Preliminary Antimicrobial, Cytotoxic and Chemical Investigations of *Averrhoa bilimbi* Linn. and *Zizyphus mauritiana* Lam.

Bijoy Karon[1], Mohammed Ibrahim[1], Ayeasha Mahmood[1], A K M Moyneenul Huq[1], M. Mohi Uddin Chowdhury[1], Md. Aslam Hossain[2] and Mohammad A. Rashid[2*]

[1]Department of Pharmacy, Southern University Bangladesh, Chittagong
[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh

Abstract

The antimicrobial activity of ethanol extracts of leaves of *Averrhoa bilimbi* and *Zizyphus mauritiana* has been evaluated against 4 Gram positive and 7 Gram negative pathogenic bacteria and 7 fungal strains by using ciprofloxacin and fluconazole as standards. Preliminary phytochemical screenings with the crude extracts demonstrated the presence of alkaloids, glycosides, saponins, tannins, steroids and reducing sugars. During antimicrobial screening by the disc diffusion method, the ethanol extracts of *A. bilimbi* and *Z. mauritiana* showed varying degrees of antimicrobial activity with zone of inhibition ranging from 10.0 - 23.33 mm and 17.67 - 24.0 mm, respectively where the growth of *Staphylococcus aureus*, *Shigella dysenteriae* and *Trichophyton* spp. was strongly inhibited. In the minimum inhibitory concentration test by serial dilution method, strong MIC (15.625 µg/ml) was found against *Pityrosporum ovale, Salmonella typhi, S. paratyphi, S. dysentariae, S. aureus, Trichophyton* spp. and *Vibrio cholerae*. *Ex-vivo* brine shrimp lethality bioassay revealed mild to moderate cytotoxic activity by the crude extractives as compared to the standard vincristine sulphate.

Key words: *Averrhoa bilimbi, Zizyphus mauritiana*, antimicrobial, cytotoxicity, MIC.

Introduction

Natural products are the valuable sources of structurally diverse chemical compounds, many of which possessing therapeutic potential for treatment of human diseases. Among the natural resources, plants have been widely studied for the discovery of antimicrobial, anticancer, antioxidants, immunomodulatory, anti-inflammatory and others therapeutic classes of chemical entities (De Smet, 1997; Web-1). The increasing prevalence of multi-drug resistant organisms as well as strains with reduced susceptibility to the available antibiotics prompted us for the search of new effective therapeutic agents from plants (Ghani, 2003).

Averrhoa bilimbi Linn. (Bengali name- Bilimbi, Family- Oxalidiaceae) is an attractive, long-lived tree, that grows in Moluccas, Indonesia and cultivated or found throughout Indonesia, the Philippines, Sri Lanka, India, Bangladesh, Myanmar, Malaysia and Zanzibar. Traditionally the leaves are used as paste on itches, swelling, rheumatism, mumps or skin eruptions, after-birth tonic and also in cold and cough, bites of poisonous creatures, etc. The syrup or the decoction of fruits is prescribed in inflammatory conditions, including hepatitis, fever, diarrhea and bilious colic (Web-2, Web-3).

Ziziphus mauritiana Lam. (Bengali name- Kul or Boroi, Family- Rhamnaceae) is an evergreen perennial tree, most commonly found in the tropical and sub-tropical regions and native to India and now widely naturalized in tropical regions from Africa to Afghanistan, China, Malaysia, Australia, Bangladesh and some Pacific regions (Web-5,6,7). It is reputed for various ethnomedical uses in asthma, cancer, constipation, wounds, boils, eruptions, fever, nausea, vomiting, piles, pulmonary ailments, alcoholic delirium, musculoskeletal disorder, etc (Web-5,6,7).

A comprehensive literature search revealed that *A. bilimbi* and *Z. mauritiana* have been subjected for preliminary phytochemical and limited antimicrobial studies against few microorganisms (Raghavendra *et al.*, 2006; Zakaria *et al.*, 2007; Mahesh *et al.*, 2008; Satish *et al.*, 2008; Raghvendral *et al.*, 2010). We herein report

* Correspondence to: Professor Dr. Md. Abdur Rashid, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh. E-mail:rashidma@univdhaka.edu

the antimicrobial activity of the extractive against a large number of microorganisms. Moreover, the results of cytotoxicity study and preliminary phytochemical screening of the above plants growing in Bangladesh have also been described for the first time.

Materials and Methods

Collection and identification: The samples of *A. bilimbi* and *Z. mauritiana* were collected from the hilly areas of Forest Research Institute, Chittagong and Rangunia, Chittagong, Bangladesh in October, 2010. The plants were identified by the scientists of the Forest Research Institute, Chittagong, where voucher specimens have been maintained.

Extraction: The aerial parts of each plant were first air-dried followed by drying into oven at 35°C and ground to coarse powder with a grinder. The powder (100 gm) of each plant was separately extracted with 95% ethanol (500 ml) at room temperature for 7 days with occasional shaking as well as with a Soxhlet apparatus. All the extractives were filtered off through filter paper and the filtrates were concentrated with a rotary evaporator at reduced temperature and pressure to yield 22.09 g (12.27%) and 22.98 g (12.77%) extractive for *A. bilimbi* and *Z. mauritiana,* respectively.

Preliminary phytochemical screenings: For preliminary phytochemical screenings all the extractives were subjected to various tests (Table-1) for determination of chemical nature of the extractives (Finer, 1983; Harborne, 1998).

Antimicrobial screening: The antibacterial and antifungal activities of the crude extracts were evaluated by the disc diffusion method (Bauer *et al*., 1996) against 4 Gram positive and 7 Gram negative pathogenic bacteria and 7 fungi (Table-2) using ciprofloxacin and fluconazole as standards. The organisms were obtained as pure culture from the Faculty of Biology, University of Chittagong, Bangladesh. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiments were carried out in triplicate and the results have been shown as mean ± SEM.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentration (MIC) of ethanol extracts of *A. bilimbi* and *Z. mauritiana* were determined by the serial tube dilution technique (Andrews, 2001) in broth medium, containing graded concentration of the plant extracts inoculated with the test organisms.

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay (Meyer *et al*. 1982) technique was applied for determination of general toxic properties of the plant extracts. DMSO solutions of the samples were applied against *Artemia salina* in a 1-day *ex-vivo* assay. For the experiment, 4 mg of ethanolic crude extract was dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/ml) were obtained by serial dilution. vincristine sulphate was used as positive control.

Statistical analysis: The primary data obtained from the experiments were manipulated as the source of responses. For each of the extracts, three samples were prepared for each of the bioassay. Data were expressed as mean ± SEM (standard error of mean). Statistical differences between extract activities were determined using ANOVA followed by Least Significant Difference (LSD) testing. Differences were considered statistically significant when $p < 0.5$.

Results and Discussion

Preliminary phytochemical screenings: The crude extractives when tested with various chemical reagents demonstrated the presence of alkaloids, glycosides, saponins, tannins, steroids and reducing sugars as shown in Table 1.

The crude extracts of plants displayed moderate to strong antibacterial and antifungal activities (Table 2). In the antimicrobial sensitivity test, the crude extract of *A. bilimbi,* at 200µg/disc, displayed moderate antibacterial activity against *B. megaterium* (14.67±0.34), *Salmonella typhi* (13.33±0.34) and *Vibrio cholerae (*13.67±0.34) while the growth of fungus *Trichophyton* spp. (23.33±0.34) and *Pityrosporum ovale* (22.67±0.67) were strongly inhibited. On the other hand, the extract of *Z. mauritiana* (at 50µg/disc) showed antibacterial activity against *S. aureus* (24.0±0.58), *S. paratyphi* (23.67±0.34) and *S. typhi* (23.33±0.34). *Z. mauritiana* was also inhabited the growth of fungus *P. ovale (*22.33±0.34) and *Microsporum* spp. (21.67±0.34) strongly.

During the MIC determination, the ethanol extracts of *A. bilimbi* and *Z. mauritiana* inhibited the growth of test

organisms at 15.625-62.50µg/ml and 15.625-62.50µg/ml, respectively (Table-3). The low MIC values of the extract of *A. bilimbi*, against *P. ovale, Trichophyton* spp. and *Z. mauritiana* against *S. aureus, S. paratyphi, S. typhi,* and *P. ovale* suggest the presence of strong antimicrobial compounds in the extractives. The other MICs above 100 µg/ml were discarded.

Table 1. Chemical groups present in extracts of *A. bilimbi* and *Z. mauritiana*

Test for	Test reagent/test name	EtOH Extracts of	
		A. bilimbi	*Z. mauritiana*
Reducing sugar	Fehling's test	+	+
	Benedict's solution	+	+
Steroids	Salkowski test	+	+
	Libermann-Burchared test	–	+
	Salkowski test	+	+
Glycosides	Libermann-Burchared test	+	+
	Ferric chloride	+	+
Tannins	Potassium dichromate test	+	+
	Mayer's test	+	–
	Dragendorff's reagent	–	+
Alkaloids	Wagner's reagent	+	+
	Hager's reagent	+	+
	Tannic acid test	+	–
Saponins	Shaking test for foaming	+	–

(+) = present; (-) = absent

Table 2. Antibacterial activity of *A. bilimbi* and *Z. mauritiana at* 200µg/disc and standard at 50µg/disc.

Test organisms	Diameter of zone of inhibition (mm)		
	EtOH Extracts of		Standard
	A. bilimbi	***Z. mauritiana***	
Gram positive bacteria			Ciprofloxacin
Bacillus subtilis	nd	17.67±0.34[a]	17.67±0.34
B. megaterium	14.67±0.34[e]	nd	21.0±1.17
B. cereus	12.0±0.58[d]	18.0±0.58[b]	18.67±0.34
Staphylococcus aureus	nd	24.0±0.58[b]	20.0±1.17
Gram negative bacteria			
Escherichia coli	11.0±0.58[b]	nd	19.0±0.58
Pseudomonas aeruginosa	10.33±0.89[b]	18.67±0.67[c]	21.67±1.21
Salmonella. typhi	13.33±0.34[c]	23.67±0.34[a]	23.67±0.89
S. paratyphi	10.0±0.58[b]	23.33±0.34[a]	17.33±0.34
Shigella dysenteriae	10.67±0.89[b]	nd	17.0±0.58
S. sonnei	nd	20.33±0.34[d]	20.67±0.34
Vibrio cholerae	13.67±0.34[b]	21.67±0.34[d]	19.33±0.34
Fungi			Fluconazole
Aspergillus niger	nd	19.33±0.34[b]	20.0±0.58
Blastomyces dermatitidis	20.33±0.67[e]	nd	16.67±0.34
Candida albicans	17.67±0.34[d]	nd	19.0±0.58
Cryptococcus neoformans	17.33±0.34[a]	nd	24.67±0.89
Microsporum spp.	nd	21.67±0.34[c]	20.33±0.34
Pityrosporum ovale	22.67±0.67[c]	22.33±0.34[b]	26.33±0.34
Trichophyton spp.	23.33±0.34[a]	nd	24.0±0.58

[a]p<0.001, [b]p<0.01, [c]p<0.02, [d]p<0.05, [e]p<0.5; nd: Not detected; The diameters of zone of inhibition are expressed as mean ± SEM (n=3); SEM: standard error of mean.

Table 3. Minimum inhibitory concentration of ethanol extracts of *A. bilimbi* and *Z. mauritiana*.

Test organisms	Minimum inhibitory concentration (µg/ml)	
	A. bilimbi (50µg/µl)	*Z. mauritiana* (50µg/µl)
Bacteria		
Bacillus subtilis	nd	62.50
B. megaterium	62.50	nd
B. cereus	-	62.50
Staphylococcus aureus	nd	15.625
Escherichia coli	-	nd
Pseudomonas aeruginosa	-	62.50
Salmonella typhi	-	15.625
S. paratyphi	-	15.625
Shigella dysenteriae	-	nd
S. sonnei	nd	31.25
Vibrio cholerae	-	62.50
Fungi		
Aspergillus niger	nd	62.50
Blastomyces dermatitidis	31.25	nd
Candida albicans	62.50	nd
Cryptococcus neoformans	62.50	nd
Microsporum spp.	nd	62.50
Pityrosporum ovale	15.625	15.625
Trichophyton spp.	15.625	nd

Table 4. Brine shrimp lethality bioassay of ethanol extracts of *A. bilimbi* and *Z. mauritiana*

Sample	LC_{50} (µg/ml)	LC_{90} (µg/ml)
Vincristine sulphate	0.44	0.67
A. bilimbi	5.81	10.28
Z. mauritiana	5.72	10.05

In brine shrimp lethality bioassay, the crude extracts were screened for probable cytotoxic activity. The concentration at which 50% and 90% mortality of brine shrimp nauplii occurred were determined and the LC_{50} and LC_{90} were found to be 5.81 & 10.28 µg/ml and 5.72 & 10.05 µg/ml for ethanol extract of *A.* and *Z. mauritiana*, respectively.

Conclusion

It is evident from the present studies that, the ethanolic crude extract of A. *bilimbi* and *Z. mauritiana* growing in Bangladesh showed significant antimicrobial and cytotoxic activities. The strong cytotoxic properties of the extracts suggested the presence of bioactive principles in the plants. Further investigation is underway to isolate the active compounds. Moreover, the bioactivities shown by the extractives of *A. bilimbi* and *Z. mauritiana* support the traditional uses of these plants against various diseases.

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