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Research Article

BIOLOGICAL INVESTIGATION OF *ENTADA RHEEDII* SPRENG. AND ISOLATION OF ENTADAMIDE A FROM ITS SEED

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ABSTRACT

The methanolic extracts of bark, leaf and seed of *E. rheedii* and their Kupchan partitionates were investigated for in vitro cytotoxic, antioxidant, thrombolytic, membrane stabilizing and antimicrobial activities. In the brine shrimp lethality bioassay, the chloroform soluble extractive from seed showed significant cytotoxicity (LC_{50} = 1.95 µg/ml and LC_{90} = 3×10^{-4} µg/ml) as compared to vincristine sulfate (LC_{50} = 0.45 µg/ml and LC_{90} = 9×10^{-6} µg/ml). Antioxidant activity was screened using DPPH assay and ethyl acetate soluble partitionate of bark revealed the highest free radical scavenging activity with IC_{50} values of 33.68 µg/ml as compared to standard, ascorbic acid (IC_{50} 4.05 µg/ml) and butylated hydroxyl toluene (IC_{50} 27.50 µg/ml). Significant membrane stabilizing activities were observed for aqueous and ethyl acetate soluble fractions of bark in hypotonic solution-induced haemolysis (94.94%) and in heat induced condition (97.39%), respectively. No significant antimicrobial and thrombolytic activities were observed for any extractive from this plant. Chemical investigation of the seed extract of *E. rheedii* provided entadamide A (1) for the first time from this plant, which was isolated previously from *E. phaseoloides*. This is the also the first report of the bioactivities of this plant.

Key words: *Entada rheedii,* entadamide A, antioxidant, cytotoxic, membrane stabilizing.

INTRODUCTION

Entada rheedii Spreng. (Fam. Mimosaceae) is a woody climbing shrub[1] that grows naturally in Africa, tropical Asia, Australia and a small part of the pacific islands. This species is commonly found in Sylhet and Chittagong hill tracts of Bangladesh[1,2], locally known as Gila lata. The seeds are very hard and have woody shell. Seed surface is smooth and olive brown or black in color[1,2].

The seeds of *E. rheedii* are used by the folkloric medicinal practitioners, locally known as 'Kabiraj and Hekims and were found to cause vivid dreaming[3] and reported to be useful as narcotic, emetic, febrifuge, alexiteric and antiperiodic[2]. It has been reported for useful in the treatment of jaundice, diarrhoea, musculo-skeletal problems and mumps[3]. They are also used as remedy for cerebral hemorrhage and oral contraceptive[2]. On the other hand, the seed kernels of *E. rheedii* are also reported to have cell viability and HIV infectivity[4]. Bark of *E. rheedii* contains saponin, which is used as a substitute of soap[5]. In Tanzania, the bark infusion is used to cure scabies[6]. In South-East Asia, bark and seeds are used to relieve pains and itch[6]. As a good fiber, bark of *E. rheedii* is used for tying and making fish lines[6].

Due to its various traditional uses, the seed kernels of *E. rheedii* have been investigated and found to contain tryptophan derivatives, tryptorheedei A and tryptorheedei B[4], triterpenoid saponins (rheediinoside A and B)[3], oleanane-type triterpene oligoglycosides (rheedeiosides A, B, C and D), thioamide glycoside and cis-entadamide A-β-d-glucopyranoside[7]. *E. rheedii* seeds are also known to contain *N*-sulfonyl-L-tryptophan (tryptorheedei A) and 3-(*N*-sulfonylindolyl)-d-lactic acid (tryptorheedei B), 5-O-β-d-glucopyranosyl-2-hydroxyphenylacetic acid, 1-O-methylglucopyranoside, homogentisic acid and 3-O-β-d-glucopyranosyl-β-sitosterol[4].

However, *E. rheedii* has not been investigated for cytotoxic, antimicrobial, antioxidant, thrombolytic and membrane stabilizing activities. As other species of *Entada* like *E. scandens* have been reported to exhibit antinociceptive, cytotoxic, and anti-diarrheal activities[8], it was presumed that *E. rheedii* may have similar properties. As a part of our ongoing research on medicinal plants of Bangladesh[9-12] we studied *E. rheedii* and herein, report some bioactivities of the extractives of different parts and re-isolation of entadamide A from its seed.

MATERIALS AND METHODS

General experimental procedure

The ^{1}H NMR spectrum was recorded using Bruker AMX-500 instrument in $CDCl_3$ and the δ values are reported relative to the residual non-deuterated solvent signal. RV10 Basic (IKA, Germany) rotary evaporator was used for concentrating extracts. Analytical thin layer chromatography (TLC) was performed on pre coated (TLC Silica gel 60 F_{254}-Merck KGaA) plates and spots were visualized by using UV light and 1% vanillin/H_2SO_4. All other chemicals, solvents and reagents were of highest analytical grade.

Plant materials

Fresh seeds, bark and leaf from healthy, disease free, plants of *E. rheedii* were collected from Sylhet, Bangladesh in March 2012 and June 2013, respectively. The exsiccated plant samples were identified by Mr. Sarder Nasir Uddin, Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka where voucher specimens representing these collections have been preserved for future reference (Accession number: DACB-38589, 38640).

Extraction and isolation

The fresh seeds were shade dried and powdered using a suitable blender (Noka super blender, China). The dried and powdered materials (735 gm) were extracted with dichloromethane- methanol (1:1) at room temperature for 15 days with occasional shaking and stirring. The whole mixture was then filtered through a clean, white cotton followed by Whatman filter paper, number 1. The filtrate thus obtained was concentrated at 40ºC with a rotary evaporator to get a brownish gummy mass (59.76 gm, yield = 8.13%). The crude extract was then stored in the refrigerator at 4°C. A partition of the concentrated extract was fractionated by the modified Kupchan partitioning protocol[13] to afford petroleum ether, chloroform, ethyl acetate and aqueous soluble materials having 49.91% 3.12%, 3.85% and 43.12% w/w yield, respectively.

The fresh bark was cleaned, sun dried and pulverized and the powdered material (1.117 kg) was extracted with 2.5 liters of dichloromethane- methanol (1:1) at room temperature for 10 days with occasional manual shaking and stirring. The whole mixture was then filtered through a clean, cotton bed followed by Whatman filter paper and concentrated with a rotary evaporator at reduced temperature (40-45ºC) and pressure to provide a brownish gummy concentrate (83.43 gm, yield = 7.47%). A portion (25 gm) of the concentrated extract was fractionated by the modified Kupchan partitioning protocol[13] to afford petroleum ether (5.39 gm), dichloromethane (7.05 gm), ethyl acetate (3.25 gm) and aqueous (8.21 gm) soluble materials. The residues were then stored in a refrigerator until further use.

The fresh leaf was cleaned, sun dried and pulverized. The powdered material (0.93 kg) was extracted with dichloromethane-methanol as before which afforded a greenish gummy concentrate of 53.43 gm (yield = 5.74%). A portion (20 gm) of the concentrated extract was fractionated by the modified Kupchan partitioning protocol[13] to afford petroleum ether (8.12 gm), dichloromethane (6.77 gm), ethyl acetate (1.35 gm) and aqueous (3.76 gm) soluble materials. The residues were then stored in a refrigerator until further use.

The ethyl acetate soluble fraction of seed (2.30gm and percent yield=3.85%) was subjected to thin layer chromatographic (TLC) analysis. Compound (**1**) appeared as a dark oval spot under UV light (254 nm) at R_f 0.622 (EtOAc-MeOH, 95:5). When the TLC plate was sprayed with 1% vanillin/sulfuric acid and heated at 110°C for 5-10 minutes, the spot became ash. Preparative thin layer chromatography (PTLC) over silica gel (Kieselgel 60 F_{254}) using 5% methanol in ethyl acetate has led to the isolation of compound **1** (2.5 mg).

Evaluation of cytotoxicity

Cytotoxicity of the extractives was assessed by brine shrimp lethality[14,15]. Dimethyl sulfoxide (DMSO, BioReagent, for molecular biology; Sigma-Aldrich, India) was used as solvent and negative control and anticancer drug vincristine sulfate (Techno Drugs Ltd, Bangladesh) was used as positive control. In this investigation, the resulting data were processed by probit analysis software (LdP Line software, USA) to determine LC_{50} and LC_{90} values.

DPPH scavenging activity

DPPH scavenging activity was determined by the method developed by Brand-Williams et al.[16] by using tert-butyl-1-hydroxytoluene (BHT) (Jayson Pharmaceuticals Ltd. Bangladesh) and ascorbic acid as positive controls. The absorbance of the samples was recorded at 517 nm with a UV-visible spectrophotometer.

Thrombolytic activity

In vitro thrombolytic activity was assessed by following previously described method[17]. Lyophilized Altepase (Streptokinase) vial (Beacon Pharmaceutical Ltd) of 15,00,000 IU and distilled water were used as positive and negative control, respectively.

Membrane stabilizing activity

Membrane stabilizing activity in hypotonic solution- and heat-induced conditions was studied by established protocol[18].

Antimicrobial screening

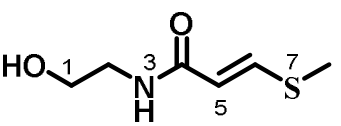
Antimicrobial activity of extracts were evaluated against 5 Gram positive (*B. cereus*, *B. megaterium, B. subtilis, Sarcina lutea, Staphylococcus aureus*) and 8 Gram negative (*Escherichia coli, Salmonella paratyphi, S. typhi, Shigella boydii, S. dysenteriae, Pseudomonas aeruginosa, Vibrio mimicus, V. parahemolyticus*) bacteria and 3 fungus (*Candida albicans, Aspergillus niger, Sacharomyces cerevisiae*) by the disc diffusion method[19]. The bacterial and fungal strains were collected as pure cultures from the State University of Bangladesh (SUB). Sterile blank discs, 400 µg/disc concentration for each extract and standard antibiotic discs (Ciprofloxacin, 30 g/disc) were used. The antimicrobial activity of the test materials was determined by measuring the diameter of zone of inhibition in millimeter with a digital slide calipers.

Properties of isolated compound

Compound (**1**): Colorless gum; ^{1}H NMR (400 MHz, $CDCl_3$); d 2.33 (3H, s, S-CH_3), 2.36 (1H, br. s, O-H), 3.41 (1H, br. s, N-H), 3.48 (2H, m, H_2-2), 3.75 (2H, t, J = 8.0 Hz, H_2-1), 5.64 (1H, d, J = 16.0 Hz, H-5), 7.65 (1H, d, J = 16.0 Hz, H-6).

RESULTS AND DISCUSSION

Entadamide A (**1**) (Figure 1) was isolated from the ethyl acetate soluble fraction of methanol extract of *E. rheedii* seed and its structure was solved on the basis of ^{1}H NMR data as well as by comparison with published values[20].



Entadamide A (**1**)

Figure 1 Structure of Entadamide A (1)

Among all the samples tested for cytotoxicity, the chloroform soluble fraction of seed revealed the highest activity (LC_{50} = 1.95µg/ml and LC_{90} = 3×10^{-4}µg/ml) as compared to vincristine sulfate (LC_{50} = 0.45µg/ml and LC_{90} = 9×10^{-6}µg/ml) (Table 1).
In DPPH scavenging assay the ethyl soluble fraction of bark demonstrated highest activity (IC_{50} = 33.68 µg/ml) as compared to BHT (IC_{50} = 27.5 µg/ml). During screening for in vitro thrombolytic activity, the petroleum ether soluble partitionate of bark revealed significant clot lysis (16.34% clot lyses, Table 2).

In the assay for membrane stabilizing activity, the ethyl acetate soluble fraction of seed displayed the highest % inhibition of hemolysis, e.g. 188.29% as compared to acetyl salicylic acid 71.91% in hypotonic solution (Table 2). On the other hand, ethyl acetate soluble fraction of bark demonstrated the highest 97.39% inhibition of hemolysis as compared to acetyl salicylic acid 42.20% in heat-induced conditions (Table 2).

Table 1: LC_{50}, LC_{90} and IC_{50} values of standard and *E. rheedii* seed, bark and leaf extractives in brine shrimp lethality and DPPH assays

Test samples	LC_{50} (µg/ml)	LC_{90} (µg/ml)	IC_{50} (µg/ml)
VS	0.45	9×10^{-6}	-
BHT	-	-	27.5
AA	-	-	4.05
SME	5.89	3.9×10^{-2}	281.34
FSPE	4.90	2×10^{-3}	555.22
FSC	1.95	3×10^{-4}	279.79
FSEA	12.56	4×10^{-3}	233.72
FSAQ	3.16	5×10^{-4}	321.88
FBPE	4.84	-	86.00
FBDCM	28.10	-	69.42
FBEA	75.45	-	33.68
FBAQ	2682.23	-	51.08
BME	7.55	-	57.86
FLPE	1302.78	-	115.35
FLDCM	99.10	-	63.80
FLEA	8436.25	-	87.71
FLAQ	12960793.13	-	43.78
LME	270.85	-	76.65

Here, VS = vincristine sulfate; BHT = *tert*-butyl-1-hydroxytoluene; AA = Ascorbic acid; SME = Methanol extract of Seed; FSPE = Petroleum ether soluble fraction of Seed; FSC = Chloroform soluble fraction of Seed; FSEA = Ethyl acetate soluble fraction of Seed; FSAQ = Aqueous soluble fraction of Seed; FBPE = Petroleum ether soluble fraction of bark; FBDCM = Dichloromethane soluble fraction of bark; FBEA = Ethyl acetate soluble fraction of bark; FBAQ = Aqueous soluble fraction of bark; BME = Methanol Extract of bark; FLPE = Petroleum ether soluble fraction of leaf; FLDCM = Dichloromethane soluble fraction of leaf; FLEA = Ethyl acetate soluble fraction of leaf; FLAQ = Aqueous soluble fraction of leaf; LME = Methanol Extract of leaf

Table 2: Thrombolytic activity in terms of % of clot lyses and % inhibition of hemolysis of erythrocyte membrane by E. rheedii seed, bark and leaf extractives

Test samples	% Clot lyses	% Inhibition of hemolysis	
		Hypotonic solution-induced (50 mM)	Heat-induced
ASA	-	71.91	42.20
SK	66.98	-	-
Water (Blank)	4.03		
SMW	10.79	42.04	31.9
FSPE	1.74	40.385	45.5
FSC	6.62	21.31	21.3
FSEA	10.58	188.29	12.62
FSAQ	7.71	28.59	37.9
FBPE	16.340	71.875	88.644
FBDCM	13.239	78.125	95.636
FBEA	9.795	90.938	97.388
FBAQ	6.952	94.938	77.118
BME	13.273	83.313	88.322
FLPE	13.713	34.375	40.144
FLDCM	11.467	42.813	28.568
FLEA	6.65	66.344	32.594
FLAQ	14.908	48.125	38.436
LME	12.773	48.750	35.556

Here, SK = streptokinase, ASA = acetyl salicylic acid

Table 3: Antimicrobial activity of test samples of Bark of *E. rheedii*

Test Organisms	**Diameter of zone of inhibition (mm)**					
	FBPE	FBDCM	FBEA	FBAQ	BME	Ciprofloxacin
Gram positive bacteria						
Bacillus cereus	08	10	13	-	11	39
Bacillus megaterium	12	16	12	-	10	36
Bacillus subtilis	-	-	-	-	-	35
Sarcina lutea	12	14	14	-	14	36
Staphylococcus aureus	12	08	10	-	06	40
Gram negative bacteria						
Escherichia coli	10	-	-	-	06	40
Salmonella paratyphi	09	11	-	-	06	31
Salmonella typhi	16	-	10	-	10	38
Shigellaboydii	13	11	13	-	11	42
Shigella dysenteriae	11	06	06	-	07	36
Pseudomonas aeruginosa	08	12	14	-	11	40
Vibrio mimicus	08	11	06	-	06	29
Vibrio parahemolyticus	10	07	14	-	07	38
Fungi						
Candida albicans	11	08	-	-	05	40
Aspergillus niger	14	08	11	-	11	34
Sacharomyces cerevisiae	14	11	08	-	11	36

On the other hand, the dichloromethane soluble extractive of the bark exhibited mild to moderate antimicrobial activity against 16 microorganisms ranging from 08 to 16 mm (Table 3) as compared to ciprofloxacin (29-40 mm). The highest activity was measured against *B. megaterium* (16 mm).

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