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Antimicrobial and toxicity studies of different fractions of the aerial parts of the *Mikania cordata*

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Abstract

The methanolic extract along with some organic soluble fractions of the aerial parts of *Mikania cordata* (Bumr.f.) B.L. Robinson were tested against five Gram-positive and eight Gram-negative bacteria and against three pathogenic fungi. Only dichloromethane fractions showed remarkable activities against all the tested bacteria and the highest activity against *Escherichia coli* with a zone of inhibition 14 ± 0.75 mm and the minimum activity against *Salmonella typhi* (zone of inhibition 8 ± 0.73). Other showed moderate or little activity. n-Haxene fractions showed significant activity against *Escherichia coli* with a zone of inhibition 10 ± 0.15. The highest antifungal activity was shown against *Candida albicans* (12 ± 1.75 mm, inhibition zone diameter) and the weakest activity against *Aspergillus niger* (9 ± 0.65mm, inhibition zone diameter) for CH_2Cl_2 fraction whereas n-Haxen fractions had no activity. Significant and highest minimum inhibitory concentration (MIC) values were observed in dichloromethane fraction against *Escherichia coli* with the value of 1.25 mg/ml. Lowest LC_{50} value 0.49 ± 1.16 of n-Haxene fraction indicated the highest toxicity incomparison with the other fractions.

Key-Words: Antibacterial, Antifungal, *Mikania cordata*, MIC, Toxicity.

Introduction

The frequency of life-threatening infections caused by pathogenic microorganisms has increased worldwide and become a prime factor of morbidity and mortality in immune compromised patients in developing countries and many infectious microorganisms are resistant to synthetic drugs and hence an alternative therapy is very much needed[1]. Finding healing powers in plants is an ancient idea. Drugs derived from unmodified natural products or semi-synthetic drug obtained from natural sources corresponded to 78% of the new drugs approved by the FDA between 1983 and 1994[2]. Interest in natural products with antimicrobial properties has revived as a result of current problems associated with the use of antibiotics. This evidence contributes to support and quantify the importance of screening of natural products.

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Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids, flaonoids, etc, which showed wide range of *in vitro* antibacterial and antifungal activities[3-4]. So, the development of new antibacterial agents, the most feasible way to combat the problem of microbial resistance and for substitution with ineffective ones. Moreover, it is presumed that the broad spectrum effectiveness of plant species may provide a suitable basis for new antimicrobial therapies[5].

Mikania cordata (Bumr.f.) B.L. Robinson belongs to the family Asteraceae, is very well known medicinal plant used against Coughs, eye sores, gastro-intestinal disorder, snake and scorpion bites[6-7]. In India, the plant, used as a vegetable, is thought to be effective in the treatment of itch and as a poultice for wounds[6]. In Bangladesh, the decoction of leaves is used in dyspepsia, dysentery, and gastric ulcer[8]. The alkaloidal fraction of the leaf ethanolic extract and the methanolic extract of the root showed anti-ulcer effects[9-11], stimulation of hepatic protein synthesis in CCl_4-induced hepatotoxicity in mice[12], and significant anti-

carcinogenic response[13]. Scandenolide obtained from *M. cordata* exhibited significant anti-inflammatory activity

In addition, considering the wide folk medicinal application of this plant, this work was set out in order to investigate the antimicrobial activity of extracts and fractions of the aerial part of the *M. cordata* against some pathogenic bacteria and fungi and both the extract and fractions were also tested against brine shrimp nauplii in order to evaluate their potential toxicity.

Material and Methods

Plant materials

The aerial part of the plant of *M. cordata* was collected from the botanical garden of Pharmacy department of Jahangirnagar University, Bangladesh during the month of January 2009. The plant material was taxonomically identified by the National herbarium of Bangladesh. A voucher specimen no. DACB 31451 is maintained in our laboratory for future reference.

Preparation of plant extract

The shade dried plant materials were pulverized into coarse powder using mechanical grinder, seiving through sieve #40, and stored in a tight container. The dried powder material (1.0 kg) was refluxed with MeOH for three hours. The total filtrate was concentrated to dryness, in vacuo at 40^0C to render the MeOH extract (320 g). This extract was suspended in H_2O and then successively partitioned with n-Hexane, and CH_2Cl_2, to afford 80 g, and 150 g of the extract, respectively.

Test microorganisms

Strains of both fungi and bacteria (Gram positive and Gram negative) were obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). Bacillus cereus ATCC 14579, *Bacillus megaterium* ATCC 13578, *Bacillus subtilis* ATCC 6059, *Staphylococcus aureus* ATCC 6538, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25922, *Pseudomonus aeruginosa* ATCC 27853, *Salmonella paratyphi* ATCC 9150, *Salmonella typhi* ATCC 13311, *Shigella boydii* ATCC 9234, *Shigella dysenteriae* ATCC 9361, *Vibrio mimicus* ATCC 33653, *Vibrio parahemolyticus* ATCC 17802, *Candida albicans* ATCC 90028, *Aspergillus niger* ATCC 1004 and *Sachoromyces cerevacae* ATCC 60782 were used as test microorganism. All these bacterial and fungal species are recommended by ATCC for their susceptibility assay. The strains are maintained and tested on Nutrient Agar media (NA) for bacteria and Sabourand dextrose agar media (SDA) for fungi.

Phytochemical analysis

Desirable amount of *M. cordata* extract was solubilized in water for phytochemical analysis. The extracted solution was tested for alkaloids, glycosides, steroids, gums, flavonoids, saponins, sugars and tannins according to the protocol described by Trease and Evans[14].

Antimicrobial activity

Crude methanolic extract along with the other organic fractions were dissolved in 10% DMSO to get a concentration of 250 µg/ml and sterilized by filtration by 0.45 µm Millipore filters. Standard antibacterial agents Ciprofloxacin (30µg/disc), were prepared. Antimicrobial tests were then carried out by modified agar diffusion method[15-16] using 100 µl of suspension containing 108 CFU/ml of bacteria, 106 CFU/ml of yeast and 104 spore/ml, spread on nutrient agar (NA), and subourand dextrose agar (SDA), respectively[17]. Bacteria were cultured overnight at 37^0C and fungi at 28^0C for 72 hour used as inoculums. Nutrient agar (20 ml) was dispensed into sterile universal bottles. These were then inoculated, mixed gently and poured into sterile petri dishes. After setting a number 3-cup borer (6 mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/wells in each Petri dish. A drop of molten nutrient agar was used to seal the base of each cup. The cups/wells were filled with 50µl of the extract concentration of 250 µg/ml and were allowed for diffuse (45 minutes). The plates were then incubated at 37^0c for 24 hours for bacteria. The above procedure was followed for fungal assays and the media used was sabourand dextrose, incubated at 25^0C for 48 hours. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicate. The extracts and phytochemicals that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial and fungal sample according to method[18].

Determination of minimum inhibitory concentration (MIC)

MIC values were also studied for microorganisms, which were determined as sensitive to the extract in disc diffusion assay. In order to determine the MIC values, extract or fractions were dissolved in 10% DMSO to make a concentration of 100 mg/ml. The extract or fractions were diluted in a simple dilution manner to make concentrations in the range of 20, 10, 5, 2.5, 1.25, 0.625, 0.312 mg/ml. 0.1 ml of the extract or fractions were then added to each hole. The MIC was taken as the lowest concentration of extracts or fractions that caused a clear to semi clear inhibition zone around the hole. All the tests were repeated in triplicates.

Determination of relative percentage inhibition

The relative percentage inhibition with respect to positive control was calculated by using the following

formula[19]. Relative percentage inhibition of the test extract = [{100 x (a - b)}/(c - b)]. Where, a: total area of inhibition of the test extract; b: total area of inhibition of the solvent; c: total area of inhibition of the standard drug. The total area of the inhibition was calculated by using area = πr^2; where, r = radius of the zone of inhibition.

Brine shrimp lethality bioassay

The toxic potnetiality of the plant crude extract and fractions were evaluated using Brine Shrimp lethality bioassay method[20] where 6 graded doses (viz,. 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml, 160µg/ml, 320µg/ml) were used. Brine shrimps (*Artemia salina* Leach) nauplii Ocean 90, USA were used as test organisms. For hatching, eggs were kept in brine with a constant oxygen supply for 48 hours. The mature nauplii were then used in the experiment. DMSO was used as a solvent and also as a negative control. The median lethal concentration LC_{50} of the test sample after 18 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentratin. Tamoxifin, a well known anticancer drug was used as a reference standard in this case.

Statistical analysis

All assays were performed in triplicate under strict aseptic conditions to ensure consistency of all findings. Data of all experiments were statistically analyzed and expressed as the mean ± standard deviation of three replicate experiments.

Results and Conclusion

Presence of alkaloids, glycosides, flavonoid and tannins were observed in the methanolic extracts of the areal part of the plant of *M. cordata* (Table 1). Tablet 2 expressed the antibacterial and antifungal activity (zone of inhibitions) of extract and fractions of the aerial part of the *M. cordata*. Among the all extract/fractions only CH_2CH_2 fractions have inhibitory function for all the test bacteria. The highest zone of inhibition was found against *Escherichia coli* (zone of inhibition 14 ± 0.75 mm), followed by *Sarcina lutea* and *Pseudomonas aeruginosa* (zone of inhibition 12 ± 0.95 and 12 ± 1.25 mm, respectively) and the weakest activity was shown against *Salmonella typhi* (zone of inhibition 8 ± 0.73). MeOH extract was active against all microflora except *Bacillus subtilis*, *Shigella boydii*, and *Vibrio parahemolyticus*. MeOH extract showed high activity against *Escherichia coli.* Inhibition range for *Salmonella typhi* and *Salmonella paratyphi* was observed very mild against all the tested extract/fractions. The highest antifungal activity was shown against *Candida albicans* (12 ± 1.75 mm, inhibition zone diameter) and the weakest activity against *Aspergillus niger* (9 ± 0.65mm, inhibition zone diameter) for CH_2Cl_2 fraction.

Minimum inhibitory concentration (MIC) values of crude extract and various fractions of the aerial parts of *M. cordata* against susceptible bacterial were represented in table 3. All the tested extracts showed significant variations in MIC values depending upon the test bacteria. *Escherichia coli* the most sensitive bacteria showed the variable MIC ranges of 1.25-10 mg/ml.

Solvents used in the current study to prepare extract and fractions were further evaluated separately as negative control for their antibacterial activity to check whether the activity is due to the extract or fractions containing active compound(s) or due to the solvents used for extraction or fractionation. Our data (not shown in table) revealed that zone of inhibition arises due to the extract or fractions not solvent.

The results of relative percentage inhibition are reported in Table 4. CH_2Cl_2 fractions showed the maximum relative percentage inhibition against *E. coli* (45.34%) for bacteria and *C. albicans* (41.56%) for fungi whereas, lowest relative percentage inhibition against *S. paratyphi* (19.43%) for bacteria and S. *cerevaceae* (19.20) for fungi. The range of relative percentage inhibition for MeOH extract and n-Haxene was 11.43%-25.31% and 10.67%-18.73%, respectively.

Phytoconstituents such as alkaloid, sesquiterpine, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections[21-22]. So, the antimicrobial activity showed by MeOH and CH_2Cl_2 fractions of *M. cordata* may be due to presence of alkaloids, saponin, tannin, phenolic compounds and flavonoids.

The brine shrimp lethality bioassay (BSLA) has been used routinely in the primary screening of the crude extracts to assess the toxicity towards brine shrimp, which could also provide an indication of possible toxicity of the test materials. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay[20]. As summarized in table 5, the toxicity exhibited by the crude MeOH extract as well as the organic soluble fractions of the plant showed potent activity against with the positive control (Tamoxifin). The toxicity of the MeOH extract and its fractions on the BSLA increased in the order of n-Hexane > MeOH > CH_2Cl_2 and were 0.49 ± 1.16, 2.91 ± 1.13, and 8.00 ± 2.56 µg/mL in their LC_{50}, respectively. The variation in BSLA results (Table 5) may be due to the difference in the amount and kind of cytotoxic substances (e.g. Alkaloids, tannins, flavonoids) present in the crude extracts. Moreover, this significant lethality of the crude plant extracts (LC_{50} values less than 100 ppm or µg/mL) to brine

shrimp is indicative of the presence of potent cytotoxic and probably insecticidal compounds which warrants further investigation. BSLA results may be used to guide the researchers on which crude plant extracts/fractions to priority for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

In conclusion, our findings support the use of this plant in the treatment of infectious diseases caused by resistant microorganisms. The plant also used to discover bioactive natural products that may serve as leads for the development of new pharmaceuticals that address hither to unmet needs. Furthermore, active plant extracts can be subjected to various chemical evaluation by several methods such as GC-MS, NMR, HPLC, Mass Spectroscopy, etc, for the isolation of the therapeutic antimicrobials.

Table 1: Phytochemical properties of *M. cordata* crude extract

Compounds	Observation
Alkaloids	+ ve
Glycosides	+ ve
Steroids	+ ve
Gums	- ve
Flavonoids	+ ve
Saponins	+ ve
Reducing sugars	- ve
Tannins	+ ve

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Table 2: *In vitro* antimicrobial activity of MeOH extract and different organic fractions of the aerial parts of the *M. cordata* on various bacterial and fungal strains by agar diffusion method

[a]Zone of inhibition in diameter (mm) (n=3)				
Fraction	**MeOH**	**CH_2Cl_2**	**n-Hexane**	**Ciprofloxacin[b]**
Gram Positive				
Bacillus cereus	8 ± 0.65	10 ± 0.15	NA	42 ± 0.65
Bacillus megaterium	9 ± 0.15	9 ± 0.35	NA	41 ± 0.25
Bacillus subtilis	NA	11 ± 0.45	NA	41 ± 0.55
Staphylococcus aureus	7 ± 0.45	10 ± 0.75	9 ± 1.19	42 ± 0.16
Sarcina lutea	9 ± 0.26	12 ± 0.95	NA	42 ± 0.75
Gram Negative				
Escherichia coli	12 ± 0.71	14 ± 0.75	10 ± 0.15	42 ± 0.75
Pseudomonas aeruginosa	11 ± 0.32	12 ± 1.25	9 ± 0.82	42 ± 0.75
Salmonella typhi	7 ± 0.53	8 ± 0.73	8 ± 0.71	41 ± 0.35
Salmonella paratyphi	6 ± 0.29	9 ± 0.19	9 ± 0.79	42 ± 0.35
Shigella boydii	NA	11 ± 0.29	NA	41 ± 0.85
Shigella dusenteriae	9 ± 0.42	10 ± 1.19	NA	41 ± 0.15
Vibrio mimicus	9 ± 0.32	10 ± 2.10	NA	42 ± 1.75
Vibrio parahemolyticus	NA	11± 0.75	NA	41 ± 1.35
Fungi				
Candida albicans	11 ± 1.02	12 ± 1.75	NA	42 ± 0.45
Aspergillus niger	NA	10 ± 0.55	NA	42 ± 0.25
Sacharomyces cerevaceae	NA	9 ± 0.75	NA	42 ± 0.95

[a] Values of the observed diameter zone of inhibition (mm) excluding cap diameter. Incubation conditions for bacteria – 24 hours at 37^0C and for fungi – 48 hours at 25^0C. Assay was performed in triplicate and results are the mean of three values ± Standard Deviation. [b] Reference standard, NA- Zone of inhibition < 5 mm consider as no activity.

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Table 3: Minimum inhibitory concentration of active extract and different fractions of *M. cordata* on various bacterial and fungal strains by agar diffusion method

Minimum inhibitory concentration (MIC) (mg/ml)			
Fraction	**MeOH**	**CH_2Cl_2**	**n-Hexane**
Gram Positive			
Bacillus cereus	> 20	10	NA
Bacillus megaterium	> 20	> 20	NA
Bacillus subtilis	NA	5	NA
Staphylococcus aureus	> 20	10	> 20
Sarcina lutea	> 20	2.5	NA
Gram Negative			
Escherichia coli	2.5	1.25	10
Pseudomonas aeruginosa	5	2.5	> 20
Salmonella typhi	> 20	> 20	> 20
Salmonella paratyphi	> 20	> 20	> 20
Shigella boydii	NA	5	NA
Shigella dusenteriae	> 20	10	NA
Vibrio mimicus	> 20	10	NA
Vibrio parahemolyticus	NA	5	NA
Fungi			
Candida albicans	5	2.5	NA
Aspergillus niger	NA	10	NA
Sacharomyces cerevaceae	NA	> 20	NA

Table 4: Relative percentage inhibition of MeOH extract and different organic fractions of the aerial parts of the *M. cordata* on various bacterial and fungal strain

Relative percentage inhibition (in %)			
Fraction	**MeOH**	**CH_2Cl_2**	**n-Hexane**
Gram Positive			
Bacillus cereus	16.34	26.53	NA
Bacillus megaterium	19.01	23.96	NA
Bacillus subtilis	NA	27.21	NA
Staphylococcus aureus	14.39	26.72	18.73
Sarcina lutea	19.43	32.56	NA
Gram Negative			
Escherichia coli	25.31	45.34	15.92
Pseudomonas aeruginosa	19.02	.	12.81
Salmonella typhi	14.02	20.56	10.67
Salmonella paratyphi	11.92	19.43	12.56
Shigella boydii	NA	26.43	NA
Shigella dusenteriae	11.43	21.23	NA
Vibrio mimicus	11.67	23.42	NA
Vibrio parahemolyticus	NA	26.43	NA
Fungi			
Candida albicans	19.89	41.56	NA
Aspergillus niger	NA	25.73	NA
Sacharomyces cerevaceae	NA	19.20	NA

Table 5: LC_{50} data of test samples of *M. cordata* and Tamoxifin

Sample	LC_{50} (µg/ml), Mean ± SE[a]
MeOH	2.91 ± 1.13
n-Haxen	0.49 ± 1.16
CH_2Cl_2	8.00 ± 2.56
Tamoxifin	1.27 ± 0.11

[a]Values of toxicity (LC_{50}) were expressed as the mean±standard error of three experiments.