EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 6
731-739
Year: 2018

EVALUATION OF THROMBOLYTIC AND MEMBRANE STABILIZING ACTIVITY OF ETHANOLIC *NYMPHAEA NOUCHALI* EXTRACT

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Article Received on 17/04/2018 **Article Revised on 07/05/2018** **Article Accepted on 28/05/2018**

ABSTRACT
The present study is aimed to investigate in vitro thrombolytic and Membrane stabilizing activity of ***Nymphaea nouchali.*** Among the different fraction crude extract of leaf and stem. ***Nymphaea nouchali*** (Burm. f.) is commonly known as Blue lotus found in Sylhet, Bangladesh. DPPH radical scavenging activity. The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. Commercially available lyophilized Alteplase (Streptokinase) vial (Beacon pharmaceutical Ltd) of 15, 00, 000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30, 000 I.U) was used for *in vitro* thrombolysis., the ethanolic extract (EE) of stem inhibited 83.36% hemolysis of RBCs as compared to 83.52% produced by acetyl salicylic acid 10.0 mg/mL. Ethanolic extract of leaf and stem tested for clot lysis, crude ethanolic extract of stem proved its superiority maximum (54.829%)of clot lysis. Sterile distilled water as a negative control also showed negligible (13.357%) clot lysis. Standard Acetyl Salicylic Acid (ASA) or Aspirin was used as standard drug for comparison with different ethanolic extracts of leaves of *N.nouchali.* A buffer is an aqueous solution that has a highly stable pH. The buffer was prepared at pH 7 using monosodium phosphate and its conjugate base, disodium phosphate. The organic soluble materials of ethanol extract of the stems along with its various organic soluble partitionates were assessed for the membrane stabilizing activity as well as the thrombolytic activity of N. nouchali in the current study.

KEYWORDS: Thrombolytic, Membrane stabilizing, blood clot, Streptokinase, Aspirin.

INTRODUCTION
Nymphaea nouchali (blue lotus or water lily) is a day - blooming nonviviparous plant with submerged roots and stems, of the genus *Nymphaea* and family Nymphaeace. It is native to southern and eastern parts of Asia. The leaves are round having darker underside with green toppings and parts of the leaves are submerged. The floating leaves have a crenellated appearance which is due to the undulating edges of the leaves and their size is about 20-23 cm which spreads around 0.9 to 1.8 m. It is a kind of water lily which is usually blue or purple in color having 4-5 sepals and 13-15 petals which have angular appearance, due to this flower look star shaped from above.[12] Leaf, stem, flower, seed almost all parts of the plant is used for different purposes. From the very beginning, as a treatment of various diseases herbal preparations are being used and as they are natural they are assumed to be safe.[6] Foods having anti thrombotic effect can be used to reduce the risk of thrombosis which is provided by the epidermiologic studies as a evidence.[7] Unlike any other developing countries, in Bangladesh thromboembolic disorders are considered to be one of the major causes of morbidity as well as mortality.[8] Alteplase, anistreplase, streptokinase, urokinase, and tissue plasminogen activators are one of the most commonly used thrombolytic agents in order to dissolve clots.[9] Thrombolytic agents that are available, still tend to have some shortcomings. For instance, it needs on larger doses to have maximal effect, limited fibrin specificity as well as bleeding tendency. Plant compounds which are considered to be effective in curing particular diseases, has renewed part in the field of herbal medicine with the advancement that has been made in phytochemistry as well in identification of plant compounds. Further investigation in this respective area will not only provide new insight but also ameliorate progress to the upliftment of the thrombolytic therapy which is ideal and individualized by maximized stable coronary arterial thrombolysis with minimal bleeding.[10, 11]

MATERIALS AND METHODS

Collection of the plant parts

In September 2016, the stems of the plant N.nouchali were collected from mirpur, Dhaka which was identified by an adept taxonomist of National Herbarium of Bangladesh mirpur, Dhaka.

Collection and identification

At first with the help of a comprehensive literature review and the website of Medicinal Plant in Bangladesh ***Nymphaea nouchali*** from Nympheaceae was selected for this investigation. The leaves of this plant were collected from Mirpur at Dhaka, in September 2016 and identified by an expert taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka. The voucher specimens of the plants have been deposited in the Herbarium for further reference was signed by the Director of Bangladesh National Herbarium. They gave me an accession number which is**73884.**

Drying, Pulverization and Preservation of plant parts

The leaves of the plants were first washed properly to remove dirty materials and then sundried for 10 days. After complete drying, the dried plants were ground into coarse powder by a grinding machine and were stored in an airtight container for further use.

Preparation of plant extracts

There are some processes which are involved in preparing plant extracts.

Extraction of Plant materials

Powdered plant materials having a weight of about 500gm were taken in one Amber colored reagent bottle and soaked in 1000ml ethanol. The bottle with its contents were sealed and kept for a period of about 7 days with occasional shaking and stirring. The whole mixture was then filtered through cotton and then through Whatman No. 1 filters paper and was concentrated with a Rotary evaporator under reduced pressure at 50°C temperature to afford a gummy or semisolid concentrates and were designated as crude extracts of ethanol crude extract (45.39 gm).

Fig1: Evaporation of the solvent with rotary evaporator.

Solvent-solvent partitioning of crude extract

An aliquot (20gm) of the concentrated ethanolic extract was fractionated by modified Kupchan method and the resultant fractions that is n-Hexane Fraction (NHF, 5.89gm), DCM Fraction (DCMF, 4.36gm), Ethyl Acetate Fraction (EAF, 2.86gm) and Aqueous Fraction (AF, 4.36gm) soluble fractions were obtained and used for the experiment purpose.Solvent-solvent portioning was done using the protocol designed by kupchan and modified by Wagenen.

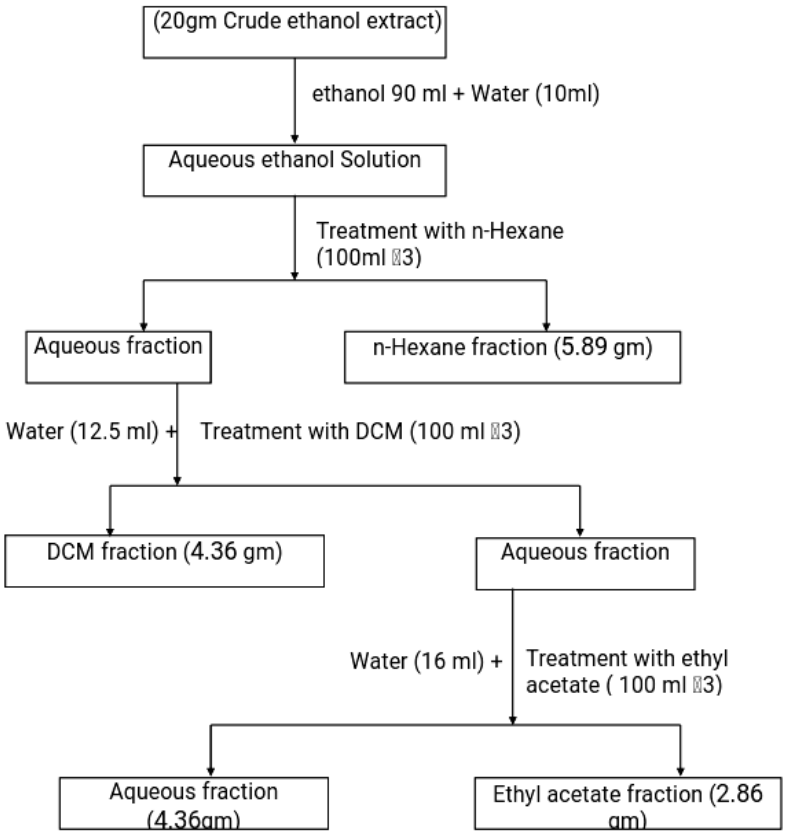

Fig 2: Schematic representation of solvent-solvent.

THROMBOLYTIC ACTIVITY TESTING METHOD

Preparation of sample

The thrombolytic activity of all extractives was evaluated by a method using streptokinase (SK) as standard substance. 10 mg of ethanolic extracts and its different fractions of leaves of *Nymphaea nouchali* were taken in different vials to which 1ml distilled water was added.

Streptokinase (SK)

Commercially available lyophilized Altepase (Streptokinase) vial (Beacon pharmaceutical Ltd) of 15, 00, 000 I.U., was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30, 000 I.U) was used for *in vitro* thrombolysis.

Blood Sample

Whole blood (n=10) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1ml of blood was transferred to the previously weighed sterile vials and was allowed to form clots.

Procedure

Aliquots (5 ml) of venous blood were drawn from healthy volunteers who were distributed in ten different pre weighed sterile vials (1 ml/tube) and incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each vial having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To each vial containing pre-weighed clot, 100 µl aqueous solutions of different partitionates along with the crude extracts was added separately. As a positive control, 100 µl of streptokinase (SK) and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control vials. All the vials were then incubated at 37 °C for 90 minutes and observed for clot lysis. After incubation, the released of fluid was removed and vials 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 as shown below.

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

MEMBRANE STABILIZING ACTIVITY TESTING METHOD

Preparation of the extract

Table1: Preparation of different extracts

Plant Part	Sample Code	Test Sample	Concentration
Leaves of *N. Nnouchali*	Hypotonic Medium	------	50 mM
	EE	Ethanolic Extract	1 mg/mL
	NHF	n-Hexane Fraction	1 mg/mL
	DCMF	DCM Fraction	1 mg/mL
	EAF	Ethyl acetate fraction	1 mg/mL
	AF	Aqueous fraction	1 mg/mL
	ASA	Acetyl Salicylic Acid	0.10 mg/mL

Solvent used: Ethanol analytical grade.

Drug

Standard Acetyl Salicylic Acid (ASA) or Aspirin was used as standard drug for comparison with different ethanolic extracts of leaves of *N.nouchali.*

Red Blood Cells (RBC) collection

Human RBCs were collected for the study. RBCs collected from the human was male, 70 kg, fare complexion and free from diseases. The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions of temperature 23±2°C and relative humidity 55±10%.

Preparation of Phosphate buffer solution

A buffer is an aqueous solution that has a highly stable pH. The buffer was prepared at pH 7 using monosodium phosphate and its conjugate base, disodium phosphate.

Phosphate buffer materials

Monosodium phosphate, Disodium phosphate, Water, pH meter, Glassware, Stirring bar.

Calculation of Phosphate buffer

A pH of about 7.4 with buffer strength of 10 mM was obtained using 0.0352% monosodium phosphate dehydrate and 0.1099% disodium phosphate anhydrate. The buffer was made by adding 0.352 gm monosodium phosphate dehydrate and 1.099 gm disodium phosphate anhydrate to 1000 mL water.

Preparation of isotonic solution

A solution that has a concentration of electrolytes, nonelectrolytes or a combination of the two that will exert equivalent osmotic pressure as that solution with which it is being compared. Either 0.16M sodium chloride (NaCl) solution (approximately 0.95% salt in water) or 0.3M nonelectrolyte solution is approximately isotonic with human red blood cells. For the preparation of 500 ml isotonic solution of 154 mM strength, 4.5045 gm NaCl was added and mixed.

Material for isotonic solution

Sodium chloride (NaCl)**,** Water**,** Glassware**,** Stirring bar.

Calculation for isotonic solution

1000ml solution of strength 1M contain = 58.5gm NaCl

500ml solution of strength 1M contain = 58.5/2 gm NaCl

500ml solution of strength 1000mM contain = 58.5/2 gm NaCl

500ml solution of strength 154mM contain = 58.5 × 154/2 × 1000 gm NaCl = 4.5045gm NaCl

Preparation of hypotonic solution

A solution of lower osmotic pressure than that of a reference solution or of an isotonic solution is called hypotonic solution. For the preparation of 500ml hypotonic solution, having strength of 50mM, 1.4625gm NaCl was added and mixed.

Materials for hypotonic solution

Sodium chloride (NaCl), Water. Glassware, Stirring bar.

Calculation for hypotonic solution

1000 ml solution of strength 1 M contain = 58.5 gm NaCl.

500 ml solution of strength 1 M contain = 58.5/2 gm NaCl.

500 ml solution of strength 1000 mM contain = 58.5/2 gm NaCl.

500 ml solution of strength 50 mM contain = 58.5 × 50/2 × 1000 gm NaCl = 1.4625 gm NaCl.

Effect on hemolysis:

Erythrocyte suspension

Whole blood was collected from male human under standard condition. EDTA was used to prevent clotting. The blood was washed three times with isotonic solution (154mM NaCl) in 10mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10min at 3000g. Thus the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic solution- induced hemolysis

The experiments were carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffer saline (pH 7.4) containing either the different ethanolic extract (1.0 mg/mL) or Acetyl Salicylic Acid (0.10 mg/mL). The Acetyl Salicylic Acid was used as a reference standard. The mixtures were incubated for 10

min at room temperature, centrifuged for 10 min at 3000 g and the absorbance (O.D.) of the supernated was measured at 540 nm using Shimadzu UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation.

% inhibition of haemolysis = 100 × {(OD1- OD2)/ OD1}

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD2 = Optical density of test sample in hypotonic solution.

RESULTS AND DISSCUSSION

Thrombolytic activity

In investigating cardio protective drugs from natural sources the extracts obtained from ***Nymphaea nouchali*** were assessed for thrombolytic activity and the results are presented in Table.

Table2: Thrombolytic activity (in terms of % of clot lysis) of leaves of *Nymphaea nouchali*.

Fractions	Sample code	Weight of empty eppendorf tube W_1 g	Weight of clot containing eppendorf tube before clot disruption W_2 g	Weight of clot containing eppendorf tube after clot disruption W_3 g	Weight of clot before lysis $W_4=W_2-W_1$ g	Weight of lysis clot $W_5=W_2-W_3$ g	% of lysis W_5 X 100% W_4	Mean % of lysis
Crude	**1**	.0.8109	1.2771	1.0474	0.4662	0.2297	49.2707	52.329
	2	0.8409	1.2975	1.0446	0.4566	0.2529	55.3876	
N-Hexane fraction(NHF)	**3**	0.8418	1.2519	1.0707	0.4101	0.1812	44.1843	45.475
	4	.0.8175	1.2193	1.0314	0.4018	0.1879	46.7646	
DCM	**5**	0.8015	1.2761	1.0420	0.4746	0.2341	49.3257	45.861
	6	0.8289	1.2959	1.0979	0.4670	0.1980	42.3983	
Ethyl acetate	**7**	0.8288	1.2183	1.0912	0.3895	0.1271	32.6316	32.350
	8	0.8290	1.2194	1.0942	0.3904	0.1252	32.0697	
AQF	**9**	0.8320	1.2801	1.0482	0.4481	0.2319	51.7518	41.871
	10	0.7939	1.2296	1.0941	0.4357	0.1355	31.0994	
Blank	**11**	0.8710	1.4641	1.3721	0.5930	0.5011	15.51	13.375
	12	0.902	1.400	1.344	0.498	0.056	11.24	
SK	**13**	0.8276	1.2978	1.0030	0.4702	0.2948	62.6967	60.765
	14	0.8145	1.2894	1.0100	0.4749	0.2794	58.8334	

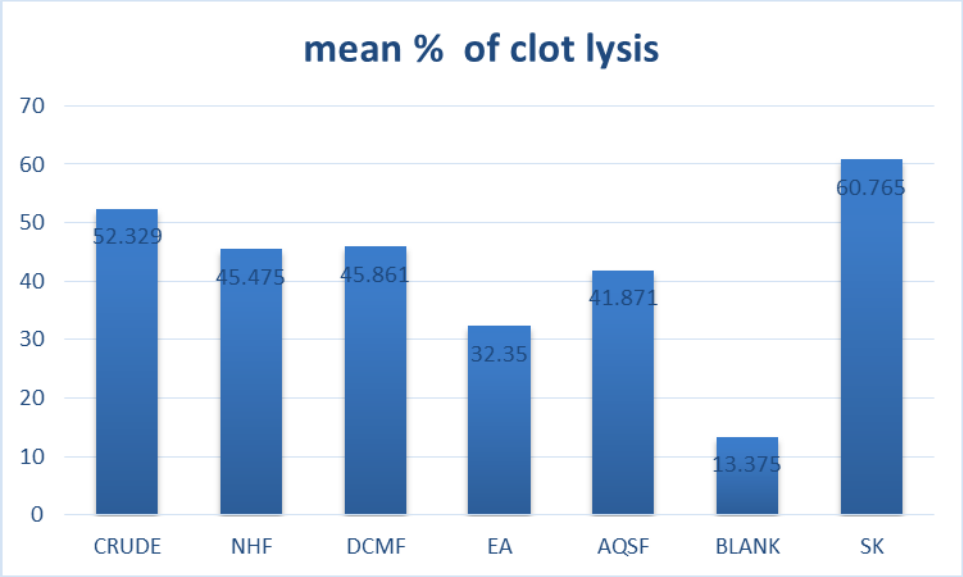

Fig 3: % of clot lysis of *Nymphaea nouchali* leaf.

Table3: Thrombolytic activity (in terms of % of clot lysis) of stem of *Nymphaea nouchali.*

Fractions	Sample code	Weight of empty eppendorf tube W_1 g	Weight of clot containing eppendorf tube before clot disruption W_2 g	Weight of clot containing eppendorf tube after clot disruption W_3 g	Weight of clot before lysis $W_4=W_2-W_1$ g	Weight of lysis clot $W_5=W_2-W_3$ g	% of lysis W_5 X 100% W_4	Mean % of lysis
crude	1	.0.8109	1.2771	1.0474	0.4662	0.2297	49.2707	54.829
	2	0.8409	1.2975	1.0446	0.4566	0.2529	60.3876	
N-Hexane fraction(NHF)	3	0.8418	1.2519	1.0707	0.4101	0.1812	44.1843	42.97
		.0.8175	1.2193	1.0314	0.4018	0.1879	41.7646	
DCM	5	0.8015	1.2761	1.0420	0.4746	0.2341	49.3257	49.362
	6	0.8289	1.2959	1.0979	0.4670	0.1980	46.3983	
Ethyl acetate	7	0.8288	1.2183	1.0912	0.3895	0.1271	37.6316	34.850
	8	0.8290	1.2194	1.0942	0.3904	0.1252	32.0697	
AQF	9	0.8320	1.2801	1.0482	0.4481	0.2319	51.7518	41.871
	10	0.7939	1.2296	1.0941	0.4357	0.1355	31.0994	
Blank	11	0.8710	1.4641	1.3721	0.5930	0.5011	15.51	13.375
	12	0.902	1.400	1.344	0.498	0.056	11.24	
SK	13	0.8276	1.2978	1.0030	0.4702	0.2948	62.6967	60.765
	14	0.8145	1.2894	1.0100	0.4749	0.2794	58.8334	

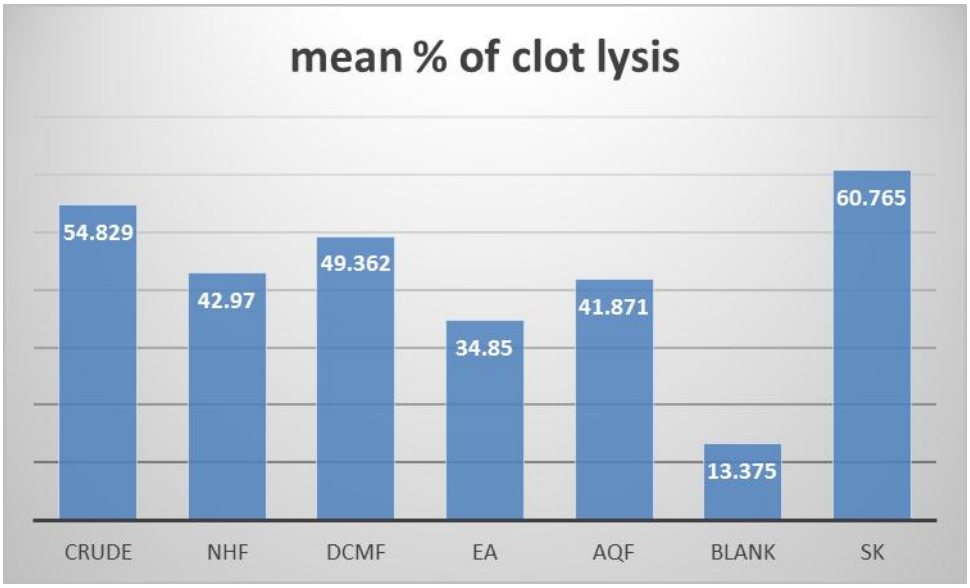

Fig4: mean % of clot lysis of *Nymphaea nouchali* stem.

Addition of 100µl SK, a positive control (30, 000 I.U.), to the clots and subsequent incubation for 90 minutes at 37°C, showed 60.765% lysis of clot. On the other hand, distilled water was treated as negative control (Blank) which exhibited a negligible percentage of lysis of clot (13.375%). The mean difference in clot lysis percentage between positive and negative control was found very significant.

In case of ***Nymphaea nouchali*** leaf crude, NHF, DCMF, EAF, AQF exhibited52.329 %, 45.475%, 45.861%, 32.350% and 41.871% thrombolytic activity. All of them exhibit moderate to postitive effect comparing with the positive standard and negative control.

In case of ***Nymphaea nouchali*** stem crude, NHF, DCMF, EAF, AQF exhibited 54.829%, 42.97%, 49.362%, 34.85% and 41.871% thrombolytic activity. All of them exhibit moderate to postitive effect comparing with the positive standard and negative control.

DCMF = Dichloromethane soluble fraction of the ethanolic extract of ***Nymphaea nouchali.***
EAE= Ethyl acetate.
AQF = Aqueous fraction of the ethanolic extract of ***Nymphaea nouchali.***
SK = Streptokinase.

MEMBRANE STABILIZING ACTIVITY TEST

The various extractives of leaves and stem of ***Nymphaea nouchali*** at concentration 1.0 mg/mL were tested to evaluate the activity against lysis of human erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.10 mg/mL).

The crude extract or ethanolic extract (EE), n-Hexane Fraction (NHF), DCM fraction (DCMF), ethyl acetate fraction (EAF) and aqueous fraction (AF) were subjected to assays for membrane stabilizing activities by following standard protocols and the obtained results were statistically represented in Tables 18 and Figure 30.

Table 4: Membrane stabilizing activity of *Nymphaea nouchali* stem.

Sample Code (N.nouchali stem)	Concentration	Mean Absorbance ± STD	% of Inhibition of Haemolysis
EE	10mg/ml	.104±.001708	83.36
NHF	10mg/ml	.180 ±.001291	71.2
DCMF	10mg/ml	.192±.001295	69.28
EAF	10mg/ml	.121±.001725	80.64
AF	10mg/ml	.150±.001291	76
ASA	10mg/ml	0.103±0.0017	83.52
Control	10mg/ml	0.625±0.0036	--

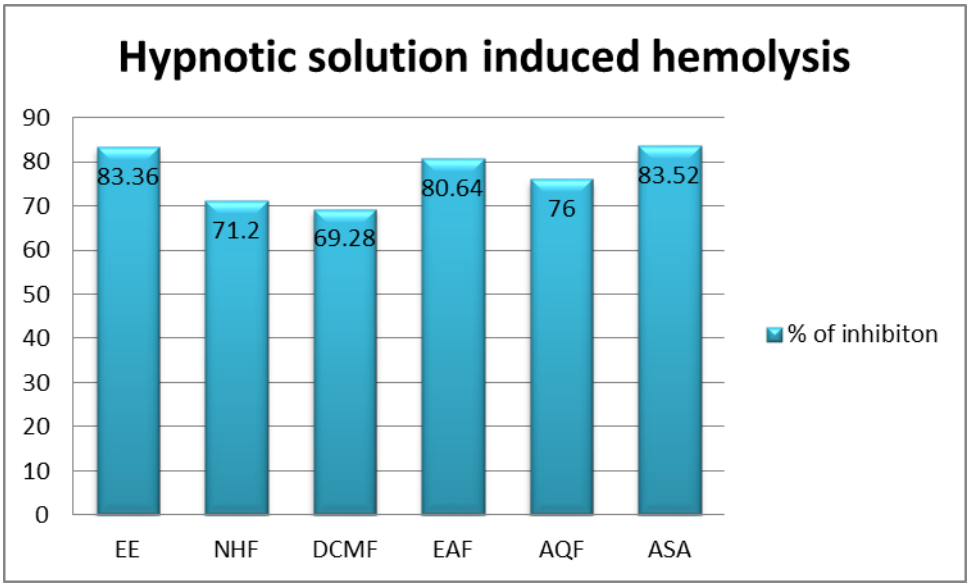

Fig5: % inhibition of hemolysis of different extractives of stem of *Nymphaea nouchali* on hypotonic solution-induced condition.

In hypotonic solution induced condition, the samples were found to inhibit lysis of erythrocyte membrane within the range of 76% to 83.36%. Among all the test samples, ethanolic crude extract of stem of ***Nymphaea nouchali*** showed high inhibition (83.36%) hemolysis of RBC as compared to Acetyl salicylic acid (83.52%).

Table5: Membrane stabilizing activity of *nymphaea nouchali* leaf.

Sample Code (N.nouchali leaf)	Concentration	Mean Absorbance ± STD	% of Inhibition of Haemolysis
EE	10mg/ml	0.153±0.0018	75.52
NHF	10mg/ml	0.190 ±0.0012	69.6
DCMF	10mg/ml	0.170±0.022	72.80
EAF	10mg/ml	0.190±0.017	69.60
AF	10mg/ml	0.301±0.002	51.84
ASA	10mg/ml	0.103±0.0017	83.52
Control	10mg/ml	0.625±0.0036	--

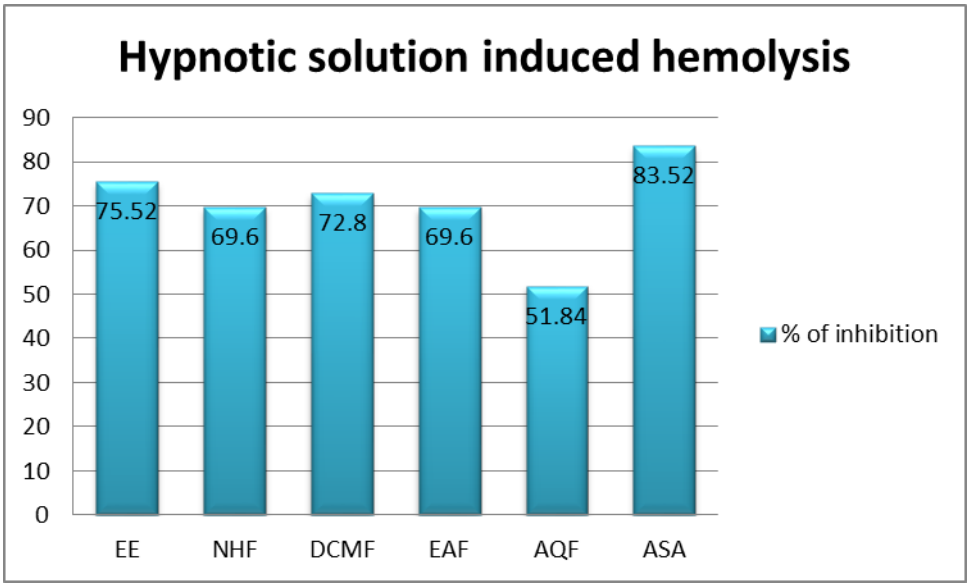

Fig6: **% inhibition of haemolysis of different extractives of leaf of *Nymphaea nouchali* on hypotonic solution-induced condition.**

In hypotonic solution induced condition, the samples were found to inhibit lysis of erythrocyte membrane within the range of 51.84% to 75.52%. Among all the test samples, crude ethanolic extract of leaf of ***Nymphaea nouchali*** showed high inhibition (75.52%) hemolysis of RBC as compared to Acetyl salicylic acid (83.52%).

DISCUSSION

The present study aimed at assessing the effect of ethanol extract of ***Nymphaea nouchali*** a widely used in folkloric medicine, in experimentally induced inflammation, using human red blood cell (HRBC) membrane stabilization as study method. In hypotonic solution induced condition among all the test samples, crude ethanol extract of stem of ***Nymphaea nouchali*** showed high inhibition (83.36%) hemolysis of RBC as compared to Acetyl salicylic acid (83.52%) and %. all the test samples of leaf, crude ethanol extract of leaf of ***Nymphaea nouchali*** showed high inhibition (75.52%) hemolysis of RBC as compared to Acetyl salicylic acid (83.52%).

CONCLUSION

The present study is aimed to investigate in vitro thrombolytic activity ***Nymphaea nouchali.*** Among the different fraction crude extract of leaf and stem showed highest thrombolytic activity by clot lysis of 52.329 and 54.829 respectively. From our findings it is observed that investigated plant revealed remarkable thrombolytic activity.

ACKNOWLEDGEMENT

I would like to express my heartiest gratitude to my respected teacher and supervisor, Saikat Ranjan Paul, Senior lecturer, Department of Pharmacy, Southeast University, for the continuous supervision, providing all facilities and congenital and comfortable atmosphere to perform the project work.

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