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Phytochemical and Biological Investigation on *Artocarpus lackoocha* Roxb.

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ABSTRACT

Mixture of three compounds lupeol acetate (**1**), alpha amyrin acetate(**2**) and beta amyrin acetate(**3**) were isolated from the methanolic extract of the stem bark of *Artocarpus lackoocha* Roxb.(Family: Moraceae).The crude methanol extract as well as its petroleum ether, carbon tetrachloride; chloroform and aqueous soluble Kupchan fractions were studied for antioxidant, antimicrobial and cytotoxic activities. Among the different fractions tested for antioxidant activity, the aqueous soluble partitionate was the most potent with IC50 value of 3.47µg/ml as compared to *tert*butyl-1-hydroxytoluene (IC50=27.54µg/ml). Antimicrobial screening of the different extractives was conducted by the disc diffusion method and the crude methanol extract as well as aqueous soluble fractions exhibited moderate antimicrobial activity with zone of inhibition ranging from 7-12 mm. In brine shrimp lethality bioassay, the aqueous soluble materials demonstrated the highest toxicity with LC50 of 1.6µg/ml. β-amyrin acetate is the first report of isolation of compounds from *Artocarpus lackoocha*.

Keywords: *Artocarpus lackoocha*, chemical constituents, antioxidant, antimicrobial, cytotoxicity.

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INTRODUCTION

Artocarpus lackoocha Roxb (Syn: A. *lacucha* Buch.-Ham.) is a member of the family Moraceae and is cultivated in Uttar Pradesh, Bengal, Khasi Hills and Western Ghats. It is called Monkey Jack in English and in Ayurveda it is called Lakuch, Kshudra Panas, Granthiphala and Pitanaasha. Bark when applied externally, draws out purulent matter; heals boils, cracked skin and pimples. Seeds are purgative, haemagglutinating. Stem is vermifug. A lectin, artocarpin, isolated from seeds, precipitates several galactoman-nans. It agglutinates rat lymphocytes and mouse as-cites cells[1]. Te edible fruit pulp is believed to acts as atonics for the liver. The raw fruits and male flowers spikes (acidic and astringent) are utilized in pickles and chutney. Ties preparation has been used as a traditional anthelmintic drug for treament of tapeworm infection in Thailand[2, 3]. Tree bark containing 8.5% tannin is chewed like betel nuts and is also used to treat skin ailments. It yields durable fiber good for cordage. Wood and roots yield a lavish color dye[4]. Two isolectins, ALA-I and ALA-II, isolated from seed extracts of A. *lakoocha* possessed several similar properties such as blood type agglutination, pH optimum, pH and temperature stability, as well as binding specificity towards asialomucins[5]. Two new stilbene derivatives, lakoochins A and B, were isolated from the roots of A. *lakoocha*. Both exhibited anti-mycobacterial activity and showed cytotoxic activity against some cell lines[6].Oxyresveratrol, isolated from heartwood of A. *lakoocha* has shown moderate anti-herpes simplex virus activity and anti-HIV activity against a wild-type human immunodeficiency virus type 1[7].Critical review of literature revealed scanty information on antibacterial, antioxidant, anthelmintic and insecticidal activity of fruit pericarp of A. *lakoocha*. Thus, Objectives of the present study were isolated chemical constituent and to determine antibacterial, antioxidant and cytotoxicity of different fractions of methanol extract of the stem bark of A. *lakoocha*.

MATERIALS AND METHOD

General experimental procedure

The ^{1}H NMR spectra were obtained using a Varian Unity 500 spectrometer (500 MHz) instrument in $CDCl_3$.For NMR studies deuterated chloroform was used and the δ values for ^{1}H spectra were referenced to the residual non deuterated solvent signals.

Plant materials

The stem bark of *Artocarpus lackoocha* Roxb (Family: Moraceae**)** was collected from Barisal, Bangladesh in the month of November, 2011. A voucher specimen had been maintained in the herbarium of the Department of Botany, University of Dhaka under the accession number DUH-

2879. The samples were cut into small pieces and sun dried for 7 days followed by oven drying for 24 hours at 40°C to facilitate grinding.

Extraction and isolation

The air-dried and powdered stem bark (3 kg) of *Arocarpus lackoocha* was soaked in 3L of methanol for 10 days at room temperature and then filtered through a cotton plug, followed by Whatman filter paper number 1. The extract was concentrated with a rotary evaporator. The solvent was evaporated to obtain a solid residue of around 96 gm. Then this was subjected to Vacuum liquid chromatography[8] for the initial rapid fractionation of the crude extract. The column was first eluted with 100% n-hexane. Then the mobile phases with progressively increasing polarity were passed through the column, until it reached to 100% ethyl acetate. Thin layer chromatographic technique[9] was used for the initial screening of the VLC fraction extracts and the compounds – **1** (9 mg), **2** (9 mg), **3** (9 mg) were isolated from the VLC extracts of 20% EtOAc in petroleum ether followed by TLC using Merck pre-coated TLC plates (Silica gel 60, F_{254}), eluting with petroleum ether/chloroform (60 : 40).

An aliquot (5.0 g) of the concentrated methanolic extract was fractionated by the modified Kupchan partitioning method [10] into petroleum ether (1.8g), carbon tetrachloride (0.9 g), chloroform (0.7g) and aqueous (1.1 g) soluble fractions for biological activity.

DPPH free radical scavenging activity:

The free radical scavenging activity of the extractives were determined by the method developed by Brand-Williams *et al.*[11] based on the scavenging activity of the stable 1, 1 diphenyl-2-picrylhydrazyl (DPPH) free radical. In short, 2.0 ml of a methanol solution of the extract at different concentrations were mixed with 3.0 ml of a DPPH methanol solution (20µg/ml) and the mixture was kept in dark for 20 minutes for reaction to occur. The absorbance of the resultant solution was determined at 517 nm and the percent inhibition was calculated from [(Ao–A1)/Ao] × 100, where Ao is the absorbance of the control and A1 is the absorbance of the test sample.

Antimicrobial screening:

The disc diffusion method[12] was used to evaluate the antimicrobial activity of the extractives against 10 bacteria and 3fungi (Table-2), collected as pure cultures, from the Institute of Nutrition and Food Sciences (INFS),University of Dhaka, Bangladesh. Solutions of known concentration (400µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents, $CHCl_3$ or CH_3OH. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amount of the test substance using micropipette and the residual solvents were completely evaporated. Standard disc of

ciprofloxacin (30µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4º C) for 24 hours to allow maximum diffusion of the test materials and ciprofloxacin. The plates were finally incubated at 37º C for 24 hours to allow maximum growth or inhibition of growth of the organisms. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiments were carried out in triplicate and the mean values were taken.

Evaluation of cytotoxicity:

Brine shrimp lethality bioassay[13, 14] techniques was applied for the determination of general toxic property of the plant extractives. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day assay. For the experiment, 4 mg of each of the extractive was dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563 and 0.781µg/ml) were obtained by serial dilution using DMSO. Vincristine sulphate (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 and0.0390µg/ml) was used as positive control.

Compound **1**:

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.69 (1H, s, H-29b), 4.56 (1H, s, H-29a), 4.47(1H, dd, *J*=12.8, 4.5 Hz, H-3), 2.05(3H,s,$OCOCH_3$),1.68 (3H, s, H-30), 1.04 (3H, s, H-25), 0.95 (3H, s,H-28), 0.85 (3H, s, H-23), 0.84 (3H, s, H-24), 0.83(3H, s,H-26), 0.79 (3H, s, H-27).

Compound **2**:

^{1}H NMR (500 MHz, $CDCl_3$): δ 0.75 (s, 3H, H-25), 0.80 (s, 3H, H-26), 0.85 (s, 3H, H-28), 0.95 (s, 3H, H-24),1.05 (s, 6H, H-23, H-27), 1.25 (s, 6H, H-29, H-30), 2.05 (s, 3H, $OCOCH_3$), 4.50 (m, 1H, C-3), 5.15 (1H, t, *J* =3.6 Hz, H-12) and 1.30-1.95 (23H).

Compound **3**:

^{1}H NMR (500 MHz, $CDCl_3$): δ 3.21 (1H,dd,*J*=11.0 Hz and 6.0 Hz, H-3), 5.17(1H,dd,*J*=3.5 Hz and 3.5 Hz, H-12),2.45(3H,s, $OCOCH_3$), 0.96 (3H,s, Me-23), 0.78 (3H,s, Me-24), 0.93 (3H,s, Me-25), 0.99 (3H,s, Me-26), 1.12 (3H,s, Me-27), 0.82 (3H,s, Me-28), 0.86(6H,s, Me-29& Me-30).

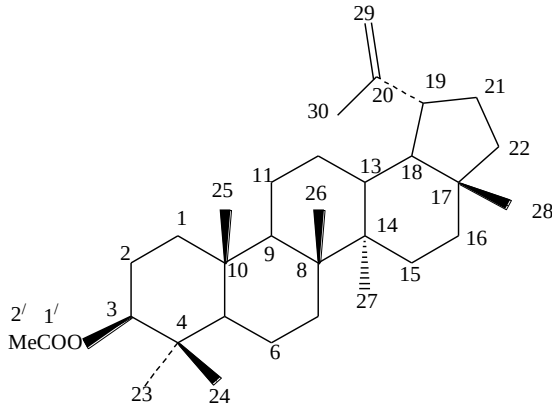
RESULTS AND DISCUSSION

Repeated chromatographic separation and purification over silica gel of the methanol extract of *Artocarpus lackoocha* provided a total of three compounds (**1-3**). The structures of isolated compounds were solved by extensive NMR data analysis, comparison with published values as well as co-TLC with authentic samples.

Compound **1** (9 mg); was isolated as white needles. The^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed the presence of eight tertiary methyl singlets at δ 0.79, 0.83, 0.84, 0.85, 0.95, 1.04, 1.68 and 2.05.Two protons appeared at δ 4.69 and 4.56 as singlets, representing the exocyclic double bond protons H-29b and H-29a, respectively. Comparing its H^{1} NMR spectral data with the literature values of reported compounds,the structure of compound **1** was elucidated as lupeol acetate.[15]

The^{1}H NMR spectrum (500 MHz, $CDCl_3$)of compound **2** showed the presence of eight tertiary methyl singlets at δ 0.75 (s, 3H, C-25), 0.80 (s, 3H, C-26), 0.85 (s, 3H, C-28), 0.95 (s, 3H, C-24),1.05 (s, 6H, C-23, C-27), 1.25 (s, 6H, C-29, C-30) on an oleanane skeleton and a characteristic triplet at δ 5.15(1H,t, C-12) was assigned to H-12, suggesting an olean-12-ene skeleton. One methine proton exhibited a double doublet at δ 4.50 assigned to H-3.The downfield shift of H-3 suggested that it was esterified and one methyl singlet at δ 2.05 showed that the compound **2** has one acetyl group. The coupling constant of this methine proton indicates that the acetyl functional group must be in axial position.

The above spectral features were similar to the ones reported for alpha-amyrin acetate. On this basis, the identity of compound **2** was confirmed as α- amyrin acetate.[16]



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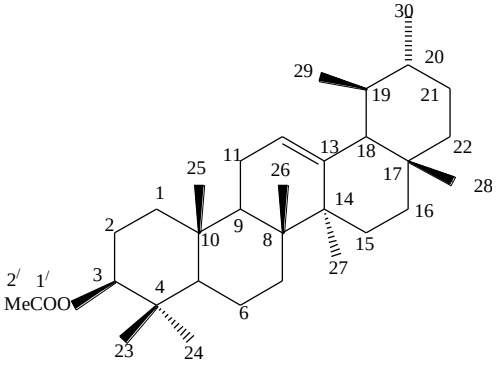

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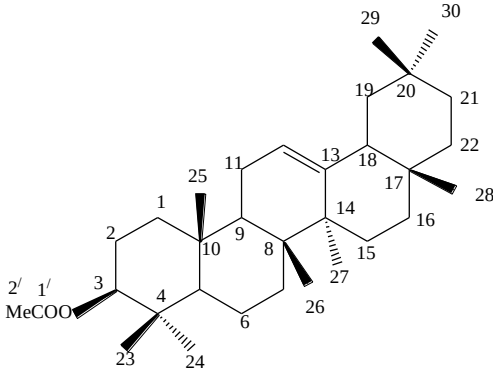

(3)

Compound **3** (9 mg); white, needle like crystal; it was visualized as a quenching spot under UV light (254 nm) on TLC plate and showed an intense pinkish color after spraying with vanillin-sulfuric acid reagent followed by heating at 110°c for 5-10 minutes. From the ^{1}H NMR spectrum, compound **3** was characterized as β-amyrin acetate.[17] The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) showed the presence of eight methyl singlets at δ 0.82 (3H,s, Me-28), 1.12 (3H,s, Me-27), 0.99 (3H,s, Me-26), 0.93 (3H,s, Me-25), 0.78 (3H,s, Me-24), 0.96 (3H,s, Me-23), 0.86 (6H,s, Me-29 & Me-30) on an oleanane skeleton i.e. the compound must be a pentacyclic compound. A characteristic double doublet at δ 5.17 (1H,dd, *J*=3.5, 3.5 Hz) was assigned to H-12, suggesting an Olean-12-ene skeleton. One methine proton exhibited a double doublet at δ 3.21 assigned to H-3.The downfield shift of H-3 suggested that it was esterified and one methyl singlet at δ 2.45 showed that the compound 3 has one acetyl group. The coupling constant of this methine proton indicates that the acetyl functional group must be in axial position. The above spectral features were similar to the ones reported for β-amyrin acetate.

The above spectral features were similar to the ones reported for β-amyrin acetate. On this basis, the identity of compound **3** was confirmed as β-amyrin acetate.[18] This is the first report of this compound from A.*lackoocha*.

Table 1. IC50 values of the standard partitionates of *Artocarpus lackoocha* in DPPH assay

Test samples	IC50 value (µg/ml)
BHT	27.54
MEF	6.43
PEF	15.54
CTF	39.25
CLF	22.46
AQ	3.47

BHT = *Tert*- butyl-1-hydroxytoluene

Among the different fractions tested for antioxidant activity (Table 1), the aqueous soluble partitionate and crude methanol extract demonstrated maximum free radical scavenging activity with IC50 value of 3.47µg/ml and 6.43 followed by the two other fractions petroleum ether (PEF, IC50 = 15.54µg/ml) and chloroform (CTF, IC50 = 22.46µg/ml) soluble fractions exhibiting significant antioxidant activity as well.

In the antimicrobial screening, the methanolic crude extract as well as its aqueous soluble fractions exhibited moderate antimicrobial activity with average zone of inhibition ranging from 7-12 mm each as compared to standard (40-42 mm) (Table 2) exhibited by ciprofloxacin. However, the chloroform soluble fraction, pet-ether soluble fraction and the negative control disc

showed no inhibition of microbial growth (data not shown in the table).

Table 3 shows the results of brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate. The LC50 values were found to be 1.6µg/ml for aqueous soluble fractions of the methanol extract revealed its toxicity to a significant degree.

Table 2. Antimicrobial activity of *Artocarpus lackoocha* extractives and ciprofloxacin

Test microorganisms	Diameter of zone of inhibition (mm)			
	MEF	AQ	CTF	Ciprofloxacin
Gram positive bacteria				
Bacillus cereus	10	10	-	42
B. megaterium	10	10	-	41
B. subtilis	8	10	8	42
Staphylococcus aureus	7	7	7	40
Sarcina lutea	9	11	10	22
Gram negative bacteria				
Escherichia coli	-	-	-	42
Salmonella paratyphi	-	-	-	42
S. typhi	-	-	-	56
Shigella boydii	-	-	-	42
Sh. dysenteriae	-	-	-	46
Fungi				
Candida albicans	7	7	7	42
Aspergillus niger	-	-	7	42
Sacharomyces cerevacae	7	7	-	42

Table 3. LC50 values of the standard and partitionate of *Artocarpus lackoocha* in brine shrimp leothalics assay.

Test Samples	LC50 values (µg/ml)
VS	0.451
MEF	3.79
PEF	54.38
CTC	2.07
CLF	6.89
AQ	1.60

VS = Vincristine sulphate, MEF = Methanolic extract of the whole plant, PEF = Petroleum ether soluble fraction of the methanolic extract of the whole plant, CTF = Carbon tetrachloride soluble fraction of the methanolic extract of the whole plant, CLF = Chloroform soluble fraction of the methanolic extract of the whole Plant, AQ= Aqueous soluble fraction of the methanolic extract of the whole Plant.

CONCLUSION:

Medicinal plants used in the folk medicine may be an interesting and unexplored source for the

development of potential new compounds. It was our attempt to identify new compounds in this plant that revealed three compounds and all of them are previously established. The crude methanol extract as well as aqueous soluble fractions of the plant exhibited moderate antimicrobial activity; the aqueous soluble materials demonstrated the highest toxicity and most potent antioxidant activity. This is only a preliminary study and a more detailed study is under progress.

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