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Research Article

PROTECTIVE EFFECT OF AQUEOUS BARK EXTRACT OF *TERMINALIA ARJUNA* AGAINST COPPER-ASCORBATE INDUCED OXIDATIVE STRESS *IN VITRO* IN GOAT HEART MITOCHONDRIA

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ABSTRACT

Objective: The present study is aimed at investigating the effect of aqueous bark extract of *Terminalia arjuna* (TA) against Cu^{+2}-ascorbate induced oxidative stress in goat heart mitochondria *in vitro.* Methods: Fresh goat heart mitochondria was prepared and oxidative stress was induced *in vitro* with Copper-ascorbate. Biomarkers of oxidative stress, activities of antioxidant enzymes, Kreb's cycle enzymes and respiratory chain enzymes were determined using standard methods. Results: Treatment of copper-ascorbate treated mitochondria with aqueous bark extract of TA increased reduced glutathione (GSH) levels, reduced lipid peroxidation (LPO) levels and protein carbonyl (PCO) content. It also protects the activities of Mn-superoxide dismutase (Mn-SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) from being altered. Furthermore, it also restores the activities of pyruvate dehydrogenase (PDH), different Kreb's cycle enzymes and mitochondrial respiratory chain enzymes. Moreover, this extract also possesses antioxidant property as established by DPPH radical scavenging activity and reducing power.

Conclusion: The present study indicates that the aqueous bark extract of TA is capable of protecting the mitochondria against copper-ascorbate-induced oxidative stress *in vitro* and hence may be considered for future use as a therapeutic antioxidant intervention.

Keywords: *Terminalia arjuna*, Antioxidant property, Oxidative stress markers, Kreb's cycle enzymes, Mitochondrial respiratory chain enzymes.

INTRODUCTION

Reactive oxygen species (ROS) such as $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ 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].

In humans, oxidative stress is thought to be involved in the development of many diseases or may exacerbate their symptoms. 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]. With the central place in cell metabolism, damage — and subsequent dysfunction — in mitochondria is an important factor in a wide range of human diseases.

Mitochondria are the powerhouse of the cell, but there may be some leakage of the high-energy electrons in the respiratory chain to form reactive oxygen species. This can result in significant oxidative stress in the mitochondria with high mutation rates of mitochondrial DNA [13]. A vicious cycle is thought to occur, as oxidative stress leads to mitochondrial DNA mutations, which can lead to enzymatic abnormalities and further oxidative stress.

Since the available drugs for the treatment of various diseases may have serious side effects when used for longer period and since many antioxidants despite their usefulness may exhibit pro-oxidant activity, it becomes a necessity to turn our attention to alternative medicine with high efficacy and no or minimal side effects. The extracts or the isolated bioactive fractions from various parts of the herbs and plants with traditionally important medicinal value may serve as potentially good source of alternative medicine against these diseases. However, these need thorough scrutiny for safety in respect of human consumption apart from understanding their mechanism(s) of action and their bio-transformation.

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 [14]. Both aqueous and ethanolic extract of the bark of TA plays a protective role against sodium-fluoride-induced hepatic and cardiac oxidative stress [15, 16]. *Terminalia arjuna* bark extract protects DNA damage against adriamycin (ADR)-induced damage [17]. 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 [18]. Methanolic extract of TA protects against gastric ulcer in rats [14]. But there are no such evidences that aqueous extract of TA can protect the mitochondria against oxidative stress.

Herein, we provide evidences that the aqueous bark extract of TA has the capability to protect against Cu^{+2}- ascorbate induced oxidative stress *in vitro* in goat heart mitochondria and antioxidant mechanism(s) may be responsible for such protections. Cu^{2+} in presence of ascorbate at low concentrations (0.2mM-2.0mM) generates **.**OH radicals. Ascorbate by virtue of its metal reducing capacity generates $OH^{\cdot}$ together with H_2O_2 in presence of Cu^{2+} [19, 20, 21].

MATERIALS AND METHODS

Materials

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 of bark of TA and qualitative and quantitative determination of some of the phytoconstituents

5gm 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. Then the supernatant was collected and

lyophilized. The yield of the aqueous extract of *Terminalia arjuna* from 5gm of TA bark powder was 10%.

Various phytoconstituents such as tannins, cumarins, quinones, saponins and terpenoids were determined qualitatively according to the methods as adopted by Ramesh *et al* [22]. On the other hand, total phenolics, total phytosterols, flavonoids and condensed tannins were determined quantitatively according to the methods of Singleton *et al.* [23] Ramesh *et al.* [22] Dewanto *et al.* [24] and Broadhurst *et al.* [25] respectively.

Determination of antioxidant properties of aqueous extract of bark of TA

The DPPH free radical scavenging activity of each sample was determined according to the method described by Joyeux *et al.* [26]. A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm. An aliquot (40 µL) of an extract was added to 3 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured after 30 min. The antiradical activity (AA) was determined using the following formula:

AA% = 100 – [(Abs: sample – Abs: empty sample)] × 100)/Abs: control

The reducing power was determined according to the method of Oyaizu [27]. 2.5 ml of extract were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50^0C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1500g for 10 min. The upper layer (5 ml) was mixed with 5 ml deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Ascorbic acid was used as standard.

Preparation of goat heart mitochondria (Caprine heart mitochondria)

Goat heart mitochondria were isolated according to the procedure of Hare [28] with some modifications. Goat heart was purchased from local Kolkata Municipal Corporation approved meat shop. After collection it was brought into laboratory in an ice packed manner. Then the heart was cleaned and cut into pieces. 5gm of tissue was placed in 10ml of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H_2SO_4 (pH 7.8)] at 25^0C. Then the tissue was blended for 1 minute at low speed by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA), after which it was centrifuged at 1500rpm for 10 minutes. The supernatant was poured through several layers of cheesecloth and kept in ice. Then it was centrifuged at 4000rpm for 5minutes. The supernatant obtained was further centrifuged at 14000rpm for 20 minutes. The supernatant obtained was discarded and the pellet was resuspended in sucrose buffer and was stored at -20^0C for further assay.

The integrity and purity of isolated mitochondria was elucidated by determining the intactness of mitochondria. The intactness of mitochondria is usually established with enzyme assays that take advantages of the fact that an intact mitochondrial membrane is not permeable to certain molecules [29]. In the present study, we determined the intactness of the isolated mitochondria by measuring the activity of enzymes cytochrome C oxidase for outer membrane integrity and NADH cytochrome C oxidoreductase for inner membrane integrity according to the method of Goyal *et al.*[42].

Preparation of in vitro stress model: Incubation of mitochondria with Cu^{2+} and/or ascorbic acid

The incubation mixture containing membrane protein (1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), and 0.2 mM Cu^{2+} and/or 1 mM ascorbic acid in a final volume of 1.0 ml was incubated at 37 ° C in incubator for 1 hour. The reaction was terminated by the addition of 40µl of 35mM EDTA [30].

Protection of Cu^{2+}-ascorbate -induced mitochondrial oxidative stress by aqueous bark extract of TA

The copper-ascorbate incubated mitochondria were treated with four different concentrations of aqueous bark extract of TA- 5mg/ml, 10mg/ml, 15mg/ml and 20mg/ml. After treatment, the biomarkers of oxidative stress like reduced glutathione content, lipid peroxidation level and protein carbonyl content were determined.

Biochemical analysis

Measurement of biomarkers of oxidative stress

The lipid peroxides in the incubated mitochondria were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege *et al.* [31] with some modification as adopted by Bandyopadhyay *et al.* [32] Briefly, the incubated mitochondria was mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80^0C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 12,000g for 10 min at room temperature was measured at 532 nm using a UV–VIS spectrophotometer (Bio-Rad, Hercules, CA, USA). The values were expressed as nmols of TBARS/mg protein.

Reduced GSH content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak *et al.* [33] with some modifications by Bandyopadhyay *et al.* [32] Incubated mitochondria was mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer to determine the GSH content. The values were expressed as nmole GSH/ mg protein.

Protein carbonyl content was estimated by DNPH assay [34]. 0.25 ml of incubated mitochondria was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The tubes were vortexed every 10 min in the dark for 1 h. Proteins were then precipitated with 30% TCA and centrifuged at 4000g for 10 min. The pellet was washed three times with 1.0 ml of ethanol: ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of $2.2X\ 10^{-4}\ M^{-1}\ cm^{-1}$. The values were expressed as nmoles /mg protein.

Determination of the activities of antioxidant enzymes

Manganese superoxide dismutase (Mn-SOD or SOD2) activity was measured by pyrogallol autooxidation method [35].To 50 µl of the mitochondrial sample, 430 µl 50 mM of Tris–HCl buffer (pH 8.2) and 20 µl 2 mM of pyragallol were added. An increase in absorbance was recorded at 420 nm for 3 min in a UV/VIS spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyragallol as determined by change in absorbance/min at 420 nm. The enzyme activity was expressed as units/min/mg of tissue protein

The glutathione peroxidase activity was measured according to the method of Paglia *et al.* [36] with some modifications as adopted by Chattopadhyay *et al.* [37]. 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 H_2O_2. The linear decrease of absorbance at 340 nm was recorded using a UV/VIS spectrophotometer. The specific activity was expressed as Units/min/mg tissue protein.

The glutathione reductase enzyme assay was carried out according to the method of Krohne- Ehrich *et al.* [38]. 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 incubated mitochondria (enzyme) into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored at 340 nm. The specific activity of the enzyme was calculated as units/min/mg tissue protein.

Determination of the activities of pyruvate dehydrogenase and some of the key mitochondrial Kreb's cycle enzymes

Pyruvate dehydrogenase activity was measured spectrophotometrically according to the method of Chretien *et al.*

[39] with some modifications by following the reduction of NAD^+ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4, 0.5 mM sodium pyruvate as the substrate and 0.5 mM NAD^+ in addition to the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Isocitrate dehydrogenase activity was measured according to the method of Duncan *et al.* [40] by measuring the reduction of NAD^+ to NADH at 340 nm with the help of a UV–VIS spectrophotometer. One ml assay volume contained 50 mM phosphate buffer, pH 7.4, 0.5 mM isocitrate, 0.1 mM $MnSO_4$, 0.1 mM NAD^+ and the suitable amount of enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Alpha-Ketoglutarate dehydrogenase activity was measured spectrophotometrically according to the method of Duncan *et al.* [40] by measuring the reduction of 0.35 mM NAD^+ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4 as the assay buffer and 0.1 mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/min/mg tissue protein.

Succinate dehydrogenase activity was measured spectrophotometrically by following the reduction of potassium ferricyanide [$K_3Fe(CN)_6$] at 420 nm according to the method of Veeger *et al.* [41] with some modifications. One ml assay mixture contained 50 mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4 mM succinate, 2.5 mM $K_3Fe(CN)_6$ and a suitable aliquot of the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Determination of the activities of some of the mitochondrial respiratory chain enzymes

NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565 nm according to the method of Goyal *et al.* [42] 1ml of assay mixture contained in addition to the enzyme, 50 mM phosphate buffer, 0.1 mg BSA, 20 mM oxidized cytochrome c and 0.5 (M) NADH. The activity of the enzyme was expressed as units/min/mg tissue protein.

Cytochrome c oxidase activity was determined spectro-photometrically by following the oxidation of reduced cytochrome c at 550 nm according to the method of Goyal *et al.* [42] One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c and a suitable aliquot of the enzyme. The enzyme activity was expressed as units/min/mg tissue protein.

Prevention of Cu^{2+}-ascorbate -induced mitochondrial oxidative stress by aqueous bark extract of TA

The copper-ascorbate incubated mitochondria were pre-treated with two different concentrations of aqueous bark extract of TA-10mg/ml and 20mg/ml. After treatment, the pyruvate dehydrogenase activity, isocitrate dehydrogenase activity, α-ketoglutarate dehydrogenase activity and succinate dehydrogenase activity were determined according to the method of Chretien *et al.* [39], Duncan *et al.* [40], Veeger *et al.* [41] respectively.

Estimation of protein

The protein content of the different samples was determined by the method of Lowry *et al.* [44]

Statistical evaluation

Each experiment was repeated at least three times. 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. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Table 1 depicts the qualitative estimation of various phytoconstituents in the aqueous bark extract of TA, which shows that tannins, cumarins and terpenoids are present whereas quinones and saponins are absent. Among the phytoconstituents determined, tannins could be quantified and it amounts to about 11.2mg/gm of aqueous TA bark extract. Due to non-availability of ZORBAX Eclipse XDB C8 column for terpenoids and SUPELCOSIL LC 18 column for cumarins, terpenoids and cumarins could not be estimated.

Table 1: Qualitative estimation of various phytoconstituents in *T. arjuna* aqueous bark extract

Phytoconstituents	Present/Absent
Tannins	Present (11.2 mg/gm)
Cumarins	Present
Quinones	Absent
Saponins	Absent
Terpenoids	Present

On the other hand, Figure 1 shows the concentration of total phenolics, total phytosterols, flavonoids and also condensed tannins in the unit of mg/gm of the aqueous TA bark extract. The extract was found to contain high amount of total phenolics.

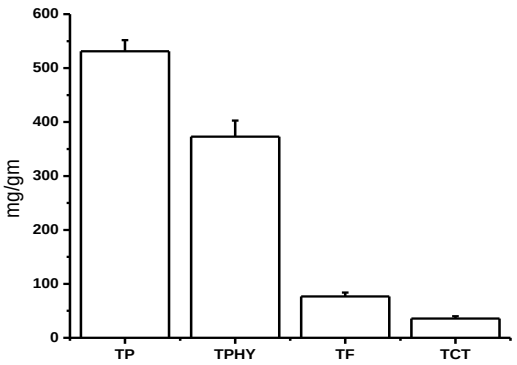

Fig. 1: Concentrations of various phytoconstituents (mg/gm) in aqueous bark extract of TA

DPPH free radical has been widely accepted as a model compound to evaluate the antioxidant abilities of various samples. This assay indicates that the aqueous extract of bark of TA showed dose-dependent DPPH radical scavenging activity. Figure 2A, shows that TA extract exhibited 8.87 ± 0.03 % inhibition of DPPH activity at the dose of 12.5mg/ml whereas ascorbic acid (used as standard) showed this value as 17.19 ± 0.02 at the same dose. Reducing power is to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample extracts. The reducing power of TA extracts is summarized in Figure 2B. From the figure, it is evident that the reducing power increased with increase in extract concentration. The data showed that all samples increased their reducing ability when the concentrations of extracts were increased.

Table 2, showed a significant increase in cardiac mitochondrial LPO level following the treatment of mitochondria with CuAs (35.33%, *P ≤ 0.001 vs. control). This elevated level of lipid peroxidation products were found to be decreased significantly (37.17% from CuAs-treated group, **P ≤ 0.001) when the mitochondria were co-treated with CuAs and TA extract (20mg/ml), indicating the ability of this aqueous extract to protect the mitochondria against oxidative stress-induced changes due to copper-ascorbate. On the other hand, significant decrease was observed in cardiac mitochondrial reduced GSH content following the treatment of mitochondria with CuAs (23.12%, *P ≤ 0.001 vs. control). This decreased level of reduced GSH content was found to be increased significantly (33.52% from CuAs-treated group, **P ≤ 0.001) when the mitochondria were co-treated with CuAs and TA extract (20mg/ml). The protein carbonyl assay showed a significant increase in cardiac mitochondrial protein carbonyl content following the treatment of mitochondria with CuAs (64.78%, *P ≤ 0.001 vs. control). This elevated level of protein carbonyl content was found to be decreased significantly (33.67% from CuAs-treated group, **P ≤ 0.001) when the mitochondria were co-treated with CuAs and TA extract (20mg/ml).

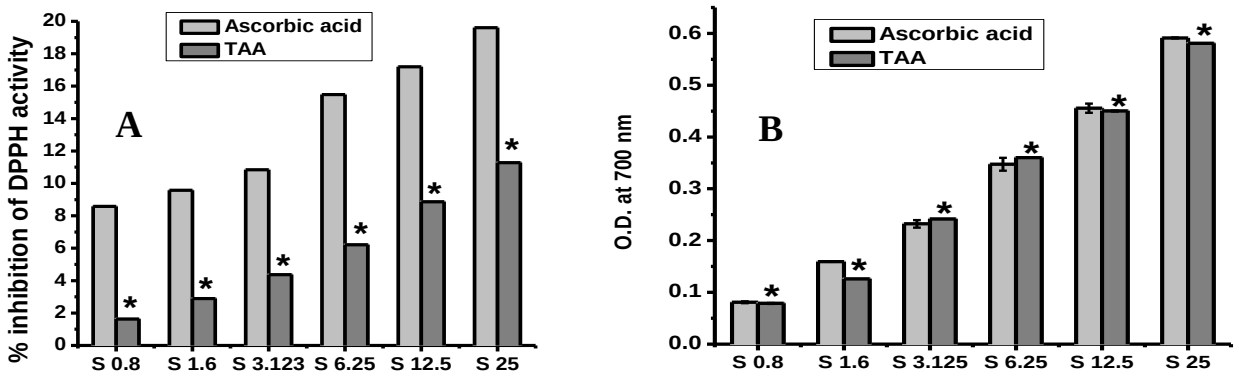

Fig. 2: (A) DPPH Radical Scavenging activity and (B) reducing power activity of aqueous bark extract of TA; the values are expressed as mean ± S.E.M; *P ≤ 0.001 compared to ascorbic acid (standard) values using ANOVA.

Table 2: Protective effect of aqueous bark extract of *T. arjuna* against copper-ascorbate induced alteration in the biomarkers of oxidative stress in goat heart mitochondria

Groups	LPO level (nmol TBARS/ mg of protein)	GSH (nmole GSH/ mg of protein)	Protein carbonyl (nmoles/ mg of protein)
Control	14.69 ± 0.9	182.52 ± 3.0	3.01 ± 0.0
CuAs	19.88 ± 1.0*¶	140.33 ± 1.8*¶	4.96 ± 0.2 *¶
T 5	14.00 ± 0.2	182.01 ± 2.0	3.25 ± 0.6
T 10	14.28 ± 0.1	182.84 ± 4.1	3.66 ± 0.0
T 15	14.59 ± 0.2	182.90 ± 2.4	3.72 ± 0.2
T 20	14.92 ± 0.2	185.17 ± 3.7	3.90 ± 0.1
CuAs-T 5	19.42 ± 1.0	142.94 ± 2.7	4.87 ± 0.0
CuAs-T 10	18.34 ± 1.2	159.44 ± 5.1	4.44 ± 0.0
CuAs-T 15	15.85 ± 1.2	172.54 ± 4.8	3.67 ± 0.1
CuAs-T 20	12.49 ± 0.6 *#§	187.37 ±3.2 *#§	3.29 ± 0.0 *#§

CuAs = copper-ascorbate treated group; CuAs-T5-20= group co-treated with copper-ascorbate and TA at the dose of 5-20mg/ml respectively; The values are expressed as Mean ± S.E.M; * P ≤ 0.001; ¶ As compared to control values using ANOVA; # As compared to CuAs-treated values using ANOVA; § As compared to control values using ANOVA.

Our dose-dependent experiments have revealed 20mg/ml of TA extract to be the best effective dose. Therefore, subsequent experiments were carried out with this best effective dose of TA bark extract.

Figure 3A, reveals a highly significant increase (58.05%,*P ≤ 0.001 vs. control group) in the activity of Mn-SOD following treatment of mitochondria with CuAs. The activity of this enzyme was found to be protected from being increased when the mitochondria were co-treated with CuAs and TA extract. TA extract decreased Mn-SOD activity 30.00 %(**P ≤ 0.001 vs. CuAs-treated group) at the dose of 10mg/ml. Figure 3B, reveals a highly significant decrease (40.34%,*P ≤ 0.001 vs. control group) in the activity of GPx following treatment of mitochondria with CuAs. The GPx activity was protected from this decrease when the mitochondria were co-treated with CuAs and TA extract. TA extract increased GPx activity 42.48 %(**P ≤ 0.001 vs. CuAs-treated group) at the dose of 10mg/ml. The aqueous extract, by itself, has no effect on the activity of GPx. On the other hand figure 3C, depicts a highly significant decrease (38.78%,*P ≤ 0.001 vs. control group) in the activity of GR following treatment of mitochondria with CuAs. The GR activity was protected from this decrease when the mitochondria were co-treated with CuAs and TA extract. TA extrct increased GR activity 39.78 %(**P ≤ 0.001 vs. CuAs-treated group) at the dose of 10mg/ml. The results indicate that the aqueous extract may have an influence on the GSH biosynthesis.

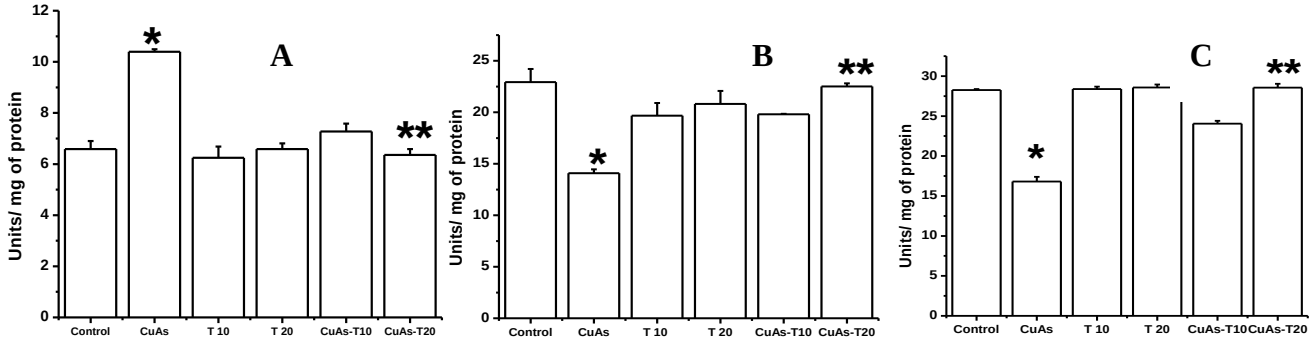

Fig. 3: Protective effect of aqueous bark extract of TA against CuAs-induced (A) increased Mn-SOD activity, (B) decrease in glutathione peroxidase activity and (C) decrease in glutathione reductase activity of goat cardiac mitochondria; CuAs = copper-ascorbate treated group; CuAs-T10= group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 10mg/ml; CuAs-T20= group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 20mg/ml; the values are expressed as Mean ± S.E.M; *P ≤ 0.001 compared to control values using ANOVA; **P ≤ 0.001 compared to CuAs-treated values using ANOVA.

Table 3, reveals that the treatment of the mitochondria with CuAs inhibits cardiac mitochondrial pyruvate dehydrogenase activity (30.65%, *P ≤ 0.001 vs. control). When the mitochondria were co-treated with CuAs and TA extract, the activity of the enzyme, however, was found to be significantly protected compared to the activity observed in the CuAs-treated group (23.26%, **P ≤ 0.001 vs. CuAs-treated group) at the dose of 20mg/ml. Isocitrate dehydrogenase activity assay reveals that treatment of the mitochondria with CuAs inhibits isocitrate dehydrogenase activity (20.84%,*P ≤ 0.001 vs. control). Isocitrate dehydrogenase is a key enzyme in cellular defense against oxidative damage as it provides NADPH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin. The activity of the enzyme was found to be completely protected when mitochondria were treated with TA extract at the dose of 20 mg/ml (28.25%, **P ≤ 0.001 vs. CuAs-treated group). Treatment of mitochondria with CuAs inhibits alpha keto glutarate dehydrogenase activity (57.75%, *P ≤ 0.001 vs. control). This enzyme was found to be able to generate ROS during its catalytic function, which is regulated by the NADH/NAD^+ ratio. The activity of the enzyme was found to be significantly protected when the mitochondria were treated with 10 mg/ml dose of the TA extract (60.04%, **P ≤ 0.001 vs. CuAs-treated group). The succinate dehydrogenase activity assay reveals that treatment of mitochondria with CuAs inhibits succinate dehydrogenase activity (33.69%,*P ≤ 0.001 vs. control). This might result in interference of the metal in electron transport chain (ETC) and thus generate copious amounts of superoxide anion radicals in the cardiac mitochondria. The enzyme activity was found to be significantly protected when the mitochondria were co-treated with 10 mg/ml dose of the TA extract (30.65%, **P ≤ 0.001 vs. CuAs-treated group).

Table 3: Protective effect of aqueous bark extract of *T. arjuna* against copper-ascorbate induced alteration in the pyruvate dehydrogenase and other Kreb's cycle enzymes in goat heart mitochondria

Groups	Pyruvate dehydrogenase (Units/mg of protein)	Isocitrate dehydrogenase (Units/ min/mg of protein)	α-ketoglutarate dehydrogenase (Units/ min/mg of protein)	Succinate dehydrogenase (Units/ min/mg of protein)
Control	233.83 ± 54.68	247.23 ± 1.11	131.26 ± 1.37	1441.9 ± 16.3
CuAs	159.71 ± 28.21*¶	194.31 ± 7.51*¶	56.19 ± 4.16 *¶	956.89 ± 3.9 *¶
T 10	233.32 ± 32.4	247.87 ± 4.7	131.55 ± 1.8	1441.00 ± 8.6
T 20	233.51 ± 20.5	247.15 ± 3.8	131.33 ± 2.1	1441.22 ± 3.1
CuAs-T 10	192.35 ± 41.09	220.66 ± 2.02*#	87.77 ± 1.78*#	1253.62 ± 6.7*#
CuAs-T 20	205.26 ± 23.64 *#§	250.58 ± 1.69§	100.01 ± 1.26§	1323.32 ± 12.4§

CuAs = copper-ascorbate treated group; CuAs-T10 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 10mg/ml; CuAs-T20 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 20mg/ml; The values are expressed as Mean ± S.E.M; * P ≤ 0.001; ¶ As compared to control values using ANOVA; # As compared to CuAs-treated values using ANOVA; § As compared to control values using ANOVA.

Table 4, shows that the treatment of the mitochondria with copper-ascorbate inhibits NADH Cytochrome c oxidoreductase activity (23.67%,*P ≤ 0.001 vs. control). However, the enzyme activity was found to be completely protected when the mitochondria were co-treated with 10 mg/ml of TA extract (31.62%, **P ≤ 0.001 vs. CuAs-treated group). Cytochrome c oxidase activity assay reveals that treatment of mitochondria with Copper-ascorbate also inhibits cytochrome c oxidase activity (61.71% decrease, *P ≤ 0.001 vs. control). The activity of this enzyme was also found to be significantly protected (106.80% increase, **P ≤ 0.001 vs. CuAs-treated group) when the mitochondria were pre-treated with 10 mg/ml dose of the TA extract.

Table 5, reveals that the treatment of the mitochondria with CuAs inhibits cardiac mitochondrial pyruvate dehydrogenase activity (31.70%, *P ≤ 0.001 vs. control), isocitrate dehydrogenase activity (21.41%,*P ≤ 0.001 vs. control), alpha keto glutarate dehydrogenase activity (57.19%, *P ≤ 0.001 vs. control) and succinate dehydrogenase activity (33.64%,*P ≤ 0.001 vs. control). But when the mitochondria were pre-treated with TA extract, the activity of the enzyme was found to be almost at the normal level. Thus, it can be said that the aqueous extract of TA bark can provide the preventive effects against CuAs induced enzymatic alterations of mitochondrial pyruvate dehydrogenase and Kreb's cycle enzymes activities.

Table 4: Protective effect of aqueous bark extract of *T. arjuna* against copper-ascorbate induced alteration in the mitochondrial respiratory chain enzymes in goat heart mitochondria

Groups	NADH Cytochrome C oxidoreductase (Units/ min/mg of protein)	Cytochrome C oxidase (Units/min/ mg of protein)
Control	10.94 ± 0.1	2.69 ±0.0
CuAs	8.35 ± 0.0 *¶	1.03 ± 0.0 *¶
T 10	10.53 ± 0.0	2.87 ± 0.1
T 20	10.24 ± 0.6	2.02 ± 0.0
CuAs-T 10	10.99 ± 0.0 *#	2.13 ± 0.0 *#
CuAs-T 20	12.34 ± 0.0§	2.67 ± 0.0§

CuAs = copper-ascorbate treated group; CuAs-T10 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 10mg/ml; CuAs-T20 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 20mg/ml; The values are expressed as Mean ± S.E.M; * P ≤ 0.001; ¶ As compared to control values using ANOVA; # As compared to CuAs-treated values using ANOVA; § As copared to control values using ANOVA.

Table 5: Preventive effect of aqueous bark extract of *T. arjuna* against copper-ascorbate induced alteration in the Pyruvate dehydrogenase and other Kreb's cycle enzymes in goat heart mitochondria

Groups	Pyruvate dehydrogenase (Units/mg of protein)	Isocitrate dehydrogenase (Units/ min/mg of protein)	α-ketoglutarate dehydrogenase (Units/ min/mg of protein)	Succinate dehydrogenase (Units/ min/mg of protein)
Control	233.83 ± 54.68	247.23 ± 1.11	131.26 ± 1.37	1441.9 ± 16.3
CuAs	159.71 ± 28.21*¶	194.31 ± 7.51*¶	56.19 ± 4.16 *¶	956.89 ± 3.9 *¶
T 10	233.27 ± 0.51	247.43 ± 4.7	131.05 ± 1.34	1441.97 ± 112.60
T 20	233.87 ± 1.09	247.90 ± 3.8	131.33 ± 0.72	1441.00 ± 98.57
CuAs-T 10	232.27 ± 0.51	246.62 ± 1.90 *#	131.05 ± 2.39*#	1441.23 ± 104.45*#
CuAs-T 20	233.99 ± 2.53 *#§	247.12 ± 1.46§	131.90 ± 1.69§	1440.45 ± 290.14§

CuAs = copper-ascorbate treated group; CuAs-T10 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 10mg/ml; CuAs-T20 = group co-treated with copper-ascorbate and aqueous bark extract of TA at the dose of 20mg/ml; The values are expressed as Mean ± S.E.M; * P ≤ 0.001; ¶ As compared to control values using ANOVA; # As compared to CuAs-treated values using ANOVA; § As compared to control values using ANOVA.

DISCUSSION

Oxidative stress is generally defined as an imbalance that favors the production of ROS over antioxidant defenses; however, the precise mechanisms by which ROS cause cellular injury remain elusive. Mitochondria are the main source of the superoxide radical and other reactive oxygen species that may generate from them [45].The main mechanisms responsible for mitochondrial ROS production are the respiratory chain, in particular its complexes I and III, [46] in the inner mitochondrial membrane, and monoamine oxidase in the outer membrane. About 1–2% of the molecular oxygen consumed during normal physiological respiration is converted into superoxide radicals [47]. Normally, ROS are decomposed or their peroxidation products are neutralized by natural defense systems mainly consisting of mitochondrial (manganese-containing) and cytosolic (containing Cu and Zn) super oxide dismutases (Mn- and Cu-Zn-super oxide dismutase, respectively); glutathione peroxidase, and phospholipid hydroperoxide glutathione peroxides [48]. The noxious action of ROS mainly consists of the peroxidation of lipids, in particular phospholipids of biological membranes, and oxidative damage to proteins and DNA [49]. Natural antioxidants that are present in medicinal plants are responsible for inhibiting or preventing the deleterious consequences of oxidative stress as they contain free radical scavengers like polyphenol, flavonoids and phenolic compounds. The present study reveals the protective effect of aqueous TA bark extract against copper-ascorbate induced oxidative stress on mitochondria. Cu^{2+} in presence of ascorbic acid (0.2mM-2.0mM) generates $OH\cdot$ radical and H_2O_2 as follows [19, 20, 21]:

$$\text{Ascorbate} + Cu^{2+} + O_2 + 2H^+ \longrightarrow \text{Dehydroascorbate} + Cu^+ + H_2O_2$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH\cdot + OH^-$$

The hydroxyl radical induce mitochondrial damage by impairment of mitochondria functionality which sensitizes cells to oxidative challenges.

Concerns over the safety of synthetic antioxidants have shifted the global interests towards exploration of antioxidant compounds from natural sources [50]. A plethora of phenolics extracted from several plant species have been reported to possess strong antioxidant activities [51]. Phenolics are ubiquitously present in plants, and when plants are consumed as foods, these phytochemicals contribute to the intake of natural antioxidants in the diets of human as well as animals. Out of all the phenolics, the flavonoids belong to a large family of compounds with different degrees of hydroxylation, oxidation and substitution. Our qualitative assessment of the phytoconstituents present in the aqueous extract of TA has shown the presence of tannins, cumarins and terpenoids. However, our quantitative assessment of the phytoconstituents present in the aqueous bark TA extract revealed a higher amount of polyphenol which may be responsible for antioxidant activity of this extract.

The free radical scavenging activity of TA aqueous bark extract through DPPH assay suggests that aqueous extract of TA contain compounds such as polyphenolic that can donate electron/hydrogen easily thereby reducing the DPPH absorption which proves its ROS scavenging activity.

Antioxidant activity depends on the metallic catalyst used to generate the reactive species [52]. In this study, to measure reductive capability, we investigated the Fe^{3+}- Fe^{2+} transformation in the presence of TA extract using the method of Oyaizu [27]. The presence of reducing compounds causes reduction of the Fe^{3+}/ferricyanide complex to ferrous ion (Fe^{2+}) [53]. From this experiment it was proved that aqueous extract of TA exhibited increasing reducing power ability with increasing concentration.

Copper-binding proteins are involved in both transport and metabolism. In addition, a relation between copper metabolism and the intracellular availability of glutathione has been defined [54]. Moreover copper-ascorbate may induce oxidative stress by enhancing tissue LPO and by altering the antioxidant system in the organs. The copper-ascorbate induced mitochondrial damage is due to generation of oxidative stress as is evident from elevated levels of LPO and protein carbonyl content and a decreased tissue level of reduced GSH. The aqueous TA bark extract is found to be effective in decreasing the lipid peroxidation level of the cardiac mitochondria. In *in vitro* studies of lipid peroxidation, transition metal ions are used to initiate peroxidation reactions. When Cu^+ ions are added to isolated mitochondria, peroxidation occurs. The oxidized form of this transition metal ion (Cu^{2+}) can also accelerate peroxidation in presence of ascorbate [55]. A highly significant increase in lipid peroxidation has been observed when Cu^{2+} and ascorbate are used together to induce lipid peroxidation rather than when used separately [55]. This experiment indicates that the aqueous TA bark extract has anti-lipoperoxidative and antioxidative properties which may be due to the presence of phytochemical(s)/phytonutrient(s).

Although GSH exerts important antioxidant functions, by acting as superoxide and hydroxyl radical-scavenger and as cofactor of the glutathione peroxidase and transferase enzymes, it is possible that the tripeptide exert also a pro-oxidant activity as result of its metal-reducing properties. Regarding this latter aspect of GSH, its interaction with copper ions leads to the swift reduction of Cu2+ into Cu+ ions in a reaction which, in the presence of additional GSH molecules, results in the formation of the Cu(I)–[GSH] complex [56]. Although the biological function of the Cu (I)–[GSH] complex has not been yet established, the complex is believed to play a role as Cu (I)-carrier to several copper-dependent proteins, including SOD, ceruloplasmin and metallothionein. In addition to such potential biological function, the Cu(I)–[GSH] complex has been postulated to serve as a mechanism to protect cells from undergoing the damage expected to arise from the ability of otherwise free copper ions to bind non-specifically to essential bio molecules and/or to catalyze free radical generation [56]. We have measured the mitochondrial level of reduced GSH, one of the important biomarkers of oxidative stress, which forms the first line of defense against oxidative stress induced damage. Decreased levels of mitochondrial reduced GSH in our studies indicate, generation of oxidative stress following exposure to copper- ascorbate, which may be responsible for the oxidative damage of bio-molecules of the mitochondria. However, co-treatment of mitochondria with the aqueous TA bark extract prevented the GSH depletion in the organelle. The results indicate toward the possibility that the aqueous extract may deliver effective antioxidant phytochemical molecules in the mitochondria.

Oxidation of proteins can generate stable as well as reactive products that can generate additional radicals on reaction with transition metal ions. Most oxidized proteins are functionally inactive and are rapidly removed; some gradually accumulate and contribute to damage [57]. The experimental results indicate that functionally active oxidized proteins that are capable of causing damage are produced following exposure of mitochondria to copper-ascorbate. The aqueous TA bark extract seems potent in either scavenging the toxic free radicals and other reactive intermediate(s) or chelating the redox-active transition/non-transition heavy metal divalent cations thereby protecting the proteins from getting oxidized.

Mn-SOD, a mitochondrial enzyme, responded to the stress with an increase in activity. The cytotoxic effect of Cu may be derived from its oxidation potential and mitochondrial Mn-SOD is more sensitive to copper than cytoplasmic Cu/Zn-SOD [58]. Cu^{2+}-ascorbate induced oxidative stress in mitochondria increases the activity of Mn-SOD. But when the mitochondria were co-treated with aqueous bark extract of TA, the activity of this enzyme was found to be protected from being increased.

Catalase, a cytosolic enzyme is not present in mitochondria, but its role is provided in mitochondria via glutathione peroxidase [59]. Glutathione peroxidase (GPx) is a Se dependent oxido-reductase which protects tissues from toxin induced oxidative damage [60]. A decreased activity of GPx following Cu^{2+}-ascorbate treatment of mitochondria as observed is expected to further aggravate the situation of oxidative stress. However, the aqueous extract of TA was found to restore the activities of this key antioxidant enzyme to normal.

Glutathione reductase (GR) is the enzyme responsible for the reduction of oxidized glutathione (GSSG) to GSH [61]. In the present study, the activity of the glutathione dependent enzyme, GR was

reduced significantly in the mitochondria with the treatment of Cu^{2+}-ascorbate. The formation of copper-sulfhydryl complex with SH groups of the GR might lead to a decrease in the activity of the enzyme [62]. It seems clear that copper-ascorbate decreases intracellular GSH level by binding to its thiol group, and also by decreasing the activity of GR. Our studies further demonstrated that following Copper-ascorbate treatment, the activity of GPx decreased. This indicates that GSH metabolizing pathway is disturbed in mitochondria.

Therefore, the rate of GSH formation from GSSG is reduced. But when the mitochondria were treated with aqueous bark extract of TA bark, then it protected the GPx activity as well as GR activity from being altered. This again proves the potentiality of the extract in regulating glutathione synthesis, thereby providing antioxidant defense against copper-ascorbate induced mitochondrial damage.

To cope with the ROS, animal and human cells express an array of antioxidant enzymes, including Mn^{2+}-dependent superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx), glutathione reductase (GR). Mn-SOD converts superoxide anions to hydrogen peroxide, which is then transformed to water by GPx. However, the activities of these antioxidant enzymes and the concentrations of small-molecular-weight antioxidants in mitochondria are altered (mostly declined) during oxidative stress. Thus, there is an increase in the fraction of ROS and free radicals that may escape these cellular defense mechanisms and exert damage to cellular constituents, including DNA, RNA, lipid, and proteins. But the applications of aqueous extract of TA restore the activities of various mitochondrial antioxidant enzymes and thus, can prevent ROS induced oxidative stress.

Mitochondria are the major source of ROS production in cells [63]. In our study we found that the activities of pyruvate dehydrogenase and the Kreb's cycle enzymes like Isocitrate dehydrogenase, alpha-keto glutarate dehydrogenase and succinate dehydrogenase were decreased after the treatment of mitochondria with copper-ascorbate. The activities of all these enzymes were protected when the mitochondria were co-treated with the aqueous TA bark extract. Succinate dehydrogenase and alpha-ketoglutarate dehydrogenase are prone to ROS attack and inactivation. They are more sensitive to H_2O_2, so the activities were inhibited by alteration of H_2O_2 generation, but there is a difference between the changes of activities of these enzymes.

Previously it was proved that the inhibition of mitochondrial respiratory complexes, especially cytochrome c oxidase, increased metabolic stress (decrease in ATP), increasing oxidative stress and nitrative stress [64]. Alteration in antioxidant concentrations may result in up-regulation of mitochondrial stress protein, HSP70. The mitochondrial stress causes more inflow of HSP70 from the cytoplasm into mitochondria. This may help to maintain the function of the respiratory complexes and suppress mitochondrial ROS production through stabilization of cytochrome c [65]. The over expression of HSP70 also mediates the folding and assembly of mitochondrial proteins, serving as an additional mechanism leading to the preservation of respiratory complex activities.

Heavy metals are also known to affect respiratory chain complexes and there is absolute substrate specificity [66]. The impairment of electron transfer through NADH:ubiquinone oxidoreductase (complex I) and ubiquinol:cytochrome c oxidoreductase (complex III) may induce superoxide formation. Mitochondrial production of ROS is thought to play an adverse role in many pathologic disorders. In our present study, copper-ascorbate induced oxidative stress inhibits NADH cytochrome c oxidoreductase and cytochrome c oxidase enzymes of ETC. The activities of these enzymes were found to be protected when the mitochondria were co-treated with aqueous TA bark extract. This strongly indicates that the extract possesses either some chelating property or is simply able to protect mitochondria from getting damaged due to ROS production, by itself being a quencher of reactive oxygen species.

Further studies are necessary to examine the protective effects of this aqueous bark TA extract *in vivo*, in a suitable animal model of ischemic stress before recommending this extract for future therapeutic use.

In conclusion it can be said that the present findings suggest that the aqueous bark extract of *Terminalia arjuna* provides protective effects against oxidative stress, induced *in vitro,* in goat heart mitochondria as is evident from the various oxidative stress bio-markers as well as activities of antioxidant enzymes and pyruvate dehydrogenase and some of the key mitochondrial Kreb's cycle enzymes (Figure 4). The activities of some of the mitochondrial respiratory chain enzymes also established the protective effect of aqueous bark extract of TA against copper-ascorbate induced oxidative stress. This aqueous extract of TA contains phytochemicals (such as phenolics, flavonoids etc) which possess antioxidant activity. So, it can be assumed that these phytochemicals may be responsible for the protective effects of the aqueous extract of TA against Cu^{2+}-ascorbate induced oxidative stress in *in vitro* model. However, further studies involving identification of the active moieties of this aqueous bark extract of TA and elucidation of the mechanism of action involved therein are in progress.

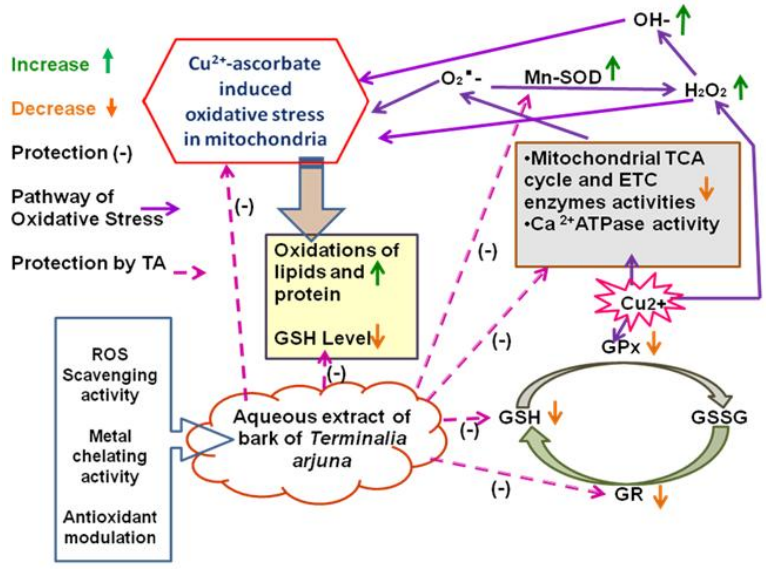

Fig. 4: Scheme showing the probable mechanism of protection offered by aqueous bark extract of *Terminalia arjuna* against Cu^{2+}-ascorbate induced oxidative stress *in vitro* in goat heart mitochondria

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