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Full Length Research Paper

Determination of *in vitro* thrombolytic activity in isolated flavonoids of plant Madras Nelli

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The study is regarded for standardization of aerial parts of the plant *Phyllanthus maderaspatensis* L., phytochemical investigations, isolation of flavonoids and evaluation for thrombolytic activity of alcoholic extract and butanolic fraction of alcoholic extract. Standardization study includes macroscopy, microscopy and determination of proximate values and phytochemical investigations include extraction of coarsely powdered herb with exhaustive alcoholic and aqueous extractions and successive extraction with petroleum ether, chloroform and alcohol. The extracts were subjected to qualitative chemical analysis, chromatographic studies [Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC)] were performed for flavonoids detection in alcoholic extract. Isolated two flavonoids from butanolic fraction of alcoholic extract by column chromatography and preparative TLC, their partial characterization was done with Proton Nuclear Magnetic Resonance (PNMR) and Fourrier Transform Infrared (FT-IR) spectral data. Thrombolytic activity of alcoholic extract and butanolic fraction of alcoholic extract (20 and 10 mg/100 µl) were evaluated in incubated blood. An attempt made to standardize aerial parts of *P. maderaspatensis* L., was successful. Two flavonoids were isolated; the alcoholic extract showed significant thrombolytic effect in comparison with thrombosis control in comparison with standard thrombolytic agent streptokinase (30000 unit/100 µl).

Key words: *Phyllanthus maderaspatensis* L., phytochemistry, flavonoids, column chromatography, thrombolytic activity.

INTRODUCTION

Herbal medicine, also known as traditional medicine, is practiced all over the world, and herbs have been the primary source of most pharmaceuticals for ages. Medicinal plants have a large number of chemical compounds that are used as medicinal agents to treat human ailments. Human health care has improved as a result of recent discoveries and advancements in medicinal and aromatic plants (Oladeji, 2016).

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Herbal medication has a long history of use and is more tolerated and accepted by patients. Medicinal plants have a renewable source, which is our only hope for ensuring a steady supply of lower-cost medications for the world's rapidly rising population. Similarly, availability is not an issue, especially in emerging countries with diverse agro-climatic, cultural, and ethnic biodiversity, such as Nepal and India (Ekor, 2014).

The creation of a blood clot inside blood arteries obstructs the flow of blood through the circulatory system, causing thrombosis. When a blood vessel is injured, the body forms a blood clot of platelets (thrombocytes) and fibrin to prevent blood loss. Even if a blood artery is not damaged, blood clots can grow in the body under certain circumstances.

When a thrombus occupies more than 75% of the surface area of an artery's lumen, blood flow to the tissue supplied is reduced enough to cause systems due to decreased oxygen and the accumulation of metabolic products such as lactic acid. When a thrombus occupies more than 90% of the surface area of an artery's lumen, anoxia, or complete deprivation of oxygen, and infraction, a mode of cell death, can occur. By accelerating the synthesis of plasmin from plasminogen, thrombolytic medicines rapidly lyse thrombi (Periayah et al., 2017).

There is limited source of drugs for treating thrombosis. Very few plants are reported for having thrombolytic activity from the literature. Macro and microscopical characters of the plant part were used for the identification of the drug.

Madras Nelli is an annual or perennial, erect to glabrous herb of 90 to 120 cm tall branches angular-red brown. Leaf arranged spirally, simple and asymmetrical, triangular, lanceolate.

Flowers are unisexual, regular six lobes with six-disc free glands. Fruits globes capsules flattened at both ends 3 mm in diameter with shiny greenish six seeded and bitter in taste.

The flavonoids have major role in thrombolytic activity. As the plant *Madras Nelli* is reported for the presence of flavonoids and also reported for treating wide range of ailments, the plant is selected for the present investigation to establish scientific evidence for having thrombolytic activity.

MATERIALS AND METHODS

Collection of plant material

The plant *Madras Nelli* was collected from Chittur district of Andhra Pardesh. It was dried under shade and made coarse powder.

Identification

The plant material collected was identified and authenticated by Assistant Prof. (Dr.) K. Madhava Chetty, Department of Botany, Shree Vinkateswara University, Tirupati Chittur district, Andhra Pradesh.

Pharmacognostical studies

Different parameters, viz: macroscopy, microscopy and proximate values were investigated. The macroscopical features and microscopical features were investigated as per standard protocols. (Kokate et al., 2002; Mukherjee, 2002)

Preliminary phytochemical screening

The extract was made from the previously powdered drug. Extracting the plant material with several solvents of increasing polarity yielded various extracts. Then, to detect various chemical ingredients, chemical tests were done as shown in Table 1. (Williams and Chika, 2019).

Detection of carbohydrates

Small quantity of acetone, alcohol and aqueous extracts were dissolved separately in distilled water and filtered. The filtrate was subjected to various tests to detect the presence of different carbohydrates: Molisch's, Fehling's, and Barfoed's tests.

Detection of proteins and free amino acids

Small quantities of alcohol and aqueous extracts were diluted separately in water and tested for the presence of proteins and free amino acids by subjecting the extracts to various tests: Biuret's, Millon's, and Ninhydrin tests.

Isolation and characterization of phytoconstituents

The isolated pure phytoconstituents are most preferred than whole extract. The type of compound to be isolated was selected based on results of preliminary phytochemical screening.

Fractionation of alcoholic extract

The preliminary phytochemical screening indicated for the presence of flavonoids in alcoholic extract. To isolate flavonoids the alcoholic extract was first fractionated with chloroform and butanol solvent. The butanolic fraction showed the presence of flavonoids (by chemical test and TLC), the butanolic fraction of alcoholic extract selected for isolation of flavonoids by column and preparative TLC.

Isolation

Column chromatography was used for isolation of phytoconstituents.

Column chromatography

Column profile: Column length: 57 cm; 80% packing length; Column diameter: Outer & inner diameters: 1.5 and 1.1 cm respectively; Adsorbent: Silica gel 60 – 120 mesh for column chromatography [from Central Drug House (P) Ltd]; Type of column packing: Dry packing; Solvent system: Chloroform: methanol - 1:1; Volume of fraction collected: 5 ml.

Isolation of two flavonoids by preparative TLC from mixture: Preparative TLC plates: The size of TLC plates which was used for the preparative TLC was 10 × 20 cm. Preparation of Plates: A thick

Table 1. Detection of secondary metabolites.

S/N	Tests	Procedure	Result for positive test
1	General test for glycosides	200 mg of extract + 5 ml of dilute sulphuric acid + warm + filter + neutralized with 5% solution of sodium hydroxide + 0.1 ml of Fehling's solution A and B and heat on a water bath for 2 min	Red precipitate
2	Detection of phytosterols and triterpenoids	extracts refluxed with ether + evaporated to dryness + residue subjected to Liebermann-Burchard's	Green colour in the upper layer indicates the presence of steroids and deep red colour indicates triterpenoids
3	Detection of tannins	Test solution + a few drops of 5% freshly prepared ferric chloride solution indicates the presence of tannins	A blue-black or green-black colour
4	Detection of flavanoids	test solution + few magnesium turnings + conc. HCl dropwise from the sides of test column	Pink, scarlet, crimson red or occasionally green to blue color appears after 5 min
5	Detection of alkaloids	Mayer's test Wagner's test Hager's test	Cream colour precipitate Brown precipitate Yellow precipitate
6	Saponin test	1 ml of extracts + 20 ml water + shake	Formation of froth above the surface

slurry of Silica gel G was prepared with distilled water and was spread on the clean and dry TLC plates. The thickness of the layer was 0.5 to 2.0 mm. To avoid cracking of thick layers, they were dried for several hours at room temperature before placing them in oven. Activation of TLC plates: For the activation, the TLC plates were heated in oven at 110°C for 30 min. Preparation of solvent system: was used as solvent system or mobile phase benzene-acetic acid in the ratio of 4.5:0.4. Preparation of sample: The solid residue obtained from pooled fractions is dissolved in alcohol to form a concentrated solution, which is applied on TLC plates. Application of sample:

The sample is applied in the form of bands (about 2 cm above from the bottom of the plate) and allowed to dry at room temperature. After drying the plates were placed in pre-saturated mobile phase in the developing chamber for development. Development of plates: After saturation of mobile phase the plates were kept into the development chamber and allowed to develop up to 3/4th of the plate.

Then the plates were removed and dried at room temperature. Identification of band for flavonoids: For the identification of flavonoids, a portion of developed plate was exposed in strong ammonia solution. The ammonia exposed plates were air dried, which showed 2 bands visible in UV light with R_f values 0.69 and 0.8. The areas corresponding to the R_f values was scrapped off. Collection of compounds (1 - 2): The scrapped portions were treated separately in alcohol. The treated portions were filtered and the filtrate was concentrated in a china dish on water bath to get the compounds. The final compound showed single spot by TLC studies.

Characterization of isolated flavonoid

The isolated steroids were characterized by further investigations with TLC studies and spectral data of IR & PNMR.

Pharmacological studies

Pharmacological investigation preparation of standard

Drug dilution was performed to the commercially available lyophilized streptokinase vial (15, 00,000 iu). Phosphate buffered saline (PBS, 5 ml) was added and mixed properly. This suspension was used as a standard drug dilution to observe the thrombolytic activity of the sample using *in vitro* model studied in laboratory.

Sample preparation

The air dried aerial part of *Phyllanthus maderaspatensis* was extracted with alcohol and total alcoholic extract fraction with n-butanol. Both extracts were concentrated and evaporated to dryness.

Thrombolytic activity

The dried extract was dissolved in distilled water. The thrombolytic activity was performed by *in-vitro* model. The blood samples (collected from slaughter house) was transferred in different pre-weighed sterile micro centrifuge tube (500 µl/tube) and incubated at 37°C for 45 min and allowed to stand. The serum was completely removed (aspirated out without disturbing the clot formed) after clot formation. Each tube having clot was again weighed to determine the clot weight.

Clot weight = weight of clot containing tube - weight of tube alone

Each microcentrifuge tube containing clot was properly labeled and 100 µl of streptokinase was taken as standard drug. 10 and 20

Table 2. Preliminary phytochemical screening of the aerial parts powder of Madras Nelli.

S/N	Test for	Pet. Ether extract	Chloroform extract	Ethanol extract	Water extract
1	Proteins and amino acids	-	-	+	+
2	Carbohydrates	-	-	+	+
3	Glycosides	-	-	-	-
4	Phytosterols and triterpenoids	+	-	-	-
5	Tannins	-	-	+	-
6	Flavanoids	-	-	+	-
7	Saponins	-	-	+	+
8	Alkaloids	-	-	-	-
10	Fats and fixed oils	-	-	-	-

+ = Present; - = absent.

mg/100 µl of isolated constituent of *Madras Nelli* and distilled water (as control) were added. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid obtained 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 test was repeated six times with all different dilution of the extract, standard drug, and control (Ali et al., 2013).

RESULTS AND DISCUSSION

Pharmacognostical studies

In powder analysis, aerial part showed for the presence of fragments of cork cells, cell wall, fibres, stomata and calcium oxalate crystal and starch grains. The powder is light green in colour, has characteristic odour and bitter taste.

Proximate values

Various physical constants of aerial part of plant were performed like loss on drying, ash values and extractive values.Loss on drying 5% is very less, which indicates lower quantities or absence of volatile constituents. It also shows that the drug is dried enough to control the bacterial growth.Total ash value and acid-insoluble ash value was found to be 10 and 2%, respectively. The very low values of acid-insoluble ash represents that the drug is less adhered with dirt and sand which in turn represent the purity of the drug.

Aqueous extractive value is more compared to alcohol extractive value may be due to tannins. Both extractive values are found to be 11.80 and 9.80%, respectively.

Preliminary phytochemical screening

The qualitative chemical investigations were carried out to check for the presence of various phytoconstituents. The tests revealed the presence of phytosterols, flavonoids, and tannins as shown in Table 2.

HPTLC fingerprinting of alcoholic extract was performed which showed 4 spots which correspond to 5 different phytoconstituents. The corresponding R_f values are 0.49, 0.60, 0.70, 0.72, 0.77, and quantification of the spots obtained was performed and the percentage area of each spot was 0.08, 9.65, 19.64,1 9.69, and 40.94%, respectively as shown in Figures 1 to 3 and Table 3.

Isolation and characterization of phytoconstiuents

Isolation of flavonoid

Two different flavonoids were isolated from butanolic fraction of alcoholic extract by column chromatography followed by preparative TLC. The isolated flavonoids were characterized by Proton NMR and IR spectra. The IR spectra of both flavonoids show peaks representing Free N-H group, C=0, C=C, C=N, N=0, stretching, bending group, and C-C bending. The P NMR spectra of both flavonoids showed peaks representing groups such as CH_3 group, mm of CH_2 groups, bars of OH group, mm CH (singlet, duplet and triplet) in aromatic ring.

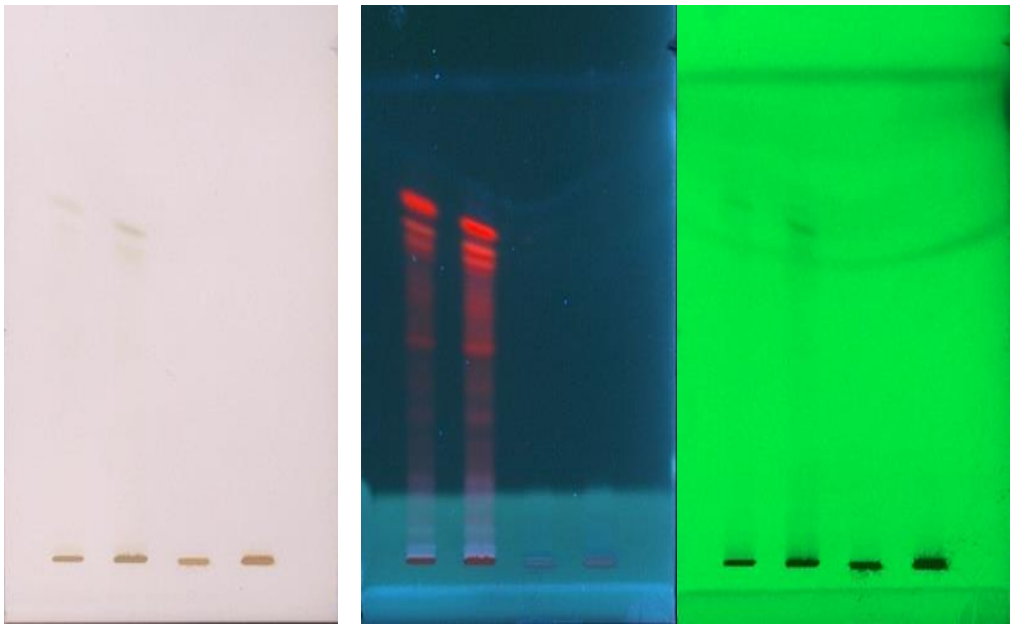
Results of column chromatography and Physical examination of Isolated Compounds are presented in Table 4 and 5 respectively.

Characterization

An attempt was made to characterize the isolated flavonoids (1 and 2) by subjecting them to IR and NMR spectral studies.

IR and PNMR spectrum of isolated steroids

IR spectra of flavonoids 1 and 2 are as shown in Figures



At 425 nm At 366 nm At 254 nm

Figure 1. HPTLC of alcoholic extract.

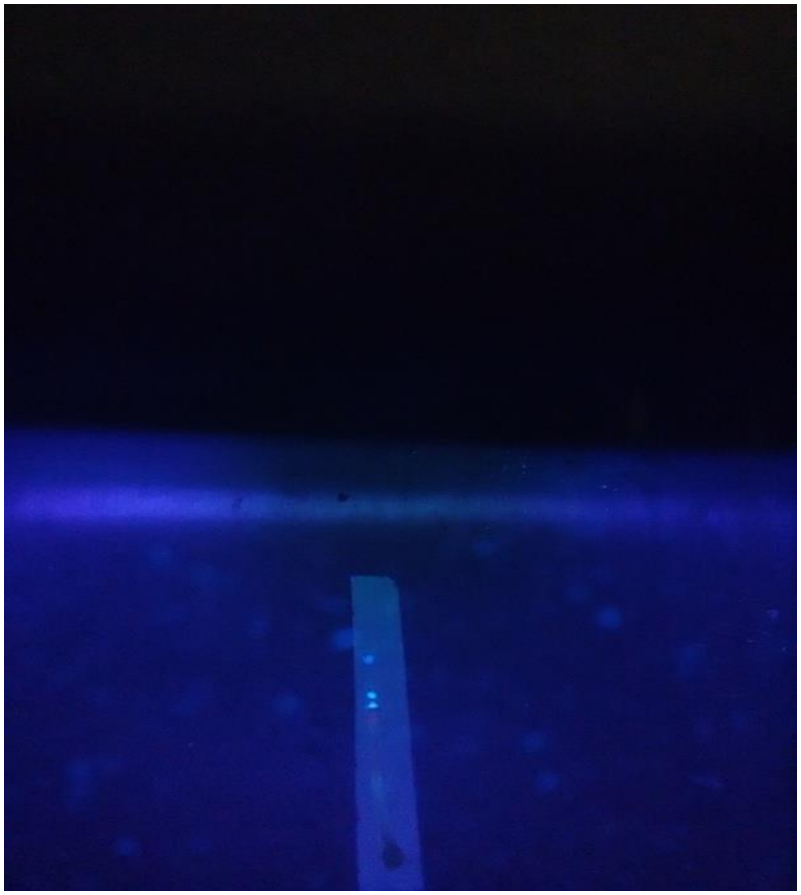


Figure 2.TLC of alcoholic extract.

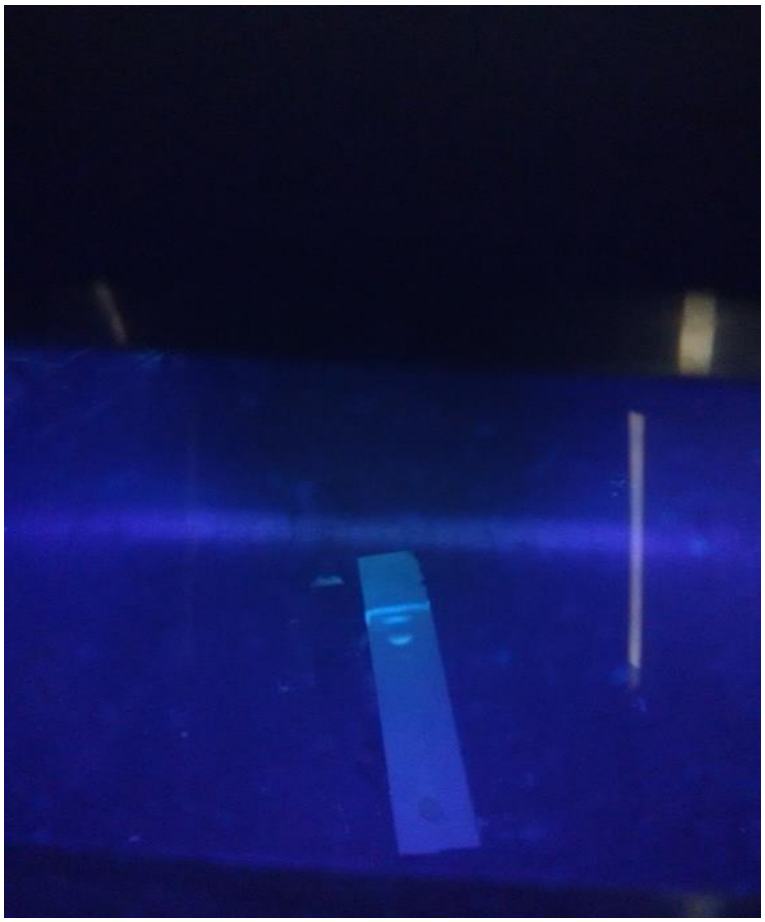


Figure 3. TLC of butanolic fraction of alcoholic extract.

Table 3. TLC of alcoholic extract and butanolic fraction of alcoholic extract for flavonoid detection.

S/N	Extract used	Number of spots	Rf value of spots
1	Alcoholic extract	3	Spot A: 0.86 Spot B: 0.73 Spot C: 0.60
2	Butanolic fraction of alcoholic extract	2	Spot A: 0.69 Spot B: 0.84

4 and 5, respectively. The interpreted data of IR spectrum is shown in Table 6.

PNMR spectra of flavonoids 1 and 2 are as shown in Figures 6 and 7, respectively. The interpreted data of PNMR spectrum is shown in Table 7.

Thrombolytic activity

The percentage of clot lysis for standard streptokinase (30000 unit/100 µl) was found to be 70.925%. For alcoholic extract (20 and 10 mg/100l), it was found to be 24.708 and 11.298%, respectively. The percentage clot lysis for butanolic fraction of alcoholic extract (20 and 10 mg/100 µl) was found to be 44.394 and 24.693%, respectively. Alcoholic extract and butanolic fraction of alcoholic extract showed thrombolytic activity. In comparison, concentration of 20 mg/100 µl of butanolic fraction of alchoholic extract having significant activity when compared with standard and with other

Table 4. Details of fractions collected in column chromatography.

S/N	Solvent system used	No. of fractions collected	Inference
1	Chloroform:Methanol (1:1)	22 (01 - 22)	No spots observed
2	Chloroform:Methanol (1:1)	22 (23 - 44)	Two spots observed in each fraction from 35 to 44

Table 5. Physical examination of isolated compounds.

S/N	Parameter	Unknown 1	Unknown 2
1	Colour	Pale pink, needle shaped	Pale pink, needle shaped
2	Odour	Characteristic	Characteristic
3	Solubility	Chloroform, methanol, ethanol	Chloroform, methanol, ethanol

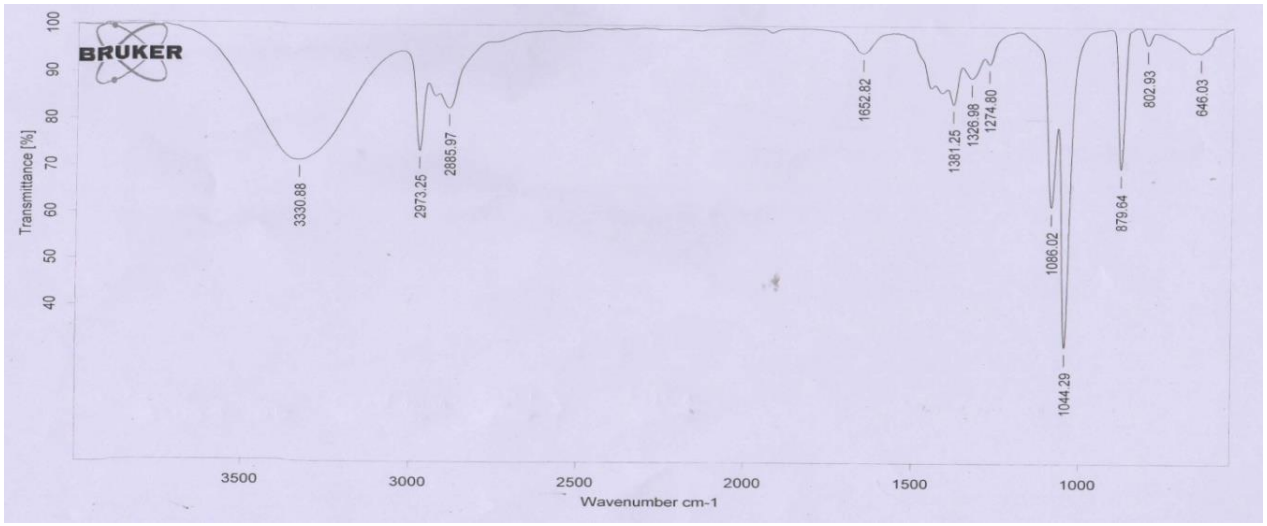


Figure 4. IR Spectrum of isolated flavonoid 1.

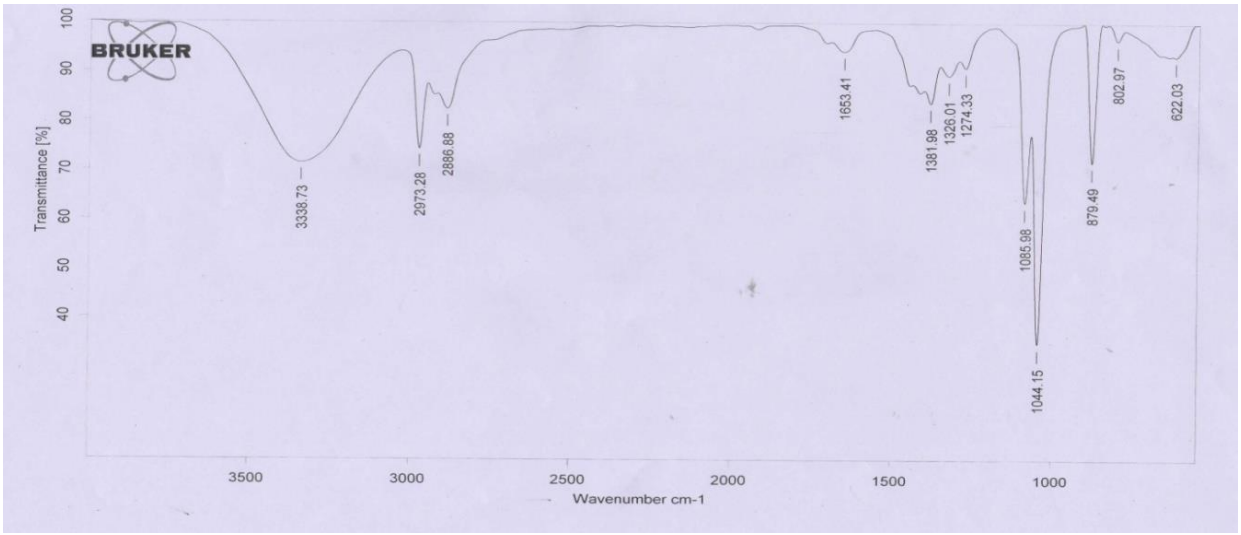


Figure 5. IR Spectrum of isolated flavonoid 2.

Table 6. IR Spectrum of isolated flavonoids 1 and 2.

S/N	Wavenumber	Functional group	Absorption frequencies (cm^{-1})	
			Isolated flavonoid 1	Isolated flavonoid 2
1	3300±100	N-H stretching	3330.85	3338.73
2	2900±100	C-H stretching	2973.25	2973.26
3	1600±50	C=C or C=O or C=N stretching	1652.82	1653.41
4	1400±50	N=O stretching	1381.25	1381.96
5	1200±800	C-C	1274.80	1274.32

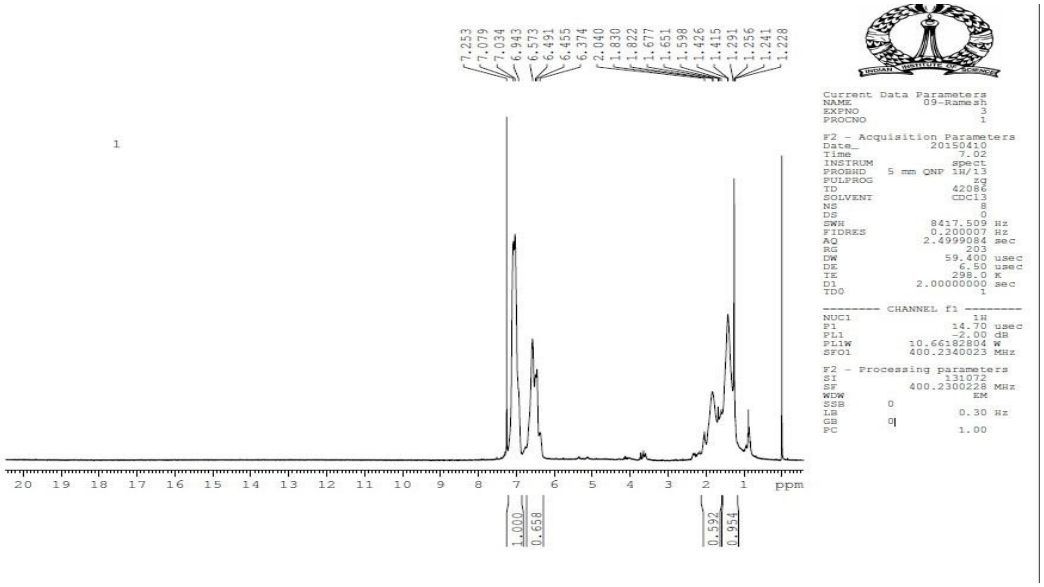


Figure 6. P-NMR Spectrum of isolated flavonoid 1.

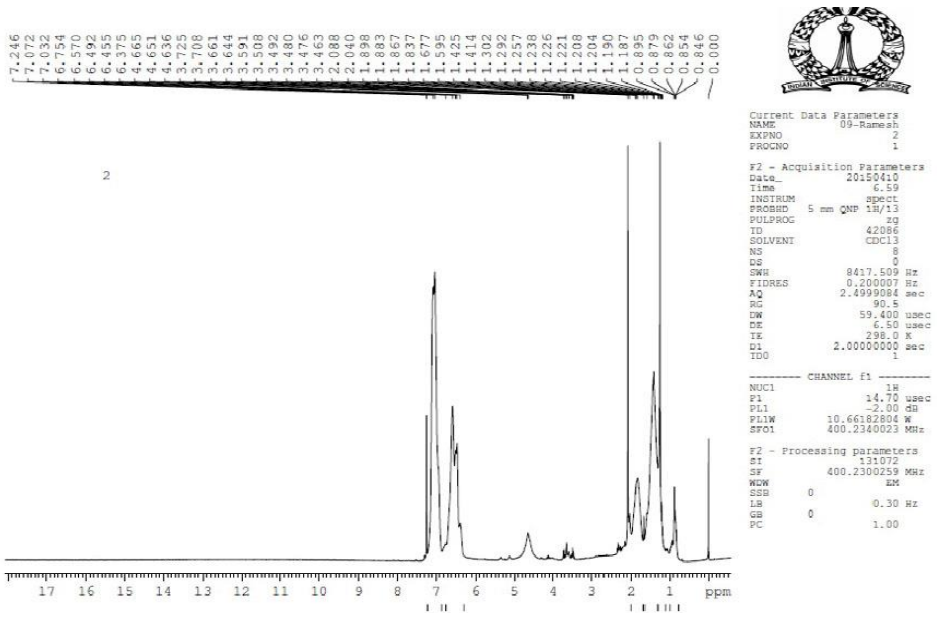


Figure 7. P-NMR spectrum of isolated flavonoid 2.

Table 7. Report of P-NMR spectrum of isolated flavonoids 1 and 2.

S/N	Isolated flavonoid 1		Isolated flavonoid 2	
	Signals	**Protons (No.)**	**Signals**	**Protons (No.)**
1	0.854-2.040	CH_3 group	0.846-2.08	CH_3 group
2	3.507-3.703	mmCH_2, groups	3.508-3.708	mm CH_2 groups
3	Absent	Absent	4.636	brs of OH group
4	6.374-6.573	mm CH – triplet of the aromatic ring	6.375-6.492	mm CH –triplet of the aromatic ring
5	6.943-7.079	mm CH – duplet of the aromatic ring	6.570-6.754	mm CH – duplet of the aromatic ring
6	7.021-7.253	mm CH – singlet of the aromatic ring	7.032-7.246	mm CH – singlet of the aromatic ring

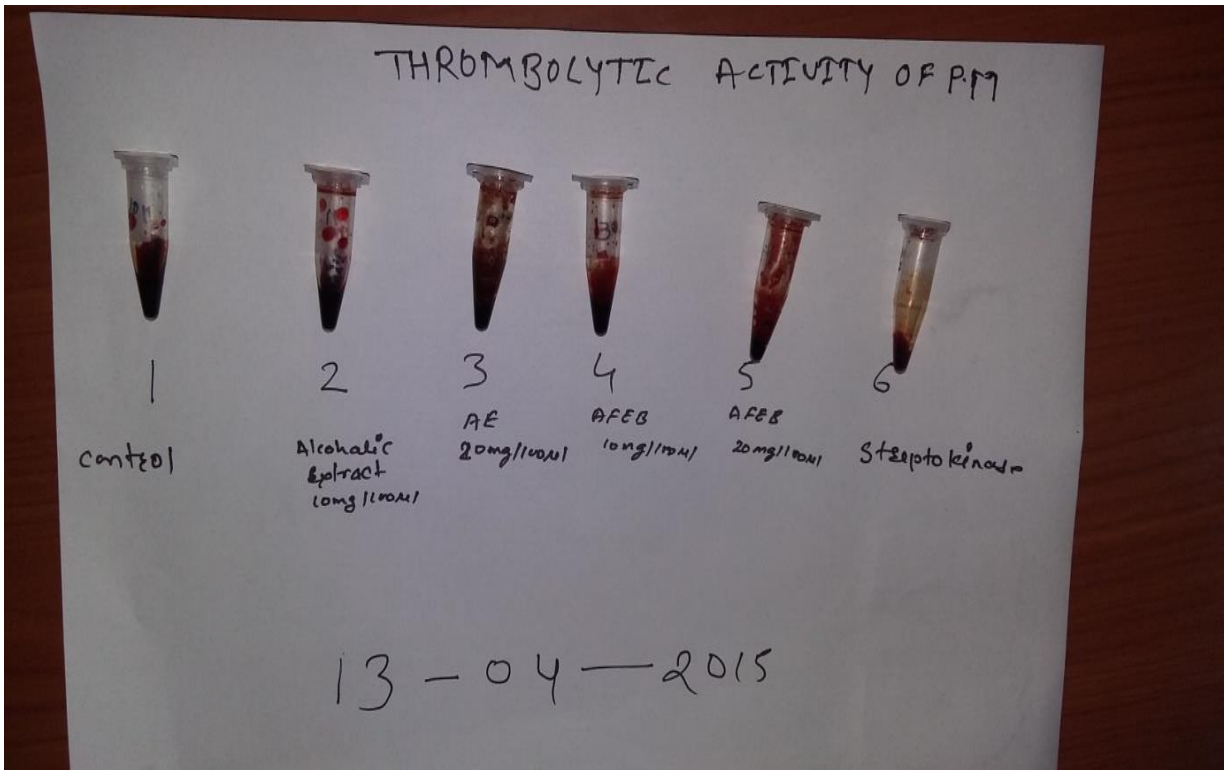


Figure 8. Thrombolytic activity of standard drug and plant extracts.

concentration as per literature, the flavonoids present in the alcoholic extract and in the butanolic fraction of alchoholic extract may be responsible for thrombolytic activity.

Details of thrombolytic activity of plant extracts are as shown in Figures 8 and 9 and Table 8.

Conclusion

An attempt was made to standardize the aerial part of Madras Nelli for morphological, microscopical and proximate values. HPTLC studies of alcoholic extract showed the presence of 5 spots which corresponds to different phytoconstituents. Isolation of two pure compounds (flavonoids) was done successfully. The isolated flavonoids were partially characterized based on P-NMR and IR spectral studies. Alcoholic extract and butanolic fractional of alcoholic extract at dose levels of10 and 20 mg/100 µl were tested for thrombolytic activity in animal blood. Butanolic fractional of alcoholic extract have shown significant activity on comparison with standard drug streptokinase.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

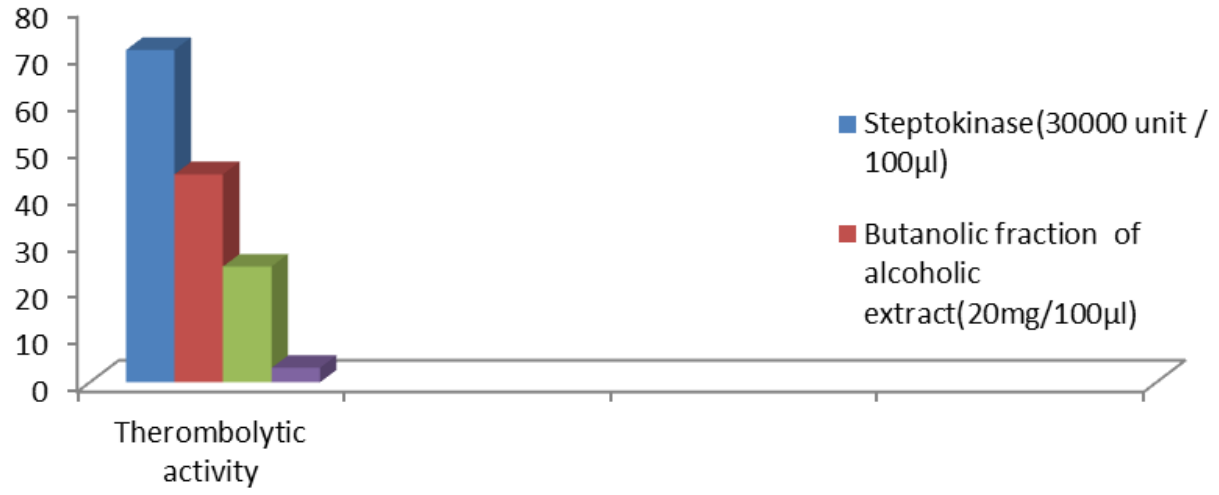


Figure 9. Percentage of clot lysis in different concentration of plant extracts, control vehicle and standard drug.

Table 8. Percentage of clot lysis in different concentration of plant extracts, control vehicle and standard drug.

Group	Treatment	Concentration	% clot lysis
I	Vehicle	100 µl	3.085
II	Streptokinase	30000 unit /100 µl	70.925
III	*Phyallanthus maderasptensis (Alcoholic Extract)*	20 mg/100 µl	24.708
IV	*Phyallanthus maderasptensis (Alcoholic Extract)*	10 mg/100 µl	11.298
V	Butanolic fraction of alcoholic extract.	20 mg/100 µl	44.394
VI	Butanolic fraction of alcoholic extract.	10 mg/100 µl	24.693

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