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Cytoprotective Properties of Antioxidant Protein from Curry leaves (*Murraya koenigii* L.) against Oxidative Stress Induced Damage in Human Erythrocytes

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Abstract

Oxidative stress induced by reactive oxygen species (ROS) cause lipid peroxidation at the human erythrocyte membrane with subsequent alterations in the ATPases function. In this study, potential cytoprotective activities of previously reported "Antioxidant Protein from Curry leaves (APC)" in erythrocytes against reactive oxygen (ROS) species generated by pro-oxidants *in vitro*. The APC prevented red blood cell lysis induced by pro-oxidants; Fe: As (2:20 µmole), Hydrogen peroxide (0.2 mM), and tertiary butyl hydroperoxide (1 mM) upto 97.5, 82.5 and 63%, respectively. Further, APC prevented Fe:As induced K^+ leakage in red blood cells up to 95%. The inhibition offered by APC on K^+ leakage was comparable to inhibition offered by quinine sulphate, a known K^+ channel blocker. Interestingly, the APC at dose dependently restored Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase activities of erythrocyte membrane when altered by ROS. The restoration of ATPase activity by APC was two times more than standard antioxidants BHA and α-tocopherol. In conclusion, Curry leaves protein antioxidant is an effective antioxidant in preventing membrane damage and associated functions mediated by reactive oxygen species. It can be further developed as an effective bioprotective antioxidant agent to cellular components.

Keywords: Antioxidant protein of curry leaves; Human erythrocytes; Lipid peroxidation; Fenton reactants; Oxidative hemolysis; K^+ leakage; Na^+K^+ ATPase; $Ca^{2+}Mg^{2+}$ ATPase

Abbreviations: APC: Antioxidant Protein of Curry leaves; RBC: Red Blood Cells; BHA: Butylated hydroxyanisole; Fe: As, Ferrous sulphate and Ascorbic acid mixture; t-BOOH: Tetra butyl hydroperoxide; TBA: Thiobarbituric acid; TBARs: Thiobarbituric acid reactive substances; MDA: Malondialdehyde; ROS: Reactive oxygen species

Introduction

Oxidative stress is detrimental to cells, as reactive oxygen species (ROS) can cause oxidative damage to lipids, proteins, DNA and other macromolecules [1-4]. Elevated levels of ROS have been implicated in etiology of many diseases like cancer, neurodegenerative disorders, cardiovascular diseases, atherosclerosis, cataract and inflammation [5-10].

Lipid peroxidation by ROS at the membrane levels generates variety of reactive substances such as aldehydes, including malondialdehyde (MDA) [11,12]. These may potentially affect membrane permeability and functioning of ion pumps and structure function relationship of membrane bound proteins resulting in the inability of the cell to maintain its ionic environment. Several structural and functional damages are caused to RBC (Red Blood Cells) by the exposure to MDA [13,14].

Under oxidative stress, hemolysis of RBCs takes place due to the action of ROS [15,16]. Further, intra cellular K^+ concentration is maintained by the cell to accomplish essential physiological task. The ROS cause disruption of cellular membrane proteins and leakage of intracellular contents [17,18]. The Na^+K^+ ATPAse and $Ca^{2+}Mg^{2+}$ ATPases are membrane bound enzymes which are involved in maintenance of ion concentration within cells. The altered activities of ATPases indicate the extent of damage to cell membranes. It has been reported that there is a permanent inhibition of ATPase activities by free radicals via lipid peroxidation [19]. A limiting factor which may control /prevent the extent of damage to cells may be the level of exogenously derived antioxidants. Dietary antioxidants have been shown to protect the cells from damage caused by oxidative stress and to fortify the defense system against degenerative diseases [8].

As reported earlier, an Antioxidant Protein of molecular weight ~ 35 KDa from Curry leaves (APC) [20]. In this study, we tested ability of APC to prevent Fenton reactants (Ferrous sulphate: Ascorbate system) induced oxidative membrane damage to RBCs. The use of human RBCs as a model for oxidative damage is because the liability of erythrocyte membrane to lipid peroxidation induced by peroxidation *in vitro* reflects the liability of other cell membranes to oxidative damage *in vivo* relating to oxidative stress [21,22]. This study for the first time shows the cytoprotective effect of APC against fenton reactant mediated alterations in RBC membrane, ATPases and K+ channels.

Materials and Methods

Materials

Ferrous sulphate, Ascorbate, Acrylamide, thiobarbituric acid, Ascorbate, α-tocopherol, O-phenanthroline, Adenosine 5`triphosphate (5`ATP) and all other chemicals were purchased from Sigma Chemical Co., USA. All other chemicals unless otherwise mentioned were of

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analytical grade and procured from Merck (Darmastadt, Germany). Solvents were distilled prior to use. Blood samples were obtained from the healthy male volunteers of Adichunchanagiri Biotechnology and cancer research institute (ABCRI), Mandya district, Karnataka, India.

Plant material

Curry leaves (*Murraya koenigii L.*) were obtained from a garden maintained by Adichunchanagiri Biotechnology and Cancer Research Institute (ABCRI), BG Nagara, Mandya district, India. Prof. GR Shivamurthy, Taxonomist, University of Mysore, India authenticated the plant. The plant was deposited at ABCRI against voucher No. ABCRI, 7/2007.

Purification of antioxidant protein of curry leaves (APC)

The APC was purified according to the method of Ningappa and Srinivas [20]. Briefly, curry leaves were washed, shade dried and powdered. Five grams of curry leaves powder was homogenized in 20 ml of 10 mM Tris buffer, pH 7.0, with addition of polyvinylpyrrolidone to remove polyphenols. The suspension was incubated overnight at 4°C with constant stirring, then filtered and centrifuged at 13,000 rpm at 4°C for 20 min. The supernatant was treated with 0.1% polyethyleneimine to precipitate nucleotides. The resulting pellet was discarded and supernatant brought to 65% saturation with ammonium sulphate. The pellet was dissolved in 20 mM Tris buffer, pH 7.4 (NH_4SO_4 extract). The NH_4SO_4 extract (21 mg) was loaded onto a Sephadex G-75 column (14 × 73 cm), pre-equilibrated and eluted with 20 mM Tris buffer, pH 7.4, at a flow rate of 1.5 ml/5 min. Protein elution was monitored at 280 nm using a spectrophotometer. The antioxidant activity of these protein samples at various concentrations ranging from 20-1000 µg was tested by TBARs assay. Active peak II (8 mg), pooled and lyophilised was further fractionated on a Sephadex G-75 column by eluting with 20 mM Tris buffer, pH 7.4, to give a homogenous preparation, APC, which showed maximum antioxidant activity.

Preparation of human erythrocytes

Human red blood cells (RBC) were separated from heparinized blood that was collected from healthy volunteers. The blood was centrifuged at 2500 rpm for 10 min to separate the RBCs from plasma. RBCs were washed three times with phosphate-buffered saline (PBS) at pH 7.4. During the last washing the cells were centrifuged at 2500 rpm for 10 min to obtain a constantly packed RBC-cell suspension.

Erythrocyte susceptibility to oxidation and protection by APC

100 µl of freshly prepared RBC suspension) was preincubated with antioxidants such as APC (0-125 µg), Trolox (0-125 µg), and α-tocopherol (0-125 µg) for 20 min at 37°C, then ferrous sulphate: ascorbate (2:20 µmole) or hydrogen peroxide (0.2 mM) or t-BOOH (1 mM) was added and final volume of 1 ml with saline. Reaction mixture was incubated at 37°C for 3 hr, centrifuged at 2500 rpm for 10 min and the extent of hemolysis was measured using spectrophotometer at 540 nm. Appropriate controls were taken and percentage inhibition of oxidative hemolysis by antioxidants using the formula.

$$\%\,\text{Oxidative Hemolysis inhibition} = \frac{\text{Absorbance of control - Absorbance of test sample}}{\text{Absorbance of Control}} \times 100$$

Preparation of human erythrocyte ghost (membrane)

Human blood was collected from healthy male volunteers. Erythrocyte ghost, free of hemoglobin and superoxide dismutase, was prepared by the method of Dodge et al. [23]. Blood was centrifuged at 2500 rpm for 15 min, the supernatant obtained was discarded and the RBC pellet was taken and kept as RBC suspension for further assays. The RBC pellet was washed three to five times with isotonic phosphate buffer (310 milli osmolar, pH 7.4) centrifuged at 2500 rpm at 4°C for 20 min. The RBC pellet obtained was suspended in hypotonic phosphate buffer and incubated overnight at room temperature for hypotonic hemolysis to take place. The contents were centrifuged at 12000 rpm at 4°C for 20 min, to remove unlysed RBC cells. Supernatant was collected and centrifuged at 12000 rpm for 20 min and washed in 0.9% saline. Erythrocyte ghost was suspended in 0.9% NaCl in aliquots and stored at -20°C for further use. The protein content of ghost was estimated by Bradford`s method [24].

Inhibition of lipid peroxidation induced by Fenton reactants (Fe:As) by APC and standard antioxidants

Erythrocyte membrane (200 µg membrane protein) were preincubated with or without various concentrations of APC (0-25 µg), BHA (400 µM), α-tocopherol (400 µM) in 0.5 ml of TBS, pH 7.4 at 37°C for 20 min. Then Ferrous sulphate: ascorbate (2: 20 µmole) were added, final volume made upto 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min. To each supernatant 1 ml of 10% TCA and 1 ml of 10% TBA was added, heated for 95°C for 15 min, cooled, centrifuged at 500 rpm for 10 min and supernatant was read at 535 nm. MDA equivalents were calculated using molecular extinction coefficient of MDA (1.56×10^5 $M^{-1}cm^{-1}$).

Intracellular K^+ leakage in erythrocytes and protection by APC in comparison with standard antioxidants

To 100 µl of RBC suspension, ferrous sulphate: ascorbate (2:20 µmole) was added and final volume made upto 1 ml with saline. At various time intervals, the amount of K^+ leakage from cells into the medium was measured by a K^+ specific micro electrode (Micro electrodes Inc. London-derry-New Hampshire, 03053, USA) connected to a pH meter. Calibration was done with 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M KCl solution. The amount of K^+ released was expressed as µM K^+.

For studying inhibitory effect of antioxidants on Fe:As induced K^+ ion leakage in erythrocytes. 100 µl ml RBC suspension was pre-incubated with or without different concentrations of APC (0-120 µg), BHA (0-120 µg), α-tocopherol (0-120 µg), Quinine sulphate (1 µM) and O-phenathroline (10 mM) for 20 min at 37°C. To this Fe:As (2:20 µmole) was added and final volume was made upto 1 ml with saline. The amount of K^+ ion leakage was monitored at 20th min as described above. Suitable controls of solvents or antioxidants or inhibitors alone were maintained. µM K^+ ion leakage induced by pro-oxidant without inhibitor or any antioxidants was expressed as 100% and % inhibition of K^+ ion leakage is calculated accordingly.

Restoration of Na^+K^+ ATPase activity by APC in comparison with standard antioxidants

Na^+K^+ ATPase activity of RBC membranes was determined following Ames [25-27]. Erythrocyte membranes (200 µg of membrane protein) were preincubated with or without various concentrations of APC (0-100 µg), BHA (0-100 µg) α-tocopherol (0-100 µg) and O-phenathroline (10 mM) in 0.5 ml of TBS, pH 7.4 at 37°C for 20 min. Then Fe: As (2: 20 µmole) were added, final volume made upto 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min. The supernatant discarded and pellet was dissolved and incubated in 0.5 ml of reaction mix (Tris 50 mM, NaCl 350 mM, KCl 35 mM, $MgCl_2$ 7.5 mM, EDTA 0.5 mM, pH 7.0) for 10 min at 37°C. At the end of the incubation period, ATP (15 mM) was added and further incubated at 37°C for 60 min, Reaction was stopped by adding 0.1 ml of 10% TCA, kept in ice water for 10 min. Na^+K^+ ATPase activity of RBC membranes were estimated by the inorganic phosphorous liberated (Pi). 700 µl of Ammonium

molybdate reagent was added followed by addition of 40 µl of ANSA reagent, incubated at 37°C for 60 min. The blue colour developed was read at 690 nm. Appropriate controls were done. The phosphorous content was calculated from calibration curve (absorbance versus phosphorous content) and enzyme activity was expressed as µmole Pi/mg membrane protein/hr.

Restoration of $Ca^{2+}Mg^{2+}$ ATPase activity by antioxidants

$Ca^{2+}Mg^{2+}$ ATPase activity of RBC membranes was determined following Ames [25-27]. Erythrocyte membranes (200 µg of membrane protein) were preincubated with or without various concentrations of APC (0-100 µg), BHA (0-100 µg), α-tocopherol (0-100 µg) and o-phenanthroline (10 mM) in 0.5 ml of TBS, pH 7.4 at 37C for 20 min. Then Fe: As (2: 20 µmole) were added, final volume made upto 1 ml with TBS, pH 7.4 and incubated at 37°C for 60 min. The supernatant discarded and pellet was dissolved and incubated in 0.5 ml of reaction mix (Tris 50 mM, NaCl 350 mM, KCl 35 mM, $MgCl_2$ 7.5 mM, EDTA 0.5 mM, pH 7.0) for 10 min at 37°C. At the end of the incubation period, ATP (15 mM) was added and further incubated at 37°C for 60 min, Reaction was stopped by adding 0.1 ml of 10% TCA, kept in ice water for 10 min. $Ca^{2+}Mg^{2+}$ ATPase activity of RBC membranes were estimated by the inorganic phosphorous liberated (Pi). 700 µl of Ammonium molybdate reagent was added followed by addition of 40 µl of ANSA reagent, incubated at 37C for 60 min. The blue colour developed was read at 690 nm. Appropriate controls were done. The phosphorous content was calculated from calibration curve (absorbance versus phosphorous content) and enzyme activity was expressed as µmole Pi/mg membrane protein/hr.

Statistical analysis

Statistical analysis was done in SPSS (Windows version 10.0.1 Software Inc., New York) using a one-sided student`s *t*-test. All results refer to mean ± SD. $P<0.05$ was considered as statistically significant as comparing to relevant controls.

Results and Discussion

Lipid peroxidation by ROS at membrane levels, affect the structure and function of membranes. High levels of ROS have been implicated in several oxidative damage related diseases [6-8]. The limiting factor that may prevent the extent of damage could be the quantum of exogenously derived antioxidants [8]. The present study reports the cytoprotective effects of APC [20] against oxidative membrane damage in RBC cells. In this study, RBC`s are used to test the extent of lipid peroxidation, as liability of erythrocyte membrane to lipid peroxidation *in vitro* reflect the liability of other cell membranes to oxidative damage *in vivo,* relating to oxidative stress [21,22]. Further, *in vivo* erythrocytes are highly exposed to oxygen and are site for radical formation under pathological conditions [28]. Anemia of chronic inflammatory diseases appears to be caused, in part by oxidative damage to erythrocytes.

A number of factors have been considered relevant to the oxidative lysis of RBC`s and in addition, it has been reported that hemolysis finally depends on the integrity of membrane proteins [15,16]. Hence it was important to study the protective role of APC to prevent oxidative hemolysis. When APC was tested for its efficiency to prevent the oxidative RBC lysis induced by pro-oxidants, there was a dose dependent prevention by APC of membrane lysis upon fenton reactants; Fe;As (2:20 µmole), hydrogen peroxide (0.2 mM) and t-BOOH (1 mM) induction (Figure 1). At 125 µg APC exhibits maximum protection to the extent of 97.5% against Fe:As induced oxidative hemolysis, on the other hand α-tocopherol and BHA at 400 µM exhibited protection by 92% and 85%, respectively (Figure 2). Upon hydrogen peroxide and t-BOOH induced oxidative hemolysis, APC at 125 µg showed about 63% protection against hydrogen peroxide induced hemolysis and about 82.5% against t-BOOH induced hemolysis. On the other hand, BHA and α-tocopherol showed 83% and 94% against hydrogen peroxide induced hemolysis and about 78% and 90% against t-BOOH induced hemolysis (Figure 2). The results indicate that APC is a potent protectant against oxidative lysis of erythrocytes when compared to standard antioxidants BHA and α-tocopherol. Similar studies have reported that oolong tea extracts and melatonin shown to inhibit hemolysis under oxidative stress [28-30]. The results suggest that oxidative membrane damage could be efficiently protected by antioxidants.

The K^+ ion leakage is one of the results of membrane damage. In order to accomplish essential physiological task, virtually all cells accumulate K^+ and excludes Na^+ from the cytoplasm. The oxidation of membrane lipids, with the formation of aldehydes results in the disruption of cellular membrane proteins and cause leakage of intracellular contents [17,18]. In this study, when erythrocyte

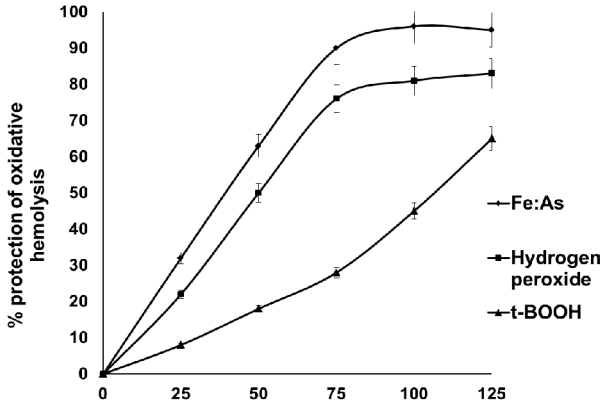

Figure 1: Dose dependent protective effect of APC on pro-oxidant induced oxidative hemolysis. 100 µl of RBC suspension was preincubated with APC (0-125 µg) at 37°C for 20 min. Then Fe:As (2:20 µmole) or hydrogen peroxide (0.2 mM) or t-BOOH (1 mM) added in 1 ml saline, incubated at 37°C for 180 min. Oxidative hemolysis induced by pro-oxidants in the absence of antioxidants was expressed as 100% lysis. Data represent the mean ± SD (n=6).

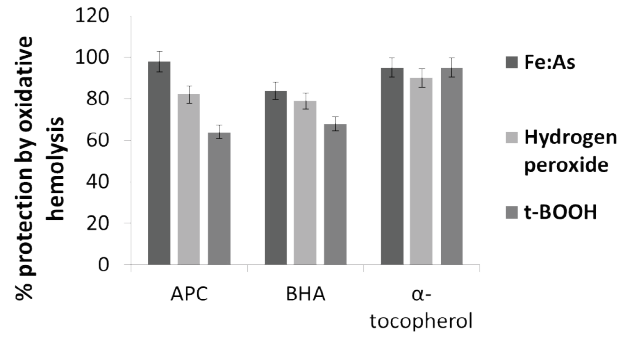

Figure 2: Protective effect of APC on pro-oxidant induced oxidative hemolysis: comparison with standard antioxidants. 100 µl of RBC suspension preincubated with APC (125 µg) or BHA (400 µM) or α -tocopherol (400 ¹M) at 37°C for 20 min. Then Fe:As (2:20 µmole) or hydrogen peroxide (0.2 mM) or t-BOOH (1 mM) added in 1 ml saline, incubated at 37°C for 180 min. Oxidative hemolysis induced by pro-oxidants in the absence of antioxidants was expressed as 100% lysis. Data represent the mean ± SD (n=6).

suspension was treated with Fe:As (2:20 µmole) the maximum damage was at 20th min, indicated by leakage of K^+ ions in the medium. When antioxidants were tested in terms of their potency to prevent K^+ ion leakage in erythrocytes, it was found that APC, BHA and α-tocopherol dose dependently prevented Fe:As induced K^+ leakage to (Figure 3). APC at 120 µg inhibited K^+ leakage in erythrocyte membrane upto 95%, whereas BHA and α-tocopherol at 120 µg inhibited up to 81% and 89%, respectively (Table 1). APC was more potent than standard antioxidants in inhibiting K^+ leakage and inhibition is comparable to inhibition offered by quinine sulphate (upto 90%), a known K^+ channel blocker. In addition, O-phenanthroline (lipophilic iron chelator) offered 92% inhibition against Fe:As induced K^+ leakage (Table 1). Inhibition of lipid peroxidation by iron chelators was shown to completely prevent cell death indicating that iron induced peroxidative damage was responsible for causing irreversible injury. The comparison studies with OH radical quenchers (BHA and α-tocopherol) and lipophilic iron chelator (O-phenanthroline) indicate that APC is effective inhibitors of OH radical formation and iron induced peroxidative damage.

Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase are membrane bound enzymes, which are involved in maintenance of respective monovalent and divalent cation concentrations within cells. The altered activities of ATPases possibly show the extent of damage in cell membrane. It has been reported that there is a permanent inhibition of ATPase activity by oxygen free radicals via lipid peroxidation [19]. When the effect of antioxidants including APC was tested on Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ upon induction by Fe:As (2:20 µmole), it was observed that all the antioxidants tested APC, BHA, and α-tocopherol dose dependently restored Na^+K^+ ATPase (Figure 4) and $Ca^{2+}Mg^{2+}$ ATPase activities (Figure 5) of erythrocyte membrane. The activities of isolated RBC membranes (Untreated membranes) were 8.5 ± 1.0 and 11.2 ± 1.2 µmole/mg membrane protein/hr for Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase activities, respectively. Treatment of RBC membranes with Ferrous sulphate: ascorbate (2:20 µmole) resulted in significant inhibition of ATPase activities, the activities were found to be 2.5 ± 0.08 and 3.4 ± 0.07 for Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase activities, respectively. ATPase activities in presence of APC (100 µg), BHA (100 µg) and α-tocopherol (100 µg) were found to be 7.9 ± 0.3, 6.6 ± 0.55 and 7.4 ± 0.35 for Na^+K^+ ATPase (Table 2) and 9.9 ± 0.9, 7.3 ± 0.6 and 8.4 ± 0.55 for $Ca^{2+}Mg^{2+}$ ATPase activities (Table 2). In comparison, the decrease in ion pump ATPase activity was significantly prevented by the pretreatment of O-phenanthroline (10 mM), which showed 8.4 ±

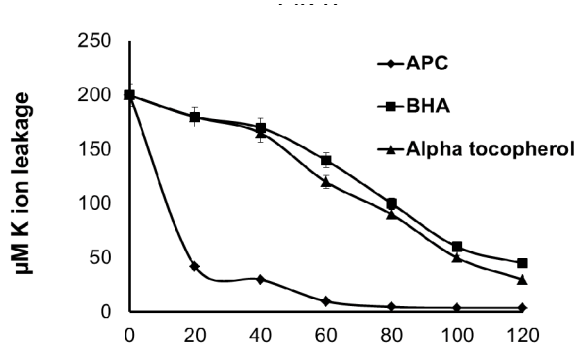

Figure 3: Dose dependent inhibition of Fe:As induced K^+ leakage by APC and standard antioxidants. 100 µl of RBC suspension was pre-incubated with or without antioxidants at indicated concentrations for 20 min at 37°C. Fe:As (2: 20 µmole) were added and final volume made upto 1 ml with saline. The amount of K^+ ion leakage was measured at 20th min with K^+ specific microelectrode connected to pH meter. Amount of K^+ released is expressed as µM $K^+/10^6$ cells. Data represent the mean ± SD (n=6).

Antioxidants/Inhibitors	Function	Concentration	% inhibition of K^+ leakage in erythrocytes (1×10^6 cells)
No Antioxidants/ Inhibitors			0
Quinine sulphate	K^+ channel Blocker	1 µM	90 ± 1.9
O-phenanthroline	iron chelator	10 mM	92 ± 1.5
APC	Antioxidant	120 µg	95 ± 1.1
BHA	Antioxidant	120 µg	81 ± 1.7
α-tocopherol	$^1O_2$ quencher	120 µg	89 ± 1.3

100 µl of RBC suspension was preincubated with antioxidants/inhibitors for 20 min at 37°C. Ferrous sulphate:ascorbate (2:20 µmole) was added and the amount of K+ leakage was measured at 20th min. K+ leakage induced by pro-oxidant without inhibitor was expressed as 100% and % inhibition calculated accordingly. Values are mean ± SD (n=6).

Table 1: Inhibitory effect of APC on pro-oxidant induced K^+ leakage in erythrocyte.

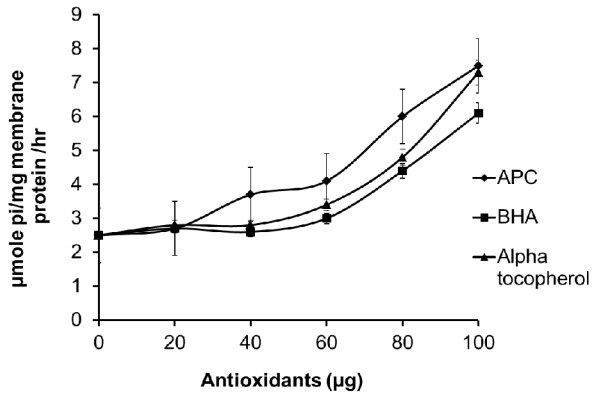

Figure 4: Restoration of Na^+K^+ ATPase activity by APC and standard antioxidants. Erythrocyte membrane (200 µg) preincubated with APC (0-100 µg) or BHA (0-100 µg) or α-tocopherol (0-100 µg) in 0.5 ml TBS, pH 7.4 and incubated at 37°C for 30 min. Fe:As (2:20 umole) were added, final volume made upto 1 ml with TBS, Incubated at 37°C for 60 min. following incubation Na^+K^+ pump ATPase of membrane were determined and enzyme activity is expressed as µmole Pi/mg protein/hr. Data represent the mean ± SD (n=6).

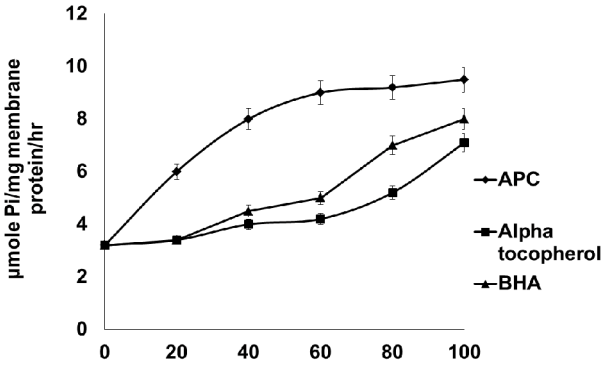

Figure 5: Restoration of $Ca^{2+}Mg^{2+}$ ATPase activity by APC and standard antioxidants. Erythrocyte membrane (200 µg) preincubated with APC (0-100 µg) or BHA (0-100 µg) or α -tocopherol (0-100 µg) in 0.5 ml TBS, pH 7.4 and incubated at 37°C for 30 min. Fe:As (2:20 umole) were added, final volume made upto 1 ml with TBS, Incubated at 37°C for 60 min. following incubation $Ca^{2+}Mg^{2+}$ pump ATPase of membrane were determined and enzyme activity is expressed as µmole Pi/mg protein/hr. Data represent the mean ± SD (n=6).

1.2 and 10.3 ± 0.9 for Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase activities, respectively (Table 2). This suggests that if iron is chelated, inhibition of ion pump ATPases activities could be diminished. Overall ATPase activities in presence of antioxidants were comparable to activities of untreated RBC membrane Based on the results obtained it can be concluded that APC is an effective antioxidant in restoration of ATPases activities upon oxidative damage to membrane.

Studies have shown that end products of lipid peroxidation, MDA is capable of cross-linking membrane components containing amino groups [17,31]. It has been reported that one potential mechanism for iron mediated damage of Ca^{2+} pump ATPase is through cross-linking by MDA [32]. The incubation of RBC membrane (200 µg) with Fe:As (2:20 µmole) as for ATPase analysis was found to induce membrane lipid peroxidation as evidenced by the formation of TBARs of about 1.56 nmole of MDA equivalents/mg membrane protein/hr. The treatment of APC (0-25 µg), BHA (0-25 µg) and α-tocopherol (0-25 µg) found to limit the formation of TBARs in a dose dependent manner (Figure 6) for protection of ion pump ATPases (Figures 4 and 5). The above results indicate that ferrous sulphate: ascorbate induced formation of TBARs in RBC ghost which closely parallels with inhibition of both ion pump ATPases (Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase) and pretreatment of APC, BHA and α-tocopherol significantly ameliorated the oxidative damage of RBC membrane by reducing MDA levels and restoring ATPases activities towards normalcy. The mechanism by which APC does it probably is by directly scavenging radicals or chelating the ferrous ions (Fenton reaction) [33].

In conclusion, the Curry leaves protein antioxidant is an effective antioxidant in preventing membrane damage and associated functions mediated by reactive oxygen species. It could be further developed as an effective bioprotective antioxidant agent to cellular components.

Acknowledgement

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Antioxidants	Final Concentration	Na^+K^+ ATPase activity*	$Ca^{2+}Mg^{2+}$ ATPase activity*
Untreated RBC Membrane		8.5 ± 1.0	11.2 ± 1.2
RBC membrane + Fe:As (2:10 µmole) + APC		2.5 ± 0.1[a]	3.4 ± 0.[a]
RBC membrane + Fe:As (2:10 µmole) + APC	100 µg	7.9 ± 0.3[b]	9.9 ± 0.9[b]
RBC membrane + Fe:As (2:10 µmole) + APC	100 µg	6.6 ± 0.5[c]	7.3 ± 0.6[c]
RBC membrane + Fe:As (2:10 µmole) + APC	100 µg	7.4 ± 0.3[b]	8.4 ± 0.5[b]
RBC membrane + Fe:As (2:10 µmole) + APC	10 mM	8.4 ± 1.2[b]	10.3 ± 0.9[b]

*The enzyme activity is expressed as µmole Pi/mg protein/hr; [a]Statistically significant (P<0.001) compared to untreated membrane; [b]Statistically significant (P<0.01) compared to Fe:As treated membrane; [c]Statistically significant (P<0.05) compared to Fe:As treated membrane; Erythrocyte membrane (200µg) preincubated with APC (0-100 µg) or BHA (0-100 µg) or a-tocopherol (0-100 µg) or O-phenanthroline (0-10 mM) in 0.5 ml TBS, pH 7.4 and incubated at 37C for 30 min. Fe:As (2:20 umoles) were added, final volume made upto 1 ml with TBS, Incubated at 37°C for 60 min. following incubation Na^+K^+ pump ATPase and $Ca^{2+}Mg^{2+}$ pump ATPase of membrane (pellet) were determined and enzyme activity is expressed as µmole Pi/mg protein/hr. Data represent the mean ± S.D (n=6).

Table 2: Restoration of ATPase activities by APC; Comparison with standard antioxidants.

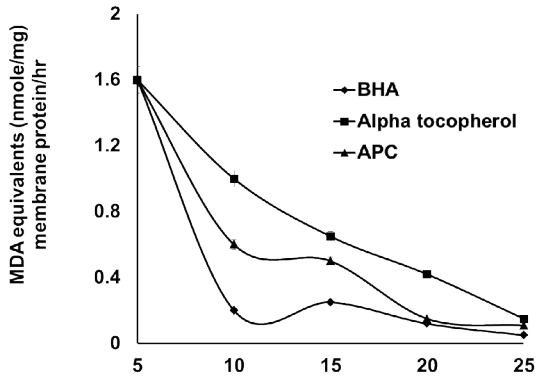

Figure 6: Inhibition of lipid peroxidation by APC and standard antioxidants. RBC ghost (200 µg) pre-incubated with antioxidants, treated with Fe:As (10:100 µmole) in 1 ml of TBS, pH 7.4. TBARs determined and calculated as equivalents of MDA using molecular extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and expressed as nmole of MDA equivalents/mg membrane protein/hr. Data represent the mean ± SD (n=6).

References

1. Butterfield DA, Koppal T, Howard B, Subramanium R, Hall N, et al. (1998) Structural and functional changes in proteins induced by free radical-mediated oxidative stress and protective action of the antioxidants N-tert-butyl-alpha-phenylnitrone and vitamin E. Ann NY Acad Sci 854: 448-462.
2. Lee HC, Wei YH (2007) Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. Exp Biol Med (Maywood) 232: 592-606.
3. Pallavi S, Ambuj Bhushan J, Rama Shanker D, Mohammad P (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany 1: 26.
4. Kashmiri ZN, Mankar SA (2014) Free radical and oxidative stress in bacteria. Int J Current microbiology and applied sciences 3: 34-40.
5. Halliwell B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet 344: 721-724.
6. Aviram M (2000) Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. Free Radic Res 33 Suppl: S85-97.
7. Kris-Etherton PM, Lichtenstein AH, Howard BV, Steinberg D, Wiztum JL (2004) Nutrition Committee of the American Heart Association Council on Nutrition, Physical Activity and Metabolism. Antioxidant vitamin supplements and cardiovascular disease. Circulation 110: 637-641.
8. Riccioni G, Mancini B, Di Ilio E, Bucciarelli T, D'Orazio N (2008) Protective effect of lycopene in cardiovascular disease. Eur Rev Med Pharmacol Sci 12: 183-190.
9. Sabry El-Bahr M (2013) Biochemistry of Free Radicals and Oxidative Stress. Science International 1: 111-117.
10. Saha H, Mitra M, Deepa Sankar P (2014) Oxidative stress and approaches to enhance abiotic stress tolerance in plants. Res J Pharm Biol Chem Sci 5: 724.
11. Atef MMA, Fatma AAI, Noha AAbd E, Samir WA (2014) Antioxidant effects of curcumin against cadmium chloride - induced oxidative stress in the blood of rats. Journal of Pharmacognosy and Phytotherapy 6: 33-40.
12. Chidiebere U, Suleiman F, Joseph OA, King ANE, Angel JU (2014) Erythrocyte osmotic fragility and lipid peroxidation following chronic co-exposure of rats to chlorpyrifos and deltamethrin, and the beneficial effect of alpha-lipoic acid. Toxicology reports 1: 373–378.
13. Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine. 3rd edn. Oxford University Press Inc., New York, USA.
14. Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11: 81-128.

15. van den Berg JJ, Op den Kamp JA, Lubin BH, Roelofsen B, Kuypers FA (1992) Kinetics and site specificity of hydroperoxide-induced oxidative damage in red blood cells. Free Radic Biol Med 12: 487-498.

16. Cleldon G, Rodriguex I, Espana J, Escobar J, Lissi E (2001) contribution of haemoglobin and membrana constituents modification to human erythrocyte damage promoted by per-oxyl radicals of different charge and hydrophobicity. Free radical research 34: 17-31.

17. Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. Clarendon Press. Oxford.

18. Rohn TT, Nelson LK, Waeg G, Quinn MT (1998) U-101033E (2,4-diaminopyrrolopyrimidine), a potent inhibitor of membrane lipid peroxidation as assessed by the production of 4-hydroxynonenal, malondialdehyde, and 4-hydroxynonenal-protein adducts. Biochem pharmacol 56: 1371-1379.

19. Hexum TD, Fried R (1979) Effects of superoxide radicals on transposrt (Na+K+) adenosine transport triphosphatase and protection by superoxide dismutase. Neurochemical research 4: 73-82.

20. Ningappa MB, Srinivas L (2008) Purification and characterization of approximately 35 kDa antioxidant protein from curry leaves (*Murraya koenigii* L.). Toxicol *In Vitro* 22: 699-709.

21. Sies H (1985) Hydroperoxides and thiol oxidants in the present study of oxidative stress in intact cells and organs. In “oxidative stress” Sies H (ed.). Academic press, New York, USA. pp: 73-78.

22. Fraga CG, Tappel AL, Leibovitz BE, Kuypers F, Chiu D, et al. (1990) Lability of red blood cell membranes to lipid peroxidation: application to humans fed polyunsaturated lipids. Lipids 25: 111-114.

23. Dodge JT, Mitchell C, Hanahan DJ (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 100: 119-130.

24. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Analytical biochemistry 7: 248-254.

25. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymology 8: 115-118.

26. Moore RB, Brummitt ML, Mankad VN (1989) Hydroperoxides selectively inhibit human erythrocyte membrane enzymes. Arch Biochem Biophys 273: 527-534.

27. Kaul S, Krishnakanth TP (1994) Effect of retinol deficiency and curcumin or turmeric feeding on brain Na+K+ adenosine transport triphosphatase activity. Molecular and cellular biochemistry 137: 101-107.

28. Tesoriere L, D'Arpa D, Conti S, Giaccone V, Pintaudi AM, et al. (1999) Melatonin protects human red blood cells from oxidative hemolysis: new insights into the radical-scavenging activity. J Pineal Res 27: 95-105.

29. Allegra M, Gentile C, Tesoriere L, Livrea MA (2002) Protective effect of melatonin against cytotoxic actions of malondialdehyde: an in vitro study on human erythrocytes. J Pineal Res 32: 187-193.

30. Zhu QY, Hackman RM, Ensunsa JL, Holt RR, Keen CL (2002) Antioxidative activities of oolong tea. J Agric Food Chem 50: 6929-6934.

31. Tappel AL (1973) Lipid peroxidation damage to cell components. Fed Proc 32: 1870-1874.

32. Pacifici RE, Davies KJ (1990) Protein degradation as an index of oxidative stress. Methods Enzymol 186: 485-502.

33. Ningappa MB, Dinesha R, Srinivas L (2008) Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (Murraya koenigii L.) extracts. Food Chemistry 106: 720-728.