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Investigation of Antimicrobial Activity of Ethanolic Leaf- Fruit Extract of *Terminalia arjuna* against Multi-Drug Resistance (MDR) Bacteria in Bangladesh

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ABSTRACT

Plants used for medicinal purpose for thousands of years and most of the world still depends on them. This study describes the chemical and biological studies of *Terminalia arjuna*, a plant belonging to the family Combretaceae .Here the ethanolic leaf-fruit extract of *Terminalia arjuna* used to observe the cytotoxicity and antibacterial activity. To ascertain the bioactivity of the extract Brine shrimp lethality test was done and it was observed that LC_{50} obtained for *T. arjuna* extract was 44.157 µg/ml, which was found to be quite lower than the previous studies, indicating that the prepared extract was rich in bioactive compounds. To observe antibacterial activity four Gram-negative and two Gram-positive bacteria were tested using agar well diffusion method. The results indicate that antibacterial activity of the extract were concentration dependent ranging from 0.5-10mg/ml. The striking and distinctive feature of observed antibacterial activity of *T. arjuna* extract is that it exhibited decent activity against the multi-drug resistant Gram- negative bacteria *Coliform spp*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* even at low concentrations(3mg/ml).Minimum Inhibitory Concentration(MIC) was predicted for the extract and it was varied from 3-20mg/ml.

KEY WORDS: *Terminalia arjuna,* Phytomedicine, Antibacterial Activity, Agar well diffusion assay, Minimum Inhibitory Concentration.

INTRODUCTION

Being a developing country, in Bangladesh the rate of mortality due to infectious diseases is very high. Antimicrobial resistance of the variable drugs is the key reason for this. Inappropriate use of readily available antibiotics, prolonged hospitalization, and poor implementation of infection control measures is the main causes of drug resistance. Moreover, powerful drugs against which antimicrobial resistance has not yet been developed are unavailable and costly. So, poor people of our country cannot afford this. In this situation, there is a crying need for an alternative treatment for gut infection, which is both effective and inexpensive. Using of medicinal plant in healing this type of infection can be an important solution to this emerging problem [1].

Phytomedicine usually indicates to the use of plants or parts of any plant for the treatment and prevention of diseases [2].Natural products of plants are rich of different bioactive compounds which are used for the treatment of diverse diseases and also it is used as template for synthetic modification of the phytomedicine. According to World Health Organization (WHO) around 4 billion people, directly or indirectly use phytomedicine for treating and prevention of diseases [3].Now-a-days, natural products of plants and their analogs still represent a large portion of all drugs in clinical use. In recent past some plant based new drugs have been introduced and commercialized in the international market during 2000-2007[4].These new drugs have been approved for the treatment of different genetic disorders and metabolic diseases.

Terminalia arjuna is a tree of Combretaceae family which also known as 'Arjuna' or 'Arjun'. This tree is usually grown up to 25 meter height and found throughout India and some regions of Bangladesh [5]. *Terminalia arjuna* is an only herb that helps uphold a healthy heart and decrease the effects of stress and anxiety. The bark of Arjuna is useful as an anti-ischemic and cardio protective agent in hypertension (High blood pressure) and in ischemic heart illness (IHD), particularly in uneasy cardiac rhythm, angina or myocardial infarction. The bark powder possesses diuretic and a broad-spectrum tonic effect in cases of cirrhosis of the liver, *Terminalia* may also be useful in treating hypercholesterolemia by reducing LDL levels [6]. Previously, it was found that the extract of bark contains arjunic acid, arjungenin and arjunetin compound which possess strong activity against *S. epidermidis* [7]. However, very little work

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is reported with the bioactivity of root, leaves and fruits extract of *T. arjuna* and so far no work has been done on a combinatorial basis like leaf-fruit extract. On this perspective this work was objected to carry out to investigate the cytotoxicity and antimicrobial activity of ethanolic leaf-fruit extract of *T. arjuna* against selected Gram- positive and Gram-negative bacteria in Bangladesh.

METHODS AND MATERIALS

Preparation of extract

T. arjuna leaves and fruits were collected from the Dhaka locality. The taxonomic identity of this plant was confirmed at Department of Botany, University of Dhaka. It was sun dried for 3 days, and then samples were powdered using mechanical grinder. Equal volume of dried leaf powder and fruit powder was taken on an erlynmeyer flask and was soaked 95% ethanol. The flask was covered with aluminum foil and then kept on a mechanical for rotator 24 hours. Next day, the solution was filtered by whattman filter paper and the filtrate was collected. Solvent evaporator was used to evaporate ethanol in the crude preparation under reduced pressure until a gummy substance was obtained. Then approximately equal amount of water was added to the gummy substance and again the solvent was evaporated until a gummy substance was found. Then the substance on the round bottom flask was freeze-dried to prepare the powdered form of plant extract. 18.05 g of *T. arjuna* extract was obtained from a mixture of 180 g containing 90 g of dried leaf powder and 90 g of dried fruit powder The prepared extract was stored in -20^0 C. Consecutive Brine shrimp toxicity and antibacterial test were carried out with this ethanolic leaf-fruit extract to *T. arjuna*

Brine shrimp cytotoxicity test

Brine shrimp lethality bioassay is a protocol to monitor cytotoxicity of a compound thus aids to assess the presence of bioactive natural products. Brine shrimp (*Artemia salina*) eggs were hatched on seawater collected from the Bay of Bengal to get the nauplii. Test sample of experimental plant was taken from prepared extracts and were dissolved in 40% ethanol.100mg *T. arjuna* ethanolic leaf-fruit extract was taken as test sample. Here, the Meyer protocol with some modification was applied [8]. 100 µl of solution containing approximately 10 living shrimps were added to each of the well of a 96-well microtitre plate. The extracts of different concentrations were added to the pre-marked wells by micropipette so that the final concentrations were 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml with a total solution of 300 µl for each extract. Vincristine sulphate was used here as positive control. Seawater used to balance the concentrations. Negative control included only solvent and a blank were maintained to check the validity of the procedure. After 24 hours of incubation, the wells were inspected under light microscope and the number of survivors was counted and noted. Then LC_{50} values, which represent the concentration of the extracts or chemical that produces death in half of the test subjects, with 95% confidence intervals were determined for each extract and the positive control to check statistical significance.

Antibacterial screening

The microbial strains (Table 1) were collected as pure cultures from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). Both Gram-positive and Gram-negative bacteria were taken for the test.

Table1. List of test Gram-positive and Gram-negative bacteria.

Gram-positive bacteria	*Staphylococcus aureus, Streptococcus faecalis*
Gram-negative bacteria	*Coliform spp., Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,*

Inoculum was developed according to 0.5 McFarland standards, that is, $1.5x10^8$ cfu/ml, by taking OD at 600 nm using spectrophotometer. Simply, 50 µl of 1% $BaCl_2$ was mixed well with 9.95 ml H_2SO_4 and suitable three single colonies of microorganism were picked up from subculture and were suspended with normal saline (0.9% NaCl solution). Then absorbance of microbial suspension was adjusted to 0.5 McFarland standards by adding proper amount of normal saline to it. This inoculum was prepared every time just before placing antibiotic disk or before applying plant extract on well.
The ethanolic extracts were checked for their antibacterial screening by agar well diffusion method [9].100 µl of inoculums was taken to each Muller Hinton agar plate using micropipette and spreaded well using sterile spreader. Using sterile tips, wells were produced on the agar plate. 100 µl of plant extract of concentrations 5 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, 60 mg/ml, 70 mg/ml and 100 mg/ml were applied in each well. Also, on another plate, wells were created as same as above 100 µl 40% ethanol was applied as a negative control. Then the plates were incubated in an incubator overnight at 37 °C. The antimicrobial potency of the extract was measured by their activity to prevent the growth of microbes surrounding the wells, which give clear zone of inhibition. Then, the bioactivity of the plant extract was determined by measuring the diameter of the zones of inhibition in millimeter using scale.

RESULTS

Mortality induction of plant extract in Brine shrimp

The lethality of brine shrimp upon treatment with *T. arjuna* ethanolic leaf-fruit was investigated by the procedure of brine shrimp bioassay. Vincristine sulphate was used as positive control. All experiments were repeated thrice and the mean result was noted. The lethal concentration LC_{50} of the test

samples after 24 hrs was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration. The degree of lethality was directly proportional to the concentration of the extract ranging from the lowest concentration (25 µg/ml) to the highest concentration (200µg/ml). In other words, mortality increased gradually with the increase in concentration of the test samples, that is, there was a positive correlation exists between the mortality with concentrations of plant extract.

From the determined LC_{50} value; it was observed that, *T. arjuna* extract LC_{50} value was 44.67 µg/ml which is lower than 50 µg/ml (Table2, Figure1). The positive control was used here to check the validity of the test and LC_{50} of the control was 0.288 µg/ml (Table 2, Figure 2)

Antibacterial Activity of Plant Extracts

Antibacterial activity was observed by many plant extracts [10-11].Before observing the antibacterial activity of *T. arjuna*, first of all the antibiogram of collected strains was done. The result obtained from such test is given on Table 3. For this test, ampicillin, chloramphenicol, erythromycin, rifampicin and tetracycline was used as reference antibiotics. Sensitivity against the antibiotics of a microorganism is determined by Kirby-Bauer method, 1995.

Table2. Effect of T. arjuna ethanolic leaf-fruit extract and Vincristrine sulphate on brine shrimp nauplii

Agent	Concentration (µg/ml)	Log C	Nauplii		% Mortality	$LogLC_{50}$ (µg/ml)	LC_{50} µg/ml
			Total	Dead			
Blank	-	-	10	0	0.00	1.645	44.67
Solvent	-	-	10	0	0.00		
T. arjuna	25	1.398	9	3	33.33		
	50	1.699	18	10	55.55		
	100	2.0	14	10	71.50		
	200	2.301	10	9	90.00		
Vincristine	1.25	0.096	10	7	70.00	-0.569	0.288
	2.5	0.397	12	10	83.33		
	5	0.698	10	9	90.00		

Table 3: Antibiogram of selected strains

Name of Microorganism		Amp. (75 µg)	Chlora (30 µg)	Ery. (15 µg)	Rif. (5 µg)	Tet. (30 µg)
Gram- positives	*Staphylococcus aureus*	R	S	R	I	I
	Streptococcus faecalis	I	S	I	I	S
Gram- negatives	*Coliform spp.*	R	R	R	R	I
	Escherichia coli	R	S	I	S	S
	Klebsiella pneumoniae	R	S	R	R	R
	Pseudomonas aeruginosa	R	S	R	R	R

R: Resistant, S: Sensitive and I: Intermediate. Amp: Ampicillin, Chlora: Chloramphenicol, Ery.: Erythromycin, Rif.: Rifampicin and Tet.: Tetracycline. Content is given as per disc of antibiotic

From the antibiogram study it was found that *Coliform spp, Klebsiella pneumoniae, Pseudomonas aeruginosa* were resistance to multidrug and *Staphylococcus aureus, Streptococcus faecalis, Escherichia coli* showed intermediate resistance and sensitivity against the drug tested. All of the strain therefore selected for the antimicrobial activity of the plant extracts. The antimicrobial activities of *T. arjuna* ethanolic leaf-fruit extract was examined in the present study. The results are given in Table 4. The zones of inhibition produced by these extracts were ranged from 8-17 mm and 8-20 mm for different concentrations for different strains. In Figure3, visible zone of inhibition for antimicrobial activities of plant extract is given.

Table 4: Antibacterial activity of *T. arjuna* ethanolic leaf-fruit extract against Gram-positive and Gram-negative bacteria

Name of Microorganism		Diameter of Zone of Inhibition (mm)									
		0 mg	0.5 mg	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg	7 mg	10 mg
Gram - positive bacteria	*Staphylococcus aureus*	-	-	8	9	11	13	13	14	15	15
	Streptococcus faecalis	-	-	-	-	8	9	10	12	13	14
Gram- negative bacteria	*Coliform spp.*	-	11	11	12	12	12	12	12	13	18
	Escherichia coli	-	-	-	-	-	-	-	8	8	8
	Klebsiella pneumoniae	-	-	8	9	10	12	12	12	12	15
	Pseudomonas aeruginosa	-	-	-	8	9	10	10	10	11	11

Diameter below 7 mm is omitted, as diameter of well is 7 mm. 0 mg is solvent as negative control.

Table5: MICs of different strains for *T. arjuna* ethanolic leaf-fruit extract.

Name of Microorganism		MIC (mg/ml) *T. arjuna* Extract
Gram (-) ves	*Staphylococcus aureus*	5-15
	Streptococcus faecalis	10-20
Gram (+) ves	*Coliform spp.*	<3
	Escherichia coli	>30
	Klebsiella pneumoniae	5-15
	Pseudomonas aeruginosa	10-20

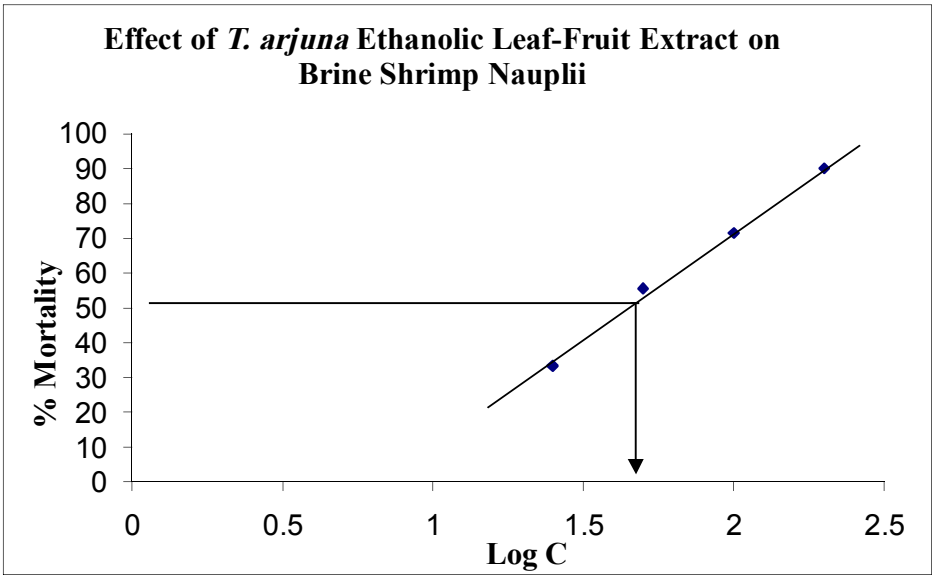

Fig.1: Determination of lethal concentration 50 (LC50) of *T. arjuna* .A straight line obtained by plotting percentage of mortality of brine shrimps nauplii against the logarithm of the concentration of plant extract (from Table 2). From the graph, log LC50 was obtained at 50% mortality. LC50 value was obtained by inversing the log LC50value. LC50 value obtained for *T. arjuna* extract was 44.67 µg/ml

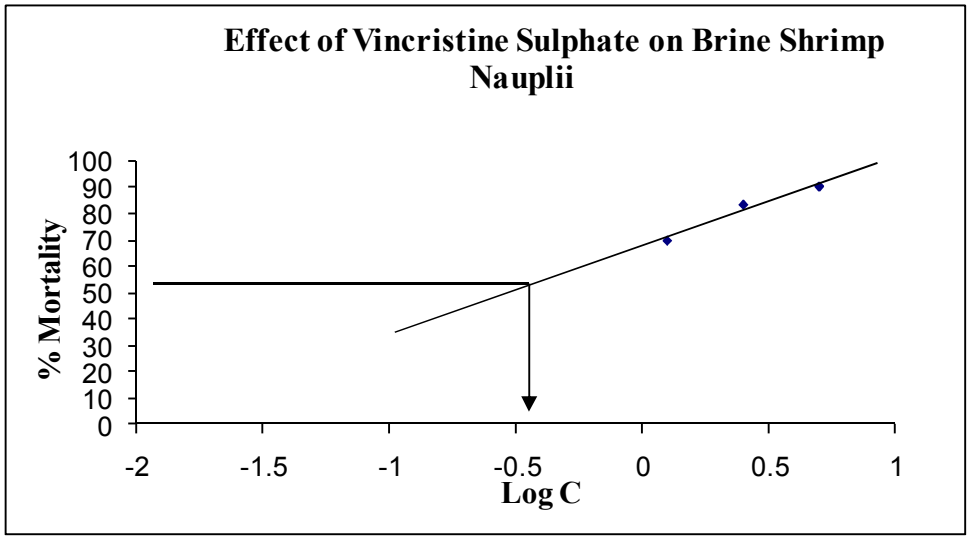

Fig. 2: Regression analysis of effect of vincristine sulphate on brine shrimp nauplii**.** Brine shrimp nauplii were treated with different concentrations of vincristine sulphate as indicated and after 24 hours number of live and dead nauplii was counted and percentage of mortality was calculated. A straight line obtained by plotting percentage of the shrimps killed against the logarithm of the concentration.

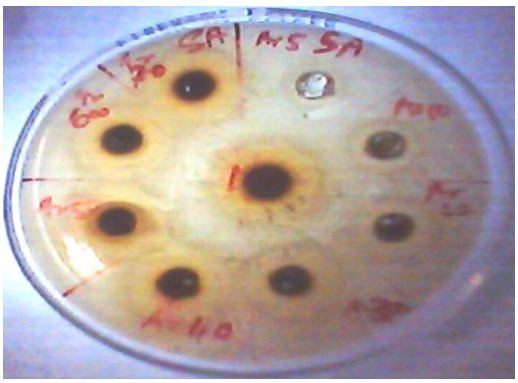

Fig.3: Antibacterial activity of *T. arjuna* ethanolic leaf-fruit extract against *Staphylococcus aureus* at different concentrations. Zone of inhibition is clearly visible and maximizes for 10 mg of sample.

From the observation it was found that extract of *T. arjuna* showed intense activity against different bacterial strain both Gram- positive and Gram-negative bacteria. From the above data, MIC could be predicted as minimum concentration required for inhibiting bacterial growth and could be defined as minimum concentration for which zone of inhibition was more than 8 mm. This could be further analyzed for determination of MIC. It was observed that, the predicted MIC of *T. arjuna* extracts ranges from <3-20 mg/ml (Table 5)

DISCUSSION

Being a developing country, in Bangladesh the rate of mortality due to infectious diseases is very high. One of the major reasons for this is the antimicrobial resistance of the variable drugs. Inappropriate use of readily available antibiotics, prolonged hospitalization, and poor implementation of infection control measures is the main causes of drug resistance [12]. Different pathogenic bacteria such as *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella spp*. are widely distributed in the hospitals and in community thus creating a serious therapeutic problem [13-14]. Moreover, powerful drugs against which antimicrobial resistance has not yet been developed are unavailable and costly. In this situation, there is a crying need for an alternative treatment for gut infectidion, which is both effective and inexpensive. To combat the situation researchers around the world are interested to the natural products compare to synthetic drug as herbal medicine is proven better therapeutics against multi-drug resistant (MDR) bacteria [15]. In this study, ethanolic extract of *T. arjuna* leaf-fruit mixture was investigated for its potential bioactivity like antibacterial activity. To ascertain any bioactivity of desired plant, it should be affirmed that whether this plant has bioactive compound or not. Brine shrimp bioactivity assay is the test to observe whether a plant has bioactive compounds by assessing its toxicity [16]. The LC_{50} obtained for *T. arjuna* extract 44.67 µg/ml. In previous study, the LC_{50} value obtained for aqueous extracts *T. arjuna* bark was 110 µg/ml [17]. The determined LC_{50} values of the *T. arjuna* leaf-fruit extract was found to be quite lower than the previous studies [17] indicating that the prepared extract was rich in bioactive compounds.

To ensure whether the collected stains were antibiotic resistant or not, the antibiogram of these strains was determined. From the observation, it was found that almost all strains showed resistance or immediately sensitive against all the standard antibiotics used (Table 3). The extract was a mixture of leaf and fruit and it was not used before and it was intended to observe for enhance activity. From the experiment, it was observed that the mixed extract of *T. arjuna* showed good activity against different strain that was used. All of the strains, both Gram-positives and Gram-negatives except *Escherichia coli* were found to be sensitive against this extract at low concentration. Most important findings of observed antibacterial activity of *T. arjuna* extract is that it showed intense activity against different multi-drug resistance (MDR) bacteria like *Coliform spp*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* even at low concentrations (3mg/ml).The study also showed better results for Gram-positive bacteria than previous study [18] due to possibly for combinatorial activity of the leaf-fruit extract. These findings suggest that the extract of this plant may become alternative source of antimicrobial drugs in developing country like Bangladesh, which will complement the existing antibiotics.

CONCLUSION

In future, Genes responsible for producing the bioactive compounds in medicinal plants can be identified which have potential therapeutic value. These genes can be induced to over express to increase overall production of the compounds. The responsible genes can be cloned into microorganisms (such as yeast) under the control of tight and regulated promoter that would produce the therapeutics. So the therapeutic product from plant can be overproduced in large scales to develop phytomedicince that might reduce cost and safe sufficient.

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