In-Vitro Antioxidant Potential of *Terminalia Arjuna* Bark Aqueous Extract on Sheep Red Blood Cells

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Abstract

An aqueous extract from the bark of *Terminalia arjuna* (TA), Marutham Pattai was evaluated for its protective (antioxidant) effect against hydrogen peroxide (H_2O_2) induced oxidation in normal sheep erythrocytes (red blood cells (RBCs). RBCs, treated with increasing concentrations of 30% H_2O_2 along with *Terminalia arjuna* aqueous bark extract (TA) were analyzed for hemolysis and lipid peroxidation. Sheep RBCs treated with increasing concentrations of glucose, to study effect of high glucose level or hyperglycemia on normal SRBC, were found to be more susceptible to lipid peroxidation than those from normal subjects. However, on treatment with TA, the oxidative modifications in both the groups (RBCs treated with glucose and not treated with glucose) were found to reduce significantly. The inhibition of lipid peroxidation was concentration dependent up to 100 µl of extract, which contained 0.75 mM gallic acid equivalent (GAE) of phenolic compounds. The total phenolic content in the extract was determined spectrophotometrically according to the Folin-Ciocalteau procedure and was expressed as mg or mM GAE. The results indicate that the aqueous bark extract of *Terminalia arjuna* (TA) contains antioxidants and protects cellular structures from oxidative damage. These findings demonstrate the potent antioxidant properties of the aqueous bark extract of *Terminalia arjuna*.

Key words: *Terminalia Arjuna* (TA), Osmofragility test, Lipid peroxidation.

1. Introduction

Several evidences suggest that oxidative stress plays a major role in the pathogenesis of both types of diabetes mellitus. Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and OH are highly toxic to cells. Cellular antioxidant enzymes and the free-radical scavengers normally protect a cell from toxic effects of the ROS. However, when generation of the ROS overtakes the antioxidant defense of the cells, oxidative damage of the cellular macromolecules (lipids, proteins, and nucleic acids) occurs, leading finally to various pathological conditions [1]. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA [2].These include cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, Schizophrenia, Bipolar disorder, fragile X syndrome, Sickle Cell Disease and chronic fatigue syndrome [3-11]. These diseases include mitochondrial dysfunctions [12].

It has been well documented that *Terminalia arjuna* (TA) bark extract contains arjunic acid, terminic acid, glycosides, tannins, saponins and flavones which may be responsible for the antioxidant activity, protective action on lipid peroxidation and the enhancing effect on cellular antioxidant defenses [13]. Both aqueous and ethanolic extract of the bark of TA plays a protective role against sodium-fluoride-induced hepatic and cardiac oxidative stress [14, 15]. *Terminalia arjuna* bark extract protects DNA damage against adriamycin (ADR)-induced damage [16]. Aqueous extract of TA play a role as the anti carcinogenic activity by reducing the oxidative stress along with inhibition of anaerobic metabolism. Ethanolic extract of TA exhibited significant antioxidant effect by altering the renal and hepatic protection against oxidative damage by diabetes [17]. Methanolic extract of TA protects against gastric ulcer in rats [13]. But there are no such evidences that aqueous extract of TA can protect the RBCs against oxidative stress.

2. Materials and Methods

Chemicals

All the fine chemicals were procured from Sigma Chemicals & Co., Ltd., St.Louis, MO, USA. All other chemicals used were of good quality and analytical grade.

Preparation of Sample

Osmotic Fragility Test

Sheep Blood was collected from the slaughter house in an (heparinized) ice cold storage bottle. One volume of the collected sheep blood sample was centrifuged. RBCs that settled in the pellet were then washed and suspended in sterile 1X phosphate buffer saline solution (pH 7.4). The suspended RBCs were then incubated with increasing concentrations of 30% H_2O_2, namely, 10, 20, and 50 mM for 2 hours. The other volume of blood was first incubated with 100µl of TA for 1 hour. The blood was centrifuged. The RBCs that settled in the pellet were then washed and suspended in phosphate buffered saline (PBS) (pH7.4). The suspended RBCs were incubated with increasing concentrations of H_2O_2, namely, 10, 20, and 50 mM for 2 hour. The percentage hemolysis was evaluated by performing osmotic fragility test as described by Dacie *et al*., (1964).

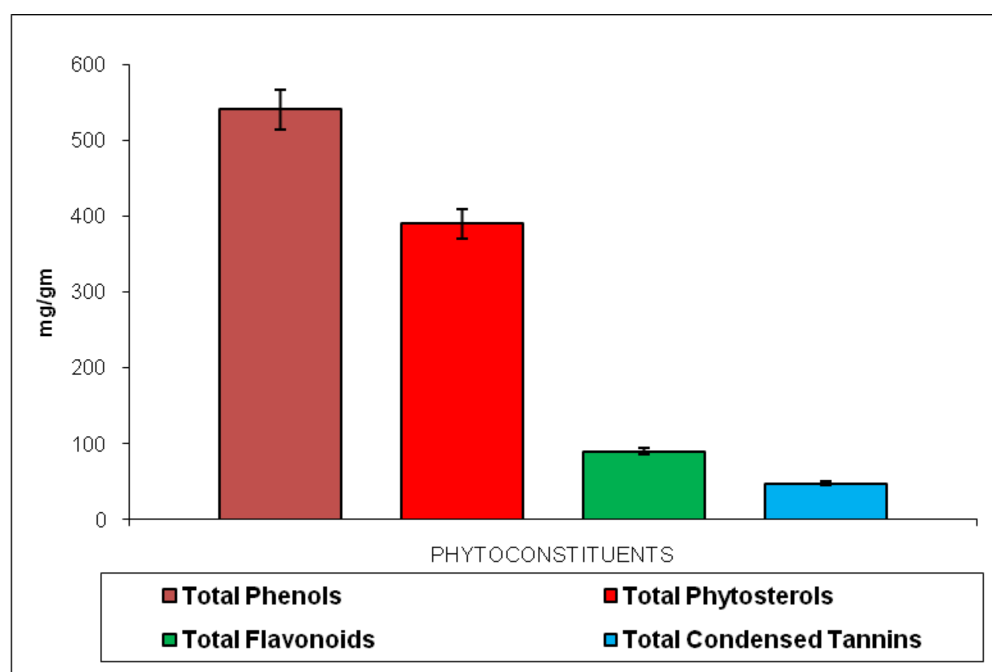
Preparation of Aqueous Bark Extract of *Terminalia Arjuna* & Qualitative and Quantitative Determination of Some of the Phytoconstituents

5gm 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 hours). Then it was centrifuged twice at 1300 g for 10 minutes. Then the supernatant was collected and lyophilized. The yield of the aqueous extract of *Terminalia arjuna* from 5 gm of TA bark powder was 10%. (Table 1)Various phytoconstituents such as tannins, cumarins, quinones, saponins and terpenoids were determined qualitatively [19]. On the other hand, total phenolics, (Graph 1) total phytosterols, flavonoids and condensed tannins were determined quantitatively [18, 19, 20, 21].

Phtoconstituents	Present/Absent
Tannins	+
Coumarins	+
Quinones	-
Saponins	-
Terpenoids	+

+Present, -Absent

Table 1. Qualitative Estimation of various phytoconstituents in *T.arjuna* aqueous bark



Graph 1. Phytoconstituents of various Concentrations (mg/gm) in aqueous bark extract of *Terminalia Arjuna* (TA)

Treatment with Glucose and *Terminalia Arjuna* (TA)

SRBCs prepared from fresh blood samples were treated with three different concentrations (namely, 20, 30, and 45 mM) of glucose for 6 hours to induce hyperglycemic condition *in vitro* and a second set of blood samples without treatment served as control. Simultaneously, a third set of SRBCs were treated similarly with three different concentrations of glucose along with 100 µl of TA (with a concentration of 0.75 mM GAE of phenolic compounds).

Determination of Activity of Antioxidant Enzymes

Estimation of Hemolysate Protein

The concentration of protein in the hemolysate was estimated by the standard method of Lowry *et al*. (1951).

Preparation of Sample

One volume of collected sheep blood was incubated with increasing concentrations of glucose, namely, 20, 30, and 45 mM for 6 hours. The other volume of blood was incubated with increasing glucose concentrations along with 100 µl of aqueous bark extract of *Terminalia arjuna* (TA) for 6 hours. Each of the incubated samples was hemolysed separately. The hemolysed blood was centrifuged at 20,000 g in a high speed centrifuge for 40 min. The supernatant or hemolysate was used for the assay. The sedimented membrane might be washed with PBS (pH 7.4) and used for estimation of membrane bound enzymes. A third volume of blood was preincubated with 100µl of aqueous bark extract of *Terminalia arjuna* (TA) for 1 hour. The RBCs were then washed and incubated with 50 mM H_2O_2 for 2 hours. A fourth volume of blood sample was incubated only with 50 mM H_2O_2 for 2 hours. Both the samples were hemolysed separately and this was used as the positive control. The supernatant from the lysed blood was used immediately to estimate the levels of thiobarbituric acids (TBARs) and the activities of antioxidant enzymes, namely, catalase, superoxide dismutase (SOD), and glutathione peroxidase, respectively. Catalase was estimated by the method of Sinha (1972). Superoxide dismutase was assayed by the method of Marklund and Marklund (1974). Glutathione peroxidase was estimated by the method of Rotruck *et al.*, (1973) with some modifications.

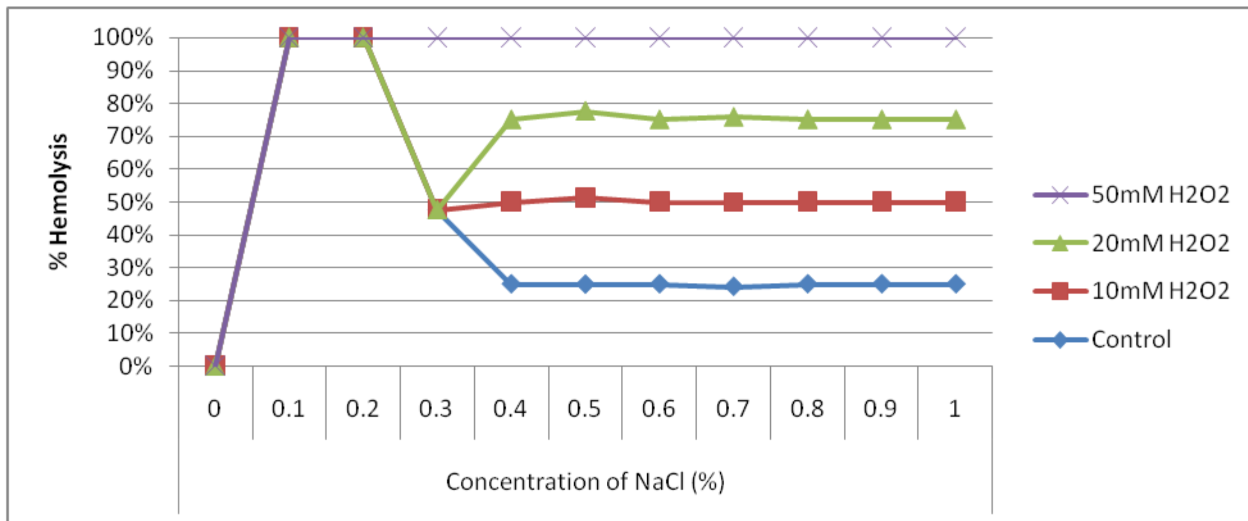
3. Statistical methods

Results are expressed as mean ± standard deviation (SD). Statistical analysis was done by one way analysis of variance (ANOVA), followed by multiple comparison by Tukey's honestly significant difference (HSD) test. The values were considered statistically significant when $p < 0.05$.

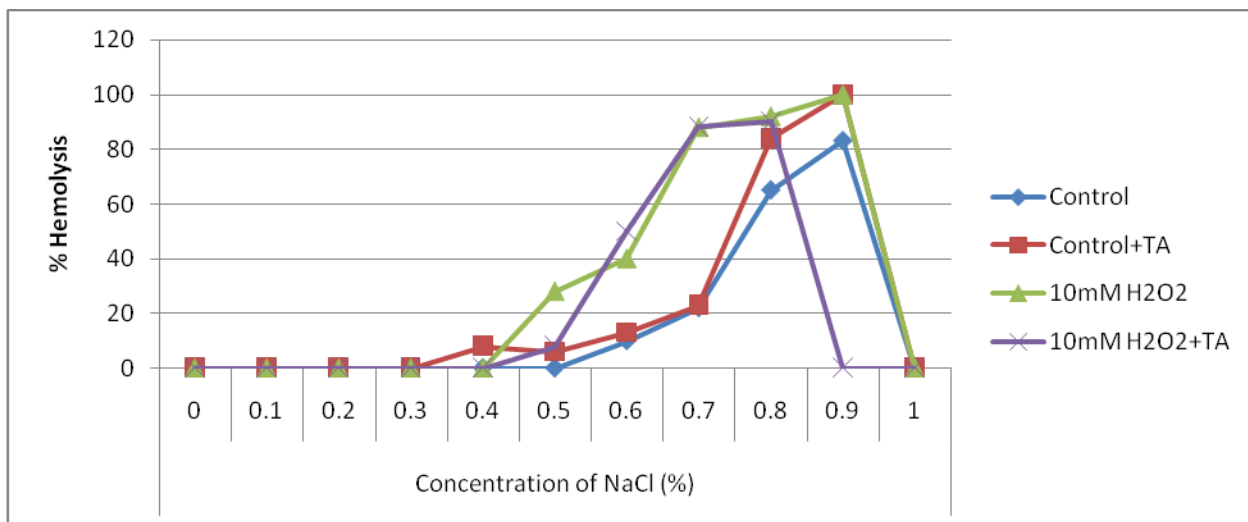
4. Results and Discussions

Effect of TA on the RBC Membrane Integrity

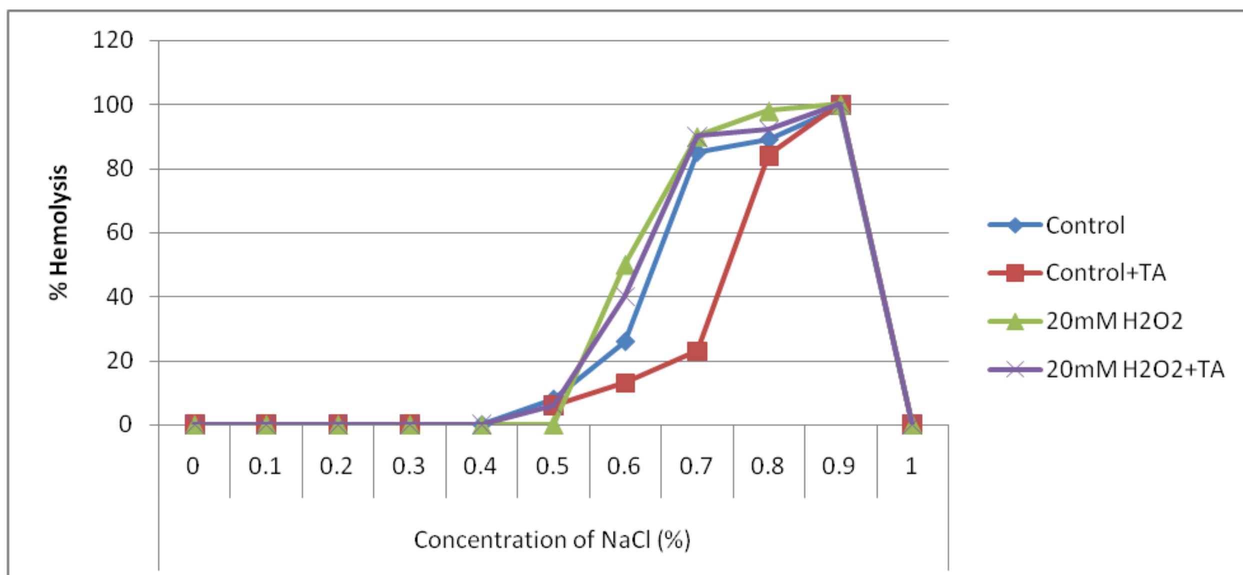
Results obtained in osmofragility test showed that there was no significant increase in the rate of hemolysis in 10 mM H_2O_2 treated blood sample as compared to the control sample suggesting that there was no significant membrane damage at this concentration. With 20 and 50 mM H_2O_2 concentrations, the rate of hemolysis was found to increase as depicted by the osmofragility curves that deviated significantly from the control curve. Maximum hemolysis was observed in blood incubated with a concentration of 50 mM H_2O_2 (Graph 2). Hemolysis was found to decrease in blood incubated with aqueous bark extract of *Terminalia arjuna* (TA) along with H_2O_2 suggesting that TA has some protective effect in maintaining the RBC membrane integrity (Graph 3,4,5).



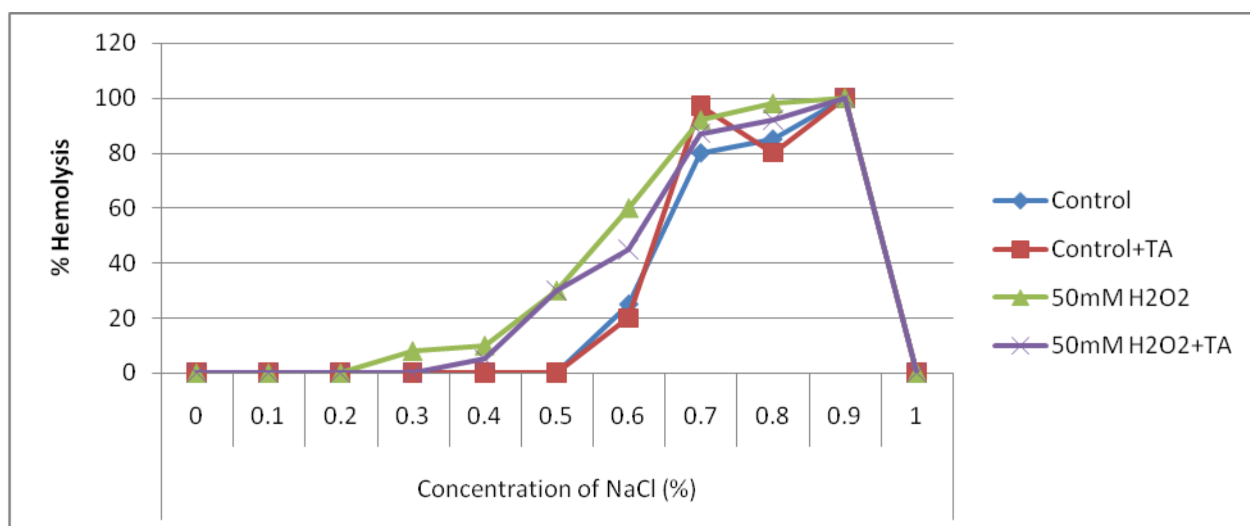
Graph 2. Rate of increasing H_2O_2 concentrations induced RBC hemolysis Values shown are mean±SD (n=6).



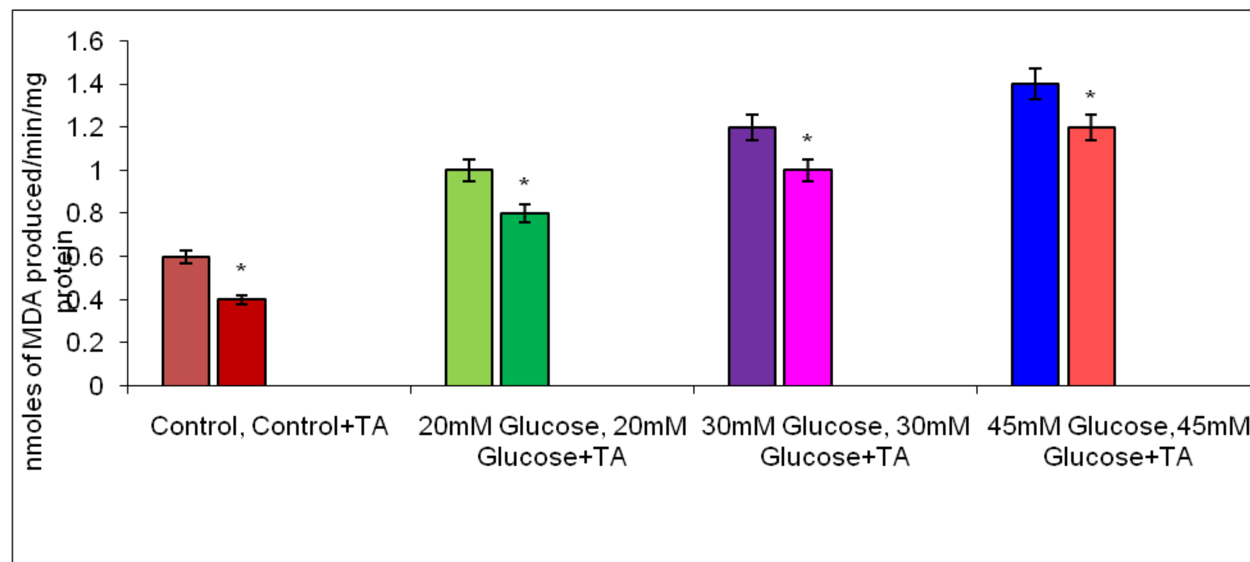
Graph 3. Rate of RBC hemolysis in control and 10mM H_2O_2 treated blood with and without preincubation with 100 µl TA. Values shown are mean±SD (n=6).



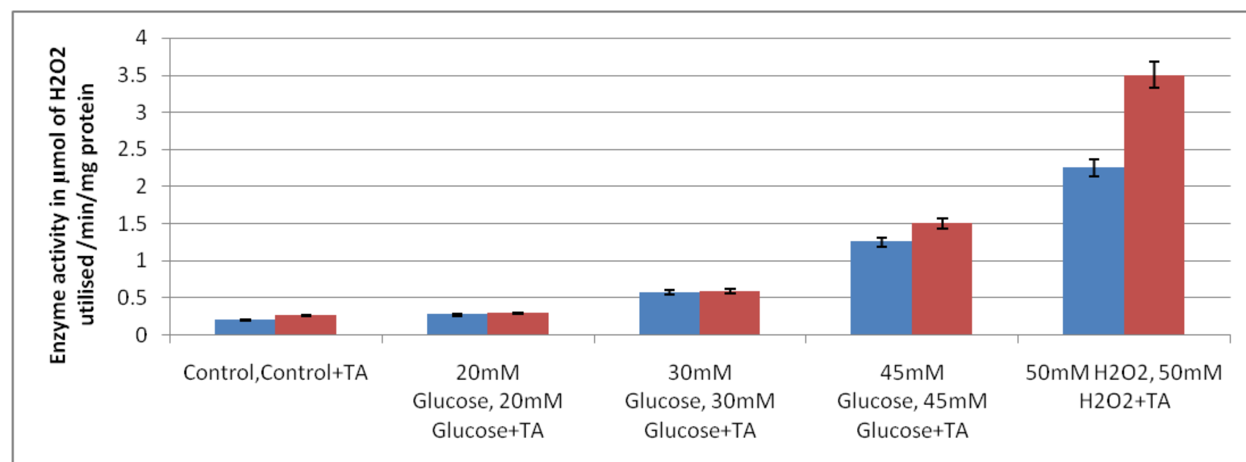
Graph 4. Rate of RBC hemolysis in control and 20mM H_2O_2 treated blood with and without preincubation with 100 µl TA. Values shown are mean±SD (n=6).



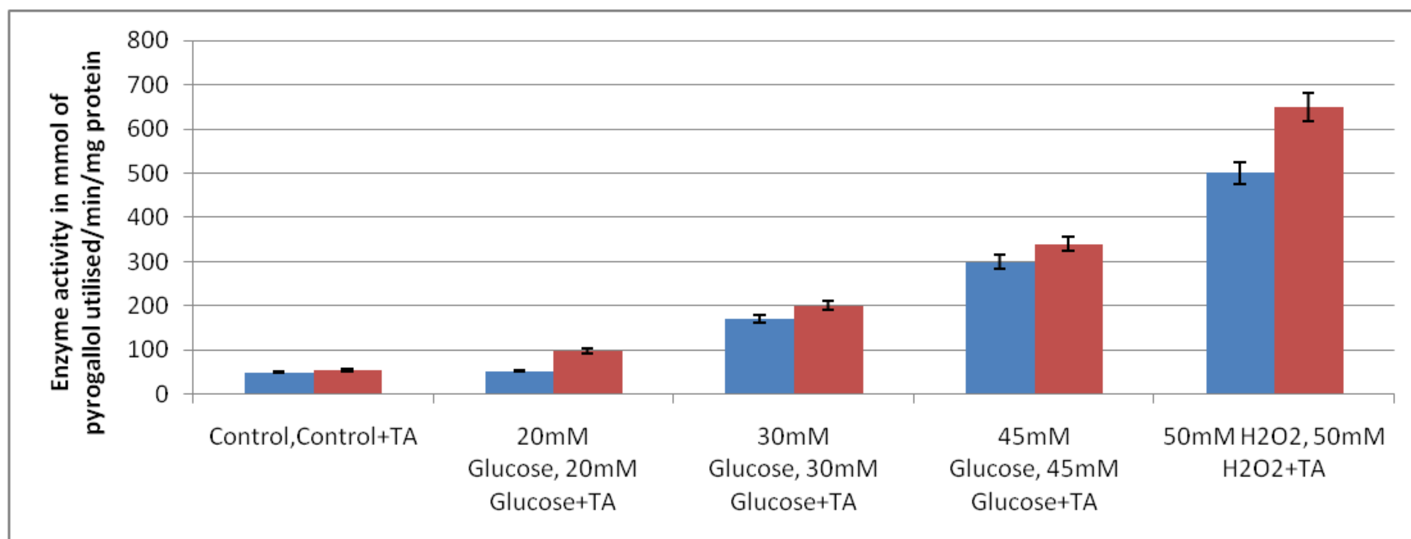
Graph 5. Rate of RBC hemolysis in control and 50mM H_2O_2 treated blood with and without preincubation with 100 µl TA. Values shown are mean±SD (n=6).



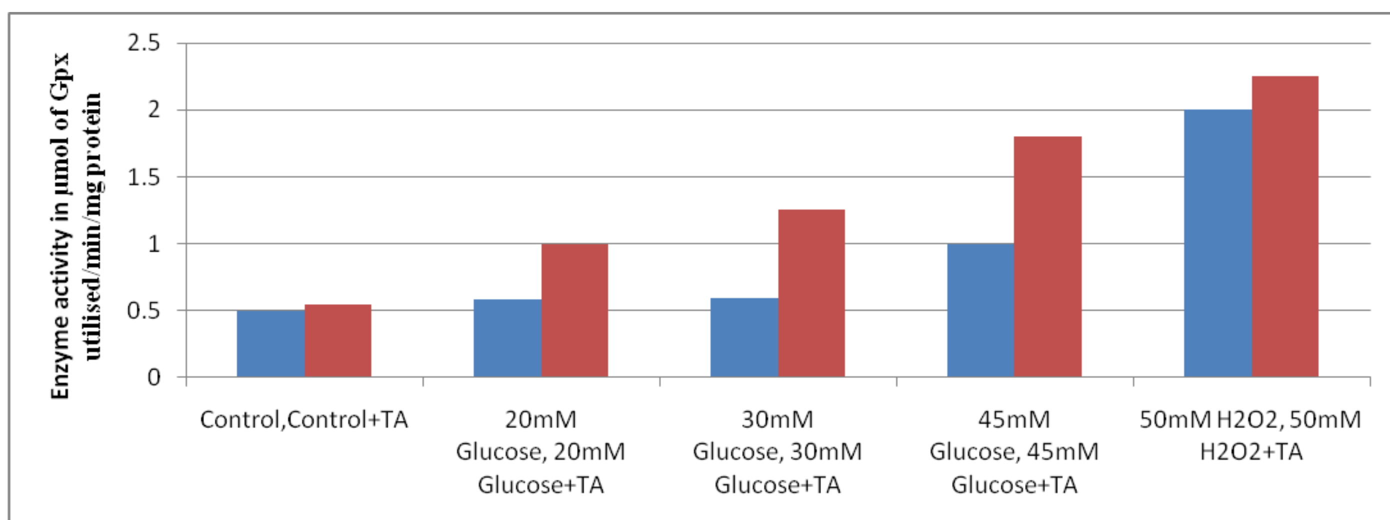
Graph 6. Effect of TA on the levels of TBA reactive substances (TBARs) in RBCs treated with increasing concentrations of Glucose (20,30,45mM). Values shown are mean±SD (n=6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Turkey's HSD test. Values not sharing a common superscript differ significantly. Significance level was p<0.05.



Graph 7. Effect of TA on Catalase activity in RBCs treated with increasing concentrations of Glucose (20,30,45mM). Values shown are mean±SD (n=6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Turkey's HSD test. Values not sharing a common superscript differ significantly. Significance level was p<0.05.



Graph 8. Effect of TA on SOD in RBCs treated with increasing concentrations of Glucose (20,30,45mM). Values shown are mean±SD (n=6). Statistical analysis was done by one way ANOVA and multiple comparison was done by Turkey's HSD test. Values not sharing a common superscript differ significantly. Significance level was p<0.05.



Graph 9. Effect of TA on Glutathione peroxidase (Gpx) activity in RBCs treated with increasing concentrations of Glucose (20,30,45mM). Values shown are mean±SD (n=6). Statistical analysis was done by one way ANOVA and multiple comparisons were done by Turkey's HSD test. Values not sharing a common superscript differ significantly. Significance level was p<0.05.

Effect of High Glucose Concentrations and TA Treatment on the Levels of TBARS

Lipid peroxidation (LPO) level in RBCs treated with glucose increased as could be seen from significantly elevated levels of TBARS in treated samples when compared with that of the control group (Graph 6). Treatment with aqueous bark extract of *Terminalia arjuna* (TA) decreased the levels of TBARS significantly in both glucose treated and untreated RBCs. Lipid peroxidation was found to be maximum in RBCs treated with 45 mM glucose concentration and there was a significant decrease ($p<0.05$) in lipid peroxidation in RBCs treated with aqueous bark extract of *Terminalia arjuna* (TA) along with a particular concentration of glucose.

Effect of High Glucose Concentrations and TA Treatment on Catalase Activity

Catalase activity was found to increase significantly on exposure to 30 and 45 mM glucose concentrations ($p<0.05$), but there was no significant change in activity at 20 mM glucose concentration. Catalase activity in RBCs exposed to 50 mM H_2O_2 served as a positive control in our study. Treatment with aqueous bark extract of *Terminalia arjuna* (TA) along with high glucose brought about significant increase in catalase activity ($p<0.05$). Similar increase in catalase activity was observed in RBCs treated with 50 mM H_2O_2 and aqueous bark extract of *Terminalia arjuna* (TA) (Graph 7). This explains the protective action of *Terminalia arjuna* against oxidative damage on RBCs.

Effect of High Glucose Concentrations and TA Treatment on SOD Activity

The activity of superoxide dismutase was assayed in the hemolysate of RBCs treated with different glucose concentrations (20, 30, and 45 mM) with or without *Terminalia arjuna* treatment. The SOD activity was found to increase significantly on exposure to 30 and 45 mM glucose concentrations ($p<0.05$), but there was no significant change in the activity at 20 mM glucose concentration. SOD activity in RBCs exposed to 50 mM H_2O_2 served as positive control in our study. Treatment with *Terminalia arjuna* along with high glucose treatment brought about further increase in SOD activity which was not significant. A significant increase in SOD activity was observed in RBCs treated first with 50 mM H_2O_2 and subsequently with *Terminalia arjuna* (Graph 8).

Effect of High Glucose Concentrations and TA Treatment on Glutathione Peroxidase (Gpx) Activity

Glutathione peroxidase activity in RBCs treated with different glucose concentrations (20, 30, and 45 mM) with or without *Terminalia arjuna* treatment was determined using the hemolysate. GPx activity was found to increase significantly on exposure to 20, 30, and 45 mM glucose concentrations ($p<0.05$). GPx activity in RBCs exposed to 50 mM H_2O_2 served as positive control in this study. Treatment with *Terminalia arjuna* along with high glucose treatment brought about an increase in GPx activity which was not significant. A significant increase in GPx activity was observed in RBCs treated with 45mM glucose and 50 mM H_2O_2 along with *Terminalia arjuna* (Graph 9).

5. Conclusion

Hyperglycemia can lead to both an increase in ROS production and attenuation of free radical scavenging compounds [23]. There are many ways by which hyperglycemia may increase free radical generation, such as glycoxidation, polyol pathway, prostanoid biosynthesis, and protein glycation [24]. There is also ample evidence that elevation in glucose concentration may depress natural antioxidant defense, such as GSH [26]. The imbalance between the generation of oxygen free radicals and an antioxidant defense system may increase oxidative stress and lead to the damage of molecules, such as DNA, proteins, or lipids.

Using SRBCs as a model, our study demonstrates that *Terminalia arjuna* can reduce membrane damage due to oxidation in cells exposed to high glucose. High glucose concentrations can induce oxidative stress due to excessive ROS production resulting from the auto oxidation of glucose, glycosylated proteins, or stimulation of cytochromeP_{450}-like activity by the excessive NADPH produced by glucose metabolism [26]. Whereas, *Terminalia arjuna* reduces membrane damage due to high glucose or increasing concentrations of H_2O_2. *Terminalia arjuna*, in general, has free radical scavenging property and it can reduce free radicals produced in the presence or absence of any antioxidant, that is, it can reduce lipid peroxidation levels even in normal red blood cells. Even, reduced osmotic fragility might be due to increased glucose utilization of the *Terminalia arjuna* treated cells [27], in their study showed that a decrease in LPO levels in RBCs exposed to increasing concentrations of polyphenol from the aqueous bark extract of *Terminalia arjuna* in H_2O_2 induced oxidative damage.

In our study, *Terminalia arjuna* proved to be an effective antioxidant and an anti-inflammatory agent in protecting SRBCs from membrane damage due to high glucose or increasing H_2O_2 concentrations induced oxidative stress. *Terminalia arjuna* bark extract was found to decrease the rate of hemolysis in H_2O_2-treated RBCs, indicating that *Terminalia arjuna* bark extract has protective effect in maintaining red blood cell membrane integrity. *Terminalia arjuna* bark extract reduces damage to the fragile RBC membrane due to oxidative stress imposed by H_2O_2. Alterations in LPO levels and antioxidant enzyme activities were observed in blood treated with increasing concentrations of glucose. The levels of TBARs were found to increase on treatment with high glucose concentrations, whereas the activity of antioxidant enzymes was found to increase on long term exposure to oxidative stress. This decrease in the activity of antioxidant enzymes in some tissues during diabetes may be due to the inactivation or inhibition of the enzymes by the increased production of oxygen free radicals during diabetes [28, 29]. In our study, on prior treatment with *Terminalia arjuna* bark extract, lipid peroxidation levels are found to decrease significantly, whereas the activity of antioxidant enzymes was found to increase further on pretreatment with *Terminalia arjuna* bark extract, indicating that *Terminalia arjuna* reduces oxidative stress to RBCs. The possible source of oxidative stress during hyperglycemic condition includes shifts in redox balance resulting from altered carbohydrate and lipid metabolism, increased generation of reactive oxygen species, and decreased level of antioxidant defenses such as GSH [23]. The exact mechanism involved in the activity of *Terminalia arjuna* is not yet known. In the present study, an increase in the level of TBARS and enzymatic antioxidants were observed in blood samples treated with high concentrations of glucose.

The observed results in the present study demonstrate the occurrence of oxidative damage to RBC membranes due to hyperglycemia. The observed increase in lipid peroxidation levels in RBCs are in agreement with similar findings in rat tissues in earlier studies [30]. Lipid peroxidation may bring about protein damage and inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, malondialdehyde and 4-hydroxynonenal [31]. Glucose was known to induce lipid peroxidation through activation of the lipoxygenase enzymes [32]. An increased level of TBARS is an index of lipid peroxidation. The present study shows that aqueous bark extract of *Terminalia arjuna* bark extract tends to bring TBARS levels in blood back to near normal, which was increased earlier on exposure to high glucose concentrations. Increased lipid peroxidation under diabetic condition can be due to the increased oxidative stress in the cells as a result of depletion of antioxidants scavenger systems as reported [33]. It was also shown that supplementation of *Terminalia arjuna* bark extract in the diet enhances the antioxidant potential in control and in diabetic rats [34].

In the present study, *Terminalia arjuna* bark extract treatment was observed to cause significant decrease in lipid peroxidation. The activities of the antioxidant enzymes, SOD and catalase were observed to increase in glucose treated SRBCs as compared to untreated SRBCs. Higher levels of lipid peroxides and increased SOD and catalase activity are indicative of an oxidative stress condition. H_2O_2 is toxic by itself and can be a precursor to other toxic species. It can react with $O2^{\bullet -}$ to form $OH^{\bullet}$ and result in increased lipid peroxidation and hence higher TBARS level [35]. This suggests the antioxidant potential of *Terminalia arjuna* bark extract which may involve some mechanism related to ROS scavenging activity. Thus, from the results obtained in the present study it can be inferred that *Terminalia arjuna* bark extract have potent antioxidant properties. The evidence that *Terminalia arjuna* bark extract can prevent oxidative stress needs to be explored further at the clinical level to determine whether supplementation can lower levels of protein glycosylation, circulating glucose levels and oxidative stress and thereby reduce the incidence of vascular disease in the diabetic patient population.

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