ORIGINAL ARTICLE

In vitro anticoagulant activity of *Nelumbo nucifera* leaf extracts on normal healthy blood plasma

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Abstract

Aim: Hemostasis is the process of formation of clots within the walls of damaged blood vessels. To prevent abnormal bleeding and to maintain intravascular blood in a fluid state, in this study we aimed to evaluate the possible anticoagulant effect of leaf extracts of *Nelumbo nucifera*. **Materials and Methods:** The aqueous, methanol, acetone, and ethyl acetate extracts of *N. nucifera* at different concentrations were tested for *in vitro* prothrombin time (PT) test. The *in vitro* anticoagulant effects of different extracts of *N. nucifera* in different concentrations 0.5, 0.25, 0.125, and 0.062 g/ml were examined using plasma, collected from blood samples of normal individuals by measuring PT. Ethylenediaminetetraacetic acid (EDTA) and saline in distilled water were used as a negative and positive control, respectively. The extract plasma was subjected to anticoagulation activity and was compared with EDTA-plasma and saline plasma. **Results:** The methanol leaf extract of *N. nucifera* was found to inhibit coagulation process in 60 min: 3 s in 0.5 g/ml. The time taken for clotting at the concentration of 0.5 g/methanol leaf extract showed the moderate effect of 10 min:20 s with respect to control while ethyl acetate extract showed the least effect of 8 min: 23 s compared to control. Overall, the concentration of 0.5 g/ml of leaf extract showed a maximum effect in all the tested extracts with respect to other concentrations 0.25, 0.125, and 0.062 g/ml. Thus, *N. nucifera* methanol, ethanol, and ethyl acetate leaf extract in different concentrations inhibits clot formation and increases the PT in a dose-dependent manner. The observed prolonged prothrombin activity could be due to the presence of certain phytochemical constituents in the crude extract. Phytochemical analysis revealed the presence of tannins, flavonoids and steroid compounds in the crude extract and further, the active principles could be isolated and evaluated for clinical or physiological purposes. **Conclusion:** *In vitro,* anticoagulant activity studies results demonstrated that leaf extract *N. nucifera* possesses pharmacologically active anticoagulant components which could be helpful in preventing blood clotting disorders. Thus, in future *N. nucifera* leaf could be used as a supplementary source of natural anticoagulant.

Key words: Calcium, clotting factors, hemostasis, phytochemical, prothrombin time

INTRODUCTION

Hemostasis is an interaction process between coagulation and anticoagulants that retains the blood within the injured vascular system during periods of injury.[1] Hemostasis comprises a complex mechanism that contains three major steps: (1) Vasoconstriction, (2) temporary blockage of a break by a platelet plug, and (3) blood coagulation, or formation of a fibrin clot. The coagulation mechanism is a complex cascade mechanism involving the conversion of precursor enzymes (zymogens, procoagulants, and proenzymes) into the active enzymes. Mostly, substances that are necessary for coagulation are present in an inert form and converted to an activated state. Once, one active enzyme is formed it converts the next inactive zymogen to its active enzyme. This series process continues until a fibrin meshwork clot is formed. Protein cofactors, membrane phospholipids surfaces and calcium ions play an active role in the development of the

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fibrin clot.[1] Cardiovascular disorders include hypertension, cerebral hemorrhage, coronary thrombosis, arteriosclerosis, and congestive heart failure are caused by blood circulatory system as blood clotting disorders constitute a serious medical problem. The prothrombin time (PT) test also known as pro-test or PT test used to screen the extrinsic pathways and detects the deficiencies in Factors II, V, VII, and X. In the presence of calcium ions thromboplastin activates the extrinsic pathway in coagulation system and the subsequent clotting time depends on the concentration of Factors II, V, VII, and X. Thus, one or more of these clotting factors (VII and X) deficiency indicated by a prolonged PT and considered as abnormal.[2-4] The normal PT is 11-15 s. Except for nonsteroidal anti-inflammatory drugs (aspirin and indomethacin) some other important synthetic anticoagulant agents are heparin, ethylenediaminetetraacetic acid (EDTA), citrate, and warfarin have anti-inflammatory and anti-platelets activity.[5]

In India, the use of plants with widespread medicinal purposes for the prevention and/or treatment of various ailments is one of the most ancient traditional remedial forms of primary health care.[6,7] Besides, the pharmaceutical properties anticoagulant drugs show serious side effects and also expensive. Hence, therefore, it is necessary to explore alternative anticoagulants. Since the plants are the safer source of medicine, this study is a preliminary attempt to investigate the *in vitro* anticoagulant activities of *Nelumbo nucifera* leaf extracts using standard experimental models in the blood samples of normal individuals.

MATERIALS AND METHODS

Collection of Plant Materials

The leaves of *N. nucifera* were collected from local ponds at Eripudur, Vellore District, Tamil Nadu in November 2016. The *N. nucifera* species were voucher specimen has been identified by the Department of Botany, D.K.M College, Vellore, Tamil Nadu, India, and deposited at the Herbarium D.K.M College for future reference. The leaves of *N. nucifera* which float on water and washed to solid debris and dust particles were remove then cut into small pieces, air dried at room temperature.

Extraction of Plant

N. nucifera leaves were air dried at room temperature and crushed into powder with an electric grinder. This plant material was soaked by suspending 10 g of powdered *N. nucifera* leaf in 100 ml of ethyl acetate, ethanol, and methanol with occasional stirring for 24 h. After 24 h, the suspension was filleted through a fine muslin cloth and then through a No. 1 Whatman filters paper. The solvent was removed at low temperature (40-50°C) under reduced pressure in a rotary evaporator to dryness. They were preserved into sterile bottle kept in a refrigerated until used for further analysis.

Phytochemicals

Each extract (ethyl acetate, ethanol, and methanol) of the leaves of *N. nucifera* was subjected to a preliminary phytochemical analysis for the detection of different phytochemical constituents present in the extract[8,9] using the different phytochemical tests. Different crude extracts were dissolved in respective solvent and used for qualitative phytochemical constituent's confirmation such as alkaloids, saponins, flavonoids, phenols, tannins, and steroids.

Alkaloids

Dragendroff's reagent test

To 1 ml of extract, a few drops of dragendroff's reagent were added to the test tube, and the development of color was noticed. Appearance of orange color indicates the alkaloids presence.

Saponins

Foam test

To 1 ml of extract, 10 ml of water was added and boiled. After few minutes, the mixture was shaken vigorously and filtered. The formation and persistence of froth (1 cm height) for 1 h indicates the presence of saponins.

Flavonoids

Sodium hydroxide test

To 1 ml of extract, 1 ml of sodium hydroxide solution was added and observed. Appearance of yellow color indicates the presence of flavonoids.

Phenolics

Ferric chloride test

To 1 ml of extract, 2 ml of distilled water was added followed by a few drops of 10% ferric chloride. The presence of phenols was indicated by the appearance of blue or green color.

Tannins

Ferric chloride test

To 2 ml of extract, 1 drop of ferric chloride was added followed by the appearance of bluish or greenish black color indicates the presence of tannins.

Steroids

Salkowski test

To extract, 2 ml of chloroform, 10 drops of acetic anhydride, and 2 drops of concentrated sulfuric acid were added. The change of color from red to blue and finally bluish indicates the presence of steroids.

Determination of PT

Collection of blood and separation of plasma

About 10 ml of blood was drawn from healthy volunteers (having no medicine consumption history) by making vein puncture. To the 9 µl volume of blood, 1 µl volume of 3.8% trisodium citrate solution uses added to avoid natural coagulation process. Immediately centrifugation was carried out for 15 min at a rate of 3000 rpm to separate the blood cells from plasma and to obtain pure platelet plasma (PPP). PPP was used for PT test.

Plasma sample was divided into four groups:

- Group I: Negative control group 0.2 ml plasma, 0.1 ml of 0.9% saline water and 0.3 ml of 25 ml $CaCl_2$
- Group II: Positive control group 0.2 ml of plasma + 0.1 ml of 50 mg/ml of EDTA + 0.3 ml of $CaCl_2$ (0.5 g/ml)
- Group III: 0.2 ml of plasma + 0.062 ml of plant extract + 0.3 ml of $CaCl_2$
- Group IV: 0.2 ml of plasma + 0.125 g/ml of plant extract + 0.3 ml of $CaCl_2$
- Group V: 0.2 ml of plasma + 0.25 g/ml of plant extract + 0.3 ml of $CaCl_2$
- Group VI: 0.2 ml of plasma + 0.1 g/ml of plant extract + 0.3 ml of $CaCl_2$.

All the tubes are tilted at an angle of 45° for every 30 s to measure the clotting time. Stop watch was used for measuring the clot formation. This time is called as PT. Tests were repeated 3 times and the average time was calculated.[10]

Tested Extracts

Ethyl acetate extract, ethanol extract, and methanol extract of leaves of *N. nucifera* were investigated for their anticoagulant activity. Each extract was prepared in the concentrations of 0.062, 0.125, 0.25, and 0.5 g/ml with dimethyl sulfoxide.

RESULTS

Phytochemical

Identification of secondary metabolites by phytochemical screening from N. nucifera extract

Table 1 summarizes the phytoconstituents identified from *N. nucifera* leaf extracts. Preliminary phytochemical analysis was performed on ethyl acetate, ethanol, and methanol extract. The crude extract showed positive results of flavonoid in ethyl acetate and methanol extracts except ethanol extract.

Tannin is present in all crude extracts on the other hand steroid is absent in the extract ethyl acetate and ethanol while present in other extracts like methanol. From the result, it is also found that alkaloids and saponins were absent in all the three crude extracts (ethyl acetate, ethanol, and methanol).

PT

Extracts of three from *N. nucifera* leaf increased the clot time in relative to the control. The results are summarized in Table 2. The methanol extract of *N. nucifera* at concentrations of 0.5, 0.25, 0.125, and 0.062 g/ml showed an increase in PT of 60 min:3 s, 31 min:36 s, 11 min:30 s, and 1 min:20 s, respectively. The ethanol extract also produced a prolonged time duration to clot as the concentration increases 0.5 g/ml (10 min:20 s), 0.25 g/ml (7 min:2 s), 0.125 g/ml (5 min:40 s), and 0.062 g/ml (2 min:7 s). While the ethyl acetate produced an inhibitory activity at least level compared to control with the following time intervals of 8 min:23 s, 6 min:49 s, 4 min:5 s, and 1 min:10 s at the concentration of 0.5 g/ml, 0.25 , 0.125, and 0.062 g/ml, respectively. From the data, it was found that methanol extract had taken a greater prolonged duration to clot formation compared to ethanol and ethyl acetate. The clot formation increases as the concentration increases from 0.062 to 0.5 g/ml.

DISCUSSION

A coagulation is a process that occurs mainly due to the complex interaction of cellular and molecular components.[11] Initially clotting involves common pathway of both intrinsic and extrinsic pathways, but lately, it found to be due to a

Table 1: Phytochemical analysis of *Nelumbo nucifera* leaf extract

Phytochemical test	Ethyl acetate	Ethanol	Methanol
Alkaloid (Dragendroff's test)	-	-	-
Flavonoids (alkaline test)	+	-	+
Saponin (foam test)	-	-	-
Tannins (ferric chloride test)	+	+	+
Phenolic compound (ferric chloride test)	+	+	+
Steroid (Salkowski test)	-	-	+

-: Absence, +: Presence

Table 2: Dose-dependent effect of *Nelumbo nucifera* leaf extract on clotting time of normal human plasma

Plant extracts (*Nelumbo nucifera*)	Experimental groups			
	Group III (0.062 g/ml)	Group IV (0.125 g/ml)	Group V (0.25 g/ml)	Group VI (0.5 g/ml)
Methanol	1 min:20 s	11 min:30 s	31 min:36 s	60 min:3 s
Ethanol	2 min:7 s	5 min:40 s	7 min:2 s	10 min:23 s
Ethyl acetate	1 min:10 s	4 min:5 s	6 min:49 s	8 min:23 s
Group I (negative control)	1 min:40 s			
Group II (positive control)	NA			

NA: Indicates absence of clot

balance between the procoagulants and anticoagulants.[11] A similar study was reported by Ikese *et al.,* 2015,[12] whose findings reported that aqueous extract of *Tridax procumbens* reduced the clotting time. The petroleum ether extract of *T. procumbens* also showed a significant reduction in clotting time as similar to our reports.[13] The results of this study are also similar to those results obtained by Kale *et al*., 2008[14] in which the ethanolic leaf extract of *T. procumbens* had specifically reduced the clotting time. A reduced clotting time of plant extract was also shown by Sowmya *et al*., 2015[15] as like our clotting time. Taj *et al*., 2011,[16] noticed a correlation between concentration of aqueous extract of *Allium cepa* and the time needed to inhibit the clot formation with a prolonged PT. That is, as concentration increases, the aqueous extract of red onion strongly inhibited the coagulation process and also increased the PT. In this investigation, an similar correlation exists between concentration of extracts and the time taken to inhibit the clot formation. This may be attributed due to the presence of several phytochemical compounds that have been noted in the extracts of leaves.

Manicam *et al*., 2010,[17] studied the anticoagulant activity of aqueous leaf extract of *Melastoma malabathricum* Linn. The study revealed that the aqueous leaf extracts prolonged the coagulation time as similar to the result obtained. Many researchers also studied the anticoagulant property of some plant extracts such as *Sutherlandia frutescens* leaf extract, *Gloriosa superba*, *Zantedeschia aethiopica* leaf extract, and *Leonotis leonurus* root extract[18] and their study revealed to have a prolonged coagulation time, as same to our present coagulation time. Further studies are desired to assess its effect and to ascertain the mode of action.

CONCLUSION

The anticoagulant activity of *N. nucifera* leaf extract was not yet reported and this report was found to be the first investigation for PT. Hence, further identification and characterization of active molecules responsible for activity was to be found out in future.

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