J Membrane Biol (2015) 248:1137–1144
DOI 10.1007/s00232-015-9831-8

Protective Effect of Sundakai (*Solanum torvum*) Seed Protein (SP) Against Oxidative Membrane Damage in Human Erythrocytes

M. Sivapriya[1] · S. S. Thammanna Gowda[2] · Leela Srinivas[2]

Received: 10 April 2015 / Accepted: 6 August 2015 / Published online: 15 September 2015
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Abstract Lipid peroxidation by ROS at the membrane level disturbs the inherit integrity of components activating subsequent alterations in the function. In this study, the protective effect of purified Sundakai (*Solanum torvum*) seed protein (SP) was tested against oxidative membrane damage in erythrocyte membrane. SP prevented oxidative RBC lysis induced by pro-oxidants; Fe:As (2:20 µmol), periodate (0.4 mM), and *t*-BOOH (1 mM) up to 86, 81, and 86 %, respectively. Further, SP prevented the Fe:As-induced K^+ leakage up to the tune of 95 %. The inhibition offered by SP on K^+ leakage was comparable to inhibition offered by quinine sulfate, a known K^+ channel blocker. SP dose dependently restored Na^+K^+ ATPase and Ca^{2+} Mg^{2+} ATPase activities in erythrocyte membrane. The restoration of ATPase activity by SP was two times more than standard antioxidants BHA and α-tocopherol. Besides, SP at 1.6 µmol restored the membrane proteins over Fe:As induction when analyzed by SDS-PAGE, which was comparable to protection offered by BHA. In conclusion, SP is an effective antioxidant in preventing oxidative membrane damage and associated functions mediated by ROS. As SP is non-toxic, it can be used as an effective bioprotective antioxidant agent to cellular components.

Keywords Sundakai seed protein (SP) · Lipid peroxidation · Pro-oxidants · Oxidative hemolysis · K^+ leakage · Na^+K^+ ATPase

✉ Leela Srinivas
sstgindia@yahoo.co.in

[1] Aurigene Discovery Technologies, Bangalore, Karnataka, India

[2] Adichunchanagiri Biotechnology and Cancer Research Institute, Mandya, Karnataka, India

Abbreviations

BHA	Butylated hydroxyanisole
Fe:As	Ferrous sulfate:ascorbic acid
t-BOOH	Tertiary butyl hydroperoxide
TBA	Thiobarbituric acid
TBARs	Thiobarbituric acid reactive substances
MDA	Malondialdehyde
°C	Degree Celsius
H	Hour(s)
Mg	Milligram
Mg	Microgram
µM	Micromolar
nm	Nanometer
%	Percentage
SD	Standard deviation

Introduction

Excess production of reactive oxygen species (ROS) under conditions of oxidative stress is detrimental to cells, as ROS can cause oxidative damage to lipids, proteins, DNA, and other macromolecules (Butterfield et al. 1998; Lee and Wei 2007; Valk et al. 2007). Elevated levels of ROS have been implicated in etiology of many diseases like cancer, neurodegenerative disorders, cardiovascular diseases, atherosclerosis, cataract, and inflammation (Halliwell 1994; Aviram 2000; Kris-Etherton et al. 2004; Riccioni et al. 2008).

Lipid peroxidation by ROS at the membrane levels disturbs the inherit integrity of lipids in membrane causing subsequent alterations in the functions. Lipid peroxides generates a variety of reactive substances such as

aldehydes, including malondialdehyde (MDA). 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 RBCs by the exposure to MDA (Halliwell and Gutteridge 1999; Esterbauer et al. 1991).

Under oxidative stress, hemolysis of RBCs takes place due to the action of ROS (Van den Berg et al. 1992). Further, intracellular K^+ concentration is zealously guarded by the cell to accomplish essential physiological task. Oxidative damage causes disruption of cellular membrane proteins and causes leakage of intracellular contents (Halliwell and Gutteridge 1989; Rohn et al. 1998). 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 oxygen-free radicals via lipid peroxidation (Hexum and Fried 1979). A limiting factor which may 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 (Clarke et al. 2008; Riccioni et al. 2008). Several natural antioxidants and phytonutrients present in dietary sources reduced the oxidative stress and oxidative stress-induced hemolysis (Tulipani et al. 2011, 2014; Widen et al. 2012; Rajamani et al. 2014; Phrueksanan et al. 2014).

Earlier we have isolated and characterized an antioxidant protein from Sundakai (*Solanum torvum*) seeds which are designated as Sundakai seed protein (SP) (Marappan and Leela 2007). Here in this study we test the ability of SP to prevent pro-oxidant-induced oxidative membrane damage to RBCs. The use of human RBCs as a model for oxidative damage is as 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 (Sies 1997; Fraga et al. 1990).

Materials and Methods

Materials

Ferrous sulfate, ascorbate, acrylamide, thiobarbituric acid, butylated hydroxyl anisole (BHA), α-tocopherol, quercetin, *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 analytical grade and procured from Merck (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. A homogenous preparation of Sundakai seed protein (SP) was purified as described previously (Marappan and Leela 2007).

Analysis of Oxidative Hemolysis

Preparation of RBC's and 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. (1963). 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 RBS 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 for overnight at room temperature for hypotonic hemolysis to take place. The contents were centrifuged at 12,000 rpm at 4 °C for 20 min to remove unlysed RBC cells. Supernatant was collected and centrifuged at 12,000 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 (Bradford Marion 1976).

Erythrocyte Susceptibility to Oxidation and Protection by SP

100 µls of freshly prepared RBC suspension (1×10^6 cells) was preincubated with antioxidants such as Sundakai seed protein (SP) (0–25 µgs), BHA (0–25 µgs), and α-tocopherol (0–25 µgs) for 20 min at 37 °C, and then ferrous sulfate:ascorbate (2:20 µmol), periodate (0.4 mM) or *t*-BOOH (1 mM) was added and final volume was made up to 1 ml with saline. Reaction mixture was incubated at 37 °C for 3 h, centrifuged at 2500 rpm for 10 min and the extent of hemolysis was measured spectrophotometrically at 540 nm. Appropriate controls were taken and percentage inhibition of oxidative hemolysis was calculated. The concentration of protein in the hemolysates was estimated by Bradford's method (1976).

Inhibition of Lipid Peroxidation Induced by Fe:As by Sp and Standard Antioxidants

Erythrocyte membrane (200 μg membrane protein) was preincubated with or without various concentrations of SP (0–25 μgs), BHA (400 μM), and α-tocopherol (400 μM) in 0.5 ml of TBS, pH 7.4 at 37 °C for 20 min. Then, ferrous sulfate:ascorbate (2:20 μmol) was added, final volume was made up to 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 were 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 ($1.56 \times 10^{+5}$ M^{-1} cm^{-1}) (Buege and Aust 1978; Yang et al. 2001).

Intracellular K^+ Leakage in Erythrocytes and Protection by Antioxidants

To 0.1 ml of RBC suspension (1.0×10^6 cells), ferrous sulfate:ascorbate (2:20 μmol) was added and final volume was made up to 1 ml with saline. At various time intervals, the amount of K^+ leakage from cells into the medium was measured by a K^+ specific microelectrode (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 $K^+/10^6$ cells.

For studying inhibitory effect of antioxidants on Fe:As-induced K^+ ion leakage in erythrocytes, 0.1 ml RBC suspension (1.0×10^6 cells) was preincubated with or without different concentrations of SP (0–120 μgs), BHA (0–120 μgs), α-tocopherol (0–120 μgs), Quinine sulfate (1 μM), and *O*-phenanthroline (10 mM) for 20 min at 37 °C. To this, Fe:As (2:20 μmol) was added, and final volume was made up to 1 ml with saline. The amount of K^+ ion leakage was monitored at 20^{th} 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 Antioxidants

Na^+K^+ ATPase activity of RBC membranes was determined following Ames (Ames 1966; Moore et al. 1989; Kaul and Krishnakanth 1994). Erythrocyte membranes (200 μgs of membrane protein) were preincubated with or without various concentrations of SP (0–100 μgs), BHA (0–100 μgs) -tocopherol (0–100 μgs) and *O*-phenanthroline (10 mM) in 0.5 ml of TBS, pH 7.4 at 37 °C for 20 min. Then, Fe:As (2:20 μmol) was added, final volume was made up to 1 ml with TBS, pH 7.4 and incubated at 37 °C for 60 min. The supernatant was discarded, and the 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 and kept in ice water for 10 min. Na^+K^+ ATPase activity of RBC membranes was estimated by the inorganic phosphorous-liberated (Pi) according to the method of Fiske and Subbarao (1921). 700 μl of Ammonium molybdate reagent was added followed by the addition of 40 μl of ANSA reagent, incubated at 37 °C for 60 min. The blue color 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 μmol Pi/mg membrane protein/h.

Restoration of $Ca^{2+}Mg^{2+}$ ATPase Activity by Antioxidants

$Ca^{2+}Mg^{2+}$ ATPase activity of RBC membranes was determined following Ames (Ames 1966; Moore et al. 1989, Kaul and Krishnakanth 1994). Erythrocyte membranes (200 μgs of membrane protein) were preincubated with or without various concentrations of SP (0–100 μgs), BHA (0–100 μgs) -tocopherol (0–100 μgs), and *O*-phenanthroline (10 mM) in 0.5 ml of TBS, pH 7.4 at 37 °C for 20 min. Then, Fe:As (2:20 μmol) was added, final volume was made up to 1 ml with TBS, pH 7.4 and incubated at 37 °C for 60 min. The supernatant was discarded, and the 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 and kept in ice water for 10 min. $Ca^{2+}Mg^{2+}$ ATPase activity of RBC membranes was estimated by the inorganic phosphorous-liberated (Pi) according to the method of Fiske and Subbarao (1921). 700 μls of Ammonium molybdate reagent was added followed by addition of 40 μl of ANSA reagent, incubated at 37 °C for 60 min. The blue color 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 μmol Pi/mg membrane protein/h.

Protection of Fe:As-Induced Alterations in RBC Membrane Protein Profile by Antioxidants

Ghost membrane (500 μg of membrane protein) was preincubated with or without SP (1.6 μmol) and BHA (400 μM) for 20 min at 37 °C, followed by the addition of ferrous sulfate:ascorbate (2:20 μmol) in a total volume of 0.5 ml of saline and incubated for 60 min at 37 °C. The above reaction mixture was taken in sample buffer containing SDS (5 %), glycerol (20 %), mercaptoethanol (2 mM), and bromophenol blue (0.06 %) and loaded on the gel at 100 volts. SDS polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970). Gels were stained using coomassie brilliant blue R-250 and destained, and bands were visualized.

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 affects the structure and function of membranes. High levels of ROS have been implicated in several oxidative damage-related diseases (Aviram 2000; Kris-Etherton et al. 2004; Riccioni et al. 2008). The limiting factor that may prevent the extent of damage could be the quantum of exogenously derived antioxidants (Clarke et al. 2008; Riccioni et al. 2008). Recently, we isolated and characterized an antioxidant protein from Sundakai (*S. torvum*) seeds termed as Sundakai seed protein (SP) (Marappan and Leela 2007). The present study reports the protective effect of SP against oxidative membrane damage in RBC cells.

In our study, RBC's are used to test the extent of lipid peroxidation, as liability of erythrocyte membrane to lipid peroxidation in vitro reflects the susceptibility of other cell membranes to oxidative damage in vivo, relating to oxidative stress (Sies 1997; Fraga et al. 1990). Moreover, in vivo erythrocytes are highly exposed to oxygen and are site for radical formation under pathological conditions (Tesoriere et al. 1999). 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 (Van den berg et al. 1992; Celedon et al. 2007). Hence it was important to study the protective role of Sundakai seed protein (SP) to prevent oxidative hemolysis. When SP was tested for its efficiency to prevent the oxidative RBC lysis induced by pro-oxidants, there was a dose-dependent prevention by SP of membrane lysis upon Fe:As (2:20 μmol), periodate (0.4 mM) and *t*-BOOH (1 mM) effect (Fig. 1). At 25 μgs, SP exhibits maximum protection to the extent of 86 % against Fe:As-induced oxidative hemolysis: on the other hand, α-tocopherol and BHA at 400 μM exhibited protection by 92 and 85 %,

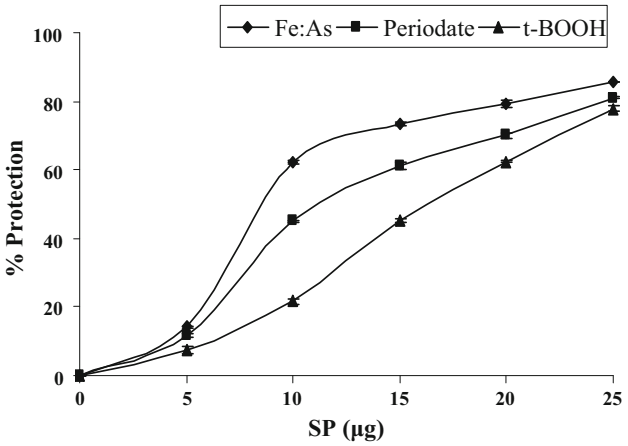

Fig. 1 Dose-dependent protective effect of Sp on pro-oxidant-induced oxidative hemolysis. 0.1 ml of RBC suspension (1×10^6 cells) preincubated with SP (0–25 μgs) at 37 °C for 20 min. Then, Fe:As (2:20 μmol) or periodate (0.4 mM) or *t*-BOOH (1 mM) added in 1 ml saline, incubated at 37 °C for 180 min. Oxidative hemolysis induced by pro-oxidants in the absence of antioxidants was expressed as 100 % lysis. Values are mean ± SD ($n = 6$)

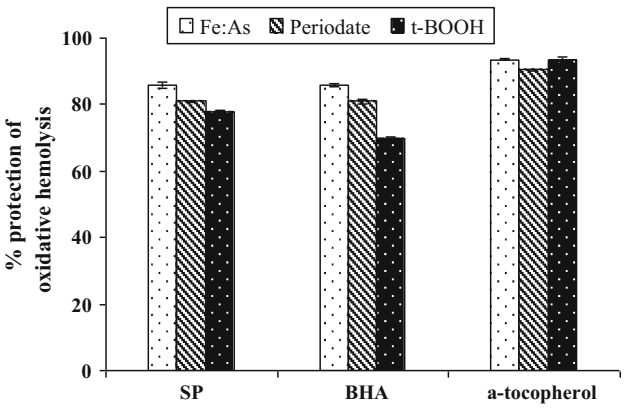

Fig. 2 Protective effect of Sp on pro-oxidant-induced oxidative hemolysis: comparison with standard antioxidants. 0.1 ml of RBC suspension (1×10^6 cells) preincubated with SP (25 μgs) or BHA (400 μM) or α-tocopherol (400 μM) at 37 °C for 20 min. Then, Fe:As (2:20 μmol) or periodate (0.4 mM) or *t*-BOOH (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. Values are mean ± SD ($n = 6$)

respectively (Fig. 2). Upon periodate and *t*-BOOH-induced oxidative hemolysis, SP at 25 µgs showed 81 and 86 %. On the other hand, BHA and α-tocopherol showed 83 and 94 % against periodate-induced hemolysis and about 78 and 90 % against *t*-BOOH-induced hemolysis (Fig. 2). The results indicate that SP 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 are shown to inhibit hemolysis under oxidative stress (Allegra et al. 2002; Tesoriere et al. 1999; Zhu et al. 2002). The results suggest that oxidative membrane damage could be efficiently protected by antioxidants.

The K^+ ion leakage is one of the consequences 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 interruption of cellular membrane proteins and cause leakage of intracellular contents (Halliwell and Gutteridge 1989; Rohn et al. 1998). In this study, when erythrocyte suspension was treated with Fe:As (2:20 µmol) 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 SP, BHA, and α-tocopherol dose dependently prevented Fe:As-induced K^+ leakage to (Fig. 3). SP at 80 µg inhibited K^+ leakage in erythrocyte membrane up to 97 %, whereas BHA and α-tocopherol at 120 µg inhibited up to 81 and 89 %, respectively (Table 1). SP was more potent than standard antioxidants in inhibiting K^+ leakage and inhibition is comparable to inhibition (up to 90 %) offered by quinine sulfate, a known K^+ channel blocker In addition *O*-phenanthroline (lipophilic iron chelator) offered 92 % inhibition against Fe:As-induced

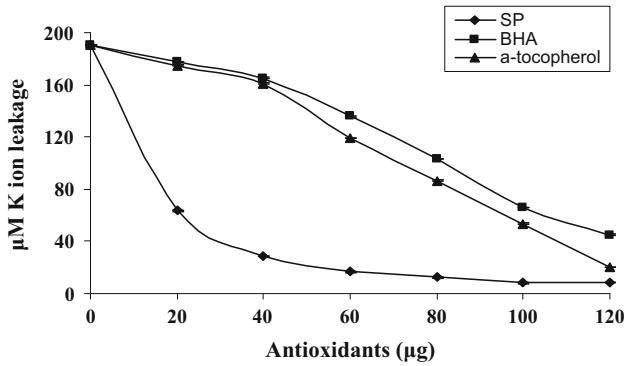

Fig. 3 Dose-dependent inhibition of Fe:As-induced K^+ leakage by antioxidants. 100 µls of RBC suspension (1×10^6 cells) was preincubated with or without antioxidants at indicated concentrations for 20 min at 37 °C. Fe:As (2:20 µmol) was added and final volume was made up to 1 ml with saline. The amount 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. Values are mean ± SD ($n = 6$)

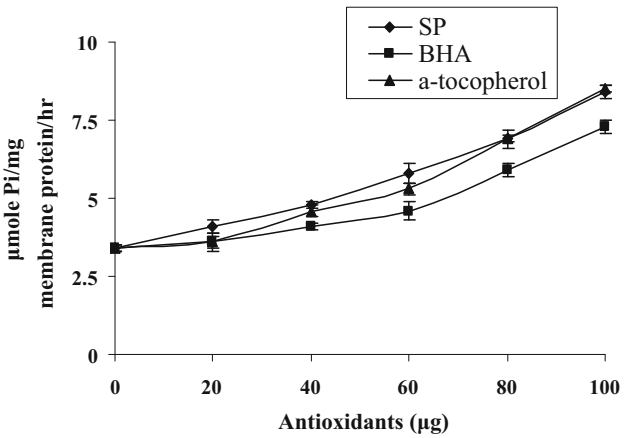

Fig. 4 Restoration of Na^+K^+ ATPase activity by SP and standard antioxidants. Erythrocyte membrane (200 µgs) preincubated with Sp (0–100 µgs) or BHA (0–100 µgs) or a-tocopherol (0–100 µgs) in 0.5 ml TBS, pH 7.4 and incubated at 37 °C for 30 min. Fe:As (2:20 µmol) was added, final volume was made up to 1 ml with TBS, Incubated at 37 °C for 60 min. Following incubation, Na^+K^+ pump ATPase of membrane (pellet) was determined and enzyme activity is expressed as µmol Pi/mg protein/h. Data represent the mean ± SD ($n = 6$)

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

Antioxidants/inhibitors	Function	Concentration	% inhibition of K^+ leakage in erythrocytes (1×10^6 cells)
No antioxidants/inhibitors			0
Quinine sulfate	K^+ channel Blocker	1 µM	90 ± 1.2
O-phenanthroline	Iron chelator	10 mM	92 ± 0.5
SP	Antioxidant	100 µgs	90 ± 1.0
BHA	Antioxidant	120 µgs	81 ± 0.9
α-tocopherol	$^1O_2$ quencher	120 µgs	89 ± 1.1

100 µl of RBC suspension (1×106 cells) was preincubated with antioxidants/inhibitors for 20 min at 37 °C. Ferrous sulfate:ascorbate (2:20 µmol) 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 percentage inhibition calculated accordingly. Values are mean± S.D ($n = 6$)

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 (Morel et al. 1990). The comparison studies with $OH^·$ radical quenchers (BHA and α-tocopherol) and lipophilic iron chelator (*O*-phenanthroline) indicate that SP 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 to cell membrane. It has been reported that there is a permanent inhibition of ATPase activity by oxygen-free radicals via lipid peroxidation (Hexum and Fried 1979). When the effect of antioxidants including SP was tested on Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ upon induction by Fe:As (2:20 μmol), it was observed that all the antioxidants tested SP, BHA, and α-tocopherol dose dependently restored Na^+K^+ ATPase (Fig. 4) and $Ca^{2+}Mg^{2+}$ ATPase activities (Fig. 5) of erythrocyte membrane. The activities of isolated RBC membranes (untreated membranes) were 8.5 ± 1.0 and 11.2 ± 1.2 μmol/mg membrane protein/h for Na^+K^+ ATPase and $Ca^{2+}Mg^{2+}$ ATPase activities, respectively. Treatment of RBC membranes with Ferrous sulfate:ascorbate (2:20 μmol) resulted in significant inhibition of ATPase activities, and 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 the presence of SP (100 μgs), BHA (100 μgs) and α-tocopherol (100 μgs) were found to be 7.6 ± 0.9, 6.6 ± 0.55 and 7.4 ± 0.35 for Na^+K^+ ATPase (Table 2) and 8.5 ± 1.0, 7.3 ± 0.6 and 8.4 ± 0.55 for $Ca^{2+}Mg^{2+}$ ATPase activities, respectively (Table 2). In comparison, the decrease in ion pump ATPase activity was significantly prevented by the pre-treatment of *O*-phenanthroline (10 mM), which showed 8.4 ± 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 reduced. Overall ATPase activities in the presence of antioxidants were comparable to activities of untreated RBC membrane. Based on the results obtained, it can be concluded that SP is an effective antioxidant in restoration of ATPases activities upon oxidative damage to membrane.

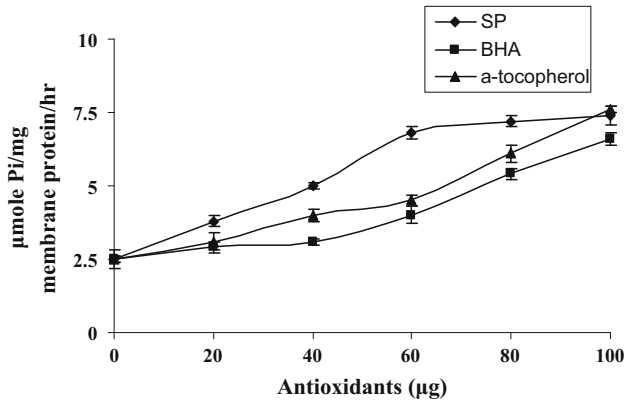

Fig. 5 Restoration of $Ca^{2+}Mg^{2+}$ ATPase activity by SP and standard antioxidants. Erythrocyte membrane (200 μgs) preincubated with SP (0–100 μgs) or BHA (0–100 μgs) or α-tocopherol (0–100 μgs) in 0.5 ml TBS, pH 7.4 and incubated at 37 °C for 30 min. Fe:As (2:20 μmol) was added, final volume was made up to 1 ml with TBS, incubated at 37 °C for 60 min. Following incubation, $Ca^{2+}Mg^{2+}$ pump ATPase of membrane (pellet) was determined and enzyme activity is expressed as μmol Pi/mg protein/h. Data represent the mean ± SD ($n = 6$)

Table 2 Restoration of ATPase activities by SP; comparison with standard antioxidants

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 μmol)		2.5 ± 0.008[a]	3.4 ± 0.007[a]
RBC membrane + Fe:As (2:10 μmol) + SP	100 μgs	7.6 ± 0.89[c]	8.5 ± 0.9[c]
RBC membrane + Fe:As (2:10 μmol) + BHA	100 μgs	6.6 ± 0.5[b]	7.3 ± 0.55[b]
RBC membrane + Fe:As (2:10 μmol) + α-tocopherol	100 μgs	7.4 ± 0.3[c]	8.4 ± 0.5[c]
RBC membrane + Fe:As (2:20 μmol) + *O*-phenanthroline	10 mM	8.4 ± 1.0[c]	10.3 ± 0.99[c]

Erythrocyte membrane (200 μgs) preincubated with 0–100 μg of Sp or BHA or α-tocopherol or *O*-phenanthroline in 0.5 ml TBS, pH 7.4 and incubated at 37 °C for 20 min. Fe:As (2:20 μmol) was added, final volume was made up to 1 ml with TBS, incubated at 37 °C for 60 min. Following incubation, Na^+K^+ pump ATPase and $Ca2^+Mg2^+$ pump ATPase of membrane (pellet) were determined, and enzyme activity is expressed as μmol Pi/mg protein/h. Data represent the mean ± SD ($n = 6$)

* The enzyme activity is expressed as μmol Pi/mg protein/h

[a] Statistically significant ($P < 0.001$) compared to untreated membrane

[b] Statistically significant ($P < 0.05$) compared to Fe:As-treated membrane

[c] Statistically significant ($P < 0.01$) compared to Fe:As-treated membrane

Studies have shown that end products of lipid peroxidation, MDA is capable of cross-linking membrane components containing amino groups (Tappel 1973; Halliwell and Gutteridge 1985). It has been reported that one potential mechanism for iron-mediated damage of Ca^{2+} pump ATPase is through cross-linking by MDA (Pacifici and Davies 1990). The incubation of RBC membrane (200 µgs) 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/h. The treatment of SP (0–25 µgs), BHA (0–25 µgs), and α-tocopherol (0–25 µgs) was found to decrease the formation of TBARS in a dose-dependent manner for protection of ion pump ATPases (Fig. 6). The above results indicate that ferrous sulfate:ascorbate (Fenton reactant) 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 SP, BHA, and α-tocopherol significantly ameliorated the oxidative damage of RBC membrane by reducing MDA levels and restoring ATPases activities toward normalcy. The mechanism by which SP does is probably by directly scavenging radicals or chelating the ferrous ions (Fenton reaction) (Marappan and Leela 2007).

Lipid peroxidation of RBC membranes alters membrane fluidity and impairs deformability of RBC cells, which results in shape and deformability of RBC. In order to assess the effect of Fe:As (2:20 µmol) induced membrane protein damage and its protection by antioxidants SDS-PAGE was carried out. The treatment of Fe:As with membrane showed disappearance of membrane proteins by SDS-PAGE. In contrast, the pretreatment of membrane with SP (1.6 µmol) and BHA (400 µM) restored the membrane protein profile upon Fe:As induction (Fig. 7). The results indicate that SP at a lower concentration effectively protected the membranes proteins when compared to standard antioxidant BHA.

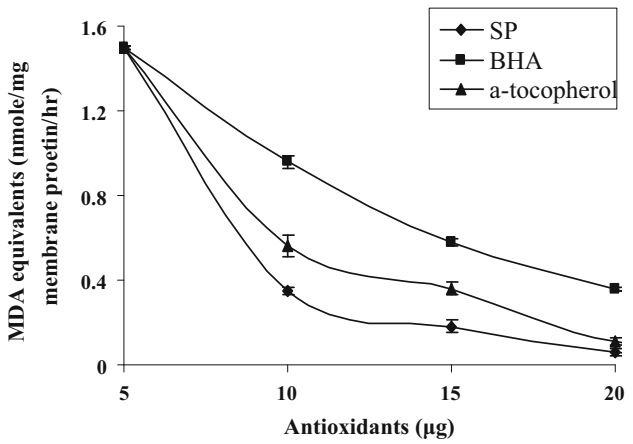

Fig. 6 Inhibition of lipid peroxidation by SP and other standard antioxidants. RBC ghost (200 µgs) preincubated with antioxidants, treated with Fe:As (10:100 µmol) in 1 ml of TBS, pH 7.4. TBARs determined and calculated as equivalents of MDA using molecular extinction coefficient of $1.56 \times 10^{+5}$ M^{-1} cm^{-1} and expressed as nmole of MDA equivalents/mg membrane protein/h. Data are mean ± SD ($n = 6$)

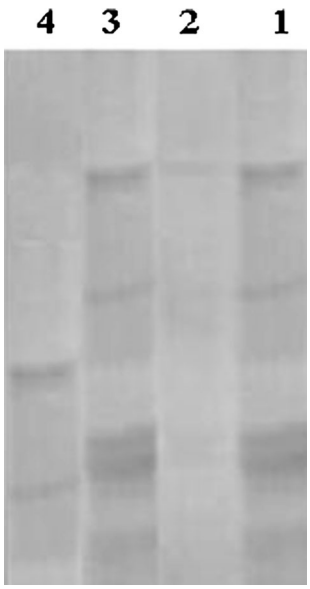

Fig. 7 SDS-PAGE of membrane proteins upon antioxidants exposure against pro-oxidants induced membrane damage. Erythrocyte ghosts (500 µg of membrane protein) pretreated with SP (1.6 µmol) or BHA(400 µM) at 37 °C for 20 min. Then treated with Fe:As (2:20 µmol) in 500 µls of TBS, pH 7.4 at 37 °C for 60 min. Reaction mixture taken in sample buffer and SDS-PAGE (10 %) was run at 100 V. The proteins were visualized by staining with coomassie blue R-250. *Lane 1* untreated ghosts, *Lane 2* ghosts + Fe:As (2:20 µmol), *Lane 3* ghosts + Fe:As (2:20 µmol) + BHA (400 µM), *Lane 4* ghosts + Fe:As (2:20 µmol) + SP (1.6 µmol)

In conclusion, the Sundakai seed antioxidant protein (SP) is potent in preventing oxidative membrane damage and associated activities mediated by ROS. As it is a non-toxic protein (Marappan and Leela 2007), it can be used as an effective bioprotective antioxidant agent to cellular components.

Acknowledgments The authors acknowledge the Adichunchanagiri Shikshana Trust and The principal, Adichunchanagiri Institute of Medical Sciences (AIMS), B.G.Nagar for providing facilities to carry out this work.

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