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Original Article

SILYMARIN PROTECTS AGAINST COPPER-ASCORBATE INDUCED INJURY TO GOAT CARDIAC MITOCHONDRIA *IN VITRO*: INVOLVEMENT OF ANTIOXIDANT MECHANISM(S)

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ABSTRACT

Silymarin, 'one of the component of the Milk thistle seeds *Silybum marianum* (L.) is used in traditional food and medicine in India. In the present study, we investigated the antioxidant activities of Silymarin against copper-ascorbate induced toxic injury to mitochondria obtained from goat heart, *in vitro*. Incubation of isolated cardiac mitochondria with copper-ascorbate resulted in elevated levels of lipid peroxidation and protein carbonylation of the mitochondrial membrane, a reduced level of mitochondrial GSH and altered status of antioxidant enzymes as well as decreased activities of pyruvate dehydrogenase and the Kreb's cycle enzymes, altered mitochondrial morphology, mitochondrial swelling and di-tyrosine level. All these changes were found to be ameliorated when the cardiac mitochondria were co-incubated with copper-ascorbate and Silymarin, *in vitro*. Silymarin, in our *in vitro* experiments, was found to scavenge hydrogen peroxide, superoxide anion free radicals, hydroxyl radicals and DPPH radical, in a chemically defined system, indicating that this compound may provide protection to cardiac mitochondria against copper-ascorbate induced toxic injury through its antioxidant activities. The results of this study suggest that Silymarin 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.

Keywords: Silymarin, Antioxidant, Copper-Ascorbate, Goat Heart Mitochondria, Oxidative Stress.

INTRODUCTION

Silymarin consists of a major active constituent, Silybinin, also known as silybin, obtained from a standardized extract of the Milk thistle seeds *Silybum marianum* (L.) Gaertn., family Asteraceae. It consists of a mixture of flavonolignans consisting of among others of silibinin, isosilibinin, silicristin, and silidianin. Silibinin itself is mixture of two diastereomers, silybin A and silybin B, in approximately equimolar ratio [1].

Silymarin has clinical applications as a potential anti-hepatotoxic drug and it is currently used for supportive treatment of liver aliments [2, 3]. Silymarin is widely used for protection against various hepatobiliary problems in Europe [4]. It is also reported to offer protection against chemical hepatotoxins such as CCl4 [5], acetaminophen [6], phalloidin, galactosamine and thioacetamide [7] and alcoholic liver diseases [8]. Due to its proven hepatoprotective and antioxidant properties, silymarin is being used as a standard agent for comparison in the evaluation of hepatoprotective effects of plant principles [9]. In addition to its antioxidant properties, it has been reported to have high anti-tumor promoting activity [10] and has been linked to the prevention of skin carcinogenesis [10]. Recent studies have also reported that silymarin is an effective antiviral treatment for hepatitis C virus (HCV) [11]. In addition, a variety of studies demonstrated that silymarin exhibits anti-carcinogenic, anti-inflammatory, anti-atherosclerotic activities [12, 13] and also immune-modulatory activity [10]. Protective effects of silymarin against gastric ulcers induced by cold-resistant stress and ischemia–reperfusion were also reported previously [14].

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

MATERIALS AND METHODS

Chemicals

Copper-chloride and ascorbic acid were purchased from Sisco Research Laboratories (SRL), Mumbai, India. All other chemicals used including the solvents, were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

Silymarin

Silymarin was gifted to us by Micro Labs Ltd. Silymarin / silybin sample of specifications Ref. No & Item code 100R0200S008 and batch no. 71315300612, manufactured by Tewa Czech Industrial Ltd, and analyzed by Micro Labs Ltd., Hosur, Tamilnadu as a brownish yellow fine powder. It was soluble in hot methanol, slightly soluble in ethyl acetate but insoluble in chloroform and water. Its 1% solution shows the pH in the rage of 4.5 to 6.0. HPLC analysis of the sample was carried out by Micro Labs Ltd.

Determination of antioxidant properties of silymarin

Hydroxyl radical (·OH) scavenging activity

Hydroxyl radical was generated in sodium phosphate buffer (0.05 mM, pH 7.4) with 1 mM ascorbate and 0.2 mM Cu^{2+} for 60 minutes in the presence and absence of DMSO (500 mM) and different concentrations of silymarin in a volume of 1 ml to determine the hydroxyl radical scavenging activity of silymarin in an *in vitro* system. The reaction was terminated in each case by the addition of 0.1 mM EDTA. Methanesulfinic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner[15] as modified by Bandyopadhyay *et al.* [16].

Superoxide anion free radical ($O_2^{\cdot-}$) scavenging activity

Superoxide anion free radical ($O_2^{\cdot-}$) scavenging activity was studied by following the rate of epinephrine oxidation in alkaline pH at 480

nm[17]. The reaction mixture had in a volume of 1 ml, 50 mM Tris-HCl buffer (pH 10), 0.6 mM epinephrine and different concentrations of silymarin. The increase in absorbance due to the formation of the adrenochrome was followed for 7 minutes spectrophotometrically and the activity was calculated from the linear part in absence and presence of silymarin.

Hydrogen peroxide (H_2O_2) scavenging activity

Hydrogen peroxide (H_2O_2) scavenging activity was assayed by studying the breakdown of H_2O_2 at 240 nm spectrophotometrically [18].

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of each sample was determined according to the method described by Dutta *et al.* [19]. A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured spectrophotometrically at 515 nm. 40 µL of silymarin solution was added to 3 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured after 30 min. The antiradical activity (AA) was determined using the following formula: AA% = 100 − [(Abs: sample − Abs: empty sample)] × 100)/Abs: control

Preparation of goat heart mitochondria (Caprine heart mitochondria)

Goat heart mitochondria were isolated according to the procedure of Dutta *et al.* [20] with some modifications. Goat heart was purchased from local Kolkata Municipal Corporation approved meat shop. After collection it was brought into laboratory in sterile plastic container kept in ice. Then, the heart tissue was cleaned and cut into pieces.

Five gm of tissue was placed in 10 ml of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-H_2SO_4 (pH 7.8)] at 25^{0}C. Then, the tissue was blended for 1 minute at low speed by using a Potter Elvenjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA), after which it was centrifuged at 1500rpm for 10 minutes. The supernatant was poured through several layers of cheesecloth and kept in ice. Thereafter, it was centrifuged at 4000rpm for 5 minutes. The supernatant, thus obtained, was further centrifuged at 14000rpm for 20 minutes. The final supernatant obtained was discarded and the pellet was resuspended in sucrose buffer and was stored at -20^{0}C for further use.

Incubation of mitochondria with copper- ascorbate

The incubation mixture containing mitochondrial membrane protein (1.6 mg/ml), 50 mM potassium phosphate buffer (pH 7.4), and 0.2 mM Cu^{2+} and 1 mM ascorbic acid in a final volume of 1.0 ml was incubated at 37 ° C in an incubator for 1 hour. The reaction was terminated by the addition of 40 µl of 35 mM EDTA [21].

Protection of Cu^{2+}-ascorbate -induced toxic injury to mitochondria by silymarin

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

Determination of mitochondrial intactness by using Janus green B stain

After incubation, the mitochondrial sample was diluted 1:200 by using 50 mM phosphate buffer (pH 7.4). Then, the mitochondria were spread and dried on slide after which a few drops of Janus green B stain was added and kept for 5 min for staining. It was then rinsed once with distilled water and mounted in a drop of distilled water with a cover slip and imaged with a laser scanning confocal system (Zeiss LSM 510 META, Germany) and the stacked images were captured. The digitized images were then analyzed using image analysis system (ImageJ, NIH Software, Bethesda, MI) and the intactness of mitochondria of each image was measured and expressed as the % fluorescence intensity [21].

Measurement of reactive nitrogen species (RNS) in mitochondria

Nitric oxide concentrations in the incubated goat cardiac mitochondria were measured spectrophotometrically at 548 nm according to the method of Fiddler[22] by using Griess reagent [23]. 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 nitric oxide concentration was expressed as µm

Biochemical analysis

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

The lipid peroxides in the incubated mitochondria were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege *et al.* [24] with some modifications as adopted by Bandyopadhyay *et al.* [16]. The incubated mitochondria were mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80^{0}C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 8000rpm for 10 min at room temperature was measured at 532 nm using a UV–VIS spectrophotometer (Bio-Rad, Hercules, CA, USA). The values were expressed as nmols of TBARS/mg protein.

The GSH content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak *et al.* [25] with some modifications by Bandyopadhyay *et al.* [16]. Incubated mitochondria were mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for colour development. The absorbance was measured at 412 nm using a UV–VIS spectrophotometer to determine the GSH content. The values were expressed as nmole GSH/ mg protein.

Protein carbonyl content was estimated by DNPH assay[26].0.25 ml of incubated mitochondrial suspension was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The tubes were vortexed every 10 min in the dark for 1 h. Proteins were then precipitated with 30% TCA and centrifuged at 2000rpm for 10 min. The pellet was washed carefully three times with 1.0 ml of ethanol: ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined spectrophotometrically at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of 2.2X 10^{-4} M^{-1} cm^{-1}. The values were expressed as nmoles /mg of protein.

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

Manganese superoxide dismutase (Mn-SOD) activity was measured by pyrogallol autooxidation method [27].To 50 µl of the mitochondrial sample; 430 µl of 50 mM Tris–HCl buffer (pH 8.2) and 20 µl of 2 mM pyragallol were added. An increase in absorbance was recorded at 420 nm for 3 min in a UV/VIS spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyragallol as determined by change in absorbance/min at 420 nm. The enzyme activity was expressed as units/ mg of protein

The glutathione reductase (GR) assay was carried out according to the method of Krohne- Ehrich *et al.* [28]. The assay mixture in the final volume of 3 ml contained 50 mM phosphate buffer, 200 mM KCl, 1 mM EDTA and water. The blank was set with this mixture. Then, 0.1 mM NADPH was added together with suitable amount of incubated mitochondria (as the source of enzyme) into the cuvette. The reaction was initiated with 1 mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored spectrophotometrically at 340 nm. The specific activity of the enzyme was calculated as units/min/mg of protein.

The glutathione peroxidase (GPx) activity was measured according to the method of Paglia *et al.* [29] with some modifications as adopted by Dutta *et al.* [30]. The assay system contained, in a final

volume of 1 ml, 0.05 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione, and 0.25 mM NADPH. The reaction was started by the addition of 0.36 mM H_2O_2. The linear decrease of absorbance at 340 nm was recorded using a UV/VIS spectrophotometer. The specific activity was expressed as Units/mg of protein.

Measurement of the activities of pyruvate dehydrogenase and some of the Kreb's cycle enzymes

Pyruvate dehydrogenase (PDH) activity was measured spectro photometrically according to the method of Chretien *et al.* [31] with some modifications by following the reduction of NAD^+ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4, 0.5 mM sodium pyruvate as the substrate and 0.5 mM NAD^+ in addition to the enzyme. The enzyme activity was expressed as units/mg of protein.

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan *et al.* [32] by measuring the reduction of NAD^+ to NADH at 340 nm with the help of a UV–VIS spectrophotometer. One ml assay volume contained 50 mM phosphate buffer, pH 7.4, 0.5 mM isocitrate, 0.1 mM $MnSO_4$, 0.1 mM NAD^+ and the suitable amount of incubated mitochondria as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Alpha-ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan *et al.* [32] by measuring the reduction of 0.35 mM NAD^+ to NADH at 340 nm using 50 mM phosphate buffer, pH 7.4 as the assay buffer, incubated mitochondria as the source of enzyme and 0.1 mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/mg of protein.

Succinate dehydrogenase (SDH) activity was measured spectro photometrically by following the reduction of potassium ferricyanide [K3Fe (CN) 6] spectrophotometrically at 420 nm according to the method of Veeger et al. [33] with some modifications. One ml assay mixture contained 50 mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4 mM succinate, 2.5 mM K3Fe(CN)6 and a suitable aliquot of the incubated mitochondria as the source of enzyme. The enzyme activity was expressed as units/mg of protein.

Measurement of di-tyrosine fluorescence intensity

Emission spectra of di-tyrosine, a product of tyrosine oxidation, were recorded in the range 380 to 440 nm (5 nm slit width) at excitation wavelength 325 nm (5 nm slit width) [34]. Emission spectra (from 425 to 480 nm, 5 nm slit width) of lysine conjugated with LPO products were recovered at excitation of 365 nm (5 nm slit width). Excitation spectra (from 325 to 380 nm, 5 nm slit width) were measured at 440 nm (5 nm slit width)[35].

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 Halestrap *et al.* [36]. The standard incubation medium for the swelling assay contained 250 mmol/L sucrose, 0.3 mmol/L $CaCl_2$ and 10 mmol/L Tris (pH 7.4). Mitochondria (0.5 mg protein) were suspended in 3.6 mL of phosphate buffer. 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 at 520 nm wavelength, changes in absorption was recorded continuously at 25^0C for 10 min. Swelling of mitochondria was evaluated according to decrease in values of in absorption at 520 nm.

Scanning electron microscopy

The mitochondrial suspension (250 µl) 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).

Estimation of protein

The protein content of the isolated mitochondria was determined by the method of Lowry *et al.* [37].

Table 1: Specifications of silymarin

Test No.	Tests	Results	Specifications
1.	Description	Brownish yellow fine powder	Brownish yellow fine powder
2.	Solubility	Complies	Slightly soluble in ethyl acetate, soluble in hot methanol, insoluble in chloroform and water
3.	Identification:		
	A) By HPLC	Complies	Retention time values of silybin and isosilybin peaks on the sample chromatogram obtained during content determination shall be equal to those of said peaks in the standard solution
4.	pH (1% suspension)	5.15	Between 4.5 to 6.0
5.	Loss on drying	0.54%	Not more than 5.0%
6.	Sulphated Ash	0.22%	Not more than 1.0%
7.	Heavy Metals	Complies	Not more than 100 ppm
8.	Microbial Test		
	a) Bacteria	70 cfu/gm	Not more than 1000 cfu/gm
	b) Fungi	< 10 cfu/gm	Not more than 100 cfu/gm
	c) Pathogenic Organisns:	Absent/10g	Should be absent/10 g
	i) Escheria coli	Absent/10g	Should be absent/10 g
	ii) Salmonella sp.	Absent/10g	Should be absent/10 g
	iii) Pseudomonas aeruginisa	Absent/10g	Should be absent/10 g
	iv) Staphylococcus aureous		
9.	Assay:		
	i) Content of Silymarine (on as such basis)	70.32%	Not less than 70%
	ii) Content of Silybin and Isosilybib (on as such basis)	30.14%	Not less than 70%

Statistical evaluation

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

RESULTS

HPLC of the silymarin shows the retention times of the peaks of silybinin A and silybinin B in the chromatogram of the test solution corresponds to those in the chromatogram of the standard solution. HPLC analysis reported the content of silymarin as 70.32% and that of silybin and isosylibin as 30.14. Specification of silymarin has been described in table 1.The chemical structures of silybin and isosylibin have been shown in figure 1.

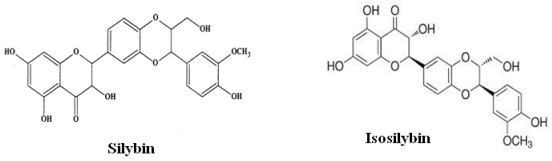

Fig. 1: Chemical structures of silybin and isosilybin.

The hydroxyl radical (•OH) scavenging ability of silymarin was studied in an *in vitro* chemically defined system using Cu^{2+} and ascorbic acid where •OH was generated which resulted in breakdown of deoxyribose to form a pink chromogen. Silymarin fraction directly scavenged •OH in a concentration-dependent manner exhibiting about 71.58% scavenging activity at a concentration of 0.50mg/ml (P<0.001 Vs. CuAs) and the minimum effective dose at which statistically significant change was observed was found to be 0.05mg/ml (Table 2).

The effect of silymarin on the superoxide anion free radical scavenging ability was studied by following the rate of superoxide anion free radical mediated epinephrine oxidation. Increasing concentrations of silymarin altered the rate of superoxide anion free radical mediated epinephrine oxidation indicating a scavenging ability silymarin for $O^{\cdot 2-}$.

About 71.76% scavenging activity was observed at a concentration of 0.50mg/ml of Silymarin which was statistically highly significant. The values in the figure are Mean **±** SE of at least four sets of experiments; P<0.001 vs. % of control epinephrine auto-oxidation (Table 2).

The H_2O_2 scavenging activity of Silymarin, if any, was also tested *in vitro* by studying the breakdown of H_2O_2 at 240 nm. Silymarin scavenged hydrogen peroxide in a dose dependent manner. The maximum scavenging activity was observed at concentration of 0.50mg/ml (P<0.001Vs. control hydrogen peroxide breakdown) (Table 2).

DPPH appears as a free radical in methanolic solution. In this medium, Silymarin scavenged this free radical in a dose-dependent manner. Maximum scavenging ability (32.32%) (P< 0.001) was exhibited at a concentration of 0.50mg/ml of silymarin(Table 2).

Table 2: Antioxidant activity of silymarin

Groups	Hydroxyl radical scavenging activity (n moles/ml of reaction mixture)	Superoxide anion free radical scavenging activity (Change in OD at 480 nm/min)	Hydrogen peroxide scavenging activity (Units/ min/mg of protein)	DPPH radical scavenging activity (Units/ min/mg of protein)
Control	0.25 ± 0.008	0.085 ± 0.001	0.75 ± 0.030	0.99 ± 0.003
CuAs	0.95 ± 0.002#	---------	---------	---------
S0.05	0.64 ± 0.007	0.066 ± 0.000	0.64 ± 0.080	0.96 ± 0.000
S0.10	0.53 ± 0.001	0.052 ± 0.003	0.59 ± 0.001	0.84 ± 0.000
S0.25	0.42 ± 0.002	0.046 ± 0.002	0.41 ± 0.030	0.77 ± 0.002
S0.50	0.27 ± 0.004 *	0.024 ± 0.006*	0.32 ± 0.002*	0.67 ± 0.000*

CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with silymarin at the dose of 0.05-0.50mg/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 CuAs-treated values using ANOVA.

Figure 2(A-C) depicts a significant decrease in the mitochondrial intactness following the incubation of mitochondria with copper-ascorbate (75.45%, P < 0.001 vs. control). This decreased level of mitochondrial intactness were found to be significantly protected from being altered (3.12 fold compared to copper-ascorbate-incubated group, P < 0.001) when the mitochondria were co-incubated with copper-ascorbate and silymarin(0.50mg/ml), indicating the ability of silymarinto protect the mitochondria against copper-ascorbate induced changes in mitochondrial swelling which may be due to oxidative stress. Figure 2(D-E) (magnification 40X) reveals the mitochondria of various groups. It depicts a decrease in the mitochondrial intactnes following the incubation of mitochondria with copper-ascorbate.

This decreased level of mitochondrial intactness was found to be protected from being altered when the mitochondria were co-incubated with copper-ascorbate and silymarin in a dose-dependent manner.

The level of NO in mitochondria in copper-ascorbate incubated group was found to be increased significantly (figure. 3) when compared to control group by 1.33 fold (#P≤ 0.001). However, a dose-dependent protection of the level of NO was observed when the cardiac mitochondria were co-incubated with copper-ascorbate and increasing concentrations of silymarin. At 0.50mg/ml, silymarinwas found to maximally protect the level of mitochondrial NO from being altered (57.57% protection, *P≤ 0.001).

A significant increase in cardiac mitochondrial LPO level following the incubation of mitochondria with copper-ascorbate (1.27 fold, #P < 0.001 vs. control) was observed. This elevated level of lipid peroxidation products were found to be protected from being increased significantly (60.00% from copper-ascorbate-treated group, *P < 0.001Vs. Cu-ascorbate) when the mitochondria were co-incubated with copper-ascorbate and silymarin(0.50mg/ml), indicating the ability of silymarin to protect the mitochondria against oxidative stress-induced changes due to copper-ascorbate (Table 3).

Copper-ascorbate caused significant decrease in cardiac mitochondrial reduced GSH content (45.77%, #P< 0.001 vs. control). This decreased level of reduced GSH content was found to be protected significantly in a dose-dependent manner. An increase of 75.42% was observed compared to copper-ascorbate-treated group, (*P < 0.001 Vs. Cu-As) when the mitochondria were co-incubated with copper-ascorbate and silymarin(0.50mg/ml) (Table 3).

Measurement of protein carbonyl content showed a significant increase in cardiac mitochondrial protein carbonyl following the incubation of mitochondria with copper-ascorbate (1.74 fold, #P < 0.001 vs. control).

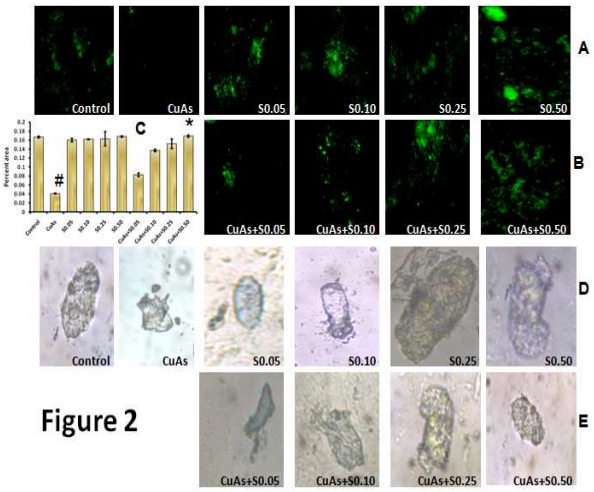

Fig. 2: Changes of intactness of mitochondria. (A-B) Janus green B stained (40X magnification), (C) graphical representation of changes of mitochondrial intactness and (D-E) Janus green B stained (Bright field microscopy; 40X magnification); CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with Silymarin at the dose of 0.05-0.50mg/ml respectively (positive control); CuAs- S0.05-0.50= group co-incubated with copper-ascorbate and silymarin at the dose of 0.05-0.50mg/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.

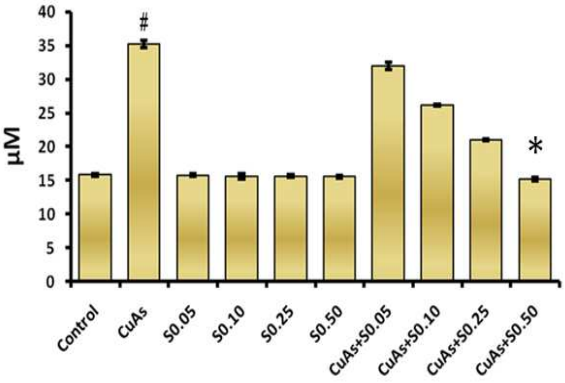

Fig. 3: Protective effect of silymarin against copper-ascorbate-induced increase in nitric oxide concentration. CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with silymarin at the dose of 0.05-0.50mg/ml respectively (positive control); CuAs- S0.05-0.50= group co-incubated with copper-ascorbate and silymarin at the dose of 0.05-0.50mg/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.

This elevated level of protein carbonyl content was found to be protected significantly (66.40% from copper-ascorbate-incubated group, *P <0.001) when the mitochondria were co-incubated with copper-ascorbate and silymarin (0.50mg/ml) (Table 3).

Table 3: Protective effect of silymarin against copper-ascorbate induced alteration in the biomarkers of oxidative stress in goat heart mitochondria

Groups	LPO level (nmol TBARS/ mg of protein)	GSH (nmole GSH/ mg of protein)	Protein carbonyl (nmoles/ mg of protein)
Control	0.75± 0.009	32.86 ± 3.0	7.50 ± 0.0
CuAs	1.7± 0.001#	17.82 ± 1.8#	17.53 ± 0.2 #
S0.05	0.74±0.003	33.81 ± 2.0	7.55 ± 0.2
S0.10	0.75±0.005	33.84 ± 0.1	7.56 ± 0.0
S0.25	0.74±0.003	32.98 ± 0.6	7.52 ± 0.2
S0.50	0.74±0.001	33.07 ± 1.7	7.50 ± 0.7
CuAs-S0.05	1.44 ± 0.002	19.97 ± 0.5	17.63 ± 0.8
CuAs-S0.10	1.20 ± 0.001	23.13 ± 5.1	11.59± 0.0
CuAs-S0.25	0.74 ± 0.002	25.50 ± 2.9	7.17 ± 0.1
CuAs-S0.50	0.68 ± 0.002*	31.26 ±1.2*	5.89 ± 0.2*

CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with silymarin at the dose of 0.05-0.50mg/ml respectively (positive control); CuAs-S0.05-0.50= group co-incubated with copper-ascorbate and silymarin at the dose of 0.05-0.50mg/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 CuAs-treated values using ANOVA.

A highly significant elevation (1.27 fold,#P < 0.001 vs. control group) was observed in the activity of Mn-SOD following incubation of mitochondria with copper-ascorbate. The activity of this enzyme was found to be protected from being increased when the mitochondria were co-incubated with copper-ascorbate and silymarin. Silymarin protected Mn-SOD activity by 56.65% (*P < 0.001 vs. copper-ascorbate-treated group) at the dose of 0.50mg/ml (Table 4). Silymarin by itself has no effect on the activity of Mn-SOD.

A highly significant decrease (64.81%, #P < 0.001 vs. control group) in the activity of GPx following the incubation of mitochondria with copper-ascorbate was observed. The GPx activity was protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and silymarin. Silymarinnot only protected but also stimulated GPx activity 2.54 fold (*P <0.001 vs. copper-ascorbate-treated group) at the dose of 0.50mg/ml (Table 4). However, Silymarin by itself has no effect on the activity of GPx.

There is also a highly significant decrease (58.03%, #P < 0.001 vs. control group) in the activity of GR following incubation of mitochondria with copper-ascorbate. The GR activity was found to be protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and silymarin. Silymarin protected GR activity by about 1.78 fold (*P < 0.001 vs. copper-ascorbate-treated group) at the dose of 0.50 mg/ml (Table 4). Silymarin by itself, however, has no effect on the activity of GR.

The incubation of the goat heart mitochondria with copper-ascorbate inhibits pyruvate dehydrogenase activity (54.11%, #P<0.001 vs. control). When the mitochondria were co-incubated with copper-ascorbate and silymarin, the activity of the enzyme, however, was found to be significantly protected from being decreased compared to the activity observed in the copper-ascorbate-incubated group (1.29 fold increased,*P<0.001 vs. copper-ascorbate-incubated group) at the dose of 0.50mg/ml (Figure 4).

Measurement of isocitrate dehydrogenase (ICDH) activity reveals that the incubation of the mitochondria with copper-ascorbate significantly inhibits isocitrate dehydrogenase activity (55.78%, #P<0.001 vs. control). The activity of the enzyme was found to be

completely protected when mitochondria were co-incubated with silymarin at the dose of 0.50mg/ml (1.54 fold increased, *P<0.001 vs. Cu-As) (Figure 4). Alpha keto glutarate dehydrogenase (α-KGDH) activity was found to be decreased when mitochondria were incubated with copper-ascorbate (54.25%, P<0.001 vs. control). The activity of the enzyme was found to be significantly protected from being decreased when the mitochondria were co-incubated with 0.50mg/ml dose of silymarin (1.27 fold higher, *P<0.001 vs. copper-ascorbate-incubated group) (Figure 4).

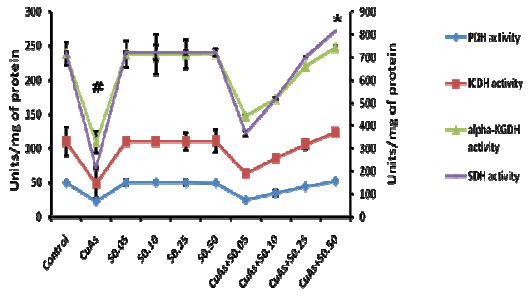

Fig. 4: Protective effect of silymarin against copper-ascorbate-induced alteration in the pyruvate dehydrogenase and other Kreb's cycle enzymes in goat heart mitochondria. CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with silymarin at the dose of 0.05-0.50mg/ml respectively (positive control); CuAs- S0.05-0.50= group co-incubated with copper-ascorbate and silymarin at the dose of 0.05-0.50mg/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.

The succinate dehydrogenase (SDH) activity was found to be significantly decreased when mitochondria were incubated with copper-ascorbate (70.17%, #P<0.001 vs. control). The enzyme activity was found to be significantly protected from being decreased when the mitochondria were co-incubated with 0.50mg/ml dose of the silymarin (2.80 fold higher, *P<0.001 vs. copper-ascorbate-incubated group) (Figure 4).

The mitochondria were added to the reaction buffer (at pH 7.2) or 0.3 mmol/L of $CaC1_2$. After that the mitochondrial absorbance at 520 nm declined, indicating mitochondrial swelling due to alteration in osmotic pressure. The extent of decrease in absorbance in the mitochondria incubated with copper-ascorbate was found to be lower compared to the control group (Figure 5A), demonstrating that incubation with copper-ascorbate caused oxidative damage of mitochondria.

The absorbance was found to be significantly increased when the goat heart mitochondria were co-incubated with copper-ascorbate and silymarin (at a dose of 0.50mg/ml) compared to mitochondria incubated with copper-ascorbate only. This indicates that silymarin has the potential to improve impaired mitochondrial function.

That copper-ascorbate induced oxidative stress has a direct effect on the oxidation level of amino acid is evident from increased di-tyrosine formation (2.00 fold increase, #P<0.001 compared to control group) (Figure 5B) as observed using fluorimetric analysis of this amino acids' basal auto-fluorescence.

Co-incubation of cardiac mitochondria with copper-ascorbate and silymarin (at the dose of 0.50mg/ml) was found to protect these molecules from losing their original configuration as indicated by recovered auto-fluorescence level for di-tyrosine formation (45.83% protection, *P<0.001 compared copper-ascorbate-incubated group). The silymarin by itself has no effect on the di-tyrosine fluorescence of cardiac mitochondria.

Table 4: Protective effect of silymarin against copper-ascorbate induced alteration in the activities of antioxidant enzymes of goat heart mitochondria

Groups	Mn-superoxide dismutase activity (Units/mg of protein)	Glutathione peroxidase activity (Units/ mg of protein)	Glutathione reductase activity (Units/ mg of protein)
Control	67.91±1.27	29.67±1.29	43.89 ± 0.11
CuAs	154.39±8.21#	10.44±0.09#	18.42 ± 0.51#
S0.05	67.24±2.40	29.45±0.82	43.87 ± 1.70
S0.10	67.50±4.50	28.93±0.84	43.15 ± 0.05
S0.25	67.93±0.003	29.55±0.05	43.88 ± 0.01
S0.50	67.29±0.19	29.53±2.17	43.04 ± 0.08
CuAs-S0.05	141.40±3.89	13.84±0.03	25.33 ± 0.12
CuAs-S0.10	125.51±7.34	20.39±0.17	32.94 ± 0.09
CuAs-S0.25	84.10±0.04	29.49±1.02	43.61± 0.01
CuAs-S0.50	65.39±0.21*	36.97±0.19*	51.27 ± 0.02*

CuAs = copper-ascorbate incubated group; S0.05-0.50= group incubated with silymarin at the dose of 0.05-0.50mg/ml respectively (positive control); CuAs-S0.05-0.50 = group co-incubated with copper-ascorbate and silymarin at the dose of 0.05-0.50mg/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 CuAs-treated values using ANOVA.

Figure 5(C-F) shows the changes brought about to the cardiac mitochondrial surface following incubation with copper-ascorbate studied through scanning electron microscopy. The figure shows a perforated surface with convoluted membranes. Moreover, the mitochondria were found to be markedly contracted, with large membrane blebs covering its surface. However, when the cardiac mitochondria were co-incubated with copper-ascorbate and silymarin (at 0.50mg/ml), the changes on the mitochondrial surface were found to be significantly protected from being taken place.

DISCUSSION

Reactive oxygen species (ROS) are generated due to redox imbalance in tissues, which is responsible for tissue damage by modification of lipid, proteins and nucleic acids. In our *in vitro* experimental system Cu-ascorbate was used as an inducer of oxidative stress in goat heart mitochondria. The mechanism of generation of OH in this system is as follows:

$$Cu^{2+} + \text{ascorbic acid} \rightleftharpoons Cu^{+} + \text{dehydroascorbic acid} + H_2O_2$$

$$Cu^{+} + O_2 \rightleftharpoons Cu^{2+} + O_2^{\cdot-}$$

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

As evident from the above reaction, O_2^{-} anion radical is generated by Cu^{2+}. The activity of superoxide dismutase which is found to be increased in mitochondrial compartments may be due to generation of this O_2^{-}anion radical. As the catalase enzyme is absent in mitochondria, so it is quite impossible to quench this H_2O_2 overload. Hence, this hydrogen peroxide generates.OH responsible for lipid peroxidation, protein carbonylation, depletion of mitochondrial GSH (that is also evident from decreased activities of GPx and GR). Additionally the generated hydroxyl radical causes declining activities of NAD linked enzymes like pyruvate dehydrogenase, α-

keto glutarate dehydrogenase, isocitrate dehydrogenase as well as FAD linked enzyme i.e. succinate dehydrogenase (all of them are associated with electron transport chain for oxidative phosphorylation.) that again will generate more superoxide anion through electron transport chain and which will follow above reaction scheme and overload of excess hydroxyl radical will be exerted on the mitochondria and finally mitochondria will be fragile as evident from our studies using scanning electron microscopy.

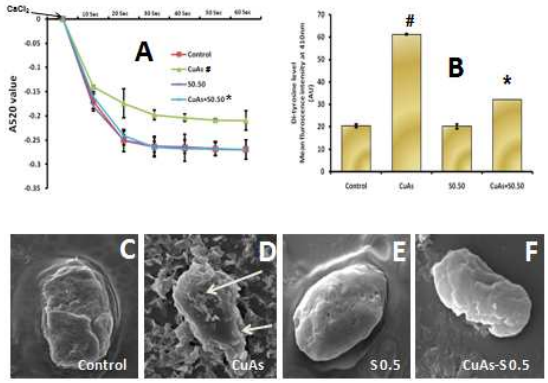

Fig. 5: Protective effect of silymarin against copper-ascorbate-induced (A) decrease in mitochondrial swelling and (B) increase in di-tyrosine level. (C-F) Scanning electron micrograph (X6000) of mitochondrial surface. Arrow heads indicate perforated surface of mitochondria. CuAs = copper-ascorbate incubated group; S0.50= group incubated with silymarin at the dose of 0.50mg/ml respectively (positive control); CuAs-S0.50= group co-incubated with copper-ascorbate and silymarin at the dose of 0.50mg/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.

Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species generated exceeds the antioxidant capability of the cell [38]. The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress [39]. Free radical scavenging enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) protect the biological systems from oxidative stress. The SOD dismutates superoxide anion free radicals (O_2^{-}) into hydrogen peroxide (H_2O_2) and O_2 [40]. Glutathione peroxidase also functions in detoxifying H_2O_2 similar to catalase. Thus, SOD and glutathione peroxidase act mutually and constitute the enzymatic antioxidative defense mechanism against reactive oxygen species [41].

The alteration in the activities of these enzymes in the present study could be attributed to the excessive utilization of these enzymes in inactivating the free radicals generated during the incubation of mitochondria with copper-ascorbate. This is further substantiated by an elevation in the levels of lipid peroxidation. Restoration in the levels of lipid peroxidation after administration of silymarin could be related to its ability to scavenge reactive oxygen species, thus preventing further damage to membrane phospholipids. Our results are in line with previous studies by Ramakrishnan *et al.* [42] who have shown that silymarin exhibits excellent antioxidant property.

Therefore, this property of silymarin might have resulted in the recoupment in the activities of the above antioxidant enzymes to normalcy. The above mentioned results of our experiments inspired us to consider it as a potent antioxidant, which is strongly evident from its ROS scavenging activities i.e. direct scavenging ability of superoxide anion free radical, free hydroxyl radical and hydrogen peroxide. On another side, it also prevents Cu-ascorbate induced alteration in mitochondria in a dose dependent manner. It also stimulates cellular antioxidant i.e. GSH synthesis, evident from result of glutathione reductase assay and reduced glutathione.

Mainly, it also prevented lipid peroxidation and protein carbonylation in Cu-ascorbate induced samples of mitochondria through removal of hydroxyl radicals. It also ameliorated oxidative stress by protecting the activities of Kreb's cycle enzymes. Reduce levels of activities of these enzymes may cause generation of superoxide anion radical using molecular oxygen as an electron sink. Superoxide anion free radicals if generated will cause hydroxyl radical generation, which in turn will result in oxidative cell damage. By altering liver cell membranes, silymarin inhibits toxin uptake and stimulates cell regeneration [8]. Silymarin's potent antioxidant activity helps to quell inflammation and replenish glutathione [8]. Glutathione is the chief antioxidant inside most living cells and is the main line of defense against free radical damage. It is found in high concentrations in the liver. In a review of viral hepatitis studies, silymarin decreased liver enzymes known as serum transaminases [43] and improved symptoms and general well-being [44]. Regarding activity against hepatitis C virus, silymarin and its components were anti-inflammatory. All compounds blocked virus-induced oxidative stress. Multiple assays suggest that numerous milk thistle compounds may help ameliorate hepatitis C disease [45]. In conclusion, silymarin can be very effective antioxidant and can protect biological systems against the oxidative stress that is found to be an important pathophysiological event in a variety of diseases including aging, cancer, diabetes, cardiovascular disorders and rheumatoid arthritis. As far as we know, this is the first report to describe an antioxidant mechanism(s) (Figure 6) of protective effect of silymarin toward copper-ascorbate-induced mitochondrial oxidative damage. Therefore, as it is stated above silymarin shows high antioxidant capacity mainly due to its phenolic compounds and inhibits lipid peroxidation in mitochondrial *in vitro* models, the present study suggests that silymarin may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement.

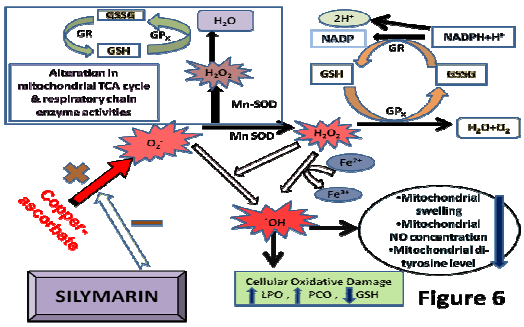

Fig. 6: Schematic diagram representing the antioxidant mechanism(s) of protection of Silymarinagainst copper-ascorbate induced oxidative damages in cardiac mitochondria.

CONFLICT OF INTERESTS

Declared None

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