PREVENTIVE EFFECTS OF TINOSPORA CORDIFOLIA EXTRACT AGAINST AFLATOXIN-B1 INDUCED OXIDATIVE STRESS IN SWISS ALBINO MICE

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MATERIALS AND METHODS

Chemicals

Crystalline AFB1 (from Aspergillus flavus), purchased from HIMEDIA (India). All other chemicals used were of analytical grade and obtained from SD fine chemicals (Mumbai, India), SRL (India), CDH (India) and Qualigens (India/Germany).

Animals

Healthy male Swiss albino mice (Mus musculus) were procured from Haryana Agricultural University, Bissar (Haryana, India). Only male mice were used because previous studies have indicated that these were more sensitive to aflatoxin treatment than female. The animals were housed under standard laboratory conditions of light (12 h light-dark cycle), temperature (25 ± 2°C), humidity (55 ± 5%) and fed with Standard mice pellet diet (Hindustan Liver Limited, India) and tap water ad libitum in animal house of Banasthali University. A prior approval was obtained from the institutional animal ethics committee for the study protocol. After 1 week of acclimatization mice were used for experimental purpose.

Preparation of Aflatoxin B1 and ethanolic extract of Tinospora cordifolia

Crystalline aflatoxin B1 was dissolved in dimethylsulfoxide and further diluted with distilled water to the required concentration. The final gavage solution of AFB1 contained 1% dimethyl sulfoxide.

The experimental plant material was collected from Krishi Vigyan Kendra, Banasthali University; India. It was identified as Tinospora cordifolia by a plant taxonomist of our department and its sample has been preserved and documented in the herbarium of our University. The hanging aerial roots were washed thoroughly with distilled water and shade-dried. Ethanolic extract of the dried roots of Tinospora cordifolia was prepared by soxhlet method using 300ml ethanol for 50g (dry weight) of dried root powder. The ethanolic extract thus obtained was dried under reduced pressure at a room temperature not exceeding 40°C to get a yield of 7% from the crude extract. The extract devoid of alcohol, was used for required concentration.
Experimental Design

Male Swiss albino mice (30 ± 5 g) were randomized into eight groups comprising of six animals in each group and were administered orally by gavage, once daily as below for 75 days-

Group I- Control (Normal saline, 0.9%)
Group II- AFB1 (2 µg/30 g body weight)
Group III- T. cordifolia (50 mg/kg body weight)
Group IV- T. cordifolia (100 mg/kg body weight)
Group V- T. cordifolia (200 mg/kg body weight)
Group VI- AFB1 + T. cordifolia (50 mg/kg body weight)
Group VII- AFB1 + T. cordifolia (100 mg/kg body weight)
Group VIII- AFB1 + T. cordifolia (200 mg/kg body weight)

The doses of Aflatoxin & plant extract were decided on the basis of previously published reports.22, 23 The mice from each groups were sacrificed by cervical dislocation on 75th day of the study. Liver and kidney sample from the sacrificed mice were quickly removed and cleaned to make them free from extraneous material and perfused with ice-cold saline for biochemical and histopathological evaluation. Post-mortem supernatant (PMS) was prepared using method of Mohandas et al.24 with some modifications. The various histo-biochemical variables viz, lipid peroxidation25, Superoxide dismutase26, Catalase27, glutathione-S-transferase28, reduced glutathione29, glutathione peroxidase30, glutathione reductase31, Ascorbic acid32 and Protein33 were performed.

Noncoagulated blood was used for hematological parameters viz, hemoglobin estimation was done using Sahli’s hemoglobin meter34, total erythrocyte count and total leukocyte count by hemocytometer34, PCV by Wintrob method35, and Differential leukocyte count by Leishman’s staining method. Mean corpuscular hemoglobin, Mean corpuscular hemoglobin content & Mean corpuscular volume were also calculated.36,37

Serum was obtained by centrifugation of blood samples at 860 × g for 20 min, and was stored at -20°C until used for analysis. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman and Frankel.38 Activity of alkaline phosphatase (ALP) was determined according to the protocol described in a laboratory practical manual.39

Histopathological examination of liver and kidney tissues were done according to the method of Luna.40

Statistical analysis

The results are expressed as mean ± standard error (S.E.M). Statistical significance between the different groups was determined by one way analysis of variance (ANOVA) using the SPSS software package.46 Post hoc testing was performed for inter-group comparisons using the Tukey multiple comparison test. The level of significance was set at P<0.05.

RESULTS

Biochemical parameters

Table 1 depicts the levels of lipid peroxide, ascorbic acid, protein and reduced glutathione in normal and experimental mice liver and kidney. The lipid peroxide activity was significantly raised (P<0.01) in aflatoxin intoxicated mice but the activities of ascorbic acid, protein and reduced glutathione were found to fall significantly (P<0.01) as compared with normal mice. Administration of all doses of RTc extract (low, medium and high), decreased the level of LPO and increased the level of ascorbic acid, protein and reduced glutathione significantly (P<0.01) when compared with aflatoxin alone receiving group of mice (group II).

Antioxidant Parameters

Table 2 shows levels of superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase in liver & kidney of normal and experimental mice. The level of SOD, CAT, GST, GPx and GR were decreased significantly (P<0.01) in liver and kidney of aflatoxin intoxicated mice group when compared with normal mice. The RTc extract treatment brought the activities of all these antioxidant enzymes back to normal level to some extent when compared with group II mice.

Hematological variables

Data presented in Table 3 shows the effect of RTc extract either alone or in combination with AFB1 on hematological variables. AFB1, alone administration caused a significant decrease (P<0.01) in hemoglobin, hematocrit, total erythrocyte count, lymphocyte count and significant increase (P<0.01) in WBC, platelet and neutrophil count when compared with control values. The levels of Hb, PCV, RBC were increased significantly (P<0.01) and it was comparable to control in the groups of mice that receive combination of different doses plant extract along with AFB1. On the other hand, the level of WBC, platelet and neutrophil count were reduced significantly (P<0.01) in the group of mice that received RTc extract and AFB1 simultaneously in comparison to group II mice.

Serological parameters

Table 4 shows SGOT, SGPT and ALP activities in the serum of normal and treated mice. The values of SGOT, SGPT and ALP were elevated significantly (P<0.01) when compared with respective values of normal mice. RTc extract supplemented mice showed decrease (P<0.01) in SGOT, SGPT and ALP values; RTc extract reversed the toxic effect of aflatoxin to some extent.

Histological/histopathological examination

Histopathological examination of liver (Fig 1) revealed no tumor and other pathological abnormalities in mice administered with all doses of plant extract and normal saline but mice administered with aflatoxin alone showed hepatocellular carcinoma. Mice supplemented with aflatoxin along with RTc (50) and RTc (100) showed normal hepatic parenchyma whereas mice administered with RTc (200) along with aflatoxin showed scattered vacuolated hepatocytes in the mid and periporal zones. Histopathological examination of kidney (Fig 2) showed normal kidney parenchyma in mice administered with different doses of plant and normal saline whereas group of mice that receive aflatoxin alone showed vacuolation, necrosis and shedding of tubular epithelial cells. Mice supplemented with different doses of plant along with aflatoxin prevent the toxic effect of aflatoxin and showed normal kidney architecture.

DISCUSSION

Carcinogenesis is a multistep process begins with cellular transformation and process by hyper proliferation leading to metastatic lesions.41 This progress can be activated by carcinogenotoxic substance such as aflatoxin that develops cancers in specific organ of experimental animals. However, relatively little is known about the mechanism of its toxicity, it was discovered that AFB1 was able to induce chromosomal damage through the release of free radicals.42 This finding suggested that oxidative damage might contributed to the carcinogenic effects of AFB1. Aflatoxin cause oxidative stress by increasing lipid peroxidation and decreasing enzymatic and non-enzymatic antioxidant in aflatoxin treated mice.42,43 Oxidative damage usually refers to impairment of the function of cellular component by reactive oxygen species such as superoxide anion (O2-), hydroxyl ion (OH -), and hydrogen peroxide (H2O2).These agents initiate cell injury by extracting hydrogen atoms from polyunsaturated fatty acid and cause a degenerative process known as lipid peroxidation.44 To protect them against oxidative stress, cell have developed antioxidant defense and repair system which prevent the accumulation of oxidatively damaged molecules. The antioxidant defense system include enzyme like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase, as well as small molecules ascorbic acid.44 A significant increase in production of MDA and decrease in the level of glutathione was observed in AFB1 administered animal. Malondialdehyde, a major end product of lipid peroxidation, cross-links with DNA, protein and nucleotides on the
same or opposite stand there by promoting carcinogenesis. Elevation in the level of lipid peroxide in the present study is in accordance with the previous reports given by Shen et al., 1994. The simultaneous administration of RTc root extract in aflatoxin treated animal has effectively controlled the rate of lipid peroxidation.

Table 1: Effect of RTc extract either alone or in combination with AFB1 on LPO, Ascorbic acid, Protein and GSH level in mice liver and Kidney

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tissues</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>
<th>(Group VII)</th>
<th>(Group VIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO (nmol s TBRs)</td>
<td>Liver</td>
<td>8.08±0.40</td>
<td>30.12±1.17</td>
<td>7.99±0.59</td>
<td>7.96±0.25</td>
<td>8.06±0.14</td>
<td>10.14±0.84</td>
<td>9.59±0.41</td>
<td>10.85±0.42</td>
</tr>
<tr>
<td>h-ig (1 mg tissue)</td>
<td>Kidney</td>
<td>8.47±0.23</td>
<td>27.10±1.22</td>
<td>8.29±0.26</td>
<td>8.18±0.14</td>
<td>8.47±0.35</td>
<td>9.75±0.52</td>
<td>9.23±0.43</td>
<td>10.2±0.33</td>
</tr>
<tr>
<td>Asc (mg ascorbic acid/g tissue)</td>
<td>Kidney</td>
<td>6.44±0.26</td>
<td>1.60±0.05</td>
<td>8.14±0.76</td>
<td>8.87±0.23</td>
<td>7.73±0.17</td>
<td>6.24±0.12</td>
<td>6.29±0.14</td>
<td>6.02±0.11</td>
</tr>
<tr>
<td>Protein (mg g-1 fresh wt)</td>
<td>Liver</td>
<td>9.27±1.35</td>
<td>42.47±2.14</td>
<td>90.92±1.49</td>
<td>92.97±1.90</td>
<td>90.55±2.29</td>
<td>86.67±2.24</td>
<td>89.54±1.74</td>
<td>82.28±1.38</td>
</tr>
</tbody>
</table>

Abbreviations:- AFB1, Aflatoxin B1; Asc, Ascorbic acid; GSH, Reduced Glutathione; LPO, Lipid peroxidase; RTc, Root of Tinospora cordifolia

Values are mean± SE of six mice. Significant differences in data are shown as a p<0.01 and b p<0.05 when compared with aflatoxin treated group (group II).NS (Statistically not significant)

Table 2: Effect of RTc extract either alone or in combination with AFB1 on antioxidant parameters in mice liver and kidney

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Tissues</th>
<th>(Group I)</th>
<th>(Group II)</th>
<th>(Group III)</th>
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<th>(Group V)</th>
<th>(Group VI)</th>
<th>(Group VII)</th>
<th>(Group VIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD(Units mg protein)</td>
<td>Liver</td>
<td>14.86±0.11</td>
<td>2.47±0.09</td>
<td>14.59±0.16</td>
<td>14.98±0.11</td>
<td>13.08±0.20</td>
<td>12.23±0.23</td>
<td>13.30±0.19</td>
<td>11.27±0.28</td>
</tr>
<tr>
<td>CAT (µmol H2O2 consumed min-1 mg-1 protein)</td>
<td>Kidney</td>
<td>12.26±0.21</td>
<td>1.52±0.02</td>
<td>13.26±0.46</td>
<td>13.58±0.34</td>
<td>13.53±0.50</td>
<td>12.16±0.23</td>
<td>12.45±0.38</td>
<td>10.84±0.30</td>
</tr>
<tr>
<td>GST (nmol CDNB conjugated min-1 mg-1 protein)</td>
<td>Liver</td>
<td>225.39±4.0</td>
<td>98.09±3.1</td>
<td>231.12±1.56</td>
<td>233.42±3.86</td>
<td>230.62±1.99</td>
<td>210.14±1.6</td>
<td>219.32±2.4</td>
<td>204.78±2.04</td>
</tr>
<tr>
<td>GPx (µg of glutathione utilized min-1 mg-1 protein)</td>
<td>Kidney</td>
<td>82.93±1.88</td>
<td>18.22±0.9</td>
<td>86.13±1.32</td>
<td>85.66±2.64</td>
<td>89.24±1.25</td>
<td>75.87±2.29</td>
<td>78.63±1.96</td>
<td>69.55±0.77</td>
</tr>
<tr>
<td>GR (nmol moles)</td>
<td>Liver</td>
<td>202.58±2.2</td>
<td>52.08±2.2</td>
<td>195±1.49</td>
<td>196.18±1.92</td>
<td>193.20±1.89</td>
<td>194.16±3.1</td>
<td>201.07±2.9</td>
<td>185.16±2.72</td>
</tr>
<tr>
<td>NADPH oxidized (min-1 mg-1 protein)</td>
<td>Kidney</td>
<td>165.53±2.0</td>
<td>67.77±1.7</td>
<td>171.23±1.71</td>
<td>175.49±2.43</td>
<td>168.20±1.47</td>
<td>163.45±2.1</td>
<td>171.32±2.3</td>
<td>158.43±2.23</td>
</tr>
</tbody>
</table>

Abbreviations:- AFB1, Aflatoxin B1; CAT, Catalase; GPx, Glutathione peroxidase; GR, Glutathione reductase; GST, Glutathione-S-transferase; RTc, Root of Tinospora cordifolia; SOD, Superoxide dismutase

Values are mean± SE of six mice. Significant differences in data are shown as a p<0.01 and b p<0.05 when compared with control (group I) and c p<0.01 and d p<0.05 when compared with aflatoxin treated group (group II).NS (Statistically not significant)
Table 3: Effect of RTc extract either alone or in combination with AFB1 on haematological variables in Swiss albino mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatments</th>
<th>(mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Group I)</td>
<td>(Group II)</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.39±0.23</td>
<td>7.71±0.45</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>43.80±0.56</td>
<td>32.52±1.52</td>
</tr>
<tr>
<td>RBC (10^6/mm³)</td>
<td>8.94±0.41</td>
<td>5.74±0.38</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>48.75±2.63</td>
<td>56.83±4.11</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>14.99±0.49</td>
<td>12.53±1.16</td>
</tr>
<tr>
<td>WBC (10³/mm³)</td>
<td>30.56±0.71</td>
<td>22.05±1.10</td>
</tr>
<tr>
<td>Platelet(10³/mm³)</td>
<td>17.21±0.78</td>
<td>23.92±0.57</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>71.73±7.8</td>
<td>173.41±6.9</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>21.4±1.14</td>
<td>24.16±1.06</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>92.04±2.81</td>
<td>62.97±1.84</td>
</tr>
</tbody>
</table>

Abbreviations: AFB1, Aflatoxin B1; Hb, Haemoglobin; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin content; MCV, Mean corpuscular volume; PCV, Packed cell volume; RTc, Root of Tinospora cordifolia; WBC, White blood corpuscles

Values are mean± SE of six mice. Significant differences in data are shown as a p<0.01 and b p<0.05 when compared with control (group I) and c p<0.01 and d p<0.05 when compared with aflatoxin treated group (group II). NS (Statistically not significant)

Table 4: Effect of RTc extract either alone or in combination with AFB1 on some serological variables in Swiss albino mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatments</th>
<th>(mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Group I)</td>
<td>(Group II)</td>
</tr>
<tr>
<td>SGOT (Units /ml)</td>
<td>10.07±0.12</td>
<td>51.50±2.32</td>
</tr>
<tr>
<td>SGPT (Units /ml)</td>
<td>11.25±0.51</td>
<td>52.17±1.10</td>
</tr>
<tr>
<td>ALP (KA Units /ml)</td>
<td>4.96±0.27c</td>
<td>14.22±0.39a</td>
</tr>
</tbody>
</table>

Abbreviations: AFB1, Aflatoxin B1; SGOT, Serum glutamate oxaloacetate transaminase; SGPT, Serum glutamate pyruvate transaminase; ALP, Alkaline phosphatase; RTc, Root of Tinospora cordifolia

Values are mean± SE of six mice. Significant differences in data are shown as a p<0.01 and b p<0.05 when compared with control (group I) and c p<0.01 and d p<0.05 when compared with aflatoxin treated group (group II). NS (Statistically not significant)
SOD is a family of metallo-enzyme that accelerate the dismutation of endogenous cytotoxic superoxide radical to $\text{H}_2\text{O}_2$ which are deleterious to polyunsaturated fatty acid and structural protein of plasma membrane. The $\text{H}_2\text{O}_2$ produced by SOD is further removed by CAT. Decline in the activities of these enzymes after AFB$_1$ administration might be due to the inactivation of these enzymes by ROS. Ethanolic root extract of T. cordifolia increases the GSH status resulting in the increase in SOD activity thereby preventing the deleterious effect of superoxide radicals. Thus, T. cordifolia indirectly influences the activity of SOD and CAT. GPx, selenium dependent enzymes removes both $\text{H}_2\text{O}_2$ and lipid peroxides by catalyzing the conversion of lipid hydroperoxide to hydroxy acid in the presence of GSH. The activity of GPx decreased during AFB$_1$ administration may be due to decrease availability of substrate (GSH) and also because of alteration in their protein structure by ROS. The increased level of GSH in T. cordifolia treated group may activate GPx thereby preventing accumulation of $\text{H}_2\text{O}_2$ GR is enzyme responsible for the conversion of glutathione disulphide (GSSG) back to reduced GSH. GSSG is formed during the detoxification of $\text{H}_2\text{O}_2$ by GPx. Reduced GR activity in AFB$_1$ treated group confirmed the higher production of GSSG due to the higher rate of detoxification of $\text{H}_2\text{O}_2$ by GPx. Similar results were also obtained in rats hepatotoxicity by Cascales et al., 1991. GST eliminates the reactive epoxide of aflatoxin by conjugation with GSH. The values showed significant reduction in AFB$_1$ treated group and non significant changes in RTc supplemented groups indicate that RTc plays important role in
maintenance of GST activity. Vitamin C was also found to be low in AFBl intoxicated mice. This may be due to the utilization of vitamin C for scavenging free radical produced by cancerous cell and AFBl, metabolism or its synthesis in body may be affected by the damaged cell in response of AFBl treatment.25

Cross Section kidney mice treated with AFBl and RTC for 75 days (40X). Groups of mice supplemented with normal saline and different doses of plant extract (low, medium and high) (a, c, d, e) shows normal kidney parenchyma whereas groups of mice that received AFBl (b) shows vacuolation, necrosis and shedding of tubular epithelial cells. Mice supplemented with different doses of plant along with AFBl, (f, g, h) revert the toxic effect of aflatoxin and show normal kidney architecture.

We observed a significant decrease in haemoglobin concentration, PCV and total RBCs resulting in a normocytic normochromic anaemia. This decrease in haemopoietic parameters may be due inhibition of protein synthesis as indicated by lower amount of total protein.26 Previous reports indicated that AFBl decreases the total iron binding capacity24 and affects the metabolism of minerals such as Cu and Zn.25 These results support our findings that aflatoxin causes normocytic normochromic anaemia. There was a significant enhancement in the WBC counts, which mainly consisted of neutrophil. This increase in WBCs and neutrophils suggested that normocytic normochromic anaemia. There was a significant increase in WBCs and neutrophils suggested that

In conclusion, we determined that aflatoxin could increase the liver and kidney carcinomas, these enzymes are released from the liver and kidney tissue into the blood stream.25 In cases such as liver and kidney carcinoma these enzymes are released from the liver and kidney tissue into the blood stream.25 The results obtained in present study indicated a significant increase in the activities of these marker enzymes in serum, which is in accordance with the previous reports.25,26,27 Treatment with RTC significantly lowered the levels of these enzymes, suggests the protective role of RTC extract.

CONCLUSION

In conclusion, we determined that aflatoxin could increase the liver and kidney enzyme levels and affect some hematological and serological parameters. Increase in these parameters may occur due to peroxidation reactions, arising in aflatoxin biotransformation, and these reactions may inflict oxidative injury to cellular components. Administration of RTC extract to mice received aflatoxin B1, resulted in a significant improvement in all biochemical, hematological, serological indices as well as a significant improvement in histopathological picture of the liver and kidney in different experimental groups. In the light of these results, RTC extract was found to induce the potent protective action in mice and plays a role in prevention of hepatic cellular injury produced by aflatoxin.

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REFERENCES


