RESEARCH ARTICLE

In-vitro Evaluation of Antithrombin and Thrombolytic Activity of Leaves Extract of *Lantana camara Linn*

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ABSTRACT

Thrombosis is possible outcomes of excessive coagulation or inhibition of anticoagulant processes. The pathophysiology of thrombosis is primarily caused by irregularities of the vascular wall, changes in blood flow, and changes in blood composition. Now a day's, various natural antithrombotic or thrombolytic medications are used for the treatment. This study evaluated the pharmacognostical, antithrombin and thrombolytic activity of leaves extract of plant *Lantana camara*. The extractive value of leaves extract of plant *L. camara* was higher (20.58%) in methylene chloride as solvent. Total ash of plant *L. camara* was about 9.00% w/w. In comparison with heparin *L. camara* extract shown 50.10, 60.23, 62.32, 68.48 and 71.32% antithrombin activity at 10, 20, 40, 60, 80 and 100 mg/mL concentration, respectively. When different extract concentrations were added in the tubes containing blood clots, *L. camara* showed a good thrombolytic activity. We conclude that the leaves extract of plant *L. camara* shows significant antithrombin and thrombolytic activity.

Keywords: Thrombosis, Leaves, *Lantana camara,* Antithrombin activity, Thrombolytic activity.

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INTRODUCTION

For heart attacks, thrombolytic therapy is most effective. Patients with cerebral venous sinus thrombosis must be managed with the help of thrombolytic medications.[1] Only three medications, streptokinase, urokinase, and tissue-type plasminogen activator, have received American approval for usage. The use of urokinase is not currently permitted in the United States. On the basis of its shown efficacy with a relatively brief infusion (2 hours), the latter drug has received the most attention.[2]

Tenecteplase and reteplase are more recent, unapproved medications. With careful evaluation of contraindications, risk stratification in acute pulmonary embolism is helpful in identifying which patients are the best candidates for thrombolysis. All currently available thrombolytic drugs have some drawbacks, such as the necessity for high doses to be most effective, a restricted ability to target specific types of fibrin, severe allergic reactions, and a propensity to cause bleeding. To address these shortcomings, efforts are being made to create enhanced recombinant versions of these medications. Nattokinase was explored as an oral thrombosis preventative since it was observed to increase plasma fibrinolytic activity.[3]

The majority of underdeveloped nations and health insurance providers do not reimburse stroke sufferers for the high cost of thrombolytic therapy. In underdeveloped nations, the main obstacles to thrombolytic therapy are prehospital delay, budgetary limitations, and a lack of infrastructure. Instead, developing nations ought to concentrate on primary and secondary stroke preventive measures until a suitable therapeutic infrastructure and a less expensive source for thrombolytic agents are accessible. However, developing country governments and health systems should exert efforts to promote their infrastructure for stroke care.[4]

It is obvious that chemicals produced from plants play a significant role. While 75% of non-therapeutic plants display bioactivity, about 79% of medicinal plants exhibit some cytotoxicity. In large doses, bioactive substances are virtually always harmful. On the other hand, herbal remedies can provide a different and superior option for treating a variety of problems if they are taken in the right amounts.[5] Despite many plant extracts being poisonous, lethality assay methods have been developed and successfully applied to biomonitor the cytotoxicity of plant materials. Thus, a practical observation for screening and fractionation for new bioactive natural moiety

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can be the *in-vivo* lethality of an extract against brine shrimp nauplii (*Artemia salina*) or any cell line. Due to their widespread plant distribution and biofunctional health-promoting qualities, phenolic compounds are of great scientific interest. Fruits are utilized as food additives to reduce lipid oxidation and are possible sources of natural phenolic antioxidants.[6]

The antioxidant capacity and phenolic profile of many fruits have been studied. Different phenolic rings make up polyphenolic chemicals, and flavonoids are one of the main subgroups of these secondary metabolites. They are widely distributed in nature and make up a sizeable portion of the human diet. Natural drug use is once again the latest topic due to some synthetic medications' declining efficacy and rising contraindications to their use. Therefore, research on phytotherapy for treating chronic diseases may provide high returns on sources of medicinal plants that are useful in preventing disease and are promoted and used in accordance with all current prevention techniques. Strong antioxidants, flavonoids have recently sparked a lot of interest due to their ability to improve health and prevent disease. The molecular makeup of flavonoids determines how effective they are as antioxidants.[7]

A lot of work has gone into finding and developing natural compounds with antiplatelet, anticoagulant, antithrombotic, and thrombolytic activities from various plant and animal sources. Epidemiologic research has shown that foods with an antithrombotic effect that has been experimentally proven can lower the risk of thrombosis. Herbs with thrombolytic activity have been researched, and some important findings have also been reported. In order to find an isolated molecule that can improve thrombosis conditions while being efficacious, safe, affordable, and nontoxic, more research is necessary.[8] In this work, we studied the pharmacognostic, antithrombin and thrombolytic activity of plant *Lantana camara*. According to our results, leaves extract of the plant *L. camara* showed significant antithrombin and thrombolytic activity.

MATERIAL AND METHODS

Materials

Antithrombin activity was carried out on human blood plasma obtained from Blood Bank, AVBRH, Sawangi (Meghe), Wardha and rabbit blood plasma. Chromogenic thrombin substrate received from Biophen, tris-HCl buffer, heparin received from Samarth life sciences and all other chemicals were of analytical grade and used as provided.

Method

Collection of plant and authentication

The leaves of *L. camara* were collected from the local area of Wardha, Maharashtra, India. Dr. Lalchand P. Dallal authenticated the plant at the botany department, Shiksha Mandal, Bajaj College of Science, Wardha (11/Botany/2021-22).

Preparation of plant material

The primary component of plant matter was once divided into good and bad stuff. Only premium components were used. The chosen materials were then cleaned of trash and other unwanted debris. Sand and other foreign debris were sieved out and then sized by hand to achieve this.

Collected leaves were washed thoroughly in tap water, followed by purified water 3-4 times. They were then dried in the shade, reducing the moisture content to roughly 10%. After that, the dried leaves were crushed into smaller particles using a mechanical grinder and sieved by sieve no. 36 (420 micron).[9] The plant material was then used for further studies.

Extraction and Fractionation of Crude Extracts

The powdered plant material underwent serial solvent extraction using hexane, ether, methylene chloride, acetone, butanol, ethanol, methanol, and water, moving from non-polar to polar solvents (Figure 1).

A soxhlet was filled with roughly 100 g of weighted plant material. One solvent at a time was poured through the glass wool from non-polar to polar until the solvent in the flask's spherical bottom was level with the heating mantle's side (600–700 mL). The sample was then given six hours to reflux in each solvent. The fractionated extracts were cooled and filtered into weighed flasks. Fractionated extracts were concentrated and then dried using a Rota evaporator.[10]

The fractionated extracts were numbered and evaluated their physiochemical and phytochemical parameters for further study.

Physiochemical Evaluation

Physicochemical parameters of leaves of plant *L. camara* such as ash, extractive value, loss on drying and fiber content etc were studied.

Preliminary Phytochemical Screening of the Extracts

Extracts from *L. camara* leaves were examined for the initial phytochemical investigation. The *L. camara* leaf extracts were tested for the presence of several phytoconstituents using the following phytochemical assays: alkaloids, glycosides, tannins, flavonoids, amino acids, carbohydrates, and triterpenoids etc.[11]

In-vitro Animal Studies

Inclusion criteria

Healthy rabbits of weigh 2–3 kg

Figure 1: Extraction Assembly

Blood Withdrawal

Healthy Rabbits in the 2–3 kg range were used to collect blood samples. Blood was drawn from the auricular marginal veins from both ears, using a 22G to 25G needle.

The skin was washed with alcohol before beginning the sampling, and the hair on the ear was removed. Due to the delicate nature of the skin on the ear, a cream containing lidocaine was used to locally anesthetize the area. The creamed area was covered with a plastic sheet and an adhesive bandage for protection. The entire thickness of the skin was numb after 45 minutes. Massage of the ear had resulted in vascular dilation.

The needle was carefully inserted and blood was collected in tubes. Once the removal of needle, cotton gauze was applied on the site of venepuncture until the bleeding stopped. The rabbits were kept under observation for the next few hours to ensure homeostasis was completed.[12]

Preparation of Plasma

The plasma was obtained by the optimized centrifugation method. The whole blood was collected into the commercially available anticoagulant-treated tube (lavender top). The tube was inverted to 10 times for mixing blood and anticoagulant. Then it was centrifuged at 2000 rpm for 20 minutes. The supernatant liquid was transferred immediately into a clean polypropylene tube using a Pasteur pipette.

Antithrombin Activity

The 1-g of methylene chloride extracts was dissolved in solvent and dilution was done accordingly. Plant extracts were initially evaluated for their potential to obstruct the thrombin-induced coagulation of healthy human plasma.

In this test, 50 L of extract was mixed with an equivalent volume of thrombin, incubated at 370°C for 5 minutes, and 50 L of normal human plasma was added from a healthy volunteer. The coagulation analyzer was used to calculate the clot time. Lalpath Laboratory received the sample and the method for the initial inspection.

The capacity of an extract to specifically inhibit thrombin was assessed using a chromogenic test to elucidate antithrombin activity. A yellow color is produced by the chromogenic test, which absorbs light at 405 nm.

It was performed by placing 50 µL of various concentrations (20, 40, 60, 80, and 100 mg/mL) of plant extract. Each concentrate was further diluted with 50 µL of tris-HCl buffer (50 mM, pH 8.0). Thrombin (5 units/mL,) was added and incubated at 37°C for 10 minutes. Then 50 µL chromogenic substrate was added and the change in absorbance at 405 nm was monitored for 10 minutes. The comparison was carried out by using heparin, 5000 unit/mL (1 unit Haparin = 0.002 mg of pure heparin). Heparin's dose was calculated using the dose conversion method HED to AED.[13,14]

The HED to AED equation (1) is

$$\text{Animal dose (mg/kg)} = \text{HED (mg/kg)} \times \text{Km ratio (H/A)} \quad \text{.....equ. (1)}$$

And factor used for calculation of rabbit dose is 1.

The maximum rate of absorbance change was calculated and recorded. Antithrombin level (B) was computed as in Eq 2.

$$B\ (\%) = \{(Ao - At)/Ao\}100 \quad \text{……. (2)}$$

Where Ao and At denote the absorbance of negative control and the test drug, respectively.

In order to serve as a control, the extraction solvent was diluted in tris-HCl buffer to the same extent as the sample. This served as a placeholder. Antithrombin activity was thought to be present if reaction rates were lower than the solvent blank.

Thrombolytic Activity

Five mL of venous blood from each rabbit was taken, divided among five pre-weighed sterile microcentrifuge tubes, and incubated for 45 minutes at 37°C. After clot formation, the serum was completely removed, avoiding damaging the clot, and then the weight of each tube containing a clot was determined by subtracting the weight of the tube by itself from the weight of the tube.

At intervals ranging from 24 to 72 hours of incubation at 37°C for optimum clot lysis, various leaves extract strengths, including 200, 400 600 and 800 g/mL, were assessed in each microcentrifuge tube with a pre-weighed clot. The pre-weighed blood clots were added to various incubation durations together with 100 L of distilled water as a control and 100 L of streptokinase (30,000 I.U.) as a benchmark. The fluid that had been evacuated was removed following incubation, and the tubes were weighed again to ascertain the weight difference following clot disruption. The percentage that separated the weights obtained before and after clot lysis was recorded. The thrombolytic capacities of each extract were evaluated (Figure 2 A, B). The experiment was repeated. Finally, %clot lysis was determined as[15,16]

$$\textbf{\% of clot lysis} = \frac{\text{weight of the clot after lysis by sample and removal of serum}}{\text{weight of the clot before lysis by sample}} \times 100$$

Statistical Tool for Analysis

Relevant statistical tools for reliability, co-relation and Regression Analysis of obtained data will be applied to draw a statistical relevance of study. *p-value < 0.05 was* regarded as a significant level.[17]

(A) (B)

Figure 2: Thrombolytic activity at various concentrations

Table 1: Extractive values of different extracts of *L. camara* leaves

Sr no.	*Extracts*	*% yield (w/w)*
1	Aqueous	14.26%
2	Methylene Chloride	20.58%
3	Methanol	18.79%
4	Ethanol	11.01%
5	Petroleum Ether	6.01%

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

Extraction

While performing extraction, following solvents were used for extraction. Out of these, methylene chloride and methanol have higher percentage yields as shown in Table 1**.**

Physiochemical Evaluation

Physiochemical parameters of the leaves of *L. camara* are tabulated in Table 2.

Preliminary Phytochemical Screening of the Extracts

Alkaloids, flavonoids, saponins, phenolic compounds, phytosterols, proteins, and amino acids make up the majority of the constituents. The primary source for classifying illicit substances is their phytochemical composition. These plants' medicinal value comes from a few chemical constituents that have a clear physiological effect. When compared to routinely used synthetic drugs, the most significant quality of these bioactive plant components is that they are more effective with few or no side effects. Aqueous, methylene chloride, methanol, ethanol and petroleum ether extracts of plants *L. camara* were tested for determination of presence of metabolites in extracts shown in Table 3.

On the basis of study indicates that the phytochemical analysis of extracts of *L. camara* leaves contains steroids, flavonoids, triterpenoids, alkaloids, carbohydrate, glycosides, tannin and polyphenols and proteins. The flavonoids which are responsible for antithrombin and thrombolytic activities are present in all the extracts but the intensity is high in methylene chloride extract. On this basis, methylene chloride extract was selected for further study.

Antithrombin Activity

The chromogenic assay results are stated in terms of reaction rate, such as mOD/minute, which is the rate at which thrombin cleaved the chromogenic substrate.

The average blank reaction rate was 0.38 OD units/minute. The *L. camara L.* extract produced results, which is significantly lower than the result from the control sample (Table 4).

Analysis was done on the fractionated extracts obtained using the methylene chloride technique mentioned above and it compared with antithrombin activity of heparin standard dose of 547.2 unit. Five samples were examined after fractions were pooled. The output is given in mOD units/minute.

L. camara methylene choride extract at various concentrations showed antithrombin activity and the activity was compared with the standard heparin (Figure 3). In comparison with heparin (std), *L. camara* extract shown 50.10, 60.23, 62.32, 68.48 & 71.32%. Antithrombin activity at 10, 20, 40, 60, 80 & 100 mg/mL concentration, respectively. It means that to achieve the 98.13% antithrombin activity, the concentration of *L. camara* has to increase.

Table 2: Physico-chemical standards of powdered *L. camara* leaves

Sr. no.	*Ash Value*	*% w/w*
1	Total Ash	8.99
2	Acid insoluble	3.50
3	Water soluble	4.01
4	Loss on drying	4.00

Table 3: Preliminary phytochemical screening of the extract

S. No	*Test*	*Aqueous*	*Methylene chloride*	*Methanol*	*Ethanol*	*Petroleum Ether*
1	Alkaloids	+	++	++	++	-
2	Steroids	+	++	++	++	++
3	Glycosides	+	++	++	++	+
4	Flavonoids	+	++	+	+	+
5	Tannins and Polyphenols	+	+	++	+	-
6	Triterpenoids	+	++	++	++	-
7	Carbohydrates	+	+	++	++	++
8	Proteins	-	-	-	-	+

Note: (-) Absence, (+) Presence and (++) present with high intensity of the colour.

Table 4: Antithrombin activity of *camara lantana L.* extract

Concentration (mg/mL)	*OD units/minute*	*% Antithrombin activity*
Control	0.38	-
20	0.191	50.10
40	0.152	60.23
60	0.144	62.32
80	0.120	68.48
100	0.109	71.32
Heparin (547.2 unit)	0.007	98.13

Thrombolytic Activity of *L. camara* Leaves Extract

When different concentration of extract was added in the different tubes containing blood clots, it looks a significant (Table 5) and it comparable to the standard drug streptokinase.

The 200 ug/mL leaf extract demonstrated mean% clot lysis of 11.4 ± 1.02, 19.7 ± 1.11, and 36.1 ± 1.25 after 24, 48, and 72 hours, respectively, of incubation at 37°C. The mean % clot lysis for the 400 ug/mL leaf extract was 27.2 ± 1.35, 42.91 ± 1.07, and 58.26 ± 1.89 at 24, 48, and 72 hours of incubation, respectively, at 37°C. The mean% clot lysis for the 600 µg/mL leaf extract was 42.08 ± 0.35, 54.17 ± 1.60, and 70.06 ± 0.25 at 24 hours, 48 hours, and 72 hours, respectively, of incubation at 37°C. The mean %clot lysis for the 800 µg/mL leaf extract was 62.02 ± 0.54, 72.42 ± 0.85, and 85.99 ± 1.24 at 24, 48, and 72 hours of incubation, respectively, at 37°C.

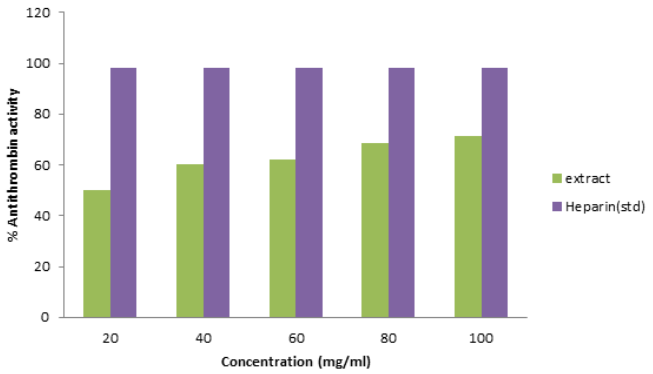

Figure 3: Antithrombin activity of *L. Camara extracts* at various concentration and heparin (Std)

Table 5: Thrombolytic activity of leaves extract of plant *L. camara*

*Concentrations (*µg/mL*)*	*Incubation Time*	*Clot Lysis % (Mean ± SD)*
200	24	11.4 ± 1.02
	48	19.7 ± 1.11
	72	36.1 ± 1.25
400	24	27.2 ± 1.35
	48	42.91 ± 1.07
	72	58.26 ± 1.89
600	24	42.08* ± 0.35
	48	54.17* ± 1.60
	72	70.06* ± 0.25
800	24	62.02* ± 0.54
	48	72.42* ± 0.85
	72	85.99* ± 1.24

* The result is significant at $p < 0.01$

CONCLUSION

This study studied the pharmacognostical, antithrombin and thrombolytic activities of leaves extract of plant *L. camara*. The extractive value of leaves extract of plant *L. camara* was higher in methylene chloride (20.58%) as compared to other solvent. In comparison with Heparin *L. camara* extract shown 50.10, 60.23, 62.32, 68.48 and 71.32% antithrombin activity at 10, 20, 40, 60, 80 and 100 mg/mL concentration, respectively. It means that for achieving the 98.13% antithrombin activity, the concentration of *L. camara* has to increase. Standard drug streptokinase shows 92.04 ± 2.89 clot lysis % and 800 ug/mL leaf extract showed mean %clot lysis of 62.02 ± 0.54, 72.42 ± 0.85 and 85.99 ± 1.24 at incubation time of 24, 48, and 72 hours, respectively at 37°C. Compared to streptokinase, different extract concentrations were added in the tubes containing blood clots, showing a good thrombolytic action. From the study we conclude that the leaves extract of plant *L. camara* shows significant antithrombin and thrombolytic activity.

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