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Estra-1, 3, 5(10) - triene-3, 17 β-diol protects mitochondria against Cu-ascorbate induced oxidative damage in *in vitro* system: A novel therapeutic approach

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ABSTRACT

Background: Cu-ascorbate is a well-established oxidative stress inducing agent in *in vitro* system. *Terminalia arjuna* is also a well known medicinal plant used as an anti-ischemic and cardiotonic agent for over three centuries in India. Estra-1, 3, 5(10) - triene-3, 17 β-diol (β-E) was identified as a component of the aqueous extract of bark of *Terminalia arjuna*. **Aims and Objectives:** To determine the antioxidant efficacy of β-E against Cu-ascorbate induced oxidative stress in isolated goat liver mitochondria in an *in vitro* system. **Methods:** Goat liver mitochondria was incubated with Cu-ascorbate and different concentrations of β-E at pH 7.4 and 37^{0}C for 60 minutes. Then the reaction was stopped upon addition of EDTA. Enzymes and DNA from incubated mitochondria were isolated to determine the alteration in their activities and the status of the biomarkers of oxidative stress. **Results:** Incubation of goat liver mitochondria with Cu-ascorbate at pH 7.4 and 37^{0}C has resulted in significant elevation of lipid peroxidation, protein carbonylation , DNA damage, activities of Mn-superoxide dismutase, xanthine oxidase along with a concomitant decrease in reduced glutathione level, activities of the Kreb's cyle and electron transport chain linked enzymes which is indicating towards the generation of reactive oxygen species (ROS) mediated mitochondrial dysfunction, that was confirmed by Janus green B staining. All of these changes were prevented from being occurring on co-incubation of mitochondria with β-E. **Conclusion:** From these above results it can be concluded that β-E possesses a significant antioxidant potential and provides protection to mitochondria against Cu-ascorbate induced oxidative damage.

KEY WORDS: Estra-1, 3, 5(10)-triene-3,17β-diol, Antioxidant efficacy, Oxidative stress, Mitochondria, Cytochrome C, *Terminalia arjuna*

1. INTRODUCTION:

Estra-1,3,5(10) triene -3,17 β-diol(β-E) is a chemical form of estrogen, the steroid hormone which performs the major physiological functions for regulating the female reproductive system. Recently some evidences are accumulating since last three to four years which suggest that this compound can also perform a major role in preventing the generation of hydrogen peroxide, that is, reactive oxygen species (ROS) in *in vitro* system in a receptor dependent pathway [1]. Some of the previous studies have also suggested that there is a relation between increasing activities of antioxidant enzymes in women with endometrial polyp [2] and it can also prevent the metal mediated oxidation of low density lipoprotein (LDL) [3] which is considered as the most common factor of atherosclerosis. Here we have identified β-E as a major fraction from the aqueous bark extract of *Terminalia arjuna* by High Pressure Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometry(GC-MS)[4]. In our earlier works we have already showed the presence of

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antioxidant potential in crude aqueous bark extract of *Terminalia arjuna* [5] . Considering the presence of antioxidant properties in this crude extract and the presence of β-E as one of the major components in this extract, it becomes necessary to elucidate the ROS scavenging activity of this compound in both biological and chemically defined system to draw the focus on its pharmacological characteristics.

ROS ($O_2^{\cdot-}$,H_2O_2,$^{\cdot}OH$) are the unusual reduced form of molecular oxygen that are generated when the cellular metabolic switch is turned to a continuous reduction mode using this molecular oxygen as an electron sink.

Mitochondria is one of the organelle of eukaryotic cell that is a major seat of generation of oxidative stress due to the presence of redox enzymes associated with electron transport chain(ETC) that constitute the major part of aerobic respiration. Again the presence of cardiolipin like phospholipids in inner mitochondrial membrane acts as a major marker to determine the intensity of oxidative damage [6]. Moreover, copper-ascorbate is an established model to induce oxidative stress *in vitro* [7]. So in our present study an attempt has been made to elucidate the antioxidant potential of aqueous solution of β-E , in both chemically defined *in vitro* system and to examine whether it can protect liver mitochondria against Cu-ascorbate induced oxidative stress mediated damages.

2. MATERIALS AND METHODS:

β-E powder was purchased from MP Biomedicals. L-epinephrine and 4',6-diamidino-2-phenylindole(DAPI) were purchased from Sigma-Aldrich. 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. DAPI was purchased from Sigma-Aldrich limited.

2.1. Preparation of β-E solution:

β-E was weighed and dissolved in absolute ethanol and diluted thousand folds with double distilled water.

2.2. Determination of ROS scavenging activity in a chemically defined system:

$^{\cdot}OH$ radical was generated in sodium phosphate buffer (0.05M, pH 7.4) with 1mM ascorbate and 0.2mM Cu^{2+} for 60 minutes in the presence of DMSO (500µM) and different concentrations of β-E in a volume of 1ml to determine the hydroxyl radical scavenging activity of the β-E in an *in vitro* system. The reaction was terminated in each case by the addition of 0.1mM EDTA. Methanesulfinic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner [8] as modified by Bandyopadhyay *et al.* [9]

Superoxide scavenging activity was studied by following the rate of epinephrine oxidation in alkaline pH at 480nm according to the method of Misra *et al.* [10]. The reaction mixture had in a volume of 1ml, 50mM Tris-HCl buffer (pH 10),0.6mM epinephrine and different concentrations of β-E. The increase in absorbance due to the formation of the adrenochrome was followed for 7 minutes and the activity was calculated from the linear part in absence and presence of β-E. The involvement of superoxide was checked with standard superoxide dismutase (SOD).

H_2O_2 scavenging activity was assayed by the method of Beer and Sizer [11] where breakdown of H_2O_2 was monitored at 240nm. The reaction mixture contained 50mM phosphate buffer (pH 7.4), 53µmoles H_2O_2 and 0.125µM to 1µM β-E.in a final volume of 3ml.

2.3. Isolation of mitochondria:

Mitochondria was isolated from the liver according to the method of Hare *et al.* with some modifications[12].Goat liver was collected from slaughter house just after sacrifice, homogenized with 50 mM Tris-sucrose buffer(pH 7.8) at 4^0C with teflon homogenizer. This 10% homogenate was first centrifuged at 600g at 4^0C to remove the nucleus as pellet. The supernatant was collected and again centrifuged at 16000g for 45 minutes at 4^0C using cold ultracentrifuge. The pellet was collected and resuspended in the same buffer and preserved at -20^0C, and the supernatant was finally discarded.

2.4. Incubation of mitochondria in in vitro system:

50% mitochondrial suspension was incubated with 0.2mM $CuCl_2$, 1mM ascorbic acid and co-incubated with 0.125-1 β-E at 37^0C and pH 7.4 for 1 hour. In each case the reaction was terminated with addition of 0.02 ml 35mM EDTA after completion of 1 hour.

2.5. Determination of viability of mitochondria

Viablity of mitochondria was determined according to the method of Mukherjee *et al.*[13]. 0.2 ml from each group of incubated mitochondrial suspension was spread uniformly on glass slide and dried. 0.1% Janus green B stain was applied to them. The slides were then kept in the dark for 40 minutes and then rinsed with distilled water carefully for removal of excess stain. Ultimately the slides were mounted with DPX and observed under Olympus BX 51 fluorescence microscope using green filter at 40X magnification (excited by application of blue filter).

2.6. Measurement of biomarkers of oxidative stress:

2.6.1. Lipid peroxidation level:

Lipid peroxidation level of mitochondria was measured by the method of Buege and Aust .[14]. 2 ml TBA-TCA-HCl was added to 0.5 mg

protein containing mitochondrial suspension and heated at 80^{0}C for 20 minutes and then centrifuged at 2000 rpm for 10 minutes to remove the protein debris. The absorbance of the colored supernatant was recorded at 532nm. Lipid peroxidation level was finally calculated in terms of nmoles TBARS/mg protein using 1.56x10^5 as molar extinction coefficient of malondialdehyde (MDA).

2.6.2. Reduced glutathione level

Reduced glutathione level (GSH) was measured from mitochondria using the Ellman's reagent (DTNB) according to the method of Sedlak and Lindsay [15]. In this method proteins of incubated mitochondria were precipitated with 10% ice cold TCA, and centrifuged at 5000 rpm for 20 minutes at 4^{0}C.To one volume supernatant , two volume 0.8(M) Tris-HCl-EDTA(pH 9.0) and one tenth volume 10mM DTNB were added and kept at room temperature for 10 minutes. Then the absorbance of each sample was recorded at 412 nm. Finally reduced glutathione was estimated from the standard curve and evaluated in terms of nmoles GSH/mg protein.

2.6.3. Protein carbonyl content

The protein carbonyl content of incubated mitochondria was determined according to the method of Levine *et al.*[16] as modified by Reznick *et al.*[17]. The sample was incubated with 10mM DNPH for 45 minutes in the dark. At the end of incubation 10% TCA was added, and the mixture was centrifuged at 7000rpm for 15minutes, after which the supernatant was discarded and the pellets were washed carefully with ethanol : ethyl acetate mixture (1:1) thrice. Then equal volume of 6(M) guanidine hydrochloride and 0.5(M) potassium dihydrogen phosphate (pH 2.5) were added to the washed pellets, mixed thoroughly and centrifuged at 7000rpm for 15 minutes. The supernatant was collected and the absorbance was determined at 375nm and the calculated values were expressed in terms of nmoles protein carbonyl/mg protein.

2.7. Measurement of antioxidant and prooxidant enzymes

2.7.1. Mn-Superoxide dismutase (Mn-SOD) activity

Mn-SOD activities of incubated mitochondria was determined according to the method of Marklund *et al.*[18] . Here 10 mM pyrogallol was used as superoxide anion radical generator. To 0.48 ml 50 mM Tris-HCl -EDTA (pH 8.2), 0.02 ml pyrogallol was added and autooxidation of pyrogallol in 0.5 ml reaction volume was monitored at 420nm at UV-Visible Bio Rad spectrophotometer. The decrease in the rate of autooxidation was monitored by introducing incubated mitochondrial suspension in this reaction mixture and as well as by adjusting the buffer volume.50% inhibition of autooxidation was considered as 1 Unit SOD activity. Finally the specific activity of Mn-SOD was expressed in terms of Units/mg protein.

2.7.2. Xanthine oxidase (XO) activity

XO activities of incubated mitochondrial suspensions was measured according to the method of Greenlee *et al.*[19]. To 0.58 ml of 50 mM phosphate buffer containing 0.1 mM xanthine pH 7.8, defined aliquots of mitochondria were added and increase in absorbance at 295 nm was monitored for 5 minutes at interval of 30 seconds. Activity of this enzyme was calculated in terms of mUnits/mg protein, using molar extinction co-efficient of uric acid.

2.8. Measurement of the enzymes associated with glutathione metabolism

2.8.1. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity of incubated mitochondria was determined in a coupled assay method as described by Paglia and Valentine [20]. To the 0.46 ml reaction mixture containing 50 mM phosphate buffer with 2 mM EDTA(pH 7.4), 0.025 mM sodium azide, 1.4 Units/ml pure glutathione reductase,0.15 mM glutathione, and 0.25 mM NADPH, 0.02 ml sample was added. The reaction was started by the addition of 0.36 mM H_2O_2 in a 0.5 ml final reaction volume. The decrease in the absorbance was monitored at 340nm by using a UV/Vis Bio Rad spectrophotometer for 90 seconds at an interval of 10 seconds. Values were expressed in terms of Units/mg protein.

2.8.2. Glutathione reductase activity

Glutathione reductase (GR) activity of incubated mitochondria was determined according to the method of Krohne-Echrich *et al.*[21]. To the 0.42 ml 50 mM phosphate buffer pH 7.6, 0.02ml 2% GSSG, 0.02ml 1% BSA, 0.02ml sample were added and finally 1mg/ml NADPH was added to start the reaction in 0.5 ml reaction volume. A decrease in the absorbance of NADPH was monitored at 340nm by UV/Vis spectrophotometer for 3 minutes at the interval of 15 seconds. Finally values were expressed in terms of Units/mg protein.

2.8.3. Determination of activities of Kreb's cycle enzymes

Pyruvate dehydrogenase activity of incubated mitochondria was measured following the reduction of NAD to NADH at 340 nm for 90 seconds according to the method of Chreiten *et al.*[22]. using UV/Vis Bio Rad spectrophotometer .The 0.5 ml reaction mixture contained 0.1 M phosphate buffer pH 7.5,0.5 mM sodium pyruvate,0.5 mM NAD and suitable aliquot of mitochondrial suspension. Specific activity was calculated in terms of Units/mg protein.

Isocitrate dehydrogenase-1 activity of incubated samples was measured according to the method of Duncan *et al.*[23] .The 0.5 ml reaction mixture contained 0.1 M phosphate buffer pH 7.5, 10 mM isocitrate, 2.5 mm $MnSO_4$ and incubated mitochondria. To start the reaction 5 mM NAD was added and the reaction was monitored

following the increase in absorbance at 340 nm for 90 seconds at an interval of 10 seconds. Specific activity was evaluated in terms of Units/mg protein.

α-keto glutarate dehydrogenase activity was determined according to the method of Duncan *et al.*[23].This assay was performed in 0.5 ml reaction mixture that contained 0.1 M phosphate buffer pH 7.5,0.5 mM α-keto glutarate and suitable aliquot of incubated mitochondria, 0.35 mM NAD.The increase in absorbance at 340 nm was monitored for 90 seconds at interval of 10 seconds. Specific activity was expressed in terms of Units/mg protein.

Succinate dehydrogenase activity of incubated mitochondria was measured through monitoring the reduction of potassium ferricyanide spectrophotometrically at 420 nm for 2 minutes according to the method of Veeger *et al.*[24].The 0.5 ml reaction mixture contained 0.1 M phosphate buffer pH 7.5,2% BSA, 2.5 mM potassium ferricyanide and 4mM succinate and suitable volume of mitochondrial suspension. The specific activities were expressed in terms of Units/mg protein.

2.9. Determination of activities of Electron transport chain (ETC) linked enzymes

NADH-cytochrome C reductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome C at 565 nm according to the method of Goyal *et al.*[25] with some modifications as done by Mitra *et al.*[26]. 0.5 ml of assay mixture contained in addition to the incubated mitochondrial suspension as the source of enzyme, 50 mM phosphate buffer pH 7.4, 0.5 µM NADH, 1 mg/ml BSA and 20 mM oxidized cytochrome C. The decrease in absorbance of cytochrome C was monitored for 90 seconds at 10 second interval. Specific activity was expressed in terms of Units/mg 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.[25] with some modifications as done by Mitra *et al.*[26]. Oxidized cytochrome C was reduced by 1 M dithiothreitol for overnight. Then OD_{550}/OD_{560} ratio was observed to determine the degree of reduction. Then in 0.5 ml of assay mixture suitable volumes of 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome C and the incubated mitochondrial suspension as the source of enzyme were taken and decrease in absorbance at 550nm was monitored for 90 seconds at interval 10 seconds. Specific activity was calculated in terms of Units/mg protein.

2.10. Determination of mitochondrial damage markers through fluorescence

Fluorescence measurements were done with the samples containing incubated mitochondrial suspensions (of 50 µg protein per ml), 10 mmol/l HEPES, 100 mmol/l KCl (pH 7.0) at 25^{0}C using Hitachi spectrouourimeter.

The fluorescence emission spectra (from 300 to 450 nm, 5 nm slit width) of tryptophan were measured by excitation at 295 nm (2 nm slit width) (Dousset *et al.*) [27].

Fluoroscence emission spectra of dityrosine, 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 *et al.*)[28].

NADH autofluorescence of incubated mitochondria was observed in the range of 400 to 500 nm (5 nm slit width) at excitation wavelength 340 nm (5 nm slit width) [29].

All of those parameters were evaluated in terms of Fluorescence intensity (FI) of the incubated samples.

2.11. Determination of mitochondrial DNA damage

2.11.1. DNA isolation and agarose gel electrophoresis

DNA isolation from mitochondria were done according to the method of Yamada *et al.*[30] with some modifications. To isolate DNA incubated mitochondrial suspensions were firstly treated with lysis buffer containing 100 mM NaCl, SDS, EDTA,Tris-HCl ,0.1mg/ml proteinase K,pH 8.0 at 55^{0}C for 4 hours.The lysates were further treated with phenol:chloroform: isoamyl alcohol mixture in the ratio of 25:24:1,centrifuged at 10000 rpm for 10 minutes and the aqueous layer was collected. To this aqueous layer half volume 7.5 M ammonium acetate and 2 volume absolute ethanol (chilled) were added and kept at -20^{0}C for 15 minutes, centrifuged at 16000 rpm for 40 minutes at 4^{0}C.The obtained stringy DNA precipitate was washed with 70% ethanol to remove salts, the alcohol was evaporated and finally dissolved in TE buffer containing10 mM Tris-HCl, 1mM EDTA, pH 8.0, Absorbance of samples was recorded at 260 and 280 nm to confirm about the purity of DNA.

2 µg DNA from each sample was loaded on to 1% agarose gel made with TAE and electrophoresed by standard protocol[31]. Finally the gel was stained by 10 mg/ml ethidium bromide solution and fluorescence of DNA bands were detected by Bio Rad gel doc.

2.11.2. DAPI staining of mitochondria

DAPI staining of mitochondrial sample was done according to the method of Mukherjee *et al.*[32]. Incubated mitochondria was uniformly spread on clean glass slides ,300 nm DAPI stain was applied and kept in the dark for 15 minutes. The excess stain was washed away by applying PBS and the slides were mounted by DPX and observed under Olympus Fluoview IX 81confocal laser scanning microscope at 40X magnification (Excitation wavelength 358 nm,emission wavelength 461 nm).

2.11.3. Determination of surface topology of mitochondria

Surface topology of mitochondria was studied according to the method of Ghosh *et al*.[33].0.5 ml of incubated mitochondrial suspension was mixed with equal volume 2% glutaraldehyde and kept at 4^0C for two nights for fixation. These samples were then dehydrated with gradual washing with increasing concentration of ethanol and n-amyl alcohol, dried and observed under scanning electron microscope at 40X magnification.

2.11.4. Determination of alteration of mitochondrial membrane permeability by analyzing the release of cytochrome C by Western blot

Concentration of released Cyt c was detected by western blot analysis according to the method as described by Ichimura *et al*.[34] with some modifications. The mitochondrial suspension was centrifuged at 10,000 × g for 30 min at 4^0C. The collected supernatant was mixed with loading buffer, boiled for 5 min and subjected to 10% SDS-polyacrylamide gel for 40 min at 200 V followed by electroblotting to PVDF membranes for 1.5 h at 2 mA/cm^2. Membranes were blocked for 1 h with 5% skimmed milk in TBST at room temperature, rinsed and subsequently probed with a polyclonal mouse anti-Cyt c antibody (1:2,000 dilution) for 1 h at room temperature. The membranes were further rinsed and incubated with alkaline phosphatase-labeled anti-mouse antibody (1:6,000 dilution) for 1 h at room temperature. After incubation with the secondary antibody, the membranes were rinsed, and cytochrome C blot was evaluated in terms of band intensity formed by NBT(Nitro blue tetrazolium) in combination with BCIP(5-bromo 4-chloro 3-indolyl phosphate) as chromogenic substrate. Intensities of band were determined by ImageJ software.

2.12. Estimation of protein

Protein concentration of mitochondria was determined by the method of Lowry *et al*.[35].

2.13. Statistical evaluation

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

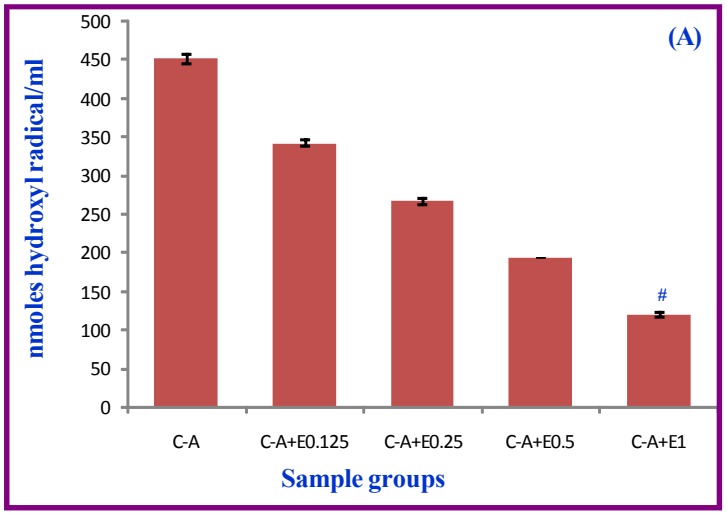
3. RESULTS

3.1. Effect of β-E on generation of ROS in *in vitro* chemically defined system

3.1.1. Scavenging of hydroxyl radical

Cu-ascorbate system has generated almost 452 nmoles hydroxyl radical per ml reaction mixture at pH 7.4. β-E were found to significantly scavenge hydroxyl radical in a dose dependent manner. At 1 µM concentration it scavenged about 73.25% hydroxyl radical (#p<0.001 vs. Cu-Asc) as evident from figure 1(A).

Figure 1(A).Effect of different concentrations of β-E on Cu-ascorbate mediated generation of hydroxyl radical in chemically defined *in vitro* system. C-A=Cu-ascorbate,E0.125=0.125µM,E0.25=0.25 µM,E0.5=0.5 µM,E1=1 µM.

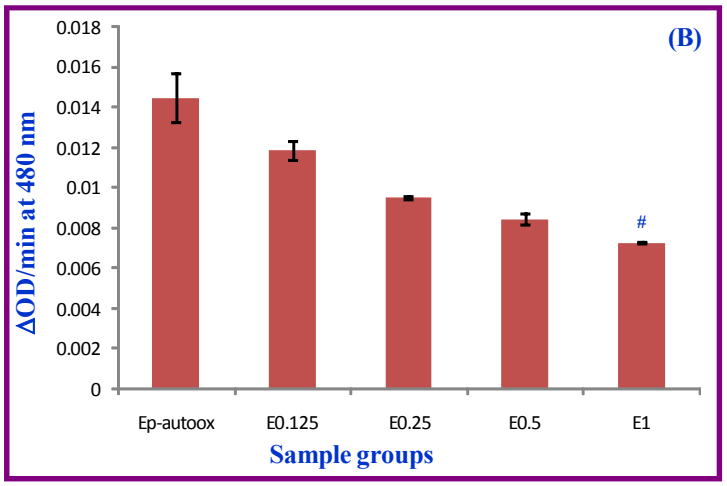


Values were expressed in terms of mean ± S.E.,#P<0.001 vs. Cu-ascorbate.

3.1.2. Scavenging of superoxide anion radical

β-E also significantly scavenged superoxide anion radical evaluated through adrenochrome formation in a chemically defined system. At 1µM β-E scavenged about 49.6% superoxide anion radical (#p<0.001 vs. epinephrine autooxidation) as evident from figure 1(B).

Figure 1(B). Effect of different concentrations of β-E on generation of epinephrine mediated superoxide anion radical in chemically defined *in vitro* system. Ep-autoox= epinephrine autooxidation, E0.125=0.125µM,E0.25=0.25 µM,E0.5=0.5 µM,E1=1 µM.



Values were expressed in terms of mean ± S.E.,#P<0.001 vs. epinephrine autooxidation.

3.1.3. Scavenging of hydrogen peroxide

As evident from table 1, β-E is not capable of scavenging hydrogen peroxide.

Table 1. Table 1 shows the hydrogen peroxide scavenging activity of β-E at its increasing concentrations in terms of change in the absorbance at 240 nm per minute, where E0.125=0.125µM, E0.25=0.25 µM,E0.5=0.5 µM,E1=1 µM.

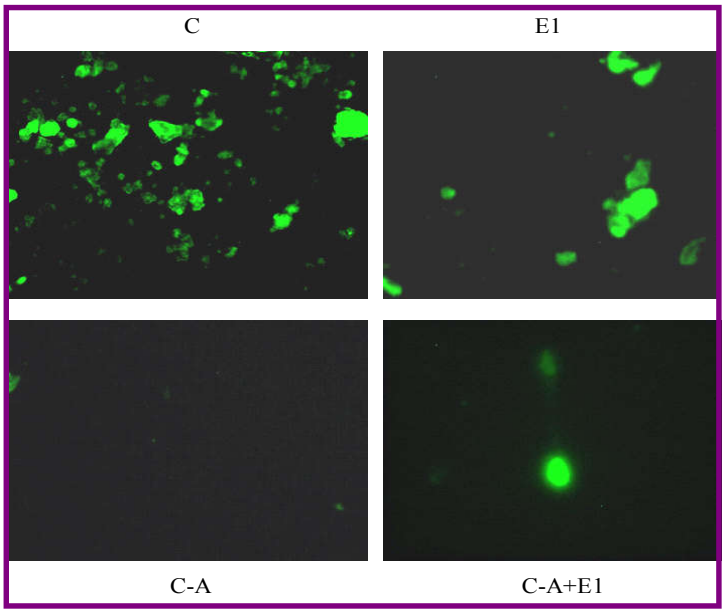
Sample	ΔOD/minute at240 nm
Hydrogen peroxide control	0.7 ± 0.03
Hydrogen peroxide+0.125 µM β-E	0.75±0.03
Hydrogen peroxide+0.25 µM β-E	0.72 ±0.05
Hydrogen peroxide+0.5 µM β-E	0.79 ± 0.07
Hydrogen peroxide+1 µM β-E	0.81±0.1

Values were expressed in terms of mean ± S.E.

3.1.4. Effect of β-E on viability of mitochondria

Innumerable green fluorescence spots confirmed the presence of viable mitochondria in control samples. Samples treated with 1 µM β-E only also showed numerous green fluorescence spots. In case of Cu-ascorbate treated samples the intensity of green fluorescence spots decreased to a minimum.But in those samples where Cu-ascorbate treated mitochondria were co-incubated with 1 µM β-E , the intensity of green fluorescence of spots increased compared to Cu-ascorbate, as evident from figure(2).

Figure 2. Effect of β-E on mitochondrial viability observed by staining with janus green B,C=Control,E1=Positive control with 1 µM β-E,C-A=Cu-ascorbate,C-A +E1=Coincubation of 1 µM β-E with Cu-ascorbate.

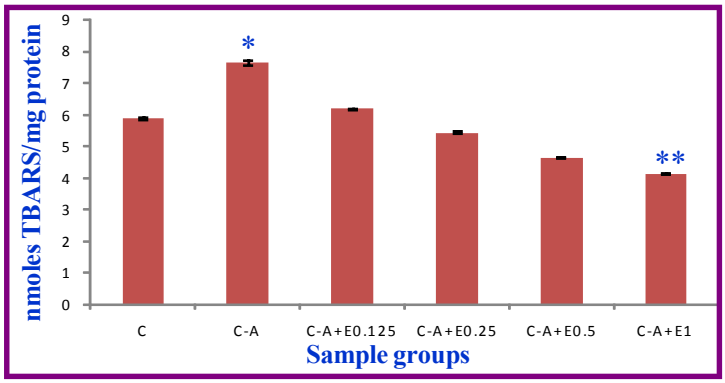


3.2. Effect of β-E on biomarkers of oxidative stress

3.2.1. Lipid peroxidation level

There was a significant rise in lipid peroxidation level observed in the Cu-ascorbate treated mitochondria compared to control(almost 30% increase,*p<0.001 vs. control)(Figure.3A). But when mitochondrial suspensions was co-incubated with increasing concentrations of β-E in presence of Cu-ascorbate , the lipid peroxidation level was significantly protected from being altered dose dependently. At 1 µM concentration lipid peroxidation level was completely restored to its basal level (45.84% decrease,**p<0.001 vs. Cu-ascorbate).Incubation of mitochondria with only increasing concentrations of β-E did not show any significant deviation of lipid peroxidation level from its basal value(data not shown).

Figure 3(A). Effect of different concentrations of β-E on lipid peroxidation (LPO) of liver mitochondria,C=Control, C-A=Cu-ascorbate,C-A+E0.125 =0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.

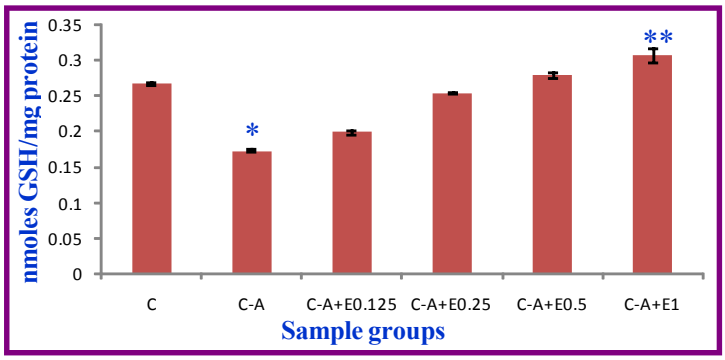


*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

3.2.2. Reduced glutathione content

Upon incubation of mitochondrial suspension with Cu-ascorbate a significant decline in reduced glutathione (GSH) level was observed(35.2% decrease,*p<0.001 vs. control) as shown in figure 3B. In the mitochondria co-incubated with increasing concentrations of β-E along with Cu-ascorbate ,a dose-dependent increase in GSH was observed. Complete protection of GSH level occurred at the dose of 0.5µM (61.22% increase vs. Cu-ascorbate,**p<0.001) and an increment of GSH level was observed at 1 µM concentration of β-E. Incubation of mitochondria with only increasing concentrations of β-E did not show any significant alteration of GSH content of mitochondria from its basal value(data not shown).

Figure 3(B). Effect of different concentrations of β-E on reduced glutathione (GSH) content in liver mitochondria,C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.

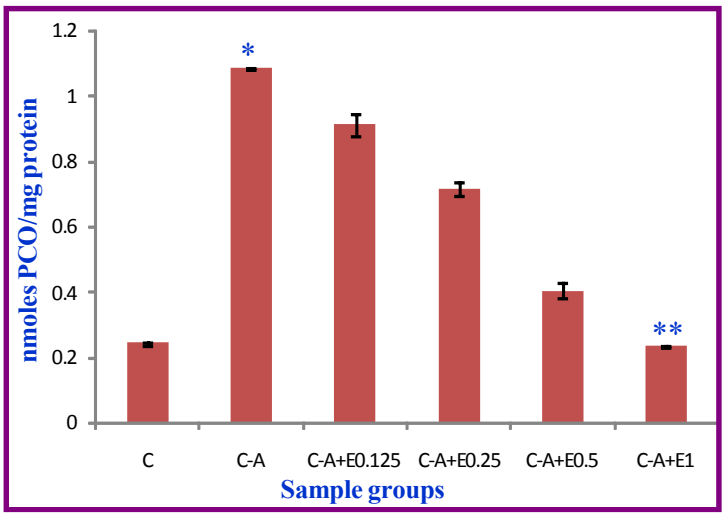


*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,#P<0.001 vs. Cu-ascorbate.*

3.2.3. Protein carbonyl content

A significant elevation in protein carbonyl content was observed in Cu-ascorbate incubated mitochondria (6 fold increase vs. control,*p<0.001) in comparison with control as evident from figure 3C. But upon co-incubation of mitochondria with increasing concentrations of β-E in presence of Cu-ascorbate this parameter was significantly protected from being altered in a dose dependent manner(78.38% decrease Vs. Cu-ascorbate,**p<0.001).When mitochondrial samples were incubated with same concentrations of β-E, there was no alteration in this parameter(data not shown).

Figure 3(C). Effect of different concentrations of β-E on protein carbonylation(PCO) of liver mitochondria,C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.

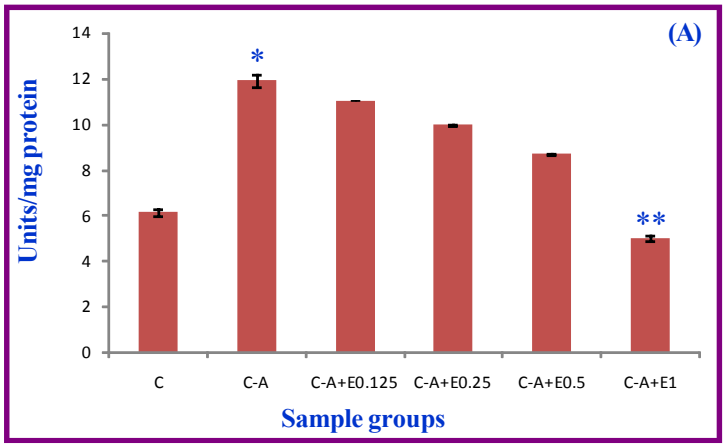


*Values were expressed in terms of mean ± S.E.,*P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

3.2. 4. Effect of β-E on antioxidant and prooxidant enzymes of mitochondria

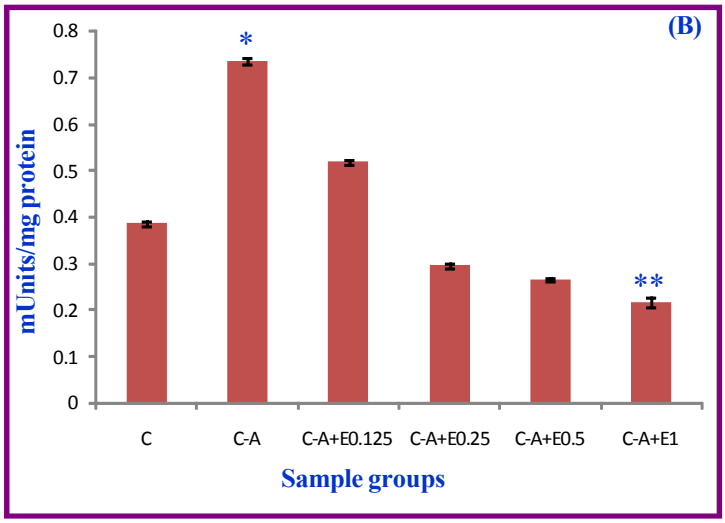
Figure 4(A and B) depicts that upon Cu-ascorbate treatment both Mn-SOD (figure 4A) and XO(figure 4B) activities of liver mitochondria was significantly elevated compared to control(93.66% increase of Mn-SOD and 90.17% increase of XO respectively vs. control,*P<0.001),whereas co-incubation of β-E with cu-ascorbate significantly protected both of these parameters of incubated mitochondria dose dependently(57.87% decrease of Mn-SOD and 70.55% decrease of XO vs. Cu-asorbate,**P<0.001).Incubation of mitochondria only with same concentrations of β-E at identical condition has not shown any significant alteration in these antioxidant(Mn-SOD) and prooxidant(XO) enzyme activities in comparison with control(data not shown).

Figure 4(A). Effect of different concentrations of β-E on Mn-superoxide dismutase (Mn-SOD) activity of liver mitochondria, C=Control,C-A=Cu-ascorbate,C-A+E0.125=0.125µM, C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.



*Values were expressed in terms of mean ± S.E.,*P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

Figure 4(B). Effect of different concentrations of β-E on xanthine oxidase (XO) activity of liver mitochondria,C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.



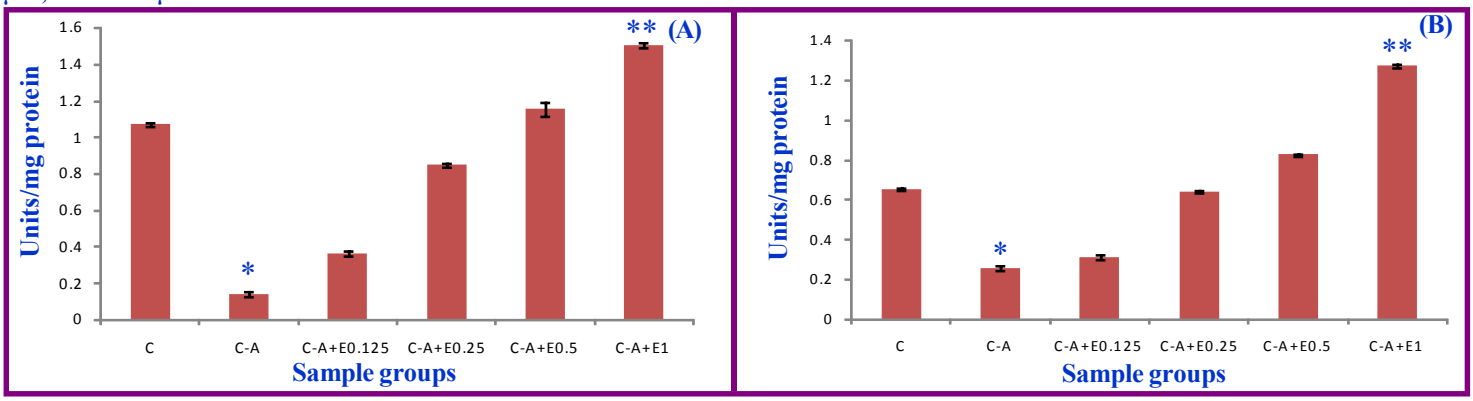
*Values were expressed in terms of mean ± S.E.,*P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

3.2.5. Effect of β-E on GSH metabolizing enzymes

3.2.5.1. Glutathione peroxidase and glutathione reductase

Figure 5(A and B) revealed a significant decrease in both of the reduced glutathione metabolizing enzymes i.e. glutathione reductase (figure 5A) and glutathione peroxidase (figure 5B) following the incubation of cu-ascorbate with liver mitochondria (79.5% and 53.33% decrease Vs. control respectively,*p<0.001). But co-incubation of

Figure 5. Effect of different concentrations of β-E on activities of glutathione metabolizing enzymes of liver mitochondria, (A)Glutathione reductase(GR),(B) Glutathione peroxidase (GPx), C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.



*Values were expressed in terms of mean ± S.E.,*P<0.001 vs. Control,**P<0.001 vs. Cu-ascorbate.*

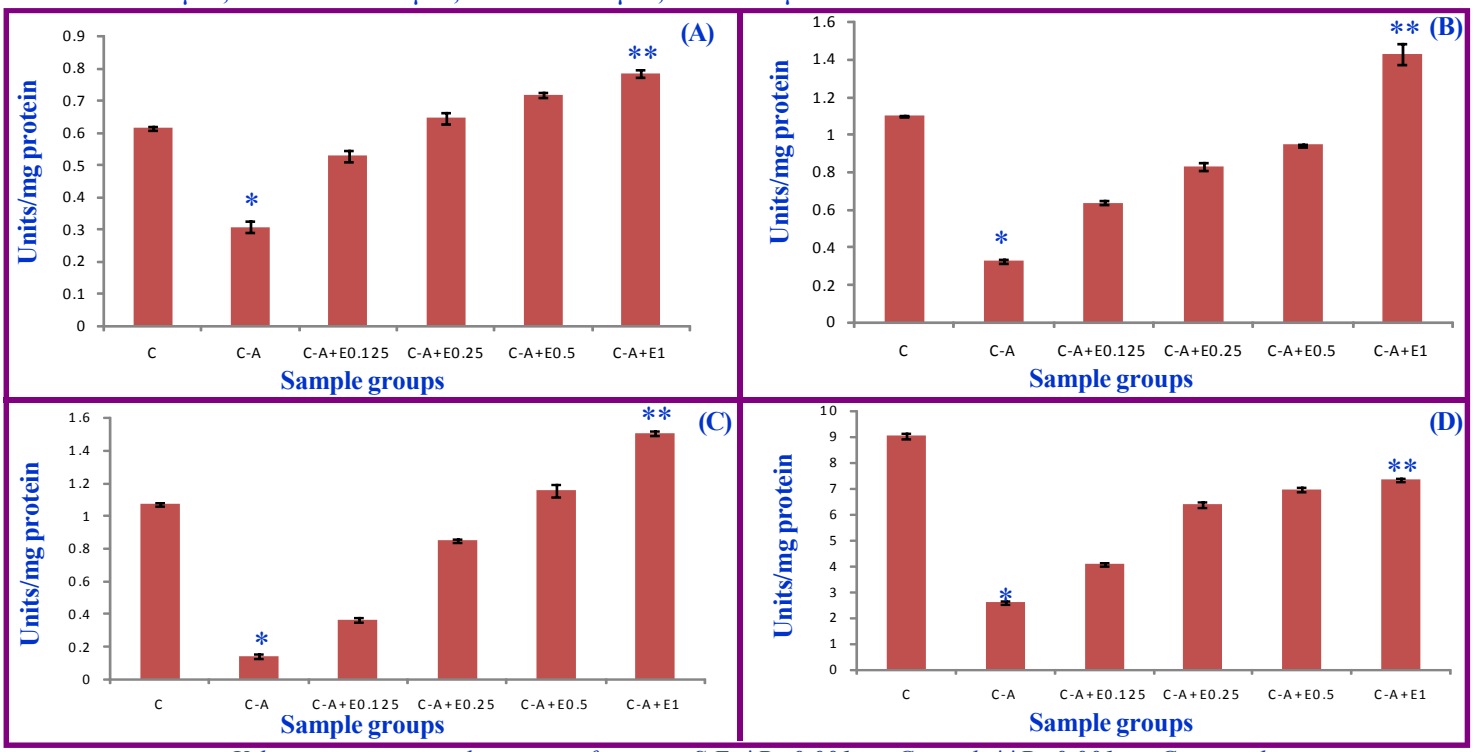
increasing concentration of β-E with Cu-ascorbate treated mitochondria has provided the significant protection to both of these enzymes from being altered dose dependently (**p<0.001 Vs. Cu-ascorbate). Incubation of mitochondria only with same concentrations of β-E did not show any significant alteration in the activities of these gluthathione metabolizing enzymes in comparison with control (data not shown).

3.2.5. 2. Effect of β-E on Kreb's cycle enzymes

Treatment of liver mitochondria with Cu-ascorbate resulted in a significant decline in the activity of pyruvate dehydrogenase (A multienzyme complex and as well as NAD linked enzyme) (50% decrease vs. control,*P<0.001) compared to control as evident from figure 6(A). The activity of this enzyme was significantly prevented from being decreased in mitochondrial samples, co-incubated with increasing concentrations of β-E and Cu-ascorbate.

Figure 6(B) depicts that upon incubation with Cu-ascorbate liver mitochondria has showed a significant decrease in the activity of isocitrate dehydrogenase-I (another NAD linked enzyme) in comparison with control (70.2% decrease vs. control,*P<0.001), Co-incubation of the same with increasing concentrations of β-E and Cu-ascorbate has shown a significant dose dependent protection of this enzyme.

Figure 6. Effect of different concentrations of β-E on activities of Kreb's cycle enzymes of liver mitochondria,(A) Pyruvate dehydrogenase,(B) Isocitrate dehydrogenase,(C) α-keto glutarate dehydrogenase,(D) Succinate dehydrogenase, C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.



*Values were expressed in terms of mean ± S.E.,*P<0.001 vs. Control , **P<0.001 vs. Cu-ascorbate*

Figure 6(C) depicted that the incubation of Cu-ascorbate with mitochondria showed a significant decline in the activity of α-keto glutarate dehydrogenase activity (87.1% decrease vs. control, * P<0.001) compared to control. But co-incubation of mitochondria with β-E and Cu-ascorbate significantly and dose dependently protected this NAD linked multienzyme complex from being decreased.

Moreover, a stimulating effect of β-E on these NAD linked Kreb's cycle enzymes was also observed.

From figure 6(D) it is evident that Cu-ascorbate incubated mitochondria showed a much reduced succinate dehydrogenase activity compared to the control (71.4% decrease vs. Control, *P<0.001), whereas activity of the succinate dehydrogenase was significantly protected from being altered in those Cu-ascorbate treated mitochondria, which were co-incubated with increasing concentrations of β-E.But incubation of increasing doses of only β-E did not alter the activities of those Kreb's cycle enzymes in comparison with control mitochondria (data not shown).

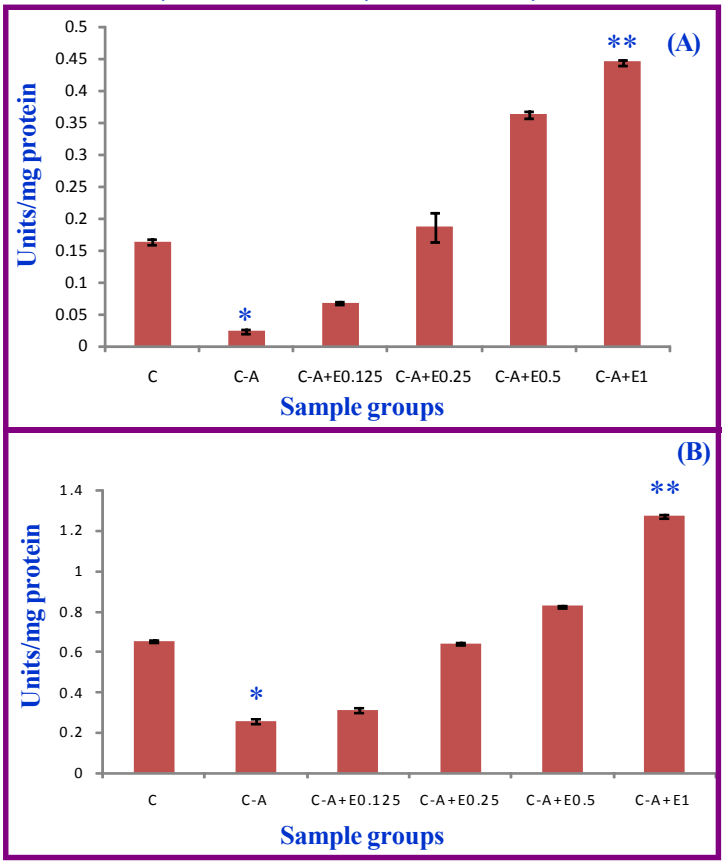
3.2.5.3. Effect of β-E on energy metabolizing electron transport chain (ETC) linked enzymes

The activity of NADH-cytochrome C reductase activity was significantly decreased in Cu-ascorbate incubated mitochondria (85.72% decrease vs. control,*P<0.001) in comparison with control as evident from figure 7(A).The activity of this enzyme was significantly protected from being decreased when mitochondrial suspensions were co-incubated with increasing concentrations of β-E.

Cytochrome C oxidase activity of mitochondria was significantly reduced upon incubation with Cu-ascorbate compared to control(60.77% decrease vs. control,*P<0.001),whereas the activity of this enzyme was significantly protected from being decreased in a dose dependent manner when mitochondria was co-incubated with increasing concentrations of β-E in presence of Cu-ascorbate, as shown in figure 7(B).

Activities of both of these energy metabolizing enzymes were stimulated in presence of β-E. But incubation of mitochondria with increasing concentrations of β-E did not show any significant deviation in the activities of these enzymes from control level (data not shown).

Figure 7. Effect of different concentrations of β-E on activities of electron transport chain(ETC) associated enzymes of liver mitochondria,(A) NADH-cytochrome C reductase,(B) Cytochrome C oxidase, C=Control, C-A=Cu-ascorbate,C-A+E0.125=0.125µM,C-A+E0.25=0.25 µM,C-A+E0.5=0.5 µM,C-A+E1=1 µM.



*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

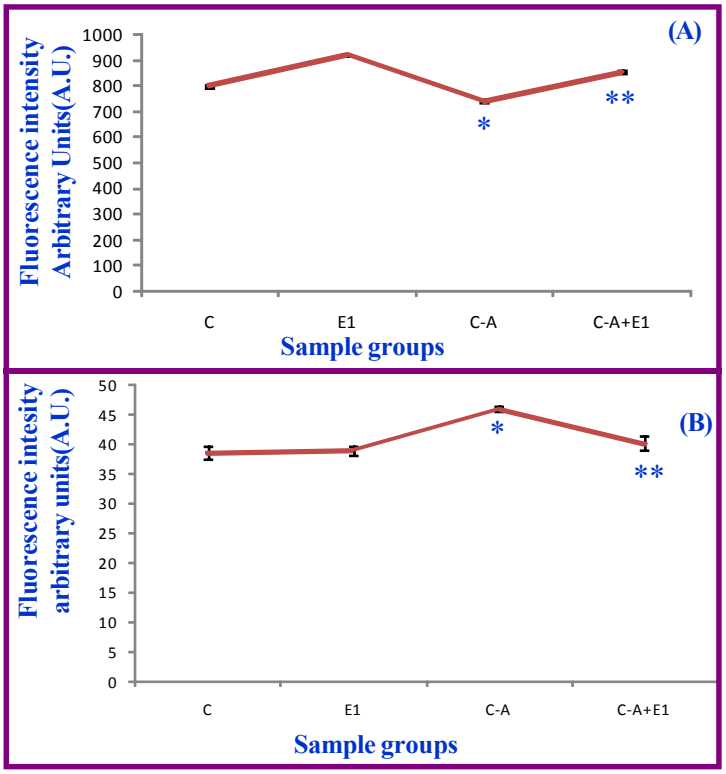
3.2.5.4. Status of protein modification of mitochondria

Status of protein modification was examined through measurement of those following parameters.

Figure 8(A) depicts that Cu-ascorbate treatment resulted in significant decrease in tryptophan level (a major marker of normal and fully functionally active mitochondria) in comparison with control (*P<0.001 vs. Control), which was protected from being altered upon co-incubation of mitochondria with β-E.

On the other hand, there was a significantly elevated level of dityrosine (a conjugate of two phenolic rings of two tyrosine molecules formed by the action of ROS) in Cu-ascorbate treated mitochondria, compared to control(*P<0.001 vs. Control),whereas co-incubation with β-E inhibited this parameter from being elevated, as evident from figure 8(B). Incubation of mitochondria only with β-E did not show any significant alteration of both of those parameters from control level.

Figure 8. Effect of β-E on the markers of protein modification of liver mitochondria,(A) Tryptophan level,(B) Dityrosine level, C=Control, E1=Positive control with 1 µM β-E,Cu-As=Cu-ascorbate,Cu-As+E1=Co-incubation of 1 µM β-E with Cu-ascorbate.

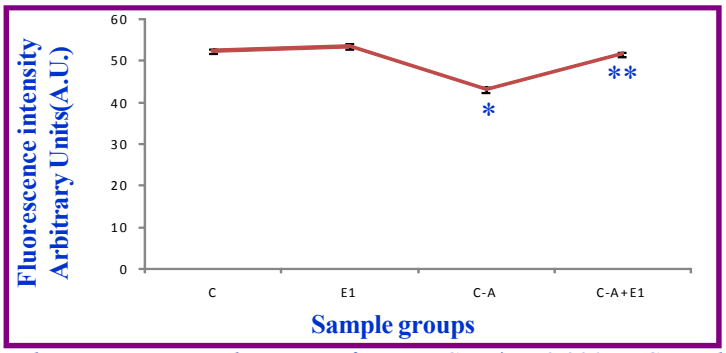


*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control, **P<0.001 vs. Cu-ascorbate.*

3.2.5.5. Redox strategy of mitochondria

Redox strategy of mitochondria was determined by NADH auto fluorescence in mitochondria, which significantly declined upon Cu-ascorbate treatment compared to control (*P<0.001 vs. Control) as evident from figure (9), whereas co-incubation of β-E protected this parameter from being reduced in presence of Cu-ascorbate. But incubation of liver mitochondria only with β-E did not cause any significant alteration in this parameter from its basal level.

Figure 9. Effect of β-E on NADH level of liver mitochondria,(A) Tryptophan level,(B) Dityrosine level, C=Control,E1=Positive control with 1 µM β-E,C-A=Cu-ascorbate,C-A+E1=Co-incubation of 1 µM β-E with Cu-ascorbate.

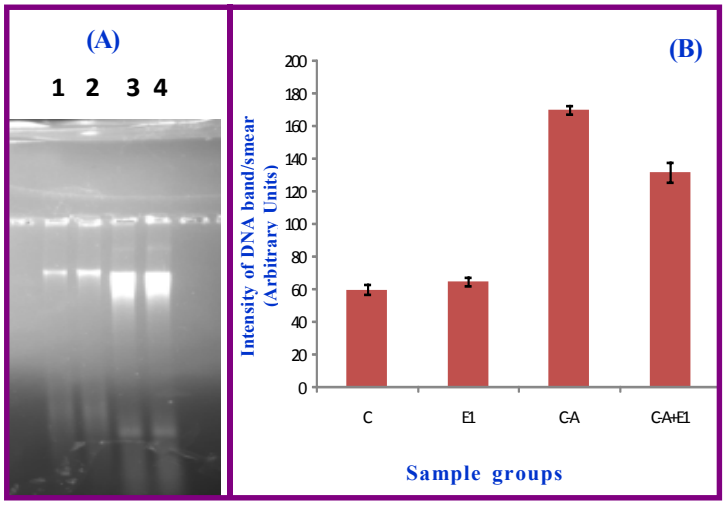


*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

3.2.5.6. Detection of DNA damage from agarose gel electrophoresis and DAPI staining

The DNA damage pattern of liver mitochondria (both treated and nontreated) are shown in figure 10. In case of Cu-ascorbate treated mitochondria,a significant smearing pattern of DNA was observed (Lane 3) compared to appearance of sharp DNA bands in control(3.7 fold increase of smearing intensity vs. control,*P<0.001) and positive control (treated only with β-E) (Lane 1 and 2 respectively).But in case of Cu-ascorbate treated mitochondria which was co-incubated with β-E, the intensity of smearing pattern of DNA was decreased to some extent in comparison with Cu-ascorbate treated mitochondria(Lane 4)as designated in figure(10A) &(10B).

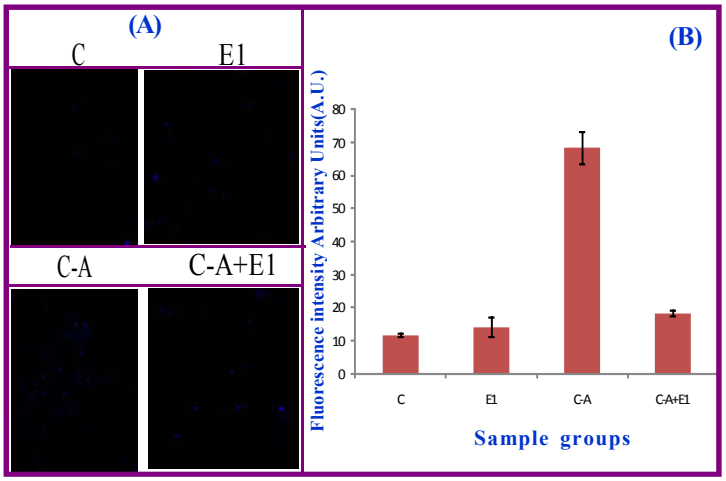
Figure 10. Effect of β-E on the damage of mitochondrial DNA,(A) Banding pattern of DNA on 1% agarose gel, lane 1=Control, lane 2= Positive control with 1 µM β-E, lane 3= Cu-ascorbate, lane 4= Co-incubation of 1 µM β-E with Cu-ascorbate.(B) Measurement of intensity of DNA band or smearing. C=Control,E1=Positive control with 1 µM β-E,C-A=Cu-ascorbate,C-A+E1=Co-incubation of 1 µM β-E with Cu-ascorbate.



*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

The blue fluorescence spots formed by intercalation of DAPI with DNA were highly abundant in the slides of Cu-ascorbate treated mitochondria in comparison to control, which was confirmed by evaluation of fluorescence intensity (9.5 fold increase vs. control,*P<0.001).But in the slides of mitochondria that was co-incubated with β-E in presence of Cu-ascorbate , the blue fluorescence spots were comparatively less abundant. In the slides of positive control, that is mitochondria incubated only with β-E, the abundance of these fluorescence spots was almost identical with the slides of control mitochondria as evident from figure(11A) and (11B).

Figure 11. (A) Confocal laser scanning electron micrograph of DAPI stained liver mitochondrial DNA,(B) Fluorescence intensities of DAPI stained mitochondria is presented by bar diagram. C=Control,E1=Positive control with 1 µM β-E,C-A=Cu-ascorbate,C-A+E1=Coincubation of 1 µM β-E with Cu-ascorbate.

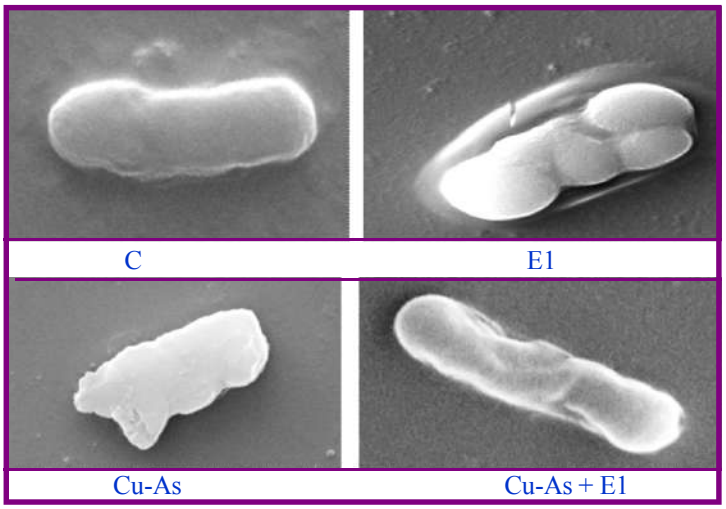


*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

3.2.5.7. Scanning electron micrograph

According to the pictures of scanning electron microscopy the surfaces of Cu-ascorbate treated mitochondria were perforated with slightly convoluted membranes compared to smooth surface topology of control and positive control mitochondria, whereas in case of β-E co-incubated with Cu-ascorbate treated mitochondria, the changes on mitochondrial surface was protected from being taken place, as was revealed from figure (12).

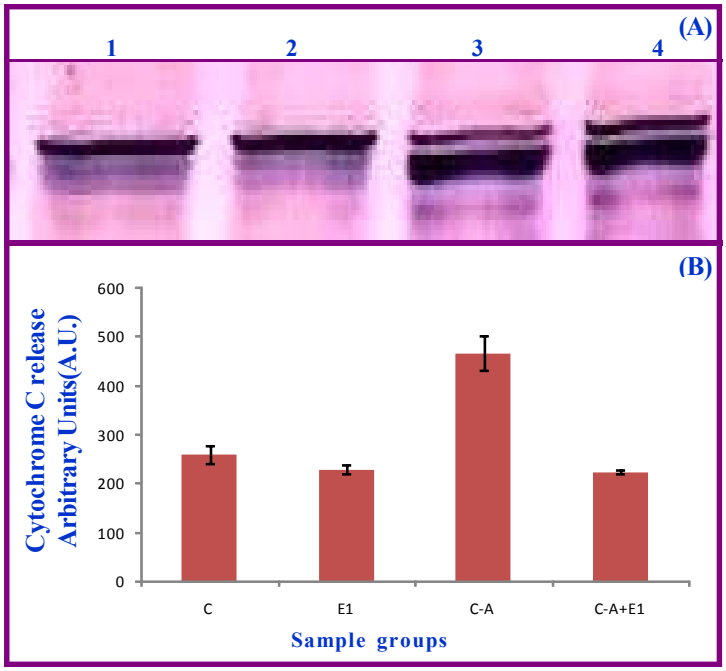
Figure 12. Scanning electron micrograph of liver mitochondria. C=Control,E1=Positive control with 1 µM β-E, Cu-As=Cu-ascorbate,Cu-As+E1=Co-incubation of 1 µM β-E with Cu-ascorbate.



3.2.5.8. Effect of β-E on the Cu-ascorbate induced alteration of mitochondrial membrane permeability

Mitochondrial membrane permeability was examined in terms of the release of cytochrome c from its membrane as was measured by western blot. In case of Cu-ascorbate treated mitochondrial sample, the band intensity of cytochrome c was increased compared to control and positive control mitochondria (79.7% increase vs. control,*P<0.001), that confirmed about the increased release of cytochrome c upon Cu-ascorbate treatment. The release of cytochrome c was protected from being taken place upon co-incubation of mitochondria with β-E, which was also confirmed by band intensity, as depicted in figure(13A and B).

Figure 13 Effect of β-E on the release of cytochrome C as well as mitochondrial permeability.(A) Banding pattern of cytochrome c in western blot, lane 1=Control, lane 2= Positive control with 1 µM β-E, lane 3= Cu-ascorbate, lane 4= Co-incubation of 1 µM β-E with Cu-ascorbate.(B) Analysis of density of cytochrome c bands in terms of bar diagram C=Control,E1=Positive control with 1 µM β-E,C-A=Cu-ascorbate,C-A+E1=Co-incubation of 1 µM β-E with Cu-ascorbate.



*Values were expressed in terms of mean ± S.E., *P<0.001 vs. Control ,**P<0.001 vs. Cu-ascorbate.*

DISCUSSION

Cu-ascorbate is a chemically defined system that generates first paramagnetic superoxide anion radical and then diamagnetic hydroxyl radical in *in vitro* system at pH 7.4 and 37^{0}C temperature the chemical environment that mimics the physiological environment[36]. This system generates almost 440 nmoles hydroxyl radical per ml solution, which is sufficient for damaging tissue proteins through carbonylation, membrane lipids through peroxidation and DNA through the formation of 7,8 dihydro-8-oxo-2' –deoxyguanine [37]. On the other hand, epinephrine can also form adrenochrome at high alkaline pH through the generation of superoxide anion radical(1B).

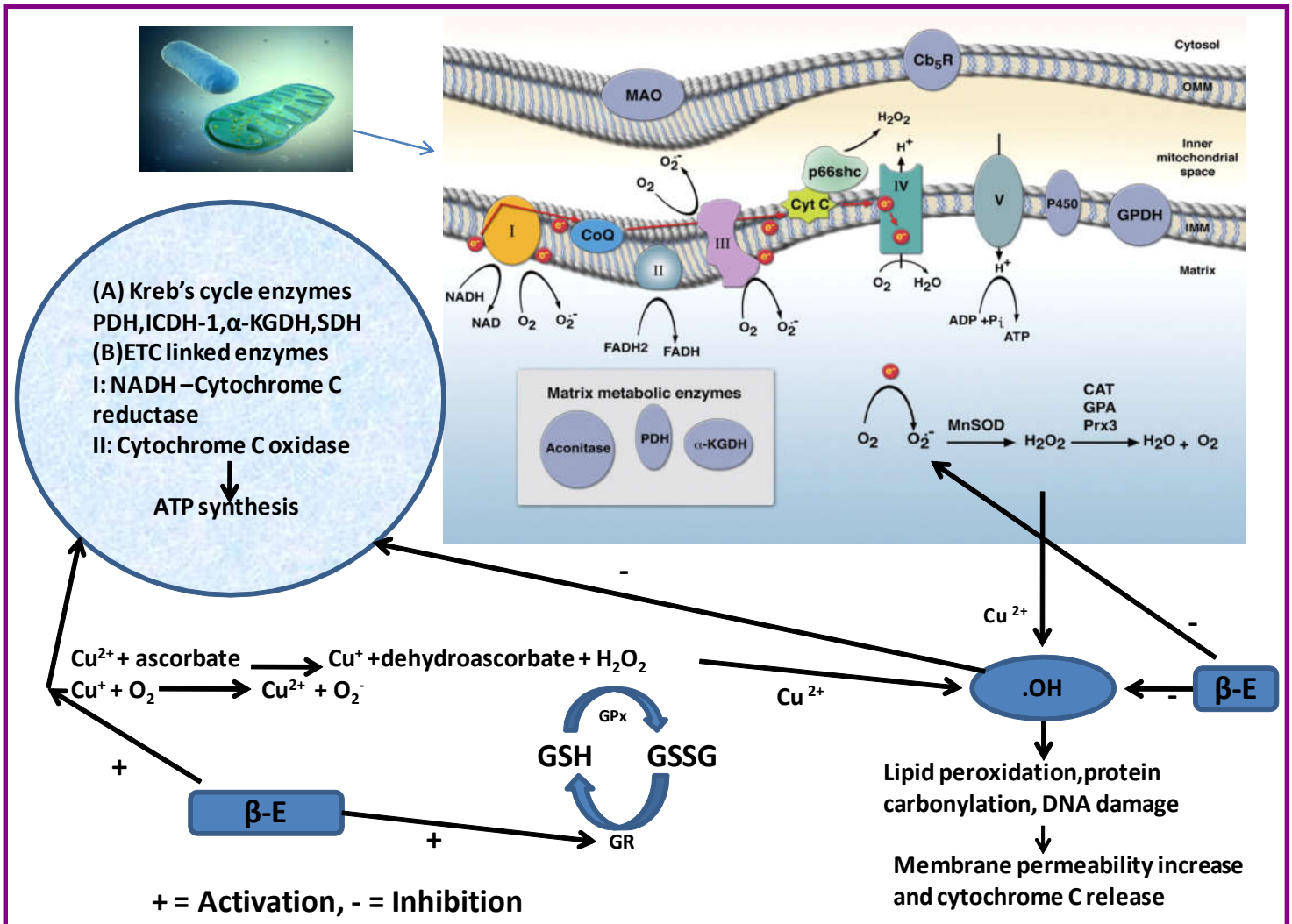


Figure 14. Scheme of possible antioxidant mechanism(s) of β-E.

Removal or withdrawal of these ROS from the medium can be a very good therapeutic approach to protect against oxidative stress induced biochemical and as well as physiological disorders. However the potentiality of β-E to scavenge the hydroxyl radical and superoxide anion radical hints at the possibility of β-E acting as a good antioxidant molecule and inspired us to determine its capability to protect mitochondria against oxidative stress induced damage.

Mitochondria is a typical organelle to mediate aerobic respiration in living system through oxygen consumption through electron transport chain(ETC) where free molecular oxygen acts as an electron sink. By accepting excess electron at its antibonding orbital molecular oxygen easily generates ROS[38] within mitochondria that make this organelle a seat of generation of ROS. Moreover xanthine oxidase(XO), the prooxidant enzyme of mitochondria generates superoxide anion radical, which is induced in Cu-ascorbate induced oxidative stress and Mn-SOD, the antioxidant enzyme activity is also increased in response to it as evident from figure(4A). If ROS induce lipid peroxidation and protein carbonylation in mitochondrial membrane, then mitochondrial membrane permeability is altered, which can cause osmotic imbalance leading to mitochondrial death. The live and death stages of mitochondria is determined by determining their viability by Janus green B staining, which gives green fluorescence with oxygen rich live mitochondria and no fluorescence with dead mitochondria as evident from figure (2). But protection of these parameters by β-E in presence of Cu-ascorbate helped us to confirm about its antioxidant ability in mitochondria. On the other hand, cytochrome c is an important member of ETC and an integral membrane protein that is embedded within the phospholipid bilayers of mitochondrial inner membrane through the hydrophobic interactions. So when lipid gets peroxidised by ROS, then the hydrophobic strength of phospholipid bilayers with cytochrome c is disturbed and it is released outside which is evident from decrease in activities of ETC linked enzymes like NADH-cytochrome C reductase

and cytochrome C oxidase in Cu-ascorbate treated samples. Furthermore the Kreb's cycle enzymes are closely associated with ETC through their coenzyme. i.e. NAD and prosthetic group FAD of succinate dehydrogenase and the decrease in their activities during oxidative stress can cause disturbance of activities of ETC linked enzymes, thereby affecting ATP synthesis (Figure 14). On the other hand, the release of cytochrome C from mitochondrial membrane also results in activation of caspases and programmed cell death[1]. Additionally the decline in the tryptophan, NADH level and concomitant elevation in the dityrosine level upon Cu-ascorbate treatment has confirmed the generation of oxidative stress and its biochemical consequences. Therefore mitochondria affected in the influence of oxidative stress acquired an altered abnormal surface topology as captured by scanning electron microscopy(Figure 12). The total scenario is inhibited from being taken place during co-incubation of mitochondria with β-E in presence of Cu-ascorbate.

Again reduced glutathione (GSH), a major mitochondrial antioxidant scavenges ROS through the formation of its oxidized form (GSSG) [39]. So during oxidative stress the level of GSH is decreased as evident from our studies. Oxidative stress can also affect the activities of glutathione metabolizing enzymes like glutathione peroxidase (GPx) and glutathione reductase (GR), thereby affecting the GSH:GSSG ratio. The major cause of depletion of GSH level is due to reduced activity of GR, and due to decreased GSH level, GPx activity is also decreased following substrate concentration kinetics and with Cu-ascorbate mediated oxidative stress (Figure 5). All of these parameters are protected from being altered in presence of β-E.

Presence of DNA in mitochondria has made it a semiautonomous organelle. Mitochondrial DNA of mammalian system also contain some genes that encode some proteins also responsible for regulation of physiological activity of mitochondria [40]. So oxidative damage induced by Cu-ascorbate in the DNA can also alter mitochondrial physiology, that can be detected by intercalating with ethidium bromide in agarose gel and also intercalating of DAPI with AT rich sequence of DNA. Reactive oxygen species induced damage of DNA appears as smearing pattern in agarose gel and this breakdown is confirmed by DAPI staining, in which the appearance of the fluorescence spots is directly proportional to the breakdown of DNA as evident from figure 11(A) and (B). Co-incubation of mitochondria with β-E in presence of Cu-ascorbate has inhibited this damage from occurring.

CONCLUSION

From the above discussion it can be concluded that beside the regulation of physiological activity of female reproductive system, β-E also possesses a significant receptor independent antioxidant activity against Cu-ascorbate induced oxidative damage in mitochondria, which may have a novel therapeutic relevance in oxidative stress mediated biochemical disorders.

Conflict of interest

The authors declare that there are no conflicts of interest.

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