IN VITRO ANTIOXIDANT ACTIVITY OF PROSOPIS CINERARIA LEAVES AGAINST H₂O₂ INDUCED OXIDATIVE STRESS IN GOAT LIVER SLICES

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ABSTRACT

Objective: To evaluate the antioxidant status of goat liver slices treated with standard oxidant H₂O₂ in the presence and the absence of methanolic leaf extract of Prosopis cineraria.

Methods: Precision-cut goat liver slices were challenged with oxidant H₂O₂ with or without the methanolic leaf extract of Prosopis cineraria. The activity of enzymic antioxidants and the levels of non-enzymic antioxidants were analysed in the homogenate of liver slices.

Results: The levels of antioxidants were drastically decreased in oxidant treated group compared to untreated control, whereas there was a significant increase in the antioxidants levels in the methanolic leaf extract treated group.

Conclusion: The organ slices represent the multicellular, structural and functional features of in vivo tissues and therefore represent as a good in vitro model. The present study revealed that leaf extract improved the antioxidant status of the goat liver slices and also protected against oxidative damage.

Keywords: in vitro models, Prosopis cineraria, Liver slices, Antioxidant activity, Hydrogen peroxide, Oxidative stress, Vanni, Enzymic antioxidants, Non-enzymic antioxidants.

INTRODUCTION

Free radicals are species that contains one or more unpaired electrons, which make them very unstable and, therefore, to attain stability, they extract electrons from other molecules, thereby causing damage to them [1]. During oxidative stress conditions, there is an imbalance between antioxidants and reactive oxygen species; hence, exogenous supply of antioxidants is important to prevent the body from free radical-induced injury [2]. Oxidative stress is considered as a major etiological and/or pathogenic agent of most degenerative diseases such as cancer, Alzheimer’s disease, diabetes and aging [3]. Antioxidants are substances that inhibit and scavenge radicals and thus protect humans against infectious and degenerative diseases [4]. Natural antioxidants scavenge the excessively generated free radicals in the human body and combat the devastating consequences of free radicals. Hence, they are considered to be very useful therapeutic agents in a large variety of human diseases [5]. Development of in vitro model systems is very important in order to evaluate the chemical and drug toxicity, reduce the cost of animal testing and also to reduce the sufferings of animals [6]. Among the in vitro models, precision-cut liver slices are very useful for studying drug metabolism and toxicity [7]. Medicinal plants are a rich source of bioactive phytochemicals or bionutrients. The recent trend has moved towards the use and ingestion of natural antioxidants present in different parts of plants due to their phytochemical constituents [8].

The candidate plant chosen for the present study is Prosopis cineraria, commonly called as Vanni (Tamil) and Jhand tree (Hindi). The tree belongs to Fabaceae family and is used as a remedy for cough, cold, asthma and rheumatism. The plant also possesses anti-inflammatory property. Previous study proved that the methanolic leaf extract of Prosopis cineraria is a potential free radical scavenger. Therefore, the present study focused on evaluating the antioxidant status of oxidatively stressed goat liver slices in the presence and the absence of methanolic leaf extract of Prosopis cineraria.

MATERIALS AND METHODS

Preparation of the extract

1g of fresh leaf sample and 10 ml of methanol were homogenized in a mortar and pestle. The extract was then centrifuged at 2000 rpm for 5 minutes and the methanol was dried at 60°C protected from light. The resulted residue was weighed and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/20 µl. Free radical scavenging activity was performed with different concentrations and the dose of 0.15 mg/5 µl of DMSO were optimized. Hence, the same dose was used for assessing enzymic and non-enzymic antioxidants in in vivo stimulated in vitro model.

Preparation of goat liver homogenate

The liver was purchased fresh from the local slaughter house. The tissue was quickly plunged into cold PBS and maintained at 4°C. The tissue was sliced into 1 mm thin slices by using the sterile scalpel. 250 mg of tissue was taken in 1.0 ml of cold PBS. The H₂O₂ (5 µl) and methanolic extract (5 µl) were added and incubated at 37ºC for one hour with mild shaking. After the incubation period, the tissues were homogenized in the same aliquot of the PBS using a Teflon homogenizer and centrifuged to remove the debris. The supernatant was then used for the estimation of various parameters to assess the antioxidant potential. The concentration of H₂O₂ used was 200µM. The experimental groups set up for the study were as follows.

1) Untreated (negative) control.
2) H₂O₂ treated (positive) control.
3) Methanolic extract of Prosopis cineraria leaves
4) Methanolic extract of Prosopis cineraria leaves+H₂O₂

Parameters analysed

The antioxidant effect of Prosopis cineraria leaves was assessed by analyzing activities of enzymic antioxidants and the levels of non-enzymic antioxidants.

Determination of the activity of enzymic antioxidants

i) Assay of superoxide dismutase (SOD)

Superoxide dismutase activity was assayed spectrophotometrically by the method of Misra and Fridovich (1972) [9]. The reaction
mixture contained 50 mm potassium phosphate buffer, 45μM methionine, 53μM riboflavin, 84μM NBT and 20 mm potassium cyanide and 100 μl of enzyme source at a final volume of 3 ml. The tubes were maintained at 25°C equipped with 15W fluorescent lamps in an aluminum foil-lined box. After incubating for 10 minutes, reduced NBT was measured spectrophotometrically at 600 nm. The maximum reduction was observed in the absence of the enzyme source. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT.

ii) Assay of catalase (CAT)
Catalase activity was determined by adopting the method of Luck (1974) [10]. Into quartz cuvette, 3 ml of H₂O₂ in phosphate buffer (0.067M, pH 7.0) was taken and the baseline was adjusted at 240 nm followed by 20μl of homogenate and mixed thoroughly. The time interval for decrease in absorbance by 0.05 units was recorded at 240 nm. The enzyme source and phosphate buffer without H₂O₂ served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

iii) Assay of peroxidase (POD)
The activity of peroxidase in the samples was assessed by the method of David and Richard (1983) [12]. The assay system contained 1 ml of potassium phosphate buffer (0.12M, pH7.2), 0.1 ml EDTA (15 mm), 0.1 ml sodium azide (10 mm), 0.1 ml of oxidized glutathione (6.3 mm) and 0.1 ml of liver homogenate. The final volume was made up to 2 ml using water and incubated for 3 minutes. Following incubation, 0.1 ml of 6.3 mm NADPH was added. The absorbance at 430 nm was recorded at an interval of 15 seconds for 2-3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430 nm.

iv) Assay of glutathione reductase (GR)
The activity of glutathione reductase was determined by the method of David and Richard (1983) [12]. The assay system contained 1 ml of potassium phosphate buffer (0.12M, pH7.2), 0.1 ml EDTA (15 mm), 0.1 ml sodium azide (10 mm), 0.1 ml of oxidized glutathione (6.3 mm) and 0.1 ml of liver homogenate. The final volume was made up to 2 ml using water and incubated for 3 minutes. Following incubation, 0.1 ml of 6.3 mm NADPH was added. The absorbance at 430 nm was recorded at an interval of 15 seconds for 2-3 minutes. One unit of enzyme activity was expressed as μmole of NADPH oxidized/minute/g liver tissue.

v) Assay of glutathione S-transferase (GST)
The assay of glutathione S-transferase activity was performed by the method of Habig et al. (1974) [13]. The enzyme activity was determined by the change in absorbance at 340 nm in a spectrophotometer. The reaction was started by the addition of 0.1 ml of liver homogenate to the mixture containing 0.1 ml of GSH, 0.1 ml of CDNB and phosphate buffer in a total volume of 2.9 ml and the readings were recorded for a minimum of three minutes. Distilled water served as a blank and the complete assay mixture without the enzyme served as the control to monitor non-specific binding of the substrates. One unit of GST activity is defined as the nmoles of CDNB conjugated per minute.

Determination of the levels of non-enzymic antioxidants

i) Estimation of ascorbic acid
Ascorbic acid present in the sample was estimated by the method of Roe and Keuther (1943) [14].0.2 to 1.0 ml of the working ascorbate solution and 0.1 ml of the liver homogenate were taken. The reaction mixture was made up to 2.0 ml with 4% TCA. To this, 0.5 ml DNPH reagent was added followed by two drops of 10% thiourea solution. The tubes were incubated at 37ºC for 3 hours. The osazone crystals formed were dissolved by the addition of 2.5 ml 85% H₂SO₄. DNPH reagent and thiourea were added to blank after the addition of H₂SO₄. After cooling the tubes, the absorbance was read at 450 nm. A standard graph was constructed using an electronic calculator set to the linear regression mode. The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

ii) Estimation of tocopherol
The levels of tocopherol in the samples were estimated spectrophotometrically by the method reported by Rosenberg (1992) [15]. The liver homogenate, standard and water of 1.5 ml were pipetted out into three centrifuge tubes namely test, standard and blank respectively. To all the tubes, 1.5 ml ethanol and xylene were added, stopped, mixed well and centrifuged. After centrifugation, the xylene layer was transferred into another tube, taking care not to include the ethanol or protein. To 1.0 ml of xylene layer, 1.0 ml of 2, 2'-dipiridyl reagent was added and mixed. This reaction mixture was taken in the spectrophotometric cuvettes and the extents of the test and the standard were read against the blank at 460 nm. Then, 0.33 ml of ferric chloride solution was added, mixed well and after exactly 15 minutes, the test and the standard were read against the blank at 520 nm. The levels of tocopherol were calculated using the formula

\[ \text{Tocopherols (μg)} = \frac{\text{Sample A520} - \text{A460}}{\text{Standard A520}} \times 0.29 \times 0.15 \]

iii) Estimation of vitamin A
The estimation of vitamin A in goat liver homogenate in the presence and the absence of leaf extracts were done by the method proposed by Bayfield and Cole (1980) [16]. One ml of liver homogenate was mixed with 1.0 ml of saponification mixture (2N KOH in 96% alcohol) and refluxed for 20 minutes at 60°C in the dark. All the steps subsequent to saponification were carried out in the dark. Vitamin A was extracted twice with 10 ml of petroleum ether (40-60°C). The extracts were pooled, washed thoroughly with water and the layers were separated using a separating funnel. When the petroleum ether fraction was clear, a pinch of sodium sulphate (_anhydrous) was added to remove the excess moisture. The volume of the extract was noted and the fraction (1.0 ml) was evaporated to dryness at 60°C. The residue was dissolved in 1.0 ml of chloroform. Aliquots of the standard (vitamin A palmitate) were pipetted out into a series of clean, dry test tubes in the concentration ranging from 0 to 7.5μg. The volume in all the tubes was made up to 1.0 ml with chloroform. TCA reagent (2.0 ml) was added rapidly, mixed well and the absorbance of blue colour was read immediately at 620 nm in a spectrophotometer. The vitamin A level was expressed as μg/g tissue.

iv) Estimation of reduced glutathione
The levels of reduced glutathione were estimated by the method proposed by Moron et al. (1979) [17]. To 0.5 ml of the liver homogenate 2.5 ml of 5% TCA was added. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1 ml) was used for the estimation of GSH. 0.1 ml of the sample was made up to 1 ml with 0.2M sodium phosphate buffer (pH 8.0). 2.0 ml of freshly prepared DTNB solution (0.6M in phosphate buffer) was added and the intensity of the yellow colour formed was read at 412 nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2-10 micromoles of GSH. The values are expressed as n moles of GSH/g tissue.

v) Determination of total thiols and protein thiols
The protein thiols and total thiols were estimated as per the procedure outlined by Sedlack and Lindsay (1968) [18].10g of the tissue was homogenized with 5 ml of 0.2M Tris-EDTA (pH 8.0). The assay mixture contained 0.2 ml of the homogenate, 1.5 ml of buffer (0.2M tris, pH 8.0, containing 0.2M EDTA) and 1.0 ml of DTNB. This mixture was then made upto 10.0 ml with absolute methanol. Two tubes containing a reagent blank without sample and another without DTNB were stopped and allowed to stand for 15 minutes with occasional shaking at room temperature. The tubes were then centrifuged at 3000g for 15 minutes and absorbance was measured at 420 nm in a spectrophotometer. The level of total thiols minus glutathione levels gives the amount of protein thiols in the tissue. Protein thiols are expressed as μg/g tissue.

Statistical analysis
Values were expressed as mean±SD. Statistical difference in mean was analysed using one way ANOVA and followed by least square mean deviation comparison tests (LSD), P<0.05 was considered statistically significant.
RESULTS AND DISCUSSION

Enzymic antioxidants

The results of the present study showed that the activities of enzymic antioxidants significantly decreased in the H₂O₂ exposed group when compared to the untreated control group. Treatment with the methanolic extract of *Prosopis cineraria* leaf caused a significant increase in the antioxidant status when compared to the untreated control group. In the oxidant and plant extract treated group, the antioxidant levels were higher than the oxidant alone treated group, which indicated the protective role of the leaf extract in oxidatively stressed goat liver slices. The activities of enzymic antioxidants are represented in table 1.

Table 1: It shows the effect of *Prosopis cineraria* leaf extract on enzymic antioxidant activities in goat liver slices exposed to H₂O₂ in vitro

<table>
<thead>
<tr>
<th>Enzymic Antioxidants (U/g tissue)</th>
<th>Treatment groups</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated control</td>
<td>H₂O₂ treated</td>
</tr>
<tr>
<td>SOD</td>
<td>14.48±0.44</td>
<td>11.06±0.67a</td>
</tr>
<tr>
<td>CAT</td>
<td>36.00±28.28</td>
<td>138.9±4.08a</td>
</tr>
<tr>
<td>POD</td>
<td>29.62±1.50</td>
<td>27.02±0.20a</td>
</tr>
<tr>
<td>GST</td>
<td>0.153±0.01</td>
<td>0.080±0.01a</td>
</tr>
<tr>
<td>GR</td>
<td>1.5±0.06</td>
<td>1.2±0.00a</td>
</tr>
</tbody>
</table>

Values are mean±SD of triplicates

Units

1-1 Unit is the amount of enzyme that gave 50% inhibition of the extent of NBT reduction in 1 minute
2-1 Unit is the amount of enzyme required to decrease the absorbance by 0.05 units at 240 nm
3-1 Unit is the change in absorbance at 430 nm per minute.
4-1 Unit is the milli moles of NADPH oxidized/minute
5-1 Unit is the nano moles of CDNB conjugated/minute.

Group comparison

a-statistically significant (p<0.05) compared to untreated control
b-statistically significant (p<0.05) compared to H₂O₂ control
c-statistically significant (p<0.05) compared to the respective plant control

Similar results have been reported by Nandy et al. (2012) [19] whose observations showed that the methanolic extract of *Leucas plukenetti* Smith. whole plant enhanced the CAT, SOD and GSH activities in the goat liver slices. The decreased levels of SOD, CAT, GSH and GPx in streptozotocin induced rats were significantly increased after the administration of methanolic extract of *Coleus vettiveroides* Jacob whole plant [20]. Goat liver slices treated with different extracts of *Moringa oleifera* showed a significant increase in the CAT activity on exposure to CC1₃ [21].

In another study, Sridharan et al. (2014) [22] reported that EAC inoculation in mice resulted in the marked elevation of LPO and reduction in the levels of non-enzymic antioxidants. A significant increase in the level of non-enzymic antioxidants was observed on administration of the methanolic extract of the leaves. When the tissue slices were exposed to both the oxidant and the methanolic leaf extract of *Prosopis cineraria*, there was a significant improvement in the levels of the non-enzymic antioxidants.

The results correlate with that of several studies reported. Goat liver slices treated with H₂O₂ showed depletion of vitamin C and the effect was counteracted by treatment of methanolic leaf extract of *Nyctanthes arbor-tristis* [29]. Ramesh and Satakopan (2010) [30] reported that the methanolic leaf extract of *Ocimum sanctum* increased the liver ascorbate levels in cadmium treated rats. Pradeep et al. (2010) [31] reported that the decreased level of vitamin C, vitamin E and GSH on ethanol and diethylnitrosamine exposure in liver of the albino Wistar rats, was improved on co-administration of ethanolic leaf extract of *Cassia fistula*.

Yamuna and Padma (2013) [32] reported that there was a significant increase in the levels of vitamins C, E, A, GSH and total protein thiols were presented in table 2. From table 2, it is clear that exposure to hydrogen peroxide caused a significant reduction in the levels of non-enzymic antioxidants. A significant increase in the level of non-enzymic antioxidants was observed on administration of the methanolic extract of the leaves. When the tissue slices were exposed to both the oxidant and the methanolic leaf extract of *Prosopis cineraria*, there was a significant improvement in the levels of the non-enzymic antioxidants.

The levels of non-enzymic antioxidants namely vitamins C, E, A, GSH, total and protein thiols are presented in table 2. From table 2, it is clear that exposure to hydrogen peroxide caused a significant reduction in the levels of non-enzymic antioxidants. A significant increase in the level of non-enzymic antioxidants was observed on administration of the methanolic extract of the leaves. When the tissue slices were exposed to both the oxidant and the methanolic leaf extract of *Prosopis cineraria*, there was a significant improvement in the levels of the non-enzymic antioxidants.

Non-enzymic antioxidants

Haniya and Padma (2013) [34] reported that hydrogen peroxide caused a decline in the vitamin A level in goat liver slices and the methanolic leaf extract of *Artemisia vulgaris* effectively reverted the antioxidant level. Kavitha et al. (2013) [35] reported that the chloroform extract of *Costus pictus* leaves increased the levels of vitamin C, A, E and GSH of goat liver slices exposed to hydrogen peroxide. Similarly H₂O₂ treatment showed vitamin A depletion in goat liver slices. Co-treatment of *Zea mays* leaf extracts reverted this depletion in leaf extract treated group and also in H₂O₂ treated group [36]. Padma and Kalaiyani (2013) [37] reported that the exposure of goat liver slices to H₂O₂ caused a decrease in the levels of vitamin C, E, A and GSH and co-exposure to the *Solanum nigrum* leaves increased the levels of these non-enzymic antioxidants.

Morrone et al. (2013) [38] proved that the aqueous extract of *Passiflora mancipata* leaves reverted the total protein thiols oxidation.
in hydrogen peroxide treated rat brain and rat liver slices. The decreased level of hepatic total thiols, CAT and GST in CCl₄ induced albino rats were increased on pre-treatment with the aqueous bark extract of *Terminalia paniculata* [39]. The level of total thiols was significantly decreased upon paracetamol treatment. The co-administration of methanolic extract of *Amaranthus viridis* reverted the effect [40]. Treatment with *B. monnieri* leaf extracts minimized the effects of CCl₄ in the liver and kidney of rats [41]. The co-administration with artemisinin and dihydroartemisinin caused a significant increase in the total and protein thiol levels compared to H₂O₂ treated goat liver slices [42].

The above findings strengthen our findings that the methanolic leaf extract of *P. cineraria* is capable of improving the antioxidant levels.

**CONCLUSION**

The results of the study with goat liver slices, confirm that the methanolic extract of *P. cineraria* leaves can improve the antioxidant status of the tissue by increasing the activities/levels of both enzymic and non-enzymic antioxidants.

**CONFLICT OF INTERESTS**

Declared None

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