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PHYTOCHEMICAL AND *IN-VITRO* THROMBOLYTIC ACTIVITY OF *PERGULARIA DEAMIA* (FORSK.) STEM

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ABSTRACT

The aim of the study was to determine the phytochemical constituents of *Pergularia deamia* (stem). Bioactive compounds are alkaloids, carbohydrate, glycosides, protein, cardiac glycosides, quinone, phenol, terpenoids, and steroids contains in all extracts of stem of *P. deamia*. *In vitro* thrombolytic model was used to evaluate the clot lysis effect of different extracts of *P. deamia*, along with aspirin as a positive control and distilled water as a negative control. An *in vitro* thrombolytic model of aqueous, methanol and hexane extracts have showed higher thrombolytic activity 59.50±4.02%, 61.11±5.00% and 62.22±5.21% against clot. Whereas the positive control aspirin exhibited 67.76±5.42%. The maximum thrombolytic activity was observed in hexane extracts of *P. deamia*. In future, to isolated the particular phytocompound to proceed new novel drug for against thrombus.

KEY WORDS: Medicinal plants, Thrombolytic activity, *Pergularia deamia*, photochemical compounds.

INTRODUCTION

Medicinal plants are the rich source of important compounds and since ancient time, plant and plant derived products are used as medicine in traditional and folk medicinal system. Initially the herbal drugs were used in the form of dried powder, gums, extracts or formulations of more than one plant products. Advanced scientific techniques brought a revaluation in herbal medicine industry and all focus is concentrate on bioactive molecule.

However, a lot of processing is required to develop a drug from the natural sources (Dasilva *et al*., 2004).

Thrombosis is the formation of a blood clot inside a blood vessel, obstructing the flow of blood through the circulatory system. When a blood vessel is injured, the body uses platelets (thrombocytes) and fibrin to form a blood clot to prevent blood loss. Even when a blood vessel is not injured, blood clots may form in the body under certain conditions. A clot that breaks free and begins to travel around the body is known as an embolus (Handin *et al*., 2005). Epidemiologic studies have provided evidence that foods with experimentally proved anti-thrombotic effect could reduce risk of thrombosis. Herbs showing thrombolytic activity have been studied and some significant observations have been reported (Anwar *et al*., 2012).

Anticoagulation therapy is the basis of management, and the proper choice of thrombolytic drugs to decrease platelet aggregation or interfere with the clotting process can be critical. Intravenous heparin, the first line of treatment for cerebral venous sinus thrombosis (CVST), is used in the anticoagulation therapy, because it is, effective and feasible. The development of modern pharmaceuticals many drugs have been developed with the purpose of dissolving clots, such as alteplase, anistreplase, streptokinase, urokinase and tissue plasminogen (TPA) etc (Khan *et al*., 2011).

The plant *P. daemia* (Asclepiadaceae) is widely distributed in the old world tropics and subtropics from southern and tropical Africa and Asia, have multiple applications in different folk medicine, including the Indian Ayurvedic system and have been documented for antifertility (Anonymous, 2014). Traditionally the plant *P. daemia* was used as anthelmintic, laxative, antipyretic and expectorant and also used to treat infantile diarrhoea and malarial intermittent fevers, stomachic, laxative, diuretic properties, cough, and sore eyes (Kirtikar and Basu 2011). The plant stem bark is also used to treat malaria and the twig is used as an antipyretic and leaf of this plant are a good remedy for cold (Dokosi, 2014). The latex of the plant is used for treating boils, sores and venereal diseases (Van Damme *et al*., 2014). The entire plant is used for pulmonary conditions, asthma, cough, piles, leprosy, and syphilis and the leaf juice is used as an expectorant, uterine tonic and emetic (Mohamed *et al*., 2009) The root is used to treat mental disorders, anemia, leprosy, piles, uterine and menstrual disorders (Yoganarasimhan, 2009). Our present study, to determine the phytochemical compounds and *in vitro* thrombolytic activity of different extracts of *P. deamia* (stem).

MATERIALS AND METHODS

Collection of plant material

Plant of *Pergularia deamia* was collected from Siruthondamadevi, Cuddalore (District), Tamilnadu, India, during November 2014. The stem of the plant were washed thoroughly in tap water followed by distilled water. It was shade dried at the room temperature. Dried stem were uniformly grinded well using mechanical grinder. The powder material was stored at air tight container.

Preparation of extracts

The powdered stem (250 g) of *P. deamia* were macerated in 250ml hexane and methanol for 3 days and then filtered through a cotton plug followed by Whatmann filter paper No.1. The extract was concentrated by boil water both and then crude extracts was stored at 4-8°C in air tight container. To one part of the plant material three parts of water was added to boil the content and then reduced original volume and filtrate was evaporated to boiling water bath. Paste form of the extracts was subjected to screening test.

Qualitative method of phytochemical analysis (Sofowara (1993)

The stem extracts were analysed for alkaloids flavanoids, pholabatannins, glycosides, phenols, saponins, lipids and fat, tannins, anthraquinones, quinones, cardiac glycosides, coumarins acids, steroids, phytosterols, proteins, carbohydrates etc.

Detection of Alkaloids

About 50 mg of solvent free extract was stirred with 3 ml of dilute hydrochloric acid and then filtered thoroughly. The filtrate was tested carefully with various alkaloid reagents as follows:

Mayer's test

To a 1 ml of filtrate, few drops of Mayer's reagent are added by the side of the test tube. The white or creamy precipitate indicated test as positive.

Wagner's test

To a 1 ml of filtrate, few drops of Wagner's reagent are added by the side of the test tube. The color change was observed. A reddish-brown precipitates confirms the test as positive.

Dragendorff's test

To a 1 ml of filtrate, 2 ml of Dragendorff's reagent are added and the result was observed carefully. A prominent yellow precipitate confirms the test as positive Detection of Carbohydrate.

Fehlings test

One ml of extract was boiled on water bath with 1 ml each of Fehling solutions A and B. The color change was observed. A red precipitates indicated presence of sugar.

Barfoed's test

To 1 ml of extract, 1 ml of Barfoed's reagent was added and heated on a boiling water bath for 2 minutes. The color change was noted and recorded. A red precipitates indicated presence of sugar.

Benedict's test

To 0.5 ml of extract, 0.5 ml of Benedict's reagent was added. The mixture is heated on a boiling water bath for 2minutes and the result was observed. A red precipitates indicated presence of sugar.

Detection of Glycosides

Legals test

Chloroform (3ml) and ammonia solution (10%) was added to 2ml plant extract. Formation of pink color indicated the presence of glycosides.

Detection of Proteins

The extract was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrateis subjected to tests for proteins and amino acids.

Millon's test

To 2 ml of filtrate, few drops of Millon's reagent are added. The result was observed. A white precipitates indicated presences of proteins.

Biuret test

An aliquot of 2 ml of filtrate was treated with drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. The pink color in ethanol layer indicated presences of proteins.

Detection of amino acid

Ninhydrin test

Two drops of ninhydrin solution (5 mg of ninhydrin in 200 ml of acetone) are added to two ml of aqueous filtrate.The color change was observed. A characteristic purple color indicated the presence of amino acids.

Detection of Phytosterols

Liberdmann-Burchard's test

The extract (5 mg) was dissolved in 2 ml acetic anhydride and one or two drops of concentrated sulphuric acid wasadded slowly along the sides of the test tube. The formation of blue green color indicated the presence of triterpinoides and phytosteroids.

Detection of Tannins

Ferric chloride test

The extract (5 mg) was dissolved in 5 ml of distilled water and few drops of neutral 5% ferric chloride solution were added. The formation of blue green color indicated the presence of tannins.

Detection of Phenols

Lead acetate test The extract (5 mg) was dissolved in distilled water and 3 ml of 10% lead acetate solution was added. A bulky white precipitates indicated the presence of phenols.

Detection of flavanoids

An aqueous solution of the extract was treated with ammonium hydroxide solution. The yellow fluorescence indicated the presence of flavanoids.

Detection of coumarins

10% NaOH (1ml) was added to 1 ml of the plant extracts formation of yellow color indicated presence of coumarines.

Detection of Saponin

Distilled water 2ml was added of each plant extracts and shaken in a graduated cylinder for 15 mins length wise. Formation of 1cm foam indicates the presence of saponin.

Detection of Quinone

Concentrated sulphuric acid (1ml) was added to 1ml of each of the plant extract. Formation of red color indicated the presence of Quinones.

Detection of Cardiac glycosides

Glacial acetic acid (2ml) and few drops of 5% ferric chloride were added to 0.5% of the extract. This was under layered with 1ml of concentrated sulphuric acid. Formation of brown ring at the interface indicated presence of cardiac glycosides.

Detection of Terpenoid

Chloroform (2ml) and concentrated sulphuric acid was added carefully to 0.5 ml of extract. Formation of red brown color at the interface indicated the presence of terpenoids.

Detection of Phlobatannins

Few drops of 10% ammonia solution were added to 0.5 ml of root extract. Appearance of pink color precipitates indicated the presence of phlobatannins.

Detection of Anthraquinones

Few drops of 2% HCL were added to 0.5 ml of root extract. Appearance of red color precipitate indicated presence of anthraquinones.

Detection of steroids and phytosteroids

To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid. Appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicated the presence of phytosteroids

Thrombolytic Activity (Prasad *et al.*, 2006)

Venous blood samples (3 ml each) were drawn from three healthy human volunteers. 500 µl of blood was transferred to each *in vitro* of four previously weighed Eppendorff tubes for each subject. In the first series, the transferred 500 µl allowed to form clots at 37 º C for 45 minutes. After clot formation, serum was completely removed and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube-weight of the tube alone). To each Eppendorff containing pre-weighed clot, 100 -1000 µl of different concentration of plant extracts or 100 µl distilled water as a negative control were added. All the tubes were then incubated at 37 ° C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the

difference in weight after clot stabilization. The obtained difference in weight was expressed as percentage of stabled or lysed clot. In the second series of experiments, simultaneous addition of 500 µl blood and 100 µl aspirin, incubated at 37° C for 45 minutes. The obtained clot weight was determined as above (Prasad *et al*., 2013)

% of clot lysis = (wt of released clot /clot wt) × 100

= (W2 – W3 / W2 – W1) × 100

W1=Empty weight of Eppendorff tube
W2=Weight of Eppendorff tube+clot
W3=Weight of clot release after addition of plant extract

RESULTS AND DISCUSSION

Table.1 showed phytochemical screening of aqueous, methanol and hexane extracts of *Pergularia deamia* stem were carried out by using the standard protocols. The presence of bioactive compounds are alkaloids, carbohydrate, protein, aminoacids, tannins, phenols, quinone, cardiac glycosides, anthraquinone, coumarins, terpenoids, flavanoids, steroids and phytosteroids are present in all the extracts. Glycosides, phlobatannis, saponin are absent in all the extracts of *P. deamia.*

Phytochemicals are basically divided into two groups that are primary and secondary metabolism. The major constituents are consists of carbohydrates, amino acids, proteins, and chlorophylls while secondary metabolites consist of alkaloids, saponins, steroids, flavonoids, tannins and so on (Kumar *et al*., 2012). Phytochemical constituents are the basic source for the establishment of several pharmaceutical industries. The constituents are playing a significant role in the identification of crude drugs (Savithramma *et al*., 2012).

The leaves of *P. deamia* contains are flavonoids, alkaloids, terpenoids, tannins, steroids and carbohydrates (karthishwaran *et al*., 2010). Althrough large number of compounds has been isolated from various parts of *P. deamia*, a few of them have been studied for biological activity was reported. The seeds of *P. deamia* contain uzarigenin, coroglaucigenin, calactin, calotropin, other cardenolides and a bitter resin, cardiotoxic action (Patel and Roeson, 1965).

Table:1 Pytochemical analysis of aqueous, methanal and hexane extracts of *P .deamia* (Forsk.) Stem

S.no	Name of the Phytocompound	Aqueous extracts	Methanol extracts	Hexane Extracts
1	Alkaloids	++	+++	++
2	Carbohydrate	++	++	+
3	Glycosides	-	-	-
4	Proteion	++	++	+
5	Aminoacid	+	-	-
6	Pytosteroids	-	++	+
7	Tannins	-	-	+
8	Phenols	+++	+++	++
9	Flavanoids	-	++	-
10	Saponins	-	-	-
11	Quinone	+	+++	+
12	Cardiac glycosides	+++	+++	+++
13	Terpenoid	+	+	+++
14	Phlobatanins	-	-	-
15	Anthraquinone	+	-	-
16	Steroid and phytosteroid	+	++	++

+++(highly) ; ++(moderate) ; +(mild) ; -(absent)

In vitro* thrombolytic activity of *P. deamia

As a part of discovery thrombolytic drugs from natural product from extracts, stem of *P. deamia* and were screened against thrombosis. The results are showed in table 2 and figure 1. 1ml of aspirin as a positive control (1mg/ml) added to (clots) and subsequent incubation for 90 minutes at 37 °C, It showed 67.76% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (3.34%). The hexane extract of stem of *P. deamia* exhibited higher degree of thrombolytic activity at all the concentration. Increased clot lysis activity was observed in 62.22% of hexane extract (1mg/ml). The minimum of clot lysis was seen in the aqueous extract of *P. deamia.*

Since ancient times, herbal preparations have been used for the treatment of several diseases. Herbal products are often perceived as safe because they are "natural" (Demrow *et al* ., 1995). Epidemiologic studies have provided evidence that foods (Plant materials) with experimentally proved antithrombolytic effect could reduce risk of thrombosis. The herbs and natural food sources and their supplements having antithrombotic (anticoagulant and antiplatelet) effect and there is evidence that consuming such food leads to prevention of coronary events and stroke (Ratnasooriya *et al*. 2012). Herbs showing thrombolytic activity

have been studied and some significant observations have been reported (Yamamoto et al., 2005). Advances of phytochemistry and identification of plant compounds which are effective in curing certain diseases has renewed the herbal medicines.

Thrombus is formed by the adhering of the damaged regions (caused by reactive oxygen species) of the endothelial cell surface, where platelets a vital role in the formation process. The process of thrombosis is initiated when the activated platelets form platelets to platelets bonds, and also bind to the leucocytes and bringing them into a complex process of plaque formation and growth (Prentice *et al*., 2011). Plasmin is a natural fibrinolytic agent, lyses clot by breaking down the fibrinogen and fibrin contained in a clot. Streptokinase forms a 1:1 stochiometric complex with plasminogen that can convert additional plasminogen to plasmin (Banerjee *et al*.,2011). Moreover, phlorotannin, isolated from marine brown algae, have a unique property in promotion of dissolution of intravascular blood clot via antiplasmin inhibition. (Prasad *et al*., 2011). Since phytochemical analysis showed that the crude extract contains tannins, alkaloid and saponin could be participated for its clot lysis activity. The *in vitro* thrombolytic activity of water extract of Green tea leaves exhibited highest thrombolytic activity (45.60 ±2.313%). Thrombolytic activity was demonstrated by the ethanol and acetone extracts showed 37.68 ±2.21% and 30.51% ± 2.551% ± 2.551% respectively (Hossain and Shabrin, 2014).

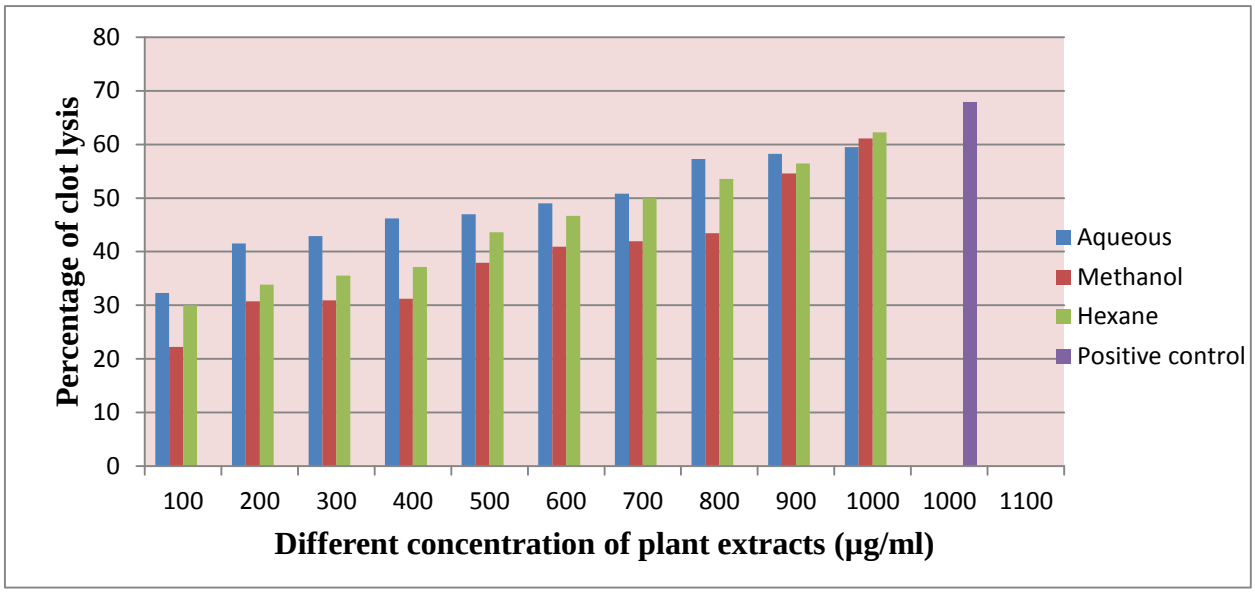
In recent past two decades towards the exploration, discovery, designing and development of natural products with antiplatelet (Briggs *et al.*, 2001), anticoagulant (Zhiguang *et al.*, 2000), antithrombotic (Rajapakse *et al*., 2005) and thrombolytic activity of the medicinl plants **(**Atiar Rahman *et al*., 2013). Few plant extracts and their products having fibrinolytic activity are identified, which includes *Lumbricus rubellus* (Jeon *et al*., 1995), *Pleurotus ostreatus (*Choi *et al.*, 1998), *Spirodela polyrhiza* (Choi, 2001), *Flammulina velutipes* (Shin, 1998) and *Ganoderma lucidum* (Choi, 2000), Ginger (*Zingiber officinale*) (Verma, 2001), Garlic (*Allium sativum*) (Bordia et al., 1998).

Table: 2 *In-vitro* thrombolytic activity of different extracts of *p. deamia* (Forsk.) stem

S.no	Concentration of plant extract (µg/ml)	% of clot thrombolysis (90 minutes)			Blank Negative (control)	Aspirin Positive (control)
		Aqueous	Methanol	Hexane		
1.	100	32.28±0.56	22.22±1.00	30.00±0.82	3.34±0.52	67.76±3.42
2.	200	41.50±0.91	30.76±1.08	33.84±1.05		
3.	300	42.90±0.92	30.95±2.22	35.55±1.24		
4.	400	46.21±1.02	31.25±2.52	37.14±1.50		
5.	500	47.00±1.24	37.93±3.56	43.63±2.00		
6	600	49.01±1.50	40.90±4.11	46.66±2.50		
7.	700	50.83±2.20	41.96±4.22	50.00±3.00		
8.	800	57.27±3.06	43.47±4.35	53.58±3.60		
9.	900	58.26±4.50	54.56±4.54	56.42±4.00		
10.	1000	59.50±5.02	61.11±5.28	62.22±4.21		

(Values are expressed as mean ± standard deviation of 3 reading)

Figure:1 Thrombolytic activity of different extracts of *P. deamia* (Forsk.) Stem



CONCLUSION

Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Herbal medicines generally deal with plants and its extracts for treatment of various ailments. These are usually considered to be safer and with no side effects. *In vitro* Thrombolytic activity of various extracts of *P. deamia* against blood clot. The result revealed that the hexane extracts of *P. deamia* possess high thrombolytic properties which were compared to aqueous and methanol extracts of plant. Clot lysis may be presence of the tannins, alkaloids, saponins and other active compounds. The plant also used

to discover bioactive natural products that may severe as leads for the development of new pharmaceutical compounds. Further more active plant extracts can be subjected to various chemical evaluations by several methods for the isolation of biological active agent. In future it may be incorporated as a thrombolytic agent for the improvement of the patients from atherothrombotic disease.

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