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Research Article
Phytochemical Analysis and *in vitro* Thrombolytic Potential of Aerial Parts of *Ipomoea aquatica* Forssk.

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Abstract

Background and Objective: Abnormal thrombosis is one of the major causes of Ischemic Cardiovascular Diseases (ICVD). In the acute stage, thrombolytic agents are required t o re-canalize the clogged vessels for restoration of blood flow. These thrombolytic agents are costly and not free from adverse effects. This study aimed to characterize the phytochemical constituents and evaluate the *in vitro* thrombolytic potential of the aerial parts of *Ipomoea aquatica* Forssk. as a potential natural source of thrombolytic agents. **Materials and Methods:** Aerial parts (stem, leaves and shoots) of *I. aquatica* were collected from Udaipur, India, air-dried, and powdered. Two types of methanolic extracts (ME-I and ME-II) for each plant part were prepared through cold extraction procedure and stored in the refrigerator after evaporation. Aqueous extract was always prepared fresh and evaluated along with ME-I for presence of phytochemicals as per the standard methodology. The ME-II was dissolved in sterile distilled water to prepare 10 mg/mL concentration and used for evaluation of *in vitro* clot lysis assay as per the standard protocol. After obtaining institutional ethical approval and informed consent, the *in vitro* thrombolytic activity of extracts of its leaves, stem and shoot (MEL-II, MES-II, and MESH-II, respectively) was assessed in fasting blood samples of thirty healthy volunteers. Streptokinase served as positive control and distilled water as negative control. Student's paired 't'-test was employed for statistical comparisons. A p-value of less than 0.05 was considered to be significant. **Results:** Qualitative phytochemical analysis revealed the presence of flavonoids, terpenoids, tannins, phlobatannin, saponins, and carbohydrates in both leaves and stem. A significant percent clot lysis activity of 32.94±1.14, 36.78±1.27 and 40.92±1.67 has been exhibited for the first time by methanolic extracts of *I. aquatica* leaves, stem and shoot, respectively as compared to the positive and negative controls. **Conclusion:** A higher thrombolytic potential of its shoots scientifically highlights the traditional wisdom of using its shoots as a vegetable. Further, *in vivo* studies are required to validate its thrombolytic activity along with isolation of anti-thrombotic molecules.

Key words: *Kalmisag*, polyphenol, thrombolytic agent, ischemic heart disease, isorhamnetin, clot lysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Thrombosis is a localized clotting of blood required to heal the injury to blood vessels and maintain normal haemostasis. However, abnormal thrombosis causes interference in the blood flow to the respective organ and leads to conditions like acute myocardial infarction and stroke. Lysis of those intravascular clots formed due to abnormal thrombosis and platelet aggregation is therefore, required to prevent ischemic damage thereby improving blood flow[1]. The current thrombolytic agents such as streptokinase (SK), urokinase (UK), alteplase, recombinant tissue type Plasminogen Activator (rt-PA) etc. have own limitations and safety issues[2]. The need for a safer and effective thrombolytic agent could be fulfilled by using natural products especially derived from plants. In this context, leads from plants mentioned for treatment of heart disease/high blood pressure/high blood sugar in traditional medicine could be more effective rather than screening the plants randomly.

Ipomoea aquatica Forssk. (Family- Convolvulaceae) is a perennial aquatic trailing vine; known as Swamp Cabbage, Swamp Morning Glory, Water Spinach, *Kalmisag, Karmi, Patuasag, Nadishaka, Naipala, Tutikura, Nalanibhaji* etc., in various languages. Long hollow stems, sub-deltoid leaves with acute, rounded or slightly toothed lobes, pale purple colored flowers in axillary cymes and ovoid capsules are some of its important identifying features[3]. It is native to tropical and subtropical old world countries and considered invasive in many states of America. Notably, its shoots are consumed as vegetable in South-East Asia, as also in Eastern and Southern States of India[4,5].

In traditional system of medicine, *I. aquatica* has been recommended for the treatment of jaundice, nervous debility, constipation, abscesses, intestinal problems, mental illness, high blood pressure etc.[5]. It is also used as blood purifier and to treat bone fracture, cuts and wounds, diabetes, jaundice, diarrhea, dysentery, fever, opium poisoning, skin diseases, snake bite etc., by various ethnic communities of India[4]. Several pharmacological activities have been reported from different parts of the plant such as antioxidant, anti-inflammatory, anticoagulant, antimicrobial, anxiolytic, analgesic, anti-ulcer, anticancer, anti-acetylcholinesterase, hepatoprotective, hypoglycemic, hypolipidemic, cytotoxic, nephroprotective, diuretic, nootropic etc.[5-10]. Notably, Sarkar[11] has recommended its use as a vegetable in the dietary regime for patients with heart disease. Recently, Juthi *et al.*[12] reported *in vitro* thrombolytic potential of 70% methanolic extract of its leaves, suggesting its possible role in CVD. But *in vitro* thrombolytic potential of 100% methanolic extract of its aerial parts (leaves, stem and shoots having leaves and stem together), which are consumed as vegetables, has still not been investigated. Therefore, the present study aimed to evaluate the *in vitro* clot lysis potential of aerial parts of *I. aquatica* as well as their preliminary qualitative phytochemical analysis.

MATERIALS AND METHODS

Collection, identification and preparation of plant material: Plants of *Ipomoea aquatica* Forssk. were collected from Fateh Sagar Lake, Udaipur, Rajasthan, India in January 2021 and identified with the help of Flora of Rajasthan[3]. It was further authenticated at Botanical Survey of India (BSI), Arid Zone Regional Centre, Jodhpur, Rajasthan (BSI/AZRC/I.12012/Tech./2020-21-(PI.Id.)/424 dated 08/02/2021, Sl. No. 3). A voucher specimen of the plant was also preserved at Department of Botany, Government Meera Girls College, Udaipur for future use. Its aerial parts, specifically, leaf, stem and shoot (leaves with stem) were dried separately under shade and ground to make fine powder and stored at 4°C in a refrigerator.

Preparation of plant extracts: The stored dried powder of plant parts was used to prepare the following suitable extracts as per the established protocol[13].

Methanolic extracts

Methanolic extract- I (ME-I): Five gram dried powder of leaves, stem and shoot each of *I. aquatica* was soaked in 50 mL methanol at room temperature with occasional stirring and filtered after 24 hrs. This process was repeated for three times with 50 mL of methanol and the resulting last filtrates were evaporated on boiling water bath and stored in refrigerator, coded as MEL-I, MES-I and MESH-I. These were used for preliminary qualitative phytochemical analysis of leaves, stem and shoot, respectively.

Methanolic extract-II (ME-II): This type of extract was prepared for evaluation of *in vitro* clot lysis activity. For this purpose, dried powder (100 g each) of leaves, stem and shoot of *I. aquatica* was soaked in 500 mL of methanol separately for eight days with occasional stirring and filtered with Whatman's filter paper no. 1. The filtrates were then evaporated on boiling water bath at a temperature of 40°C and stored at 4°C in refrigerator. These extracts were coded as MEL-II (Methanolic extract of Leaves-II; % yield = 10.85), MES-II (Methanolic extract of Stem-II; % yield = 11.07%) and MESH-II (Methanolic extract of Shoot- II; % yield = 10.63).

Aqueous extract: Aqueous extract was freshly prepared for preliminary qualitative analysis of certain phytochemicals. It was prepared by soaking 400 mg dried powder of leaves, stem and shoot each in 20 mL distilled water and then boiling for 20 min. It was then filtered with Whatman's filter paper no. 1 and used immediately.

Qualitative phytochemical analysis: Plant extracts (Aqueous and ME-I) or dried powder of leaves, stem and shoot of *I. aquatica* were screened for qualitative phytochemical analysis of amino acids, carbohydrates, terpenoids, steroids, cardiac glycosides, phlobatannins, flavonoids, phenols, tannins and saponins as per standard methods[13-15].

Evaluation of *in vitro* thrombolytic activity: The methodology demonstrated by Prasad *et al.*[16] was adopted for evaluation of thrombolytic activity. After obtaining institutional ethical approval (Ref.PMU/PMCH/IEC/2019, dated 26.12.2019) and informed consent, the *in vitro* thrombolytic activity of MEL-II, MES-II and MESH-II was assessed. Fasting blood samples of thirty healthy volunteers without gender bias were collected, taking into consideration that they were not receiving any drugs. The entire experiment was performed in accordance with the Declaration of Helsinki, 2004.

A concentration of 10 mg/mL of all three extracts, MEL-II, MES-II and MESH-II, was prepared by suspending 100 mg crude methanolic extract in 10 mLl of sterile distilled water and shaking vigorously on a vortex mixer. It was kept overnight and used for clot lysis assessment the next day after filtering with a syringe filter. For positive control, Streptokinase (STPase manufactured by Cadila Pharmaceuticals, Ahmedabad, India) was prepared by dissolving 15,00,000 IU of lyophilized STPase in 5 mL of sterile distilled water and mixed thoroughly. One hundred microlitre (30,000 IU) of this stock was used for the test purpose.

Ten milliliter blood samples were drawn from each volunteer. For clot formation, 500 µL blood was immediately poured in pre-weighed sterile microcentrifuge tubes and incubated at 37°C for 45 min. Weight of clot was determined after removing serum by centrifugation at 2000 rpm for 10 min and subtracting weight of tube from weight of tube with clot. Then 100 µl of plant extracts (MEL-II, MES-II and MESH-II), sterile distilled water (negative control) and streptokinase (positive control) were added to the tubes separately and kept for incubation at 37°C for 90 min. The fluid obtained after clot lysis was carefully removed and re-weighing of tubes was done to find out the weight of clot after lysis by subtracting it from initial weight of tube with clot. Percent clot lysis was calculated by following formula:

$$\text{Clot lysis (\%)} = \frac{\text{Weight of clot after lysis}}{\text{Weight of clot}} \times 100$$

Estimation of blood lipids: Lipid fractions such as serum Total cholesterol (TC), Triglycerides (TG) and High-Density Lipoprotein Cholesterol (HDL-C) were measured using standard enzymatic kits (Reckon Diagnostics P. Ltd., Baroda, India). Serum Very Low-Density Lipoprotein Cholesterol (VLDL-C), Low-Density Lipoprotein Cholesterol (LDL-C) and Non-High-Density Lipoprotein Cholesterol (Non-HDL-C) were calculated by applying standard formula as follows[17,18]:

$$\text{VLDL-C} = \text{Triglycerides}/5$$

$$\text{LDL-C} = \text{Total cholesterol} - (\text{HDL-C} + \text{VLDL-C})$$

$$\text{Non-HDL-Cholesterol} = \text{Total Cholesterol} - \text{HDL-C}$$

Statistical analysis: Results of the clot lysis experiment were expressed as Mean±SEM (Standard Error of the Mean) for three replicates and Student's Paired t-test was carried out for statistical comparisons using Microsoft Excel (2010). A p-value of less than 0.05 was considered to be significant. Microsoft Excel (2019) was used to determine the correlation of highest clot lysis activity among all the extracts with various atherogenic lipid fractions (TC, TG, LDL-C and Non-HDL-C).

RESULTS

Phytochemical screening: The qualitative phytochemical analysis has shown presence of various primary and secondary metabolites such as carbohydrate, saponin, flavonoid, tannin, phlobatannin and terpenoid in leaves, stem and shoot of *I. aquatica*. However, amino acid, cardiac glycoside and steroid were not detected in the stem (Table 1).

***In vitro* thrombolytic activity:** A significant ($p<0.0001$) *in vitro* clot lysis activity of 32.94±1.14% and 36.78±1.27% was demonstrated by methanolic extracts (10 mg/mL) of leaves and stem of *I. aquatica* respectively in comparison to positive control SK and sterile distilled water as negative control in the present study (Table 2). Interestingly, a slightly higher thrombolysis activity (40.92±1.67%) was exhibited by

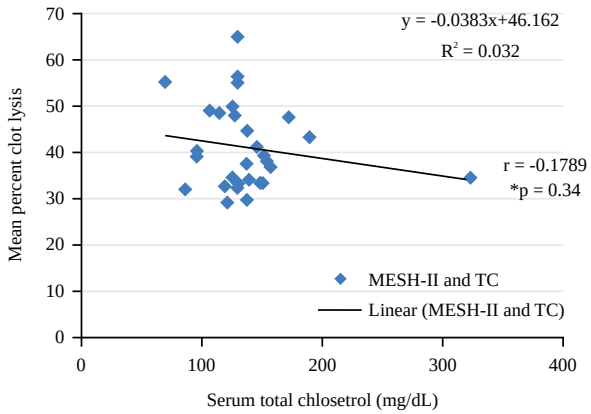


Fig. 1: Correlation between serum total cholesterol (TC) and clot lysis activity of methanolic extract of *I. aquatica* shoot (MESH-II)

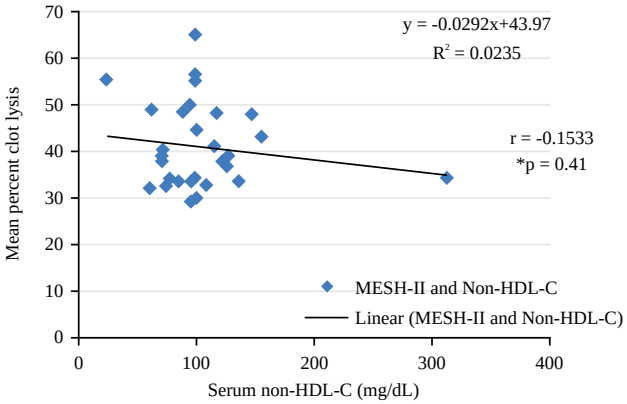


Fig. 2: Correlation between serum non-HDL-C and clot lysis activity of methanolic extract of *I. aquatica* shoot (MESH-II)

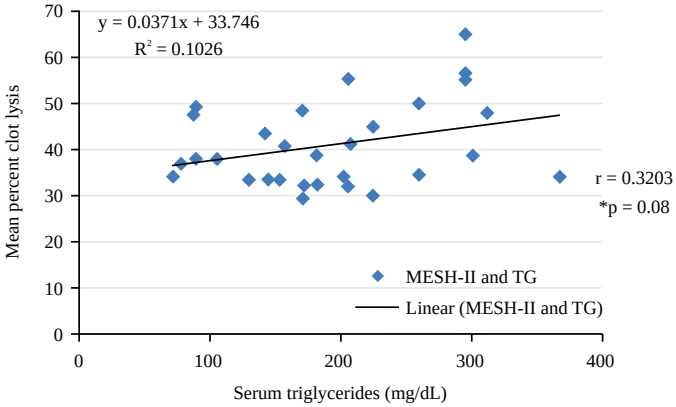


Fig. 3: Correlation between serum triglycerides (TG) and clot lysis activity of methanolic extract of *I. aquatica* shoot (MESH-II)

methanolic extract of shoots of *I. aquatica* (MESH-II) than both MEL-II and MES-II. Table 2 also shows that there was significant difference in percent clot lysis between positive and negative control. A clot lysis potential of distilled water from 3.25% to 4.72% depicts insignificant role of water in the clot lysis exhibited by methanolic extracts of various plant parts of *I. aquatica*.

Correlation analysis between blood lipids and thrombolytic activity: A negative correlation was observed with *in vitro* clot lysis activity of *I. aquatica* shoot extract (MESH-II) along with TC (Fig. 1) and Non-HDL-C (Fig. 2) and a mild positive correlation was observed with TG (Fig. 3) and LDL-C (Fig. 4); though statistically not significant.

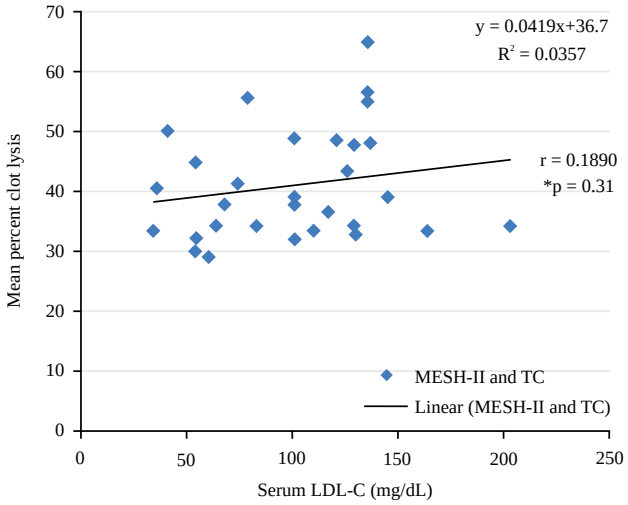


Fig. 4: Correlation between serum LDL-C and clot lysis activity of methanolic extract of *I. aquatica* shoot (MESH-II)

Table 1: Qualitative preliminary phytochemical analysis of leaves, stem and shoot of *I. aquatica*

Phytochemical test	Leaves	Stem	Shoot
Carbohydrate	+	+	+
Amino acid	+	-	+
Saponin	+	+	+
Flavonoid	+	+	+
Phenol	+	+	+
Tannin	+	+	+
Phlobatannin	+	+	+
Terpenoid	+	+	+
Cardiac glycoside	+	-	+
Steroid	+	-	+

+: Present and -: Absent

Table 2: *In vitro* percent clot lysis activity of methanolic extracts of *I. aquatica* leaves (MEL-II), stem (MES-II) and shoot (MESH-II), Streptokinase (30000 IU) and distilled water

	Percent clot lysis (Mean±SEM)		
Plant extract (10 mg/mL)	Plant extract (I)	Streptokinase (II)	Distilled water (III)
MEL-II	32.94±1.14^{a,b}	49.82±0.88^{a,c}	4.72±0.26^{b,c}
MES-II	36.78±1.27^{a,b}	53.95±0.49^{a,c}	5.67±0.35^{b,c}
MESH-II	40.92±1.67^{a,b}	55.76±0.80^{a,c}	3.25±0.11^{b,c}

p-value: a- I v/s II; p<0.0001, b- I v/s III, p<0.0001, c- II v/s III, p<0.0001 and Values are expressed as Mean±SEM (n = 30)

DISCUSSION

There is an important role of a normal thrombolytic process in body for the management of Ischemic Cardiovascular Diseases (ICVD). Plants have been traditionally employed to treat a wide array of ailments. Plants which are consumed in diets for their nutritional purpose also possess medicinal benefits. Hence, they are considered as 'Functional Food'[19]. In this context, development of some health-beneficial dietary plant products could be useful for management of ICVD.

Ipomoea aquatica is consumed as a vegetable in various Indian states[4]. In the present study, the *in vitro* clot lysis potential of its leaves, stem and shoot was demonstrated as 32.94%, 36.78% and 40.92%, respectively (Table 2). Similarly, several edible plants have shown *in vitro* thrombolytic potential ranging between 30 to 45%. For example, 39.30% clot lysis by leaves of *Leea indica*, 38% by fruits of *Punica granatum*, 43.25% by fruits of *Coriandrum sativum*, 40.9% by leaves of *Spinacia oleracea*, 41.40% by leaves of *Moringa oleifera* etc.[20]. Recently, 28.96% clot lysis activity of 70% methanolic leaf extract (10 mg/mL) of *I. aquatica* in comparison with 68.79% clot lysis by streptokinase is reported by Juthi *et al.*[12]. In this regard, results of the present study indicate better thrombolytic potential of 100% methanolic extracts of aerial parts of *I. aquatica* (Table 2). Other species of the genus *Ipomoea* have also shown appreciable *in vitro* clot lysis potential such as *I. pes-tigridis*, *I. alba*, *I. sepiaria* and *I. hederifolia*[21-24] indicating members of this genus may possess shared phytochemical

constituents responsible for thrombolytic effects. This reinforce the potential of *I. aquatica* as a candidate for further investigation in the development of plant-based thrombolytic therapeutics.

Nutritionally, *I. aquatica* leaves are rich in iron, magnesium, calcium, manganese, phosphate, sulphate and nitrates and vitamins A, B1, B2, B6, B12, C and K[25]. The ethanolic extract (80%) of steamed water spinach possesses carotenoids (7.1 mg/g), total chlorophylls (35 mg/g), total polyphenols (35.6 mg/g) and 40.4 mg/g of total flavonoids[26]. Notably, carotenoids and polyphenols are well-known for their potential to scavenge free radicals, which play an important role in pathogenesis of CVDs[27].

In addition to its nutritional value, this plant has significant pharmacological and therapeutic properties due to abundance of naturally present phytochemicals. Shah *et al.*[28] have demonstrated an extensive range of phytochemicals, for example, carbohydrates, protein, glycosides, alkaloids, phenolics, tannins, saponins, quinines, flavonoids and terpenoids in the *I. aquatica* leafy vegetable. Shoot and leaves of *I. aquatica* have shown the presence of Gallic acid, Ferulic acid, Vanillic acid, Chlorogenic acid, Quercetin, Rutin and catechin, whereas Caffeic acid, Ellagic acid, Sinapic acid, Kaempferol, Myricetin, Naringin, β-sitosterol and Lycopene have been isolated from shoot alone[29-33]. Several other bioactive molecules such as Apigenin, Farnesol, β-carotene, Violaxanthin, Lutein, Vitamin E and Ascorbic acid have also been reported from *I. aquatica* leaves[34-36]. These metabolites are well-known for their antioxidant, anti-inflammatory, antiplatelet, hypolipidemic, antihyperglycemic and cardiovascular protective effects[19,37,38]. The presence of such compounds in the extract may be associated with the observed thrombolytic activity. However, the exact anti-thrombotic molecule present in *I. aquatica* needs to be explored.

Abnormal lipid profile and coagulation parameters, high blood glucose, obesity etc. are considered culprits behind the origin of CVD[39]. The thrombolytic potential observed in the present study is consistent with other reports describing cardiovascular benefits associated with this plant. *I. aquatica* have shown hypolipidemic, hypoglycemic, antioxidant and anticoagulant potential in scientific studies[9,40,41]. Recently, ethanolic extract of aerial parts of *I. aquatica* have shown a significant delay in clotting time and further, *in silico* analysis has revealed three major phytochemicals, namely, tamarixetin, kaempferol and isorhamnetin showing the strongest binding affinity against human coagulation Factor X[9]. In view of the above-mentioned activities, thrombolytic potential as demonstrated by the plant extract in the present study is an added advantage. It may represent a multifactorial pharmacological effect arising from the synergistic interaction of its several bioactive constituents.

Interestingly, a moderate positive correlation was observed with levels of TG and LDL-C and clot lysis activity of methanolic shoot extract of *I. aquatica* (Fig. 3-4), indicating better thrombolytic protection by *I. aquatica* even in the conditions of elevated lipids. LDL-C is one of the key molecules responsible for atherogenesis[42] and protection against the negative effects of LDL-C by enhancement of the thrombolytic process through natural compounds/herbs could be a better strategy to prevent cardiovascular diseases. However, the negative correlation observed between thrombolytic activity of shoot with Total Cholesterol and Non-HDL-C levels (Fig. 1-2) suggests that shoot extract could lyse the blood clot irrespective of cholesterol levels.

Toxicological studies in animals have shown a wide safety profile of *I. aquatica* in both acute and sub-acute studies[43]. Moreover, people are consuming it from time immemorial as vegetable without any noticeable side effects. Another important observation of the study is the demonstration of higher thrombolytic potential by its shoots as compared to leaf and stem alone (Table 2). This suggests towards a synergistic medicinal effect of both leaves and stem together. Despite demonstrating promising thrombolytic activity and providing preliminary phytochemical insights, limitations of the present study include its exclusively *in vitro* nature. Therefore, the observed activity cannot be directly extrapolated to its clinical efficacy and need long term *in vivo* and clinical studies. Moreover, the detailed quantitative characterization of individual phytoconstituents using advanced analytical techniques is required to identify the specific bioactive compounds responsible for the thrombolytic effect. The underlying mechanisms of thrombolysis, further need investigation on fibrinolytic pathways, plasminogen activation, fibrin degradation, or platelet aggregation.

CONCLUSIONS

In the present study, the methanolic extract of aerial parts (leaves, stem and shoot) of *I. aquatica*, collected from Udaipur, India have shown significant *in vitro* thrombolytic activity as compared to the standard thrombolytic agent. This is an important finding for a vegetable which is recommended as diet for patients of heart disease. The plant possess a wide range of phyto-constituents including phenols, flavonoids, carotenoids, terpenoids, vitamins etc. along with hypolipidemic, antioxidant, anticoagulant and hypoglycemic potential. The addition of clot lysis potential to its therapeutic

armamentarium could make it an ideal choice to be included in diet as a nutraceutical herb. However, *in vivo*, long term studies are warranted for establishing its thrombolytic potential as well as isolation and characterization of bioactive anti-thrombotic molecules and the detailed mechanism of thrombolytic action need to be researched.

SIGNIFICANCE STATEMENT

Thrombolysis-enhancing edible plants could be very helpful in prevention and onset of cardiovascular diseases which mostly occur due to impaired thrombolysis. The present study has shown that *Ipomoea aquatica* possesses significant *in vitro* thrombolytic potential. This conventional vegetable could be utilized in dietary component of persons pre-disposed to cardiovascular risk. However, it needs long term *in vivo* and clinical studies.

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