Protective effects of *Emblica officinalis* (Amla) on metal-induced lipid peroxidation in human erythrocytes

Vijay Kumar Krishnamoorthy[1] and Irfan Ahmad Rather[2]*
[1]Department of Biotechnology, Mohamed Sathak College of Arts and Science, Chennai, India
[2]Department of Applied Microbiology and Biotechnology, School of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk, Korea

Abstract: The protective potential of *Emblica officinalis* (amla) was investigated on metal-induced lipid per oxidation in human erythrocytes. Increases in the levels of MDA and catalase activity were assessed as lipid per oxidation. In addition, glutathione peroxidase (GPX), glutathione (GSH), and ascorbic acid levels were assessed as antioxidant indices. Preliminary investigation of the extract exhibited a significant reduction in lipid per oxidation and an increase in antioxidant abilities, such as a decrease in MDA, GPx and GSH ($P<0.05$). A significant reduction in erythrocyte hemolysis induced by hydrogen peroxide was observed using amla extract ($P<0.05$). These findings show that amla extract has significant protective potential against lipid per oxidation.

Keyworsd: Amla, lipid peroxidation, antioxidative activity, erythrocytes

INTRODUCTION

Lipid per oxidation has received great consideration due to the possible toxicological and health hazards of exposure to certain metals (Sunderman 1986). This process occurs through the peroxidation of unsaturated fatty acids in almost all living systems of aerobic nature. An oxidative breakdown of polyunsaturated fatty acids due to free radical damage to cellular components leads to the development of cellular toxicity. Some of the serious concerns of lipid per oxidation are that it damages the kidneys, liver, and brain (Poli *et al*., 1987; Cojocel *et al*., 1989; Uysal *et al*., 1989). Metals such as $HgCl_2$ and sodium arsenate play avital role in inducing oxidative stress in human erythrocyte cells, which leads to hemolysis (Ribarov and Benov, 1981).

Antioxidants play a crucial role in initiating metal-induced lipid damage, and plants are natural resources that are rich in antioxidants. Evidence suggests that plant-rich diets have been used in the prevention of several diseases (Willett *et al*., 2006). Therefore, a number of medicines that are currently available in the market have been isolated from medicinal plants (Wachtel-Galor & Benzie, 2011). Thus, humans and animals rely on plants and plant products for their beneficial, dietary, and medical properties. In addition, there is a great demand for herbal drugs (Dubey *et al*., 2004). They are cost effective, readily available, and very effective in many cases (Watt & Pretorius, 2001). There is great interest in scientific efforts to inspect the beneficial effects of herbal plants or plant parts. Many research studies have already been done on many plants; however, these plants are not widely used for their professed bioactivities.

In this study, we evaluated the extract of *Emblica officinalis* (amla) for the treatment of metal-induced metal lipid per oxidation and toxicity. *Emblica officinalis*is considered as one of the most important plants of great medical value in the Ayurveda. Amla has been used in Thai traditional recipes to slow down aging, treat the common cold, and as a liver tonic, anti-pyrectic, and hair tonic. In addition, it is also used to prevent peptic ulcers. It lowers cholesterol levels apart from inhibiting platelet aggregation. In India, it is the main source of Indian Gooseberry juice and oil. It is nature's richest source of vitamin C (720mg/100g of fresh pulp or up to 900mg/100g of pressed ferine). In Egypt, the fruits are used as memory stimulators and restoratives for body organs (Ali *et al*., 2013). Considering the beneficial effects of amla (Nain *et al*., 2012; Chen *et al*., 2011; Anilakumar *et al*., 2013), there is a great need to utilize it for other beneficial bioactivities.

The tree normally ranges from one to eight meters in height with fine branches bearing greenish-yellow flowers. The fruit has a spherical shape and yellow-greenish color. In India, both dried and fresh fruits are used as medicine for a number of diseases as a traditional treatment. However, in several other treatments, roots, leaves, and seeds are also used.

MATERIALS AND METHODS

Sample collection, extraction and treatment

Emblica officinalis (amla) fruit was purchased from a local market, in Chennai, India. The fruits were washed and cut into segments and air dried. The air dried samples were then ground into powder and stored in sterilized zip-lock bags.

**Corresponding author:* e-mail: erfaan21@gmail.com

The powered sample of *Emblica officinalis* was used for extraction using the Soxhlet extraction method. Briefly, the sample was extracted in a Soxhlet apparatus using methanol as a solvent. The extracted sample was filtered through Whatman No. 1 filter paper. Further, the filtrate was dried under reduced pressure using rotatory evaporator. The extracted sample was stored at 4^{o}C for further use.

The blood samples were collected in heparini zed tubes via venous arm puncture. The sample was centrifuged at 3000rpm for fifteen minutes in order to separate the plasma. This was followed by the removal of the buffy coat and the washing of packed cells thrice with physiological saline. The erythrocyte membrane was prepared by following the method of Dodge *et al*. (1968) that was modified by Quist (1980).

For metal-induced oxidative stress, erythrocytes were divided into four groups: A control group without any treatment, a negative control group that was treated with 0.5mM of arsenic, a positive control group that was treated with 0.8mg/ml of amla extract and a test group that was treated with 0.8mg/ml of the extract prior to 0.5 mM of arsenic induction.

Chemicals and reagents

The chemicals and reagents used were all of analytical grade. Triton -X-100 and thio-barbituric acid were purchased from Sigma Aldrich. Other reagents, such as Tri-chloro-acetic acid, chloroform, phosphate buffer, sodium oxide, DTNBs and so on, were obtained from a local supplier.

Lipid per oxidation

In order to induce lipid per oxidation, 100µL of hydrogen peroxide (100mM) was mixed with 200µL of suspended erythrocytes. The solution was incubated at 37^{o}C for one hour. Further, 2mL of thio-barbituric acid stock reagent containing 0.375% TBA, 15% TCA and 0.2 M HCl) was added in order to halt the reaction. The mixture was incubated on a boiling water bath for one hour. It was then cooled and centrifuged for five minutes at 3000rpm. The supernatant was collected, and its absorbance was measured at 532nm (Grottoa *et al.,* 2009).

Catalase activity

A method described by Sinha *et al*. (1972) was used to measure the catalase activity. Briefly, 1.5mL of the reaction mix was used. It contained 1000µL of phosphate buffer (0.01M, pH 7), 100µL of erythrocytes, and 400µL of hydrogen peroxide (2M). A 2mL of dichromate-acetic acid (1: 3) reagent was added in order to halt the reaction. The absorbance was measured at 620nm.

Glutathione peroxidase (GPx) activity

A method described by Rotruck *et al*., (1973) was used to measure the GPx activity. Briefly, 0.4mL of phosphate buffer (0.4M, pH 7), 0.1mL of sodium azide (10mM), 0.1mL of hydrogen peroxide, 0.2mL of reduced glutathione aliquot of hemolysate, and distilled water was taken as a reaction mixture with a volume of 2mL. The mixture was incubated at 37^{o}C for three minutes. The reaction was clogged by adding 0.5mL of TCA (10%). The supernatant was removed by centrifugation at 3200rpm for twenty minutes. Further, 3mL and 0.1mL of disodium hydrogen phosphate and DTNB reagent were added to the mixture. The developed color was read at 412nm. The glutathione peroxidase activity was expressed in µg of GSH consumed/gHb/min.

Reduced glutathione (GSH) activity

GSH was analyzed using the method described by Beutler *et al*., (1963). A 0.2mL hemolysate and 1mL of TCA (10%) was mixed and centrifuged at 1500rpm for fifteen minutes. 1mL of the supernatant was collected, to which 0.1mL of DNTB and 3mL of phosphate solution was added. The developed color was measured at 412nm and was expressed as mg of GSH/dl.

Estimation of ascorbic acid

The method of Omaye *et al*. (1979) was used in order to estimate the level of ascorbic acid. Briefly, 1mg of packed cells were precipitated with 2mL of 5% ice-cold TCA. The reaction was centrifuged for twenty minutes at 3500rpm, and 1ml of the supernatant was collected and mixed with 0.2mL of DNPH thiourea copper sulfate reagent (DTC). The mixture was incubated for three hours at room temperature. Further, 5mL of 65% of ice-cold $H_2SO_4$was added to the solution and incubated at room temperature for additional thirty minutes. The developed color was measured at 520nm, and the result was expressed as µg of ascorbic acid/gHb/mL.

STATISTICAL ANALYSIS

The data was expressed as mean ±SEM from three different readings and comparison was performed by Student's t-test. The results were considered significant when $P<0.05$.

RESULTS

The current work demonstrates the protective effect of crude extract of amla plants on metal (arsenic) induced lipid per oxidation and its antioxidant properties. Incubation of erythrocytes with metal (0.5mg/mL) for five minutes showed a significant increase in intracellular malondialdehyde (MDA) compared to the control ($P<0.05$). This suggests that there was an increase in lipid per oxidation. Similarly, there was a concomitant increase in the catalase activity ($P<0.05$). When the erythrocytes were pre-incubated with 0.8mg/mL of the crude extract prior to metal (arsenic) induced lipid per oxidation (test group), it significantly reduced the levels of MDA

Table 1: Protective effect of extract on arsenic induced lipid per oxidation

		Control	(-) Con	(+) Con	Test
Non-enzymatic antioxidants	LPO	57.45±13.27	126.43±13.27$^{@}$	22.99±0.06	34.42±13.27
	GSH	0.92±0.45	0.39±0.15^{a*}	0.92±0.45	0.79±0.03^{b*}
	Vit. C	0.96±0.05	0.78±0.05	0.922±0.02	0.76±0.05
Enzymatic antioxidants	GPx	0.68±0.44	0.42±0.03	0.50±0.29	0.68±0.44
	Catalase	72.15±11.95	20.09±2.48^{a}	44.48±8.02	59.48±8.95$^{b@}$

Value expressed as: LPO (namomoles of MDA released/gHb); GSH (microgram/gHb); GPx (micromoles of gHb/min); Catalase (micromoles of H_2O_2/gHb/min); Vit. C (micro gram/gHb). No treatment (Control); (-) con (Treated with metal); (+) con (treated with amla extract); test (treated with metal and amla extract). Values are statistically significant when *=P<0.05; #=P<0.01; @=P<0.005

Table 2: Protective effect of extract on $HgCl_2$ induced lipid per oxidation

		Control	(-) Con	(+) Con	Test
Non-enzymatic antioxidants	LPO	80.43±1622	172.4±16.25$^{a@}$	57.46±16.26	90.35±5.94b$^{@}$
	GSH	0.46±0.05	0.73±16.25$^{a@}$	1.49±0.15	1.05±0.12$^{b@}$
	Vit. C	0.92±0.05	0.81±0.52	0.922±0.02	0.76±0.15
Enzymatic antioxidants	GPx	0.52±0.05	0.28±0.07^{a*}	1.00±0.39	0.56±0.21$^{b\#}$
	Catalase	68.39±7.19	53.50±6.9$^{a\#}$	64.06±10.65	58.97±9.85$^{b@}$

compared to the negative control group, in which levels of MDA increased. This marked protection afforded by the crude extract of 0.8mg of amla against arsenic-induced RBC damage suggests the proactive effects of the crude extract on lipid per oxidation. Treatment of erythrocytes with metal (arsenic) significantly reduced enzymatic antioxidant and non-enzymatic antioxidant in human erythrocytes compared to the control and the group that was treated with crude amla extract (test group). In addition, metal (arsenic) treated human erythrocytes showed reduced levels of vitamin C compared to the control and positive control groups. However, the test group that treated with metals and crude extract showed an increase in vitamin C compared to the negative control group.

In addition, exposure of erythrocytes with $HgCl_2$showed a drastic increase in LPO as indented by the MDA released when compared with the erythrocytes that were incubated with physiological saline and the test group. The depletion of the non-enzymatic antioxidant systems, like GSH (P<0.01), and vitamin C (P<0.05) was significant with metal exposure. The preventive effect of *in vitro* amla extract on metal-induced oxidative stress might be due to the strong antioxidant efficacy of the vitamin C content present in the amla extract.

Plant and plant products have been used as sources of medicine in pharmaceutical preparation for ages. In this work, we confirmed the possible antioxidant status of methanolic extract of amla. The effects are mainly expressed through increases in the specific activities of both enzymatic and non-enzymatic antioxidants that were determined to be GPX and GSH, as well as the decrease of the central lipid per oxidation processes, which was demonstrated by the decrease in levels of MDA compared to the control and negative control groups.

DISCUSSION

The increased specific activity of GPx could be explained by the possible increased levels of its substrate glutathione (GSH), which was previously reported to be stimulated by the flavonoids (Mandel *et al*., 2005) through trans activation of a catalytical subunit promoter known as the γ-glutamyl cysteine synthetase (Myhrstad *et al*., 2002). Plant-based natural products are of great importance. They have a wide range of properties and mechanisms of action. A number of plants with phytochemical components have been studied and reported to have antioxidant properties. The antioxidant efficacies are primarily attributed to the presence of polyphenols, which and are known as inhibitors of lipid per oxidation. Amari *et al*. (2014) and Wojdylo *et al*. (2007) also reported the antioxidant property of some plant extracts. Synthetic antioxidants are considered to have higher potential than plant-based antioxidants; however, their use is limited due to their high toxicity, and the yare used only at restricted levels (Gulcin *et al*., 2010). Our results ratify that the crude extract of amla has high potential as an antioxidant and inhibitor of lipid per oxidation. This could be due to the fact that some polyphenolic compounds are present in the extract. Amla is known to contain a number of polyphenolic compounds with bioactive properties (Krishnaven & Mirunalini 2010). Polyphenolic compounds have been of great interest in the food industry. Therefore, amla could add to the quality of foods.

CONCLUSION

In the current study, we showed the possible antioxidant effects of methanolic extract of amla *in vitro*. These effects were mainly expressed through a general increase in the specific activities of both antioxidant enzymes (GPx) and a decrease in MDA concentration, as a marker of the lipid per oxidation processes.

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