

Research Article

Isolation, Characterization and Evaluation of Anti-microbial Activity of *Ravenia spectabilis* (Rutaceae)

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ABSTRACT

Stem bark of the plant *Ravenia spectabilis* (family: Rutaceae) was extracted and the extract was fractionated by using standard chromatographic techniques. This yielded a total of four compounds among which one was an alkaloid that was isolated and identified by spectral data (1D and 2D NMR). The alkaloid has been characterized as arborinine [RS-1]. The other compounds isolated from the plant (RS-2, RS-3 & RS-5) could not be elucidated due to insufficient spectral data. Two VLC fractions of crude extracts from which pure compounds were isolated, were screened for their antibacterial activities against a wide range of both gram-positive and gram-negative bacteria by disc diffusion method. Antifungal activities against some clinically isolated fungi were also observed. The results obtained were compared with those for a standard antibiotic, kanamycin. The zones of inhibition produced by the VLC fraction-16 were found to be 08-12 mm at a concentration of 200 µg/disc. Fraction-16 exhibited moderate activity against *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella paratyphi*, *Vibrio mimicus* and mild activity against some other studied bacteria like *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*. Fraction-11 showed no activity and found to be resistant against bacteria and fungi studied. Fraction -16 showed moderate activity against *Aspergillus niger* and *Sacharomyces cerevaeae* fungi and mild activity against *Candida albicans*.

Keywords: *Ravenia spectabilis*, NMR, arborinine, disc diffusion, kanamycin

INTRODUCTION

The importance of plants in search of new drugs is not diminishing, and may be increasing. For example, recently ricin, a toxin produced by the beans of *Ricinus communis*, has been found to effectively couple to tumor targeted monoclonal antibodies and has proved to be a very potent antitumor drug^{1,2}. Further, HIV inhibitory activity has been found in some novel coumarins (complex angular pyranocoumarins) isolated from *Calophyllum lanigerum*³. Recently developed genetic engineering in plants has further increased their importance in the field of medicine, for example, in the production of antibodies by expression of an appropriate gene in the plant. By using this technique it is possible to modify the activity or regulate the properties of the key enzymes responsible for the production of secondary metabolites. Thus, by knowing the potential resources it is possible to increase the content of the important active compounds⁴.

Bangladesh is a good repository of medicinal plants belonging to various families, including Rutaceae. The Rutaceous plants contain a wide range of pharmacologically active compounds⁵, including anti-inflammatory, anti-implantation, anti-neoplastic and anti-mutagenic activities. A number of Rutaceous plants have been used in medicine⁶. Among the different variety of pharmacological activities reported to be exerted by the

Rutaceous plants include antimicrobial activities. The root bark of *Toddalia aculeata* is considered in Hindu medicine to be a potent antimalarial drug. The bark of *Raputia alba* is an excitant and febrifuge and that of *R. magnifica* is used to expel intestinal worms⁷. The acetophenones sessiliflorene and sessiliflorols (A & B) showed inhibitory activity against herpes simplex virus types I and II *in vitro*⁸. Antibacterial and antifungal activity has been reported for a number of secondary metabolites. The alkaloid berberine produced by Rutaceae⁹ has inhibitory activity against *Candida albicans*¹⁰. The flavonoid quercetin inhibits the growth of *Candida albicans* and a number of gram positive and gram negative bacteria e.g. *Salmonella typhimurium*, *E. coli*, *Klebsiella* spp, *S. aureus*, *S. pyogens* etc.¹¹ and is present in many Rutaceous plants¹². Fungicidal activity has been reported for two prenylated and geranylated acetophenones isolated from *Melicope lunu-ankenda*¹³. The coumarins, seselin¹⁴ and psoralen¹⁵ also have antifungal activity.

In Bangladesh there are about 25 plants belonging to the family Rutaceae. Although a large number of plants included in Rutaceae have been investigated all over the world very little is known regarding the chemistry and pharmacology of the Rutaceous species grown in Bangladesh. Therefore an attempt has been taken to study the chemical constituents of the *Ravenia spectabilis* and

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Table 1: Different solvent systems used for VLC analysis of pet ether extract

Fraction no.	Solvent system	Volume collected (ml)
1	Petroleum ether : Ethyl acetate 98 : 2	100
2	Petroleum ether : Ethyl acetate 95 : 5	100
3	Petroleum ether : Ethyl acetate 92 : 8	100
4	Petroleum ether : Ethyl acetate 90 : 10	100
5	Petroleum ether : Ethyl acetate 88 : 12	100
6	Petroleum ether : Ethyl acetate 85 : 15	100
7	Petroleum ether : Ethyl acetate 82 : 18	100
8	Petroleum ether : Ethyl acetate 80 : 20	100
9	Petroleum ether : Ethyl acetate 78 : 22	100
10	Petroleum ether : Ethyl acetate 75 : 25	100
11	Petroleum ether : Ethyl acetate 70 : 30	100
12	Petroleum ether : Ethyl acetate 65 : 35	100
13	Petroleum ether : Ethyl acetate 60 : 40	100
14	Petroleum ether : Ethyl acetate 50 : 50	100
15	Petroleum ether : Ethyl acetate 40 : 60	100
16	Petroleum ether : Ethyl acetate 30 : 70	100
17	Petroleum ether : Ethyl acetate 20 : 80	100
18	Ethyl acetate 100%	100
19	Ethyl acetate : Methanol 98 : 2	100
20	Ethyl acetate : Methanol 95 : 5	100
21	Ethyl acetate : Methanol 90 : 10	100
22	Ethyl acetate : Methanol 80 : 20	100
23	Ethyl acetate : Methanol 50 : 50	100
24	Ethyl acetate : Methanol 20 : 80	100
25	Methanol 100%	100

evaluate its pharmacological (mainly antibacterial and antifungal) profiles.

Ravenia spectabilis is a shrub of South America. It is included under the family Rutaceae and the subfamily Rutoideae and tribe Cusparieae¹⁶. In Bangladesh this species is cultivated in the garden. Flowers of this plant are orange-red. Alkaloids are the most frequently encountered natural organic compounds in *Ravenia spectabilis*. *Ravenia spectabilis* grown in Bangladesh has not been studied before. The results of previous investigations show the presence of different alkaloids in this plant. Such plants include – paraensine, ravesilone, spectabiline, ravenine, atanine, γ -fagarine, arborinine, etc.^{17,18,19}.

The present investigation may provide some interesting

List of Test Bacteria

Gram positive	Gram negative
<i>Bacillus cereus</i>	<i>Escherichia coli</i> (BTCC-172)
<i>Bacillus megaterium</i>	<i>Pseudomonas aeruginosa</i> (BTCC-1252)
<i>Bacillus subtilis</i>	<i>Salmonella paratyphi</i>
<i>Staphylococcus aureus</i>	<i>Salmonella typhi</i>
<i>Sarcina lutea</i>	<i>Shigella boydii</i>
	<i>Shigella dysenteriae</i>
	<i>Vibrio mimicus</i>
	<i>Vibrio parahemolyticus</i>

compounds, which may be pharmacologically active. If significant results are obtained these can be used as remedies for the treatment of some diseases. Since this plant is available in Bangladesh, this may be a cost-effective treatment. So, the objective is to explore the possibility of developing new drug candidates from this plant for the treatment of various diseases.

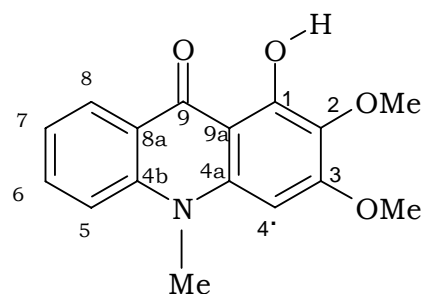


Figure 1: Structure of RS-1 (1-hydroxy-2,3 dimethoxy-N-methyl-acridinone)

The structure was further confirmed by comparison of the ¹H and ¹³C NMR with that published in the literature²⁰.

MATERIALS AND METHODS

Collection and preparation of the plant material

Plant sample of *Ravenia spectabilis* Engl. (Family: Rutaceae) was collected from the Balda Garden, Dhaka. A voucher specimen (DACB- 28065) for this plant has

Table 2: Comparison between the ¹H NMR spectral data of RS-1 (400 MHz, CDCl₃) and Arborinine (400 MHz, CDCl₃)²⁰

Protons	RS-1 δ _H in ppm (CDCl ₃)	Arborinine δ _H in ppm (CDCl ₃)
OMe-2	δ 3.93 <i>s</i>	δ 3.93 <i>s</i>
OMe-3	δ 4.02 <i>s</i>	δ 4.01 <i>s</i>
H-4	δ 6.30 <i>s</i>	δ 6.23 <i>s</i>
N-Me	δ 3.85 <i>s</i>	δ 3.80 <i>s</i>
H-5	δ 7.51 <i>d</i> (<i>J</i> =8.7 Hz)	δ 7.49 <i>d</i> (<i>J</i> =8.7 Hz)
H-6	δ 7.73 <i>dd</i> (<i>J</i> =8.7, 7.2 Hz)	δ 7.70 <i>ddd</i> (<i>J</i> =8.7, 7.1, 1.7 Hz)
H-7	δ 7.30 <i>dd</i> (<i>J</i> =8.0, 7.2 Hz)	δ 7.27 <i>ddd</i> (<i>J</i> =7.8, 7.2, 0.6 Hz)
H-8	δ 8.47 <i>d</i> (<i>J</i> =8.0 Hz)	δ 8.40 <i>dd</i> (<i>J</i> =8.0, 1.6 Hz)

been deposited in the National Herbarium, Dhaka, Bangladesh. Stems of this plant were at first cut into small pieces and dried in the sunlight and air for several days. The bark is finally oven dried for 24 hours and then ground to a coarse powder.

Extraction of the plant material

The air dried and powdered plant material (700 gm) was successively extracted with petroleum ether, ethyl acetate and methanol by hot extraction using Soxhlet apparatus. The extracts were concentrated with a rotary evaporator at low temperature (40-50^oC) and reduced pressure.

Investigation of the pet ether extract

The extracts were screened using standard TLC procedure and from the findings of the study pet ether extract was selected for further study.

Vacuum Liquid Chromatography (VLC) of pet ether extract

The column was packed with fine TLC grade silica gel (kieselgel 60H). A short column having 15 cm length and 5 cm in diameter was packed with the silica gel upto height of 6 cm under reduced pressure. The column was washed with petroleum ether to facilitate compact packing. The sample was prepared by adsorbing 1.5 gm of petroleum ether extract onto silica gel (kieselgel 60, mesh 70-230), allowed to dry and subsequently applied on top of the adsorbent layer. The column was then eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate of increasing polarity, then by ethyl acetate and finally with ethyl acetate and methanol mixtures of increasing polarity. Solvent systems used as

Table 3: Comparison between the ¹³C NMR spectral data of RS-1 (100 MHz, CDCl₃) and Arborinine²⁰

Carbons	RS-1 δ _C in ppm (CDCl ₃)	Arborinine δ _C in ppm (CDCl ₃)	Carbons	RS-1 δ _C in ppm (CDCl ₃)	Arborinine δ _C in ppm (CDCl ₃)
C-1	δ 159.4	δ 159.3	C-9	δ 180.9	δ 180.9
C-2	δ 131.0	δ 130.3	C-4a	δ 140.6	δ 140.6
C-3	δ 159.2	δ 156.3	C-4b	δ 142.1	δ 140.6
C-4	δ 86.8	δ 86.9	C-8a	δ 120.8	δ 120.8
C-5	δ 114.6	δ 114.8	C-9a	δ 106.0	δ 105.9
C-6	δ 134.0	δ 134.1	OMe-2	δ 60.8	δ 61.0
C-7	δ 121.5	δ 121.6	OMe-3	δ 56.1	δ 56.2
C-8	δ 126.8	δ 126.7	N-Me	δ 34.2	δ 34.3

mobile phases in the VLC analysis of petroleum ether extract were listed in Table 1. A total of 25 fractions were collected in 100 ml fractions.

Analysis of VLC fractions by TLC

All the VLC fractions were screened by TLC under UV light and by spraying with vanillin-sulfuric acid reagent. Depending on the TLC behavior fractions 8, 9, 10, 11, 15 and 16 were selected for further investigation.

Isolation and purification of compounds from the selected VLC fractions

Four compounds (RS-1, RS-2, RS-3 and RS-5) have been isolated and purified from the different VLC fractions adopting various techniques.

Table 4: HSQC and HMBC data for RS-1

Protons	¹ <i>J</i>	² <i>J</i> / ³ <i>J</i>
N-Me	δ 34.2	δ 142.1
O-Me	δ 60.8	δ 131.0
O-Me	δ 56.1	δ 159.2
H-4	δ 86.7	δ 106.0, 131.0, 140.6, 159.4

Isolation of compound RS-3 and RS-5

Fractions 11 was subjected to PTLC (Stationary phase - Silica gel PF₂₅₄, Mobile phase - Toluene: Ethyl acetate = 78: 22, single development, Thickness of plates - 0.5 mm). From the developed plates two characteristic bands were scraped and eluted with solvents. Thus VLC fraction 11 on purification by PTLC gave RS-3 and RS-5.

Isolation of compound RS-1 and RS-2

Fraction 15 and 16 were bulked together as they showed

Table 5: Antibacterial activity of two VLC fractions (Fraction 11 and 16) of *Ravenia spectabilis*

Test organism		Diameter of Zone of Inhibition (mm)		
		VLC fraction-11 (200 µg/disc)	VLC fraction-16 (200 µg/disc)	Kanamycin (30 µg/disc)
Gram Positive Bacteria	<i>Bacillus cereus</i>	-	11	22
	<i>Bacillus megaterium</i>	-	11	23
	<i>Bacillus subtilis</i>	-	10	22.5
	<i>Staphylococcus aureus</i>	-	10	23
	<i>Sarcina lutea</i>	-	8	20
	<i>Escherichia coli</i>	-	12	23
Gram Negative Bacteria	<i>Pseudomonas aeruginosa</i>	-	9	24
	<i>Salmonella paratyphi</i>	-	12	18
	<i>Salmonella typhi</i>	-	8	19
	<i>Shigella boydii</i>	-	8	20
	<i>Shigella dysenteriae</i>	-	11	22
	<i>Vibrio mimicus</i>	-	8	22
	<i>Vibrio parahemolyticus</i>	-	-	25
	<i>Candida albicans</i>	-	9	25.5
Fungi	<i>Aspergillus niger</i>	-	11	19.5
	<i>Sacharomyces cerevacaee</i>	-	12	18

“-” Indicates no sensitivity

similar TLC feature. This combined fraction was subjected to PTLC (Stationary phase - Silica gel PF₂₅₄, Mobile phase - Toluene: Ethyl acetate = 72: 28, multiple developments, thickness of plates - 0.5 mm). From the developed plates two sets of identical bands were separately scraped and eluted with different solvents. Thus VLC fractions 15 and 16 on purification by PTLC gave RS-1 and RS-2.

Test for purity of the isolated compounds

The purity of each of the isolated compounds was monitored by TLC using different solvent systems. Commercially available pre-coated plates with silica gel (Kieselgel 60 PF₂₅₄) on plastic or aluminium sheets were used for this purpose. Moreover, purity was also tested by spraying the developed plates with different spray-reagents, particularly with vanillin-sulfuric acid solution followed by heating at 110°C for several minutes.

Characterization of the samples by NMR

The compounds isolated and purified from the VLC fractions 11, 15 and 16 were sent for NMR (400 MHz Bruker NMR spectrometer with TMS as the internal reference) at the Analytical Lab of BCSIR (Bangladesh Council of Scientific and Industrial Research) and the compounds were characterized by taking ¹H (400MHz), ¹³C (100MHz), HMBC, HSQC, COSY45 spectrums.

Antibacterial & Antifungal Screening

Two VLC fractions of crude extract (fraction 11 and 16) were tested for antimicrobial (antibacterial and antifungal) activity against a total of 13 (5 gram positive and 8 gram negative bacterial strains (Table 2) and three fungal strains (*Candida albicans*, *Aspergillus niger* and *Sacharomyces cerevacaee*) collected as pure cultures from

the Institute of Nutrition and Food Science (INFS), University of Dhaka. Standard disc of kanamycin (30 µg/disc) was used for comparison purpose.

RESULTS AND DISCUSSION

Characterization of RS-1 as 1-hydroxy-2,3-dimethoxy-10-methyl-9(10H) - Acridinone or Arborinine

RS-1 was obtained as yellow gum. It was yellow in color on TLC plate in daylight. It appeared as a dark quenching spot on TLC plate under UV light at 254 nm. With Dragendorff's reagent it gave an orange color. The structure of this compound was determined by ¹H-, ¹³C-NMR and 2D NMR studies.

The ¹H NMR spectrum (400 MHz, CDCl₃, Table 2) of RS-1 revealed signals characteristic of a polycyclic acridone alkaloid where two of the three hydroxy groups are etherified with methyl groups. It also showed signal characteristic to an N-methyl group. The spectrum also showed a highly characteristic ABCD spin system.

Signal for four aromatic protons in an ABCD spin system at δ 7.51, δ 7.73, δ 7.30 and δ 8.47 are present in the spectrum which could be assigned to four adjacent protons H-5, H-6, H-7 and H-8 of ring C, respectively. The sharp singlet at δ 6.30 was attributable to the aromatic proton at C-4 of ring A. The three proton singlet at δ 3.85 could be assigned to N-CH₃. Two sharp singlets at δ 3.92 and 4.02 are assigned to the two methoxy groups. The ¹³C NMR spectrum (100 MHz, CDCl₃) of RS-1 (Table 3) showed two methoxyl carbons resonating at δ 56.1 and 60.8. The HMBC experiment (Table 4) revealed the position of these methoxyl groups at C-2 and C-3 respectively. The N-CH₃ signal resonated at δ 34.2, which supported the fact that the C-4 position was unsubstituted. The signal at δ 180.92 clearly shows the C-9 position has

ketonic functionality. The other ^{13}C assignments are shown in Table 3.

The HSQC and HMBC experiments showed the expected ^1J , ^2J and ^3J connectivity.

Thus the compound was identified as 1-hydroxy-2,3-dimethoxy-10-methyl-9(10H)-acridinone or Arborinine (Figure 1).

In vitro Antibacterial and Antifungal Screening

VLC fraction -16 of crude extract exhibited mild to moderate activity against most of the test bacteria and fungi (Table 5). On the other hand fraction -11 showed no antibacterial and antifungal activity against all test bacteria and fungi.

The zones of inhibition produced by the VLC fraction-16 ranged from 08-12 mm at a concentration of 200 $\mu\text{g}/\text{disc}$. Fraction-16 exhibited moderate activity against *Bacillus cereus*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella paratyphi*, *Vibrio mimicus* and mild activity against some other test bacteria like *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhi*. Fraction-11 showed no activity and found to be resistant against test bacteria and fungi. Fraction -16 showed moderate activity against *Aspergillus niger* and *Sacharomyces cerevacae* fungi and mild activity against *Candida albicans*.

The outcome of the present study demands more extensive work to be initiated so that the antibacterial and antifungal activities of the plant can be established. Moreover, the alkaloid responsible for producing such activities should be more completely characterized, isolated and modified structurally to develop novel antibiotic compound that can be used against the resistant organism.

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