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Antimicrobial and Preliminary Cytotoxic effects of Ethanol extract and its fractions of *Anthocephalus cadamba* (Roxb.) Miqstem bark

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Abstract

Anthocephalus cadamba(Roxb.) Miq, Rubiaceae, is an important Bangladeshi medicinal plant for its traditional uses against different types of diseases. Therefore, the present study investigated for its potential antimicrobial and cytotoxic activities of petroleum ether (pet. ether) and chloroform fractions of ethanol extract of *Anthocephalus cadamba* stem bark. The antimicrobial potentialities were evaluated by disc diffusion method against four Gram-positive, six Gram-negative bacteria and six species of fungi at a concentration 500 µg/disc.Kanamycin 30 µg/disc was used as the standard drug. The chloroform fraction showed maximum activity against almost all bacteria. *Shigella dysenteriae, Escherichia coli* and *Pseudomonus aeruginosa* showed better sensitivities to chloroform fraction with the zone of inhibition 24.1, 22.3 and 22.1 mm respectively while pet. ether fraction showed mild sensitivities. *Bacillus cereus* and *Klebsiella pneumonia* were two bacteria amongst ten showed lowest activities to the extracts. In case of antifungal activity screening, the chloroform fraction induced significantzone of inhibition16.6, 15.7 and 13.2 mm by *Aspergillus flavus, Candida albicans and Aspergillus niger*.These two fractions were subjected to brine shrimp lethality test to evaluate cytotoxicity. The LC_{50}values for pet. ether, chloroform fractions and standard vincristine sulphate were 17.78, 15.66 and 12.02µg/ml respectively.The present study revealed that chloroform fractions of ethanol extracts of stem bark possess potent antimicrobial activities along with moderate cytotoxicities that may lead to new drug development.

Key-Words: *Anthocephalus cadamba* (Roxb.) Miq,antimicrobial activity, disc diffusion method, *Artemia salina* Leach,brine shrimp lethality

Introduction

Resistance to antimicrobial agents is a major global public health problem[1]. Infectious diseases account for approximately one-half of all death in tropics. Despite the progress made in the understanding of microorganisms and their control in industrialized nations, incidents due to drug resistant microorganisms and the emergence of unknown disease causing microbes, posed enormous public health concern[2]. This resistance is largely due to indiscriminate use of antimicrobial drugs, immunosuppressive agents, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection[3-6].

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Furthermore some antibiotics have undesirable side effects which limit their application and also in developing countries, these synthetic drugs are expensive and inadequate for the treatment of diseases due to adulterationsSo, there is a serious need to develop new antimicrobial agents that are very effective with minimal unwanted side effect. Plants represent a potential source of novel antibiotic prototypes[7].Today, nearly 88% of the global populations turn to plant derived medicines as their first line of action for maintaining health and combating diseases[8].According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs[9]. Thus, many plants that are of medicinal importance have been investigated by various researchers[10-13].The success story of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of microorganisms[14].

Pharmacology is simply toxicology at a lower dose and toxicology is simply pharmacology at a higher dose[15].A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in crude extracts is the brine shrimp lethality (BSLT). This method provides a front-line screen that can be backed up by more specific and expensive bioassays once the active compounds have been isolated. It appears that BSLT is predictive of cytotoxicity and pesticidal activity[16]. Cytotoxicityvia the brine shrimp lethality test is studied in order to reveal new anticancer compounds. Taxol™, a new antitumor drug approved by FDA for treatment of ovarian, breast and non-small-cell lung carcinomas and originally isolated from the bark of *Taxus brevifolia* was discovered in this way[17].

Anthocephalus cadamba (Roxb.) Miq (local name – Kadam, Kadamba; Family - Rubiaceae) is moderate sized to tall tree with broadly elliptic and strongly veined opposite leaves and beautiful globose pinkish white flower heads. It is found to grow wild in jungles in almost all areas of Bangladesh, Nepal, India, Myanmar, Sri Lanka, the Philippines, Indonesia and also planted as a timber and shade tree. Its bark is used as hypoglycemic, anthelmintic, tonic, astringent and also in the treatment of snakebite and malarial fever. Its bitter and pungent bark is used in Ayurvedic medicine for uterine complaints, blood diseases, leprosy and dysentery. Decoction of leaves is used as a gargle in case of aphthae and stomatitis[18-20]. Previous phytochemical investigations with this plant led to the isolation of cinchotannic acid, quinovic acid, cadambagenic acid, saponins, β-sitosterol, fats and reducing sugars, a secoiridoid, 3-O-caffeoylsweroside and two phenolic apioglucosides, kelampayoside A and kelampayoside B, indole alkaloids, cadambine and 3α-dihydrocadambine, cadamine, a glycosidal alkaloid, 3β-dihydrocadambine and 3β-isodihydrocadambine; hentriacontanol[18]. The crude extracts from *A. cadamba* have been shown to possess biological activities viz., anti-inflammatory[21], antihepatotoxic activities[22], analgesic activities[23], hypoglycemic activities[24], antimicrobial and anthelmintic activities[25], wound healing and antioxidant activities[26],antidiarrhoeal properties[27]. In continuation of our phytochemical and pharmacological screening of Bangladeshi medicinal plants we report on the antimicrobial and preliminary cytotoxic activities of pet.etherand chloroform fractions of crude ethanol extracts from *A.cadamba*stem bark.

Material and Methods

Collection of plant material

The stem barks of *Anthocephalus cadamba* (Roxb.) Miq were collected from the trees growing in the Natore city of Bangladesh, in May, 2013 and identified at the National Herbarium, Mirpur, Dhaka-1216,Bangladesh (Accession No. DACB31749).

Extraction and fractionation

The barks were washed with running water to remove any dust impurities, cut into small pieces, dried at room temperature for about 15 days. The materials (400 gm) were finely powdered and extracted with 95% ethanol(2 litres)through occasional stirring for 8 days[28, 29]to obtain ethanol extract and were concentrated under reduced pressure using rotary evaporator. This extract was made aqueous with distilled water in a separating funnel and further fractionated with organic solvents in order of increasing polarity viz., pet. ether and chloroform to obtain pet. ether fraction and chloroform fraction.

Microorganisms with strain number

Four Gram-positive bacterial strains, viz., *Bacillus cereus* (ATCC-14603), *Bacillus megaterium* (QL-38), *Staphylococcus aureus*(ATCC-259233) and *Streptococcus-β-haemolyticus*(ATCC-10389); six Gram-negative bacterial strains, viz., *Shigella dysenteriae* (AL-35587), *Escherichia coli* (FPFC-1407), *Salmonella typhii* (ATCC-14028), *Proteus vulgaris*, *Pseudomonus aeruginosa* (ATCC-27853) and *Klebsiella pneumoniae* and six fungal strains, viz., *Aspergillus flavus*, *Candida albicans* (ATCC-2091), *Aspergillus niger*, *Fusarium oxysporum*, *Mucor species*and *Aspergillus fumigatus*were employed in this test. These were procured from the mother stock of the Enteric Microbiology Laboratory, ICDDR,B, Dhaka, Bangladesh.

Growth media and conditions

Nutrient agar media (Difco laboratories) P^H 7.2, Sabouraud dextrose agar media (Biolife Vole Monza) P^H 5.6 and artificial sea water (3.8% sodium chloride solution) p^H 8.4were used for antibacterial screening, antifungal activity determination and brine shrimp lethality bioassay respectively.

Disc diffusion assay

Antimicrobial activity was determined as diameter of zone of inhibition expressed in millimeterat a concentration 500 µg/discusing disc diffusion method[30].Nutrient agar media and Sabouraud dextrose agar media were distributed in sterilized petridishes for ten pathogenic bacteria and six fungal strains respectively. This was accomplished by placing 10 µl of each separate pet. ether and chloroform soluble fractions of stem bark extracts on a small (6 mm diameter) filter paper discs. These discs were placed on an agar growth medium containing a confluent lawn of microorganisms. The concentration of microorganism was also 10 µl/petridishes. Negative controls were

prepared using respective solvent and kanamycin 30µg/disc was used as positive control. These plates were then kept at 4ºc for 24 hours to allow maximum diffusion followed by incubation at 37ºc for 24 hours for maximum growth of the organisms[31]. The absence of bacterial and fungal growth around the disc indicated that the plant extracts contain antimicrobial properties against that particular organism.

Brine shrimp lethality bioassay

The brine shrimp (*Artemia salina* Leach) lethality bioassay offers an advantage in standardization and quality control of botanical products. This test is well correlated with antitumor activity (cytotoxicity) and can be used to monitor the activity of bioactive natural products. The main reason why this salt-water anostracan crustacean is used widely for toxicity testing of plant extracts is due to the commercial availability of dormant eggs (cysts), which are harvested in huge amounts in salt lakes and pans. The larvae hatched from the cysts are used worldwide in aquaculture and in aquariology as live food for juvenile fish. Dormant brine shrimp eggs remain viable for many years and are therefore a suitable biological source for rapid, simple and inexpensive bioassays[32].

The assay was carried out according to the principle and protocol developed by Meyer *et al*[33, 34], with little modifications. Brine shrimp eggs (*Artemia salina* Leach, Carolina, Biological Supply Company, Burlington, NC, USA) were purchased from the locality and placed on one side of a small tank which filled with boiled, filtered sea water, covered with aluminum foil and fully aerated. After 48 hours of incubation at room temperature and under illumination, the resulting nauplii (larvae) were attracted to the other side of the tank with a light source and collected with pipette. Samples for testing were prepared by initially dissolving 50 mg of crude extracts in 5 ml of dimethyl sulfoxide (DMSO) and further diluted with sea water to produce the required concentrations 5, 10, 20, 40, 80µg/ml.Ten brine shrimps were transferred to each sample vial and tests for each concentration were done in triplicate. The total volume of the solution in each vial was adjusted to 5 ml by adding artificial saline. Artificial sea water medium containing DMSO was considered as negative control while standard vincristine sulphate was used as positive control. The vials were maintained in the laboratory at room temperature (25-29°C) under illumination. Survivors were counted after 24 hours and the percentage mortality at each vial and control were determined using the equation:

% of mortality = (No. of dead nauplii / initial no. of live nauplii) x 100

Microsoft Excel was used to determine the concentration at which lethality to brine shrimp represents 50% (LC_{50})[35].LC_{50} values less than 100 µg/ml were considered significant[36].

Results and Discussion

The results in Table 1 showed that pet. ether and chloroform fractions of ethanolic stem bark extract prevented the growth of all tested pathogenic bacteria. The chloroform soluble fractions exhibited the maximum zone of inhibition 24.1, 22.3 and 22.1 mm against Gram negative strain *Shigella dysenteriae*, *Escherichia coli* and *Pseudomonusaeruginosa* while minimum antibacterial activity was shown by*Bacillus cereus* and *Klebsiella pneumoniae*to pet. ether fraction with the zone of inhibition 6.6 and7.1 mm. The results revealed that pet. ether fraction had minimum antibacterial activity against ten bacterial strains as compared to chloroform fraction.

Antifungal activities of pet.etherand chloroform fractions of *A. cadamba* stem bark extracts were investigated against six fungal strains (Table 2). Chloroform fractions of stem bark extracts showed highest sensitivities against *Aspergillus flavus, Candida albicans*and*Aspergillus niger* with the zone of inhibition 16.6, 15.7 and 13.2 mm whereaspet. ether fractionate exhibited comparatively mild inhibitory effects.

The findings of the present study explain why the plant extracts are used in traditional folk medicine and confirm the activity of *A. cadamba* as a broad spectrum antimicrobial agent since inhibited the growth of Gram-positive and Gram-negative bacteria as well as some fungal species.

The data of this experiment showed that the responses of microorganisms to tested substances varied in term of sensitivity among the strains. The differences in susceptibility may be explained by the differences in cell wall composition and/or in genetic content of plasmids that can be easily transferred among microbial strains[37]. It may also be explained by differences in the mechanism by which the active principles of the plant extracts exert their effect[38]. This is very interesting in view of the perspective of new antibacterial discovery from this plant, when considering the medicinal importance of the tested microorganisms.

The brine shrimp lethality assay has been used routinely in the primary screening of the crude extracts as well as isolated compounds to assess the toxicity towards brine shrimp, which could also provide an indication of possible cytotoxic properties of the test materials. Brine shrimp nauplii have been previously utilized in various bioassay systems. Among these

applications have been the analyses of pesticidal residues, mycotoxins, stream pollutants, anesthetics, dinoflagelate toxins, morphin-like compounds, carcinogenicity of phorbol esters and toxicants in marine environment. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay[33, 34].

In the present study it was found that both the active fractions pet. ether and chloroform derived from ethanolic stem bark extracts of *A. cadamba* showed prominent results in brine shrimp cytotoxic assay in a dose dependent manner (Table 3 & Figure 1).The lethal concentration (LC_{50}) of the test samples after 24 hours was obtained by a plot of the percentage mortalities of shrimps against the logarithm of the sample concentration (toxicant concentration) and an approximate linear correlation was observed. LC_{50} values ranged from 15.66to 17.78µg/ml in comparison with vincristine sulphate having LC_{50} value 12.02 µg/ml. Pet. ether fraction of stem bark showed lowest lethality with LC_{50} value 17.78µg/ml while chloroform fractionate showed highest cytotoxic activity with LC_{50} value 15.66µg/ml.

Previous studies on this plant revealed that Chandrashekar and Prasanna, 2009 reported the antimicrobial activity of leaves of *Anthocephalus cadamba* Linn[39]. Hossain *et al*. 2011 also reported on preliminary cytotoxicity and antimicrobial activity of methanol extract and its fractions of the stem bark of this plant[40] and similar findings were also reported by Hassan *et al.* 2013[41].However, to the best of our knowledge studies on antimicrobial and preliminary cytotoxic effects of ethanol extract and its fractions of *Anthocephalus cadamba*(Roxb.)Miqstem bark still not reported in literature. The fact that the ethanolic extract of *A. cadamba* and its fractions showed prominent activity against most of the test microorganisms with moderate brine shrimp lethality has added another plant in bank of herbal medicines.

Conclusion

The ethanol extract and its fractions of*A. cadamba* stem bark showed the highest antimicrobial activity confirming the traditional use of this plant in the various bacterial and fungal diseases. Cytotoxic potential against *Artemia salina* Leach has also established that the crude extract/fractions could be safely used as antimicrobial agent. The obtained results might be considered sufficient for further studies geared towards the isolation and identification of the active principles and to evaluate possible synergistic effects among the extract components for their antimicrobial and cytotoxic properties.

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Table 1: *In vitro* antibacterial activities of pet.ether and chloroform fractionates of crude ethanol extracts from *Anthocephalus cadamba* (Roxb.) Miqstem bark with standard kanamycin

Tested bacterial strains	Diameter of zone of inhibition (mm)		
	Pet.ether fractionate(500 µg/disc)	Chloroform fractionate (500 µg/disc)	Kanamycin (30 µg/disc)
Gram (+ve) bacteria			
Bacillus cereus	6.60	8.10	28.5
Bacillus megaterium	10.1	15.3	27.6
Staphylococcus aureus	11.3	13.2	27.5
Streptococcus-β-haemolyticus	7.80	12.3	29.8
Gram (-ve) bacteria			
Shigella dysenteriae	11.7	24.1	28.3
Escherichia coli	11.2	22.3	29.9
Salmonella typhii	10.2	17.1	27.7
Proteus vulgaris	9.50	14.2	26.1
Pseudomonus aeruginosa	9.30	22.1	29.1
Klebsiella pneumoniae	7.10	8.50	28.4

Table 2: *In vitro* antifungalactivities of pet.ether and chloroform fractionates of crude ethanol extracts from *Anthocephalus cadamba* (Roxb.) Miqstem bark with standard kanamycin

Tested fungal strains	Diameter of zone of inhibition (mm)		
	Pet.ether fractionate(500 µg/disc)	Chloroform fractionate (500 µg/disc)	Kanamycin (30µg/disc)
Aspergillus flavus	10.1	16.6	25.8
Candida albicans	10.3	15.7	27.1
Aspergillus niger	9.50	13.2	28.5
Fusarium oxysporum	7.40	10.2	28.1
Mucor species	7.90	10.1	29.5
Aspergillus fumigatus	8.50	12.3	27.3

Table 3: Results of brine shrimp lethality bioassay on pet.ether and chloroform fractionates of crude ethanol extracts from *Anthocephalus cadamba* (Roxb.) Miqstem bark and for standard vincristine sulphate

Test samples	Conc. µg/ml	Log of conc.	No. of nauplii taken	No. of nauplii dead			Average no. of nauplii dead	Percent (%) of mortality	LC_{50}µ g/ml
				Vial 1	Vial 2	Vial 3			
Pet.ether fractiona-te	5	0.69	10	2	2	2	2.00	20.0	17.78
	10	1.0	10	3	3	4	3.33	33.3	
	20	1.3	10	6	5	5	5.33	53.3	
	40	1.6	10	6	6	6	6.00	60.0	
	80	1.9	10	7	8	7	7.33	73.3	
Chlorofor-m fractiona-te	5	0.69	10	1	2	2	1.66	16.6	15.66
	10	1.0	10	3	4	4	3.66	36.6	
	20	1.3	10	6	5	6	5.66	56.6	
	40	1.6	10	9	6	6	7.00	70.0	
	80	1.9	10	9	8	7	8.00	80.0	
Vincristi-ne sulphate	5	0.69	10	2	2	3	2.33	23.3	12.02
	10	1.0	10	5	4	4	4.33	43.3	
	20	1.3	10	8	7	5	6.66	66.6	
	40	1.6	10	7	9	8	8.00	80.0	
	80	1.9	10	8	9	9	8.66	86.6	
Control	20 DMSO	00	10	0	0	0	0	0	---

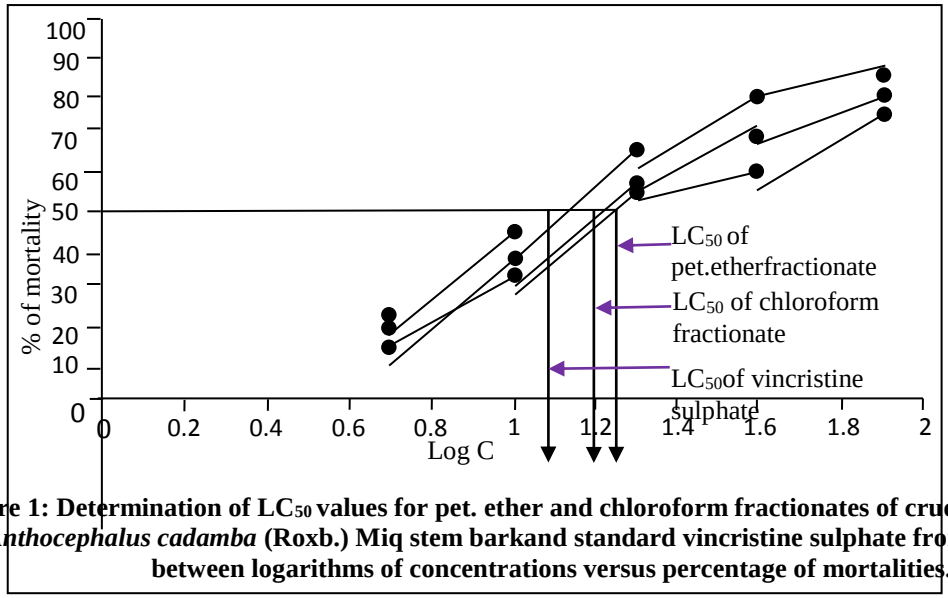

Figure 1: Determination of LC_{50} values for pet. ether and chloroform fractionates of crude ethanol extracts from*Anthocephalus cadamba* (Roxb.) Miq stem barkand standard vincristine sulphate from linear correlation between logarithms of concentrations versus percentage of mortalities.

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