



RESEARCH ARTICLE

IN VITRO ANTIOXIDANT AND ANTITHROMBOTIC ACTIVITY OF *CLEOME VISCOSA*

Sharmila, G. and *Brindha Durairaj

Department of Biochemistry, PSG College of Arts and Science, Coimbatore-641014

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ABSTRACT

For the past few decades plants have been the basis of treatment for many human diseases. In this study traditional plant *Cleome viscosa*, annual weed was selected and its free radical scavenging potential, total antioxidant capacity and antithrombotic activity were evaluated. Preliminary phytochemical screening of *Cleome viscosa* leaf extracts revealed the presence of phytochemical constituents (alkaloids, flavonoids, phenols etc.). From the results obtained the hydroethanolic extract exhibited maximum activity when compared with aqueous extract. Hence, an attempt has been made to evaluate the inhibitory effect of hydroethanolic extract on *in vitro* antithrombotic activity. The results obtained revealed that *Cleome viscosa* has exhibited considerable antithrombotic activity which may be due to the phytochemical constituents present in it.

INTRODUCTION

In India, rich knowledge about medicinal importance of plants is available to the common people. About 3,500 plant species are used as a source of crude drug in India. Some plant species are considered as a weed, but they are medicinally important too. In various traditional and modern methods of therapy, plants products are also used (Packialakshmi and Oviya, 2014). Thus natural products have been a major source of drugs for centuries (Ravinder *et al.*, 2011). Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans (Hasler and Blumber, 1999). They protect plants from disease and damage and contribute to the plant's colour, aroma and flavour (Gibson *et al.*, 1998). Oxidative stress plays an important role in the pathogenesis of various diseases such as atherosclerosis, alcoholic liver cirrhosis and cancer. The reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide formed in the biological system due to several metabolic activities in cells. It is also known to initiate the lipid peroxidation of phospholipids in cell membranes, thereby propagating a chain reaction and ultimately results in cell damage (Braca *et al.*, 2002). Antioxidants are substances that counteract oxidative damage from ROS (Pulido *et al.*, 2000). A blood clot (thrombus) developed in the circulatory system due to failure of hemostasis causes vascular blockage and while recovering

leads to serious consequences in atherothrombotic diseases such as myocardial or cerebral infarction, at times leading to death (Lee, 1995). Thrombolytic agents are used all over the world for the treatment of these diseases. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Adams and Cory, 1998). *Cleome viscosa* which is also known as wild mustard, dog mustard belongs to the family Cleomaceae (Edeoga *et al.*, 2009). Cleomaceae are a small family of flowering plants in the order Brassicales (Raghavan, 1993). These genera were previously included in the family Capparaceae, but were raised to a distinct family when DNA evidence suggested that they are more closely related to Brassicaceae than they that of the Capparaceae (Stevens, 2001). The present study therefore investigates the *in vitro* antioxidant, free radical scavenging potential, total antioxidant activity and antithrombotic activity of *Cleome viscosa* leaf extract.

MATERIALS AND METHODS

Plant material

The leaves of *cleome viscosa* were collected from different localities of Coimbatore district, Tamil Nadu in September 2016 and authenticated by the Botanical Survey of India (BSI) at "Tamil Nadu Agriculture University" Coimbatore, Tamil Nadu. A voucher specimen (No.BSI/SRC/5/23/2016/Tech/2114) has been deposited at the Herbarium of the Botany

*Corresponding author: Brindha Durairaj,
Department of Biochemistry, PSG College of Arts and Science, Coimbatore-641014

department of "Tamil Nadu Agriculture University" for future reference.

Preparation of extract

The air-dried and powdered leaves (5g each) were macerated with 50% ethanol, water, acetone, ethanol, ethyl acetate for 3 days, with occasional stirring. After 3 days, the suspensions were filtered through a fine muslin cloth and it is used for further analysis.

Qualitative screening of phytochemical constituents

The qualitative phytochemical screening of different solvents of *Cleome viscosa* leaf extracts were carried out by using standard procedure (Harborne, 1973) to test the presence of secondary metabolites including alkaloids, anthocyanin, flavonoids, glycosides, phenols, proteins, saponins, steroids and tannins.

Determination of enzymic and non enzymic antioxidants

Antioxidant ability of *Cleome viscosa* by using different *in vitro* systems including enzymic, non enzymic antioxidant, free radical scavenging assays (Hydroxyl and nitric oxide radical) and total antioxidant activity were screened. Various concentrations of *Cleome viscosa* were compared with standard and were evaluated for its antioxidant potential.

Determination of glutathione reductase

The glutathione reductase determination was carried out as per the method reported by Beutler, 1984 (Beutler, 1984). The reaction mixture contained 1.5 ml of buffer, 0.5 ml of EDTA, 0.2 ml of GSSG and 0.1ml of NADPH. The reaction initiated by the addition of 0.2ml of enzyme extract. The enzyme activity is calculated intermediate of micromoles of NADPH oxidized /min/mg protein. The activity of the enzyme was determined by observing the change in absorbance at 340nm.

Determination of carotenoids

Weigh 5 to 10 g of the sample and saponify for about 30 minutes in a shaking water bath at 37°C after extracting the alcoholic KOH. Transfer the saponified extract into a separating funnel (Packed with glass wool and calcium carbonate) containing 10 to 15ml of petroleum ether and mixed gently which take up the carotenoid pigments into the petroleum ether layer. Transfer the lower aqueous phase to another separating funnel and petroleum ether extract containing the carotenoid pigments to an amber colored bottle. Repeat the extraction of the aqueous phase similarly with petroleum ether, until it is colorless. Discard the aqueous layer. To the petroleum ether extract added a small quantity of sodium sulphate to remove turbidity. Note the final volume of the petroleum ether extract and diluted if needed by a known dilution factor. The absorbance at 450nm was noted in a spectrophotometer using petroleum ether as a blank. The results are expressed as mean \pm SD for three replicates.

Free radical scavenging potential

Hydroxyl radical scavenging activity

The reaction mixture contains deoxyribose (2.8mM), FeCl buffer (67mM, pH 7.8) and various concentrations of the

extracts in a final volume of 3.0ml. The tubes were illuminated under incandescent lamp for 15min. The optical density at 530nm was measured before and after illumination the inhibition of superoxide radical was determined by comparing the absorbance values of the control with those of treatments. Ascorbic acid was used as standard (Rajeshwar *et al.*, 2005).

Nitric oxide radical scavenging activity

Nitric oxide was generated from sodium nitroprusside and measured by Greiss reaction. Sodium nitroprusside in standard phosphate buffer solution was incubated with different concentration (100 - 500 μ g) of the ethanol extract dissolved in phosphate buffer (0.025M, pH 7.4) and the tubes were incubated at 25°C for 5hr. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5hr., 0.5ml of incubation solution was removed and diluted with 0.5 ml of griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% naphthylethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthylethylene diamine was read at 546 nm and percentage inhibition calculated (Madan *et al.*, 2005).

Ferric thiocyanate (FTC) method

A mixture consisting of 4mg of the sample in 4ml of absolute ethanol, 4.1ml of 2.52% linoleic acid in absolute ethanol, 8 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of distilled water was added to a vial with screw cap and then kept in an incubator at 40°C in dark place. To 0.1ml of this mixture 9.7ml of 75% ethanol and 0.1ml of 30% ammonium thiocyanate were added. After 3 minutes, 0.1ml of 0.02M ferrous chloride in 5% hydrochloric acid was added to reaction mixture (the absorbance of red color indicated the antioxidant activity). Absorbance was observed at 500nm for every 24 hours until the absorbance of the control reached maximum level. Both control and standard were subjected to the same procedures. The solvent was used for control and for the standard 4mg of the sample was replaced by 4mg of vitamin C (Kikuzaki and Nakatani, 1993).

TBA method

At low pH, and high temperature (100° C), melonaldehyde binds TBA to form a red complex. 2ml of 20% trichloroacetic acid and 2ml of 0.67% TBA solutions were added to 2ml of the mixtures containing the sample prepared in FTC method. This mixture was kept in water bath (100°C) for 10 minutes and after cooling to room temperature, was centrifuged at 3000rpm for 20 minutes. Antioxidant property was calculated based on the absorbance of the supernatant at 532nm on the final day of assay (Ottolenghi, 1959).

FRAP assay

The standard solution and extract solution was prepared in the concentration range of (100 -800 μ g) in different test tubes from that 1.0 ml of sample is taken to different tubes. Add 2.5 ml of phosphate buffer (0.2M, pH - 6.6). To that tubes add 2.5 ml of 1% potassium ferricyanide. Incubate all the tubes at 50°C for 20 min. After incubation add 2.5 ml of 10% TCA and centrifuge the tubes at 3000 rpm for 10mins. Collect 2.5ml of upper layer and add 2.5 ml distilled water and add 0.5 ml of

0.1% FeCl₃ to all the tubes. The intensity of red color formation was read at 700 nm in a UV Visible spectrophotometry. Maintained the control instead of sample solution, make with distilled water (Oyaizu, 1986).

Antithrombotic assay

2.5 ml of venous blood drawn from healthy volunteers was distributed in 5 different pre weighed sterile micro centrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 minutes. 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 each micro centrifuge tube containing pre-weighed clot, 100 µl of different extracts of plant samole is added. To the commercially available lyophilized streptokinase vial (Lupiflo, Lupin Limited, Mumbai, India) 2.5 ml of PBS was added and thoroughly mixed. This suspension was used as a stock from which 100µl was added to the micro centrifuge tube as a positive control. For negative control, 100µl of distilled water were added. All the tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the 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 (Prasad *et al.*, 2006).

RESULTS

Table 1 show in water, extract alkaloids, anthocyanin, proteins, saponins and tannins are present. In hydroethanolic extract alkaloids, flavonones, phenol and steroids are present. Ethanol extract shows no phytochemical constituents in qualitative screening. In ethyl acetate only glycosides is present. In acetone extract only alkaloids and steroids are present. However, compared with different solvent extracts hydroethanol and aqueous extract were found to contain more selected active phytoconstituents.

Table 1. Phytochemical screening of *Cleome viscosa* leaf extract

Biochemical Tests	Water	Hydro ethanol	Ethanol	Ethyl acetate	Acetone
Alkaloids	+	+	-	-	+
Anthocyanin	+	-	-	-	-
Flavonones	-	+	-	-	-
Glycosides	-	-	-	+	-
Phenol	-	+	-	-	-
Proteins	+	-	-	-	-
Saponins	+	-	-	-	-
Steroids	-	+	-	-	+
Tannins	+	-	-	-	-

'+' indicates presence, '-' indicates absence

From Table 2 it was revealed that *Cleome viscosa* found to possess effective GST activity. Gayathri *et al.*, 2013 reported that the catalase and glutathione peroxidase content of *Cleome viscosa* leaf extract to be (97±0.7) and (23.25 min/g).

From carotenoids estimation hydroethanol extract shows 0.627±0.015 and aqueous extract shows 0.377±0.015. The β-carotene content of fresh cape gooseberry was 1074.67 ± 6.41 mg·100 g⁻¹ sample, which is higher than that previously reported by Ramadan and Morsel, (2007).

Table 2. Enzymic and non-enzymic antioxidants of *Cleome viscosa* leaf extract

% inhibition		
Leaf extracts	Glutathione reductase (U/g sample)	Carotenoids (mg/g sample)
Hydroethanol	2.333±0.233	0.627±0.015
Water	1.333±0.233	0.377±0.015

The values are expressed as Mean ± SD. (n=3)

From the results of hydroxyl radical scavenging activity it may be postulated that the hydroethanolic extract was found to have 95% of inhibition at 100µg/ml concentration than the standard and water extract. IC₅₀ value of hydroethanolic extract was found to be 9.39 ±16 aqueous extract to be 59.25 ± 48.3 and for standard ascorbic acid as 54.62 ±16.6. Lakshmi and Bindu, (2013) reported that the % inhibition by methanol extract of *Cleome viscosa* higher when compared with *Cleome burmanni*.

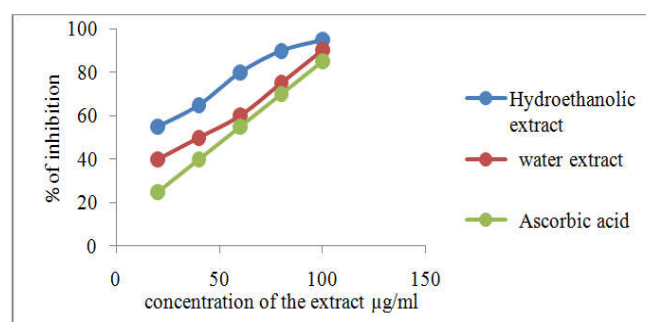


Figure 1. Inhibition of hydroxyl radical by *Cleome viscosa* leaf extract

From the results of nitric oxide it may be postulated that the hydroethanolic extract was found to have 88% of inhibition at 1mg/ml concentration when compared with water extract and standard at the same concentration. IC₅₀ value of hydroethanolic extract was found to be 49.46±52, aqueous extract to be 61.37±1.04 and for standard it was found to be 48.54 ± 45. Lakshmi and Bindu, 2013 reported that the methanol extract of *Cleome viscosa* shows higher % of inhibition when compared with *Cleome burmanni*.

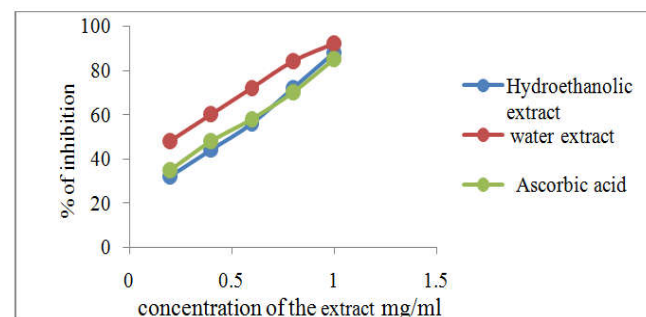


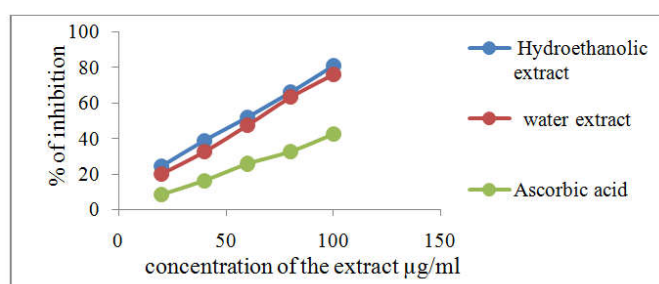
Figure 2. Inhibition of nitric oxide radical by *Cleome viscosa* leaf extract

Table 3 shows the total antioxidant activity by FTC and TBA methods. The results reveal that there was a significant decrease in the absorbance when compared with that of the control, suggesting high level of antioxidant potential. Hydroethanolic leaf extract of *Cleome viscosa* shows low absorbance value than the control which indicated a high level of antioxidant activity.

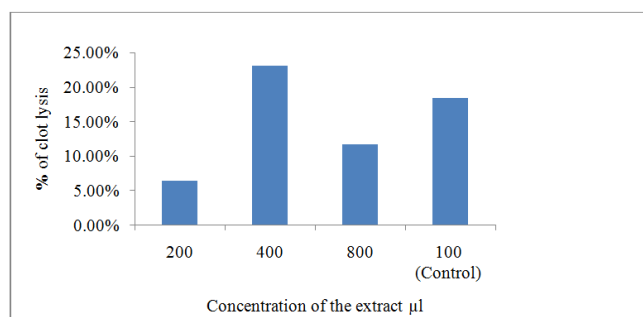
Table 3. Antioxidant activity of hydroethanolic leaf extract of *Cleome viscosa* by FTC and TBA method

Samples	FTC								TBA
	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7	Day-8	Day-9
Control	0	0.080	0.105	0.143	0.175	0.223	0.232	0.251	0.260
Vitamin -C	0	0.038	0.041	0.048	0.068	0.073	0.078	0.086	0.087
Hydroethanol	0	0.036	0.050	0.071	0.085	0.140	0.164	0.185	0.206
Water	0	0.012	0.025	0.038	0.051	0.078	0.098	0.120	0.144

Figure 3 shows results for FRAP assay. From figure it may be postulated that the hydroethanolic extract was found to have 80.74% of inhibition at 100 μ g/ml concentration when compared with ascorbic acid and water extract. IC₅₀ value of hydroethanolic extract was found to be 508.2 \pm 1.52, aqueous extract to be 101.15 \pm 16.6 and for standard it was found to be 1107.83 \pm 2.4. Balakrishnan and Kokilavani (2011) reported that when the sample concentration increases the scavenging effect also increases in dose dependent manner.

**Figure 3. FRAP Total antioxidant activity of *Cleome viscosa* leaf extract**

In thrombolytic activity using *in vitro* clot lysis assay method, the crude hydroethanol extract showed average clot lysis of 6.4%, 23.1% and 11.61% while the standard streptokinase showed 18.43% which is shown in figure-4. The percentage of clot lysis was found to be significant when compared with the vehicle control.

**Figure 4. Antithrombotic activity of *Cleome viscosa* leaf extract**

DISCUSSION

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans (Hasler *et al.*, 1999). *Cleome viscosa* showed positive result for alkaloids, anthocyanin, flavonoids, glycosides, phenols, proteins, saponins, steroids and tannins in the hydroethanol and water extracts. Many of these compounds have been recognized to possess wide range of medicinal properties such as antioxidant, antihepatotoxic and anticancer effects. In the present study, the enzymic and non enzymic antioxidants were studied. Glutathione-S-transferase (GST) is

a cytosolic enzyme that plays a vital role in detoxification and excretion of xenobiotics (Gayathri *et al.*, 2013). From the results, it was revealed that *Cleome viscosa* found to possess effective GST activity. Carotenoids are fat-soluble pigments that have significant antioxidant potential, with the main carotenoids being lycopene and β -carotene (Cardoso *et al.*, 2011). Hydroethanolic extract of *Cleome viscosa* exhibited highest carotenoid content than aqueous extract. These activities were due to the presence of phytochemical constituents in it. From the results of hydroxyl radical and nitric oxide scavenging activity it may be postulated that the hydroethanolic extract was found to have maximum inhibition at corresponding concentration than the standard and water extract. From FTC and TBA methods there was a significant decrease in the absorbance value when compared with that of the control suggesting high level of antioxidant potential. The higher absorbance value indicates the lower level of antioxidant capacity. Hence the leaf extract possess strong antioxidant activity when compared with that of the control. FRAP assay was assessed and found to possess maximum % of inhibition.

Cardiovascular disease caused by blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in the recent years (Sai *et al.*, 2012). The result of antithrombotic activity shows that the *Cleome viscosa* plant extract at 400 μ l was found to have highest % of clot lysis. These findings clearly indicate that the extract has a very good antithrombotic activity.

Conclusion

The phytochemical constituents can be derived from any part of the plant and may contain active components. The present investigation confirmed the antithrombotic activity of hydroethanolic leaf extract of *Cleome viscosa* shows the maximum activity. Further works on the *in vivo* antithrombotic activity of the hydroethanolic leaf extract of *Cleome viscosa* needs to be assessed.

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