Evaluation of Phytochemical Screening, *In Vitro* Membrane Stabilization and Thrombolytic Activity of Ethanolic Extracts of *Callicarpa longifolia* Lam. Leaves

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Abstract

The current study was carried out on ethanolic extracts of the leaves of Callicarpa longifolia, an evergreen scrub that belongs to the family Lamiaceae. The aim of our study was to examine and investigate the phytochemicals present in the plant leaves, and to evaluate the thrombolytic and membrane stabilizing activity of the extracts. In phytochemical screening, it was found that the leaves contain various phytochemicals, such as glycosides, alkaloids, flavonoids, saponins, tannins, and steroids. Thrombolytic activity tests were carried out using in vitro clot lysis assay method, and the ethanolic extracts were found to have considerable thrombolytic property atadose of 10mg/ml. Clot lysis percentage was found to be 22.68±1.92%, taking streptokinase as standard which had the value of 46.13±3.87%. The ethanolic extracts also exhibited membrane stabilizing activity on human erythrocytes with 25.73±0.87% inhibition of hypotonic solution induced hemolysis. Our study indicated that Callicarpa longifolia leave extracts possess various phytochemicals along with significant membrane stabilizing and thrombolytic properties.

Keywords: *Callicarpa longifolia, Lamiaceae, phytochemicals, thrombolytic activity, membrane stabilizing activity, clot lysis*

Introduction

Wide arrays of bioactive compounds are produced by the plants and many of them have monumental medicinal significance. Medicinal plants have been utilized as natural remedies in many clinical conditions and as tonics for maintaining good health since the early era of human civilization. Even today, plants remain the source of many drugs in both traditional and modern medicine. Traditional medicine still remains one of the primary health care systems in many developing countries. Recently, more intensive studies have been devoted to natural therapies.The World Health Organization (WHO,1980) encouraged the use of traditional medicines especially inareas where modern medical treatment is not readily accessible. Evaluation and investigation of the local flora for various biological activities is there fore critical and far-reaching. Bioactive phytochemicals are often utilized as the lead compounds in the pursuit of new drug development, and there fore their isolation and characterization remain pivotal. The most important of these bioactive phytochemicals are alkaloids, glycosides, tannins, saponins, flavonoids, and phenolic compounds (James et al., 2008).

Thrombosis or blood clotting is the underlying cause of some serious medical conditions. Cerebralvenous sinus thrombosis (CVST) is a common disorder which is associated with significant morbidity and mortality. Ischemic heart disease, ischemic stroke, myocardial infarction, pulmonary embo-

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lism, etc. stem from thrombosis. Heparin and thrombolytic drugs like tissue plasminogen activator (t-PA), streptokinase, urokinase, etc. play a critical role in the management of patients with CVST and other conditions associated with thrombosis (Chowdhury et al., 2011).

Anti-inflammatory properties of many natural products have been studied extensively by Oyedapoet al., (1997). The method measures the effect of plant extracts on the stabilization of erythrocyte membrane exposed to hypotonic solution and heat. Since the erythrocyte membrane very much resembles the lysosomal membrane, the action of a drug on the stabilization of erythrocyte membranes can be extrapolated to predict the drug's effect on the stabilization of lysosomal membranes (Oyedapo et al., 2010).

The genus Callicarpa (beauty berry) incorporates about 140 species of shrubs and small-trees which belongs to the Lamiaceae family. Callicarpa plants have widely been exploited in indigenous medicine in the treatment of inflammation, rheumatism, fractures, hematuria, hematemesis, women amenorrhea, gastrointestinal bleeding, scrofula, etc (Tu et al., 2013).

Method and Materials

Plant Collection and Extraction

Mature fruiting *Callicarpa longifolia* plants were procured for this study. The voucher specimens were deposited in the National Herbarium of Bangladesh, Mirpur, Dhaka. The leaves were separated, washed, and then air-dried at room temperature. The dried plant materials were powdered intoc oarse particles. Powdered plant materials weighing about 200 grams were taken in anamber reagent bottle, and were soaked in one liter of ethanol for two weeks for extraction. The mixture was then filtered with Whatman Grade 1 filter paper. A rotary evaporator was used to concentrate the filtrate, and the percentage yield of the extracts was calculated.

Phytochemical Screening Methods

Detection of Glycosides: Two milliliters of extract solution was taken in a test tube. One milliliter mixture of Fehling solution was added to it, and the test tube was placedin a water-bah at 60°C. For mation of brick red color indicates that glycosides are present (Mamun et al., 2017).

Detection of Alkaloids: About 0.5g of extract was added to 5 ml of 1% aqueous hydrochloric acid, and the mixture was stirred on a water bath. One milliliter of the filtrate was treated with a few drops of Mayer's reagent, and a second one milliliter portion was treated with Dragendorff's reagent. Formation of orange-red color suggests the presence of alkaloids (Mamun et al., 2017).

Detection of Flavonoids: In 5ml of 95% ethanol, a small amount of the extracts was dissolved. A few drops of concentrated hydrochloric acid and 0.5 g of magnesium metal were added to the solution. If pink, crimson or magenta color is developed with in a minute or two, flavonoids are present (Mamun et al., 2017).

Detection of Tannins: About 5 g of plant extract was added to 10 ml of distilled water. The mixture was stirred, filtered, and a small amount of ferric chloride reagent was added to the filtrate. Development of dark green or deep blue color indicates the presence of tannins (Mamun et al., 2017).

Detection of Saponins: A few milligrams of the plant extract was taken in a test tube. A small amount of sodium bicarbonate and water were added to it. If stable characteristic honey comblike froth is obtained upon vigorous shaking, saponins are present (Mamun, et. al, 2017; Harborne, 1973).

Detection of Steroids (Salkowski's test): Dried *C. longifolia* leaf extract of about 100 mg was dissolved in 2ml of chloroform. Concentrated sulfuric acid was carefully added to form a bottom layer. If steroidal ring is present in the extract, areddish-brown color appears at the interface (Gowri & Vasantha, 2010).

Membrane Stabilizing Activity

Membrane stabilizing activity of *Callicarpa longifolia* leave extracts was evaluated by utilizing hypotonic solution induced hemolysis of human erythrocytes, as described by Omale and Okafor (2008) with minor modifications (Omale & Okafor, 2008; Islam, 2015).

Collection of Blood Samples: Human erythrocytes or red blood cells (RBCs) were used for the study. Seven milliliters of blood was drawn from each healthy male volunteers (aged 20 to25 years), who were disease free and did not have a history of receiving oral contraceptives or anticoagulant therapy (protocol approved by Institutional Ethics Committee). Ethylene diamine tetraaceticacid (EDTA), which acts here as an anticoagulant, was added to the collected RBCs. The mixture was kept in a test tube understandard conditions with temperature 23±2°C and relative humidity 55±10%.

Preparation of Erythrocyte Suspension: Seven milliliters of blood was collected using syringes containing the anticoagulant EDTA from each male volunteers through puncture of the antecubital vein. The blood samples were centrifuged at 3000g for 10 minutes, and were washed three times with 0.9% sodium chloride isotonic solution. After washing, the volume was measured and reconstituted as a 40%(v/v) suspension with isotonic buffer solution (pH 7.4) which contained 1L of distilled water, $NaHPO_4$. $2H_2O$ 0.26 g, Na_2HPO 41.15 g, and NaCl 9 g(10mM sodium phosphate buffer). The prepared suspension was used as the stock erythrocyte (RBC) suspension.

Hypotonic Solution-Induced Hemolysis: Aliquots of 0.5 ml were taken from the stock erythrocyte (RBC) suspension, and were mixed with 5ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (2.0 mg/ml) or the standard acetyl salicylic acid (0.1mg/ml).The control samples contained 0.5 ml of RBC mixed with hypotonic–buffered saline without the extracts or standard. The mixtures were incubated for10 min at room temperature, and after than centrifuged for10 min at 3000 rpm. Absorbance of the obtained supernatantsat 540 nm was measured using UV spectrometer. The following equation was used to calculate the percentage inhibition of hemolysis.

%Inhibition of hemolysis =(OD1-OD2)/OD2 x 100

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control), and

OD2 = Optical density of test sample in hypotonic solution

In vitro Thrombolytic Activity

Thrombolytic activity of *Callicarpa longifolia* leaf extracts was evaluated using the method described by Prasad et al (2006). Five milliliters of phosphate buffered saline (PBS) was added to 1,500,000I.U. streptokinase vial and mixed properly.This suspension was used as the stock from which 100 µL (30,000 IU) aliquots were taken for evaluating in vitro thrombolysis. Venous blood of 5 ml, drawn from healthy volunteers (n=3), was distributed in pre-weighed Eppendorf tubes (0.5 ml/tube).The blood specimens were centrifuges at 2,500 rpm for 10 minutes,and then incubated at 37 ºC for 45 minutes. After clot formation, serum was aspirated out completely without disturbing the clot. Each tube having the clot was again weighed in order to determine the clot weight. To three of the Eppendorf tubes containing the pre-weighed clot, 100 µl of aqueous ethanol extracts (10 mg/ml) was added. As a positive control, 100 µl of streptokinase was added to one of the remaining Eppendorf tubes containing the clot. As a negative nonthrombolytic control, 100 µl of distilled water was adde dinstead. All tubes were properly labelled. The Eppendorf tubes were then incubated at 37ºC for 90 minutes. After the incubation period, the tubes were observed for clot lysis. The fluid released during incubation was removed, and the tubes were weighed again. Percentage of clot lysis was calculated from the difference between the weight taken be fore and after clot lysis.

Results

Phytochemical Screening

The preliminary phytochemical screening of ethanol extracts was done to ascertain the presence or absence of bioactive constituents. The results of phytochemical screening of *Callicarpa longifolia*- for the bioactive components are presented in table-1.

The preliminary phytochemical evaluation of ethanolic extract of *Callicarpa longifolia* confirmed the presence of alkaloids, glycosides, steroids, flavonoids, tannins, and saponins.

Table 1

Phytochemicals	Presence
Glycosides	+
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	+
Steroids	+

*key: + (present), - (absent)

Membrane Stabilizing Activity

Effect of *Callicarpa longifolia* plant extract on hypotonic solution of erythrocyte membrane is shown in table 2.

Table 2

Sample	% inhibition of hypotonic solution induced hemolysis
Ethanolic extract	25.73 ± 0.87
Standard (aspirin)	77.27 ± 0.32

The crude ethanolic extracts of *Callicarpa longifolia* leaves were subjected to assay for membrane stabilizing activities by following standard protocol. The results, shown in table 2, suggested that the extracts (at concentration 2mg/ml) adequately protected the human erythrocytes against hypotonic solution,when compared to the standard drug aspirin (acetylsalicylic acid) (0.10mg/ml). In hypotonic solution, the extracts were found to inhibit 25.73±0.87% hemolysis of erythrocytic membrane, while in the same condition, aspirin inhibited 77.27±0.32% hemolysis of erythrocytes.

Thrombolytic Activity

Thrombolytic activity of *Callicarpa longifolia* plant extracts is summarized in table 3.

Table 3

Sample	% of clot lysis
Control	13.35 ± 2.26
Ethanolic extract	46.13 ± 3.87
Standard (aspirin)	22.68 ± 1.92

Figure 1 shows the effect of the extracts on clot lysis. The percentage (%) clot lysis was statistically significant ($p<0.001$) when compared to the control. The plant leaf extracts showed moderate clot lysis activity of 22.68±1.92% where as the standard streptokinase showed 46.13 ± 3.87% clotlysis activity.

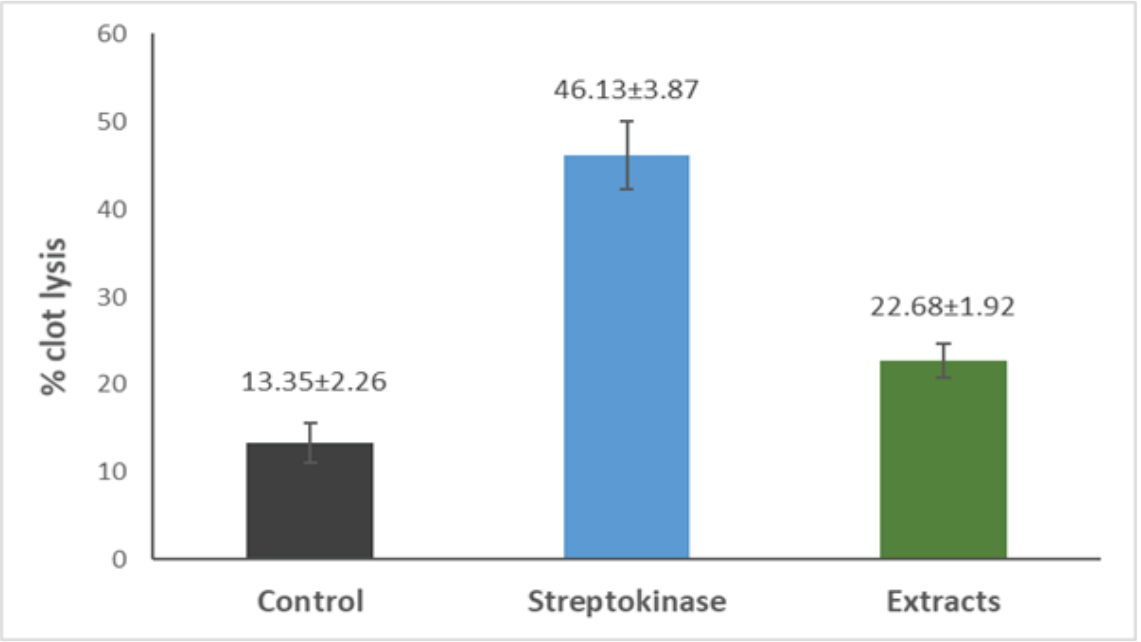

Figure 1: Thrombolysis activity of ethanolic extract of *Callicarpa longifolia* compared with streptokinase and control. Thrombolytic activities were measures by percentage clot lysis; results are shown as mean±SEM of three parallel measurements.

Discussion

In our present study, the phytochemical screening of ethanol extracts of the leaves of *Callicarpa* longifolia was performed. *C.longifolia* has found to be rich in alkaloids, glycosides, *flavonoids,* steroids, phenols, saponins, and tannins. The various phytochemical compounds detected in C. *longifolia* leaves are known to have outmost importance in medicinal science. For instance, *flavonoids* have been recognized for their ability to modify body'sreaction to allergies and viruses. For this reason, they are often described as nature's biological response modifiers. Flavonoids are known to have anti-allergic, anti-inflammatory, anti-microbial, and anti-cancer properties (Aiyelaagbe & Osamudiamen, 2009). Plant steroids are known to possess cardiotonic, insecticidal and antimicrobial properties. They are also used in nutrition and cosmetics (Callow, 1936).

Tannins are well-known for their antiviral, antibacterial and anti-tumor activities. Certain tannins have been reported to inhibit HIV replication selectively Tannins also have diuretic property (Callow, 1936). Saponins act as mild detergents and are utilized in intracellular histochemical staining. Saponins are also used to allow antibody access in intracellular proteins. In medicine, they are used in hypercholesterolemia, hyperglycemia, obesity, etc., and as an antioxidant, anticancer, and anti-inflammatory agent. They are also known to have antifungal properties (Haslem, 1989).

Table 2 summarizes the membrane stabilizing activity of the ethanolic extracts *C. longifolia. The results suggest* that the extracts are moderately potent on human erythrocyte, protecting it against hypotonic induced erythrocyte lysis to some extent. The activity was comparable to that of standard anti-inflammatory drug, aspirin. It has been previously reported that flavonoids can show marked-stabilizing effects on lysosomes both *invitro* and invivo in experimental animals (Caneghem, 1972; Sadique et al. 1989; Middleton, 1996) while saponins and tannins have the ability to bind cations and other biomolecules, and are able to stabilize the erythrocyte membrane (Oyedapo, 2001; El-Shanbrany et al., 1997). High flavonoid and tannin content can therefore be attributed to the membrane stabilizing activity of the leaf extract of *C. longifolia* observed in this investigation. It is known from earlier investigations that various plant extracts are capable ofs tabilizing the erythrocyte membranes and can exert anti-in flammatory activity (Sadique et al., 1989; Olugbenga et al., 2005).

It has also been reported that phenolic compounds can inhibit the activity of prostaglandin cyclooxygenase and can there by exert anti-inflammatory action (Richter et al., 2003). Our investigation suggests that *C. longifolia* extracts at a concentration of 2mg/ml can readily inhibit the lysis of human erythrocyte membrane induced by hypotonic solution, which is comparable to the standard acetyl salicylic acid. Since the erythrocyte membrane is similar to lysosomal membrane, it is probable that *C. longifolia* leaf extracts also possess some anti-inflammatory activity.

Platelets play a critical role in the development of atherothrombosis. Platelets adhere to the damagedregions, caused by reactive oxygen species, of the endothelial surface of the blood vessels.Theactivated platelets form platelets to platelets bonds, bring leucocytes in the region, and promote plaque formation and growth (Prentice, 1999). Plasmin, acting as a natural fibrinolytic agent, dissolves clot by breaking down the contained fibrinogen and fibrin. Streptokinase forms a1:1stoichiometric complex with plasminogen allowing the convertion additional plasminogen to plasmin (Banerjee et al., 2004). Phlorotannins, obtained from marine browna lgae, promotes the lysis of intravascular blood clotvia anti plasmininhi bition (Prasad et al., 2007). Since Callicarpa longifolia extracts contain tannins, alkaloids and saponins, they may participate in the clot lysis activity observed in the present study.

Conclusion

In Bangladesh, *Callicarpa longifolia* is an indigenous flowering plant, grows in the hill tracts in the southeastern part of the country (Species Description – Flora of Bangladesh). Our present study indicates that *C. longifolia* leaves contain some important phytochemical agents and possess various biological activities. The crude ethanolic extracts of *C. longifolia* leaves have shown potential membrane stabilizing as well as significant thrombolytic properties. The plant, therefore, canbe a good candidate for further research. The plant can thoroughly be investigated in order to discover bioactive components that, in future, may serve as the lead for development of new drugs.

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