The Origins of Tannins and Flavonoids in Black-Wattle Barks and Heartwoods, and their Associated 'Non-Tannin' Components

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1. The distributions of flavonoid, carbohydrate, amino acid and imino acid components in the leaves, twig bark, stem bark, root bark and heartwoods of the black-wattle tree were compared by paper chromatography after their isolation from specific portions of the tree. 2. Wattle leaves contain mainly myricitrin, (+)-gallocatechin, an unknown myricetin glycoside and leuco-delphinidin tannins, together with smaller amounts of (+)-catechin, quercitrin and other flavonol glycosides. These are prominent in the twig bark, but decline progressively with age in the stem bark and are absent from root bark. 3. The non-phenolic components of the mature stem bark were shown to be (+)-pinitol, sucrose, glucose, fructose, L(−)-pipecolic acid, *trans*-4-hydroxy-L(−)-pipecolic acid, α-alanine, arginine, aspartic acid, glutamic acid, L(−)-proline, serine, a 'steroid' alcohol and a long-chain β-diketone. 4. Wattle bark and heartwood 'tannins' consist of the analogues of closely related prototypes with common origins in the vascular tissues of the bark. Leaf 'tannins' are superimposed on the bark components mainly during the initial stages of bark growth. 5. Origins of the pipecolic acids and the transformations of carbohydrates in the sap- and heart-woods are discussed.

Although the heartwood tannins of the black-wattle tree (*Acacia mearnsii*) are composed of a seemingly complex mixture of flavonoid compounds, most of these are either analogues (flavan-3-ol, dihydroflavonol, flavanone, flavonol and chalcone) or polymers (true tannins) of the predominant component, (+)-mollisacacidin [(+)-3′,4′,7 - trihydroxy - 2,3 - *trans* - flavan - 3,4 - *trans* - diol] (Roux & Maihs, 1960; Roux & Paulus, 1960, 1961*a,b,c*, 1962; Drewes & Roux, 1964). Such fundamentally simple interrelationships, devolving on a single pattern of phenolic hydroxylation, suggests their derivation from common precursors, and possibly their common origins from vascular tissues.

Among the bark tannins, on the other hand, Drewes & Roux (1963) recognized the presence of four similar but more elaborate patterns in a far more complex flavonoid mixture. These appear to have different origins.

Since it is unlikely that bark and other tannins are translocated once they have formed in certain tissues, owing to their relatively high molecular weight, their origins have now been sought by comparative examination of leaf, twig-bark, stem-bark and root-bark tannins, and also by comparison of their non-phenolic components. The carbohydrate, imino acid, amino acid and petroleum-soluble components of wattle bark have been examined in detail.

EXPERIMENTAL AND RESULTS

Non-phenolic components ('non-tannins') of black-wattle bark

Amino acids and imino acids. Fresh stem bark from young (5–6-year-old) black-wattle trees was cut across the

grain into thin slivers. These were air-dried for 4 days. The dry bark (2 kg.) was successively extracted twice with hot petroleum (b.p. 60–70°) (4 l., solution *A*) to remove fats and waxes, with warm (60–70°) acetone (2 l.) to remove some of the polyphenolic tannins, and finally with warm ethanol (three extractions, 6 l.). The ethanolic solution was concentrated under vacuum to a viscous brown residue. This was diluted with water (2 l.) and the brown precipitate filtered off. The aqueous solution was extracted five times with ethyl acetate and concentrated to small volume (200 ml.). Residual tannins were removed by shaking with pre-chromed hide powder (100 g.) (Atkin & Thompson, 1937). The clear tannin-free solution was passed through a sulphonated polystyrene cation-exchange resin (Permutit Zeo-Karb 225) column (2·5 cm. × 32 cm.). Amino acids and imino acids were adsorbed, whereas carbohydrates were removed by elution with water. The nitrogenous acid fraction was removed by elution with dilute (6 N) NH₃ and the eluates were concentrated to dryness under reduced pressure.

The residue was taken up in water (20 ml.) and the mixture resolved by chromatography on sheets (22½ in. × 18¼ in.) of Whatman no. 3 paper in butan-1-ol–acetic acid–water (6:1:2, by vol.) for 24 hr. Bands were cut at R_F 0·08, 0·16, 0·24 and 0·33 after locating them with ninhydrin and isatin spray reagents. The components of bands at R_F 0·16 and 0·24 overlapped and were resolved by further chromatography for 48 hr. in the same solvent system.

(−)-*Pipecolic acid*. The eluates of the band at R_F 0·33 gave a characteristic mauve with ninhydrin that showed a red–purple fluorescence under ultraviolet light, and gave a pale blue–green with isatin. The solids recovered from the eluates were dissolved in boiling ethanol (50 ml.) and filtered from an insoluble residue. On concentrating and treating with charcoal, (−)-pipecolic acid (250 mg.) crystallized in white micro-crystals, m.p. 260–264°. Three recrystallizations from aq. ethanol gave m.p. 277° (decomp.), $[\alpha]_D^{25}$ − 27·4° (c 0·98 in water). The mixed m.p. with authentic (−)-pipecolic acid from *A. oswaldii* leaves was 275–276°.

Clark-Lewis & Mortimer (1961) found m.p. 273–275°, $[\alpha]_D$ − 25°.

L-(−)*Proline*. The solids from the band at R_F 0·24 were dissolved in boiling aq. 95% (v/v) ethanol (10 ml.), treated with charcoal and filtered from an insoluble residue. The solution was cooled to 0° and ether (3 ml.) added. After 3 days at 0° a white amorphous mass separated. This was reprecipitated from ethanol with ether, giving a white amorphous powder (110 mg.) that settled at 225° with decomposition, $[\alpha]_D^{26}$ − 83·1° (c 1·66 in ethanol). The compound gave a yellow changing to grey with ninhydrin and a bright blue with isatin.

The picrate was prepared (cf. Signaigo & Adkins, 1936) by dissolving the proline (20·9 mg.) and picric acid (41·8 mg.) in a minimum volume of hot acetic acid (1 ml.). To the mixture was added ether (4 ml.) and the solution cooled to 0°. Long yellow needles crystallized over 2 days. These were recrystallized (32 mg.) from ethanol, m.p. 153–154°. The mixed m.p. with the picrate of authentic (−)-proline prepared by similar means was 152–154°.

Kapfhammer & Eck (1927) found $[\alpha]_D$ − 84·9° for (−)-proline, and m.p. 152–154° for its picrate.

trans-4-*Hydroxy*-L(−)-*pipecolic acid*. Solids recovered from the eluates of the band at R_F 0·16 were dissolved in aq. 95% (v/v) ethanol (10 ml.), and an insoluble brown

precipitate was removed by filtration. The solution was treated with charcoal and concentrated to small volume (2 ml.), when a crystalline precipitate (245 mg.) appeared during a week at room temperature. This was recrystallized three times from aq. ethanol, m.p. 292°, $[\alpha]_D^{25}$ − 12·0° (c 0·78 in water). The mixed m.p. with authentic trans-4-hydroxy-L-(−)-pipecolic acid from *A. oswaldii* was 292–293°. The compound gave a characteristic green with ninhydrin that faded through khaki to grey, showing a brick-red fluorescence under ultraviolet light. There was no reaction with isatin.

Virtanen & Kari (1955) and Virtanen & Gmelin (1959) found m.p. 270°, $[\alpha]_D$ − 12·5°; Clark-Lewis & Mortimer (1961) recorded m.p. 294°, $[\alpha]_D$ − 13·0°.

α-Alanine, arginine, aspartic acid, glutamic acid and serine. The band at low R_F (0·08) contained a partially resolved mixture of amino acids, each present in lower concentration than the above compounds. The amino acids were identified by chromatography, with reference compounds, in five solvent systems that are designed for their separation (cf. Levy & Chung, 1953; Hardy, Holland & Nayler, 1955): phenol–*m*-cresol–borate buffer (pH 9·3) (25:25:7, by vol.); butan-1-ol–acetone–water (2:2:1, by vol.); butan-1-ol–ethanol–water (2:2:1, by vol.); butan-1-ol–ethanol–water–propionic acid (10:10:5:2, by vol.); butan-1-ol–ethyl methyl ketone–water–cyclohexylamine (10:10:5:2, by vol.). α-Alanine, arginine, aspartic acid, glutamic acid and serine were found to be present by comparison of R_F values, and by colour reactions with ninhydrin and isatin.

Petroleum (b.p. 60–70°)-soluble components. Solution *A* (4 l.) was concentrated to small volume (200 ml.), when a white precipitate (4 g., 0·2% yield) formed. This was examined by thin-layer chromatography on silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany) with benzene–acetone (17:3, v/v), and spraying with conc. H₂SO₄–40% formaldehyde (20:1, v/v). The precipitate consisted of six components, shown as grey–black spots, of which two at R_F 0·97 (long-chain β-diketone) and 0·63 ('steroid' alcohol) predominated.

Long-chain β-diketone. The crude petroleum extract was dissolved in light petroleum (b.p. 40–60°) (200 ml.) and shaken with aq. 5% (w/v) NaOH solution and then with water, and the petroleum was dried over anhydrous Na₂SO₄ for 2 hr. The residue obtained on evaporation was triturated with fresh petroleum (b.p. 40–60°) and the residual solids were crystallized from ethanol–petroleum (2:1, v/v) in white micro-crystals, m.p. 80–82° (Found: C, 80·1; H, 13·5. $C_{32}H_{64}O_2$ requires C, 79·9; H, 13·4%), $\lambda_{max.}$ 278 mμ (log ε 2·99) in ethanol.

The infrared spectrum in Nujol showed bands at 3350 cm.⁻¹ (rounded, O–H stretching), 3100 cm.⁻¹ (shoulder, O–H stretching chelate H-bonds), 2780 cm.⁻¹ (weak, C–H stretching), 1730 cm.⁻¹ (strong, ketonic C=O stretching vibrations), 1400 cm.⁻¹ [weak, C–H deformation (?)], 1175, 1100 and 1060 cm.⁻¹ [strong, C–O stretching and O–H deformation (?)] and at 730 and 720 cm.⁻¹ (strong, C–H out-of-plane deformations of alkenes). The n.m.r. spectrum showed heavily split protons at $\tau = 5·8$ p.p.m. (vinyl proton), and over the range 7·2–9·5 p.p.m. (methylene protons) with a sharp peak at 8·72 p.p.m. (probably methyl and hydroxyl proton resonances superimposed). Both spectra reflect the well-known enolic character of β-diketones.

The same compound (200 mg.), m.p. and mixed m.p.

80–82°, was obtained by treating the crude petroleum-soluble material (1 g.) with cuprous acetate, and by regenerating the β-diketone from the crystalline complex (cf. Horn & Lamberton, 1962; Horn, Kranz & Lamberton, 1964). The copper complex had infrared-absorption bands in the carbonyl region at 1725 (medium) and 1600 cm.$^{-1}$ (strong) in Nujol. The m.p. (80–82°) is higher than that of isomeric long-chain β-diketones from plant waxes (cf. Horn & Lamberton, 1962; Horn et al. 1964).

'Steroid' alcohol. The solids (1 g.) of the whole crude petroleum extract were resolved on ten preparative 20 cm. × 20 cm. thin-layer chromatographic plates, with Kieselgel G (1 mm. thick) and benzene–acetone (17:3, v/v) as solvent. The chromatograms were developed to the top of the plates (2 hr.). The steroid was located by spraying a narrow strip along the edge of the plate with phosphomolybdic acid in 20% (v/v) ethanol (Bergmann, Ikan & Harel, 1964). The band, corresponding to a blue on a yellow background, was removed by scraping the substrate off the plate and eluting with petroleum (b.p. 60–70°). A solid was recovered that crystallized from ethanol in white needles (98 mg.), m.p. 160–162° (Found: C, 82·2; H, 11·5. Calc. for C$_{22}$H$_{38}$O: C, 83·0; H, 12·0%). The Liebermann–Burchard reaction gave a violet-to-green coloration.

The n.m.r. spectrum of the steroid had the following characteristics: $\tau = 9·45$ and 9·21 p.p.m. (singlets, tertiary methyl resonances), 8·98 p.p.m. (doublet, probably secondary methyl), 7·5–9·2 p.p.m. (methylene proton resonances), 5·5–6·7 p.p.m. (multiplet, heavily split tertiary proton), 4·87 p.p.m. [multiplet, two vinyl protons (?)]. The infrared spectrum in chloroform solution had the characteristics: 3600 cm.$^{-1}$ (weak, O–H stretching), 3420 cm.$^{-1}$ (weak rounded, O–H stretching), 2970 and 2870 cm.$^{-1}$ (strong, C–H stretching), 1710 cm.$^{-1}$ (weak, ketonic C=O stretching vibration), 1450 and 1380 cm.$^{-1}$ (medium, OH deformations), 1090 cm.$^{-1}$ (weak), 1035 cm.$^{-1}$ (medium) and 972 cm.$^{-1}$ (medium).

The n.m.r. and infrared spectra of the above compound and of a 'steroid' alcohol, m.p. 160–161°, from black-wattle wood (Stephen, 1952) were superimposable. A mixed m.p. of the 'steroid' alcohols from wattle bark and heartwood showed no depression, 161–162°.

Acetate of the 'steroid' alcohol. The steroid (51 mg.) was dissolved in pyridine (0·3 ml.) and acetic anhydride (0·4 ml.). After 30 min. at room temperature the acetate was precipitated, and was recovered from water. Crystallization from ethanol–petroleum (b.p. 60–70°) (9:1, v/v) gave colourless plates (45 mg.), m.p. 175–176° (Found: C, 80·7; H, 10·8; CO·CH$_3$, 7·3. Calc. for C$_{24}$H$_{40}$O$_2$: C, 79·9; H, 11·2; CO·CH$_3$, 11·9%). The mixed m.p. with the 'steroid' acetate from black-wattle heartwood (Stephen, 1952) showed no depression, 173–175°. Keppler (1957) found m.p. 160–161° for the steroid from wattle wood, and m.p. 167–169° for its acetate.

The n.m.r. spectrum of the acetate is identical with that of the alcohol, except that an acetyl protons signal at $\tau = 7·97$ p.p.m. in the former replaces the hydroxyl proton signal at 8·48 p.p.m. in the latter, and the single proton at 5·5–6·7 p.p.m. is shifted to 5·0–5·6 p.p.m. in the acetate.

Carbohydrate components. Freshly stripped black-wattle bark (2 kg.) was dried at room temperature for 4 days and extracted with boiling aq. 90% (v/v) acetone (3 l.) for 2 hr. The solid extract (500 g.), obtained by evaporation under vacuum, was warmed to 60° with water (250 ml.). The insoluble residue was filtered and the solids were recovered from the filtrate by evaporation under vacuum. The dry powdered residue was refluxed with dry acetone for 2 hr. The tannins dissolved slowly and the carbohydrates were precipitated in white crystalline lumps.

Paper chromatography on Whatman no. 1 paper in butan-1-ol–acetic acid–water (6:1:2, by vol.) (24 hr. downward migration) showed the presence of sucrose (3·3 cm., migration, red), together with traces of glucose (5·6 cm., blue) and fructose (8·9 cm., red), with naphtharesorcinol-H$_3$PO$_4$ spray reagent (Bryson & Mitchell, 1951), and the presence of pinitol (7·5 cm., buff against brown background) with lead tetra-acetate spray (Buchanan, Dekker & Long, 1950). The mixture consisted mainly of sucrose and (+)-pinitol.

Sucrose. Sucrose (500 mg.) crystallized first from a methanol–acetone (4:1, v/v) solution (10 ml.) of the crystalline lumps, and was recrystallized from aq. ethanol as large cubic crystals, m.p. 189–190°, $[\alpha]_D^{20} + 66·4°$ (c 0·92 in water). The mixed m.p. with authentic sucrose showed no depression, m.p. 190–192°. Hydrolysis of the natural sucrose gave glucose and fructose (paper chromatogram).

(+)-Pinitol. Concentration of the methanol–acetone mother-liquor gave a white crystalline precipitate. This was recrystallized from the same mixture to give white micro-crystals (420 mg.), m.p. 185–186°, $[\alpha]_D^{25} + 67·9°$ (c 0·67 in water) (Found: C, 43·0; H, 7·3; O·CH$_3$, 16·2. Calc. for C$_7$H$_{14}$O$_6$: C, 43·3; H, 7·3; O·CH$_3$, 16·0%). The mixed m.p. with (+)-pinitol from Adesmia spp. (Appel & Lobos, 1962) and from wattle heartwood gave no depression, 186–188°. Anderson, MacDonald & Fischer (1952) found m.p. 185–186°, $[\alpha]_D^{23} + 66·8°$ (c 2·5 in water) for (+)-pinitol. The di-isopropylidene derivative (cf. Anderson et al. 1952) of (+)-pinitol from wattle bark had m.p. 102–103°.

Amino acid and imino acid contents of the seeds, seed pods, flowers, leaves, twig bark, stem bark, root bark, stem heartwood and root heartwood

The above portions of the black-wattle tree were extracted with aq. 80% (v/v) methanol and examined by two-dimensional chromatography with butan-1-ol–acetic acid–water (6:1:2, by vol.) (24 hr. downward migration), and then butan-1-ol–ethyl methyl ketone–water (2:2:1, by vol.) under the same conditions. Chromatograms were sprayed with ninhydrin and heated at 120° for 5–10 min. With the exception of variations in the root and stem heartwoods (cf. Table 1), pipecolic acid, proline and 4-hydroxypipecolic acid were present in all instances. In the flowers and seeds they were accompanied by very high concentrations of a complex mixture of amino acids.

The pipecolic acids and amino acids were present also in the leaves of the green wattle (A. decurrens) and silver wattle (A. dealbata).

Semi-quantitative estimation of pipecolic acids and proline in black-wattle heartwoods, barks and leaf extracts

Drillings of the stem and root heartwoods, and root bark, stem bark and twig bark that had been cut across the grain into thin fragments, as well as leaves that had been macerated in a Waring Blendor (1 g. each), were extracted

Table 1. *Percentage concentration of pipecolic acids and proline in fresh black-wattle barks, heartwoods and leaves*

Values in parentheses are on a dry wt. basis. N.D., Not detectable.

Portion of tree	(−)-Pipecolic acid	(−)-4-Hydroxypipecolic acid	(−)-Proline
Leaves	1·0 (1·8)	0·6 (1·1)	0·2 (0·4)
Twig bark	0·8 (1·3)	0·3 (0·5)	0·2 (0·3)
Stem bark	0·2 (0·3)	0·2 (0·3)	0·1 (0·2)
Root bark	0·6 (1·0)	0·3 (0·5)	0·2 (0·3)
Stem heartwood	0·04 (0·04)	0·05 (0·05)	N.D.
Root heartwood	0·02 (0·03)	N.D.	N.D.

with aq. 80% (v/v) ethanol (5 ml.) for 24 hr. A 0·05 ml. sample from each was applied to two-dimensional chromatograms (18¼ in. × 18¼ in. sheets of Whatman no. 1 paper) and these were developed as above. Duplicate chromatograms were sprayed with ninhydrin (pipecolic acids) and isatin (proline).

Standard solutions of L(−)-proline, *trans*-4-hydroxy-L(−)-pipecolic acid and L(−)-pipecolic acid were prepared by dissolving each (1·0 mg.) in water (5 ml.) and spotting suitable volumes (0·1, 0·2 and 0·3 ml.) on two-dimensional chromatograms. Comparison of the sprayed chromatograms with those from the various black-wattle extracts, when run at suitable dilution, enabled semi-quantitative estimation of these three compounds to be made (Table 1).

Carbohydrate content of flowers, leaves, stem bark, stem sapwood and stem heartwood

The aq.-ethanolic extracts (as above) of the above portions of the black-wattle tree were examined by chromatography as for wattle bark. In the heartwood fructose, glucose and sucrose were absent, but pinitol was present. The sapwood contained mainly fructose, accompanied by sucrose and glucose in low concentration. The bark and leaves contained mainly sucrose and pinitol, accompanied by glucose and fructose in low concentration. The flowers had the same composition of sugars as the sapwood, accompanied by a low concentration of pinitol.

Polyphenolic components of black-wattle leaves, barks and heartwoods

Black-wattle leaf polyphenols. The fresh leaves (500 g.) were covered with methanol (1·5 l.) and the mixture was macerated in a Waring Blendor. The methanolic extract was sucked off from the leaf debris, and shaken three times with light petroleum (b.p. 30–50°) to remove chlorophyll and waxes. Two-dimensional chromatograms of the extract were fumed with NH_3 to show flavonol glycosides, and also sprayed with ammoniacal $AgNO_3$ and bis-diazotized benzidine. These reagents showed the following components: a prominent streak from the origin in the direction of the partitioning run (R_F 0·0–0·55 in water-satd. butan-2-ol, and 0·0 in 2% acetic acid); the prominent components an unknown myricetin glycoside (R_F 0·45, 0·07), an unknown compound (R_F 0·48, 0·17), myricitrin (R_F 0·66, 0·17) and (+)-gallocatechin (R_F 0·50, 0·30); and the less prominent components quercitrin (R_F 0·76, 0·17), (+)-catechin (R_F 0·70, 0·34) and an unknown compound (R_F 0·40, 0·20). Minor unknown components, including flavonol glycosides,

were also present in the high-R_F region of the second direction (2% acetic acid). The more prominent components and tannins were all phloroglucinol derivatives (ochre with the benzidine reagent), and many were readily recognized from the work by Drewes & Roux (1963) on immature bark.

The methanolic solution was reduced in volume (400 ml.) and was applied to 80 sheets of Whatman no. 1 paper (5 ml./sheet) and the chromatograms were developed in 20% acetic acid by upward migration. Bands were cut at R_F 0·31 (unknown glycoside), 0·45 [myricitrin, quercitrin and (+)-gallocatechin], 0·50 [(+)-catechin and (+)-gallocatechin] and 0·70 [flavonol glycosides (?)]. These were stripped with 70% (v/v) ethanol and the eluates worked up as for immature bark (Drewes & Roux, 1963) by further separation in water-satd. butan-2-ol.

Myricitrin. Myricitrin (R_F 0·51 and 0·67 in water-satd. butan-1-ol and water-satd. butan-2-ol respectively) predominated and crystallized from water over 3 weeks (120 mg.). The crystals were separated mechanically from an accompanying brown sludge. Recrystallization from water gave pale-yellow crystals, m.p. and mixed m.p. with authentic myricitrin from *A. mearnsii* bark (Drewes & Roux, 1963) 204–206° (decomp.). The infrared spectra of these two compounds (KBr disk) were superimposable. The compound gave a typical olive green with ferric alum spray on chromatograms.

Quercitrin. Quercitrin (R_F 0·64 and 0·79 in water-satd. butan-1-ol and water-satd. butan-2-ol respectively) was present in less than a tenth of the concentration of myricitrin and did not crystallize although pure by chromatography. The compound gave a characteristic lime green with ferric alum spray on chromatograms. It showed $\lambda_{max.}$ (in ethanol) 256 and 348 mμ. Hydrolysis (cf. Drewes & Roux, 1963) gave quercetin and rhamnose.

(+)-Catechin and (+)-gallocatechin. The catechins were identified by chromatographic comparison with authentic compounds (cf. Roux, Maihs & Paulus, 1961) by using bis-diazotized benzidine (ochre) and ferric alum (green and blue respectively). (+)-Catechin was present in less than a third of the concentration of (+)-gallocatechin. The positive rotation of these compounds could be assigned on the basis of the known resolution of their epimers and enantiomers in these solvent systems (Roberts & Wood, 1953).

Unknown flavonol glycosides. The leaves are rich in minor flavonol glycosides (chromatographic mobility in 2% acetic acid, dark under ultraviolet light and yellow on fuming with NH_3). However, one of the unknown flavonol glycosides at R_F 0·48 and 0·07 (water-satd. butan-2-ol and 2%

acetic acid) is a major flavonoid leaf component, which gives an olive green typical of myricetin glycosides with ferric alum. Hydrolysis, followed by paper chromatography, showed it to be a myricetin glucoside or galactoside. It is accompanied (R_F 0·64, 0·07) by a forward-running component at low concentration, forming a pair, as with myricitrin and quercitrin.

Leaf tannins. These give a blue with ferric alum, an ochre with benzidine spray and yield delphinidin chloride on treatment with propan-2-ol-HCl. These reactions indicate their poly-leuco-delphinidin characteristics.

Polyphenols of twig bark. Immature stem bark was examined by Drewes & Roux (1963) and was shown to contain relatively high concentrations of myricitrin, quercitrin, (+)-catechin, (+)-gallocatechin and the (+)-leuco-fisetinidin and (+)-leuco-robinetinidin analogues and related tannins.

The twig bark contained all these as well as the unknown myricetin glycoside (R_F 0·48, 0·07) as very prominent components. These 'leaf components' were accompanied by (−)-robinetinidol (low concentration) and components A (polymeric leuco-robinetinidin and leuco-fisetinidin, low concentration), F (complex leuco-fisetinidin), B, C, D and E (polymeric leuco-robinetinidins)—all typical bark components (Roux & Evelyn, 1958). The typical leaf and bark components were present in equivalent concentrations with (+)-catechin and (+)-gallocatechin in higher relative concentrations than in wattle leaves. These catechins were present in similar relative concentrations.

Polyphenols of mature stem bark. Mature bark from the base of a 6-year-old tree was extracted with methanol, and the extractives (250 mg./sheet) were separated on five 18¼ in. × 22½ in. sheets of Whatman no. 3 paper in 2% acetic acid. An appropriate band (R_F 0·24) was cut out, stripped with 70% ethanol as before, and examined by two-dimensional chromatography for myricitrin and quercitrin. These and other flavonol glycosides could not be detected. (+)-Catechin occurred at about three times the concentration of (+)-gallocatechin, and the relative concentration of (−)-robinetinidol had increased.

Polyphenols of root bark. The composition of tannins from the fresh bark of a large root (1 in. diam. at sampling position) was almost indistinguishable from that of mature stem bark. Flavonol glycosides were absent. (+)-Catechin had about six times the relative concentration of (+)-gallocatechin, and component F (complex leuco-fisetinidin) was more prominent than in stem bark.

Polyphenols of branch, stem and root heartwoods. These had identical compositions, (+)-mollisacacidin, (−)-fisetinidol, (+)-fustin, (−)-butin, butein, fisetin, 'dimeric', trimeric and other polymeric leuco-fisetinidin tannins (cf.

Roux & Paulus, 1960, 1961a,b,c, 1962) being readily recognized by means of suitable spray reagents.

Leuco-anthocyanidin content of leaves and of twig, stem and root barks

The methanolic extracts of root bark, stem bark (sampled at various positions), twig bark and leaves were evaporated to dryness and anthocyanidins were generated from the solids (1–2 mg.) of each by the method of Pigman, Anderson, Fischer, Buchanan & Browning (1953). The resulting red solutions were streaked on Whatman no. 1 paper and the chromatograms were developed with 90% formic acid–3N-HCl (1:1, v/v) (Roux, 1957) for 2 hr. The anthocyanidins developed from the various extracts were compared (Table 2).

DISCUSSION

The non-phenolic components of black-wattle bark were initially studied in some detail, and their presence or absence in other portions of the tree was examined by paper chromatography.

Thus the predominant carbohydrate components of wattle bark were sucrose and (+)-pinitol, accompanied by lower concentrations of glucose and fructose. Among the nitrogen-containing compounds, the imino acids L(−)-pipecolic acid, trans-4-hydroxy-L(−)-pipecolic acid and L(−)-proline predominated, and were accompanied by the amino acids α-alanine, arginine, aspartic acid, glutamic acid and serine. As gauged from the nitrogen content (0·28%) these constitute about 3% of the dry extract. The petroleum-soluble fraction of the bark (0·22% on bark weight) consists of at least six components, among which a long-chain β-diketone and a 'steroid' alcohol predominate.

Among these non-phenolic compounds only sucrose, glucose and fructose were previously recognized as bark components by means of paper chromatography (Stephen, 1952; Roux, 1952), although the 'steroid' alcohol (Stephen, 1952) and pipecolic acids (Clark-Lewis & Mortimer, 1961) have been isolated from black-wattle wood (A. mearnsii, formerly A. mollissima).

The pipecolic acids are present in all parts of the black-wattle tree (cf. Table 1), but exist in declining concentration in the sequence: leaves, twig bark,

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Table 2. *Anthocyanidins generated from black-wattle barks and leaves from a 6-year-old tree*

Anthocyanidin	Colour	Root bark (6–12 in. below ground level)	Bark (ground level)	Bark (5 ft. above ground)	Bark (base of main branch)	Twig bark	Leaves
Delphinidin	Red–purple	−	+	+	+	+	+
Cyanidin	Red–blue	−	−	−	−	−	−
Robinetinidin	Violet–pink	+ + +	+ + +	+ + +	+ + +	+ + +	−
Fisetinidin	Red–pink	+ + +	+	+	+	+	−

stem bark. Their high concentration in the leaves and green twig bark suggests that the leaves are probably the focal point of their biosynthesis, although the root bark again has higher proportions than the stem bark or heartwoods. Their presence in the sap- and heart-woods is probably through translocation from the leaves and possibly the roots. Their associated amino acids, α-alanine, arginine, aspartic acid, glutamic acid and serine, and also proline, are almost absent from the heartwoods (cf. Table 1), but strongly elaborated in the flowers and seeds. The above amino acids, together with others, have been found associated with 4-hydroxypipecolic acid in *Armeria maritima* (Fowden, 1958), and with pipecolic acid in the green bean (Grobbelaar & Steward, 1953). Radioactive tracer studies have shown that lysine is the precursor of pipecolic acid in the developing green bean (Grobbelaar & Steward, 1953), and the latter is probably converted into 4-hydroxypipecolic acid by direct hydroxylation (Fowden, 1960; Schenk, Schuette & Mothes, 1962). The concurrence of these in the leaves of the black wattle and elsewhere in the tree indicates a similar sequence in this and in related *Acacia* spp. (Bentham, 1864), the pipecolic acids having been isolated from the leaves of *A. oswaldii* (Clark-Lewis & Mortimer, 1961) and heartwood of *A. excelsa* (Clark-Lewis & Mortimer, 1959) and now shown to be present in *A. decurrens* and *A. dealbata*.

($+$)-Pinitol as a major carbohydrate constituent of black-wattle bark extract was previously overlooked owing to its relatively poor and non-specific response to most spray reagents, and its similar R_F to accompanying carbohydrates. ($+$)-Pinitol is a component common to many other species among the Leguminosae [cf. Appel & Lobos (1962) (*Adesmia* spp.), Clark-Lewis, Katekar & Mortimer (1961) (*A. intertexta=A. orites*), Stephen (1952) (*A. mearnsii*), Rimington (1935) (*A. longissima*, *A. stolonifera* and *A. lasiopetala*) and Plouvier (1949, 1950a,b)], and its simultaneous presence in the leaves, barks, sapwood and heartwood of the black wattle suggests a possible origin in the leaves, vertical translocation in the vascular tissues of the bark followed by radial translocation to the sapwood and heartwood. Other carbohydrates, sucrose (predominantly), glucose and fructose, have the same concentration in the leaves and bark, but inversion of the sucrose predominates in the sapwood. The absence of these carbohydrates from the heartwood, leaving only ($+$)-pinitol, suggests that the products of inversion, glucose and sucrose, are either consumed or undergo transformation during tannin formation at the sapwood–heartwood interface.

The mutual presence of the 'steroid' alcohol, ($+$)-pinitol and the pipecolic acids in bark and heartwood finds a parallel among their flavonoid components, where ($+$)-mollisacacidin, its analogues and associated polymeric tannins are similarly common to both (Roux & Paulus, 1960, 1961a,b,c, 1962; Drewes & Roux, 1963). ($+$)-Leuco-robinetinidin and its analogues, however, constitute the predominant pattern of bark components (Roux & Evelyn, 1958; Roux & Maihs, 1960; Maihs, 1961; Drewes & Roux, 1963), thus showing that a related but different enzymic system must coexist in the bark (cf. Table 3). Such an increase in the degree of phenolic hydroxylation in the bark as compared with heartwood flavonoids is a ubiquitous phenomenon. The two remaining and minor patterns of flavonoid bark components (analogues of delphinidin and cyanidin) (Roux & Maihs, 1960; Drewes & Roux, 1963), namely ($+$)-gallocatechin, myricitrin, and uncharacterized myricetin glycoside and leuco-delphinidins, with small amounts of ($+$)-catechin and quercitrin, undoubtedly originate in the leaves, where they predominate (cf. Tables 2 and 3). These typical leaf components (analogues of delphinidin and cyanidin) and the typical bark components (leuco-fisetinidin and leuco-robinetinidin analogues) are present in equivalent proportions in the chlorophyll-rich twig bark, but the leaf components decrease while the bark components increase rapidly and progressively with increasing age of the bark. Thus in bark of the small branches the relative concentrations of leaf components are already much decreased, are almost absent in mature stem bark, which usually still contains some chlorophyll (6–8-year-old trees), and are absent from the root bark, where no photosynthetic processes are possible (cf. Tables 2 and 3).

Thus leuco-fisetinidin analogues of the heartwood and the chemically related leuco-robinetinidin–leuco-fisetinidin mixture of the bark appear to arise from similar enzymic systems with common origins in the vascular tissues of the bark. The immature barks, however, have the typical leaf components superimposed, their concentrations varying according to bark age (or thickness) and with origins in the early photosynthetic processes of leaves and twig bark or, perhaps to a very limited extent, in similar processes in the mature chlorophyll-containing bark.

Exceptions to this simple differentiation are ($+$)-catechin, which increases progressively in relative concentration in the sequence: leaves, twig bark, branch bark, stem bark, root bark; and ($+$)-gallocatechin, where the reverse sequence applies. These may undergo vertical translocation from the leaves owing to their relatively low molecular weights (approx. 300) and low affinity for cellulose substrates.

Separate enzymic systems must exist in annually recurrent but temporary structures of the tree, namely the flowers and seeds, since they contain

Table 3. *Relative distribution of components in wattle leaves, barks and heartwoods*

Portion of wattle tree	Component									
	(+)-Catechin	Quercitrin	(+)-Gallo-catechin	Myricitrin	Flavonol glycoside	Leuco-delphinidin tannin	(+)-Leuco-robinetinidin	(−)-Robinetinidol	(+)-Dihydro-robinetin	Robtin
Leaves	(+)	(+)	++	++++	++++	+++++	—	—	—	—
Twig bark	++	++	++	++++	++++	+++++	—	++	Trace	Trace
Stem bark	++	Trace	+(+)*	Trace	—	+++++	Trace	+++	Trace	Trace
Root bark	++	—	(+)	—	—	++++	Trace	+++	Trace	Trace
Stem heartwood	—	—	—	—	—	—	—	—	—	—
Root heartwood	—	—	—	—	—	—	—	—	—	—

Portion of wattle tree	Component									
	Robinetin	Robtein	Polymeric leuco-robinetinidin tannins	(+)-Leuco-fisetinidin	(−)-Fisetinidol	(+)-Fustin	Butin	Fisetin	Butein	Polymeric leuco-fisetinidin tannins
Leaves	—	—	—	—	—	—	—	—	—	—
Twig bark	Trace	Trace	+++	Trace	Trace	Trace	Trace	Trace	Trace	+(+)
Stem bark	Trace	Trace	+++++	Trace	Trace	Trace	Trace	Trace	Trace	+++(+)
Root bark	Trace	Trace	+++++	Trace	Trace	Trace	Trace	Trace	Trace	++++
Stem heartwood	—	—	—	+++++	(+)	+(+)	(Trace)	(Trace)	Trace	++++++
Root heartwood	—	—	—	+++++	(+)	+(+)	(Trace)	(Trace)	Trace	++++++

* Concentration declines with increasing age of the bark.

mixtures of amino acids, flavonoids (Petrie, 1923) and sugars of different composition. However, green seed pods contain typical leaf flavonoids and tannins.

The long-chain β-diketone isolated from wattle bark is a typical component of cuticle waxes of many trees including *Acacia* spp. (Horn & Lamberton, 1962; Horn *et al.* 1964).

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