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In vitro antimicrobial and cytotoxicity screening of *Terminalia arjuna* ethanol extract

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Abstract

The present study was carried out to evaluate the antibacterial and cytotoxic activity of 50% ethanol extract of bark from *Terminalia arjuna* on selected four Gram positive and eight Gram negative bacterial strains. The bark extract of *Terminalia arjuna* showed potential antimicrobial activities against all of the selected strains of microorganisms and the greatest activity was observed against *Shigella dysenteriae*. For antimicrobial test, Disc diffusion technique was used and the zone of inhibition of microorganisms was measured in mm. *In vitro* cytotoxicity test was also studied by Brine Shrimp Lethality Bioassay and results illustrated significant ($p<0.05$) cytotoxicity against *A. salina,* that were expressed as LC_{50}. *Terminalia arjuna* ethanol extract showed brine shrimp cytotoxicity with lethal concentration 50 (LC_{50}) value of 50.11 µg/ml.

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Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many of which based on their use in traditional medicine. It has been noted that the original source of many important pharmaceuticals currently in use have been plants used by indigenous people (Balick *et al.*, 1996). Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Barrett *et al.*, 1999). The potential of medicinal plants can be assessed by finding new chemical entities of wide structural diversity. These new chemical substances can also serve as templates for producing more effective drugs through semi-synthetic and total synthetic procedure. According to World Health Organization (WHO), about 74% of 119 plant-derived pharmaceutical medicines or biotechnology medicines are used in modern medicine in ways that correlate directly with their traditional uses (Newman *et al.*, 2003; Barrett *et al.*, 1999). In order to promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use (Nair *et al.*, 2006). Some phytochemicals produced by plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs (Nascimento *et al.*, 2000). Secondary plant metabolites are largely unexplored in 'conventional' animal production systems. In the past, plant metabolites were generally considered as sources of antinutritional factors. Recent bans and restrictions on the use of animal antibiotic growth promoters stimulated interest in bioactive secondary metabolites of plant source as alternative performance enhancers (Greathead, 2003).

Terminalia arjuna (family - Combretaceae), a large tree, is found throughout the South Asian region. It is one of the most versatile medicinal plants having a wide spectrum of biological activity (Ramesh *et al.*, 2004). There are very few reports regarding to its antimicrobial and cytotoxic effects using various parts of this plant. Some scientists used leaves and bark aqueous extracts to screen its antimicrobial activity (Ramya *et al.*, 2008; Miraj *et al.*, 2008) and cytotoxicity by using brine shrimp lethality assay (BSLA) (Miraj *et al.*, 2008).

The aim of the present work was to evaluate the cytotoxic and antimicrobial assays to support the pharmacological effects and phytochemical investigation of this plant as well. Although numerous studies have shown the medicinal values of this plant, there still remains ample scope for further in depth research. So far, for the first time an attempt was taken to investigate the antimicrobial and cytotoxic effect of *Terminalia arjuna* by using 50% ethanol extract of the bark. Accordingly, we disclose herein the antimicrobial and cytotoxic effects of the bark of *Terminalia arjuna* to further establish the scientific basis of the traditional uses of this plant.

Materials and methods

Plant materials and preparation of test sample

The barks of *Terminalia arjuna* were collected from Khamarpara, a village of Magura, Bangladesh. The plant was identified by the Bangladesh National Herbarium, Dhaka and the specimens were stored in there for the further reference (Voucher Specimen No. DACB-35235). The stem barks of the *T. arjuna* were cut into small pieces and then water washed carefully. After washing, the fresh barks were air dried and then oven dried at 40°C temperature. The dried barks are then grinded to make powder, which were then screened to get fine powder. 1500g of barks were dried in oven and finally 500 g of fine powder was obtained. 500 g of dried bark powder were soaked in 50% ethanol. These suspensions were filtered with thin and clean cloth and then filtered by filter paper. The suspensions were evaporated by BUCHI Rota

vapor R-114 [BUCHI, Germany], connected with BUCHI water bath B-480 at 50°C. In this case, 175mbar (to remove ethanol), 72mbar (to remove water) pressure and 160rpm rotation speed were maintained constantly. Finally, small amount of liquid were evaporated from the semi-solid extracts by using a freeze-drier (HETOSICC, Heto Lab Equipment, Denmark) and 75 g of ethanol extracts were obtained.

Phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Molisch's reagent, flavonoids with the use of Mg and HCl; tannins with ferric chloride solutions and saponins with ability to produce stable foam and steroids with Libermann-Burchard reagent. Gum was tested using Molish reagent and concentrated sulphuric acid; reducing sugars with Benedict's reagent; terpenoids with chloroform and conc. sulphuric acid. These were identified by characteristic color changes using standard procedures (Ghani, 2003).

Test organisms

In total, twelve strains of pathogenic bacteria including four Gram-positive bacteria - *Bacillus megaterium, Bacillus subtilis, Staphylococcus areus, Sarcina lutea,* and eight strains of Gram negative bacteria - *Salmonella paratyphi, Salmonella tiphi, Vivrio parahemolyticus, Vivrio mimicus, Escherichia coli, Shigella dysenteriae, Pseudomonas aureus, and Shigella boydii* were used to assess the antibacterial activity (Table 1). The strains were collected from the Microbiology Department, University of Dhaka, Bangladesh. All bacterial cultures were maintained in NA slants/ plates; stored at 4°C and periodically sub-cultured.

Antibacterial assay

The antimicrobial activity for different extracts was determined by the disc diffusion method (Bauer *et al*., 1966). Both gram positive and gram-negative bacterial strains were used for the test. The bacterial strains used for the investigation are listed in Table 1. Solutions of known concentration (mg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out three times and the mean of the reading is required.

Cytotoxicity screening

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds by the method Meyer (Meyer *et al*., 1982). Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of the brine shrimp were collected and hatched in artificial seawater (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. The test samples (extract) were prepared by dissolving them in distilled water (20mg/ml). 2.5, 5, 10, 20, 40, and 80µl of solutions for each test sample were taken in 6 vials and 4ml of sea water was added

to each vial containing 30-35 brine shrimp nauplii. So the concentrations of the test sample in the vials were 12.5, 25, 50, 100, 200, and 400µg/ml respectively. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard Colcichine was used as positive control. Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Statistical analysis of cytotoxicity screening data

Data from the experiments were analyzed using the Statistical Package for Social Science(SPSS) software for windows version 17 (SPSS Inc., Chicago, Illinois, USA). All the triplicate data were expressed as Mean ± SD as appropriate. Statistical analysis of the results was performed by using the ANOVA (analysis of variance) followed by Bonferroni post hoc and Dunnett test. The limit of significance was set at $p<0.05$. The LC_{50} values were calculated from linear regression analysis.

Results

Phytochemical screening

Phytochemical screening of the crude extract revealed the presence of tannins, flavonoids, saponins, gums, steroids, alkaloids, reducing sugar and terpenoids. The intensity of the component content was high in most of the tested groups except saponins and terpenoids (Table 2).

Antimicrobial screening

The ethanol extract of the *Terminalia arjuna* were screened against twelve human pathogenic bacteria to check antibacterial activities by disc diffusion method which showed valuable zone of inhibition. The specific zone of inhibition against various types of pathogenic bacteria was shown in table 1.

Brine shrimp lethality assay

Following the procedure of Mayer, the lethality of the extracts of *T. arjuna* to brine shrimp was determined on *A. salina* after 24 hours of exposure of the samples and the positive control, colcichine. The results of the different extracts of *T. arjuna* (% mortality at different concentrations and LC_{50} values) were shown in Table 3 and Fig. 1-2. The percent mortality increased with an increase in concentration. The 50% ethanol extract of *T. arjuna* and colcichine showed almost 100% mortality to brine shrimp at 400 µg/ml. The LC_{50} obtained from the best-fit line slope were found to be 50.11 µg/ml and 12.59 µg/ml for ethanol extract and standard respectively.

Discussion

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. For this reason, it is necessary to have methods available which eliminate unnecessary separation procedures. Chemical screening is thus performed to allow localization and targeted isolation of new or useful constituents with potential activities. This procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is thus economically very important.

Flavonoids, (a large group of naturally occurring plant phenolic compounds including flavones, flavonols, isoflavones, flavonones and chalcones), also known as nature's tender drugs, possess numerous biological/ pharmacological activities. Recent reports of antiviral, anti-fungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcinogenic, hepatoprotective, and cytotoxic activities of flavonoids have generated interest in studies of flavonoid-containing plants. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been utilized in Chinese medicine and the cosmetic industry as a form of crude plant extracts (Aguinaldo *et al*., 2005; Moon *et al*., 2006; Veitch, 2007; Jiang *et a*l., 2008; Kim *et al*., 2004; Wu *et al*., 2008).

Table 1. Antimicrobial activity of *T.* arjuna ethanol (50%) extract.

Plant Name: *Terminalia arjuna*	Kanamycine 30 µg/disc	Ethanol 50% (1000µg/disc)
Gram positive Bacteria		
Bacillus megaterium	32±0.5	16±0.8
Sarcina lutea	26±0.0	16±0.8
Bacillus subtilis	36±0.2	15±0.5
Staphylococcus areus	36±0.0	16±1.5
Gram negative Bacteria		
Salmonella paratyphi	38±0.8	18±1.6
Salmonella tiphi	36±0.6	18±1.2
Vivrio parahemolyticus	30±0.9	14±0.5
Vivrio mimicus	34±0.6	18± 0.7
E.coli	42±0.0	18±1.3
Shigella dysenteriae	28±0.0	20±0.5
Pseudomonas aureus	31±0.9	18±1.0
Shigella boydii	30±1.8	18±0.9

Data were measured in mm and presented as Mean ± SD of triplicate.

Table 2. Qualitative analysis of the phytochemicals of *T.* arjuna ethanol (50%) extract.

Tanins	+++
Flavonoids	+++
Saponins	++
Gum and Carbohydrate	+++
Steroids	+++
Alkaloids	+++
Reducing sugar	+++
Terpenoids	++

Symbol (+++) indicates higher content and (++) moderate content.

Table 3. Results of Brine Shrimp Lethality Assay for *T.* arjuna ethanol (50%) extract.

		% mortality at different Concentration						
	Sample	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	LC_{50} µg/ml
T. arjuna	Control	6.18±0.57	8.98±0.57	10.65±0.57	11.76±0.57	11.76±0.57	20.00±0.57	
	Colchine	44.4±0.57*	52.94±0.57*	58.94±0.57*	76.47±0.57*	87.50±0.57*	100±0.57*	12.59
	Ethanol Extract	13.3±0.57*	23.54±0.57*	23.41±0.57*	55.61±0.57*	78.67±0.57*	94.78±0.57*	50.11

Data were expressed as mean±SD and analyzed by one way ANOVA, Post hoc and Dunnett test. All of the results were compared with the standard (colchicine); $P<0.05$.

Triterpenoids have a range of unique and potentially usable biological effects and reference to the use of plants with high saponin / triterpenoid content can be found in the first written herbarium. Triterpenoids are studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and for their cytostatic effects. The disadvantage of using triterpenoids is the toxicity associated with their hemolytic and cytostatic properties. Hand in hand with ongoing extraction and isolation of natural products therefore, is the development of synthetic derivatives with lower toxic and higher therapeutic potential (Dzubak *et al*., 2006).

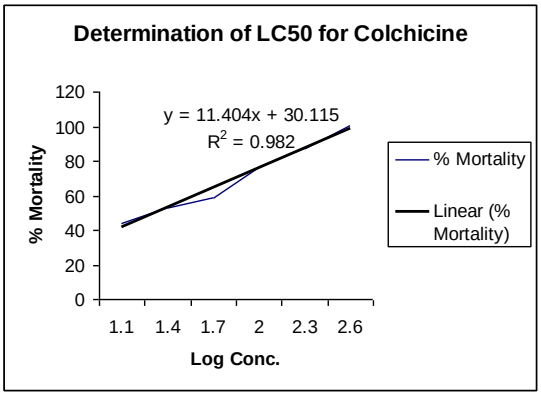

Fig. 1. Determination of LC_{50} of Cholchicine against brine shrimp nauplii.

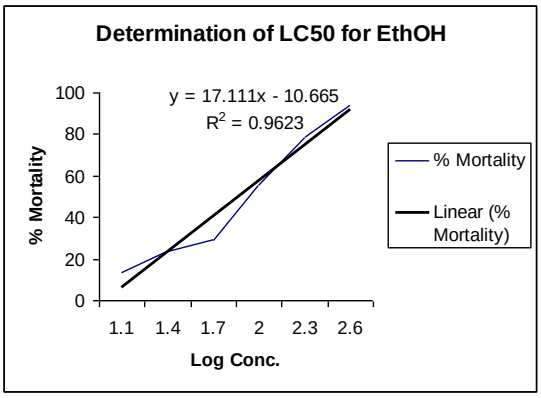

Fig. 2. Determination of LC_{50} of Ethanol extract of *T. arjuna* against brine shrimp nauplii.

The antimicrobial potential of plant was compared according to their zone of inhibition against the several pathogenic organisms. Though bioactive products of *T.* arjuna have been used in treatment of various aliments since time immemorial, role of phytochemical in inhibition of growth of microorganisms has gained less prominence (Sasidharan *et al.*, 1998). The *T.* arjuna ethanol extract showed their potential anti-bacterial activity against both Gram positive and Gram negative bacterial species (Table 1) which is consistent with other investigation (Ramya *et al.*, 2008) though they investigated with bark aqueous extract. Other scientists found its antimicrobial activity against *Vibrio cholerae* by using its 100% ethanol extract (Fakruddin *et al.*, 2011). Among the different microorganisms tested maximum inhibition was found in *S. dysenteriae* followed by *S. paratyphi, S. tiphi, V. mimicus, E.coli, P. aureus, S. boydii, B. megaterium, S. areus, S. lutea, B. subtilis* and *V. parahemolyticus* remained less sensitive to aqueous extracts of *T. arjuna*. Based on the statistical analysis, this inhibition is moderately significant than the standard broad spectrum antibiotic (30 µM of Kanamycin). From the result, regarding to the antimicrobial activity, it can be concluded that the ethanol extract of bark of *T.* arjuna possesses prospective broad spectrum anti-microbial potency against the given test organisms.

The brine shrimp lethality assay (BSLA) has been used routinely in the primary screening of the 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, morphine-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 (McLaughlin *et al.*, 1991; Meyer *et al.*, 1982; Sam, 1993).

The variation in BSLA results (Table 3) may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids or triterpenoids) present in the extracts. The cytotoxic effect of the *T.* arjuna was consistent with some other investigators though they investigated this activity by using the leaves of this plant (Miraj *et al.*, 2008). 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 part of the plant

extracts/fractions to prioritize for further fractionation and isolation of these bioactive compounds. Other cytotoxicity tests and specific bioassays may be done on the isolated bioactive compounds later.

The present study indicates that the extract of *T.* arjuna (50% ethanol) has got profound cytotoxic and antimicrobial effect and may have potential use in medicine. From the previous studies and our current investigation it may be concluded that the flavonoids and tannins are responsible for aforementioned activity. This novel finding will aid us to conduct bioactivity guided isolation and characterization of leading compounds in due course.

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