

A Possible Anti-Atherothrombosis Activity Via Cytoprotective Trait of The *Clerodendrum viscosum* Leaf Methanol Extract

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Abstract

Previous reports saying that, the shrub, *Clerodendrum viscosum* Vent. has a number of important biological activities. Oxidative stress and inflammatory events trigger cardiovascular diseases (CVD), known as one of the major consequences in health problems. This study evaluated a possible clotlysis activity of *C. viscosum* leaf (MECVL). The hot MECVL undergone for antiradical (DPPH*: 1,1-diphenyl-2-picrylhydrazyl radical), egg albumin protection and inhibition of hemolysis of human erythrocytes (HRBCs), and clotlysis analysis, taking ascorbic acid, acetyl salicylic acid and streptokinase as standards, respectively. Additionally, a preliminary phytochemical study was also conducted, suggesting the presence of alkaloids, glycosides, flavonoids, reducing sugars, and gums in MECVL. The MECVL concentration dependently scavenged the DPPH*, inhibited protein denaturation, protected HRBCs and caused clotlysis. In the DPPH assay, MECVL at 100 µg/mL scavenged DPPH radical by 47.1 ± 0.8%. The highest activity was observed at the highest concentration of the MECVL (500 µg/mL), with which it inhibited protein denaturation, protected HRBCs and caused clotlysis by 81.5 ± 0.1%, 89.4 ± 0.1% and 81.9 ± 4.8%, respectively. Although in all cases the standards exhibited better activities, but the effects of MECVL should be considered significant (p<0.05) in comparison to the negative control. The extract exhibited an anti-atherothrombosis capacity possibly via antioxidant, anti-inflammatory and membrane stabilization pathways, recommending *C. viscosum* as a promising phytotherapeutic tool, especially on pathology of CVD.

Keywords: Antioxidant; Anti-inflammatory; Membrane stabilization; Cardioprotective

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Introduction

The use of plant products is increasing antique, eventually increasing day by day. At present, thousands of plant metabolites are being successfully used in the treatment of a variety of diseases. According to an estimate, 80% of the world's population is found to rely upon plants for medication [1]. For example, more than 47% of all drugs, used in Russia are obtained from plant sources [2]. In Bangladesh about 250 plant species are used in herbal medicines.

Clerodendrum viscosum Vent. is a slightly woody shrub (2-4 feet in height) under the family Verbenaceae. It is a terrestrial shrub

having square, blackish stem and simple, opposite, decussate, petiolate, exstipulate, coriaceous, hairy leaves with a disagreeable odour [3]. Flowers are bluish-purple often white in pyramid shaped terminal panicles. It has blunt quadrangular stems and branches, leaves are usually three at a node, sometimes opposite oblong or elliptic, serrate, flowers are blue, many in long cylindrical thyrus and the fruits are four lobed purple durpe, somewhat succulent with one pyrene in each lobe [4]. The plant tropical habitat and commonly distributed throughout the plains of India, mainly in West Bengal [5]. It is known as Hill glory bower and *Clerodendrum* in English; Bhandira and Sthauneya in Sanskrit; Bhates and Titabhamt in Hindi; Periyalam, Peruku and perigalam in Malayalam, and Ghentu or Vatia in Bengali.

Different species of *Clerodendrum* genus have been traditionally used over centuries for their promising biological activities in – oxidative stress, inflammation, furunculosis, echymosis, gastritis, burns, wounds, headaches, dizziness, furuncles, hysteroptosis, dyspepsia, dropsy, fever, jaundice, typhoid, syphilis, gonorrhoea, ophthalmia, antidiabetic, antibacterial, anti-malaria, organ damage and so on [5-7].

In Indian folk medicine, parts of *C. viscosum* are used in the treatment of bronchitis, asthma, fever, wound, blood disorders, inflammation, burning sensation and epilepsy. Various parts of the plant are used by tribes in colic, scorpion sting and snake bite, tumors and certain skin diseases. The leaves are slightly bitter, cure inflammation, skin diseases and small pox [7]. The bitter substance, clerodin is widely used as a tonic and an anthelmintic agent in the North India. The leaf extract is also reported for its haem synthesis, anti-piles, anti-diabetic, and pain-killer activities. Leaves and roots contain clerodolone, clerodone, clerodol, and a sterol now designated as clerosterol and are used for external applications on tumours [6,7]. In a phytochemical study, the species *C. viscosum* revealed the presence of saponin, clerodin (a bitter diterpene) and some enzymes. Leaves also contain fixed oils, while roots by luperol, β -sitosterol, clerosterol (5,25-sigmastadien-3 β -ol, clerodolone as lup-20(30)-en-3 β -diol-12-one), clerodone (3 β -hydroxylupan-12-one) and a steroidal glycoside. The plant is also evident to contain alkyl sterols, 2-(3,4-dehydroxyphenyl) methanol 1-O- α -2-rhamnopyranosyl-(1 \rightarrow 3)- β -D-(4-O-caffeoyl) glycopyranoside (acteoside), triterpenes, steroids and flavonoids [7].

A clot inside the blood vessel is called atherombosis, which is one of the major consequences of cardiovascular diseases. Unfortunately, recently used anti-atherothrombotic drugs have a number of serious adverse effects [8], which is a stimulation to search of new and safe agents. According to [9], oxidative stress due to the overproduction of reactive oxygen and nitrogen species (ROS/RNS) triggers inflammatory and chronic human diseases, including aging and atherosclerosis.

In previous studies, *C. viscosum* has been found to show organo-protective along with antioxidant and anti-inflammatory capacities. Thus, this study was shot out to screen a possible anti-atherothrombosis effect of the crude methanol leaf extract of *C. viscosum* in human clotted blood. In this regard, after a preliminary phytochemical investigation, antioxidant and anti-inflammatory potentials were re-checked along with a newly perfumed membrane stabilization test.

Materials and Methods

Research ethics

This study was conducted under the Department of Pharmacy, Southern University Bangladesh (SUB) with the ethical number #PH-9991010. For human blood, ten students of the same/different departments of the SUB were willingly participated in this study. A specialist from a reputed diagnostic centre was invited to collect 4-5 mL blood through venous puncture.

Plant collection, identification, drying and grinding

The whole plant was collected from the Batali Hill-Tracts (Chittagong), Bangladesh and was identified at Forest Research Institute (Chittagong), Bangladesh. A voucher specimen was deposited with the number: BFRIH-2034.

The leaves were washed with running tap water and subjected for shade-drying (temperature not exceeding 50°C). Dried leaves were then ground into coarse powder with the help of a grinder and stored in an amber color airtight container until the extraction commenced.

Preparation of crude extract

Approximately 110 g of powdered materials was subjected to hot extraction with 700 mL of absolute methanol for 10 h using a Soxhlet extractor (Quickfit, England). The extract was then concentrated under an air cooler. The yield value was 15.89%.

To carry out the antioxidant assay, the crude extract was dissolved in methanol (used for extraction), while in distilled water (DW, with a 10min hand shake) following to an overnight standing for the other tests. For antioxidant test, we used a concentration range from 3.125-100 μ g/mL, while for other assays it was 125-500 μ g/mL. The standards were diluted in DW.

Source of reagents and chemicals

The standards and the other necessary reagents and chemicals were purchased from the Sigma Chem Ex. Co. St. Louis, Missouri, United States of America.

Preliminary phytochemical analysis of the methanol extract of *Clerodenrum viscosum*

Phytochemical study was conducted according to the methods described by [10].

Antioxidant capacity assay (DPPH radical scavenging test)

The test for DPPH• scavenging activity was done using a modified method described by Islam et al. [11]. Briefly, 0.3 mL of sample was added to a 2.7 mL ethanolic solution of DPPH (0.5 mM). After 30 min, the absorbance was measured using a spectrophotometer at 517 nm. A similar concentration of TRO served as the positive control, while only 0.3 mL methanol was considered as a negative control group. The blank contained no sample. The DPPH radical scavenging potential was calculated using the following equation:

$$\% \text{ inhibition of DPPH}^{\bullet} \text{ scavenging} = [(A_{br} - A_{ar})/A_{br}] \times 100$$

Where, A_{br} and A_{ar} represent the before and after absorbance of DPPH free radicals in reaction mixtures.

Evaluation of anti-inflammatory activity (egg albumin denaturation test)

The anti-inflammatory (*in vitro*) of methanol extract of *C. viscosum* (MECVL) was carried out according to [12]. Briefly, 1% egg albumin was constituted in phosphate buffer saline solution (PBS, pH 7.4). The assay mixture contains 1 mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hypo-saline (0.36%) and 0.5 mL of the test

sample. Acetyl salicylic acid (ASA) and DW were taken as positive and negative (NC) controls. After the incubation at 37°C for 30 min, reaction mixtures were centrifuged and the supernatant was collected for spectrophotometric analysis at 560 nm. Activity was measured by the following equation:

$$\% \text{ inhibition of protein denaturation} = 100 - \left[\frac{\text{absorbance of test solution}}{\text{absorbance of control}} \right] \times 100$$

Evaluation of membrane stabilization capacity (HRBC assay)

This test was conducted by the hypo-saline induced hemolysis (HL) method described by a modified method [13]. In this occasion, fresh human RBC was reconstituted as 10% suspension human red blood cell reconstitution (HRBC) in isosaline (0.9% NaCl, pH 7.4). The assay mixture contains 1 mL phosphate buffer (pH 7.4, 0.15 M), 2 mL hypo-saline (0.36%), 0.5 mL HRBC suspension (10% v/v) with 0.5 mL of the test sample. The ASA and DW were taken as positive and negative controls. After incubation at 37°C for 30 min, the reaction mixtures were centrifuged and the supernatant was collected for spectrophotometric analysis at 560 nm. Activity was measured by the following equation.

$$\% \text{ inhibition HL} = 100 - \left[\frac{\text{absorbance of test solution}}{\text{absorbance of control}} \right] \times 100$$

Anti-atherothrombosis assay (Clotlysis test in human blood)

The thrombolytic activity of the MECVL was evaluated [12] using streptokinase (SK) 100 IU and previously said NC, respectively. Briefly, blood was collected from 10 healthy volunteers and distributed into pre-weighed (W1) micro-centrifuge tubes (0.5 mL/tube) and incubated at 37°C for 45 min and then weight (W2) was taken. The weight of clotted blood (ΔW) was taken by subtracting the pre-weight and the weight of clotted blood containing tube. Then 100 μ L of the treatments were added to the clot containing tubes marked. The MECVL was tested at concentrations of 125-500 μ g/mL. On the other hand, 100 μ L of streptokinase (100 IU/tube) and 100 μ L of NC were added to the control marked tubes. All the tubes were then incubated at 37°C for 90 min. After incubation, fluid released was removed carefully without disrupting the clot, and tubes were again weighed for getting the weight variation among the pre-weight and final weight (W3) that was achieved for clotlyses.

Statistical analysis

Values are mean \pm SD (standard deviation). The data were analyzed by means of analysis of variance (ANOVA) followed by *t*-Student–Newman–Keuls's as *post-hoc* test using the GraphPad Prism software (version 6.0) with 95% confidence interval at $p < 0.05$.

Results

Preliminary phytochemical analysis revealed the presence of alkaloids, glycosides, flavonoids, reducing sugars and gums (Table 1).

According to the Table 2, the MECVL showed significant ($p < 0.05$)

DPPH radical scavenging capacity in comparison to the NC group. However, the activity was lower than the ascorbic acid (AA, standard). The extract and ascorbic acid at 100 μ g/mL exhibited highest scavenging of DPPH radical by $47.1 \pm 0.8\%$ and $94.8 \pm 1.1\%$, respectively. A concentration-dependent radical scavenging capacity was observed by the MECVL and AA. The NC produced a negligible radical scavenging capacity.

Both MECVL and the standard, acetyl salicylic acid (ASA) concentration-dependently inhibited protein denaturation capacity in egg albumin assay. At 500 μ g/mL, MECVL and the ASA produced % inhibition of protein denaturation by $81.5 \pm 0.1\%$ and $89.1 \pm 0.1\%$, respectively. In comparison to the NC group, the MECVL and ASA significantly ($p < 0.05$) inhibited egg albumin denaturation. The IC_{50} s calculated for the MECVL and ASA were $127.7 \pm 0.5 \mu$ g/mL and $91.9 \pm 0.6 \mu$ g/mL, respectively (Table 3).

Table 4 says that, not only the ASA, but also MECVL concentration-dependently and strongly inhibited HL of HRBC when compared to the NC group. At 500 μ g/mL, ASA and MECVL exhibited HL inhibition by $92.2 \pm 0.1\%$ and $89.4 \pm 0.1\%$, respectively. IC_{50} s calculated for the MECVL and ASA were $123.8 \pm 0.6 \mu$ g/mL and $82.2 \pm 0.6 \mu$ g/mL, respectively.

Table 5 suggests that, the MECVL has clotlysis capacity as compared to the NC group. The highest clotlysis capacity of the MECVL was observed with 500 μ g/mL concentration of $81.9 \pm 4.8\%$, while the standard drug, streptokinase (SK) by $84.5 \pm 3.9\%$ with 100 μ L. The NC produced negligible clotlysis capacity.

Discussion

Nevertheless, till date considerable attention has been given on plant-derived products. There is no doubt that, these kinds of medicaments are increasingly popular throughout the world. In fact, plant foods are one of the common daily consumption to us [14]. Previous phytochemical studies suggest that *C. viscosum* contains glycosides, flavonoids, steroids and some sugars. To date, seven sugars have been demonstrated in *C. viscosum* such as raffinose, lactose, maltose, sucrose, galactose, glucose and fructose [6,7]. Our study demonstrates the presence of alkaloids, reducing sugars and gums along with the previously reported phytochemical groups.

Table 1 Chemical groups identified in methanol extract of *Clerodendrum viscosum*.

Chemical groups	Consequences
Alkaloids	++++-
Glycosides	++
Steroids	--
Tannins	--
Flavonoids	+
Saponins	-
Reducing sugars	++
Gums	+

(+): Presence; (-): Absence; Numbers represent the number of tests performed.

Table 2 DPPH radical scavenging capacity of crude extract and controls.

Parameters		% scavenge of DPPH radicals		
		MECVL	AA	NC
Concentration (µg/mL)	3.125	10.2 ± 0.6 ^a	13.3 ± 1.3 ^a	-
	6.25	24.5 ± 1.1 ^a	32.3 ± 0.8 ^{a,b}	-
	12.5	29.3 ± 2.1 ^a	50.5 ± 1.5 ^{a,b}	-
	25	33.0 ± 1.0 ^a	90.8 ± 0.8 ^{a,b}	1.8 ± 0.4
	50	34.6 ± 1.3 ^a	93.6 ± 0.6 ^{a,b}	-
	100	47.1 ± 0.8 ^a	94.8 ± 1.1 ^{a,b}	-
IC ₅₀ [CI; R ²]		8.3 ± 0.2 [4.5 – 15.3; 0.8]	9.5 ± 0.4 [7.1 – 12.8; 0.9]	-

Values are mean ± standard deviation (SD); ^ap <0.05 compared to the NC; ^bp <0.05 compared to the MECVL at same concentration; ANOVA followed by *t*-Student–Newman–Keuls's as *post-hoc* test; AA: ascorbic acid; MECVL: methanol extract of *C. viscosum* leaf; NC: negative control (methanol); IC₅₀: Half minimal inhibitory concentration; CI: Confidence interval; R²: Coefficient of determination.

Table 3 Anti-inflammatory assay in egg albumin.

Test groups	
NC	
ASA (µg/mL)	125
	250
	500
MECVL (µg/mL)	125
	250
	500

Values are mean ± standard deviation (SD) (n = 5); ANOVA followed by *t*-Student–Newman–Keuls's as *post-hoc* test; *p <0.001 when compared to the NC; ASA: acetyl salicylic acid; MECVL: methanol extract of *C. viscosum* leaf; NC: negative control (DW); IC₅₀: Half minimal inhibitory concentration; CI: Confidence interval; R²: Coefficient of determination.

Table 4 Membrane stabilization test in re-constituted human erythrocytes.

Test groups		% hemolysis inhibition	IC ₅₀ [CI; R ²]
NC		0.0 ± 0.0	-
ASA (µg/mL)	125	66.7 ± 0.1*	82.2 ± 0.6
	250	84.8 ± 0.1*	[27.5 –
	500	92.2 ± 0.1*	245.7; 0.9]
MECVL (µg/mL)	125	46.1 ± 0.1*	123.8 ±
	250	69.8 ± 0.1*	0.6 [27.9 –
	500	89.4 ± 0.1*	549.3; 0.9]

Values are mean ± standard deviation (SD) (n = 5); ANOVA followed by *t*-Student–Newman–Keuls's as *post-hoc* test; *p <0.001 when compared to the NC; ASA: acetyl salicylic acid; MECVL: methanol extract of *C. viscosum* leaf; NC: negative control (DW); IC₅₀: Half minimal inhibitory concentration; CI: Confidence interval; R²: Coefficient of determination.

Flavonoids are well-known for their promising biological activities, including immunomodulatory, anticancer, cyto- and organo-protective, anti-diabetic and so on [14]. Phytochemicals containing active hydroxyl group (-OH), especially the essential oils and flavonoids may act as multi-edged sword in various diseases, has been considered as perfect weapons in the treatment of diseases [14]. In our study, MECVL concentration-dependently exerted antioxidant and anti-inflammatory activities. Furthermore, it also exerted a strong membrane stabilization capacity in HRBCs. The determined IC₅₀s, confidence interval (CI) and coefficient of determination (R²) values suggest that MECVL has significant

cytoprotective capacity. The AA and ASA are established antioxidant and anti-inflammatory agents, respectively. The AA can scavenge ROS and stimulate physiological defense systems, while ASA has a shunting effect on pro-inflammatory as well as inflammatory mediators. However, substance having radical scavenging and antagonistic capacity of inflammatory mediators may impart cellular protection. For an example, the diterpene phytol is evident to scavenge OH radical from the cell surface of *Saccharomyces cerevisiae*, thus protects them from OH-induced damaging effect. This diterpene is also evident to protect rat erythrocytes from hydrogen peroxide-induced oxidative stress,

Table 5 Thrombolytic activity of crude extract and controls.

Treatments	Per tube (100 μ L)	% clotlysis	IC ₅₀ [CI; R ²]
NC	-	2.7 \pm 1.5	-
SK	100 IU	84.5 \pm 3.9*	-
MECVL	125 μ g/mL	36.8 \pm 4.1*	138.7 \pm 0.5
	250 μ g/mL	62.8 \pm 4.7*	[44.4 – 433.7;
	500 μ g/mL	81.9 \pm 4.8*	0.9]

Values are mean \pm standard deviation (SD) (n = 5); ANOVA followed by t-Student–Newman–Keuls's as post-hoc test; *p<0.001 when compared to the NC; SK: streptokinase; MECVL: methanol extract of *C. viscosum* leaf; NC: negative control (DW); IC50: Half minimal inhibitory concentration; CI: Confidence interval; R2: Coefficient of determination.

which is a powerful oxidizer that liberates OH radical [14,]. Moreover, examples of phytochemicals with antioxidant and anti-inflammatory capacities are vast [10,14].

Thrombus formation inside the blood vessel obstacles the passages of required amount of oxygen and nutrients to the cells has been detected as one of the major consequences of cardiological complications, including stroke [8]. The SK is a widely used fibrinolytic drug, which binds and hydrolytically activates human plasminogen. The ultimate result is the clotlysis. On the other hand, oxidative stress due to the overproduction of reactive oxygen and nitrogen species (ROS/RNS) triggers inflammation and chronic human disease, including aging. The ROS/NOS-mediated oxidative stress can be identified in most of the key steps in the pathophysiology of atherosclerosis and the consequential clinical manifestations of cardiovascular disease due to it involves lipid metabolism, plaque rupture, thrombosis, myocardial injury, apoptosis, fibrosis and failure [9]. In this study, we found MECVL has a clotlysis capacity of human clotted blood. In comparison to the NC group, MECVL exhibited a significant (p<0.05) clotlysis capacity. The MECVL may act through either way.

Conclusion

The findings in phytochemical screening of MECVL are the agreement of previous studies. MECVL concentration-dependently exhibited antioxidant, anti-inflammatory, membrane stabilization and clotlysis activities. Antioxidant and anti-inflammatory-mediated cytoprotective effect may be linked to its membrane stabilization and anti-atherothrombosis effects. Further researches are welcomed to isolate and investigate responsible phytochemicals and their molecular mechanisms.

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Conflict of Interest

None declared.

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