International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 5, 2016

Original Article

AQUEOUS BARK EXTRACT OF *TERMINALIA ARJUNA* PROTECTS AGAINST PHENYLHYDRAZINE INDUCED OXIDATIVE DAMAGE IN GOAT RED BLOOD CELL MEMBRANE BOUND AND METABOLIC ENZYMES

SUDESHNA PAUL[1], DEBOSREE GHOSH[1,4], ARNAB KUMAR GHOSH[1], DEBAJIT BHOWMICK[3], DEBASISH BANDYOPADHYAY[2], AINDRILA CHATTOPADHYAY[1*]

[1]Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700006, India, [2]Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700009, India, [3]Centre for Research in Nano Science and Nanotechnology, Acharya Prafulla Chandra Sikhsha Prangan, University of Calcutta, JD-2, Sector-III, Salt Lake City, Kolkata 700098, India, [4]Department of Physiology [UG and PG], Hooghly Mohsin College, Chinsurah, Hooghly, West Bengal, India
Email: aindrila63@gmail.com

Received: 12 Dec 2015 Revised and Accepted: 15 Mar 2016

ABSTRACT

Objective: The objective of the present study is to determine the phenylhydrazine (PHZ) induced oxidative stress mediated alteration in the metabolic status and morphology of the red blood cells (RBC) and amelioration of the same by aqueous bark extract of *Terminalia arjuna* (TA).

Methods: Fresh goat blood collected from local Kolkata Corporation approved slaughter house, was used for the present study. Packed cells were prepared from the freshly collected goat blood and were divided into four groups as follows for further studies i. e Group I: Control (CON), Group II: TA bark extract treated, named T5C (5 mg/ml, incubation mixture; positive control), Group III: PHZ treated (1 mM), Group IV: PHZ treated+TA bark extract at a dose of 5 mg/ml, named as P+T5. ROS, superoxide anion radical, and hydroxyl radical scavenging activity were determined. Intracellular iron and intracellular nitrate concentration were estimated. Activities of various membrane-bound enzymes like Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase and Ach-E were determined. Moreover, the activities of some metabolic enzymes like glucose 6-phosphate dehydrogenase (G6PDH), hexokinase, aldolase, lactase dehydrogenase were also studied. In addition, the morphological structure of RBCs was also determined.

Results: PHZ treatment caused significant alterations in RBC morphology as well as altered the activities of membrane-bound as well as metabolic enzymes. All these changes following oxidative stress were found to be ameliorated when the RBCs were co-treated with PHZ and aqueous bark extract of TA. However, aqueous bark extract of TA alone did not exhibit any such changes in RBC.

Conclusion: The aqueous bark extract of TA ameliorates PHZ-induced oxidative damages in goat RBC possibly by an antioxidant mechanism(s). The aqueous bark extract of TA may have future therapeutic relevance in oxidative stress-induced damages in RBCs.

Keywords: Antioxidant enzymes, Aqueous bark extract, Oxidative stress, Phenylhydrazine, Red blood cells, *Terminalia arjuna*

INTRODUCTION

Red blood cells (RBCs) are one of the most susceptible biological tissues to oxidative stress due to the presence of high concentration of polyunsaturated fatty acids (PUFA) in the membrane and the oxygen transport associated with redox active hemoglobin molecules, which are promoters of ROS. Due to their susceptibility to oxidation, RBC are often used as cellular models to investigate the oxidative damage.

Phenylhydrazine (PHZ), one of the most investigated intracellular free radical generating probes promotes oxidative damage in erythrocytes. It can penetrate to the 0_2-binding site of the haemoglobin molecule and react with it. PHZ oxidation results in the formation of superoxide anion free radicals and hydrogen peroxide [1-4]. These ROS and intermediates of PHZ form complexes with haemoglobin [5-7] which can denature the hemoglobin molecule [8]. Iron is probably released from the denatured hemoglobin, which may promote the conversion of superoxide anion free radicals and hydrogen peroxide into the very reactive hydroxyl radical through the Haber-Weiss reaction [9].

Since there are no mitochondria in erythrocytes, these cells depend on less efficient pathways for production of high-energy compounds, the anaerobic glycolytic (Embden-Meyerhof) pathway, which is also known as the hexose monophosphate shunt or the phospho-gluconate pathway. Under normal circumstances, about 90% of glucose entering the red cell is metabolized by the anaerobic pathway and 10% by the aerobic pathway [10]. It has been reported that G6PDH and other metabolic enzymes like hexokinase and aldolase activity is reduced, and LDH activity is increased following oxidative stress [10].

Membrane-bound enzymes are important in maintaining the normal physiology of erythrocytes [11, 12]. Free-radicals induced degenerative changes in erythrocytes which disturbed the structural integrity of the membrane that in turn affected the activities of membrane-bound enzymes like Na^+/K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase [11] and AchE [12] activity. The level of nitric oxide (NO), another oxidative stress biomarker also increased during conditions of oxidative stress. NO is responsible for the conversion of oxyhemoglobin to methemoglobin (MHb) [13]. Increased NO status also indicates the increased nitric oxide synthase (NOS) activity in the cell.

Terminalia arjuna (TA) belonging to the family Combretaceae, is an important medicinal plant widely used in the preparation of Ayurvedic formulations for over three centuries in India. It is primarily used in the treatment of coronary artery diseases, heart failure and hypercholesterolemia [14-16]. It has also been found to have antibacterial and antimutagenic activities [17-19]. It has been earlier reported from this laboratory that aqueous bark extract of TA provides protection to RBCs against oxidative stress *in vitro* through antioxidant mechanisms [20].

The aim of our present study was to elucidate whether this aqueous bark extract of TA is capable of protecting the membrane-bound enzymes and the enzymes of the metabolic pathway of anaerobic

oxidation in goat RBCs from phenylhydrazine-induced oxidative stress *in vitro* and whether antioxidant mechanisms are associated with such protection.

MATERIALS AND METHODS

Chemicals used

Powder of bark of *Terminalia arjuna* (TA) was purchased from Herby House, Kolkata India. All the other chemicals used including the solvents were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

Preparation of aqueous extract from TA bark powder

Five gm of TA bark powder was dissolved in 25 ml of double distilled water. After proper mixing it was kept with cotton plugging for overnight (approximately 16 h*). Then, it was centrifuged twice at 1300g for 10 min. The supernatant, thus obtained, was collected, frozen and lyophilized. The yield of the aqueous extract of TA from 5 gm of TA bark powder was 10%. The lyophilized material herein was termed as aqueous bark extract. Different concentrations of the extract dissolved in double distilled water were used for the present study, and any leftover extract was always discarded.

Processing of goat blood to obtain the whole RBCs

Goat blood was collected from local Kolkata Corporation approved slaughterhouse in Acid Citrate Dextrose buffer. Packed RBCs were obtained by centrifugation at 3000rpm for 10 min at 4 °C. The plasma and the buffy coat were removed by aspiration and the whole RBCs, thus obtained, were washed thrice with 0.9% NaCl solution.

In vitro incubation of whole RBCs with the PHZ and aqueous bark extract of TA

The following experiments were carried out with whole RBCs divided into four groups.

Group I: Control (CON)

Group II: TA treated, named T5C (5 mg/ml, incubation mixture; positive control)

Groups III: PHZ treated (PHZ)

Group IV: PHZ treated+TA at a dose of 5 mg/ml, named as P+T5

Five hundred µl of whole RBCs with 1 mM phenylhydrazine (PHZ), a concentration of 5 mg/ml of aqueous bark extract of TA and 50 mM sodium phosphate buffer (pH 7.4) in a final volume of 1.0 ml were incubated at 37 °C in a shaking water bath for 1 hour. The incubation was terminated by addition of 100µl of 16 mM EDTA and the treated red blood cells were washed thrice with 0.9% NaCl solution prior to lysis and preparation of membrane there from.

Preparation of hemolysate from incubated whole RBCs for the assay of antioxidant enzymes

For the assay of antioxidant enzymes, after lysis of the washed erythrocytes in deionized water, the suspension was centrifuged at 7000 rpm. for 25 min at 4 °C. The supernatants, thus obtained, were then stored at–20 °C to be used later for the assay.

Preparation of erythrocyte membrane

Haemoglobin-free erythrocyte membrane (either control or treated) was prepared according to a method of Arduini *et al.* [34]. The washed erythrocytes were subjected to hypotonic lysis in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0) and centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant, thus obtained, was discarded and the pellet was washed at least five times in the same buffer until a colorless pellet was obtained. The erythrocyte ghosts were suspended in the same buffer and stored at–20 °C for future use.

Determination of ROS scavenging activity of aqueous TA bark extract in a chemically defined system

The RBCs were washed with phosphate-buffered saline (PBS) to remove traces of the original medium. Washed RBCs were incubated with 2′,7′–dichlorofluorescein diacetate (DCFDA) at a final concentration of 1µM and was kept 30 min in the dark, in a conventional incubator (37 °C, 5% CO2). The DCFDA containing medium was removed, and the RBCs were washed twice with PBS. The level of ROS was assessed immediately by flow cytometry [21].

Hydroethidine (HE) was used as a superoxide indicator. HE is oxidized selectively by superoxide to ethidium, whose fluorescence intensity within the cell is proportional to the total production of superoxide anion free radicals [22]. The oxidation of HE is not accomplished by hydroxyl radical, singlet O_2, H_2O_2 or nitrogen radicals.

The hydroxyl (·OH) radical was generated in sodium phosphate buffer (0.05 mM, pH 7.4) with 1 mM PHZ for 60 min in the presence and absence of dimethyl sulfoxide (DMSO) (500µM) and different concentrations of aqueous bark extract of TA in a volume of 1 ml to determine the ·OH scavenging activity of the aqueous bark extract of TA in an *in vitro* system. The reaction was terminated in each case by the addition of 16 mM EDTA. Methane sulfonic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner [23] as modified by Bandyopadhyay *et al.* [24]

Estimation of iron content of RBCs by Atomic absorption spectrophotometry (AAS)

The iron content of the RBCs was determined by atomic absorption spectrophotometry as per the protocol mentioned in the cookbook of the Sophisticated Analytical Instrument Facilities' (SAIF) and "Thermo Scientific mCE 3000 Series Atomic Absorption Spectrometer" available at the Chemical Engineering Department of University College of Science and Technology, University of Calcutta and at the Bose Institute, Kolkata. After *in vitro* incubation of whole RBCs, the cells were centrifuged at 3000 rpm for 10 min. The supernatant, thus obtained, was collected in a conical flask. Concentrated nitric acid was then carefully added to it and the conical flask with its contents was placed on the hot plate and heated at 65–70 °C for digestion of the RBCs and heated until white fumes come out. The contents of the conical flasks were then carefully and quantitatively transferred into 25 ml volumetric flasks, and, finally, the volume made up to 25 ml with double distilled water. The iron content of the samples was then measured using an atomic absorption spectrophotometer [25].

Measurement of the activities of glucose-6-phosphate dehydrogenase (G6PDH), hexokinase, aldolase and lactate dehydrogenase (LDH) of RBCs

Glucose-6-phosphate dehydrogenase activity was determined in hemolysate by measuring the increase in absorbance spectrophotometrically at 340 nm. The reaction mixture containing 0.006 M NADP and 0.1 M glucose-6-phosphate in 2.7 ml of 0.055 M Tris HCl buffer, pH7.8 with 0.0033M $MgCl_2$were individually incubated for 7-8 min to reach temperature equilibrium and then hemolysate was added and the change in absorbance recorded at 340 nm for 5 min [26]. The enzyme activity was expressed as units/minute/mg of protein.

The activity of hexokinase was determined from RBC hemolysate by using 0.67 M glucose as the substrate, 0.05 M Tris $Mgcl_2$ buffer (pH 8), 16.5 mM ATP, 6.8 mM NAD and glucose 6 phosphate dehydrogenase (300 IU/ml of Tris $MgCl_2$ buffer). The change in absorbance was observed spectrophotometrically at 340 nm [27]. The enzyme activity was expressed as units/minute/mg of protein.

The activity of aldolase was measured in hemolysate based on the fact that 3-phosphoglyceraldehyde reacts with hydrazine to form a hydrazone which absorbs at 420 nm. The reagents used were 0.012 M fructose-bis-phosphate, 0.1 mM EDTA containing 0.0035 M hydrazine sulphate, pH 7.5 and hemolysate as the source of the enzyme. The change in absorbance was recorded spectrophotometrically at 240 nm for 10 min [28]. The enzyme activity was expressed as units/minute/mg of protein.

The activity of lactate dehydrogenase was determined spectrophotometrically by measuring the oxidation of NADH (0.1 mM) to NAD+at 340 nm using 1.0 mM sodium pyruvate as a

substrate, according to the method Dhanesha *et al.* [29]. The enzyme activity was expressed as units/minute/mg of protein.

Determination of the activities of sodium-potassium ATPase (Na^+/K^+ATPase), magnesium-ATPase (Mg^{2+}ATPase) and calcium ATPase (Ca^{2+}ATPase) in RBCs

The activities of Na^+/K^+ATPase, Mg^{2+}ATPase, and Ca^{2+}ATPase, were determined simultaneously from the membrane fraction of RBCs. Na^+/K^+Mg^{2+}ATPase activities were determined from the hemolysate in the presence of 180 µM EGTA; total ATPase activity was measured in the presence of calcium, and only Mg^{2+}ATPase activity was determined in the presence of 180 µM EGTA and 0.5 mM ouabain. Mg^{2+}ATPase activity was deducted from the combined activity of Na^+/K^+, Mg^{2+}ATPase activity to obtain only Na^+/K^+ATPase activity. ATPase activities were expressed as µg of inorganic phosphate (Pi)/mg of the protein [30].

Determination of the activities of acetylcholine esterase (AChE) and measurement of nitric oxide concentration in RBCs

The activity of AChE of RBCs of the different experimental groups was determined from the suspended membrane fractions of hemolyzed RBCs according to the method developed by Ellman *et al.*, [31]. Acetylcholine iodide was used as the substrate which when acted on by AChE breaks down to thiocholine and acetate. Thiocholine is allowed to react with dithiobisnitrobenzoate (DTNB) which results in the development of a yellow colour. The changes in the intensity of yellow colour over a period were estimated using a UV/VIS spectrophotometer, which represents the activity of AChE. One tenth of acetylcholine iodide and 0.3 mM DTNB solution was prepared in phosphate buffer of pH 7.4 for the assay of the enzyme. The concentration of nitric oxide (NO) was measured using Griess reagent. (1% sulfanilamide–0.1% N-1-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) (Sigma, St. Louis, MO, USA). Hemolysates were mixed with an equal amount of Griess reagent [32]. The generation of NO was determined by measuring the absorbance at 540 nm in a spectrophotometer of the purple azo compound formed from the reaction between nitrates formed in samples and Griess reagent i. e, sulfanilamide and N-1napthylethylenediamine dihydrochloride (NED). Potassium nitrate (Sigma) dilutions, ranging from 0 to 35 mM, were used to create a standard curve. Data are presented as the concentration of nitrite (mM) per mg protein amount (mg).

Determination of the activity of methaemoglobin reductase (MHbR), measurement of methaemoglobin concentration and Heinz body in RBCs

The activity of MHbR was determined from hemolysate by the method adopted by Board *et al.*, [33]. Potassium ferricyanide is reduced to potassium ferrocyanide by MHbR and the change in absorbance corresponding to the formation of $NAD^+$is measured spectrophotometrically at 340 nm. Tris HCl EDTA buffer (pH 8.0) was used for the assay.

Heinz body content was determined in RBCs directly from the turbidimetric measurement as adapted by Bates and Winterbourn, (1984) [34]. Whole RBCs were incubated with 5 mM sodium phosphate buffer, pH 7.4 for 15 min at room temperature, in dark and absorbance was recorded spectrophotometrically (Biorad) at 700 nm.

Morphological studies of erythrocytes by using Atomic force microscopy (AFM)

Control and the treated whole RBCs were fixed using 3 % glutaraldehyde for 30 min and rinsed with phosphate buffered saline. The samples were dehydrated with series of ethanol and sample was drawn on a glass coverslip to prepare a film. The film was then air dried and was ready for AFM analysis.

AFM imaging and measurement

AFM study was performed with model Veeco di Innova(Company name Bruker) Multimode system. All the images were taken using tapping mode in air (ambient temperature) with RTESPA tip silicon probes at resonant frequency 276-318 kHz. 100 µm L scanner was used. Scan area size is 5 µm X 5 µm. The images obtained were analyzed by Nanoscope Software version 1.40 in offline . The following parameters were measured:

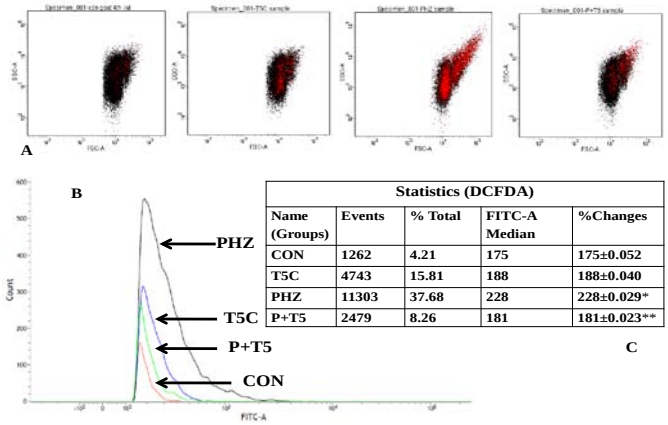
The form and the size of RBCs were measured; these measurements included 3D image, diameter, radius, concave depth and roughness of the erythrocytes.

Statistical analysis

Data are presented as means±SEM Significance of mean values of different parameters between the treatment groups were analyzed using one-way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Flow cytometry analysis of RBCs exposed to PHZ (1 mM) for 1hour exhibited a significant increase by 30.29%;(*P≤ 0.001 vs. control) in the intracellular ROS level compared to the control (Fig. 1A, B and C). Such intracellular ROS levels were prevented from being increased by 20.61%; (**P≤ 0.001vs. PHZ-treated group) significantly on co-treatment with aqueous bark extract of TA (5 mg/ml) and PHZ (1 mM) (Fig. 1). TA itself was not involved in ROS generation, as was observed from DCF fluorescence intensity when the RBCs were treated only with aqueous bark extract of TA.

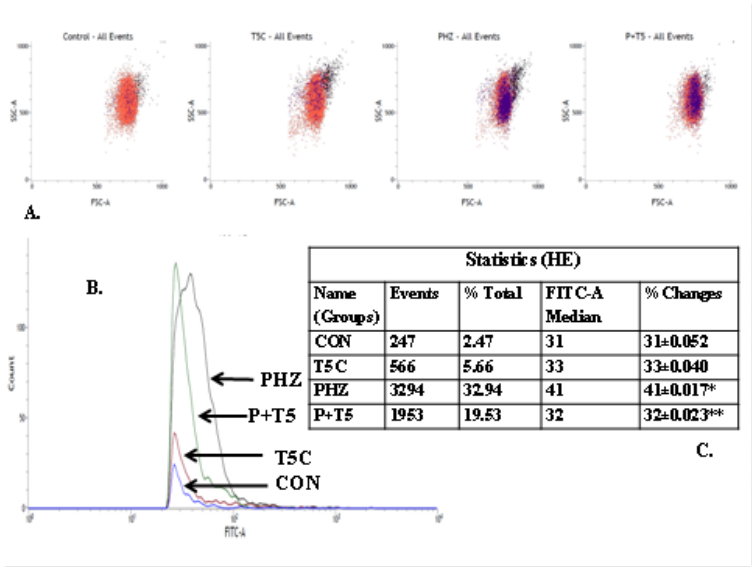


Statistics (DCFDA)				
Name (Groups)	Events	% Total	FITC-A Median	%Changes
CON	1262	4.21	175	175±0.052
T5C	4743	15.81	188	188±0.040
PHZ	11303	37.68	228	228±0.029*
P+T5	2479	8.26	181	181±0.023**

Fig. 1: A, B and C Protective effect of aqueous bark extract of *Terminalia arjuna* on the ROS scavenging activity *in vitro* against phenylhydrazine-induced oxidative stress in goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA.

HE is selectively oxidized to ethidium by superoxide, anion free radical. The intensity of ethidium fluorescence reflects the level of $O_2^{\cdot-}$-produced in the cell since $O_2^{\cdot-}$-cannot easily transverse the cell membrane. As shown in fig. 2A, B and C, the fluorescence intensity of the PHZ (1 mM) treated RBCs were increased by 32.26% (*P≤ 0.001 vs. control), while in RBCs co-incubated with PHZ (1 mM) and aqueous bark extract of TA (5 mg/ml), the fluorescence intensity was prevented from being increased by 21.95 % (*≤P0.001vs . PHZ-treated group). However, TA aqueous bark extract alone was not involved in superoxide radical generation, as was observed from ethidium fluorescence intensity in the RBCs.



Statistics (HE)				
Name (Groups)	Events	% Total	FITC-A Median	% Changes
CON	247	2.47	31	31±0.052
T5C	566	5.66	33	33±0.040
PHZ	3294	32.94	41	41±0.017*
P+T5	1953	19.53	32	32±0.023**

Fig. 2: A, B and C Protective effect of aqueous bark extract of *Terminalia arjuna* on the superoxide radical scavenging activity *in vitro* against phenylhydrazine-induced oxidative stress in goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P ≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

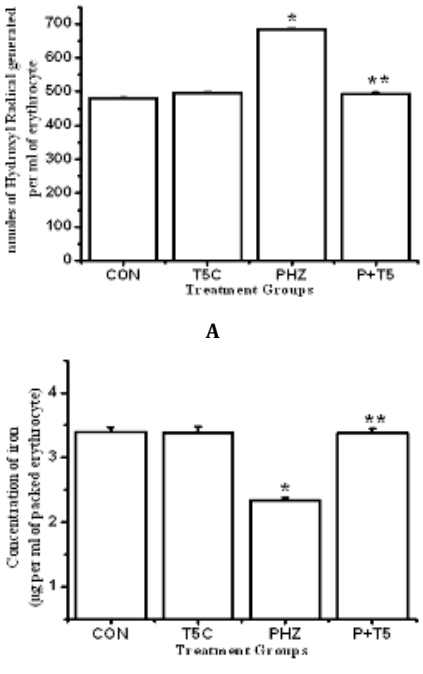


Fig. 3: Protective effect of aqueous bark extract of *Terminalia arjuna* on: A. Hydroxyl radical scavenging activity *in vitro* and B. intracellular iron concentration, against phenylhydrazine-induced oxidative stress in goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

The hydroxyl radical scavenging ability of aqueous TA bark extract was studied in an *in vitro* standard model system using PHZ where ·OH was generated. fig. 3A indicated that PHZ (1 mM) produced about 684 nmoles ·OH per ml incubation mixture in 1hr and aqueous bark extract of TA directly scavenged OH· by about 71%≤ (**P 0.001vs. PHZ-treated group) at a concentration of 5 mg/ml (fig. 3A).

Fig. 3B demonstrates decreased the intracellular iron concentration of RBCs treated with 1 mM PHZ (30.97%≤0.001 vs. control). However, when the RBCs were co-incubated with aqueous bark extract of TA (5 mg/ml) and PHZ (1 mM), the intracellular iron content of RBCs was found to be protected from being decreased by1.21 folds; (**P≤0.001 vs. PHZ-treated group). Aqueous TA bark extract alone had no significant effect on the concentration of iron in the RBCs.

Fig. 4A reveals that on the treatment of the RBCs with PHZ, the activities of G6PDH, hexokinase and aldolase decreased respectively by 40.78 %, 63.79% and 63.31% (*P≤ 0.001 vs. control) and that of lactate dehydrogenase (LDH) increased 1.68 folds (*P 0.001 vs. control). When the RBCs were co-treated with PHZ (1 mM) and aqueous bark extract of TA at a dose of 5 mg/ml, the activities of G6PDH, hexokinase and aldolase were found to be significantly protected from being decreased by 57.38 %, 1.75 folds, and 1.70 folds respectively (**P≤0.001 vs. PHZ-treated group) and in case of LDH the enzyme activity was significantly protected from being increased by 62.30% (**P ≤0.001 vs. PHZ-treated group).

Fig. 4B reveals that treatment of RBCs with PHZ(1 mM) decreased the activities of Na^+/K^+ATPase, Mg^{2+}ATPase, Ca^{2+}ATPase by 42.83%, 26.15% and 32.64% respectively (*P 0.001 vs. control). Co - treatment of RBCs with PHZ(1 mM) and aqueous bark extract of TA (5 mg/ml) were found to protect the activities of these three ATPases from being decreased by 69.55%, 34.19%, and 47.06% respectively (**P≤ 0.001 vs . PHZ-treated group). However, TA aqueous bark extract alone did not significantly alter the activity of any of the enzymes studied.

Treatment of RBCs with PHZ (1 mM) for 1 hour decreased the AchE enzyme activity by 1.55 folds and increased the NO concentration by 54.87% (*P≤ 0.001 vs. control). The enzyme activity of the AChE and

intracellular NO concentration were found to be protected significantly from being altered, when the RBCs were co-treated with PHZ and 5 mg/ml of the aqueous bark extract of TA (50.29% increase in acetylcholine esterase enzyme activity and 1.21 folds decrease in intracellular NO concentration, ≤*P0.001 vs. PHZ - treated group) (fig. 5A and B).

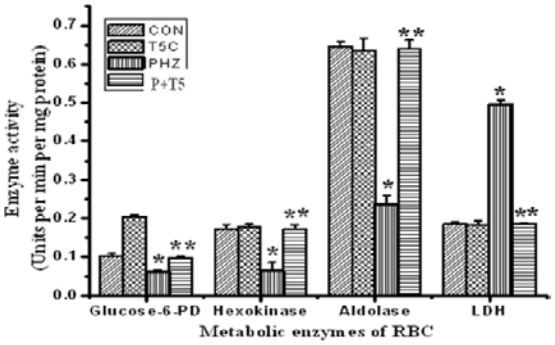


Fig. 4A: Protective effect of aqueous bark extract of *Terminalia arjuna* against PHZ induced changes in activities of glucose-6-phosphate dehydrogenase (G6PDH), hexokinase, aldolase and lactate dehydrogenase (LDH) of goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

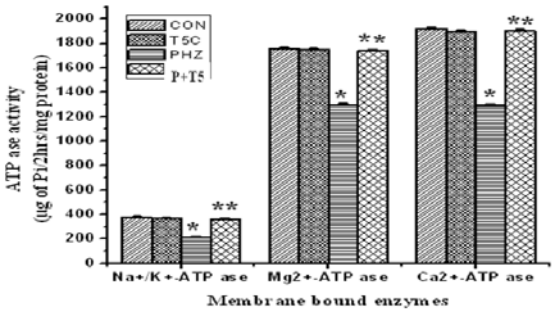
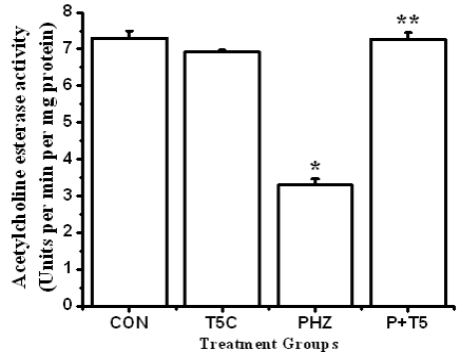


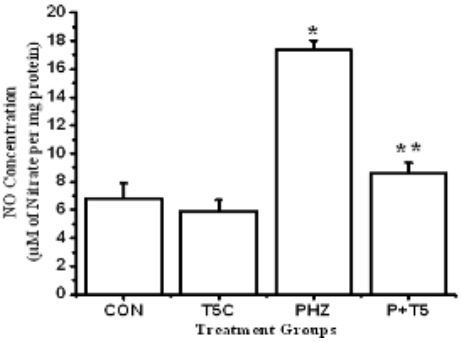
Fig. 4B: Protective effect of aqueous bark extract of *Terminalia arjuna* against PHZ induced changes in activities of sodium-potassium ATPase (Na^+/K^+ATPase), magnesium-ATPase (Mg^{2+}ATPase) and calcium ATPase (Ca^{2+}ATPase) of goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

Fig. 6 A, B and C illustrates an increase in methaemoglobin concentration and turbidity index by 31.18%, 67.21% and a significant decrease in methaemoglobin reductase activity of RBCs by 58.33% respectively,* P≤ 0.001 vs. control) following exposure of RBCs to PHZ (1 mM). However, co-treatment of RBCs with PHZ (1 mM) and the present dose of aqueous bark extract of TA significantly decreased the methaemoglobin concentration as well as turbidity index and protected the methaemoglobin reductase activity (23.19 %, 36.27% decrease, and 1.42 folds increase respectively, **P≤ 0.001 vs. PHZ) from being altered. The results indicate that the aqueous bark extract of TA alone has no influence on the methaemoglobin concentration, turbidity index, and methaemoglobin reductase enzyme activity.



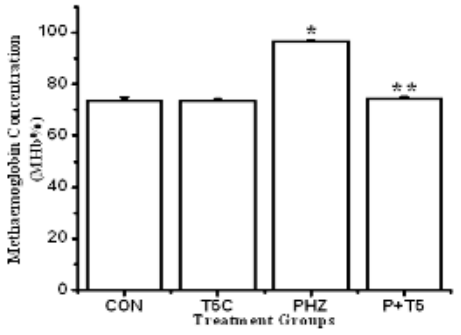
A



B

Fig. 5: Protective effect of aqueous bark extract of *Terminalia arjuna* against PHZ induced alterations on A. activity of acetylcholine esterase (AChE) and B. nitric oxide concentration in goat RBCs

CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA



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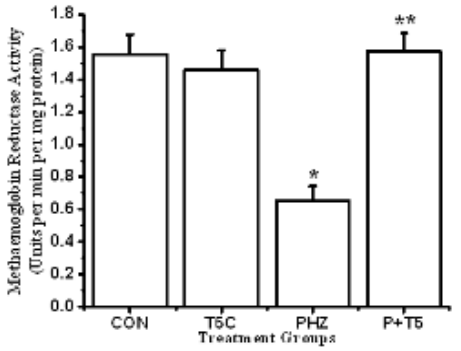
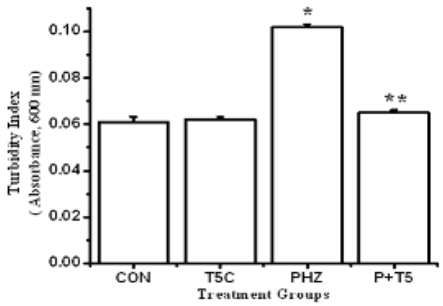


B



C

Fig. 6: Protective effect of aqueous bark extract of *Terminalia arjuna* on A. methaemoglobin concentration, B. methaemoglobin reductase activity, and C. turbidity index, against phenylhydrazine induced oxidative stress in goat RBCs CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

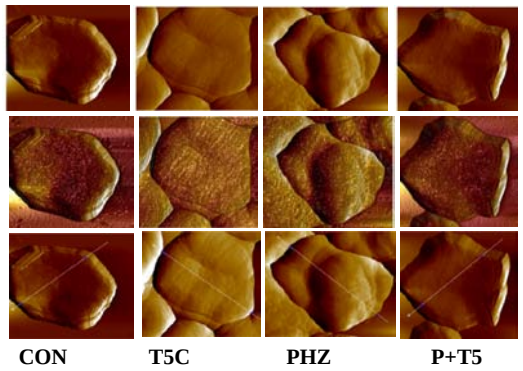
Measurement of morphological parameters of RBCs by AFM indicated that PHZ treatment altered the shape of RBCs due to an increase in the diameter and radius (by 36.76% and 36.06% respectively, *P≤ 0.001 vs. control) and a reduced concave depth and roughness (by 36.28% and 46.80% respectively, *P≤0.001 vs. control) (Fig.7A and B).

However, co-treatment with PHZ (1 mM) and aqueous bark extract of TA (5 mg/ml) protected the RBCs from these alterations in diameter, radius, depth, and roughness.

DISCUSSION

Phenylhydrazine (PHZ) in the presence of hemoglobin autoxidizes to form both superoxide anion free radical and hydrogen peroxide which ultimately give rise to hydroxyl radical. In our present study, there was a significant increase in the level of ROS production in the RBCs following treatment with PHZ (fig. 1A, B and C). It was previously reported that PHZ intoxication leads to hemolysis resulting in severe hemolytic anemia and generates ROS [37]. Our present studies, however, demonstrated that co-treatment of goat RBCs with PHZ and aqueous bark extract of TA at present does prevented the enhancement of ROS production in these cells (fig. 1A, B and C).

A



B

Treatment Groups	Diameter (µm)	Radius (µm)	Concave depth(nm)	Roughness (nm)
Control	3.40±0.057	1.70±0.058	576±0.087	1579±0.057
T5C	4.01±0.046	2.00±0.046	430±0.029	1269±0.028
PHZ	4.65±0.040*	2.33±0.035*	367±0.058*	840±0.046*
P+T5	3.08±0.035**	1.54±0.029**	441±0.046**	937±0.035**

Fig. 7: A and B protective effect of aqueous bark extract of *Terminalia arjuna* against phenylhydrazine-induced oxidative stress on morphological changes of goat RBCs using atomic force microscopy. 1st panel (2D image) 2nd panel (3D image) 3rd-panel measurement of diameter, radius, concave depth and roughness (scan area size is 5 µm X 5 µm) of the RBC CON = Control; T5C = *Terminalia arjuna* bark extract (5 mg/ml); PHZ=Phenylhydrazine; P+T5= Phenylhydrazine+*Terminalia arjuna* bark extract (5 mg/ml); the values are expressed as mean±SE, *P≤0.001 compared to control group; **P≤ 0.001 compared to PHZ treated group using ANOVA

An elevated level of ·OH is indicative of an elevated level of oxidative stress following treatment of goat RBCs with PHZ (fig. 3A). Phenylhydrazine in the presence of haemoglobin oxidizes to form hydrogen peroxide that generates ·OH through Haber–Weiss Reaction [38]. The latter initiates the peroxidation of unsaturated fatty acids in endogenous phospholipids [39]. Besides PHZ, other oxidants like iron with hydrogen peroxide and hydrogen peroxide alone, participates in oxidative reactions through the generation of highly reactive. OH this readily damages lipids, proteins and other components of the cell membrane. Our studies reveal (fig. 3A) that co-treatment of goat RBCs with PHZ and aqueous bark extract of TA reduces the amount of· OH formation in the RBCs, demonstrating the ability of the extract to reduce the level of oxidative stress. Additionally, it has already been reported from our laboratory that the aqueous bark extract of TA exhibited a strong hydroxyl radical scavenging potential [40]. However, the extract did not exhibit any H_2O_2 scavenging activity and metal ion chelating effect *in vitro* (40).

Damaging effects of PHZ like other oxidants have been reported by various workers [41]. It has also been demonstrated that normal RBCs treated with PHZ has a mimetic characteristic as those found in severe β-thalassemia [42]. Phenylhydrazine-induced peroxidation of oxyhemoglobin and MHb leads to the formation of both superoxide (O^-) and phenyl radicals [43]. These radicals can denature haemoglobin with the consequential release of iron from denatured haemoglobin which can induce lipid peroxidation of the cell membrane if the cell is depleted of GSH. This eventually causes RBC haemolysis [43]. Increased MHb concentration increased

superoxide anion free radicals and decreased the intracellular iron concentration of RBCs and increased turbidity index following treatment with PHZ indicated elevated levels of oxidative stress (fig. 6A, fig. 2A, B and C and fig. 6C). The GSH concentrations were found to be depleted following PHZ treatment of RBCs in our earlier observation [20]. In the present study, co-treatment of RBCs with PHZ and aqueous bark extract of TA inhibited MHb formation, prevented superoxide anion free radical generation and protected the hemoglobin structure thereby reducing the turbidity index which prevented the hemolysis of RBCs.

The present study further reveals that MR activity is decreased following treatment of RBCs with PHZ. This results in accumulation of MHb in the RBCs (fig. 6A and B respectively.) Co-treatment of RBCs with PHZ and aqueous bark extract of TA prevents the rise in MHb level.

The RBCs depend solely on the anaerobic conversion of glucose by the Embden-Meyerhof pathway for the generation and storage of high energy phosphates, which is necessary for the maintenance of a number of vital functions. Fig. 4A shows that the activities of the metabolic enzymes G6PDH, hexokinase, aldolase and LDH are altered by the treatment of RBCs with PHZ. However, co-treatment of the RBCs with aqueous bark extract of TA and PHZ, protected the activities of these enzymes from being altered consequently protecting the metabolic status of the RBCs.

Erythrocytes are highly susceptible to oxidative damage due to the presence of heme iron. Polyunsaturated fatty acids (PUFA) and oxygen may initiate the reactions that induce oxidative changes in the red blood cells [44, 45]**.** Peroxidation of PUFA in membrane lipids has been suspected to be a major mechanism of oxidant injury leading to membrane dysfunction and subsequently to alterations in cellular functions [45,46]. Lipid peroxidation and perturbed lipid composition are known to disturb structural integrity of the membrane that might, in turn, affect the activity of membrane-bound enzymes like ATPases [47]. Membrane-bound enzymes are important in maintaining the normal physiology of erythrocytes. The decrease in the activities of the membrane ATPases in this study may be due to the elevation in free radical formation and decrease in the GSH content. Free radicals can induce degenerative changes in erythrocytes that can affect dynamic properties of the membranes such as fluidity and permeability and consequently the activities of the membrane-bound enzymes. It has been reported earlier that oxidative products inhibit Na^+/K^+-ATPase and Mg^{2+}-ATPase activities [48]. Lipid peroxidation decreases the affinity of Na^+/K^+-ATPase for $Na^+$and K^+ions because the active sites of these enzymes are directly attacked by ROS. It may be suggested that inhibition of erythrocyte membrane Na^+/K^+-ATPase in PHZ treated goat RBCs lead to disturbance or impairment of Na^+/K^+-pump in membrane due to alterations in the composition of the erythrocyte membranes, which in turn results in decreased activities of other membrane-bound enzymes. Na^+/K^+-pump functions for the maintenance of the ionic milieu of normal blood cells and essential for the normal functioning of the cells. Co-treatment of the RBCs with aqueous bark extract of TA and PHZ restored the activities of ATPases to some extent. This might be due to the potential role of this aqueous bark extract of TA to scavenge the free radicals produced by PHZ-induced toxicity.

The present study demonstrated that *in vitro* administration of PHZ resulted in inhibition of AChE activity in erythrocytes and co-administration of aqueous bark extract of TA and PHZ helps in partial protection of AChE activity. In blood, normal erythrocyte function depends on the intactness of the erythrocyte membrane, which is the target for PHZ toxicity [43]. Inhibition of AChE activity by PHZ is well documented in the literature [49]. In addition to inhibition of AChE activity, PHZ has been shown to induce lipid peroxidation and oxidative stress in various tissues [50]. The cells have several ways to alleviate the effects of oxidative stress either by repairing the oxidative damage or by directly diminishing the occurrence of oxidative damage by means of enzymatic and non-enzymatic antioxidants which have been shown to scavenge free radicals and ROS [51]. Numerous reports have documented protective actions of aqueous bark extract of TA in various models of oxidative stress due to its high efficacy as a free radical scavenger and indirect antioxidant [20, 40, 52]. It has been reported that aqueous bark extract of TA also directly neutralizes the precursor of •OH, namely hydrogen peroxide [40]. The •OH is widely accepted as the most damaging molecule endogenously produced in aerobic organisms, and many studies have confirmed the TA's ability to detoxify •OH [53, 54]. The •OH mutilates any molecule in the vicinity of where it is produced [55]. Exogenous administration of aqueous TA bark extract neutralized the formation of ROS, reducing thereby, the molecular and physiological damage. In addition, increased intracellular level of NO in PHZ treated RBCs were observed. Increased concentration of NO has been reported to cause accumulation of nitrite, an end product of nitric oxide metabolism, which reacts with superoxide radicals ultimately leading to nitrosative stress [56]. Co-treatment of RBCs with PHZ and aqueous bark extract of TA prevented this increase in NO concentration thereby indicating once again TA's ability to provide protection against oxidative insult. The aqueous bark extract of TA also reduces the generation of NO by inhibiting the activity of its rate limiting enzyme, nitric oxide synthase (NOS) [57].

Atomic force microscopy (AFM) is an advanced tool to analyze the structure of the erythrocyte membrane and its membrane proteins [58]. In our previous studies, it was observed that PHZ altered the RBC membrane structure by causing an alteration in the membrane protein content [20]. In our current study, the diameter, radius, concave depth and roughness of the RBCs were measured with the help of AFM analysis. With PHZ treatment, the structure of RBCs was altered; concave depth and roughness were also decreased (fig. 7A and B). On the contrary, simultaneous treatment of RBCs with aqueous bark extract of TA and PHZ protected the RBCs from these morphological alterations (fig. 7A and B).

CONCLUSION

Thus, it can be concluded from the above findings that PHZ at the present dose triggered oxidative stress-induced morphologic and metabolic alterations and functional impairment in the goat red blood cells. The aqueous bark extract of TA is capable of providing protection against these PHZ induced oxidative stress mediated alterations in the RBCs possibly through its antioxidant mechanisms. The results of the present study hints at the possibility that the aqueous bark extract of TA with the promising antioxidant potential to combat oxidative stress-induced damages may have future therapeutic relevance in situations of hemolytic anemia and other diseases involving oxidative stress in general.

ACKNOWLEDGEMENT

Sudeshna Paul gratefully acknowledges the receipt of an UGC Non-NET JRF under the University of Calcutta. DG was a Senior Research Fellow (SRF) under INSPIRE program of Department of Science and Technology, Government of India, presently works as an Assistant Professor at Department of Physiology, Hooghly Mohsin College, Chinsurah, Hooghly. AKG is a CSIR RA. DB is supported by the funds available to him by his institute. Prof. DB also extends grateful thanks to UGC, Govt. of India, for the award of a research project under Centre with Potential for Excellence in a Particular Area (CPEPA) Scheme of UGC, Govt. of India, at University of Calcutta. Dr. AC is supported by funds available to her from UGC Minor Research Project, Govt. of India and Department of Science and Technology, Govt. of India (DST-WOS-A). We are grateful to Centre for Research in Nanoscience and Nanotechnology (CRNN), University of Calcutta and Department of Biotechnology-Interdisciplinary Program in Life Sciences (DBT-IPLS), the University of Calcutta for allowing us to use some of the facilities.

CONFLICT OF INTERESTS

Declared none

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