Dhaka Univ. J. Sci. 58(2): 191-193, 2010 (July)

Phytochemical and Biological Investigations of *Casuarina equisetifolia*

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Received on 25. 05. 2009. Accepted for Publication on 06. 01. 2010.

Abstract

6, 7- dimethoxy coumarin(1) and **Scopoletin (2)** were isolated from the chloroform soluble fraction of a methanol extract of the fresh leaves of ***Casuarina equisetifolia*** (Family: Casuarinaceae). The carbon tetrachloride and choloroform soluble fractions of crude methanol extract were subjected to antimicrobial screening and the petroleum ether, carbon tetrachloride and chloroform soluble fraction of methanol extract were subjected to brine shrimp lethality bioassay. All of the partitionates showed poor to mild inhibitory activity to microbial growth while the chloroform soluble fraction showed significant cytotoxicity having LC_{50} 3.16 µg/ml. This is the first report of occurrence of **compounds (1)** and **(2)** from ***C. equisetifolia.***

Key words: *Casuarina equisetifolia*, Casuarinaceae, Scopoletin, 6, 7- dimethoxy coumarin, Brine shrimp lethality bioassay, antimicrobial screening

I. Introduction

Casuarina equisetifolia (Bengali name-Jhau; Family-Casuarinaceae) is evergreen shrubs and trees growing to 35 m tall. The foliage consists of slender, much-branched green to grey-green twigs bearing minute scale-leaves in whorls of 5–20.It grows in sea shore areas such as Chittagong native to Australasia, southeastern Asia, and islands of the western Pacific Ocean[1,2,3].This plant contains antibacterial, anticancer, and antitumor agent. Gallic acid, protocatechuic acid, hydroquinons, fuglanin, afzelin (+) –catechin, (-) – epicatechin, and (+) – gallacatechin were isolated from the tittle fruits and wood.Tryptophen, leucin, valine,tyrosine and glycine were isolated from leaves[4,5].Here, the preliminary antimicrobial and cytotoxicity activities of the organic extracts and the isolation of **Compound(1)** and **Compound (2)** from the chloroform soluble material of the methanol extract are reported for the first time.

II. 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 referred to the residual nondeuterated solvent signals.

Plant Material

The plant of *Casuarina equisetifolia* was collected from Chittagong in the month of July 2007. It was identified by Bangladesh National Herbarium, Dhaka. A voucher specimen has been deposited in the Bangladesh National Herbarium, Dhaka (DACB - 33735) for the collection. The leaves of the plant were cut into small pieces and then air dried for several days. The pieces were then dried in oven for 24 hours at considerably low temperature to affect grinding. The oven dried leaves were then ground into a coarse powder using a grinding machine.

Extraction and Isolation

The air dried and powdered plant material (950 gm) was soaked in 2.5 litre of methanol for eight days for the purpose of cold extraction. The extract was filtered through fresh cotton bed and finally Whatman No.1 filter paper. The filtrate was concentrated with a rotary evaporator at low temperature (40^0-50^0C) and reduced pressure. The weight of the crude extract was 27.522 gm. The concentrated methanol extract was fractionated by the modified Kupchan partitioning method [6] into petroleum ether, carbon tetrachloride and chloroform soluble fractions. Evaporation of solvents afforded petroleum ether, carbon tetrachloride and chloroform extract. The chloroform soluble fraction(2.937gm) was fractionated by column chromatography (CC) over silica gel (70-230 mesh) using petroleum ether, ethyl acetate and methanol mixtures of increasing polarities to give 38 fractions, collecting in each 100ml. Depending on the TLC behaviour, fractions were mixed and a portion (100mg) of the mixed column fractions from 11 to 15 of chloroform ($CHCl_3$) fraction was subjected to column chromatography for fractionation by column chromatography (CC) over silica gel (70-230 mesh) using petroleum ether, followed by mixtures of petroleum ether and dichloromethane of increasing polarity, then by dichloromethane and finally dichloromethane and methanol mixtures of increasing polarity to give 35 fractions, collecting in each 15ml . Preparative thin layer chromatography (stationary phase- silica gel $F_{254,}$ mobile phase – 57.5 % petroleum ether in dichloromethane and 52.5 % petroleum ether in dichloromethane, thickness of plates-0.5 mm) of fractions 16 and 18 afforded **compound (1) and compound (2)** respectively.

6, 7- dimethoxy coumarin(1): white crystalline mass; ^{1}H NMR (400 MHz, $CDCl_3$)**:** δ 6.27 (1H, d,$J_{3,4}$=9.4Hz, H-3),7.60 (1H, d,$J_{4,3}$= 9.4Hz, H-4), 6.85 (1H, s, H-5), 3.94 (3H,s,OCH_3-6), 3.91 (3H,s,OCH_3-7),6.84(1H,s,H-8).

Scopoletin (2): white gum; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.57 (1H, d,J = 9.6 Hz, H-4), 6.83 (1H, s, H-8), 6.91 (1H, s, H-5), 6.25 (1H, d,J = 9.6 Hz, H-3), 3.95 (3H,s, OMe-6).

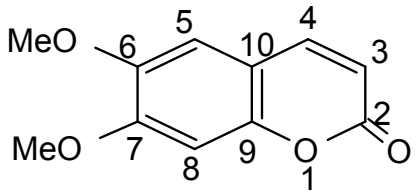
Bioassay. The antimicrobial activity of the crude extracts was determined by the disc diffusion method [7,8].The

extracts were dissolved separately in chloroform and applied to sterile filter paper discs at a concentration of 500 µg/ disc. Kanamycin disc (30µg/disc) was used as standard in each study. For cytotoxicity screening, DMSO solutions of the compounds were applied against *Artemia salina* for 24 hours in vivo simplified assay. In this experiment, the extracts were dissolved in DMSO and by serial dilution technique, 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. Then each of these test solutions was added to test tubes containing 10 shrimps in simulated brine water (5 ml).After 24hrs,the median lethal concentration(LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. Vincristine sulphate was used as positive control in this assay to compare the cytotoxicity of the extracts.

III. Results and Discussion

Compound(1) and **Compound (2)** were isolated from the chloroform soluble fraction of a methanol extract of the fresh leaves of ***Casuarina equisetifolia*** 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.[9,10,11]

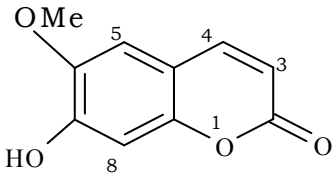
The ^{1}H NMR spectrum (400 MHz , $CDCl_3$) of **compound(1)** revealed the presence of four protons in the aromatic /olefinic region and two O- methyl groups. The ^{1}H NMR of **compound(1)** in $CDCl_3$ displayed signals characteristics of coumarin. The lactone ring protons showed the AB pattern for H-3 (δ 6.27, d, $J_{3,4}$ = 9.4 Hz) and H-4 (δ 7.60, d, $J_{4,3}$ = 9.4 Hz). In the ^{1}H NMR spectrum of **compound (1),** the presence of two aromatic singlets at δ 6.85 (1H, s) ppm and δ 6.84 (1H, s) ppm were attributable to H-5 and H-8 respectively. The resonance of two - three protons singlets at δ 3.94 ppm and δ 3.91 ppm in the ^{1}H NMR spectrum proved the presence of two O-methyl groups. The absence of any coupling between the two aromatic protons at C-5 and C-8 necessitated the accommodation of these two o– methyl group at C-6 and C-7.



Compound (1)

On this basis, **compound (1)** was identified as 6, 7 – dimethoxy coumarin.The identity of this compound as 6, 7 – dimethoxy coumarin was further substantiated by comparison of its spectral data with previously reported values.[11]This is the first report of its occurrence in ***C. equisetifolia.***

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of **compound (2)** displayed signals characteristics of 6, 7-dioxygenated coumarin. The spectrum revealed two doublets at δ 6.25 (^{1}H, d, J=9.6 Hz*)* and δ 7.57 (^{1}H, d, J=9.6 Hz*)* characteristic of H-3 and H-4 protons respectively of the pyrone ring of a coumarin . The presence of two aromatic proton singlets at δ 6.91 and δ 6.83 were attributable to



Compound (2)

H-5 and H-8 respectively. In this spectrum a three-proton singlet at δ 3.95 was assigned for a methoxyl group at C-6.

On this basis, **compound (2)** was identified as **Scopoletin** the identity of this compound as **Scopoletin** was further substantiated by comparison of its spectral data with previously reported values.[9,10]This is the first report of its occurrence from ***C. equisetifolia.***

In the antimicrobial screening, the extracts of the ***Casuarina equisetifolia*** exhibited poor and mild antimicrobial activity against most of the test organisms cited in table-1.The zone of inhibition produced by the methanol extract, carbon tetrachloride and chloroform soluble fractions ranged from 07 – 8 mm, 07 – 10 mm and 08 – 11 mm respectively at a concentration of 500 µg/disc.

Following the procedure of Meyer [12, 13], the lethality of crude extract of methanol, carbon tetrachloride and chloroform fractions were screened by brine shrimp lethality bioassay for probable cytotoxic activity. The LC_{50} obtained from the best-fit line slope were found to be 56.23µg/ml, 16.59µg/ml and 3.16µg/ml for methanol extract carbon tetrachloride and chloroform fractions, respectively cited in table-2.In comparison with the positive control (vincristine sulphate), the cytotoxicity exhibited by chloroform soluble fractions of methanol extract was significant. The results of antimicrobial and cytotoxicity screening were found to be consistent with the folk uses of ***C.equisetifolia*** by local people.

Table. 1. Antimicrobial activity of *C.equisetifolia* extracts (500 µg/disc) and Kanamycin (30 µg/disc)

Test microorganisms	Diameter of zone of inhibition (mm)			
	MeOH	CCl_4	$CHCl_3$	KAN
Gram Positive				
Bacillus cereus	NA	10	9	39
B. megaterium	8	7	10	32
B. subtilis	7	NA	9	20
Staphylococcus aureus	7	8	8	22
Gram Negative				
Escherichia coli	NA	8	9	23
S. typhi	NA	NA	9	20
Shigella boydii	7	7	10	26
Vibrio mimicus	7	9	11	24
Fungi				
Candida albicans	NA	8	9	24
Aspergillus niger	8	NA	11	32

MeOH: methanol extract; **CCl_4**: carbon tetrachloride fraction; **$CHCl_3$**: chloroform fraction; **KAN**: kanamycin; **NA**: No Activity.

Table. 2. LC_{50} data of *C.equisetifolia* extracts and vincristine sulfate.

Samples	LC_{50} (µg/ml)
VS	0.33
MeOH	56.23
CCl_4	16.59
$CHCl_3$	3.16

The values of LC_{50} are expressed in µg/ml. **VS:** vincristine sulphate (Std.); **MeOH**: methanol extract; **CCl_4**: carbon tetrachloride fraction **$CHCl_3$**: chloroform fraction

Acknowledgements

The authors wish to thank the Bangladesh Council of Scientific and Industrial Research under Ministry of Science and Information & Communication Technology, Government of the Peoples' Republic of Bangladesh.

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