SUPPLEMENTARY MATERIAL

GC/MS profiling, *in vitro* anti-leptospiral and haemolytic activities of *Boesenbergia rotunda* (L.) Mansf. used as a medicinal plant by Nicobarese of Andaman and Nicobar Islands

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GC/MS profiling, in vitro anti-leptospiral and haemolytic activities of *Boesenbergia rotunda* (L.) Mansf. used as a medicinal plant by Nicobarese of Andaman and Nicobar Islands

Leaves of the plant *Boesenbergia rotunda* are used by the Nicobarese tribe of Andaman and Nicobar Islands, India, to prepare traditional medicine for treating fever, headache and body ache. In the present investigation, methanol fraction of these leaves were analysed by GC/MS that revealed the presence of 25 compounds. The anti-leptospiral activity of methanol crude extract was determined by both microdilution and macrodilution methods. The MICs of the extract were tested against 24 pathogenic leptospiral strains and ranged between 62.5-125 µg/ml in both microdilution and macrodilution. The range of MBCs was 250 µg/ml and 500 µg/ml in macrodilution and microdilution respectively. The crude extract was subjected to cytotoxic studies and found to have negligible or no haemolytic activity, exhibiting IC₅₀ values of greater than 4mg/ml. Further in-vivo studies are needed to investigate the pharmacological and toxicological properties of *Boesenbergia rotunda*, before it can be considered as a new anti-leptospiral agent.

Keywords: *Boesenbergia rotunda*, anti-leptospiral activity, Car Nicobar Island, Nicobarese

Experimental

Materials

Plant Material

Leaves of *B. rotunda* are used in curing for fever, headache and body ache by Nicobarese as part of their herbal medicinal practices. Nicobarese are indigenous tribal population residing in scattered Islands of the Nicobar district of Andaman and Nicobar Islands. Leaves of *B. rotunda* were collected from the forest area of Chukchucha village (N 09°12.092' E 092°48.883'), with the help of local Traditional knowledge practitioners (TKPs) of the Car Nicobar Island, during March 2012. The plant material brought to the research laboratory, Regional Medical Research Centre (ICMR), Port Blair, was later identified at the Botanical Survey of India, Port Blair, and its voucher specimen maintained in the Institute library (Voucher No. ANH/CN/27).

Leptospiral strains

In the present study, a total of 24 leptospiral reference strains were used (obtained from the Leptospira Reference Laboratory, Port Blair). All strains were maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30°C (Faine et al. 1999).

Methods

Extraction of plant material

Leaves of plants were separately dried under shade, pulverized by a mechanical grinder and passed through a 40-mesh sieve, in order to obtain a fine powder. Hundred grams of coarsely powdered dry leaves were extracted by cold percolation method with 95% methanol as solvent, by keeping it for 72 hours at room temperature. The whole plant extract was collected in a conical flask, filtered, and the solvent evaporated to dryness under reduced pressure in an evaporator (Eppendroff 5304) at 40-45°C. The residues were stored at 4°C for future use.

Susceptibility testing – Anti-leptospiral activity

Testing of each combination of leptospiral strain and the plant compound was performed in parallel. Three parallel tests were performed at different times to determine the reproducibility of results. Cumulative efficacy was described as the MIC₉₀ and MBC₉₀.

Microdilution method

The antileptospiral activity of compounds was studied using Broth microdilution testing method with AlamarBlue dye, as previously described (Murray & Hospenthal 2004). Briefly, broth microdilution testing was performed with 96-well round-bottom microtiter plates. Each plate included positive controls (bacteria without any antimicrobial compound), negative controls (medium only), and serial two fold dilutions of crude extract. The experiment was carried out in EMJH medium.

Antimicrobial-containing wells consisted of final concentrations of each compound, ranging from 7.81 to 1000 µg/ml. For inoculum preparation, *Leptospira* cultures were grown for 7 days (2×10^8 cells/ml) at 30°C. Following the addition of 100-µl inoculum (containing 2×10^6 leptospiral organisms/ml) to the antimicrobial-containing wells and positive control wells, the plates were incubated at 30°C. The final volume of each well was 200 µl. After 3 days of incubation, 20 µl of concentrated (10X) Alamar Blue was added to all wells and incubated for 48 h at 30 °C. On the fifth day of incubation, the colour in each well was noted. The MICs were observed visually, by comparing wells with lowest concentration of antimicrobials, which did not show any colour change (from blue to pink/purple). The plate was also read by spectrophotometer at 570 nm.

Macrodilution method

Broth macrodilution MICs and MBCs were carried out as previously described (Murray & Hoshenthal 2004), with minor modification. Crude extract and antibiotics-containing tubes were prepared by serial two fold dilutions of antimicrobials in EMJH medium, in order to obtain final concentrations of 1000 to 7.81 µg/ml (200 to 0.01 U/ml for penicillin G and 200 to 0.01 µg/ml for Doxycycline). Leptospiral strains were added to each tube, at a concentration of 10⁶ organisms/ml (final volume, 2 ml), and tubes were incubated at 30°C for 7 days. The antimicrobial concentration present in the tube without visual growth was observed using Petroff-Hauser counting chamber, under dark field microscope, and recorded as the MIC. MBC testing was performed by transferring 10 µl of the fluid from each tube without visible growth into 2 ml of fresh EMJH medium. The lowest antimicrobial concentration that did not yield any growth by visual inspection, post 3 weeks of incubation at 30°C was recorded.

Anti-haemolytic activity

In vitro haemolytic activity was performed using spectrophotometer method (Yang et al. 2005). Briefly, 0.5 ml of the cell suspension was mixed with 0.5 ml of plant crude extracts (156.25, 312.5, 625, 1250, 2500 and 5000 µg/ml concentrations in phosphate buffer saline). 5% of SDS and PBS served as positive and negative controls respectively. After 1hr of incubation at 37°C, cell suspensions were centrifuged for 10 minutes at 1500 x g and supernatants were transferred to a flat bottom 96-well plate. The absorbance (A) was read at 492nm by ELISA reader (Tecon). Since the crude extract was coloured, each concentration of the extract had separate blank. For preparation of blanks, plant extracts were added to separate tubes without blood and were treated in the same manner as that of the tests. For each concentration, the experiment was performed in triplicate.

The percentage of haemolysis was calculated using the formula, Haemolysis (% of control) = $(A_{ts} - A_{sc}) / (A_{wc} - A_{sc}) \times 100$. Where, A_{ts} is the absorbance of test sample, A_{sc} is absorbance of the control (saline control) and A_{wc} is the absorbance of the control (water control).

Preliminary phytochemical analysis

The plant extracts were screened for the presence of different classes of secondary metabolites including alkaloids, flavonoids, triterpenes, sterols, tannins and saponins using previously described methods (Harborne, 2007).

GC-MS analysis

Gas Chromatography/Mass spectrometry (GC/MS) analysis was performed at the South India Textile Research Association (SITRA) – Coimbatore – Tamilnadu. Methanol extracts were diluted with HPLC grade methanol. GC/MS analyses were carried out using a Thermo GC – Trace Ultra VER: 5.0, THERMO MS DSQ II, equipped with DB 35 – MS capillary standard non-polar column having polydimethylsiloxane as stationary phase, column length 30m, internal diameter 0.25 mm and film thickness 0.25µm. The temperature of injector was 250°C and 1.0 µL of the sample was injected in a split mode with a split ratio of 30:1. Helium (He) was used as a carrier gas, and the flow rate of gas was 1.0 µL/min. The temperature program was: initial temperature of 50°C and held for 1 min, ramping at a rate of 5°C/min up to 280°C, which was finally held at this temperature for 5 min. For mass spectra determination, MSD was operated in electron ionization (EI) mode, with the ionization energy of 70 eV, while the mass range scanned was 3-500 m/z. The temperature of ion source was 230°C, while it was 150°C for MS quadropole. The identification of components was based on the comparison of their mass spectra with those of NIST mass spectral library (Massada, 1996).

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Table S1. Susceptibility of 24 serovars of *Leptospira* spp. to crude extract of plant *B. rotunda* and antibiotics by microdilution and macrodilution

S. No.	Genotype/Strain	<i>Boesenbergia rotunda</i>				Doxycycline				Penicillin			
		MIC ₉₀ (µg/ml)		MBC ₉₀ (µg/ml)		MIC ₉₀ (µg/ml)		MBC ₉₀ (µg/ml)		MIC ₉₀ (µg/ml)		MBC ₉₀ (µg/ml)	
		Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro
1	L. interrogans/Ballico	125	125	250	500	3.9	7.81	62.5	125	1.95	3.9	31.25	62.5
2	L. interrogans/ Bankinang 1	125	125	250	500	3.9	7.81	62.5	125	3.9	7.81	62.5	62.5
3	L. interrogans/ Paidjain	62.5	62.5	250	500	3.9	3.9	62.5	62.5	1.95	3.9	31.25	62.5
4	L. interrogans/ Hond utrech-IV	62.5	125	250	500	3.9	7.81	62.5	125	3.9	15.6	62.5	125
5	L. interrogans/ Djasiman	125	125	250	500	3.9	3.9	62.5	62.5	3.9	7.81	62.5	125
6	L. interrogans/ Moskva V	62.5	62.5	250	500	7.81	7.81	125	125	1.95	3.9	62.5	62.5
7	L. interrogans/ CH-31	62.5	62.5	250	500	3.9	3.9	62.5	125	1.95	3.9	62.5	62.5
8	L. interrogans/ Hebdomadis	125	125	250	500	3.9	3.9	62.5	62.5	1.95	3.9	62.5	125
9	L. interrogans/ Pomona	125	125	250	500	7.81	7.81	125	125	3.9	7.81	62.5	125
10	L. interrogans/ RGA	62.5	62.5	250	500	7.81	7.81	125	125	1.95	3.9	62.5	125
11	L. interrogans/ Salinem	125	125	250	500	7.81	7.81	125	125	1.95	3.9	31.25	62.5
12	L. interrogans/ Hardjoprajtno	125	125	250	500	7.81	7.81	125	125	3.9	7.81	62.5	62.5
13	L. borgpetersenii/ Poi	62.5	62.5	250	500	3.9	3.9	62.5	62.5	1.95	3.9	31.25	62.5
14	L. borgpetersenii/ Sari	125	125	250	500	3.9	7.81	62.5	125	7.81	15.6	125	125
15	L. borgpetersenii/ Perepelician	62.5	62.5	250	500	3.9	3.9	62.5	62.5	3.9	7.81	62.5	125
16	L. noguchii/ CZ-214-K	125	125	250	500	3.9	7.81	62.5	125	1.95	3.9	62.5	62.5
17	L. noguchii/ 507	125	125	250	500	3.9	3.9	62.5	62.5	3.9	7.81	62.5	125
18	L. santarosai/ 1343-K	62.5	125	250	500	7.81	7.81	125	125	1.95	3.9	62.5	62.5
19	L. krischneri/ Nicalaeva	125	125	250	500	3.9	3.9	62.5	62.5	1.95	3.9	62.5	62.5
20	L. krischneri/ 3522 C	62.5	125	250	500	3.9	3.9	62.5	62.5	1.95	3.9	62.5	125
21	L. fainei/ But-6	125	125	250	500	7.81	7.81	125	125	3.9	7.81	62.5	125
22	L. meyeri/ Peludo	125	125	250	500	3.9	7.81	62.5	125	1.95	3.9	62.5	125
23	L. inadai/ L-14	125	125	250	500	3.9	3.9	62.5	62.5	3.9	7.81	62.5	125
24	L. biflexa/ Patoc-1	125	125	250	500	7.81	7.81	125	125	7.81	15.6	125	125

Table S2. Chemical composition of *B. rotunda* leaves methanol extract analysed by GC/MS

S.No.	SI	RSI	Compound Name	Mol Formula	Mol. Wt	Area %
1	409	945	(E)-5-Methyl-2-methylene-2-hexen-1-ol	C ₈ H ₁₄ O	126	0.22
2	768	970	(3R*,5S*,6R*)-3-Isopropyl-6-methyl-6-phenyl-5-(p-toluidino)-1,2,4-trioxane	C ₂₀ H ₂₅ NO ₃	327	0.31
3	871	975	2-Propenoic acid, 3-phenyl-, methyl ester, (E)- (CAS)	C ₁₀ H ₁₀ O ₂	162	0.46
4	267	876	1-(tert-Butyl)dimethylsilyloxy-3-hydroxypropan-2-one	C ₉ H ₂₀ O ₃ Si	204	0.41
5	497	986	1-(3'-Fluorophenyl)-4-phenylbut-3-en-2-one	C ₁₆ H ₁₃ FO	240	2.93
6	521	931	(2E,4E,6R,7R,10S)-4,6,10-Trimethyl-2,4-dodecadien-7-ol	C ₁₅ H ₂₈ O	224	0.19
7	315	922	3-[4'-Methoxybenzyl]-2(5H)-furanone	C ₁₂ H ₁₂ O ₃	204	0.19
8	177	917	Z,Z-10,12-Hexadecadienal	C ₁₆ H ₂₈ O	236	0.4
9	864	973	2-Propenoic acid, 3-(4-methoxyphenyl)-, methyl ester, (E)-	C ₁₁ H ₁₂ O ₃	192	2.28
10	605	920	(E and Z)-1-(3,4-Dimethoxyphenyl)butadiene	C ₁₂ H ₁₄ O ₂	190	0.63
11	657	971	5-methoxy-3-oxo-2,3-dihydro-1H-indene-1-carboxylic acid	C ₁₁ H ₁₀ O ₄	206	13.37
12	290	938	tert-Butyl 4-hydroxy-2-methylhexanoate	C ₁₁ H ₂₂ O ₃	202	0.4
13	902	956	Methyl 3-(p-methoxyphenyl)acrylate	C ₁₁ H ₁₂ O ₃	192	8.8
14	434	988	6,6,7,7,9,9,10,10-Octadeuterio-undecane-1-carboxylic acid	C ₁₂ H ₁₆ D ₈ O ₂	200	61.91
15	182	794	BenzylN-[(N-Benzenesulfonyl)á-tryptophyl]-N-[(trimethylsilyl)methyl]carbamate	C ₂₇ H ₃₀ N ₂ O ₄ SSi	506	0.18
16	569	855	2-Propenoic acid, 3-(4-hydroxy-3-methoxyphenyl)-, methyl ester (CAS) C ₁₁ H ₁₂ O ₄	C ₁₁ H ₁₂ O ₄	208	0.23
17	744	954	(E)-4-(3',4'-DIMETHOXYPHENYL)BUT-3-EN-1-OL	C ₁₂ H ₁₆ O ₃	208	3.31
18	181	942	4,9-Dimethyl-7-oxabicyclo[4.3.0]nonan-8-one	C ₁₀ H ₁₆ O ₂	168	0.61
19	521	918	[4-(Tetrahydropyran-2-yloxy)phenoxy]-tert-butyldimethylsilane	C ₁₇ H ₂₈ O ₃ Si	308	0.56
20	200	966	2,2-Bis(methylthio)-4-phenyl-2,5-dihydrofuran	C ₁₂ H ₁₄ OS ₂	238	0.33
21	125	965	(+)-(1S,2S)-1,7,7-Trimethyl-2-iodomethylbicyclo[2.2.1]heptane	C ₁₁ H ₁₉ I	278	0.34
22	92	981	6-Acetoxy-3,3a,4,5,6,8a-hexahydro-(3aà,5à,8aà)-2H-cyclohepta[b]furan	C ₁₁ H ₁₆ O ₃	196	1.25
23	321	870	6,7,8,9-Tetrahydro-3-hydroxy-6,9a-methanocycloocta[b]furan-2,4(5H)-dione	C ₁₁ H ₁₂ O ₄	208	0.22
24	59	962	1-Dimethyl(phenyl)silyl-5-phenylpentan-3-yl benzoate	C ₂₆ H ₃₀ O ₂ Si	402	0.29
25	108	884	(-)-(1S,2S,6R,9R)-4-Isopropylidene-1-methyltricyclo[4.3.0.0(2,9)]nonan-8-one	C ₁₃ H ₁₈ O	190	0.19

Figure S1. Chromatogram of methanol extract of *B. rotunda* leaves