Research Article

Andrographolide, One of the Major Components of *Andrographis paniculata* Protects against Copper-Ascorbate Induced Oxidative Damages to Goat Cardiac Mitochondria *In-Vitro*

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ABSTRACT

Andrographolide is a labdane diterpenoid that is the main bioactive component of the medicinal plant *Andrographis paniculata*. Andrographolide is an extremely bitter substance extracted from the stem and leaves of *A. paniculata*. The present studies revealed that andrographolide also possesses potent antioxidant properties which is evident from its capacity to scavenge hydroxyl radical, superoxide anion radical, hydrogen peroxide and DPPH radical in chemically defined system, *in vitro*. Moreover, andrographolide was found to provide protection against Cu-ascorbate induced elevation in the levels of lipid peroxidation and protein carbonylation as well as reduction in the levels of GSH in goat cardiac mitochondria incubated *in vitro.* This compound was further found to protect against Cu-ascorbate induced alterations in the activities of Mn-SOD, glutathione reductase and the activities of some of the Kreb's cycle enzymes. Andrographolide was also found to protect against Cu-ascorbate induced mitochondrial swelling, mitochondrial membrane cardiolipin content, di-tyrosine level as well as mitochondrial DNA damage. The results of this study suggest that andrographolide 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: Andrographolide, Cu-ascorbate, Cardiac mitochondria, *In-vitro*.

INTRODUCTION

Andrographis paniculata (Nees) family Acanthaceae, is a small gregarious herb which grows abundantly in moist, shady, waste grounds and in dry forests of India and China. It also grows in many other Asian countries and is used as a traditional herbal medicine in Hong Kong, the Philippines, Malaysia, Indonesia, and Thailand. Commonly known as 'Kalmegh' in Hindi and 'Chuanxinlian' or 'Lanhelian' in Chinese, it is widely used in Indian system of medicine and Traditional Chinese Medicine as a natural bitter, stomachic, tonic, antipyretic, Anthelmintic, febrifuge and cholagogue for liver disorders, general debility and colic pains. In India it is extensively used as a hepato-stimulant and hepatoprotective agent. It is referred to as 'king of bitters'. Its 'Blood purifying' property, results in its use in diseases where 'Blood abnormalities' are considered cause of disease.[1,2]

A. paniculata has been reported as having choleretic, hypoglycemic, hypocholesterolemic and adoptogenic activities. *A. paniculata* extract and andrographolide has been evaluated for its multifarious activities ranging from analgesic, anti-inflammatory, antibacterial, antifungal, antiviral, anti-HIV and antineoplastic activities.[3] *A. paniculata* contains andrographolide as a major bitter constituent. Other constituents includes deoxyandrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographolide, andrographoside, andrographosterol and flavonoids. [4] Andrographolide has been reported to have a wide range of biological activities, such as those that are anti-inflammatory, anti allergic, antiplatelet aggregation, hepatoprotective, and anti-HIV.[5-10] In addition to these activities, the ability of ethanol or an aqueous extract of *A. paniculata* to decrease blood glucose levels in normal rats or streptozotocin diabetic rats had been documented.[11]

In biological systems, andrographolide can interact with many inter- and intracellular constituents as a bipolar compound, thus ensuing inmany biological responses. Earlier study had shown that various analogues of andrographolide such as 3,19-isopropylidene andrographolide have protective effects against leukaemia and colon cancer cells and 14-acetylandrographolide have protective effects against leukaemia, ovarian and renal cancer cells.[12] On the other hand various andrographolide derivatives had anti dyslipidemic, LDL-oxidation and antioxidant activity and AL-1 (another andrographolide analogue) had both hypoglycemic and beta cell protective effects which translated into antioxidant and NF-κB inhibitory activity.[13-14] Recent study has shown that andrographolide possesses antioxidative properties against cigarette smoke-induced lung injury probably via augmentation of Nrf2 activity and may have therapeutic potential for treating chronic obstructive pulmonary disease (COPD).[15]

But till date there is no report available, to the best of our knowledge and belief, about the protective effect of andrographolide against mitochondrial oxidative stress.

Herein, we provide evidences perhaps for the first time, the protective ability of andrographolide 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

Thiobarbituric acid (TBA), eosin, nicotinamide adenine dinucleotide (NAD), Direct Red-80, 2,2-dithiobis-nitro benzoic acid (DTNB), cytochrome c, nitro blue tetrazolium (NBT), and glutathione (GSH) were obtained from Sigma, St. Louis, MO, USA. Hematoxylin, hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were obtained from Merck Limited, Delhi, India. All other chemicals used including the solvents, were of analytical grade obtained from Sisco Research Laboratories (SRL), Mumbai, India, Qualigens (India/Germany), SD fine chemicals (India), Merck Limited, Delhi, India.

Isolation and extraction of andrographolide from the dry fruit of *Andrographis paniculata*

A. paniculata contains andrographolide as a major bitter constituent. Andrographolide is a bitter diterpene lactone which contains a lactone ring connected to a decaling ring system via an unsaturated C2 moiety. It is found in different parts of the plant both in the free state and in the form of glycoside. Andrographolide can be isolated from the dried aerial part of the plant or from dried leave by defattying with petroleum ether (60-800c) and then further soxhlet extraction with ethyl alcohol (95%). Partial evaporation and cooling of alcoholic extract yield heavy crystals of andrographolide. The crystals can be repeatedly washed with benzene and recrystallized in ethanol.[16] Hydrolysis of andrographolide under cleavage of the lactone ring yields salts of andrographolic acid which can be reconverted into andrographolide by acidification. The sample of andrographolide used for the present study was a gift sample of andrographolide (67%) received from Micro Lab. Limited, Bangalore, India.

Determination of antioxidant properties of andrographolide

Hydroxyl radical scavenging activity

Hydroxyl radical was generated in sodium phosphate buffer (0.05mM, pH 7.4) with 1mM ascorbate and 0.2mM Cu^{2+} for 60 minutes in the presence and absence of DMSO (500mM) and different concentrations of andrographolide in a volume of 1mL to determine the hydroxyl radical scavenging activity of andrographolide in an *in vitro* system. The reaction was terminated in each case by the addition of 0.1mM EDTA. Methane sulfinic acid (MSA) formed during incubation was measured by the method of Babbs and Steiner (1990) as modified by Bandyopadhyay et al., (2004).[17, 18]

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 480nm.[19] The reaction mixture had in a volume of 1mL, 50mM Tris-HCl buffer (pH 10), 0.6mM epinephrine and different concentrations of andrographolide. The increase in absorbance due to the formation of the adreno-chrome was followed for 7 minutes spectrophotometrically and the activity was calculated from the linear part in absence and presence of andrographolide. The involvement of $O_2^{\cdot-}$ was checked with standard SOD.

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 240nm spectrophotometrically.[20] The reaction mixture contained 50mM phosphate buffer (pH 7.4), 1mM H_2O_2 and 62.5mM, 125mM, 250mM and 500mM andrographolide in a final volume of 3mL.

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., (2014).[21] A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured spectrophotometrically at 515nm. 40µL of andrographolide solution was added to 3mL of methanolic DPPH solution. The change in absorbance at 515nm 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

Goat heart mitochondria were isolated according to the procedure of Dutta et al., (2013) with some modifications.[22] Goat heart was purchased from local Kolkata Municipal Corporation approved meat shop. After collection it was brought into laboratory in sterile plastic container kept in ice. Then, the heart tissue was cleaned and cut into pieces. Five gm of tissue was placed in 10mL of sucrose buffer [0.25(M) sucrose, 0.001(M) EDTA, 0.05(M) Tris-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. Then it was centrifuged at 4000rpm for 5minutes. The supernatant obtained was further centrifuged at 14000rpm for 20 minutes. The supernatant obtained was discarded and the pellet was resuspended in sucrose buffer and was stored at -20ºC for further use. Each experiment was repeated three times with the mitochondria prepared from a fresh batch of heart.

Incubation of mitochondria with copper- ascorbate

The incubation mixture containing mitochondrial membrane protein (1.6mg/mL), 50mM potassium phosphate buffer (pH 7.4), and 0.2mM Cu^{2+} and 1mM ascorbic acid in a final volume of 1.0mL was incubated at 37°C in an incubator for 1 hour. The reaction was terminated by the addition of 40µl of 35mM EDTA.[23]

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

The goat heart mitochondria were co-incubated with copper-ascorbate and four different concentrations of andrographolide. 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, mitochondria DNA damage 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 50mM phosphate buffer (pH 7.4). Then, the mitochondria were spread and dried on slide to prevent being washed away while staining. After that a few drops of Janus green stain were put on the slide and was left for 5-10 min for staining. After 5 min of staining, the mitochondria were rinsed once with distilled water so that complete stain was not gone and a diluted stain remained. Then the mitochondria were mounted in a drop of distilled water with a cover slip and imaged with a 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 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., (1978) with some modification as adopted by Dutta et al., (2014).[24,21] The incubated mitochondria were mixed with thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and heated for 20 min at 80^0C. 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 532nm 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., (1968) with some modifications by Dutta et al., (2014).[25,21] Incubated mitochondria were mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412nm 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] 0.25mL of incubated mitochondrial suspension was taken in each tube and 0.5mL DNPH in 2.0M HCl was added to the tubes. The tubes were vortexed every 10 min in the dark for 1 hour. Proteins were then precipitated with 30% TCA and centrifuged at 2000rpm for 10 min. The pellet was washed carefully three times with 1.0mL of ethanol: ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0mL of 6.0M guanidine HCl in 20mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined spectrophotometrically at 370nm. The protein carbonyl content was calculated using a molar absorption coefficient of 2.2 X 10^{-4} $M^{-1}cm^{-1}$. The values were expressed as nmoles/mg of protein.

Measurement of reactive nitrogen species (RNS) in mitochondria

Nitric oxide concentrations in the incubated goat cardiac mitochondria were measured spectrophotometrically at 548nm according to the method of Fiddler (1977) by using Griess reagent (1879).[27,28] The reaction mixture contained 100µL of Griess Reagent, 700µL of the sample (i.e., incubated mitochondrial suspension) and 700µL of distilled water. The nitric oxide concentration was expressed as µM/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 or SOD2) activity was measured by pyrogallol autooxidation method.[29] To 50µl of the mitochondrial sample; 430µl of 50mM Tris–HCl buffer (pH 8.2) and 20µl of 2mM pyragallol were added. An increase in absorbance was recorded at 420nm 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 420nm. The enzyme activity was expressed as units/mg of protein

The glutathione reductase (GR) assay was carried out according to the method of Krohne- Ehrich et al., (1977).[30] The assay mixture in the final volume of 3mL contained 50mM phosphate buffer, 200mM KCl, 1mM EDTA and water. The blank was set with this mixture. Then, 0.1mM NADPH was added together with suitable amount of incubated mitochondria (as the source of enzyme) into the cuvette. The reaction was initiated with 1mM oxidized glutathione (GSSG). The decrease in NADPH absorption was monitored spectrophotometrically at

340nm. The specific activity of the enzyme was calculated as units/mg of protein.

The glutathione peroxidase (GPx) activity was measured according to the method of Paglia et al., (1967) with some modifications as adopted by Dutta et al., (2014).[31,32] The assay system contained, in a final volume of 1mL, 0.05M phosphate buffer with 2mM EDTA, pH 7.0, 0.025mM sodium azide, 0.15mM glutathione, and 0.25mM NADPH. The reaction was started by the addition of 0.36mM H_2O_2. The linear decrease of absorbance at 340nm 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 spectrophotometrically according to the method of Chretien et al., (1995) with some modifications by following the reduction of NAD^+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4, 0.5mM sodium pyruvate as the substrate and 0.5mM NAD^+ in addition to the enzyme.[41] The enzyme activity was expressed as units/mg of protein.[33]

Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan et al., (1979) by measuring the reduction of NAD^+ to NADH at 340nm with the help of a UV–VIS spectrophotometer.[34] One mL assay volume contained 50mM phosphate buffer, pH 7.4, 0.5mM isocitrate, 0.1mM $MnSO_4$, 0.1mM 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., (1979) by measuring the reduction of 0.35mM NAD^+ to NADH at 340nm using 50mM phosphate buffer, pH 7.4 as the assay buffer, incubated mitochondria as the source of enzyme and 0.1mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/mg of protein.[34]

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide [$K_3Fe(CN)_6$] spectrophotometrically at 420nm according to the method of Veeger et al., (1969) with some modifications.[35] One mL assay mixture contained 50mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4mM succinate, 2.5mM $K_3Fe(CN)_6$ 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 mitochondrial swelling

Mitochondrial swelling was assessed by measuring the changes in absorbance of the suspension at 520nm (Δ) by spectrophotometry according to Halestrap et al., (1990).[36] The standard incubation medium for the swelling assay contained 250mmol/L sucrose, 0.3mmol/L $CaCl_2$ and 10mmol/L Tris (pH 7.4). Mitochondria (0.5mg protein) were suspended in 3.6mL of phosphate buffer. 1.8mL 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 520nm wavelength, changes in absorption was recorded continuously at 25°C for 10 min. Swelling of mitochondria was evaluated according to decrease in values of absorption at 520nm.

Measurement of di-tyrosine fluorescence intensity

Emission spectra of di-tyrosine, a product of tyrosine oxidation, were recorded in the range 380 to 440nm (5nm slit width) at excitation wavelength 325nm (5nm slit width).[37] Emission spectra (from 425 to 480nm, 5nm slit width) of lysine conjugated with LPO products were recovered at excitation of 365nm (5nm slit width). Excitation spectra (from 325 to 380nm, 5nm slit width) were measured at 440nm (5nm slit width).[38]

Determination of mitochondrial (mt) DNA damage with agarose gel electrophoresis

The incubated mitochondria with or without copper-ascorbate and copper-ascorbate plus andrographolide and andrographolide only were lysed with 3mL of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) containing 0.5% SDS and 0.3mg/mL of proteinase K overnight at 37°C. Mitochondrial DNA was isolated using extraction with 1M NaCl for 10 min at room temperature and purified twice with chloroform/isoamyl alcohol, 24:1. Then, the samples were precipitated and dissolved in TE buffer, and the DNA, thus obtained, gave an average 260/280 absorbance ratio of 2-2.5. The obtained DNA samples were then mixed with 6X loading dye and resolved in 0.8% agarose gel. The gel was stained with ethidium bromide and DNA bands detected in a Gel-Doc apparatus (Biorad, Hercules CA).[21]

Scanning electron microscopy

The mitochondrial suspension (250µl) 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).[21]

Estimation of protein

The protein content of the isolated mitochondria was determined by the method of Lowry et al., (1951).[39]

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 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. Pair wise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

1-2 % of andrographolide has been reported form *A. paniculata*. The chemical structure of andrographolide has been shown in figure 1.

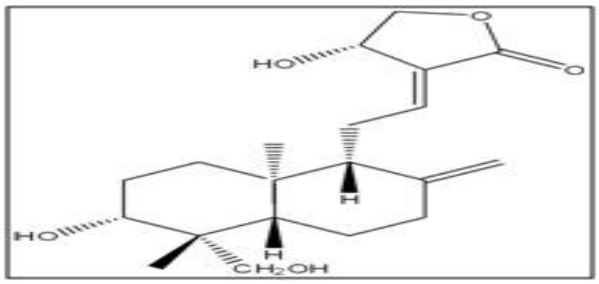

Figure 1: Chemical structure of andrographolide

The hydroxyl radical ($^{\bullet}OH$) scavenging ability of andrographolide was studied in an *in vitro* standard model system using Cu^{2+} and ascorbic acid where $^{\bullet}OH$ was generated. Table 1 indicated that andrographolide directly scavenged $^{\bullet}OH$ in a concentration dependent manner exhibiting about 81.72% ($P \leq 0.001$) scavenging activity at a concentration of 1mg/mL.

The superoxide anion ($O_2^{\bullet-}$) free radical scavenging ability of andrographolide was studied by following the rate of superoxide mediated epinephrine oxidation (Table 1). Increasing concentrations of andrographolide altered the rate of superoxide mediated epinephrine oxidation indicating $O_2^{\bullet-}$ scavenging ability of this molecule. About 62.35% ($P \leq 0.001$) scavenging activity was observed at a concentration of 1mg/mL of andrographolide which is statistically highly significant.

The H_2O_2 scavenging activity of the andrographolide, if any, was also tested *in vitro* by studying the breakdown of H_2O_2 at 240nm. Table 1 clearly indicates that andrographolide possesses the ability to scavenge H_2O_2, *in vitro* in a dose-dependent manner.

The DPPH free radical has been widely accepted as a model compound to evaluate the antioxidant abilities of various samples. This assay indicated that the andrographolide has dose-dependent DPPH radical scavenging activity. Table 1 shows that andrographolide exhibited 0.71±0.000% ($P \leq 0.001$) inhibition of DPPH activity at the dose of 1mg/mL, which is highly significant.

Table 1: Antioxidant activity of andrographolide

Groups	Hydroxyl radical scavenging activity (nmoles/ml of reaction mixture)	Superoxide anion free radical scavenging activity (Change in OD at 480nm/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	0.97 ± 0.003
CuAs	0.93± 0.002$^{\#}$	---------	---------	---------
A0.125	0.55 ± 0.002	0.064 ± 0.000	0.62 ± 0.080	0.96 ± 0.000
A0.25	0.41± 0.001	0.055 ± 0.003	0.58 ± 0.001	0.90 ± 0.000
A0.50	0.27 ± 0.001	0.040 ± 0.002	0.43 ± 0.030	0.83 ± 0.002
A1	0.17 ± 0.034*	0.032 ± 0.006*	0.39 ± 0.002*	0.71 ± 0.000*

CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively; The values are expressed as Mean ± S.E.; $^{\#}P < 0.001$ as compared to control values using ANOVA; * $P < 0.001$ as compared to copper-ascorbate incubated values using ANOVA.

Figure 2(A-E) depicts a significant decrease in the mitochondrial intactness following the incubation of mitochondria with copper-ascorbate (73.49%, $P \leq 0.001$ vs. control). This decreased level of mitochondrial intactness were found to be significantly protected from being altered (3.69 fold compared to copper-ascorbate-incubated group, $P \leq 0.001$) when the mitochondria were co-incubated with copper-ascorbate and andrographolide (1mg/mL), indicating the ability of andrographolide to protect the mitochondria against copper-ascorbate induced changes in mitochondrial swelling which may be due to oxidative stress.

Table 2, showed a significant increase in cardiac mitochondrial LPO level following the incubation of mitochondria with copper-ascorbate (3.05 folds, $P \leq 0.001$ vs. control). This elevated level of lipid peroxidation products were found to be significantly protected from being increased (75.88% compared to copper-ascorbate-incubated group, $P \leq 0.001$) when the mitochondria were co-incubated with copper-ascorbate and andrographolide (1mg /mL), indicating the ability of andrographolide to protect the mitochondria against oxidative stress-induced due to copper-ascorbate.

On the other hand, significant decrease was observed in cardiac mitochondrial GSH content following the incubation of mitochondria with copper-ascorbate (47.95%, $P \leq 0.001$ vs. control). The GSH content was found to be significantly protected from being decreased (1.01 fold from copper-ascorbate -incubated group, $P \leq 0.001$) when the mitochondria were co-incubated with

copper-ascorbate and andrographolide (1mg/mL) (Table 2).

The protein carbonyl assay showed a significant increase in cardiac mitochondrial protein carbonyl content following the incubation of mitochondria with copper-ascorbate (1.28 fold, *P ≤ 0.001* vs. control). This elevated level of protein carbonyl content was found to be significantly protected from being increased (71.72% compared to copper-ascorbate -incubated group, *P ≤ 0.001*) when the mitochondria were co-incubated with copper-ascorbate and andrographolide (1mg/mL) (Table 2).

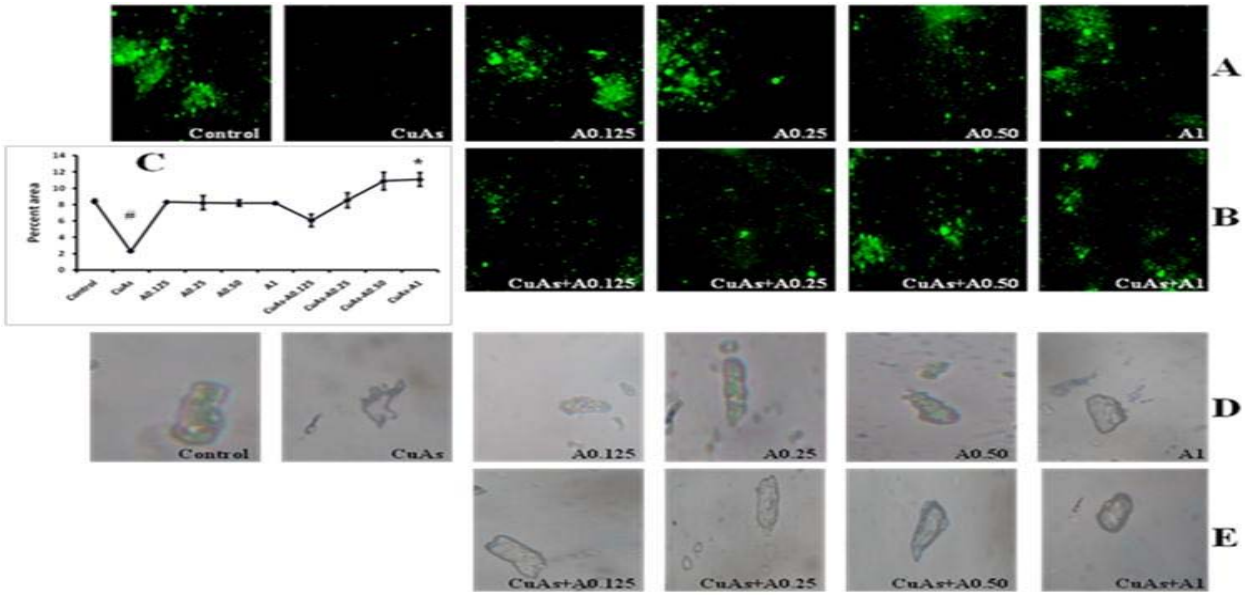

Figure 2: Changes of intactness of mitochondria. (A-B) Janus green B stained (40X magnification), (C) graphical representation of changes of mitochondrial intactness and (D-E) Janus green B stained (Bright field microscopy; 40X magnification); CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively (positive control); CuAs- A0.125-1= group co-incubated with copper-ascorbate and andrographolide at the dose of 0.125-1mg/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.

Table 2: Protective effect of andrographolide extract 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	0.42± 0.001	32.51± 1.3	7.89 ± 0.1
CuAs	1.7± 0.001#	16.92 ± 0.1#	18.00 ± 1.2 #
A0.125	0.43±0.003	32.31 ± 0.7	7.52 ± 0.9
A0.25	0.42±0.009	33.14 ±1.0	7.56 ± 0.2
A0.50	0.44±0.002	32.98 ± 0.2	7.72 ± 0.2
A1	0.43±0.000	32.07 ± 0.9	7.19 ± 0.3
CuAs- A0.125	1.52 ± 0.03	18.31 ± 0.0	17.00 ± 1.8
CuAs- A0.25	1.28 ± 0.011	24.04 ±2.0	14.62 ± 1.3
CuAs- A0.50	0.82 ± 0.005	28.98 ± 0.6	10.11 ± 1.1
CuAs- A1	0.41± 0.008*	34.07 ± 1.9*	5.09 ± 1.4*

CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively (positive control); CuAs- A0.125-1= group co-incubated with copper-ascorbate and andrographolide at the dose of 0.125-1mg/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 level of NO in mitochondria in copper-ascorbate incubated group was found to be increased significantly (Figure 3A) when compared to control group by 2.02 fold (*P ≤ 0.001* vs. control). 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 andrographolide. At 1mg/ml, andrographolide was found to maximally protect the level of mitochondrial NO from being altered (73.23% protection, *P ≤ 0.001*).

Fig. 3B reveals a highly significant increase (2.66 folds, *P ≤ 0.001* vs. control group) in the activity of Mn-SOD following incubation of mitochondria with copper-ascorbate. The activity of this enzyme was found to be significantly protected from being increased when the mitochondria were co-incubated with copper-ascorbate and andrographolide (73.74% protection; *P ≤ 0.001* compared to copper-ascorbate -incubated group, at the dose of 1mg/mL).

Figure 3B also reveals a highly significant decrease (49.84%, *P ≤ 0.001* vs. control group) in the activity of GPx following incubation of mitochondria with copper-ascorbate. The GPx activity was found to be significantly protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and andrographolide (*P ≤ 0.001* compared to copper-ascorbate -incubated group, at the dose of 1mg/mL).

Figure 3B further depicts a highly significant decrease (39.44%, *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 significantly protected from being decreased when the mitochondria were co-incubated with copper-ascorbate and andrographolide (*P ≤ 0.001* compared to copper-ascorbate -incubated group, at the dose of 1mg/mL).

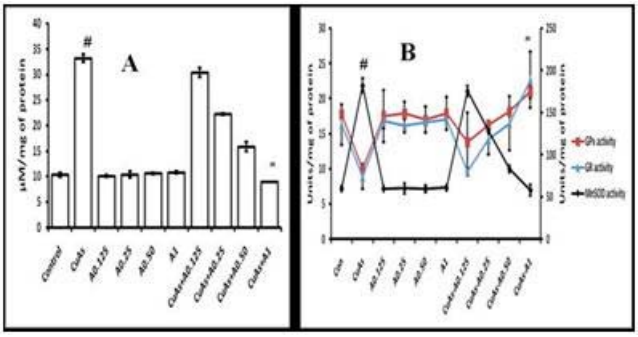

Figure 3: Protective effect of andrographolide against copper-ascorbate-induced (A) increase in nitric oxide concentration and (B) alteration in the activities of antioxidant enzymes of goat heart mitochondria. Con= Control; CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively (positive control); CuAs- A0.125-1= group co-incubated with copper-ascorbate and andrographolide at the dose of 0.125-1mg/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 incubation of the goat heart mitochondria with copper-ascorbate inhibits pyruvate dehydrogenase activity (47.58%, *P ≤ 0.001* vs. control). When the mitochondria were co-incubated with copper-ascorbate and andrographolide, 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 (1.13 fold increased, *P ≤ 0.001* vs. copper-ascorbate-incubated group) at the dose of 1mg/ml (Table 3).

Measurement of isocitrate dehydrogenase (ICDH) activity reveals that the incubation of the mitochondria with copper-ascorbate significantly inhibits isocitrate dehydrogenase activity (35.69%, *P ≤ 0.001* vs. control). The activity of the enzyme was found to be completely protected when mitochondria were co-incubated with andrographolide at the dose of 1mg/ml (*P ≤ 0.001* vs. Cu-As) (Table 3).

Alpha keto glutarate dehydrogenase (α-KGDH) activity was found to be decreased when mitochondria were incubated with copper-ascorbate (60.10%, *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 1mg/ml dose of andrographolide (1.55 fold higher, *P ≤ 0.001* vs. copper-ascorbate-incubated group) (Table 3).

The succinate dehydrogenase (SDH) activity was found to be significantly decreased when mitochondria were incubated with copper-ascorbate (57.74%, *P ≤ 0.001* vs. control). The enzyme activity was found to be significantly protected from being decreased when the mitochondria were co-incubated with 1mg/ml dose of the andrographolide (*P ≤ 0.001* vs. copper-ascorbate-incubated group) (Table 3).

After the mitochondrial sample was added to the reaction buffer (at pH 7.2) or 0.3mmol/L of $CaC1_2$, the mitochondrial absorbance at 520 nm declined, indicating mitochondrial swelling due to alteration in osmotic pressure. The extent of increase in absorbance in mitochondria incubated with copper-ascorbate was found to be lower compared to the control group (Figure 4A), demonstrating that incubation with copper-ascorbate caused mitochondrial dysfunction. The absorbance was found to be significantly decreased when the goat heart mitochondria were co-incubated with copper-ascorbate and andrographolide (at a dose of 1mg/mL) compared to mitochondria incubated with copper-ascorbate only. This indicates that andrographolide has the potential to improve impaired mitochondrial function.

The effect of the free radical-generating system (in this case copper-ascorbate system) on protein structure was examined by measuring di-tyrosine fluorescence. That copper-ascorbate induced oxidative stress has a direct effect on the oxidation level of amino acid is evident from increased di-tyrosine formation (1.2 fold increase, *P ≤ 0.001* compared to control group) (Figure 4B) as observed using fluorimetric analysis of this amino acids' basal auto-fluorescence. Co-incubation of cardiac mitochondria with copper-ascorbate and andrographolide (at the dose of 1mg/mL) was found to protect these molecules from losing their original configuration as indicated by recovered auto-fluorescence level for di-tyrosine formation (63.64% protection, *P ≤ 0.001* compared CuAs-incubated group). The andrographolide, by itself, has no

effect on the di-tyrosine fluorescence of cardiac mitochondria.

Detection of mtDNA damage is an important indicator of mitochondrial stress. Increased mtDNA damage has been associated with declining mitochondrial function, and with numerous diseases and aging. Incubation of cardiac mitochondria caused damage to mtDNA. However, when the mitochondria were co-incubated with copper-ascorbate and andrographolide (at 1mg/mL dose), the mtDNA damage was found to be almost completely protected (Figure 4C).

Figure 4D shows the changes brought about in cardiac mitochondrial surface following incubation with copper-ascorbate and 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 andrographolide (at 1mg/mL), the changes on the mitochondrial surface were found to be significantly protected from being taken place.

Table 3: Protective effect of andrographolide extract against copper-ascorbate induced alteration in the activities of Kreb's cycle enzymes and respiratory chain enzymes in goat heart mitochondria

Groups	Pyruvate dehydrogenase (Units/mg of protein)	Isocitrate dehydrogenase (Units/mg of protein)	α-ketoglutarate dehydrogenase (Units/mg of protein)	Succinate dehydrogenase (Units/mg of protein)
Control	50.15±1.30	110.12±2.18	1504.91±12.67	528.16±30.12
CuAs	26.29±0.25#	70.82±0.18#	600.46±20.91#	223.19±12.89#
A0.125	50.24±0.01	111.98 ±1.20	1502.65±27.71	527.18±11.28
A0.25	50.21±1.97	111.24±0.92	1503.27±19.08	528.17±0.98
A0.50	51.28±2.00	111.56±2.41	1504.76±15.61	529.10±2.65
A1	50.27±0.76	111.81±2.01	1504.77±23.40	529.28±11.02
CuAs-A0.125	35.00±1.08	78.19±0.01	603.51±20.87	230.18±11.90
CuAs-A0.25	42.38±0.87	89.91±0.84	827.21±19.54	381.92±10.21
CuAs-A0.50	51.58±2.09	122.67±1.29	1165.28±29.03	512.77±2.98
CuAs-A1	56.29±0.001*	142.71±2.92*	1532.79±24.65*	601.27±26.71*

CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively (positive control); CuAs- A0.125-1= group co-incubated with copper-ascorbate and andrographolide at the dose of 0.125-1mg/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.

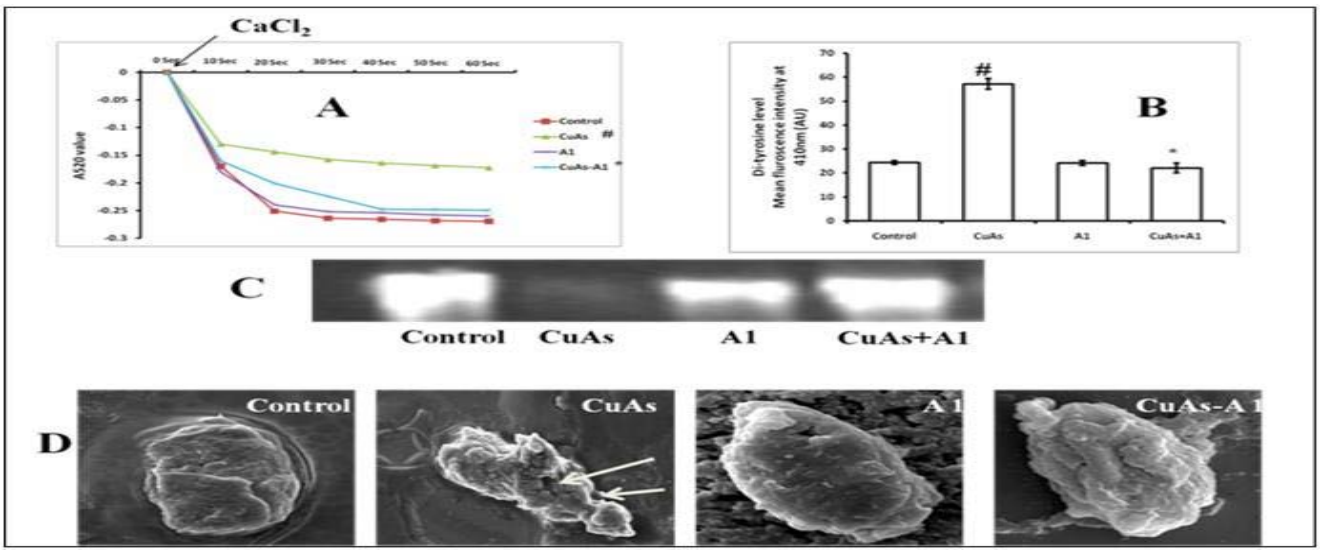

Figure 4: Protective effect of andrographolide against copper-ascorbate-induced (A) decrease in mitochondrial swelling, (B) increase in di-tyrosine level and (C) mitochondrial DNA damage. (D) Scanning electron micrograph (X6000) of mitochondrial surface. Arrow heads indicate perforated surface of mitochondria. CuAs = copper-ascorbate incubated group; A0.125-1= group incubated with andrographolide at the dose of 0.125-1mg/ml respectively (positive control); CuAs- A0.125-1= group co-incubated with copper-ascorbate and andrographolide at the dose of 0.125-1mg/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.

DISCUSSION

Oxidative stress is generally defined as an imbalance that favors the production of ROS over antioxidant defenses; however, the precise mechanisms by which ROS cause cellular injury remain elusive. Mitochondria are the main source of the superoxide radical and other reactive oxygen species that may generate from them.[40] The main mechanisms responsible for mitochondrial ROS production are the respiratory chain, in particular its complexes I and III, in the inner mitochondrial membrane, and monoamine oxidase in the outer membrane.[41] The noxious action of ROS mainly consists of the peroxidation of lipids, in particular phospholipids of biological membranes, and oxidative damage to proteins and DNA.[42]

Copper-binding proteins are involved in both transport and metabolism. In addition, a relation between copper metabolism and the intracellular availability of glutathione has been defined.[43] Moreover copper-ascorbate may induce oxidative stress by enhancing tissue LPO and by altering the antioxidant system in the organs. The copper-ascorbate induced mitochondrial damage is due to generation of oxidative stress as is evident from elevated levels of LPO and protein carbonyl content and a decreased tissue level of reduced GSH. The biological function of the Cu (I)–[GSH] complex has not been yet established, the complex is believed to play a role as Cu (I)-carrier to several copper-dependent proteins, including SOD, ceruloplasmin and metallothionein. In addition to such potential biological function, the Cu(I)–[GSH] complex has been postulated to serve as a mechanism to protect cells from undergoing the damage expected to arise from the ability of otherwise free copper ions to bind non-specifically to essential bio molecules and/or to catalyze free radical generation.[44] Oxidation of proteins can generate stable as well as reactive products that can generate additional radicals on reaction with transition metal ions. Most oxidized proteins are functionally inactive and are rapidly removed; some gradually accumulate and contribute to damage.[45]

Mn-SOD, a mitochondrial enzyme, responded to the stress with an increase in activity. The cytotoxic effect of Cu may be derived from its oxidation potential and mitochondrial Mn-SOD is more sensitive to copper than cytoplasmic Cu/Zn-SOD.[46] Cu^{2+}-ascorbate induced oxidative stress in mitochondria increases the activity of Mn-SOD. Glutathione peroxidase (GPx) is a Se dependent oxido-reductase which protects tissues from toxin induced oxidative damage.[47] A decreased activity of GPx following Cu^{2+}-ascorbate treatment of mitochondria as observed is expected to further aggravate the situation of oxidative stress. Glutathione reductase (GR) is the enzyme responsible for the reduction of oxidized glutathione (GSSG) to GSH.[48] In the present study, the activity of the glutathione dependent enzyme, GR was reduced significantly in the mitochondria with the treatment of Cu^{2+}-ascorbate. Our studies further demonstrated that following incubation of goat cardiac mitochondria with copper-ascorbate, the activity of GPx was found to be decreased. This indicates that GSH metabolizing pathway is disturbed in mitochondria. To cope with the ROS, animal and human cells express an array of antioxidant enzymes, including Mn^{2+}-dependent superoxide dismutase (Mn-SOD), glutathione peroxidase (GPx), glutathione reductase (GR). Mn-SOD converts superoxide anions to hydrogen peroxide, which is then transformed to water by GPx. However, the activities of these antioxidant enzymes and the concentrations of small-molecular-weight antioxidants in mitochondria are altered (mostly declined) during oxidative stress. Thus, there is an increase in the fraction of ROS and free radicals that may escape these cellular defense mechanisms and exert damage to cellular constituents, including DNA, RNA, lipid, and proteins bringing about organ dysfunction.

Mitochondria are the major source of ROS production in cells.[49] In our study, we found that the activities of pyruvate dehydrogenase and the Kreb's cycle enzymes like Isocitrate dehydrogenase, alpha- keto glutarate dehydrogenase and succinate dehydrogenase were decreased after incubating the mitochondria with copper-ascorbate. Succinate dehydrogenase and alpha-ketoglutarate dehydrogenase are prone to ROS attack and inactivation. They are more sensitive to H_2O_2, so the activities were inhibited by alteration of H_2O_2 generation. Andrographolide can prevent copper-ascorbate-induced oxidative damages in goat cardiac mitochondria possibly by scavenging H_2O_2, superoxide anion free radical and or hydroxyl radicals. Whether andrographolite has the capability to chelate transition metal ions remains a subject of future investigations.

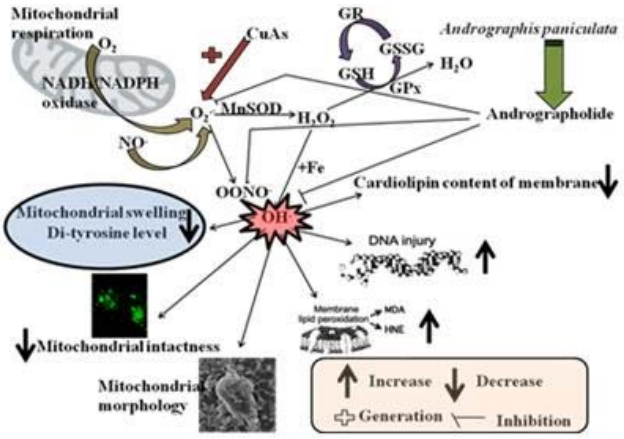

Figure 5: Schematic diagram representing the antioxidant mechanism(s) of protection of andrographolide against copper-ascorbate induced oxidative damages in cardiac mitochondria

CONCLUSION

Andrographolide 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. To the best of our knowledge and belief, this is the first report to describe an antioxidant mechanism(s) (Figure 5) of protective effect of andrographolide toward copper-ascorbate-induced mitochondrial oxidative damage. Therefore, as it is stated above andrographolide shows high antioxidant capacity mainly due to its phenolic groups and inhibits lipid peroxidation in mitochondrial *in vitro* models, the present study suggests that andrographolide may be used in preventing free radical-related diseases as a dietary natural antioxidant supplement.

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