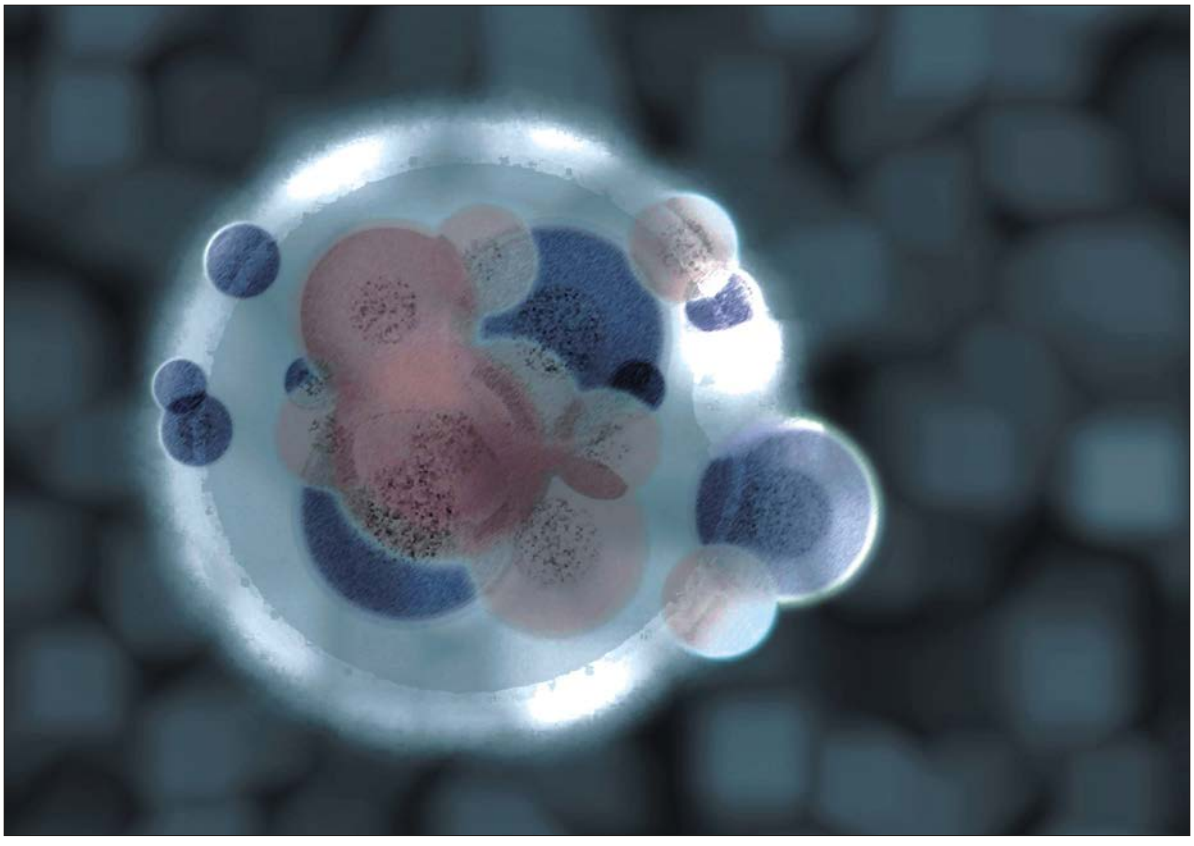

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Biological Investigation of *Macrotyloma uniflorum* Linn. Extracts Against Some Pathogens

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Abstract: In the present research, attempt was taken to explore the antimicrobial potency of the crude extracts of the *Macrotyloma uniflorum* plant. The extractives of the plant were subjected to screening for inhibition of microbial growth by the disc diffusion method. The zones of inhibition demonstrated by the dichloromethane, ethyl acetate, 1-butanol and aqueous extracts ranged from 11-16, 10-24, 10-14 and 10-12 mm, respectively at a concentration of 500 μg disc^{-1}. The ethyl acetate extract showed promising antibacterial activities against all the gram-positive and gram-negative bacteria whereas dichloromethane extract showed moderate activities and the 1-butanol and aqueous extracts did not show any significant antimicrobial activities. In addition, the antifungal activities of all the extractives were tested, using the food poisoning technique. Only dichloromethane extract has been proved to be active against all fungi tested with a higher inhibition activity than standard nystatin. The overall results provide promising baseline information for the potential use of the crude extracts from *M. uniflorum* in the treatment of bacterial and fungal infections.

Key words: Gram-positive, antibacterial, antifungal, *M. uniflorum*, mycelial growth, inhibition

INTRODUCTION

The plant kingdom has been the safeguard for the survival of the humans throughout recorded history. The importance of medicinal plants and traditional health systems in solving the health care problems is gaining increasing attention and because of this resurgence of interest, the research on plants of medicinal importance is rapidly increasing at the international level. However, this is occurring while natural habitats in countries of origin are being lost. Medicinal plants have long been the subjects of human curiosity and need. It is estimated that there are about 2,500,000 species of higher plants and the majority of these have not been examined in detail for their pharmacological activities (Ram *et al*., 2004). The anticancer and antimicrobial properties of certain Bangladeshi medicinal plants were reported based on folkloric information and few attempts were made to study the inhibitory activity against certain pathogenic bacteria and fungi (Rahman *et al*., 2007; Costa Lotufo *et al*., 2005; Chowdhury *et al*., 2003; Rahman *et al*., 2001).

Infectious diseases are one of leading cause of premature death and in recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world due to indiscriminate use of antibiotics to treat infections in humans but also for livestock production (Ahmad and Beg, 2001). New therapeutic agents are in great demand and many infectious diseases are known to be treated with herbal medicines derived from the plant sources from regions throughout earth. Plants have shown great promise in the treatment of intractable infectious diseases (Idu *et al*., 2007). Even today, plant materials continue to play major role in primary health care and higher plants have been shown to be potential sources for the new antimicrobial and chemotherapeutic agents with possibly novel mechanisms of action (Hamil *et al*., 2003; Karaman *et al*., 2003; Motsei *et al*., 2003; Sibanda and Okoh, 2008).

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Macrotyloma uniflorum Linn (Bengali name-Kurti kalai; English name-horse gram; Family-Fabaceae) is a herbaceous plant with annual branches, suberect or twining, leaflets 2.5-5 cm and widely distributed throughout Bangladesh but abundant in Rajshahi and Dinajpur districts (Kirtikar and Basu, 1998). It is famous for its medicinal uses because different parts of the plants are used for the treatment of heart conditions, asthma, bronchitis, leucoderma, urinary discharges and for treatment of kidney stones (Ghani, 2003). Indeed, *M. uniflorum* could play a role in antioxidation (Reddy *et al*., 2005) as when this plants were exposed to toxic levels of lead, several enzymes showed a pivotal role against oxidative injury. *Macrotyloma uniflorum* has the greatest potential for further utilization as nutraceuticals, forage and food for malnourished and drought-prone areas of the world (Morris, 2008). Herbal medicine is part and parcel of the much needed health care in most of the developing countries including Bangladesh. As a part of investigations on the medicinal plants of Bangladesh, *M. uniflorum* and isolated Kaempferol-3-O-β-D-glucoside, β-sitosterol and stigmasterol was investigated (Kawsar *et al*., 2003) and we recently reported the cytotoxicity assessment of this plant (Kawsar *et al*., 2008). In this study, we report on the preliminary antibacterial and antifungal activities of crude extracts of the aerial parts of *M. uniflorum* Linn. against some human and plant pathogens for the first time.

MATERIALS AND METHODS

Plant material: *Macrotyloma uniflorum* (Fabaceae) was collected from the village, Susunda of Muradnagar, Comilla, Bangladesh in March, 2002. The botanical identification was made by Prof. Salar Khan (University of Dhaka) and voucher specimen was deposited at the Bangladesh National Herbarium (BNH) (DACB Accession No. 28264). The whole plants were cleaned, air-dried and followed by drying in an oven at 40°C. The dried plants were powdered by grinding in a cyclotec-grinding machine (200 mesh).

Preparation of extracts: The powdered plant was (3.5 kg) was successively extracted with aqueous 80% ethanol (18 L×3 times, 24 h) at room temperature. The extract was filtered and the filtrate was evaporated to dryness at 40°C under vacuum and finally freeze-dried to obtain crude ethanolic extract of 484 g (13.82%) as solid material. The ethanol extract (484 g) was suspended in water (~2000 mL) and the suspension was transferred into a separating funnel. The aqueous suspension was successively partitioned with dichloromethane (CH_2Cl_2, *ca.* 2000 mL×3), ethyl acetate (EtOAc, *ca.*1500 mL×3) and 1-butanol (1-BuOH, *ca.*1500 mL×3). The CH_2Cl_2, EtOAc, 1-BuOH and aqueous extracts were evaporated separately and lastly freeze-dried. The extractive yield (%) of all the extracts is shown in Table 1. The extracts were tested for their antibacterial and antifungal inhibition activities.

Table 1: Percentage of different extracts from *M. uniflorum* plant

Extracts	Amount (g)	Yield[a] (%)
Dichloromethane (CH_2Cl_2)	40.0	1.14
Ethyl acetate (EtOAc)	48.0	1.37
1-butanol (1-BuOH)	110.0	3.14
Aqueous (H_2O)	58.5	1.67

[a]Percentage extract yield (w/w) was estimated as dry extract/dry material weight×100

Microorganisms: The bacterial and fungal strains used in this research were obtained from the Microbiology Laboratory, Department of Microbiology, University of Chittagong, Bangladesh. The bacterial strains comprised: *Bacillus subtilis* BTCC 17, *Bacillus cereus* BTCC 19, *Bacillus megaterium* BTCC 18, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Salmonella typhi* AE 14612, *Shigella dysenteriae* AE 14396 and *Shigella sonnei* CRL (ICDDR,B). The fungal pathogens were *Fusarim equiseti* (*corda*) *Sacc*., *Macrophomina Phaseolina* (Tassi) Goid, *Colletotrichum corchori* (Ikata Yoshida), *Botrydiplodia theobromae* pat, *Curvularia lunata* (wakker becdijin) and *Alternaria alternata* (Fr.) Kedissler.

Preparation of stock culture and bacterial suspension: Standard Nutrient Agar (NA) medium was used for growing bacterial strains throughout the research. In a hard glass screw cap test tube, sterile slants of NA were prepared. Older cultures were transferred to freshly prepared NA slants separately for each species via sterilized bacterial loop. In such a way, four test tubes were freshly prepared for each bacterial pathogen. These test tubes of inoculated slants were incubated at (35±2)°C in an incubator. After 18-24 h of incubation each culture was used throughout for antibacterial screening studies. For preservation of the stock culture, one set of culture slants were kept in polythene bag, properly tied and preserved as stock culture at 10°C. Subcultures were maintained at 3 to 4 week intervals to ensure culture viability. Bacterial cultures, after 24 h of incubation, were transferred to sterilized distilled water and mixed and these bacterial suspensions were used in the pour plate technique during sensitivity testing.

Antibacterial assay: The antibacterial activities of the different extracts were detected by disc diffusion method (Bauer *et al*., 1966). Paper discs of 4 mm in diameter and

glass petri dish of 90 mm in diameter were used throughout the experiment. Paper discs were sterilized in an autoclave and dried at 100°C in an oven. The discs were then soaked with test extracts at the rate of 500 μg (dry weight) per disc for antibacterial effect analyses. One drop of bacterial suspension was taken in a sterile petri dish and approximately 20 mL of sterilized melted NA (~45°C) was poured into the plate and then mixed thoroughly with the direction of clock wise and anticlock wise motion. After solidification of the seeded NA medium, paper discs, after soaking with test extracts (2% in DMSO; dimethyl sulphoxide) were placed at the centre of the inoculated pour plate. A control plate was also maintained in each case with DMSO. Firstly, the plates were kept for 4 h at low temperature (4°C) and the test extracts diffused from disc to the surrounding medium by this time. The plates were then incubated at (35±2)°C for growth of test organisms and were observed at 24 h intervals for two days. The activity was expressed in terms of inhibition zone diameter in mm. Each experiment was repeated three times and ampicillin (FISONS Bangladesh Ltd.) was used as reference or positive control while DMSO without sample was used as negative control. Antibacterial activity is given in Table 2 and 3.

Medium and cultures: The antifungal activities of the different extracts were investigated against six plant pathogenic fungi. The investigation was based on food poisoning technique (Grover and Moore, 1962). Potato Dextrose Agar (PDA) was used as basal medium for test fungi. DMSO was used as a solvent to prepare the desired solution (2%) of the extracts initially. Glass Petri dishes were cleaned and sterilized in an autoclave after which sterile, molten (~ 45°C) PDA was poured into each plate in 10-12 mL aliqouts. After a few minutes the medium solidified. Then small portions of mycelium of each fungus pathogen were placed at the centre of each PDA plate via sterilized needles. In such a way, each fungus species was transferred into a number of petri dishes. After a few days, the mycelium grows in the entire petri dish and these are then ready for antifungal activity testing of plant extracts.

Test for antifungal activity: A required amount of medium (PDA) was taken in conical flasks separately and was sterilized by autoclave (121°C, 15 psi) for 15 min. After autoclaving, calculated amounts of test extracts (2%) was added to the sterilized medium in conical flask and the flasks were shaken thoroughly to mix the extract with the medium before pouring. The medium with (2%) of extract was then transferred in 10 μL aliquots into sterilized glass petri dishes individually. Proper control was maintained separately with sterilized PDA medium without extract and three replications were prepared for each treatment. After solidification of medium, the fungal inoculum (5 mm mycelial block) was placed on the centre of the petri dishes in the inverted position.

All the plates were incubated at room temperature on a laboratory bench top for three days at (25±2)°C in triplicate. After three days of incubation, the diameter of fungal mycelial growth was measured. The average of two measurements was taken as mycelial colony diameter of the fungus in mm. Nystatin was used as positive control. Antifungal activity of the extracts is presented in the Table 3. The percentage inhibition of mycelial growth of test fungus was calculated by following equation:

$$\text{Inhibition (\%)} = (C\text{-}T/C)\times 100$$

where, C was the diameter of the fungal colony in control and T was the diameter of the fungal colony in treatment.

RESULTS AND DISCUSSION

The extractives of *M. uniflorum* demonstrated varying degrees of inhibition to growth of microorganisms. The dichloromethane, ethyl acetate, 1-butanol and aqueous extracts were subjected to screening for inhibition of microbial growth against 8 bacteria and 6 fungal strains. The *in vitro* growth inhibition of all gram-positive bacterial strains are mentioned in Table 2 and it was found that the ethyl acetate extract showed significant antibacterial activity against *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* by showing zones of inhibition of 22, 17 and 16 mm, respectively. The ethyl acetate extract

Table 2: Zone of inhibition observed against Gram-positive bacteria by the extracts

	Zone of inhibition (mm)[a] 500 μg disc^{-1}			
Microbial strains	Dichloromethane	Ethyl acetate	1-butanol	Aqueous
Bacillus subtilis	11	22	11	10
Bacillus cereus	12	17	-	12
Bacillus megaterium	-	8	-	-
Staphylococcus aureus	16	16	10	12
Ampicillin[b] 500μg disc^{-1}	19	18	16	22

[a]Values are mean of three replicates, -no. inhibition zone, 10-15: moderately active, >16: significantly active, [b]Standard antibiotic

Table 3: Zone of inhibition observed against Gram-negative bacteria by the extracts

	Zone of inhibition (mm)[a] 500 μg disc^{-1}			
Microbial strains	Dichloromethane	Ethyl acetate	1-butanol	Aqueous
Escherichia coli	12	15	11	-
Salmonella typhi	-	24	12	10
Shigella dysenteriae	14	17	-	12
Shigella sonnei	15	10	14	11
Ampicillin[b] 500 μg disc^{-1}	19	18	16	22

[a]Values are mean of three replicates, -no. inhibition zone, 10-15: moderately active, >16: significantly active, [b]standard antibiotic

Table 4: Antifungal activities of the different extracts

	Inhibition of fungal mycelial growth[a], 500 μg(dw) sample mL PDA (%)			
Microbial strains	Dichloromethane	Ethyl acetate	1-butanol	Aqueous
Fusarim equiseti	32	14	10	15
Macrophomina phaseolina	12	10	15	-
Colletotricum corchori	20	21	-	-
Botrydiplodia theobromae	13	-	16	18
Curvularia lunata	18	14	19	-
Alternaria alternata	22	15	23	15
Nystatin[b] (500 μg disc^{-1})	30	35	30	32

[a]Values are mean of three replicates, -no. inhibition zone, [b]standard antibiotic

also showed more activity (22 mm) against *Bacillus subtilis* than the standard antibiotic, ampicillin (18 mm) and the dichloromethane extract was active against only one bacterium, *Staphylococcus aureus* (16 mm). The 1-butanol and aqueous extracts were less active against *Bacillus subtilis* and *Staphylococcus aureus* than standard antibiotic. However, *Bacillus megaterium* bacteria did not show any signs of inhibition against any extracts.

The screening data presented in Table 3 against gram-negative bacteria suggests that the ethyl acetate extract showed significant inhibition against *Salmonella typhi* (24 mm) but good inhibition against *Shigella dysenteriae* (17 mm) and *Escherichia coli* (15 mm). Contrastively, the dichloromethane extract showed little growth inhibition for any bacteria. 1-butanol and aqueous extracts also showed good activity against a few bacteria but none of them was stronger than the standard antibiotic. The antibacterial activity increased as the polarity of the solvents increased and the maximum antibacterial activity was found to be caused by the ethyl acetate extract.

The dichloromethane extract gave significant (32%) antifungal activity against *Fusarim equiseti* which was higher inhibition than the standard antibiotic, nystatin (30%). The same extract showed moderate to good inhibition of mycelial growth against *Alternaria alternate* (22%), *Colletotricum corchori* (20%) and *Curvularia iunata* (18%) as well. The ethyl acetate extract showed significant antifungal activity against *Colletotricum corchori* (21%). 1-butanol and aqueous extracts showed good antifungal activity against *Alternaria alternata* (23%) and *Botrydiplodia theobromae* (18%), respectively (Table 4). The overall results indicated that out of six fungi tested against *M. uniflorum* plant extracts, minimum average inhibition was observed in the case of *Colletotricum corchori* while *Fusarim equiseti* showed maximum average inhibition.

The antibacterial activities of the plant extracted in different solvents varied greatly because there were many factors that influence the active compounds present in the plant. Here the polarity of the extracting solvents was different and it greatly influenced the antimicrobial properties (Table 2, 3). This may be due to the better solubility of the active compounds in organic solvent (De Boer *et al*., 2005; Parekh *et al*., 2006). The extractive yield of different solvents is given Table 1. Maximum yield obtained was with 1-butanol (3.14%) while the minimum was with dichloromethane (1.14%) (Table 1).

Traditional healers use primarily water as solvents but in this study showed that the plant extracts, extracted in different organic solvents, showed profoundly distinct antibacterial activities compared to aqueous extracts. These observations can be rationalized in terms of the polarity of the compound being extracted by each solvent and, in addition to their intrinsic bioactivity by their ability to dissolve or diffuse in the media used in the assay. The growth media also seem to play an important role in the determination of the antibacterial activity (Lin *et al*., 1999). Amongst the gram-positive and gram-negative bacteria, gram-negative bacterial strains were

more susceptible to the extracts as compared to gram-positive bacteria. Generally gram-negative bacteria are more resistant than gram-positive bacteria (Kelmanson *et al*., 2000; Rabe and Staden, 1997) but the result of this present work is different. Here the maximum antibacterial activity was seen against gram-negative bacteria (Table 3). The biological activities exhibited by the different extracts of *M. uniflorum* is most likely due to the presence of different chemical components in the extracts.

CONCLUSION

Extracts of *M. uniflorum* Linn. in this study demonstrated a broad-spectrum of activity against both gram-positive and gram-negative bacteria and fungi. The results of present study support the traditional usage of the studied plant and suggest that the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents after further pharmacological study.

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