

Original Article

EVALUATION OF IN VITRO ANTI-THROMBOLYTIC ACTIVITY AND CYTOTOXICITY
POTENTIAL OF TYPHA ANGUSTIFOLIA L LEAVES EXTRACTS

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ABSTRACT

Objective: The aim of this study was to investigate the in vitro thrombolytic activity and cytotoxicity of *T. angustifolia* leaf extract.

Methods: An in vitro thrombolytic model was used to evaluate the clot lysis effect of different extracts of *T. angustifolia* Linn along with Streptokinase as a positive control and distilled water as a negative control. The cytotoxic activity of different extracts of *T. angustifolia* leaves was evaluated by Brine Shrimp Lethality Bioassay.

Results: An in vitro thrombolytic model of aqueous, methanol and chloroform extracts have shown 51.76±2.5%, 58±2.32 and 18±1.84 clot lysis respectively, whereas the positive control Streptokinase shown 79.6±1.10 and negative control shown negligible 2.44±0.62. The aqueous, methanol and chloroform extracts have shown brine shrimp lethality with LC₅₀ value of 40µg/ml, 30µg and 104 µg/ml respectively. It was found that aqueous and methanol extracts of *T. angustifolia* possess potential thrombolytic activity as well as cytotoxicity.

Conclusion: The present investigation revealed that the aqueous and methanol extracts of *T. angustifolia* possess thrombolytic properties as well as cytotoxicity effects and it can be further used for treatment of cardiovascular diseases and cancer.

Keywords: Antithrombolytic, Brine Shrimp, Cytotoxicity, Typha Angustifolia.

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"[1] 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 [2]. Primary bioassay screens are most important for the initial screening of plants for bioactive principles and are often the first step in drug development [3]. Medicinal plants have acquired significant importance in the field of biotechnology for their developing applications [4]. 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.

Cardiovascular disease caused by blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in the recent years.[4] Thrombolytic agents are used to dissolve clot and in the management of thrombosis in patients[5]. Thrombolytic agents such as tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK)[6] etc, are used all over the world for the treatment [7] but their use is associated with hyper risk of haemorrhage [8], anaphylactic reaction and lacks specificity. Remarkable efforts have been made towards the discovery and development of natural constituents from various plant and animal sources which have antiplatelet [9,10] anticoagulant [11,12], antithrombotic [13] and thrombolytic activity [14-16]. The brine shrimp lethality bioassay was used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds [17]. The brine shrimp lethality bioassay is efficient, rapid and inexpensive tests that require in a relatively small amount samples. The technique is easily mastered, costs little, and utilizes small amount of test material[18]. Meyer et al.(1982)[19] have successively studied for *in-vivo* lethality bioassay-guide fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba* [20], cis-annonacin from *Annona muricata* [21] and ent- kaur-16-en-19-oic acid from *Elaeoselinum foetidum* [22].

Typha angustifolia of the family typhaceae is commonly known as Elephant grass or cattail. This plant is characterized by its fast

growth and high biomass [23]. Several parts of the plant are edible, including dormant sprouts on the roots and bases of the leaves, ripe pollen, the stem and the starchy roots [24,25]. The traditional uses of pollen grains of *T. angustifolia* for treatment of kidney stones, abnormal uterine bleeding, abscesses, tapeworm infection diarrhea and dysentery is well known [26]. Modern research on pollen grains of *angustifolia* mainly reveals that it contains sterols, terpenoids, flavonoid glycosides [27], cerebrosides and long chain hydrocarbons that possess various pharmacological activities like immunosuppression[28], antiplatelet aggregation [29], antimicrobial [30,2], cholesterol lowering activity and antiatherogenic effect[31]. The rhizome flour of *Typha angustifolia* used in the treatment of human IBD (Inflammatory bowel disease) is also studied by Andréa et al (2012)[32]. The present study has been designed to evaluate the antithrombolytic activity and cytotoxicity of different extracts of *T. angustifolia* Linn leaves.

MATERIALS AND METHODS

Collection and extraction of plant materials

Aerial part (leaves) of *T. angustifolia* was collected in and around Gulbarga University campus, Gulbarga, Karnataka, India in the month of March 2013. The collected plant materials were washed with running tap water, allowed to air dry and were dried in shade for two to four weeks. Precaution was taken to avoid direct sun light contact of leaves otherwise it will destroy the active compounds of plant leaves. After drying, the plant leaves were grinded finely and stored in airtight container. The air dried leaf powders (50 g) were successively extracted by Soxhlet extraction with solvents of increasing polarity i.e., petroleum ether (60°-80°C), chloroform, methanol and distilled water. The extracts were dried and stored in a sterile container for further use.

Clot lysis

The clot lysis was carried out as per the method reported by Prasad et al 2006[33]. In brief, 2.5 ml of venous blood drawn from healthy volunteers was distributed in 5 different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 minutes. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again

weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). To each microcentrifuge tube containing pre-weighed clot, 100 μ l of different extracts of *Typha angustifolia* (Linn) is to be added. To the commercially available lyophilized streptokinase vial (Lupiflo, Lupin Limited, Mumbai, India) 2.5 ml of PBS was added and thoroughly mixed. This suspension was used as a stock from which 100 μ l was added to the microcentrifuge tube as a positive control. For negative control, 100 μ l of distilled water were added. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the fluid released 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. The experiment was repeated 4 times with the blood samples of 5 volunteers.

Brine Shrimp Lethality Bioassay

Brine shrimp cytotoxicity bioassay is very simple bench-top assay used to measure cytotoxicity of plant extracts as well as synthetic compounds [34, 35]. It was carried out with the method as described

by Meyer et al. (1982) [19] to investigate the cytotoxicity of the extracts. The different extracts of *Typha angustifolia* were dissolved in DMSO to obtain a stock solution of 10 mg/ml from which appropriate (1-160 μ g/ml) dilutions were made to observe the cytotoxic activity. Simple zoological organism (*Artemia salina*) was used as a convenient monitor for cytotoxic screening. The commercially available eggs were hatched in a small partitioned tank containing artificial seawater (3.8% NaCl, pH 8.5) under constant aeration for 24h under the light and allowed to grow further for 48 h to get shrimp larvae called nauplii. With the help of Pasteur pipette add 10 brine shrimps to the vial containing 5ml of artificial sea water. After 24 h, the vials were inspected using a magnifying glass, and the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 sec of observation [19].

Statistical analysis

Results are expressed as Mean \pm SEM. The statistical analysis was carried out using one way ANOVA analysis. The *p*-value of 0.05 or less was considered significant for all experiment.

Table 1: brine shrimp lethality tests of different extracts of *T. angustifolia*

S. No.	Conc in μ g/ml	Aqueous Extract	Methanol Extract	Chloroform Extract
1.	1	13.33 \pm 5.77	17 \pm 5.77	3.33 \pm 5.77
2.	2.5	16.67 \pm 5.77	20 \pm 10	6.67 \pm 5.77
3.	5	20 \pm 00	26.67 \pm 5.77	13.33 \pm 5.77
4.	10	23.33 \pm 5.77	36.67 \pm 5.77	16.67 \pm 5.77
5.	20	36.67 \pm 5.77	46.67 \pm 5.77	26.67 \pm 5.77
6.	40	50 \pm 00	53.33 \pm 5.77	30 \pm 00
7.	80	56.67 \pm 11.5	63.33 \pm 5.77	46.67 \pm 5.77
8.	160	73.33 \pm 15.3	86.67 \pm 5.77	56.67 \pm 5.77
	LC ₅₀	40 μ g/ml	30 μ g/ml	104 μ g/ml

Values are expressed as Mean \pm SEM, Sample volume 3(n=3), *p*<0.05.



Fig. 1: Dissolved clots after treating with different extracts of *T. angustifolia*

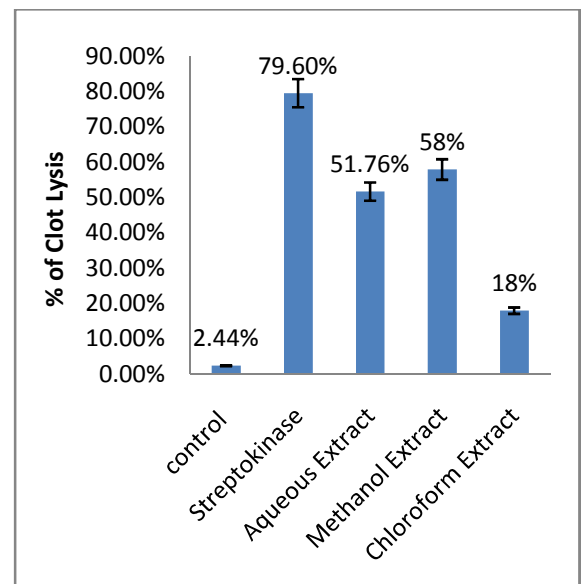


Fig. 2: Percentage of Clot lysis by distilled water, Streptokinase and different extracts of *T. angustifolia*

RESULTS

The aqueous and methanol extracts showed 51.76 \pm 2.5% and 58 \pm 2.33% respectively where as chloroform extract shows 18 \pm 1.84%. Addition of 100 μ l Streptokinase has showed 79.6 \pm 1.1% clot lysis (Fig 1 & Fig 2). However, distilled water (negative control) shown only negligible clot lysis (2.44 \pm 0.62%). The mean difference in clot lysis percentage between positive and negative control was significant (*p* value < 0.0001). The mean percentage of clot lysis by different extracts of *T angustifolia* was statistically more significant

(*p* value < 0.0001) when compared to those of both positive control streptokinase and negative control water.

In the brine shrimp test, among three extracts evaluated the chloroform extracts having LC₅₀ > 100 μ g/mL which is nontoxic or exhibited weak toxicity. On the other hand, methanol (LC₅₀ 30 μ g/mL) and aqueous (LC₅₀ 40 μ g/mL) extracts have shown significant toxic effects.

DISCUSSION

Medicines derived from plants origin will have a long history of use for the prevention and treatment of various diseases. Approximately 30% of the pharmaceuticals formulations are prepared from plants across the world [36] and are considered to be less toxic and freer from side effects than the synthetic one [37]. Efforts have been carried in recent past two decades towards the exploration, discovery, designing and development of natural products with antiplatelet [10], anticoagulant [12], antithrombotic [13] and thrombolytic activity of the plants [38]. Few plant extracts and their products having fibrinolytic activity are identified, which includes *Lumbricus rubellus* [39], *Pleurotus ostreatus* [40], *Spirodela polyrhiza* [41], *Flammulina velutipes* [42], and *Ganoderma lucidum* [43], Ginger (*Zingiber officinale*) [44], Garlic (*Allium sativum*) [45] as well as from *Bacillus* sp. in Korean and Japanese fermented foods, chungkook-jang [46] and natto [47,48] respectively.

Generally blood clots are formed from fibrinogen by thrombin. Antithrombotic or thrombolytic drugs can block the pathway of thrombus formation. The fundamental task of thrombolytic therapy is the degradation of fibrin by plasmin, which can be activated by the activators from inactive plasminogen. [49]. *T. angustifolia* is known for antibacterial activity against six strains notably *Enterobacter aerogenes*, *Salmonella typhimurium*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* [2,30]. However, there are findings of bacterial contaminants of plants which have plasminogen receptors that bind plasminogen. Cell surface bound plasminogen is easily activated to plasmin, which could lead to fibrinolysis [50]. Bacterial plasminogen activator: staphylokinase, streptokinase, act as cofactor molecules that contribute to exosite formation and enhance the substrate presentation to the enzyme. Staphylokinase activates plasminogen to dissolve clots, which also destroys the ECM and fibrin fibers that hold cells together [51,52]. By comparing with this positive & negative control, a significant thrombolytic activity was observed after treating the clots with aqueous and methanol extract where as chloroform extract of *T. angustifolia* result indicates less potential to lyse the clot. Thrombolytic activity (Clot lysis) of *T. angustifolia* extract may be the result of the combinatorial effect of the active compounds present or by the individual compounds. Further research on cell viability tests and *in vivo* studies, will have an important implications in the treatment of cardiovascular diseases which is increasing at an alarming rate. Since the commercially available drugs used for the cardio vascular diseases are expensive and not accessible to the greater section of the society, application of this study may be a boon for them.

Herbal preparations will be a better option, if taken in an appropriate dose for curing various ailments and if taken in higher/lethal dose plant extracts could be harmful [53,54]. Toxicity of plant extract is a major concern of scientists and medical practitioners. Several methods of lethality tests have been successfully used to biomonitor the isolation of cytotoxic, antimalarial, insecticidal and antifeedant compounds from plant extracts [55]. Several lethality tests have been designed and one such method is the lethality test wherein Brine Shrimp Lethality (LC₅₀, 24 hr.) test is used to determine cytotoxicity of different plant extracts.

Brine shrimp lethality bioassay is a rapid and comprehensive bioassay for the study of bioactive compound of the natural and synthetic origin. Brine shrimp cytotoxic assay not only reveals the cytotoxicity of the natural products and synthetic compounds but it also supports anticancer, antiviral, insecticidal and pesticidal potential [56]. This test is based on the potential effects of different extracts of *T. angustifolia* to become lethal to *A. salina* nauplii due to its toxic expression. According to Meyer *et al.* (1982) [19], extracts derived from natural products will have LC₅₀ ≤ 1.0 mg/mL are known to possess toxic effects. In this study, the table (Table 1) shows that the LC₅₀ values of the aqueous, methanol and chloroform extracts is 0.04, 0.03 and 0.104 mg/mL after 24 h, respectively. Thus, these results prove that the aqueous and methanol extracts of *T. angustifolia* were significantly toxic compared to chloroform extract. A good correlation has been found between brine shrimp cytotoxicity and cytotoxicity against KB

(human nasopharyngeal carcinoma) cells [17]. Toxicity of the leaf and seed extracts of *Cassia alata* by using brine shrimp cytotoxicity assay was demonstrated by Awal *et al.*, (2004) [57], where as cytotoxic evaluation of components of *Bolax gummifera* was studied by using brine shrimp cytotoxicity assay by Mongelli *et al.*, (2003) [58]. While Chowdhury *et al.*, (2004) [59] described that the cytotoxic potential of extracts and purified components of *Stachytarpheta urticaefolia* by using Brine shrimp assay. Hence, *T. angustifolia* leaf extract may be further explored for the development of natural product-based pharmaceutical products.

CONCLUSION

Through our study it was found that aqueous and methanol extracts of *T. angustifolia* possesses thrombolytic properties as well as cytotoxicity effects. However, *in vivo* clot dissolving properties and active component(s) responsible for cytotoxicity of *T. angustifolia* are yet to be discovered. By the above obtained results, it can be suggested that the application of the *T. angustifolia* component may be accessible for greater section of the society for the treatment of cardiovascular diseases and cancer.

CONFLIT OF INTREST

The authors declare that there is no conflict of interest.

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