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Phytochemical and Biological Evaluation of MeOH Extract of *Casuarina equisetifolia* (Linn.) Leaves

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FA and SMMH designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors JI, SMSH, MMR managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The present study was aimed at investigating the phytoconstituents in order to correlate the folkloric claims with the bioactive compounds present in *Casuarina equisetifolia.* Also evaluate the antioxidant, antibacterial and cytotoxic property of *Casuarina equisetifolia.*
Methodology: In the present study, the leaf extracts were investigated for different phytochemical groups using specified reagents. Antioxidant activity by following DPPH free radical scavenging study, antibacterial activity by disc diffusion method and cytotoxic activity by Brine shrimp (*Artemia salina*) lethality bioassay procedures.
Results: The qualitative phytochemical screening revealed that the extract contains alkaloids, glycosides, tannins, steroids, etc. The extract showed strong antioxidant activity in DPPH free radical scavenging study (IC_{50}: 25.89µg/mL), while, it showed moderate cytotoxic activity in Brine shrimp (*Artemia salina*) lethality bioassay study (LC_{50}: 77.98µg/mL). It also showed mild antibacterial activity against both gram positive and gram negative bacteria.

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Conclusion: The present study tends to suggest the antioxidant, cytotoxic and mild antibacterial activity of MeOH extract of *Casuarina equisetifolia.*

Keywords: Casuarina equisetifolia; antioxidant; antibacterial; cytotoxic.

ABBREVIATIONS

DPPH=2, 2-diphenyl-1-picrylhydrazyl; LC_{50}=Lethal concentration for 50% of the population; IC_{50}=Inhibition concentration for 50% of the sample.

1. INTRODUCTION

Plants are the major source of medicine; most of the modern medicines are originated from plants. Today, about 80% of the World's population still depends on the traditional medicine practices for the management of various diseases [1,2] and day by day it becoming more popular. Medicinal plants are good source of many potent and powerful drugs [3].

Present days phytochemical, pharmacological, toxicological screening on different plant extracts are going on and there is a growing interest in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity [4,5]. The toxicological effects of most of the crude extracts are often overlooked based on the facts that plant medicines have better compatibilities with the human body and produce fewer side effects, although the adverse effects, sometimes life-threatening, allegedly arising from herbal remedies consumption [3].

Casuarina equisetifolia family Casuarinaceae is a perennial tree, widely distributed all over the world. Its leaves are traditionally used for the treatment of mouth infection, rheumatism, stomachache, urinary tract infection [6]. The plant is traditionally also used to treat constipation, cough, diabetes, diarrhea, dysentery, gonorrhea, nervous disorders, stomach acne, throat infections and ulcer [7-9]. The plant is reported to have various biological activities like anti-asthmatic [10], antimicrobial [11], antioxidant [12-14], antifungal [15], hepatoprotective [16], nitrogen fixation [17], antidiarrheal [18], antidiabetic, antihyperlipidemic [19], and antiulcerogenic [20].

Seventy-six compounds comprising of monoterpene hydrocarbons (29.3%), oxygenated monoterpenoids (16.2%), sesquiterpene hydrocarbons (2.7%), oxygenated derivatives (1.0%), aliphatic (40.6%) and non-terpenoid (7.2%) compounds were observed in the leaf oils. The major compounds were pentadecanal (32.0%) and 1,8-cineole (13.1%). Significant quantities of α-phellandrene (7.0%), apiole (7.2%) and α-terpinene (6.9%) were present. The fruit oil was devoid of sesquiterpene hydrocarbon compounds. The main constituents were caryophyllene-oxide (11.7%), translinalol oxide (11.5%), 1,8-cineole (9.7%), α-terpineol (8.8%) and α-pinene (8.5%). All the eleven compounds identified in the oil occurred at levels between 3.4 and 11.7%. Both caryophyllene-oxide and trans-linalool oxide were absent in the leaf oil [21]. Phytoconstituents reported in *C. equisetifolia* include β-sitosterol, campesterol, stigmasterol, cholesterol, cholest-5-en-3-β-ol derivatives, casuarine, catechin, citrulline, cupressuflavone, epicatechin, gallicin, gentisic acid, isoquercitrin, juglanin, kaempferol, proanthocyanidins, rutin, trifolin [22,23].

The present study was aimed at investigate the safety profile as the plant having enormous biological activity and traditional uses. Cytotoxicity potential of the plant on brine shrimp (*Artemia salina*) larvae can reveal the safety profile. Since toxicological evaluation of plant extracts seeks to determine its possible collateral effects to ensure the safety of its use, brine shrimp larvae being sensitive to toxic substances are commonly used for toxicity assays in pharmacology. This paper present for the first time, cytotoxicity of *Casuarina equisetifolia* leaf extracts. This biological screening may open a new window for herbal use of this plant.

2. MATERIALS AND METHODS

2.1 Plant Collection

Leaves of *Casuarina equisetifolia* were collected from National Botanical Garden, Dhaka, Bangladesh on November, 2013 and were authenticated at department of botany, Chittagong University, Bangladesh. A voucher specimen (Acc. No. SBU1031) was also deposited for future reference in department of pharmacy and botany, Chittagong University, Bangladesh.

2.2 Extraction of Plant

400g of leaves of *Casuarina equisetifolia* were cut into small pieces and was macerated in MeOH at 37°C for 15 days accompanying occasional shaking and stirring. The whole mixture was then underwent a coarse filtration by clean, white cotton, followed by a filtration through Whatmann filter paper. The filtrate was then evaporated through rotary evaporator followed by desiccation to get the dried crude extract (yield:12%). This extract was used for phytochemical and biological screening [24].

2.3 Phytochemical Screening

MeOH extract of *Casuarina equisetifolia* was tested for different chemical groups according to the described methods [25-28].

2.4 Anti Oxidant Activity

Anti oxidant activity of *Casuarina equisetifolia* was tested using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) free radical scavenging assay method modified by Gupta et al. [29,30]. In short, different concentrations (1, 5, 10, 50, 100 and 500μg/mL) of extract and ascorbic acid was prepared as standard in ethanol and was taken in test tubes. Then 2mL of 0.004% of DPPH solution was added to each test tube, shaken well and allowed to stand for 30 min for reaction to occur. The absorbance was then determined at 517nm. Then % inhibitions were plotted against concentration and IC_{50} was calculated from the graph. The experiment was performed in triplicate and average absorption was noted for each concentration.

2.5 Antibacterial Activity

Antibacterial activity of *Casuarina equisetifolia* was tested by the disc diffusion method [31,32]. Sterilized Whatmann No. 1 filter paper was cut into circular disc diameter of 6mm. Calculated amount of the test sample was infused with disc, allowed to dry and placed onto inoculated nutrient agar seeded plates aseptically. The plates were incubated with the

extract of Casuarina equisetifolia at 37°C for 24 hours. Then the diameters of the zone of inhibition were measured in millimeters. Antibacterial activity test was carried out in triplicate. Sample disc used 500µg/disc of extract solution; Standard disc used of ciprofloxacin (30µg/disc) from Ovoid Ltd. served as positive control and blank discs was used as negative control [33].

2.6 Brine Shrimp Lethality Bioassay

The Brine shrimp Nauplii were obtained by hatching eggs in artificial sea water after incubation at 37°C for 48h with continuous oxygen's supply. The nauplii were allowed to stand for another 48h in seawater to ensure survival and maturity before use. Six conc. of plant extract (10, 20, 40, 80, 160, and 320µg/mL) in DMSO. Each extract preparation was dispensed into clean test tube 10ml volumes and tested in triplicate. The concentration of DMSO in the vials was kept below 20µL/mL. For control, same procedure was followed except test sample. After marking the test tubes properly, 10 living nauplii were added to each of the 20 vials with the help of the Pasteur pipette. The test tube containing the sample and control were then incubated at 37°C for 24h in a water bath, after which tube was examined and the surviving nauplii counted. From this, the percentage of mortality was calculated at each concentration [34-38]

2.7 Statistical Analysis

A significant difference between the control group and experimental groups was determined by the Student's *t*-test.

3. RESULTS

3.1 Phytochemical Screening

Phytochemical analysis showed that *Casuarina equisetifolia* contains flavonoids, alkaloid, saponin, tannin, steroid among others. The results are as given in Table 1.

Table 1. Test result for chemical groups of *Casuarina equisetifolia*

S/no.	Compound groups	Present/absent
1	Flavonoid	+
2	Alkaloid	+
3	Saponin	+
4	Tannin	+
5	Steroid	+
6	Sugar	+
7	Gum	+

ME=Methanolic extract, +=Present, -=Absent

3.2 DPPH Free Radical Scavenging Activity

In comparison to ascorbic acid *Casuarina equisetifolia* showed strong antioxidant (DPPH free radical scavenging activity**)** activity where the IC_{50}=25.71µg/mL Table 2, Fig. 1.

Table 2. DPPH free radical scavenging activity of *Casuarina equisetifolia*

Concentration (µg/mL)	% inhibition		IC_{50}	
	Ascorbic acid Mean±SD	*Casuarina equisetifolia* Mean±SD	*Casuarina equisetifolia*	Standard ascorbic acid
1	4.01±0.001	11.48±0.004	25.71±0.003*	5.98±0.007*
5	48.03±0.005	16.84±0.002		
10	80.14±0.002	17.92±0.004		
50	93.04±0.003	65.05±0.002		
100	94.03±0.007	85.45±0.002		
500	95.14±0.009	90.17±0.006		

*Values represent the mean±SD; Number of readings in each group =3, * p<0.001 vs. control*

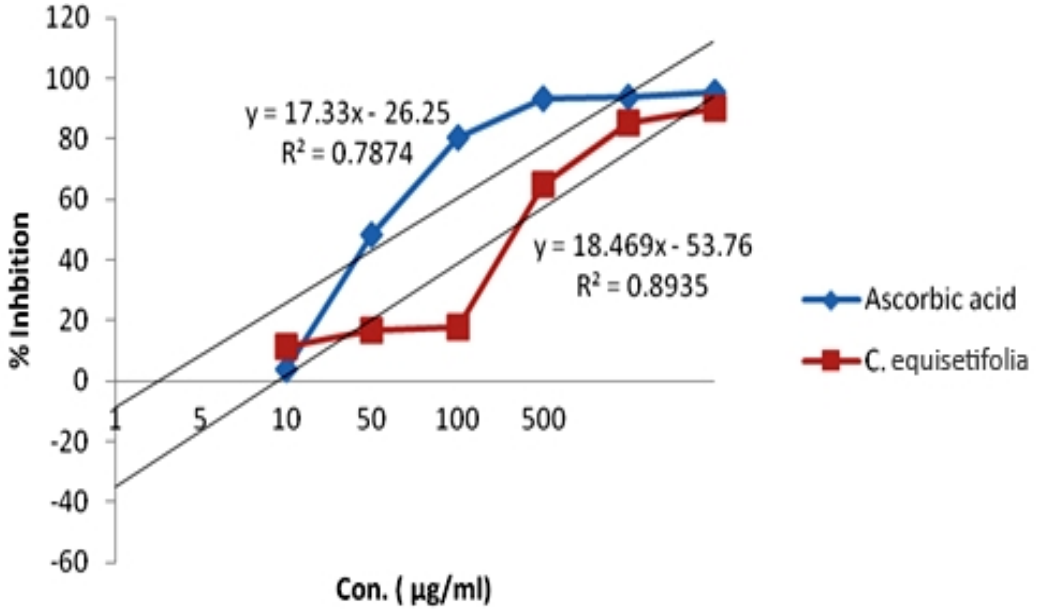

Fig. 1. DPPH free radical scavenging activity of *Casuarina equisetifolia,* values represent the mean±SD; Number of readings in each group =3

3.3 *In vitro* Antibacterial Activity

Methanolic extracts of the leaves of *Casuarina equisetifolia* showed mild antibacterial activity against test organism Table 3.

3.4 Brine Shrimp lethality bioassay

Methanolic extracts of leaves of *Casuarina equisetifolia* showed moderate Cytotoxic Activity comparable to standard sample in Brine Shrimp lethality bioassay test where the LC_{50} was found at 95.87µg/mL Table 4, Fig. 2.

Table 3. *In vitro* Antibacterial activity of *Casuarina equisetifolia*

Bacterial strains		Diameter of zone of inhibition (mm)	
		Sample (250µg/disc) Mean±SD	Ciprofloxacin HCl (30µg/disc) Mean±SD
Gram positive	*Bacillus subtilis*	8.02 ±0.23	30.01±0.21
	Bacillus cereus	6.11 ±0.12	32.21±0.23
Gram negative	*Pseudomonas aureus*	7.23 ±0.27	35.06±0.46
	E. coli	7.14 ±0.33	35.04±0.44

Values represent the mean±SD; Number of readings in each group =3.

Table 4. Brine Shrimp lethality bioassay of *Casuarina equisetifolia*

Conc. of *Casuarina equisetifolia*	Log conc.	% mortality	LC_{50} (µg/mL) Mean±SD
10	1	10	95.87±0.86*
20	1.301	20	
40	1.602	20	
80	1.903	60	
160	2.204	60	
320	2.505	70	

*Values represent the mean±SD; Number of readings in each group=3, * p<0.001 vs. control*

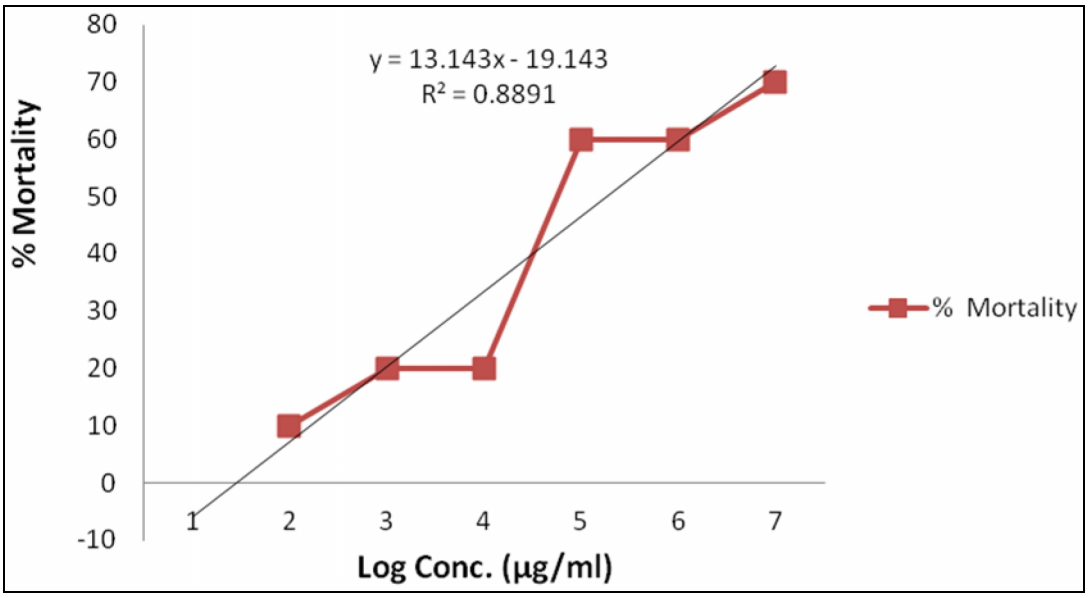

Fig. 2. Brine Shrimp lethality bioassay of *Casuarina equisetifolia,* values represent the mean±SD; Number of readings in each group=3

4. DISCUSSION

Flavonoid, tannin, alkaloid, Saponin, steroid, sugar, and gum has been Preliminary confirmed by phytochemical screening in the plant extract. The major phenol compounds that were isolated from leaves of *Casuarina equisetifolia* were gallic, protocatioic, chlorogenic, p. hydroxy benzoie, p. coumaric, syringic, vanillic and salicylic acid [39]. These phenolic compounds may be responsible for antioxidant activity. *Casuarina equisetifolia* has tannic acid confirmed from phytochemical screening could have for the antioxidant action Fig. 1 [40]. Polyphenolic compounds, like flavonoids, tannins and phenolic acids have the antioxidant activity. With the increasing dose the antioxidant activity was increased significantly ($P<0.001$). Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [41]. An antioxidant is any substance that, when present at low concentrations significantly prevents oxidation of cell content like protein, lipid, carbohydrates and DNA. *Casuarina equisetifolia* shows more than five times IC_{50} than standard ascorbic acid and may be a good source of natural antioxidant.

Extracts are regarded as non-toxic if its LC_{50} is greater 100µg/mL in brine shrimp lethality assay [38]. The mortality percentage and LC_{50} (lethal concentration for 50% of the population) were determined using statistical analysis and the graph of Logarithm of concentration against percentage lethality [40]. Methanol extract of leaves of *Casuarina equisetifolia* has moderate cytotoxicity in comparison to standard sample Fig. 2. According to the literature, compounds that present brine shrimp (Artemia salina) toxicity, in general also have cytotoxic properties against cells of solid tumors found in humans [42]. The presence of flavonoids, glycosides, alkaloids and saponin in plants probably responsible for this activity because the biological activities of plants may be due to the presence of these diverse group of chemical compounds [43]. However, increasing the concentration of *Casuarina equisetifolia* leaf extract increasing the percent mortality rate significantly, $P<0.001$.

Methanol extract of *Casuarina equisetifolia* showed mild effect against the Gram positive and Gram-negative organisms. Kishore and Rumana [44] reported that the presence of alkaloids, proteins, glycosides, saponins, flavonoids and tannins of *C. equisetifolia* bark extract might be responsible for the antimicrobial activity. The antimicrobial effect of methanol extract of *Casuarina equisetifolia* against these organisms may be responsible for phenolic compounds, alkaloids, saponin and flavonoids present in the leaf extract.

5. CONCLUSION

Cytotoxic activity study reveals that *Casuarina equisetifolia leaves are safe for human use and as Casuarina equisetifolia* having good antioxidant it may be used as natural antioxidant.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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