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RESEARCH NOTE

Complete 2D NMR assignment and antifungal activity of ishwarane isolated from *Hortonia*, a genus endemic to Sri Lanka

Rukmal Ratnayake[1], Susanthi Jayasinghe[1], B.M. Ratnayake Bandara[1], Raymond J. Andersen[2] and Veranja Karunaratne[1*]

[1] *Department of Chemistry,University of Peradeniya, Peradeniya, Sri Lanka.*

[2] *Department of Earth & Ocean Sciences, University of British Columbia, Vancouver, B.C., Canada.*

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Abstract: The tetracyclic sesquiterpene, ishwarane, was isolated from the leaves of the representative species of the genus *Hortonia*, *H. angustifolia, H. floribunda* and *H. ovalifolia* collected in Sri Lanka. The complete 2D NMR assignments are reported. Ishwarane exhibited antifungal activity against *Cladosporium cladosporioides*.

Keywords: Antifungal activity, *Hortonia*, ishwarane, 2D NMR assignments, Sri Lanka.

INTRODUCTION

The genus *Hortonia* is endemic to Sri Lanka and belongs to the Order Laurales and Family Monimiaceae. Members of this family are weeds or shrubs and rarely climbers. This family comprises of about 39 genera and 440 species which are widely spread in the Southern Hemisphere, mainly tropical and subtropical regions of the Americas[1]. Members of Family Monimiaceae are present in Sri Lanka, Oceania, Polynesia, Australia, Malaysia, Madagascar and South America[1, 2]. 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 Monimiaceae[2].

Dassanayake (1996) lists three distinct Monimiaceae species {*H. floribunda* Wight ex Arn., *H. angustifolia* (Thw.) Trimen and *H. ovalifolia* Wight} in Sri Lanka[3]. Some phytogeographers consider the genus *Hortonia* to have originated in Gondwanaland about 100-200 million years ago[4]. The present study was initiated with a view to isolating the bioactive compounds from the plants of genus *Hortonia* in Sri Lanka.

Although three species of *Hortonia* are found in a range of environments from sea level (*H. angustifolia*) to the montane (*H. floribunda* and *H. ovalifolia*) regions, there have been no reports of the medicinal use of these plants in Sri Lanka. It has been previously reported that the bioassay-guided fractionation of the dichloromethane extracts of all three species yielded two mosquitolarvicidal butenolides[5]. Herein we report the isolation, complete 1D and 2D NMR assignment and the antifungal activity of the sesquiterpene hydrocarbon ishwarane from all three species of *Hortonia*.

METHODS AND MATERIALS

Analytical Thin Layer Chromatography (TLC) was performed on Merck Kieselgel 60 F_{254} aluminium foil plates. TLC plates were visualized by spraying with anisaldehyde followed by heating. Medium Pressure Liquid Chromatography (MPLC) and flash chromatography were performed on Merck Kieselgel 60 (230-400 mesh ASTM). ^{1}H and ^{13}C NMR, COSY, DEPT, HETCOR, HMQC and HMBC spectra were recorded on a VARIAN (^{1}H 500 and ^{13}C 125 MHz) in $CDCl_3$ with TMS as the internal standard. EIMS 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.

Specimens of *H. angustifolia* were collected from Kanneliya (Southern Province), *H. floribunda* from Hakgala (Central Province), and *H. ovalifolia* from the foothills of Adam's Peak (Central Province)

*Corresponding author

in September 1998. The plants were identified by Dr. D.S.A. Wijesundara, Director, Royal Botanic Gardens, Peradeniya and voucher specimens were deposited at the National Herbarium, Peradeniya.

Air-dried, powdered leaves of *H. angustifolia* were extracted repeatedly into CH_2Cl_2 (3 x 500 mL) at 27 °C. The combined CH_2Cl_2 extract was concentrated *in vacuo*, to obtain 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: CH_2Cl_2-hexane, 1:1) to give ishwarane (100 mg) (0.4%) as a colourless oil. Similarly Ishwarane, was isolated from the leaves of *H. floribunda* and *H. ovalifolia* in comparable yields using identical chromatographic conditions. ^{1}H NMR (500 MHz, C_6D_6); ^{13}C NMR (125 MHz, C_6D_6): See Table 1; HRESIMS $[M]^+$: 204.18785 (calculated for $C_{15}H_{24}$, 204.18780).

Antifungal assay was carried out on pure ishwarane (1 mg). An aqueous solution of benomyl, 50% w.p. {methyl 1-[(butylamino) carbonyl]-H-benzimidazol-2-ylcarbamate} (1 mg) were used in the assays for comparison purposes. TLC plates (silica gel $PF_{254=366}$, 0.5 mm x 20 cm x 20 cm) were spotted with pure ishwarane and the aqueous solution of Benor eluted with CH_2Cl_2, and then air dried (overnight) to evaporate all traces of remaining solvents. A spore suspension of *Cladosporium cladosporioides* in Czapek-dox nutrient solution (CNS) was sprayed on to the plates and incubated in a moist chamber at 28±2 °C for two days. Diameter of the inhibition zones were measured[6].

Conidia (5-7 d old) of *C. cladosporioides* were suspended in sterile distilled water and was passed through glass wool to remove mycelia. The conidia were washed three times by centrifugation (3 min at 3000 rpm) and resuspension of the resulting pellet in sterile distilled water. Conidia in the suspension were adjusted to about $5x10^2$ mL^{-1}. A series of solutions of the compound was prepared in 10% EtOH in water. Drops (10 µL) from each were placed on clean glass slides. Care was taken to apply the drops over a uniform area on the glass slides. The control was prepared without the compound. The glass slides were then incubated in a moist chamber for 6 h at room temperature. Germination was stopped at the end of the incubation period by adding a drop of lactophenol.

Table 1: 1D and 2D NMR spectroscopic data of ishwarane

Carbon	^{13}C	DEPT	^{1}H	multiplicity (*J*, Hz)	^{1}H-^{1}H	HMBC
1	33.9	CH_2	a 0.93 b 1.52	m m	1b, 2b 1a	2 2,5, 9, 10, 12
2	24.3	CH_2	a 1.44 b 1.48	m m	1b, 3a 1a	10 ---
3	31.1	CH_2	a 1.00 b 1.24	br s m	3b, 2a 3a	1, 4, 5, 2 1, 5
4	38.9	CH	1.52	m	15	15, 2
5	35.93	C	----------		-----	-----
6	34.9	CH_2	a 1.55 b 1.67	br d br s	6b, 7 6a	7, 11,14 7, 8, 11, 14
7	20.3	CH	0.48	br s	8, 6a	13, 5
8	23.4	CH	0.85	m	7, 9a	7, 13, 11
9	36.2	CH_2	a 1.22 b 1.93	br d d (11.9)	9b, 8 9a	7, 8 , 11 5, 8, 10,11,12
10	44.2	C	---------		-------	----------
11	22.7	C	---------		--------	----------
12	39.5	CH_2	a 0.97 b 2.05	br d d (11.9)	12b 12a	1,5,7,9, 10, 11,13 5, 7, 8, 9, 10, 11,13
13	20.5	CH_3	1.18	s	8	7, 8, 11, 12
14	17.0	CH_3	0.79	s	6b	4, 5, 6, 10
15	16.8	CH_3	0.74	d (8)	4	3, 4, 5

Four randomly selected areas on each slide were observed under a microscope and germinated and non-germinated conidia were counted. Percentage germination was determined by using average values of the four replicates[7].

RESULTS AND DISCUSSION

Ishwarane was first isolated from the plants *Aristolochia indica*[8] and *Cembopentalum penduliflorum*[9]. It has been isolated from the essential oils of *Bixa orellana*[10], *Corallocarpus epigaeus*[11] and *Piper fulvescens*[12]. Its structure was elucidated 35 years ago by chemical transformations and spectroscopic techniques[8,9,13] (Figure 1). The oxygenated derivatives ishwarol[14] and ishwarone[15] have been reported from the essential oils of *Aristolochia indica* and more recently, ishwarol B was isolated from the essential oils of *Cedrelopsis grevei*[16].

Ishwarane ($C_{15}H_{24}$, M^+ 204.1) was isolated from the CH_2Cl_2 extracts of the leaves of all three species of *Hortonia* in identical yields by MPLC followed by column chromatography and was subjected to a full set of 1D and 2D NMR experiments (Table 1).

Interestingly, it showed antifungal activity against *C. cladosporioides* (Table 2). A recent report showed that the essential oil from *Piper guineense* from Nigeria containing ishwarane inhibited the growth of *Pseudomonas aeruginosa* UCH 655 strain[17]. The occurrence of ishwarane in all the representative species in the genus *Hortonia* is of chemotaxonomic significance.

Table 2: Antifungal activity of ishwarane against *C. cladosporioides*

Compound	Diameter (mm) of the Inhibition zone [a]	% Germination [b]
Ishwarane	23	2.5
Control (Benor)	37	0

[a] In the TLC bioassay

[b] Spore germination assay at 6.25 ppm. Concentrations higher than 6.25 ppm produced zero germination. The control benomyl {methyl-1- (butylcarbonyl)-2- benzimidazolecarbamabamate} produced 0% germination at all concentrations.

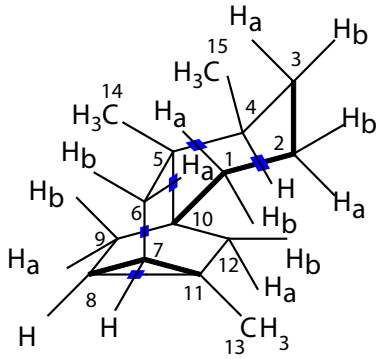

Figure 1: Ishwarane (numbering of carbons according to Joulain and Konig[18])

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