

Research Article



Protection of DNA and Erythrocytes from Free Radical Induced Oxidative Damage by Methanolic Extract of Amla (*Phyllanthus emblica*)

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ABSTRACT

Amla (*Phyllanthus emblica*) is well known for its rich Vitamin C and polyphenolic contents which contribute to its antioxidant activity. The present study was focused on the total phenolic, proanthocyanidin, flavonoid contents and also protective effect of phenolic compounds present in methanolic extract of amla against free radical induced oxidative damage on DNA and erythrocytes. The extract was also tested for its inhibitory action on α -glucosidase and its reducing power. The total phenolic content was evaluated to 108.4 mg gallic acid equivalent (GAE)/g. The amount of flavonoids and proanthocyanidin content were recorded as 40.1 mg quercetin equivalents (QE)/g and 60.4 mg catechin equivalents (CE)/g respectively. The extract prevented the DNA from damage, after treatment of λ DNA with H_2O_2 and methanolic extract of amla. The oxidative hemolysis induced by hydrogen peroxide in rat erythrocytes was inhibited by amla extract in a dose dependent manner. The methanolic extract of amla exhibited good antioxidant properties which was comparable with standard antioxidant used.

Keywords: Hemolysis, Lambda DNA, Oxidative damage, Proanthocyanidin, Reducing power.

INTRODUCTION

The phytochemicals in fruits, vegetables, spices and traditional herbal medicinal plants have major protective role against many human chronic diseases such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular diseases, atherosclerosis and inflammation¹. All these diseases were associated with oxidative stresses caused by excess free radicals and other reactive oxygen species (ROS). Vitamins like C and E are essential to prevent the oxidative stress, but the majority of antioxidants available in fruits or vegetables may be from compounds such as phenolic acids and flavonoids and show protection against ROS by neutralizing these highly reactive radicals, rather than from vitamin C and E^{2,3}. In recent years much attention has been drawn on the use of natural dietary antioxidants as an effective protection against oxidative damage.

Erythrocytes are also found continuously exposed to both endogenous and exogenous source of ROS. They are highly prone to oxidative damage due to the presence of high concentration of hemoglobin and oxygen, potent promoters of oxidative stress⁴. Due to these activities erythrocytes are used as a cellular model to study about biomembrane integrity in relation to oxidative damage. Many researchers have reported that phenolic compounds present in plants shows strong antioxidant activity which reduces the cellular damage caused by free radicals⁵. Phenolic compounds are well known for its free radical scavenging property thereby protecting the cell from ROS. The antioxidant property of phenolics is mainly due to their redox properties, which allows them to act as reductants, hydrogen donors and singlet oxygen quenchers. Also they have a metal chelation property⁶. The presence of conjugated ring structures and hydroxyl

groups enable the phenolic compounds to perform antioxidant activity by scavenging superoxide anions, lipid peroxy radicals and singlet oxygen⁷.

Amla (*Phyllanthus emblica*) of family *Phyllanthaceae* has been used in traditional Ayurvedic medicines for treating several diseases like scurvy, common cold, cancer and heart disease⁸. The Amla fruit is the richest source of vitamin C, superoxide dismutase and also packed with other compounds like polyphenols which includes gallic acid, tannins, flavonoids and minerals like iron and zinc, vitamins like carotenes and vitamin B complex. Amla is reported to have hypolipidemic, hypoglycemic activities⁹. It also possess good antimicrobial, anti-inflammatory and anti cancer properties. The fruit can also improve metal induced clastogenic effects^{10,11}. Amla has strong antioxidant property which may be due to the existence of flavonoids and several gallic acid derivatives including epigallocatechin gallate and tannins^{12,13}. In the present study, the protective role of amla extract in preventing DNA damage and erythrocyte hemolysis was studied. In addition the contents of phenolics, flavonoids, proanthocyanidins, reducing power and α -glucosidase inhibitory activity of the extract were assessed.

MATERIALS AND METHODS

Chemicals

λ DNA was purchased from Bangalore Genei, India. α -glucosidase and p-nitro phenyl- α -D-glucopyranoside were obtained from Sigma Aldrich, USA. Butylated hydroxyanisole (BHA), Vanillic acid, Folin-Ciocalteu reagent, Gallic acid, Trichloro acetic acid, Potassium ferricyanide, Hydrogen peroxide, Ethidium bromide was purchased from SRL, India. All other chemicals were of analytical grade.



Preparation of Methanolic Extract of Amla

Fresh fruits of amla were collected from the Amirthi forest of Vellore, India and were freed from foreign matter such as dust or other organic matter. The fruits were then commuted to reduce its size and were dried at 40°C. Following which the fully dried pieces were grinded to obtain powder form. The 10 gms of powder obtained was extracted with 100 ml methanol for 24 hours and filtered using 0.45 mm filter paper. The residue was extracted twice with methanol as explained above. The methanolic extract thus obtained was then concentrated at 40°C, which was then freeze dried for complete removal of solvent and stored at 4°C for further use.

Determination of total phenolic, flavonoid, and proanthocyanidin contents

Total phenolic contents in the extract were determined by Folin-Ciocalteu method¹⁴. 100 mg of extract was mixed in 100 ml of methanol. The extract sample 0.5 ml was mixed with 0.5 ml of 0.2 N Folin – Ciocalteu reagent. After 2 minutes 0.5 ml (100 mg/ml) of sodium carbonate was added and left in room temperature for 2 hours. The absorbance of mixture was measured at 765 nm. Gallic acid was used as standard. The results obtained were expressed as milligrams of Gallic acid equivalents.

The total flavanoid content in the extract was measured using colorimetric method¹⁵. The extract 0.25 ml was added to 1.25 ml of distilled water. To this 5% sodium nitrate 75 µl and 150 µl of 10% aluminium chloride were added and mixed. 0.5 ml of 1M NaOH was added to above mixture, after 6 minutes and the total volume were made up to 2.5 ml by adding distilled water. The solution was mixed well and absorbance was measured against reagent blank at 510 nm. The flavonoid content was expressed as milligrams of Quercetin equivalents (QE)/gram dry mass.

Proanthocyanidin content was estimated using Vanillin assay¹⁶. 0.5 ml of extract was added to 3 ml of 4% vanillin-methanol reagent followed by 1.5 ml of HCl. The solution was mixed well and left at room temperature for 15 minutes. The absorbance was read at 500 nm against a reagent blank. The proanthocyanidin content was expressed in catechin equivalents (CE) per gram dry mass.

Determination of Reducing Power

The reducing power of amla extract was determined using the method described by Yen and Chen¹⁷. Different concentrations of methanolic extract 1 ml (20, 40, 60, 80, 100 µg/ml) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of 1% potassium ferricyanide. The sample was mixed well and incubated at 50°C for 20 minutes. 2.5 ml of Trichloroacetic acid (10%) was added to the mixture to stop the reaction. It was then centrifuged at 3000 rpm for 10 min and 2ml of supernatant was taken and mixed with 2 ml of distilled water. Ferric chloride 0.4 ml (0.1% w/v) was added to all

the tubes and absorbance was measured at 700 nm. BHA was used as positive control.

Inhibitory Action of Extract on α Glucosidase Enzyme

The reaction mixture contained 50 µl of p-nitro phenyl- α -D-glucopyranoside, different concentrations of extract 50 µl (2, 4, 6, 8, 10µg/ml in phosphate buffer). The reaction was initiated by adding 100 µl of α - glucosidase enzyme and volume was made up to 2 ml with 0.1 M phosphate buffer. The increase in absorbance was measured at 405 nm and the enzyme reaction was compared without adding the extract.

Prevention of λ DNA damage by Amla extract

The prevention of λ DNA damage by amla extract was done according to the method described by Ghanta *et al.*,¹⁸. Two sets of reaction were performed. λ DNA (0.5 µg), with (2 µg) and without extract was mixed with 1mM FeSO₄.7H₂O, 25 mM H₂O₂ in tris buffer (10mM, pH 7.4) making the reaction volume to 20 µl. One set was incubated for 1 hour and the other for 2 hours at 37°C. λ DNA mixed only with extract was also tested. Samples were run on 1% agarose gel prepared in Tris acetate EDTA buffer (pH 8.5) at 50 V at room temperature and visualized in gel documentation.

Erythrocyte preparation

Male wistar albino rats in the body weight range of 200–220 gms were used for the study. All the animal experiments were performed with the approval of ethical committee of VIT University, Vellore, India. The animals were provided with standard conditions and diet. The animals were sacrificed under anesthesia and blood was collected in heparinized tubes by heart puncture. Erythrocytes were collected from the blood and stored according to the method of Yuan and Yang *et al.*,^{19, 20}. The blood samples were centrifuged (1500 g, 5 minutes, at 4°C) and erythrocytes were separated from buffy coat and plasma. Erythrocytes were then washed thrice in 20mM phosphate buffered saline (10 volumes) carefully removing supernatant and buffy coat after each wash. Erythrocytes thus obtained were stored at 4°C and used within in 6 hours for tests.

Extract action in inhibiting hemolysis of rat erythrocytes

The assay was performed according to the procedure described by Tedesco *et al.*,²¹. The free radical initiator used was hydrogen peroxide. 200 µl of 10% (v/v) erythrocyte suspension in PBS was mixed with 50 µl of extract with varying concentrations (5-25 µg extract in PBS pH 7.4). To this mixture 100µl of 200 µMH₂O₂ (in PBS pH 7.4) was added. The mixture was incubated at 37°C for 30 minutes following which it was centrifuged at 2000 g for 10 minutes. 200 µl of resulting supernatant was added with 800 µl of PBS and absorbance was measured at 410 nm. The inhibitory effect of extract was compared with a standard antioxidant BHA. Similarly, the erythrocytes were treated with hydrogen peroxide and without extract



to obtain a complete hemolysis. The absorbance of the supernatant was measured as the above condition.

Statistical Analysis

The experiments were conducted in triplicate and data obtained was represented as mean \pm SD. Duncan's new multiple range tests was used to find the difference of means.

RESULTS

Total Phenolic, flavonoid and proanthocyanidin content

Polyphenol compounds form a major group of which accounts for the antioxidant activity of amla. The total phenolic content present in the extract was found to be 108.4 mg GAE/g. The flavonoid and proanthocyanidin contents were measured to be 40.1 mg QE/g and 60.4 mg CE/g respectively.

Reducing power of Amla extract

The reducing power of a compound is due to its property to donate electrons. Polyphenols are reported as good electron donors thus reducing Fe^{3+} /ferricyanide complex to ferrous²². The result obtained from the study showed an increase in reducing power as the extract concentration increases (Fig 1). There by proved the reducing power of amla extract in dose dependent manner.

α -Glucosidase enzyme inhibition by Amla extract

α -Glucosidase action is associated with Type-2 diabetes affecting glucose metabolism of the body leading to an increased postprandial glucose level. Several studies have reported that by inhibiting the enzyme action, glucose uptake can be regulated resulting in normal glucose levels, thereby can maintain the metabolism involved^{23, 24}. The study was done to prove the inhibitory action of amla extract on α -glucosidase action. The results obtained showed that inhibition percentage was seen increasing on increasing extract concentration (Fig 2). 2 μ g extract exhibited 60.6% inhibition where as 10 μ g inhibited 89% of the enzyme activity.

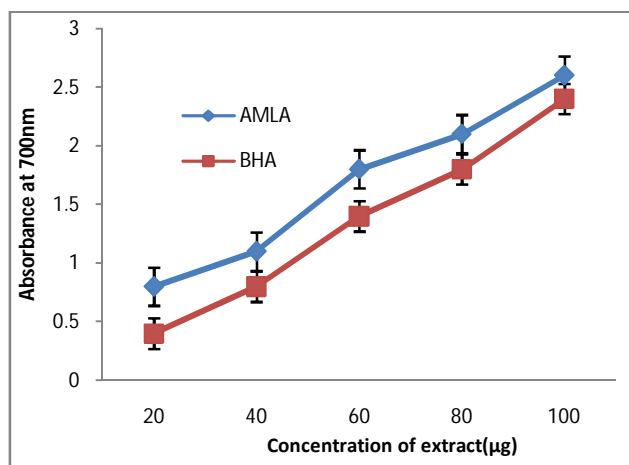


Figure 1: Reducing power of methanolic extract of amla. Values are mean \pm SD (n=3)

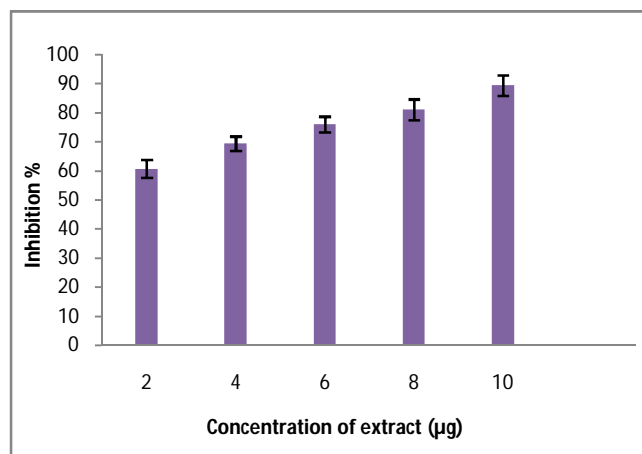


Figure 2: α -Glucosidase enzyme inhibition by amla extract. Values are mean \pm SD (n=3)

Rat erythrocyte hemolysis inhibition

Presence of free radicals results in release of hemoglobin pigment into the medium and the intensity of colour produced by hemoglobin was studied for evaluating the limit of erythrocyte damage. H_2O_2 was used to induce hemolysis and protective activity of extract was compared with a known antioxidant, BHA. Fig 3 shows the inhibitory action of extract against erythrocyte hemolysis in dose dependent manner. The IC_{50} values for amla and BHA was 18.5 and 24.5 μ g respectively (Fig. 3). Amla extract showed a better rate of inhibition compared to the known control. Thus the inhibitory action of amla extract to rat erythrocyte hemolysis was proved.

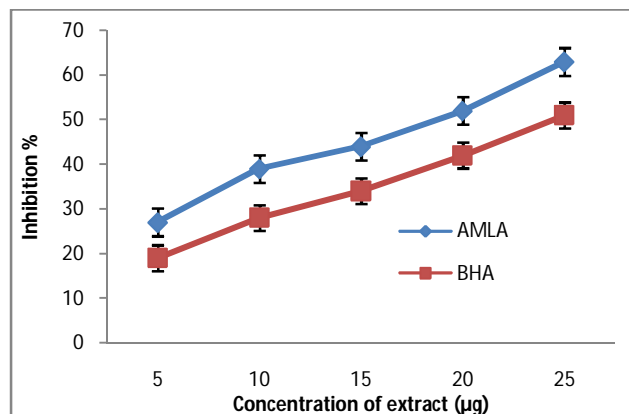


Figure 3: Protective effect of amla extract against H_2O_2 induced hemolysis of rat erythrocytes. Values are mean \pm SD (n=3)

Prevention of λ DNA damage using extract

The extract was tested for its ability to prevent DNA damage caused by free radicals from $FeSO_4$ and H_2O_2 using agarose gel respectively. DNA with $FeSO_4$ and H_2O_2 resulted in a decreased band intensity at the end of 1st hour (Fig 4 A, lane 2) and the band was seen further disintegrated towards the end of 2nd hour (Fig 4B, lane 2) indicating damage. In the presence of amla extract, λ DNA damage due to free radicals were not observed showing its protective activity (Fig 4A and B, lane 3). λ DNA in the presence of extract alone was seen unaffected indicating

that extract by itself do not contribute to any DNA damage (Fig 4A and B, lane 4). Thus the result proves the ability of extract in preventing DNA from oxidative damage.

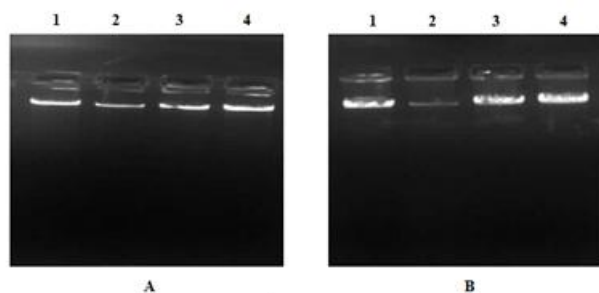


Figure 4: Agarose gel electrophoresis image of λ DNA damage inhibition by methanolic amla extract. (A). After 1 hr incubation of reaction mixture (B). After 2 hr incubation of reaction mixture. Lanes 1: 0.5 μ g λ DNA alone; Lane 2: 0.5 μ g λ DNA + 1 mM FeSO₄.7 H₂O +25 mM H₂O₂; Lane 3: 0.5 μ g λ DNA + 1 mM FeSO₄.7H₂O +25 mM H₂O₂ + 2 μ g methanolic amla extract; Lane 4: 0.5 μ g λ DNA + 2 μ g methanolic amla extract.

DISCUSSION

Phyllanthus emblica also known as amla possess enormous medicinal values that it can be used as anti-inflammatory, anti mutagenic agent and free radical scavenger. All these biological properties of amla are derived mainly from the ascorbic acid and polyphenol compounds present in it. The antioxidant activities of poly phenols is due to the presence of hydroxyl group (-OH) in aromatic ring, which helps in mediating redox reaction, and thereby scavenges free radicals²⁵. The reducing property of a compound signifies its potent antioxidant activity. The transformation of Fe³⁺ to Fe²⁺ has been looked for, on adding different extract concentrations. The poly phenols present in extract served as electron donors resulting in reducing ferricyanide complex to ferrous form¹⁷. The results obtained coincided to the fact that reducing power of amla extract increases with increase in concentration.

Erythrocytes were chosen for the study as it was found to be the prime targets for free radical attack due to the presence of high membrane concentration of poly unsaturated fatty acids and oxygen transport associated with hemoglobin molecules, which are highly susceptible to peroxidation²⁶. Even when hemolysis gives an indication to the extent of damage caused by free radicals and inhibitory action of antioxidants, very few studies have been reported on it. In the present study, Amla extract has been assessed for its protective action on erythrocytes from H₂O₂ induced hemolysis. The results proved that antioxidant activity of Amla extract helps in inhibiting hemolysis. Free radical induced DNA damage is seen increasing for which amla extract has been studied to rule out its preventive action on oxidative DNA damage. Studies have proved its potential in protecting DNA from damage. DNA along with free radicals resulted

in disintegration of it whereas on adding extract to the mixture, DNA was found intact without any damage.

Type-2 diabetes caused due to impairment in insulin secretion results in postprandial hyperglycemia. α -glucosidase levels need to be controlled in order to restore the normal glucose levels²⁸. α -glucosidase inhibitors are present in several natural food sources. The extract from amla was studied for its inhibitory activity on α -glucosidase enzyme and the result revealed its usefulness in regulating glucose homeostasis. Thus dietic supplement of amla can be useful for hyperglycemic patients.

Based on the results observed from various assays, it is proved that methanolic amla extract is rich in polyphenols exhibiting antioxidant properties and that it prevents λ DNA damage and inhibits erythrocyte hemolysis in addition to its valuable medicinal properties. As amla has got various excellent properties, it can be added as a functional food ingredient or as nutritional product for health benefits.

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