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Antihemolytic Activity of *Clerodendrum viscosum* Vent. is Mediated by its Antioxidant Effect

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SH, MAB and MR designed the study, performed the statistical analysis. Author SH interpreted the results. Authors MR and SH wrote the protocol, and wrote the first draft of the manuscript. Authors MR, AR and NF managed the analyses of the study. Authors AR and NF managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The objective of this study was to evaluate the protective effect of polar extract of *Clerodendrum viscosum* vent. against *in vitro* hemolysis of human erythrocytes and its association with the antioxidant activity of *C. viscosum*.
Study Design: Extraction of *C. viscosum* dried root, *in vitro* antihemolytic activity assay, lipid peroxidation assay, phytochemical analysis, estimation of polyphenols and flavonoids.
Place and Duration of Study: Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka, Bangladesh, between January 2011 and May 2011.
Methodology: *C. viscosum* polar extract-pretreated erythrocytes were hemolysed by hypotonia and oxidizing agent, H_2O_2. The liberated hemoglobin was determined as a measure of hemolysis. Total reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging activity of extract were compared with those of vitamin C. Anti-

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lipid peroxidation activity of *C. viscosum* polar extract also was determined by exposing rat brain cortex tissue to Fenton's reagent (H_2O_2+$FeSO_4$)- induced oxidative stress. Then *C. viscosum* extract was subjected to estimation of total polyphenols and total flavonoids following qualitative phytochemical analysis.

Results: *C. viscosum* polar extract significantly inhibited *in vitro* hemolysis. Total reducing power and DPPH-radical scavenging activity were higher than those of vitamin C. In cortical tissue homogenate, *C. viscosum* polar extract significantly reduced (~38%) the levels of lipid peroxides (LPO). Phytochemical analysis revealed the presence of substantial amount of polyphenols, flavonoids and other antioxidant chemicals in the extract.

Conclusion: Present investigation demonstrates that antihemolytic activity of *C. viscosum* polar extract is mediated by its antioxidant effect.

Keywords: Hemolysis; lipid peroxidation; C. viscosum; antioxidant.

1. INTRODUCTION

Hemolysis refers to destruction of erythrocytes with liberation of hemoglobin in the plasma. Hemolysis occurs in a variety of pathological conditions such as autoimmunity against RBC-surface antigen, mechanical disruption of RBC, malaria/clostridium infection, thalassemia and sickle cell disease [1]. Erythrocytes are exclusive blood cells that deliver oxygen to our body, act as vendor of nutrients [2] and participate in detoxification of a great variety of toxic xenobiotics [3]. Furthermore, RBCs are very susceptible to oxidative stress due to high cellular concentration of oxygen and hemoglobin, high polyunsaturated fatty acid content while oxidative stress on RBC is implicated to hemolysis [4]. During transportation, radical-scavenging and detoxification function erythrocytes continuously experience oxidant injury on the heme iron, the globin chain and on other essential cellular molecules [3]. Therefore, dietary supplementation with natural antioxidants such as dietary polyphenols, flavonoids, and carotenoids might strengthen the antioxidative defense system of RBCs to cope over free radical challenge.

Clerodendrum viscosum vent belongs to Lamiaceae family which is locally known as Vita and enjoy an abundant distribution in Bangladesh. In folk medicine, *Clerodendrum viscosum* is widely used as blood purifier [5] though there is no pharmacological evaluation in support of antihemolytic activity of *Clerodendrum viscosum*. Keeping this in view, the present study was undertaken to investigate antihemolytic activity of *C. viscosum* and its association with antioxidant activity of *C. viscosum*.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials and Extraction

Wild *C. viscosum* was collected from Savar, Dhaka, Bangladesh. Plant samples were then identified and authenticated from the Department of Botany, Jahangirnagar University, Bangladesh (Nomen no. 410408). The plants roots were dried in sun, powdered using mechanical grinder, extracted by soxhlet apparatus (50ºC) using 100% methanol to get polar extract and evaporated to dryness under reduced pressure using rotary evaporator (50ºC).

2.2 Antihemolytic Activity

The ability of *C. viscosum* polar extract to prevent hypotonia induced and hydrogen peroxide (H_2O_2) induced hemolysis was assayed as a measure of *in vitro* antihemolytic effect. Erythrocytes were collected from venous blood of healthy volunteers in a heparinized test tube and centrifuged at 800g for 5 minutes. The plasma and buffy coat were removed by aspiration. The erythrocytes were then washed three times with cold (4ºC) phosphate-buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and used immediately after preparation. Hypotonia-induced osmotic fragility of erythrocytes was determined by the method of Malamos et al. [6]. In Brief, RBCs were incubated without (control) and with (test) extract at a final concentration of 100µg/ml for 40 minutes at 37ºC. RBCs were then suspended at 2% hematocrit in phosphate-buffered saline of various osmolarities. The suspension was centrifuged after 1 min, and the hemoglobin in the supernatant was measured spectrophotometrically at 540 nm. Isotonic (0.9%) NaCl, and 0% NaCl were used for 0% and 100% hemolysis respectively. The result was calculated as % of hemolysis inhibition. H_2O_2-induced antihemolytic activity was determined by the method of Sivonová et al. [7]. RBCs were preincubated without (control) or with (test) extract at a final concentration of 300µg/ml. RBCs were then suspended at 5% hematocrit in phosphate-buffered saline and exposed to H_2O_2-induced oxidative stress. The result was calculated as % hemolysis.

2.3 Anti-Lipid Peroxidation Activity

Brain cortex tissues were separated from brain of five Long Evans male rats (~ 25 weeks of age) and homogenized (in 50 mM phosphate buffer, pH 7.4). Fenton's reagent [H_2O_2 (45 mM) + $FeSO_4$ (2mM)] were used to induce *in vitro* oxidative stress in the brain cortical homogenates without (control) or with (test) *C. viscosum* polar extract at a final concentration of 100µg/ml. Malondialdehyde content in the homogenates were determined by previously described (Shahdat, et al, 2012) method as indicator of oxidative stress [8]. Lipid peroxidation of brain was calculated as nmol /mg of protein and total protein in the tissue homogenate was estimated by the Lowry method [9].

2.4 Antioxidant Potential Assessment

In vitro antioxidant potential assessment of *C. viscosum* polar extract was carried out by total reducing potential assay and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity assay.

2.4.1 Total reducing potential assay

The reducing power of *C. viscosum* polar extract was determined by the method as described by Oyaizu [10]. The reducing power of extract was expressed as ascorbic acid equivalents (µg of ascorbate equivalent/mg of extract).

2.4.2 DPPH scavenging activity assay

DPPH-free radical scavenging activity of *C. viscosum* polar extract was measured by the method of Braca et al. [11]. DPPH-free radical scavenging activity of *C. viscosum* was calculated as % of radical inhibition and expressed as IC_{50} that is the concentration of *C. viscosum* required to scavenge 50% of DPPH used.

$$\% \text{ Radical Inhibition} = \{(\text{Control OD} - \text{Sample OD})/ \text{Control OD})\} \times 100$$

2.5 Phytochemical Analysis

C. viscosum polar extract was subjected to the qualitative phytochemical analysis by previously described methods: polyphenols, terpenoid and alkaloid by Harborne [12], tannin and flavonoids by Trease and Evans [13], steroid by Sofowora [14], and glycoside by Siddique and Ali [15].

2.6 Quantitative Estimation of Total Polyphenol and Flavonoid

Total polyphenol content of *C. viscosum* polar extract was determined by the Folin and Ciocalteu's method [16] against pyrogallol standard. The concentration of total phenol compounds in extracts was determined as pyrogallol equivalents (µg of pyrogallol/mg of extract). Total flavonoid content of *C. viscosum* polar extract was measured by aluminum chloride colorimetric assay [17] against quercetin standard. The concentration of total flavonoid in the extract was determined as quercetin equivalents (µg of quercetin/mg of extract).

2.7 Statistical Analysis

The results are expressed as mean ± SEM (Standard error of mean). The statistical programs used were StatView® 4.01 (MindVision Software, Abacus Concepts, Inc, Berkeley, CA, USA) and Graphpad Prism® (version 4.00; Graphpad Software Inc., San Diego, CA, USA). A level of $P<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

The results of the present study clearly indicate that both *in vitro* hypotonia and H_2O_2 caused hemolysis of erythrocytes and *C. viscosum* polar extract significantly inhibited the hemolysis, as indicated by the increased % of inhibition following osmotic and H_2O_2-induced stress (Fig. 1A & 1B). Hypotonia induced hemolysis occurs due to destabilization of membrane [18]. Antihemolytic activity, therefore, relies on the membrane stabilizing effects. Percent of hemolysis inhibited by *C. viscosum* (57.2 ± 8.7) was parallel to that of vitamin C (50.7 ± 1.4). This reflects the comparability of *C. viscosum* with vitamin C in term of antihemolytic activity (Fig. 1B). H_2O_2 penetrates cellular membranes and in the presence of metal ions H_2O_2 triggers hydroxyl radical ($^{\cdot}OH$) formation. This process leads to the lipid peroxidation and leakage of the hemoglobin [19]. Intracellular events leading to oxidant-induced cell hemolysis include oxidation of hemoglobin, fragmentation of oxidized protein, lipid peroxidation and alteration of cellular deformability [20]. We speculate that the inhibition of one or more of these event(s) is one of the mechanism by which *C. viscosum* polar extract inhibits H_2O_2-induced hemolysis.

To support the proposition, we induced oxidative stress in the cortical tissue with the use of Fenton's reagent (H_2O_2+Fe_2SO_4). Fenton's reagents lead to the formation of hydroxyl radicals ($^{\cdot}OH$) which in turn causes lipid peroxidation, alters membrane properties and disrupts the function of membrane-associated proteins. All of these may lead to disruption of cellular functions [21]. As expected, the Fenton's reagent increased the levels of lipid peroxide (LPO) in the brain tissues. Treatment of *C. viscosum* polar extract alone did not increase the level of LPO (data not shown) while co-incubation of the oxidative-stressed tissue with extract significantly reduced the level of LPO by ~39% (Fig. 1C). Thus, the effects

of *C. viscosum* on the brain tissue were consistent with those of its effect on hypotonia- and H_2O_2-induced hemolysis.

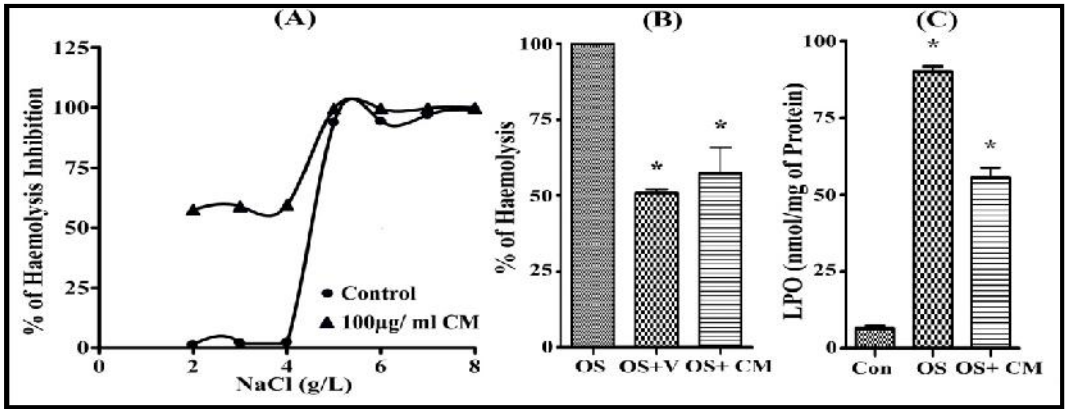

Fig. 1. Antihemolytic and anti-lipid peroxidation activity of *C. viscosum* polar extract
*(A) Hypotonia induced hemolysis. Data was presented as % of hemolysis inhibition in the presence (100µg/ml CM) or absence (control) of C. viscosum extract. (B). H_2O_2-induced hemolysis. Here, OS= RBCs with H_2O_2-induced oxidative stress; OS+V= RBCs with H_2O_2-induced oxidative stress plus vitamin C; OS+CM= RBCs with H_2O_2-induced oxidative stress plus polar extract of C. viscosum. The bars represent the mean ± SEM (n = 3). (C). Effect of C. viscosum on lipid peroxidation of rat cerebral cortex homogenate. Here, Con = Control; OS = rat brain cortex with Fenton's reagent-induced oxidative stress; OS+CM = rat brain cortex with Fenton's reagent-induced oxidative stress plus polar extract of C. viscosum. The bars represent the mean ± SEM (n = 3). *P<0.05 (One-way ANOVA).*

The antioxidant potential of *C. viscosum* polar extract was further confirmed by the estimation of total reducing power and DPPH-radical scavenging activity assay. Total reducing potential of *C. viscosum* was 70 ± 4.0 µg of ascorbate equivalent/mg of extract. Furthermore, *C. viscosum* root extract showed a TEAC (Trolox Equivalent Antioxidant Capacity) value of 0.400 ±0.002 [22]. During scavenging the free radicals of DPPH, the IC_{50} values were 98±1.4 µg/ml and 121.6±1.4 µg/ml for *C. viscosum* and vitamin C, respectively. A lower IC_{50} value indicates a higher radical scavenging activity, thus again demonstrating that the *C. viscosum* possesses significantly higher free radical scavenging activity than that of vitamin C. Priyankar et al. (2012), found the IC_{50} value of *C. viscosum* root extract as 31.04 ± 1.06 µg/ml that strengthen our observation [22].

Table 1. Qualitative phytochemical analyses of *C. viscosum* extract

Polyphenols	Flavonoids	Tannins	Terpenoids	Steroids	Glycosides	Alkaloids
+	+	+	+	+	+	-

'+' indicates the presence while '-' indicates the absence of respective phytochemical.

Antihemolytic activity and/or anti-lipid peroxidation capacity of extract denote the probable presence of antioxidant phytochemicals in the *C. viscosum* extract. We, therefore qualitatively and quantitatively analysed the phytochemicals of the extract. Qualitative analysis of extract showed the presence of polyphenols, flavonoids, tannins, terpenoids, steroids and glycosides but not alkaloids (Table 1). Quantitative analysis was performed for total polyphenols and total flavonoids. Total polyphenol content of *C. viscosum* extract was

85±3.0 µg of pyrogallol equivalent/mg of extract while total flavonoid content of *C. viscosum* was 28 ±1.0 µg of quercetin equivalent/mg of extract. Besides our study, Priyankar et al., reported significant content of polyphenol and flavonoid in *C. viscosum* root extract [22]. However, polyphenols have redox properties while flavonoids are free radical scavenger or metal ion chelator. Polyphenolic structure of flavonoids confers their interaction with biological membranes and scavenging of free radicals, as well as chelation of transition metals directly in the membranes [23]. Furthermore, flavonoids and a host of other secondary plant metabolites are reported to have membrane stabilizing effect [24]. Therefore, it can be speculated that flavonoids and other chemical components are responsible for the observed membrane stabilizing action of the *C. viscosum* polar extract and inhibition of oxidative stress that is manifested as both antihemolytic and anti-lipid peroxidation activity.

4. CONCLUSION

The occurrence of atherosclerosis, thrombosis and consequent myocardial infarction, hemorrhagic shock, thrombotic shock has increased many folds in populations of Bangladesh where economic burden is an existing reality [25]. Chronic arsenic exposure from drinking water in many areas of Bangladesh has been acting as root of the vascular inflammation leading to cardiovascular disease through exertion of oxidative stress on erythrocytes and other deadly diseases such as cancer [26]. Thus hemolysis related to these clinical cases is expected to be benefited by the *C. viscosum* extract; however, further studies are essential.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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