Trigonelline [99%] protects against copper-ascorbate induced oxidative damage to mitochondria: an in vitro study

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ABSTRACT

Fenugreek [Trigonella foenum graecum] (Linn.); family Papilionaceae, commonly known as Methi in Hindi and Bengali is a small annual herb which is cultivated throughout the world. It is commonly used as a dietary ingredient (spice) in India, Middle East, Egypt, North Africa 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 and the pure compound was found to scavenge reactive oxygen species (ROS), in vitro. Oxidative stress was generated, in vitro, by copper-ascorbate in mitochondria isolated from goat tissues like, heart, liver, brain, lung and kidney and the changes brought about in the levels of biomarkers of oxidative stress, activities of antioxidant and pro-oxidant enzymes, levels of reactive oxygen and nitrogen species, activities of Kreb’s cycle and respiratory chain enzymes, cardiolipin content, NADH autofluorescence, di-tyrosine fluorescence, mitochondrial swelling and mitochondrial morphology was studied. Trigonelline [99%] when co-incubated was found to protect against copper-ascorbate induced oxidative stress mediated changes in mitochondria and antioxidant mechanisms appear to be associated with such protection. A dose-dependant protection was also evident. The results of the current study suggests that trigonelline [99%] may be considered as a future therapeutic antioxidant and may be used singly or as a co-therapeutic in the treatment of diseases associated with mitochondrial oxidative stress.

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INTRODUCTION

Antioxidants can protect the human body from damages associated with free radicals. Although many synthetic antioxidants (e.g., butylated hydroxytoluene) have been used to decrease the excessive amounts of free radicals and their safety still raises concerns due to their potential side effects. It has been reported that synthetic antioxidants have displayed some toxic and hazardous effects on organisms. For this reason, antioxidants, especially peptides, from natural sources and foods have gained growing interests in recent days.

Currently one of the strategies used to improve health is the use of functional foods. Human health is benefited by functional food beyond the effect of nutrients alone. Fenugreek (Trigonella foenum graecum) is cultivated throughout the world and is being used as a nutritional regimen. Fenugreek seeds have been extensively used for its phytochemical constitution and pharmacological activities. It shows the presence of alkaloids such as trigonelline trimethylamine, neurin, choline, gentianine, carpine and betain. Many flavonoid glycosides such as queretin, rutin, vitexin and isovitexin are also present in this plant. Fenugreek plant parts and its seeds both show antidiabetic activity.

The most common ROS are hydrogen peroxide, hydroxyl radical, superoxide anion, hydrogen peroxide, and nitric oxide. These radicals are formed as a result of metabolic reactions in the mitochondria. Mitochondrial oxidative stress occurs when there is a net increase in reactive oxygen species with respect to the capacity of the cell to produce antioxidants. Oxidative stress is associated with diseases such as atherosclerosis, diabetes, cancer, neurodegenerative diseases, liver cirrhosis and the aging process. The most common ROS are hydrogen peroxide, hydroxyl radical, superoxide anion, hydrogen peroxide, and nitric oxide.
superoxide anion free radical (\(O^2^-\)), hydrogen peroxide (\(H_2O_2\)) and hydroxyl radical ('OH) that are partially reduced species. Peroxynitrite anion (ONOOO\(^-\)), generated by the reaction between nitric oxide ('NO) and \(O^2^-\), is another reactive species that has gained great biological importance. Reactive species are continuously generated inside the organism as a consequence of exposure to exogenous chemicals in our environment and/or to the endogenous metabolic processes involving enzymes and bio-energetic electron transfer. Among all of the compounds that are present in fenugreek seed, trigonelline was successfully isolated according to the method of Bhaskaran and Mohan. It is documented that trigonelline had 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. Recently, the studies carried out at our laboratory demonstrated that Sugaheal, the trigonelline and 4-hydroxyisoleucine enriched fraction (TF4H) isolated from Fenugreek seed, had a beneficial effect against mitochondrial oxidative stress. Herein, we provide evidences, perhaps for the first time, that trigonelline [99\%] has the ability to protect against copper-ascorbate induced oxidative damage to mitochondria obtained from goat tissues, in vitro, and antioxidant mechanism(s) may be responsible for such protections.

**MATERIALS AND METHODS**

**Chemicals**

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. 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 mitochondria from goat tissues (heart, liver, brain, lung and kidney)**

The mitochondria from goat tissues like heart, liver, brain, lung and kidney were isolated according to the method described earlier with some modifications. Goat tissues were purchased from local Kolkata Municipal Corporation approved meat shop. After collection, the tissues were brought into laboratory in sterile plastic container kept in ice. Then, the tissues were cleaned and five gm of each tissue was placed in 10ml of sucrose buffer (0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H\(\text{SO}_4\) (pH 7.8)) at 25°C. The tissues were then blended for 1 minute at low speed by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA). The homogenate was centrifuged at 1500rpm for 10 minutes. The supernatant, thus obtained, was kept in ice. This was then centrifuged at 4000rpm for 5minutes. The supernatant obtained was further centrifuged at 20000rpm for 40 minutes. The final supernatant was discarded and the pellet was resuspended in sucrose buffer and was either used fresh or stored at -20°C until further analysis. Each experiment was repeated three times with the mitochondria prepared from a fresh batch of goat tissues.

**Incubation of mitochondria with Cu\(^{2+}\) and ascorbic acid**

To determine the dose-dependent effect of copper, ascorbate and trigonelline three different sets of experiments were carried out. In the first set, the incubation mixture contained in addition to mitochondrial protein (1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), and different concentrations of Cu\(^{2+}\) (0.04, 0.08, 0.1, 0.2 mM). In the second set, apart from mitochondria, the incubation mixture contained different concentrations of ascorbic acid (0.4, 0.8, 1, 2mM). In the third set of experiments, mitochondria were incubated with different concentrations of ascorbic acid (0.4, 0.8, 1, 2mM) and 0.2mM Cu\(^{2+}\), in a final volume of 1.0 ml, at 37°C in an incubator for 1 hour. In each case, the reaction was terminated by the addition of 40µl of 35mM EDTA.

**Protection of Cu\(^{2+}\)-ascorbate-induced mitochondrial oxidative damage by trigonelline [99\%]**

The mitochondria, obtained from different goat tissues, in separate assays, were co-incubated with copper-ascorbate and four different concentrations of trigonelline [99\%] (0.1, 0.2, 0.4, 0.8mg/ml) (Concentration of stock trigonelline [99\%] solution was 1mg/ml). After the incubation period of 1 hour, the reaction was terminated as described above, and, the intactness of mitochondria, the biomarkers of oxidative stress like lipid peroxidation level, reduced glutathione and protein carbonylation level, the activities of antioxidant enzymes and Kreb’s cycle enzymes, mitochondrial swelling, tryptophan level, di-tyrosine level, mitochondrial membrane potential were determined as described below.

**Determination of hydroxyl (OH) radical scavenging activity of trigonelline [99\%]**

Incubation mixture contained sodium phosphate buffer (0.05mM, pH 7.4) with 1mM ascorbate and 0.2mM Cu\(^{2+}\) for 60 minutes in the presence and absence of DMSO (500µM) and different concentrations of trigonelline [99\%] (0.1, 0.2, 0.4, 0.8mg/ml) in a volume of 1ml to determine the hydroxyl radical scavenging activity of the trigonelline [99\%] in an in vitro system. Each of the reaction mixture contained 6µg/ml...
Protein carbonyl (PCO) content was estimated by the method described earlier with some modifications. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer to determine the GSH content. The values were determined spectrophotometrically at 532 nm. The PCO content was calculated using an extinction coefficient of 2.2X 10^-4 M^-1 cm^-1. The values were expressed as nmoles/mg of protein.

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 548 nm according to the method described earlier by using Griess reagent. 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.

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 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 of each image was measured and expressed as the % fluorescence intensity. Besides, 100µl of mitochondrial suspension, after incubation, were mixed with 20µl of Janus Green B solution and incubated at room temperature, in dark. The mitochondria, stained with Janus Green B were analyzed by using flow cytometry (BDFACS Versa, USA).

Measurement of mitochondrial lipid peroxidation (LPO) level and reduced glutathione (GSH) and protein carbonyl (PCO) content
The level of lipid peroxidation in the mitochondria was measured in terms of thiobarbituric acid reactive substances (TBARS) using the method described earlier. 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 sample was determined spectrophotometrically at 532 nm. The level of TBARS was calculated using an extinction coefficient of 1.56 X 10^5 M^-1 cm^-1.

The glutathione content (as acid soluble sulphydryl) was estimated by its reaction with DTNB (Ellman’s reagent) following the method described earlier with some modifications. Incubated 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.

Protein carbonyl (PCO) content was estimated by the method described earlier. 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 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 4000 g 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 2.2X 10^-4 M^-1 cm^-1. The values were expressed as nmole/mg of protein.

Determination of the activities of antioxidant enzymes
Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method. To 50µl of the mitochondrial suspension, 430µl of 50 mM of Tris–HCl buffer (pH 8.2) and 20µl of 2 mM pyrogallol 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 pyrogallol as determined by change in absorbance per 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 described earlier with some modifications. 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 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.

The glutathione reductase (GR) activity was measured according to the method described earlier. 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. Then, 0.1 mM NADPH was added together with suitable amount of incubated mitochondrial suspension as the source of enzyme into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSGG). 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 described earlier 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 suitable amount of mitochondria as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Isocitrate dehydrogenase (ICDH) activity was measured according to the method described earlier 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 MnSO₄, 0.1 mM NAD⁺ and the suitable amount of incubated mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Alpha-ketoglutarate dehydrogenase (a-KGDH) activity was measured spectrophotometrically according to the method described earlier 35 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, 0.1 mM a-ketoglutarate as the substrate and the suitable amount of incubated mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide [K₃Fe(CN)₆] at 420 nm according to the method of described earlier with some modifications 36,17. 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 the suitable amount of incubated mitochondrial suspension 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 described earlier 37. One ml of assay mixture contained in addition to the incubated mitochondrial suspension 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 described earlier 37. 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 as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

**Determination of activities of respiratory complex enzymes of liver and brain mitochondria during coupling and uncoupling condition to assess mitochondrial status**

NADH-Cytochrome C oxidoreductase activity of liver and brain mitochondria was measured spectrophotometrically by following the reduction of oxidized cytochrome C at 565 nm according to the method of described earlier 37. One ml of assay mixture contained in addition to the suitable amount of mitochondrial suspension 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 liver and brain mitochondria was determined spectrophotometrically by following the oxidation of reduced cytochrome C at 550 nm according to the method of described earlier 37. 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 as the source of enzyme. The enzyme activity was expressed as units/mg of mitochondrial protein. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

ATP synthase activity of the liver and brain mitochondria, measured in the direction of ATP hydrolysis (ATPase activity), was determined by the continuous spectrophotometric assay of described earlier except that 2 mM EGTA replaced EDTA in the reaction medium. Aliquots of sonicated mitochondrial sample as a source of enzyme (20-40 µg in 20 µl sample volume) were added to the reaction medium containing 60 mM sucrose, 50 mM triethanolamine-HCl, 50 mM KCl, 4 mM MgCl₂, 2 mM ATP, 2 mM EGTA, 1 mM KCN, pH 8.0 (KOH) with 100 µM NADH, 5 units/ml pyruvate kinase and 5 units/ml lactate dehydrogenase. The total volume in the cuvette was 1 ml. The linear reaction was followed for 2 min at 340 nm and 37 °C. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as an uncoupler (0.1mM).

**Determination of mitochondrial membrane cardiolipin content**

Mitochondrial membrane cardiolipin content was determined by using NAO (10-nanoyl acridine orange) dye (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). To 100µl of incubated mitochondria (10mg protein/ml), 10µl of NAO (10µM) was added and the final volume was adjusted with 50mM phosphate buffer. The samples were incubated for 2 min, and then centrifuged at 1000g for 5 min. The stained mitochondrial sample was analysed and an excitation wavelength of 488 nm and an emission wavelength of band pass filter 586/42 nm were used to measure the mitochondrial membrane potentiality with help of flow Cytometry (BDFACS Versa, USA).

**Determination of mitochondrial nicotinamide adenine dinucleotide-reduced (NADH) level**

The mitochondrial nicotinamide adenine dinucleotide-reduced (NADH) was monitored by measuring its autofluorescence with excitation and emission wavelengths of 360 nm and 450 nm, respectively according to the method described earlier 39. Mitochondria (2 mg protein) were added to 1.8mL of phosphate buffer containing 6mmol/L succinate and the autofluorescence of NADH was determined.

**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) 40. Emission spectra (from 425 to 480 nm, 5 nm slit width) of lysine conjugated with LPO products were...
Measurement of mitochondrial swelling
Mitochondrial swelling was assessed by measuring the changes in absorbance of the suspension at 520 nm (Δ) by spectrophotometry according to the method described earlier. The standard incubation medium for the swelling assay contained 250 mmol/L sucrose, 0.3 mmol/L CaCl₂ and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 mL of phosphate buffer. 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 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).

Spectral scan to identify the mechanism(s) of possible interaction between trigonelline and copper, ascorbate and/or copper-ascorbate system
The spectra of solutions of only copper (0.2mM), only ascorbic acid (1mM), only trigonelline (0.8mg/ml), copper (0.2mM) and trigonelline (0.8mg/ml), ascorbic acid (1mM) and trigonelline (0.8mg/ml) and copper-ascorbate (0.2mM/1mM) and trigonelline (0.8mg/ml) were recorded at wavelengths ranging from 200-800nm to determine the absorption maxima of the different component(s) using a UV-VIS scanning spectrophotometer (Systronics; Model: Type 2202 which company).

Estimation of protein
The protein content of the different samples was determined by the method described earlier.

Statistical evaluation
Each experiment was repeated at least three times. Data are presented as mean ± SE. Significance of mean values of different parameters between the incubation 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. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Hydroxyl radical generation by copper-ascorbate system
Fig. 1A depicts that copper when added to the incubation medium, in vitro, containing mitochondria obtained from goat heart, liver, brain, lung and kidney, cannot produce hydroxyl radical significantly whereas ascorbic acid itself can generate hydroxyl radical in very small amount which is not enough to cause oxidative damages in mitochondria. However, a pro-oxidant effect of ascorbic acid was evident at the concentration of 2mM. But copper and ascorbic acid at the concentrations of 0.2mM and 1mM, respectively, in combination can produce hydroxyl radical significantly in these mitochondria (i.e., heart,97.80%; liver,1.68 fold; brain,90.91%; lung,96.82%; and kidney,87.89%, respectively, with respect to control, P < 0.001). So, it can be concluded that neither copper nor ascorbic acid alone are potent enough to produce hydroxyl radical but they can produce significantly higher amount of ‘OH in mitochondria when incubated in combination, in vitro.

Effect of trigonelline [99%] on the hydroxyl radical scavenging activity
The hydroxyl radical scavenging ability of trigonelline [99%] was studied in an in vitro standard model system using Cu²⁺ and ascorbic acid where 'OH was generated. Fig. 1B demonstrates that Cu²⁺-ascorbic acid produced significant amount of 'OH, in vitro, in mitochondria obtained from goat heart, liver, brain, lung and kidney tissues (227.04, 289.88, 253.77, 239.65 and 223.54 nmoles 'OH/mg of protein, respectively [P < 0.001 vs. control mitochondrial]) and trigonelline [99%] scavenged 'OH in a concentration-dependent manner. Trigonelline [99%], at the concentration of 0.8mg / ml, scavenged maximum amount of 'OH, in vitro (P < 0.001 vs. CuAs).

Effect of trigonelline [99%] on the status of nitric oxide concentration
The concentration of NO in mitochondria in copper-ascorbate-incubated mitochondria isolated from goat heart, liver, brain, lung and kidney were found to be significantly increased compared to the control mitochondria by 66.40%, 37.72%, 50.53%, 63.10%, 40.55%, respectively (P < 0.001). However, when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] (0.8mg/ml), the level of NO was found to be dose-dependently protected from being increased in heart, liver, brain, lung and kidney mitochondria by 49.33%, 55.26%, 54.77%, 33.06% and 64.66%, respectively (P < 0.001) compared to CuAs-incubated mitochondria (Fig. 1C). Trigonelline [99%], alone, at increasing concentration did not show any effects on NO level of mitochondria.
Figure 1. (A) Hydroxyl radical generation by different concentrations of copper alone, different concentrations of ascorbic acid alone and different concentrations of ascorbic acid with a fixed concentration of copper; Protective effect of trigonelline [99%] against copper-ascorbate-incubated increase in (B) hydroxyl radical generation and (C) nitric oxide concentration in mitochondria isolated from goat heart, liver, brain, lung and kidney. Con= control mitochondrial group; Cu0.04-0.2= mitochondrial groups incubated with copper at the dose of 0.04-0.2mM/ml respectively; Asc0.4-2= mitochondrial groups incubated with ascorbic acid at the dose of 0.4-2mM/ml respectively; Cu0.2-Asc0.4= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 0.4mM/ml; Cu0.2-Asc0.8= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 0.8mM/ml; Cu0.2-Asc1= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 1mM/ml; Cu0.2-Asc2= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 2mM/ml; CuAs = copper-ascorbate incubated mitochondrial group; T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and 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 ± S.E.; #P < 0.001  as compared to control values using ANOVA; *P < 0.001 as compared to copper-ascorbate incubated values using ANOVA.
Effect of trigonelline [99%] on the intactness of mitochondria

Fig. 2(A-E) depicts a significant decrease in the mitochondrial intactness following the incubation of mitochondria with copper-ascorbate. The mitochondrial intactness were found to be significantly protected from being decreased in a dose-dependent manner when the mitochondria were co-incubated with 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.

Figure 2. (B-E) Protective effect of trigonelline [99%] against copper-ascorbate incubated changes in intactness of mitochondria isolated from goat heart, liver, brain, lung and kidney. 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.
Effect of trigonelline [99%] on the biomarkers of oxidative damage

Fig. 3A, showed a significant increase in mitochondria isolated from goat heart, liver, brain, lung and kidney LPO level following the incubation of mitochondria with CuAs (2.10 fold, 69.45%, 2.81 fold, 84.37% and 2.12 fold, respectively, P<0.001 vs. control mitochondria). The level of lipid peroxidation were found to be protected from being increased significantly in mitochondria isolated from goat heart, liver, brain, lung and kidney (63.58%, by 49.78%, 61.05%, 39.83% and 58.23%, respectively, from CuAs-incubated mitochondria, P ≤ 0.001) when the mitochondria were co-incubated with CuAs and trigonelline [99%] (0.8mg/ml), indicating the ability of this trigonelline [99%], a bioactive compound isolated from Trigonella foenum graecum to protect the mitochondria against oxidative stress-induced changes due to copper-ascorbate. However, trigonelline [99%], alone, at increasing concentration did not show any effects on lipid peroxidation level.

A significant decrease was observed in reduced glutathione (GSH) content in mitochondria isolated from goat heart, liver, brain, lung and kidney following incubation of mitochondria with CuAs (56.68%, 29.04%, 33.46%, 35.62%, and 49.65%, respectively P < 0.001 vs. control mitochondria). This decrease in GSH content in mitochondria isolated from goat heart, liver, brain, lung and kidney were found to be significantly protected from being decreased (3.9fold, 94.36%, 86.45%, 50.42% and 98.77%, respectively, from CuAs-incubated mitochondria, P = 0.001) when the mitochondria were co-incubated with CuAs and trigonelline [99%] (0.8mg/ml). However, Trigonelline [99%], alone, at increasing concentration did not show any effects on reduced GSH content (Fig. 3B).

The measurement of protein carbonyl content demonstrated a significant increase in mitochondria isolated from goat heart, liver, brain, lung, and kidney following incubation of mitochondria with CuAs (2.35 fold, 85.23%, 3.45 fold, 3.30 fold and 83.36%, respectively, P ≤ 0.001 vs. control mitochondria). This elevated level of protein carbonyl content was found to be protected from being increased when the mitochondria were co-incubated with CuAs and trigonelline [99%] (0.8mg/ml) significantly in mitochondria isolated from goat heart, liver, brain, lung and kidney (76.75%, 55.05%, 76.81%, 55.25% and 56.47%, respectively, from CuAs-incubated mitochondria, P = 0.001). However, Trigonelline [99%], alone, at increasing concentration did not show any effects on protein carbonyl content (Fig.3C).
Figure 3. Protective effect of trigonelline [99%] against copper-ascorbate-incubated (A) increase in lipid peroxidation level, (b) decrease in reduced glutathione content and (C) increase in protein carbonylation level in mitochondria isolated from goat heart, liver, brain, lung and kidney. 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 ± S.E.; *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 antioxidant enzymes

Fig. 4A, reveals a highly significant increase (3.91 fold, 83.74%, 2.29 fold, 2.10 fold and 3.63 fold, respectively, P<0.001 vs. control mitochondria) in the activity of MnSOD following incubation of mitochondria isolated from heart, liver, brain, lung and kidney with CuAs. The activity of this enzyme was found to be protected from being increased when the mitochondria were co-incubated with CuAs and trigonelline [99%]. The activity of MnSOD was found to be 73.74%, 68.18%, 80.68%, 47.96% and 74.85% (P<0.001) less than the copper-ascorbate-incubated mitochondria when the mitochondria isolated from goat heart, liver, brain, lung and kidney tissues, respectively, were co-incubated with trigonelline (0.8mg/ml). However, Trigonelline [99%], alone, at increasing concentration did not show any effects on MnSOD activity.

A highly significant decrease (38.26%, 53.86%, 55.31%, 53.88% and 63.72%, respectively, P≤0.001 vs. control mitochondria) was found in the activity of GPx following incubation of mitochondria isolated from heart, liver, brain, lung and kidney with CuAs. The GPx activity was found to be 2.40 fold, 2.84 fold, 2.10 fold, 94.96%, and 3.74 fold, respectively, (P≤0.001 compared to CuAs-incubated mitochondria) higher when the mitochondria were co-incubated with CuAs and 0.8mg/ml of trigonelline [99%]. However, Trigonelline [99%], alone, at increasing concentration did not show any effects on GPx activity (Fig. 4B).

On the other hand, Fig. 4C further depicts a highly significant decrease (30.19%, 47.27%, 54.91%, 49.63% and 39.17%, respectively, P≤0.001 vs. control mitochondria) in the activity of GR following incubation of mitochondria isolated from heart, liver, brain, lung and kidney with CuAs. The GR activity was also protected from being decreased when the mitochondria isolated from goat heart, liver, brain, lung and kidney tissues were co-incubated with CuAs and trigonelline [99%]. Here also, GR activity was 2.10 fold, 3.05 fold, 4.40 fold, 97.18% and 2.28 fold higher (P<0.001) compared to CuAs-incubated mitochondria when the mitochondria were co-incubated with CuAs and 0.8mg/ml of trigonelline [99%]. However, Trigonelline [99%], alone, at increasing concentration did not show any effects on GR activity.
Figure 4. Protective effect of trigonelline [99\%] against copper-ascorbate-incubated (A) increase in manganese superoxide dismutase (MnSOD) specific activity, (b) decrease in glutathione peroxidase (GPx) specific activity and (C) decrease in glutathione reductase (GR) specific activity in mitochondria isolated from goat heart, liver, brain, lung and kidney. 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 ± S.E.; \# 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 pyruvate dehydrogenase and some of the mitochondrial Kreb’s cycle enzymes

Fig. 5(A-D) reveals that the incubation of the mitochondria isolated from heart, liver, brain, lung and kidney with CuAs inhibits PDH activity (36.64\%, 52.93\%, 38.66\%, 41.65\% and 23.78\%, respectively), ICDH activity (57.33\%, 44.38\%, 31.74\%, 37.90\% and 35.49\%, respectively), \(\alpha\)-KGDH activity (43.69\%, 53.87\%, 58.62\%, 48.27\% and 34.33\%, respectively) and SDH activity (32.31\%, 24.26\%, 46.94\%, 45.37\%, 42.98\%, respectively) (P < 0.001 vs. control mitochondria). When the mitochondria were co-incubated with CuAs and trigonelline [99\%], the activities of PDH (78.78\%, 2.24 fold, 52.84\%, 66.50\% and 43.11\%, respectively), ICDH (3.10 fold, 2.19 fold, 52.62\%, 56.61\% and 83.68\%, respectively), \(\alpha\)-KGDH (88.11\%, 3.52 fold, 2.90 fold, 89.97\% and 36.55\%, respectively) and SDH (82.27\%, 83.00\%, 2.30 fold, 2.71 fold and 2.71 fold respectively) enzymes, however, was found to be significantly protected from being decreased in mitochondria isolated from goat heart, liver, brain, lung and kidney (P < 0.001 vs. CuAs-incubated mitochondria) at the dose of 0.8mg/ml. Trigonelline [99\%], alone, at increasing concentration did not show any effects on pyruvate dehydrogenase and other Kreb’s cycle enzymes activities.
Fig. 5A

Con CuAs T0.1 T0.2 T0.4 T0.8 CuAs/T0.1 CuAs/T0.2 CuAs/T0.4 CuAs/T0.8

Pyruvate dehydrogenase activity (Units/mg of protein)

Heart Liver Brain Lung Kidney

Fig. 5B

Con CuAs T0.1 T0.2 T0.4 T0.8 CuAs/T0.1 CuAs/T0.2 CuAs/T0.4 CuAs/T0.8

Isocitrate dehydrogenase activity (Units/mg of protein)

Heart Liver Brain Lung Kidney

Fig. 5C

Con CuAs T0.1 T0.2 T0.4 T0.8 CuAs/T0.1 CuAs/T0.2 CuAs/T0.4 CuAs/T0.8

Alpha-ketoglutarate dehydrogenase activity (Units/mg of protein)

Heart Liver Brain Lung Kidney
Effect of trigonelline [99%] on the activities of respiratory chain enzymes
Mitochondria isolated from heart, liver, brain, lung and kidney, when incubated with copper-ascorbate, was found to be decreased NADH cytochrome C oxidoreductase (51.87%, 45.44%, 79.38%, 66.21% and 51.10%, respectively) and cytochrome C oxidase (82.49%, 61.67%, 69.75%, 76.34% and 41.15%, respectively) activities (P = 0.001 vs. control mitochondria). The activities of NADH cytochrome C oxidoreductase (4.48 fold, 5.70 fold, 9.73 fold, 3.39 fold, 4.77 fold, respectively) and cytochrome C oxidase (13.37%, 5.52 fold, 5.92 fold, 5.55 fold, 2.20 fold, respectively) enzymes were found to be protected from being decreased (P≤ 0.001 vs. CuAs-incubated mitochondria) when the mitochondria were co-incubated with trigonelline [99%] with 0.8mg/ml dose. Here, the degree of protection afforded was found to be greater in case of liver and brain mitochondria. Trigonelline [99%], alone, at increasing concentration did not show any effects on respiratory chain enzymes activities (Fig. 6A-B).

Assessment of functional status of mitochondria isolated from liver and brain by determining the activities of respiratory complex enzymes during coupling and uncoupling condition
Fig. 6 showed a significant decrease in mitochondria isolated from goat liver (Fig. 6C) and brain (Fig. 6F), ATP synthase activity following the incubation of mitochondria with CCCP (28.81% and 23.32%, respectively, P ≤ 0.001 vs. 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 with CCCP.

Fig 6G reveals that in case of CCCP pre-incubation of mitochondria isolated from goat liver caused significant decreases in the activities of cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthase (47.40%, 67.59% and 43.82%, respectively, P≤ 0.001 vs. control mitochondria) following incubation of mitochondria isolated from goat liver with copper-ascorbate. The activities of these enzymes were found to be significantly protected from being decreased when the mitochondria isolated from goat liver were co-incubated with copper-ascorbate and trigonelline [99%] (79.96%, 2.16 fold and 36.98% protection, respectively; P≤ 0.001 compared to copper-ascorbate-incubated mitochondria). However, trigonelline [99%] alone has no effect on enzyme activity.
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significantly protected from being decreased when the mitochondria isolated from goat liver were co-incubated with copper-ascorbate and trigonelline [99%] (1.00 fold and 2.08 fold protection, respectively; \( P < 0.001 \) compared to copper-ascorbate-incubated mitochondria), but the activity of ATP synthase was not found to be significantly protected when the mitochondria isolated from goat liver were co-incubated with copper-ascorbate and trigonelline [99%] (23.77% protection, \( P < 0.001 \) compared to copper-ascorbate-incubated mitochondria). On the other hand, Fig. 6E reveals highly significant decreases in the activities of NADH cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthase, (37.43%, 50.77% and 48.34%, respectively, \( P < 0.001 \) vs. control mitochondria) following incubation of mitochondria isolated from goat brain with copper-ascorbate. The activities of these enzymes were found to be significantly protected from being decreased when the mitochondria isolated from goat brain were co-incubated with copper-ascorbate and trigonelline [99%] (74.95%, 1.21 fold and 1.09 fold protection, respectively; \( P < 0.001 \) compared to copper-ascorbate-incubated mitochondria). However, trigonelline [99%] alone has no effect on enzyme activity.

Fig. 6H reveals that in case of CCCP pre-incubation of mitochondria isolated from goat brain caused significant decreases in the activities of NADH cytochrome C oxidoreductase, cytochrome C oxidase and ATP synthase (47.14%, 57.66% and 55.44%, respectively, \( P < 0.001 \) vs. control mitochondria) following incubation of mitochondria isolated from goat brain with copper-ascorbate. The activities of NADH cytochrome C oxidoreductase and cytochrome C oxidase were found to be significantly protected from being decreased when the mitochondria isolated from goat brain were co-incubated with copper-ascorbate and trigonelline [99%] (92.34% and 1.34 fold protection, respectively; \( P < 0.001 \) compared to copper-ascorbate-incubated mitochondria), but the activity of ATP synthase was not found to be significantly protected when the mitochondria isolated from goat brain were co-incubated with copper-ascorbate and trigonelline [99%] (11.17% protection, \( P < 0.001 \) compared to copper-ascorbate-incubated mitochondria).
Figure 6. Protective effect of trigonelline [99%] against copper-ascorbate-incubated decrease in (A) NADH cytochrome C oxidoreductase and (B) NADH cytochrome C oxidase specific activities in mitochondria isolated from goat heart, liver, brain, lung and kidney. Effect of CCCP on the activities of NADH cytochrome C oxidoreductase, NADH cytochrome C oxidase, ATP synthase of liver (C) and brain (F) mitochondria; *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 (D, G) in absence of CCCP and (E, H) in presence of CCCP; 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 ± S.E.; *P < 0.001 compared to control values using ANOVA.
Effect of trigonelline [99\%] on the mitochondrial cardiolipin content

The fluorescence variations of NAO may also be used to reveal topological modifications of cardiolipin in the space of a biological structure [44]. In case of analysis carried out through flow cytometry (Fig. 7A) it was observed that when the mitochondria isolated from heart, liver, brain, lung and kidney were incubated with copper-ascorbate, the mitochondrial membrane was damaged. So, the dimerization between cardiolipin and NAO dye was decreased which decreases the fluorescence (32.61%, 33.10%, 44.24%, 57.45% and 34.97%, respectively. P < 0.001 compared to control mitochondria). However, when the mitochondria were co-incubated copper-ascorbate and trigonelline [99\%] (0.8mg/ml), the fluorescence was found to be increased by 39.94\%, 44.66\%, 76.03\%, 91.97\% and 47.85\% in case of mitochondria isolated from heart, liver, brain, kidney and lung respectively. This indicates that trigonelline [99\%] has potent capacity to protect the mitochondrial membrane cardiolipin content significantly (P < 0.001 compared to CuAs-incubated mitochondria). Trigonelline [99\%], alone, did not show any effects on mitochondrial membrane cardiolipin content.

Effect of trigonelline [99\%] on the NADH auto-fluorescence level

Fig. 7B reveals that a significant decrease was found in the level of NADH, compared to control, when mitochondria isolated from heart, liver, brain, kidney and lung were incubated with copper-ascorbate (20.87\%, 18.73\%, 36.57\%, 58.07\% and 23.66\%, respectively, P < 0.001 vs. control mitochondria). However, NADH level was found to be significantly protected from being decreased when the mitochondria isolated from goat heart, liver, brain, lung and kidney tissues were co-incubated with CuAs and trigonelline [99\%] (0.8mg/ml) (96.38\%, 78.70\%, 68.75\%, 2.40 fold and 74.78\% protection, respectively; P < 0.001 vs. CuAs-incubated mitochondria), indicating the activity of trigonelline [99\%] to protect against oxidative stress-induced disturbances in mitochondrial energy metabolism.

Effect of trigonelline [99\%] on the di-tyrosine fluorescence intensity

The effect of the free radical-generating system on protein structure was examined by measuring di-tyrosine fluorescence. That copper-ascorbate induced oxidative stress has a direct effect on the oxidation level of amino acids. Co-incubation of mitochondria isolated from heart, liver, brain, kidney and lung with CuAs and trigonelline [99\%] ameliorates these changes, thereby, restoring these molecules to their original configuration as indicated by recovered auto-fluorescence level for formation of di-tyrosine. Fig. 7C depicts a highly significant increase (2.24 fold, 36.67\%, 31.71\% and 44.25\%, respectively, P < 0.001 vs. control mitochondria) in the di-tyrosine fluorescence following incubation of mitochondria isolated from heart, liver, brain, kidney and lung with CuAs. This di-tyrosine fluorescence level was protected from being increased when the mitochondria were co-incubated with CuAs and trigonelline [99\%] by 56.75\%, 22.81\%, 39.57\%, 17.93\% and 28.40\%, respectively (P < 0.001 vs. CuAs-incubated mitochondria) at the dose of 0.8mg/ml. Trigonelline [99\%], alone, has no effect on the di-tyrosine fluorescence of mitochondria.

Figure 7. Protective effect of trigonelline [99\%] against copper-ascorbate-incubated (A) decrease in mitochondrial cardiolipin content, (B) decrease in mitochondrial NADH auto-fluorescence level and (C) increase in mitochondrial di-tyrosine level in mitochondria isolated from goat heart, liver, brain, lung and kidney. Con=control mitochondrial group; CuAs = copper-ascorbate incubated mitochondrial group; T0.1-0.8= mitochondrial groups co-incubated with copper-ascorbate and 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 ± S.E.; *P < 0.001 compared to control values using ANOVA. *P < 0.01 compared to control values using ANOVA.
Effect of trigonelline [99%] on the 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 treated mitochondria isolated from heart, liver, brain, kidney and lung compared to that observed in the control mitochondria (Fig. 8A-E), 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).

Figure 8. Protective effect of trigonelline [99%] against copper-ascorbate-incubated (A-E) increase in mitochondrial swelling in mitochondria isolated from goat heart, liver, brain, lung and kidney. Con=control mitochondrial group; CuAs = copper-ascorbate incubated mitochondrial group; T0.8= mitochondrial group incubated with trigonelline [99%] at the dose of 0.8mg/ml (positive control); CuAs- T0.8= mitochondrial group co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.8mg/ml; 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.

Scanning electron microscopy

Fig. 9(A-E) shows the changes brought about to the mitochondrial surface, following incubation of mitochondria with copper-ascorbate, studied through scanning electron microscopy. When the mitochondria isolated from heart, liver, brain, kidney and lung were incubated with copper ascorbate showed a perforated surface with convoluted membranes. The mitochondria were markedly contracted with large membrane blebs covering the mitochondrial 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 trigonelline [99%] at the dose of 0.8mg/ml.
Inhibition of PDH and Kreb’s cycle enzymes of mitochondria isolated from liver and brain tissues by copper-ascorbate incubation

Fig. 10(A-D) depicts that in liver mitochondrial in vitro system, copper itself cannot inhibit the activities of PDH and Kreb’s cycle enzymes significantly whereas ascorbic acid itself can inhibit the activities of PDH and Kreb’s cycle enzymes in very small amount which is not enough to cause oxidative damages in mitochondria, but it also showed a pro-oxidant effect at the dose of 2mM. But copper at the concentration of 0.2mM and ascorbic acid at the concentration of 1mM, in combination can inhibit the activities of PDH and Kreb’s cycle enzymes significantly in mitochondria isolated from goat liver(48.17%, 53.92%, 35.70%, 35.44%, respectively in respect to control, $P \leq 0.001$). So, it can be said that neither copper nor ascorbic acid alone are potent to inhibit the activities of PDH and Kreb’s cycle enzymes but they can inhibit the activities of PDH and Kreb’s cycle enzymes in combination in the mitochondrial in vitro system, significantly.
Fig. 10(A-D) depicts that in brain mitochondrial in vitro system, copper itself cannot inhibit the activities of PDH and Kreb’s cycle enzymes significantly whereas ascorbic acid itself can inhibit the activities of PDH and Kreb’s cycle enzymes in very small amount which is not enough to cause oxidative damages in mitochondria, but it also showed a pro-oxidant effect at the dose of 2mM. But copper at the concentration of 0.2mM and ascorbic acid at the concentration of 1mM, in combination can inhibit the activities of PDH and Kreb’s cycle enzymes significantly in mitochondria isolated from goat brain (50.57%, 48.36%, 57.46%, 46.91%, respectively in respect to control, P = 0.001). So, it can be said that neither copper nor ascorbic acid alone are potent to inhibit the activities of PDH and Kreb’s cycle enzymes but they can inhibit the activities of PDH and Kreb’s cycle enzymes in combination in the mitochondrial in vitro system, significantly.
Figure 11. Effects of copper, ascorbic acid individually and in combination on the specific activities of (A) pyruvate dehydrogenase (PDH), (B) isocitrate dehydrogenase (ICDH), (C) α-ketoglutarate dehydrogenase (α-KGDH) and (D) succinate dehydrogenase (SDH) in mitochondria isolated from goat brain. Con = control mitochondrial group; Cu0.04-0.2= mitochondrial groups incubated with copper at the dose of 0.04-0.2mM/ml respectively; Asc0.4-2= mitochondrial groups incubated with ascorbic acid at the dose of 0.4-2mM/ml respectively; Cu0.2-Asc0.8= mitochondrial groups incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 0.8mM/ml; Cu0.2-Asc1= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 1mM/ml; Cu0.2-Asc2= mitochondrial group incubated with copper at the dose 0.2mM/ml and ascorbic acid at the dose 2mM/ml.

Mechanism(s) of inhibition of PDH and Kreb’s cycle enzymes of mitochondria isolated from liver and brain tissues by copper-ascorbate and their protection by trigonelline [99%]

On the other hand fig. 12(E-H) depicts that when the mitochondria isolated from goat brain were incubated with copper-ascorbate then the Vmax values of PDH (0.346Units), ICDH (0.265Units), α-KGDH (0.142Units) and SDH (0.236Units) enzymes were decreased than the Vmax values of PDH (0.612Units), ICDH (0.345Units), α-KGDH (0.339Units) and SDH (0.609Units) enzymes respectively in control mitochondria (43.46%, 23.19%, 58.11% and 61.25%, respectively, P < 0.001 vs. control mitochondria) and Km value of PDH (0.746µM) enzyme was increased than the Km value of PDH (0.153µM) in control mitochondria and Km values of ICDH (0.111µM), α-KGDH (0.104µM) and SDH (3.32 µM) were decreased than the Km values of ICDH (0.163µM), α-KGDH (0.186µM) and SDH (8.63 µM) enzymes in control mitochondria (3.89 fold increase, and 31.90%, 44.09%, 61.53% decrease respectively, P ≤ 0.001 vs. control mitochondria). But when the mitochondria isolated from goat brain were co-incubated with copper-ascorbate and trigonelline [99%] then the Vmax values of PDH (0.571 Units), ICDH (0.292 Units), α-KGDH (0.268 Units) and SDH (0.435 Units) enzymes were found to be protected (65.03%, 10.19%, 88.73% and 84.32%, respectively, P < 0.001 vs. control mitochondria) from being altered than the Km values of these enzymes in Copper ascorbate treated mitochondria and Km values of PDH (0.137µM), ICDH (0.137µM), and SDH (6.17 µM) enzymes were also found to be protected from being altered than the Km values of these enzymes in control mitochondria (81.64%, 23.42%, 16.67% and 85.84%, respectively, P ≤ 0.001 vs. control mitochondria).
We know in case of uncompetitive inhibition of enzyme the Vmax value will be decreased as well as Km value may either be increased or be decreased. So, from our study it was established that copper-ascorbate in combination can inhibit the PDH and the Kreb’s cycle enzymes activities in an uncompetitive manner. But when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%] then the activities of PDH and Kreb’s cycle enzymes were found to be protected from being altered.

Identification of the possible interaction between trigonelline and copper, ascorbate and/or copper-ascorbate system
The possible interaction between trigonelline and copper, ascorbate and/or copper-ascorbate system had been studied by spectral scanning. Copper individually cannot produce any effect as observed from the spectral analysis, even minute changes in absorbance is observed in case of ascorbic acid and trigonelline combination. When trigonelline was incubated with copper ascorbate then the nature of spectra had been changed as the curve shifts to the left suggesting decrease in absorbance with small change in wavelength on application of trigonelline (Fig. 13).

Figure 12. Protective effect of trigonelline [99%] against copper-ascorbate-incubated uncompetitive inhibition of the activities of pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (ICDH), α-ketoglutarate dehydrogenase (α-KGDH) and succinate dehydrogenase (SDH) in mitochondria isolated from goat liver (A-D) and brain (E-H). CuAs = copper-ascorbate incubated mitochondrial group; CuAs- T0.8 = mitochondrial group co-incubated with copper-ascorbate and trigonelline [99%] at the dose of 0.8mg/ml.

Figure 13. Spectral scan of copper, ascorbic acid and trigonelline [99%] individually and mixture of copper-ascorbic acid, ascorbic acid-trigonelline [99%], copper-trigonelline [99%] and copper-ascorbic acid-trigonelline [99%].
Mitochondria are considered as power houses of a cell as it produces ATP by a process called oxidative phosphorylation. 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 of the stain is applied to stain the mitochondria, it enters in the mitochondria. Since mitochondrial inner membrane contains cytochrome oxidase enzyme, which 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.

Lipid peroxidation products are not produced on purpose and inhibition of lipid peroxidation by antioxidants should be beneficial for maintenance of health and reducing disease risk\(^5\). In addition, a relation between copper metabolism and the intracellular availability of glutathione has been defined\(^3\). 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 mitochondrial level of GSH. Trigonelline [99\%] was found to be effective in protecting against the elevation in lipid peroxidation level of the goat mitochondria obtained from goat tissues. Free radicals have been postulated to play a key role in starting the chain of disease-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\(^5\). Mitochondrial disorder is characterized by destruction of membrane structural integrity essential to mitochondrial functions, leading to the loss of mitochondrial membrane fluidity\(^5\) which is caused by oxidation of mitochondrial lipid.

Thiols are thought to play a pivotal role in protecting cells against peroxidation. Cellular defense mechanism against superoxide anion free radical includes a series of linked enzyme reaction to remove this oxygen based free radical and repair radical induced damage. First of these enzymes is superoxide dismutase, which converts superoxide anion free radical to \(\text{H}_2\text{O}_2\), a non-radical ROS. The superoxide anion free radicals escape quenching in the mitochondria of older tissues because the ability of mitochondrial protecting mechanisms against disorganizing effects of free radicals decreases during senescence\(^8\). The GPx plays an important role in curtailing the quantity of cellular destruction inflicted by LPO products by catalyze the transformation of \(\text{H}_2\text{O}_2\) within the cell to harmless by products\(^5\). The GPx is required to repair LPO initiated by superoxide anion free radicals 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. In heart, liver, brain, lung and kidney mitochondria, the activity of both the enzymes decreases as an effect of mitochondrial disorders. The decreased activity is indicative of increased peroxidation of membrane lipids and increased oxidative stress observed in mitochondrial dysfunctions.

Studies of mitochondrial respiratory parameters provide an important insight in understanding mitochondrial physiology and the potential role of mitochondrial pathologies in cellular damages\(^6\). Mitochondrial uncoupling can arise from oxidative damage of the mitochondrial membrane as induced by increased ROS concentrations\(^5\).

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. 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\(^5\). The impairment of electron transfer through NADH: ubiquinone oxidoreductase (complex I) and ubiquinol: cytochrome c oxidoreductase (complex III) may induce superoxide anion free radical 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 uncouplar and trigonelline [99\%] can protect the mitochondria from the inhibitory effect of copper-ascorbate but not the uncoupling effect of CCCP.

Our studies demonstrate that trigonelline [99\%] can protect against copper-ascorbate-induced oxidative damages in mitochondria isolated from different tissues of goat i.e. heart, liver, brain, kidney and lung, in a dose-dependent manner. However, the protective efficiency of trigonelline [99\%] in mitochondria isolated from different goat tissues can be ordered as brain mitochondria > Liver mitochondria > Heart mitochondria > Kidney mitochondria > Lung mitochondria. From the present study it was observed that protective efficiency of trigonelline [99\%] against copper-ascorbate-induced oxidative stress is higher in goat brain and liver mitochondria. So, some of critical analysis have been carried out with mitochondria isolated from brain and liver tissues of goat.
In our present study it was established that neither Cu²⁺ (0.04-0.2mM) nor ascorbic acid (0.4-1mM) alone can produce oxidative stress but in combination Cu²⁺ (0.2mM) and ascorbic acid (1mM) can produce oxidative stress significantly which further confirms our previous work illustrating that Cu²⁺ in presence of ascorbic acid (0.2mM-2.0mM) generates •OH radical and H₂O₂, which induce mitochondrial damage by impairment of mitochondria functionality which sensitizes cells to oxidative challenges. On the other hand, the activities of Kreb’s cycle enzymes 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. The O₂•⁻ accumulation may be exacerbated by a difference in the rate of O₂•⁻ accumulation and conversion to H₂O₂ and ‘OH (most stable diamagnetic free radical) in stressed mitochondria. Alternatively, O₂•⁻ may be metabolized promptly with other reactive species such as nitric oxide (NO). The NO is shown to interact with O₂•⁻ to generate peroxynitrite anions (ONOO⁻) and nitrogen oxides, which could attenuate the formation of the highly reactive •OH 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. The electron transfer chain of mitochondria is also a well-documented source of H₂O₂. Proteins containing the amino acids tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine can undergo free radical–mediated amino acid modification by oxidation/reduction reactions in a neutral aqueous milieu.

Figure 14. Schematic diagram representing the antioxidant mechanism(s) of protection of trigonelline [99%] against copper-ascorbate incubated oxidative damages in mitochondria isolated from goat heart, liver, brain, lung and kidney.
Under our experimental conditions, •OH probably react with various amino acids at or near the active site(s) of the Kreb’s cycle enzymes which may result in distortions in three dimensional conformation of the enzymes. Our studies revealed that trigonelline [99%] is capable of scavenging •OH, in vitro. So, it may be possible that when mitochondria were co-incubated with copper-ascorbate and trigonelline [99%], the activities of Kreb’s cycle enzymes were found to be protected from being inhibited because of scavenging of this oxygen free radical.

From our study it was established that in case of copper-ascorbate-incubated mitochondria the activities of Kreb’s cycle enzymes were inhibited in an uncompetitive manner. Neither copper nor ascorbic acid singly can inhibit activities of Kreb’s cycle enzymes but copper-ascorbate in combination can inhibit the enzyme activities significantly. So, copper-ascorbate, as a complex, can bind to the enzymes to cause uncompetitive inhibition. From our spectral study it was evidenced that a reaction might have occurred between ascorbic acid and trigonelline [99%] as well as copper-ascorbate and trigonelline [99%]. So, when the mitochondria were co-incubated with copper-ascorbate and trigonelline [99%], the activities of Kreb’s cycle enzymes were found to be protected from being inhibited because of the probable reaction between copper-ascorbate and trigonelline [99%]. This reaction may prevent the binding of copper-ascorbate to the enzymes and thus, can provide a protection against copper-ascorbate-induced inhibition of the activities of Kreb’s cycle enzymes.

CONCLUSION

In conclusion, it can be said that trigonelline [99%] obtained from Trigonella foenum graecum (Linn.) protects mitochondria isolated from different goat tissues against copper-ascorbate induced oxidative stress mediated injury via antioxidant mechanism(s) (Fig. 14). Trigonelline [99%] may find its extensive use against mitochondrial dysfunction related various diseases at a specific pharmacological dose. It may as well find its place in alternative medicine or integrative medicinal interventions. Till date there has been no report on the side effects of trigonelline [99%] indicating that this molecule may be considered a safe antioxidant which may be used singly or in combination in situations involving oxidative stress.

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