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ANTITHROMBOTIC, CYTOTOXIC AND ANTIBACTERIAL ACTIVITIES OF METHANOL EXTRACT OF *FICUS SAGITTATA* (VAHL) LEAVES.

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ABSTRACT

Extract from the leaves of *Ficus sagittata* (Vahl) were screened for their antithrombotic, cytotoxic and antimicrobial exercises. The cytotoxicity was surveyed with the brine shrimp lethality bioassay and antithrombotic impact with human blood by clot lysis method. The brine shrimp lethality bioassay was utilized to assess cytotoxicity (LC_{50} = 226.85 µg/ml) contrasted with Vincristine sulfate (LC_{50} = 0.74 µg/ml). It was also assessed as antithrombotic activity when contrasted with streptokinase. It has significant antithrombotic movement (56.13±2.72%) contrasted with standard streptokinase (81.32±1.46%). The extract indicated zone of inhibition against Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram negative bacteria (*Salmonella typhi, Salmonella paratyphi, Escherichia coli, Pseudomonas aeruginosa*) at 1000 µg/disk. Gram positive bacteria *Bacillus subtilis* demonstrated no action against *F. sagittata* leaves extract. Relative percentage inhibition of the extract against each bacterium also calculated. These results indicate that F. sagittata have favorable antithrombotic, cytotoxic and antibacterial effects and capacities of *F. sagittata* extract to be processed for pharmaceutical use.

KEYWORD: *Ficus sagittata*, Antithrombotic, cytotoxic, antibacterial.

1. INTRODUCTION

Traditional knowledge regarding medicinal plants and their use by indigenous cultures is not only useful for maintenance of cultural traditions and biodiversity but also for community healthcare and drug development in the present and future. Therapies with synthetic tropical applications have many side effects and cannot be afforded by the people due to higher cost of the drug. For overcoming this problem plants growing around us are utilized without scientific validation. The use of higher plants and their extracts to treat infections is an age-old practice. Traditional medicinal practice has been known for centuries in many parts of the world. Herbal medicines are gaining interest because of their cost effective and eco-friendly attributes.[1]

Thrombosis is the fundamental pathophysiological process that underlies the acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks; which are the main causes of morbidity and mortality in developed countries.[2] This disease is characterized by the development of a blood clot (thrombus) in the circulatory system of the body due to the failure of homeostasis which leads to vascular blockade and while recovering causes fatal consequences, such as myocardial or cerebral infarction, as well as death.[3] Therefore, anticoagulation therapy is the basis of management, and the proper choice of antithrombotic drugs.[4]

Availability of the eggs, the ease of hatching them into larvae, the rapid growth of the nauplii, and the relative ease of maintaining a population under laboratory conditions have made the brine shrimp a simple and effective animal test in biological sciences and in toxicology. Combined with a reference standard, the brine shrimp test offers a bioassay that can be rapid, simple, bench-top, and more importantly, inexpensive and reproducible.[5]

Increasing bacterial resistance is prompting resurgence in research of the antimicrobial role of herbs against resistant strains. A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds.[6,7] Medicinal plant extracts offer considerable potential for the development of new agents effective against infections that are currently difficult to treat.[8,9] Previous studies have shown that several substances such as peptides, unsaturated long chain aldehydes, essential oils and alkaloid constituents of plant extracts have potential therapeutic properties.[10]

Ficus sagittata (Vahl) 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 outcompeting and killing the tree in which it is growing. F. sagittata is native to warm temperate and tropical regions of Asia (Bangladesh, India etc). The plant is sometimes harvested from the wild for local medicinal use. It is cultivated for its ornamental value.[11,12,13]

The purpose of the present study focuses on the scientific investigation of antithrombotic, cytotoxic and antibacterial activity of *Ficus sagittata* leaves.

2. MATERIAL AND METHOD

2.1 Plant material

Fresh leaves of *F. sagittata* were collected from Bandarban, Chittagong, Bangladesh in the month of March 2015. It was authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2 Preparation of Extract

The leaves were dried for a period of 10 days under shade and ground. The ground leaves (450 gm) were soaked in sufficient amount of ethanol for one week at room temperature with occasional shaking and stirring then the whole mixture was filtered and the filtrate thus obtained was concentrated using a water bath to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (yield value, 5.3%). The extract prepared was for pharmacological screening.

2.3 Chemicals and equipment

To the commercially available lyophilized streptokinase (SK) vial (Square Pharmaceuticals Ltd. Dimethyl sulfoxide) of 1500000 I.U., 5mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 μL (30,000 I.U.) was used for *in vitro* thrombolysis. Methanol purchased from Merck (Germany). Dimethyl sulfoxide (DMSO) and Vincristine sulfate (2mg/vial; Techno Drugs Limited Bangladesh). Kanamycin (30ìg/disc, Oxoid, England) was used as a standard antibiotic disc.

2.4 *In vitro* Antithrombotic activity

2.4.1 Blood specimen

Whole blood (1.5 ml) was drawn from healthy human volunteers (n = 12) without a history of oral contraceptive or anticoagulant therapy. A new consent, approved by Mohammed Abu Sayeed, Assistant professor & Head of Department of Pharmacy, International Islamic University Chittagong, Bangladesh, for collection of blood samples from Human volunteers. Blood collection were conducted by Md. Shariful Islam (Lab technician, Department of Pharmacy, IIUC) and preservation were conducted by Abdul Karim (Lab technician, Department of Pharmacy, IIUC), who stored the clot containing Eppendorf tube in the refrigerator in Microbiology lab, Department of Pharmacy, IIUC. A 500 µl of blood was transferred to each of the three previously weighed Eppendorf tube tubes to form clots.

2.4.2 Statement on informed consent of the donors

The volunteer donors were supplied a consent form which informed the title of the research project, name and detail contact of investigators as well as purpose of the research. Description of the research mentioning step-by-step brief of the proposed research, inclusion and exclusion criteria of the donors, whether donors will receive any therapy or not, volume of blood to be taken, possible discomfort of the puncture sites, time required for the blood sampling. Benefits of the volunteer described. It was indicated to the consent form that the volunteers might refuse to donate blood at any time. Donor whether could withdraw his sample data was disclosed. The sample was restricted for that individual study not for future research projects was presented in the consent form. Potential harm, injuries, discomforts or inconvenience associated with donors in this study was added as informed consent statement. If there was known harm to the donors, the potential harm, current knowledge regarding the probability of the occurrence of the harm, clinical importance of the harm; and any relevant knowledge regarding the probability of reversibility. Treatment alternative and possibility of the research was described. Confidentiality statement was included in the consent form in the way that “confidentiality will be respected and no information that discloses the identity of the participant will be released or published without consent unless required by law of states. Finally identification of investigators was provided in case of further query. The consent form was concluded with major questions on above disclosures in Yes/NO form followed by the signature (with date) of the donor.

2.4.3. *In Vitro* Antithrombotic Study procedure

Experiments for clot lysis were carried as reported earlier.[14-16] Briefly, 1.5 ml venous blood drawn from the healthy volunteers was distributed in three different pre weighed sterile Eppendorf tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each Eppendorf tube containing pre-weighed clot, 100 µl of methanol extract of F. sagittata leaves were added separately. As a positive control, 100 µl of SK and as a negative non-antithrombotic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis. The experiment was repeated with the blood samples of the 12 volunteers.

2.5 Cytotoxicity assay

Brine shrimp lethality bioassay was carried out with the method as described by Meyer et al.[17,18] to investigate the cytotoxicity of methanol extract of F. sagittata leaves. The dried extract preparations were re-dissolved in DMSO to obtain a solution of 10 mg/ml which was subjected to serial dilution to get the concentrations between 12.5 µg/ml- 400 µg/ml. Standard drug Vincristine Sulphate (VS) was used as positive control at concentrations of 5 µg/ml - 0.312 µg/ml. A 5.0 ml of artificial sea water was added into all the test tubes. Simple zoological organism (Artemia salina) was used as a convenient monitor for cytotoxic screening. The eggs of the brine shrimps were collected from local aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (Prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 24 h under the light. The hatched shrimps were allowed to grow by 48 h to get shrimp larvae called nauplii. After 48 h, active nauplii were attracted to one side in a glass petri dish by using a micropipette. The nauplii were then separated from the eggs by aliquoting them in another glass petri dish containing artificial sea water and used for the assay. Suspension containing 10 nauplii was added into each test tube and was incubated at room temperature (25±1°C) for 12 h under the light. The tubes were then examined after 24 h and the number of surviving larvae in each tube was counted with the aid of a 3X magnifying glass. Experiments were conducted along with VS in a set of three tubes per dose. The concentration that would kill 50% of the nauplii (LC_{50})

was determined from a linear regression equation using the software "Microsoft excels 2007".

2.6 *In vitro* Antibacterial activity

2.6.1 Microorganisms

Seven bacterial species, gram-positive *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and gram-negative *Salmonella typhi*, *Salmonella paratyphi*, *Escherichia coli*, *Pseudomonas aeruginosa*. These microbes were obtained from the department of Pharmacy, International Islamic University Chittagong.

2.6.2 Media preparation and maintenance of bacteria

All of the bacterial strains were grown and maintained on Nutrient agar (Merck, India) media at 37 °C and pH (7.4±0.2). The bacteria were subculture overnight.

2.6.3 Preparation of concentration

In the study of the antibacterial activity, all the extracts were diluted in their solvent. So methanol extract diluted in methanol and other also. The concentrations corresponding to the extracts given in Table 2 are expressed in terms of µg/ disk.

2.6.4 Preparation of discs

The discs of about 5 mm in diameter were cut by punching machine from Whatman No.1 filter paper. The discs were taken in a petri dish and sterilized by autoclaving, dried in oven at 180^0C.

2.6.5 Antibacterial screening by disk diffusion technique

The antibacterial effects were tested by the disc diffusion method[19-22] with some minor modification. The filter paper discs (5 mm in diameter) were individually impregnated with 21 µl of 700 µg/disk and 30 µl of 1000 µg/disk of leaves extract of F. sagittata and then placed onto the agar plates which had previously been inoculated with the test microorganisms (within 15 min). The Petri dishes were kept at 4 °C for 3 h before incubation at 37 °C for 24 h. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate. Blank disc impregnated with distilled water was used as negative control and disc of Kanamycin (30 µg / disc) as positive control.

2.6.6 Determination of relative percentage inhibition: The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula.[23][24]

Relative percentage inhibition of the test extract:

$$\frac{100 \times (x - y)}{(z - y)}$$

Where,

x = total area of inhibition of the test extract

y = total area of inhibition of the solvent (methanol)

z = 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 zone of inhibition.

2.7 Statistical analysis

The results were expressed as mean±SD from triplicate experiment for zone of inhibition from triplicate experiments for Antibacterial activity. All other results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using one way factorial ANOVA tests using SPSS Data Editor for Windows, Version 22.0 (SPSS Inc., USA) followed by Dennett's tests on each group except negative control for antibacterial activity. The results obtained were compared with the control groups for antithrombotic activity by using Tukey test and $P < 0.01$, $P < 0.001$ and $P < 0.0001$ was considered to be statistically significant in Dennett's and Tukey tests. GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) was used for graphical presentation.

3. RESULTS

3.1. *In Vitro* Antithrombotic activity: In antithrombotic activity assay, addition of 100µl streptokinase as positive control (30,000 I.U.) to the clots and subsequent incubation for 90 minutes at 37°C, showed 81.32±1.46 % lysis of clot. On the other hand, distilled water treated as negative control exhibited a negligible percentage of lysis of clot (6.81±0.97%). The mean difference in clot lysis percentage between positive and negative control was found statistically very significant (P<0.0001). In this study, the crude methanol extract of *F. sagittata* exhibited antithrombotic activity (56.13±2.72%) (Figure1).

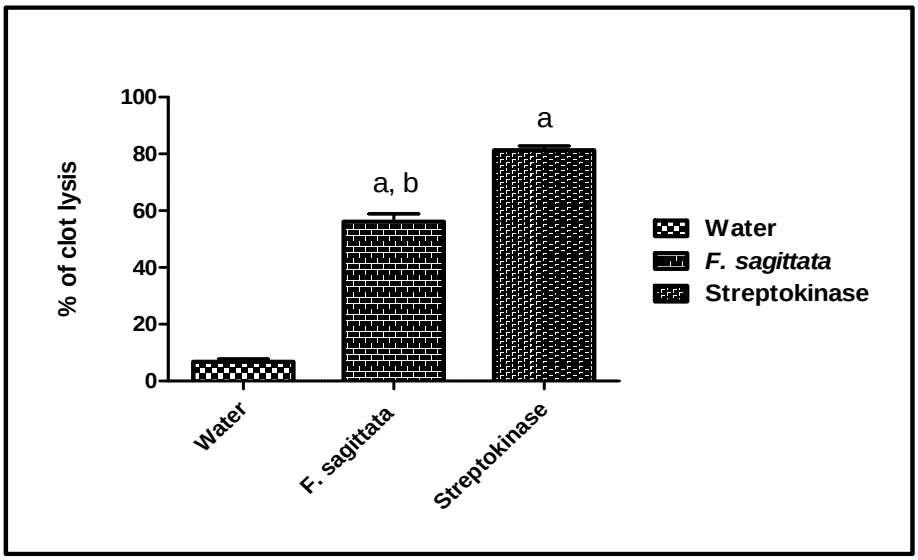

Figure 1: Antithrombotic activity of methanol extract of F. sagittata leaves.

Values are mean ± SEM (n = 12); [a]P <0.0001, Tukey test as compared to negative control (Water), [b]P < 0.001, compared to positive control (Streptokinase). Statistical representation of the effective clot lysis percentage by drugs preparations, positive thrombolytic control (streptokinase), and negative control (sterile distilled water) processed by Tukey test by using SPSS for windows, version 22.0.

3.2. *In vitro* Brine Shrimp Lethality Bioassay

In brine shrimp lethality bioassay, the methanolic extract of F. sagittata leaves showed optimistic result in comparison with the positive control Vincristine Sulphate. By plotting concentration versus percent (%) of mortality for all test samples showed an approximate linear correlation. From the graph, the median lethal concentration (LC_{50}) Cytotoxic effect of the extract is summarized in the Figure 2. The LC_{50} for methanol extract of F. sagittata leaf were found to be 226.85µg/ml respectively, and that of Vincristine Sulphate was 0.74µg/ml. DMSO was used as negative control to validate the test method.

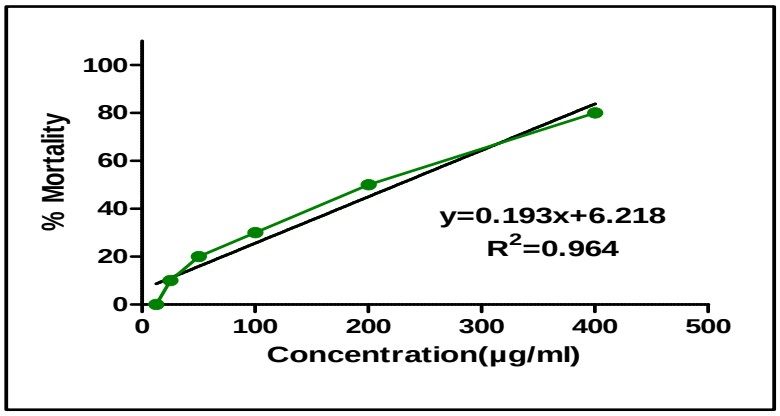

Figure 2: Effects of various concentrations of methanol extract of F. sagittata leaves on the viability of brine shrimp nauplii after 24 hrs incubation.

Percent mortality of brine shrimps of methanol extract of F. sagittata. Data are shown as mean ± SEM of ten shrimps for each concentration.

3.3 *In vitro* Antibacterial activity

Antibacterial activity results of F. sagittata leaves extract are given in Table 1. The extract at two different concentrations 1000 µg/disc and 700 µg/disc showed significant ($P < 0.01$ and $P < 0.001$) as compared with standard Kanamycin 30µg/disc showed zone of inhibitions against Gram positive *Staphylococcus aureus* (8.0±0.50, Nil), *Bacillus subtilis* (Nil), *Bacillus cereus* (8.0±0.50, Nil), *Salmonella typhi* (7.5±0.50, Nil), *Salmonella paratyphi* (7.8±0.29, Nil), *Escherichia coli* (10.2±1.26, 8.3±0.29), *Pseudomonas aeruginosa* (11.0±1.50, 7.5±0.50) respectively. The extract showed the highest zone of inhibition against the Gram negative Pseudomonas aeruginosa (11.0±1.50) at concentration 1000 µg/disc. However, *Staphylococcus aureus* showed the no antibacterial activity to the extract F. sagittata leaves. Relative percentage inhibition of the test extract presented in Table 2.

Table 1: Results of antibacterial activity testing of F. sagittata leaves.

Name of the bacteria	Negative control (Methanol)	F. sagittata		Kanamycin
	30 µl/disc	1000 µg/disc	700 µg/disc	30 µg/disc
Gram Positive				
Staphylococcus aureus	-	**8.0±0.50[a]**	-	22.2±0.76
Bacillus subtilis	-	-	-	18.2±0.29
Bacillus cereus	-	8.0±0.50[b]	-	25±0.50
Gram Negative				
Salmonella typhi	-	7.5±0.50[b]	-	25.3±0.58
Salmonella paratyphi	-	**7.8±0.29[b]**	-	20.3±0.29
Escherichia coli	-	**10.2±1.26[a]**	8.3±0.29[a]	23.5±0.50

Pseudomonas aeruginosa	-	11.0±1.50[b]	7.5±0.50[b]	25.5±0.50

Values are mean inhibition zone (mm) ± S.D of three replicates. The different superscripted (a, b) values have significantly different ([a]P < 0.01 and [b]P < 0.001) as compared with standard (Kanamycin) in same row in Dunnett's test by SPSS. - - - = no zone of inhibition.

Table 2: Relative percentage inhibition of Methanol extract of F. sagittata leaves with their doses compare to standard antibiotics.

	Relative percentage inhibition (%)	
Name of the bacteria	**Methanol extract of F. sagittata leaves**	
	1000 µg/disc	**700 µg/disc**
Gram Positive		
Staphylococcus aureus	13	-
Bacillus subtilis	-	-
Bacillus cereus	10.2	-
Gram Negative		
Salmonella typhi	8.8	-
Salmonella paratyphi	14.7	-
Escherichia coli	18.8	12.5
Pseudomomas aeruginosa	18.6	8.7

Values calculated from their mean values.

1. DISCUSSION

Herbal preparations are used since ancient times to maintain health and regain healthy state of mind. Advances in phytochemistry and identification of plant compounds, which are effective in curing certain diseases have renewed the interest in herbal medicines. About 30% of the pharmaceuticals are prepared from plants worldwide.[25] A number of studies have been conducted by various researchers to find out the herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke.[26] There are several antithrombotic drugs obtained from various sources. Some are modified further with the use of recombinant technology[27] in order to make these antithrombotic drugs more site specific and effective. Side effects related to these drugs have been reported that lead to further complications.[28] Sometimes the patients die due to bleeding and embolism.[29]

Cytotoxicity assay has been considered as pre-screening assay for antimicrobial, antitumor, antimalarial and insecticidal activities. Therefore it is suggested to be a convenient probe for

the pharmacological activities of plant extracts.[30,31] The brine shrimps lethality assay was used to assess the cytotoxicity of leaves of F. sagittata. The LC_{50} value for the crude extracts was found to be very high signifying that the extract is safe at the therapeutic doses.

The results indicated that the methanol extract of F. sagittata leaves showed better antibacterial activities towards the gram-negative bacteria at higher concentration while gram-positive bacteria are less sensitive to the extract probably due to permeability barrier provided by cell wall or to the membrane accumulation.[32]

2. CONCLUSION

Methanol extract of F. sagittata leaves indicated antithrombotic, cytotoxic and antibacterial impact, it can be expected that distinctive dynamic auxiliary metabolites were available in this concentrate and maybe some of these mixes may work in a synergistic way. On the other hand, further studies are important to illustrate the component lying with these impacts. On the other hand, this is the first write about this example and it may serve as a stride with respect to the natural and pharmacological exercises of this specimen.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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