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SHORT COMMUNICATION

DISTRIBUTION OF LEUCOANTHOCYANIDINS, PHENOLIC GLYCOSIDES AND IMINO-ACIDS IN LEAVES OF *SALIX* SPECIES

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Abstract—The distribution has been studied of leucoanthocyanidins, phenolic glycosides and piperidine-based imino-acids in the leaves of nineteen *Salix* species. Leucoanthocyanidins were detected in eighteen species, the exception being *S. nigricans*. Leucocyanidin only was present in six species, the remainder containing both leucocyanidin and leucodelphinidin. Phenolic glycosides were detected in all of the species tested. Piperidine imino-acids were found in nine species, all of which contained pipecolic acid, with the exception of *S. alba* and *S. fragilis*, which contained 5-hydroxypipecolic acid and an unknown imino-acid, probably based on piperidine.

INTRODUCTION

THE GENUS *Salix* contains about 300 species. Though there is general agreement as to the number of species, the identification of plants of this genus is often difficult, because of the freedom with which they hybridize, the fact that the sexes occur on different trees and the variability of many of the species in such features as leaf shape.[1] Because of these difficulties, combined with the large number of varieties, a chemical classification of the genus may be advantageous. The most widely studied constituents in the genus *Salix* are the phenolic glycosides, their structure and distribution in the Salicaceae having been reviewed by Thieme.[2–5] From his results distinct differences between species were apparent. In his studies, however, the phenolic glycosides were detected by paper chromatography.[6] A more sensitive, thin-layer chromatographic procedure was developed by Audette, Blunden, Steele and Wong[7] for use as a screening method for phenolic glycosides in the genus *Salix*. Other compounds that have been reported in the genus include leucoanthocyanidins[8–9] and imino-acids.[10,11] Leucoanthocyanidins in *Salix* have received only passing attention, but

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[2] H. THIEME, *Pharmazie* **11**, 770 (1963).
[3] H. THIEME, *Pharmazie* **20**, 436 (1965).
[4] H. THIEME, *Pharmazie* **20**, 570 (1965).
[5] H. THIEME, *Planta Medica* **13**, 431 (1965).
[6] H. THIEME, *Pharmazie* **19**, 471 (1964).
[7] R. C. S. AUDETTE, G. BLUNDEN, J. W. STEELE and C. S. C. WONG, *J. Chromatog.* **25**, 367 (1966).
[8] E. C. BATE-SMITH and N. H. LERNER, *Biochem. J.* **58**, 126 (1954).
[9] E. C. BATE-SMITH and C. R. METCALFE, *J. Linn. Soc. (Bot)*. **55**, 669 (1957).
[10] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.* **9**, 1548 (1955).
[11] G. BLUNDEN, S. B. CHALLEN and B. JAQUES, *Nature* **212**, 514 (1966).

these compounds have been shown by Bate-Smith to be of chemotaxonomic value in a number of genera. Virtanen and Kari[10] when studying the free amino-acids of pollen from wind-pollinated plants found pipecolic acid (piperidine-2-carboxylic acid) in the pollen of *S. caprea*, but not in the green parts. Blunden, Challen and Jaques[11] detected 5-hydroxy-pipecolic acid and another unidentified imino-acid, probably based on piperidine, in the leaves and leaf galls of *S. fragilis*.

In this present study the distribution of leucoanthocyanidins, phenolic glycosides and piperidine-based imino-acids in the leaves of nineteen *Salix* species has been studied to determine whether these compounds have any chemotaxonomic value within the genus.

RESULTS AND DISCUSSION

Leucoanthocyanidins present in *Salix* leaves were converted to anthocyanidins by heating with HCl, and the compounds so formed were examined by paper chromatography. Of

TABLE 1. DISTRIBUTION OF ANTHOCYANIDINS DERIVED FROM LEUCOANTHOCYANIDINS IN THE LEAVES OF *Salix* SPECIES

Species	Delphinidin*	Cyanidin
Salix alba var. Cardinalis	−	++++
S. americana	−	+++
S. babylonica var. Fardon Weeping	++	++++
S. callicarpaea	++	++
S. calodendron	+++	++
S. caprea var. *lanata*	+	++
S. cinerea	+++	++
S. daphnoides var. *acutifolia*	+	++
S. decipiens	+++	++
S. fragilis	−	++++
S. × *geminata*	+	++
S. incana	+	+
S. nigricans	−	−
S. pentandra var. Lumley	−	+
S. phylicifolia	+++	+
S. purpurea var. Goldstones	−	+
S. × *rubra*	−	+
S. triandra var. Black Maul	+	+
S. viminalis	+++	++

* ++++ very strong, +++ strong, ++ moderate, + weak.

the nineteen species tested, eighteen gave a positive reaction, the exception being *S. nigricans* (Table 1). Cyanidin only was detected in six species, whereas the other twelve contained both cyanidin and delphinidin. In the species containing both, the relative proportions of cyanidin and delphinidin varied considerably, for example, in *S. phylicifolia* delphinidin was the major component, in *S. babylonica* cyanidin predominated, whereas in *S. callicarpaea* the two compounds were in approximately equal proportions. The yield of anthocyanidins produced varied greatly from species to species, being high in such species as *S. babylonica*, *S. fragilis* and *S. alba*, but being low in *S.* × *rubra*, *S. incana*, *S. purpurea*, *S. pentandra* and *S. triandra*. With the latter, high loadings were required on the chromatograms to obtain positive results. In the case of *S. nigricans*, however, even with a high loading, no anthocyanidins were detected.

Phenolic glycosides were detected in the leaves of all nineteen species of *Salix* tested (Table 2), although the yields in some species were exceptionally low. Identification of an individual phenolic glycoside was based on its chromatographic position and the colour it produced on thin-layer chromatograms after spraying with 4 per cent H_2SO_4 in EtOH and heating. Salicin, salicortin and salidrosid were detected in all species. Picein and salireposide were not detected in any of the leaf extracts, an observation in agreement with the conclusions of Thieme[2] and Rabaté.[14] Phenolic glycosides were detected in the leaves of *S. alba*, *S. viminalis*, *S. caprea* and *S. cinerea*, which were reported by Thieme to be free from phenolic glycosides.[5] The yields in these species were very low, but they were detectable by thin-layer chromatography. Certain species used in our work have been examined previously

TABLE 2. DISTRIBUTION OF PHENOLIC GLYCOSIDES IN THE LEAVES OF *Salix* SPECIES

Species	Salicin	Salicortin	Salidrosid	Fragilin	Tremuloidin	Vimalin	Triandrin	Grandidentatin	Populin	Picein	Salireposide
Salix alba var. Cardinalis	+	+	+	+	±	+	±	+	−	−	−
S. americana	+	+	+	−	−	±	−	±	−	−	−
S. babylonica var. Farndon Weeping	⊕[13]	+	+	+	+	+	+	±	−	−	−
S. callicarpaea	+	+	+	+	+	+	±	±	+	−	−
S. calodendron	+	+	+	+	−	+	±	±	−	−	−
S. caprea var. *lanata*	+	+	+	−	−	±	−	−	−	−	−
S. cinerea	+	+	+	−	−	+	±	−	−	−	−
S. daphnoides var. *acutifolia*	⊕[14]	+	+	−	−	−	+	±	+	−	−
S. decipiens	+	+	+	+	−	+	+	+	+	−	−
S. fragilis	⊕[4]	⊕[4]	+	⊕[4]	⊕[4]	±	±	±	⊕[4]	−	−
S. × *geminata*	+	+	+	−	+	±	+	+	±	−	−
S. incana	⊕[14]	+	+	+	+	+	−	+	+	−	−
S. nigricans	⊕[12]	+	+	+	+	−	−	−	−	−	−
S. pentandra var. Lumley	⊕[4]	⊕[4]	+	+	+	+	±	+	±	−	−
S. phylicifolia	+	+	+	+	−	−	−	+	−	−	−
S. purpurea var. Goldstones	⊕[4]	⊕[4]	+	+	⊕[4]	+	+	+	⊕[14]	−	−
S. × *rubra*	+	+	+	+	+	−	−	+	+	−	−
S. triandra var. Black Maul	+	+	⊕[4]	+	+	±	+	±	−	−	−
S. viminalis	+	+	+	+	−	±	+	±	−	−	−

Key: + positive result, ± uncertain, ⊕ compound previously reported.

for the presence of phenolic glycosides. The presence of the reported compounds has been confirmed, but in all cases other phenolic glycosides have been detected. In addition to the compounds identified, a number of leaf extracts, particularly, *S. decipiens*, *S. purpurea*, *S. nigricans*, *S. pentandra* and *S.* × *rubra* produced unidentified sulphuric acid positive spots on thin-layer chromatograms in the region of the known phenolic glycosides.

The qualitative distribution of compounds was similar in most species, although there were distinct quantitative differences, for example, salidrosid was the predominant glycoside in *S. triandra* and *S. caprea*, whereas it was only a minor constituent in *S. fragilis* and *S. callicarpaea*. Similarly, salicin was the major component in *S. nigricans*.

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[14] J. RABATÉ, *Bull. Soc. Chim. Biol.* **17**, 439 (1935).

The distribution patterns of phenolic glycosides in the nineteen species examined have limited chemotaxonomic interest. Compounds such as vimalin, triandrin, grandidentatin and salidrosid, previously thought to have a restricted distribution, have now been shown to occur in several of the species tested, with salidrosid being detected in all nineteen species. The presence/absence of individual phenolic glycosides do not seem to show any particular pattern. With the exception of *S. callicarpaea*, which was collected in August, all the leaf samples were collected in September when phenolic glycoside levels are low, and it is possible that if samples collected in May were used, when the levels are at their maximum,[5] additional phenolic glycosides may be found.

Piperidine imino-acids were detected in the leaves of nine out of the nineteen species of *Salix* examined (Table 3). Pipecolic acid was present in all the species giving positive results with the exceptions of *S. alba* and *S. fragilis*, which contained both 5-hydroxypipecolic acid

TABLE 3. DISTRIBUTION OF PIPERIDINE IMINO-ACIDS IN THE LEAVES OF *Salix* SPECIES

Species	5-hydroxy-pipecolic acid	Unknown imino-acid	Pipecolic acid
Salix alba var. Cardinalis	+	+	–
S. americana	–	–	–
S. babylonica var. Farndon Weeping	–	–	–
S. callicarpaea	–	–	–
S. calodendron	–	–	+
S. caprea var. *lanata*	–	–	+
S. cinerea	–	–	+
S. daphnoides var. *acutifolia*	–	–	–
S. decipiens	–	–	+
S. fragilis	+	+	–
S. × *geminata*	–	–	–
S. incana	–	–	–
S. nigricans	–	–	+ (faint)
S. pentandra var. Lumley	–	–	–
S. phylicifolia	–	–	–
S. purpurea var. Goldstones	–	–	–
S. × *rubra*	–	–	–
S. triandra var. Black Maul	–	–	+ (faint)
S. viminalis	–	–	+ (faint)

and an unknown imino-acid, probably based on piperidine. The yields of piperidine imino-acids were often low, only trace quantities of pipecolic acid being detected in *S. triandra*, *S. viminalis* and *S. nigricans*. Virtanen and Kari[10] found pipecolic acid in the pollen of *S. caprea*, but were unable to detect it in the green parts. We have detected it in the leaves of this species, the difference in results probably being due to the use of a more sensitive technique based on thin-layer chromatography. Differences in imino-acid content are apparent between the nineteen species of *Salix* tested. However, considering the very small quantities present in some species, great care would be needed before making chemotaxonomic conclusions from negative results.

EXPERIMENTAL

All the leaf samples used in this work, with the exception of *Salix callicarpaea*, were collected at the Long Ashton Research Station, Bristol, in September 1965 and 1967. *S. callicarpaea* leaves were collected from the Lake Myvatn area, Iceland, in August, 1965. All the leaf samples were carefully dried in a circulating air oven at a temperature not exceeding 50° and then stored in sealed tins until used.

Examination for Leucoanthocyanidins

The leucoanthocyanidins present in *Salix* leaves were converted into anthocyanidins by the method of Bate-Smith[15] and examined by paper chromatography.[15,16] Powdered leaf (0·5g) was heated with 5 ml 2 N HCl for 20 min at 100°. The mixture was filtered, the filtrate shaken with a small quantity of *n*-pentanol, the alcoholic layer removed and used for chromatographic examination on Whatman No. 1 paper in the solvents (*a*) water–acetic acid–12 N HCl (10:30:3 v/v), (*b*) *n*-butanol–2 N HCl (1:1 v/v) and (*c*) formic acid–12 N HCl–water (5:2:3 v/v).

Examination for Phenolic Glycosides

The dry, powdered leaves (10 g) were extracted in a Soxhlet apparatus with 95 per cent ethanol for 10 hr, the ethanol recovered, and the residue extracted with water (4 × 20 ml). This aqueous solution was extracted with ethyl acetate by continuous liquid–liquid extraction for 6 hr, the ethyl acetate was recovered under reduced pressure and the residue re-dissolved in water (4 × 20 ml). The aqueous solution was treated with 10 ml strong lead subacetate solution B.P. and the resultant precipitate was removed by centrifugation. The clear, decanted liquid was treated with H_2S and the precipitate formed was filtered off. The filtrate was concentrated to about 20 ml and extracted with ethyl acetate (5 × 10 ml). The ethyl acetate solution was concentrated to dryness and the residue redissolved in water for chromatographic examination.

The phenolic glycoside extracts were separated by two-way thin-layer chromatography, using the technique of Audette, Blunden, Steele and Wong[7] and located by spraying with 4 per cent H_2SO_4 in EtOH, and heating at 110° for 10–15 min.

Examination for Imino-Acids

Extracts of *Salix* leaves for imino-acid evaluation were prepared by boiling 5 g powdered leaf with 3 × 50 ml water and filtering. This extract was passed through a column of Amberlite i.r. 120 as described by Virtanen and Kari.[17] The purified extract was concentrated to about 5 ml, 2–3 drops of conc. HCl were added and the precipitated material was removed by filtration. The filtrate was studied by two-way thin-layer chromatography, using the method of Blunden and Challen.[18] The imino-acids were located by spraying with 0·1 per cent w/v ninhydrin solution in acetone and heating at 100° for 10 min.

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[16] J. B. HARBORNE, *J. Chromatog.* **1**, 473 (1958).
[17] A. I. VIRTANEN and S. KARI, *Acta Chem. Scand.* **9**, 170 (1955).
[18] G. BLUNDEN and S. B. CHALLEN, *J. Chromatog.* **24**, 224 (1966).