

PHYTOCHEMICAL SCREENING, *IN VITRO* CYTOTOXIC AND ANTIMICROBIAL ACTIVITY OF METHANOLIC EXTRACT OF LEAVES OF *FICUS SAGITTATA VAHL*.

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Received: 25 August 2015, Revised and Accepted: 14 November 2015

ABSTRACT

Objective: The present study was aimed to evaluate the brine shrimp cytotoxic lethality and antimicrobial activity of methanolic extract of leaves of *Ficus sagittata vahl*. Phytochemical screening was also evaluated.

Methods: To determine these activities, *F. sagittata vahl* was tested against various Gram-positive and Gram-negative bacterial organism through disc diffusion method and cytotoxic activity by brine shrimp lethality assay. For antimicrobial activity, various concentrations of 1000 µg/disc, 800 µg/disc, 500 µg/disc, and 300 µg/disc were used. For cytotoxic activity, various concentration of 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml were used.

Results: The methanolic extract showed remarkable antimicrobial activity against all the organism but it showed the highest activity against *Escherichia coli* and its zone of inhibition was 23 mm at a concentration of 1000 µg/disc compared to 33 mm that of positive control kanamycin. The crude methanolic extract showed strong cytotoxic activity with lethal concentration value of 31.73 µg/ml compared to that of 0.839 µg/ml exhibited by the positive standard vincristine sulfate. The phytochemical screening revealed the presence of phenols, tannins, flavonoids, alkaloids, carbohydrate, saponin, glycosides, and steroids.

Conclusion: From our evaluation, we conclude that, *F. sagittata vahl* possesses the potentiality for further research as an antimicrobial and cytotoxic agent.

Keywords: Antimicrobial, Cytotoxic, Vincristine Sulphate, Disc diffusion, Kanamycin, Phytochemical screening.

INTRODUCTION

Medicinal plants are an important therapeutic aid for various diseases [1]. Medicinal plants have been used for many years in daily life to treat diseases 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 [2]. Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids, and flavonoids which showed a wide range of *in vitro* antibacterial and antifungal activity [3,4]. In the recent years, the development of resistance of pathogens against antibiotics has become a difficult issue caused by the indiscriminate use of modern antibiotics [5].

Ficus Linn. species, commonly known as "Fig," is considered as a keystone species in tropical rain forests as it plays very fundamental role in ecosystem, due to its fruits which are eaten by insects, birds, and animals throughout the year. It is one of the largest genera in the angiosperms with ca. 750 species. The genus is distributed throughout the world primarily in subtropical and tropical regions. It is distributed in India (Andaman Islands, Assam, Meghalaya, Sikkim), Bhutan, China, Bangladesh, Myanmar, Indochina, Malaysia. The main concentration of the species lies in Asian-Australian region with about 500 species which is about 66% of the world species. The maximum diversity of the genus exhibits in Asiatic mainland, New Guinea and Borneo. Many species of *Ficus Linn.* are very common in different biogeographic regions. Although the great majority of the species grow in lowlands, some of them reach up to about 2,000 m altitudes. *Ficus* is also considered one of the most diversified genera with regard to its habits (deciduous and evergreen trees, shrubs, herbs, climbers, and creepers)

and life forms (free standing tree, epiphytes, semi-epiphytes in the crevices, rheophytes, and lithophytes) [6]. There are different chemical constituents available in *Ficus* species such as flavanoid, glycosides, alkaloids, phenolic acids, steroids, saponins, coumarins, tannins, triterpenoids - oleanolic acid, rusolic acid, α-hydroxy ursolic acid, protocatechuic acid, and maslinic acid. The nonenzymatic constituents include phenolic compounds, flavanoids, vitamin C. The enzymatic constituents present ascorbate oxidase, ascorbate peroxidase, catalase, and peroxidase. The phenolic compounds present gallic acid and ellagic acid. Furanocoumarins that were reported are psoralen, bergapten [7].

Ficus sagittata vahl (Family: Moraceae) is a climbing shrub when young, often starting life as an epiphyte. As it grows older, it can become a tree. It often starts life as an epiphyte in the branch of a tree and can eventually send down aerial roots that, once they reach the ground, provide extra nutrients that help the plant grow more vigorously. These aerial roots can completely encircle the trunk of the host tree, constricting its growth - this, coupled with the more vigorous top growth, can lead to the fig out competing and killing the tree in which it is growing. The plant is sometimes harvested from the wild for local medicinal use. It is cultivated for its ornamental value. It is common in lowland to montane forest, at elevations up to 1,600 meters. Fig trees have a unique form of fertilization, each species relying on a single, highly specialized species of wasp that is itself very dependent on that fig species to breed. The trees produce three types of flower; male, a long-styled female and a short-styled female flower, often called the gall flower. All three types of flower are contained within the structure we usually think of as the fruit [8].

Based on the phytochemical evaluation of *Ficus* species done by different researcher we intended to do the phytochemical screening

of *F. sagittata vahl*. The presence of different compounds such as flavonoids and steroids led us to carry out the antimicrobial and *in vitro* cytotoxic activity of *F. sagittata vahl*.

Brine shrimp lethality evaluation is a bench top bioassay method for evaluating anticancer, antimicrobial and other pharmacological activity of natural products. Natural products extracts, fractions, or pure compounds can be tested for their bioactivity by this method [9].

METHODS

Plant materials

The leaves of the plant were collected from local area of Boalkhali in Chittagong during the month of September 2014. It was identified and authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Extract preparation

The collected plant was washed thoroughly with water and air dried for a week at 35-40°C and pulverized in electric grinder. The obtained powder was successively added to methanol with vigorous shaking at 55-60°C temperature. The extracts were made to dry by rotary evaporator under reduced pressure.

Bacterial media preparation (Agar media)

A value of 36 gm Nutrient Agar Media was mixed with distilled water and then sterilized in autoclave at 15 lb pressure for 15 minutes. The sterilized media were poured into petri-dishes.

Antibacterial assay

The methanol crude extract was screened at four concentrations (1000 µg/disc, 800 µg/disc, 600 µg/disc, and 400 µg/disc) against seven bacteria using the disc diffusion method [10]. Dried and sterilized filter paper discs (5-mm diameter) were then impregnated with known amounts of the test substances using a micropipette. Discs containing the test material were placed on nutrient agar medium (Merck) uniformly seeded with the pathogenic test microorganisms. Standard antibiotic discs (kanamycin, 30 µg/disc) and blank discs (impregnated with solvents) were used as positive and negative controls, respectively. These plates were then, kept at 4°C for a 1-hr diffusion of the test material. There was a gradual change in concentration surrounding the discs. The plates were then, incubated at 37°C for 24 hrs to allow organism growth. The test materials having antibacterial activity inhibited microorganism growth, and a clear, distinct zone of inhibition surrounding the discs was visualized. The antibacterial activity of the test agents was determined by measuring the diameter of the zone of inhibition expressed in millimeters.

In vitro cytotoxic activity

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds [11,12]. Here, simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The dried cyst of the brine shrimp was collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) with strong aeration for 48 hours day/dark cycles to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method [11]. The test sample (extract) were prepared by dissolving them in dimethyl sulfoxide (DMSO (not more than 50 µL in 5 mL solutions) plus sea water (3.8% NaCl in water) to attain concentrations of 10, 50, 100, 200 µg/ml. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Standard vincristine sulfate was used as positive control. Then, matured shrimps were applied to each of all experimental and control vials. After 24 hrs, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percentage of the mortality of brine shrimp nauplii was calculated for each concentration using the following formula:

$$\% \text{ Mortality} = N_t \times N_0 / 100$$

Where, N_t = Number of killed nauplii after 24 hrs of incubation,
 N_0 = Number of total nauplii transferred, i.e., 10.

The Median lethal concentration (LC_{50}) was then determined by regression analysis.

Phytochemical screening

The crude extracts were screened for the presence of various chemical constituents such as alkaloids, carbohydrates, proteins, cardiac glycosides, steroids, saponins, flavanoids, terpenoid, tannins, and phenols [13-17].

1. Test for alkaloids (Wagner's reagent): A fraction of extract was treated with 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish brown precipitate (or coloration).
2. Test for carbohydrates (Molisch's test): Few drops of Molisch's reagent were added to 2 ml portion of extracts. This was followed by addition of 2 ml of conc. H_2SO_4 down the side of the test tube. The mixture was then allowed to stand for 2-3 minutes. Formation of a red or dull violet color at the interphase of the two layers was a positive test.
3. Test for proteins (Millon's test): Crude extract when mixed with 2 ml of Millon's reagent, white precipitate appeared which turned red on gentle heating that confirmed the presence of protein.
4. Test for cardiac glycosides (Keller Kelliani's test): 5 ml of extract was treated with 2 ml of glacial acetic acid in a test tube and a drop of ferric chloride solution was added to it. This was carefully underlayered with 1 ml concentrated sulphuric acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violet ring may appear below the ring while in the acetic acid layer, a greenish ring may form.
5. Test for steroids (Salkowski test): To 2 ml of extract, 2 ml of chloroform, and 2 ml of concentrated sulphuric acid were added and shaken, red color at lower layer indicated the presence of steroids.
6. Test for saponins: About 0.5 g of the extract was shaken with water in a test tube. Frothing which persisted for 15 minutes indicates the presence of saponins.
7. Test for flavonoids: 1 ml of the extract, a few drops of dilute sodium hydroxide was added. An intense yellow color was produced in the plant extract, which become colorless on addition of a few drops of dilute acid indicates the presence of flavonoids.
8. Test for terpenoids: Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish color indicated the presence of terpenoids.
9. Test for phenols and tannins: Crude extract was mixed with 2 ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

RESULTS AND DISCUSSION

Antimicrobial assay

The results of the antimicrobial assay of the *F. sagittata vahl*. (methanol extract) indicated that this plant exhibited antimicrobial activity against the tested microorganisms at concentrations of 300, 500, 800 and 1000 µg/disc. The potential sensitivity of the extract was obtained against all the microorganisms tested, and the zone of inhibition was recorded and presented below in the tabulation drawn (Table 1). The extract showed no zone of inhibition against *Salmonella paratyphi*. The extract showed strong activity against *Escherichia coli* with a zone of inhibition of 23 mm at concentration of 1000 µg/disk.

A comparison of zone of inhibition of crude extract with the control is shown in the Fig. 1.

In vitro cytotoxic activity

In brine shrimp lethality bioassay, the methanolic extract of *F. sagittata vahl*. leaves showed a positive result in comparison with the positive control vincristine sulfate. By plotting the concentration versus percent (%) of mortality for all test samples showed an approximate linear correlation.

Table 1: Antimicrobial activity of the leaves extract of *Ficus sagittata vahl.*

Name of the Bacteria	Zone of inhibition in mm				
	Kanamycin disc (standard)	<i>Ficus sagittata vahl.</i> leaves (methanol extract)			
	30 µg/disc	300 µg/disc	500 µg/disc	800 µg/disc	1000 µg/disc
<i>Bacillus subtilis</i>	29	0	9	9	12
<i>Staphylococcus aureus</i>	30	0	12	12	13
<i>Escherichia coli</i>	32	12	14	15	23
<i>Pseudomonas aeruginosa</i>	27	9	9	14	22
<i>Salmonella typhi</i>	30	6	6	11	12
<i>Bacillus cereus</i>	28	5	6	15	15
<i>Salmonella paratyphi</i>	29	0	0	0	0

Table 2: *In vitro* cytotoxic activity of leaves extract of *Ficus sagittata vahl.*

Concentration (µg/ml)	Log C	Total nauplii	No. of nauplii dead	No. of nauplii live	% of mortality	LC ₅₀
5	0.698	10	01	09	10	31.73 µg/ml
10	1	10	02	08	20	
50	1.698	10	03	07	30	
100	2	10	07	03	70	
150	2.176	10	09	01	90	
200	2.301	10	10	00	100	

Table 3: Phytochemical screening of leaves extract *Ficus sagittata vahl.*

Constituents	Results
Alkaloids	+
Carbohydrates	+
Cardiac glycosides	+
Steroids	+
Flavanoids	+
Saponins	+
Protein	-
Terpenoids	+
Phenols	+
Tennins	+

Key: +: Present, -: Absent

From the graph, the median LC₅₀ was determined to check the toxic level of the extract. The crude extract of *F. sagittata vahl.* leaf showed significant cytotoxic activity against brine shrimp nauplii and LC₅₀ value was 31.73 µg/ml compared with standard vincristine sulfate (LC₅₀ = 0.839 µg/ml) (Table 2 and Fig. 2). DMSO was used as negative control to validate the test method.

Phytochemical screening

From phytochemical screening different constituents were detected such as alkaloids, carbohydrates, cardiac glycosides, steroids, saponins, flavanoids, terpenoids, phenols, and tennins (Table 3).

In the present investigation, the antimicrobial activity of the leaves extract of *F. sagittata vahl* was assayed against seven potentially pathogenic microorganisms *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *S. paratyphi*, and *E. coli* at different concentrations of the extract to understand the most effective activity. The leaves extract of *F. sagittata vahl.* showed a broad-spectrum antibacterial activity with a zone of inhibition of 0-23 mm. For methanol extract, the maximum zone of inhibition was obtained for *E. coli* at a concentration of 1000 µg/disc.

Brine shrimp lethality bioassay is an easy and straight forward bench top screening method for predicting important pharmacological activities such as enzyme inhibition, ion channel interference, antimicrobial and cytotoxic activity [18]. The extract showed LC₅₀ at a moderate concentration with very quick response indicating that the

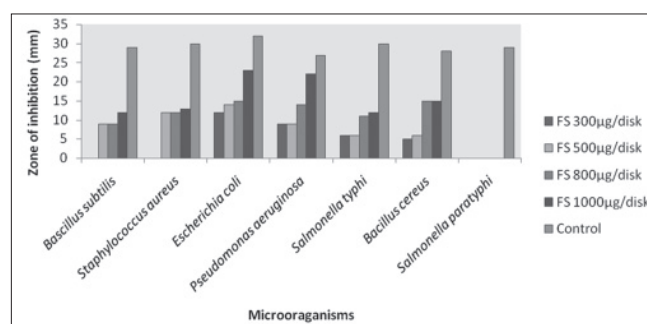


Fig. 1: Comparison of the zone of inhibition of different concentration of *Ficus sagittata vahl.* with the standard control

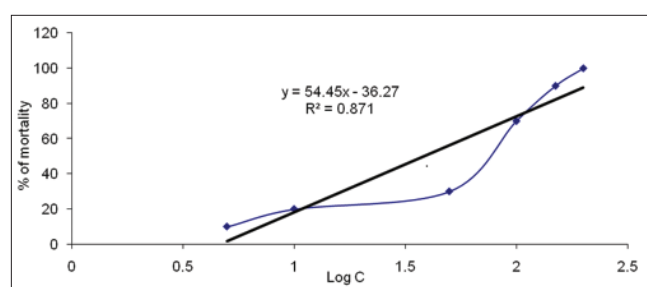


Fig. 2: Determination of lethal concentration of methanol extract of *Ficus sagittata vahl.*

extract is significantly potent. Further investigation is required to find the responsible compound(s) for the cytotoxic activity observed for *F. sagittata vahl.*

The discovery of effective antibiotics, vaccines, and other products or methods has decreased the devastating impact of infectious diseases and improved quality of life. However, the efficacy of many antibiotics is being threatened by the emergence of microbial resistance to existing chemotherapeutic agents because of their indiscriminate and inappropriate use [19]. The use of some antibiotics is associated with side effects, including allergy, immune suppression, and hypersensitivity [20]. Many populations who live in developing countries are deprived of the advantages of modern medicine because

of its high cost; hence, poor people are more vulnerable to infectious diseases. Besides these, co-infection with multiple diseases is an obstacle to infection prevention and treatment. For all these reasons, there is a pressing need to identify new, safe, and cost-effective antimicrobial agents that would help to alleviate the problems of infectious diseases. Plant-derived natural products represent an attractive source of antimicrobial agents because they are natural and affordable, especially for rural societies [21]. Acceptance of medicines from such plant origins as an alternative form of healthcare is increasing because they are serving as promising sources of novel antibiotic prototypes. Moreover, these compounds may have different mechanisms of action than conventional drugs and could be of clinical importance to improve health care [22-24]. Some of the phytochemical compounds, e.g., glycoside, saponin, tannin, flavonoids, terpenoid, and alkaloids, have been reported to have antimicrobial activity [25,26].

Therefore, if a plant extract is found to show significant antimicrobial activity, an acceptable level of toxicity must be considered. In the present investigation, moderate brine shrimp cytotoxicities were found for the extract compared with the standard drug vincristine sulfate. However, these activities might be due to the presence of bioactive or inhibitory compounds or factors in the fractions or synergism by the existence of some compounds or factors in the fractions. Because a variety of constituents, such as saponin, tannin, phenols, flavonoids, and alkaloids were present in the extract studied, further extensive investigations are required to determine the active antimicrobial and cytotoxic properties present in the leaf extracts.

CONCLUSION

Our results may suggest an impressive pathway to discover a potent antimicrobial agent from *F. sagittata vahl*. The present study indicates that the plant contains antimicrobial compounds that can be further developed as phytomedicine for the therapy of infection. It can also be investigated as a possible source of antitumor drugs. This is only a preliminary study and to make final comment, the extract should be thoroughly investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentials. This study may be helpful for further research works.

ACKNOWLEDGMENTS

The authors acknowledge to the Department of Pharmacy of International Islamic University Chittagong (IIUC) for laboratory facilities. The authors are also thankful to Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh, for his valuable help in identifying and authenticating the plant.

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