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In-vitro thrombolytic and anti inflammatory activity of *Cipadessa baccifera* (Roth) Miq. and *Elytraria acaulis* (L.f) Lindau

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Abstract
The present study was mainly focused on *in-vitro* thrombolytic and anti inflammatory activity of petroleum ether, toluene and methanolic extracts of *Cipadessa baccifera* petroleum ether and methanolic extracts of *Elytraria acaulis.* The extracts of *Cipadessa baccifera* showed 54.64% of clot lysis and the *Elytraria acaulis* exhibited the maximum 66.53% of 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 79.86% respectively. During this study petroleum ether and methanolic extracts of *Elytraria acaulis* showed the inhibition of hypotonicity induced HRBC membrane lysis was taken as a measure of the anti inflammatory activity. The percentage of membrane stabilisation for petroleum ether and methanolic extracts and Diclofenac sodium were done at different concentrations. The maximum membrane stabilization showed in the methanolic extract and petroleum ether extract of *Elytraria acaulis* extracts was found to be 57.14% and 44.89% at a dose of 60 mg/ml. Therefore, our studies support the isolation and the use of active constituents from *Elytraria acaulis* in treating inflammations. But in case of *Cipadessa baccifera* did not showed the inhibition of hypotonicity induced HRBC membrane lysis. As a result *Cipadessa baccifera* did not take part in anti-inflammatory activity.

Keywords: thrombolytic, anti-inflammatory activity, *Cipadessa baccifera*, *Elytraria acaulis*

Introduction
A blood clot (thrombus) developed in the circulatory system due to failure of hemostasis causes vascular blockage and while recovering leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death (Lee HS, 1995). Thrombolytic agents that include tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK) etc. are used all over the world for the treatment of these diseases. In India, though SK and UK are widely used due to lower cost (Mucklow JC, 1995 and Collen, 1990) as compared to other thrombolytic drugs, their use is associated with hyper risk of hemorrhage (Rouf SA *et al*., 1996). Severe anaphylactic reaction and lacks specificity. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted (Jennings, 1996). Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini *et al*., 2012 and Wu DH).
The selective antiplatelet agents and thrombin inhibitor are most potent though safety is yet a main concern. The new studies and investigations in this area will give new imminent that encourage the advancement of the ideal thrombolytic therapy. The use of herbal for treatment of disease has been in practice since ancient times. Herbal medicines are considered safer due to their natural activity (Demrow HS *et al*., 1995). It has been reported from studies that herbal products showing their thrombolytic activity significantly (Giuseppina B *et al*., 2004). 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. The anti thrombotic activity of herbs and their natural compound has reported previously (Yamamoto J *et al*., 2005).
Inflammation was described as "the succession of changes which occurs in a living tissue when it is injured provided that the injury is not of such a degree as to at once destroy its structure and vitality" (Sanderson,1871), or "the reaction to injury of the living microcirculation and related tissues (Spector WG, 1963). Inflammatory response to tissue injury involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (Vane JR, 1995) which are aimed at host defense and usually activated in most disease conditions. HRBC or erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes. Stabilization of human red blood cell membrane (HRBC) by hypo tonicity induced membrane lysis can be taken as an in vitro measure of anti inflammatory activity of the drugs or plant extracts.

Stabilization of lysosomal membrane is important in limiting the inflammatory response by inhibiting the release of lysosomal constituents of activated neutrophil such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extra cellular release or by stabilizing the lysosomal membrane (Chou CT, 1992).

Cipadessa baccifera belongs to Meliaceae family which is an evergreen shrub growing up to a height of 3 meters. It is very good for treatment for snake poison and particularly for the Cobra poison. Treatment includes drinking one ounce of leaf juice and applying paste of leaves in the case of cobra bite. Daily intake of one or two leaves will make a permanent resistance against cobra poison. It is also very good for Hemiplegia (condition in which one-half of a patient's body is paralyzed). It also will dilute the clotted Blood. Intake of half ounce of leaf juice every morning will help Hemiplegia patients to get cured.

Elytraria acaulis belongs to family Acanthaceae, which is a small shrub that grows in shady dry places. *Elytraria acaulis*is is widely distributed in South Africa and India. *Elytraria acaulis*is traditionally used in the treatment of asthma, migraine, leucorrhoea, snake bite etc. The *Elytraria acaulis* extracts are effective in decreasing blood glucose level, increases oral glucose tolerance test, moderately alternating body weight and there is a marked reduction in the liver glycogen levels and reduction in glycated haemoglobin levels. (Praveen Kumar R, 2014).

Materials and Methods

Plant Material

Mature and healthy plants of *Elytraria acaulis* (L.f) Lindau belonging to the family Acanthaceae was collected from Thiruvannamalai district of Tamil Nadu, India. The mature and healthy leaves of *Cipadesssa baccifera* collected from Jnanabharathi Campus, Bangalore University, Bangalore. The plants were identified by Prof. Seetharam from Biological Science Department, Bangalore University, Bangalore.

Preparation of solvent extract

Leaves of *Cipadessa baccifera* and *Elytraria acaulis* was collected and washed thoroughly, shade dried, pulverized mechanically and sieved. 50g of each shade dried and finely powdered of *Cipadessa bacciferra* and *Elytraria acaulis* were filled in the thimble separately and extracted successively with 200-300 ml of petroleum ether, toluene, chloroform, methanol and ethanol using a soxhlet extractor until colourless extract obtained on the top of the extractor. Each of the solvents extracts were concentrated separately under reduced pressure using rotary flash evaporator (Thippeswamy *et al*., 2012). The concentrated extracts were subsequently dried at room temperature under a steam of cold air and kept in air-tight containers at 4^0C until tested. The dried organic plant extracts were re-suspended in DMSO for the final concentration of 20 mg/ml and filtered through 0.45µm membrane filter for sterilization and subjected to thrombolytic and anti-inflammatory activity.

Streptokinase solution preparation

Commercially available lyophilized Streptokinase C from Streptococcus Sp. vial procured from Sigma Aldrich, >15,00,0000 Unit/mg. 5 ml of Phosphate buffered saline (PBS) was added and mixed properly. This suspension was used as s stock from which 100ml (30,000 I.U) was used. In this study, Streptokinase (SK), a known haemolytic drug is used as a positive control.

In vitro Thrombolytic Activity

2 ml venous blood drawn from healthy volunteers was distributed in three different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, serum was completely removed (aspirated out 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). To each micro centrifuge tube containing preweighed clot, 100 µl of ethanol extract (10 mg/ml) of was added. As a positive control, 100 µl of streptokinase and as a negative nonthrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as constant oxygen supply for 48 hours. (Swetha, 2007)

Anti-inflammatory activity by HRBC membrane stabilization method

The human red blood cell membrane stabilization method (HRBC) has been used as a method to study the *in-vitro* anti-inflammatory activity. Blood was collected from healthy human volunteer who was not taken any NSAIDS for 2 weeks prior to the experiment. The collected blood was mixed with equal volume of sterilised Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% NaCl in water) and centrifuged at 3,000 rpm. The packed cells were washed with isosaline (0.85%, pH 7.2) and 10% (v/v) suspension was made with isosaline (Gandhisan *et al*., 1991).

Various concentrations of extracts were prepared (20, 40, 60, 80, and 100mg/ml) using dimethyl sulphoxide DMSO and to each concentration 1 ml of phosphatebuffer (0.15M, pH 7.4), 2 ml of hyposaline (0.36%)and 0.5 ml of HRBC suspension were added. It isincubated at 37^0C for 30 min and centrifuged at 3,000rpm for 20 min. The hemoglobin content in the supernatant solution was estimated spectrophotometrically at 560 nm.

Diclofenac sodium, of different concentration 20 mg/ml is mixed with 5ml of hypotonic saline. The control sample was 0.03 ml RBC suspension mixed with hypotonic buffered solution alone. The mixtures were incubated at 10 minutes at room temperature, centrifuged for 10 minutes at 3000rpm and absorbance of the supernatant was measured spectrophotometrically at 540 nm. The experiment was carried out in triplicate and the percentage inhibition of membrane stabilization was calculated (Sadique *et al*, 1989). The percentage of HRBC membrane stabilization or haemolysis was calculated using the formula % inhibition of Haemolysis= 100× OD1- OD2/ OD1 Where, OD1 and OD2 are absorbance of Diclofenac and test extracts respectively.

Result and Discussion

Table 1: Thrombolytic activity of *Cipadessa baccifera*

Solvents	Concent-ration mg/ml	Weight of clot in gm	Weight of Tube with clot after lysis gm	Weight of lysis	% of clot lysis	Average % of clot lysis	% clot lysis in *Cipadessa baccifera*
Petroleum ether	20	1.93	1.02	0.91	52.8	55.58	54.64
	40	1.22	0.73	0.49	59.8		
	60	1.23	0.69	0.54	56.0		
	80	1.19	0.67	0.52	56.3		
	100	1.3	0.69	0.61	53.0		
Toluene	20	1.87	1.0	0.87	53.4	56.8	
	40	1.16	0.69	0.47	59.4		
	60	1.18	0.69	0.49	58.4		
	80	1.25	0.71	0.54	56.8		
	100	1.23	0.69	0.54	56.0		
Methanol	20	2.2	1.04	1.16	47.2	51.34	
	40	1.14	0.69	0.45	60.5		
	60	2.3	0.70	1.6	30.43		
	80	1.14	0.67	0.47	58.77		
	100	1.12	0.67	0.45	59.82		

Table 2: Thrombolytic activity of *Elytraria aculis*

Solvents	Concentration mg/ml	Weight of clot in gm	Weight of Tube with clot after lysis gm	Weight of lysis	% of clot lysis	Average % of clot lysis	% clot lysis in *Elytraria aculis*
Methanol	20	2.27	1.2	1.07	55.29	68.23	66.53
	40	0.99	0.70	0.29	70.70		
	60	1.06	0.79	0.27	74.52		
	80	0.95	0.63	0.32	59.85		
	100	1.20	0.97	0.23	80.80		
Petroleum ether	20	1.98	1.02	0.96	51.50	64.84	
	40	0.95	0.69	0.26	65.55		
	60	1.08	0.70	0.38	75.60		
	80	0.93	0.62	0.31	57.66		
	100	0.98	0.73	0.25	74.00		

The average percentage of clot lysis of was found to be 54.64% in *Cipadessa baccifera* and the maximum 66.53% clot lysis was observed in *Elytraria acaulis*. The maximum 79.86% was observed in Streptokinase as a positive control. Most thrombolytic agents work by activating the enzyme plasminogen, which clears the cross-linked fibrin mesh. This makes the clot soluble and subject to further proteolysis by other enzymes and restores blood flow over occluded blood vessels. Thus thrombolytic agents are useful for the treatment of myocardial infarction, thrombo embolic strokes, deep vein thrombosis and to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain and leg).

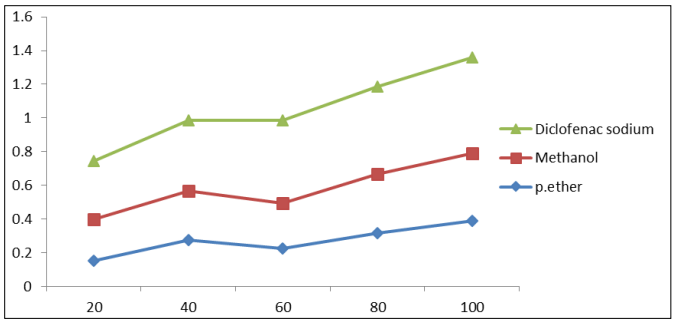

Fig 1: Showing the anti-inflammatory activity of *Elytraria acaulis.*

Membrane lysis is taken as a measure of *in vitro* anti-inflammatory activity. *In vitro* anti inflammatory activity of the *Elytraria acaulis* were concentration dependent, the maximum membrane stabilization showed in the extracts of methanol and petroleum ether of *Elytraria acaulis* was found to be 57.14% and 44.89% at a dose of 60 mg/ml. the results of the present study have shown that the leaves of *Elytraria acaulis* possess anti-inflamatory acitivity. The study also provides emipiral evidence for the use of the leaves of *Elytraria acaulis* in folkloric treatment of inflammatory disorders and pain. In case of Cipadessa baccifera did not showed the inhibition of hypotonicity induced HRBC membrane lysis. As a result *Cipadessa baccifera* did not take part in anti-inflammatory activity.

Conclusion

The present investigation provides the confirmation of anti-inflammatory activity due to the strong occurrence of secondary metabolites like polyphenolic compounds such as falvonoids, tannins, terpenoids, phenols and saponins. The scientific evidence to support traditional medicinal uses and they indicate a promising potential for the development of an antimicrobial, antioxidant, thrombolytic and anti-inflammatory agent of plant. The study might provide foundation to identify various pharmaceutical products in future also the research will be required to explore these plants

to food and pharmaceutical industry in upcoming days.

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