**ABSTRACT**

**Aim:** Aflatoxin B$_1$ (AFB$_1$) and fumonisin B$_1$ (FB$_1$) are important food-borne mycotoxins. Co-contamination of foodstuffs with these two mycotoxins is well-known and has been implicated in a possible development of hepatocellular carcinoma in humans living in regions of the world where exposures to these mycotoxins in grain are greatest. The aim of the current study was to evaluate the potential protective effects of an aqueous extract of *Corchorus olitorius* (*C. olitorius*, moroheiya) against cytotoxicity of AFB$_1$ and/or FB$_1$ in H4IIE-luc rat hepatoma cells, using assays to measure cell viability and disruption of DNA integrity. Although this transactivation assay was originally developed to specifically respond to aryl hydrocarbon agonists, this cell line was used because of its hepatic origin.

**Methods:** H4IIE-luc cells were incubated with different concentrations of AFB$_1$ and/or FB$_1$ for 24 and 48 h with or without aqueous extract of *C. olitorius*. **Results:** Both mycotoxins decreased cell viability and increased DNA damage. Cytotoxicity was more pronounced when cells were exposed simultaneously to AFB$_1$ and FB$_1$. **Conclusion:** Aqueous extract of *C. olitorius* protected cells against cytotoxicity of mycotoxins. *C. olitorius* contains a water-soluble, natural chemo-preventative agent for cancer that should be isolated and identified.

**Key words:** Anticancer; cytotoxicity; DNA; liver; moroheiya; mycotoxins

**INTRODUCTION**

Co-occurrence of various mycotoxins in foodstuffs and animal feed is common because each toxigenic fungus can produce more than one mycotoxin and foodstuff can be colonized by several fungi either while growing in the field or during storage or transport.**[1]** Processed products are often composed of various raw materials which might be...
contaminated with mycotoxins. Poor harvest practices and inadequate conditions during drying, handling, packaging, storing and transporting can contribute to the growth of fungi and an increased risk of production of mycotoxins. The importance of co-occurrence of mycotoxins lies in the changes that might occur in the combined toxicity of mycotoxins. In addition, the existence of relationships in the occurrences of mycotoxins allows predictions of the presence of individual mycotoxins from the presence of others.

Among these mycotoxins, aflatoxin B1 (AFB1) is the predominant contaminant in cereals and oilseed and presents a significant risk due to being hepatotoxic and carcinogenic to humans and animals. AFB1 is classified by the International Agency of Research on Cancer (IARC) as a Group 1 carcinogen. This mycotoxin is also mutagenic, teratogenic, and immunosuppressive in farm, and laboratory animals, and primarily affects cell-mediated immunity. AFB1 is also able to induce reactive oxygen species (ROS) possibly requiring activation of cytochrome P450.

Fumonisins, mainly produced by Fusarium verticillioides and F. proliferatum, are mycotoxins commonly found on corn. The most toxic and abundant of these is fumonisin B1 (FB1), which causes esophageal and hepatic cancer in humans and liver and kidney cancer in rodents. IARC evaluated FB1, and classified it as probably carcinogenic to humans (Group 2B). Moreover, FB1 modulates immunity in animals and decreases viability of lymphocytes in poultry.

Humans and animals are constantly exposed to small concentrations of these mycotoxins, either individually or in combination. Mycotoxicoses occur seasonally in areas that have not implemented effective prophylactic measures. While interactions between mycotoxins had been discussed, few studies have been conducted with these combinations.

Cochorus olitorius (C. olitorius, Tiliaceae family) is indigenous to the Middle East, including Egypt and South Africa. Young leaves of C. olitorius are regarded to be a healthy vegetable in East Asia and Japan, typically known as moroheiya. Its health benefits have been reported to include antitumor activity by inhibiting tumorigenesis, antioxidant properties, and antibacterial activity. Young leaves of C. olitorius are rich in calcium, potassium, phosphate, iron, ascorbic acid, carotene and other nutrients, and contain a large amount of mucilaginous polysaccharides. It has also been reported that compounds such as carotenoids, flavonoids, and vitamin C, isolated from leaves of C. olitorius, exhibit significant antioxidant characteristics. In addition, leaves of C. olitorius have been reported to have ethno-medicinal importance as a demulcent and febrifuge and also possess anti-inflammatory, analgesic, and antimicrobial activities.

The aim of the current research was to assess possible protective effects of C. olitorius extracts against cytotoxic effects and disruption of DNA integrity induced by FB1 and AFB1 in the rat hepatoma cell line (H4IIE-luc).

METHODS

Chemicals
Aflatoxin B1 and FB1 (98% purity) were purchased from Sigma Chemicals (St. Louis, MO, USA). The DNA extraction kit (DNeasy Blood and Tissue Kit) was obtained from Qiagen (Hilden, Germany). A DNA ladder, polymerase chain reaction (PCR) master mix containing 100 base pairs and RNase free water were obtained from Fermentas Inc. (Glen Burnie, MD, USA). Supertherm Taq polymerase was purchased from JMR Holdings (London, UK). Forty primers were obtained from Operon Technologies (Alameda, CA, USA). All solvents used were analytical grade from Burdick and Jackson (Muskegon, MI, USA).

Plant materials
Stems and leaves of C. olitorius were collected from a residential garden in the city of Potchefstroom, North West Province, South Africa. The plant material was freeze-dried, pulverized, and 1 g was infused with 10 mL water for 24 h at room temperature. After centrifugation, the supernatant was freeze-dried and stored at 4 °C until used.

Cytotoxicity
Rat hepatoma cells (H4IIE-luc) were used as the mammalian model. This cell line had been stably transfected with a firefly luciferase reporter gene under control of the dioxin response element and the aryl hydrocarbon receptor mechanism.

These cells were originally developed as a reporter gene assay to determine the presence of, and to semi-quantify the concentrations of certain groups of persistent organic pollutants including mixtures.

H4IIE-luc cells were seeded at a density of 10,000 cells/mL media (Dulbecco's Modified Eagle's Medium, Sigma: D2902; St. Louis, MO, USA) in the inner 60 wells of a 96-well microplate. A volume of 250 μL of culture medium, supplemented with 0.044 mol/L NaHCO3 and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), was added to each well. To avoid edge effects and to create a homogenous microclimate across all wells containing cells, outer cells received 250 μL Dulbecco's phosphate buffered saline (PBS) (Tewksbury, MA, USA). Two sets of plates were incubated, at 37 °C in humidified air with 5% CO2, with one set for 24 h and the other set for 48 h. After incubation,
the medium was removed and replaced with medium containing C. olitorius extract at either of two concentrations (20 or 40 μg/mL) and incubated for another 24 h. The medium was replaced with medium containing varying concentrations of AFB1 (50, 25, 2.5, 0.25, 0.025 μmol/L) dissolved in methanol, or of FB1, (200, 100, 10, 1, 0.1 μmol/L) dissolved in methanol. A combination of the already mentioned concentrations of AFB1 and FB1 were also tested: 50 μmol/L AFB1 + 200 μmol/L FB1; 25 μmol/L AFB1 + 100 μmol/L FB1, and so on. Exposure to mycotoxins was carried out in triplicates. Cells in six wells in each plate were exposed only to the aqueous extract of C. olitorius and cells in 11 wells were not exposed to anything except the growth medium.

To determine the viability, based on metabolic activity of cells, a colorimetric assay was performed using the yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Montigny-le-Bretonneux, France). In this assay, MTT is converted to formazan (blue) by mitochondrial reductase enzymes in living cells. A final concentration of 500 μg/mL MTT was added to each well and incubated for 30 min. Blue formazan crystals that were formed by reduced MTT were dissolved with dimethylsulfoxide and absorbance of the formazan was measured spectrophotometrically at 560 nm. The amount of blue formazan produced is proportional to the amount of viable cells, and the percentage of viable to dead cells was calculated by comparison with a control (untreated and solvent control). Viability among various C. olitorius treatments described above were compared to the viability of cells treated only with mycotoxins by applying the same protocol described before, but omitting aqueous extract of C. olitorius.

**Extraction of DNA**

Harvested cells were washed with PBS to remove the nonadherent dead cells. The adherent cells were PBS by trypsinizing (0.25% trypsin, 0.1% versene EDTA; purchased from Thermo Scientific, Rockford, IL, USA) and activity was stopped by addition of media. The cell suspension was centrifuged at 3,000 g for 5 min at room temperature. Genomic DNA was extracted from cells according to the Qiagen instruction manual and concentrations determined spectrophotometrically by use of the NanoDrop ND-1,000 Spectrophotometer. Purity of DNA was assessed by examining the 260/280 nm ratio.

**Random amplification of polymorphic DNA-polymerase chain reaction analysis**

Amplification of DNA fragments was carried out using an iCycler (Bio-Rad, Herts, UK) thermal cycler using 20 primers from the Operon Biotechnologies (BioCampus Colonge Nattermannalle, Germany). PCR amplification was conducted in 25 μL reaction volumes containing 10 ng genomic DNA, 12.5 pmol/L master mix (×2) (Thermo Fisher Scientific, Carlsbad, CA, USA), 1.0 units of Supertherm Taq polymerase and 50 pmol/L primer. The PCR reactions were carried out in a thermocycler (Bio-Rad C1000, Bio-Rad, Hercules, CA, USA), programed with a first denaturation for 5 min at 95 °C, followed by 40 cycles for 30 s denaturation at 95 °C, 30 s annealing at 37 °C and 1 min extension at 72 °C. Final extension at 72 °C for 5 min was allowed before holding at 4 °C for 5 min. Reaction products were stored at -80 °C prior to electrophoresis.

**Gel electrophoresis**

Amplified products together with marker (100 bp DNA) were resolved by gel electrophoresis (60 V/cm for 135 min) on 2% agarose gel in tris-acetate-EDTA buffer containing 0.001 mg/mL ethidium bromide purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gels were photographed by Gel Documentation System (Gensnap) software (Syngen, UK).

**Band analysis**

The gels for control and exposed DNA were run for each of the 20 primers [Table 1]. A DNA ladder of 100 bp was also run in each gel. The bands for PCR products were analyzed by TotalLab Quant (V11.5: TL100-LX59-7YF4-EX). The fluorimetric profiles of each amplification reaction were studied both qualitatively and quantitatively by comparing profiles from control and DNA exposed to the extracts. Each change observed in random amplification of polymorphic DNA (RAPD) profiles of treated groups (disappearances and appearance of bands in comparison to the control RAPD profiles) was given the arbitrary score of +1. The mean was then calculated for each experimental group exposed to the mycotoxins for varying time periods. Template genomic stability (%) was calculated as “100 - (100a/n)” where “a” is the average number of changes in DNA profiles and “n” is the number of bands selected in control DNA profiles.

**Statistical analysis**

All data were statistically analyzed with the Graphpad Prism 4.02 Inc. (La Jolla, CA, USA). The significance of the

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
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<tbody>
<tr>
<td>D01</td>
<td>ACGCCGAAGG</td>
<td>D11</td>
<td>AGGCCCATTG</td>
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<td>D02</td>
<td>GGACCCCAAC</td>
<td>D12</td>
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<td>D13</td>
<td>GGGGTGACGA</td>
</tr>
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<td>D04</td>
<td>TCGGTGAGG</td>
<td>D14</td>
<td>CTCCCACAG</td>
</tr>
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<td>D05</td>
<td>TACGGCGACA</td>
<td>D15</td>
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</tr>
<tr>
<td>D06</td>
<td>ACCGTGACGG</td>
<td>D16</td>
<td>AGGGCTGCTA</td>
</tr>
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<td>TGTGCCAGGG</td>
<td>D17</td>
<td>TTCCAGGCTCG</td>
</tr>
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<td>D08</td>
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<td>GGTCTACACC</td>
<td>D20</td>
<td>ACCCGGTCAC</td>
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</tbody>
</table>
Incubation of H4IIE-luc cells with AFB_1 + FB_1 for 24 h resulted in greater cytotoxicity to cells as measured by the MTT assay, with significant toxicity at the higher concentration (250 μg/mL). Addition of C. olitorius extract to cells resulted in a reduction of cytotoxicity. At lesser concentrations of AFB_1 (1.25 μmol/L) + FB_1 (12.5 μmol/L), protective effects of aqueous extracts of C. olitorius on viability of cells was more pronounced relative to the cells that did not receive plant extract [Figure 2].

The EC_{50} values for AFB_1 were 6.9 and 1.8 after 24 and 48 h of exposure, respectively. When C. olitorius extract was added, the EC_{50} values were 4.3 and 2.49 after 24 or 48 h of exposure, respectively [Table 3]. At the lesser concentration, FB_1 did not cause measurable cytotoxicity. However, the MTT assay revealed cytotoxicity at the greater concentration (200 μmol/L) although all doses studied were less than those required to obtain an EC_{50}.

Only 5 of 10 oligonucleotide primers, primers D07, D09, D13, D15, and D16, used to measure responses of molecular-genetic parameters of cells among various treatments, gave detectable bands [Figure 3]. A total of 75 DNA sequences, ranging from 144 to 2,000 bp, were observed. All of the bands were "polymorphic" given 100% polymorphism for control cells and the other treatments for the 2 time periods using all primers. Quantitative analysis of these bands, expressed as a percentage of band loss, showed a time-dependent relationship [Figure 3 and Table 4]. Similarly, in the case of losses of bands after the shorter period of exposure (24 h), 12 of 75 bands (16%) had disappeared [Figure 3a]. At the longer duration of exposure (48 h), 21 of 75 bands (28%) had disappeared [Figure 3b]. Protective effects of C. olitorius extract were observed after 24 h, when 25 of 75 bands (33.3%) were "polymorphic" given 100% polymorphism for control cells and the other treatments for the 2 time periods using all primers.

Table 2: Summary of Wilcoxon matched pair tests to compare the viability of rat hepatoma H4IIE-luc cell line treated with C. olitorius extract

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Exposure time</th>
<th>C. olitorius extract concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 μg/mL</td>
</tr>
<tr>
<td>FB_1</td>
<td>24 h</td>
<td>0.04*</td>
</tr>
<tr>
<td>AFB_1</td>
<td>24 h</td>
<td>0.9</td>
</tr>
<tr>
<td>FB_1 + AFB_1</td>
<td>24 h</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*P ≤ 0.05. AFB_1: aflatoxin B_1; FB_1: fumonisin B_1; C. olitorius: Cochlospermum olitorius

Table 3: EC_{50} values of AFB_1, FB_1, and AFB_1 + FB_1 alone or in combination with the C. olitorius extract after 24 and 48 h and exposure measured by the MTT bioassay using H4IE-luc rat hepatoma cells

<table>
<thead>
<tr>
<th>Mycotoxin and/or plant extract treatments</th>
<th>Time exposure (h)</th>
<th>Cytotoxicity (EC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB_1</td>
<td>24</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>ND</td>
</tr>
<tr>
<td>AFB_1</td>
<td>24</td>
<td>6.90</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.95</td>
</tr>
<tr>
<td>FB_1 + AFB_1</td>
<td>24</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>6.8</td>
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<tr>
<td>FB_1 + C. olitorius (20 μg/mL)</td>
<td>24</td>
<td>542.8</td>
</tr>
<tr>
<td>FB_1 + C. olitorius (40 μg/mL)</td>
<td>24</td>
<td>26646</td>
</tr>
<tr>
<td>AFB_1 + C. olitorius (20 μg/mL)</td>
<td>24</td>
<td>4.32</td>
</tr>
<tr>
<td>AFB_1 + C. olitorius (40 μg/mL)</td>
<td>24</td>
<td>2.42</td>
</tr>
<tr>
<td>FB_1 + AFB_1 + C. olitorius (20 μg/mL)</td>
<td>24</td>
<td>18.5</td>
</tr>
<tr>
<td>FB_1 + AFB_1 + C. olitorius (40 μg/mL)</td>
<td>24</td>
<td>21.77</td>
</tr>
</tbody>
</table>

AFB_1: aflatoxin B_1; FB_1: fumonisin B_1; C. olitorius: Cochlospermum olitorius; ND: not detectable; MTT: methylthiazole tetrazolium
Figure 1: Cytotoxicity of (a) AFB$_1$ at concentrations of 0.25-50 μmol/L without and with \textit{C. olitorius} extract, (b) FB$_1$ at concentration of 1-200 μmol/L without and with \textit{C. olitorius} extract, and (c) AFB$_1$ at concentrations of 0.25-50 μmol/L together with concentrations of 1-200 μmol/L FB$_1$, on proliferation of H4IIE-luc cell line determined by MTT bioassay. Data represent mean ± SEM of triplicates (significance of the differences among treatment groups: *$P< 0.05$; **$P< 0.01$; ***$P< 0.001$). AFB$_1$: aflatoxin B$_1$; FB$_1$: fumonisin B$_1$; \textit{C. olitorius}: Cochorois olitorius; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: standard error mean.
Figure 2: RAPD profiles of genomic DNA from cell line of rat, hepatoma (H4IIE-luc) cells, following exposure to FB₁ and/or AFB₁ for various time periods. (a) PCR products with primer OPD 07. (b) PCR products with primer OPD 09. Lane 1: the DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (1 μmol/L); lane 4: cells plus FB₁ (200 μmol/L); lane 5: cells plus AFB₁ (0.25 μmol/L); lane 6: cells plus AFB₁ (50 μmol/L); lane 7: cells plus mixture (1 μmol/L FB₁ + 0.25 μmol/L AFB₁); lane 8: cells plus mixture (200 μmol/L FB₁ + 0.25 μmol/L AFB₁); lane 9: cells plus C. oltiorius (40 μg/mL); lane 10: C. oltiorius (40 μg/mL) plus FB₁ (1 μmol/L); lane 11: C. oltiorius (40 μg/mL) plus FB₁ (200 μmol/L); lane 12: C. oltiorius (40 μg/mL) plus AFB₁ (0.25 μmol/L); lane 13: C. oltiorius (40 μg/mL) plus AFB₁ (50 μmol/L); lane 14: C. oltiorius (40 μg/mL) plus (1 μmol/L FB₁ + 0.25 μmol/L AFB₁); and lane 15: C. oltiorius (40 μg/mL) plus (200 μmol/L FB₁ + 50 μmol/L AFB₁). (c) PCR products with primer OPD 13. (d) PCR products with primer OPD 16. Lane 1: DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (1 μmol/L); lane 4: cells plus AFB₁ (0.25 μmol/L); lane 5: cells plus mixture (1 μmol/L FB₁ and 0.25 μmol/L AFB₁); lane 6: cells plus C. oltiorius (40 μg/mL); lane 7: C. oltiorius (40 μg/mL) plus FB₁ (1 μmol/L); lane 8: C. oltiorius (40 μg/mL) plus AFB₁ (0.25 μmol/L); and lane 9: C. oltiorius (40 μg/mL) plus mixture (1 μmol/L FB₁ and 0.25 μmol/L AFB₁). (e) PCR products with primer OPD 16. Lane 1 and 10: DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (200 μmol/L); lane 4: cells plus AFB₁ (50 μmol/L); lane 5: cells plus mixture (200 μmol/L FB₁ and 50 μmol/L AFB₁); lane 6: cells plus C. oltiorius (40 μg/mL); lane 7: C. oltiorius (40 μg/mL) plus FB₁ (200 μmol/L); lane 8: C. oltiorius (40 μg/mL) plus AFB₁ (50 μmol/L); and lane 9: C. oltiorius (40 μg/mL) plus mixture (200 μmol/L FB₁ and 50 μmol/L AFB₁). AFB₁: aflatoxin B₁; FB₁: fumonisin B₁; C.: Cochorus oltiorius; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction. (a) OPD 07 for lesser (24 h) exposure; (b) OPD 09 for greater (48 h) exposure; (c) OPD 13 for greater (48 h) exposure; (d) OPD 16 for lesser (24 h) exposure; (e) OPD 16 for greater (48 h) exposure

had disappeared, while for the 48 h and exposure, 30 of 75 bands (40%) had disappeared.

In cases where bands were gained after exposure to C. oltiorius extract at the shorter duration of exposure, 21 new bands out of 75 (28%) were amplified. A similar trend was observed during the longer exposure, where 25 of 75 bands (33.3%) appeared [Figure 3c]. Protective effects of C. oltiorius extract were observed as new bands appeared during the 24 h, since 32 of 75 bands (42.7%) appeared;
during the longer exposure, 23 of 75 bands (30.7%) disappeared [Figure 3d].

When OPD 9 primer was used, a maximum of 10 RAPD-PCR bands disappeared when cells were exposed to the mixture of FB1 and AFB1 + aqueous extract of C. olitorius for 48 h [Table 4]. However, when with OPD 15 was used as the primer, the maximum appearance of new bands showed the same number of bands lost (10) that was observed in cells exposed to AFB1 + aqueous extract of C. olitorius after 24 h.

There was a significant difference in stability of the DNA template between control and each of the treated groups [Figure 4]. However, no significant difference was observed in stability of the DNA template between control and cells exposed to the aqueous extract of C. olitorius alone. The protective effect of the aqueous extract of C. olitorius on DNA was observed in the cells exposed to FB1 and AFB1.

### DISCUSSION

Aflatoxin B1 and FB1 are the most frequently observed mycotoxins in food and animal feed. In African and European countries, both mycotoxins are found in maize. Toxicity and carcinogenicity of AFB1, which has been classified as Group 1 carcinogen are thought to be directly linked to its bioactivation, resulting in a reactive form of AFB1, the 8, 9-epoxide. Bioactivation of AFB1 occurs primarily by a microsomal cytochrome P450-dependent epoxidation of the terminal furan ring of AFB1, which is responsible for binding to cellular macromolecules such as DNA, RNA and other protein constituents. The MTT assay is more sensitive and reproducible than testing intact animals and is valuable in determining the modes of action of toxins. In the current study, H4IIE-luc cells responded to FB1 and AFB1 as well as a mixture of the two mycotoxins. Cytotoxic effects of FB1 have been previously observed for murine microglial cells and primary astrocytes, rat glioblastoma cells, human keratinocytes and esophageal epithelial cells, primary keratinocytes and esophageal epithelial cells,

#### Table 4: Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from H4IIE-luc rat hepatoma cell line following exposure to FB1 and/or AFB1 alone and in combination with the C. olitorius extract for 24 and 48 h

<table>
<thead>
<tr>
<th>Primer</th>
<th>Change in the RAPD profile</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
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<th>T9</th>
<th>T10</th>
<th>T11</th>
<th>T12</th>
<th>T13</th>
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</thead>
<tbody>
<tr>
<td>OPD 7 (24 h)</td>
<td>Appeared</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>4</td>
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<td>0</td>
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<td>OPD 9 (48 h)</td>
<td>Appeared</td>
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<td>1</td>
<td>0</td>
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<td></td>
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T1: control; T2: FB1 (1 μmol/L); T3: FB1 (200 μmol/L); T4: AFB1 (0.25 μmol/L); T5: AFB1 (50 μmol/L); T6: 1 μmol/L FB1 + 0.25 μmol/L AFB1; T7: 200 μmol/L FB1 + 50 μmol/L AFB1; T8: C. olitorius 40 μg/mL; T9: C. olitorius 40 μg/mL + 1 μmol/L FB1; T10: C. olitorius 40 μg/mL + 200 μmol/L FB1; T11: C. olitorius 40 μg/mL + 0.25 μmol/L AFB1; T12: C. olitorius 40 μg/mL + 50 μmol/L AFB1; T13: C. olitorius 40 μg/mL + (1 μmol/L FB1 + 0.25 μmol/L AFB1); T14: C. olitorius 40 μg/mL + (200 μmol/L FB1 + 50 μmol/L AFB1); AFB1: aflatoxin B1; FB1: fumonisin B1; C. olitorius: Cochorois olitorius; RAPD: random amplification of polymorphic DNA

![Figure 4: Stability (%) of DNA templates, as determined RAPD-PCR in rat hepatoma cells (H4IIE-luc) following exposure to FB1 and/or AFB1 for 24 or 48 h.](image-url)
In the current study, EC_{50} could not be calculated for FB1 because viability of cells exposed to 200 μmol/L was reduced only 41.6%, which is consistent with previously published results.[44] In yet another study, FB1 was only weakly cytotoxic.[57] The EC_{50} for AFB1 was 1.87 μmol/L, which is similar to that observed previously by others,[58-60] who reported EC_{50} values ranging from 0.065 μmol/L for B-CMV1A2 cells to 14 μmol/L in BE12-6 cells. Exposure of H4IIE-luc cells to greater concentrations of AFB1 and FB1 resulted in lethality that was a concentration- and time-dependent. This effect was greater in cells treated with AFB1 or AFB1 + FB1. The interaction of FB1 and AFB1 in the induction of DNA damage and its correlation with biomarkers of cellular oxidative status has previously been reported to occur in vivo.[4,8,22,61] These reports suggested that genotoxicity and carcinogenicity of AFB1 were enhanced by exposure to FB1.[8] The in vivo results indicated that these effects were due to the production of ROS, which resulted in lipid peroxidation.[14,61]

AFB1 is a well-known genotoxicant. When the mechanism by which the aqueous extract of C. olitorius protected H4IIE-luc rat hepatoma cells against genetic damage caused by AFB1 and/or FB1 was investigated by use of RAPD analysis, there were statistically significant differences in the profiles of expression of the investigated genes, between the control and the treated cell lines at all concentrations tested.[58] Differences in the profile between the control and the treated samples were due to point mutations and/or base modifications of the genome caused by AFB1 and/or FB1.[62] Changes were observed for all primers which for primers were used. In our study, both qualitative and quantitative analyses showed that both mycotoxins increased instability of DNA templates of cells, in time- and concentration-dependent manners. This result supports the conclusion that both mycotoxins are direct-acting, genotoxicants that have the potential to attack hotspots present in DNA. The number of stable bands increased as a function of time and dose. Inconsistency in profiles of bands in RAPD analyses might have been observed because the two mycotoxins are acting directly as genotoxicants. However, they might act as genotoxicants through generation of free radicals during metabolism of the toxins through reactions of either electrophiles or nucleophiles with DNA. This interaction creates changes in their sequences that ultimately results in the formation of new priming sites and/or disappearances of existing priming sites for the RAPD primers. Thus, it gives different RAPD profiles for cells exposed to toxins.[63]

Random amplification of polymorphic DNA-PCR suffers from inherent limitations such as a lack of reproducibility and occurrence of pseudo-bands which prevent its routine application.[64] However, if conditions of the assays are properly optimized, these limitations can be resolved.[65,66] By optimizing conditions of the analysis, cloning the PCR products and further sequencing the products, RAPD can be useful in analyzing the nature and mode of action of the genotoxicants.[65,66] While in the present study RAPD could detect toxin-induced DNA damage, further studies would be needed before it could be used regularly as a tool in the detection of alterations in DNA sequence due to the genotoxicants.

Previous studies have demonstrated that certain compounds in the diet can offer protection against toxicity of mycotoxins.[67] Natural vitamins, carotenoids, polyphenol and trace elements are potentially beneficial in protection against mycotoxicosis.[68] Green leafy vegetables are known to be dietary sources of minerals, trace elements and phytochemicals that contribute to health.[69] Molecular evidence has suggested that trace elements and antioxidant molecules in green, leafy vegetables lessen risks of cancer and cardiovascular diseases through mechanisms that modulate free radical attack on nucleic acids, proteins, and polysaturated fatty acids.[70] C. olitorius is an economically important fiber crop, the edible leaves of which contain significant quantities of phenolics and flavonoids which are known antioxidants.[51,71-74] Although in the current study the active compound(s) in the aqueous extract of C. olitorius were not isolated or identified, flavonoids are possible candidates among the active compound(s) in C. olitorius. C. olitorius contains abundant amounts of a number of flavonoids that could act as antioxidants, including: 5-cafeoylquinic acid, 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucone), quercetin 3-(malonylgalactoside), ascorbic acid, a-tocopherol, and chlorophyll.[29] Furthermore, C. olitorius contains relatively high levels of quercetin glycosides. Several novel flavonol glycosides named corchorusides A and B, in addition to a major component, capsugenin-25, 30-O-β-glycosides have been isolated from C. olitorius.[26] Recently, several flavonoids, such as rutin, and quercetin and phenolic compounds, including gallic acid, chlorogenic acid, p-cumaric acid, ferulic
In conclusion, both AFB₁ and FB₁ induced oxidative stress, which resulted in cytotoxicity and fragmentation of DNA of H4IIE-luc rat hepatoma cells after various durations of exposure to these toxins singly or in combination. Exposure to these mycotoxins resulted in appearance of new bands in the RAPD analysis, in addition to DNA damage. Treatment with an aqueous extract of *C. olitorius* resulted in a significant improvement in viability of cells and reduced damage to DNA in H4IIE-luc cells exposed to mycotoxins. Due to these effects, *C. olitorius* is suggested to be a traditional edible plant containing potential chemo-preventive agents for human cancers. However, additional studies on the uptake, metabolism, and disposition of the active ingredients in *C. olitorius* need to be further studied. Currently, the active ingredient(s) are unknown, and it is also not known whether these constituents that are effective *in vitro* can have similar effects *in vivo*.

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**REFERENCES**


8. Abdel-Wahhab MA, Hassan NS, El-Kady AA, Khadratty WA, El-Nekeety AA, Mohamed SR, Sharaf IA, Mannaa FS, Red ginseng extract protects against aflatoxin B₁ and fumonisins-induced hepatic pre-cancerous lesions in rats. *Food Chem Toxicol* 2010;48:733-42.


37. Atienza FA, Jha AN. The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: a critical review. Mutat Res 2006;613:76-102.


39. Koudajo JD, Mobio TA, Baudrinmont I, Moukha S, Dano SD, Creppy EE. Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B₁ in human intestinal cell line Caco-2. Toxicology 2005;213:56-65.


43. Osuchowski MF, Sharma RP. Fumonisin B₁ induces necrotic cell death in BV-2 cells and murine cultured astrocytes and is antiproliferative in BV-2 cells while N2A cells and primary cortical neurons are resistant. Neurotoxicology 2005;26:981-92.


52. Koudajo JD, Dano SD, Moukha S, Mobio TA, Creppy EE. Effects...
of combinations of Fusarium mycotoxins on the inhibition of macromolecular synthesis, malondialdehyde levels, DNA methylation and fragmentation, and viability in Caco-2 cells. Toxicon 2007;49:306-17.


