CHAPTER 6
Chapter 6. *Achyranthes aspera* L. ameliorates aflatoxin B₁-induced peroxidative hepatic damage in rats

Although the mechanism underlying the hepatotoxicity of aflatoxins is not fully understood, several reports suggest that toxicity may ensue through the generation of intracellular reactive oxygen species (ROS) like superoxide anion, hydroxyl radical and hydrogen peroxide (H₂O₂) during the metabolic processing of AF-B₁ by cytochrome P₄₅₀ in the liver (Towner *et al.*, 2003; Sohn *et al.*, 2003). These species may attack soluble cell compounds as well as membranes, eventually leading to the impairment of cell functioning and cytolysis (Berg *et al.*, 2004). Peroxidative damages induced in the cell are encountered by elaborate defense mechanisms, including enzymatic and non-enzymatic antioxidants (Janssen *et al.*, 1993). Biological compounds with antioxidant properties contribute to the protection of cells and tissues against deleterious effects of ROS and other free radicals.

Reactive oxygen species (ROS) are short lived reactive molecules that can modify cellular components including nucleic acid, proteins and lipids (Eitenne Manneville and Hall, 2002). ROS are involved in the etiology of numerous diseases, such as atherosclerosis, cardiovascular disease, ischemic disease and aging processes (Halliwell *et al.*, 1992; Gutteridge, 1993; Halliwell and Gutteridge, 1995). ROS and lipid peroxidation has been considered to play an important role in the pathogenesis of chronic fluoride toxicity (Bhanuprakash reddy *et al.*, 2003). ROS-antioxidant imbalance leading to defective endometrium for supporting embryo. ROS - antioxidant- imbalance leading luteal regression and lack of luteal hormonal support for continuation of pregnancy (Agarwal *et al.*, 2004).
Silymarin is a purified extract of *Silybum marianum* Gaertn, composed mainly of flavonolignans like silybin, silibinin and its diastereoisomers isosilybin, silydianin and silychristin (Franschini *et al.*, 2002). Silymarin is frequently used in the treatment of liver diseases where it is capable of protecting liver cells directly by stabilizing the membrane permeability through inhibiting lipid peroxidation (LPO) and preventing liver glutathione depletion (Gazak *et al.*, 2004; Soto *et al.*, 2003; Valenzuela *et al.*, 1989). These findings prompted us to evaluate the effect of *Achyranthes aspera* supplementation in AF-B1-induced hepatotoxicity in rats and compare its antihepatotoxic efficacy with an established drug-silymarin.

6.1. Materials and Methods

6.1.1. Drugs and chemicals

AFB1, bovine serum albumin and 1, 1, 3, 3-tetraethoxypropane were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

6.1.2. Experimental design

Animals were divided into six groups of six rats each as follows. Group I served as control. Group II rats were given AF-B1 (1mg/kg) as a single oral dose on the 8th day of the experimental period. Group III animals received *Achyranthes aspera* (100mg/kg) orally for first seven days. Group IV animals received silymarin (25mg/kg body mass) orally for seven days. Group V rats received *Achyranthes aspera* (dosage and duration were as group III) followed by AF-B1 administration (1mg/kg) on day 8. Group VI rats received silymarin (dosage and duration as group IV) followed by AF-B1 administration (1mg/kg body mass) on day 8. 1g *Achyranthes aspera* and 1g silymarin were dissolved in 5ml of olive oil. Five mg AF-B1 was
dissolved in 1 ml dimethyl sulfoxide and further diluted with distilled water to the required concentration.

The final gavage solution of AF-B1 contained 1% dimethyl sulfoxide. At the end of the 10-day experimental period (72h after AF-B1 administration), the animals were killed by decapitation. Blood samples were collected and the serum was separated for enzyme assays. The liver was excised immediately, rinsed in ice-cold physiological saline and homogenized in Tris-HCl buffer (0.1M, pH 7.4) to give a 10% homogenate aliquots of the tissue homogenate were suitably processed for the assessment of following biochemical parameters.

6.1.3. Biochemical parameters

The activities of Lactate dehydrogenase (LDH), alkaline phosphatase (ALKP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in liver and serum were estimated by the method of King (1965a, b, c). Estimation of alkaline phophatase was done by the procedure explained in chapter 4 (4.2.4.3). Lipid peroxidise (LPO) was determined by the procedure of Hogberg et al. (1974), malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of oxidative stress. Superoxide dismutase (SOD) was assayed according to the method of Marklund and Marklund (1974).

The unit of enzyme activity is defined as the enzyme required giving 50% inhibition of pyrogallol autooxidation. Catalase (CAT) was assayed by the method of Maehly and Chance, 1954. In this method, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of H2O2, with the formation of perchloric acid as an unstable intermediate. Chromic acetate thus produced was measured colorimetrically at 610nm.
Glutathione peroxidase (GPx) was assayed by the method of Beutler and Kelly (1963). Glutathione reductase (GR) that utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form was assayed by the method of Staal et al., (1969). Glucose-6-phosphate dehydrogenase (G6PD) was assayed by the method of Beutler (1983) wherein the increase in absorbance was measured when the reaction was started by the addition of glucose-6-phosphate. Glutathione-S-transferase (GST) was assayed by the method of Habig et al., (1974). Total reduced glutathione (GSH) was determined by the method of Beutler and Kelly (1963). Vitamin C was estimated by the method of Rao and Kuether (1943). Vitamin E was estimated according to the procedure of Baker et al., 1980.

6.1.3.1. Determination of lactate dehydrogenase activity (LDH). King, 1965 a, b, c.

Reagents

1. 0.1M glycine buffer: 7.505g of glycine and 5.85g of sodium chloride was dissolved in one litre of distilled water.

2. Buffered substrate: In 125ml of glycine buffer and 75ml of 0.1N sodium hydroxide are added to 4g of lithium lactate and mixed well. The prepared substrate was stored in cold room.

3. Nicotinamide adenine dinucleotide (NAD+): 10mg of NAD+ was dissolved in 2ml of water

4. 2, 4-Di nitrophenylhydrazine: 20mg of DNPH was dissolved in 100ml of 1N hydrochloric acid.

5. 0.4N sodium hydroxide: 1.6g of sodium hydroxide was dissolved in 100ml of distilled water.
6. Standard pyruvate solution: 11mg of sodium pyruvate was dissolved in 100ml of distilled water. 1ml of this solution contained 1ug of pyruvate.

**Procedure**

Buffered substrate of about 1ml and 0.2ml of serum and liver homogenate were pipetted out into separate test tubes and placed in a water bath at 37°C. Blank and control were set up. 0.2ml of NAD+ was added and shaken well. Tubes were incubated at 37°C for 15 minutes exactly. After the incubation period the reaction was stopped by adding 1ml of DNPH reagent. To the control 0.2ml of NAD+ was added after arresting the reaction. It was kept at 37°C for another 15 minutes. 5ml of 0.4N sodium hydroxide was added and colour developed was read at 420nm within 5 minutes. A set of standard pyruvate solution 0.2, 0.4, 0.6, 0.8, 1.0ml was also treated in a similar manner. The colour developed was read at 420nm in a spectrophotometer. The activity of LDH was measured as μ moles x 10^-1 of pyruvate liberated / minute.

6.1.3.2. Estimation of alanine transferase activity (ALT) .King. 1965 a, b, c.

**Reagent**

1. Serum glutamate oxaloacetate transferase (SGOT)buffered Substrate : 13.3g of aspartic acid was dissolved in 90ml of 1 N NaOH and 0.146g of α-ketoglutaric acid was added and dissolved by adding few drops of 1N NaOH. The pH was adjusted to 7.4 and the final volume of 500ml was obtained with phosphate buffer.

2. Dinitro phenyl hydrazine (DNPH) Reagent: 99g of DNPH was dissolved in 50ml conc. HCl and made up to 500ml with water.

3. 0.4 N NaOH
4. Stock pyruvate standard: 220mg of sodium pyruvate was dissolved in 100ml of phosphate buffer.

5. Working standard: 1ml of Stock solution was diluted to 100ml with phosphate buffer

Procedure

0.5ml of SGOT buffered substrate was taken in two test tubes and incubated at room temperature for few minutes. To the test tubes, 0.1ml of serum and liver homogenate were added and incubated at 37°C. Blank and control were set up. 0.5ml of DNPH was added to the above mixture, mixed well and incubated at room temperature for 20 minutes. Finally 5ml of 0.4N NaOH was added left for 10 minutes and the OD was read using 520nm in a spectrophotometer. The activity of ALT was measured by $\mu$ moles x $10^{-2}$ pyruvate liberated / minute.

6.1.3.3. Estimation of aspartate aminotransferase activity (AST). King., 1965 a, b, c.

Reagents

1. Phosphate buffer (pH 7.4) : 11.3 g of dry anhydrous Na$_2$HPO$_4$ and 2.7g of dry anhydrous potassium dihydrogen phosphate were dissolved in one litre of distilled water.

2. SGPT buffered substrate: 9g of alanine was dissolved in 90ml of water and added about 2.5ml of IN NaOH to adjust the pH 7.4. 0.146g of $\alpha$ -keto glutaric acid was dissolved by adding a few drops of NaOH and adjusted to pH 7.4. The final volume was made up to 500ml with phosphate buffer.

3. DNPH reagent: 99mg of DNPH was dissolved in 50ml of conc. HCl and made up to 500ml with water.

4. 0.4N NaOH
5. Stock pyruvic acid standard: 200mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.

6. Working standard: 1ml of the stock solution was diluted to 10ml with phosphate buffer.

**Procedure**

0.5ml of SGPT substrate was taken in test tube and incubated at room temperature for a few minutes. 0.5ml of serum was added, incubated for 30 minutes at 37°C. Blank and controls were prepared. To all tubes 0.5ml of DNPH reagent was added, mixed well and incubated at room temp for 20 minutes. Then 5ml of 0.4N NaOH was added, waited for 10 minutes. The OD was read using a green filter (540 nm) in a spectrophotometer. The activity of AST was measured by μ moles x 10^-2 pyruvate liberated / minute.

6.1.3.4. Estimation of Lipid peroxidase (LPO). Hogberg *et al.*, 1974

**Reagents**

1. 8.1% Sodium dodecyl sulphate; 2. 20% Glacial acetic acid ;3. 0.8% Thiobarbituric acid (TBA); 4. n-Butanol and pyridine (5:1 v/v); 5. Standard solution: 1, 1, 3, 3'- tetra methoxy propane- 0.5 nmol/ml.

**Procedure**

To 0.2ml of tissue homogenate, 0.2ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid were added. The pH was adjusted to 3.5 with sodium hydroxide, then 1.5ml of 0.8% aqueous solution of thiobar butyric acid was added to the mixture and the volume was made up to 4ml with distilled water. The reaction mixture was heated in an oil bath at 95° C for 1h. After cooling by tap water, 1ml of distilled water and 5ml of n-butanol pyridine mixture were added and shaken
vigorously. Centrifuged at 4000 rpm for 10 minutes, the organic layer was removed and absorbance was read at 535 nm. The level of TBARS in tissue was expressed as n mol/100 mg protein.


Reagents
1. 1.50 mM phosphate buffer pH 7.4; 2. 20 mM L-Methionine; 3. 1% (v/v) Triton X100; 4. 10 mM hydroxylamine hydrochloride; 5. 50 μM EDTA; 6. 50 μM riboflavin; 7. Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine hydrochloride.

Procedure
Pipetted out 1.4 ml of aliquot of the reaction mixture in a test tube. 100 μl of the liver homogenate was added followed by preincubation at 37°C for 5 minutes. 80 μl of riboflavin was added and the tubes were exposed for 10 minutes to 200 w Philips fluorescent lambs. The control tube contain equal amount of buffer instead of sample. The sample and its respective control were run together. The absorbance of the colour formed was measured at 543 nm in a spectrophotometer. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition (U/ mg protein).


Reagents
1. Phosphate buffer, 0.01 M, pH 7.0
2. Hydrogen peroxide, 0.2 M
3. Potassium dichromate, 5 %
4. Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this 1ml was diluted again with 4ml of acetic acid.

5. Standard hydrogen peroxide, 0.2mM

Procedure
To 0.1ml tissue homogenate, 0.9ml phosphate buffer and 0.4ml H₂O₂ were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0ml of dichromate-acetic acid mixture. The tubes were kept in a boiling water bath for 10 minutes, cooled and the color developed was read at 530nm. Standard H₂O₂ in a concentration range of 20-100 μmoles were processed as for the test. The activity of catalase was expressed as U/mg protein for tissues (U-μ moles of H₂O₂ utilized/sec.).


Reagents
1. Phosphate buffer, 0.4 M, pH 7.0; 2. Sodium azide solution, 10 mM; 3. 10 % Trichloroacetic acid (TCA); 4. 0.4mM Ethylene diamine tetra acetic acid (EDTA); 5. 0.2 mM H₂O₂ ; 6. 2 mM Glutathione (GSH).

Procedure
The reaction mixture in a total volume of 1ml contained 0.2ml of phosphate buffer, 0.2ml EDTA, 0.1ml of sodium azide and 0.5ml of the tissue homogenate. 0.2ml of glutathione and 0.1ml of H₂O₂ were added to this mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10 % TCA. The tubes were centrifuged and the supernatant was used for taking OD at 412 nm in a spectrophotometer. A blank was treated similarly to which 0.2ml of the enzyme
was added after the incubation. The activity of glutathione peroxidase was expressed as U/mg protein for tissues (U-μ moles of GSH utilized/min.).


Reagent
1. 0.12M phosphate buffer, pH 7.2; 2. 15mM EDTA; 3. 10mM sodium azide; 4. 6.3mM oxidized glutathione; 5. 9.6mM NADPH

Procedure

Enzyme extraction

1g of sample was homogenized with 0.12M phosphate buffer. Centrifuged at 5000rpm for 10 minutes supernatant was used as the source of the enzyme.

Assay

The assay system contained 1ml of 0.12 M potassium phosphate buffer, 0.1ml of 15mM EDTA, 0.1ml of 10mM sodium azide; 0.1ml of 6.3mM oxidized glutathione and 0.1ml of the tissue homogenate and made up to 2.0ml with water, kept for 3 min at room temperature. Then 0.1ml of NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2-3 minutes. Control was prepared using water instead of oxidized glutathione. The enzyme activity was expressed as μmoles of NADPH oxidized/minutes/g/sample.


Reagents
1. 0.1M Tris HCl buffer, ph 8.2

A: 0.1M solution of Tris (12.1g/l water)
B: 0.1M HCl: 50ml of solution A and 21.9ml of B were diluted to a total of 200ml.

2. 0.2mM NADP solution.

3. 0.6mM Glucose-6-phosphate solution.

4. 0.1M magnesium chloride solution.

Procedure

Tris HCl buffer about 0.4ml, 0.2ml of NADP, 0.2ml of magnesium chloride, 1ml water and 0.2ml enzyme were taken in a cuvette. The reaction was started by the addition of 0.2ml of Glucose-6-phosphate and the increase in OD was measured at 340nm. The activity was expressed in terms of units/mg protein, in which one unit is equal to the amount of enzyme that brought about a change in OD of 0.01/min.


Reagents

1. Phosphate buffer 0.3M, pH 6.5; 2. GSH 30mM; 3. 30mM 1-chloro 2, 4 dinitrobenzene (CDNB) in 95% ethanol.

Procedure

1.0ml of phosphate buffer, 0.1ml of 1-chloro 2, 4 dinitrobenzene (CDNB) and 0.1ml of tissue homogenate were taken in a test tube. The volume was adjusted to 2.9ml with water. The reaction mixture was preincubated at 37°C for 5 minutes and the reaction started by the addition of 0.1ml of 30mM glutathione. The absorbance was taken after 5 minutes at 540nm. A system devoid of enzyme served as the blank. The specific activity of GST is expressed as μmoles of CDNB- GSH conjugates formed/min/mg protein.

Reagents

1. 10% TCA; 2. 1% Sodium citrate; 3. Ellman's reagent: 34mg of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) in 10ml of 1% sodium citrate; 4. 0.3M Disodium hydrogen phosphate (pH 8.0); 5. Standard solution: 10mg of reduced glutathione in 100ml distilled water.

Procedure

To 0.5ml of tissue homogenate, 1.5ml of TCA and 1.0ml of distilled water were added and centrifuged. 2.0ml of supernatant along with 2.0ml of blank containing distilled water also taken. A set of standards (20-100µg) were taken and made upto 2.0ml with water. To all the tubes 4.0ml of 0.3M disodium hydrogen phosphate and 1.0ml of DTNB reagent were added and colour developed was read at 412nm in a spectrophotometer. The values were expressed as mg/100mg tissues for liver.


Reagents

1. 4% trichloracetic acid; 2. 9N sulphuric acid; 3. 2% 2, 4-dinitrophenyl hydrazine reagent (DNPH); 4. 10% thiourea solution; 5. 85% sulphuric acid; 5. Stock standard solution: 100mg ascorbic acid was dissolved in 100ml of 4% TCA; 6. Working standard: 10ml of the stock solution was diluted to 100ml with 4% TCA.

Procedure

Sample of about 1g was ground and homogenized in 4% TCA upto 10ml, centrifuged at 2000 rpm for 10 minutes. The supernatants obtained were treated with a
pinch of activated charcoal, shaken well and kept for 10 minutes. The mixture was centrifuged again to remove the charcoal residue. The volume of the clear supernatants obtained was noted.

Aliquots of 0.5 & 1ml of these supernatants were taken for the assay. The assay volumes were made up to 2.0ml with 4% TCA. 0.2ml to 1ml of the working standard solution containing 20-100µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4% TCA. 0.5ml of DNPH reagent was added to all the tubes, followed by 2 drops of 10% thiourea solution, incubated at 37°C for 3h. The osazones formed were dissolved in 2.5ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of sulphuric acid. After incubation for 30 minutes at room temperature, the absorbance was read spectrophotometrically at 540nm. The amount of vitamin C was measured as U/ mg protein.

6.1.3.13. Estimation of α-tocopherol (Vitamin E). (Baker et al., 1980)

Reagents

1. Absolute alcohol; 2. Xylene; 3. 2, 2' dipyridyl reagent− 1.2g in 1 litre of n-propanol;
4. Ferric chloride solution – 1.2g of FeCl3.6H2O was dissolved in one litre of ethanol. The solution was stored in a brown bottle; 5. Standard solution of D, L- α- tocopherol acetate.

Enzyme extraction

The sample was homogenized in a blender. Accurately 2.5g of the homogenized tissue was weighed into a conical flask. 50ml of 0.1N sulphuric acid
was added slowly without shaking. It was stoppered and allowed to stand overnight. The next day, contents of the flask were shaken vigorously and filtered through whatman No.1 filter paper, discarding the initial 10-15ml of the filtrate. Aliquots of the filtrate were used for the estimation.

**Procedure**

Into a 3 stopper centrifuge tube (test, standard and blank) 1.5ml of sample, 1.5ml of the standard and 1.5ml of water were pipetted. To the test and blank 1.5ml of ethanol was added and to the standard 1.5ml of water was added. 1.5ml of xylene was added to the above tubes, stoppered, mixed well and centrifuged.

Transferred 1.0ml of xylene layer into another stopper tube, taking care to avoid the mixing of ethanol or protein. 1 ml of 2, 2’-dipyridyl reagent was added to each tube, stoppered and mixed. 1.5ml of the mixtures was pipetted into cuvettes and the extinction of test and standard was read against the blank at 460nm in a spectrophotometer. Then in turn, beginning with the blank, 0.33ml of ferric chloride solution was added. Mixed well and after exactly 15 minutes test and standard was read against the blank at 520nm. The amount of vitamin E was measured as U/mg protein.

6.1.4. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were assessed by one-way analysis of variance using the SPSS software package for Windows. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; significance at P-values <0.001, <0.01, <0.05 have been given respective symbols in the tables.
6.2. Results

Abnormal activities of serum and liver enzymes in rats were observed due to the cellular damage caused by AF-B₁ treatment (Table 6.1). The activities of serum LDH, AST, ALT and ALP were increased by 2.98-, 2.33-, 2.01- and 2.78-fold, respectively, in group II animals when compared with control. Activities of these marker enzymes were significantly (P<0.001) decreased in the liver of AFB₁ administered animals. The *Achyranthes aspera* (group V) and silymarin (group VI) pre-treated animals restored these enzyme levels to nearly that of control values (P<0.001) indicating the hepatoprotective role of *Achyranthes aspera* L.
Table 6.1. Alterations in serum and tissue enzyme activities in AFB1-induced animals and the effect of *A. aspera* and silymarin pre-treatment (values are expressed as mean ± SD for six animals in each group)

<table>
<thead>
<tr>
<th>Experimental group of rats</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum enzyme assays (U/mg protein)</td>
<td>12.29±1.09****</td>
<td>4.09±0.42</td>
<td>4.21±0.34</td>
<td>4.52±0.49***</td>
<td>4.57±0.41***</td>
<td>4.13±0.36</td>
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<tr>
<td>LDH</td>
<td>(297.5)</td>
<td>(232.6)</td>
<td>(96.15)</td>
<td>(94.23)</td>
<td>(109.52)</td>
<td>(107.9)</td>
</tr>
<tr>
<td>AST</td>
<td>0.52±0.03</td>
<td>0.50±0.06</td>
<td>0.49±0.05</td>
<td>0.57±0.05***</td>
<td>0.65±0.07</td>
<td>0.63±0.06</td>
</tr>
<tr>
<td>ALT</td>
<td>1.39±0.14****</td>
<td>0.65±0.07</td>
<td>0.62±0.04</td>
<td>0.69±0.05***</td>
<td>0.71±0.08***</td>
<td>0.69±0.12</td>
</tr>
<tr>
<td>ALP</td>
<td>4.51±0.35****</td>
<td>1.92±0.16</td>
<td>1.93±0.18</td>
<td>2.18±0.19***</td>
<td>2.16±0.23***</td>
<td>1.98±0.20</td>
</tr>
<tr>
<td>Liver enzyme assays (U/mg protein)</td>
<td>6.74±0.49****</td>
<td>10.08±0.87</td>
<td>10.17±0.98</td>
<td>9.10±0.71***</td>
<td>9.30±0.82***</td>
<td>10.15±1.12</td>
</tr>
<tr>
<td>LDH</td>
<td>(66.4)</td>
<td>(99.31)</td>
<td>(99.31)</td>
<td>(90.11)</td>
<td>(91.62)</td>
<td>(90.75)</td>
</tr>
<tr>
<td>AST</td>
<td>0.16±0.02</td>
<td>0.09±0.01****</td>
<td>0.15±0.02</td>
<td>0.17±0.02</td>
<td>0.14±0.01***</td>
<td>0.15±0.01***</td>
</tr>
<tr>
<td>ALT</td>
<td>0.12±0.01</td>
<td>0.06±0.01****</td>
<td>0.13±0.02</td>
<td>0.12±0.01</td>
<td>0.11±0.02***</td>
<td>0.11±0.01***</td>
</tr>
<tr>
<td>ALP</td>
<td>1.75±0.19</td>
<td>0.68±0.06****</td>
<td>1.71±0.14</td>
<td>1.77±0.16</td>
<td>1.61±0.14***</td>
<td>1.63±0.13***</td>
</tr>
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</table>

- Enzyme units: LDH: μmol×10−1 of pyruvate liberated/min; AST, ALT: μmol×10−2 of pyruvate liberated/min; ALP: μmol×10−2 of phenol liberated/min. Comparisons are made between:
  a-group I and group II, III, IV, V, VI;
  b-group II and group V, VI;
  c-group V and group VI.

The symbols represent statistical significance: *P<0.05, **P<0.01, ***P<0.001.

- 1 – Control
- II – Aflatoxin (1mg/kg) treated
- III – *Achyranthes aspera* (100mg/kg) treated
- IV – Silymarin (25mg/kg) treated
- V – *Achyranthes aspera* (100mg/kg) + Aflatoxin (1mg/kg) treated
- VI – Silymarin (25mg/kg) + Aflatoxin (1mg/kg) treated
Table 6.2. Alterations in lipid peroxidation and antioxidant enzymes in the liver of AF-B1-induced animals and the effect of *A. aspera* and silymarin pretreatment (values are expressed as mean ± SD for six animals in each group)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO</td>
<td>1.08±0.13</td>
<td>3.74±0.41***</td>
<td>1.12±0.11</td>
<td>1.05±0.12</td>
<td>1.21±0.17***</td>
<td>1.19±0.20***</td>
</tr>
<tr>
<td></td>
<td>(346.29)</td>
<td>(346.29)</td>
<td>(103.70)</td>
<td>(97.22)</td>
<td>(112.03)</td>
<td>(110.18)</td>
</tr>
<tr>
<td>CAT</td>
<td>340.82±31.71</td>
<td>210.11±17.25***</td>
<td>349.36±29.42</td>
<td>338.54±25.62</td>
<td>324.73±26.32***</td>
<td>317.76±33.56***</td>
</tr>
<tr>
<td></td>
<td>(61.64)</td>
<td>(61.64)</td>
<td>(102.50)</td>
<td>(99.33)</td>
<td>(95.27)</td>
<td>(93.23)</td>
</tr>
<tr>
<td>SOD</td>
<td>7.55±0.87</td>
<td>4.21±0.45***</td>
<td>7.62±0.81</td>
<td>7.46±0.75</td>
<td>7.15±0.51***</td>
<td>7.09±0.57***</td>
</tr>
<tr>
<td></td>
<td>(55.76)</td>
<td>(55.76)</td>
<td>(100.92)</td>
<td>(98.80)</td>
<td>(94.70)</td>
<td>(93.90)</td>
</tr>
<tr>
<td>GPx</td>
<td>5.49±0.61</td>
<td>3.09±0.28***</td>
<td>5.36±0.56</td>
<td>5.52±0.58</td>
<td>5.27±0.32***</td>
<td>5.19±0.45***</td>
</tr>
<tr>
<td></td>
<td>(56.28)</td>
<td>(56.28)</td>
<td>(97.63)</td>
<td>(100.54)</td>
<td>(96)</td>
<td>(94.53)</td>
</tr>
<tr>
<td>GR</td>
<td>0.28±0.03</td>
<td>0.16±0.02***</td>
<td>0.27±0.03</td>
<td>0.28±0.04</td>
<td>0.30±0.03***</td>
<td>0.27±0.02***</td>
</tr>
<tr>
<td></td>
<td>(57.14)</td>
<td>(57.14)</td>
<td>(96.42)</td>
<td>(99.86)</td>
<td>(107.14)</td>
<td>(96.42)</td>
</tr>
<tr>
<td>G6PD</td>
<td>2.12±0.20</td>
<td>1.21±0.13***</td>
<td>2.05±0.19</td>
<td>2.10±0.18</td>
<td>1.94±0.21***</td>
<td>1.85±0.19***</td>
</tr>
<tr>
<td></td>
<td>(57.07)</td>
<td>(57.07)</td>
<td>(96.69)</td>
<td>(99.05)</td>
<td>(91.50)</td>
<td>(87.26)</td>
</tr>
<tr>
<td>GST</td>
<td>1.11±0.14</td>
<td>0.61±0.07***</td>
<td>1.21±0.13</td>
<td>1.17±0.12</td>
<td>1.00±0.12***</td>
<td>1.02±0.06***</td>
</tr>
<tr>
<td></td>
<td>(54.95)</td>
<td>(54.95)</td>
<td>(109)</td>
<td>(105.40)</td>
<td>(90.09)</td>
<td>(91.89)</td>
</tr>
</tbody>
</table>

- **Units**: LPO: nmol of MDA formed/mg protein; CAT: μmol of H₂O₂ consumed/min/mg protein; SOD: units/mg protein; GPx: μg of GSH utilized/min/mg protein; GR: nmol of NADPH oxidized/min/mg protein; G6PD: nmol of NADPH formed/min/mg protein; GST: nmol of 1-chloro-2, 4-dinitrobenzene–GSH conjugate formed/min/mg protein. Comparisons are made between: a-group I and group II, III, IV, V, VI; b-group II and group V, VI; c-group V and group VI.

The symbols represent statistical significance: *P<0.05, **P<0.01, ***P<0.001.

- 1 – Control
- II – Aflatoxin (1mg/kg) treated
- III – *Achyranthes aspera* (100mg/kg) treated
- IV – Silymarin (25mg/kg) treated
- V – *Achyranthes aspera* (100mg/kg) + Aflatoxin (1mg/kg) treated
- VI - Silymarin(25mg/kg) + Aflatoxin (1mg/kg) treated
Table 6.3. Alterations in non-enzymatic antioxidant status in the liver of AF-B<sub>1</sub>-induced animals and the effect of A. aspera and silymarin pre-treatment (values are expressed as mean ± SD for six animals in each group)

<table>
<thead>
<tr>
<th>Tissue antioxidants •</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>5.17±0.57</td>
<td>3.34±0.31***</td>
<td>5.07±0.51</td>
<td>5.18±0.52</td>
<td>4.94±0.39b***</td>
<td>4.89±0.52***</td>
</tr>
<tr>
<td>(6.57)</td>
<td>(98.06)</td>
<td>(100.19)</td>
<td>(95.55)</td>
<td>(94.58)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>3.12±0.38</td>
<td>1.78±0.13***</td>
<td>3.16±0.31</td>
<td>3.19±0.33</td>
<td>3.08±0.25b***</td>
<td>3.11±0.46***</td>
</tr>
<tr>
<td>(57.05)</td>
<td>(101.28)</td>
<td>(102.24)</td>
<td>(98.71)</td>
<td>(99.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td>1.44±0.09</td>
<td>1.07±0.09**</td>
<td>1.41±0.14</td>
<td>1.48±0.14</td>
<td>1.43±0.12b***</td>
<td>1.40±0.17***</td>
</tr>
<tr>
<td>(74.30)</td>
<td>(97.91)</td>
<td>(102.77)</td>
<td>(99.30)</td>
<td>(97.22)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

• Units: GSH, Vitamin C and Vitamin E—μg/mg protein.

Comparisons are made between: a-group I and group II, III, IV, V, VI; b-group II and group V, VI; c-group V and group VI.

The symbols represent statistical significance: *P<0.05, **P<0.01, ***P<0.001.

Values in parenthesis(%) indicates comparison beween test and aflatoxin groups and aflatoxin group with control group.

• 1 – Control

II – Aflatoxin (1mg/kg) treated

III – Achyranthes aspera (100mg/kg) treated

IV – Silymarin (25mg/kg) treated

V – Achyranthes aspera (100mg/kg) + Aflatoxin (1mg/kg) treated

VI - Silymarin(25mg/kg) + Aflatoxin (1mg/kg) treated
Table 6.2 shows the effects of *Achyranthes aspera* and silymarin on AFB$_1$-induced LPO and antioxidant status. The 3.46-fold rise in LPO seen in the AFB$_1$ group was maintained at near normal levels by *Achyranthes aspera* and silymarin pre-treatment. A significant decrease (P<0.001) in the activities of enzymatic antioxidants (SOD, CAT, GPx, GR, G6PD and GST) was seen in the AF-B$_1$ treated animals (group II). *Achyranthes aspera* and silymarin pretreated rats did not show any decrease in the activities of antioxidants. GSH, vitamin C and vitamin E levels were decreased by 57.74%, 42.95% and 25.69% respectively in the AF-B$_1$ administered rats (Table 6.3). *Achyranthes aspera* and silymarin pre-treatment restored the levels of these non-enzymatic antioxidants towards the control level (P<0.001, P<0.01; as against group II), thereby indicating that *Achyranthes aspera* and silymarin pre-treatment protects the liver against oxidative stress-induced depletion of antioxidants.

6.3. Discussion

The hepatotoxic effect of AF-B$_1$ has been well documented in a variety of animal species (Mishra and Das, 2003; Cheng *et al.*, 2001; Wogan, 1992). Increased activities of serum ALT, AST, LDH and ALP are well known diagnostic indicators of hepatic injury. In cases such as liver damage with hepatocellular lesions, these enzymes are released from the liver into the blood stream (Plaa and Hewitt, 1986). The results obtained by us indicate a significant increase in the activities of these marker enzymes in serum, which is in accordance with the previous reports (Wang *et al.*, 1991; Liu *et al.*, 2001). Pre-treatment with *Achyranthes aspera* significantly lowered the levels of these enzymes, and the values were comparable with that of the control animals and silymarin pre-treated group. This suggests the hepatoprotective role of *A. aspera*. 
In the present study, a decrease in the activities of amino transferases, LDH and ALP was observed in the liver following AFB$_1$ administration. Amino transferases (ALT and AST) being an important class of enzymes linking carbohydrate and amino acid metabolism, have established a relationship between the intermediates of citric acid cycle. These enzymes are markers of liver injury since liver is the major site of metabolism. The marked decrease in the activity of hepatic LDH with AFB$_1$ treatment indicates impaired liver function (Fig.6.1,2). ALP is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites (Plaa and Hewitt, 1986).

![Fig6.1. Alterations in serum enzyme (U/mg protein) activities in AFB1-induced animals and the effect of A. aspera, silymarin pre-treatment. 1: AF Control, 2: A. aspera (100mg), 3: silymarin (25mg), 4: A. aspera (100mg)+AF, 5: Silymarin (25mg)+AF](image)

Several researchers have reported decreased activities of ALT, AST, LDH and ALKP in liver during AF-B$_1$ treatment, which corroborate with our study (Kalengayi and Desmet, 1975; Yin et al., 1980). The AF-B$_1$-induced decreases in the liver enzymes were significantly inhibited by A. aspera pre-treatment in a manner similar...
to that observed with silymarin, a known hepatoprotectant. The protection rendered by silymarin may be due to their antioxidant effect and their ability to act as a radical scavenger, thereby protecting membrane permeability (Sudharsan et al., 2005; Nagaraj et al., 2000; Soto et al., 2003; Franschini et al., 2002).

**Fig 6.2.** Alterations in liver enzyme (U/mg protein) activities in AFB1-induced animals and the effect of A. aspera, silymarin pre-treatment. 1: AF Control, 2: A. aspera (100mg), 3: Silymarin (25mg), 4: A. aspera (100mg)+AF, 5: Silymarin (25mg)+AF

AF-B1-induced free radicals production has been referred to as a possible contributor to hepatotoxicity (Towner et al., 2003). LPO is one of the main manifestations of oxidative damage initiated by ROS and it has been linked with altered membrane structure and enzyme inactivation. It is initiated by the abstraction of a hydrogen atom from the side chain of polyunsaturated fatty acids in the membrane (Niki et al., 2005). The present data reveals that AFB1 administration produced a marked oxidative impact as evidenced from the significant increase in LPO. The increase in lipid peroxides might result from increased production of free radicals and a decrease in antioxidant status (Fig 6.3). The oxidative stress observed in our study is in accordance with other reports (Shen et al., 1994; Rastogi et al., 2001) where it has been implicated in AF-B1-induced hepatotoxicity. In this study, A.
aspera and silymarin pre-treatment significantly reduced the AFB\textsubscript{1}-induced LPO by their ability to scavenge the free radicals.

GSH and GST play a critical role in the protection of tissues from the deleterious effects of activated AF-B\textsubscript{1} (Larsson \textit{et al.}, 1994). GSH is a tripeptide containing cysteine that has a reactive \(-\text{SH}\) group with reductive potency. It can act as a non-enzymatic antioxidant by direct interaction of \(-\text{SH}\) group with ROS or it can be involved in the enzymatic detoxification of ROS, as a cofactor or a coenzyme (Janssen \textit{et al.}, 1993). GST catalyzes the conjugation of AFB\textsubscript{1}-8, 9-epoxides with GSH to form AFB\textsubscript{1}- epoxide–GSH conjugates thereby decreasing the intracellular glutathione content (Raney \textit{et al.}, 1992). This observation supports our finding where we observed a significant decline in the levels of GSH and GST in AF-B\textsubscript{1}-induced animals (Fig.6.4).
Silymarin has already been reported to improve the GSH level (Valenzuela et al., 1989). The restoration of intracellular GSH content and GST activity to normal levels by A. aspera and silymarin pre-treatment indicates that they play a vital role in mitigating AFB₁-induced oxidative stress and subsequent damage to the liver. Antioxidant enzymes like SOD, CAT and GPx form the first line of defense against ROS and a decrease in their activities was observed with AFB₁ administration (Verma and Nair, 2001; Rastogi et al., 2001). The above finding corroborates with our results where we have observed a decline in SOD, CAT and GPx activities.

SOD is a family of metallo-enzymes that is known to accelerate the dismutation of endogenous cytotoxic superoxide radicals to H₂O₂ which are deleterious to polyunsaturated fatty acids and structural proteins of plasma membrane (Johnson and Giulivi, 2005). The hydrogen peroxide produced by SOD is further
removed by catalase (CAT). Decline in the activities of these enzymes after AFB₁ administration might be due to the inactivation of these enzymes by ROS.

*A. aspera* increases the GSH status resulting in the increase in SOD activity thereby preventing the deleterious effect of super oxide radicals. Thus *A. aspera* indirectly influences the activities of SOD and CAT. Selenium dependent GPx removes both H₂O₂ and lipid peroxides by catalyzing the conversion of lipid hydroperoxide to hydroxy acids in the presence of GSH. The activity of GPx, which is a constituent of GSH redox cycle decreased during AFB₁ administration. The reduction in the activity of GPx on AFB₁ administration may be due to decrease in the availability of substrate (GSH) and also because of alterations in their protein structure by ROS (Janssen *et al.*, 1993).

The increased intracellular GSH content following *A. aspera* and silymarin pretreatment may activate GPx thereby preventing the accumulation of H₂O₂. The decrease in the levels of glutathione metabolizing enzymes (G6PD and GR) in AFB₁ administered rats occurs as a result of impaired flux of glucose-6-phosphate through hexose monophosphate shunt and decreased supply of reduced nicotinamide adenine dinucleotid phosphate (NADPH) for the conversion of GSSG to GSH in the presence of GR. Under conditions of oxidative assault, the NADP⁺/NADPH ratio will switch in favour of NADP⁺, indicating decreased G6PD activity. The present study also showed a similar finding in the levels of these enzymes, indicating increased onslaught of oxidative radicals. Treatment with *A. aspera* and silymarin significantly improved the activities of GR and G6PD.

Vitamin E, a fat-soluble molecule present in the interior of membranes protects against LPO while ascorbate, a water-soluble antioxidant reduces oxidized
α-tocopherol and lipid peroxides (Singh et al., 2005). GSH depletion can additionally explain the decreased concentration of vitamin C observed in the present study. This vitamin enters the cell mainly in the oxidized form where it is reduced by GSH. *A. aspera* and silymarin pre-treatment prevented the significant decline in the level of vitamin C. The increased ascorbate and GSH content in the *A. aspera* and silymarin pre-treated animals regenerates vitamin E and establishes a synergistic effect among them thereby enhancing the antioxidant protection (Lee, 1999).

It showed a trend similar to that of silymarin, a known hepatoprotective agent in protecting liver from AFB1-induced toxicity. Thus, it may be concluded that *Achyranthes aspera* ameliorates AFB1-induced toxicity due to its combined antioxidant potential as well as hepatoprotective action.