Aqueous bark extract of *Terminalia arjuna* protects against phenylhydrazine induced oxidative damage in goat red blood cell membrane protein, phospholipid asymmetry and structural morphology: a flow cytometric and biochemical analysis

Sudeshna Paul1,2, Arnab Kumar Ghosh1, Debosree Ghosh1, Mousumi Dutta1,2, Elina mitra2, Monalisa Dey2, Debajit Bhowmick2, Tridib Das2, Syed Benazir Firdaus3, Sanatan Mishra1,2, Debasis Bandyopadhyay4, Aindrila Chattopadhyay4,*

1Department of Physiology, Vidyasagar College, Kolkata 700 006, India.
2Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009, India.
3Centre for Research in Nano Science and Nano technology, Acharya Prafulla Chandra Sikhsa Prangan, University of Calcutta, JD-2, Sector-III, Salt Lake City, Kolkata 700098, India.

Received on: 27-10-2014; Revised on: 04-11-2014; Accepted on: 23-12-2014

**ABSTRACT**

**Background:** Phenylhydrazine (PHZ) is one of the most investigated intracellular free radical generating probes which promote oxidative damage in erythrocytes and mimics the β thalassemia model. *Terminalia arjuna* (TA) bark, an indigenous plant used in ayurvedic medicine in India, primarily as a cardiotonic is also used in treating diabetes, anemia, tumors, hypertension, cancer, renal and hepatic disorders. TA has the hypcholesterolemic, hypolipidemic, antimutagenic, anti-inflammatory, radioprotective and anti dysenteric activity. It is also used as anti coagulant and antioxidant. **Objective:** To evaluate the effect of aqueous bark extract of *Terminalia arjuna* (TA), against phenylhydrazine induced oxidative damage, *in vitro*, in goat red blood cell (RBC) membrane protein, phospholipid asymmetry and structural morphology. **Methods:** Oxidative stress biomarkers and activities of antioxidant enzymes were determined by biochemical analysis. RBC membrane protein damage was evaluated using SDS-PAGE followed by densitometric analysis. Phospholipid asymmetry of the RBC membrane was determined by thin layer chromatography followed by phosphate estimation. Iron concentration was estimated by using atomic absorption spectrophotometry (AAS). Morphological analysis of the RBCs were performed using scanning electron microscopy (SEM) and flow cytometry. **Results:** Aqueous bark extract of TA protected the RBCs from phenylhydrazine (PHZ) induced oxidative damages *in vitro* as evident from its effect on lipid peroxidation level, reduced glutathione and protein carbonyl content, and activities of antioxidant enzymes – superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). Membrane protein damage, phospholipid traslocation, and morphological alterations of RBCs induced by PHZ were also found to be protected by aqueous bark extract of TA. **Conclusion:** The present study indicated that the aqueous bark extract of TA, protects against PHZ induced oxidative stress mediated damages in goat RBCs in vitro, possibly through its antioxidant activity and its bioactive constituents may contribute toward such protection.

**KEYWORDS:** Antioxidant, bark extract, oxidative stress, phenylhydrazine (PHZ), red blood cells (RBCs), *Terminalia arjuna*.

**INTRODUCTION**

Red blood cells (RBCs) are one of the most susceptible biological tissues to oxidative stress due to the presence of both high concentration of polyunsaturated fatty acids (PUFA) in the membrane and the oxygen transport associated with redox active hemoglobin molecules, which are promoters of reactive oxygen species (ROS). Lipid peroxidation involves the cleavage of unsaturated fatty acids at their double bonds producing short-chained aldehydes. The occurrence of lipid peroxidation causes alteration in RBC membrane structure, function and alteration of membrane bound receptors and enzymes [1].

Phenylhydrazine (PHZ) in the presence of hemoglobin autooxidizes to form both O₂⁻ and H₂O₂ which ultimately give rise to hydroxyl radical [2] each with the capacity to initiate the peroxidation of unsaturated fatty acids in endogenous phospholipids [3]. Treatment of normal RBCs with PHZ, produces features that are characteristic of RBC phenotypes in severe β-thalassemia (4), namely, rigid and mechanically unstable membranes in conjunction with selective association of oxidized α-globin chains with the membrane skeleton. As such, PHZ was suggested to serve as an *in-vitro* model for beta-thalassemic RBC phenotypes [4]. During β thalassemia this type of condition is created due to the iron overload and hampers the antioxidant system of the RBC membrane. Trace metals, especially iron are implicated as causative agents in excessive generation of free radical which are capable of causing oxidative damage to erythrocytes [5].

*Terminalia arjuna* (Combretaceae family) a deciduous and evergreen
Sudeshna Paul et al. / Journal of Pharmacy Research 2014,8(12),1790-1804

**tree** [6], standing 20-30m in height is found in abundance throughout Indo-sub-Himalayan tracts of Uttar Pradesh, South Bihar, Madhya Pradesh, Delhi and Deccan region near ponds and rivers [7]. Among different species of Terminalia, the bark of TA has its own characteristic features [8]. The active constituents of TA include tannins, triterpenoid, saponins (arjunolic acid, arjunic acid, arjungenium, arjunglycosides), flavonoids (arjuncoumarin, luteolin), oxalic acid, ellagic acid, gallic acid, oligomeric proanthocyanidins(OPCs), phytosterols, polyphenols, calcium, magnesium, zinc and copper [9]. TA is also an important medicinal plant widely used in the preparation of Ayurvedic formulations for over three centuries primarily as a cardia tonic in India [10]. Clinical evaluation of this plant indicates that it can be of benefit in the treatment of coronary artery diseases, heart failure and possibly hypercholesterolemia [9,11,12,13]. It has also been found to be antibacterial and antimitogenic [14, 15]. But, most of the beneficial works on this plant have been carried out on the alcoholic extract of its bark. However, its aqueous bark extract also showed novel protection mechanism in several *in vitro* systems like RBC, liver tissue, heart mitochondria as discussed in our previous publications[16,17]and its antioxidant mechanisms were also established [16,17]. The significance of using this aqueous extract of TA bark for our studies lies in the fact that, it is the form in which it is consumed directly by the tribals and rural people of India as medicines for their various health problems.

Hence, our present study was aimed at exploring whether this aqueous bark extract of TA, is capable of protecting the cytoskeletal architecture of the RBC membranes from phenylhydrazine induced oxidative stress in goat RBCs 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 lyophilized bark extract of TA**

Five gm of TA bark powder was dissolved in 25ml of double distilled water. After proper mixing it was kept with cotton plugging for overnight (approximately 16 hours). Then, it was centrifuged twice at 1300g for 10 minutes. The supernatant, thus obtained, was collected and lyophilized. The yield of the aqueous extract of TA from 5gm 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 discarded.

**Processing of goat blood to obtain whole RBCs**

Goat blood was collected from local Kolkata Corporation approved slaughter house in Acid Citrate Dextrose buffer. Packed RBCs were obtained by centrifugation at 3000rpm for 10 minutes 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**

Five hundred µl of whole RBCs with 1mM phenylhydrazine (PHZ) 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 16mM EDTA and the PHZ treated red blood cells were washed thrice with 0.9% NaCl solution prior to lysis and preparation of membrane there from.

**Determination of the effective dose of aqueous TA bark extract against PHZ induced oxidative stress in whole RBCs** *in vitro*

The whole RBCs were incubated in following groups as shown below:

- **Group I:** Control (CON)
- **Group II:** PHZ treated (PHZ)
- **Group III:** PHZ treated + TA at a dose of 1 mg/ml (P+T1)
- **Group IV:** PHZ treated + TA at a dose of 2 mg/ml (P+T2)
- **Group V:** PHZ treated + TA at a dose of 5 mg/ml (P+T5)

After the incubation, the biomarkers of oxidative stress like lipid peroxidation level, reduced glutathione content and protein carbonyl content were determined in whole RBCs. The activity of the antioxidant enzymes, viz., superoxide dismutase (SOD) and catalase (CAT) were also determined.

From this dose dependent study, the best effective dose of aqueous TA bark extract was determined. After determination of the best effective dose of aqueous TA bark extract, subsequent experiments were carried out with the best effective dose. Then the experiments were carried out with whole RBCs divided in to four groups. These were the followings:

- **Group I:** Control (CON)
- **Group II:** TA treated, named T5C (5mg/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

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

In another set of preparations, after lysis of the washed erythrocytes in deionized water, the suspension was centrifuged at 7000 rpm. for 25
The PCO content of RBCs were estimated by DNPH assay. Protein Carbonyl (PCO) content was determined at 532nm. The malondialdehyde (MDA) concentration of cells and heated for 20 minutes at 80ºC. The absorbance of the sample was determined spectrophotometrically at 340 nm using a 5,5'-dithio-bis-(2-nitro benzoic acid) (DTNB) according to the method of Sedlak and Lindsay. [20].

**Preparation of erythrocyte membrane**

Haemoglobin-free erythrocyte membrane (either normal or treated) was prepared according to method of Arduini et al. [18]. 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 ºC. The supernatant, thus obtained, was discarded and a 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 ºC for future use.

**Measurement of biomarkers of oxidative stress in goat red blood cells**

**Lipid Peroxidation level**

The level of lipid peroxidation in whole RBCs were measured in terms of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust [19]. Two ml of TBA-TCA-HCl reagent (15% TCA, 0.375% TBA and 0.25(N) HCl) was added to the incubation mixture and heated for 20 minutes at 80ºC. The absorbance of the sample was determined at 532nm. The malondialdehyde (MDA) concentration of the sample was calculated using an extinction coefficient of 1.56 X 10⁻⁴ M⁻¹ Cm⁻¹.

**Reduced Glutathione (GSH) content**

The GSH content of whole RBCs and in membrane of RBCs were determined spectrophotometrically at 412nm using 5,5’-dithio-bis-(2-nitro benzoic acid) (DTNB) according to the method of Sedlak and Lindsay [20].

**Protein Carbonyl (PCO) content**

The PCO content of RBCs were estimated by DNPH assay [21]. The sample was incubated with 10mM DNPH for 45 minutes in the dark. At the end of incubation, 20% TCA was added, and the mixture was centrifuged at 7000rpm for 15 minutes, after which the supernatant was discarded and the pellets were washed carefully with ethanol:ethyl acetate mixture (1:1) thrice. Then 6(M) guanidine hydrochloride and 0.5(M) potassium dihydrogen phosphate (pH 2.5) were added to the washed pellets, mixed thoroughly and centrifuged at 7000rpm for 15 minutes. The supernatant, thus obtained, was collected and the absorbance was determined at spectrophotometrically 375nm.

**Measurement of the activities of antioxidant enzymes**

The activity of Cu-Zn superoxide dismutase (SOD 1) was measured by method of pyrogallol autooxidation method Marklund et al. [22]. To extract Cu-Zn SOD, the hemoglobin present in the hemolysate was removed according to the method of Mc Cord and Fridovich. [23]. The hemolysate was pre-warmed at 37ºC and treated with ethanol-chloroform (2:1, v/v) and mixed thoroughly to obtain a thick precipitate. Deionized water was then added, mixed and again incubated at 37ºC for 15 minutes with occasional stirring. The mixture was then centrifuged and the colorless supernatant, thus obtained, was used for measurement of the SOD activity.

The activity of CAT was measured spectrophotometrically by following the rate of decomposition of H₂O₂ at 240nm by the method of Beers and Sizer [24].

**Measurement of the levels of GSH and GSSG as well as determination of GSSG:GSH ratio**

The GSH content of whole RBCs were determined spectrophotometrically at 412nm using 5,5’-dithio-bis-(2-nitro benzoic acid) (DTNB) according to the method of Sedlak and Lindsay [20].

The GSSG content of whole RBCs was measured by the method of Sedlak and Lindsay [20]. The reaction mixture contained 0.1 mM sodium phosphate buffer, EDTA, NADPH and 0.14 units per ml GR. The absorbance was measured at 340 nm using a UV-VIS spectrophotometer to determine the GSSG content. The values were expressed as nmols GSSG/mg protein. The GSSG:GSH ratio was thereafter calculated.

**Determination of the activities of glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase(GST)**

The GPx activity was measured according to the method of Paglia and Valentine [25]. The assay system contained, in a final volume of 1 ml, 0.05 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione, and 0.25 mM NADPH. The reaction was started by the addition of 0.36 mM H₂O₂. The linear decrease of absorbance at 340 nm was recorded using a UV/VIS spectrophotometer. The specific activity was expressed as nmols GSSG/mg protein.

The GR activity was measured according to the method of Krohn-Ehrich et al. [26]. The assay mixture in the final volume of 3 ml contained 50 mM phosphate buffer, 200 mM KCl, 1 mM EDTA and water. The blank was set with this mixture. Then, 0.1 mM NADPH was added together with suitable amount of sample (as the source of enzyme) into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored spectrophotometrically at 340 nm. The specific activity of the enzyme was expressed as units/min/mg tissue protein.
Phospholipid analysis by thin layer chromatography (TLC)

The GST activity of the RBCs was measured spectrophotometrically according to Habig et al. The enzymatic reaction was measured by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione. One unit of enzyme will conjugate 10.0 nmol of CDNB with reduced glutathione per minute at 25 °C. The rate where the reaction is linear was noted spectrophotometrically at 340 nm. The molar extinction of CDNB is 0.0096 µM⁻¹/cm. The enzyme activity was expressed as units/min/mg of protein.

Measurement of membrane phospholipid asymmetry

Phospholipase treatment of phenylhydrazine treated red blood cells

The other half of the PHZ treated RBCs, as described above, were processed for phospholipase A₂ (PA₂) treatment as reported by Heast et al. except 50 mM Tris buffer (pH 7.4) was used instead of glycylglycine buffer. Tris buffer (1 ml) and 10 units of pancreatic PA₂ were added to normal saline washed PHZ treated RBCs. The incubation was performed in a 2 ml microfuge tube on a shaking water bath at 37°C for 1 hr. Control experiments were carried out in the absence of PA₂. In order to terminate the phospholipase action, 20 μl of 16 mM EDTA was added to the incubation medium and the cells were washed three times with cold 0.9% NaCl solution.

Lipid extraction:

Phospholipid was extracted from the washed packed cells by the method of Rose and Oklander. One ml of 5 mM EDTA solution was added to the packed cells and mixed thoroughly. Under this condition, the lysis of the cells was completed in about 10 min. The cell lysate was chilled on ice and 3.5 ml cold isopropanol was added to it. The sample was kept on ice for 15 min and then at room temperature for 1 hr with frequent vigorous shaking. One and a sixth ml of chloroform was added to the samples and again placed on ice for 15 min followed by incubation at room temperature for 1h. The samples were centrifuged at 1500 × g for 10 min and the organic layer was carefully removed and dried under nitrogen.

Phospholipid analysis by thin layer chromatography (TLC)

The dried extracts of phospholipase A₂ (PA₂) treated cells, thus obtained, were re-dissolved in chloroform: methanol (1:1, v:v) and 0.5 mg of lipid extract was applied on TLC plates (20 × 20, silica gel 60 from E. Merck). The plates were developed with chloroform: methanol: acetic acid: water (65:43:4:4, v/v), dried over hot air and stained with iodine. The different phospholipids were identified by comparing with the standard and the spots corresponding to various phospholipids were scraped from the plates. After digestion of the phospholipids with perchloric acid on a sand bath at 180 -200 °C, the released inorganic phosphate was measured as described below.

Measurement of inorganic phosphate

The inorganic phosphate was determined by the method of Ames and Dubin. One seventh of an ml of ascorbic-molybdate mixture was added to the sample in the test tube and kept for 20 minutes at 45°C. The absorbance was then recorded spectrophotometrically at 820 nm against a blank containing water instead of sample. The ascorbic-molybdate mixture, which should be freshly prepared, contains 1 part of 10% ascorbic acid to 6 parts of 0.42% ammonium molybdate 4H₂O in 1(N) H₂SO₄.

Evaluation of oxidative damage on RBCs membrane proteins by SDS-PAGE:

Electrophoresis was carried out according to the method of Laemmlli using a polyacrylamide gel. Both treated and untreated membrane preparations containing the same amount of protein were solubilized by boiling for 5 min in SDS sample buffer containing 1% SDS, 10% (v/v) glycerol, 63 mM Tris-HCl (pH 6.5) and 1% β-mercaptoethanol. Equal amounts of membrane proteins (50 μg) were loaded on the gel. Gels were fixed and stained with 0.1% Coomassie blue R-250. Densitometric analysis of the membrane proteins was also carried out.

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 cook book of the Sophisticated Analytical Instrument Facilities’ (SAIF) and “Thermo Scientific μCE 3000 Series Atomic Absorption Spectrometer” available at the Bose Institute, Kolkata was used for estimation. After in vitro incubation of whole RBCs, the cells were centrifuged at 3000 rpm for 10 minutes. 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 were placed on the hot plate and heated at 65–70°C for digestion of the RBCs. The contents of the conical flasks were then carefully and quantitatively transferred into 25 ml volumetric flasks, and, finally the volume was made up to 25 ml with double distilled water. The iron content of the samples was then measured using an atomic absorption spectrophotometer.

Determination of osmotic fragility of RBCs:

Osmotic fragility was determined according to the method of Hunter et al.

Morphological studies of RBCs:

Scanning Electron Microscopy

Another set of treated whole RBCs were fixed using 3% glutaralde-
Microscopic imaging by BD pathway
For imaging studies, BD Pathway 855 instrument was used. The RBCs were seeded into a 96 well bio-imaging plate and incubated on the stage for 15 min to allow the cells to settle and Nipkow spinning disc based confocal images were captured by a CCD Camera in transmission mode. A 3×3 confocal stacked montage image was captured for each well [35].

Flow cytometric analysis
The data on morphological changes of RBCs were obtained through flow cytometry using a BD FACS Aria III with a 488 nm blue laser and a total of 30,000 events were recorded for each sample. The FSC data were collected on a linear scale, whereas for SSC data log scale was used. The height, width and area parameter of the voltage pulse of both FSC and SSC were recorded. On the BD FACS Aria III, a 85 µm nozzle was used and the custom sheath pressure was set at 14 psi with a minimum sample flow rate of 1. Flow cytometry data, in terms of (i) area, (ii) height and (iii) width distribution of both FSC and SSC were extracted from the raw experimental data of all events with the help of Flow Diva software (version 6.1.3). Software output also includes basic statistics (mean, median, standard deviation, coefficient of variance, etc.) of the respective distribution [35].

Estimation of protein content
Protein content of whole RBCs, packed cell suspensions and hemolysates were determined according to the method of Lowry et al. [36]. The protein content of RBC membranes was determined according to the method of Bradford [37].

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

RESULTS

Changes in biomarkers of oxidative stress:

Lipid peroxidation (LPO) level
The level of LPO in oxidative stress induced RBCs was measured in terms of thiobarbituric acid reactive substances (TBARS) in presence and absence of different concentrations of aqueous TA bark extract as shown in Figure 1A. The level of LPO was found to be significantly increased in whole RBCs incubated with PHZ (1.25 folds; *P < 0.001 vs. control). However, this elevated level of LPO products were prevented from being increased, from phenylhydrazine treated group dose-dependently (**) when they were co-treated with increasing concentrations of aqueous TA bark extract and PHZ (1 mM), indicating the ability of this aqueous extract to protect the RBCs against oxidative stress-induced changes in LPO due to PHZ. Aqueous TA bark extract exhibited maximum protection at the dose of 5 mg/ml (55.05% protection; **P<0.001 vs. PHZ). The extract alone has no significant effect on LPO.

Reduced glutathione (GSH) content
On the other hand, Figure 1B showed a significant decrease of GSH content in whole RBCs incubated with PHZ compared to control (2 folds; *P = 0.001 vs. control). However, this decrease in the GSH content were found to be significantly prevented from being decreased, in a dose dependent manner, when they were co-treated with different concentrations of aqueous TA bark extract and PHZ (1 mM). The aqueous TA bark extract exhibited a maximum protection at a concentration of 5 mg/ml (2.48 folds protection; **P = 0.001 vs. PHZ).

Protein carbonyl (PCO) content
The PCO content (Figure 1C) showed a significant increase in whole RBCs incubated with PHZ compared to control (2.84 folds; *P < 0.001 vs. control). However, this increased PCO content were prevented from being increased significantly, dose dependently, when they were co-treated with increasing concentrations of aqueous TA bark extract and PHZ (1 mM). Almost complete protection was found at a concentration of 5 mg/ml aqueous TA bark extract (**) .

Figure 1. Protective effect of aqueous bark extract of *Terminalia arjuna* on PHZ induced oxidative stress biomarkers in goat RBCs (A) LPO, (B) GSH and (C) Protein carbonyl.
Changes in the activities of antioxidant enzymes viz., superoxide dismutase (SOD) and catalase (CAT):

Figure 2A reveals a highly significant increase (1.17 folds, \( P < 0.001 \) vs. control group) in the activity of Cu-Zn-SOD following treatment of whole RBCs with PHZ. The activity of this enzyme was found to be protected from being increased in a dose dependent manner when the RBCs were co-treated with increasing concentrations of aqueous TA bark extract and PHZ (1mM). Aqueous TA bark extract, at the dose of 5mg/ml, maximally protected the Cu-Zn-SOD activity (53.92% protection; \( **P < 0.001 \) vs. PHZ treated group).

Our dose-dependent studies have revealed that aqueous TA bark extract, at a dose of 5mg/ml, exhibited maximum protection against PHZ induced changes in biomarkers of oxidative stress in RBCs. Therefore, subsequent experiments were carried out with this best effective dose of aqueous TA bark extract.

Figure 2. Protective effect of aqueous bark extract of \textit{Terminalia arjuna} against PHZ on the activities of antioxidant enzymes of goat RBCs: (A) SOD and (B) CAT.

Changes in status of GSH, GSSG, GSSG: GSH ratio:

Figure 2B demonstrates a significant decrease in CAT activity, compared to control, when the RBCs were incubated with PHZ (1mM) [30.16% decrease; \( *P < 0.001 \) vs. control]. The activity of this enzyme was found to be protected from being decreased, dose-dependently, when the RBCs were co-treated with increasing concentrations of aqueous TA bark extract and PHZ (1mM). The aqueous TA bark extract exhibited maximum protection of enzyme activity at a concentration of 5mg/ml (45.50% protection; \( **P < 0.001 \) vs. PHZ treated group).

Our dose-dependent studies have revealed that aqueous TA bark extract, at a dose of 5mg/ml, exhibited maximum protection against PHZ induced changes in biomarkers of oxidative stress in RBCs. Therefore, subsequent experiments were carried out with this best effective dose of aqueous TA bark extract.

Figure 3A, 3B and 3C shows that there occurred a significant decrease in GSH, increase in GSSG level as well as an increase in GSSG:GSH ratio following treatment of RBCs with PHZ (1mM)
Changes in activities of glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST):

Figure 4A reveals a highly significant decrease (37.50%, *P < 0.001 vs. control group) in the activity of GPx following treatment of whole RBCs with PHZ (1mM). The GPx activity was protected from being decreased when the whole RBCs were co-treated with aqueous TA bark extract (5mg/ml) and PHZ (1mM) [55% protection; **P < 0.001 vs. PHZ-treated group). However, aqueous TA bark extract alone has no significant effect on the GSH and GSSG levels of RBCs.

On the other hand Figure 4B, depicts a highly significant decrease (50%, *P < 0.001 vs. control group) in the activity of GR following treatment of whole RBCs with PHZ (1mM). The GR activity was almost completely protected from being decreased when the RBCs were co-treated with aqueous TA bark extract (5mg/ml) and PHZ (1mM). Figure 4C, illustrates a significant increase in GST activity (44.83%, *P < 0.001 vs. control group) following treatment of whole RBCs with PHZ (1mM). However, the GST activity was significantly protected from being increased when the RBCs were co-treated with aqueous TA bark extract (5mg/ml) and PHZ (1mM).

Figure 3. Protective effect of aqueous bark extract of *Terminalia arjuna* against PHZ-induced alteration in the value of (A) GSH, (B) GSSG and (C) GSSG:GSH ratio in goat RBCs.

CON=Control; T5C = *Terminalia arjuna* bark extract; PHZ=Phenyldiazine; P+T5= Phenylhydrazine + *Terminalia arjuna* bark extract; the values are expressed as Mean ± SE, *P≤0.001 compared to control group; **P≤0.001 compared to PHZ treated group using ANOVA.
Changes in the phospholipid asymmetry of RBC membranes:
In normal RBCs the phospholipid distribution across the membrane bilayer is asymmetrical, sphingomyelin and phosphatidylcholine (PC) being predominately present in the outer bilayer and entire phosphatidylserine (PS) and most of phosphatidylethanolamine (PE) are present on the inner side of the membrane. In our experiment, we found significant amount of PC and practically no PE and PS, in normal RBCs after pancreatic phospholipase A$_2$ treatment followed by TLC (Figure 5A). When PHZ exposed RBCs were treated with the same pancreatic phospholipase A$_2$, there was an increase in PS and PE with reduction of PC indicating a translocation of PS and PE from the inner to the outer surface and PC from outer to inner surface of the membrane. However, when the cells were co-treated with aqueous TA bark extract (5mg/ml) and PHZ (1mM), it showed a reduction in the translocation of PS and PE from inner to outer and PC in the opposite direction (Figure 5A).

Changes in RBC membrane proteins:
Phenylhydrazine produced a generalized decrease in the intensity of all the major cytoskeletal protein bands (lane 3 with respect to lane 1, Figure 5B) when analyzed through SDS-PAGE. A partial protection of spectrin bands and almost complete protection of other four protein bands were observed when the RBCs were co-treated with aqueous TA bark extract (5mg/ml) and PHZ (1mM) (lane 4). These observations were confirmed when the protein gels were scanned in a Versadoc Ultrascan Laser Densitometer for protein profile (Figure 5C). The scan shows a reduction of spectrin bands along with bands 3, 4.1, 4.2 and actin on PHZ treatment. Most of the bands on the gel corresponding to different membrane proteins showed varying degrees of decrease in intensity. Spectrin along with the bands 3 and actin showed a marked decrease thereby indicating a correlation between increased lipid peroxidation and degradation of cytoskeletal proteins. On the other hand, bands 4.1 and 4.2 (Figure 5B) showed a considerable decrease in their susceptibility to degradation under oxidative threat. However, when the RBCs were co-treated with aqueous TA bark extract (5mg/ml) of TA and PHZ (1mM), it gave almost complete protection to all the bands in the gel.

Figure 5A. Protective effect of aqueous bark extract of _Terminalia arjuna_ against PHZ -induced alteration on the phospholipids of RBC membrane.

CON = Control; T5C = _Terminalia arjuna_ bark extract; PHZ=Phenyldrazine; P+T5= Phenyldrazine + _Terminalia arjuna_ bark extract; the values are expressed as Mean ± SE, *P<0.001 compared to control group; **P< 0.001 compared to PHZ treated group using ANOVA.

5B. SDS-PAGE of RBC membrane protein treated with aqueous bark extract of _Terminalia arjuna_ and PHZ. Identical amount of protein were applied to each lane (50µg).

CON = Control; T5C = _Terminalia arjuna_ bark extract; PHZ=Phenyldrazine; P+T5= Phenyldrazine + _Terminalia arjuna_ bark extract; [PC= Phosphatidylcholine, PS= Phosphatidylserine , PE= Phosphatidylethanolamine]; the values are expressed as Mean ± SE, *P≤0.001 compared to control group; **P<0.001 compared to PHZ treated group using ANOVA.

Lane 1.CON = Control; lane 2.T5C = _Terminalia arjuna_ bark extract; Lane 3.PHZ=Phenyldrazine; Lane4. P+T5= Phenyldrazine + _Terminalia arjuna_ bark extract;
Changes in iron concentration of RBCs

Figure 6A demonstrates increased concentration of iron in RBCs treated with 1mM PHZ (64.23%, *p<0.001 vs. control). However, when the RBCs were co-incubated with aqueous TA bark extract (5mg/ml) and PHZ (1mM), the iron content was found to be reduced significantly (37.87% reduction; **p<0.001 vs. PHZ-treated group). Aqueous TA bark extract alone had no significant effect on the concentration of iron in the RBCs.

Changes in osmotic fragility of RBCs

Red blood cells on incubation with PHZ (1mM) for an hour showed increased membrane fragility at low concentrations of NaCl compared to the cells of control group. When RBCs were co-incubated with aqueous TA bark extract (5mg / ml) and PHZ (1mM), the cells showed decreased membrane fragility. Aqueous TA bark extract alone had no effect on the fragility of the cell membrane compared to control (Figure 6B).

Changes in morphology of RBCs:

Scanning Electron Microscopy (SEM)

The morphology of RBCs of the control, PHZ-treated as well as aqueous TA bark extract protected groups were assessed using SEM as shown in Figure 6C (1st and 2nd panel). Untreated RBCs appeared as typical discocytes while exposure to PHZ (1mM) resulted in a significant change in the cell shape with distinct echinocyte formation. The oxidative damage to cell membrane leads to alterations in cell rigidity and shape. The oxidative stress leads to echinocyte formation because of membrane damage leading to altered function.

Analysis of RBC morphology using BD Pathway:

Figure 6C (3rd panel) demonstrates the pictograph of the control, PHZ (1mM) treated and aqueous TA bark extract (5mg / ml) protected RBCs, obtained using BD Pathway at 60X, which showed altered morphology of cells treated with PHZ. The cellular morphology was found to be protected from being altered when RBCs were co-treated
with aqueous TA bark extract and PHZ at concentrations indicated above. Aqueous TA bark extract alone caused no changes in the RBC morphology compared to the controls (Figure 6C, 3rd panel).

**Flow cytometric analysis**

From the flow cytometry study (Figure 7) of the RBCs of control, PHZ (1mM) treated and aqueous TA bark extract (5mg / ml) protected groups, alterations were observed in the %rCV of forward scatter (FSC) and side scatter (SSC) parameters of the cells after incubation with PHZ. This is also evident from the contour plot of the same. The changes were found to be prevented from occurring when RBCs were co-incubated with aqueous TA bark extract and PHZ at the concentrations as mentioned above.

A significant decrease in the %rCV of the side scatter area (SSC-A) and side scatter height (SSC-H) was observed in the PHZ (1mM) treated RBCs compared to control (34.12% and 35.80% decreases respectively; p<0.001 vs control). This decrease of %rCV of SSC–A and SSC-H was found to be prevented from being taken place when the RBCs were co-treated with aqueous TA bark extract and PHZ at the concentrations as mentioned above (43.69% and 47.47% increases respectively; **p<0.001, vs PHZ). There were no significant changes in any of the SSC parameters studied in flow cytometry following incubation of RBCs with aqueous TA bark extract only. No alteration in the %rCV of the sides catter width (SSC-W) of the RBC population of all four groups was found.

On the other hand, an increase of 3.87% and 2.81% in %rCV of forward scatter area (FSC-A) and forward scatter height (FSC-H) of the PHZ treated RBCs were recorded compared to control RBCs (*p<0.001 vs control). This increase in the %rCV of FSC–A, FSC-H was found...
The antioxidant defense system of RBCs has earlier been reported to be significantly prevented from being taken place when the RBCs were co-incubated with aqueous TA bark extract (5mg/ml) and PHZ (1mM) (2.66% and 1.64 increase respectively; **p<0.001 vs PHZ). There were no significant changes in any of the FSC parameters studied in flow cytometry when the RBCs were incubated with aqueous TA bark extract only. No changes were observed in case of forward scatter width (FSC-W) of the RBC population of all four groups studied.

**DISCUSSION**

The peroxidative damage to RBCs is evident from the alterations observed in the biomarkers of oxidative stress as illustrated in Figure 1, which is probably initiated by reactive oxygen species (ROS) like, superoxide anion free radical ($\mathrm{O}_2^-$), hydrogen peroxide ($\mathrm{H}_2\mathrm{O}_2$) and hydroxyl radical (·OH). The actual damaging species is probably ·OH generated by a transition metal catalyzed ‘Fenton reaction’. Iron may be released due to the oxidation and destruction of hemoglobin on treatment with PHZ which leads to increased hemolysis of the RBCs as evident from the results presented in Figure 6A and B. The released iron in the redox active form (Figure 6A) may play a central role in the oxidation of membrane proteins and formation of methemoglobin, which may result in hemolysis of RBCs (Figure 6B).

The antioxidant defense system of RBCs has earlier been reported to be severely affected by PHZ [39]. Phenylhydrazine generates H$_2$O$_2$ in the presence of hemoglobin [39]. A decrease in CAT activity with a concomitant increase in SOD activity is indicative of a situation in which there remains a fairly high possibility of accumulation of intercellular H$_2$O$_2$. Hydrogen peroxide in presence of redox active transition metal like iron may be converted to the ·OH, the most dreaded of the ROS [40,41]. The ·OH is capable of reacting with anything in its vicinity with diffusion-controlled rate to cause oxidative damage of membrane lipids, proteins and cellular enzymes [42,43]. The decreased CAT and increased SOD activities with consequential oxidative damage due to accumulating ROS has also been demonstrated in other models of oxidative stress [44].

The GSH and GSH-related enzymes play a key role in protecting the cell against oxidative stress [45]. The GSH is a tri-peptide composed of L-cysteine, L-glutamic acid and glycine-cysteinyl moiety. The ROS are reduced by GSH in the presence of GPxs. The GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GR at the expense of NADPH [46]. The GSH redox cycle consists of GSH, GPx, GR and GST, which are the major components of the antioxidant defense system of RBCs [47]. Coordinated activities of these enzymes maintain intracellular thiol status. The GSH plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by GSTs and GPxs. Glutathione reductase reduces GSSG to biologically active GSH and NADPH serving the role of a cofactor of GR reaction. However, GPxs detoxifies peroxides using GSH as an electron donor, producing GSSG as an end product. The altered levels of GSH and GSSG as well as consequential alterations in GSGG: GSH ratio along with changes in the activities of GP Px, GR and GST of RBCs following treatment with PHZ (1mM) were clearly indicative of total damage of the most important antioxidant defence system of RBCs.

Co-incubation of RBCs with PHZ and aqueous TA bark extract at the indicated dose protected the RBCs from being damaged due to oxidative stress as evident from our studies of biomarkers of oxidative stress, protein carbonylation, status of GSH, GSSG and GSSG: GSH ratio and the activities of antioxidant enzymes. The phospholipids are a suitable target in the course of oxidative process due to the presence of esterified polyunsaturated fatty acids in their molecular structure [48]. The phospholipid distribution across the RBC membrane bilayer is asymmetrical, phosphatidylcholine(PC) being predominantly present in the outer layer and entire phosphatidylserine (PS) and most of phosphatidylethanolamine (PE) on the inner side of the membrane [49]. In our study, the free radical generated by PHZ, attack the fatty acid side chains of the membrane phospholipids and induce a significant translocation of PS and PE from the inner to the outer leaflet and PC from the outer to the inner leaflet of the red cell membrane. Co-incubation of RBCs with aqueous TA bark extract and PHZ at the indicated concentrations tend to protect the cells from such translocation of phospholipids.

Oxidative stress in the PHZ treated RBCs leads to oxidative modifica-
tion and breakdown of several membrane proteins as evident from SDS-PAGE analysis of the RBC membrane proteins (Figure 5C). Red blood cells treated with PHZ clearly shows alterations in a number of important membrane cytoskeletal proteins when compared to normal membrane proteins from control RBCs (Figure 5C). SDS-PAGE analysis of membrane proteins of RBCs co-incubated with aqueous TA bark extract and PHZ further shows a striking improvement in the intensity of RBC cytoskeletal membrane protein bands with a reduction of low molecular weight protein aggregates. The densitometric scan of SDS-PAGE clearly shows that aqueous TA bark extract has a protective role. It may be due to the fact that the iron content in RBCs being reduce significantly reduces the propagation of lipid peroxidation and accumulation of TBARS by scavenging the reactive oxygen or nitrogen intermediates thereby reducing protein degradation and, thus, stabilizing the RBC membrane proteins.

Moreover, RBCs on incubation with PHZ showed enhanced osmotic fragility at low concentrations of NaCl compared to RBCs of control group. Co-incubation with aqueous TA bark extract (5mg/ml) and PHZ (1mM) decreased the osmotic fragility of the RBCs.

Protective effect of aqueous TA bark extract against PHZ treatment on the structural integrity and morphology of RBCs as evaluated using SEM is shown in Figure 6 (1st and 2nd panel). Untreated RBCs appeared as typical discocytes while exposure to PHZ resulted in a significant change in the cell shape with distinct echinocyte formation. The morphological changes induced by PHZ were greatly prevented when the cells were co-treated with aqueous TA bark extract. Oxidative damage to cell membrane following oxidative stress leads to alterations in cell rigidity and shape with consequential loss of function [50]. Microscopic pictograph of the RBCs using BD Pathway at 60X showed altered morphology of RBCs treated with PHZ. Co-treatment with aqueous TA bark extract and PHZ showed protective effect of TA on the morphology of RBCs. Aqueous TA bark extract alone caused no changes in the RBCs compared to control (Figure 6, 3rd panel).

Forward scatter correlates with the cell size and side scatter depends on the internal granularity of the particle/cell (i.e. number of cytoplasmic granules, membrane size), and in this manner cell populations can often be distinguished based on differences in their size and density. Flow cytometry study revealed marked alterations in the morphology of RBCs following PHZ treatment.

A decrease in % rCV of side scatter area (SSC-A) and side scatter height (SSC-H) of PHZ treated RBCs compared to the respective parameters of control RBCs showed occurrence of less variations of RBCs population in the PHZ treated group. SSC parameters correspond to granularity of the cells studied and are directly proportional to the presence of granules or components inside the cell or internal cellular complexity. In our case, it may be that the RBCs treated with PHZ changed their shape and size which occurred due to formation of methemoglobin and Heinz body which is reflected from the changes in the % rCV of the SSC parameters studied. The structural changes from hemoglobin to methemoglobin may be the main reason for alteration of the granularity of the RBCs.

An increase in % rCV of SSC-A and SSC-H of the aqueous TA bark extract (5mg/ml) and PHZ (1mM) co-treated RBCs compared to that of PHZ treated group was observed which is due to occurrence of more variations of RBCs in the sample and because of the protection provided by aqueous TA bark extract to the RBCs against PHZ induced hemolysis and methemoglobin formation. No marked change in the SSC-W of the aqueous TA bark extract and PHZ co-treated RBCs were observed.

An increase in % rCV of forward scatter area (FSC-A) and forward scatter height (FSC-H) of PHZ treated RBCs compared to the respective parameters of control RBCs reveal occurrence of more variations of RBCs population in the PHZ treated group which may be due to the presence of partially damaged or hemolysed RBCs in PHZ group. Enhanced FSC-A, FSC-H in flow cytometry study denotes increase in the area and height of the specific bell shaped signal generated from the cell while passing through the laser. A decrease in the % rCV of FSC-A and FSC-H of the aqueous TA bark extract (5mg/ml) and PHZ (1mM) co-treated RBCs compared to that of PHZ treated group was observed which is due to occurrence of less variations of RBCs in the sample because of the protection provided by aqueous TA bark extract to the RBCs against PHZ induced hemolysis. Forward scatter correlates with the cell size. So, it can be said that cell size of RBCs increased in PHZ treated groups of RBC population due to the shape changes and enhanced membrane fragility and hence susceptibility to hemolysis although no marked change in the FSC-W of the aqueous TA bark extract and PHZ co-treated RBCs were observed. Study of RBCs using flow cytometry showed remarkable changes in the granularity of the cells following PHZ treatment.

CONCLUSION
The present findings suggest that the aqueous TA bark extract provides protective effects against oxidative stress, induced by PHZ in vitro, in goat RBCs as is evident from the alterations in the level of oxidative stress bio-markers, the activities of antioxidant enzymes, membrane protein degradation, phospholipid translocation and the morphological changes. The aqueous TA bark extract has been reported to contain various bioactive compounds such as phenolics, flavonoids, proanthocyanidins and many others which are known to
possession antioxidant activity. It can be assumed that these bioactive compounds may be responsible for the protective effects of the aqueous TA bark extract against PHZ induced oxidative stress in goat RBCs in vitro. Moreover, earlier studies from our laboratory reported that this aqueous TA bark extract do possess a strong antioxidant potential and it is unique in specifically scavenging ‘OH and O₂⁻’ [16]. The current studies indicate that aqueous TA bark extract may have future therapeutic relevance in situations of beta-thalassemia, in particular, and other diseases involving oxidative stress, in general.

ACKNOWLEDGEMENTS

Sudeshna Paul gratefully acknowledges the receipt of an UGC Non-NET JRF under University of Calcutta. AKG is a DST Purse Extended-SRF. DG is a Senior Research Fellow (SRF) under INSPIRE program of Department of Science and Technology, Government of India. MD is a Woman Scientist under Women Scientists Scheme-A (WOS-A), Department of Science and Technology, Govt. of India. EM was a Project Fellow under a UGC Major Research Project awarded to Prof. DB. MD was a UPE Project Fellow of UGC, under University of Calcutta. DB is supported by the funds available to him by his institute. TD is supported by the funds from CRNN, University of Calcutta. SBF is a URF of UGC, under University of Calcutta. SM is supported from the funds available to Prof. DB from Teacher’s Research Grant (BI 92) of University of Calcutta. Prof. DB also extends grateful thanks to UGC, Govt. of India, for 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 the funds available to her from UGC Minor Research Project, Govt. of India and also from the funds available to her from Department of Science and Technology, Govt. of India under DST-WOS-A Scheme. We are grateful to Centre for Research in Nanoscience and Nanotechnology (CRNN), University of Calcutta, for allowing us to use some of the facilities.

REFERENCES:

47. Ahmed RG, Incerpi S, Ahmed F, Gaber A. The developmental


Source of support: UGC, Govt. of India; and DST, Govt. of India., Conflict of interest: None
**AQUEOUS BARK EXTRACT OF TERMINALIA ARJUNA PROTECTS AGAINST PHENYLHYDRAZINE INDUCED OXIDATIVE DAMAGE IN GOAT RED BLOOD CELL MEMBRANE BOUND AND METABOLIC ENZYMES**

**SUDESHNA PAUL1, DEBOSREE GHOSH1,4, ARNAB KUMAR GHOSH1, DEBAJIT BHOWMICK3, DEBASHISH BANDYOPADHYAY2, AINDRILA CHATTOPADHYAY1**

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

Email: aindrila65@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 (CDN), Group II: TA bark extract treated, named TSC (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+TS. 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, Mg2+-ATPase and Ca2+-ATPase and Ach-E were determined. Moreover, the activities of some metabolic enzymes like glucose 6-phosphate dehydrogenase (G6PDH), hexokinase, aldolase, lactate 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*
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 hr). Then, it was centrifuged twice at 1300 g 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 lyopholized 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 3000 rpm 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 P+T5

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 hemolysate from incubated whole RBCs for the assay of antioxidant enzymes

The hemolysate were 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 6.0) 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 1 μM 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 O2, H2O2 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 Bandypadhyay 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 mCIE 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, pH 8.0 with 0.0053 M MgCl2 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 MgCl2 buffer (pH 8), 16.5 mM ATP, 6.8 mM NAD and glucose 6-phosphate dehydrogenase (300 HI/ml of Tris MgCl2 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 hydratase to form a hydratase which absorbs at 420 nm. The reagents used were 0.012 M fructose-bis-phosphate, 0.1 M EDTA containing 0.0035 M hydratase 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
substance, according to the method Dhanesh 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 dithio bisnitrobenzoate (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). Hemolyses 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-1-naphthyl ethylenediamine dinitrochloride (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\(^+\)s 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. The following parameters were measured:

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

**Statistical analysis**

Data are presented as mean±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 1 hour 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 significantly increased by 20.61%; **(P=0.001 vs. 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.

---

**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**

<table>
<thead>
<tr>
<th>Name (Groups)</th>
<th>Events</th>
<th>% Total</th>
<th>DCFDA</th>
<th>% Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>1263</td>
<td>4.21</td>
<td>158</td>
<td>1754.0±0.052</td>
</tr>
<tr>
<td>TSC</td>
<td>6743</td>
<td>15.81</td>
<td>168</td>
<td>1964.0±0.040</td>
</tr>
<tr>
<td>PHZ</td>
<td>11361</td>
<td>37.48</td>
<td>238</td>
<td>2234.0±0.029**</td>
</tr>
<tr>
<td>P+T5</td>
<td>2479</td>
<td>8.26</td>
<td>181</td>
<td>1814.0±0.025**</td>
</tr>
<tr>
<td>CON</td>
<td>1263</td>
<td>4.21</td>
<td>158</td>
<td>1754.0±0.052</td>
</tr>
</tbody>
</table>

**Table:** Statistics (DCFDA)
HE is selectively oxidized to ethidium by superoxide, anion free radical. The intensity of ethidium fluorescence reflects the level of O$_2^-$ produced in the cell since O$_2^-$ 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 % (**P≤0.001 vs. 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.

**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=Phenyldihydrazine; 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 $\cdot$OH was generated. Fig. 3A indicated that PHZ (1 mM) produced about 684 nmoles $\cdot$OH per ml incubation mixture in 1hr and aqueous bark extract of TA directly scavenged $\cdot$OH by about 71% (**P≤ 0.001 vs. PHZ-treated group) at a concentration of 5 mg/ml (fig. 3A).

**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=Phenyldihydrazine; 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. 3B demonstrates decreased the intracellular iron concentration of RBCs treated with 1 mM PHZ (30.97%±0.01 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 by 1.21 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, Mg2+ATPase, Ca2+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, *P≤0.001 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 (Mg2+ATPase) and calcium ATPase (Ca2+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.
Additionally, it has already been reported from our laboratory that the extract to reduce the level of oxidative stress. Reduces the amount of \( \cdot \text{OH} \) formation in the RBCs, demonstrating the co-treatment of goat RBCs with PHZ and aqueous bark extract of TA highly reactive \( \cdot \text{OH} \) this readily damages lipids, proteins and other oxidants like iron with hydrogen peroxide and hydrogen peroxide fatty acids in endogenous phospholipids [39]. Besides PHZ, other Phenylhydrazine in the presence of haemoglobin oxidizes to form \( \cdot \text{OH} \). This radical initiates the peroxidation of unsaturated fatty acids in endogenous phospholipids [39]. It has also been demonstrated that normal RBCs treated with PHZ has a mimetic characteristic as those found in severe \( \beta \)-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

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).

An elevated level of \( \cdot \text{OH} \) 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 \( \cdot \text{OH} \) 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. \( \cdot \text{OH} \) 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 \( \cdot \text{OH} \) 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 \( \text{H}_2\text{O}_2 \) 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 \( \beta \)-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

---

**Table 1**

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Diameter (µm)</th>
<th>Radius (µm)</th>
<th>Concave depth (nm)</th>
<th>Roughness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.40±0.057</td>
<td>1.70±0.058</td>
<td>576±0.087</td>
<td>1579±0.057</td>
</tr>
<tr>
<td>T5C</td>
<td>4.01±0.046</td>
<td>2.00±0.046</td>
<td>430±0.29</td>
<td>1269±0.028</td>
</tr>
<tr>
<td>PHZ</td>
<td>4.65±0.040*</td>
<td>2.33±0.035*</td>
<td>367±0.058*</td>
<td>840±0.046*</td>
</tr>
<tr>
<td>P+T5</td>
<td>3.08±0.035**</td>
<td>1.54±0.029**</td>
<td>441±0.046**</td>
<td>937±0.035**</td>
</tr>
</tbody>
</table>

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.

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.
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]. The present study demonstrated that co-treatment of 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 membrane-bound 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 membrane integrity of RBCs.

Erythrocytes are highly susceptible to oxidative damage due to the presence of heme iron. Polysaturated fatty acids (PUFA) and oxygen may initiate the reactions that induce oxidative changes in the red blood cells [44, 45]. Peroxidation of PUFA in the 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 Mg2+-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 also 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 neutralizes any molecule in the vicinity of where it is produced [55]. Exogenous administration of aqueous TA bark extract neutralized the formation of ROS, thereby reducing, the membrane and physiological damage. In addition, increased intracellular lipid 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. DB 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-Inter disciplinary Program in Life Sciences (DBT-IPLS), the University of Calcutta for allowing us to use some of the facilities.

CONFLICT OF INTERESTS
Declared none

REFERENCES
Chattopadhyay et al.

Int J Pharm Pharm Sci, Vol 8, Issue 5, 62-70


LEAD INDUCES OXIDATIVE STRESS IN RAT HEART AND LIVER TISSUE HOMOGENATES: AN IN VITRO STUDY

PAUL, S.,1 GHOSH, D.,1 GHOSH, A. K.,1 MITRA, E.,1 DEY, M.,1 CHATTOPADHYAY, A.2 AND BANDYOPADHYAY, D.1*

1Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009; *Principal Investigator, Centre with Potential for Excellence in Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009; 2Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700 006.

E. mail: debasish63@gmail.com, Cell 09433072066

Received: September 24, 2013; Accepted: October 24, 2013

Abstract: Environmental lead toxicity is an old but persistent public health problem throughout the world and children are more susceptible to lead than adults because of their hand to mouth activity, increased respiratory rates and higher gastrointestinal absorption per unit body weight. The propensity of lead to catalyze oxidative reactions and generate reactive oxygen species has been demonstrated in multiple studies in vivo. Dose-response study of lead acetate on the biomarkers of oxidative stress i.e., levels of lipid peroxidation (LPO) and reduced glutathione (GSH), activities of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT), and some pro oxidant enzymes i.e., xanthine oxidase (XO) and xanthine dehydrogenase (XDH) have been carried out in vitro in heart and liver tissues of male Wistar rats. The in vitro study provides us with a wide knowledge about the correlation of increased concentration of lead and the activities of the different enzymes which are related to the management of oxidative stress in the vital organs of the body.

Key words: Lead, Oxidative stress, Reactive oxygen species

INTRODUCTION

Lead poisoning (also known as plumbism, colica pictonium, saturnism, Devon colic, or painter’s colic) is a medical condition caused by increased levels of the heavy metal, lead in the body [1]. Lead interferes with a variety of body processes and is toxic to many organs and tissues including the heart, bones, intestines, kidneys, and reproductive and nervous systems [2,3]. It interferes with the development of the nervous system and is therefore particularly toxic to children, causing potentially permanent learning and behavior disorders [1]. Symptoms include abdominal pain, headache, anemia, irritability, and in severe cases seizures, coma, and death [3]. Lead is one of the few natural substances that have no known use in the human body. At even very low level, lead has been shown to cause health problems [1]. The difficulty with lead is that once it is mined from the earth, there is no known way to destroy or make it harmless. This makes it extremely important that we reduce overuse of lead and dispose it safely. Lead poisoning can also occur due to consumption of drinking water that is supplied through lead pipes. When ingested, it can act as a poison as the metal is converted into salt and is absorbed [4,5]. Such kind of poisoning can occur in workers working in soldering, plumbing, alloys, and toys, ceramic, paint, and foil paper industries. Recent studies suggest that the toxic impact of lead is mainly through increased production of reactive oxygen spe-
Melatonin protects against lead acetate induced oxidative stress-mediated changes in morphology and metabolic status in rat red blood cells: a flow cytometric and biochemical analysis

Debosree Ghosh1,2, Sudeshna Paul1,2, Shamreen Naaz1, Debajit Bhowmik1, Mousumi Dutta1,2, Arnab K. Ghosh1, Syed Benazir Firdaus1,2, Aindrila Chattopadhyay3, Russel J. Reiter4 and Debashis Bandyopadhyay1*  
1 Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009, West Bengal, India  
2 Department of Physiology, Hooghly Mohsin College, P.O. - Chinsura, Dist.- Hooghly, Pin-712 101, West Bengal, India  
3 Centre for Research in Nano Science and Nano technology, Acharya Prafulla Chandra Roy Siksha Prangan, University of Calcutta, Kolkata 700 098, West Bengal, India  
4 Centre for Research in Nano Science and Nano technology, Acharya Prafulla Chandra Roy Siksha Prangan, University of Calcutta, Kolkata 700 098, West Bengal, India  
5 Department of Physiology, Government General Degree College, Ambigeria, Kharagpur II, WB, India  
#Principal Investigator, Centre with Potential for Excellence in a Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009, West Bengal, India

Received on: 27-03-2016; Revised on: 11-05-2016; Accepted on: 09-06-2016

ABSTRACT

Background: Lead is an abundantly occurring heavy metal known to be toxic in higher quantities for humans and other animals. Moreover, lead exposure has previously been shown to cause damage to red blood cell. However, a detailed study with a plausible mechanism is still lacking. The pineal hormone, melatonin, is well known for its antioxidant and free radical scavenging properties. Thus, we examined the effect of melatonin pre-treatment on lead acetate-induced toxicity in rat RBCs in vivo as well as in vitro and tried to find out the mechanism behind such protection. Main methods: Rats were injected intra-peritoneally with lead acetate (15mg/kg/day) for seven consecutive days in presence/absence of melatonin (10mg/kg body weight). RBCs isolated from whole blood were haemolysed and was used for measuring changes in biochemical parameters and altered red blood cell morphology. Results & Discussion: Rats injected intra-peritoneally with lead acetate (15mg/kg/day) for seven days exhibited an altered status of lipid peroxidation level, reduced glutathione content, protein carbonyl and oxidized glutathione levels along with inhibition of superoxide dismutase and catalase activities in RBCs indicating generation of oxidative stress. Data obtained using light microscopy, scanning electron microscopy and flow cytometry indicate deterioration of RBC morphology along with marked alterations in granularity. Also, lead acetate-induced alterations of RBC enzyme activities are time and concentration-dependent and when co-incubated with melatonin, these changes were restored. Conclusion: The present study demonstrates the potential ability of melatonin to provide protection against lead acetate-induced injury to RBCs through its antioxidant properties in addition to removal of non-competitive inhibition of some of the enzymes.

KEYWORDS: Flow cytometry, lead, melatonin, oxidative stress, red blood cells.

INTRODUCTION

Red blood cells (RBCs) play a pivotal role in the maintenance of normal metabolic status of healthy individuals. In addition to supplying oxygen to each cell in vertebrates, they also aid in the elimination of carbon dioxide. RBCs have always been an important model to study because of its crucial role in various physiological and biochemical processes. Exposure to toxins often results in changes in the shape of RBCs due to biochemical alterations in the structural and functional components of the cell. Such changes in RBC morphology can be used as clinical markers of anaemia and other types of blood diseases. Morphologically altered RBCs are functionally compromised and are susceptible to haemolysis. On the other hand, in some cases, structurally altered RBCs in circulation are considered as indicative of deteriorative changes in the bone marrow. RBCs are highly flexible
Melatonin and aqueous curry leaf extract in combination protects against lead induced oxidative stress mediated injury to rat heart: a new approach

Debosree Ghosh1,2, Sudeshna Pau1, Aindrila Chattopadhyay3, Debasis Bandyopadhyay1*

1 Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009 India.
2 Department of Physiology, Hooghly Mohsin College, P.O. - Chinsura, Dist.- Hooghly, Pin-712 101, West Bengal, India.
3 Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700 006, West Bengal, India.

#Principal Investigator, Centre with Potential for Excellence in a Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009 India.

Objective(s): The objective(s) of the present study was to investigate whether a combination of melatonin and aqueous curry leaf extract is capable of providing protection against lead acetate induced oxidative stress mediated injury to cardiac tissue of experimental rats. Methods: Male Wistar rats were used as an animal model for the present study. After acclimatization to laboratory condition, in the first set of experiments rats were divided in to different groups. In the second, rats were divided in to four groups, i.e., control, positive control, lead acetate treated and melatonin + curry leaf extract in combination protected. Rats were treated intraperitoneally (i.p.) with lead acetate (15 mg / kg body weight) for a period of seven consecutive days. Rats of the protected group were pre-treated with melatonin also for seven days. The control rats were treated with vehicle only while the positive control rats were treated with melatonin + aqueous curry leaf extract only. After the treatment period, rats were sacrificed; blood and cardiac tissue collected and processed for analysis. Results: Treatment of rats with lead acetate caused accumulation of lead in the cardiac tissue and alterations in the biomarkers of organ damage and oxidative stress. It caused deteriorative changes to the cardiac tissue morphology and collagen content which were evaluated using light microscopy, electron microscopy and confocal microscopy. Involvement of oxidative stress is evident from the alterations in the level of lipid peroxidation and protein carbonyl content, activities of the antioxidant as well as pro-oxidant enzymes and some of the enzymes of the citric acid cycle and Electron Transport Chain (ETC) following lead acetate treatment. All changes were protected when the rats were pre-treated (fed orally) with melatonin (10 mg / kg body weight) and aqueous extract of curry leaves (50 mg/kg body weight) in combination for seven days. Conclusion: The results of the current studies indicate protective effect of the combination of melatonin (a natural antioxidant in pure form) and the aqueous extract of curry leaves to mitigate lead acetate-induced oxidative stress in experimental rats possibly through their synergistic antioxidant mechanism(s). This study opens up avenues for development of an effective drug formulation against lead induced oxidative stress mediated cardiac damage in people who get environmentally or occupationally exposed to lead.

KEY WORDS: Curry leaves, lead acetate, melatonin, oxidative stress, tissue injury, synergistic antioxidant mechanism(s).

INTRODUCTION:
Metal lead is a ubiquitous highly toxic environmental pollutant1. Air, water and soil get contaminated by the toxic metal through processes like mining, metallurgy, its extensive uses in industries (i.e., paint, dye, pottery, jewellery, mint, water pipeline manufacturing, battery, bullets etc.) and recycling. The heavy metal is unnecessary in living system and if it enters the body, causes extensive range of physiological, biochemical deteriorative changes1. The toxic metal is known from long time and the mechanism of lead induced toxicity i.e., ‘plumbism’ has been addressed by many and has been concluded to be multifactorial1. Recent studies revealed that lead induces toxicity by mediating oxidative stress 2,3,4. Our studies also reveal the same 5,6. Lead induced oxidative stress may be held responsible for the reported occupational health hazards experienced by workers who get exposed to lead regularly in industries. It is an area of extensive research around the globe.

Melatonin is a neuro-hormone secreted from the pineal gland. It is present in unicells as well as in human and plants. It helps to maintain circadian rhythms 7, boosts immune system 7, induces sleep, regu-
Aqueous Extract of *Murraya koenigii* in Combination with Melatonin Provides Better Protection Against Lead Induced Alterations in Blood Corpuscles and Lipid Profile of Male Wistar Rats

**Abstract**: This study was designed to evaluate the effect of aqueous Curry leaf extract (CuLE) against lead induced alterations in the counts of the blood corpuscles and lipid profile of male Wistar rats as well as to investigate the effect of CuLE in combination with melatonin against lead induced alterations in the counts of the blood corpuscles and lipid profile of male Wistar rats.

Treatment of rats with lead acetate at a dose of 15 mg / kg body weight intraperitoneally (i.p) for a period of seven consecutive days caused alterations in the total count of erythrocyte and leucocyte, hemoglobin content, mean corpuscular hemoglobin content, neutrophil count, small lymphocyte count, eosinophil count, Erythrocyte Sedimentation Rate (ESR), total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, Total cholesterol: HDL cholesterol, and LDL cholesterol / HDL cholesterol. All these changes were ameliorated when the rats were pre-treated with CuLE at a dose of 50 mg / kg (fed orally) for a similar period of time. As the animals were pre-treated with CuLE at a dose of 50 mg / kg (fed orally) in combination with melatonin at a dose of 10 mg / kg (fed orally), for a similar period of time, we observed a better protection against the lead induced changes. The results of the current studies indicate that CuLE has the ability to mitigate heavy metal-induced alterations in blood tissue but CuLE in combination with melatonin provides a better protection in the situation. This is probably brought about through the synergistic activity of the antioxidant phytochemicals of CuLE and Melatonin and may have future therapeutic relevance to occupational and environmental lead exposure induced hemato-pathological state.

**Key words**: Curry leaves, Melatonin, blood, lipid profile, antioxidant, lead acetate, Cholesterol

Curry leaves are widely used popular spice herb in South East Asian Countries. It belongs to the family Rutaceae. It is used as a flavouring agent and has wide spread medicinal application as is evident from the Vedas and ayurvedic literature. The phytochemicals present in the Curry leaves are stable, void of cytotoxicity and any kind of side effects if used in a definite dose. Hence curry leaves extract can be dealt with extensively for the purpose of drug development and experimental as well as pharmaceutical interventions. Curry leaves are used in traditional system of Medicine, as an antiemetic, antidiarrhoeal, dysentery, blood purifier, tonic, stomachic etc. Curry leaves have been reported to be potent antioxidants with free radical scavenging capabilities.

Melatonin is a small indole amine, released from the pituitary gland in mammals and is found to be present from unicells to plants and insects. It is the key regulator of biological clock and is recognized and well documented as a potent antioxidant against various models of stress. Melatonin has been reported to be scavenger of the dangerous free radicals which are generated in state of oxidative stress. Melatonin has been reported to be a potent sleep promoter and also has use in neural disease, depression, chronic fatigue syndrome (CFS), migraine and other headaches, irritable bowel syndrome (IBS), bone loss (osteoporosis), epilepsy, as an anti-aging agent, for menopause, excessive acid secretion, gastric ulcers, cancer treatment, diabetes and for impotency.

Lead is a heavy metal, persistent environmental toxin and present widely in our environment and gets added daily to the sink with human activities. The metal is highly toxic and unnecessary in living system. Lead finds its way to our body primarily through routes like food and inhalation of contaminated air. Children playing with crayons or toys may also ingest lead. We get either environmentally or occupationally exposed to this heavy metal. Lead enters our system and gets stored in the soft organs and also inside blood cells and disrupts the normal processes of our various organs and organ system at cellular, molecular, biochemical and physiological level. Lead induced toxicity has been reported to be multifactorial. Lead has been reported to bring deteriorative changes in various tissues. Continued, unaided exposure to this toxic heavy metal can be life threatening. Occupational exposure to lead is unavoidable for people engaged in industries like paints, dye, battery, bullet, metallurgy etc. Lead induced changes in blood cell counts and lipid profile may go unnoticed and asymptomatic for several period of time and may ultimately culminate to fatal situation for the individual. Chelation therapies available for lead toxicity has limited success and hence the search for a better, potent, effective, safe, adaptable, easy and affordable remedy against lead induced damages continues.

Our earlier studies reveal that aqueous extract of *Murraya koenigii*, containing primarily the polar antioxidant phytochemicals possess protective effect against lead...
Amelioration of gastrotoxic effect of indomethacin by piperine in male Wistar rats: a novel therapeutic approach

Nirajan Ghosal1, Syed Benazir Firdaus1, Sudestha Paul2,3, Shamreem Naa3, Aindrila Chattopadhyay1, Prachi Shukla1, Garima Jain1, Sanjib Pattar4, Vinod D. Rangar4 and Debasish Bandyopadhyay1∗

1Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009, India
2Department of Physiology, Govt. General College, Madpur, Paschimmedinipur, West Bengal
3Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700 009, India
4S.L.T. Institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya (Central University), Koni, Bilaspur-495 009, CG, India
5RN Tagore International Institute of Cardiac Sciences, 124, Mukundapur, EM Bypass, Kolkata 700 099, India

Received on:23-02-2016; Revised on: 27-03-2016; Accepted on: 09-05-2016

ABSTRACT

Background: Gastric ulcer, an alarming disease among middle aged person in India and middle East Asia, generally develops due to long term use of Non-Steroidal Anti Inflammatory Drugs (NSAIDs), consumed as pain killers. It is well established that oxidative stress and free radical mediated injury play a pivotal role in generation of gastric ulcer. Antioxidant activity and gastro protective effects of black pepper are well known for long time. Piperine, a major alkaloid present in black pepper also possesses free radical scavenging activity. Objective: The aim of this study is to evaluate the ameliorative role of piperine against indomethacin, a classical NSAID, induced gastric damage. Methods: Indomethacin was orally administered in male albino Wistar rats to generate gastric ulcers. These rats were orally fed with graded doses of aqueous solution of piperine prior to indomethacin administration. Oxidative stress biomarkers, activities of antioxidant and pro-oxidant enzymes, PGE2 level, activities of mitochondrial enzymes and histology of gastric tissues were studied. Results: From the obtained data, it was seen that, Indomethacin treatment altered all the above mentioned parameters whereas, piperine pre-treated animals were protected against indomethacin induced ulceration. Conclusion: Hence, piperine possess the potentiality to be used as an antiulcer drug against indomethacin induced gastric ulcer or can be used as an co-therapeutic agent among those patients undergoing NSAID treatment.

KEYWORDS: Antioxidant, Gastric ulcer, Indomethacin, Non-Steroidal Anti Inflammatory Drugs (NSAIDs), oxidative stress, Piperine.

INTRODUCTION

Gastric mucosa is one of the first lines of defence that protects our body from the deleterious effects of ingested exogenous xenobiotics and microbial organisms [1]. One important factor that erodes the epithelial lining of stomach and evidently forms gastric ulcer is the administration of Non-Steroidal Anti Inflammatory Drugs (NSAID) [2]. Gastric ulceration has been associated with the use of NSAIDs [3]. Other risk factors associated with gastric ulceration include advanced age, previous history of ulceration, long term use of steroids, higher doses of NSAIDs or the use of more than one NSAIDs at a time, concomitant administration of anticoagulants, and co-existing serious systemic disorders [3]. Gastrointestinal bleeding is related to the type of NSAID being used and its dosage. All therapeutically useful NSAIDs exert their anti-inflammatory action by decreasing the synthesis of Prostaglandins (PGs) through non-selective inhibition of cyclooxygenase enzymes [4], which consequently leads to reduction in bicarbonate ion secretion and reduced mucous production [5]. In most of the cases, it ultimately leads to the generation of ulcer. NSAIDs also cause vasoconstriction which leads to hypoxia and consequent formation of ulcer. Among several NSAIDs, indomethacin is a popular choice for the treatment of arthritis and associated clinical conditions. It is a potent non-specific anti-inflammatory drug which was introduced in 1963 to treat various pathological conditions like rheumatoid arthritis, degenerative joint diseases, ankylosing spondilitis, gout, acute musculoskeletal disorders, inflammation and oedema following surgical technique and pain associated with
Orally administered aqueous bark extract of *Terminalia arjuna* protects against adrenaline-induced myocardial injury in rat heart through antioxidant mechanisms: an *in vivo* and an *in vitro* study

Sanatan Mishra¹², Shamreen Naaz², Arnab K. Ghosh², Sudeshna Paul¹², Nirajan Ghosal², Mousumi Dutta¹², Debasish Bandopadhyay², Aindrila Chattopadhyay¹

¹Department of Physiology, Vidyasagar College, Kolkata 700 006, India,
²Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009, India.

Received on:11-04-2016; Revised on: 19-05-2016; Accepted on: 27-06-2016

**ABSTRACT**

Objective: The present study is aimed to evaluate the cardioprotective effects of the most effective dose of aqueous bark extract of *Terminalia arjuna* (TA) on adrenaline-bitartrate induced myocardial damages in male albino rats. Methods: After sacrifice of rats, the left ventricular portion of heart tissues were used for determination of biomarkers of oxidative stress, activities of antioxidant enzymes, Kreb’s cycle enzymes and respiratory chain enzymes by using standard methods. Results: Treatment of rats with adrenaline-bitartrate induced alterations in the activities of serum lactate dehydrogenase total (LDH-T), lactate dehydrogenase-1 (LDH-1), serum glutamate oxaloacetate transaminase (SGOT), tissue and serum nitric oxide (NO) concentration. Moreover, it caused elevation in the level of lipid peroxidation and protein carbonylation, a decrease in glutathione content as well as altered the activities of antioxidant enzymes and the enzymes of Kreb’s cycle and respiratory chain. Tissue histomorphological studies also showed considerable damage following adrenaline treatment. Pre-treatment of rats with aqueous bark extract of TA significantly protected against these myocardial damages. Conclusion: The present studies suggest that the effective dose of aqueous bark extract of TA may be beneficial in ameliorating adrenaline-induced oxidative stress mediated myocardial injury.

Keywords: Adrenaline, antioxidant, cardiac damages, oxidative stress, rats, *Terminalia arjuna*.

**INTRODUCTION**

Adrenaline, a catecholamine synthesized by adrenal medulla, is generally considered as a hormone involved in “fight or flight” mechanism¹. However, its role in the genesis of oxidative stress in humans is being increasingly recognized and is considered more dangerous in bringing about myocardial ischemia as well as myocardial infarction. In early phase of myocardial infarction systemic circulatory catecholamine level is vigorously increased² and is released from ischemic region of myocardium³. In addition, auto-oxidation of catecholamine results in generation of cytotoxic free radicals⁴. It has been reported that during myocardial infarction, the components of the natural antioxidant defense system (i.e. GSH, superoxide dismutase and catalase) are depleted and subsequently the myocardial tissue becomes vulnerable to oxidative stress as a result of which cardiac-myopathy occurs. Adrenaline acts by binding to a variety of adrenergic receptors present in human system. Epinephrine is a nonselective agonist of all adrenergic receptors, including the major sub-type α₁, α₂, β₁, β₂ and β₃⁵. The binding of epinephrine to these receptors triggers a number of metabolic changes. Endogenous plasma adrenaline concentrations in resting adults have been reported normally to be less than 10 ng/L, but may increase 10-fold during exercise and 50-fold or more during time of stress⁶. Therefore, adrenaline is an endogenous stress inducer.

Aerobic metabolism leads to production of reactive oxygen species (ROS), which is continuously removed by antioxidant defense system of an organism. A balance between production and removal of ROS is necessary to maintain normal physiology. Any impairment in antioxidant defense system or over production of ROS results in oxidative stress. ROS are known to cause numerous cellular anomalies including protein damage, deactivation of enzymes, and alteration of DNA and lipid peroxidation of membranes.
Protection against lead-induced oxidative stress in liver and kidneys of male Wistar rats using melatonin and aqueous extracts of the leaves of Murraya koenigii - A novel combinatorial therapeutic approach

Debosree Ghosh¹, Syed Benazir Firdaus, Arnab Kumar Ghosh, Sudeshna Paul, Debashis Bandyopadhyay*¹
¹Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology; 92 APC Road, Kolkata, India
*Principal Investigator. Centre with Potential for Excellence in a Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009 India.

Received on:23-02-2014; Revised on: 06-03-2014; Accepted on:15-03-2014

ABSTRACT
Treatment of rats with lead acetate (15 mg / kg body weight) intraperitoneally for seven consecutive days caused significant damage in rat liver and kidneys indicated by the altered levels of lipid peroxidation, reduced and oxidised glutathione content, the activities of hepatic and renal antioxidant, pro-oxidant enzymes, mitochondrial Kreb’s cycle and respiratory chain enzymes. Histomorphological changes were also induced in both the tissues by lead acetate which was evaluated using histological studies and microscopy. Collagen content of the liver and kidney tissues were also altered with lead treatment and those were evaluated using acid sirius stain and confocal microscopy and quantified using Image J software. All these changes were ameliorated when the rats were pre-treated with melatonin (10 mg / kg BW, fed orally) and aqueous extract of curry leaves (CuLE) (50 mg / kg BW, fed orally) in combination. The current studies indicated that co-treatment of melatonin and CuLE protected the rat hepatic and renal tissues against lead-induced oxidative stress possibly through their antioxidant activity. The results of the current studies may have future applications in developing a potent pharmaceutical agent with minimum or no cytotoxic side effects against lead induced hepatotoxicity and renal toxicity.

KEY WORDS: lead acetate, rat, liver, kidneys, melatonin, curry leaves, co-treatment

INTRODUCTION

The largest glandular organ in the body is the liver. It performs many vital functions to eliminate toxins and harmful substances from the body. Almost every organ in the body is supported by this vital organ. A person cannot survive without a healthy liver. Weight of an average adult liver is about three pounds. The liver receives about 1.5 quarts of blood every minute via the hepatic artery and portal vein.¹

On the other hand, the kidneys are a pair of vital organs that do many functions and maintain the blood clean and chemically balanced. In humans each kidney is about 4 or 5 inches long. Each kidney contains around a million nephrons. Each nephron acts as a microscopic filter for blood. As much as 90% of kidney functional disorder can occur without experiencing any symptoms or problems.²

Our earlier studies reveal that heavy metals like lead, cadmium, arsenic accumulate in highly perfused soft organs and induce generation free radicals therein.³,⁴,⁵

Lead has been recognised as the most common cause of heavy metal poisoning. Lead is number 2 on the ASTDR’s “Top 20 List.” Now most people are aware that lead is a poison yet people in occupations like paint, dye, crayon, cosmetics, battery, metallurgy, bullet manufacturing and glamour industries get regularly exposed to lead occupationally. While others get exposed unknowingly, environmentally. The mechanism of lead toxicity has been revealed to be oxidative stress in earlier studies including studies from our laboratory.⁶,⁷,⁸,⁹

Antioxidant potential of the herbal extracts in the amelioration of metal-induced oxidative stress need thorough investigation because these natural antioxidants are components of many edible substances and has the potential for safe future use by humans. Melatonin is present in all organisms and in many food items i.e., cereals, green vegetables and fruits¹⁰. Pharmacologically administered melatonin is well tolerated in humans with no reported side-effects.¹¹ Melatonin’s ability to act synergistically with other natural antioxidants at low doses in a number of models of oxidative stress has been identified recently¹²,¹³. The current study is aimed to examine whether melatonin exhibits any...
Antioxidant characteristics. Gastric ulcer generally develops due to long term use of Non-Steroidal Anti Inflammatory Drugs (NSAIDs), consumed as pain killers. It is well established that oxidative stress and free radical mediated injury play a major role in generation of gastric ulcer. This influenced us to investigate the ameliorative role, if any, of Sugaheal® against indomethacin (IMN), a classical NSAID, induced gastric damage. **Objective:** Evaluation of the protective effect of Sugaheal® against indomethacin induced gastric ulceration. **Methods:** Indomethacin was orally administered in male Wistar rats to generate gastric ulcers. These rats were orally fed with aqueous solution of Sugaheal® prior to indomethacin administration. Oxidative stress biomarkers, activities of antioxidant enzymes, activities of mitochondrial enzymes and gastric tissue morphology were studied through histological analysis. **Results:** From those obtained data, it was seen that, indomethacin treatment altered all the above mentioned parameters whereas; Sugaheal® pre-treatment prevented those deleterious changes. Biochemical and histological data supported these findings. **Conclusion:** Hence it can be said that besides being a potent anti-diabetic drug, Sugaheal® also possess the potentiality to be used as an antiulcer drug against indomethacin induced gastric ulcer.

**KEYWORDS:** Antioxidant, Gastric Ulcer, Indomethacin, Non-Steroidal Anti Inflammatory Drugs (NSAIDS), Oxidative Stress, Sugaheal®.

**INTRODUCTION**
Gastric mucosa is one of the first lines of defence that protects our body from the deleterious effects of ingested exogenous xenobiotics and microbial organisms[4]. Gastric ulcer develops because of imbalance between aggressive and protective factors. Several endogenous aggressive factors like hydrochloric acid, pepsin, refluxed bile, leukotrienes and reactive oxygen species (ROS), and several exogenous factors including non steroidal anti-inflammatory drugs (NSAIDs), stress, alcohol and *Helicobacter pylori* infection are major causative agents for mucosal damage and ulceration[24]. Among them one important factor that degenerates the epithelial lining of stomach and evidently forms gastric ulcer is the administration of non-steroidal anti-inflammatory drugs (NSAID)[7]. Other risk factors associated with gastric ulceration include advanced age, previous history of ulceration, long term use of steroids, higher doses of NSAIDs or the use of more than one NSAIDs at a time, concomitant administration of anticoagulants, and co-existing serious systemic disorders[8]. All therapeutically useful NSAIDs exert their anti-inflammatory action by preventing the synthesis of prostaglandins (PGs)[9] through non-selective inhibition of cyclooxygenase enzymes, which consequently leads to reduction in bicarbonate ion secretion and reduced mucous production[10]. In most of the cases, it ultimately leads to the generation of ulcer. Among various NSAIDs, indomethacin is one of the popular medicines used to treat various pathological conditions like rheumatoid arthritis, degenerative joint diseases, ankylosing spondilitis, gout, acute musculoskeletal disorders, inflammation and oedema following surgery and pain associated with primary dysmenorrhea[10]. Indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid) has prominent anti-inflammatory and analgesic-antipyretic properties. It is a potent inhibitor of the cyclooxygenases (COX) 1 and 2; also decreases the motility of
Aqueous bark extract of *Terminalia arjuna* protects against adrenaline-induced hepatic damage in male albino rats through antioxidant mechanism(s): a dose response study

Sanatan Mishra[1,2], Mousumi Dutta[1,2], Sadhan Kumar Mondal, Monalisa Dey[1], Sudeshna Paul[1,2], Aindrila Chattopadhyay[2], Debasish Bandyopadhyay[1,*]

1Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009, India. # Principal Investigator, Centre with Potential for Excellence in Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009, India

2Department of Physiology, Vidyasagar College, Kolkata 700 006, India

Received on:21-07-2014; Revised on:17-08-2014; Accepted on:18-09-2014

ABSTRACT

**Background:** The present study aimed to evaluate the hepatoprotective effects of different doses of aqueous bark extract of *Terminalia arjuna* (TA) on adrenaline-induced hepato-toxicity in male albino rats. **Methods:** A total number of 48 adult healthy male albino rats were divided into eight groups comprising of 6 animals each. Group-I served as normal control group. All other groups (group-II and group-VI to VIII) were administered adrenaline sub-cutaneously at a dose of 0.3mg/kg body weight. Group- III to V was treated with different doses of aqueous bark extract of TA at doses of 10mg/kg bw/administered orally (For Group-III), 20mg/kg bw/administered orally (For Group-IV), 40mg/kg bw/administered orally (For Group-V). Group-VI, group-VII and group-VIII were also co-treated with different doses of aqueous bark extract of TA at doses of 10, 20 and 40 mg/kg bw/ administered orally, respectively. **Results:** Treatment of rats with adrenaline induced alterations in the activities of serum lactate dehydrogenase total (LDH T), lactate dehydrogenase-5(LDH 5), serum glutamate pyruvate transaminase (SGPT), caused elevation in the level of lipid peroxidation and protein carbonylation, a decrease in glutathione content as well as altered the activities of antioxidant enzymes and the enzymes of Kreb’s cycle and respiratory chain. Tissue histomorphological studies also showed considerable damage following adrenaline treatment. Pre-treatment of rats with aqueous bark extract of TA significantly protected against these hepatic damage. **Conclusion:** The present studies suggest that the aqueous bark extract of TA may be beneficial in ameliorating adrenaline-induced oxidative stress mediated damages in the rat liver.

KEYWORDS: Adrenaline, antioxidant, hepatotoxicity, oxidative stress, rats, *Terminalia arjuna*.

INTRODUCTION

Reactive oxygen species (ROS), reactive nitrogen species (RNS) and other free radicals causes hepatic tissue injury by causing lipid peroxidation, depletion of cellular antioxidants and through oxidation of critical cellular proteins as well as by bringing alterations in the activities of the antioxidant enzymes[1]. Adrenaline, a catecholamine, is generally considered as a hormone involved in “fight or flight” mechanism[2]. However, its role in the genesis of oxidative stress in humans is being increasingly recognized and is considered more dangerous in bringing about hepatic disorders. Adrenaline acts by binding to a variety of adrenergic receptors present in our system. Epinephrine is a non-selective agonist of all adrenergic receptors, including the major subtypes a1, a2, b1, b2, and b3. The binding of epinephrine to these receptors triggers a number of metabolic changes[3]. Endogenous plasma adrenaline concentrations in resting adults have been reported normally to be less than 10 mg/L, but may increase by 10-fold during exercise and by 50-fold or more during times of stress. Therefore, adrenaline is an endogenous stress inducer.

*Terminalia arjuna* (TA) Wight & Arn. belonging to the family Combretaceae, is distributed throughout the greater part of India, Burma and Sri Lanka. Different parts, particularly its fruit and bark are used as a human consumable component in water, milk and other drinks to maintain good health[4]. Chemical analyses showed that the entire plant is full of compounds like tannin, saponin, ester, sugar, steroids, acids and minerals etc[5]. Experimental and clinical studies revealed the beneficial effects of this plant against various diseases by exerting its effect as gastroprotective[6] as well as anti-mutagenic activities[7]. Its aqueous bark extract showed a novel protection mechanism in several *in vitro* systems like RBC, liver tissue, heart mitochondria as discussed in our previous publications[8,9,10] and its antioxidant mechanisms were also established[10]. But till date there is no evidence regarding the hepato-protective activity of the bark

---

*Corresponding author.
Dr. Debasish Bandyopadhyay
Oxidative Stress and Free Radical Biology Laboratory, Department of Physiology, University of Calcutta, University College of Science and Technology
92 APC Road, Kolkata 700 009, India
Protective effect of antioxidant rich aqueous curry leaf (Murraya koenigii) extract against gastro-toxic effects of piroxicam in male Wistar rats

Syed Benazir Firdaus a, Debosree Ghosh a, Aindrila Chattyopadhyay b, Mousumi Dutta a, Sudekshna Paul a, Jagannath Jana c, Anjali Basu a, Gargi Bose a, Hiya Lahiri a, Bhaswati Banerjee d, Sanjib Pattar e, Subhrangshu Chatterjee c, Kuladip Jana d, Debasis Bandyopadhyay a,∗

a 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
b Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700 009, India
c Biomolecular NMR and Drug Design Laboratory, Department of Biophysics, Kolkata 700 009, India
d Department of Molecular Medicine and Bose Institute, P-1/12 CIT Scheme VII M, Kolkata, India
e NN Tagore International Institute of Cardiac Sciences, 124, Mukundapur, EM Bypass, Kolkata 700 099, India

A R T I C L E   I N F O

Article history:
Received 12 March 2014
Received in revised form 6 June 2014
Accepted 10 June 2014
Available online 3 July 2014

Keywords:
Antioxidant
Curry leaves
Oxidative stress
Gastro-toxicity
Gastric ulcer
Piroxicam

A B S T R A C T

Piroxicam (chemically 4-hydroxy-2-methyl-N-2-pyridinyl-2H-1,2-benzothiazine-3-carboxamide), a classical non-steroidal anti-inflammatory drug (NSAID) is orally administered to arthritic patients. Inhibition of prostaglandin E2 (PGE2) synthesis and subsequent free hydroxyl radical generation in vivo exert gastro-toxic side effects on piroxicam treatment. Leaves of curry plant are rich in antioxidants with prolific free radical scavenging activities. This led us to investigate the efficiency of the use of curry leaves in ameliorating piroxicam induced gastric damage. Piroxicam was orally (30 mg per kg body weight) administered in male albino Wistar rats to generate gastric ulcers. These rats were orally fed with graded doses of aqueous extract of curry or Murraya koenigii leaves (Cu LE) prior to piroxicam administration. Oxidative stress biomarkers, activities of antioxidant and pro-oxidant enzymes, mucin content and nature, PGE2 level, activities of mitochondrial enzymes and histomorphology of gastric tissues were studied. Piroxicam treatment altered all the above mentioned parameters whereas, curry leaf extract pre-treated animals were protected against piroxicam induced alterations. Hence, the protective action of the antioxidant rich Cu LE was investigated to propose a new combination therapy or dietary management to arthritic patients using piroxicam.

© 2014 Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Piroxicam, a classical NSAID, is a choice for most clinicians in arthritis and similar clinical conditions. The possible risk of gastro-toxic effects and ulceration of gastric mucosa imposed by this drug [1] has recently restricted its use. This drug induced gastric damage is possibly...
Lead induced oxidative stress: a health issue of global concern

Debasish Bandyopadhyay1*, Debosree Ghosh1, Aindrila Chattopadhyay1, Syed Benazir Firdausi, Arnab Kumar Ghosh1, Sudeshna Pauf1, Debjit Bhownik1, Sanatan Mishra1, Krishnendu Dalu1
1 Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700009, India.
2 Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700006.
3 Centre for Research in Nano Science and Nano technology, Acharya Prafulla Chandra Roy Siksha Prangan, University of Calcutta, Kolkata 700 098.
#Principal Investigator, Centre with Potential for Excellence in a Particular Area (CPEPA), University of Calcutta, University College of Science and Technology, 92 APC Road, Kolkata 700 009.

Received on:21-07-2014; Revised on: 25-08-2014; Accepted on:03-09-2014

ABSTRACT

Background/aims: Lead is recognized as one of the major environmental toxins, most widely distributed in the nature. The effect of lead induced toxicity leads to severe health ailments. The most significant one being neural, cardiac, hepatic and renal damages. Method: Studies including our investigations revealed that the underlying story of lead induced oxidative stress mediated damage has been documented to be lead induced generation of free radicals which causes oxidative damages leading to oxidative stress onslaughts. Occupational and environmental exposure to lead is unavoidable for some people in certain parts of the world. Therefore, the only way out remains to provide a protective remedy against lead induced oxidative stress. Results: Researches around the globe are targeting amelioration of lead induced toxicity by administering antioxidant(s). We have found that melatonin or aqueous extract of curry leaves provides protection against lead induced oxidative damages. Aqueous curry leaves extract and melatonin if used in combination, acts synergistically, complements each other and thus provides a better and stronger protection against lead induced toxicity. Conclusions: Antioxidant supplementation can provide a magic remedy against lead induced oxidative damages. Thus, curry leaves and melatonin can be used to design a potent drug formulation to provide protection against lead induced oxidative damages. It is void of side effects and has no reported cyto-toxicity.

KEY WORDS: Antioxidants, curry leaf extract, free radicals, lead, melatonin, oxidative stress, toxicity

INTRODUCTION

Lead is a toxic heavy metal, most widely distributed in the earth’s crust. Lead induced toxicity has been an important and well studied issue of public health concern around the globe for years. The history of lead poisoning has been well monitored from the time it was spotted. Man came to know about lead as early as 4,000 BC. The Hebrews used lead and the Phoenicians mined lead ore in Spain around 2,000 BC. The earliest documentations of lead toxicity have been found in Egyptian papyrus scrolls1. Lead compounds were often used for homicidal purposes. Hippocrates, in 370 BC, was the first person to report lead colic2. An average of 60,000 tonnes of lead was produced by the Romans per year for 400 years. They used various lead compounds for glazing pottery, and metallic lead for cooking utensils and piping. Lead utensils were used for boiling and condensing grape juice and for preserving and sweetening of wine1. Lot of efforts have been made from the parents of children affected with lead toxicity, researchers, scientists, policy makers and physicians to prevent use of lead. Finally, the battle against the silent killer has been won which is evident from the decreased reports of lead toxicity and decreased rate of reported death due to lead poisoning around the world4. In the 1960s, thousands of children with lead encephalopathy were hospitalized each year in the United States and about one in four died5. On the other hand, only one child died from lead poisoning in the past decade6. Over the last three decades, blood lead loads (BLL) of children and adults have decreased as a result of bans on lead in gasoline, paint, and solder used in canned foods7. But still it is a doubt that whether this drop in the rate of death due to lead poisoning should be considered as a victory against lead poisoning7. Though there has been some success in the worldwide ban of leaded gasoline, widespread lead exposure from industrial emissions and lead-contaminated paint and consumer products remains common in children around the world8. The Chemistry

Lead has been known from very early times. In India, it came into use during the Vedic period. Lead is chemically a heavy metal. Atomic