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Original Article

Isolation of Bioactive Principles and Studies of Antimicrobial, Cytotoxic and Antioxidant Activities of the stem bark of *Baccaurea ramiflora* (Euphorbiaceae)

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ABSTRACT: Phytochemicals are extensively found at different levels in many medicinal plants. Stem bark of *Baccaurea ramiflora* (common name Latkan) belonging to the family of Euphorbiaceae, has been studied for isolation of its secondary metabolites and evaluation of its bio-activities of the crude extract and fractions with especial emphasis on the antimicrobial, cytotoxic and antioxidant activities. The concentrated chloroform extract was partitioned with petroleum ether, ethyl acetate and methanol and the remaining chloroform crude extract was kept for assessing antimicrobial, cytotoxic and antioxidant activities. After successful separation and purification of the different VLC fractions of the chloroform extract of stem bark *B. ramiflora*, two secondary metabolites namely BR-F5-B1 and BR-F5-B2 were isolated. The structure of this two compounds were elucidated as phytol (**1**) and betulinic acid (**2**) by comparing with their ^{1}H NMR spectral data with published values.
The disc diffusion method was used to evaluate the antimicrobial activities. The methanol soluble fraction of *B. ramiflora* stem bark did not show any activity against the testing bacteria. The petroleum soluble fraction (PESF) and crude extract showed mild to moderate antibacterial activities against both gram positive and gram negative bacteria as compared to control antibiotic disc.The ethyl acetate soluble fraction (EASF) revealed mild activity. *Staphylococcus aureus*, *Shigella boydii* and *Vibrio mimicus* showed the maximum sensitivity to the crude extract of *B. ramiflora* when compared to control (Ciprofloxacin 30µg/disc). The crude extract showed mild activity against certain fungi. In the DPPH assay, different fractions of chloroform extract of *B. ramiflora* demonstrated significant antioxidant activity.In the brine shrimp lethality bioassay the LC_{50} value of crude extract of *B. ramiflora* was 11.09 µg/ml, which demonstrated moderate cytotoxic principles present in the crude extract.

Keywords: *Baccaurea ramiflora*, Phytochemical, Antibacterial, Antifungal, Cytotoxic, Antioxidant

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INTRODUCTION

Baccaurea ramiflora (Family: Euphorbiaceae), locally known as Latkan, is a versatile plant with many uses. It is also known as Leteku (Hindi), Bhubi (Bengali), Mafai (Thai) and Burmese grape (English). The whole plant of *B. ramiflora* is used as an antiphlogistic and anodyne against rheumatoid arthritis, cellulitis, abscesses and to treat injuries in Chinese Dai medicine[1].Young leaves of *B. ramiflora* are utilized as vegetable, flavoring agent with curries and minced meat in Bangladesh[2]. The plant is also used as medicine by the tribes in Northern Thailand. In India, fresh bark is chewed or juice is used orally for constipation. Leaves of *B. ramiflora* exhibited hypoglycemic and hypolipidemic activities. Seeds exhibited analgesic activity and seeds are crushed to cure diarrhea[3]. *B. ramiflora* fruit juice, served a multifunctional role both as reducing agents for Ag^+ ions to Ag0 and as stabilizers for the synthesized Ag[4]. Phytosterols, carbohydrates, gums and mucilage have been found from the bark of *B. ramiflora*[5].

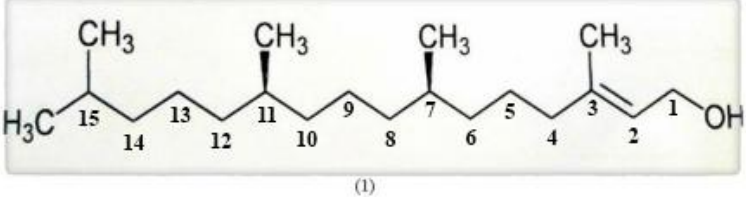
Three picrotoxane sesquiterpenes including one new glycoside ramifloside and two known constituents, sapidolide A and picrotoximaesin were isolated from the berries of *B. ramiflora* which showed antifungal activity[6]. The fruits of *B. ramiflora* has hemolytic, antioxidant and cytotoxic activities[7]. Chilling treatment reduced germination, causing CO and NO generation, and increased heme activity of *B. ramiflora* seeds[8]. *B. ramiflora* has been screened for larvacidal activity against four mosquito vector species[9]. Epidihydrotutin is a new sesquiterpene lactone which has isolated from root of *B. ramiflora*[10]. It exhibited good antimicrobial activity against *Shigella boydi*i, *Vibrio spp.* and *Salmonella spp*.[11]. The leaf of *B. ramiflora* is known to contain a glycoside 6′-O-vanilloyltachioside 4′-O- 6-Ovanilloyl)-β-D-glucopyranosyl tachioside D[12]. Essential oil components are also found from *B. ramiflora*[13]. So far no detailed phytochemical and biological studies have been carried out on the stem bark of this plant. In the present study, isolation of two compounds and antimicrobial, cytotoxic and antioxidant activities of the chloroform extract of the stem bark of *B. ramiflora* have been discussed.

MATERIALS AND METHODS

The stem bark of *B. ramifolia* was collected from the forest of Norshingdi district of Bangladesh. It was taxonomically identified by a botanist of the University of Dhaka. After proper identification the stem bark was cut into small pieces and dried naturally. The sun dried stem bark was ground mechanically and extracted in a soxhlet apparatus with chloroform.The extract was then concentrated *in vacuo* using a Buchi rotavapor. The $CHCl_3$ extract was then fractionated by vacuum liquid chromatography (VLC) over silica gel. Pure compounds were then isolated from different fractions using chromatographic techniques like flash column chromatography and preparative thin layer chromatography. This provided two compounds, BR-F5-B1 and BR-F5-B2. The *in vitro* antibacterial and antifungal activities of the crude extract as well as different fractions were determined by disc diffusion methods[14]. Fourteen bacterial strains and five fungi were collected from the Department of Microbiology and Institution of Nutrition and Food Sciences, University of Dhaka. Brine Shrimp lethality bioassay[15] has been used for the assessment of cytotoxicity of the test samples. This bioassay can indicate not only cytotoxicity but also pharmacological activity of test materials.

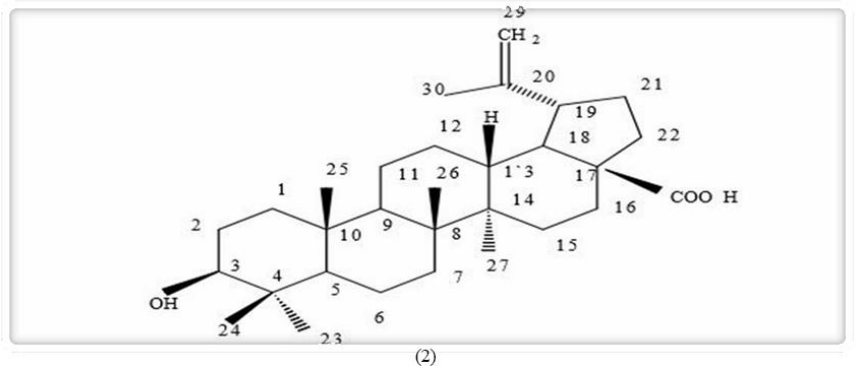
RESULTS AND DISCUSSION

Two compounds designated as BR-F5-B1 and BR-F5-B2 were isolated and characterized by ^1H NMR spectroscopy. The ^1H NMR (400MHz, $CDCl_3$) spectrum of the isolated compound BR-B5-B1 revealed a one-proton triplet at δ_H 5.36, the position and multiplicity of which was indicative of C-2 olefenic proton. The signal for H-1 of the carbonyl proton was evident from a doublet of two proton intensity at δ_H 421. The ^1H NMR also displayed signals at δ_H 0.87, 0.86, 0.82 and 1.52 assignable to methyl groups at C-16, C-7, C-11 and C-15 respectively . The ^1H NMR spectrum showed singlet centered at δ_H 1.64 which could be attributed to allylic methyl (3-methyl) protons at C-3. The doublet at δ_H 2.08 was suggestive of two proton intensity at C-4 . On the other hand, the signals at δ_H 1.36, 1.06 , 1.30 and 1.13 were assignable to methylene and methane protons at C-5, C-6, C-7, C-8, C- 9,C-10 ,C-11 and C-12, C-13, C-14 positions respectively. Therefore, BR-B5-B1 was identified as phytol (**1**) by comparing its ^1H-NMR data with published data[17].



(1)

The ^{1}H NMR (400MHz, $CDCl_3$) spectrum of compound BR-F5-B2 exhibited signals for five tertiary methyls [δ_H: 0.65, 0.75, 0.90, 0.96, 0.98], a vinyl methyl [δ_H: 1.97 (br d, J=0.5 Hz)], a secondary carbinol [δ_H: 3.16 (dd, J=9.5 and 6.0 Hz)] and [δ_H: 2.95 (ddd, J=9.0,6.0 and 0.5 Hz)] an exomethylene group [δ_H: 4.55 (1H, d, J=0.4 Hz)] and [δ_H: 4.65 (1H, d, J=0.4 Hz). Comparing with published values[18] of betulinic acid, these data allowed to identify the compound as betulinic acid **(2)**.



(2)

To assess the antimicrobial activities of *B. ramiflora*, different fractions including PESF, EASF and MESF and crude extract were screened for antibacterial and antifungal activities. Filter paper disks prepared from fractions and crude extract show mild to moderate activity against specific gram positive and gram negative bacteria. On the other hand EASF show mild antimicrobial activity. The zone of inhibition of the control (Ciprofloxacin) was 27 mm to 41 mm against gram positive and gram negative bacteria. PESF showed zone of inhibition in a diameter of 10-19 mm against gram positive bacteria and 8-18 mm against gram negative bacteria. Crude extract exhibited zone of inhibition 6-22 mm and 6-25 mm diameter against gram positive and gram negative bacteria. *Staphylococcus aureus*, *Shigella boydii* and *Vibrio mimicus* show the maximum sensitivity to the crude extract of *B. ramiflora* plant. Comparison of different fractions and crude extract with control revealed mild to moderate antibacterial activities of *B. ramiflora* (stembark) against both gram positive and gram negative bacteria. So the stem bark of *B. ramiflora* can be utilized for formulating antibacterial agent.

Table 1: Data for the determination of zone of Inhibition of gram positive bacteria

Bacterial strain (gram positive)	**Diameter of zone of inhibition in (mm)**				
	Crude extract (400 µg/ml)	**PESF (400µg/ml)**	**EASF (400µg/ml)**	**MESF (400µg/ml)**	**Ciprofloxacin (30µg/disc)**
Bacillus megaterium	17	13	9	----	38
Sarcinana lutea	7	-	----	----	41
Bacillus polymyx	19	11	8	----	36
Staphylococcus aureus	22	19	11	----	34
Bacillus cereus	14	10	-	----	35
Bacillus subtilis	-	----	----	----	33

Table 2: Data for the determination of zone of Inhibition of gram negative bacteria

Bacterial strain (gram negative)	Diameter of zone of inhibition in (mm)				
	Crude extract(400 µg/ml)	PESF (400µg/ml)	EASF (400µg/ml)	MESF (400µg/ml)	Ciprofloxacin (30µg/disk)
Salmonella typhi	15	8	-	----	39
Shigella boydii	25	18	10	----	38
Escherchia coli	18	-	----	----	32
Kiebsiella sp	8	----	6	----	28
Vibrio mimicus	23	16	9	----	31
Pseudomonus sp.	-	----	----	----	27

N.B.: (----) means No zone of inhibition

In antifungal activity assay, *B. ramiflora* also showed mild antifungal activity comparing with control. Griseofulvin was used here as a positive control. The zone of inhibition of griseofulvin lied between 13 mm to 18 mm in this experiment. MESF did not show any antifungal activity accept one fungal species. But most of the cases crude extract, EASF and PESF exhibited mild activity. The crude extract show promising antifungal activity against *Candida arrizae*. The zone of inhibition of crude extract estimated here 6 mm to 10 mm against the fungal strains.

Table 3: Data for the antifungal activity of different solvent soluble fractions of crude extract.

List of fungal strains	Diameter of zone of inhibition (mm) after 24 hours				
	MESF (400µg/ml)	EASF (400µg/ml)	PESF (400µg/ml)	Crude extract (400µg/ml)	Griseofulvin (25 µg/disc)
Candida albicans	----	6	6	7	13
Aspergillus niger	----	----	6	6	16
Rhizopus oryzae	----	7	----	9	18
Candida arrizae	6	7	6	10	13
Saccharromyces cervisia	----	6	7	9	18

N.B.: (----) means No zone of inhibition

The methanolic extract of crude sample from *B. ramiflora* (stem bark) was subjected to screening for antioxidant activity using DPPH. In this assay, butylated hydroxyl toluene (BHT) was used as control. IC_{50} value of control (BHT) and different fractions of crude sample was calculated and determined from graphical presentation (Figure 1). The chloroform soluble fraction of *B. ramiflora* showed good free radical scavenging activity than n-hexane or carbon tetrachloride soluble materials. The better IC_{50} value of control (BHT) was 17.34 µg/ml, whereas that of chloroform, hexane and carbon tetrachloride soluble fractions were 55.64, 100.5 and 84.23 µg/ml, respectively. This represented significant antioxidant activity of *B. ramiflora*.

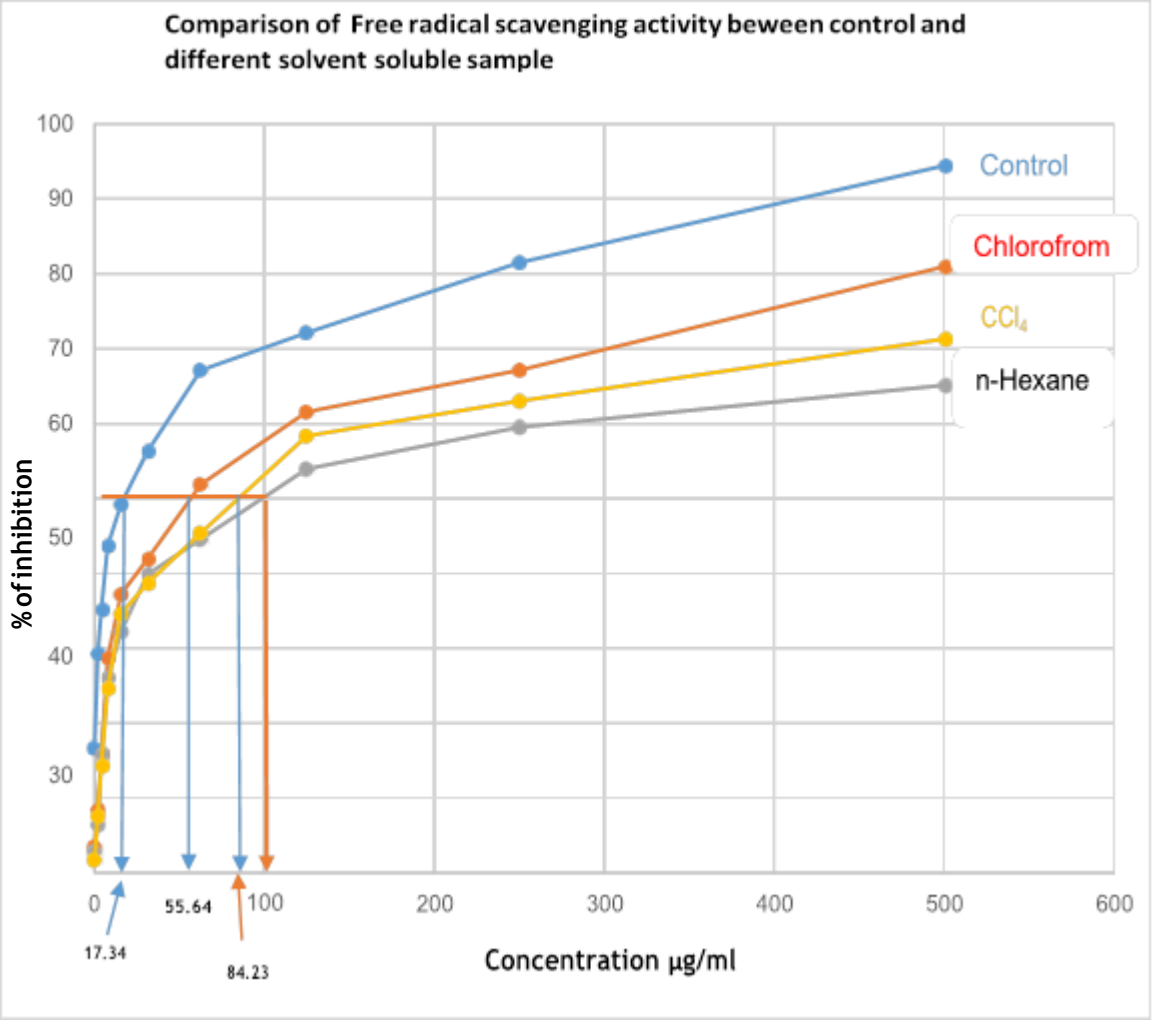


Figure 1: Comparison of IC_{50} value between control (BHT) and different solvent soluble fraction of methanolic extract

Brine Shrimp lethality bioassay was performed to assess the cytotoxicity of *B. ramiflora* stem bark. The LC_{50} value of control and test sample were determined from graphical presentation. LC_{50} value of control was 4.61 µg/mL and LC_{50} value of sample (crude extract) was 11.09 µg/mL which showed moderate cytotoxic activity (Table 4).

Table 4: Data for the Determination of LC_{50} of brine shrimp lethality bioassay for plant crude extract of *B. ramiflora* and Control (VS)

Test tube no.	Concentration (µg/ml)	Log of concentration	Percent (%) of mortality of brine shrimp				LC_{50} value of control from graph	LC_{50} value of sample from graph
			Experiment - 01(crude)	Experiment-02(crude)	Mean (crude)	Control (VS)		
1	400	2.602	90	90	90	100	4.61 (µg/ ml)	11.09 (µg/ml)
2	200	2.301	90	90	90	100		
3	100	2.000	80	70	75	90		
4	50	1.699	70	70	70	80		
5	25	1.398	60	60	60	80		
6	12.50	1.097	60	50	55	70		
7	6.25	0.796	40	30	35	60		
8	3.125	0.495	30	30	30	40		
9	1.56	0.193	10	10	10	30		
10	0.000	-----	0	0	0	0		

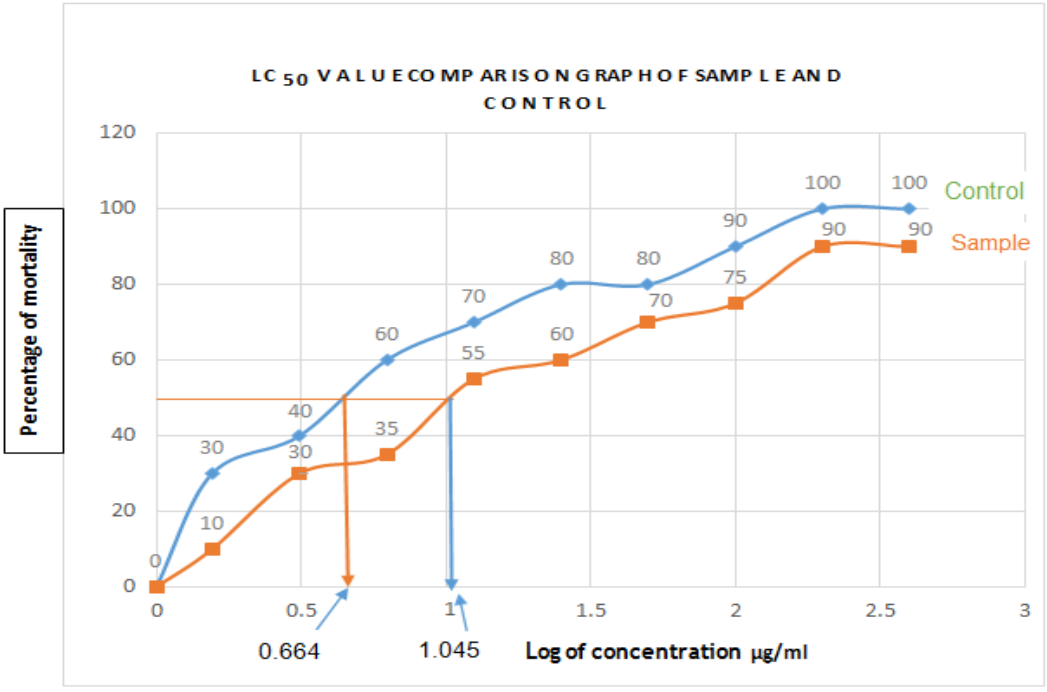


Figure 2: Graphical representation of the LC_{50} value of control and sample

So, in the bioassay for brine shrimp cytotoxicity, the crude extract of B. ramiflora showed moderate cytotoxicity compared to control which indicats that the compounds present in sample are biologicall active. Test samples showed different mortality at different concentration and the rate of mortality of brine shrimp was found to be increased with the increasing concentration for each sample. The degree of lethality was directly proportional to the concentration of the extract. The laboratory test also showed that B. ramiflora was also effective against specific bacteria and fungi. So both of this support the traditional uses of this plant in various infectious diseases and also supports the literature findings.

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