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Hot Water Extract of *Moringa oleifera* Leaves Protects Erythrocytes from Hemolysis and Major Organs from Oxidative Stress *in vitro*

Marzan Sarkar, Sujan Bhowmick, Jakir Hussain, Mahmudul Hasan, Shahdat Hossain

Laboratory of Alternative Medicine and Behavioral Neurosciences, Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.

*Corresponding author: shahdat@dhaka.net

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Abstract: *Moringa oleifera* (drumstick plant) is one of the most popular plants, which is believed to have enormous health benefits. Because of its health benefits, the demand of this plant is increasing day by day in different parts of the Indian subcontinent. *Moringa* leaves are used as a vegetable item in some parts of Bangladesh. In this study, we investigated the anti-hemolytic and anti-oxidative effects of hot water extract of *M. oleifera* leaves. The extract contained considerable amounts of different antioxidants: β-carotene (87.50±12.00 µg/g of extract), and phytochemicals such as total polyphenols (53.52±1.10 mg Gallic acid equivalent/g of extract) and flavonoids (10.40±1.40 mg catechin equivalent/g of extract). The antioxidant potential of the extract was assessed by its DPPH (2, 2-diphenyl-1-picrylhydrazyl-)-free radical scavenging activity, with *M. oleifera* leaf extract showing considerable anti-oxidative potentials. To evaluate the anti-hemolytic effect of the extract, freshly prepared erythrocytes were incubated with Fenton's reagents in the absence or presence of the extract. We observed that erythrocytes pretreated with the extract exhibited a reduced degree of *in vitro* hemolysis. To support the anti-oxidative properties, tissues were treated with or without extract in the presence of Fenton's reagents, and the levels of lipid peroxide (LPO) were determined. The *Moringa oleifera* leaf extract significantly decreased the levels of LPO in brain, liver and heart tissues. Our studies suggest that the *Moringa oleifera* leaf extract can mediate, at least partially, cytoprotective effects through scavenging of free radical, reduction of intracellular oxidative stress, and hence prevented the *in vitro* hemolysis of erythrocytes.

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INTRODUCTION

As Bangladesh is an underprivileged country (ranked as a third world country), our people are unable to buy drugs in many cases. For this reason, we always intend to search the dietary alternatives to many diseases. Traditional medicinal plant is widespread in different parts of the world (Shawa et al., 2015) and *M. oleifera* is such a dietary medicinal plant source. In many regions of our country the people use the leaves of this plant as vegetables (local name Sajina). Nowadays, plants provide raw materials for new sources of drugs and pharmaceutical products. A wide variety of naturally occurring constituents such as polyphenolics, terpenoids and pro-vitamins have received much attention as alternative therapeutic agents to fight against various oxidative stress (OS)-induced diseases (Ivanova, et al., 2005); (Gulcin, et al., 2012); (Gulcin, et al., 2013). Oxidative damage and hemolysis caused by reactive oxygen species (ROS) have a major role in the expansion of diseases such as thalassemia, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anemia. Red blood cells (RBCs) are the primary targets of free radicals, owing to their high membrane concentrations of polyunsaturated fatty acids (linoleic and arachidonic acids, in particular) and O_2 transport associated with redox active hemoglobin molecules, which are effective promoters of ROS. Oxidation depletes membrane protein content, deforms RBCs inappropriately, and interrupts microcirculation (Yang, et al., 2006; Rice-Evans, et al., 1986; Yu, et al., 2001; Flynn, et al., 1983). The ROS is also implicated in hemolysis. Hemolysis is the breakage of the red blood cells' (RBCs') membrane, triggering the release of the hemoglobin and other internal components into the adjacent fluid. Hemolysis is visually detected by screening a pink to red tinge in serum or plasma (Lemery, et al., 1998; Ko, et al., 1998). The generation of ROS beyond the antioxidant capability of a biological system gives rise to oxidative stress, which signified as an imbalance between oxidants and reductants leading to a disruption of redox signaling, control and/or molecular impairment (Lykkesfeldt and Svendsen,

2007). The antioxidants are defined as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and, thus, to significantly delay or inhibit the oxidation of these substrates (Droge, 2002). Oxidative insult has been reported as the contributing factor in various diseases such as inflammatory diseases, ischemic heart diseases, hypertension, hypercholesterolemia, stroke, various liver diseases, hemochromatosis, neurodegenerative disorders, and smoking-related illnesses (Yildirim, et al., 2016; Lobo, et al., 2016; Saravanan and nalini, 2007; Parola and Robino, 2001; Stefanis, et al., 1997; Houglum, et al., 1997) and in the diminishing of erythrocyte functions including hemolysis (Hossain, et al., 2015). The aim of this study was to explore the antihemolytic and antioxidative activity of *M. oleifera* leaves hot water extract as *Moringa* leaves has been used as vegetables in some parts of our country for many years.

METHODS AND MATERIALS

Plant Collection

Moringa oleifera leaves were collected from the Botanical Garden of Jahangirnagar University, Savar, Dhaka. Then the leaves were authenticated by a Botanist of Botany department of the same University.

Preparation of Hot Water Extract for Antioxidant and Antihemolytic Activity

M. oleifera leaves were extracted with boiling water for 30 minutes and allowed to steep with continuous swirling. Extracts were filtered through Whatman number 1 filter paper, centrifuged at 2000×g for 30min, aliquoted, and stored at -20^0 C for analyses.

Chemicals Used

Chemicals, 1,1,3,3-Tetraethoxypropane (TEP), bovine serum albumin (BSA), hydrogen peroxide (H_2O_2), and $FeSO_4$ were purchased from Sigma Aldrich (St. Louis, USA). Absolute ethanol was purchased from Hong Yang Chemical Corporation (China). Folin reagent and sodium dodecyl sulfate were purchased from Merck (Darmstadt, Germany).Gallic Acid (Sigma Aldrich, USA), Catechin (Merck, Germany), Thiobarbituric acid (Sigma, USA), DPPH- (2, 2-diphenyl-1-picrylhydrazyl-), Pyridine, Butanol etc.

Estimation of Total Protein in Hot Water Extract

Moringa leaves were soaked with 0.1N NaOH in glass screw capped test tubes for 24h with brief sonication in an ultrasound bath sonicator filled with ice water (at maximum output). Then, the test tubes were vortexed and heated at 80∘C in the block heater for 2min. After centrifugation at 2000×g, total protein was measured from the supernatant by Lowry method [Lowry et al., 1951] and was calculated as mg of protein per gram of hot water Extract.

Estimation of Total Lipid in Hot Water extract

The total lipid content of Moringa leaves hot water extract was measured gravimetrically. Total lipid was extracted according to the method of Folch, et al., (1957). The extract was soaked in glass screw capped test tubes with a solution consisting of chloroform : methanol (2:1) for 24h with brief sonication in an ultrasound bath sonicator filled with ice water (at maximum output). Then, the test tubes were vortexed and incubated at 4∘C for 24h. After centrifugation at 2000×g, the chloroform layer was collected. The procedure was repeated two more times. All chloroform layers were combined together and evaporated to dryness. The amount of total lipid was calculated from the pre- and post-weights of the test tubes and expressed as mg of lipid per gram of hot water extract.

Estimation of β-Carotene Content

β-Carotene content was determined as described by Nagata and Yamashita, (1992) but with slight modification. 100mg hot water extract was vigorously shaken with 10mL solution consisting of acetone: hexane (4:6) for 10 min and then filtered through Whatman number 4 filter paper. The absorbance of the filtrate was measured at 453, 505, 645, and 663nm spectrophotometrically. β-Carotene content was calculated according to the following equation:

$$\beta\text{-Carotene (mg/100mL)} = 0.216 \times A_{663} - 1.22 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$$

Finally, the concentration of β-carotene was expressed as ng of β-carotene/g of hot water extract.

Estimation of Total Polyphenol and Flavonoid Contents

Total polyphenol content of *M. oleifera* leaves hot water extract was determined by Folin and Ciocalteu's method against Gallic acid standard (Akter, et al., 2015) and the concentration in the extract was expressed as Gallic acid equivalents (μg of Gallic acid/mg of extract). The total flavonoid content of the extract was measured by aluminum chloride colorimetric assay against catechin standard (Akter, et al., 2015) and its concentration in the extract was expressed as catechin equivalent (μg of catechin /mg of extract).

DPPH-Free Radical Scavenging Activity

DPPH radical scavenging activity was measured by determining the decrease in absorbance of the methanolic DPPH solution at 517 nm in the presence of water extract, as previously described

(Choi, et al., 2007). In brief, the hot water extract was diluted with methanol. 100 µl of the diluted extract with different concentrations was added to100 µl of a 0.4mM methanolic solution of DPPH. The reaction mixtures were vortexed and allowed to stand for 30 min at room temperature in the dark before the absorbance at 517 nm was measured using methanol as blank. Free radical scavenging activity was expressed as IC_{50}, that is, the concentration of the extract required to decrease the absorbance of DPPH (0.2 mM, final concentration) by fifty percent. The unit of the concentration of the extract was adjusted to µg of the moringa leaves/ml of hot water extract.

Animals

Five-week-old male *Wistar albino* rats purchased from icddr,b (Dhaka, Bangladesh) were housed in an air conditioned animal room with a 12:12h dark :light cycle under controlled temperature (23 ± 2∘C) and humidity (50 ± 10%). The rats were provided with a normal pellet diet with water ab libitum. All animal experiments were performed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Jahangirnagar University compiled from the Guidelines for Animal Experimentation of the Bangladesh Association for Laboratory Animal Science.

Preparation of Erythrocytes for Hemolysis Assay

After deep anesthesia with pentobarbital, blood from rat was collected from the inferior vena cava with a heparinized syringe. Then, erythrocytes were isolated from the blood as described by Hashimoto, et al. 2015. The resultant purified erythrocytes were subjected to hemolysis.

***in vitro* Hemolysis Assay**

The extent of erythrocyte hemolysis was determined as described previously (Hossain, et al., 2015; Kondo, et al., 1997). In brief, erythrocyte suspensions at 2% hematocrit were incubated with freshly prepared Fenton's reagent [H_2O_2 (45mM) + $FeSO_4$ (2mM)] at 37∘C for 1h. At the end of incubation, erythrocytes were pelleted down by centrifuging the samples at 300×g for10min.Then, the supernatant was aspirated and the extent of hemolysis was quantified by determining the amounts of released hemoglobin (Hb) in the supernatant at 540 nm against hemoglobin standard.

Preparation of Tissue Homogenates

After drawing blood, rats were initially perfused with ice-cold saline to remove blood from the brain. Then, the brain, liver and heart were separated from rats, re-perfused with saline, and homogenized in phosphate buffer (100mM, pH 7.4) containing 1% phenylmethylsulfonylfluoride (PMSF).The homogenate was centrifuged at 1000×g to remove unbroken tissues and debris and the resultant homogenates were assigned as brain, liver or heart, tissue homogenates, respectively, which were stored at -20^0 C until analysis.

Antioxidative Stress Activity of Hot Water Extract

in vitro antioxidative stress activity of extract was evaluated by determining the levels of lipid peroxide (LPO) in the brain, liver and heart tissue whole homogenates *in vitro*. Oxidative stress was directly induced in the above tissue homogenates by Fenton's reagent, as described previously (Hossain, et al., 2015). For the determination of antioxidative stress activity, the above tissue homogenates were divided into (1) tissue whole homogenates (0.1 ml) alone (Control); (2) tissue whole homogenate plus Fenton's reagents (OS); and (3) tissue whole homogenates plus Fenton's reagents (OS) plus 100 μl of hot water extract (10 mg/ml) (OS +E), all of which were incubated at 37∘C for 2h. Then, the levels of LPO were measured to examine whether extract exhibits any effects on production of LPO, that is, the effects against the oxidative stress *in vitro*.

Statistical Analysis

All results were expressed as mean ± standard error of the mean (SEM). The significance of the difference in means among different groups for antihemolytic or antioxidative stress activity was determined by one-way ANOVA, followed by Fisher's PLSD test for post hoc comparisons using Graph Pad Prism software version 5.0 (Graph Pad Software Inc., San Diego, CA, USA). $P< 0.05$ was considered statistically significant.

RESULTS

Total Protein, Total Lipid, β-Carotene, and Antioxidant Phytoconstituents of *M. oleifera* leaves hot water extract

The total protein content of the leaves in hot water extract was 154.11 ± 4.20 mg/g of extract, while the total lipid content was 67.0 ± 2.80 mg/g of extract. The β-carotene content was 87.5 ± 12.00 ng/g of extract. In addition, hot water extract *M. leaves* also had considerable amounts of antioxidant phytochemicals, such as total polyphenols (53.52 ± 1.10 mg gallic acid equivalent/g of extract) and total flavonoids (10.40 ± 1.40 mg catechin equivalent/g of extract). These outcomes advocate that the *M. oleifera* leaves hot water extract could be used as a decent source of antioxidants.

DPPH-Free Radical Scavenging Activity of hot water extract of *M. oleifera* leaves

Due to the presence of antioxidant components in leaves hot water extract, we checked whether this extract has DPPH-free radical scavenging ability or not. We observed that leaves hot water extract was able to reduce the purple color of DPPH and abolished the absorption peak at 517nm, indicating DPPH-free radical scavenging ability of the extract. Results also showed that DPPH-free radical scavenging activity was increased with the increase in the concentration of the extract. However, the IC_{50} (concentration required to scavenge 50% of 0.4mM of DPPH) for hot water extract was 1570mM (GAE) (equivalent to ~267 µg /mL of extract) and that of control (gallic acid) was 3.6mM (equivalent to 6.25 µg /ml GA). These results indicate that the hot water extract, which contains antioxidants, possesses considerable antioxidative potential.

Protective Effects of *M. oleifera* Leaves Hot Water Extract Against Erythrocyte Hemolysis i*n vitro*

Mammalian erythrocytes are a unique and interesting cellular model for research on oxidative stress, induced by either reactive nitrogen species (RNS) or reactive oxygen species (ROS), as well as for studies of the molecular mechanisms underlying the protective effects of the antioxidants. Therefore, we investigated whether *M*. leaves hot water extract possesses any protective effects against oxidative stress-induced hemolysis in vitro.

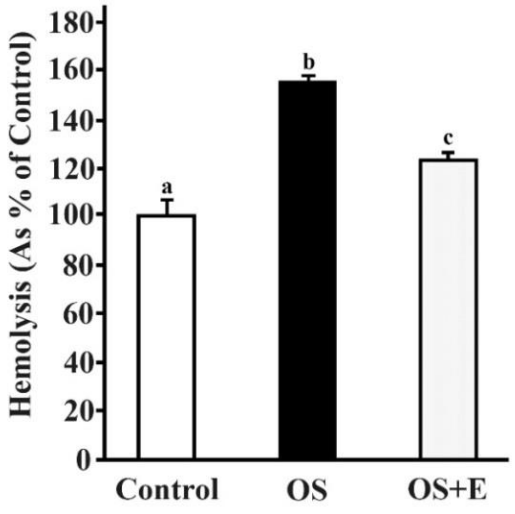

Fig. 1: Effects of *M. oleifera* hot water extract on Fenton's reagents-induced hemolysis of erythrocytes *in vitro*. Results are expressed as mean ± SEM, each with duplicate determinations. Bars with different letters are significantly different at $P< 0.05$. Data were analyzed with one-way ANOVA followed by Fisher's PLSD for post hoc comparison. Control, OS: oxidative stress, and OS+E: oxidative stress and *M. oleifera* hot water extract

For this purpose, erythrocytes were incubated with *Morinaga* leaves hot water extract and oxidative stress that leads to hemolysis was induced simultaneously by the addition of Fenton's reagent. Results showed that incubation of erythrocytes in the presence of Fenton's reagent resulted in an extensive hemolysis (Figure 1), suggesting that Fenton's reagent are almost similarly effective for inducing erythrocyte hemolysis *in vitro*. Most importantly, erythrocytes pretreated with the hot water extract had reduced degree of Fenton's reagent-induced hemolysis, confirming that extract effectively protects erythrocytes from oxidative stress-induced hemolysis *in vitro*. We also observed that hot water extract itself did not cause lysis of any erythrocytes *in vitro* (data not shown).

Hot Water Extract of *M. oleifera* Leaves Reduces Oxidative Stress in Brain, Liver and Heart Tissues *in vitro*

We next induced oxidative stress in several tissue homogenates particularly in the brain, heart, and liver tissue homogenates *in vitro* with the use of Fenton's reagent and examined whether oxidative stress in these tissues could be inhibited by *M. oleifera* leaves hot water extract. Lipid peroxide (LPO) levels were measured in these tissue samples as an indicator of oxidative stress in the absence or presence of leaves hot water extract. As expected, Fenton's reagent induced oxidative stress significantly increased LPO levels in the brain, heart, and liver tissues (Figure 2).

In contrast, LPO levels significantly reduced in the above tissues when these were pretreated with leaves hot water extract. All these results collectively suggest that *M*. Leaves hot water extract had a significant effect in inhibiting oxidative stress on brain, heart, and liver tissues.

DISCUSSION

Plants are the branded sources of polyphenols like phenolic acids, flavonoids, steroids and tannins, which confer them with many therapeutic properties (Suhartonoa, et al., 2012; Djeridane, et al., 2010; Oliveira, et al., 2008; George, et al., 1999; Chung, et al., 1998). The concentrations of these polyphenols vary depending on many environmental factors and the extraction procedures employed.

The *M. oleifera* leaves hot water extract showed high total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources and show significant antioxidant activity (Ebrahimzadeh, et al., 2010). DPPH is a stable nitrogen centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to accomplish this reaction can be considered as antioxidants as well as radical scavengers (Nabavi, et al., 2008). The phenol and flavonoid contents of

this plant may be liable for its good DPPH-scavenging activity (Parola, et al., 2001). The correlation between total phenol contents and antioxidant activity has been extensively studied in different foodstuffs such as fruit and vegetables (Ghasemi, et al., 2009; Nabavi, et al., 2009).

Hemolysis refers to the demolition of erythrocytes with the liberation of hemoglobin in the plasma (Dhaliwal, et al., 2004). Hemolysis happens in a variety of pathological conditions such as autoimmunity against an RBC surface antigen, mechanical disruption of RBC, malaria/clostridium infection, thalassemia and sickle cell disease (Giardina, et al., 1995). Erythrocytes are fashionable blood cells that deliver oxygen to our body, act as a vendor of nutrients (Sivilotti, 2004) and participate in detoxification of a great variety of poisonous xenobiotics (Sivilotti, 2004). Additionally, 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 (Halliwell, 2007). 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 (Pandey and Rizvi, 2010).

Oxidative stress is basically an imbalance between the production of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization of its own antioxidants (Halliwell, 2007; Sies, 1986). Here we have observed that *M. oleifera* leaves hot water extract contains considerable phytoconstituents that confer the antioxidative defense of major organs like liver, brain and heart tissues from oxidative stress *in vitro*. Therefore, dietary supplementation with natural antioxidants such as dietary polyphenols, flavonoids, and carotenoids might strengthen the antioxidative defense system of RBCs, liver, brain and heart tissues to cope over free radical challenge.

Due to the safety and restrictions of synthetic antioxidants, naturally originated antioxidants provide an interesting alternative to minimize the oxidative damage caused oxidative agents (Ghasemzadeh, et al., 2010). Reactive oxygen species (ROS) can lead to hemolysis and ultimately to diseases such as thalassemia and sickle cell anemia. Their action can be counteracted by the anti-hemolytic activity of therapeutic agents. The goal of our study was to identify plants that most efficiently counteract ROS-caused hemolysis (Khalili, et al., 2014) as well as to prevent oxidative stress.

CONCLUSION

The present study clearly demonstrates the rich repertoire of flavonoids and polyphenolic compounds in *Moringa* leaves hot water extract. These phytoprinciples in the native extract might have shown a synergistic, multi-modal therapeutic action by scavenging the free radicals generated in vitro and thus protect the erythrocytes against free radical-induced hemolysis, thus confirming the antihemolytic ability of *Moringa* leaves hot water extract. The results of the present study also encourages for further investigations on the therapeutic efficacy of *Moringa oleifera* against oxidative stress-induced damage of not only erythrocytes but also other tissues and organs using animal and human cell line models.

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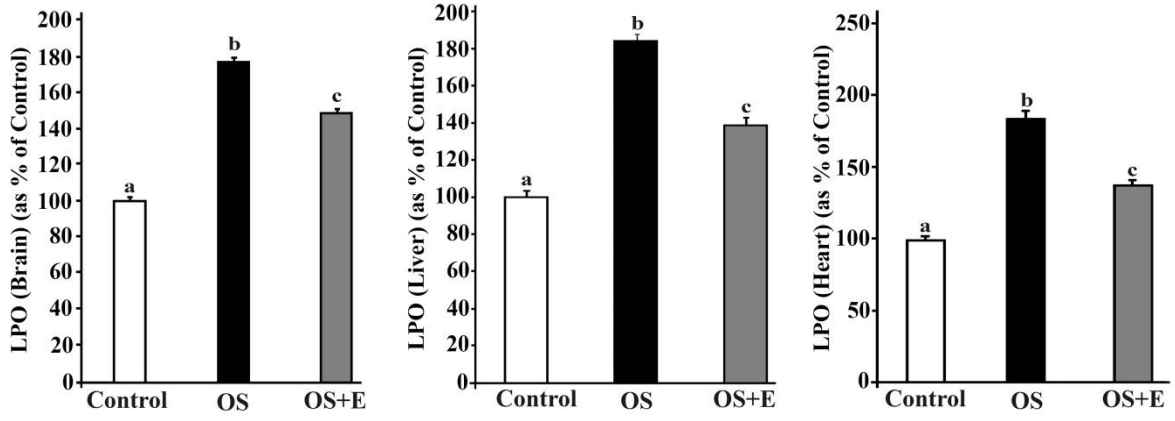

Fig. 2: Effects of *M. oleifera* extract on Fenton's reagent-induced oxidative stress in brain, liver and heart tissues. For a tissue, the bars with different letters are significantly different at $P< 0.05$. Data were subjected to one-way ANOVA followed by Fisher's PLSD post hoc test for multiple comparisons. LPO: lipid peroxide, Control, OS: oxidative stress, and OS+E: oxidative stress and *M. oleifera* hot water extract.

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