




Research Article

Ethyl acetate extract of *Terminalia arjuna* bark shows antioxidant activity and protects against oxidative stress in rat liver mitochondria

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Abstract

The antioxidant properties of *Terminalia arjuna* (TA) bark have been identified in this present study. The antioxidant properties of ethyl acetate extract of TA bark were established by DPPH radical scavenging activity, reducing power, H₂O₂ scavenging activity, scavenging activity of hydroxyl radical, superoxide radical, and nitric oxide radical. This present study also showed the protective effects of ethyl acetate extract against copper-ascorbate-induced oxidative stress in rat liver mitochondria *in vitro*. The ethyl acetate extract of TA bark altered the content of reduced glutathione (GSH), levels of lipid-peroxidation (LPO), and protein carbonylation (PCO) ($P < 0.001$) against copper-ascorbate treated rat liver mitochondria, *in vitro*. Thus, the results indicate that the ethyl acetate extract of TA bark can be considered as a therapeutic agent in the future.

Keywords Terminalia arjuna, Ethyl acetate extract, Oxidative stress, Antioxidant, Rat liver mitochondria

Introduction

In humans, oxidative stress is responsible for various diseases such as Schizophrenia, Bipolar disorder, fragile X syndrome, cancer, Sickle Cell Disease, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure (Halliwell, 2007; Valko et al., 2007; Singh et al., 1995; Ramond et al., 2011; Boskovic et al., 2011). As mitochondria are the source of energy in cellular metabolism, the leakage of electrons from the respiratory chain is responsible for the generation of oxidative stress (Lim et al., 2010). This mitochondrial oxidative stress results in the mutation of mitochondrial DNA which is the causative factor of various enzymatic abnormalities. This situation can lead to further oxidative stress.

It has been well documented that the aqueous bark extract of *Terminalia arjuna* (TA) possesses potential antioxidant activity which can protect against oxidative stress mediated damages and thus, can enhance the cellular antioxidant defences. Both aqueous and ethanolic extract of the aqueous bark extract of TA plays a protective role against sodium-fluoride-induced hepatic and cardiac oxidative stress, DNA

damage, anticarcinogenic activity (Sinha et al., 2007; Sinha et al., 2008). The methanolic bark extract of TA protects against gastric ulcer in rats (Devi et al., 2008). The aqueous extract of TA also contains benzoic acid protects against copper-ascorbate induced oxidative stress (Dutta, 2020).

To maintain good physical health bark, fruits, leaf can be taken through consumable component such as water, milk etc. (Kiritiker and Basu, 1987). Different bioactive compounds (such as tannin, saponin, ester, sugar, steroids etc.) have been identified chemically in the various parts of the plant (Kiritiker and Basu, 1935). Earlier studies revealed the gastroprotective and anti-mutagenic effects of this plant against various diseases (Devi et al., 2007; Kaur et al., 2002). The protective mechanism(s) of the aqueous bark extract has been established in several *in vitro* systems such as RBC, liver tissue, heart mitochondria. Now a day such extracts have been used at a higher rate as an alternative and complementary medicine for pharmaceutical industrial research (Newman et al., 2007). Addition of non-polar organic solvent to aqueous bark extract causes removal of less polar organic compounds from aqueous

fraction which is retained in its upper organic fraction. Ethyl acetate is a type of organic solvent that can retain less polar organic compounds from an aqueous fraction.

But there is no such evidence of the protective effect of the ethyl acetate extract of TA bark against the oxidative stress-mediated damages in liver mitochondria and which bioactive compound of the ethyl acetate extract of TA bark is responsible for this protective effect.

Materials and Methods

Materials

Powder of bark of *Terminalia arjuna* (TA) was purchased from Herby House, Kolkata, India. Cupric chloride, ascorbic acid and all the 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.

Preparation of ethyl acetate extract of TA bark and qualitative and quantitative determination of some of the phytoconstituents

5gm of TA bark powder was dissolved in 25ml of ethyl acetate. After proper mixing, it was kept with cotton plugging overnight (approximately 16 hours). Then it was centrifuged twice at 1300g for 10 minutes. Then the supernatant was collected and lyophilized. The yield of the ethyl acetate extract of TA from 5gm of TA bark powder was 15%.

Total phenolics, flavonoids, condensed tannins, and total phytosterols of the ethyl acetate extract of TA were determined quantitatively according to the standard methods (Singleton et al., 1999; Dewanto et al., 2002; Broadhurst et al., 1978; and Ramesh et al., 1998).

Measurements of antioxidant activities of the ethyl acetate extract of TA bark in a chemically defined system

DPPH free radical scavenging activity

The DPPH free radical scavenging activity of each sample was determined according to the method described by Joyeux et al., 1995. The antiradical activity (AA) was determined using the following formula:

$$AA\% = 100 - [(Abs: sample - Abs: empty sample) \times 100] / Abs: control$$

Reducing power activity

The reducing power was determined according to the method of Oyaizu (1986). Ascorbic acid was used as standard.

H₂O₂ scavenging activity

H₂O₂ scavenging activity was assayed by studying the breakdown of H₂O₂ at 240nm.

The reaction mixture contained 50mM phosphate buffer (pH 7.4), 10,000μM H₂O₂, and different concentrations of ethyl acetate extract of TA bark in a final volume of 3ml.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by the formation of methanesulfinic acid (MSA) generated during incubation was measured by the method of Babbs and Steiner (1990).

Superoxide scavenging activity

Superoxide anion-free radical scavenging activity was studied by following the rate of epinephrine oxidation in alkaline pH at 480nm. The reaction mixture contained 50mM Tris-HCl buffer (pH 10), 0.6mM epinephrine, and different concentrations of the ethyl acetate extract of bark of TA. 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 the absence and presence of the ethyl acetate extract of bark of TA.

Nitric oxide scavenging activity

The method as described earlier (Ebrahimzadeh et al., 2008) was used to determine the antiradical activity of the ethyl acetate extract of TA bark against nitric oxide radical. A volume of 2 mL of sodium nitroprusside was prepared in 0.5 mM phosphate buffer BHT or rutin at various concentrations (0.2-1.0 mg/mL). The mixture was incubated at 25°C for 150 min. An aliquot of 0.5 mL of the solution was added to 0.5 mL of Griess reagents [1.0 mL of sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthylethylenediamine chloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm. The amount of nitric oxide radical was calculated using the equation:

$$NO \text{ radical scavenging activity} = [(Abs \text{ control} - Abs \text{ sample}) / (Abs \text{ control})] \times 100, \text{ where Abs control is the absorbance of NO radical + methanol; Abs sample is the absorbance of NO radical + sample extract or standard.}$$

Preparation of liver mitochondria

Liver mitochondria were isolated according to the procedure of Dutta and Paul (2018) with some modifications. Two grams of tissue was placed in 10 ml of sucrose buffer [0.25 (M) sucrose, 0.001 (M) EDTA, 0.05 (M) Tris-HCl (pH 7.8)] at 25°C. This mixture was homogenized for 1 min at a low speed. Then the homogenate was centrifuged at 1500 rpm for 10 min (at 4°C). The supernatant was then collected and kept

in ice. Then, the supernatant was centrifuged at 4000 rpm for 5 min (at 4°C). The supernatant, thus, obtained was further centrifuged at 14,000 rpm for 20 min at 4°C. The final pellet was collected and resuspended in sucrose buffer and was stored at -20°C for further assay.

Incubation of placental mitochondria with Cu²⁺ and ascorbic acid

Final volume (1.0 ml) of the incubation mixture contained mitochondrial protein (1.6 mg/ml), potassium phosphate buffer (50 mM, pH 7.4), and Cu²⁺ (0.2 mM) and ascorbic acid (1 mM), and the mixture was incubated at 37°C in an incubator for 1 hour. The reaction was terminated by the addition of 40µl of 35mM EDTA.

Biochemical analysis

Measurement of biomarkers of oxidative stress

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 modifications as adopted by Roy et al., (2021). The values were expressed as nmols of TBARS/mg protein.

Reduced GSH content (as acid-soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsay, 1968 with some modifications by Parvin et al., 2019. The values were expressed as nmole GSH/ mg protein.

Protein carbonyl content was estimated by DNPH assay (Levine et al., 1994) with some modifications (Dutta and Paul, 2019). The values were expressed as nmoles /mg protein.

Estimation of protein

The method of Lowry et al., (1951) was used to measure the protein content in the samples.

Statistical evaluation

Each experiment was repeated at least three times. Data are presented as means ± S.E.M. Significance of mean values of different parameters between the treatments groups was analyzed using one-way analysis of variances (ANOVA). Statistical tests were performed using Microcal Origin version 7.0 for Windows.

Results and Discussion

The imbalance between the productions of ROS and antioxidant defences is responsible for oxidative stress (Dutta and Paul, 2018). Superoxide radicals and many other radicals are being generated in mitochondria (Chance et al., 1968). Complexes I and III of the mitochondrial respiratory chain and monoamine oxidase are responsible for mitochondrial ROS production (Raha and Robinson, 2000). The noxious actions

of ROS mainly include lipid peroxidation, and oxidative damages to proteins and DNA (Wanasundara and Shahidi, 1998). The natural antioxidants containing various free radical scavengers like polyphenol, flavonoids, and phenolic compounds are present in medicinal plants and are responsible for inhibiting the deleterious consequences of oxidative stress. The present study reveals the protective effect of the ethyl acetate extract of TA bark against copper-ascorbate-induced oxidative stress on mitochondria.

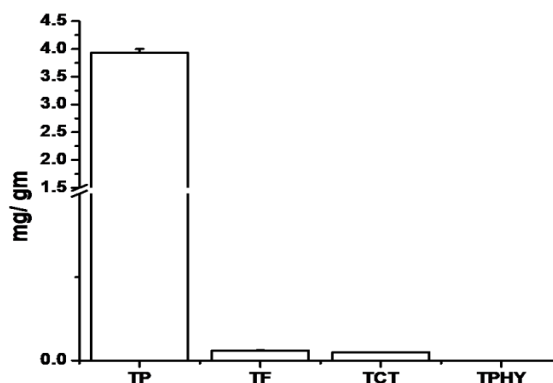


Fig. 1 Concentrations of various phyto-constituents (mg/gm) in ethyl acetate extract of bark of TA.

Fig.1 shows the concentration of total phenolics, total flavonoids, condensed tannins, and also total phytosterols in the unit of mg/gm of the ethyl acetate extract of bark of TA. The extract was found to contain a high amount of total phenolics. Earlier studies showed that the phenolics isolated from various natural sources possess strong antioxidant activities (Zahin et al., 2009). Phenolics are ubiquitously present in plants, and when plants are consumed as foods, these phytochemicals contribute to the intake of natural antioxidants in the diets of humans as well as animals. The flavonoids also have high hydroxylation, oxidation, and substitution capacity. Our quantitative assessment identified the presence of polyphenols, flavonoids, and condensed tannins in the ethyl acetate extract of TA bark. A higher amount of polyphenol was identified in the extract which may be responsible for the antioxidant activity of this extract.

DPPH free radical scavenging activity has been used as a model to identify the antioxidant abilities of various samples. This assay indicates that the ethyl acetate extract of TA bark showed dose-dependent DPPH radical scavenging activity. Fig.2A shows that ethyl acetate extract of TA bark exhibited 3.75 ± 0.1 % inhibition of DPPH activity at the dose of 25mg/ml whereas ascorbic acid (used as standard) showed this value as $5.63 \pm 0.3\%$ at the same dose. The free radical scavenging activity of the ethyl acetate extract of TA bark through DPPH assay suggests that the ethyl acetate extract of TA bark contain compounds such as polyphenolic that can donate electron/hydrogen easily thereby reducing the DPPH absorption which proves its ROS scavenging activity.

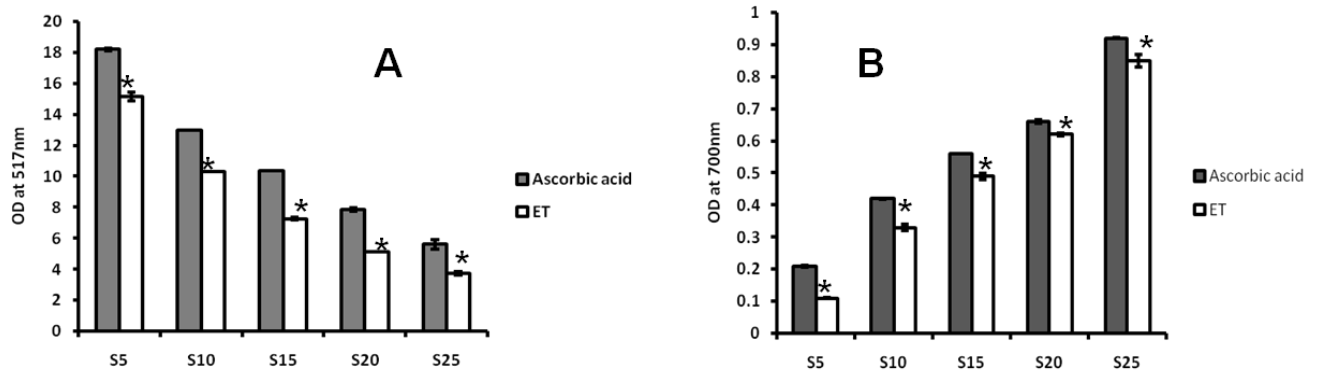


Fig. 2 (A) DPPH radical scavenging activity and (B) reducing power activity of ethyl acetate extract of bark of TA; the values are expressed as mean \pm S.E.M; *P \leq 0.001 compared to ascorbic acid (standard) values using ANOVA.

In the case of reducing power activity, the antioxidant (present in the sample) reduces Fe (III) to Fe (II). The reducing power of the ethyl acetate extract of TA bark has been summarized in Fig. 2B. From the figure, it is evident that the reducing power increased with an increase in extract concentration. The data showed that all samples increased their reducing ability in a dose-dependent manner. Antioxidant activity depends on the

metallic catalyst used to generate the reactive species (Krishnaswamy et al., 2013). The reducing compounds cause the transformation of Fe³⁺/ferricyanide complex to ferrous ion (Fe²⁺) (Sousa et al., 2008). From this experiment, it was proved that the ethyl acetate extract of TA bark exhibited increasing reducing power ability with increasing concentration.

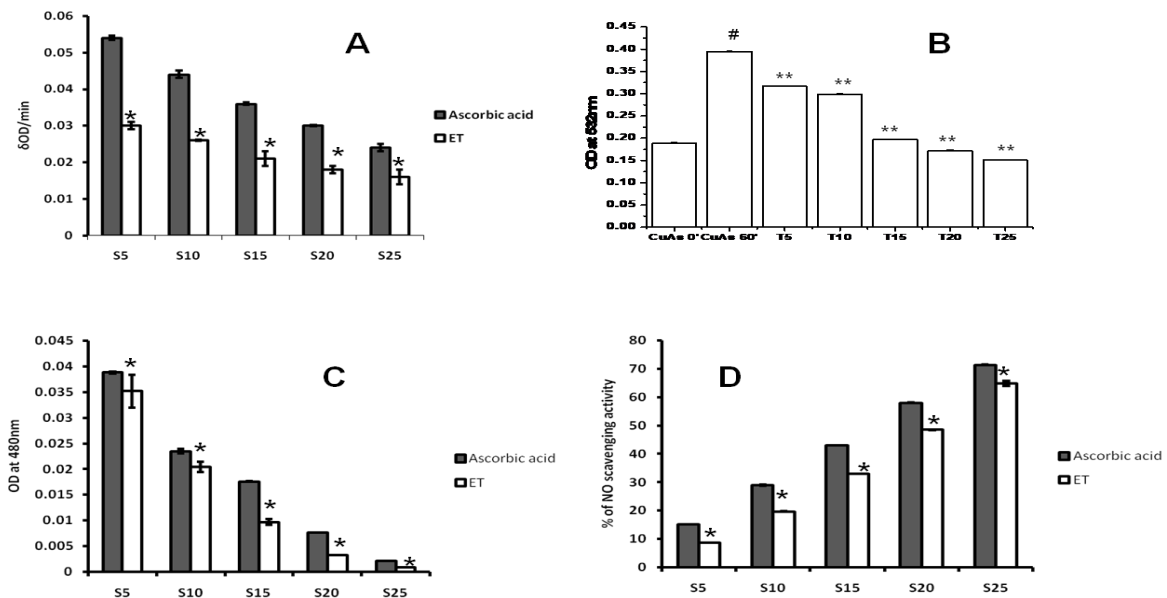


Fig. 3 (A) H₂O₂ radical scavenging activity, (B) hydroxyl radical scavenging activity, (C) superoxide radical scavenging activity and (D) nitric oxide radical scavenging activity of ethyl acetate extract of bark of TA; the values are expressed as mean \pm S.E.M; *P \leq 0.001 compared to ascorbic acid (standard) values using ANOVA; #P \leq 0.001 compared to CuAs0' values using ANOVA; **P \leq 0.001 compared to CuAs60' values using ANOVA.

The H₂O₂ scavenging activity of the ethyl acetate extract of bark of TA, if any, was also tested *in vitro*. Fig.3A indicates that the ethyl acetate extract of TA bark possesses H₂O₂ scavenging activity, significantly in comparison to the standard ascorbic acid.

Deoxyribose degradation inhibition assay established hydroxyl radical scavenging activity of the ethyl acetate extract of TA bark. In this study, 19.54%, 24.11%, 50.00%, 56.60% and 61.68% of scavenging activity were observed with 5mg, 10mg, 15mg, 20mg and 25mg/ml of the ethyl acetate extract of TA bark (Fig.3B).

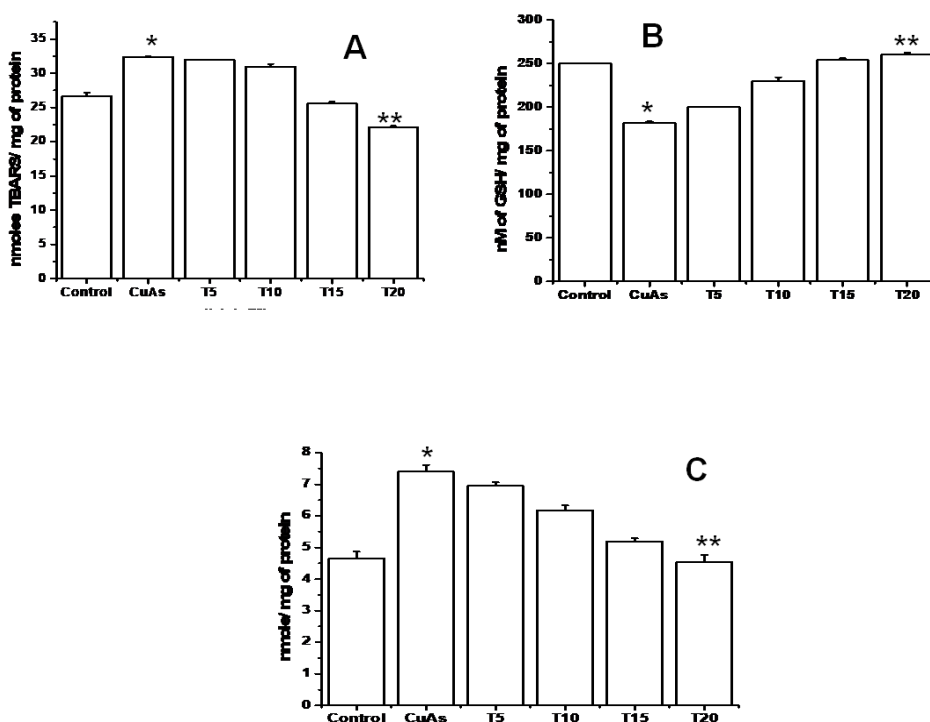


Fig. 4 Protective effect of ethyl acetate extract of TA bark against CuAs-induced (A) increased lipid peroxidation level, (B) decrease in reduced glutathione content and (C) increase in protein carbonylation of rat liver mitochondria; CuAs = copper-ascorbate treated group; T5-T20= groups co-treated with copper-ascorbate and ethyl acetate extract of TA bark at the dose of 10-20mg/ml, respectively; the values are expressed as Mean ± S.E.M; *P ≤ 0.001 compared to control values using ANOVA; **P ≤ 0.001 compared to CuAs-treated values using ANOVA.

By estimating the rate of superoxide-mediated epinephrine oxidation, the superoxide-free anion radical scavenging ability of the ethyl acetate extract of TA bark was studied (Fig.3C). The ethyl acetate extract of TA bark showed superoxide free anion radical scavenging ability in a dose-dependent manner. About 98.80% scavenging activity of superoxide anion free radical by the ethyl acetate extract of TA bark was observed at a concentration of 25mg/ml which is statistically highly significant.

Thus, these studies suggest that this ethyl acetate extract of TA bark does possess a strong antioxidant potential and it is unique in specifically scavenging OH· and superoxide anion radical. Many plants with traditional medicinal importance are known to possess antioxidant properties because of their

numerous chemical constituents. It has been found that polyphenols, flavonoids, tannins, etc. are radical scavengers and are viewed as promising therapeutic drugs for the future.

In vitro, the scavenging activity of nitric oxide radical, generated, is a measure of antioxidant activity of plant-derived bioactive compounds. The scavenging activity of nitric oxide by the ethyl acetate extract of TA bark was investigated and the result was shown in Fig.3D. The percentage of scavenging activity of nitric oxide by the ethyl acetate extract of TA bark and ascorbic acid (standard antioxidant). The percentage of scavenging activity of nitric oxide by the ethyl acetate extract of TA bark was comparable to standard antioxidant ascorbic acid. Nitric oxide plays an important role in controlling vasodilatation and blood pressure and also has a potential role

in the pathogenesis of pain, inflammation, neural signal transmission, immune response (Cross and Wilson, 2003). Inhibition of nitric oxide radical, *in vitro*, is a measure of antioxidant activity of plant-derived bioactive compounds. The antioxidant activity was further confirmed by using the hydroxyl radical scavenging assay. In this study, dose-dependent hydroxyl radical scavenging activities of the ethyl acetate extract of TA bark were observed. Superoxide anions (O_2^-) are the most common free radicals whose concentration increases under conditions of oxidative stress and are generated either by autooxidation processes or by enzymes and produce other cell damaging free radicals and oxidizing agents (Ng et al., 2000).

Fig.4A, showed a significant increase in liver mitochondrial LPO level following the treatment of mitochondria with copper-ascorbate (21.52%, *P ≤ 0.001 vs. control). This elevated level of lipid peroxidation products was found to be decreased significantly (31.60% from copper-ascorbate-treated group, **P ≤ 0.001) when the mitochondria were co-treated with copper-ascorbate and the ethyl acetate extract of TA bark (20mg/ml), indicating the ability of this the ethyl acetate extract of TA bark to protect the mitochondria against oxidative stress-induced changes due to copper-ascorbate. On the other hand, a significant decrease was observed in mitochondrial reduced GSH content following the treatment of mitochondria with copper-ascorbate (27.22%, *P ≤ 0.001 vs. control). This decreased level of reduced GSH content was found to be increased significantly (43.07% from copper-ascorbate-treated group, **P ≤ 0.001) when the mitochondria were co-treated with copper-ascorbate and the ethyl acetate

extract of TA bark (20mg/ml) (Fig.4B). The protein carbonyl assay showed a significant increase in liver mitochondrial protein carbonyl content following the treatment of mitochondria with copper-ascorbate (53.94%, *P ≤ 0.001 vs. control). This elevated level of protein carbonyl content was found to be decreased significantly (38.66% from copper-ascorbate-treated group, **P ≤ 0.001) when the mitochondria were co-treated with copper-ascorbate and the ethyl acetate extract of TA bark (20mg/ml) (Fig.4C).

Antioxidants can maintain good health and also can minimize the death risk by inhibiting the lipid peroxidation process (Niki, 2011). In addition, the earlier study revealed a relation between copper metabolism and the intracellular availability of glutathione (Luza and Speisky, 1996). The copper-ascorbate-induced mitochondrial damage is due to the generation of oxidative stress as is evident from elevated levels of LPO and protein carbonyl content and a decreased mitochondrial level of GSH. The ethyl acetate extract of TA bark was found to be effective in protecting against the elevation in lipid peroxidation level of the mitochondria isolated from rat liver. Free radicals have been postulated to play a key role in starting the chain of disease-related disorganization in target cells that contain mitochondria, using high levels of oxygen and thereby releasing large amounts of oxygen radicals exceeding the homeostatic protection of cells (Pamplona and Barja, 2007). Mitochondrial disorder is characterized by the destruction of membrane structural integrity essential to mitochondrial functions, leading to the loss of mitochondrial membrane fluidity (Chen and Yu, 1994) which is caused by oxidation of mitochondrial lipid.

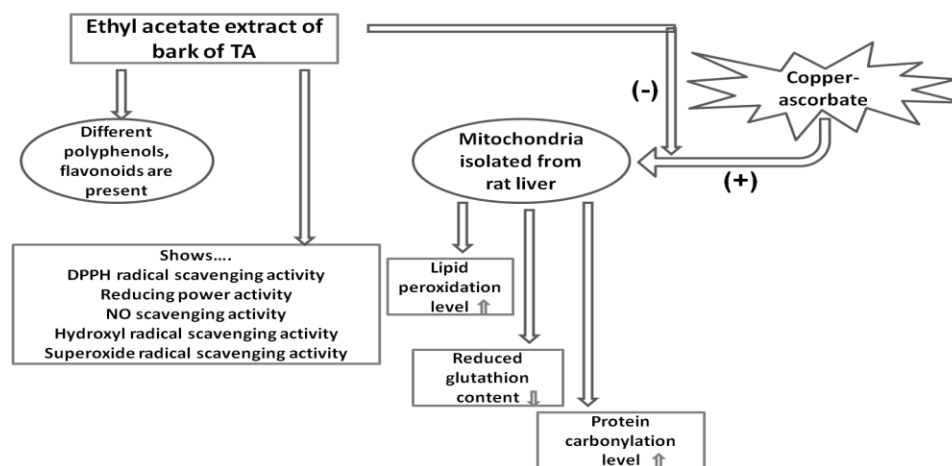


Fig. 5 Scheme showing the probable mechanism of protection offered by ethyl acetate extract of TA bark against copper-ascorbate induced oxidative stress *in vitro* in rat liver mitochondria

Conclusion

In conclusion, it can be established that the ethyl acetate extract of TA bark provides protective effects against oxidative stress, induced *in vitro*, in rat liver mitochondria as it is evident from the various oxidative stress bio-markers. Various phytochemicals (such as phenolics, flavonoids etc) are present in the ethyl acetate extract of TA bark. This extract possesses antioxidant activity. So, it can be assumed that these phytochemicals may be responsible for the protective effects of the ethyl acetate extract of TA bark against copper-ascorbate-induced oxidative stress (Fig.5).

Acknowledgment

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Conflict of Interest

The author hereby declares no conflict of interest.

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