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Original Paper

In vitro Clot Lysis Activity of *Boerhavia diffusa L.* Leaves

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ABSTRACT

Boerhavia diffusa L. (Family – Nyctaginaceae), is commonly known as Horse Purslane, Hogweed, Punarnava, Raktapunarnavaa etc. in various languages. It is a perennial creeping weed having small pinkish flowers and distributed throughout the India, tropical and subtropical Asia, Africa and America. Leaves and roots of B. diffusa are edible in many regions of India. It is widely used for the treatment of several human ailments for example, anemia, asthma, blood impurity, body pain, cough, rheumatism, gall bladder stone, joint pain, heart disease, enlargement of liver and spleen etc. in both codified and non-codified traditional systems of medicine. The plant has also shown to possess antioxidant, anti-inflammatory, antidiabetic, adaptogenic, hypolipidemic, antimicrobial, hepatoprotective, antiproliferative, anticonvulsant, nootropic, immunomodulatory activities in various in vitro and in vivo studies.

Abnormal thrombus formation is one of the major reasons behind ischemic heart disease and treatment with synthetic thrombolytic agents has many side effects. As B. diffusa is recommended in traditional medicine for treatment of vascular diseases, the leaves of the plant were assessed for preliminary qualitative phytochemical analysis and in vitro thrombolytic potential. Preliminary phytochemical screening of leaves has shown presence of flavonoids, terpenoids, cardiac glycosides, saponins, tannins, steroids, phenol, phlobatannin, carbohydrates and amino acids. A significant percent clot lysis activity of 38.42±1.28 was exhibited by methanolic extract of leaves of B. diffusa in comparison with the standard drug streptokinase as positive control (44.72±0.87 %) and sterile distilled water as a negative control (3.74±0.37%). This is the first report of in vitro clot lysis potential of B. diffusa leaves and it could be recommended to use this plant as a dietary nutraceutical. Further studies to find out the bioactive molecules and molecular mechanism responsible for its thrombolytic potential are warranted.

KEYWORDS: Cardiovascular diseases, Nutraceutical, Thrombolysis, Punarnavine, Boeravinone

INTRODUCTION

Abnormal thrombus formation due to the imbalance or failure in homeostasis in the blood vessels may cause serious events such as heart attack, stroke, ischemic heart disease, deep vein thrombosis, pulmonary embolism etc.[1]. Due to injury of blood vessels in arterial system, recruitment of platelets to the vascular endothelial membrane occurs and their hyper-aggregation leads to formation of thrombus resulting in cardio-vascular diseases; the leading cause of morbidity and mortality in world[2,3]. There is need for the lysis of the dangerous intravascular clots in order to improve blood flow to prevent ischemic damage[4]. For this purpose, there are some widely used synthetic thrombolytic agents such as streptokinase (SK), tissue plasminogen activator (t-PA), urokinase (UK), alteplase etc. These synthetic thrombolytic drugs are used in acute catastrophic situation only and possess serious side effects of bleeding, anaphylactic reactions and shock[5].

Plants are thought to be less harmful and free of adverse effects than syntheticthrombolytic drugs[6]. Many plants have shown thrombolytic and antiplatelet potential[7].

Figure 1: *Boerhavia diffusa* L. Plant

Boerhavia diffusa L. (Figure 1); member of family – *Nyctaginaceae* is a perennial diffusely branched, creeping herb with stout rootstock. It is known as Horse Purslane, Pigweed, Hogweed, *Punarnava, Raktapunarnavaa, Shophaghni, Kathila, Varshabhu, Shilatika, Kuthillaka* etc. in different languages. The name Punarnava is derived from two words *Punar* (once again) and *Nava* (new, renew or young), meaning, one which becomes new or young again. This name indicates about rejuvenating property of *B. diffusa*[8]. It is distributed in India, tropical and subtropical Asia, Africa and America. Leaves, shoot and roots of the plant are considered edible in various ethnic communities of India[9-11]. It is important to note that *Boerhaavia* Miller is an orthographic variant which was described in 1754 after Linnaeus proposed the scientific name *Boerhavia* in 1753; hence the *Boerhavia diffusa* L. is the accepted nomenclature of the plant[12].

B. diffusa is recommended in both codified and non-codified systems of medicine for the treatment of various human ailments namely anemia, asthma, blood impurity, body pain, cough, diabetes, dropsy, gall bladder stone, heart disease, joint pain, oedema, kidney problem, night blindness, rheumatism, skin disease, snake bite, stomachache, urinary trouble, menorrhagia, malaria, insomnia, enlargement of liver and spleen etc.[11,13,14].

B. diffusa is classified as *rasayana* in Ayurveda and possesses ethnopharmacological and therapeutic importance. Some Ayurvedic formulations such as *Punarnavasava, Sukumara ghrita, Punarnavadyarishta, Punarnavadi mandura, Sothaghna Lepa, Maha Narayan Taila, Punarnavastaka kvatha curna, Punarnava guggulu, Punarnavadi kvatha curna* and *Varuni* have the major ingredient of *B. diffusa.* All these ingredients are used for the treatment of various human ailments like heart disease, sciatica, inflammation, diseases of abdomen, spleen, liver disorders, arthritis etc.[15-16] The root of B. *diffusa* has laxative and diuretic properties which are due to the presence of an active constituent, Punarnavine[10].

Besides, various pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, anticonvulsant, antidiabetic, hypolipidemic, cytotoxic, diuretic, nootropic etc. have been reported from *B. diffusa* in various scientific studies[8,17]. Looking to its immense pharmacological potential and uses in traditional medicine, the present study has been carried out to evaluate *in vitro* thrombolytic potential of leaves of *B. diffusa*.

MATERIALS AND METHODS

Collection, Identification and Preparation of Plant Material

Leaves of *B. diffusa* were collected from an open land in Arvind Nagar, Sunderwas, Udaipur, Rajasthan, India. Plant was identified by Dr. Vartika Jain and a voucher specimen was preserved at Herbarium, Department of Botany, Government Meera Girls College, Udaipur. Further authentication of the plant was done at Botanical Survey of India (BSI), Arid Zone Regional Centre, Jodhpur, Rajasthan (BSI/AZRC/I.12012/Tech./2020-21- (Pl.Id.)/424 dated 08/02/2021, Sl. No. 1). Leaves were dried under the shade and ground to make a fine powder to prepare suitable plant extracts.

Preparation of Plant Extracts

1. Methanolic Extracts:

   a. *Methanolic Extract- I* (ME-I) – Five gram dried powder o f leaves of *B. diffusa* was soaked in 50 ml methanol for 24 hours at room temperature with occasional stirring and filtered. This process was repeated for three times each with 50 ml of methanol. This extract was used for qualitative phytochemical analysis of leaves.

   b. *Methanolic Extract- II* (ME-II) – This was prepared using cold maceration technique in which 100 g dried powder of leaves of *B. diffusa* was soaked in 500 ml of methanol for eight days. It was then filtered with Whatman's filter paper

no. 1 and solvent was evaporated on boiling water bath at 40ºC. The crude methanolic extract, thus obtained was named as ME-II and stored at 4ºC in refrigerator. ME-II was used for evaluation of *in vitro* clot lysis activity.

2. Aqueous Extract:

This extract was freshly prepared for preliminary qualitative phytochemical analysis. To prepare this, 400 mg dried powder of leaves *B. diffusa* was soaked in 20 ml distilled water and then boiled for 20 minutes. After boiling, it was filtered with Whatman's filter paper no. 1 and aqueous extract was ready to be used.

A. Qualitative Phytochemical Analysis

Plant extracts (Aqueous/ME-I) or dried leaves powder of *B. diffusa* were used for preliminary qualitative phytochemical screening of amino acids, carbohydrates, terpenoids, steroids, cardiac glycosides, phlobatannins, flavanoids, phenols, tannins, and saponins as per standard methodology[18-22].

B. Evaluation of in vitro Thrombolytic Activity

Assessment of *in vitro* thrombolytic activity of ME-II was carried out as per the methodology demonstrated by Prasad *et al.*[23]. For this purpose, institutional ethical approval was sought (Ref.PMU/PMCH/IEC/2019, dated 26.12.2019) and informed consent was obtained from ten healthy volunteers who were not taking any medication, or oral contraceptives or anticoagulant therapy.

Preparation of Plant Extract

For evaluation of in vitro thrombolytic activity, 100 mg crude methanolic extract (ME-II) was suspended in 10 ml of sterile distilled water to prepare a concentration of 10mg/ml. This was shaken vigorously on a vortex mixer and kept overnight at room temperature. The next day, using a syringe filter of 0.22µ pore size, it was filtered and 100 µl volume (1 mg/ml) of this extract was used for evaluation of thrombolytic activity.

Preparation of Streptokinase as Positive Control

As a well-known standard thrombolytic agent, Streptokinase was used as a positive control. STPase (commercially available lyophilized SK, manufactured by Cadila Pharmaceuticals, Ahmedabad, India) of 15,00,000 IU was dissolved in 5 ml of sterile distilled water and mixed thoroughly. This was always prepared fresh and 100 µl (30,000 IU) was used for determination of *in vitro* thrombolytic activity.

Evaluation of in vitro Clot Lysis Activity

Ten ml. fasting blood samples were drawn from the volunteers and 500 µl was poured in previously weighed sterile micro-centrifuge tubes. For clot formation, the tubes were incubated at 37ºC for 45 min. To separate the serum from blood clot, tubes were then centrifuged at 2000 rpm for 10 min and serum was removed carefully with the help of micropipette. Clot weight was then determined by reweighing the tubes as: Weight of micro-centrifuge tube having clot – Weight of micro-centrifuge tube. Addition of either 100 µl of plant extract, sterile distilled water (negative control) or streptokinase (positive control) was performed to the tubes having the pre-weighed clot and again incubated at 37ºC for 90 min to observe the clot lysis. The fluid obtained after clot lysis was removed carefully. Weight of clot, after lysis was determined by again weighing the tubes as: Weight of micro-centrifuge tube having clot – Weight of micro-centrifuge tube after clot lysis. Percent clot lysis was determined as: Weight of clot after lysis / Weight of clot × 100.

Statistical Analysis

Results of *in vitro* clot lysis activity are expressed as Mean ± SEM (standard error of the mean) for three replicates and statistical comparisons were performed using Student's Paired t-test using Microsoft Excel (2010). Differences between means were considered to be significant when p value was < 0.01.

RESULTS AND DISCUSSION

Preliminary qualitative phytochemical screening revealed the presence of primary metabolites such as carbohydrates and amino acids along with presence of secondary metabolites such as flavonoids, tannins, phlobatannins, phenols, saponins, cardiac glycosides, steroids and terpenoids in the leaves of *B. diffusa* collected from Udaipur, Rajasthan (Table 1). Interestingly, methanolic extract of aerial parts of *B. diffusa* prepared by continuous hot extraction method has shown absence of cardiac glycosides, saponins and steroids[24].

Table 1: Preliminary Qualitative Phytochemical Analysis of Leaves of *B. diffusa*

S. No.	Phytochemical Test	*B. diffusa* leaves
01.	Carbohydrate	+
02.	Amino acid	+
03.	Saponin	+
04.	Flavanoid	+
05.	Phenol	+
06.	Tannin	+
07.	Phlobatannin	+
08.	Terpenoid	+
09.	Cardiac glycoside	+
10.	Steroid	+

+ = Present

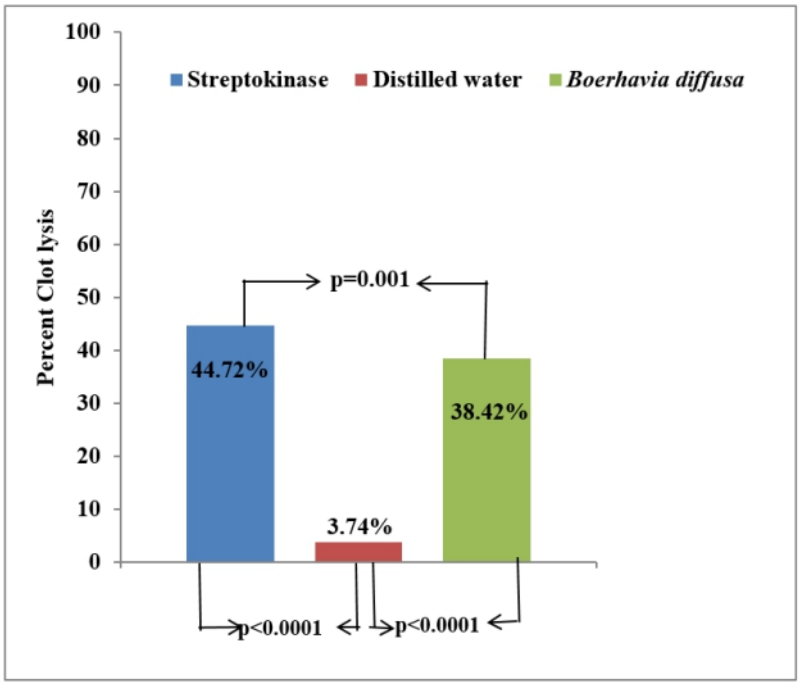

Figure 2. *In vitro* Thrombolytic Activity of *Boerhavia diffusa* Leaves

A statistically significant *in vitro* clot lysis activity of methanolic extract of *B. diffusa* leaves as compared to SK as positive control and distilled water as a negative control was observed in the present study for the first time (Figure 2).

Methanolic extract of leaves of *B. diffusa* in a concentration of 1 mg/ml (100 µl) exhibited 38.42±1.28 % *in vitro* clot lysis whereas streptokinase demonstrated 44.72±0.87 % clot lysis.

10.26±2.06% *in vitro* thrombolytic potential of methanolic extract of aerial parts of *B. diffusa*. In this regard, current results obtained from leaves of *B. diffusa* are more specific.

There are many plant species which have shown similar *in vitro* thrombolytic activity for example, hydro-alcoholic extracts of bulbs of *Allium cepa* and seeds of *Linum usitatissimum* have demonstrated 38.93 % and 35.65 % clot

Table 2: Percent Clot Lysis Activity of *B. diffusa* leaves, Streptokinase (30000 IU) as Positive Control and Distilled Water as Negative Control

S. No	Plant extract / SK / DW	Percent clot lysis (Mean±SEM)
1.	Methanolic extract of *B. diffusa* leaves	38.42±1.28 [a,b]
2.	Streptokinase (SK)	44.72±0. 87 [c]
3.	Distilled water (DW)	3.74±0.37

Values are expressed as Mean ± SEM
p value:
a- 1 v/s 2 p=0.001
b- 1 v/s 3 p<0.0001
c- 2 v/s 3 p<0.0001

Negative control of sterile distilled water exhibited negligible clot lysis of 3.74±0.37 % indicating that water had no role in the *in vitro* clot lysis (Table 2). Apu et al.[24] have shown

lysis activity respectively[6]. 39.30 ± 0.96% clot lysis by leaves of *Leea indica*[25], 38.86 ± 2.05 % clot lysis by fruits of *Tribulus terrestris*[26], 36.89 ± 1.94% clot lysis by chloroform soluble

fraction of *Brassica nigra* flowers[27] etc. and therefore, the present results obtained with leaves of *B. diffusa* are quite promising.

Plants of *Nyctaginaceae* family are rich in many bioactivities. Interestingly, the ornamental species of this family, *Bougainvillea spectabilis* were evaluated for *in vitro* thrombolytic potential and its leaves have shown 17.73% clot lysis at a concentration of 800 µg/ml after 24 hours of incubation[28]. In this regard, 38.42±1.28 % clot lysis of *B. diffusa* leaves (from the same family) after incubation of 90 minutes is a very important finding.

Ferreres et al.[29] reported that leaves of *B. diffusa* contains diverse classes of bioactive compounds including phenolics namely, quercetin, kaempferol, 3,4-dihydroxy-5-methoxycinnamoylrhamnoside, quercetin 3-O-robinobioside, quercetin 3-O-(2″-rhamnosyl)-robinobioside, kaempferol 3-O-(2″-rhamnosyl)-robinobioside, kaempferol 3-O-robinobioside, eupalitin 3-O-galactoside, 3,5,4′-trihydroxy-6,7-dimethoxyflavone 3-O-galactosyl (1 → 2) glucoside and caffeoyltartaric acid. Sharma and Sahai[30] have isolated various phytochemical constituents such as uridine triacetate, boeravinone B, eupalitin 3-O-β-Dgalactopyranoside, quercetin 3-O-α-D-rhamnoside, β-amyrin, β -amyrin acetate, 3-O- β-D-glucopyranosyl sitosterol, and β-sitosterol from leaves of *B. diffusa.* Boeravinone B is one of the important rotenoid of the plant which have shown significant in vivo anti-inflammatory activity at a dose of 50 mg/kg in carrageenan-induced rat paw model[31] as well as anticancer potential.[32] Several of these bioactive compounds have shown to possess antioxidant, anti-inflammatory, anti-platelet, and anti-coagulant potential and could be behind the thrombolytic action of the plant as observed in the present study. [33]

Besides, several minerals *viz*. sodium, potassium, calcium, manganese, iron, copper, zinc, magnesium, selenium etc. and vitamin C and E are also present in leaves of *B. diffusa*[34,35]. Recently, quantitative analysis of various phytochemicals such as flavonoid (14.14%), alkaloid (7.00%), oxalate (3.51 mg/g), saponin (14.00%), tannin (20.20 mg/100 g), total phenol (11.86 mg/100 g) and phytate (225.64 mg/100 g) contents have also been reported from ethanolic extract of *B. diffusa* leaves[36].

Antioxidant and hepatoprotective property of leaves of *B. diffusa* has been reported[34]. Ethanolic extract of *B.diffusa* leaves (500mg/kg body weight) have also shown to reduce blood sugar and total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol, phospholipids and increase in HDL-cholesterol in streptozotocin induced male wistar diabetic rats[37]. Juice of its leaves and lyophilized decoction has displayed anti-inflammatory activity by inhibiting abdominal writhing in mice[38].

Many dietary plants and natural food resources are proven to be useful in alleviating cardiovascular diseases and comparatively safer than modern medicine[33]. *B. diffusa* is also edible and full of nutritive and therapeutic phytochemicals. Besides, it has also shown antioxidant, hypolipidemic, hypoglycemic, and anti-inflammatory activities. In this regard, the present report of thrombolytic potential of *B. diffusa* is another feather in its cap and it could be recommended as a dietary supplement for prevention of cardiovascular disorders and associated symptoms.

CONCLUSION

Due to shortcomings of standard thrombolytic drugs, herbal drugs could be employed for treatment of thrombosis induced abnormalities. The present study has demonstrated that methanolic extract of *B. diffusa* leaves possess a significant 38.42±1.28 % clot lysis activity for the first time. Natural plant based products have a number of medicinal properties because of presence of various phytochemical compounds. *B. diffusa* leaves have also shown to possess various primary and secondary metabolites in the qualitative phytochemical screening. However, studies are required to isolate the bioactive compound responsible for the thrombolytic action and large scale *in vivo* studies for evaluation of thrombolytic potential of *B. diffusa* leaves are also warranted.

CONFLICT OF INTEREST: None

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