

## Research Article



## Trigonelline [99%] Protects against Copper-Ascorbate Induced Oxidative Damage to Aortic Mitochondria *In vitro*: Involvement of Antioxidant Mechanism(s)

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### ABSTRACT

Fenugreek [*Trigonella foenum graecum* (Linn.); family Papilionaceae] is commonly known as Methi in Hindi and Bengali. It is commonly used as a dietary ingredient (spice) in India and other parts of the world. It has long been used for several medicinal purposes in folk medicine. In the present study, trigonelline was isolated from the fenugreek seeds at 99% purity. Oxidative stress was generated, *in vitro*, by copper-ascorbate in mitochondria isolated from goat aortic tissue and the changes brought about in the levels of biomarkers of oxidative stress, activities of antioxidant and the levels of reactive nitrogen species, activities of Krebs's cycle enzymes and respiratory chain enzymes, cardiolipin content, di-tyrosine fluorescence, mitochondrial swelling and mitochondrial morphology were studied. When mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] then protections were found against copper-ascorbate induced oxidative stress mediated changes in mitochondria in a dose-dependent manner and antioxidant mechanisms appear to be associated with such protections. The results of the current study suggest that trigonelline [99%] may be considered as a future therapeutic antioxidant for the treatment of diseases associated with mitochondrial oxidative stress.

**Keywords:** Antioxidant property, Fenugreek, Goat aortic tissue, Mitochondria, Oxidative stress, Trigonelline [99%].

### INTRODUCTION

Heart failure is a leading cause of morbidity and mortality in industrialized countries.<sup>1</sup> It is also a growing public health problem in the developing countries as well, mainly because of aging of the population and the increase in the prevalence of heart failure in the elderly. Previous basic, clinical, and population sciences have advanced the modern treatment of heart failure. Despite extensive studies, the fundamental mechanisms responsible for the development and progression of heart failure due to abnormalities of aortic blood flow have not yet been fully elucidated. The aorta is the main artery in the human body, originating from the left ventricle of the heart and extending down to the abdomen, where it splits into two smaller arteries (the common iliac arteries). The aorta distributes oxygenated blood to all parts of the body through the systemic circulation.<sup>2</sup> The aorta is the largest artery in the body. Aorta has to withstand high blood pressure because of which it needs thick walls. So, aorta requires a huge amount of energy to maintain its normal physiological functions. Mitochondria play an important role to provide required amount of energy to aortic tissue. The large amounts of ATP required for the maintenance of aerobic life are generated predominantly by oxidative phosphorylation in mitochondria. Slightly greater than 5% of molecular oxygen gets converted in to reactive oxygen species (ROS) like, superoxide anion free radical, hydrogen peroxide and the hydroxyl radical ( $\cdot\text{OH}$ ).<sup>3</sup> So, generation of greater than normal ROS in

aortic mitochondria may cause alteration in aortic functions that may lead to cardiac dysfunction.

Studies have shown a growing demand for food that has health benefits beyond simply supplying essential nutrients to maintain nutritional status. Due to growth in consumer demand for more balanced diets, the food industry is investing in the development of functional foods.<sup>4</sup> In recent years, numerous studies describing the therapeutic properties of extracts from different parts of various medicinal plants have been developed. Indeed, the use of such extracts as complementary and alternative medicine has lately increased, and also serves as an interesting source of drug candidates for the pharmaceutical industrial research.<sup>5</sup>

*Trigonella foenum graecum* (Linn.) family Papilionaceae, commonly known as Fenugreek (Methi in Hindi and Bengali) is a small annual herb, cultivated throughout the world. Fenugreek seeds have been extensively evaluated for its phytochemical constitution and pharmacological activities.<sup>6</sup> It shows the presence of alkaloids such as trigonelline trimethylamine (derivative of vitamin B6), neurin, choline, gentianine, carpaine and betain. Many flavonoid glycosides such as quercetin, rutin, vitexin and isovitexin are also present in this plant. Fenugreek plant and its seeds both show antidiabetic activities. The alkaloid trigonelline has been reported to be responsible for the antidiabetic activity of fenugreek which is possibly due to their antioxidant activities.<sup>7</sup> Among all of these compounds, trigonelline was successfully isolated in



crystal form by Bhaskaran and Mohan.<sup>8</sup> It has been documented that trigonelline has a beneficial effect for diabetes through decreasing blood glucose and lipid levels, increasing insulin sensitivity index and insulin content, up-regulating antioxidant enzyme activity, and decreasing lipid peroxidation.<sup>9</sup> Recently, we have demonstrated that Sugaheal®, the 4-hydroxyisoleucine and trigonelline enriched fraction (TF4H (28%)) isolated from the seed of *Trigonella foenum graecum* (Linn.) had a beneficial effect against copper-ascorbate induced alteration in mitochondria in a dose-dependent manner.<sup>10</sup> But till date there is no report available, to the best of our knowledge and belief, about the protective effect of trigonelline [99%] against oxidative stress mediated damages in aortic mitochondrial. Herein, we provide evidences, perhaps for the first time, that trigonelline [99%] has the ability to protect against copper-ascorbate induced oxidative damages to goat aortic tissue mitochondria, *in vitro*, and antioxidant mechanism(s) may be responsible for such protections.

## MATERIALS AND METHODS

### Materials

Cupric-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.

### Isolation and purification of trigonelline [99%] from *Trigonella foenum graecum*

Trigonelline [99%] hydrochloride was isolated from *Trigonella foenum graecum* according to the method described earlier.<sup>11</sup> The resultant 200mg trigonelline hydrochloride was dissolved in 10ml of water and treated with 2ml of strong base anion resin to neutralize the acid and the released base is recrystallized in water, isopropyl alcohol to get 40mg of pure trigonelline in crystal form (i.e. trigonelline [99%]), which was used for the experiments described below.

### Preparation of goat aortic tissue mitochondria

Goat aortic tissue mitochondria were isolated according to the procedure of Hare et al with some modifications.<sup>12</sup> Goat aorta was purchased from local Kolkata Municipal Corporation approved meat shop. After collection the tissue was brought into laboratory in sterile plastic container kept in ice. Then, the tissue was cleaned and five gm of the tissue was minced properly and was placed in 10ml of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H<sub>2</sub>SO<sub>4</sub> (pH 7.8)] at 25°C. The minced 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 2000rpm for 10 minutes. The supernatant, thus obtained, was further centrifuged at 20,000rpm for 40 minutes. The final supernatant was discarded and the pellet was re-suspended in sucrose buffer and was either used fresh or stored at -20°C for further analysis.

### Incubation of aortic tissue mitochondria with Cu<sup>2+</sup> and ascorbic acid, in absence or presence of Trigonelline [99%]

The incubation mixture containing mitochondrial protein (6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), 0.2 mM Cu<sup>2+</sup> and 1 mM ascorbic acid, in absence or presence of different concentrations (0.1, 0.2, 0.4, 0.8mg/ml) of Trigonelline [99%], 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 40µl of 35mM EDTA.<sup>13</sup> Following incubation, (a)(a) the intactness of mitochondria, (b) the nitric oxide (NO) concentration (c) the levels of biomarkers of oxidative stress like lipid peroxidation (LPO), reduced glutathione (GSH) and the protein carbonyl (PCO) content, (d) the activities of mitochondrial antioxidant enzymes, (e) some of the Krebs's cycle enzymes, (f) respiratory chain enzymes, (g) mitochondrial membrane cardiolipin content, (h) mitochondrial swelling, (i) di-tyrosine level, were determined as described below.

### (a) Determination of mitochondrial intactness by using Janus green B stain

Following incubation, the mitochondria were spread on a slide. After that a few drops of Janus green B stain were put on the slide and was left for 5 min in moist chamber. The mitochondria were rinsed once with distilled water so that the stain was not gone and a diluted stain remained. Then, the mitochondria were mounted in a drop of distilled water with a cover slip and imaged with a confocal system (BD Pathway 855, USA). The digitized images were then analyzed using image analysis system (ImageJ, NIH Software, Bethesda, MI) and the intactness of mitochondria in each image was measured and expressed as the % fluorescence intensity.<sup>14</sup>

### (b) Measurement of reactive nitrogen species (RNS) in mitochondria

The concentration of nitric oxide (NO), one of the RNS, in the incubated mitochondria were measured spectrophotometrically at 548nm according to the method of Fiddler (1977) by using Griess reagent.<sup>15, 16</sup> The reaction mixture in a spectrophotometer cuvette (1 cm path length) contained 100 µL of Griess Reagent, 700 µL of the sample (i.e., incubated mitochondrial suspension) and 700 µL of distilled water. The NO concentration was expressed as µM/mg of protein.

### (c) Measurement of mitochondrial lipid peroxidation (LPO) level and reduced glutathione (GSH) and protein carbonyl (PCO) content

The level of LPO in the mitochondria were measured in terms of thiobarbituric acid reactive substances (TBARS) using the method of Buege and Aust (1978).<sup>17</sup> Two ml of TBA-TCA-HCl reagent (15% TCA, 0.375%TBA and 0.25(N) HCl) was added to the incubation mixture and was heated for 20 minutes at 80°C. The absorbance of the -yellow chromogen was determined spectrophotometrically at 532nm. The amount of TBARS was calculated using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1}\text{Cm}^{-1}$ .

The GSH (as acid soluble sulfhydryl) content was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak et al. (1968) with some modifications by Dutta et al. (2014).<sup>18, 19</sup> Following incubation, as described above, mitochondria were 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 of protein.

The PCO content was estimated by the method of Levine et al. (1994).<sup>20</sup> One fourth of a millilitre 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 contents of 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 spectrophotometrically at 370 nm. The PCO content was calculated using a molar absorption coefficient of  $2.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The values were expressed as nmoles/mg of protein.

### Determination of the activities of antioxidant enzymes

Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method.<sup>21</sup> To 50  $\mu\text{l}$  of the mitochondrial suspension, 430  $\mu\text{l}$  of 50 mM of Tris-HCl buffer (pH 8.2) and 20  $\mu\text{l}$  of 2 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 peroxidase (GPx) activity was measured according to the method of Paglia et al. (1967) with some modifications as adopted by Dutta et al. (2014).<sup>22, 23</sup> The assay system contained, in a final volume of one 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  $\text{H}_2\text{O}_2$ . 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.

The glutathione reductase (GR) activity was measured according to the method of Krohne- Ehrich et al. (1977).<sup>24</sup> The assay mixture in a final volume of 3 ml contained 50 mM phosphate buffer (pH 7.0), 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 mitochondrial suspension (6mg/ml protein) as the source of enzyme into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored spectrophotometrically at 340 nm. The specific activity of the enzyme was expressed as units/mg of protein.

### Determination of the activities of pyruvate dehydrogenase (PDH) and some of the Kreb's cycle enzymes

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically according to the method of Chretien et al. (1995) with some modifications by following the reduction of  $\text{NAD}^+$  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  $\text{NAD}^+$  in addition to the incubated mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of protein.<sup>25</sup>

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan et al. (1979) by measuring the reduction of  $\text{NAD}^+$  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  $\text{MnSO}_4$ , 0.1 mM  $\text{NAD}^+$  and the suitable amount of incubated mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of protein.<sup>26</sup>

Alpha-ketoglutarate dehydrogenase ( $\alpha$ -KGDH) activity was measured spectrophotometrically according to the method of Duncan et al. (1979) by measuring the reduction of 0.35 mM  $\text{NAD}^+$  to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4 as the assay buffer, 0.1 mM  $\alpha$ -ketoglutarate as the substrate and the suitable amount of incubated mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of protein.<sup>26</sup>

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] at 420 nm according to the method of Veeger et al. (1969) with some modifications.<sup>27,28</sup> One ml assay mixture contained 50 mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4 mM succinate, 2.5 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  and the suitable amount of incubated mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of protein.



### Determination of the activities of 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. (1995).<sup>29</sup> One ml of assay mixture contained in addition to the incubated mitochondrial suspension (6mg/ml protein) as the source of 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/mg of protein.

Cytochrome C oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome C at 550 nm according to the method of Goyal et al. (1995).<sup>29</sup> One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome C and a suitable aliquot of the incubated mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

### Determination of activities of respiratory complex enzymes of aortic mitochondria during coupling and uncoupling condition to assess mitochondrial status

NADH-Cytochrome C oxidoreductase activity of mitochondria was measured spectrophotometrically by following the reduction of oxidized cytochrome C at 565nm according to the method of Goyal et al. (1995).<sup>29</sup> One ml of assay mixture contained in addition to the suitable amount of mitochondrial suspension (6mg/ml protein) as the source of 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/mg of mitochondrial protein. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

Cytochrome C oxidase activity of mitochondria was determined spectrophotometrically by following the oxidation of reduced cytochrome C at 550 nm according to the method of Goyal et al. (1995).<sup>29</sup> One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome C and a suitable aliquot of the mitochondrial suspension (6mg/ml protein) as the source of enzyme. The enzyme activity was expressed as units/mg of mitochondrial protein. The CCCP was used as an uncoupler (0.1mM).

ATP synthase activity of the mitochondria, measured in the direction of ATP hydrolysis (ATPase activity), was determined by the continuous spectrophotometric assay of Rosing et al. (1975) except that 2 mM EGTA replaced EDTA in the reaction medium.<sup>30</sup> Aliquots of sonicated mitochondrial sample as a source of enzyme (representing 20-40  $\mu$ g mitochondrial protein in 20  $\mu$ l sample volume) were added to the reaction medium containing 60 mM sucrose, 50 mM triethanolamine-HCl, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 2 mM ATP, 2 mM EGTA, 1 mM KCN, 100  $\mu$ M NADH, 5 units/ml pyruvate kinase and 5

units/ml lactate dehydrogenase. The final volume in the cuvette was 1 ml. The linear reaction was followed spectrophotometrically for 2 min at 340 nm and at 37 °C. The CCCP was used as an uncoupler (0.1mM).

### Determination of mitochondrial membrane cardiolipin content

Mitochondrial membrane cardiolipin content was determined by using 10-nanoyl acridine orange (NAO) dye (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). To 100 $\mu$ l of incubated mitochondria (10mg protein/ml), increasing amounts of NAO (10 $\mu$ M) was added and the final volume was adjusted with 50mM phosphate buffer (pH 7.4). The samples were incubated for 2 min followed by centrifugation at 1000g for 5 min. The stained mitochondria were subjected to an excitation wavelength of 488 nm and an emission wavelength of band pass filter 586/42 nm to measure the mitochondrial membrane potential with help of flow cytometer (BDFACS Versa, USA).<sup>31</sup>

### Measurement of di-tyrosine fluorescence intensity

Emission spectra of di-tyrosine, a product of tyrosine oxidation, were recorded in range 380 to 440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm slit width) (Giulivi and Davies, 1994).<sup>32</sup> Emission spectra (from 425 to 480 nm, 5 nm slit width) of lysine conjugated with LPO products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width) (Dousset et al., 1994).<sup>33</sup>

### Measurement of mitochondrial swelling

Mitochondrial swelling was assessed by measuring the changes in absorbance of the suspension at 520 nm ( $\Delta$ ) by spectrophotometry according to Halestrap et al. (1990).<sup>34</sup> The standard incubation medium for the swelling assay contained 250 mmol/L sucrose, 0.3 mmol/L CaCl<sub>2</sub> and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 mL of phosphate buffer (pH 7.4). A quantity of 1.8 mL of this suspension was added to both sample and reference cuvette and 6 mmol/L succinate was added to the sample cuvette only, and the absorption at 520 nm was recorded continuously at 25°C for 10 min. Swelling of mitochondria was evaluated by the changes in the values of absorption at 520 nm.

### Scanning electron microscopy

The incubated mitochondrial suspension was centrifuged, and the supernatant was removed. The pellet was fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pellet was dehydrated for 10 min at each concentration of a graded ethanol series (50, 70, 80, 90, 95 and 100%). The pellet was immersed in pure tert-butyl alcohol and was then placed into a 4°C refrigerator until the tert-butyl alcohol solidified. The frozen samples were dried by placing them into a vacuum bottle. Mitochondrial morphology was evaluated by scanning



electron microscopy (SEM; Zeiss Evo 18 model EDS 8100).<sup>10</sup>

### Estimation of protein

The protein content of the mitochondrial suspension was determined by the method of Lowry et al. (1951).<sup>35</sup>

### Statistical evaluation

Each experiment was repeated at least three times. Data are presented as mean  $\pm$  SE. Significance of mean values of different parameters between the incubated mitochondria 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 incubations. 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 trigonelline [99%] on the intactness of aortic tissue mitochondria

Figure 1(A – J) depicts degree of mitochondrial intactness following incubation of the same with copper-ascorbate, trigonelline [99%] and copper-ascorbate plus trigonelline compared to control. The mitochondrial intactness were found to be significantly protected from being decreased in a dose-dependent manner when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%], indicating the ability of trigonelline [99%] to protect the mitochondria against copper-ascorbate induced changes in mitochondrial intactness which may be due to oxidative stress.

### Effect of trigonelline [99%] on the status of nitric oxide (NO) concentration

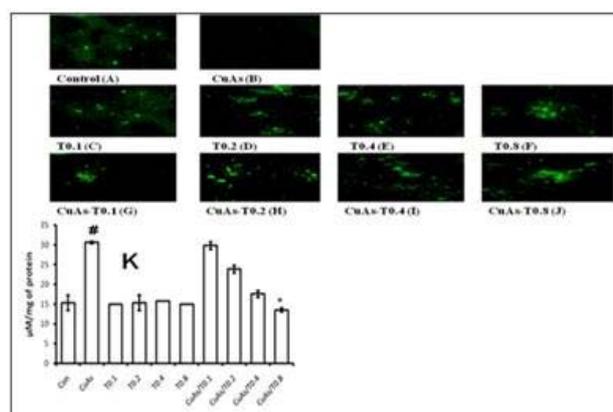
The concentration of NO in copper-ascorbate-incubated mitochondria isolated from goat aortic tissue was found to be significantly increased compared to the control mitochondria by 1 fold ( $P < 0.001$ ). However, when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%], the level of NO was found to be protected from being increased dose-dependently. At the dose of 0.8mg/ml, trigonelline [99%] was found to completely protect the NO concentration from being increased ( $P < 0.001$  compared to copper-ascorbate treated group) within the mitochondria. However, trigonelline [99%] alone (positive control), at increasing concentration, did not show any effect on NO level of mitochondria (Figure 1K).

Table 1 shows a significant increase in aortic mitochondrial LPO level following incubation of mitochondria with CuAs (1.03 fold,  $P < 0.001$  compared to control mitochondria). The level of lipid peroxidation products in mitochondria were found to be protected from being increased dose-dependently when the mitochondria were co-incubated with CuAs and increasing doses of trigonelline [99%]. Trigonelline at the dose of 0.8mg/ml completely protected the

mitochondrial LPO level from being increased. However, trigonelline [99%] alone (positive control), at increasing concentrations did not show any effect on LPO level.

A significant decrease was observed in reduced glutathione (GSH) content in aortic mitochondria following incubation of the same with CuAs (59.2%,  $P < 0.001$  compared to control mitochondria). This decrease in GSH content was found to be dose-dependently protected from being decreased when the mitochondria were co-incubated with CuAs and increasing concentrations of trigonelline [99%]. Complete protection (1.49 fold from CuAs-incubated mitochondria,  $P < 0.001$ ) was observed with trigonelline [99%] at the concentration of 0.8mg/ml. However, trigonelline [99%] alone (positive control), at increasing concentration did not show any effects on mitochondrial GSH content (Table 1).

The measurement of protein carbonyl (PCO) content demonstrated a significant increase in aortic mitochondria following incubation of the same with CuAs (1.63 fold,  $P < 0.001$  compared to control mitochondria). This elevated level of PCO was found to be dose-dependently protected from being increased when the mitochondria were co-incubated with CuAs and increasing concentrations of trigonelline [99%]. Almost complete protection (60.00% from CuAs-incubated mitochondria,  $P < 0.001$ ) was observed with trigonelline [99%] at the concentration of 0.8mg/ml. However, Trigonelline [99%] alone (positive control), at increasing concentrations did not show any effect on PCO content (Table 1).



**Figure 1:** (A-J) Changes of intactness of aortic mitochondria; Protective effect of trigonelline [99%] against copper-ascorbate-incubated (K) increase in nitric oxide concentration in mitochondria; CuAs = copper-ascorbate-incubated mitochondrial group; T0.1-0.8= mitochondrial groups incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs- T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively. The values are expressed as Mean  $\pm$  SE; #  $P < 0.001$  compared to control values using ANOVA. \*  $P < 0.001$  compared to copper-ascorbate-incubated values using ANOVA.

**Table 1:** Protective effect of trigonelline [99%] against copper-ascorbate induced alteration in the biomarkers of oxidative stress in goat aortic mitochondria CuAs = copper-ascorbate-incubated mitochondrial group; T0.1-0.8= mitochondrial group incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs- T0.1-0.8= mitochondrial group co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively; The values are expressed as Mean  $\pm$  SE; <sup>#</sup>  $P < 0.001$  compared to control values using ANOVA. \* $P < 0.001$  compared to copper-ascorbate.

Groups	LPO level (nmol TBARS/ mg of protein)	GSH (nmole GSH/ mg of protein)	Protein carbonyl (nmoles/ mg of protein)
Control	0.38 $\pm$ 0.001	24.89 $\pm$ 1.3	3.50 $\pm$ 0.1
CuAs	0.8 $\pm$ 0.001 <sup>#</sup>	11.92 $\pm$ 0.1 <sup>#</sup>	9.20 $\pm$ 0.21 <sup>#</sup>
T0.1	0.33 $\pm$ 0.003	22.31 $\pm$ 0.7	3.52 $\pm$ 0.9
T0.2	0.32 $\pm$ 0.009	24.14 $\pm$ 1.0	3.56 $\pm$ 0.2
T0.4	0.34 $\pm$ 0.002	22.98 $\pm$ 0.2	3.72 $\pm$ 0.2
T0.8	0.33 $\pm$ 0.000	23.07 $\pm$ 0.9	3.19 $\pm$ 0.3
CuAs- T0.1	0.65 $\pm$ 0.03	14.31 $\pm$ 0.0	7.00 $\pm$ 1.8
CuAs- T0.2	0.50 $\pm$ 0.011	17.04 $\pm$ 2.0	5.62 $\pm$ 1.3
CuAs- T0.4	0.42 $\pm$ 0.005	20.98 $\pm$ 0.6	4.01 $\pm$ 1.1
CuAs- T0.8	0.32 $\pm$ 0.008 <sup>*</sup>	26.07 $\pm$ 1.9 <sup>*</sup>	2.09 $\pm$ 1.4 <sup>*</sup>

incubated values using ANOVA.

#### Effect of trigonelline [99%] on the activities of antioxidant enzymes, viz., Mn-SOD, GPx and GR

Table 2 reveals a highly significant increase (60.00%,  $P < 0.001$  compared to control mitochondria) in the activity of MnSOD following incubation of mitochondria isolated from goat aortic tissue with CuAs. The activity of this enzyme was found to be dose-dependently protected from being increased when the mitochondria were co-incubated with CuAs and increasing concentrations of trigonelline [99%]. The activity of MnSOD was found to be almost completely protected from being increased when mitochondria were co-incubated with trigonelline [99%] at the concentration of 0.8mg/ml (55.91% protection compared to mitochondria incubated with CuAs;  $P < 0.001$ ). However, trigonelline [99%] alone (positive control), at increasing concentrations did not show any effect on MnSOD activity.

A highly significant decrease (48.41%,  $P < 0.001$  compared to control mitochondria) was found in the activity of GPx following incubation of aortic mitochondria with CuAs. The GPx activity was found to be dose-dependently protected from being decreased when the mitochondria were co-incubated with CuAs and increasing concentrations of trigonelline [99%]. The GPx activity was found to be completely protected (1.02 fold compared to mitochondria incubated with CuAs;  $P < 0.001$ ) from being decreased when the mitochondria were co-incubated with trigonelline [99%] at the concentration of 0.8mg/ml.

However, trigonelline [99%] alone (positive control), at increasing concentrations was found to have no effect on GPx activity (Table 2).

On the other hand, table 2 further shows a highly significant decrease (66.67%,  $P < 0.001$  compared to control mitochondria) in the activity of GR following incubation of mitochondria isolated from goat aortic tissue with CuAs. The GR activity was found to be dose-dependently protected from being decreased when the mitochondria were co-incubated with CuAs and increasing concentrations of trigonelline [99%]. Trigonelline [99%] provided complete protection (2.56 fold, compared to mitochondria incubated with CuAs;  $P < 0.001$ ) to GR activity of aortic mitochondria at the concentration of 0.8mg/ml when the same was co-incubated with CuAs. At increasing concentrations, trigonelline [99%] alone (positive control), however, was found to have no effect on GR activity.

**Table 2:** Protective effect of trigonelline [99%] against copper-ascorbate induced alteration in the activities of antioxidant enzymes in goat aortic mitochondria CuAs = copper-ascorbate-incubated mitochondrial group; T0.1-0.8= mitochondrial group incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs- T0.1-0.8= mitochondrial group co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively; The values are expressed as Mean  $\pm$  SE; <sup>#</sup>  $P < 0.001$  compared to control values using ANOVA. \* $P < 0.001$  compared to copper-ascorbate

Groups	Mn-superoxide dismutase activity (Units/ mg of protein)	Glutathione peroxidase activity (Units/ mg of protein)	Glutathione reductase activity (Units/ mg of protein)
Control	10.48 $\pm$ 0.5	25.89 $\pm$ 0.03	13.50 $\pm$ 0.41
CuAs	22.50 $\pm$ 0.101 <sup>#</sup>	12.92 $\pm$ 0.1 <sup>#</sup>	4.17 $\pm$ 0.001 <sup>#</sup>
T0.1	10.33 $\pm$ 0.003	25.31 $\pm$ 0.7	13.62 $\pm$ 0.009
T0.2	10.32 $\pm$ 0.009	24.11 $\pm$ 1.03	13.56 $\pm$ 0.12
T0.4	10.34 $\pm$ 0.23	25.98 $\pm$ 0.02	13.72 $\pm$ 0.22
T0.8	10.33 $\pm$ 0.112	25.07 $\pm$ 0.009	13.19 $\pm$ 0.03
CuAs- T0.1	20.65 $\pm$ 0.031	13.31 $\pm$ 0.016	6.80 $\pm$ 0.08
CuAs- T0.2	15.50 $\pm$ 0.011	18.04 $\pm$ 0.05	9.66 $\pm$ 0.3
CuAs- T0.4	11.42 $\pm$ 0.005	22.98 $\pm$ 0.06	12.01 $\pm$ 0.01
CuAs- T0.8	9.32 $\pm$ 0.008 <sup>*</sup>	27.67 $\pm$ 1.39 <sup>*</sup>	14.89 $\pm$ 0.44 <sup>*</sup>

incubated values using ANOVA.

#### Copper-ascorbate induced alterations in the activities of pyruvate dehydrogenase (PDH) and some of the Krebs cycle enzymes and protection by trigonelline [99%]: a mechanism-based study

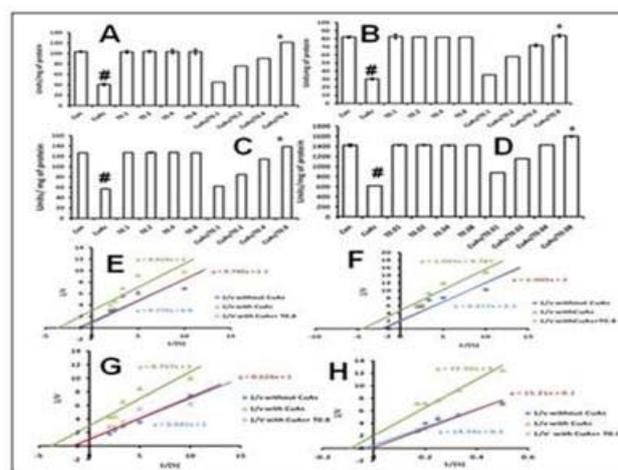
Figure 2(A-D) reveals that the incubation of the mitochondria isolated from goat aorta with CuAs inhibits PDH (61.90% compared to control;  $P < 0.001$ ), ICDH (63.14% compared to control;  $P < 0.001$ ),  $\alpha$ -KGDH (54.69% compared to control;  $P < 0.001$ ) and SDH activities (57.14% compared to control;  $P < 0.001$ ), respectively. When the

mitochondria were co-incubated with CuAs and different concentrations of trigonelline [99%], the activities of PDH, ICDH,  $\alpha$ -KGDH and SDH were found to be dose-dependently protected from being altered. In case of all the enzymes studied, the protection by trigonelline [99%] was found to be maximum at the dose of 0.8mg/ml. However, trigonelline [99%] alone, at increasing concentrations did not show any effect on the activities of these enzymes activities.

Figure 2(E-H) shows that when the mitochondria isolated from goat aorta were incubated with copper-ascorbate the  $V_{max}$  values of PDH (0.219  $\mu$ M/min), ICDH (0.168  $\mu$ M/min),  $\alpha$ -KGDH (0.699  $\mu$ M/min) and SDH (0.139  $\mu$ M/min) enzymes were found to be decreased compared to the  $V_{max}$  values of control reactions (i.e.,  $V_{max}$  of enzymes of control mitochondria) [PDH (0.250  $\mu$ M/min; 12.40%  $P<0.001$ ); ICDH (0.172  $\mu$ M/min; 2.33%  $P<0.001$ ),  $\alpha$ -KGDH (0.765  $\mu$ M/min; 8.63%  $P<0.001$ ) and SDH (0.200  $\mu$ M/min; 43.88%  $P<0.001$ ), respectively.]. The  $K_m$  value of ICDH (0.227  $\mu$ M) in mitochondria incubated with Copper-ascorbate was found to be increased compared to the  $K_m$  value of the same enzyme in control mitochondria (0.168  $\mu$ M; 35.12% increase,  $P<0.001$  compared to control mitochondria). In contrast, the  $K_m$  values of PDH (0.273 $\mu$ M),  $\alpha$ -KGDH (0.265  $\mu$ M) and SDH (11.25  $\mu$ M) were found to be decreased in mitochondria incubated with copper-ascorbate compared to the  $K_m$  values of PDH (0.963  $\mu$ M; 71.65% decrease compared to control,  $P<0.001$ ),  $\alpha$ -KGDH (0.340  $\mu$ M; 22.06% decrease compared to control,  $P<0.001$ ) and SDH (28.68  $\mu$ M; 60.77% decrease compared to control,  $P<0.001$ ) in control mitochondria. But when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] at the concentration of 0.8mg/ml, the  $V_{max}$  values of PDH (0.329  $\mu$ M/min), ICDH (0.173  $\mu$ M/min),  $\alpha$ -KGDH (.757  $\mu$ M/min) and SDH (0.336  $\mu$ M/min) enzymes were found to be significantly protected from being altered (32.13%, 2.98%, 8.30% and 1.42 fold, respectively,  $P<0.001$  compared to mitochondria incubated copper-ascorbate). Furthermore, the  $K_m$  values of PDH (0.672  $\mu$ M; 1.46 fold,  $P<0.001$  compared to mitochondria incubated with copper-ascorbate), ICDH (0.548  $\mu$ M; 1.41 fold,  $P<0.001$  compared to mitochondria incubated with copper-ascorbate),  $\alpha$ -KGDH (0.624  $\mu$ M; 1.35 fold,  $P<0.001$  compared to mitochondria incubated with copper-ascorbate) and SDH (150.59  $\mu$ M; 12.39 fold,  $P<0.001$  compared to mitochondria incubated with copper-ascorbate) enzymes were also found to be protected from being altered when the goat aortic mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] at the concentration of 0.8mg / ml.

It is well known that in case of uncompetitive inhibition of enzymes, the  $V_{max}$  value decreases and the  $K_m$  value can either increase or decrease. Thus, the results of our study demonstrate that copper-ascorbate in combination can inhibit the activities of PDH and the Krebs cycle enzymes in an uncompetitive manner. However, when the mitochondria were co-incubated with copper-ascorbate

and trigonelline [99%] (0.8mg/ml), the activities of these enzymes associated with energy metabolism were found to be significantly protected from being altered.



**Figure 2:** Protective effect of trigonelline [99%] against copper-ascorbate-incubated (A-D) alteration and (E-H) uncompetitive inhibition of the activities of the pyruvate dehydrogenase and other Krebs cycle enzymes in mitochondria isolated from goat aortic tissue. Con=control mitochondrial group; CuAs = copper-ascorbate-incubated mitochondrial group; T0.1-0.8= mitochondrial groups incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs- T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively; The values are expressed as Mean  $\pm$  SE; #  $P<0.001$  compared to control values using ANOVA. \* $P<0.001$  compared to copper-ascorbate incubated values using ANOVA.

### Effect of trigonelline [99%] on the activities of respiratory chain enzymes

The activities of NADH cytochrome C oxidoreductase (65.79% decrease;  $P<0.001$  compared to control mitochondria) and cytochrome C oxidase (65.45% decrease;  $P<0.001$  compared to control mitochondria) were found to be significantly decreased when the mitochondria isolated from goat aortic tissue, were incubated with copper-ascorbate. The activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase enzymes were found to be protected, dose-dependently, from being decreased, when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml). Here also, the protection by trigonelline [99%] was found to be maximum at the dose of 0.8mg/ml in case of NADH cytochrome C oxidoreductase (1.86 fold;  $P<0.001$  compared to CuAs-incubated mitochondria) as well as cytochrome C oxidase (2.68 fold;  $P<0.001$  compared to CuAs-incubated mitochondria) Trigonelline [99%] alone, at increasing concentrations did not show any effect on the activities of these respiratory chain enzymes (Figure 3(A-B)).

### Assessment of functional status of mitochondria isolated from goat aortic tissue by determining the activities of respiratory complex enzymes during coupling and uncoupling condition

Figure 3C demonstrates a significant decrease in goat aortic mitochondrial ATP synthase activity following the incubation of mitochondria with CCCP (42.11%,  $P < 0.001$  compared to control mitochondria without CCCP). But there were no significant changes in mitochondrial NADH cytochrome C oxidoreductase and cytochrome C oxidase activities following the incubation of mitochondria isolated from goat aortic tissue with CCCP.

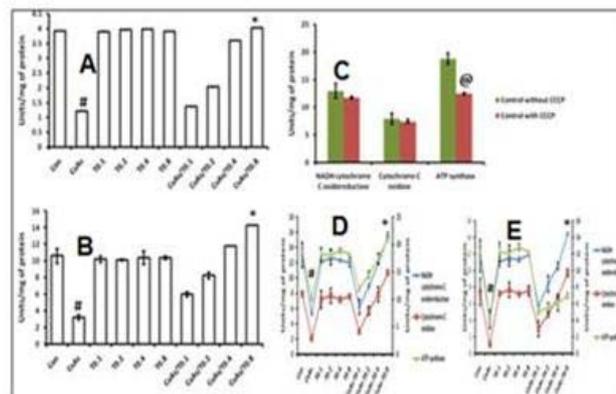
Figure 3D reveals highly significant decreases in the activities of NADH cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthase following incubation of aortic tissue mitochondria with copper-ascorbate (2.11 fold, 72.50% and 44.44%, respectively,  $P < 0.001$  compared to activities of these enzymes control mitochondria). The activities of these enzymes were found to be significantly and dose-dependently protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml) (1.72 fold, 3 fold and 1.16 fold protection, respectively;  $P < 0.001$  compared to copper-ascorbate-incubated mitochondria). However, trigonelline [99%] alone has no effect on enzymes activities.

Figure 3E reveals that when mitochondria were pre-incubated with CCCP prior to incubating them with copper-ascorbate the activities of NADH cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthase were found to significantly decreased (66.67%, 65.91% and 80.77%, respectively,  $P < 0.001$  compared to control mitochondria). The activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase were found to be significantly and dose-dependently protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml) following pre-incubation with CCCP (2.33 fold and 50.00% protection, respectively;  $P < 0.001$  compared to copper-ascorbate-incubated mitochondria). In contrast, the activity of ATP synthase was not found to be significantly protected when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml) following pre-incubation with CCCP (37.78% protection;  $P < 0.001$  compared to copper-ascorbate-incubated mitochondria).

### Effect of trigonelline [99%] on the mitochondrial cardiolipin content

The fluorescence variations of 10-nanoyl acridine orange (NAO) may also be used to reveal topological modifications of cardiolipin in the space of a biological structure (Fernandez et al., 2004). In case of analysis carried out through flow cytometry (Fig. 4A), it was observed that when the mitochondria isolated from aortic tissue were incubated with copper-ascorbate, the mitochondrial membrane was damaged. So, the

dimerization between cardiolipin and NAO dye was decreased which decreases the fluorescence (74.55%, respectively,  $P < 0.001$  compared to control mitochondria). However, when the mitochondria, were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml), the fluorescence was found to be protected from being decreased by 2.57 fold ( $P < 0.001$  compared to CuAs-incubated mitochondria). This indicates that trigonelline [99%] at the concentration of 0.8mg/ml has the ability to strongly and significantly protect the mitochondrial membrane cardiolipin content. Trigonelline [99%] alone, at the concentration of 0.8mg/ml, did not show any effect on mitochondrial membrane cardiolipin content.

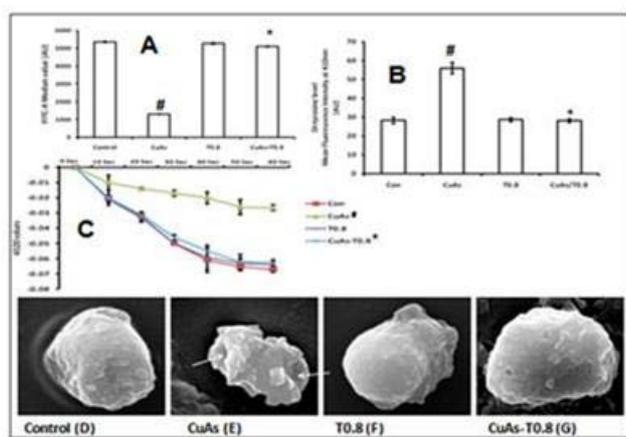


**Figure 3:** Protective effect of trigonelline [99%] against copper-ascorbate-incubated alteration in (A-B) the respiratory chain enzymes and (C-E) effect of CCCP on the activities of NADH cytochrome C oxidoreductase, NADH cytochrome C oxidase, ATP synthase of liver (F) and brain (I) mitochondria;  $^{\oplus}P < 0.001$  compared to control values without CCCP. Effect of trigonelline [99%] against copper-ascorbate-induced decrease in activities of NADH cytochrome C oxidoreductase, NADH cytochrome C oxidase, ATP synthase in goat liver and brain mitochondria (G, J) in absence of CCCP and (H, K) in presence of CCCP; CuAs = copper-ascorbate incubated mitochondrial group; T0.1-0.8= mitochondrial groups incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs-T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively; The values are expressed as Mean  $\pm$  SE;  $^{\#}P < 0.001$  compared to control values using ANOVA.  $^*P < 0.001$  compared to copper-ascorbate incubated values using ANOVA.

### Copper-ascorbate induced alterations on the di-tyrosine fluorescence intensity: protection by trigonelline [99%]

The effect of the free radical-generating system on protein structure of mitochondria isolated from goat aortic tissue was examined by measuring di-tyrosine fluorescence. That copper-ascorbate induced oxidative stress has a direct effect on the oxidation level of amino acid is evident from increased di-tyrosine formation studied through the measurement of basal auto-fluorescence of the amino acids. Co-incubation of

mitochondria with copper-ascorbate and trigonelline [99%] (0.8mg/ml) ameliorates these changes, thereby, restoring these molecules to their original configuration as indicated by recovered auto-fluorescence level for formation of di-tyrosine. Figure 4B depicts a highly significant increase (96.43%,  $P < 0.001$  compared to control mitochondria) in the di-tyrosine fluorescence following incubation of mitochondria with copper-ascorbate. This di-tyrosine fluorescence level was found to be significantly protected from being increased when the mitochondria were co-incubated with CuAs and trigonelline [99%] (0.8mg/ml) by 50.91% ( $P < 0.001$  compared to CuAs-incubated mitochondria). Trigonelline [99%], alone, at the concentration of 0.8mg/ml (positive control) was found to have no effect on the di-tyrosine fluorescence of mitochondria.



**Figure 4:** Protective effect of trigonelline [99%] against copper-ascorbate-incubated (A) decrease in mitochondrial cardiolipin content, (B) increase in mitochondrial di-tyrosine level, (C) increase in mitochondrial swelling in mitochondria isolated from goat aortic mitochondria, and (D-G) alterations on mitochondrial surface [Scanning electron micrograph (X6000)] in mitochondria isolated from goat heart, liver, brain, lung and kidney. Arrow heads indicate perforated surface of mitochondria; Con=control mitochondrial group; CuAs = copper-ascorbate incubated mitochondrial group; T0.1-0.8= mitochondrial groups incubated with trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively (positive control); CuAs- T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.1-0.8mg/ml respectively; The values are expressed as Mean  $\pm$  SE;  $^{\#}P < 0.001$  compared to control values using ANOVA.  $^*P < 0.001$  compared to copper-ascorbate incubated values using ANOVA.

#### Protective effect of trigonelline [99%] against copper-ascorbate induced mitochondrial swelling

It is well established that energized mitochondria supplemented with high  $Ca^{2+}$  concentration swell in the presence of inorganic phosphate. In presence of 0.3 mmol/L of  $CaCl_2$  (pH 7.2), the absorbance at 520 nm declined, indicating mitochondrial swelling due to

alteration in osmotic pressure. The extent of absorbance was found to be increased in the copper-ascorbate-incubated mitochondria isolated from goat aortic tissue compared to that observed in the control mitochondria (Fig. 4C), demonstrating that copper-ascorbate incubation caused mitochondrial dysfunction. Compared with the copper-ascorbate incubated mitochondria, the absorbance of mitochondria co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml) was found to be significantly protected from being increased, indicating its protective action against copper-ascorbate-incubated mitochondrial damage ( $P < 0.001$ ).

#### Scanning electron microscopy

Figure 4(D - G) shows the changes brought about to the mitochondrial surface, following incubation with copper-ascorbate, studied through scanning electron microscopy. When the mitochondria isolated from goat aortic tissue were incubated with copper-ascorbate, a perforated surface with convoluted membranes was observed. The mitochondria were markedly contracted with large membrane blebs covering the surface. These copper ascorbate-induced changes in the mitochondrial surface were found to be significantly protected from being taken place when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml).

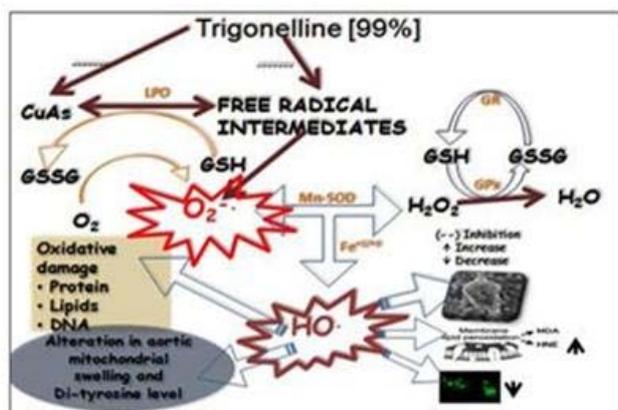
#### DISCUSSION

Free radicals have been postulated to play a key role in starting the chain of age-related disorganization in target cells which contain mitochondria, using high levels of oxygen and thereby releasing large amounts of oxygen radicals exceeding the homeostatic protection of cells.<sup>36</sup> Mitochondria, being the major source of oxidant production, can be considered the 'biological clock' for the aging process.<sup>37</sup> Mitochondrial aging is characterized by destruction of membrane structural integrity essential to mitochondrial functions, leading to the loss of mitochondrial membrane fluidity.<sup>38</sup>

Mitochondria are considered as power houses of a cell as it produces ATP by a process called oxidative phosphorylation.<sup>39</sup> The live mitochondria can be observed under a light microscope if stained with Janus green. This stain is bluish green in colour when oxidized and colourless when reduced. When a dilute solution is applied to stain the mitochondria, it enters the mitochondria. Since inner mitochondrial membrane contains cytochrome oxidase enzyme that can keep the stain in oxidized state, the mitochondria appear stained while in rest of the cytoplasm the stain gets reduced and thus appears colourless.

The inhibition of lipid peroxidation by antioxidants should be beneficial for maintenance of health and reducing disease risk.<sup>40</sup> In addition, a relation between copper metabolism and the intracellular availability of glutathione has been defined.<sup>41</sup> 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 GSH. Trigonelline [99%] was found to be effective in decreasing the lipid peroxidation level of the mitochondria.



**Figure 5:** Schematic diagram representing the antioxidant mechanism(s) of protection of trigonelline [99%] against copper-ascorbate incubated oxidative damages in mitochondria isolated from goat aortic endothelial tissue.

Thiols are thought to play a pivotal role in protecting cells against peroxidation.<sup>42</sup> Cellular defense mechanism against superoxide includes a series of linked enzyme reaction to remove superoxide and repair radical induced damage. First of these enzymes is superoxide dismutase (SOD), which converts superoxide to  $H_2O_2$ . Superoxides escape quenching in the mitochondria of older tissues because the ability of mitochondrial protective mechanism(s) against disorganizing effects of free radicals decreases during senescence.<sup>43</sup> Glutathione peroxidase (GPx) catalyzes the transformation of  $H_2O_2$  within the cell to harmless by products, thereby curtailing the quantity of cellular destruction inflicted by LPO products.<sup>44</sup> The GPx is required to repair LPO initiated by superoxide in the phospholipid bilayer for maintenance of mitochondrial membrane cardiolipin content which is responsible for mitochondrial membrane integrity. The reaction of this enzyme causes the oxidation of GSH, which is in turn reduced by GR at the expense of NADPH. Thus, NADPH plays a crucial role in the full functioning of both the enzymes.

Mitochondrial physiology and mitochondrial pathologies in cellular damages can be understood by the study of mitochondrial respiratory parameters.<sup>45</sup> Mitochondrial uncoupling can arise from oxidative damage of the mitochondrial membrane as induced by increased ROS concentrations.<sup>46</sup> Return of protons to the mitochondrial matrix either through ATP synthase (coupled respiration) or proton leak mechanisms (uncoupled respiration) can decrease proton motive force (PMF) and thereby acutely decrease ROS emission. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a chemical uncoupler which can provide its effects only on the activity of ATP synthase. Our study provided the evidence that when mitochondria were incubated with CCCP it can only decrease the activity of ATP synthase but not the

activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase. This indicates that mitochondria remained viable during both coupling and uncoupling conditions.

Heavy metals are also known to affect respiratory chain complexes and there is absolute substrate specificity.<sup>47</sup> 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. When the mitochondria were co-incubated with copper-ascorbate and different doses of trigonelline [99%] then it was observed that trigonelline [99%] can protect the activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase from being decreased due to copper-ascorbate but activity of ATP synthase was not protected in presence of CCCP. So, from our study it is established that copper-ascorbate is an inhibitor but not the uncoupler and trigonelline [99%] can protect the mitochondria from the inhibitory effect of copper-ascorbate but not the uncoupling effect of CCCP.

From our study it can be evidenced that trigonelline [99%] can protect against copper-ascorbate-induced oxidative damages in mitochondria isolated from goat aortic tissue, in a dose-dependent manner.

Mitochondrial Kreb's cycle enzymes activities were inhibited when the mitochondria were incubated with copper-ascorbate. So, the electrons which are not being utilized by Kreb's cycle enzymes have been transported to the free oxygen in mitochondrial matrix. Thus, superoxide anion free radical has been generated.  $O_2^{\bullet-}$  accumulation may be exacerbated by a difference in the rate of  $O_2^{\bullet-}$  accumulation and conversion to  $H_2O_2$  and  $OH^{\bullet}$  (most stable diamagnetic free radical) in stressed mitochondria.<sup>48</sup> Alternatively,  $O_2^{\bullet-}$  may be metabolized promptly with other reactive species such as nitric oxide (NO). NO is shown to interact with  $O_2^{\bullet-}$  to generate peroxynitrite anions (ONOO<sup>-</sup>) and nitrogen oxides, (Nicco) which could attenuate the formation of the highly reactive  $OH^{\bullet}$  and oxidative damage to mitochondrial membrane cardiolipin content as well as membrane swelling in mitochondria isolated from different goat tissues. The impairment of electron transfer through complex I and complex III may induce superoxide anion free radical formation.<sup>49</sup> The electron transfer chain of mitochondria is also a well-documented source of  $H_2O_2$ . Under our experimental conditions,  $OH^{\bullet}$  free radicals probably react with various amino acids of the Kreb's cycle enzymes which may be responsible for distortions of functional sites of the enzymes. For this reason in case of copper-ascorbate-incubated mitochondria the activities of Kreb's cycle enzymes were inhibited in an uncompetitive manner. But when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] then the Kreb's cycle enzymes were found to be protected from being inhibited. These enzymes are sensitive to reactive

oxygen species (ROS) and decrease in the activities of these enzymes could be critical in the metabolic deficiency induced by oxidative stress.<sup>50, 51</sup>

## CONCLUSION

In conclusion, trigonelline [99%] can be very effective antioxidant and can protect biological systems against the oxidative stress that is found to be an important pathophysiological event in a variety of diseases including aging, diabetes, cardiovascular disorders. To the best of our knowledge and belief, this is the first report to describe an antioxidant mechanism(s) (Figure 5) of protective effect of trigonelline [99%] toward copper-ascorbate-induced mitochondrial oxidative damages. Therefore, as it is stated above trigonelline [99%] shows high antioxidant capacity inhibits lipid peroxidation in mitochondrial *in vitro* models, the present study suggests that trigonelline [99%] may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement.

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