

## MEMBRANE STABILIZATION AS A MECHANISM OF ANTI-INFLAMMATORY AND THROMBOLYTIC ACTIVITIES OF ETHANOLIC EXTRACT OF ARIAL PARTS OF *SPONDIASIS PINANATA* (FAMILY: ANACARDIACEAE)

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### Abstract

*Spondiasis pinanata* is a medicinal herb belonging to the family of Anacardiaceae, also known as wild mango, hog-plum which has a history of ethnomedicinal properties. The crude ethanolic extract and its different partitionates of the leaves of *S. Pinanata* were evaluated for its possible thrombolytic and anti-inflammatory activities. The activities of the leaf extract were evaluated by using standard drugs; Streptokinase (SK), used for thrombolytic activity, and acetyl salicylic acid (ASA), for anti-inflammatory activity. In this study, SK showed 69.23%, the ethanolic soluble fraction of leaves (ESF) 43.28%, n-Hexane soluble fraction (HXSf) 58.06%, chloroform soluble fraction (CSF) 27.66% and aqueous soluble fraction (AQSF) 22.81% activity against thrombosis. For human red blood cell (HRBC) membrane stabilization, ASA and leaves extractives soluble fractionates significantly inhibits the haemolysis of HRBC membrane which was induced by hypnotic solutions, with their inhibition results of ASA(81.90±29%), AQSF (33.49±0.51%), ESF(58.23±0.64%), CSF(49.66±0.77%), HXSf(70.35±0.50%) respectively. While, ASA and leaves extractives soluble fractionates protected significantly heat-induced lysis of human red blood cell membrane, with their values of ASP being (74.12±0.26%), AQSF (30.34±0.42%), ESF (51.53±0.61%), CSF (46.22±0.57%), HXSf (61.11±0.69%) respectively. Hence, the results of the present study revealed that the traditional uses of this plant leaves is a remedy for thrombosis and unstable blood red cell membrane.

**Keywords:** *Spondiasis pinanata*, streptokinase, acetyl salicylic acid, thrombolytic activity, membrane stabilizing activity.

## Introduction

Medicinal plants are one of the important contributors to the most of the medicinal preparations as raw plant materials, refined crude extracts and mixtures etc. Several thousands of plants have been identified containing medicinal values and are used to treat different ailments in various cultures worldwide [1]. *S. pinnata* (Family-Anacardiaceae) is widely use as a medicinal plant in Bangladesh, also known as wild mango, hog-plum, available in the forests of Chittagong, Chittagong Hill Tract, Cox's Bazar, Dhaka, Tangail, Sylhet and Dinajpur, also found in village shrubberies throughout the country. Aerial parts have been found to contain 24-methylene cycloartenone, stigmast-4-en-3-one,  $\beta$ -sitosterol, glycoside of  $\beta$ -sitosterol and lignoceric acid. Fruits contain water-soluble polysaccharides, composed of mainly L-arabinose, D-galactose and galacturonic acid [2]. Presence of  $\beta$ -amyrin and oleanolic acid, glycine, cystine, serine, alanine and leucine have also been detected in the fruits of this plant [3]. It is useful in dysentery, diarrhea, stomach ache, rheumatism, swollen joints and is also given to prevent vomiting [4]. A paste of it is used as an embrocation for both articular and muscular rheumatism [5]. A decoction of the bark is given in gonorrhoea. The root is considered to be useful in regulating menstruation. It has also excellent antioxidant activities. The plant is reported to have anti-tubercular and flavoring properties [6]. The unripe fruit is good for rheumatism and sore throat. Ripe fruit is tonic, aphrodisiac and astringent to the bowels; cures burning sensation. Therefore, the research activities have been focused on the phytochemical investigation of the plants which have ethnobotanical information associated with them. As a part of the endeavor for the study on the medicinal properties of ethanolic extract of aerial parts of *S. Pinanata* for thrombolytic and membrane stabilizing activities, the results obtained from these significant observations have been reported [7].

## Materials and methods

### Plant materials

For this present investigation the plant *S. Pinanata* was collected from, Dhaka, Bangladesh in August 2014 and was identified by Bangladesh National Herbarium, Mirpur, Dhaka.

### Preparation of extract

The collected plant leaves were shade dried for several days and then oven dried for 24 hours at

40°C to facilitate grinding and stored in a tight container. The dried powder material (400gm) was soaked in 1000ml of 90% ethanol for two weeks. The whole mixture was then filter by a piece of clean, white cotton materials. Then the filtrate was filtered again through Whatman filter paper, total filtrate was concentrated using a rotatory evaporator to an optimum temperature of 40-50°C get the crude extract of *S. Pinanata*. The concentrated aqueous ethanol extract was partitioned by the Kupchan method (Van Wageningen et al., 1993) and the resultant partitionates, i.e. Chloroform, hexane and aqueous soluble materials were used for our current investigation.

### *In vitro* thrombolytic activity study

#### Sample preparation

The crude extract was suspended in 10ml of sterile distilled water and shaken vigorously on a sonicator, and then the suspension was kept overnight and decanted to remove soluble supernatant, which was filtered through a filter paper. The solution was then ready for *in vitro* evaluation of clot lysis activity.

#### Thrombolytic assay

The thrombolytic activity of all extractives were evaluated by the method developed by Daginawala [8], using streptokinase (SK) as standard substance. Whole blood (5 ml) were drawn from healthy volunteers, and transferred in different pre-weighed sterile tubes (1 ml/tube) to form clots and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot formed and each tube having clot, was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone) [9-11]. The tubes containing pre-weighed clot was properly labelled, 100  $\mu$ l crude extract and aqueous solutions of different fractionates were added to the tubes separately. As a positive control, 100  $\mu$ l of SK and as a negative non thrombolytic control, 100  $\mu$ l of isotonic solution was separately added to the clot containing tubes. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released fluid was removed and vials were again weighed to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis as shown below:

$$\% \text{ of clot lysis} = (\text{wt. of released clot} / \text{clot wt.}) \times 100.$$

### ***In vitro* membrane stabilizing activity study**

The membrane stabilizing activity of the extractives were determined with human erythrocytes by following the method developed by Omale [12].

### ***Preparation of the erythrocyte suspension***

The erythrocyte membrane resembles to lysosomal membrane and as such, the effect of extracts on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. The membrane stabilizing activity of the extracts was assessed by using hypotonic solution-induced and heat-induced erythrocyte haemolysis. To prepare the erythrocyte suspension, 5 ml of whole blood were withdrawn from healthy human volunteer by hypodermal syringes into anticoagulant 3.1% Na-citrate containing tubes. The blood was centrifuged through centrifugation for 10min at 3000 rpm and blood cells were washed three times with solution {(154 mM NaCl) in 10 mM sodium phosphate buffer (pH7.4)}[13,14].

### ***Hypotonic solution Induced hemolysis***

The experiment was carried out with hypotonic solution. The test sample contained stock erythrocyte (RBC) suspension(0.5ml) with 5ml of hypotonic solution[50mM NaCl in 10mM sodium phosphate buffer saline,(pH 7.4),different fractions of ethanolic extract (2mg/ml) and acetyl salicylic acid (0.1mg/ml)].The acetyl salicylic acid was used as reference standard. The mixtures were centrifuge for 10 at 3000 rpm, and incubated for 10 min at a room temperature 37°C. The absorbance of supernatant content haemoglobin was measured at 540nm using UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation.

$$\% \text{ inhibition of haemolysis} = 100 \times \left\{ \frac{OD_1 - OD_2}{OD_1} \right\}$$

Where,

OD<sub>1</sub>= optical density of hypotonic buffered saline solution alone (control);

OD<sub>2</sub>= optical density of the test sample in hypotonic solution.

### ***Heat induced haemolysis***

Isotonic buffer solution containing 2mg/ml of different fractions of *S. Pinanata* were put into two duplicates of centrifuging tube [15]. Two sets of control tubes contained 5ml of sterile vehicle and 5 ml of acetyl salicylic acid (0.1mg/ml) respectively Erythrocyte suspension (30ul) was added to each

tube and mixed gently by inversion. One pair of tubes was incubated at 54°C for 20 min in a water bath. The other pair was maintained of 0-5°C in an ice bath. The reaction mixture was centrifuged for 10 min at 3000 rpm and the absorbance of the haemoglobin content supernatant was measured at 540nm. The percentage inhibition or acceleration of hemolysis was calculated according to the equation.

$$\begin{aligned} \% \text{ inhibition of haemolysis} &= 100 \times \left\{ 1 - 100 \times \frac{OD_2 - OD_1}{OD_3 - OD_1} \right\} \\ &= 100 \times \left\{ \frac{OD_3 - OD_1 - OD_2 + OD_1}{OD_3 - OD_1} \right\} \\ &= \frac{OD_3 - OD_2}{OD_3 - OD_1} \times 100 \end{aligned}$$

Where,

OD<sub>1</sub>= absorbance of test sample unheated;

OD<sub>2</sub>= absorbance of test sample heated;

OD<sub>3</sub>= absorbance of test control sample.

## **Results and discussion**

### ***Thrombolytic Activity***

Thrombosis is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system. When a blood vessel is injured, the body uses platelets (thrombocytes) and fibrin to form a blood clot to prevent blood loss. Even when a blood vessel is not injured, blood clots may form in the body under certain conditions [16]. Several thrombolytic drugs obtained from various sources are used for the treatment of thrombosis. Thrombolytic agents are used to disrupt already formed blood clots in clinical settings where ischemia may be fatal (acute myocardial infarction, pulmonary embolism, ischemic stroke, and arterial thrombosis). Thrombolytic drugs dissolve blood clots by activating plasminogen, which forms a cleaved product called plasmin. Plasmin is a proteolytic enzyme that is capable of breaking cross-links between fibrin molecules, which provide the structural integrity of blood clots. Because of these actions, thrombolytic drugs are also called plasminogen activators and fibrinolytic drugs. There are three major classes of fibrinolytic drugs: tissue plasminogen activator (tPA), streptokinase (SK), and urokinase (UK). While drugs in these three classes all have the ability to effectively dissolve blood clots. From *In vitro* thrombolytic activity study revealed that addition of 100µl streptokinase (SK) for fibrinolytic drugs as a positive control (30,000IU) to the clots and subsequent incubation for 90 minutes at 37°C showed 69.23% lysis of clot on the other hand sterile distilled water were treated as negative control which exhibited a negligible percentage of lysis of clot 3.03%, ethanolic soluble fraction (ESF) 43.28%, hexane soluble fraction (HXSf) 58.06%, chloroform extract (CSF) 27.66%, and aqueous

soluble fraction (AQSF) 22.81% showed lysis of clot respectively. In this study observed that the hexane soluble fraction of *S. Pinanata* showed highest thrombolytic activity. Hence the obtained results were compared with the control groups, where P values < 0.01, considered statistically significant (p indicates probability).

#### **Statistical analysis**

The significance between percentages of clot lysis of crude extract by means of weight difference was tested by the paired t-test analysis. Statistical representation of the effective clot lysis percentage by different fractionate of crude leave extract results were compared, positive thrombolytic control (streptokinase) and negative control (sterile water), p values < 0.01 was considered statistically significant (p indicates probability), has been shown in table and figure 1.

#### **Membrane stabilizing activity**

Inflammation is a complex biological response of vascular tissues to harmful stimuli. It is also a protective attempt by the organism to remove the injurious stimuli and initiate the healing process [17]. At the onset of an inflammation, the cells undergo activation and release inflammatory mediators. These mediators include histamine, serotonin, slow reacting substances of anaphylaxis (SRS-A), prostaglandins and some plasma enzyme systems such as the complement system, the clotting system, the fibrinolytic system and the kinin system [18]. These mediator molecules work collectively to cause increased vasodilatation and permeability of blood vessels. Thus, leading to increased blood flow, exudation of plasma proteins and fluids, and migration of leukocytes, mainly neutrophils, outside the blood vessels into the injured tissues [19]. Inflammation can be classified as either acute or chronic inflammation [20]. Acute inflammation is the initial response of the body to injurious stimuli and is achieved by increased movement of plasma and leukocytes from the blood into the injured tissues. The process of acute inflammation is initiated by cells already present in the tissues. This is characterized by marked vascular changes, including vasodilatation and increased capillary permeability which are induced by the actions of the various inflammatory mediators [21]. Chronic inflammation is a prolonged inflammatory response that leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissues from the inflammatory

process [22]. *S. Pinanata* extract of 2mg/ml at different partitionates protected the human erythrocyte membrane against lysis induced by hypotonic solution and heat. During inflammation, there are lysis of lysosomal membrane which release their component enzymes that produce a variety of disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their beneficial effects by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes [23]. Exposure of red blood cells (RBCs) to injurious substances such as hypotonic medium and heat, results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin [24].

Since human red blood cell (HRBC) membranes are similar to lysosomal membrane components, the inhibition of hypotonicity and heat induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory activity of *S. Pinanata* extract. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Injury to red cell membrane will render the cell more susceptible to secondary damage through free radical induced lipid peroxidation [25]. Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators. *S. Pinanata* extract perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation. Phytochemical results of this study showed that *S. Pinanata* is abundantly rich in flavonoids, alkaloids, steroids and terpenoids. These phytochemicals occur in leaves of the plant. Many reports have shown that plant flavonoids possess potent anti-inflammatory and anti-oxidant properties [26-28]. Their anti-inflammatory activities are probably due to their inhibitory effect on enzymes involved in the production of the chemical mediators of inflammation and metabolism of arachidonic acid [29,30]. From *In vitro* membrane stabilizing activities revealed that, at 2mg/ml, different partitionates of crude extracts of *S. Pinanata* significantly protected the haemolysis of HRBC membrane induced by hypotonic solution and heat as compared to the standard ASA. Therefore, by hypotonic solution, values of ASA (81.90±0.29%), AQSF (33.49%±0.51), ESF (58.23%±0.64), CSF (49.66±0.77) and HSF (70.35±0.50%) respectively while in heat with the values ASA (74.12±0.26), AQSF (30.34%±0.42), ESF (51.53%±0.6), CSF (46.22±0.57%), HXSF (61.11±0.69%) respectively. All data were demonstrated in the figure 2, 3 and table 2.

### Statistical analysis

Three replicates of each sample were used for each test to facilitate statistical analysis and the Data were presented as mean  $\pm$  standard deviation (SD). In this study showed that, hexane soluble fraction of *S. Pinanata* showed 70.35 $\pm$ 0.35% by hypnotic solution and in heat 61.11 $\pm$ 0.69 respectively, highest protection of haemolysis of HRBC membrane. So the observed results can be postulated that the inhibition of erythrocyte lysis property of *S. Pinanata*, indicate the possible mechanism of action of its anti-inflammatory activity.

### Conclusion

An *In vitro* thrombolytic and membrane stabilizing study, it is clear that ethanolic crude extract of different fractionates of *S. Pinanata* has significant thrombolytic and membrane stabilizing activities. It may be assumed that these extracts can be considered as good source of thrombolytic and membrane stabilizing agents. Many anti-inflammatory plants and agents modify inflammatory responses by accelerating the destruction or antagonizing the action of the mediators of inflammatory reaction. Foods and fruits rich in flavonoids and other phenolic compounds have been associated with decreased risk of developing inflammatory and other related diseases [31, 32]. These reports, suggest that the flavonoids in extract of *S. Pinanata* might be a major anti-inflammatory constituent. However, more detailed phytochemical analysis will be necessary to isolate and characterize the active compounds responsible for the thrombolytic and membrane stabilizing activities as well as to understand the exact mechanisms of action of these activities.

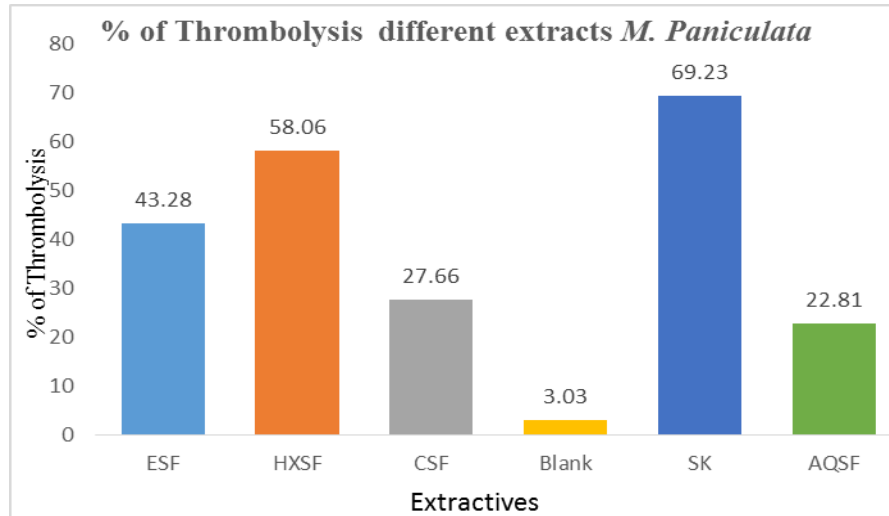
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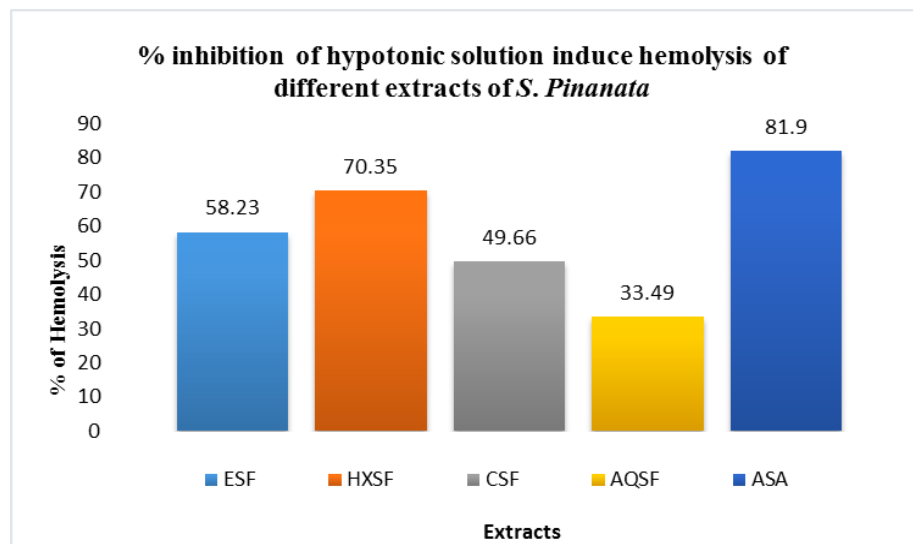
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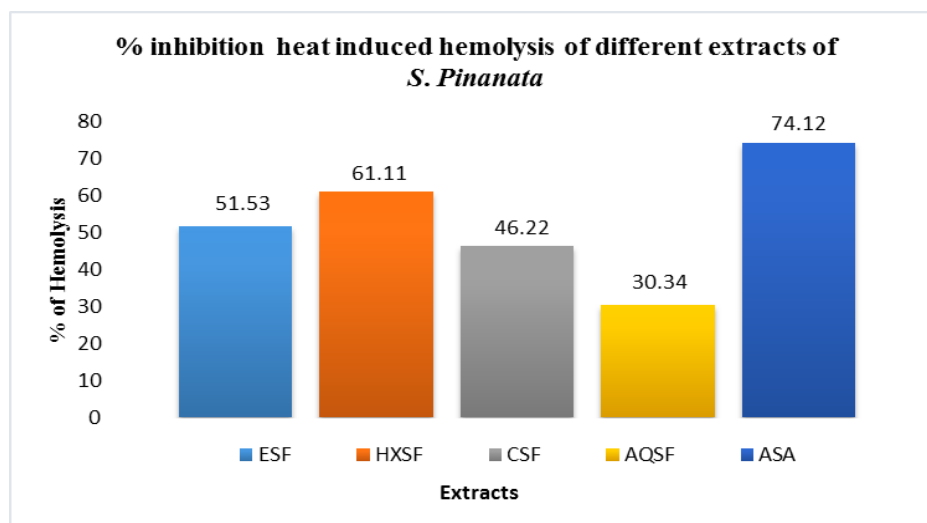
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**Figure 1.** Thrombolytic Activity (in terms of % of clot lysis) of different crude extracts of *S. Pinanata*.



**Figure 2.** Inhibition of Hypnotic solution induced hemolysis of different crude extracts of *S. Pinanata*.



**Figure 3.** Inhibition of heat induced hemolysis of different crude extracts of *S. Pinanata*.

**Table 1.**Thrombolytic Activity of different crude extract of *S. Pinanata*.

Fractions	Weight of empty vial (A) g	Weight of vial with clot (B) g	Weight of clot (B-A)g	Weight of vial with clot after lysis (D) g	Weight of clot lysis (B-D)g	% of clot lysis
ESF	5.14	5.91	0.67	5.62	0.29	43.28*
HXSF	5.29	6.22	0.93	5.68	0.54	58.06*
CSF	5.28	5.75	0.47	5.62	0.13	27.66*
Blank	5.20	5.53	0.33	5.52	0.01	3.03*
SK	4.65	5.04	0.39	4.77	0.27	69.23*
AQSF	5.47	6.04	0.57	5.91	0.13	22.81*

\*P<0.01, crude extracts are significant as compared to positive control and negative control

**Table 2.** Percentage (%) inhibition of heat and hypotonic solution induced haemolysis of erythrocyte membrane by standard and different fractions of *S. Pinanata*.

Samples	% Inhibition of haemolysis	
	Heat induced	Hypotonic solution induced
Hypotonic medium	-	-
ESF	51.53±0.61	58.23±0.64
HXSF	61.11±0.69	70.35±0.50
CSF	46.22±0.57	49.66±0.77
AQSF	30.34±0.42	33.49±0.51
ASA	74.12±0.26	81.90±0.29

Values are expressed as mean ± SD (standard deviation)

ESF=ethyl soluble fraction, HXSF=hexane soluble fraction, CSF=chloroform soluble fraction, AQSE=aqueous soluble fraction, ASA=acetyl acetic acid.