these lines were available. Several lines of most of the other species in the collection were examined and no major intra-species differences in polyphenolic content were noted. Flowers from commercial stocks of *Cichorium intybus* and *C. endivia* were used as sources of cichoriin.

Authentic compounds. Scopoletin (7-hydroxy-6-methoxycoumarin) and *iso*quercitrin (quercetin 3-glucoside) were kindly made available by Dr T. Swain of the Low Temperature Station, Cambridge. A synthetic specimen of cichoriin (aesculetin 7-glucoside) came from Professor A. Robertson, University of Liverpool, through the courtesy of Dr F. M. Dean. Authentic scopolin (6-*O*-methylaesculetin 7-glucoside) and 'methyl cichoriin' were isolated by paper chromatography from the roots of *Scopolia lurida*, *S. chinensis*, *S. carniolica* (Späth, 1937) and *Atropa belladonna* (Moore, 1911). Fabiatrin (6-*O*-methylaesculetin 7-xylosylglucoside) was obtained from the leaves of *Fabiana imbricata* (Edwards & Rogerson, 1927; Chaudhury, Holland & Robertson, 1948). The other compounds were either purchased from L. Light and Co. Ltd., or prepared from purchased compounds by well-established chemical procedures (e.g. Wawzonek, 1951).

Chromatographic survey of polyphenols. Small quantities of freshly collected corolla, leaf or tuber tissue of *Solanum* species were extracted with boiling 95% ethanol (or methanolic 1% HCl, if anthocyanins were present) and the extracts concentrated by evaporation at room temperature and applied as spots to two-dimensional chromatograms. After development with butan-1-ol–acetic acid–water (4:1:5, by vol.), the papers were dried and developed in the second direction with water. Polyphenols on the air-dried papers were detected by means of their colour reactions (Geissman, 1955).

Separation of the polyphenols of Solanum pinnatisectum. About 300 g. of freshly collected corollas of *S. pinnatisectum* were extracted with boiling 95% ethanol for 20 min. The filtered solution was washed twice with equal volumes of light petroleum, b.p. 60–80°, and concentrated to a small volume *in vacuo* at about 50°. The concentrate was applied as a band to 12 sheets of Whatman no. 3 filter paper, and these were developed with water by descent. The dried chromatograms showed the presence of six major bands (Table 1) corresponding in their colour reactions and R_F values to the main spots observed on two-dimensional chromatograms. Each band was then eluted separately with 70% methanol, and the eluates concentrated and purified by repeated chromatography, as necessary, in one or more of solvents *A*, *B*, *C* or *D* of Table 1. The purity of each band was tested by chromatographic and spectral analysis.

Hydrolysis of the polyphenols. Purified eluates of bands 2–6 were evaporated to dryness and heated for 1 hr. with N-HCl. The cooled solutions were extracted twice with small volumes of ethyl acetate, and the aqueous residues were washed with solutions of di-*n*-octylmethylamine in chloroform (10%, v/v), then with chloroform, and finally were concentrated to a small volume. These concentrates were examined for the presence of sugars by paper chromatography (Lederer & Lederer, 1957). Identifications were confirmed by chromatography and co-chromatography with authentic sugars. The results are given in footnotes to Table 1.

The ethyl acetate extracts were evaporated to dryness, and the compounds present identified by spectral and chromatographic comparison with authentic compounds (see below).

The eluate of band 1 was hydrolysed with N-NaOH at room temperature for 2 hr. The acid formed was extracted into ether, and the ether extracts were washed with water, dried and evaporated. The residue was identified by its chromatographic and spectral properties.

Identification of the polyphenols. The six polyphenolic components and their hydrolysis products were identified by comparison with authentic compounds by their colour reactions on paper, their R_F values in five solvent systems and their ultraviolet-absorption spectra as shown in Table 1. The ultraviolet spectra were measured on a Unicam SP. 500 spectrophotometer.

Identification of the anthocyanidins of Solanum pinnatisectum *tubers.* The tuber skin was extracted with methanolic 1% HCl and the extract was concentrated and heated for 40 min. with an equal volume of 2N-HCl at 100° under xylene. The cooled solution was extracted with pentan-1-ol, after removal of the xylene layer. This extract was evaporated to dryness and the anthocyanidins in the residue were identified by spectral and chromatographic comparison with authentic compounds (Bate-Smith, 1954; Harborne, 1958*a*, *b*).

RESULTS

The six major polyphenolic constituents of *Solanum pinnatisectum* corollas were identified as chlorogenic acid, scopolin, cichoriin, aesculin, rutin (quercetin 3-rutinoside) and *iso*quercitrin. The presence of seven other very minor constituents were detected on two-dimensional chromatograms, but they were not present in sufficient amounts for further study. One of these compounds, however, had the same R_F and colour reactions as aesculetin. The identity of the six major constituents was confirmed by examination of the products of hydrolysis. The combined results are presented in Table 1. Cichoriin has previously been found only in the

Table 1. R_F *values and spectra of constituents of* Solanum pinnatisectum *and their hydrolysis products*

In all cases, unknown compounds were co-chromatographed with authentic material and no separation of the mixture into two components was observed. The solvent systems used were: *A*, water; *B*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *C*, butan-1-ol–aq. 2N-ammonia soln. (1:1, v/v); *D*, aq. 15% acetic acid; *E*, butan-1-ol–ethanol–water (4:1:5, by vol.).

Band no.	Compound	R_F values in solvent					Colour in ultraviolet light	
		A	*B*	*C*	*D*	*E*	Untreated	Treated with NH_3
1	Chlorogenic acid	0·67	0·56	0·00	0·66	0·34	Blue	Green
2	Scopolin	0·64	0·53	0·44	0·85	0·47	Violet–blue	Violet–blue
3	Cichoriin	0·61	0·53	0·10	0·78	0·49	Pale pink	Orange–yellow
4	Aesculin	0·56	0·53	0·13	0·76	0·51	Light blue	Bright blue
5	Rutin	0·24	0·43	0·23	0·56	0·45	Ochre	Yellow–brown
6	*iso*Quercitrin	0·07	0·61	0·23	0·41	0·58	Ochre	Yellow–brown
'Methyl cichoriin'	Fabiatrin	0·79	0·45	0·23	0·85	0·42	Violet–blue	Violet–blue
	Products of hydrolysis							
1	Caffeic acid	0·25	0·81	0·00	0·45, 0·56	0·67	Blue	Light blue
2*	Scopoletin	0·29	0·82	0·35	0·59	0·80	Violet–blue	Bright blue
3*	Aesculetin	0·28	0·75	0·13	0·56	0·68	Blue	Light blue
4*	Aesculetin	0·28	0·76	0·13	0·56	0·68	Blue	Light blue
5†	Quercetin	0·00	0·71	0·00	0·05	0·70	Bright yellow	Yellow
6*	Quercetin	0·00	0·71	0·00	0·04	0·67	Bright yellow	Yellow
—	6-Hydroxy-7-methoxycoumarin	0·21	0·83	0·54	0·51	0·81	Pale pink	Orange–yellow

Band no.	Compound	Light absorption maxima		
		In 95% ethanol	In 95% ethanol with 2 drops of 2N-NaOH/3 ml.	In 95% ethanol with 2 drops of ethanolic 5% $AlCl_3$/3 ml.
1	Chlorogenic acid	219, 245, 300, 330	227, 275, 402	—
2	Scopolin	227, 250, 288, 339	230, 245, 278, 342	—
3	Cichoriin	228, 255, 290, 345	249, 277, 304, 395	—
4	Aesculin	224, 252, 293, 338	243, 272, 305, 383	—
5	Rutin	258, 305, 357	279, 415	363, 400
6	*iso*Quercitrin	257, 300, 360	281, 415	—
'Methyl cichoriin'	Fabiatrin	226, 249, 290, 340	230, 255, 274, 343	—
	Products of hydrolysis			
1	Caffeic acid	218, 243, 299, 326	265	—
2	Scopoletin	229, 253, 300, 346	242, 272, 392	—
3	Aesculetin	230, 260, 303, 351	259, 290, 350, 420	363
4	Aesculetin	262, 300, 350	257, 285, 345, 420	365
5	Quercetin	257, 372	Unstable	430
6	Quercetin	256, 371	Unstable	430
—	6-Hydroxy-7-methoxycoumarin	230, 256, 297, 348	252, 275, 311, 397	—

* Glucose was identified as the other product of hydrolysis of these bands.

† Glucose and rhamnose were identified as the other products of hydrolysis of this band.

blossoms of *Cichorium intybus* (Compositae; Merz, 1932). It gives, however, a most distinctive pink on paper in ultraviolet light (see Table 1), which with ammonia changes to orange–yellow. The only other phenolic substances which show similar colour reactions are, so far as the author is aware, 6-hydroxy-7-methoxycoumarin and *iso*ferulic acid (3-hydroxy-4-methoxycinnamic acid). In addition, the absorption maxima at about 250 mμ of cichoriin and 6-hydroxy-7-methoxycoumarin in alkali are much greater than those at about 390 mμ, whereas the reverse is true for aesculin [6-glucoside of aesculetin (6:7-dihydroxycoumarin)] and scopoletin.

Band 3 was identified as cichoriin by comparison with a synthetic specimen and also with extracts of the flowers of *Cichorium intybus* and *C. endivia*. Scopolin and aesculin accompany cichoriin in both these plants, a point not previously recorded. Band 2 was compared with authentic scopolin, isolated from the roots of *Scopolia* species (Späth, 1937) and *Atropa belladonna* (Moore, 1911). Scopolin occurs in the *Scopolia* plants, accompanied by a second coumarin, which has been described as a diglucoside of scopoletin, under the misleading name of 'methyl cichoriin' (cf. McIlroy, 1951). This second substance gives xylose, glucose and scopoletin on hydrolysis. It is also spectrally and chromatographically identical with the known 7-xyloglucoside of scopoletin (fabiatrin), which occurs in the leaves of *Fabiana imbricata* (Chaudhury *et al.* 1948).

The polyphenols of other parts of the *Solanum pinnatisectum* plant were examined more briefly. The leaves contain the same six compounds. The tubers, however, have only scopolin and chlorogenic acid. The skin of the tuber is pigmented with anthocyanin, and the aglycones delphinidin, petunidin and malvidin were detected in a hydrolysate of a skin extract.

Only two other species of tuberous *Solanum* plants, *S. jamesii* and *S. sambucinum*, contain coumarins in their flowers. The latter species has the same major polyphenols in its flowers as has *S. pinnatisectum*. In *S. jamesii* aesculin is absent but another component, a *p*-coumaric acid ester, is present. Flowers of domestic varieties of the cultivated potato do not contain coumarins, although these are present in the tubers, particularly in virus-infected material (Andrea, 1948; Baruah & Swain, 1959).

DISCUSSION

The presence of three derivatives of 6:7-dihydroxycoumarin (aesculetin) in *Solanum pinnatisectum* and two other related species is not surprising in view of the fact that aesculin, scopolin and scopoletin have been found severally in other plants of the Solanaceae family (Späth, 1937; Mors & Ribeiro, 1957). However, this is the first time that cichoriin has been found in solanaceous plants. The restricted distribution of these compounds in flowers of the tuberous *Solanum* plants is of interest. The three species which contain coumarins were formerly placed in the same taxonomic group, i.e. the *Pinnatisecta* series (Hawkes, 1944; Correll, 1952). Later Hawkes (1956) placed *Solanum sambucinum* in the *Cardiophylla* series, although he admits that it is intermediate in character between plants of the two series. Coumarin production can thus be provisionally considered as being characteristic of the flowers of *Pinnatisecta* species. These plants, which are unusual in the polyphenols they contain, are very distinct in their appearance and habit. Also they will readily hybridize only with species of the same series and are considered to be more primitive than the other species of the tuber-bearing *Solanum* plants.

The co-occurrence of cichoriin, aesculin and scopolin in the same plant is of biogenetic interest. It suggests that aesculetin in some form is a common precursor and that methylation, followed by glucosidation, are the final steps in synthesis. That the same three coumarins are present in a plant of quite a different family, namely *Cichorium intybus* (Compositae), suggests that the enzymes involved are not restricted in their distribution to plants of the Solanaceae. That a close biosynthetic relationship exists between aesculin and cichoriin, the 6- and 7-glucosides of aesculetin, is apparent from preliminary experiments in which aesculetin was fed to leaves of solanaceous plants which do not normally produce coumarins; the two glucosides were readily detected chromatographically.

SUMMARY

1. Three coumarins, aesculin, cichoriin and scopolin, have been found in the flowers of *Solanum pinnatisectum*.

2. The other major polyphenols present are chlorogenic acid, rutin and *iso*quercitrin.

3. The coumarins also occur in two other closely related species of the tuberous *Solanum* plants.

4. Their co-occurrence suggests that they are synthesized in the plant from aesculetin.

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The SH-Group Content of Human Foetal and Sickle-Cell Haemoglobins

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In a previous paper (Allison & Cecil, 1958) it was shown that the values obtained by various workers for the SH-group content of normal adult human haemoglobin (Hb A) by amperometric titration with silver nitrate were too high. Moreover, the high values obtained with silver nitrate had led to an incorrect interpretation of the results obtained with mercuric chloride (the interpretation depends on a knowledge of which of the possible mercaptides has been formed). The matter was resolved by using a univalent organic mercurial, phenyl mercuric hydroxide, as the titrating agent.

In this paper estimations of the SH content of human foetal haemoglobin (Hb F) and sickle-cell haemoglobin (Hb S) are reported. In the light of the results obtained by Allison & Cecil (1958) with Hb A it was considered sufficient to use only the method of amperometric titration with phenyl mercuric hydroxide.

Hommes, Santema-Drinkwaard & Huisman (1956) found six half-cysteine residues per mole of Hb F by amino acid analysis. Hommes, Dozy & Huisman (1958), using amperometric titration with silver nitrate, concluded that there are four titratable SH groups in both the native and denatured protein. They also found that after treatment with thioglycollic acid, the protein gave two components by paper electrophoresis, whereas the untreated protein gave only one. They interpreted this observation as meaning that the protein consists of two peptide chains joined by one disulphide bond and that this was reduced by the thioglycollic acid.

When blood from patients suffering from homozygous sickle-cell anaemia is deoxygenated the haemoglobin forms liquid crystals within the erythrocytes and gives them the characteristic sickle shape (Pauling, Itano, Singer & Wells, 1949; Perutz & Mitchison, 1950; Perutz, Liquori & Eirich, 1951). It has been shown (Thomas & Stetson, 1948; Ingbar & Kass, 1951; Riggs, 1953) that the addition of reagents that react with SH groups prevents this behaviour. Similarly, Allison (1957) found that the increase in viscosity associated with deoxygenation of solutions of Hb S is prevented by the addition of 2 moles of *p*-chloromercuribenzoate per mole. This effect of SH-group reagents has given rise to the idea that the difference in properties of Hb A and Hb S might be due to differences in the SH-group content.

Ingbar & Kass (1951) found three SH groups per mole of native Hb S by amperometric silver titration as compared with two for Hb A. Ingram (1957*a*) found that four silver atoms and 2·2 mercury atoms were bound per mole of native protein, the same result as he had previously obtained for Hb A (Ingram, 1955). Hommes *et al.* (1956) found that denatured Hb S and Hb A both bound eight atoms of silver per mole. Murayama (1957) reported that native Hb S bound four atoms of silver per mole at 0° and 1·9 at 38° but that 2·9 moles of *p*-chloromercuribenzoate were bound at both temperatures.