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Investigation of Thrombolytic and Antioxidant Potentials of *Centella Asiatica*

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ABSTRACT

The aim of the present investigation was to find out the antioxidant as well as the thrombolytic properties of *Centella asiatica* which is known as "Thankuni" in Bangladesh and used as a folk medicine. The methanolic extract of *Centella asiatica* plant was partitioned into petroleum ether soluble fraction (PSF), Chloroform soluble fraction (CSF), Ethyl acetate soluble fraction (ESF) and aqueous soluble fraction (ASF). The extracts were evaluated for their thrombolytic and antioxidant potentials and the results were compared with standard drugs; streptokinase for thrombolytic. In thrombolytic investigation, the aqueous soluble fraction (ASF) showed highest percent of clot lysis (50.53%) as compared to (63.74%) exhibited by the standard streptokinase. The presence of antioxidant potentials of *Centella asiatica* were evaluated by antioxidant capacity and reducing power to free radical. The total antioxidant capacity of Methanol extract of *Centella asiatica* (MCA), PSF, CSF, ESF and ASF were observed as mg of extract, respectively. The DPPH free radical in term of IC_{50} values of the extractives was found 108.25 µg/ml, 909.03 µg/ml, 292.44 µg/ml, 104.61 µg/ml and 173.09 µg/ml for MCA, PSF, CSF, ESF and ASF respectively in comparison to 400.02 µg/ml for BHT. The result obtained in this study found that *Centella asiatica* plant possesses potential thrombolytic and antioxidant effect.

Keywords: *Centella asiatica*, thankuni, antioxidant, thrombolytic effect.

INTRODUCTION

Free radicals are considered for aging and age related diseases. [1] Cancer, arthritis, Alzheimer's disease, diabetes etc can be happened when free radicals damage cell. [2,3] Extreme necrosis may happen because of free radicals. [4] Better medicinal effects can be achieved by targeted antioxidants. According to some reviewers, it is found that; antioxidant could decline side effects and augmented survival times. [5]

Thrombosis can reduce the blood supply to specific organ and different parts of body. [6] In UK, around 25000 people die because of thrombosis. [7]

About 500 medicinal plants have been reported to occur in Bangladesh. Almost 80% of rural populations are dependent on medicinal plants for their primary health care.

In recent times, focus on plant research has increased all over the world and a large body of evidence has been accumulated to highlight the immense potential of medicinal plants used in various traditional systems of medicine. *Centella asiatica* (CA) is an important medicinal herb which is used in the orient as medicine in the Ayurvedic tradition of India for thousands of years. It is commonly known as Indian pennywort (English), Thankuni (Bangladesh), Brahmi-buti (Hindi), Jal Brahmi or Mandookaparni. The herb is also used by the people of Java and other

Indonesian islands. In China, known as gotu kola, it is one of the reported “miracle elixirs of life” known over 2000 years ago.

Centella asiatica leaves are entire, crenate, orbicular and reniform. Leaves are 1.5-6.5cm in diameter, petioles 7.5-15cm in length, stipules are short forming sheathing base. It bears an umbel inflorescence with 3-4 pink sessile flowers. The stems are red and show long internodes. This plant is found in marshy areas all over Bangladesh, India, Sri Lanka, Madagascar and Africa up to an altitude of 650m.

In the nineteenth century, CA and its extracts were incorporated into the Indian pharmacopoeia, wherein in addition to wound healing, it was recommended for the treatment of mental and neurological disorders, various skin diseases such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhoea, fever, amenorrhea, and diseases of the female genitourinary tract. It is also used as vegetables. [8] In our current study, we investigated to find out the antioxidant and thrombolytic effect of *Centella asiatica*.

MATERIALS AND METHODS

Collection and Preparation of the plant material: *Centella asiatica* leaf was collected from rural areas of Tangail in July 2017. The plant was identified by Bangladesh National Herbarium (DACB accession number: 46,671) . After collecting the leaves was cleaned then cut into small pieces & were air dried for several days. After drying, the net weight of the leaf was reduced to about 500 gm from 10 kg. The dried leaves were then ground in coarse powder.

Plant extraction: The powdered materials (500gm) were soaked in 2.5 liters of methanol at room temperature for 14 days. The container with its content was sealed with cotton plug and aluminum foil and occasionally shaken during this period. The extracts were filtered through clean cotton cloth and subsequently with cotton bed and finally with filter paper. The filtrates were concentrated with a rotary evaporator under reduced pressure at 50°C and evaporated to dryness. An aliquot of the methanol extract was successively fractionated with petroleum ether, chloroform and ethyl acetate according to modified Kupchan partitioning protocol. The resultant fractions were evaporated to dryness to yield pet ether (PSF), chloroform (CSF), ethyl acetate (ESF) and aqueous (ASF) soluble materials. These residues were then stored in a refrigerator until further use.

Separation by solvent- solvent partition: The crude methanol extract was diluted with water and successively extracted (3 times each) with petroleum ether, chloroform and ethyl acetate. The concentrated and dried fractions were measured. PSF was denoted for Pet-ether soluble fraction, CSF for chloroform soluble fraction, ESF for Ethyl acetate soluble fraction and ASF for remaining aqueous soluble fraction.

Preliminary Phytochemical Investigations of Crude Extract: Freshly prepared crude extracts of *Centella asiatica* were qualitatively tested for the presence of various chemical constituents including alkaloids, flavonoids, reducing sugars, tannins and saponins by following standard procedures.

Specific test for Chemical Characterization of Crude Extract: The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents. These were identified by characteristic color changes using standard procedures.

Molisch’s Test for Carbohydrates: Two drops of Molisch’s reagent were added to about 5 mg of the extract in 5 ml aqueous solution in a test tube. 1 ml of conc. H_2SO_4 was allowed to flow down the side of the inclined test tube so that the acid formed a layer beneath the aqueous solution without mixing with in a red ring was formed at the common surface of the two liquids which indicated the presence of carbohydrate. On standing or shaking a dark-purple solution was formed. Then the mixture was shaken and diluted with 5 ml of water. Dull violet precipitate was formed immediately.

Fehling Test for Reducing Sugar: 2 ml of aqueous extract of the plant material was added to 1 ml of equal volume of Fehling's solution A and B. Then they were boiled for few minutes. A red or brick red color precipitate was formed in the presence of reducing sugar.

Test for Glycosides: A small amount of extract was dissolved in water and alcohol then boiled with Fehling's solution. Any brick-red precipitation was noted. Another portion of extract was dissolved in water and alcohol and boiled with a few drops of dilute H_2SO_4. The acid was neutralized with NaOH solution and boiled with Fehling's solution. A brick-red precipitation was produced in this experiment which showed the presence of glycosides in the extract.

Tests for Alkaloids: 300 mg of extract was digested with 2M of hydrochloric acid. This acidic filtrate was mixed with amyl alcohol at room temperature and the alcoholic layer was examined for the appearance of pink colour which indicates the presence of alkaloids. The respective color and precipitate formation was observed by Mayer's reagent, Hager's reagent, Wagner's reagent and Dragendorff's reagent. In case of Mayer's reagent formation of white and cream color precipitate indicated the presence of alkaloids. In case of Hager's reagent Formation of yellow crystalline precipitate indicated the presence of alkaloids. In case of Wagner's reagent Formation of brownish-black ppt indicated the presence of alkaloids. In case of Dragendorff's reagent formation of orange or orange-red precipitate indicated the presence of alkaloids.

Test for Saponins: 300 mg of extract was boiled with 5ml of water for two minutes. The mixture was cooled and mixed vigorously and left for three minutes. The formation of frothing indicates the presence of saponins. It was taken as preliminary evidence for the presence of saponins.

Test for Flavonoids: A dilute ethanolic solution of the test sample (0.5ml) was added to 0.5ml of aqueous NaOH solution (5% NaOH) in a test tube. The development of yellow to orange color indicates the presence of flavonoids. Again, dilute ethanolic solution of test sample was taken in a test tube. Then 4-5 drop of concentrated sulfuric acid was added. Yellow to orange color indicates the presence of flavonoids. The presence of flavonoids was determined using 1% aluminium chloride solution in ethanol, concentrated hydrochloric acid, and magnesium chloride solution. Immediate development of a red color indicated the presence of flavonoid.

Test for Tannins: An aliquot of the extract was added to sodium chloride to make 2% strength. It was filtered and mixed with 1% gelatin solution. Precipitation was taken as evidence for the presence of tannins.

Test for Triterpenes: 300 mg of extract was mixed with 5 ml of chloroform and warmed for 30 min. the chloroform solution is then treated with a small volume of conc. Sulphuric acid and mixed properly. The appearance of red colour indicates the presence of triterpenes.

Test for steroids: It was measured by Salkowski test. About 2mg of the extract was taken in a test tube and 2ml of chloroform was added. The test tube was shaken slowly to dissolve the extract. Concentrated H_2SO_4 was poured slowly by the side of the test tube so that it formed a separate layer at the bottom. Forming a purple colored ring at the junction of the two liquids indicates the presence of steroids.

Test for alcohol: A small amount of test sample was dissolved in 0.5ml of dioxane. The solution was added to 0.5ml of citric nitrate reagent diluted to 1ml with dioxane and shaken well. A yellow-red colour indicates the presence of alcohol.

Test for phenol: 2 drops of neutral ferric chloride solution was added to 1ml of diluted aqueous solution of the test sample. A greenish purple color indicates the presence of Phenolic compounds.

Test for substituted amide (-NHCOR): The compound was boiled with 6N HCl for 7-8minutes and cooled under tap water and then 10% $NaNO_2$ solution was added. This

solution was transferred to a test tube containing alkaline β-naphthol solution. Orange red color indicates the presence of substituted amide (-NHCOR).

Antioxidant activity: The free radical scavenging activity of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were determined by the method of Brand-Williams *et al*., 1995.2.0 ml of methanolic solution of the extract at different concentration were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). Then the antioxidant potential was assayed of purple colored methanol solution of DPPH radical by plant extract as compared to the tert-butyl-1-hydroxytoluene (BHT) by UV spectrophotometer.

Control preparation of antioxidant activity measurement: Tert-butyl-1-hydroxytoluene (BHT) was used as positive control. Calculated amount of BHT was dissolved in methanol to get a mother solution having a concentration of 1000 µg/ml. Then serial dilution was made using the mother solution to get different concentration from 500.0 to 0.977 µg/ml.

Test sample preparation: Calculated amount of petroleum ether, chloroform and aqueous fraction of methanolic extract of *Centella asiatica* was dissolved in methanol to get a mother solution having a concentration of 1000 µg/ml. Then serial dilution was made using the mother solution to get different concentration from 500.0 to 0.977 µg/ml.

DPPH solution preparation: 20 mg of DPPH was weighed and dissolved in methanol to get a mother solution having a concentration of 20µg/ml. The solution was prepared in the amber reagent bottle and kept in light-proof box.

Assay of Free Radical Scavenging Activity: 2.0 ml of a methanol solution of the sample (control/ extracts) at different concentration from 500.0 to0.977 µg/ml were mixed with 3.0 ml of DPPH methanol solution (20µg/ml). After 30 minutes reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I %) was measure by using below formula-

$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$

Where A_{blank} is the absorbance of control reaction (containing all reagents except the test material)

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

RESULTS

Identification of bioactive compounds: On phytochemical screening with specific reagent; the crude methanol extract of plant *Centella asiatica* showed the positive test for important chemical constituents such as alkaloids, glycosides, flavonoids, phenols and saponins.

Table 1. Phytochemical screening of methanol extract of *Centella asiatica*

Constituents present (+ve result)	Constituents absent (-ve result)
Carbohydrate, Tannins, Alkaloids, Flavonoids, Saponin, Phenols, Glycosides, Coumarin,	Terpenoids, Resin

***In-vitro* thrombolytic activity of *Centella asiatica*:** The percentage of weight loss of clot after the application of crude ethanolic *Centella asiatica* extract solution was taken as the functional indication of thrombolytic activity. The study was implemented on human volunteer with 5ml blood sample and value of weight loss (in %) was calculated to examine with the following formula:

% clot lysis = (Weight of the released clot / Weight of clot before lysis) × 100

The crude ethanolic extract of *Centella asiatica* showed significant thrombolytic activity which supports the traditional use of this plant in various diseases. It was found that % of clot lysis by crude methanolic extract of *Centella asiatica* (leaf) and its different fractions were 23.25%, 50.53%, 31.08%, 49.50%, 25.57% respectively to

Methanol Soluble fraction, Petroleum ether soluble fraction, Chloroform soluble fraction, Ethyl acetate soluble fraction, Aqueous soluble fraction.

Table 2.*In-vitro* investigation of Thrombolytic activity of *Centella asiatica* leaves

Fractions	W_1 (gm)	W_2 (gm)	W_3 (gm)	W_4(gm)	W_5 (gm)	% ofclot lysis
SK	0.52	1.00	0.55	0.48	0.45	63.74%
MSF	4.140	4.7873	4.6368	0.6473	0.1505	23.25%
PSF	4.250	4.605	4.4256	0.355	0.1794	50.53%
CSF	4.227	4.801	4.6226	0.574	0.1784	31.08%
ESF	4.227	4.913	4.5734	0.686	0.3396	49.50%
ASF	4.226	4.6260	4.5237	0.4	0.1023	25.57%

Here, W_1= Weight of empty vial, W_2= Weight of clot containing vial before clot disruption, W_3= Weight of clot containing vial after clot disruption, W_4= Weight of clot before clot disruption clot = (W_2-W_1), W_5= Weight of clot after clot disruption clot = (W_2-W_3), % of clot lysis = (W_5/W_4) ×100%, MSF = Ethanol Soluble fraction, PSF= Petroleum ether soluble fraction, CSF = Chloroform soluble fraction, ESF= Ethyl acetate soluble fraction, ASF = Aqueous soluble fraction of *Centella asiatica* leaf and Standard (SK)= Streptokinase.

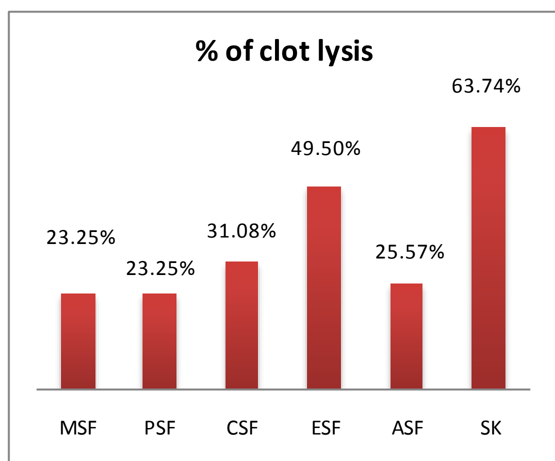


Figure 1.Percent (%) of clot lysis by different fraction of *Centella asiatica.*

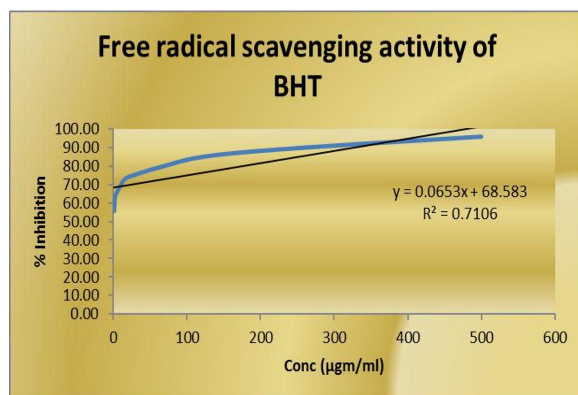
Antioxidant potentials: The presence of antioxidant potentials of *Centella asiatica* were evaluated by various determinants such as total phenolic content, antioxidant capacity, flavonoid content and reducing power of free radical.

Free radical scavenging activity (DPPH): The Methanol extract of leaf of *Centella asiatica* (MSF) and different partitionates i.e. chloroform (CSF), Pet-ether (PSF) and aqueous (ASF) soluble fraction of the methanol extract of whole plant of *Centella asiatica* were subjected to free radical scavenging activity by the method of Williams *et. al.* [9] Here, tert-butyl-1-hydroxytoluene (BHT) was used as reference standard.

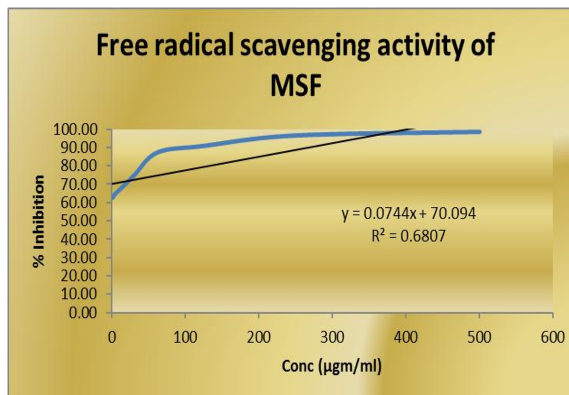
The antioxidant activity of IC_{50} values in DPPH method are differed in different extractives.

Table 3. IC_{50} values of the standard and partitionates of leaves of *Centella asiatica.*

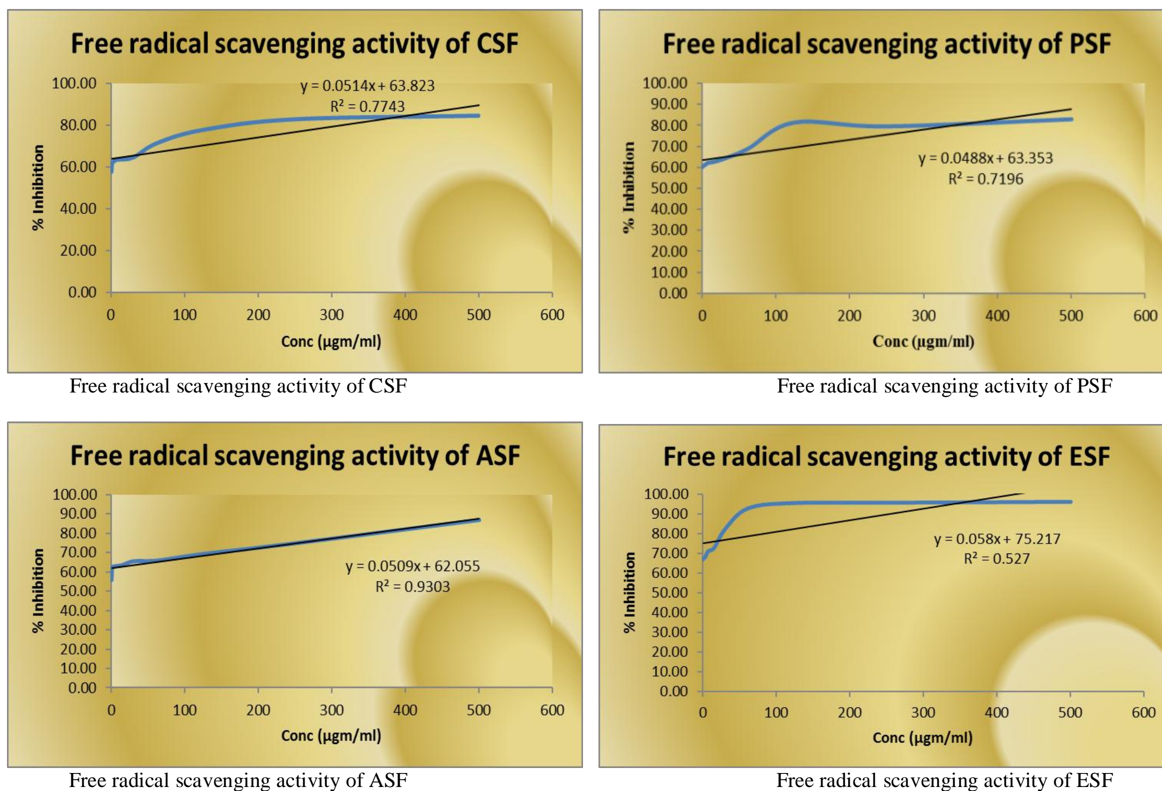
Plant part	Sample code	Test Sample	IC_{50} (µg /ml)
Leaf of *Centella asiatica*	MSF	Methanol extract Leaf of *Centella asiatica*	108.25
	CSF	Chloroform soluble fraction	292.44
	PSF	Pet-ether soluble fraction	909.03
	ASF	Aqueous soluble fraction	173.09
	ESF	Ethyl acetate soluble fraction	104.61
	BHT (Std)	tert-butyl-1-hydroxytoluene	400.02



Free radical scavenging activity of BHT



Free radical scavenging activity of MSF



Free radical scavenging activity of CSF

Free radical scavenging activity of PSF

Free radical scavenging activity of ASF

Free radical scavenging activity of ESF

Figure 2.(A-F) Free radical scavenging activity of BHT, MSF, CSF, PSF, ASF and ESF.

DISCUSSION

Formation of blood clot (thrombus), one of the major causes of blood circulation problem can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This may result in damage, destruction (infarction), or even death of the localized tissues (necrosis) in that area. Thrombus is formed from fibrinogen by thrombin and is lysed by plasmin, which is activated from plasminogen by tissue Plasminogen activator (tPA). All thrombolytic agents activate the enzyme plasminogen that clears the cross linked fibrin mesh. Fibrinolytic drugs can dissolve thrombi in acutely occluded coronary arteries thereby can restore blood supply to ischaemic myocardium and can limit necrosis. Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial in fraction. It reduces mortality as effectively as the non-antigenic alteplase in most in farct patients. Tissue-type Plasminogen activator (t-PA) is generally considered as being suitable and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant drawbacks. Large doses are required for effective affect. The plant kingdom represents an enormous reservoir of biologically active compounds with diversified structures having disease preventive properties. Nearly 50% of drugs used in medicine are of plant origin, and only a small fraction of plants with medicinal activity has been assayed. Therefore, much current research devoted to the phytochemical investigation of higher plants which have ethno-botanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity like thrombolytic potentials which could overcome the shortcomings of the current thrombolytic agents. Cerebral venous sinus thrombosis (CVST) is a common disorder which accompanied by significant morbidity and mortality. Heparin, an anti-coagulating agent, is the first line of treatment for CVST, because of its efficacy, safety and feasibility. Thrombolytic drugs like tissue plasminogen activator (t-PA), urokinase, streptokinase etc. play a crucial role in the

management of patients with CVST. Thus, the aim of the present study was to investigate the thrombolytic activity of ethanolic extracts and its different fractions of leaves of *Centella asiatica.*

Antioxidants present in plants were providing health-promoting ingredients in human diet and also responsible for the prevention and treatment of radical-mediated disorders. Therefore the interest in the field of antioxidant effects of compounds derived from plants, which could be relevant to their nutritional incidence and their role in health and disease is increasing day by day. Different synthetic antioxidant such as tert-butyl-1-hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) etc. used as food additives to increase self-life are known to have toxic and carcinogenic effects on human health and also produces abnormal effects on enzyme system. Therefore, the interest in natural antioxidant, especially derived from plant source, has greatly increased in recent years.

CONCLUSION

Centella asiatica is used in a wide range of pharmacological activity within the ethnobotanical frame worldwide. The crude methanol extract of *Centella asiatica* were subjected to different phytochemical investigations such as phytochemical screening, thrombolytic and antioxidant activity. The plant has potential thrombolytic and antioxidant activity.

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