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RESEARCH ARTICLE

# ANTIOXIDANT AND THROMBOLYTIC ACTIVITY OF CHLOROFORM EXTRACT OF *BACOPA MONNIERA* (L.)

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**The present study was designed to investigate antioxidant and thrombolytic properties of chloroform extract of *Bacopa monniera* (L.), along with phytochemical study for the presence of chemical constituents. Antioxidant potential was evaluated using DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assays. In DPPH scavenging method, scavenging of DPPH was observed in different concentrations (20, 40, 60, 80, 100, 200, 400, 800 µg/ml). Plant extract found to demonstrate significant scavenging activity. Chloroform extract of *Bacopa monniera* (L.) showed significant clot lytic properties in different blood samples. The mean percent clot lytic activity of chloroform plant extract of *Bacopa monniera* (L.) was found to be 48.39%.**

**Key words:** *Bacopa monniera* (L.), DPPH scavenging method, antioxidant potential, clot lytic activity.

## INTRODUCTION

Different approaches to drug discovery from plants can be enumerated like random selection followed by chemical screening, random selection followed by one or more biological assays, follow-up of biological activity reports, follow-up of ethnomedical (traditional medicine) use of plants, use of appropriate plant parts as such in powdered form or preparation of enriched/standardized extracts (herbal product development), use of a plant product, biologically potent, as a lead for further chemistry, and single new compounds as drugs (Samuelsson, 1999).

The future of plants as sources of medicinal agents for use in investigation, prevention, and treatment of diseases is very promising (Sofowora, 1982). The objective of the later approach is the targeted isolation of new bioactive plant products *i.e.* lead substances with novel structures and novel mechanisms of action.

Plants are well known for their medicinal value since years (Jain *et al* 2011; Dey *et al* 2012; Deb *et al* 2013; Arjariya and Nema, 2014). *Bacopa monniera* (L.) (Family: Scrophulariaceae), also referred to as *Bacopa monnieri*, *Herpestis monniera*, water hyssop, and "Brahmi," has been used in the Ayurvedic system of medicine for centuries (**Figure 1**).

**Fig. 1.** *Bacopa monniera* (L.) leaves

Traditionally, it was used as a brain tonic to enhance memory development, learning, and concentration, and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used in India and Pakistan as a cardiac

tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction. Recent research has focused primarily on Bacopa's cognitive-enhancing effects, specifically memory, learning, and concentration, and results support the traditional Ayurvedic claims. Research on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome, and gastric ulcers also supports the Ayurvedic uses of Bacopa. In present work, *Bacopa monniera* chloroform extracts were evaluated for its thrombolytic and antioxidant potential.

## MATERIALS AND METHOD

### Collection and identification of plant

The plant *Bacopa monniera* (L.) was collected from University of Chittagong and identified by Syedul Alam (Sohel), Research Assistant, Bangladesh Forest Research Institute, Chittagong. A voucher specimen that contains the identification characteristics of the plant was submitted to the herbarium for future reference.

### Preparation of plant extract

The fresh *Bacopa monniera* (L.) plant was washed with water immediately after collection. The collected leaves were chopped into small pieces, air dried at room temperature (25±2°C for about 15 days and ground into powder form and stored in an airtight container. Powder (200 mg) was macerated in 900 ml pure chloroform for 7 days at room temperature with occasional stirring.

After 7 days, chloroform extract was filtered off through a cotton plug and finally with a Whatman No. 1 filter paper. The extract was concentrated under reduced pressure within 50-55°C through rotatory vaccum evaporator (Bibby Sterlin Limited, England). The concentrated extracts were collected in a Petri dish and allow to air dry for complete evaporation of chloroform.

The whole process was repeated three times and finally, 23.649 g blackish-green colored, concentrated stem extract was obtained (yield 16.30% *w/w*) which was kept in refrigerator at 4°C (Ghani, 2003).

### Phytochemical investigation

The freshly prepared crude chloroform extract was qualitatively tested for the presence of chemical constituents. These were identified by characteristic color changes using standard procedures (Trease and Evans, 1989; Sofowara, 1993; Ghani, 2003).

### DPPH radical scavenging assay

The antioxidant activity of *Bacopa monniera* (L.) chloroform extracts and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2, 2-diphenyl-1- picrylhydrazyl (DPPH)-free radical activity according to the method of Brand-Williams with slight modifications (Brand-Williams *et al* 1995). DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can generate stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant by measuring the decrease in the absorbance of DPPH at 517 nm (Rice-Evans *et al* 1997). Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule has an absorbance at 517 nm which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Braca *et al* 2001).

### Experimental procedure

*Bacopa monniera* (L.) chloroform extract with different concentrations (20, 40, 60, 80, 100, 200, 400 and 800 $\mu$g/ml) were prepared in methanol. Ascorbic acid with different concentrations (20, 40, 60, 80, 100, 200, 400 and 800 $\mu$g/ml) were prepared in methanol. DPPH solution (0.004%) was prepared in methanol. Three millilitre of this DPPH solution was mixed with 5 ml of extract solution and standard solution separately. These solution mixtures were kept in dark for 30 min. The degree of DPPH purple decolorization to DPPH yellow indicated the scavenging efficiency of the extract (Kumar *et al* 2008).

The absorbance of DPPH solution (Control solution 'A') was measured at 517 nm using UV-Visible Spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan). The absorbance of the mixture was determined at 517 nm using UV-Visible Spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan). Ascorbic acid was served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical-scavenging activity.

The (%) scavenging activity against DPPH was calculated using the following equation:

Scavenging activity (%) = [(A – B) / A] × 100

where A is the absorbance of control (DPPH solution without the sample), B is the absorbance of DPPH solution in the presence of the sample (extract/ ascorbic acid). Then, % scavenging activity or % inhibition was plotted against log concentration and from the graph $IC_{50}$ (Inhibition concentration 50) value was calculated by linear regression analysis.

**Standard and test solution preparation**

Stock solution of plant extract and ascorbic acid was prepared (5 mg/ml). Eight screw cap tubes were labeled as 20, 40, 60, 80, 100, 200, 400 and 800 µg/ml. Methanol (5 ml) was taken in each screw cap tube. 20, 40, 60, 80, 100, 200, 400 and 800 µl of methanol were discarded (put off) from the screw cap tubes according to label, using micropipette. 20, 40, 60, 80, 100, 200, 400 and 800 µl stock solution of concentration 5 mg/ml were added (put) accordingly, using micropipette.

***In vitro* thrombolytic activity**

***Streptokinase (SK)***

To the commercially available lyophilized SK vial (Durakinase, Dongkook Pharma. Co. Ltd., South Korea) of 15, 00,000 IU, 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 IU) was used for *in vitro* thrombolysis.

***Specimen***

Whole blood (4 ml) was drawn from healthy human volunteers (*n* = 10) without a history of oral contraceptive or anticoagulant therapy (using a protocol approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences, Nagpur). 500 µl (0.5 ml) of blood was transferred to each of the ten previously weighed microcentrifuge tubes to form clots.

***Herbal preparation***

100 mg *Bacopa monniera* (L.) Chloroform extract was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22-micron syringe filter. 100 µl of this aqueous preparation of herb was added to the microcentrifuge tubes containing the clots to check thrombolytic activity.

***Clot lysis***

Venous blood (4 ml) drawn from healthy volunteers was distributed in three different pre weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 min. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone).

To one microcentrifuge tube containing pre-weighed clot, 100 µl of aqueous extract of *Bacopa monniera* (L.) was added. As a positive control, 100 µl of SK and as a negative non thrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, fluid released 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 experiment was repeated 10 times with the blood samples of 10 volunteers (Tapsell *et al* 2006).

**RESULTS AND DISCUSSION**

**Phytochemical Screening**

Phytochemical screening of *Bacopa monniera* (L.) extract under this study explored the presence of medicinally active secondary metabolites - carbohydrates and gum, alkaloid, and tannins (Zhang and Guo, 2001; Zhao *et al* 2003). This investigation also indicated the absence of reducing sugar, steroid, glycoside and flavonoid.

**DPPH radical scavenging assay**

DPPH free radical scavenging method was used for the assay of *Bacopa monniera* (L.) chloroform extract and the scavenging activity was compared with the standard antioxidant ascorbic acid (Vitamin C). The DPPH free radical scavenging activity of the *Bacopa monniera* (L.) chloroform extract and ascorbic acid is shown in **Table 1, 2, Figure 2, 3**. Both ascorbic acid and *Bacopa monniera* (L.) chloroform extract showed dose dependent activity. Among the eight different concentrations used in the study (20, 40, 60, 80, 100, 200, 400 and 800 µg/ml)

**Table 1.** DPPH free radical scavenging activity of ascorbic acid (Standard)

| Ascorbic acid | | | | $IC_{50}$ |
|---|---|---|---|---|
| **Concentration (μg/ml)** | **Log concentration** | **Absorbance** | **% Scavenging activity** | |
| Control | – | 0.568 | - | |
| 20 | 1.30 | 0.183 | 67.78 | |
| 40 | 1.60 | 0.154 | 72.89 | |
| 60 | 1.78 | 0.126 | 77.82 | 1.61 μg/ml |
| 80 | 1.90 | 0.098 | 82.75 | |
| 100 | 2.00 | 0.073 | 87.15 | |
| 200 | 2.30 | 0.061 | 89.26 | |
| 400 | 2.60 | 0.039 | 93.13 | |
| 800 | 2.90 | 0.023 | 95.95 | |

**Table 2.** DPPH free radical scavenging activity of *Bacopa monniera* (L.) chloroform extract

| ***Bacopa monniera* (L.) chloroform extract** | | | | $IC_{50}$ |
|---|---|---|---|---|
| **Concentration (μg/ml)** | **Log concentration** | **Absorbance** | **% Scavenging activity** | |
| Control | – | 0.568 | - | |
| 20 | 1.30 | 0.313 | 44.89 | |
| 40 | 1.60 | 0.201 | 64.61 | |
| 60 | 1.78 | 0.198 | 65.14 | 16.48 μg/ml |
| 80 | 1.90 | 0.192 | 66.20 | |
| 100 | 2.00 | 0.109 | 80.81 | |
| 200 | 2.30 | 0.106 | 81.34 | |
| 400 | 2.60 | 0.097 | 82.92 | |
| 800 | 2.90 | 0.036 | 93.66 | |

ascorbic acid showed 67.78%, 72.89%, 77.82%, 82.75%, 87.15%, 89.26%, 93.13% and 95.95% scavenging activity respectively where highest scavenging activity was 95.95% at concentration 800 μg/ml (**Table 1**). On the other hand, *Bacopa monniera* (L.) extract showed 44.89%, 64.61%, 65.14%, 66.20%, 80.81%, 81.34%, 82.92% and 93.66% scavenging activity at the above mentioned eight different concentrations where highest scavenging activity of *Bacopa monniera* (L.) chloroform extract was 93.66% at concentration 800 μg/ml (**Table 2**).

% of scavenging activity or % of inhibition was plotted against log concentration and from the graph $IC_{50}$ (Inhibition concentration 50) value was calculated by linear regression analysis. $IC_{50}$ value of ascorbic acid and *Bacopa monniera* (L.) chloroform extract was found 1.61 and 16.48 μg/ml respectively (**Table 2, Figures 2-4**).

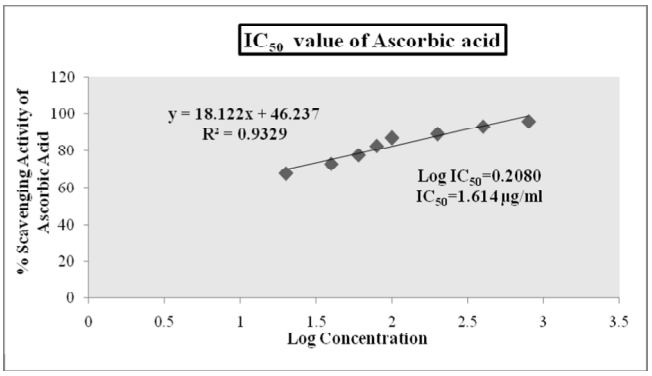


**Fig. 2.** DPPH free radical scavenging activity of ascorbic acid

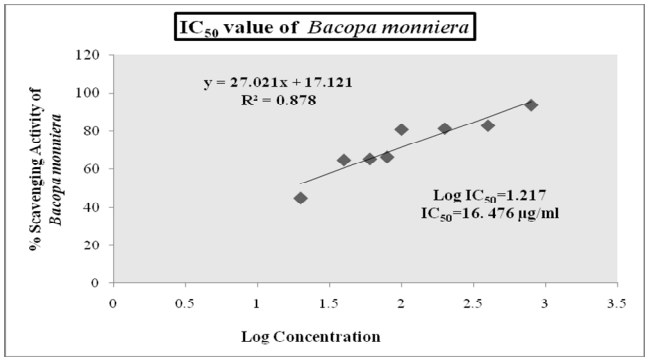


**Fig 3.** DPPH free radical scavenging activity of *Bacopa monniera* (L.) chloroform extract

***In vitro* thrombolytic activity**

Addition of 100 μl streptokinase (Durakinase, Dongkook Pharma. Co. Limited, South Korea), a positive control (30,000 IU) to the clots along with 90 min incubation at 37°C, showed 85.77% clot lysis. On the other hand, clots when treated with 100 μl sterile distilled water (negative

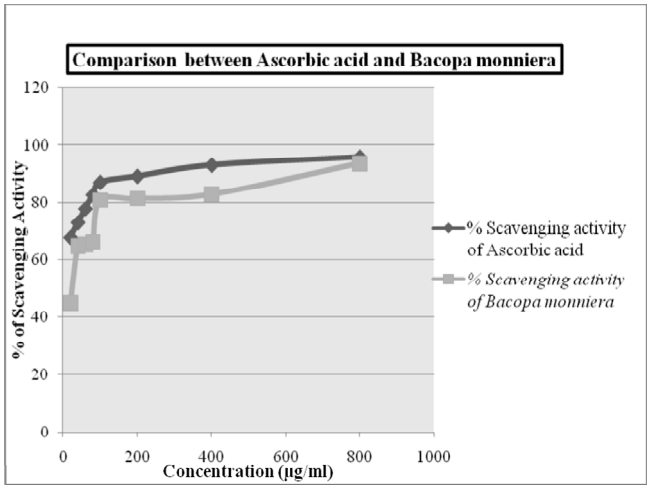


**Fig. 4.** Relative % scavenging activity or % inhibition of standard antioxidant ascorbic acid and *Bacopa monniera* (L.) chloroform extract

control) showed only negligible clot lysis which was only 4.70%. The mean difference in clot lysis percentage between positive and negative control was very significant (***p value < 0.001). But when 100 µl *Bacopa monniera* (L.) chloroform extract was added to 10 different clots, 48.39% clot lysis were obtained and when compared with the negative control (water) the mean clot lysis percentage differences was significant (***p value < 0.001). Percent clot lysis obtained after treating with water, streptokinase and *Bacopa monniera* (L.) chloroform extract shown in **Figure 5**.

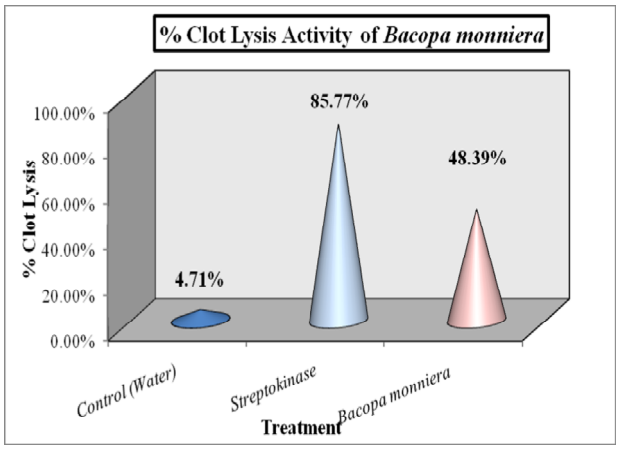


**Fig. 5.** Comparative % *in vitro* thrombolytic effect of *Bacopa monniera* (L.) chloroform extract, streptokinase and water (negative control)

Statistical representation (Student's *t*-test) of the effective clot lysis percentage by *Bacopa monniera* (L.) chloroform extract, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) is tabulated in **Table 3.**

**Table 3.** Effect of *Bacopa monniera* (L.) chloroform extract on *in vitro* thrombolysis

| Sample | % Clot lysis |
|---|---|
| Control (water) | 4.70 |
| Streptokinase | 85.77 |
| *Bacopa monniera* (L.) chloroform extract | 48.39 |

Percentage of clot lysis of 10 different blood samples after treated with water, streptokinase and *Bacopa monniera* (L.) chloroform extract is shown in **Table 4**.

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

All the tubes were incubated at 37°C for 90 min.

After Incubation, fluids released was removed and tubes were again weighted to observe the difference in weight after clot disruption. Differences obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

The experiment was carried out with blood sample of 10 volunteer.

**Table 4.** Comparison of the data of % of clot lysis using SPSS 11.5

Group statistics (Control *vs* Streptokinase)

Group statistics

| | Control, Streptokinase | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| **% of clot lysis** | Control | 10 | 4.7990 | .65113 | .20590 |
| | Streptokinase | 10 | 85.7720 | 1.12211 | .35484 |

Independent samples test

| | | | % of Clot lysis | |
|---|---|---|---|---|
| | | | **Equal variances assumed** | **Equal variances not assumed** |
| **Levene's test for equality of variances** | F | | 5.950 | |
| | Significance | | .025 | |
| **t-test for equality of means** | T | | -197.372 | -197.372 |
| | $D_f$ | | 18 | 14.444 |
| | Significance (2-tailed) | | .000 | .000 |
| | Mean difference | | -80.9730 | -80.9730 |
| | Std. error difference | | .41026 | .41026 |
| | 95% Confidence interval of the difference | Lower | -81.83491 | -81.85038 |
| | | Upper | -80.11109 | -80.09562 |

Group Statistics (Control *vs Bacopa monniera*)

Group statistics

| | **Control, Streptokinase** | **N** | **Mean** | **Std. deviation** | **Std. error mean** |
|---|---|---|---|---|---|
| **% of Clot lysis** | Control | 10 | 4.7990 | .65113 | .20590 |
| | *Bacopa monniera* | 10 | 48.3860 | 4.62638 | 1.46299 |

Independent samples test

| | | | % of Clot lysis | |
|---|---|---|---|---|
| | | | **Equal variances assumed** | **Equal variances not assumed** |
| **Levene's test for equality of variances** | F | | 14.049 | |
| | Significance | | .001 | |
| **t-test for equality of means** | T | | 24.834 | 24.834 |
| | $D_f$ | | 18 | 10.055 |
| | Significance (2-tailed) | | .000 | .000 |
| | Mean difference | | 37.3860 | 37.3860 |
| | Std. error difference | | 1.50541 | 1.50541 |
| | 95% Confidence interval of the difference | Lower | 34.22326 | 34.03424 |
| | | Upper | 40.54874 | 40.73776 |

Independent samples test

Group statistics (*Bacopa monniera* (L.) *vs* Streptokinase)

Group statistics

| | **Control, Streptokinase** | **N** | **Mean** | **Std. deviation** | **Std. error mean** |
|---|---|---|---|---|---|
| **% of Clot lysis** | Streptokinase | 10 | 85.7720 | 1.12211 | .35484 |
| | *Bacopa monniera* | 10 | 48.3860 | 4.62638 | 1.46299 |

Independent samples test

| | | | % of Clot lysis | |
|---|---|---|---|---|
| | | | **Equal variances assumed** | **Equal variances not assumed** |
| **Levene's Test for Equality of Variances** | F | | 14.049 | |
| | Significance | | .001 | |
| **t-test for Equality of Means** | T | | 24.834 | 24.834 |
| | $D_f$ | | 18 | 10.055 |
| | Significance (2-tailed) | | .000 | .000 |
| | Mean difference | | 37.3860 | 37.3860 |
| | Std. error difference | | 1.50541 | 1.50541 |
| | 95% Confidence interval of the difference | Lower | 34.22326 | 34.03424 |
| | | Upper | 40.54874 | 40.73776 |

All values are expressed as Mean ± SEM (n=20); ***P<0.001 significant compared to negative control

## CONCLUSION

*Bacopa monniera* (L.) extract was found to exhibit significant scavenging activity which was found to increase with concentration of the extract with $IC_{50}$ value of 16.48 µg/ml as compared to $IC_{50}$ value of the reference ascorbic acid was 1.61 µg/ml. Further, chloroform extract of *Bacopa monniera* (L.) showed significant ($P < 0.001$) clot lytic properties. Mean percent clot lytic activity of chloroform plant extract of *Bacopa monniera* (L.) was found to be 48.39% which was significant as compared with the water (negative control) and standard enzyme streptokinase (positive control).

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