Cytoprotective Activity of *Citrus aurantifolia* fruits Extract Against Aflatoxin-B$_1$ induced Cytotoxicity

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**ABSTRACT**

*Citrus aurantifolia*, is one of the herbal medicines in India that has been commonly used for treating various diseases, including liver disorders. Based on its use in traditional oriental medicine, the current study was carried out to evaluate the cytoprotective effects of *C. aurantifolia* using Aflatoxin B$_1$ (AFB$_1$)-induced liver injury in a rat model. Wistar albino rats were divided into five groups of six animals each. Group I served as the control. Group II was treated with vehicle, dimethyl sulfoxide (DMSO) a single intraperitoneally (i.p) on day 5. Group III animals received AFB$_1$-alone (1mg/Kg body weight) intraperitoneally in DMSO as a single dose on day 5. Group IV and V received MeCA and AqCA (500mg/Kg body weight, per oral) for 5 days and AFB$_1$ (1mg/kg body weight) intraperitoneally in DMSO as a single dose on day 5. At the end of the 8$^{th}$ day, the liver was collected and used to determine the hepatoprotective activity. Genomic DNA fragmentation was observed by agarose gel electrophoretic pattern in the rat livers. The ultra-structure of the liver cells was studied by electron microscopy. *C. aurantifolia* treatment significantly protected nucleic acid levels. We also found that the treatment significantly inhibited DNA fragmentation. Nucleus structures were well maintained. These results demonstrate that *C. aurantifolia* has a cytoprotective effect against AFB$_1$-induced liver injury. In this study, we focused on the cytoprotective effects of *C. aurantifolia* against AFB$_1$-induced cytotoxicity.

**Keywords:** Aflatoxin B$_1$, *Citrus aurantifolia*, cytoprotective, DNA fragmentation, Transmission Electron Microscope.

**INTRODUCTION**

Mycotoxins are toxic fungal metabolites. Among the mycotoxins, the aflatoxin, AFB$_1$ is the most potent, and is nearly universally found in many foods and feeds. AFB$_1$-induced cellular oxidative damage and its roles in the cytotoxicity have attracted much attention (1). Oxidative damage usually refers to the impairment of cellular components such as enzymes, nucleic acids, membrane lipids and proteins, by...
Reactive Oxygen Species (ROS) (2). ROS such as Superoxide (O$_2^-$), Hydrogen peroxide (H$_2$O$_2$) and Hydroxyl (OH•) could be generated via various pathways during metabolic processes of many xenobiotics (3). OH• readily reacts with cellular components to cause oxidative damage, such as Lipid Peroxidation (LPO) and DNA base damage (4, 5). Direct covalent binding between activated metabolites of AFB$_1$-and cellular macromolecules such as DNA, RNA and protein has been proposed as a major event in hepatotoxicity (6,7,8).

**C. aurantifolia** is one of the Asian herbal medicines that have been prescribed for the treatment of various diseases including hepatic disorders in Ayurveda, Siddha and Unani systems of medicine. The Indian Medical Practitioners Co-operative Pharmacy and Store Limited (IMPCOPS), at Tamil Nadu, India daily uses about 50,000 fruits for various medicinal preparations. Some of the preparations, which include a major constituent of *C. aurantifolia* fruits, used for liver disorders are Jambeeradi panakam, Sherbath madani, Annabeti centuram, Palakarai parpam, Pravala bhasma, Kaseesa bhasma, Sankha bhasma and Varatika bhasma. In addition, C. aurantifolia is widely used as a flavoring adjuvant in drug preparations and as a food ingredient for its taste and digestive property. There is a lack of well-controlled experiments investigating the hepatoprotective effect of *C. aurantifolia* extract. However, there are a few studies that evaluate the effects of useful herbal medicines against AFB$_1$ induced toxicity. The purpose of this research was to investigate whether *C. aurantifolia* could prevent AFB$_1$-induced hepatotoxicity.

**Methods**

**Chemicals**

AFB$_1$ was purchased from M/s. Sigma Chemical Co. (St. Louis, M.O) and all other chemicals used were of analytical grade and were obtained from Sisco Research Laboratory, India.

**Plant material and extraction**

*C. aurantifolia* fruits were collected in the Minjur area in Tamil Nadu, India and authenticated by the Chief Botanist of Captain Srinivasa Murti Drug Research Institute for Ayurveda (CCRAS), where a voucher specimen of the plant was deposited (voucher No.15). These fruits were cut into smaller pieces, shade dried and coarsely powdered and the methanolic extract (MeCA) used was prepared using a Soxhlet apparatus. Water extract of *C. aurantifolia* (AqCA) was prepared by cold percolation method. The methanolic and aqueous extracts were then concentrated in a rotary evaporator at reduced pressure and at a temperature lower than 60°C; the extracts were then stored at 4°C until they were needed for use.

**Animals and treatment**

Male albino rats (n = 6) of Wistar strain 14-16 weeks of age and weighing between 140 and 160 g were used for this study. The animals were fed on pellet diet (TANVAS pellet) and water *ad libitum*. All animal experimentation protocols were approved by the Institutional Animal Ethics Committee (IAEC No. 07/024/03).

Animals were divided into five groups of six animals each. Group I (control) received saline. Group II was treated with DMSO (vehicle) as a single i.p. dose on day 5. Group III animals received AFB$_1$ alone (1 mg/kg body weight i.p.) in DMSO as a single dose on day 5. Group IV received MeCA (500 mg/kg body weight p.o.) for 5 days and AFB$_1$ (1 mg/kg body weight i.p.) in DMSO as a single dose on day 5. Group IV received MeCA (500 mg/kg body weight p.o.) for 5 days and AFB$_1$ (1 mg/kg body weight i.p.) in DMSO as a single dose on day 5. Group IV received MeCA (500 mg/kg body weight p.o.) for 5 days and AFB$_1$ (1000 µg/Kg body weight i.p.) in DMSO as single dose on day 5. At the end of the 8th day, overnight fasted rats were perfused with cold PBS, and the rats’ livers were excised and fixed immediately for observation under a transmission electron microscope (TEM). Ten percent (w/v) liver homogenates were prepared in Tris-HCl buffer (0.1M, pH 7.4) for biochemical analysis.

**Nucleic acid and protein**

The liver homogenates were assayed to estimate protein (9), Deoxyribonucleic acid (DNA) (10) and Ribonucleic acid (RNA) levels (11).

**Genomic DNA fragmentation**

Genomic DNA fragmentation was observed by agarose gel electrophoretic (12) pattern in the rat livers.

**Transmission electron microscope**

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The ultra-structure of the liver cell was studied by electron microscopy according to the method described by Webster and Bronk (13).

Statistical analysis
The results were expressed as Mean ± SD for the six animals within each group. The data was subjected to one way-ANOVA followed by Tukey’s multiple comparison tests (SPSS windows student version 1996). P values less than 0.05 were taken as statistically significant.

Results and Discussion
DNA, RNA and Proteins

One of the main known biological activities of aflatoxin is its inhibition of protein synthesis (14). This may arise directly from inactivation of biosynthetic enzymes, or indirectly by alteration of DNA template activity, or inhibition of RNA synthesis and maturation, translation, and/or interference with amino acid transport. Exogenous and endogenous radical sources of AFB1 may damage proteins around and inside cells (15). The protein damage by free radicals is often complex and irreversible. This oxidative modification of proteins could provide an attractive universal signal for the altered structural and/or metabolic function of the proteins and consequent removal from the system by proteolysis (16).

Table 1. Effect on protein, DNA and RNA of rat liver in control and experimental animals

<table>
<thead>
<tr>
<th>Parameters (mg/g wet tissue)</th>
<th>Control Group I</th>
<th>DMSO Group II</th>
<th>AFB1 Group III</th>
<th>MeCA + AFB1 Group IV</th>
<th>AqCA + AFB1 Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>163.65 ± 10.83</td>
<td>168.57 ± 13.69</td>
<td>92.12 ± 14.24</td>
<td>155.82 ± 13.13</td>
<td>144.55 ± 16.97</td>
</tr>
<tr>
<td>DNA</td>
<td>8.79 ± 0.66</td>
<td>8.87 ± 0.73</td>
<td>6.18 ± 0.72</td>
<td>8.18 ± 0.76</td>
<td>7.69 ± 0.59</td>
</tr>
<tr>
<td>RNA</td>
<td>6.79 ± 0.61</td>
<td>6.67 ± 0.69</td>
<td>4.20 ± 0.41</td>
<td>6.15 ± 0.53</td>
<td>5.77 ± 0.49</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for 6 animals in each group.
Comparisons were made between: a - Group I vs Groups II, III, IV and V
b - Group III vs Groups IV and V
Symbols represent statistical significance: @ = p < 0.001, # = p < 0.01, © = p < 0.05 and NS = Not significant

The damaging effects of free radicals are believed to be nullified in part. The significant protection (p < 0.001) of DNA and RNA in the extract pretreatment might be expected to inhibit the interaction of AFB1 with DNA (Table 1). Ascorbic acid which is abundantly present in C. aurantifolia was reported to inhibit the binding of AFB1 to DNA (17).

The damaging effects of free radicals are believed to be nullified in part by naturally occurring antioxidants when they are administered prior to the introduction of free radical inducing xenobiotic species. GPx also plays an important role in protecting cellular proteins against oxidation (18). From the enzymic and non-enzymic antioxidant status, it is clear that the extracts have abundant antioxidant activity which could protect the protein against damage.

DNA fragmentation
AFB1 induced cellular oxidative damage and its roles in the cytotoxicity have attracted much attention (1,2). OH– reacts with the nucleic acid bases of DNA producing base and sugar radicals. The base radicals in turn react with the sugar moiety causing breakage of the sugar-phosphate backbone, resulting in strand break formation. Besides infliction of
oxidative damage on DNA by free radicals, there are also other indirect mechanisms by which radicals can cause destruction of the genome. Lipid peroxides can enter the nucleus and react with ferrous ions causing the generation of alkyl radicals that cause damage to DNA (19). Intracellular calcium levels can also increase as a result of oxidative damage to membranes, therefore providing the opportunity for calcium to enter the nucleus where it can activate nuclease that cause DNA strand breaks (20). MeCA and AqCA treated groups show little DNA damage when compared to the AFB1 treated group (Fig.1).

Figure 1. DNA fragmentation by agarose gel electrophoresis.
Lane 1 (L1) - DNA marker;
Lane 2 (L2) – Group I (control) rat liver;
Lane 3 (L3) – Group II (DMSO treated) rat liver;
Lane 4 (L4) – Group III (AFB1- induced DNA damage) rat liver;
Lane 5 (L5) – Group IV (Pretreated with MeCA and AFB1- induced DNA damage) rat liver;
Lane 6 (L6) - Group V (Pretreated with AqCA and AFB1- induced DNA damage) rat liver

This study suggests that the extracts have the ability to scavenge free radicals generated by AFB1 thus providing protection of DNA. Ascorbic acid is an excellent source of electrons, and as a result, it can donate electrons to free radicals such as OH• and O2•- to quench their reactivity (21).

Transmission electron microscope
The liver injury caused by AFB1 could also be recognized by a TEM. Using a TEM, we were able to visually analyze the condition of the rat hepatocytes. Most notably with the rats treated only with AFB1, we saw a reduction in the size of the nucleus, multiple electron dense bodies in the cytoplasm, and mild vacuolation in the mitochondria and also DNA was scattered and aggregated in the hepatocytes. However, extract pretreatment, afforded good protection against AFB1- induced liver damage; Figure 2 clearly shows the well maintained structure of the DNA, cytoplasm, and nucleus of the hepatocytes in the control rats.
Figure 2. Effect of AqCA and MeCA against AFB<sub>1</sub>-induced histopathological changes rat liver
(A) Section of control rat liver shows central nucleus and nucleolus. Nucleus has got double membrane. Nucleolus is clear. Nucleoplasm is arranged diffusely on the peripheral. Cytoplasm shows large number of mitochondria. Rough endoplasmic reticulum is prompt. Occasional lysosomes are present. Peripheral aggregation of DNA shows a few at centre and evenly distributed.
(B) Section of DMSO received rat liver showing normal architecture similar to control.
(C) Section of AFB<sub>1</sub> received rat liver nucleus shows shrinkage as indicated by wavy outline, and nucleoplasm is disposed in irregular clumps. Cytoplasm shows multiple electron dense bodies and there is mild vacuolation in the mitochondria. DNA is scattered and aggregated.
(D) Section of rat liver pretreated with MeCA followed by the challenge with AFB<sub>1</sub> shows nucleus and nucleolus. Cytoplasm shows mitochondria and has fine granules. Rough endoplasmic reticulum and electron dense particles are also seen. Multiple aggregates of DNA are seen in centre in addition to peripheral.
(E) Section of rat liver pretreated with AqCA followed by the challenge with AFB<sub>1</sub> shows central nucleus. Nucleolus appears in reduced amount. Mitochondria are reduced in size. Rough endoplasmic reticulum is also seen. Increase electron dense particles in cytoplasm and lysosomes are present. Thick accumulation of DNA is seen at periphery, uneven in distribution and decreased in amount.
Conclusion
The present investigation demonstrates that these extracts have ameliorative effects against AFB$_1$-induced hepatotoxicity in rats. Furthermore, we believe that these restorative effects may be due to the abundance of flavonoids and free radicals quenching properties.

References

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