Research Article

Elephantopus scaber Linn. extract protects against copper ascorbate induced injury to goat heart mitochondria *in-vitro*: Involvement of antioxidant mechanisms

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Abstract

Objective: *Elephantopus scaber* Linn., family Asteraceae, is a small herb found in Neotropics, Europe, Asia, Africa and Australia. The plant parts of this herb have been used traditionally for the treatment of a number of diseases in many countries. In the present study, we investigated the antioxidant activities of *E. scaber* methanolic and aqueous extract of root and aerial part against copper-ascorbate induced toxic injury to mitochondria obtained from goat heart, *in vitro*. **Material and methods:** Incubation of isolated cardiac mitochondria with copper-ascorbate resulted in elevated levels of lipid peroxidation and protein carbonylation of the mitochondrial membrane, a reduced level of mitochondrial GSH and altered status of antioxidant enzymes as well as decreased activities of pyruvate dehydrogenase and the Kreb's cycle enzymes. All of these parameters were protected from being altered when the cardiac mitochondria were co-incubated with *E. Scaber* different extracts, *in vitro*. **Results and conclusion:** A significant increase in cardiac mitochondrial LPO level following the incubation of mitochondria with copper ascorbate (63.92%, $P < 0.001$ vs. control) and protein carbonyl assay (64.78%, $P < 0.001$ vs. control). A significant decrease in cardiac mitochondrial reduced GSH content (61.89%, $P < 0.001$ vs. control), (66.69%, #$P < 0.001$ vs. control group) in the activity of GPx , (63.91%, #$P < 0.001$ vs. control group) in the activity of GR was observed. The result of present study suggested that the *E. scaber* extract may be used as a potential therapeutic antioxidant agent in future.

Keywords: *Elephantopus scaber*, copper ascorbate, antioxidant, mitochondria, protein carbonylation

Introduction

The lectotype species of *Elephantopus* genus i.e. *Elephantopus scaber* Linn family Asteraceae, is a common wild weed that forms undergrowth in shady places (Kirtikar and Basu 1991). It is a genus of about 32 species centered in the Neotropics, Europe, Asia, Australia and Africa (Kurokowa and Nakashini, 1970; Taylor et al., 1995; Hui and But, 1998; Singh et al., 2005; Than et al., 2005; Wright et al., 2007). Review of ethnomedical history of *E. scaber* clearly indicates its use in various part of the world for the treatment of variety of disease conditions such as anticancer, antimicrobial, antioxidant, antiplatelet, antidiabetic, hepatoprotective, anti-inflammatory etc. Many of these ethnomedical uses have been scientifically validated by the results of biological activity studies.

The methanolic extract of *E. scaber* root has proved to be an efficient antioxidant in the *in vitro* model. The superoxide generation by the reaction of photoreduced riboflavin and oxygen is inhibited by the methanolic extract of *E. scaber* root. The generation of malondialdehyde and related substances from lipid extract react with thiobarbituric acid was found to be inhibited by the methanolic extract of *E. scaber* root. The degradation of deoxyribose to TBARS by the hydroxyl radicals generated from Fe^{3+}-ascorbate- EDTA-H_2O_2system was markedly decreased by the methanolic extract of *E. scaber* root (Sheeba et al., 2012). However the hydroalcoholic, hexane, ethyl acetate and methanol extract

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of whole plant of *E. scaber* were tested for its antioxidant potential and were found to possess concentration dependent inhibition using DPPH, superoxide and hydroxyl radicals scavenging activity (Gangarao et al., 2012).

But till date there is no report available, to the best of our knowledge and belief, about the antioxidant activity of *E. scaber* against mitochondrial oxidative stress. Herein, we provide evidences for the first time, the antioxidant capability of different extract of *E. scaber* against copper-ascorbate induced toxic injury to goat heart mitochondria, *in vitro*, and antioxidant mechanism(s) may be responsible for such protections.

Materials and Methods

Chemicals

Copper-chloride and ascorbic acid were purchased from Sisco Research Laboratories (SRL), Mumbai, India. All 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.

Collection and authentication of plant material

The whole plant of *E. scaber* was collected in the month of October-November 2012, from the forest of Achanakmar, Chhattisgarh, India. The collected plant was authenticated by the Dr. G. P. Sinha, Scientist D, Ministry of environment and forests, Botanical survey of India, Allahabad, Uttar Pradesh.BSI/CRC/TECH/2014-15/voucher specimen has been preserved in our laboratory for future reference.

Preparation of plant extract

The whole plant material was washed well with water to separate the adhering soil material. The collected entire plant containing roots and aerial parts was dried in the shade. The total loss on drying was found to be 29.6%. After complete drying the roots and aerial parts were separated from each other. Dried roots consisted of thin, long roots attached to rhizomes while the aerial parts contained leaves, stems and flowers. 1 kg of the dried roots and rhizomes of *E. scaber* were commented to form coarse powder. The coarsely powdered crude drug material was first defatted with petroleum ether (40-60^{0}C) in Soxhlet extractor. Defatted dried marc of the crude drug was further subjected to sequential extraction with diethyl ether and with methanol. 500 gm of coarse powder of root and rhizomes of *E. scaber* was macerated with water for 24 hrs (24x3). The same extraction procedure was followed for the extraction of aerial parts to afford petroleum ether, diethyl ether, methanol and water extracts. All the extracts obtained were concentrated and dried under reduced pressure using rotary evaporator. The total four extract has been undertaken for study along with code is as follows-

ME-Methanolic extract of root of *E. scaber*

AQUE- Aqueous extract of root of *E. scaber*

MEL- Methanolic extract of aerial part of *E. scaber*

AQUEL- Aqueous extract of aerial part of *E. scaber*

Preparation of goat heart mitochondria (Caprine heart mitochondria)

Goat heart mitochondria were isolated according to the procedure of Dutta *et al.*, (2014) with some modifications. Goat heart was purchased from local Kolkata Municipal Corporation approved meat shop. After collection it was brought into laboratory in sterile plastic container kept in ice. Then, the heart tissue was cleaned and cut into pieces. Five gm of tissue was placed in 10ml of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H2SO4 (pH 7.8)] at 25°C. Then the tissue was blended for 1 minute at low speed by using a Potter Elvenjemglass 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°C for further use.

Incubation of mitochondria with copper- ascorbate

The incubation mixture containing mitochondrial membrane protein (1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), and 0.2mM Cu2+ and 1 mM ascorbic acid in a final volume of 1.0 ml was incubated at 37°C in an incubator for 1 hour. The reaction was terminated by the addition of 40 µl of 35 mM EDTA (Chattopadhyay et al., 1992).

Protection of Cu^{2+}-ascorbate-induced toxic injury to mitochondria by *E. scaber* extract

The goat heart mitochondria were co-incubated with copper ascorbate and different concentrations of *E. scaber* extract. After incubation, the intactness of mitochondria, the biomarkers of oxidative stress like lipid peroxidation level, reduced glutathione and protein carbonyl content, activities of antioxidant enzymes, Kreb's cycle enzymes, mitochondrial swelling and di-tyrosine level were determined.

Determination of mitochondrial intactness by using Janus green B stain

After incubation, the mitochondrial sample was diluted 1:200 by using 50 mM phosphate buffer (pH 7.4). Then, the mitochondria were spread and dried on slide after which a

few drops of Janus green B stain was added and kept for 5 min for staining. It was then rinsed once with distilled water and mounted in a drop of distilled water with a cover slip and imaged with a laser scanning confocal system (Zeiss LSM 510 META, Germany) and the stacked images were captured. The digitized images were then analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) and the intactness of mitochondria of each image was measured and expressed as the % fluorescence intensity (Dutta et al., 2014).

Measurement of mitochondrial oxidative stress biomarkers

Lipid peroxidation (LPO) level

The lipid peroxides in the incubated mitochondria were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust (1978) with some modifications as adopted by Bandyopadhyay *et al.* (2004). The incubated mitochondria were mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 800C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 8000rpm 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 glutathione (GSH)

The GSH content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak *et al.* (1968) with some modifications by Bandyopadhyay *et al.* (2004). Incubated mitochondria were mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for colour 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 (PCO) content

Protein carbonyl content was estimated by DNPH assay (Levine et al., 1994). 0.25 ml of incubated mitochondrial suspension 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 2000rpm for 10 min. The pellet was washed carefully three times with 1.0 ml of ethanol:ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined spectrophotometrically 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 of protein.

Measurement of the activities of Mn-superoxide dismutase (Mn-SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) of goat cardiac mitochondria

Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method (Marklund and Marklund, 1974). To 50 µl of the mitochondrial sample; 430 µl of 50 mM Tris–HCl buffer (pH 8.2) and 20 µl of 10 mM 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/ mg of protein.

The glutathione reductase (GR) assay was carried out according to the method of Krohne- Ehrich *et al.* (1977). The assay mixture in the final volume of 3 ml contained 50 mM phosphate buffer, 200 mMKCl, 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 (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 calculated as units/min/mg of protein.

The glutathione peroxidase (GPx) activity was measured according to the method of Paglia and Valentine (1967) with some modifications as adopted by Dutta *et al.* (2014). 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/mg of protein.

Measurement of pyruvate dehydrogenase and Kreb's cycle enzymes activities

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically according to the method of Chretien *et al.* (1995) 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/mg of protein.

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan and Fraenkel (1979) 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,

0.5mMisocitrate, 0.1 mM MnSO4, 0.1 mM NAD+ and the suitable amount of incubated mitochondria as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Alpha-ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan *et al.* 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, incubated mitochondria as the source of enzyme and 0.1 mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/mg of protein.

Succinate dehydrogenase (SDH) activity was measured following the reduction of potassium ferricyanide [$K_3Fe(CN)_6$] spectrophotometrically at 420 nm according to the method of Veeger *et al.,* (1969) 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 K3Fe(CN)6 and a suitable aliquot of the incubated mitochondria as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Statistical analysis

Each experiment was repeated at least three times. Data are presented as means SEM. Significance of mean values of different parameters between the treatments groups were analyzed using one way post hoc tests (Tukey's HSD test) of analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pair wise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

Results

Effect of *E. scaber* extracts on the oxidative stress biomarkers

Lipid peroxidation level

A significant increase in cardiac mitochondrial LPO level following the incubation of mitochondria with copper ascorbate (63.92%, P < 0.001 vs. control) was observed. This elevated level of lipid peroxidation products were found to be protected from being increase significantly (40.11% from copper-ascorbate-treated group, P < 0.001Vs. Cu-ascorbate) when the mitochondria were coincubated with leaves methanolic extract of *E. scaber* (0.2 mg/ml), indicating the ability of this extract to protect the mitochondria against oxidative stress-induced changes due to copper-ascorbate (Figure 1).

Reduced glutathione level

Copper-ascorbate caused significant decrease in cardiac mitochondrial reduced GSH content (61.89%, P < 0.001 vs. control). This decreased level of reduced GSH content was found to be protected significantly in a dose dependent manner in presence of methanolic extract of *E. scaber*. A 1.17 fold increase from copper-ascorbate-treated group, (P < 0.001 Vs. Cu-As) when the mitochondria were co-incubated with leaves methanolic extract (0.2mg/ml) (Figure 2).

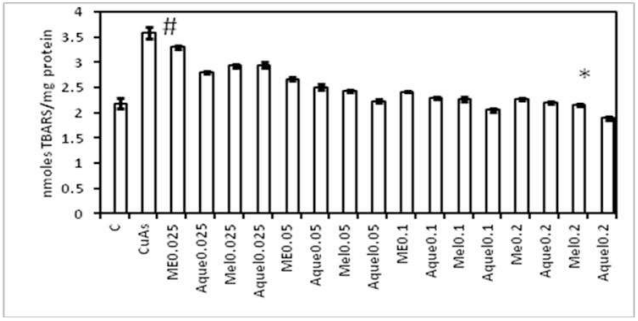

Figure 1. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in LPO level (nmol TBARS/mg of protein). C= Control group, CuAs = copper-ascorbate incubated group; ME, Aque (0.025-0.2)=Methanolic and aqueous extract of root of *E. scaber*, Mel, Aquel (0.025-0.2)=Methanolic and aqueous extract of leaves of *E. scaber*. The values are expressed as Mean±SEM (n=6); #P < 0.001 as compared to control values using ANOVA; * P < 0.001 as compared to CuAs-treated values using ANOVA.

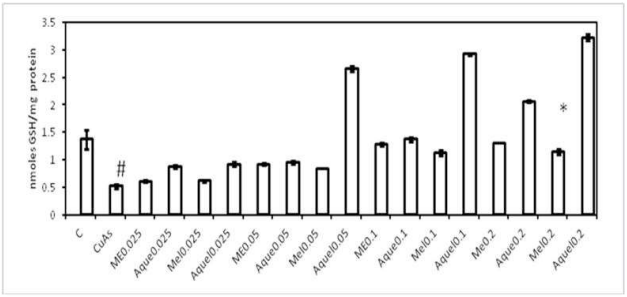

Figure 2. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in GSH (nmole GSH/mg of protein). C= Control group, CuAs = copper-ascorbate incubated group; ME, Aque (0.025-0.2)=Methanolic and aqueous extract of root of *E. scaber*, Mel, Aquel (0.025-0.2)=Methanolic and aqueous extract of leaves of *E. scaber*. The values are expressed as Mean±SEM.; #P < 0.001 as compared to control values using ANOVA; * P < 0.001 as compared to CuAs-treated values using ANOVA.

Protein carbonyl level

The protein carbonyl assay showed a significant increase in cardiac mitochondrial protein carbonyl content following the incubation of mitochondria with copper-ascorbate (64.78%, P < 0.001 vs. control).This elevated level of protein carbonyl content was found to be protected significantly (95.40% from copper-ascorbate-incubated group, P <0.001) when the mitochondria were co-incubated with methanolic extract of *E. scaber* root (0.2mg/ml) (Figure 3).

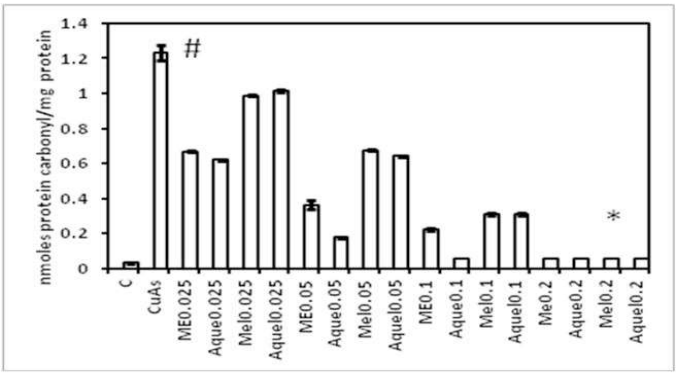

Figure 3. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in Protein carbonyl (nmoles/mg of protein). C= Control group, CuAs = copper-ascorbate incubated group; ME, Aque (0.025-0.2)=Methanolic and aqueous extract of root of *E. scaber*, Mel, Aquel (0.025-0.2)=Methanolic and aqueous extract of leaves of *E. scaber*. The values are expressed as Mean±SEM.; #P < 0.001 as compared to control values using ANOVA; * P < 0.001 as compared to CuAs-treated values using ANOVA.

Effect of *E. scaber* on the activities of antioxidant enzymes

Mn-superoxide dismutase (Mn-SOD) activity

A highly significant elevation (3.76 fold, #P < 0.001 vs. control group) was observed in the activity of Mn-SOD following incubation of mitochondria with copper-ascorbate. The activity of this enzyme was found to be protected from being increased when the mitochondria were co-incubated with aqueous extract of root of *E. scaber*. The aqueous extract of root of *E. scaber* protected Mn-SOD activity from being increased (*P <0.001 vs. copper-ascorbate-treated group) at the dose of 0.2 mg/ml (Table 1).

Glutathione peroxidase (GPx) activity

A highly significant decrease (66.69%, #P < 0.001 vs. control group) in the activity of GPx following the incubation of mitochondria with copper-ascorbate was observed. The GPx activity was protected from being decreased in a dose dependent manner when the mitochondria were co-incubated

Table 1. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in the antioxidant enzymes activities in goat heart mitochondria

Groups	Mn-superoxide dismutase activity (Units/mg of protein)	Glutathione peroxidase activity (Units/g of protein)	Glutathione reductase activity (Units/g of protein)
C	1.73±0.049	1.3±0.00015	1.2±0.00015
CuAs	8.24±0.155#	0.43±0.000033#	0.43±0.000033#
ME0.025	7.92±0.0664	0.43±0.000033	0.5±0.000057
Aque0.025	7.12±0.1068	0.53±0.000033	0.5±0.000057
Mel0.025	7.63±0.0606	0.63±0.000033	0.5±0.000057
Aquel0.025	7.56±0.1682	0.567±0.000033	0.5±0.000057
ME0.05	4.52±0.0523	0.92±0.000018	0.63±0.000033
Aque0.05	6.14±0.2001	0.83±0.000033	0.63±0.000033
Mel0.05	7.56±0.1761	0.73±0.000033	0.9±0.000057
Aquel0.05	5.52±0.0504	0.83±0.000033	0.9±0.000057
ME0.1	2.28±0.1112	1.2±0.0000577	1.1±0.000057
Aque0.1	1.47±0.0448	1.17±0.000088	0.93±0.000033
Mel0.1	2.29±0.1446	1.1±0.000057	1.1±0.000057
Aquel0.1	2.32±0.0819	1.27±0.000033	1.1±0.000057
Me0.2	1.80±0.0405	2.03±0.00014	1.9±0.000057
Aque0.2	0.42±0.050*	1.5±0.00017*	1.9±0.000057
Mel0.2	1.43±0.0638	1.67±0.000088	2.13±0.000088*
Aquel0.2	0.48±0.0097	1.77±0.000033	2.17±0.000088

C= Control group, CuAs = copper-ascorbate incubated group; ME, Aque (0.025-0.2)=Methanolic and aqueous extract of root of *E. scaber*, Mel, Aquel (0.025-0.2)=Methanolic and aqueous extract of leaves of *E. scaber*. The values are expressed as Mean±SEM.; #P < 0.001 as compared to control values using ANOVA; * P < 0.001 as compared to CuAs-treated values using ANOVA.

with aqueous extract of root of *E. scaber*. The aqueous extract of root of *E. scaber* not only protected but also stimulated GPx activity 2.46 fold (*P <0.001 vs. copper ascorbate treated group) at the dose of 0.2 mg/ml (Table 1). However, the extract by itself has no effect on the activity of GPx**.**

Glutathione reductase (GR) activity

There is also a highly significant decrease (63.91%, #P < 0.001 vs. control group) in the activity of GR following incubation of mitochondria with copper-ascorbate. The GR activity was found to be protected from being decreased dose dependently when the mitochondria were co-incubated with methanolic extract of leaves (MEL). MEL protected GR activity by about 3.91 fold (*P < 0.001 vs. copper-ascorbate treated group) at the dose of 0.2 mg/ml (Table 1). MEL by itself, however, has no effect on the activity of GR.

Effect of *E. scaber* extract on the activities of pyruvate dehydrogenase and some Kreb's cycle enzymes

Pyruvate dehydrogenase activity

The incubation of the goat heart mitochondria with copper ascorbate inhibited pyruvate dehydrogenase activity (57.23%,#P<0.001 vs. control). When the mitochondria were co-incubated with aqueous extract of root (AQUE), the activity of the enzyme, however, was found to be significantly protected from being decreased compared to the activity observed in the copper ascorbate-incubated group (5.79 fold increase,*P<0.001 vs. Copper ascorbate-incubated group) at the dose of 0.2 mg/ml (Figure 4).

Isocitrate dehydrogenase activity

Isocitrate dehydrogenase activity assay reveals that the incubation of the mitochondria with copper-ascorbate resulted in a decrease in isocitrate dehydrogenase activity (52.63%, P < 0.001 vs. control). The activity of the enzyme was found to be completely protected when mitochondria were incubated with AQUE at the dose of 0.2 mg/ml (2.44 fold increased, P <0.001 vs. Cu-As) (Figure 5).

Alpha ketoglutarate dehydrogenase activity

Alpha ketoglutarate dehydrogenase activity was decreased after copper-ascorbate incubation of mitochondria (36.37%, P < 0.001 vs. control). This enzyme was found to be able to generate ROS during copper-ascorbate treatment). The activity of the enzyme was found to be significantly protected from being decreased when the mitochondria were incubated with 2 mg/ml dose of the AQUE (1.62 fold higher, P < 0.001 vs. copper-ascorbate-incubated group) (Figure 6).

Succinate dehydrogenase activity

The succinate dehydrogenase activity assay reveals that incubation of mitochondria with copper-ascorbate inhibited succinate dehydrogenase activity (57.33%, P <0.001 vs. control). The enzyme activity was found to be significantly protected from being decreased dose-dependently, when the mitochondria were co-incubated with 2 mg/ml dose of

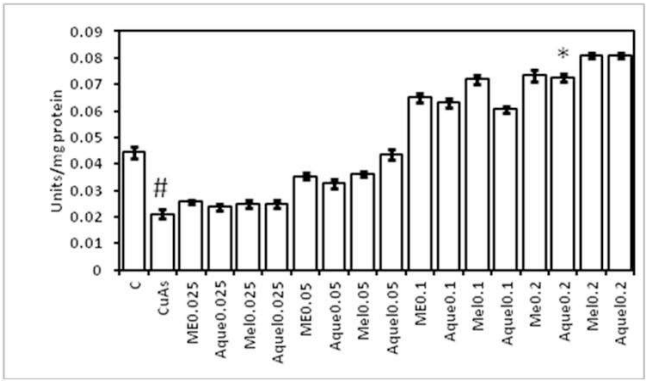

Figure 5. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in Isocitrate dehydrogenase activity (Units/ mg of protein)

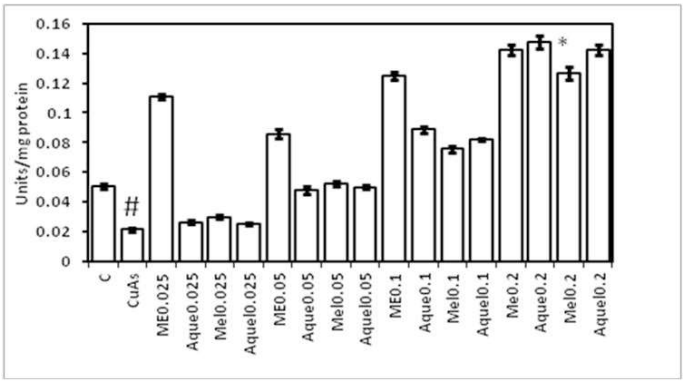

Figure 4. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in Pyruvate dehydrogenase activity (Units/mg of protein)

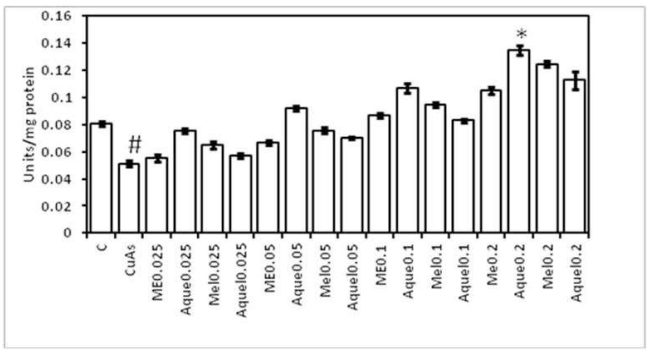

Figure 6. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in Alpha ketoglutarate dehydrogenase activity (Units/ mg of protein)

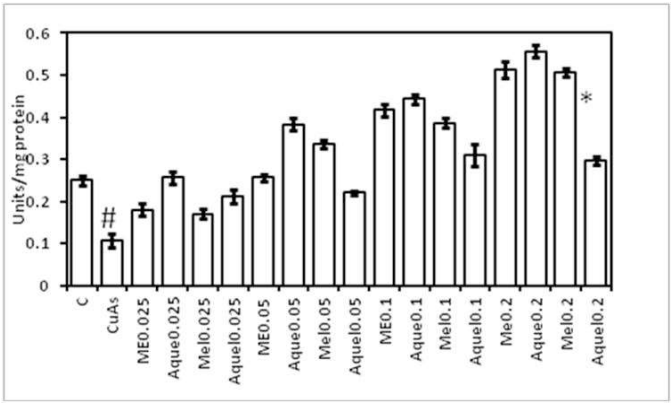

Figure 7. Protective effect of *E. scaber* extract against copper-ascorbate induced alteration in Succinate dehydrogenase activity (Units/ mg of protein)

the MEL (3.74 fold higher, $P < 0.001$ vs. copper-ascorbate incubated group) (Figure 7).

Mitochondrial viability by confocal microscopy

Figure 2 depicts a significant decrease in the mitochondrial intactness following the incubation of mitochondria with copper ascorbate (67.41%, $P = 0.001$ vs. control). This decreased level of mitochondrial intactness were found to be significantly protected from being altered (2.07 fold compared to copper-ascorbate-incubated group, $P = 0.001$) when the mitochondria were co-incubated with copper-ascorbate and MEL (0.2mg/ml), indicating the ability of MEL to protect the mitochondria against copper-ascorbate induced changes in mitochondrial swelling which may be due to oxidative stress.

Discussion

Reactive oxygen species (ROS) are generated due to redox imbalance in tissues, which is responsible for tissue damage by modification of lipid, proteins and nucleic acids. In our *in vitro* experimental system Cu-ascorbate was used as an inducer of oxidative stress in goat heart mitochondria.O_2^- anion radical is generated by Cu^{2+}. The activity of superoxide dismutase which is found to be increased in mitochondrial compartments may be due to generation of this O_2^-anion radical. As the catalase enzyme is absent in mitochondria, so it is quite impossible to quench this H_2O_2 overload. Hence, this hydrogen peroxide generates. OH responsible for lipid peroxidation, protein carbonylation, depletion of mitochondrial GSH (that is also evident from decreased activities of GPx and GR). Additionally the generated hydroxyl radical causes declining activities of NAD linked enzymes like pyruvate dehydrogenase, a-ketoglutarate dehydrogenase, isocitrate dehydrogenase (a key enzyme in cellular defense against oxidative damage as it provides NADH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin.) as well as FAD linked enzyme i.e. succinate dehydrogenase (all of them are associated with electron transport chain for oxidative phosphorylation) that again will generate more superoxide anion through electron transport chain and which will follow above reaction scheme and overload of excess hydroxyl radical will be exerted on the mitochondria and finally mitochondria will be fragile as evident from the figure of SEM.

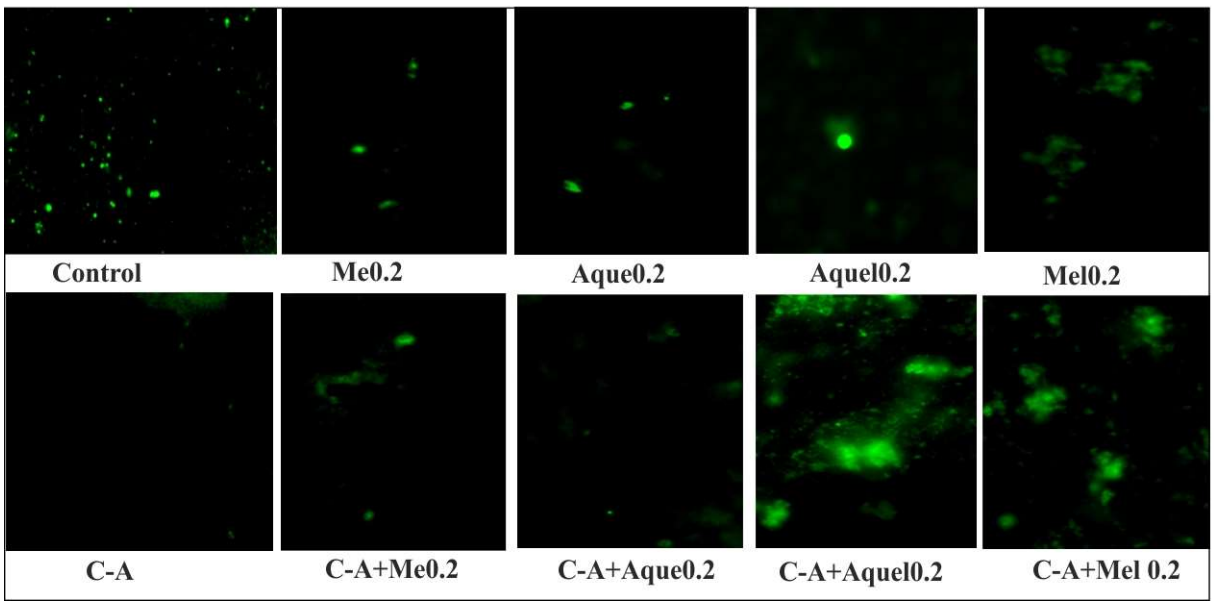

Figure 8: Changes of intactness of mitochondria. Janus green B stained (40X magnification) and (D) graphical representation of changes of mitochondrial intactness; C-A = copper-ascorbate incubated group; ME0.2= group incubated with methanolic extract of root of *E. scaber* at the dose of 0.2mg/ml respectively; CA-ME0.2= group co-incubated with copper-ascorbate and methanolic extract of root of *E. scaber* at the dose of 0.2mg/ml respectively; The values are expressed as Mean ± S.E.; # P = 0.001 compared to control values using ANOVA. *P = 0.001 compared to copper ascorbate incubated values using ANOVA.

Cu-ascorbate generates 440 nmoles hydroxyl radical per 1 ml solution at pH 7.424, which is sufficient to induce oxidative damage of cell and organelle (here mitochondria) membrane. In order to protect against this damage antioxidant compounds are necessary. Hydroxyl radical, the major stable diamagnetic ROS (reactive oxygen species) is usually generated in most of the diseases occurring in human body and damages cells and cell organelles in various diseases. Neutralization of this ROS by antioxidant compounds may be a significant initiative to cure several types of diseases including diabetes also. Our aim of this study is to evaluate the mechanism of how the *E. scaber* extract protects goat heart mitochondria against Cu-ascorbate induced oxidative stress which may indicate its significance for using it as medicine in future.

The alteration in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the incubation of mitochondria with copper-ascorbate. This is further substantiated by an elevation in the levels of lipid peroxidation. Restoration in the levels of lipid peroxidation after administration of leaves methanolic extract of *E. scaber* could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane phospholipids.

Therefore, this property of *E. scaber* might have resulted in the recoupment in the activities of the above antioxidant enzymes to normalcy. The above mentioned results of our experiments inspired us to consider it as a potent antioxidant, which is strongly evident from its ROS scavenging activities. On another side, it also prevents Cu-ascorbate induced alteration in mitochondria in a dose dependent manner. It also stimulates cellular antioxidant i.e. GSH synthesis, evident from result of glutathione reductase assay and reduced glutathione. The methanolic and aqueous extracts of *E. scaber* were known to possess sequiterpene lactones which might be responsible antioxidant potential of *E. scaber.*

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Conflict of Interests

Declared None

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