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Bioactivity and chemistry of the genus *Hortonia*

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The dichloromethane extracts of the leaves, stem bark, bark and the roots of the three species of the primitive endemic genus *Hortonia*, *H. angustifolia*, *H. floribunda* and *H. ovalifolia*, collected from nine geographical locations ranging from lower elevations (84–420 m) to higher (2000 m) showed comparable HPLC profiles and mosquito larvicidal and antifungal activities; protein analysis of the leaves of the three species of *Hortonia* showed identical peaks and bands. The two major metabolites (4*S*)-4-methyl-2-(11-dodecynyl)-2-butenolide (**2**) and (4*S*)-4-methyl-2-(11-dodecenyl)-2-butenolide (**3**), which were previously reported from all three plants, showed potent larvicidal activities. Compound **2** was excessively high in the extracts of the stem bark and the roots of all three species amounting to approximately 38 and 60%, respectively. A minor new butenolide (**4**), (4*S*)-4-methyl-2-((2*R*)-hydroxy-11-dodecenyl)-2-butenolide, with much reduced larvicidal activity and ishwarane (**1**), which showed antifungal activity, were also isolated from all three plants. Treatment of compound **2** with H₂/Pd–C afforded the completely reduced compound **5**, which showed no larvicidal activity, indicating that unsaturation in both **2** and **3** is necessary for their bioactivity. The foregoing evidence showed that there are major similarities between the three species of *Hortonia*.

Keywords: genus *Hortonia*; dichloromethane extracts; HPLC analysis; mosquito larvicidal activity; antifungal activity; protein analysis; new butenolide; similarity between species

1. Introduction

Hortonia is a genus endemic to Sri Lanka belonging to the family Monimiaceae, order Laurales. This family comprises of about 39 genera, five of which occur in Brazil, and 440 species are widely spread in the Southern Hemisphere, mainly in tropical and subtropical regions of the Americas (Leitão, Soares, de Barros, Brito, and Monache, (2000)). This family is represented in Sri Lanka, Oceania, Polynesia, Australia, Malaysia, Madagascar and South America (Claros, da Silva, Vasconcellos, de Brito, and Leitao (2000), Dassanayake, (1996)). They are very rare in Africa and absent in India. *Siparuna* and *Mollinedia*, comprising of 150 and 94 species respectively, are the two major genera of

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Monimiaceae Claros, et al., (2000). The family Monimiaceae comprises of trees or shrubs and rarely climbers.

Hortonia is considered to have originated in Gondwanaland about 100–120 million years ago (Jayasekara, 1997). Wight described three species of *Hortonia* (*H. floribunda*, *H. ovalifolia*, and *H. acuminata*) from the wet zone of Sri Lanka (Wight, (1853)). Thawaites considered all three species to be varieties of *H. floribunda*, having numerous intermediate forms (Thawaites (1864)). Trimen, on the other hand, considered *H. floribunda* and *H. angustifolia* as two different species (Trimen (1885)). An oval-leaved species collected at Adam's Peak, in central Sri Lanka, was classified as a variety under *H. floribunda*. The latest revision of the family Monimiaceae by Dassanayake in 1996 lists three distinct species (*H. floribunda* Wight ex Arn., *H. angustifolia* (Thw.) Trimen and *H. ovalifolia* Wight) in Sri Lanka (Dassanayake, 1996). The present study was initiated with a view to studying the bioactive profile and the chemistry of the genus *Hortonia* in Sri Lanka.

2. Experimental section

2.1. General experimental procedures

Optical rotations were measured with a Bellingham + Stanley ADP 220 polarimeter using CHCl_3 as solvent. UV spectra were obtained in CH_2Cl_2 using a Shimadzu (UV-160) spectrometer. IR spectra were recorded on a Shimadzu (IR-408) spectrometer as a film on NaCl. The ^1H and ^{13}C NMR spectra were recorded in CDCl_3 at 300 and 75 MHz, respectively, on a Varian VXR300S spectrometer and were referenced to the solvent (^1H 7.26 ppm; ^{13}C 77.0 ppm). HMQC and HMBC experiments were carried out at 500 MHz on a Varian Inova. All two-dimensional NMR experiments were carried out using standard and in-house modified Varian pulse sequences. EIMS spectra were recorded on a Fisons VG Autospec mass spectrometer operating at 70 eV (direct insertion). Perfluorokerosine was used as the internal reference for HRMS measurements.

Medium-pressure liquid chromatography (MPLC) and flash chromatography were performed on Merck Si gel 60 (230–400 mesh). Thin layer chromatography (TLC) was performed on Merck Si gel 60 F_{254} plates using CH_2Cl_2 as eluent.

2.2. Plant material

Specimens of three species, *H. angustifolia*, *H. floribunda*, *H. ovalifolia* were collected from nine different geographical locations of Sri Lanka ranging from lower elevations to the montane zone: *H. angustifolia* from Pittabeddara (84–137 m), Atwaltota (127 m), Wewelkandura (140 m), Kotapola (140 m), Kanneliya (165 m), and Deniyaya (420 m); *H. floribunda* from Kelabokka (1120 m), and Hakgala (1995 m); and *H. ovalifolia* from the foot hills of Adam's Peak (2007 m) in September 1998.

Air-dried and powdered leaves, stem bark, bark and root (50 g each) collected from each location were separately extracted into CH_2Cl_2 at 27°C for 2×24 h. The filtrates were concentrated in a rotavap below 30°C. *Hortonia angustifolia* yielded a blackish green CH_2Cl_2 extract of leaves (5.5 g), a brown semi-solid extract of stem bark (0.43 g), light brown oily bark extract (0.51 g), and light yellow oily extract of root (1.2 g). The other two plants also yielded the extracts in comparable yields. Each of the extracts was subjected to the mosquito larvicidal assay using second instar larvae of *Aedes aegypti*.

2.3. HPLC analysis of leaf extracts

The CH₂Cl₂ extracts of leaves collected from nine locations were subjected to HPLC analysis (Waters 2690, separation module; Waters 996, photodiode array detector, UV-visible light spectrophotometer with optical resolution of 1.2 nm per diode and wavelength from 190 to 800 nm). Each of the CH₂Cl₂ extract of leaves (1.0 mg) was dissolved in 5 mL CH₃CN and filtered using sep-pak C₁₈ cartridge; 10 µL of each extract was injected to the HPLC (C₈ symmetry, reverse phase, 5 µm, 3.9 × 150 mm column) using the following solvent mixtures of CH₃CN/H₂O (1 : 1 until 7 min; 3 : 2 until 28 min; 1 : 1 until 40 min). The PDA detector was set at 200–400 nm.

2.4. Mosquito larvicidal assay

Concentrations of 500 ppm for plant extracts, 25 ppm for fractions and 10–0.625 ppm for pure compounds were used for the mosquito larvicidal bioassay against the second instar larvae of *Aedes aegypti* (Bandara, Kumar, Jacobsson and Molleyres, (2000)). Percentage mortality was determined after 24 h and 48 h for these active extracts, fractions and pure compounds, and LC₅₀ values were calculated using probit analysis (MSTAT package). Control mortalities were adjusted using Abbot's formula (Matsumura (1985)).

2.5. Antifungal assay

Antifungal assay was carried out on extracts and pure compounds using the TLC bioassay technique with benlate (methyl-1(butylcarbonyl)-2-benzimidazolecarbamate, Du Pont, USA: 50% active ingredient) (1 mg) as the standard fungicide against the fungus *Cladosporium cladosporioides* (Klarman and Stanford, (1968)).

2.5.1. Spore germination assay

Conidia (5–7 days old) of *C. cladosporioides* were used in a spore germination bioassay to determine the percentage of germination of the fungal spores against the test compounds Anon, (1943).

2.6. Protein extraction and gel electrophoresis

Each leaf material was subjected to protein extraction and gel electrophoresis. Each of the leaf materials (0.1 g) was quickly weighed and frozen in liquid N₂ and extracted with 0.5 mL of boiling Laemmli sample buffer containing 4% sodium dodecylsulphate and 1 mM phenylmethylsulphonylfluoride (Laemmli (1970)). The entire harvesting procedure to this point was completed within 2 min to minimise degradation of protein extracts. This mixture was immediately placed in a boiling water bath for 10 min and then centrifuged at 15,000 rpm for 20 min. The supernatant was collected and introduced to a gel. Gel electrophoresis of the extracted protein was carried out at 150 mV and the gel was stained for 20–30 min.

2.7. Extraction and isolation of compounds

Ishwarane (1). Air-dried, powdered leaves (350 g) of *H. angustifolia* were extracted repeatedly into CH₂Cl₂ (3 × 500 mL) at 27°C. The combined CH₂Cl₂ extract was

concentrated in a rotavap, to obtain a brown oil (25 g). The oil was subjected to MPLC on Si gel (step gradient hexane to hexane/methanol (95 : 5)) to give several fractions. The first fraction (2.26 g) showed antifungal activity. This was subjected to flash chromatography on silica gel (30 g) (eluent: CH₂Cl₂-hexane, 1 : 1) to give ishwarae (1) (100 mg) as a colourless oil which was identified by comparing its ¹H/¹³C NMR data with those reported for the compound (Govindachari, Mohammed, and Pratha (1970), Cory and McLaren (1977), Piers and Hall (1977)).

(4S)-4-methyl-2-(11-dodecenyl)-2-butenolide (2) and (4S)-4-methyl-2-(11-dodecynyl)-2-butenolide (3) (Ratnayake et al., (2001)). The next combined fraction (2.15 g), which elicited mosquito larvicidal activity, was subjected to Si gel flash chromatography (eluent: step gradient from hexane to CH₂Cl₂/hexane (4 : 1)) to obtain the mosquito larvicidal pure butenolides (2) (328 mg; LC₅₀ = 0.41 ppm) and (3) (78 mg; LC₅₀ = 0.47 ppm).

Compounds 1, 2 and 3 were similarly isolated from the leaves of *H. floribunda* and *H. ovalifolia* in comparable yields using identical chromatographic conditions. In addition, compounds 1, 2 and 3 were also isolated from the bark and roots of the three species (Table 2).

(4S)-4-Methyl-2-((2R)-hydroxy-11-dodecenyl)-2-butenolide (4). A second, later-eluted combined fraction (8.41 g) showed larvicidal activity at 50 ppm. This fraction was subjected to MPLC (step gradient: EtOAc-hexane (1 : 49) to MeOH), and flash chromatography of a resultant larvicidal fraction (eluent: EtOAc-hexane, 3 : 7) gave compound 4 (6 mg) as a light brown oil. $[\alpha]_D^{27} = +191.66^\circ$ (*c* 0.0006 g cm⁻³ in CHCl₃); UV (CHCl₃); $\lambda_{\max}$ (nm, log ϵ), 299.6 (0.0341) and 242.6 (2.0419); IR: $\nu_{\max}$ (cm⁻¹) 3440, 3100, 3000, 2930, 2860, 1750, 1640, 1450, 1370, 1320, 1200, 1070, 1020, 950, 855; ¹H NMR (300 MHz, CDCl₃): δ ~1.25 (14H, br s, H-8, 9, 10, 11, 12, 13 and 14), 1.40 (3H, d, *J* = 6.7 Hz, H-5), 2.03 (2H, m, H-15), 2.74 (2H, m, H-6), 4.55 (1H, m, H-7), 4.65 (1H, m, H-4), 4.92, 4.99 (1H each, dd, *J* = 10, 1.5 Hz, *J* = 17, 1.5 Hz, H-17), 5.80 (1H, dd, *J* = 17, 10 Hz, H-16), 6.56 (1H, m, H-3); ¹³C NMR (75 MHz, CDCl₃): δ 14.1 (C-5), 27.8 (C-6), 28.9 (C-8), 29.1 (C-9), 29.2 (C-10), 29.3 (C-11), 29.4 (C-12 and 13), 29.7 (C-14), 33.8 (C-15), 71.4 (C-7), 77.8 (C-4), 114.1 (C-17), 130.9 (C-2), 139.2 (C-16), 149.7 (C-3), 168.6 (C-1); EIMS *m/z* (rel. int. %): 280 (12), 263 (2), 254 (10), 233 (6), 212 (12), 198 (30), 177 (14), 164 (36), 155 (58), 137 (64), 123 (44), 111 (60), 95 (72), 81 (100), 70 (94), 57 (78); HRMS: for C₁₇H₂₈O₃ requires 280.2038; found 280.2041. LC₅₀ = 1.60 ppm against 2nd instar larvae of *A. aegypti*.

Compound 4 was isolated as a minor metabolite in comparable yields from the leaves of the other two species of *Hortonia* (*H. floribunda* and *H. ovalifolia*), using an identical chromatographic procedure as that described earlier.

2.8. Preparation of compound 5

Compound 2 (39 mg) dissolved in EtOAc (5 mL) was subjected to hydrogenation in the presence of Pd-C catalyst at 27°C for 16 h. The mixture was filtered and evaporated to give compound 5 (31 mg) (yield 79%) as a white powder. The hydrogenated product was UV active and it was inactive against anisaldehyde spraying reagent. M.p. 48°C; $[\alpha]_D = 35.29^\circ$ (*c* 0.0004 g cm⁻³ in CHCl₃); UV (CHCl₃); $\lambda_{\max}$ (nm, log ϵ) 240.5 (1.201); IR (KBr); $\nu_{\max}$ (cm⁻¹) 2925, 2850, 1750, 1395, 1210, 1180; ¹H NMR (300 MHz, CDCl₃) δ 0.88 (3H, t, *J* = 6.2 Hz, H-17), 1.25 (18H, br s, H-8, 9, 10, 11, 12, 13, 14, 15 and 16), 1.36 (1H, m, H-2), 1.41 (3H, d, *J* = 6.2 Hz, H-5), 1.48 (2H, m, H-7), 2.46 (2H, m, H-6), 2.57 (2H, m, W_{1/2} = 18 Hz, H-3), 4.46

(1H, dq, $J = 6.2, 14$ Hz, H-4) EIMS m/z (rel. int.%): 269 (7) [$M^+ + 1$], 251 (3), 233 (2), 149 (4), 135 (3), 125 (3), 113 (65), 100 (100), 81 (33), 55 (60), 41 (77).

3. Results and discussion

Although the three endemic species of *Hortonia* are found in a range of environments from low elevations (*H. angustifolia*) to the montane regions (*H. floribunda* and *H. ovalifolia*), there have been no reports of the medicinal or economic uses of these plants in Sri Lanka. The CH_2Cl_2 extracts of the leaves of the three species exhibited significant mosquito larvicidal activity against the 2nd instar larvae of *A. aegypti* (100% mortality at 62.5 ppm). We had previously reported that the bioassay-guided fractionation of the dichloromethane extracts of all three species yielded compounds **2** and **3** (Ratnayake et al., (2001)). The same extracts, after careful chromatography, yielded the antifungal ishwarane (**1**) and the new, minor butenolide (**4**).

Butenolide (**4**), obtained as a colourless oil, had an elemental composition of $\text{C}_{17}\text{H}_{28}\text{O}_3$ based on its HRMS. The $^1\text{H}/^{13}\text{C}$ /APT/HMQC NMR data obtained for **4** showed 17 carbon resonances with 27 attached hydrogen atoms along with four degrees of unsaturation. The IR spectrum of compound **4** showed absorptions at 3440 and 1320 cm^{-1} indicating the presence of an alcohol group and an α, β -unsaturated- γ -lactone carbonyl at 1750 cm^{-1} ; the UV absorption at λ_{max} 242 nm corroborated with the latter. The downfield ^1H and ^{13}C NMR resonances were assigned to a double bond (δ 6.56, m, (δ 149.7, 130.9)), an aliphatic methine residue attached to an oxygen atom (δ 4.65, m, (δ 77.8)), and a α -lactone carbonyl (δ 168.6). The ^1H and ^{13}C NMR data were assigned unambiguously by COSY, HMQC and HMBC experiments confirming that compound **4** possessed a hydroxyl group (δ 4.55, 1H, m, C-7 (δ 71.4)) and a terminal double bond (δ 4.92, 4.99, 1H each, dd, $J = 10, 1.5$ Hz, $J = 17, 1.5$ Hz, C-17 (δ 114.1); 5.80 (1H, dd, $J = 17, 10$ Hz, C-16 (δ 139.2)) on a C_{12} side-chain. All four sites of unsaturation required by the molecular formula were accounted for by the terminal double bond and the α, β -unsaturated γ -lactone ring.

The connectivities in the ring were clearly established by the HMBC data. In the HMBC of compound **4** (Figure 1), H-3 (δ 6.56) showed two-bond correlations between C-2 (δ 130.9) and C-4 (δ 77.8) and a three-bond correlation via the ring double bond to C-1 (δ 168.6) thus establishing the position of the carbonyl group, and that H-3 was flanked by the side chain (C_{12}) attached to C-2 and the secondary methyl bearing oxymethine (C-4). Furthermore, the HMBC correlation observed between H-4 (δ 4.65) and C-2 (δ 130.9), together with that between the C-6 methylene (δ 2.74) in the side chain and C-1 (δ 168.6), established that a C_{12} side chain was situated at C-2. The position of the hydroxyl group at C-7 was confirmed by a COSY correlation between H-6 (δ 2.74) and H-7 (δ 4.55) and by HMBC couplings between H-7 and C-2 and H-6 and C-7. Compound **3** isolated previously by us from the same extracts of *Hortonia* differs from **4** through the absence of an hydroxyl group at C-7. Previous chemical studies on *Litsea japonica* (Lauraceae) have resulted in the isolation of two related butenolides, hamabiwalactones A and B, which also possess a side-chain at C-2 and a methyl group at C-4, differing from compound **4** by the presence of a double bond between C-6 and C-7 and the absence of a hydroxyl group at C-7 (Tanaka, Nakamura, Ichino, Ito and Tanaka, (1990)). In addition, plants of the family Annonaceae elaborate a wide variety of butenolides of biological importance (Zeng et al. (1996)). The stereochemistry at C-4 and C-7 in compound **4** was assigned *S* and *R* configuration,

Table 1. Mosquito larvicidal activity (LC₅₀) of the dichloromethane extracts of plants of the genus *Hortonia* found in different geographical locations from low elevations to montane.

Location	LC ₅₀ ppm		
	Leaves	Stem bark	Roots
<i>H. angustifolia</i>			
Pitabeddara (84–137 m) ^a	38.4 ^b (28.7) ^c	0.99 (0.42)	0.52 (0.11)
Atwaltota (127 m)	34.6 (22.2)	0.51 (0.31)	0.36 (0.23)
Wewelkandura (140 m)	32.4 (24.9)	0.63 (0.32)	0.61 (0.12)
Kotapola (140 m)	64.2 (35.6)	0.70 (0.29)	0.70 (0.41)
Kanneliya (165 m)	111.8 (62.3)	0.86 (0.30)	0.78 (0.43)
Deniyaya (420 m)	124 (82.4)	0.65 (0.37)	0.66 (0.25)
<i>H. floribunda</i>			
Kelabokka (1120 m)	71.3 (33.4)	0.76 (0.23)	0.41 (0.22)
Hakgala (1995 m)	67.2 (28.7)	8.86 (2.87)	ND
<i>H. ovalifolia</i>			
Adam's Peak (2007 m)	70.2 (32.4)	1.14 (0.51)	0.95 (0.32)

^aElevation above sea-level. ^b24 HAI. ^c48 HAI.

ND = not determined.

Table 2. Comparison of yields of compounds from the dichloromethane extracts of the three *Hortonia* species.

Species	Compound	% Yield ^a		
		Leaf	Bark	Roots
<i>H. angustifolia</i>	1	0.40	0.32	1.60
	2	1.31	42.75	60.62
	3	0.31	0.29	1.35
<i>H. floribunda</i>	1	0.37	0.30	1.52
	2	1.23	38.92	56.62
	3	0.29	0.22	1.22
<i>H. ovalifolia</i>	1	0.56	0.40	1.43
	2	0.98	51.23	58.52
	3	0.36	0.38	1.28

^awith respect to the plant extract; dry weights of: leaves = 350 g, bark = 1600 g, roots = 600 g.

(Table 3). Ishwarane (**1**), an essential oil found in plants and fruits (Oyedeki, Adeniyi, Ajayi, and König, (2005)), although not mosquito-larvicidal, showed significant antifungal activity (Table 4). Importantly, compound **2** was found in excessively high yields (Table 2), particularly in the stem bark (38.92–51.23%) and roots (56.62–60.62%) based on the weight of the extract of all three plants, making *Hortonia* a rich repository of biological activity. Butenolide (**4**) on the other hand, showed much less larvicidal activity (LC₅₀: 1.66 ppm) in comparison to compounds **2** and **3** (Figure 2). Thus, it appears that side chain oxygenation might be responsible for the reduced larvicidal activity of **4**. On the other hand, when the major butenolide (**2**) was subjected to hydrogenation, the resultant, fully

Table 3. Mosquito larvicidal activity of compounds **1–3** against *A. aegypti*.

Concentration (ppm)	1	2 ^a	3 ^b
10.0	0	–	–
2.50	–	100 ^c	100
1.25	–	100	100
0.625	–	70 (100) ^d	70 (100)
Control	0 (0)	0 (0)	0 (0)

^aLC₅₀ at 24 h = 0.41 ppm.
^bLC₅₀ at 24 h = 0.47 ppm.
^cMean of four replicates (ten larvae each) was used in all experiments.
^dMortality after 48 h given in brackets; control mortalities were adjusted using Abbot's formula.

Table 4. Antifungal activity of compounds **1–3** against *C. cladosporioides*.

Compound	Diameter (mm) of the inhibition zone ^a	% Germination ^c
1	23 (37) ^b	2.5 ^d
2	32 (44)	0.5
3	29 (44)	1.0

^aIn the TLC bioassay.
^bDiameter of the inhibition zone of control benlate.
^cSpore germination assay.
^dAt 6.25 ppm (concentrations higher than 6.25 ppm produced zero germination. The control benlate produced 0% germination at all concentrations).

reduced compound **5** was inactive, suggesting that unsaturation in compounds **2** and **3** are necessary for the larvicidal activity.

In an attempt to explore the similarities between primary metabolites in the three species of *Hortonia*, a protein analysis of the leaves was carried out. For this study, leaf specimens were collected from the following seven locations representing the three species: Kanneliya, Kelebokka, Deniyaya, Pitabeddara, Kotapola, Hakgala and Adam's Peak. Proteins were extracted from fresh leaf material from the seven specimens of *Hortonia* species under identical conditions according to an established protocol (Laemmli, (1970)). Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) showed that all leaf samples of *Hortonia* species exhibited identical protein bands. The molecular weights of the protein bands from the leaf extracts appeared below and above that of the standard bovine serum albumin (BSA, 68 KD).

The latest revision of the family Monimiaceae by Dassanayake considered leaf morphology as a key character for the speciation of the genus *Hortonia* (Dassanayake, (1996)). Leaves narrowly elliptic, broadly elliptic or narrowly ovate were described as *H. ovalifolia*, while *H. floribunda* had leaves lanceolate to narrowly ovate, and leaves lanceolate to narrowly elliptic were the key features of *H. angustifolia*. *Hortonia floribunda* is a tree, 15 m or taller and is found in the montane zone, at about 1300 m and higher. It grows in the shade, often near streams and flowers from March to November. *Hortonia ovalifolia* is a small tree, 7 m tall, found only in Adam's Peak, at about 2000 m and higher elevation, and flowers between March and August. *Hortonia angustifolia* is a small tree or

Figure 2. LC_{50} values of compounds (2)–(5).

shrub growing typically to about 5 m, abundant in moist lowland, from sea coast to an elevation of about 700 m (Dassanayake (1996)).

However, Money et al. had considered *Hortonia* to be a single species in Sri Lanka [21]. Renner claims that *H. ovalifolia* and *H. angustifolia* are identical to *H. floribunda*, the most basal group, probably because of its bisexual flowers, a trait absent from all the other Monimiaceae (Renner (1998)). The bioactivity similarities between the primary and secondary metabolites, particularly the similarities between the relative abundance of the latter, of the three species of the genus *Hortonia*, lends credence to the claims of both Money and Renner.

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