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Research Article

CENTELLA ASIATICA (LINN.) URBAN AMELIORATES OXIDATIVE STRESS ON ERYTHROCYTE MEMBRANE

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ABSTRACT

The effect of Centella asiatica on erythrocyte membrane under normal and oxidative stressed condition was investigated in the present study. 100% ethanol, 50% ethanol and water extract of Centella asiatica were screened for in vivo study by in vitro hypotonia-induced and time dependant H2O2-induced antihemolytic activity assay. During in vivo study, test group rats (n=7) were orally administered with 50% ethanol extract of Centella asiatica at a dose of 250 mg/kg/day for 6 weeks while control group rats were administered with normal saline. Then, levels of lipid peroxide (LPO) of erythrocyte ghost membrane from control and test group rats were estimated under normal and in vitro H2O2-induced oxidative stressed condition. All of the three extract prevent hypotonic solution induced hemolysis while 50% ethanol extract showed highest H2O2-induced antihemolytic activity in a time dependant manner. The LPO levels of the erythrocyte ghost membrane under H2O2-induced oxidative stressed condition were significantly decreased as a result of C. asiatica administration but not in normal condition. Therefore, the extract of C. asiatica has the ability to ameliorate oxidative stress on erythrocytes membrane at least upon oxidative challenge.

Keywords: *Centella asiatica*, erythrocyte, oxidative stress, antihemolytic activity.

INTRODUCTION

Erythrocytes, as transporter, are exposed to a wide variety of physiological environments ranging from vascular environment like hyperglycemia to local tissue environment such as free radical rich inflamed tissues with atherosclerotic lesions [1]. Erythrocytes serve as carrier system for the transport and delivery of pharmacological substances as well as participate in the free radical metabolism and detoxification of the toxic electrophilic xenobiotics [2,3]. During transportation, radical-scavenging and detoxification function erythrocytes continuously experience oxidative injury on heme iron, globin chain and on other cellular molecule [4]. Furthermore, erythrocytes are reported to have a higher degree of susceptibility to oxidative stress due to higher content of cellular oxygen, hemoglobin and polyunsaturated fatty acid [5]. Limited biosynthetic capacity of mature erythrocyte causes poor enzymatic antioxidant repair system to overcome the oxidative damage entirely [6]. Oxidative stress on erythrocyte causes cytoskeleton rearrangement with loss of membrane lipid asymmetry, depletion of thiol pool and increased methemoglobin production. All of these events lead to change in erythrocyte's adhesiveness, aggregation and morphology. Available evidence supports the causal concern of oxidative stress on erythrocytes with vascular inflammation, atherosclerosis, hemorrhagic shock, thrombotic shock, hypertension and drug toxicity [1,2,7,8]. Supplementation with dietary antioxidant is considered as a promising mean to encounter such oxidative stress associated pathological conditions.

Centella asiatica (Linn.) Urban is a prostrate stoloniferous plant that is endogenous to Bangladesh and known as 'Manduka Parni' in Ayurveda.*Centella asiatica* and its extracts is official to Indian pharmacopoeia and is recommended for the treatment of various skin conditions such as leprosy, lupus, varicose ulcers, eczema, psoriasis, diarrhoea, fever, amenorrhea, and diseases of the female genitourinary tract in addition to wound healing [9]. *Centella asiatica* is effectively being used in the treatment of fever, jaundice, dysentery, mental illness and blood purification within the frame of traditional medicine in Bangladesh [10]. Besides food value, *Centella asiatica* has been reported to have a wide range of pharmacological activity including antiulcer, anticancer, immunomodulatory, neuroprotective, sedative, antioxidant and wound healing activity. Though a few study showed vascular effect of *C. asiatica* but no study putted special emphasis on erythrocytes except hematological parameters. Therefore the present study was carried out to evaluate the effect of *C. asiatica* extract on erythrocytes membrane under normal and oxidative stressed condition.

MATERIALS AND METHODS

Plant material collection and extract preparation

Wild *C. asiatica* was collected from Manikgang and authenticated by Department of Botany, Jahangirnagar University, Savar, Dhaka (Voucher No. DBJU: 0062). The fresh leaves with petioles were air dried in shadow and grinded by mechanical grinder. Fine powders were then used for the exhausted extraction using 100% ethanol, 50% ethanol and water as extraction solvent. The solvent was evaporated under reduced pressure using rotary evaporator. The extract was then preserved in a refrigerator (4ºC) till further use.

Chemicals

MDA (1, 1, 3, 3-tetraethoxypropane), BSA (Sigma Chemical Co. St. Louis, USA), absolute ethanol (Hong Yang Chemical Corporation, China), Folin reagent, hydrogen peroxide and SDS (Merck, Dermstadt, Germany) were used in the present study.

In vitro hypotonia induced hemolysis

Erythrocytes were collected from venous blood of healthy volunteers in a heparinized tube and isolated according Dodge et al., (1963) to [11]. Hypotonia-induced osmotic fragility was determined as described by Targeon (1993) [12]. In brief, erythrocytes were incubated without

(control) and with (test) extract at a final concentration of 100µg/ml for 40 minutes at 37ºC. Erythrocytes were then suspended at 2% haematocrit 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. The result was calculated as % of hemolysis inhibition.

Time dependant antihemolytic activity

Time dependant antihaemolytic activity of 100% ethanol, 50% ethanol and water extract of *C. asiatica* was assessed by the method of Sivonová et al., (2004) at time interval of 30 minutes from 0 hour to 2 hours [13]. Briefly, erythrocytes were preincubated for 60 minutes without (control) or with (test) extract at a final concentration of 100µg/ml and exposed to H_2O_2-induced oxidative stress. The result was calculated as % of inhibition of hemolysis. Pyrogallol at a final concentration of 100µg/ml was used as a reference standard.

Animals and treatment

In-bred male Long Evan rats (~160-250 gram) of about 27 weeks were used in this experiment. The rats were housed under normal laboratory conditions (22° ± 2°C, 12:12-hr light: dark cycle) with free access to a balanced diet and purified drinking water. The rats were randomly divided into control (n=7) and test group (n=7). Test group rats were orally administered with 50% ethanol extract of *C. asiatica* at a dose of 250 mg/kg of body weight per day while control group rats were administered with normal saline. The extract was fed once daily for 6 weeks. All animal experiments were cared according to ethical norms approved by the BCSIR (Bangladesh Council of Scientific and Industrial Research) guide regarding laboratory animals.

Estimation of membrane lipid peroxide (LPO)

The animals were sacrificed under anesthesia and blood was collected in heparinized tubes. Erythrocytes were isolated according to Dodge et al. (1963) [11]. The erythrocyte ghost membranes were prepared by repeated washing of erythrocyte with lysis buffer (5mM PBS; 1 mM EDTA; pH 8.0). Malondialdehyde (MDA) content of erythrocyte ghost membrane was measured using previously described method (Shahdat et al., 2012) as indicator of oxidative stress [14]. The result was expressed as nmol/mg of protein and membrane protein content was estimated by Lowry method (1951) using bovine serum albumin (BSA) as a standard [15].

Estimation of lipid peroxide (LPO) of in vitro oxidative stressed erythrocyte membrane

Erythrocyte ghost membrane from control and test group rats were subjected to in vitro oxidative stress by exposing them to 10µl of 30% H_2O_2 for 30 minutes at 37°C. MDA content of erythrocyte ghost membrane was measured according to Shahdat et al., 2012 [14]. Membrane protein content was estimated by Lowry method (1951) using BSA as a standard. The result was expressed as nmol/mg of protein [15].

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.

RESULTS

In vitro hypotonia induced antihemolytic activity

The ability of *C. asiatica* extracts to prevent hypotonia induced hemolysis was used to screen in vitro membrane stabilizing effect of different extract. All the extracts of *C. asiatica* were found to prevent hypotonic solution induced hemolysis in comparison with control (Table-1).

Table 1: Hypotonia induced antihemolytic activity of different extract of *C. asiatica*.

NaCl (g/l)	% of Hemolysis			
	Control	100% EE	50% EE	WE
1	96.98	93.98	79.66	83.44
2	94.37	92.08	76.51	79.66
3	91.18	78.53	86.45	78.53
4	41.94	7.20	19.98	11.07
5	2.39	1.04	1.71	0.95
6	1.80	0.95	1.26	1.17
7	1.62	0.90	1.31	1.58

Here, EE=ethanol extract of *C. asiatica;* WE= Water extract of *C. asiatica*.

Time dependant antihemolytic activity

In case of time dependant antihaemolytic activity, pyrogallol showed highest degree of hemolysis inhibition at 0, 0.5, 1, 1.5 and 2 hours of H_2O_2-exposure compared to 100% ethanol, 50% ethanol and water extract of *C. asiatica*. Antihemolytic activity of pyrogallol was followed by 50% ethanol extract of *C. asiatica*. 100% ethanol and water extract exhibited a similar ($P>0.05$) antihaemolytic effect at each time interval except 2 hours of H_2O_2-exposure when percent of hemolysis inhibition by 100% ethanol extract was significantly higher ($P<0.05$) than that of water extract of Here, EE= ethanol extract of C. asiatica; WE= Water extract of C. asiatica; PG= Pyrogallol. '*' indicates significant ($p<0.05$) difference with respect to water extract of C. asiatica; '**' indicates significant ($p<0.05$) difference with respect to 100% ethanol extract and water extract of C. asiatica; '#' indicates significant ($p<0.05$) difference with respect to 100% ethanol, 50% ethanol and water extract of C. asiatica. Data point was expressed as mean ± SEM (Standard error of mean). Data was analyzed by one way ANOVA was followed by Tukey's least square differences test for post hoc comparisons.

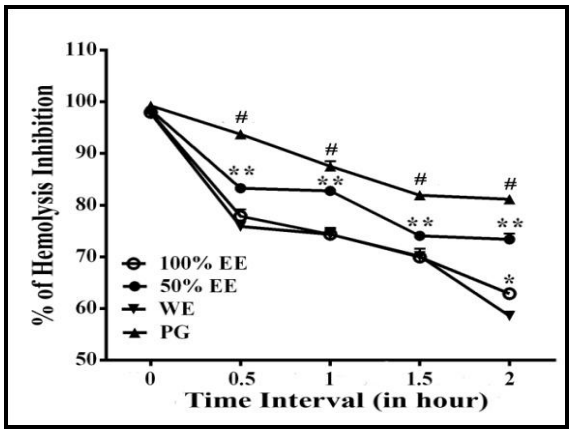

Fig. 1: Time dependent antihemolytic activity of three different extract of C. asiatica.

Estimation of membrane lipid peroxide (LPO)

Levels of lipid peroxide (LPO) in erythrocyte ghost membrane of control and test group rats were 1.23±0.20 nmol/mg and 0.88± 0.15 nmol/mg of protein respectively. Thus, oral administration of 50% ethanol extract *C. asiatica* extract decreased the levels of lipid peroxide (LPO) of erythrocyte ghost membrane but not significantly ($P>0.05$) (Fig. 2.A).

Estimation of lipid peroxide (LPO) of in vitro oxidative stressed erythrocyte membrane

Oxidative stress on erythrocyte ghost membrane raises the LPO levels of both control and test group rats ~ 5 times relative to basal LPO levels. In vitro oxidative stress induced LPO levels of erythrocyte ghost membrane from control and test group rats were 7.60 ± 1.12 nmol/mg and 3.10 ± 0.70 nmol/mg of protein respectively. Thus, in vitro oxidative stress induced LPO levels of erythrocyte ghost membrane of *C. asiatica* extract fed test group rats were significantly lower than that of control group rats (Fig. 2.B).

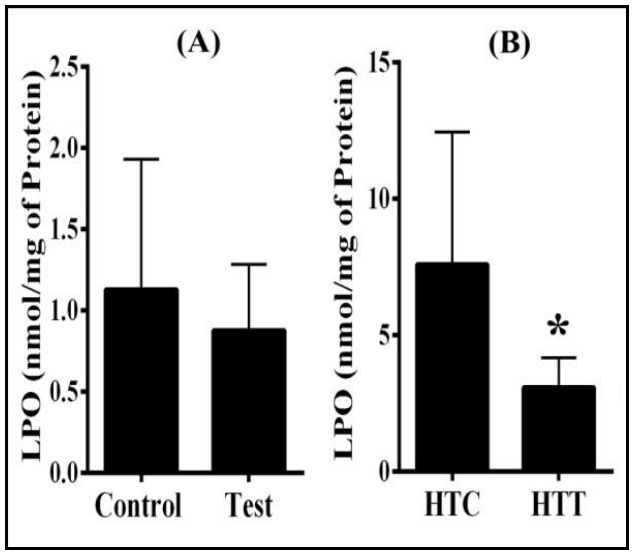

Fig. 2: (A). Effect of 50% ethanol extract of C. asiatica on LPO levels of erythrocyte ghost membrane.

Test group rats were fed with 50% ethanol extract of C. asiatica while control group with normal saline. **(B). Effect of 50% ethanol extract of C. asiatica on oxidative stressed erythrocyte ghost membrane.** Here, HTC= control group rat's erythrocyte ghost membrane treated with hydrogen peroxide treated; HTT= test group rat's erythrocyte ghost membrane treated with hydrogen peroxide treated test group rat erythrocyte ghost membrane. '*' indicates significant ($p<0.05$) difference with respect to HTC. Data was expressed as mean ± SEM (Standard error of mean). Data was analyzed by student's t-test.

DISCUSSION

Erythrocytes constitute ~ 43% of total blood cells of the circulation [16]. Cell membrane, being the surface of a cell, experience first attack of the reactive oxygen species (ROS) under oxidative stressed condition. Erythrocytes are considered as a model system to study the membrane related biochemical investigations [17]. The purpose of this study was to evaluate the effect of *C. asiatica* extract on erythrocytes membrane under normal and oxidative stressed condition.

In the present study, 100% ethanol, 50% ethanol and water extract of *C. asiatica* were subjected to hypotonia induced antihemolytic activity assessment. Hypotonia induced hemolysis occurs due to destabilization of membrane [18]. Hypotonia induced antihemolytic activity, therefore, relies on the membrane stabilizing effects. All the extract showed membrane stabilizing capacity in hypotonia induced antihemolytic activity (Table 1). With this finding in hand, we screened the extract that might provide highest protection to erythrocytes under oxidative stress as well as the inhibition kinetics by in vitro time dependant antihemolytic activity assay. Among the three extracts, highest level of antihemolytic activity was exhibited by 50% ethanol extract of *C. asiatica* (Fig. 1). H_2O_2 penetrates cellular membranes and in the presence of metal ions H_2O_2 triggers hydroxyl radical (•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. asiatica* extract inhibits H_2O_2-induced hemolysis.

Effect of the *C. asiatica* extract feeding on the normal levels of lipid peroxide of erythrocyte ghost membrane was investigated by the estimation of LPO levels in isolated erythrocyte ghost membrane. Oral administration of 50% ethanol extract of *C. asiatica* decreased the levels of lipid peroxide of the erythrocyte ghost membrane about the reduction was not statistically significant ($P>0.05$; Fig. 2.A). Erythrocytes ghost membranes from both control and test group rats were also subjected to oxidative stress by exposing them to hydrogen peroxide. Such oxidative stress by hydrogen peroxide treatment raises the LPO levels of erythrocyte ghost membrane of both control and test group rats by ~ 5 times compared to normal LPO levels. But, increase in LPO levels of erythrocyte ghost membrane from test group rats was significantly lower than that of control group rats ($P<0.05$; Fig. 2.B). Thus, erythrocyte membranes from test group rats exhibited a significant prevention against oxidative stress compared to control group rat's erythrocyte membranes. From these findings we proposed that, feeding of 50% ethanol extract of *C. asiatica* might modify the erythrocyte membrane qualitatively that was explored only under oxidative stressed condition. Antioxidant phytoconstituent counteract the oxidative stress while the antioxidant activity of *C. asiatica* has already been documented. The similarity between the findings from time dependant antihemolytic activity and LPO levels of oxidative stressed erythrocyte ghost membrane also strengthen our proposition.

CONCLUSSION

In this study, we are reporting the effect of *C. asiatica* extract on erythrocytes membrane under normal and oxidative stressed condition for the first time. Our study demonstrated that the extract of *C. asiatica* has the ability to ameliorate oxidative stress on erythrocytes membrane at least upon oxidative challenge. Future researches are essential to explore the exact mechanism(s) underlies such amelioration as well as disease related therapeutic intervention.

Conflict of interest

The author declares that there is no conflict of any competing interest.

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