

PROTECTIVE EFFECT OF AQUEOUS BARK EXTRACT OF *TERMINALIA ARJUNA* ON Cu^{2+} -ASCORBATE INDUCED OXIDATIVE STRESS *IN VITRO*: INVOLVEMENT OF ANTIOXIDANT MECHANISM(S)

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ABSTRACT

Cu^{2+} -ascorbate induced oxidative stress in goat red blood cell- an established model of oxidative stress *in vitro* was used for the present investigation. *Terminalia arjuna* (TA) an important medicinal plant has been found to be of therapeutic benefit in the treatment of cardiovascular diseases and reactive oxygen species (ROS) play a critical role in the pathogenesis of various cardiovascular diseases. The cardioprotective effects of TA are thought to be caused by the antioxidant nature of several of the constituents of TA extract. In a preliminary attempt to characterize the antioxidant properties of TA, our present studies indicate that aqueous TA bark extract is capable of protecting against oxidative stress as is evident from its effect on the biomarkers of oxidative stress, the antioxidant enzymes and the ROS scavenging activities. Aqueous TA bark extract decreased the level of lipid peroxidation, increased the reduced glutathione content and decreased the protein carbonyl content in Cu^{2+} -ascorbate treated RBCs. The activities of antioxidant enzymes, catalase and superoxide dismutase (SOD), were also found to be protected by this aqueous bark extract. Aqueous bark extract of TA was found to scavenge hydroxyl radical in a chemically defined system. It also exhibited superoxide anion radical scavenging activity.

Keywords: *Terminalia arjuna*, Oxidative stress, Copper-ascorbate, Red blood cell membrane, Antioxidant.

INTRODUCTION

Terminalia arjuna (TA) belonging to the family Combretaceae, is an important medicinal plant widely used by the tribal and rural people for alleviating angina and other cardiovascular conditions for over three centuries in India. Experimental studies have revealed that the stem bark exerts significant inotropic and hypotensive effect, increasing coronary artery flow and protecting myocardium against ischemic damage.¹ It has also been detected to have mild diuretic, antithrombotic, prostaglandin E_2 enhancing and hypolipidemic activity.² Considering its antiischemic activity and its potential to correct dyslipidemia, reduce left ventricular mass and increase left ventricular ejection fraction, it becomes essential to examine the mechanism of its protective function. Since Reactive Oxygen Species (ROS) is thought to play a critical role in the pathogenesis of various cardiovascular diseases, it may be presumed that the cardioprotective effects of TA might be exerted through an antioxidant mechanism. Thus, in order to elucidate the mechanism of cardioprotection provided by aqueous bark extract of TA, the present study was aimed at exploring the antioxidant potential of the aqueous bark extract of *Terminalia arjuna in vitro*.

In the present study the aqueous bark extract of TA was prepared in a manner that is followed by tribals and rural people for their consumption as medicine.

Red blood cells 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 ROS. Due to their susceptibility to oxidation red blood cells are often used as cellular models to investigate oxidative damage. Moreover, copper-ascorbate is an established model to induce oxidative stress *in vitro*.³ Hence, to explore the antioxidant potential of aqueous bark extract of TA, *in vitro*, copper-ascorbate induced oxidative stress in goat red blood cells was used as the model of oxidative stress.

MATERIALS AND METHODS

Chemicals used

Powder of bark *Terminalia arjuna* (TA) was purchased from Herby House, Kolkata. 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 of *Terminalia arjuna*

Terminalia arjuna bark powder was dissolved in distilled water, kept for 15 minutes, centrifuged at 2500rpm for 10 minutes and the supernatant was collected and used for the present study.

Processing of goat blood to obtain whole red blood cells

Goat blood was collected in Acid Citrate Dextrose buffer. Packed red blood cells were obtained by centrifugation at 3000rpm for 10 minutes at 4°C. The plasma and the buffy coat were removed by aspiration and the red cells thus obtained were washed thrice with 0.9% NaCl solution.

In vitro incubation

2ml of 50% suspension of washed erythrocytes were incubated with different concentrations of TA, 0.2mM Cu^{2+} and 1mM ascorbic acid in a shaking waterbath at 37°C for 1 hour. The incubation was terminated by addition of 80 μl of 35mM EDTA and the Cu^{2+} -ascorbate treated red blood cells were washed thrice with 0.9% NaCl solution.

Measurement of biomarkers of oxidative stress in goat red blood cells (RBCs)

The level of lipid peroxidation in the goat red blood cells were measured in terms of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust.⁴ 2ml 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 80°C. The absorbance of the sample was determined at 532nm. The malondialdehyde concentration of the sample was calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{Cm}^{-1}$.

The reduced glutathione content of erythrocytes was determined spectrophotometrically at 412nm using 5,5'- dithio-bis-(2-nitro

benzoic acid) (DTNB) according to the method of Sedlak and Lindsay.⁵

The protein carbonyl content of erythrocytes was determined according to the method of Reznick et al.⁶ as modified by Bandyopadhyay et al.¹⁹ The sample was incubated with 10mM DNPB 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 was collected and the absorbance was determined at 375nm.

Measurement of antioxidant enzymes

Cu-Zn superoxide dismutase was measured by the method of Martin⁷ as modified by Das et al.²¹ To extract Cu-Zn SOD, the hemoglobin present in the hemolysate was removed according to the method of Mc Cord and Fridovich.⁸ The hemolysate was prewarmed at 37°C 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 37°C for 15 minutes with occasional stirring. The mixture was then centrifuged and the colorless supernatant thus obtained was used for the SOD assay.

Catalase activity was measured by following the rate of decomposition of H₂O₂ at 240nm by the method of Beers and Sizer.⁹

Determination of ROS scavenging activity in a chemically defined system

OH[•] radical was generated in sodium phosphate buffer (0.05mM, pH 7.4) with 1mM ascorbate and 0.2mM Cu²⁺ for 60 minutes in the presence and absence of DMSO (500µM) and different concentrations of aqueous *Terminalia arjuna* bark extract in a volume of 1ml to determine the hydroxyl radical scavenging activity of the aqueous bark extract of *Terminalia arjuna* in an *in vitro* system. The reaction was terminated in each case by the addition of 0.1mM EDTA. Methanesulfinic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner¹⁰ as modified by Bandyopadhyay et al.²⁰

Deoxyribose degradation assay was performed according to the method of Halliwell and Gutteridge¹¹ as modified by Chattopadhyay et al.²² In brief, deoxyribose (3mM) was incubated with 200 µM CuCl₂ and 1mM ascorbic acid buffered with 50mM phosphate buffer (pH 7.4) in a total volume of 1mL with or without the aqueous bark extract of *Terminalia arjuna* for 60 minutes. The reaction was terminated by the addition of 2ml TBA-TCA-HCl reagent (0.375% TBA, 15% TCA and 0.25(N) HCl) and the mixture heated at 100°C for 15 minutes. The absorbance of the pink chromogen formed was measured at 532nm.

Superoxide scavenging activity was studied by following the rate of epinephrine oxidation in alkaline pH at 480nm.¹² The reaction mixture had in a volume of 1ml, 50mM Tris-HCl buffer (pH 10), 0.6mM epinephrine and different concentrations of aqueous *Terminalia arjuna* bark extract. The increase in absorbance due to the formation of the adrenochrome was followed for 7 minutes and the activity was calculated from the linear part in absence and

presence of aqueous *Terminalia arjuna* bark extract. The involvement of superoxide was checked with standard SOD.

H₂O₂ scavenging activity was assayed by studying the breakdown of H₂O₂ at 240nm.¹³ The reaction mixture contained 50mM phosphate buffer (pH 7.4), 10,000µM H₂O₂ and 62.5 µM, 125µM, 250 µM and 500µM aqueous bark extract of *Terminalia arjuna* in a final volume of 3ml.

Quantitative estimation of phytoconstituents of aqueous extract of bark of *Terminalia arjuna*

Total phenolics, flavonoids and proanthocyanidins were determined according to the methods of Singleton et al.¹⁴, Dewanto et al.¹⁵ and Broadhurst et al.¹⁶ respectively.

Measurement of protein content

The protein concentration of the samples was estimated according to the method of Bradford¹⁷ and/or Lowry et al.¹⁸

Statistical analysis

Data were expressed as Mean ± SE. Microcal Origin Version 7.0, a computer program was used to compute statistical analysis. 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.

RESULTS

Effect of aqueous *Terminalia arjuna* bark extract on biomarkers of oxidative stress in goat red blood cells

The level of lipid peroxidation in the oxidative stress induced red blood cells were measured in terms of thiobarbituric acid reactive substances (TBARS) in presence and absence of different concentrations of aqueous *Terminalia arjuna* bark extract and is shown in Table 1. The level of LPO was found to be significantly increased by 81.85% (p ≤ 0.001 Vs Control) in the red blood cells incubated with copper-ascorbate. However, aqueous *Terminalia arjuna* bark extract at increasing concentrations (10mg, 20mg, 30mg and 40mg/ ml incubation mixture) was found to decrease the level of LPO in copper-ascorbate incubated red blood cells. Aqueous *Terminalia arjuna* bark extract alone has no significant effect on lipid peroxidation.

The effect of different concentrations of aqueous *Terminalia arjuna* bark extract on the reduced glutathione content of oxidative stress induced red blood cells, *in vitro*, by copper-ascorbate treatment is depicted in Table 1. Copper-ascorbate treatment decreased the glutathione content of red blood cells by 81.28% (p ≤ 0.001) when compared to control red blood cells. Aqueous *Terminalia arjuna* bark extract at increasing concentrations was found to reduce this decrease in GSH content. A complete protection of this decrease in reduced glutathione was observed at a concentration of 40mg/ml incubation mixture of aqueous *Terminalia arjuna* bark extract.

The protein carbonyl content of oxidative stress induced red blood cells were found to be significantly increased by 85.34% (p ≤ 0.001) (Table 1). However, aqueous *Terminalia arjuna* bark extract at increasing concentrations was found to significantly reduce the protein carbonyl content in copper-ascorbate incubated red blood cells

Table 1: Effect of aqueous *Terminalia arjuna* bark extract on biomarkers of oxidative stress in goat red blood cells.

Groups	LPO Level (nmoles TBARS/ mg of protein)	% Control	GSH content (nmoles of GSH/ mg of protein)	% Control	Protein carbonyl content (nmoles/ mg of protein)	% Control
Control RBC	0.151 ± 0.006	100.0	1.300 ± 0.058	100	1.637 ± 0.065	100
Cu ²⁺ - ascorbate treated RBC	0.274 ± 0.001*	181.0	0.243 ± 0.003*	18.7	3.033 ± 0.013*	184.76
Cu ²⁺ - ascorbate treated RBC + TA 10(mg/ml)	0.256 ± 0.001	169.5	0.277 ± 0.003	21.3	2.687 ± 0.029	164.02
Cu ²⁺ - ascorbate treated RBC + TA 20(mg/ml)	0.201 ± 0.001	133.0	0.607 ± 0.043	46.7	2.410 ± 0.015	146.95
Cu ²⁺ - ascorbate treated RBC + TA 30(mg/ml)	0.186 ± 0.002	123.2	0.970 ± 0.023	74.6	2.033 ± 0.044	124.54
Cu ²⁺ - ascorbate treated RBC + TA 40(mg/ml)	0.156 ± 0.0003**	103.0	1.267 ± 0.033**	97.7	1.620 ± 0.060**	99.38

Data were analysed by one way ANOVA. *P < 0.001 compared to control group; **P < 0.001 compared to Cu²⁺-ascorbate treated group

Effect of aqueous Terminalia arjuna bark extract on the activities of antioxidant enzymes of oxidative stress induced goat red blood cells

Increased SOD activity (170% Vs Control) was observed in oxidative stress induced red blood cells. This increase in SOD activity was prevented with increasing concentrations of aqueous Terminalia arjuna bark extract. At a concentration of 40mg/ml, the aqueous

Terminalia arjuna bark extract completely prevented the increase of SOD activity [Figure 1(A)].

Catalase activity in oxidative stress induced red blood cells decreased by 39% as is evident from Figure 1(B). This reduction in activity was prevented with increasing concentrations of aqueous Terminalia arjuna bark extract. Complete restoration of catalase activity was obtained with 30mg/ml of aqueous Terminalia arjuna bark extract.

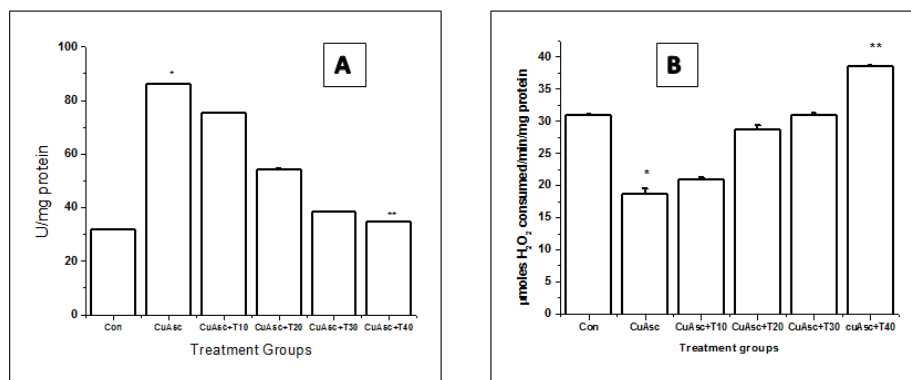


Fig 1: Effect of aqueous Terminalia arjuna bark extract on the activities of antioxidant enzymes of oxidative stress induced goat red blood cells: SOD (a) and catalase (b)

Effect of aqueous Terminalia arjuna bark extract on the ROS scavenging activity in vitro

The hydroxyl radical scavenging ability of aqueous Terminalia arjuna bark extract was studied in an in vitro standard model system using Cu²⁺ and ascorbic acid where OH[•] was generated. Table 2 indicated that Cu²⁺-ascorbic acid produced 442nmoles OH[•]/ml incubation mixture and aqueous Terminalia arjuna bark extract directly scavenged OH[•] in a concentration dependent manner exhibiting about 92% scavenging activity at a concentration of 20mg/ml aqueous Terminalia arjuna bark extract.

The hydroxyl radical scavenging activity was further confirmed by using the Deoxyribose degradation assay (Table 2). In this study

24.78%, 58.95% and 88.23% of scavenging activity were observed with 5mg, 10mg and 20mg/ml of the aqueous Terminalia arjuna bark extract.

The effect of aqueous Terminalia arjuna bark extract on the superoxide scavenging ability was studied by following the rate of superoxide mediated epinephrine oxidation (Table 3). Increasing concentrations of aqueous Terminalia arjuna bark extract altered the rate of superoxide mediated epinephrine oxidation indicating a scavenging ability of O₂^{•-}. About 71% scavenging activity was observed at a concentration of 400µg/ml of aqueous Terminalia arjuna bark extract which is highly significant.

Table 2: Hydroxyl Radical Scavenging ability of aqueous bark extract of Terminalia arjuna in vitro.

Groups	Hydroxyl radical scavenging activity (nmoles of OH [•] generation)	% Control	Deoxyribose degradation Absorbance at 532nm	% Control
Control	442.14 ± 2.06	100.00	0.967 ± 0.005	100.00
Control + TA 2 (mg/ml)	395.32 ± 1.05	89.41	0.867 ± 0.019	89.47
Control + TA 5 (mg/ml)	296.13 ± 2.24	66.98	0.729 ± 0.028	75.31
Control + TA 10 (mg/ml)	103.58 ± 0.41	23.43	0.388 ± 0.013	40.08
Control + TA 20 (mg/ml)	31.07 ± 0.62*	7.02	0.114 ± 0.006*	11.78

Data were analysed by one way ANOVA; *P < 0.001 compared to control group

Table 3: Effects of aqueous bark extract of Terminalia arjuna on superoxide anion radical generation and H₂O₂ scavenging.

Groups	Superoxide scavenging activity (Epinephrine autooxidation rate) (∂ OD/ min)	% Control	H ₂ O ₂ (∂ OD/ min) at 240nm
Control	0.042 ± 0.0006	100.00	0.790
Control + TA	0.035 ± 0.000	82.44	0.790
Control + TA	(50 µg/ml TA)		(62.5 µM TA)
Control + TA	0.028 ± 0.00005	67.74	0.795
Control + TA	(100 µg/ml TA)		(125 µM TA)
Control + TA	0.023 ± 0.001	54.36	0.793
Control + TA	(200µg/ml TA)		(250 µM TA)
Control + TA	0.012 ± 0.0002*	28.32	0.792*
Control + TA	(400 µg/ml TA)		(500 µM TA)

Data were analysed by one way ANOVA. *P < 0.001 compared to control group

The H₂O₂ scavenging activity of the aqueous bark extract of Terminalia arjuna, if any, was also tested in vitro by studying the breakdown of H₂O₂ at 240nm. Table 3 clearly indicates that the

aqueous bark extract of Terminalia arjuna does not possess any H₂O₂ scavenging activity.

Superoxide mediated epinephrine oxidation is significantly stimulated by transition metals such as Cu²⁺. However, aqueous bark extract of *Terminalia arjuna* did not inhibit metal ion stimulated epinephrine oxidation, suggesting that it has no metal chelating effect (Table 4).

Table 4: Effect of aqueous bark extract of *Terminalia arjuna* on the metal induced epinephrine autooxidation *in vitro*.

Groups	Metal chelating activity (θ OD/min)
Control	0.044 ± 0.0005
Control + Cu ²⁺	0.069 ± 0.0015
Control + Cu ²⁺ + TA 50 (µg/ml)	0.075 ± 0.0022
Control + Cu ²⁺ + TA 100 (µg/ml)	0.078 ± 0.0026
Control + Cu ²⁺ + TA 200 (µg/ml)	0.085 ± 0.0032*

Data were analysed by one way ANOVA.

*P < 0.001 compared to control group

Quantitative estimation of phytoconstituents of aqueous extract of bark of *Terminalia arjuna*

Table 5 shows that the aqueous extract of TA prepared by the procedure as described above contains total phenolics flavonoids and proanthocyanidins. The quantity of total proanthocyanidins was found to be more than 5 fold higher compared to total flavonoid content in our experimental conditions.

Table 5: Quantitative estimation of phytoconstituents of aqueous bark extract of *Terminalia arjuna*.

Phytoconstituents	Value (mg/g)
Total phenolics content	60.00 ± 1.155
Total flavonoids content	1.62 ± 0.008
Total proanthocyanidins content	8.46 ± 0.009

DISCUSSION

Tribal and rural people of India still heavily depend on traditional medicine for their health care despite the penetrating progress in modern allopathic medicine. Among the traditional medicines, the use of aqueous bark extract of *Terminalia arjuna* for cardiovascular diseases occupy a significant position. The importance of the present study lies in the fact that the aqueous bark extract of *Terminalia arjuna* have been prepared in a manner that is followed by the tribals and rural people for their consumption as medicine for their cardiovascular problems.

Cu²⁺-ascorbate is an established model of oxidative stress *in vitro*.³ A compound might exert its antioxidant actions by inhibiting generation of ROS or by directly scavenging the free radicals. Additionally, an antioxidant may act by raising the level of the endogenous antioxidant defenses. Our present studies clearly indicate that aqueous *Terminalia arjuna* bark extract is capable of protecting against oxidative stress as is evident from its effect on lipid peroxidation, reduced glutathione content, protein carbonyl content and the activities of the antioxidant enzymes like catalase and superoxide dismutase of goat red blood cells, thereby acting as an indirect antioxidant. Additionally, the aqueous bark extract of *Terminalia arjuna* exhibited a strong hydroxyl radical as well as superoxide anion radical scavenging potential hence acting as a direct antioxidant. However, aqueous *Terminalia arjuna* bark extract did not exhibit any H₂O₂ scavenging activity and metal ion chelating effect.

Thus, these studies suggest that this aqueous bark extract of *Terminalia arjuna* do possess a strong antioxidant potential and it is unique in specifically scavenging OH[•] and superoxide anion radical. Many plants with traditional medicinal importance are known to possess antioxidant property because of its numerous chemical constituents. It has been found that flavonoids, tannins, coumarins, xanthenes, proanthocyanidins etc. are radical scavengers and viewed as promising future therapeutic drugs for free radical mediated pathological conditions. Since this preparation of aqueous bark extract of *Terminalia arjuna* have been found to be rich in phenolics, flavonoids and proanthocyanidins and since the results

of our present study indicate that the aqueous bark extract of *Terminalia arjuna* do possess a strong antioxidant potential, we may presume that this antioxidant property may be due to the presence of these numerous chemical constituents which is either acting singly or in combination with synergistic responses. Further, studies relating to the identification of the chemical constituents involved in exhibiting the property of this aqueous bark extract of *Terminalia arjuna* are in progress.

In conclusion, it can be said that the present studies reveal that aqueous bark extract of *Terminalia arjuna* has the ability to provide protection to red blood cells against copper-ascorbate induced oxidative stress *in vitro* which is evident from the measurement of oxidative stress biomarkers and the status of the activities of the antioxidant enzymes of goat red blood cells and ROS scavenging ability *in vitro* in a chemically defined system. We conclude that this aqueous extract can provide protection against oxidative stress through antioxidant mechanism(s) due to presence of various bioactive compounds such as phenolics, flavonoids, proanthocyanidins etc.

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