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Evaluation of *ex vivo* Thrombolytic Activity and *in vitro* Anti-inflammatory Activity of *Thespesia populnea* Leaf Extract

Vineela Satuluri*, Vidyadhara Suryadevara, Vijetha Pendyala, Narasimhareddy M

Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur-522019, Andhra Pradesh, India

ABSTRACT

Thrombotic disorders like myocardial and cerebral infarction are fatal blood clotting related diseases. Synthetic therapeutics used in such disorders has serious adverse effects, so there is a need to investigate some more safe natural thrombolytic agents. Present study is a preliminary work towards such endeavors. During this study analysis of thrombolytic activity and anti-inflammatory activity of *Thespesia populnea* leaf extract using a simple and quick *in vitro* clot lysis assay was performed. Various concentrations of leaf extract i.e. 200µg/ml; 400µg/ml and 600µg/ml were tested at various time intervals including; 24, 48 and 72 hours duration of incubation at 37°C for observing maximum clot lysis. The result findings indicated that concentrations of leaf extract enhanced the percentage of clot lysis in dose dependent manner along with the incubation time factor. However; streptokinase SK a reference standard and water were used as a positive and negative control showed clot lysis maximum 96.35% and 35.22% in 72 hours of incubation respectively. Alcohol extract of whole plant of *Thespesia populnea* (Family: Malvaceae) was assessed for its anti-inflammatory activity by *in vitro* methods. *In vitro* anti-inflammatory activity was evaluated using albumin denaturation assay at different concentrations. Diclofenac sodium was used as standard drug. The results showed that *Thespesia populnea* alcohol extract at a concentration range of 400-1600µl significantly protects from protein denaturation.

Keywords: Thrombolytic activity, *Thespesia populnea*, thrombotic disorders, streptokinase and albumin denaturation.

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***Corresponding author**: Mrs. Vineela Satuluri
Address: Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur-522019, Andhra Pradesh, India
Tel.: +91-9491896306
E-mail ✉: vineela.cology@gmail.com
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INTRODUCTION

Nature had been known as stockyard of medicinal agents since the time immemorial. Herbal products are extensively perceived as safe because they are "natural" having less or no side effects. Medicinal plants contain large number of secondary metabolites which have potential therapeutic properties that can be utilized in

the treatment of human diseases. Primary bioassay screens are most important for the initial screening of plants for bioactive principles and are often the first step in drug development. Medicinal plants have acquired significant importance in the field of biotechnology for their developing applications. Hence, in the recent years the researchers are focusing on formulation of ayurvedic herbal medicines on the basis of their traditional uses and its known effectiveness in the treatment of various ailments. [1]

Thrombosis is the formation of a potentially deadly blood clot inside a blood vessel artery (arterial thrombosis) or vein (venous thrombosis), obstructing the flow of blood through the circulatory system. Once formed, a clot can slow or block normal blood flow, and even break loose and travel to an organ. This can result in significant injury including heart attack, stroke and venous thromboembolism, the top three cardiovascular killers. Thrombolytic drugs are used to dissolve blood clots in a procedure termed thrombolysis. Antithrombotic drugs are used to dissolve blood clots and are mainly of three type's antiplatelet agents, fibrinolytic drugs and anticoagulants. Depending upon the thrombus formed, effective antithrombotic therapy can be instituted, i.e., arterial thrombosis is treated with antiplatelet agents and venous thrombosis can be treated with anticoagulants mainly but they do not cause clot lysis that has already been formed. They prevent thrombus extension, recurrence and embolic complications. For the lysis of already formed thrombus, fibrinolytic drugs are used (Streptokinase, Urokinase, Alteplase, Reteplase, Tenectaplase, etc). But due to various side effects such as bleeding complications, hemorrhagic stroke allergy and rarely anaphylaxis, researchers are bound to discover newer drugs. Traditional systems of medicine are popular in developing countries and up to 80% of the population relies on traditional medicines or folk remedies for their primary health care need. [2]

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are damaged by microbes, physical agents or chemical agents, the injury is in the form stress. Inflammation of tissue is due to response to stress. It is a defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Loss of function occurs depends on the site and extent of injury. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion. When tissue cells become injured they release kinins, prostroglandins and histamine. These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the injured site. These substances also act as chemical messengers that attract some of the body's natural defense cells a mechanism known as chemotaxis. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, 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 tissue from the inflammatory process. Several experimental protocols of inflammation are used for evaluating the potency of drugs. The management of inflammation related diseases is a real issue in the rural community; the population in these areas uses many alternative drugs such as substances produced from medicinal plants. [3]

The aim of present study was to screen extracts of *Thespesia populinea* (TP) for its clot lysis property (thrombolytic activity) by using ex vivo and anti-inflammatory activity by an *in vitro* procedure. Plant derived drugs remain an important resource, especially in developing countries, to combat serious diseases. Approximately 60–80% of the world's population still relies on traditional medicines for the treatment of common illnesses. Medicinal plants have a long-standing history in many locations in India and continue to provide useful and applicable tools for treating ailments. With the advancement in phytochemistry and identification of new plants compounds having significant efficacy against certain diseases, it has been proved by research conducted on herbal medicines. TP (Malvaceae) is a large tree found in tropical regions and coastal forests of India. The bark, leaves, and flowers are useful in cutaneous infections such as scabies, psoriasis, eczema, ringworm, guinea worm and the leaves of this plant used as anti-inflammatory for poultice as a folk medicine. It has been reported that leaf extracts of TP have medicinal properties e.g. antinociceptive, antibacterial [4], anti-diabetic [5-7], anti-steroidogenic activity [8], anti hepatotoxic and antioxidant. [9]

MATERIALS AND METHODS

Collection of Plant Material

The leaves of TP were collected from the medicinal garden of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur and were authenticated by Dr. M. Ammani, Professor, Nagarjuna University, India.

Preparation of Standard

Commercially available lyophilized Streptokinase vial (Cadila pharmaceutical Ltd.) of 15, 00,000 I.U. was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U) was used for *Ex vivo* thrombolysis. [10]

Method of Extraction

30 grams of dried leaf powder of TP plant was weighed and taken in an iodine flask and macerated with sufficient amount of n-hexane for about 12-24 hours. Then the powder is separated and dried. To this powder alcohol and water was added in 9:1 ratio and kept a side for about 72 hours. Then after 72 hours the powder was separated and the filtrate was collected and evaporated until dried extract was formed. The yield obtained was 4.68 grams and stored in cool and dry place.

Phytochemical constituents

To the collected leaf extract the chemical analysis was performed to evaluate the phytochemicals present in it. The following chemical tests were performed.

Carbohydrates: To 1 ml of leaf extract, barfoed's reagent is added and boiled. A red copper oxide precipitate is formed.

Proteins: To 1 ml of leaf extract, 2 ml of sodium hydroxide solution is added. Add 4-5 drops of 1% $CuSO_4$ solution and warm the mixture for 5 minutes. Bluish violet colour indicates the presence of proteins.

Aminoacids: To 1 ml of leaf extract add 3-4 drops of ninhydrin solution and boil. Blue colour indicates the presence of amino acids.

Flavanoids: 1 ml extract was added to concentric sulfuric acid. Pink coloured or red coloured disappears on standing.

Tannins: 1 ml extract was added in 2 ml of water in test tube, 2-3 drops of dil. Ferric chloride solution was added and observed for green to blue green or blue black or dark green.

Saponins: To 1 ml of aqueous extract was added few volume of distilled water in a test tube. The solution was shaken vigourously and observed for stable persistant froth for 20 minutes.

Alkaloids: Evaporate 10 ml of concentric ethane solution, the dry residue was added to 1.5 ml Hcl acid. After that 1-2 drops mayers reagent and wagner was added and yellow-white precipitate indicates alkaloids.

Coumarins: Evaporate 5 ml of ethanolic solution, dissolve residue in 1-2 ml distilled water and divide the volume into 2 parts. Take half volume as witness to another volume 0.5 ml 10% NH_4OH. Put 2 spots on filter paper and examine under UV intense fluorescence indicates presence of coumarins.

Sterols and Steroids: 10 ml of ethanolic extract evaporated, residue was dissolved in 0.5 ml of hot acetic anhydride and treated with reagent of libermann burchardt. The appearance of interphase, a ring of blue green showed a positive reaction.

Glycosides: To 1 ml of leaf extract add 2-3 drops of sulphuric acid, and add 5% NaOH solution for neutralisation. Add Fehling's A and B solutions. Red colour is produced.

Thrombolytic Method

Goat blood was collected from the Slaughter house, Guntur and immediately citrated using 3.1% sodium citrate solution [11] and transferred in different pre-weighed sterile clotting tubes and incubated at 37°C for 45 minutes for clotting to occur. Clot lysis activity was determined as described in previous studies. [12] After clot formation, serum was completely removed (aspirated out without disturbing the clot formed). Each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone). Each clotting tube containing clot was properly labelled and 200µg/ml, 400µg/ml, 600µg/ml of plant extract was added to the tubes. As a positive control, 100µl of SK and as a negative non thrombolytic control, 100µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 72 hours and observed for clot lysis. After incubation, fluid obtained 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.

Percentage of clot lysis (%) = (weight of clot before lysis – weight of clot after lysis) × 100

Albumin Denaturation Method

The anti-inflammatory activity of TP was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima *et al.*, [13] and Sakat *et al.*, [14] followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min, after cooling the samples the turbidity was measured at 660 nm. (UV Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate.

The Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition = (Abs Control –Abs Sample) × 100/ Abs control

RESULTS AND DISCUSSION

The present study was undertaken to evaluate the thrombolytic and anti-inflammatory activity of alcoholic extract of TP leaves. Then the phytochemical constituents present in the alcoholic extract were observed by performing chemical tests. The results of preliminary phytochemical screening were given in Table 1.

The thrombolytic activity was checked in TP leaf extract by clot lysis method. The results of thrombolytic activity were given in Table 2. In our results it was found that the concentrations of leaf extract enhanced in the clot lysis in a dose dependent manner along with

the incubation time factor. The results indicated a maximum of 82.21% clot lysis at 600µg/ml concentration in 72 hrs of incubation as mentioned in Figure 1. The results indicated clearly that concentrations of leaf extract enhanced the percentage of clot lysis. However; streptokinase SK a reference standard and water were used as a positive and negative control that showed clot lysis maximum 96.35% and 35.22% in 72 hours of incubation respectively.

Table 1: Results of preliminary phytochemical screening

Chemical tests	Alcoholic extract
Carbohydrates	Present
Protiens	Absent
Amino acids	Absent
Flavonoids	Present
Tannins	Absent
Saponins	Present
Alkaloids	Absent
Phenols	Present
Sterols	Present
Glycosides	Present

Table 2: Percentage of clot lysis

S. No	Ingredients	Concentration (µg/ml)	%Clot Lysis
1	Leaf extract	200	58.56 ± 0.540***
2	Leaf extract	400	76.34 ± 1.749***
3	Leaf extract	600	82.21 ± 0.764***
4	Streptokinase	100µl	96.35 ± 0.304***
5	Water	100µl	35.22 ± 0.934

Table 3: Percentage of albumin denaturation

S. No	Ingredients	Concentration (µl)	Optical Density	Percent of inhibition
1.	1ml albumin + leaf extract	400	0.1	47.36 ± 0.00134***
2.	1ml albumin + leaf extract	800	0.08	57.89 ± 0.00123***
3.	1ml albumin + leaf extract	1600	0.05	73.68 ± 0.00142***
4.	1ml albumin + Diclofenac sodium	800	0.19	97.23 ± 0.00177***
5.	1ml albumin + Water	800	0.03	0

Data presented as Mean ± S.D (n=3), were analyzed by paired t-test analysis and statistically Significant ***P<0.001 when compared to control group.

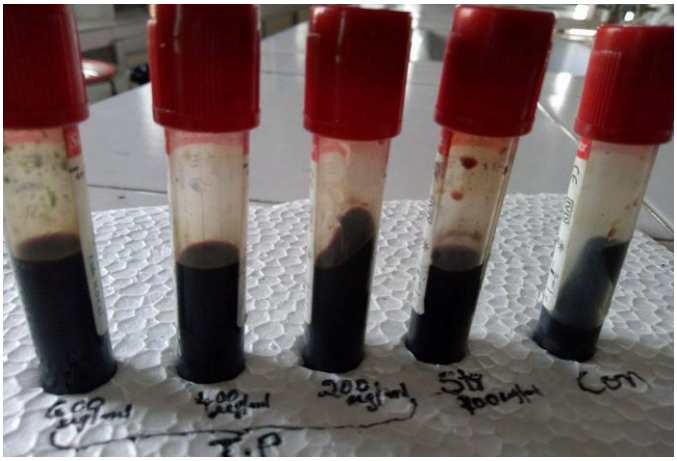

Fig. 1: Photograph of clotting tubes indicating clot lysis in streptokinase and different concentrations of extracts of TP

Some studies indicate that thrombolytic activity probably due to the diverse composition of plant extracts like phytoconstituents including rich sources of alkaloids, flavonoids, tannins and terpenoids. Apart from natural plant product, there is also a report that marine algae having a product called Seanol (phlorotannin – active compound), possessing the ability in promotion of dissolution of intravascular blood clot via antiplasmin inhibition. In this study, a simple model was used for figuring out the potential of our TP extract by the above mentioned method' nonetheless, there are a number of other sophisticated and modern technology available for finding out the mechanism of clot lysis by applying any agents. Among some are widely practiced like, radiolabelling of factors that are actively involved in clot lysis mechanism, Magnetic resonance imagaing technique (MRI), a latest technique and ultrasound frequency. [15] This effect may be possibly due to phytoconstituents present in the plant extracts affecting activation of plasminogen both by fibrin-dependent and fibrin-independent mechanisms similar to Streptokinase which causes extra production of plasmin which breaks down fibrin the major constituent of thrombi, to dissolve unwanted blood clots.

The anti- inflammatory activity was checked in TP leaf extract by albumin denaturation method. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. [16] Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. Anti-inflammatory activity was measured by measuring the absorbance of treatment groups and converting it into total inhibition of protein denaturation. The results of thrombolytic activity were given in Table 3. In the present study for in-vitro anti-inflammatory test, the 1600µl of ethanol extract of TP showed 73.68 as mean inhibition of protein denaturation value and whereas for Diclofenac Sodium, it was found to be 97.23 ± 0.00177. By performing the albumin denaturation method it was found that the leaf extract of TP contains anti-inflammatory activity.

In conclusion, it can be claimed that TP possesses significant anti-inflammatory activity as well as thrombolytic activity. In addition, positive result in thrombolytic activity test led us to the interference that the plant extract may contain bioactive compounds, which may aid ongoing cardiovascular drug discovery from the floristic resources. Hence, further studies are suggested to be undertaken to pin point the exact

compounds and to better, understand its actions scientifically.

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