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Research Article

EVALUATION OF *IN-VITRO* THROMBOLYTIC ACTIVITY USING *BOUGAINVILLEA SPECTABILIS* AQUEOUS LEAF EXTRACT UNDER DIFFERENT CONCENTRATIONS

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ABSTRACT

Aim: The current investigation was executed to study the *in-vitro* thrombolysis of *Bougainvillea spectabilis* leaves using aqueous extract under different concentrations and time intervals to compare the efficacy of herbal products versus synthetic drugs used for treatment of thrombotic disorders. The long term usage of medications leads to serious adverse effects on the body. Materials & Methods: Streptokinase (S.K) and distilled water were used as positive and negative controls respectively. Fresh leaves were collected, dried and used to make crude extract by aqueous preparation and boiling method. Two extracts were used for the study; fresh and dried leaves under two concentrations each; 0.2ml and 1ml. Both were incubated at time intervals of 24, 48 and 72 hours at 37ºC. Results: In the present study the following results were obtained using fresh leaf extract under 0.2ml concentration at 24, 48 and 72hrs showing clot lysis of 6%, 16.42% and 27% respectively. While under 1ml, it displayed a lysis of 24.07%, 52.46% and 81% accordingly. Using dried leaf extract under 0.2ml, at same intervals, clot lysis of 4%, 12% and 21% was observed. While under 1ml, displayed lysis of 17%, 32.32% and 70%. Discussion: Based on the results it was found that the maximum clot lysis was obtained from 72 hours of incubation at 1ml concentration showing 81% lysis for fresh leaves in comparison to dry leaves with 70% lysis. Conclusion: It can be concluded that the concentration of leaf extracts enhanced the percentage of clot lysis in a dose dependent manner along with incubation time factor.

Keywords: Thrombolysis, *Bougainvillea spectabilis*, Streptokinase, Haematology, Physiology and Phytopharmacology.

INTRODUCTION

A blood clot or a "thrombus" can develop in the blood circulatory system because of homeostasis, which constructs vascular blockage that can be responsible for health threatening results. Atherothrombotic diseases, caused due to arteriosclerosis like myocardial infractions can even lead to death[1]. Blood clots usually arise from interaction of various mechanisms that include the activation of the coagulation factors, fibrinolytic systems, disruption of the vascular endothelium and the generalized activation of the cellular mechanisms resulting in clotting on the surface of the monocytes and platelets in circulation[2]. Plasmin, Plasminogen, plasminogen activator and fibrin are involved in the interaction process[3]. The thrombolytic activity of plasma is physically very important[4]. Prothrombin time (PT) is a measure of the extrinsic coagulation pathway and PT measures the length of time it takes for clotting to occur when certain substances are added to the liquid portion of blood in a test tube[5-7]. Usually used thrombolytic agents are Anistreplase, Streptokinase, Alteplase, tPA (Tissue Plasminogen Activator), uPA (Urokinase Plasminogen Activator), Heparin and Warfarin are medications that are used as anticoagulants. All available thrombolytic agents have shortcomings, including the need for large dosages to be maximally effective, limited fibrin specificity and bleeding tendency. Thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and myocardial infractions (heart attack) are the main causes of morbidity and mortality in developed countries. Aspirin and Heparin are significantly effective for activation of lysis and prevention of reocclusion. Yet, this drug can produce hemorrhagic events and upper gastrointestinal bleeding as major setbacks[8]. Day by day the concept and methods of the uses of natural products in treating mankind have undergone remarkable changes[9, 10]. The uses of herbs for treatment of diseases has been in practice since ancient times. Due to the natural activity of herbal products they are considered safer[11]. Extracts of natural products provide a useful source of bioactive compounds with the advancement of phytochemistry and identification of new plants which can be used to develop as drugs directly or provide novel structure templates that shows significant efficacy against diseases and used in traditional medicines. Primary bioassay screens are important for the initial screening of plants for bioactive chemical constituents and are often the first step in drug development. In herbal medicament there is great relevance to the Nyctaginaceae family of plants. Important species among the genus *Bougainvillea* are three, which are; *B. peruvinana, B. glabra* and *B. spectabilis* from a horticulture perspective. It is proclaimed that leaf extracts of *Bougainvillea spectabilis* contains several medicinal properties e.g., anti-viral, anti-inflammatory, antibacterial, anti-diabetic, anti-fertility, amylase inhibitory, anti-hyperlipidemic, radical scavenging, anti-atherogenic, thrombolytic, analgesic, antipyretic, antihelmintic, antiulcer, regulation of menstruation-vaginal discharge and larvicidal principles. There is favourable biological activity in momentous effective constituents of D-pinitol which can be isolated from the leaves of *B. spectabilis*. Crude extracts of this plant have been a folk remedy in Northern Nigeria for ages even today to cure multiple diseases. The main objective of the proposed research

was a preliminary study to analyze and evaluate the thrombolytic activity of *Bougainvillea spectabilis* leaf extracts which is also a famous ornamental plant. It belongs to a genus of thorny ornamental vines, bushes and trees with flower-like spring leaves and about eighteen species are known. The actual flower of the plant is small and generally white and bright colours are associated with the plant like pink, magenta, purple, red, orange, white or yellow are leaves.

MATERIALS AND METHODS

Collection Of Bougainvillea Leaves

Fresh green leaves of Bougainvillea were collected in sufficient quantity from the JBAS College sports ground Teynampet, Chennai, during the month of January. These were neatly cleaned, sealed and taken for authentication of the species (*B.spectabilis*) to the Plant Biology and Biotechnology department of the J.B.A.S. College. Later a portion of these fresh leaves were kept for shade dry, for a week's time. Another set of fresh leaves were collected for the purpose of, on spot sample preparation.

Collection Of Blood Samples

For the sample, whole blood (3ml) was drawn from healthy volunteers (n=5) by phlebotomist (intravenous puncture) from persons without a history of using oral contraceptives. 0.5ml of blood was transferred to each of the five previously weighed micro centrifuge tubes or Eppendorf tubes to form clots[12]. This was further used to analyze clot formation and clot lysis.

Preparation Of The Leaf Extracts

For this a portion of leaves which had been kept for shade drying up to a week's time were retrieved and from it 500gms of dried leaves were weighed and added to 100ml of distilled water. The aqueous extract was prepared in 5% concentration by the boiling method of tea in water bath by constant agitation of the mixture for 15 minutes. After the extract preparation, the coarse suspended particles of leaves were cautiously removed by two methods, which was passing first through a strainer and then through a filter paper of 0.22µm size. In case of the fresh leaves sample, they were collected and immediately the extract was obtained by performing the similar technique of boiling method of tea[13]. This was also filtered like the previous mixture and the extract obtained from both the dried and fresh leaves were stored in small sterile vials in a refrigerator for later use.

Preparation Of The Solution

Commercially available lyophilized STPase (Streptokinase/ S.K) vial of 15,00,000 I.U., was bought from Smith Stocking & Co-NC Bose Road; Cadila Pharmacy, Chennai. Then 5ml of sterile distilled water was added to the vial and mixed well to form a completely accommodated solution. This solution served as stock from which 0.1ml (30,000 I.U) was used for *in vitro* thrombolysis during the procedures[14].

Bioassay; Procedure For Clot Lysis Of Crude Leaf Extracts

After preparation of the crude leaf extracts and S.K. solution, 0.5ml of each blood sample were distributed in six different tubes at a time, meant for different concentrations, positive control (S.K) and negative control (distilled water). These samples were left to incubate at 37ºC for 90 minutes for clot formation. After the clot formation had occurred, the serum was finely and completely aspirated without disturbing the clot and the tubes were weighed again to check the clot weight for analysis[15].

Clot weight = Weight of the tube containing the clot – Weight of the empty tube

Each Eppendorf tube containing the clot was properly labelled and 0.1ml of plant extracts were added to the tubes with the help of a micro pipette, after clot formation of 90 minutes. While preparing the extracts, two different concentrations (0.2ml and 1ml) of the two types of leaves (fresh and dry) were made. 0.2ml of fresh and dry leaves were prepared by pipetting out 0.2ml using a micro pipette, of the original extract prepared and was made to 100ml by dissolving it in distilled water. Similarly 1ml of fresh and dry leaves were also prepared. This was done to observe how far maximum clot lysis occurred at different time durations at maintained temperature with a low and a high concentration of the extracts. Also, as a positive control, 0.1ml of S.K was added to one of the labelled tubes and as a negative control, 0.1ml of distilled water was added separately to a previously labelled tube by using a 100µl micro pipette. All these tubes were then incubated at 37ºC for three different time intervals (24 hours, 48 hours and 72 hours). Each day the clot lysis was recorded by carefully aspirating the serum formed from the lytic activity of the controls and the extracts and then weighing the current weights of the tubes in a weighing machine. This will eventually give us the clot disruption rate from the total clot weight of each individual, which was noted as percentage of the clot lysis.

Weight of released clot = Weight of clot before extract addition – Weight of clot after extract addition.

Percentage (%) of clot lysis = [Weight of released clot / Weight of clot] × 100

RESULTS AND DISCUSSION

In the current study, result findings suggest that; the positive control, Streptokinase showed a clot lysis activity of 61.43%, by the first dosage that is, at the end of 24 hours and a clot lysis of 75.55% (76%) by the next dosage that is, at the end of 48 hours and a clot lysis of 90.97% (91%) by the end of the final dosage of 72 hours of incubation on an average as presented in table 1. This investigation proved to us that, Streptokinase as a synthetic drug had a clot lysis efficacy of almost 100%, within three days of dose dependent usage. While the clots were treated with the negative control, distilled water, it showed a clot lysis activity of 06.77% (7%) for 24 hours, 24.44% of clot lysis for 48 hours and 41.54% (42%) of clot lysis for 72 hours of incubation on an average as presented in table 1.

In this study, investigation of thrombolytic activity of *Bougainvillea spectabilis* leaf extracts were carried out using a simple and rapid *in-vitro* clot lysis model with extracts prepared from two types of leaves; fresh and dry each under two different concentrations 0.2ml and 1ml. It was noticed that the concentrations of the leaf extracts enhanced in a dose dependent manner along with incubation time factor.

In the case the fresh leaves extract, using 0.2ml concentration after 24 hours of incubation showed a clot lysis of 05.64% (6%) as depicted in table 1. At 48 hours of incubation there was a clot lysis of 16.42% and at 72 hours of incubation it showed lysis of 26.57% (27%) on an average. This analysis percentage depicted a gradual increase throughout the procedure and showed a significance of ≤ 0.05 when correlated against streptokinase (for 0.2ml) by a strong negative correlation of -0.561 for 24 hours and -0.049 for 72 hours as depicted in tables 2 and 3.

In the case the fresh leaves extract, using 1ml concentration after 24 hours of incubation showed a clot lysis of 21.07% as shown in

table 1. At 48 hours of incubation there was a clot lysis 52.46% and at 72 hours of incubation it showed a lysis of 80.78% (81%) on an average. The percentage lysis was a steep rise on comparison with the correlation of 1ml of the fresh leaf extract and the streptokinase was 0.039 and was significant at ≤ 0.05 for 24 hours and was -0.037 at 72 hours and significant as depicted in tables 2 and 3.

Table 1. Percentage of clot lysis of in-vitro thrombolytic activity using positive control, negative control & crude extract of *Bougainvillea spectabilis* under incubation of 24, 48 & 72 hours.

S.No:	Content	Incubation Time	Clot Lysis
1.	Positive Control Standard (S.K)	24 hours	61.46%
		48 hours	75.55%
		72 hours	90.97%
2.	Negative Control (Distilled water)	24 hours	06.77%
		48 hours	24.44%
		72 hours	41.54%
3.	Fresh Leaves:0.2ml	24 hours	05.64%
		48 hours	16.42%
		72 hours	26.57%
4.	Fresh Leaves: 1ml	24 hours	21.07%
		48 hours	52.46%
		72 hours	80.78%
5.	Dried Leaves:0.2ml	24 hours	03.81%
		48 hours	11.74%
		72 hours	20.98%
6	Dried Leaves:1ml	24 hours	16.72%
		48 hours	32.32%
		72 hours	69.62%

Table 2. Thrombolytic activity of different samples by time factor in terms of Mean ± Standard Deviation (n=5).

SAMPLES	MEAN ± S.D		
	24 hrs	48 hrs	72 hrs
S.K	61.46 ± 6.83	75.55± 4.33	90.97 ± 5.01
Fresh Leaves (0.2ml)	5.64 ± 1.91	16.42± 1.19	26.57 ± 3.83
Dried Leaves (0.2ml)	3.81 ± 1.52	11.74± 2.44	20.98 ± 2.52
Fresh Leaves (1ml)	21.07 ± 1.14	52.46± 2.85	80.78 ± 2.81
Dried Leaves (1ml)	16.72 ± 0.92	32.32± 3.93	69.62 ± 5.49

Table 3. Correlation calculated with the different samples and controls with respect to the highest and lowest concentrations.

SAMPLES	CORRELATION	
	24 hrs	72 hrs
S.K & Fresh Leaves (0.2ml)	-0.561	-0.049
S.K & Dried Leaves (0.2ml)	-0.667	-0.077
S.K & Fresh Leaves (1ml)	0.039	-0.037
S.K & Dried Leaves (1ml)	0.634	-0.317
Fresh & Dried Leaves (0.2ml)	0.028	0.043
Fresh & Dried Leaves (1ml)	0.025	0.065

Table 4. *t* – Test values that were derived from the different samples of different time intervals.

SAMPLE	*p* – VALUE OBTAINED	
	24 hrs	72 hrs
S.K & Fresh Leaves (0.2ml)	0.00029	0.00185
S.K & Dried Leaves (0.2ml)	0.00026	0.00124
S.K & Fresh Leaves (1ml)	0.00067	0.00156
S.K & Dried Leaves (1ml)	0.00052	0.00046
Fresh & Dried Leaves (0.2ml)	0.01581	0.01472
Fresh & Dried Leaves (1ml)	0.00045	0.00702

In case of thrombolysis observed in dried leaves extract, using 0.2ml concentration, it exhibited a clot lysis of 03.81% (4%) after 24 hours of incubation as presented in table 1. At 48 hours of incubation there was a clot lysis of 11.74% (12%) and at 72 hours of incubation a lysis of 20.98% (21%) was seen on an average. This lysis percentage depicted a gradual increase throughout the procedure and showed a significance of ≤ 0.05 when correlated against streptokinase (for 0.2ml) by a strong negative correlation of -0.667 for 24 hours and -0.077 for 72 hours showing an inverse correlation of the two as presented in tables 2 and 3.

While in the case of 1ml concentration of dried leaves extract, they exhibited a clot lysis of 16.72% (17%) at 24 hours of incubation with respect to table 1, which increased to 32.32% at 48 hours of incubation and further a lysis of 69.62% (70%) was seen at the 72nd hour of incubation on an average. The percentage lysis was a normal rise on comparison with the correlation of 1ml of the dried leaf extract and the streptokinase was a strong positive correlation of 0.634 and was significant for 24 hours and had a strong negative correlation of -0.317 and was also noted to be significant as shown in tables 2 and 3. This proved to be just as good as the fresh leaves extracts but was lower to the total lysis percentage. Although the amount of clot lysis was lower in

comparison to the fresh leaves, for slower therapy using natural compounds without emergency, it is thus proved to be quite efficient enough.

Due to Atherothrombotic diseases there is development of occlusion and in some critical conditions people die to embolism[16]. A number of researches conducted earlier indicated that herbs and natural plant products possessed thrombolytic activity and thus preferred over synthetic drugs[17]. Modern day research has unraveled more investigation in phytopharmacology and created a new field in discovery of plant derivative drugs. It is also assessed that 30% of today's pharmaceutical products are derived from plant derivatives, India being in the lead[18].

S.K. was the positive control and distilled water was the negative control used and it was clear in comparison that clot disruption did not occur efficiently with water than that of S.K. An incubation time factor was kept to analyze the clot dissolution activities as well at three intervals (24, 48 and 72 hours). Results indicated a significant clot lysis even under 0.2ml of concentration. Yet, the percentage of clot lysis was recorded to be maximum in case of the higher concentration with maximum time duration.

Maximum percentage of lysis was observed with S.K. followed by fresh leaves extract under 1ml concentration with equally good lysis and lastly by dried leaves extract under 1ml concentration. 0.2ml concentration of both extracts showed almost negligible lysis. When tested for the correlation of the extracts with the drug, 1ml concentration of fresh and dried leaves showed a strong positive correlation for 24 hours and a strong negative correlation with 72 hours. While both the extracts had a strong negative correlation at both time intervals with the 0.2ml concentrations. Equally there was a significant difference visible with fresh leaves when compared with the dried leaves, which can be explained due to richness of the antithrombotic enzymes that were active in fresh leaves, while these were dried out and all most inactive in case of the dried leaves which led to the slower rate of clot lysis in them.

The significance was also noted using the *t*-test, and the *p*-values that were greatly significant for both 24 hours and 72 hours. With least probability of error on repetition with 72 hours as presented in table 4. This is a breakthrough and proves the objective that with maximum concentration of the crude extract, and longer time duration for rest, there is an enhanced clot lysis observed.

CONCLUSION

In conclusion from the recorded data, it can be demonstrated that the findings may have significant implications in cardiovascular health. In addition, this finding may imply the possibility of developing novel thrombolytic compounds from *Bougainvillea spectabilis* extracts. Further studies are ongoing to isolate and characterize the compounds responsible for thrombolytic activity. Also, studies need to be conducted to isolate the bioactive components or secondary metabolites and study thoroughly for more precise and accurate findings on a variety of these metabolites and their activities. This *in-vitro* study made is only a primary investigation but it creates opportunity to invent natural alternatives to synthetic drugs.

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