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EVALUATION OF THROMBOLYTIC EFFICACY OF HERBS: *JUSTICIA ADHATODA* AND *ACACIA NILOTICA* BY *IN-VITRO* CLOT LYSIS TECHNIQUE

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Abstract

Justicia adhatoda (Acanthaceae) and *Acacia nilotica* (Fabaceae) are commonly found in India, These plants are listed for their high phenolic content and wide range of pharmacological properties. Leaves and Stem part of *J.adhatoda* and Bark of *Acacia nilotica* were evaluated for their efficacy as Thrmbolytics in the present study. Herbs were extracted successively with Pet.ether, Chloroform, Methanol, ethanol and water. Extracts were standardized and estimated for their phenolic content by Folin-Ciocalteu technique. Thrombolytic activity of all the extracts was evaluated by *In-vitro* clot lysis technique. All the extracts exhibited thrombolytic activities; however Methanolic extracts had better efficacy as compared to other solvent extracts. Efficacy was highest in case of methanolic extracts of *J.adhatoda* (leaves) followed by *A.nilotica* (bark) and *J.adhatoda* (stem). Phenolic content of the plants were also in the same order. Increased thrombolytic activity was observed with combination of extracts of *J.adhatoda* (stems and leaves) over individual extracts. From the results we could observe that phenolic compound responsible for thrombolytic activity and the study supports the choice of these drugs being and as effective drugs in treating coronary heart diseases and also the approach of using combination of herbs rather individual herbs in traditional system of medicine.

Keywords: *Justicia adhatoda, Acacia nilotica*, Total phenolic content, Folin-Ciocalteu, Thrombolytic activity, Clot lysis.

Introduction

Myocardial infraction commonly known as heart attack. This occurs when blood flow stops to a part of the heart causing damage to the heart muscle. According to recent data, approximately 30% of the urban population and 15% of the population living in rural areas suffer from high blood pressure and heart attacks. Every year 2.4 million Indians die due to heart diseases. Mostly occurs in people aged over 50 and it becomes more common with increasing age.(1) The series of chemical reactions that culminates in the formation of fibrin threads is called as clotting or coagulation. Thrombosis is the process of formation of clotting in an undamaged blood vessel. The clot itself is called thrombus which usually occurs on inner walls of blood vessels or heart. (2) Thrombus appears in blood circulatory system due to hemostasis, due to damaged blood vessels and causes vascular blockage and leads to atherothrombotic diseases such as acute myocardial infraction disseminated intravascular coagulation, deep vein thrombosis and pulmonary embolism. (3) Thrombolytic agents are chemical substances that are injected into the body to dissolve blood clots that are already formed and to restore circulation. They either directly or indirectly activate plasminogen, which is the key factor and initiates thrombolysis. (2)

In allopathic system of medicine, drugs like Emanate (anistrepals), retavase (reteplase), streptase (streptokinase), t-PA (class of drugs that includes activase), TNKase (tenecteplase and Abbokinase have been used for various conditions involving thrombolytic activity. These drugs causes various adverse reactions such as bleeding and irregular heart rhythms, skin rashes, dizziness, head injuries, muscle aches, uncontrolled high blood pressure and uneven heart rate.(4) Many herbal drugs are very effective used treating such disease since ages. Most herbs are environmental friendly, economical and cure diseases completely. They are abundantly available and do not cause any serious side effects like allopathic medicines hence they can be used to treat the disease in a better way. (5)

In this regard, the present study aims to evaluate Thrombolytic properties of *Jasticia adhatoda* (vasaca) leaves and stems, and *Acacia nilotica* (gum Arabic tree) bark. Traditionally these plants are indicated for heart problems but there are no scientific data to support such claims. It's been observed that many of the Allopathic drugs individual as thrombolytics are phenolic moiteis. There we attempt to explore the possibilities of these plants being used for treating heart diluents may be due to phenolic constituents present in these herbs.(6)

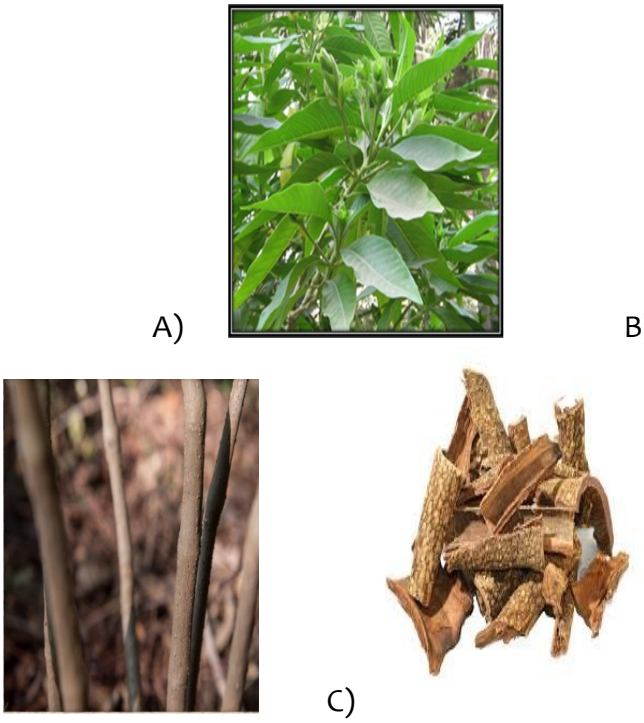

Plate No. 1. (A) *J. adhatoda* Leaves (B) *J. adhatoda* Stems (C) *Acacia nilotica* Bark

Materials and Methods

Collection of plant material

Dried leaves of *Justicia adhatoda* and *Acacia nilotica* (bark) was collected from Medi herb

and *Justicia adhatoda* (stem) was collected from natural remedies Bangalore. Identification and authentication of plant materials was done by Dr. Siddamallayya and Dr. Rama Rao, at National Ayurvedic Dietetics Research Institute, Bangalore. Letter No: 1158.

Successive solvent extraction of plant materials

Accurately weighed powdered drug was soxhlated successively with Petroleum ether (60-80°c), Chloroform, Methanol, 95% alcohol and final refluxation with water for 8 hours. The extracts were collected and each time before next extraction powdered material was air dried and weight was noted. All the extracts were filtered through Whattmann filter paper number 1 and then concentrated at low temperature (40-50°C), and air dried. Percentage yield, color and consistency of the extracts were noted.

Preliminary phytochemical screening for extracts

All the solvent extracts of *J.adhatoda* (Leaves and Stems) and *A.nilotica* (Bark) were analysed for the

Presence of phytoconstituents by Qualitative chemical tests by standard procedures.

Total phenolic content (T P)

Total phenol of the extracts was determined by Folin ciocalteau method using Gallic acid as standard.

The standard stock solution of Gallic acid 100mg/ml was prepared.

0.5ml, 1.0ml, 1.5ml, 2.0ml and 2.5ml of above stock solution was pipetted into 25ml of volumetric flask. Methanol and Ethanol extracts of *Justicia adhatoda* (Leaves & Stem) and *Acacia nilotica* (Bark) were dissolved separately in methanol to get 1mg/ml concentration. 0.5ml of each test extract solution was pipetted into 25ml volumetric flask (taken in triplicate). Blank was prepared with 1ml of solvent methanol (Without test extract or standard). 1.5ml of Folin ciocalteau reagent was added to volumetric flasks of test extract, standard and blank. After 5 min, 4ml of 20% $NaHCO_3$ solution was added to the volumetric flasks of test extract, standard and blank. Volume was made upto 25ml with distilled water. The samples were incubated at room temperature in dark for 45 min. After 45 min absorbance was measured at 765nm using UV spectrophotometer. Calibration curve was construed for standard Gallic acid. Based on the absorbance of test extract, concentration of phenolics in the extracts was expressed in terms of gallic acid equivalent (GAE) (mg of GA/g of extract). The concentration of Total Phenolic Compounds in the extract was determined by using the formula: (7)

Formula

T = Total phenolic content mg/g of plant extract

C = Concentration of gallic acid from the calibration curve

T = CV/M V = Volume of the extract in ml

M = wt of the pure plant methanol extract (7)

***In-vitro* thrombolytic activity of extracts of selected plants by clot lysis method**

Materials required:

Preparation of Phosphate buffered saline (pH -7.4) : Dissolved 2.38gm of disodium hydrogen phosphate, 0.19gm of potassium dihydrogen phosphate and 8.0gm of sodium chloride in sufficient water to produce 1000ml.

Standard preparation: To the commercially available lyophilized streptokinase vial (STPASE, 15,00,000I.U, manufactured by Cadila Healthcare) 5 ml phosphate buffered saline (PBS) was added and mixed properly. From the above stock solution 100ml was pipetted and dilutions were made with sterile distilled water in the ratio (1:0, 3:4, 1:2, and 1:3) to get 30,000 I.U, 22,500 I.U, 15000 I.U, and 7,500 I.U.

Sample preparation: Extracts were taken at concentration of 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml and 1000µg/ml concentration in respective solvents.

Procedure

Collection of blood sample: Blood sample was collected from the rats by Retro orbital. 500µl of blood was taken in to labelled microcentrifuge tubes which were previously weighed [W_1] and sterilized. Tubes were incubated at 37°C for 45 min to facilitate clot formation.

Procedure: Blood was transferred in different pre weighed sterile microcentrifuge tube (500µl/tube) and incubated at 37°C for 45 minutes. Once clot is formed serum was completely removed (aspirated out without disturbing the clot formed) and each tube

having clot was again weighed [W_2] to determine the clot weight [W_2-W_1]. 100µl of different concentrations of standard (streptokinase) was added. 100µl of test samples of both *A.nilotica* and *J.adhatoda* at different concentration were added to each of labelled tubes. Water was added to one of the tubes containing clot to serve as negative control. Standard, Test and Negative control tubes were incubated at 37°C for 90 minutes. All the samples were taken in triplicates. (8)

Statistical analysis

The mean clot lysis percent of streptokinase and extracts with different concentrations was compared with water using the repeated measures ANOVA with Tukey post test. Data are expressed as mean± SEM. A p value ≤ 0.05 was considered to be statistically significant. (8)

Results and discussion

Preliminary phytochemical screening of the extracts

***Justicia adhatoda* (leaves):** Pet. Ether extract showed the presence of saponins and flavonoids. Chloroform extract contained alkaloids. Methanol and Ethanol extract exhibited presence of alkaloids, glycosides, saponins, flavonoids and Phenols. In aqueous extract saponins, alkaloids, glycosides were present.

***Justcia adhathoda* (stem):** Pet. Ether extract showed the presence of alkaloids, saponins and flavonoids. Chloroform extract contained alkaloids. Methanol and Ethanol extract exhibited presence of alkaloids, saponins, phenols and carbohydrates. In aqueous extract alkaloids and Saponins were present.

***Acacia nilotica* (bark):** Pet. Ether and chloroform extracts showed the presence of alkaloids. Alcoholic extract exhibited presence of alkaloids, tannins, steroids and Phenols. In aqueous extract alkaloids and saponins were present.

Table No. 01: Total phenolic content for leaves and stems of *Justicia adhatoda* and bark of *Acacia nilotica*

Phenolic content was in the order of Methanolic extracts of *J.adhatoda* (leaves) 82.1% > Methanolic extracts of *A.nilotica* (bark) 79.63% > Methanolic extracts of *J.adhatoda* (stem) 55.5%

Screening of successive extracts of selected plants for *in-vitro* thrombolytic activity

Pet ether, chloroform, methanol, ethanol and water extracts of ***J.adhatoda* (leaves), *J.adhatoda* (stem)** and ***A.nilotica* (bark)** were screened for *in-vitro* thrombolytic activity at 200,400,600,800 and 1000µg/ml concentration. Streptokinase was used as the standard at 30000, 22500, 15000 and 7500 I.U the concentration. The experiment was performed in triplicate.

Conclusions

Results of the study give a scientific support for the traditional claims for the plants *J.adhatoda* and *A.nilotica* as thrombolytic agents. According to this study a significant and linear relationship exist between the thrombolytic activity and phenolic contents, indicating that phenolic compounds could be major contributors for the thrombolytic activity.

These drug extracts can be useful as safe, economic alternative remedy as thrombolytic agents individually or in combination.

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Table No. 01: Total phenolic content for leaves and stems of *Justicia adhatoda* and bark of *Acacia nilotica*

SL. NO	Solvents	*Justicia adhatoda* (leaves)*	*Justicia adhatoda* (Stems)*	*Acacia nilotica* (Bark)*
1	Methanol	82.1±0.31%	55.5±0.15%	79.63±0.68%
2	Ethanol	62.4±0.49%	32.63±0.23%	75.16±28%

*Values are expressed in terms of Mean± SEM of results done in Triplicates

Phenolic content was in the order of Methanolic extracts of *J.adhatoda* (leaves) 82.1% > Methanolic extracts of *A.nilotica* (bark) 79.63% > Methanolic extracts of *J.adhatoda* (stem) 55.5%

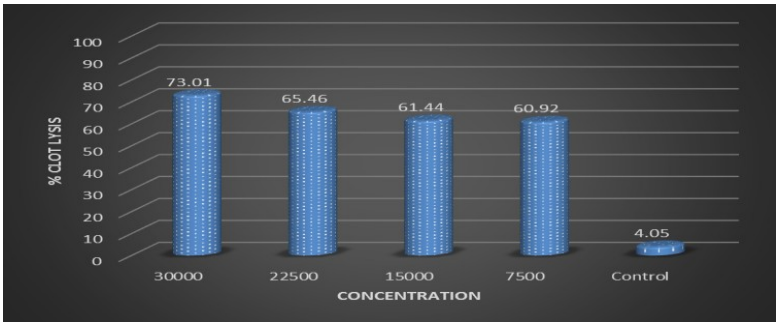
Plate No. 02: Photograph of Clot lysis of blood

30000 22500 15000 7500 Control

The % clot lysis at varying concentrations is represented in **Table No. 2, 3, 4, 5, 6.**

Table No. 02: Thrombolytic activity of standard streptokinase

Concentration I.U	% Clot lysis
30000	73.01±0.011***
22,500	65.46±0.023***
15,000	61.44±0.026***
7,500	60.92±0.011***
Control	4.05±0.011



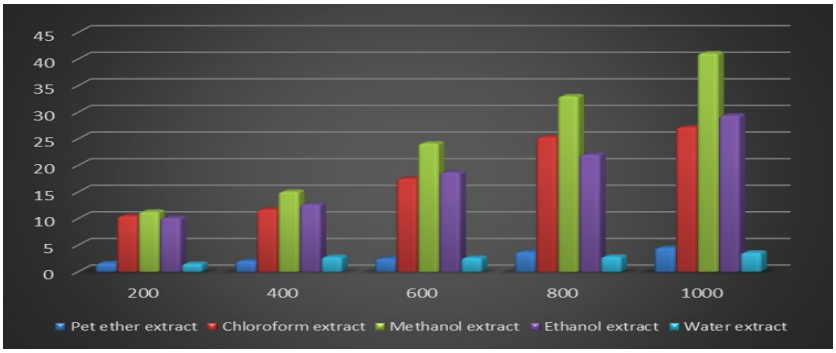
Streptokinase exhibited maximum clot lysis of 73.01%, 65.46%, 61.44%, and 60.92% clot lysis for 30,000I.U, 22,500I.U, 15,000I.U, 7500I.U. concentrations, respectively various research works are undertaken in case of thrombolytic drugs.

Table No. 03: Thrombolytic activity of Justicia adhatoda (leaves)

Concentration µg/ml	% Clot lysis				
	Pet ether	Chloroform	Methanol	Ethanol	Water
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
200	1.62±0.035	10.45±0.007	11.28±0.005	10.17±0.009	1.56±0.02
400	2.56±0.046	11.68±0.002	15.05±0.002	12.68±0.006	2.85±0.24
600	2.47±0.025	17.5±0.005	24.13±0.007	18.63±0.015	2.64±0.62
800	3.71±0.012	25.31±0.008	33.10±0.001	22.02±0.009	2.85±0.018
1,000	4.383±0.07^{ns}	27.14±0.008***	41.03±0.007***	29.37±0.004***	2.166±0.183

Values expressed as Mean ±SEM (n=5) ns - p>0.05, * -P<0.05, **- p<0.01, *** - p<0.001

Fig No. 02: Thrombolytic activity of *J.adhatoda* (Leaves)



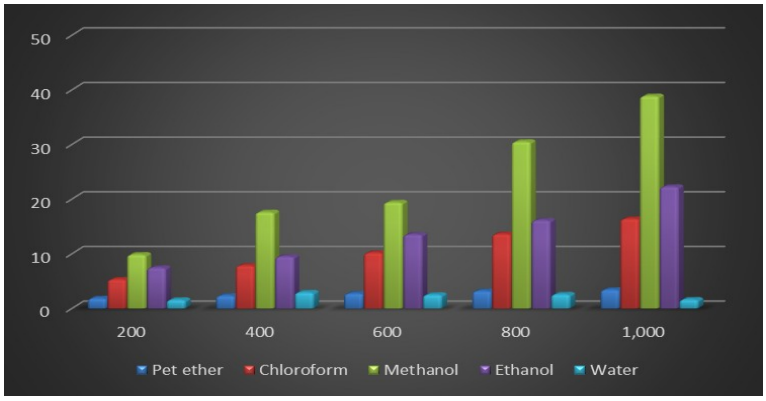
Methanolic extract of *J.adhatoda* (leaves) exhibited maximum clot lysis of 41.07% at 1000µg/ml clot lysis activity respectively, which highly significant ($P < 0.001$) were comparing with negative control (normal saline**).**

Table No. 04: Thrombolytic activity of *Justicia adhatoda* (stem)

Concentration µg/ml	% Clot lysis				
	Pet ether	Chloroform	Methanol	Ethanol	Water
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
200	1.84±0.02	5.28±0.002	9.84±0.05	7.42±0.06	1.56±0.08
400	2.31±0.61	7.82±0.006	17.6±0.012	9.45±0.52	2.92±0.81
600	2.71±0.14	10.15±0.052	19.4±0.011	13.5±0.16	2.49±0.16
800	3.16±0.23	13.58±0.011	30.5±0.015	16.1±0.11	2.56±0.11
1,000	3.40±0.112^{ns}	16.37±0.001***	38.843±0.070***	22.25±0.042***	1.61±0.149

Values expressed as Mean ±SEM (n=5) ns - p>0.05, * -P<0.05, **- p<0.01, *** - p<0.001

Fig No. 03: Thrombolytic activity of *J.adhatoda* (stem)



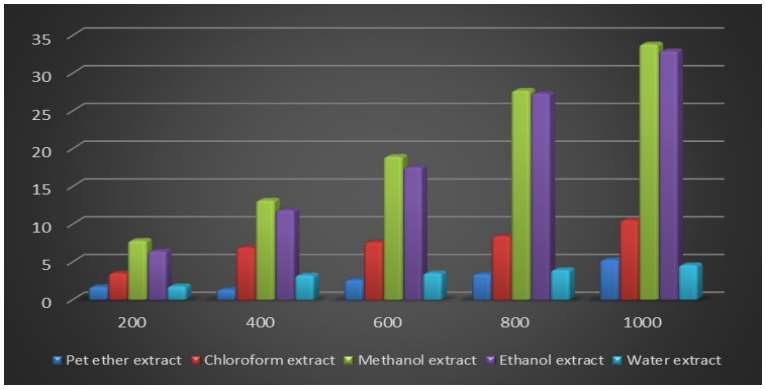
Methanolic extract of *J.adhatoda* (Stems) exhibited maximum clot lysis of 38.93% at 1000µg/ml clot lysis activity respectively, which highly significant ($P < 0.001$) were comparing with negative control (normal saline).

Table No. 05: Thrombolytic activity of *Acacia nilotica* (Bark)

Concentration µg/ml	% Clot lysis				
	Pet ether	Chloroform	Methanol	Ethanol	Water
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
200	1.75±0.12	3.52±0.081	7.83±0.003	6.42±0.02	1.82±0.12
400	1.33±0.012	6.93±0.001	13.16±0.05	11.85±0.16	3.24±0.29
600	2.62±0.48	7.64±0.32	18.96±0.012	17.54±0.04	3.52±0.18
800	3.39±0.62	8.42±0.104	27.7±0.104	27.3±0.02	3.94±0.61
1,000	4.256±0.01^{ns}	10.38±0.004***	33.5±0.0017***	32.93±0.009***	2.18±0.1621

Values expressed as Mean ±SEM (n=5) ns - $p>0.05$, * -$P<0.05$, **- $p<0.01$, *** - $p<0.001$

Fig No. 04: Thrombolytic activity of *A.nilotica* (Bark)



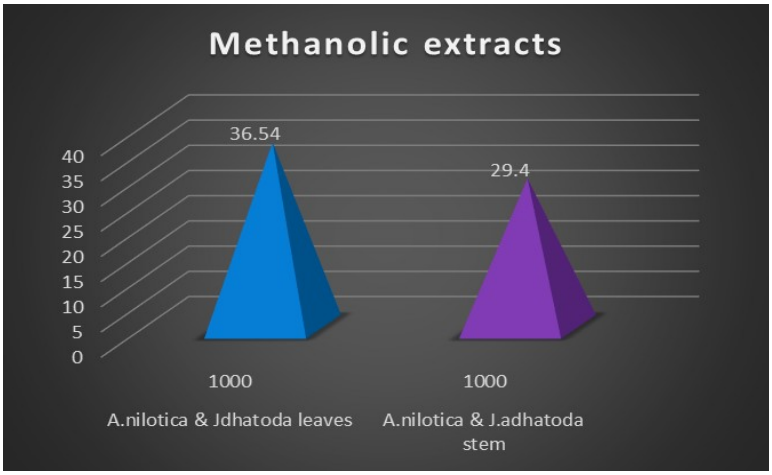
Methanolic extract of *A.nolotica* (Bark) exhibited maximum clot lysis of 33.85% at 1000µg/ml clot lysis activity respectively, which highly significant (P < 0.001) were comparing with negative control (normal saline).

Table No.06: Thrombolytic activity of *J.adhatoda* and *A.nilotica* of combined extracts

Combination of extracts	Concentration µg/ml	Clot lysis
		Methanolic extracts
		Mean±SEM
A.nilotica & *J.adhatoda* (leaves)	(500+500)µg/ml	36.32±0.091***
A.nilotica & *J.adhatoda* (stems)	(500+500) µg/ml	29.41±0.077***

Values expressed as Mean ±SEM (n=5) ns - p>0.05, * -P<0.05, **- p<0.01, * - p<0.001**

Fig No. 05: Thrombolytic activity of *J.adhatoda* and *A.nilotica* of combined extracts



Among various extracts and extract combinations of *A.nilotica* and *J.adhatoda*, methanolic extracts of the herbs had highest activity. There was not must significant difference in Thrombolytic activity of individual extracts as compared to that of extract combination.