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CHEMOTAXONOMIC STUDIES OF *CROTON* SPECIES IN SRI LANKA

B. M. RATNAYAKE BANDARA AND W. R. WIMALASIRI
Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka.

AND

S. BALASUBRAMANIAM
Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

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Abstract : Isolation of (−)−hardwickiic acid and β−amyrin from *Croton aromaticus* and 5−hydroxy−3,7,4' −trimethoxyflavone and sitosterol from *Croton lacciferus*, and the distribution of these compounds in seven species of *Croton* are described. The contention that *C. aromaticus* and *C. lacciferus* are two distinct species is supported by the present phytochemical study.

1. Introduction

The family Euphorbiaceae consists of about 7300 species which occur mainly in the tropics and extending into the temperate regions of the northern and southern hemispheres.[1,1] Next to *Euphorbia,* the genus *Croton* is the largest species (700 species) of Euphorbiaceae, and many *Croton* species find application in ethnomedical preparations. Ten species of the genus *Croton* are found in Sri Lanka and no endemic species have been reported: *Croton aromaticus* Linn., *Croton bonplandianus* Haines, *Croton lacciferus* L., *Croton hirtus* L., *Croton moonii* Thw., *Croton nigroviridis* Thw., *Croton oblongifolius* Roxb., *Croton officinalis* Klotzsch, *Croton tiglium* L. and *Croton zeylanicus* Muell. As a part of our studies on medicinal plants, the chemical constituents of some *Croton* species have been examined. This report describes the isolation of (−)−hardwickiic acid and β −amyrin from *C. aromaticus,* the isolation of 5−hydroxy−3,7,4' − trimethoxyflavone and sitosterol from *C. lacciferus* and the investigation of seven species of *Croton* for the presence of these four compounds.

2. Results and Discussion

2.1 Extractives of *Croton aromaticus*

The dried roots of *C. aromaticus* were extracted with hot petroleum. The petrol extract when chromatographed over silica gel and eluted with 25%

CH_2Cl_2–petrol gave a white crystalline solid (0.79% yield) m.p. 103–104°, $(\alpha)_D$–112.5° and a slightly less polar white solid (1.2×10^{-2}% yield), m.p. 197–198°, $(\alpha)_D$ + 87.1°. The ^1H NMR spectrum of the more polar compound (1) suggested the presence of a furan ring (δ 7.27, 7.13 and 6.18) and a carboxylic group (δ 11.57, D_2O exchangeable). The acid (1) was reacted separately with excess CH_2N_2 and excess $LiAlH_4$ to obtain the methyl ester (2) and the alcohol (3), respectively. The spectral data of 1, 2 and 3 were identical to those reported in the literature.[5,7,13] The compound (1) was thus identified as (–)–hardwickiic acid which had been isolated from *Hardwickia pinnata* (Leguminosae)[13], *Copaifera officinalis* (Leguminosae)[7] *Printzia laxa* (Compositae)[5] and *Heteropappus atlaicus* (Compositae).[6] The insecticidal properties of hardwickiic acid against *Aphis craccivora* has been reported in a recent communication.[5]

The low polar compound (4) obtained from the above column had physical data (m.p., $(\alpha)_D$, IR and ^1H NMR) consistent with those reported[9] for β–amyrin. The identity of the compound (4) as β–amyrin was confirmed by direct comparison with an authentic sample.

2.2 Extractives of *Croton lacciferus*

Crushed fresh leaves of *C. lacciferus* were extracted with methanol under reflux conditions. The hot methanol extract when chromatographed over silica gel and eluted with 2% EtOAc–benzene gave a yellow crystalline solid (5) m.p. 147–148° and a white crystalline solid (6) m.p. 136–137°, $(\alpha)_D$–36°. The UV λ_{max} of the yellow compound (5) at 345 nm and positive Gibbs test indicated the presence of a flavone system containing a phenolic group with the *para* position unsubstituted. ^1H NMR spectrum suggested the presence of three methoxy groups (δ 3.89 and 3.87), two aromatic hydrogens in a *meta* relationship (δ 6.42, 6.32, J = 2Hz) and four hydrogens of a *para* substituted benzene ring (δ 8.07, 6.49, J = 9Hz). These data and the molecular formula $C_{18}H_{16}O_6$ (high resolution mass) are consistent with those for a trimethoxy derivative of keampferol. The structure of this compound was established as 5–hydroxy–3,7,4' –trimethoxy flavone (5) by comparison of its physical data (m.p., $(\alpha)_D$, IR, ^1H NMR and mass) with those reported for the flavone (5) which had been isolated from the fern *Chelianthes farinosa* (Gymnogrammoideae).[8]

The compound (6) obtained from the leaf extract of *C. lacciferus* was found to be sitosterol by direct comparison of its physical data[9] with those of an authentic sample.

2.3 Phytochemical screening of *Croton* species

The presence of (–)–hardwickiic acid (1),β–amyrin (4) and sitosterol (6) has

been reported in some *Croton* species previously: (1) in *C. oblongifolius*[2] and *C. californicus*[12], (4) in *C. caudatus*[14] and (6) in *C. caudatus*[14] and *C. oblongifolius.*[15] These results together with the present findings from the investigation of *C. aromaticus* and *C. lacciferus* prompted us to screen locally available *Croton* plants for the presence of compounds (1), (4), (5) and (6).

Leaves and roots of seven *Croton* species were collected from different parts of Sri Lanka. The dried leaves and roots were separately extracted with hot chloroform. The TLC's of these extracts were compared with those of the corresponding extracts from *C. aromaticus* and *C. lacciferus* and the authentic samples of (−)–hardwickiic acid (1) and the trimethoxyflavone (5).

A greyish blue spot with a conical shape, corresponding to (−)–hardwickiic acid (1) was observed on TLC ($CHCl_3$, anisaldehyde) in the root extracts of *C. aromaticus* only. The trimethoxyflavone (5) was observed in the leaf extracts of *C. lacciferus* and *C. oblongifolius* as a yellow spot on TLC (5% EtOAc–benzene, anisaldehyde). Whether or not β–amyrin (4) and sitosterol (6) were present in the extracts could not be determined using the TLC method as the corresponding spots were obscured and/or inconspicuous. Hence an HPLC analysis of the extracts was carried out.

Chloroform solutions of the pure compounds, extracts, and extracts mixed with the four compounds were separately injected to an HPLC instrument with attachment to an RI detector, and the chromatograms recorded. The detection of the compounds in each extract was based on their retention times and enhancement of peaks in the chromatogram of the sample containing the extract and four compounds. The results are given in Table 1.

In conformity with the results of the TLC studies, the HPLC analysis also revealed the presence of (−)–hardwickiic acid (1) in *C. aromaticus* (roots) and the flavone (5) in *C. lacciferus* (leaves) and *C. oblongifolius* (leaves). However, the HPLC results further suggested that trace amounts of (−)–hardwickiic acid (1) were present in *C. lacciferus* (roots), *C. oblongifolius* (leaves) and *C. officinalis* (roots). Similarly the presence of the flavone (5) in the leaf and the root extracts of *C. tiglium* was detected only in the HPLC study. While all the plants except *C. oblongifolius* contained sitosterol (6), β–amyrin (4) was found in *C. aromaticus, C. bondplandianus, C. lacciferus* and *C. officinalis.*

2.4 Chemotaxonomic significance

Trimen[16] regarded *C. aromaticus* as a variety of *C. lacciferus* although other botanists[1,10] consider the two taxa as distinct species. In a previous study,[4] it has been found that the roots of *C. lacciferus* contained several kauranoids while these compounds were absent in the roots of *C. aromaticus.* Together with the results of the present study (Table 1), it is clear that the nature and

Table 1. Distribution[a] of (−)−hardwickiic acid (1), β−amyrin (4), 5−hydroxy−3,7,4'−trimethoxy−flavone (5) and sitosterol (6) in *Croton* species

Plant	Site of collection	Plant part[b]	1	4	5	6
Croton aromaticus	Nakkala	LF	−	−	−	+[c]
	Kithulhitiyawa	RT	++[c]	++	−	−
Croton bonplandianus	Madduvil	LF	−	++	−	++
	Kalpitiya	RT	−	−	−	++
Croton hirtus	Nakkala	LF	−	−	−	++
	Mihintale	RT	−	−	−	++
Croton lacciferus	Dawulagala	LF	−	+	++	++
		RT	+	−	−	+
Croton oblongifolius	Maha Oya	LF	+	−	++	−
Croton officinalis	Peradeniya	LF	−	+	−	+
	Nochchiyagama	RT	+	++	−	−
Croton tiglium	Peradeniya	LF	−	−	++	++
		RT	−	−	++	++

[a] From HPLC analysis

[b] LF = leaves, RT = roots

[c] + = less than 0.6% in the extract, ++ = more than 0.6% in the extract, as calculated from the HPLC chromatograms.

the distribution of chemical constituents in *C. aromaticus* and *C. lacciferus* are different. The view that these two taxa be maintained as two distinct species is thus supported by the phytochemical studies.

3. Experimental

3.1 Investigation of *Croton aromaticus*

Dried roots (4.3 kg) of *C. aromaticus* collected at Kithulhitiyawa near Dambulla, were crushed and extracted exhaustively with petroleum (bp 60–80°C) under reflux conditions for 5 days. The removal of solvent on a rotavapor gave a brown semi-solid (110 g).

3.1.1 Isolation of β–amyrin (4)

Flash chromatography of the petroleum extract over silica gel (TLC grade, Merck) with 25% CH_2Cl_2–petroleum afforded a white crystalline solid mp 197–198°C (CH_2Cl_2–petroleum) (lit.[9] 197–197.5°C); $(\alpha)_D$ + 87.1° ($CHCl_3$, c, 0.98) (lit.[9] 88.4°). This compound was found to be identical with an authentic sample of β–amyrin (mp, mmp, Co–TLC, IR and ^1H NMR).

3.1.2 Isolation of (–)–hardwickiic acid (1)

Further elution of the above column with 25% CH_2Cl_2–petroleum followed by TLC ($CHCl_3$) furnished a white crystalline solid (3.09 g), mp 103–104°C (n–hexane), (lit.[13] 106–107°C); $(\alpha)_D$–112.5°($CHCl_3$, c, 0.48) (lit.[13] 114.7°). This compound was identified as $(\alpha)_D$–hardwickiic acid from the following spectroscopic data and chemical conversions; IR $\bar{\nu}_{max}$(KBr) 3340–2200(OH), 1670(CO), 1400, 1255, 1240 and 775 cm^{-1}; ^1H NMR δ ($CDCl_3$, 60 MHz) 11.57 (1H,brs, D_2O exchangeable, CO_2H), 7.27 (1H, t, J = 2 Hz, 16–H), 7.13(1H,brs, 15–H), 6.83 (1H, t, J = 4Hz, 3–H), 6.18 (1H, brs, 14–H), 2.70–1.20 (15H, m), 1.23 (3H, s, 18–H), 0.83(3H, d, J = 8Hz, 17–H), 0.77(3H, s, 20–H); MS m/z (rel. int.) 316(M^+, 43), 301(20), 283(100), 221(40), 203(34), 137(22), 125(75), 96(37), 81(59).

3.1.3 Methylation of (–)–hardwickiic acid (1) into ester (2)

(–)–Hardwickiic acid (1) (27 mg) was dissolved in Et_2O (1 ml) and treated with excess ethereal CH_2N_2 for 30 min. After removal of the solvent, the product was purified by TLC (20% CH_2Cl_2–petroleum) to give the ester (2) as a colourless oil (26 mg, 92%), $(\alpha)_D$–95.1° ($CHCl_3$, c, 1.02); IR ν_{max} (neat) 1710(CO), 1430, 1245 and 1230 cm^{-1}; ^1H NMR δ(CCl_4)

7.30 (1H, t, J = 2 Hz, 16–H), 7.13 (1H, brs, 15–H), 6.57 (1H, t, J = 4 Hz, H–3), 6.17 (1H, brs, 14–H), 3.62 (3H, s, OMe), 2.70–1.20 (15H, m), 1.23 (3H, s, 18–H), 0.84 (3H, d, J = 8 Hz, 17–H), 0.77 (3H, s, 20–H).

3.1.4 Reduction of (–)–hardwickiic acid (1) into alcohol (3)

$LiALH_4$ (25 mg) was added portion-wise to a solution of 1 (20 mg) in Et_2O and stirred at room temperature for 12h. Usual work up gave a residue which was purified using TLC (50% CH_2Cl_2–petroleum) to give the alcohol (3) as a colourless oil (19 mg, 80%), $(\alpha)_D$ –34.5° ($CHCl_3$, c, 0.58); IR ν_{max} (neat) 3600–3050(OH), 1450, 1380 and 1020 cm^{-1}; ^1H NMR δ (CCl_4) 7.27 (1H, t, J = 2Hz, 16–H), 7.13 (1H, brs, 15–H), 6.17 (1H, brs, 14–H), 5.50 (1H, t, J = 4 Hz, 3–H), 3.93 (2H, brs, 19–H), 2.70–1.20 (15H, m), 1.07 (3H, s, 18–H), 0.81 (3H, d, J = 8Hz, 17–H), 0.75 (3H, s, 20–H).

3.2 Investigation of *Croton lacciferus*

Fresh leaves (250 g) of *C. lacciferus* collected at Dawulagala near Peradeniya, were crushed and exhaustively extracted with MeOH under reflux conditions for 5 days. The removal of MeOH on a rotavapor gave a semi-solid (29 g).

3.2.1 Isolation of 5–hydroxy–3,7,4'–trimethoxyflavone (5)

Silica gel column chromatography (2% EtOAc–C_6H_6) of the MeOH extract (20 g) followed by TLC (5% EtOAc–C_6H_6) gave a yellow crystalline solid (142 mg) which was identified as the flavone (5), mp 147–148°C ($CHCl_3$–MeOH)(lit.[8] 144–147°C); UV λ_{max}^{MeOH} (log ϵ)345(4.34), 268(4.40), 210(4.50) nm; IR ν_{max}(KBr) 1655(CO), 1585, 1490, 1460 cm^{-1}; ^1H NMR δ ($CDCL_3$) 12.60 (1H, s, 5–OH), 8.07, 6.49 (2H each, d, J = 9Hz, 2' –H, 3' –H, 5' –H, 6' –H), 6.42, 6.32 (1H each, d, J = 2Hz, 6–H, 8–H), 3.89 (3H, s, OMe), 3.87 (6H, s, 2xOMe); MS m/z (rel. int.) 328 (M+, 100), 327 (94), 285 (63), 242 (18), 150 (15), 148 (150, 135 (20), 119 (12); $C_{18}H_{16}O_6$ (M+) 328.0957 (cal. 328.3246).

3.2.2 Isolation of sitosterol (6)

Further elution of the above column with 2% EtOAc–C_6H_6 gave a white crystalline solid (56 mg), mp 136–137° C (lit.[9] 136–137°C); (α)D–36° ($CHCl_3$, c, 1.30) (lit.[9] –35°). This compound was found to be identical to an authentic sample of sitosterol (mp, mmp, Co–TLC, Ir and ^1H NMR).

3.3 Phytochemical screening

Leaves and roots of *C. aromaticus, C. bonplandianus, C. hirtus, C. lacciferus, C. oblongifolius, C. officinalis* and *C. tiglium* were collected from different

parts of Sri Lanka (Table 1). The air-dried leaves and roots were separately extracted with chloroform under reflux conditions for 24 h. The residues produced from concentrating the extracts were used in the following TLC and HPLC analyses.

3.4.1 TLC analysis

Each chloroform extract of the above plants, the petroleum extract of the roots of *C. aromaticus* and (–)–hardwickiic acid (1) were spotted on TLC plates (silica gel), and the plates were developed in $CHCl_3$, dried in air and sprayed with anisaldehyde. A greyish blue spot with a conical shape corresponding to (–)–hardwickiic acid (1), was observed only in the root extracts of *C. aromaticus*.

Each chloroform extract of the *Croton* plants, the MeOH extract of the leaves of *C. lacciferus* and the flavone (5) were spotted on TLC plates. A yellow spot (5% EtOAc–C_6H_6, anisaldehyde spray) corresponding to the flavone (5) was observed in the leaf extracts of *C. lacciferus* and *C. oblongifolius*.

3.4.2 HPLC analysis

The presence of (–)–hardwickiic acid (1), β–amyrin (4), flavone (5) and sitosterol (6) in the $CHCl_3$ extracts of the *Croton* species was studied using a high performance liquid chromatography (HPLC) instrument equipped with Waters Associates chromatography pump, a Differential Refractometer (R 401, Waters) and a normal phase column (450 x 10 nm, Partisil M9 10/15) connected to a guard column (CO–PELL ODS, Whatman). Eighty percent$CHCl_3$–n–hexane was used as the mobile phase maintaining a flow rate of 2.5 ml/min. Retention time of each compound was determined by injecting a $CHCl_3$ solution of each compound (0.05 mg, in 25 μl); the retention times of 1, 4, 5 and 6 were found to be 22.0 17.6, 13.2 and 24.0 min, respectively. $CHCl_3$ solutions of each extract (0.04 mg/μl) and the mixture of pure compounds (0.002 mg/μl for each compound) were prepared for subsequent injections. For each extract two HPLC runs were made by separately injecting the $CHCl_3$ solution of the extract (100 μl) and another solution prepared by mixing the $CHCl_3$ solutions of the extract (100 μl) and another solution prepared by mixing the $CHCl_3$ solutions of the extract (100μl) and the compound mixture (25 μl). The presence of the compounds in each extract signified by their retention times, was confirmed by observing the peak enhancements in the chromatogram corresponding to the combined solution of the extract and the mixture of four compounds. The results are given in Table 1.

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