IJPSR (2011), Vol. 2, Issue 4 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 December, 2010; received in revised form 21 January, 2011; accepted 02 March, 2011

ANTIMICROBIAL AND TOXICITY STUDY OF DIFFERENT FRACTIONS OF *DILLENIA INDICA* LINN. BARK EXTRACT

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Keywords:

Antibacterial,

Antifungal,

Dillenia indica,

Toxicity

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ABSTRACT

The methanolic extract along with some organic soluble fractions of the bark of *Dillenia indica* Linn. were tested against four Gram-positive and seven Gram-negative bacteria and against three pathogenic fungi. n-Hexane and dichloromethane fractions showed remarkable activities against all the tested bacteria but n-Hexane fraction showed highest activity against *Shigella dysenteriae* and its zone of inhibition was 15.51±0.75 mm. Other showed moderate or little activity. Methanol crude extract showed highest activity against fungus *Candida albicans* with a zone of inhibition 13.13±1.75mm. Lowest minimum inhibitory concentration (MIC) values were observed in n-hexane fraction against *Shigella dysenteriae* and *Staphylococcus aureus* and were 0.312 in both cases. Lowest LC_{50} value 19.02±1.16 of n-hexane fraction indicated the highest toxicity in comparison with the other fractions.

INTRODUCTION: Finding healing powers in plants is an ancient idea. Medicinal plants have been used for years in daily life to treat disease all over the world. Drugs derived from unmodified natural products or drugs semi-synthetically obtained from natural sources corresponded to 78% of the new drugs approved by the FDA between 1983 and 1994 [1]. This evidence contributes to support and quantify the importance of screening natural products. 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 activity [2-3].

Two reasons to bust up clinical microbiologist to develop antimicrobial agents using plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal of antimicrobial drugs prescribed by the physicians; several are already being tested in human. Scientists realize that the effective life span of any antibiotic is limited, so new sources especially plant sources are also being investigated.

Second, the public become increasingly aware of the problems with the over prescription and misuse/abuse of traditional antibiotics [3]. So the development of new antibacterial agents, the most feasible way to combat the problem of microbial resistance and for substitution with ineffective ones was done. Moreover, it is presumed that the broad spectrum effectiveness of plant species may provide a suitable basis for new antimicrobial therapies [4].

The genus *Dillenia* has sixty species, of which *Dillenia indica* Linn., belongs to the family Dilleniaceae, is the most common edible species. Originally from Indonesia, this evergreen tropical tree is now found from Bangladesh, India, and Nepal to China. The common names include Chulta (Bengali, Hindi), Bhavya (Sanskrit) and Elephant apple (English). It is a spreading tree and has beautiful white fragrant flowers, toothed leaves, and globose fruits with small brown seeds [5]. The leaf, bark and fruit of this plant are used as traditional medicine. The juice of *D. indica* leaves; bark and fruits are mixed and given orally (5-15ml, two to five times daily) in the treatment of cancer and diarrhea [6]. The fruit juice of this plant has anti-leukemic effect [7], cardiotonic effect, used as cooling beverage in fever and also employed in cough mixture [8]. The leaves and bark are used as a laxative and as astringent. Bruised bark is applied as a cataplasm for patients with arthritis [8]. The solvent extracts of fruits and leaves of *D. indica* are reported to have antioxidant activity [9]. CNS depressant activities [10] and anti-inflammatory activity [11] in mice and antimicrobial activity [12] were found from the alcoholic extract of the leaves of D. *indica*.

In addition, considering the wide medicinal application of this plant, this work was set out in order to investigate the antimicrobial activity of extracts and fractions of the bark of *Dillenia indica* 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 as toxic agents.

MATERIALS AND METHODS:

Plant materials: The bark of the plant of *Dillenia indica* Linn was collected from the botanical garden of Pharmacy department of Jahangirnagar University, Bangladesh during January 2009. The plant material was taxonomically identified by the National herbarium of Bangladesh. A voucher specimen no.- JU/33231 is maintained in our laboratory for future reference.

Preparation of plant extract: The plant material was shade-dried with occasional shifting and then powdered with a mechanical grinder, passing through sieve #40, and stored in a tight container. The dried powder material (1kg) was refluxed with

MeOH for three hours. The total filtrate was concentrated to dryness, *in vacuo* at 40^{o}C to render the MeOH extract (360 g). This extract was suspended in H_2O and then successively partitioned with n-Hexane, CH_2Cl_2, and EtOAC to afford the n-Hexane (90 g), CH_2Cl_2 (100 g), and EtOAC (60 g), fractions and the H_2O residue (110 g).

Test microorganisms: Strains, including fungi and bacteria both Gram positive and negative were obtained from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B). *Bacillus cereus* ATCC 14579, *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 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 (NA) for bacteria and Sabourand dextrose agar (SDA) for fungi.

Phytochemical analysis: Desirable amount of *Dillenia indica* Linn bark 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 [13].

Antimicrobial activity: The dried plant extracts (MeOH, n-hexane, CH_2Cl_2, EtOAC and aqueous) of *Dillenia indica* bark was 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 Amoxicillin (10µg/disc), Kanamycin (30µg/disc) and antifungal agent Ketoconazole (50µg/disc) were prepared. Antimicrobial tests were then carried out by agar diffusion method[14] and modified by Olurinola[15] 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), subourand dextrose agar (SDA), respectively [16]. Bacteria were cultured overnight at 37^{o}C and fungi at 28^{o}C 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 allow for diffuse (45 minutes). The plates were incubated at 37^{o}C for 24 hours for bacteria.

The above procedure was followed for fungal assays and the media used was sabourand dextrose, incubated at 25^{o}C for 48 hours. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicate. The extract/fractions that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Concentration (MIC) for each bacterial and fungal sample according to method [17].

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 100mg/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, 0.32mg/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.

Brine Shrimp Lethality Bioassay: The toxic potentiality of the plant crude extract and fractions were evaluated using Brine Shrimp lethality bioassay method [18] where 6 graded doses (viz, 5μg/ml, 10μg/ml, 20μg/ml, 50μg/ml, 100μg/ml, 200μg/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 nature 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 24 hours was obtained by a plot of percentage of the dead shrimps against the logarithm of the sample concentration. Vincristine sulfate 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 DISCUSSION: The results (**Table 1**) manifest the presence of glycoside, steroids, flavonoids, tannin, saponins and reducing sugars from crude extract of the *D. indica* bark.

TABLE 1: PHYTOCHEMICAL PROPERTIES OF *DILLENIA INDICA* LINN. CRUDE EXTRACT

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

Table 2 expressed the antibacterial and antifungal activity (zone of inhibitions) of extract and fractions of the *D. indica* bark. The inhibition pattern varied with the type of solvent used for extraction or fractionation and the microorganism tested for susceptibility assay. CH_2CH_2 and n-Hexane fractions have inhibitory function for all the test bacteria and *Shigella dysenteriae* was found the most susceptible bacterium to the all fractions.

EtOAc fractions were active against all microflora except *Sarcina lutea, Vibrio parahemolyticus* and *Vibrio mimicus.* EtOAc fractions showed high activity against *Salmonella paratyphi.* MeOH extract was found inhibitory against all the tested bacteria but the activity was found lower than CH_2CH_2 and n-Haxen fractions. Aqueous fraction was found completely inactive against all the test organisms. Inhibition range for *Vibrio mimicus* and *Vibrio parahemolyticus* was observed very mild against MeOH extract and n-Haxen fractions.

The highest antifungal activity was shown against *Candida albicans* (13.13± 1.75 mm, inhibition zone diameter) for MeOH extract and the weakest activity against *Aspergillus niger* (7.32 ± 0.65mm, inhibition zone diameter) for n-hexane fraction. Minimum inhibitory concentration (MIC) values of crude extract and various fractions of the *D. indica* bark against susceptible bacteria were represented in **table 3**.

TABLE 2: *IN VITRO* ANTIMICROBIAL ACTIVITY OF MEOH EXTRACT AND DIFFERENT ORGANIC FRACTIONS OF THE *DILLENIA INDICA* LINN BARK ON VARIOUS BACTERIAL AND FUNGAL STRAINS BY AGAR DIFFUSION METHOD

	[a]Zone of inhibition in diameter (mm) (n=3)							
Fraction	**MeOH**	**CH_2Cl_2**	**EtOAc**	**n-Hexane**	**Aqeous**	**Kanamycin[b]**	**Amoxicilline[b]**	**Ketoconazole[b]**
Gram Positive								
Bacillus cereus ATCC 14579	8.12±0.75	9.41±1.25	9.77±2.05	10.80±0.15	NA	30.16±0.95	22.89±0.65	
Bacillus subtilis ATCC 6059	9.24±0.25	10.23±2.05	9.46±1.15	12.8± 0.85	NA	23.19± 0.45	21.59±0.25	
Staphylococcus aureus ATCC 6538	9.14±1.67	8.09± 0.25	10.00±0.25	14.8± 1.25	NA	26.61± 0.55	22.71±0.55	
Sarcina lutea ATCC 9341	7.21±0.75	9.21± 0.85	NA	10.53±1.75	NA	24.23± 0.15	22.23±0.16	
Gram negative								
Escherichia coli ATCC 25922	9.36±0.75	12.00±0.75	7.67±0.15	10.52±0.25	NA	22.43± 0.45	17.45±0.75	
Pseudomonus aeruginosa ATCC 27853	10.19±0.55	10.46±1.75	11.50±0.65	12.53±0.73	NA	25.34± 0.25	19.81±0.65	
Salmonella paratyphi ATCC 9150	8.39±1.75	9.77± 0.35	12.83± 0.95	9.83±0.45	NA	25.56± 1.05	14.45±0.65	
Salmonella typhi ATCC 13311	7.41±2.75	10.67± 0.25	10.27± 0.75	10.87±2.75	NA	25.89± 0.75	16.75±0.25	
Shigella dysenteriae ATCC 9361	10.19±0.95	12.06± 0.85	11.83± 1.75	15.51±0.75	NA	25.83± 0.95	20.49±1.65	
Vibrio mimicus ATCC 33653	7.02±1.75	9.80± 0.75	NA	6.12±1.15	NA	28.78± 0.05	22.67±0.45	
Vibrio parahemolyticus ATCC 17802	7.10±1.75	9.53± 0.85	NA	7.11±1.15	NA	26.50± 0.15	14.32±0.55	
Fungi								
Candida albicans ATCC 90028	13.13±1.75	12.87±0.95	8.29± 1.15	9.72±0.55	NA			22.53± 0.15
Aspergillus niger ATCC 1004	12.19±0.75	10.83±1.75	11.45±0.75	7.32±0.65	NA			23.72± 0.05
Sachoromyces cerevacae ATCC 60782	10.08±1.35	NA	11.74±0.45	9.71±0.45	NA			19.67± 0.35

[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- No activity

TABLE 3: MINIMUM INHIBITORY CONCENTRATION OF ACTIVE EXTRACT AND DIFFERENT ORGANIC FRACTIONS OF THE *DILLENIA INDICA* LINN BARK ON VARIOUS BACTERIAL AND FUNGAL STRAINS BY AGAR DIFFUSION METHOD

	Minimum Inhibitory Concentration (MIC) (mg/ml)			
Sample	**MeOH**	**CH_2Cl_2**	**EtOAc**	**n-Hexane**
Gram Positive				
Bacillus cereus ATCC 14579	5	2.5	2.5	1.25
Bacillus subtilis ATCC 6059	2.5	1.25	2.5	1.25
Staphylococcus aureus ATCC 6538	2.5	10	1.25	0.312
Sarcina lutea ATCC 9341	10	10		10
Gram negative				
Escherichia coli ATCC 25922	5	1.25	10	5
Pseudomonus aeruginosa ATCC 27853	1.25	1.25	1.25	0.625
Salmonella paratyphi ATCC 9150	10	2.5	0.625	5
Salmonella typhi ATCC 13311	> 20	1.25	1.25	10
Shigella dysenteriae ATCC 9361	1.25	0.625	0.625	0.312
Vibrio mimicus ATCC 33653	> 20	>20		>20
Vibrio parahemolyticus ATCC 17802	>20	>20		>20

Fungi				
Candida albicans ATCC 90028	0.312	0.625	10	2.5
Aspergillus niger ATCC 1004	0.625	1.25	0.625	5
Sachoromyces cerevacae ATCC 60782	1.25		1.25	2.5

All the tested extracts showed significant variations in MIC values depending upon the test bacteria. *Shigella dysenteriae* and *Pseudomonus aeruginosa* the most sensitive bacteria showed the variable MIC ranges of (0.312-1.25 mg/ml) and (0.625-1.25 mg/ml) respectively. MIC was not observed against *Vibrio mimicus* and *Vibrio parahemolyticus* for all extract and fractions. All extract and fractions showed moderated activity against *Bacillus subtilis.*

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 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 [18]. As summarized in **table 4**, 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 (vincristine sulphate).

The toxicity of the MeOH extract and its fractions on the BSLA increased in the order of n-Hexane> CH_2Cl_2 > MeOH > EtOAc > H_2O and were 19.02±1.16, 34.92±2.56, 45.32±2.13, 104.65±3.03 and >200 µg/mL in their LC_{50}, respectively. The variation in BSLA results (Table 2) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids, triterpenoids, or coumarins) 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.

TABLE 4: LC_{50} DATA OF TEST SAMPLES OF *DILLENIA INDICA* LINN BARK AND VINCRISTINE SULPHATE

Sample	LC_{50} (µg/ml) Mean ± SE[a]
MeOH	45.32 ± 2.13
n-hexane	19.02 ± 1.16
CH_2Cl_2	34.92 ± 2.56
EtOAc	104.65 ± 3.03
H_2O	> 200
Vincristine sulphate	1.225 ± 0.11

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

Phytoconstituents such as saponin, phenolic compounds and glycosides have been reported to inhibit bacterial growth and to be protective to plants against bacterial and fungal infections [19-20]. So, the antimicrobial activity showed by methanol extract and different organic soluble fractions of *D. indica* bark may be due to presence of saponin, tannin phenolic compounds and flavonoids. Moreover, we can find a good correlation between brine shrimp lethality assay and the bactericidal activity of extracts and various fractions. Both n-Hexane and CH_2CH_2 fractions shows good to moderate antibacterial activity against all tested bacterium and also have significant lethal activity

towards nauplii. Thus once BSLA could be used to screen extract or fractions for antibacterial activity, cytotoxicity would be extremely relevant and paradoxically qualify the extract or fractions to further use as antibiotics.

CONCLUSION: 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 evaluations by several methods such as GC-MS, NMR, HPLC, Mass Spectroscopy, etc, for the isolation of the therapeutic antimicrobials.

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