Original Article

Aqueous extract of *Corchorus olitorius* decreases cytotoxicity of aflatoxin B$_1$ and fumonisin B$_1$ in H4IIE-*luc* cells

Mohamed I. M. Ibrahim[1], Rialet Pieters[2], Sekena H. Abdel-Aziem[3], Anna M. van der Walt[2], Cornelius C. Bezuidenhout[2], John P. Giesy[4,5,6,7,8,9], Mosaad A. Abdel-Wahhab[1]

[1]*Department of Food Toxicology and Contaminants, National Research Centre, Dokki, Cairo 12622, Egypt*
[2]*Unit of Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa*
[3]*Department of Cell Biology, National Research Centre, Dokki, Cairo 12622, Egypt*
[4]*Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada*
[5]*Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA*
[6]*Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong, China*
[7]*School of Biological Sciences, University of Hong Kong, Hong Kong, China*
[8]*State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, Jiangsu, China*
[9]*Department of Biology, Hong Kong Baptist University, Hong Kong, China*

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 *Cochorus 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

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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.[2] 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.[3] The importance of co-occurrence of mycotoxins lies in the changes that might occur in the combined toxicity of mycotoxins.[4] In addition, the existence of relationships in the occurrences of mycotoxins allows predictions of the presence of individual mycotoxins from the presence of others.[1]

Among these mycotoxins, aflatoxin B_1 (AFB$_1$) is the predominant contaminant in cereals and oilseed and presents a significant risk,[5] due to being hepatotoxic and carcinogenic to humans and animals.[4,6-8] AFB$_1$ is classified by the International Agency of Research on Cancer (IARC) as a Group 1 carcinogen.[9] This mycotoxin is also mutagenic, teratogenic, and immunosuppressive in farm, and laboratory animals,[10-12] and primarily affects cell-mediated immunity.[13] AFB$_1$ is also able to induce reactive oxygen species (ROS),[8,14-16] 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 B_1 (FB$_1$), which causes esophageal and hepatic cancer in humans and liver and kidney cancer in rodents.[17-19] IARC evaluated FB$_1$ and classified it as probably carcinogenic to humans (Group 2B).[20] Moreover, FB$_1$ modulates immunity in animals and decreases viability of lymphocytes in poultry.[21]

Humans and animals are constantly exposed to small concentrations of these mycotoxins, either individually or in combination.[22] Mycotoxicoses occur seasonally in areas that have not implemented effective prophylactic measures.[23] While interactions between mycotoxins had been discussed,[24] 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.[25,26] Its health benefits have been reported to include antitumor activity by inhibiting tumorigenesis,[27] antioxidant properties,[28] and antibacterial activity.[29] 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.[28,30] It has also been reported that compounds such as carotenoids, flavonoids, and vitamin C, isolated from leaves of *C. olitorius,* exhibit significant antioxidant characteristics.[30] In addition, leaves of *C. olitorius* have been reported to have ethno-medicinal

importance as a demulcent and febrifuge[31] and also possess anti-inflammatory, analgesic, and antimicrobial activities.[32,33] 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 FB$_1$ and AFB$_1$ in the rat hepatoma cell line (H4IIE-*luc*).

METHODS

Chemicals
Aflatoxin B_1 and FB$_1$ (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.[34-37] 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[38] including mixtures.[39]

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 NaHCO$_3$ 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% CO$_2$, 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 AFB_1 (50, 25, 2.5, 0.25, 0.025 µmol/L) dissolved in methanol, or of FB_1 (200, 100, 10, 1, 0.1 µmol/L) dissolved in methanol. A combination of the already mentioned concentrations of AFB_1 and FB_1 were also tested: 50 µmol/L AFB_1 + 200 µmol/L FB_1; 25 µmol/L AFB_1 + 100 µmol/L FB_1, 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-dimethyltiazol-2yl)-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.[40] 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 by 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 removed 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.[41]

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 (Synegen, 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.[42]

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

Table 1: Sequences of the primers used to amplify DNA of H4IIE-*luc* rat hepatoma cells

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
D01	ACCGCGAAGG	D11	AGCGCCATTG
D02	GGACCCAACC	D12	CACCGTATCC
D03	GTCGCCGTCA	D13	GGGGTGACGA
D04	TCTGGTGAGG	D14	CTTCCCCAAG
D05	TGAGCGGACA	D15	CATCCGTGCT
D06	ACCTGAACGG	D16	AGGGCGTAAG
D07	TTGGCACGGG	D17	TTTCCCACGG
D08	GTGTGCCCCA	D18	GAGAGCCAAC
D09	CTCTGGAGAC	D19	CTGGGGACTT
D10	GGTCTACACC	D20	ACCCGGTCAC

differences among treatment groups was determined with two-way analysis of variance. The assumptions of parametric statistics were confirmed. Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. All statements of significance were based on a probability of $P \leq 0.05$.

RESULTS

The results of cell viability assay revealed that H4IIE-*luc* cells that were treated with both concentrations (20 and 40 μg/mL) of *C. olitorius* extract were statistically significantly more viable after 24 h exposure than the ones that were not treated with the plant extract. However, there was no significant protection by the plant extract against FB_1 after 48 h of exposure. Furthermore, *C. olitorius* extract could not protect the cells from the AFB_1 concentration series or the combination exposure ($AFB_1 + FB_1$) irrespective of the exposure period (24 or 48 h) [Table 2]. A dose-dependent decrease of cell viability after exposure to increasing amounts of AFB_1 was observed only after 48 h [Figure 1a]. After 24 h exposure, the response was not linear (hormetic effect). Except for the lowest concentration, cytotoxicity was more pronounced after 48 h. However, protective effects of the *C. olitorius* extract were observed after both 24 h and 48 h of exposure to AFB_1. After 48 h, viability, expressed as a percentage of H4IIE-*luc* cells affected by FB_1, was approximately 40% less than that of cells exposed to FB_1 alone. After 48 h, there was also no dose-dependence, but cytotoxicity was less pronounced. Protective effects of 20 or 40 μg/mL *C. olitorius* extract were observed. After both 24 and 48 h of exposure, production of MTT formazan was greater in the presence of both concentrations of *C. olitorius* extract at all tested doses of FB_1 compared to those in the absence of *C. olitorius* extract [Figure 1b]. No significant differences were found between 20 and 40 μg/mL of *C. olitorius* extract after 24 h of exposure.

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 sum of the two mycotoxin concentrations 12.5 and 125 μmol/L [Figure 1c]. The cells were least viable when they were exposed to the mixture of 250 μmol/L mycotoxin. Addition of *C. olitorius* extract to cells resulted in slightly greater viability. 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 greater 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%)

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

Mycotoxins	Exposure time	*C. olitorius* extract concentrations	
		20 μg/mL	40 μg/mL
FB_1	24 h	0.04*	0.04*
	48 h	0.69	0.89
AFB_1	24 h	0.9	0.5
	48 h	0.35	0.89
$FB_1 + AFB_1$	24 h	0.69	0.5
	48 h	0.22	0.08

*$P \leq 0.05$. AFB_1: aflatoxin B_1; FB_1: fumonisin B_1; *C. olitorius: Cochorus 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 H4IIE-*luc* rat hepatoma cells

Mycotoxin and/or plant extract treatments	Time exposure (h)	Cytotoxicity (EC_{50}) H4IIE-*luc*
FB_1	24	ND
	48	ND
AFB_1	24	6.90
	48	1.95
$FB_1 + AFB_1$	24	14.5
	48	6.8
FB_1+ *C. olitorius* (20 μg/mL)	24	542.8
FB_1+ *C. olitorius* (40 μg/mL)	24	26646
AFB_1+ *C. olitorius* (20 μg/mL)	24	4.32
AFB_1+ *C. olitorius* (40 μg/mL)	24	2.42
$FB_1 + AFB_1$+ *C. olitorius* (20 μg/mL)	24	18.5
$FB_1 + AFB_1$+ *C. olitorius* (40 μg/mL)	24	21.77

AFB1: aflatoxin B_1; FB_1: fumonisin B_1; *C. olitorius: Cochorus olitorius*; ND: not detectable; MTT: methylthiazole tetrazolium
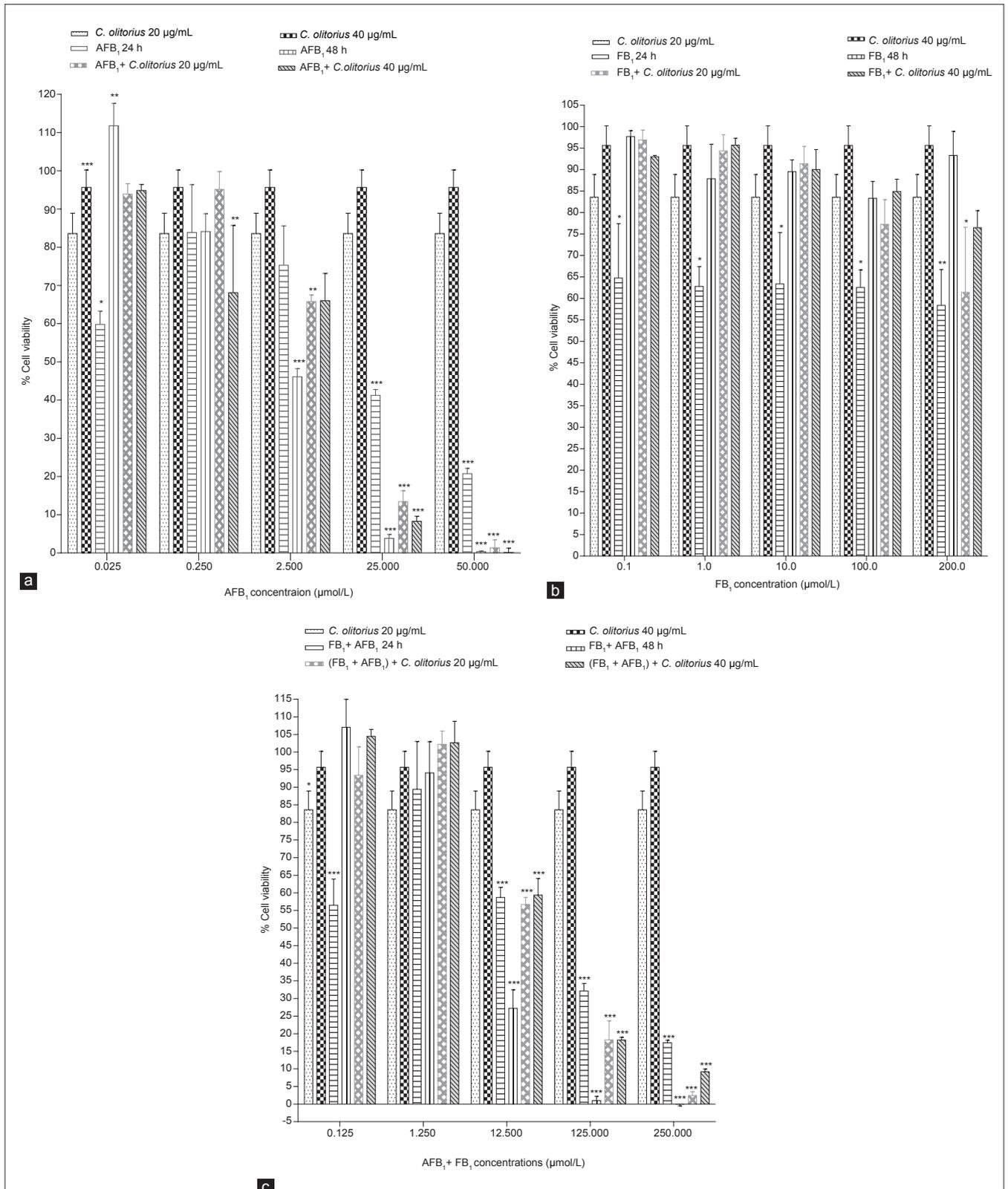
Figure 1: Cytotoxcity of (a) AFB$_1$ at concentrations of 0.25-50 μmol/L without and with *C. olitorius* extract, (b) FB$_1$ at concentration of 1-200 μmol/L without and with *C. olitorius* extract, and (c) AFB$_1$ at concentrations of 0.25-50 μmol/L together with concentrations of 1-200 μmol/L FB$_1$, 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$_1$; *C. olitorius*: *Cochorus olitorius*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: standard error mean
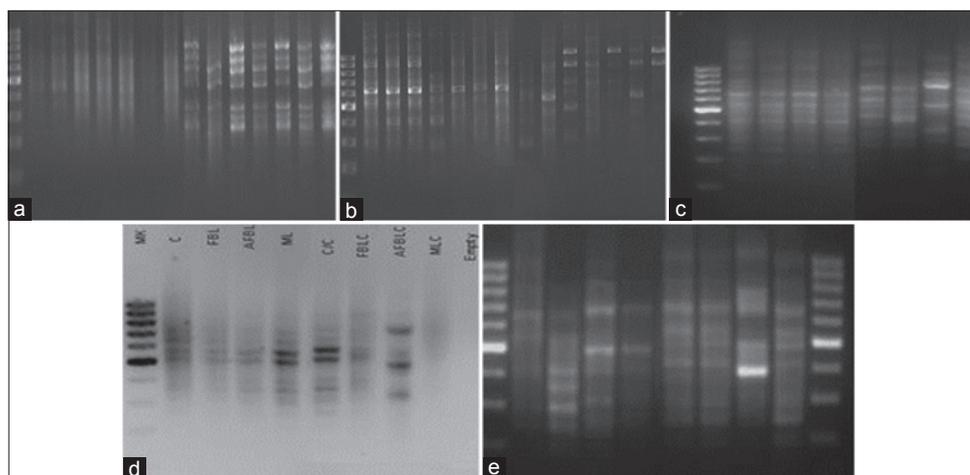
Figure 2: RAPD profiles of genomic DNA from cell line of rat, hepatoma (H4IIE-*luc*) cells, following exposure to FB_1 and/or AFB_1 for various time periods. (a) PCR products with primer OPD 07. (b) PCR products with primer OPD 09. Lane 1: the DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB_1 (1 μmol/L); lane 4: cells plus FB_1 (200 μmol/L); lane 5: cells plus AFB_1 (0.25 μmol/L); lane 6: cells plus AFB_1 (50 μmol/L); lane 7: cells plus mixture (1 μmol/L FB_1 + 0.25 μmol/L AFB_1); lane 8: cells plus mixture (200 μmol/L FB_1 + 50 μmol/L AFB_1); lane 9: cells plus *C. olitorius* (40 μg/mL); lane 10: *C. olitorius* (40 μg/mL) plus FB_1 (1 μmol/L); lane 11: *C. olitorius* (40 μg/mL) plus FB_1 (200 μmol/L); lane 12: *C. olitorius* (40 μg/mL) plus AFB_1 (0.25 μmol/L); lane 13: *C. olitorius* (40 μg/mL) plus AFB_1 (50 μmol/L); lane 14: *C. olitorius* (40 μg/mL) plus (1 μmol/L FB_1 + 0.25 μmol/L AFB_1); and lane 15: *C. olitorius* (40 μg/mL) plus (200 μmol/L FB_1 + 50 μmol/L AFB_1). (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 FB_1 (1 μmol/L); lane 4: cells plus AFB_1 (0.25 μmol/L); lane 5: cells plus mixture (1 μmol/L FB_1 and 0.25 μmol/L AFB_1); lane 6: cells plus *C. olitorius* (40 μg/mL); lane 7: *C. olitorius* (40 μg/mL) plus FB_1 (1 μmol/L); lane 8: *C. olitorius* (40 μg/mL) plus AFB_1 (0.25 μmol/L); and lane 9: *C. olitorius* (40 μg/mL) plus mixture (1 μmol/L FB_1 and 0.25 μmol/L AFB_1). (e) PCR products with primer OPD 16. Lane 1 and 10: DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB_1 (200 μmol/L); lane 4: cells plus AFB_1 (50 μmol/L); lane 5: cells plus mixture (200 μmol/L FB_1 and 50 μmol/L AFB_1); lane 6: cells plus *C. olitorius* (40 μg/mL); lane 7: *C. olitorius* (40 μg/mL) plus FB_1 (200 μmol/L); lane 8: *C. olitorius* (40 μg/mL) plus AFB_1 (50 μmol/L); and lane 9: *C. olitorius* (40 μg/mL) plus mixture (200 μmol/L FB_1 and 50 μmol/L AFB_1). AFB_1: aflatoxin B1; FB_1: fumonisin B1; *C. olitorius*: *Cochorus olitorius*; 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
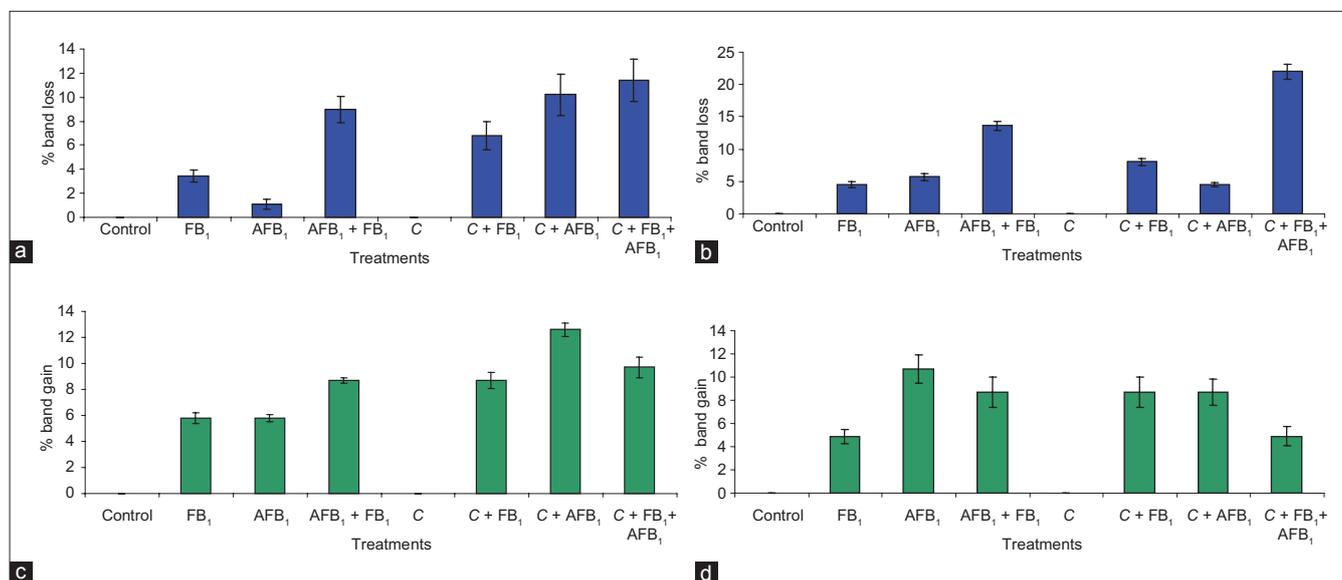

Figure 3: Genomic damage: the percentage of altered bands in each treatment at high concentration detected by RAPD-PCR. (a) Average band loss at lesser concentrations for 24 h; (b) average band loss at greater concentrations for 48 h; (c) average band gains at lesser concentrations for 24 h; and (d) average band gain at greater concentrations for 48 h. AFB_1: aflatoxin B_1; FB_1: fumonisin B_1; C: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

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. olitorius* 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. olitorius* 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 disappeared when cells were exposed to the mixture of FB_1 and AFB_1 + 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 AFB_1 + 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 FB_1 and AFB_1.

DISCUSSION

Aflatoxin B_1 and FB_1 are the most frequently observed mycotoxins in food and animal feed. In African and European countries, both mycotoxins are found in maize.[43] Toxicity and carcinogenicity of AFB_1, which has been classified as Group 1 carcinogen are thought to be directly linked to its bioactivation, resulting in a reactive form of AFB_1, the 8, 9-epoxide. Bioactivation of AFB_1 occurs primarily by a microsomal cytochrome P450-dependent epoxidation of the terminal furan ring of AFB_1, which is responsible for binding to cellular macromolecules such as DNA, RNA and other protein constituents.[44-47] 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 FB_1 and AFB_1 as well as a mixture of the two mycotoxins. Cytotoxic effects of FB_1 have been previously observed for murine microglial cells and primary astrocytes,[48] rat glioblastoma cells,[49,50] human keratinocytes and esophageal epithelial cells,[51] primary

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 FB_1 and/or AFB_1 alone and in combination with the *C. olitorius* extract for 24 and 48 h

Primer	Change in the RAPD profile	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
OPD 7 (24 h)	Appeared	0	3	5	4	4	0	6	0	0	1	1	1	3	0
	Disappeared	0	0	0	0	0	0	0	0	2	0	0	0	0	0
OPD 9 (48 h)	Appeared	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Disappeared	0	0	0	1	3	3	2	0	0	1	5	0	4	10
OPD 13 (48 h)	Appeared	0	0	0	0	3	3	0	0	0	0	0	0	0	0
	Disappeared	0	0	3	0	0	0	2	0	4	6	4	4	6	5
OPD 15 (24 h)	Appeared	0	0	0	1	4	6	3	0	5	2	10	3	0	0
	Disappeared	0	3	1	0	0	0	0	0	0	0	0	0	0	4
OPD 16 (48 h)	Appeared	0	2	0	1	0	0	0	0	4	6	3	6	7	5
	Disappeared	0	0	0	0	2	5	8	0	0	0	0	0	0	0

T1: control; T2: FB_1 (1 µmol/L); T3: FB_1 (200 µmol/L); T4: AFB_1 (0.25 µmol/L); T5: AFB_1 (50 µmol/L); T6: 1 µmol/L FB_1 + 0.25 µmol/L AFB_1; T7: 200 µmol/L FB_1 + 50 µmol/L AFB_1; T8: *C. olitorius* 40 µg/mL; T9: *C. olitorius* 40 µg/mL + 1 µmol/L FB_1; T10: *C. olitorius* 40 µg/mL + 200 µmol/L FB_1; T11: *C. olitorius* 40 µg/mL + 0.25 µmol/L AFB_1; T12: *C. olitorius* 40 µg/mL + 50 µmol/L AFB_1; T13: *C. olitorius* 40 µg/mL + (1 µmol/L FB_1 + 0.25 µmol/L AFB_1); T14: *C. olitorius* 40 µg/mL + (200 µmol/L FB_1 + 50 µmol/L AFB_1). AFB_1: aflatoxin B_1; FB_1: fumonisin B_1; *C. olitorius*: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA
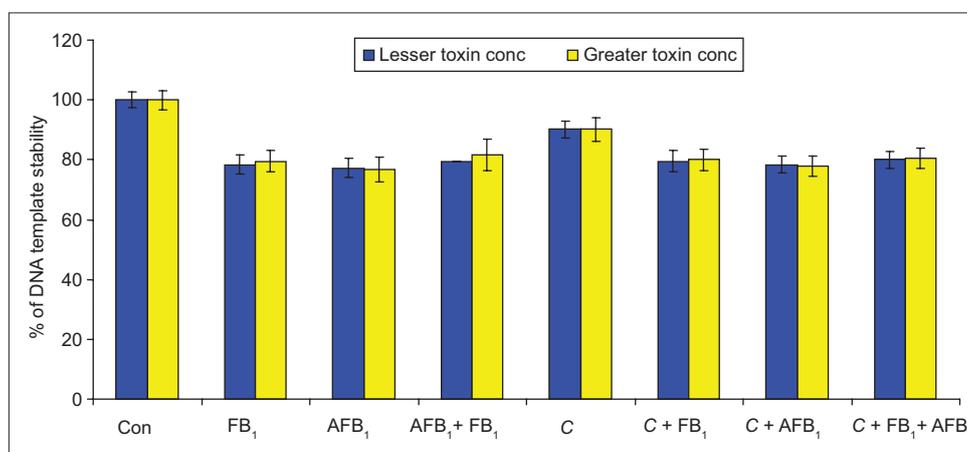

Figure 4: Stability (%) of DNA templates, as determined RAPD-PCR in rat hepatoma cells (H4IIE-*luc*) following exposure to FB_1 and/or AFB_1 for 24 or 48 h. Con: control; AFB1: aflatoxin B_1; FB1: fumonisin B_1; C: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

rat hepatocytes and rat liver *in vivo*.[52] In human and rat glioblastoma cells and mouse hypothalamic cells, production of ROS was increased after exposure to 10-100 μmol/L of FB_1 for 48-144 h.[53] Exposure to 10-100 μmol/L of FB_1 for 72 h had no effect on production of ROS in human fibroblasts, or in primary cultures of rat astrocytes exposed to the same concentrations of FB_1 for as long as 6 days.[54,55] Exposure to concentrations as high as 20 μmol/L of FB_1 did not significantly reduce the viability of IPEC-J2 cells.[56]

In the current study, EC_{50} could not be calculated for FB_1 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, FB_1 was only weakly cytotoxicity.[57] The EC_{50} for AFB_1 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 AFB_1 and FB_1 resulted in lethality that was a concentration- and time-dependent. This effect was greater in cells treated with AFB_1 or AFB_1 + FB_1. The interaction of FB_1 and AFB_1 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 AFB_1 were enhanced by exposure to FB_1.[8] The *in vivo* results indicated that these effects were due to the production of ROS, which resulted in lipid peroxidation.[4,61]

AFB_1 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 AFB_1 and/or FB_1 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.[33] 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 AFB_1 and/or FB_1.[62] Changes were observed for all genes for which 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 polyunsaturated 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.[33,71-74] 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-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside), 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-diglucopyranoside 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

acid, and ellagic acid have been isolated from extracts of *C. olitorius*.[75] Consequently, protective effects of the aqueous extract of *C. olitorius* against cytotoxicities of AFB_1 and FB_1 in H4IIE-*luc* might be due to the antioxidant capacity and the abundant occurrence of the flavonoid compounds. Another candidate for the active compound(s) is chlorophyll. Numerous *in vitro* studies have indicated that derivatives of chlorophyll, including chlorophyllide A and B and pheophorbide A and B can attenuate chemical genotoxicity by forming a molecular complex with pro-mutagens,[74-76] which might involve strong chlorophyll-AFB_1 and/or FB_1 interaction via their planar unsaturated cyclic rings.[75] Derivatives of pheophorbide A and B provided additional protection not only by direct trapping, but also by increasing glutathione S-transferase activity against hepatic AFB_1 metabolites.[76]

In conclusion, both AFB_1 and FB_1 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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