Phytochemical and Biological Investigations of *Phyllanthus reticulatus*

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ABSTRACT: Scopoletin (**1**) was isolated from the chloroform soluble fraction of a methanol extract of the stem bark of *Phyllanthus reticulatus* (Family: Euphorbiaceae). The petroleum ether, carbon tetrachloride and choloroform soluble fractions of this methanol extract were subjected to antimicrobial screening and brine shrimp lethality bioassay. All of the partitionates showed moderate to strong inhibitory activity to microbial growth while the chloroform soluble fraction showed strongest cytotoxicity having LC_{50} 1.99 µg/ml.

Key words: *Phyllanthus reticulatus*, Euphorbiaceae, Scopoletin, Brine shrimp lethality bioassay, Antimicrobial

INTRODUCTION

Phyllanthus reticulatus (Bengali name- Panjuli; Family- Euphorbiaceae) is a large glabrous or pubescent and climbing shrub which grows all over Bangladesh.[1,2] The fruit is an astringent to the bowels and is used in inflammation. The leaves are employed as a diuretic and cooling medicine. The juice of the leaves is used to care diarrhoea in infants. The stems are used to treat sore in eyes and the powdered leaf is used in sores, burns, suppurations and chafing of the skin.[3] Previous phytochemical investigations resulted in the isolation of tannic acid, friedelin, epifriedelinol, betulin, taraxerone, beta- sitosterol, glochidonol, octacosanol, taraxeryl acetate and 21-alpha-hydroxyfriedelan-3-one.[2] Here, the preliminary antimicrobial and cytotoxicity activities of the organic extractives and the isolation of a coumarin, scopoletin from the chloroform soluble material of the methanol extract are reported.

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MATERIALS AND METHODS

General experimental procedure. The ^{1}H NMR spectra were recorded using a Bruker AMX-400 (400 MHz) instrument. For NMR studies deuterated chloroform was used and the δ values for ^{1}H spectra were referenced to the residual nondeuterated solvent signals.

Plant Material. Stem bark of *P. reticulatus* was collected from Dhaka in the month of September 2005. A voucher specimen for this collection has been deposited in the Bangladesh National Herbarium, Dhaka (accession No. 31375).

Extraction and Isolation. The powdered stem bark (550 g) of *P. reticulatus* was soaked in 1.5 L methanol for 7 days and filtered through a cotton plug followed by Whatman filter paper number 1. The extract was then concentrated by using a rotary evaporator. A portion (5 g) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning method[4] into petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions. Evaporation of solvents afforded petroleum ether (0.50 g), carbon tetrachloride (0.65 g), chloroform (1.30 g) and aqueous soluble materials.

The chloroform soluble fraction was fractionated by column chromatography (CC) over silica gel (60-120 mesh) using *n*-hexane, ethyl acetate and methanol mixtures of increasing polarities to give 70 fractions, collecting each 25 ml. Preparative thin layer chromatography (stationary phase- silica gel F_{254}, mobile phase - 30 % ethylacetate in toluene, thickness of plates-0.5 mm) of fractions 58-60 afforded compound **1**.

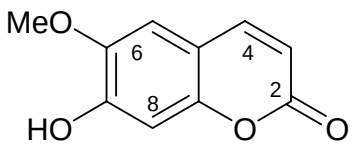
Scopoletin (**1**): white gum; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58 (1H, d, *J = 9.4* Hz, H-4), 6.91 (1H, br.s, H-8), 6.83 (1H, s, H-5), 6.25 (1H, d, *J* = 9.4 Hz, H-3), 6.10 (1H, br. s, OH-7), 3.25 (1H, br.s, OMe-6)

Bioassays. The antimicrobial activity of the crude extracts was determined by the disc diffusion method.[5] The samples were dissolved separately in chloroform and applied to sterile filter paper discs at a concentration of 400 µg/ disc. Kanamycin disc (30 µg/disc) was used as standard in each study. DMSO solutions of the plant extracts were assayed for cytotoxicity against *Artemia salina* in a 1-day *in vivo* assay, the experimental details of which could be found elsewhere.[6] For the experiment 4 mg of each of the Kupchan fractions was dissolved in DMSO. Solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ml were obtained by serial dilution technique. The median lethal concentration LC_{50} of the test samples after 24 hrs was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Here vincristine sulphate was used as a standard.

RESULTS AND DISCUSSION

Compound **1** was isolated from the chloroform soluble fractions of a methanolic extract of the stem bark of *P. reticulatus* by repeated chromatographic separation and purification over silica gel. The structure of the isolated compound was determined by ^{1}H NMR data analysis as well as by comparison with previously reported values.[7]

The ^{1}H-NMR spectrum (400 MHz, $CDCl_3$) displayed a clear AB quartet (*J*=9.4 Hz) centered at δ 6.25 (1H) and 7.58 (1H), which were typical for H-3 and H-4 of a coumarin nucleus.[7] The relatively short peak at δ 7.58 indicated a long range zig zag coupling of H-4 with H-8 appeared at δ 6.91 over five bonds. The spectrum also showed a singlet at δ 6.83 and a broad singlet at δ 6.10, each of one proton intensity. These could be assigned to H-5 and a hydroxyl group proton at C-7.



A three proton singlet in the spectrum at δ 3.95 revealed the presence of a methoxyl group. Comparison of the chemical shifts of the methoxyl and hydroxyl groups allowed to place these substituents at C-6 and C-7, respectively. On this basis, compound **1** was characterized as 7-hydroxy-6-methoxy coumarin (scopoletin). The identity of compound **1** as scopoletin was confirmed by comparison of its spectral data with reported values.[7] Although it has previously been reported from many plants,[8] but this is the first report of its isolation from *P. reticulatus.*

In the antimicrobial screening, the extractives of the *P. reticulatus* exhibited significant antimicrobial activity. The zone of inhibition produced by the pet ether, carbon tetrachloride and chloroform soluble fractions of methanolic extract ranged from 14-19 mm, 14-20 mm and 10-18 mm, respectively (Table 1). Following the procedure of Meyer,[6] the lethality of the pet ether (PE), carbon tetrachloride (CT) and chloroform (CF) soluble fractions of the methanolic extract to brine shrimp was determined on *A. salina*. Table 2 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate. The LC_{50} obtained from the best-fit line slope were found to be 2.34, 3.89 and 1.99 µg/ml for pet ether, carbon tetrachloride and chloroform, respectively (Table 2). In comparison with the positive control (vincristine

sulphate), the cytotoxicity exhibited by the pet ether and chloroform soluble fractions of methanolic extract was significant.

The results of antimicrobial and cytotoxicity screening were found to be consistent with the folk uses of *P. reticulatus* by local people.

Table 1. Antimicrobial activity of *P. reticulatus* extractives

Test microorganisms	Diameter of zone of inhibition (mm)			
	PE	CT	CF	KAN
Gram positive bacteria				
Bacillus cereus	15	18	17	25
Bacillus megaterium	17	16	10	30
Bacillus subtilis	15	15	15	23
Staphylococcus aureus	14	15	14	25
Sarcina lutea	15	18	18	24
Gram negative bacteria				
Escherichia coli	14	14	15	22
Pseudomonas aeruginosa	15	15	15	20
Salmonella paratyphi	18	20	14	25
Salmonella typhi	18	18	15	25
Shigella dysenteriae	15	15	13	25
Vibrio mimicus	16	17	17	28
Vibrio parahemolyticus	18	18	16	25
Fungi				
Candida albicans	15	16	15	25
Aspergillus niger	19	20	18	25
Sacharomyces cerevacae	15	17	16	20

PE (400 µg/disc): pet ether soluble fraction of the methanolic extract; CT (400 µg/disc): carbon tetrachloride soluble fraction of the methanolic extract; CF (400 µg/disc): chloroform soluble fraction of the methanolic extract; KAN: standard kanamycin disc (30 µg/disc); diameter of zone of inhibition less than 8 mm was considered inactive.

Table 2. LC_{50} data of test samples of *P. reticulatus.*

Sample	LC_{50} (µg/ml)
VS	0.32
PE	2.34
CT	3.89
CF	1.99

VS: vincristine sulphate (Std.), PE: pet ether soluble fraction of the methanolic extract, CT: carbon tetrachloride soluble fraction of the methanolic extract. CF: chloroform soluble fraction of the methanolic extract.

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