Protective effects of *Amaranthus hybridus* against aflatoxin B_1 and fumonisin B_1-induced genotoxicity in H4IIE-*luc* cells

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ABSTRACT

Aim: Protective effects of aqueous extract of *Amaranthus hybridus* against aflatoxin B_1 (AFB_1) and/or fumonisin B_1 (FB_1) on the H4IIE-*luc* cell line were determined by use of the methyl thiazol tetrazolium viability assay and disruption of DNA integrity. **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 *A. hybridus*. **Results:** AFB_1 decreased the viability of cells after 24 and 48 h of exposure. EC_{50} values for AFB_1 were 10.5 and 1.8 μmol/L for the two periods, respectively. When the 48 h exposure to mycotoxin repeated with a pre-treatment of 20 and 40 μg/mL extract of *A. hybridus*, the EC_{50} changed to 3.88 and 7.67 μmol/L, respectively. H4IIE-*luc* cells exposed to FB_1 for 24 h responded more than those incubated for 48 h. Cells treated with a combination of AFB_1 and FB_1 were less viable with a significant decrease in the greater concentration. The mixture of AFB_1 and FB_1 resulted in a significant threat to H4IIE-*luc* as indicated by the absence or appearance of new bands in random amplified polymorphic DNA analysis, which demonstrated damage to DNA. The protective effects were probably due to greater content of total phenolics, carotenoids, β-carotene, folic-, linolenic-, linoleic and palmitic acids, as well as calcium, magnesium, iron, zinc, and selenium observed in the extract. **Conclusion:** Exposure to 40 μg/mL of extract of *A. hybridus* protected cells from damage to DNA by stabilizing DNA.

Key words: Aflatoxin B_1; *Amaranthus hybridus;* cytotoxicity; DNA; fumonisin B_1; hepatoma cells; methyl thiazol tetrazolium assay

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INTRODUCTION

Mycotoxins are secondary metabolites of fungi which are associated with certain disorders in animals and humans. Contamination of grains by mycotoxins is a worldwide problem affecting staple crops such as corn (maize) and small grains (such as wheat), as well as tree nuts, peanuts, sorghum, and others.[1] Some mycotoxins are now linked with the incidence of certain types of cancer, and it is this aspect that has evoked global concern over feed and food safety.[2] Aflatoxins (AFs), ochratoxins, trichothecenes, zearalenone, fumonisins (FBs), tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance[3] and are known to be hepatotoxic, genotoxic, immunosuppressive, nephrotoxic, teratogenic, and carcinogenic.[4] The Food and Agricultural Organization of the United Nations has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins.[5] However, in Africa, the presence of mycotoxins in food is often overlooked due to the population's ignorance, lack of regulatory mechanisms, poor facilities for storing large volumes of food products, and the introduction of contaminated commodities into the human food chain during chronic food shortage caused by droughts, wars, and political and economic instability.[6]

At present, the interactions between AFB_1 and FB_1 with regard to their toxic and carcinogenic properties were discussed in several reports. A synergistic effect between exposure to mycotoxins and some important diseases in Africa, such as malaria, kwashiorkor, liver cancer, and human immunodeficiency virus (HIV)/acquired immune deficiency syndrome has been suggested.[7] Concerns about mycotoxins have increased during the last few decades because of their implications to human and animal health and productivity, as well as the economics of their management, and how they influence international trade.[8] This has led to the development of maximum tolerated limits for mycotoxins in various countries. The European Union has legislated maximum permitted levels of 2 ng/g dry mass for AFB_1 and 4 ng/g for total AFs (B_1, B_2, G_1, and G_2) in various products.[9] Considering the extremely potent carcinogenicity of AFs, most developed nations regulate limits of AFs as small as reasonably achievable. Several studies have shown that AFB_1 and FB_1 are cytotoxic, inhibiting the viability of different cellular models, mostly liver and kidney cells.[10-16]

The *Amaranthus* plant has been used extensively in the rural South Africa as a traditional food and is commonly known as *morogo*. *Amaranthus* species are good sources of β-carotene, polyphenols, Vitamin C, calcium, and iron.[17,18] Moreover, a joint publication of the United Nations Development Program and the Food and Agriculture Organization, expressed the view that wild-growing food plants are an affordable and practical source of nutrition to improve the nutritional status of rural HIV-affected households.[19] The present study was carried out to assess whether the aqueous extracts of *A. hybridus* can protect rat hepatoma cells against FB_1 and AFB_1 induced cytotoxicity and disruption of DNA integrity.

METHODS

Chemicals

FB_1 and AFB_1 (98% purity) and other standards were purchased from Sigma Chemicals Co (St. Louis, MO, USA). The DNA extraction kit (DNeasy blood and tissue kit) was obtained from Qiagen (Hilden, Germany). The one hundred base pair (bp) DNA ladder, polymerase chain reaction (PCR) master mix, and DNase/RNase free water were obtained from Fermentas Inc., (Glen Burnie, Maryland, USA). Supertherm *Taq* polymerase was purchased from JM Holding (UK). Forty primers were obtained from Operon Technologies (Alameda, CA, USA). All solvents were of analytical grade and were purchased from Burdick and Jackson (Muskegon, MI, USA).

Plant materials

Stems and leaves of *A. hybridus* were collected from a residential garden in the city of Potchefstroom, North-West Province, South Africa. The plant material was freeze-dried and pulverized and 1 g dry mass of the lyophilized plant powder 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.

Determination of chemical composition of the extract

Extraction of total phenolic contents in the plant was carried out in triplicate, according to the modified method of van der Walt *et al*.[18] and Kähkönen *et al*.[20] Total phenolic content was expressed as mean ± standard deviation (SD) and gallic acid equivalents were expressed in mg/100 g dm.

Total carotenoid content for the plant was extracted and analyzed in triplicate as described by Edwards *et al*.[21] and modified by van der Walt *et al*.[18] Total carotenoid content is presented as mean ± SD in mg/100 g dm.

Quantification of beta-carotene

Beta-carotene was extracted according to the modified method described by Lakshiminarayana *et al*.[22] and was quantified by high-pressure liquid chromatography coupled to a photodiode array detector capable of ultraviolet-visible absorption spectrum according to the methods of de Ancos *et al*.[23] The content was expressed as mean ± SD in mg/100 g dm.

Folic acid

Folic acid was quantified at the South African Bureau of Standards (Pretoria, South Africa). A standard method for the microbiological assay of folic acid in foods and pharmaceutical products was followed according to Barton-Wright[24] and AOAC.[25]

Quantification of fatty acids

Fatty acids were identified and quantified by use of gas chromatography coupled with mass spectrometry system with split-less injection. An Agilent 6890 gas chromatograph ported to a 5973 mass selective detector (CA, USA) was used according to the method described by van der Walt *et al*.[17]

Mineral and trace element analysis

Minerals and trace elements were quantified by use of an Agilent 7500c inductively coupled argon plasma mass spectrometer as described by van der Walt *et al*.[18] Three separate samples were analyzed and values were reported as the mean ± SD in mg/100 g dm.

Cytotoxicity measurements

The mammalian model was rat hepatoma cells (H4IIE-*luc*) that had been transfected stably with a firefly luciferase reporter gene under control of the dioxin response element and thus the aryl hydrocarbon receptor mechanism.[26] These cells had originally been developed as a reporter gene assay to detect and semi-quantify the levels of certain groups of persistent organic pollutants.[26] Since these cells are essentially still mammalian cells, they were useful to assess whether extracts of selected *A. hybridus* can be protective against AFB_1 and FB_1 or their mixture.

Cells were seeded with a density of 1.0×10^4 cells/mL media in the inner 60 wells of a 96-well microplate. Growth medium was Dulbecco's modified Eagle's medium (Sigma, D2902) supplemented with 0.044 mol/L $NaHCO_3$ and 10% fetal bovine serum (Gibco). The volume in each well was 250 μL. The outer cells received 250 μL Dulbecco's phosphate buffered saline (PBS) to create a homogenous microclimate across all wells containing cells and incubation conditions were 37 °C in a humidified 5% CO_2:air mixture. The plates were seeded and after an initial 24 h incubation medium was replaced with medium containing varying concentrations of AFB_1 (50, 25, 2.5, 0.25, 0.025 μmol/L) and 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 plus 200 μmol/L FB_1; 25 μmol/L AFB_1 plus 100 μmol/L FB_1; and so on. Two exposure periods 24 h and 48 h were investigated. In order to evaluate the protective effect of extracts of *A. hybridus*, this experiment was repeated with the following adjustments: After the initial 24 h incubation period, the media was replaced with media containing either 20 or 40 μg/mL *A. hybridus* and incubated for another 24 h which was followed by the mycotoxin exposure routine, but for only the 48 h period. The controls included (1) 11 wells with cells and nutrient medium only for the duration of the entire experiment (when media was replaced, their media was replaced with fresh nutrient medium) and (2) 6 wells with cells and plant extract containing media only. The mycotoxin exposures were dosed in triplicate.

The viability of H4IIE-*luc* cells was determined using the methyl thiazol tetrazolium (MTT) salt assay in which the mitochondria of live cells metabolize the yellow MTT into blue formazan.[27] A final concentration of 500 μg/mL MTT was incubated for 30 min and blue formazan crystals dissolved with dimethyl sulfoxide. The absorbance was measured spectrophotometrically at 560 nm. The amount of formazan gives an estimation of the proportion of viable cells. The percentage of viable to dead cells was calculated by comparison with a control (untreated and solvent control). The MTT assay assessed the viability of H4IIE-*luc* cells that were subjected to the two *A. hybridus* extract concentration treatments compared to the viability of cells that were not treated with *A. hybridus* extracts prior to mycotoxin exposure.

DNA extraction

Cells were harvested by first washing away non-adherent dead cells with PBS before trypsinizing (0.25% trypsin, 0.1% versene ethylenediaminetetraacetic acid) adherent cells. Enzyme activity was stopped by the addition of media. The cell suspension was centrifuged for 5 min (300 g) at room temperature. The genomic DNA was extracted from the cells, according to the Qiagen instruction manual. The concentration of DNA was determined by photometry (NanoDrop ND-1000 Spectrophotometer) and the purity of the DNA was judged by examining the ratio of absorbency at 260/280 nm.[28]

Random amplified polymorphic DNA-polymerase chain reaction analysis

Amplification of DNA fragments was carried out on an ICycler (Bio-Rad, UK) thermal cycler using 20 primers purchased from the Operon Biotechnologies (BioCampus Cologne Nattermannalle, Germany). PCR amplification was conducted in a 25 μL reaction volume containing 10 ng genomic DNA, 12.5 pmol Master mix (2X) (Fermentas Life Science, USA), 1.0 units of Supertherm *Taq* polymerase, and 50 pmol primer. PCR reactions were carried out in a thermocycler (Bio-Rad C1000) programmed with initial denaturation period for 5 min at 95 °C, followed by 40 cycles denaturation (95 °C for 30 s), primary annealing at 37 °C for 1 min and extension at 72 °C. Amplification was terminated by a final extension period of

72 °C for 5 min. Reaction products were stored at -80 °C prior to electrophoresis.

Gel electrophoresis

Amplified products together with a marker (100 bp DNA) were resolved by gel electrophoresis 60 V/cm for 135 min on 2% agarose gel in TAE buffer containing 0.001 mg/mL ethidium bromide. Gels were photographed by a Gel Documentation system (Gensnap) equipped with its software (Synegen, UK).

Band analysis

Gels of control and exposed DNA samples were run for each of the 20 primers [Table 1]. A DNA ladder of 100 bps was also run in each gel. Bands in 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 the control and DNA exposed to the extracts. Each change observed in random amplified polymorphic DNA (RAPD) profiles of the treated groups (disappearances and appearance of bands in comparison to the control RAPD profiles) was given the arbitrary score of +1. The average was then calculated for each experimental group exposed to the mycotoxins for varying time periods. Genomic stability (%) was calculated as "100 - (100 a/n)" where "a" is the average number of changes in DNA profiles and "n" is the number of bands selected in control DNA profiles.[29]

Statistical analysis

Values for EC_{50} and cell viability were statistically analyzed with the Graphpad Prism 4.02 Inc., (La Jolla, CA, USA). Significance of differences among treatment groups was determined with the Waller-Duncan k-ratio.[30] All statements of significance were based on a probability of $P < 0.05$.

RESULTS

The extract was rich in polyphenols (total phenolic contents: 2181.2 mg/100 g dm, total carotenoids (113.6 mg/100 g dm) and β-carotene (18.4 g/100 g dm) [Figure 1a]. The results of the lipid profile showed significant amounts of the fatty acids, linolenic, linoleic and palmitic acids [Figure 1b]. The extract had moderate concentrations of palmitoleic, stearic, and lignoceric acids whereas behenic, arachidic, and myristic acids were found in low concentrations. The extract was rich in folic acid (72 mg/100 g dm), calcium, magnesium [Figure 1c], iron, zinc, and selenium [Figure 1d].

Cytotoxicity of AFB_1, FB_1, and mixture with or without the extract of *A. hybridus* on H4IIE-*luc* cell line as measured by the tetrazolium dye-based MTT assay are shown in Figure 2a-d. There was a significant difference in viability between cells treated with 20 μg/mL *A. hybridus* and those not treated before exposure to AFB_1 for 48 h [Table 2]. Other combinations did not have any statistically significant difference. Percentage inhibition of the cells incubated for 24 h with FB_1 showed more cytotoxicity than those incubated for 48 h. On the other hand, FB_1 at the concentration of 200 μmol/L decreased cell viability to 41.6% [Figure 2b]. The protective effect of 20 μg/mL *A. hybridus* extract was decreased by increasing FB_1 dose to 100 μmol/L [Figure 2b]. *A. hybridus* extract at 40 μg/mL was more efficient at protection against all concentrations of FB_1.

Overall, AFB_1 was more cytotoxic than FB_1 for both exposure periods [Figure 2a and b]. Exposure of H4IIE-*luc* cells to AFB_1 led to a dose-and time-dependent decrease in cell viability. At 25 μmol/L AFB_1, viability was inhibited to 58.7% and 96.1% for the 24 and 48 h exposure periods respectively. Pre-treating the cells to 40 μg/mL *A. hybridus* had a more protective effect than pre-treatment of 20 μg/mL [Figure 2a].

The combination of AFB_1 and FB_1 was more cytotoxic than AFB_1 alone which indicated that FB_1 increased the cytotoxicity. This was true for both exposure periods [Figure 2a-c]. However, this general trend was not corroborated by the EC_{50} values (EC_{50} = concentration by which viability was declined to 50%) [Table 3]. They were in fact slightly greater for the combined mycotoxins than exposure to AFB_1 alone, meaning that 50% effect was reached at a greater mycotoxin concentration. Extract of *A. hybridus* alone (20-100 μg/mL) had no significant influence on the viability of cells [Figure 2d].

Table 1: Sequences of the primers used to amplify cell line of hepatoma (H4IIE-*luc*) 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

Table 2: Summary of the *P* values of the Wilcoxon matched pair tests to compare viability of cells exposed to FB_1, AFB_1, and their mixture and those treated with *A. hybridus* extracts prior to 48 h mycotoxin exposure

Mycotoxins	*A. hybridus* extract	
	20 μg/mL	40 μg/mL
FB_1	0.04*	0.69
AFB_1	0.5	0.08
FB_1 + AFB_1	0.69	0.2

*$P < 0.05$. FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; *A. hybridus: Amaranthus hybridus*

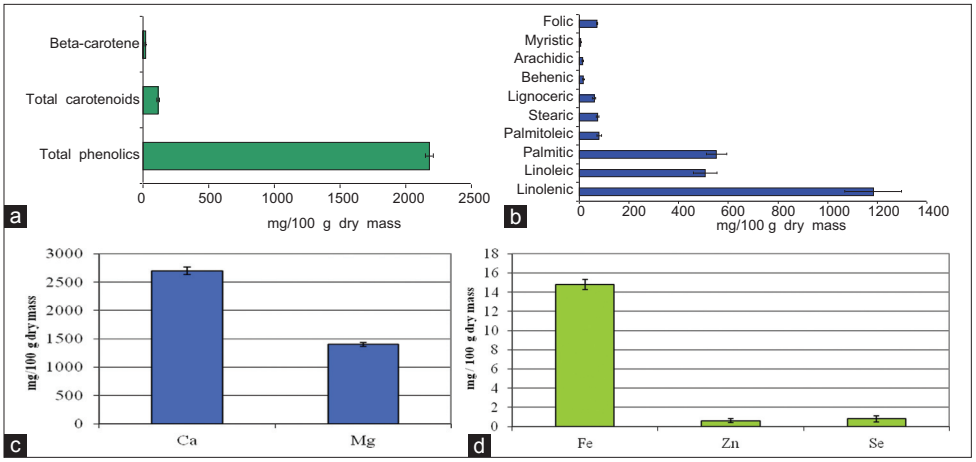

Figure 1: (a) Total phenolics, total carotenoids, and β-carotene content; (b) fatty acid profiles and folic acid content; (c) calcium and magnesium concentration; and (d) trace elements (iron, zinc, and selenium) concentration of *Amaranthus hybridus* extract

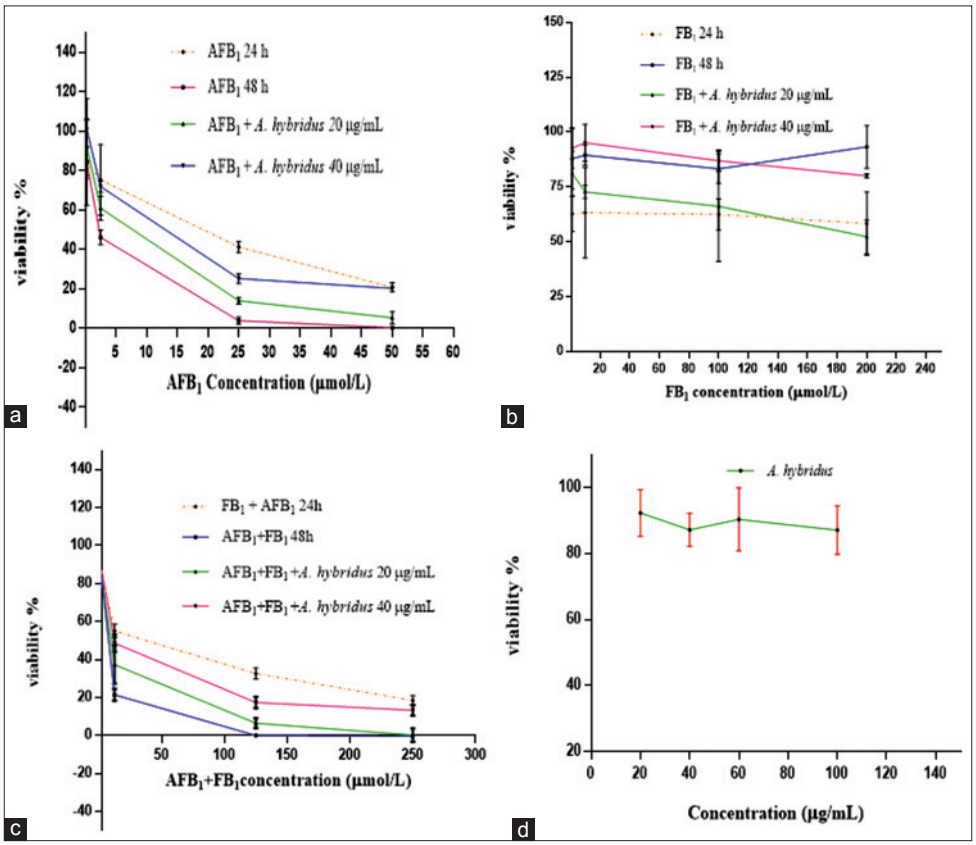

Figure 2: The cytotoxic effects of AFB_1 at different concentrations (µmol/L) without/with *Amaranthus hybridus* extract: (a) FB_1 at different concentrations (µmol/L) without/with A*maranthus hybridus*; (b) AFB_1 plus with FB1 without/with A*maranthus hybridus*; (c) A*maranthus hybridus* extract only; and (d) on proliferation of H4IIE-*luc* cell line determined by MTT bioassay. FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; MTT: methyl thiazol tetrazolium

EC_{50} values for AFB_1 were 10.5 and 1.8 µmol/L after 24 and 48 h of exposure, respectively. When the plant extract was added at 20 and 40 µg/mL, the EC_{50} values were 3.88 and 7.67 µmol/L after 48 h of exposure, respectively. On the other hand, the EC_{50} for the combined mycotoxins (AFB_1 + FB_1) was 24.02 and 5.86 µmol/L after 24 and 48 h, respectively. While the 50% inhibition of proliferation for AFB_1 plus FB_1 with plant extract were 7.30 and 14.0 µmol/L after the addition of 20 or 40 µg/mL *A. hybridus* extract, respectively. No discernible cytotoxicity was observed in cells, based on the MTT assay, when cells were exposed to FB_1 at lesser concentrations. Whereas, at greater concentrations sufficient cell mortality was exhibited in the MTT assay that indicates cytotoxicity at dosages of 200 µmol/L but the quantities tested were still substantially less than those required to obtain EC_{50}.

Genetic variability among treated cells was evaluated using 10 oligonucleotide primers. Only five primers, D07, D09, D13, D15, and D16, gave positive and detectable bands [Figure 3]

Table 3: The EC_{50}-values of AFB_1 and/or FB_1 alone or in combination with *A. hybridus* at two exposure periods using the H4IIE-*luc* cell line

Mycotoxin/ plant extract	Pre-treatment concentration of *A. hybridus* (μg/mL)	Mycotoxin exposure time (h)	EC_{50} (μmol/L)
FB_1	-	24	ND
	-	48	ND
	20	48	ND
	40	48	ND
AFB_1	-	24	10.55
	-	48	1.84
	20	48	3.88
	40	48	7.67
FB_1 + AFB_1	-	24	24.02
	-	48	5.86
	20	48	7.30
	40	48	14.00
A. hybridus			5592 μg/mL

FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; ND: not detectable; *A. hybridus: Amaranthus hybridus*

since they amplified a total of 69 different bands ranging from 144 to 2000 bp. All 69 bands were "polymorphic" given 100% polymorphism for control cells, FB_1, AFB_1, AFB_1 plus FB_1, plant extract at 40 μg/mL, 40 μg/mL plant extract plus FB_1, 40 μg/mL extract plus AFB_1 and 40 μg/mL extract plus 0.025 and 50 μmol/L AFB_1, respectively, for all primers used. Of the 69 scorable bands, 18 (26%) were similar "monomorphic" to the control and the 40 μg/mL *A. hybridus* treatment (D09-700, D09-525, D09-363, D13-363, D15-1080, D15-869, D15-646, D15-547, D15-447, D15-325, D15-229, D15-176, D16-183, D16-1267, D16-813, D16-679, D16-536, and D16-417; 1 band (1.4%) was similar for control and *A. hybridus* extract at 40 μg/mL in all treatments after the addition of the extract at 40 μg/mL for all treatments (D16-646).

Quantitative analysis of these bands, expressed as a percentage of band loss, shows a time-dependent relationship. The increase in band loss is related to the increase in time period [Table 3 and Figure 4]. Similarly, in case of band loss at the short exposure period (24 h), 26 out of 66 bands (39.4%) disappeared [Figure 4a]. At the 48 h exposure period, 44 out of 65 bands vanished which representing 75.4% [Figure 4b].

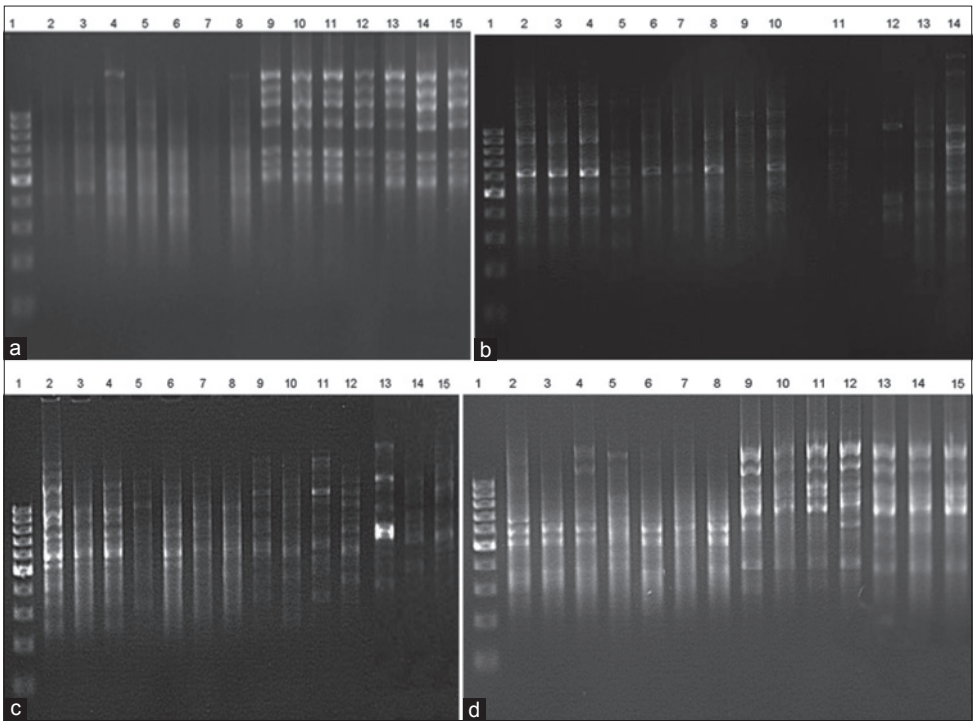

Figure 3: (a-d) RAPD profiles of genomic DNA from cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB_1 and/or AFB_1 for various time periods. Four pictures represents PCR products with primer OPD07 (a), OPD09 (b), OPD15 (c), and OPD16 (d), respectively, at less and greater concentration. The DNA marker (100 bp) in lanes 1 and 2 represents cells only; lane 3 represents cells plus FB_1 (0.1 μmol/L); lane 4 represents cells plus FB_1 (200 μmol/L); lane 5 represents cells plus AFB_1 (0.025 μmol/L); lane 6 represents cells plus AFB_1 (50 μmol/L); lane 7 represents cells plus the mixture of FB_1 and AFB_1 (0.1 + 0.025 μmol/L); lane 8 represents cells plus the mixture of FB_1 and AFB_1 (200 + 50 μmol/L); lane 9 represents cells plus *Amaranthus hybridus* extract (40 μg/mL); lane 10 represents *A. hybridus* extract (40 μg/mL) plus FB_1 (0.1 μmol/L); lane 11 represents *Amaranthus hybridus* extract (40 μg/mL) plus FB_1 (200 μmol/L); lane 12 represents *Amaranthus hybridus* extract (40 μg/mL) plus AFB_1 (0.025 μmol/L); lane 13 represents *Amaranthus hybridus* extract (40 μg/mL) plus AFB_1 (50 μmol/L); lane 14 represents *Amaranthus hybridus* extract (40 μg/mL) plus the mixture of FB_1 and AFB_1 (0.1 + 0.025 μmol/L), and lane 15 represents *Amaranthus hybridus* extract (40 μg/mL) plus the mixture of FB_1 and AFB_1 (200 + 50 μmol/L). FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

A protective effect of the extract at 40 µg/mL was observed for the short exposure period and 14 out of 69 bands (20.29%) disappeared, as compared to the 28 out of 69 bands (40.58%) for the longer period which disappeared.

Meanwhile, bands also appeared at the short exposure period, 43 new bands out of 66 bands were amplified which represents 65.2%. In the same trend, at the longer exposure period, 31 out of 65 appeared which represents 47.7% [Figure 4c]. The protective effect of the 40 µg/mL plant extract was observed as the production of new bands appeared at the short exposure period (24 h) since 22 out of 66 bands emerged which represents 33.3% while at the long exposure period, 6 out of 66 bands occurred and represented 9.09% [Figure 4d].

Profiles of RAPD-PCR and the number of bands that appeared or disappeared in the DNA of hepatoma H4IIE-*luc* at various exposure periods are shown in Table 4. A maximum of 9 bands vanished in the mixture of FB_1 at 200 µmol/L and AFB_1 at 50 µmol/L plus the plant extract-treated cells for 48 h with OPD 15 primer. Whereas, the maximum appearance of 7 new bands were observed in the AFB_1 at 50 µmol/L plus plant extract-exposed cells at 48 h with OPD 15 too.

The percentage of DNA template stability in the treated cells in comparison to the controls at various concentrations is presented in Figure 5. The results showed that there was a significant difference in the DNA template stability between the control and all the treated groups, no significant difference was observed in the DNA template stability between the

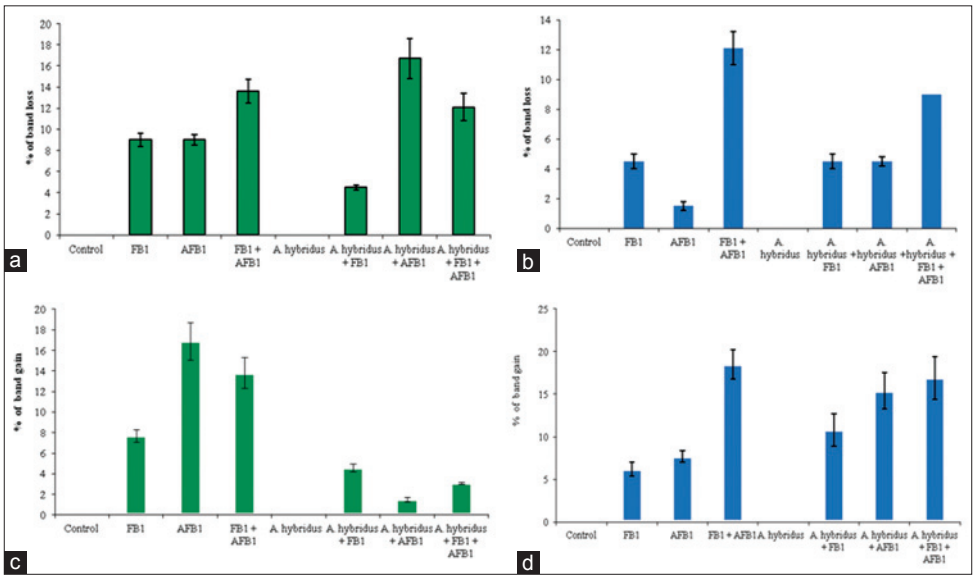

Figure 4: Genomic damage. The percentage of altered bands in each treatment of low and high concentration of FB_1 and AFB_1 detected by RAPD-PCR. (a) Average band loss after 24 h; (b) average band loss after 48 h; (c) average band gains after 24 h; and (d) average band gain after 48 h. FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

Table 4: Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB_1 and/or AFB_1 for various time periods

Name of primer	Change in RAPD profile	Control	FB_1 (LC)	FB_1 (HC)	AFB_1 (LC)	AFB_1 (HC)	AFB_1 + FB_1 (LC)	AFB_1 + FB_1 (HC)	AE	AE + FB_1 (LC)	AE + FB_1 (HC)	AE + AFB_1 (LC)	AE + AFB_1 (HC)	AE + AFB_1 + FB_1 (LC)	AE AFB_1 + FB_1 (HC)
D7	A	0	3	5	4	4	0	6	0	0	2	1	0	1	0
	D	0	0	0	0	0	0	0	0	1	0	0	0	0	0
D9	A	0	1	0	0	0	0	0	0	0	0	0	1	5	0
	D	0	0	0	1	3	3	2	0	0	6	3	0	0	2
D13	A	0	0	0	0	3	3	0	0	1	0	3	0	2	1
	D	0	0	3	0	0	0	2	0	0	0	0	2	0	0
D15	A	0	0	0	1	4	6	3	0	2	1	7	0	0	0
	D	0	3	1	0	0	0	0	0	0	0	0	5	6	9
D16	A	0	2	0	1	0	0	0	0	0	0	0	0	0	1
	D	0	0	0	0	2	5	8	0	2	1	2	3	0	0

RAPD: random amplified polymorphic DNA; FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; AE: *Amaranthus* extract (40 µg/mL); LC: low concentration (1 µmol/L for FB_1 and 0.25 µmol/L for AFB_1); HC: high concentration (200 µmol/L for FB_1 and 50 µmol/L for AFB_1); A: appeared; D: disappeared

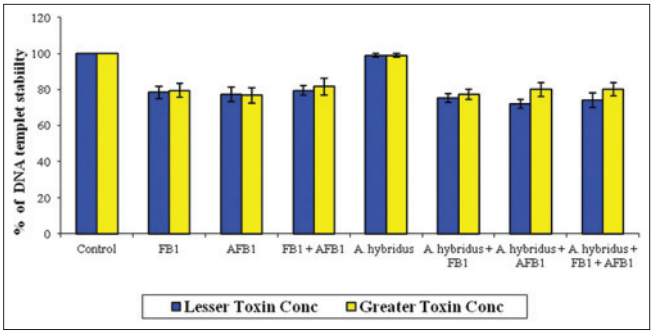

Figure 5: Template DNA stability (%) in cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB_1 and/or AFB_1 for various time periods evaluated in RAPD-PCR. FB_1: fumonisin B_1; AFB_1: aflatoxin B_1; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

control and cells treated with 40 μg/mL *A. hybridus* extract. The protective effect of the extract at 40 μg/mL appeared as marked in the stability of DNA in all treatments.

DISCUSSION

Polyphenols are a class of phytochemicals that contribute to the total antioxidant capacity of dark green leafy vegetables.[31] They have aromatic rings and achieve their antioxidant activities mainly through the donation of hydrogens.[32] In the current study, *A. hybridus* extract was found to be enriched in phenolic compounds in amounts comparable to those of conventional and commercially-grown non-conventional vegetables.[17] Total phenolic concentrations reported herein were similar to those reported in commercial spinach.[33] Total phenolic concentrations in leaves of commercially-produced *Ipomoea batata,* which is also eaten as *morogo* in South Africa were similar.[34]

Carotenoids are pigment molecules responsible for the color of many fruits and vegetables, have important functions in photosynthesis and are abundant in plant leaves. Carotenoid and beta-carotene concentrations reported in the current study were comparable with that of baby spinach reported previously.[35] Bioavailability of carotenoids in dark green leafy vegetables is reduced by the leaf matrix.[36] Notwithstanding this limitation, and distinct from being Vitamin A precursors, carotenoids also exhibit considerable antioxidant capacity based on their symmetrical linear 40-carbon tetraterpene structure, which features alternating double and single carbon-carbon bonds.[23,34] Folic acid concentration in *A. hybridus* reported in the current study was similar to that reported previously in African vegetables.[18] In the present study, six saturated fatty acids, one monounsaturated fatty acid, and two poly-saturated fatty acids were isolated. These results were in accordance with those reported by Weather[37] who suggested that dark green leafy vegetables generally contained small amounts of fat predominantly in the form of polyunsaturated fatty acids.

Calcium (Ca^{2+}) plays a vital role in regulating cellular transmembrane trafficking of elements and molecules.[38] Dark-green leafy vegetables, therefore, are primary sources of minerals and trace elements.[39] In the present study, the extract was enriched in calcium and magnesium, and trace elements iron, zinc, and selenium. Mineral and trace element content of plant leaves is a function of the environment and in leafy vegetables would be strongly influenced by the chemical composition of the soil and the climate.[40,41] The current results were similar to those reported previously,[42] which suggests that wild *morogo* should be considered an important source of calcium, magnesium, iron, and zinc, particularly for households that are not in a position to access conventional vegetables, whether for economic or demographic reasons.

Exposure of H4IIE-*luc* cells to AFB_1 resulted in the death of cells in a concentration and time-dependent manner. H4IIE-*luc* cells were more sensitive to AFB_1, and AFB_1 plus FB_1 mixture as compared to the control and FB_1 alone. The results showed that treatment with the plant extract 24 h prior to mycotoxin exposure succeeded to blocks the AFB_1 toxicity in H4IIE-*luc* cells line. This may be associated with the content of vitamins, antioxidants and minerals in the plant extract. This result suggested that natural vitamins, provitamins, carotenoids, chlorophyll, phenolics, and synthetic compounds with antioxidant properties could potentially be effective against the toxic consequence of these mycotoxins.[43]

Toxic effects of FB_1 were more pronounced after 24 h than 48 h exposure. However, the cytotoxic effect of FB_1 was eliminated at lesser concentrations, suggesting the rapid metabolism of this mycotoxin.[44] These results were similar to those observed during an *in vivo* study that proved the elevation of sphinganine was reversible after short-term exposure.[45] Disruption of sphingolipid metabolism as a specific cytotoxic response to FB_1 exposure and sphingosine reached its maximum concentration after 48 h.[46,47] Several reports indicate that FB_1 inhibited cell proliferation in different cell lines H4TG, MDCK, NIH3T3, and LLC-PKI.[47,48] Among 15 mammalian cell lines, MDCK and H4TG were found to be the most sensitive to FB_1 with EC_{50}-values of 2.5 and 4 μg/mL, respectively, after 4 days exposure.[49]

AFB_1 is a well-known genotoxicant able to alter the genetic constitution of an organism by inducing insults of various types. Changes in profiles were observed between control and all mycotoxin treatments. Differences in profiles of bands between the control and treated samples might be due to AFB_1 and/or FB_1-induced point mutations and/or base

modifications elicited in the genome.[50] All primers used in this study could detect changes in all treatments that might be due to a latent phase required for the appearance of adequate number of cells with genetic damage.

Alterations observed in the present study included the absence and/or presence of bands in all treatment groups. The appearance and disappearance of bands might be associated with genetic rearrangements or clastogenic effects of the toxicant. Such alterations in the genome might subsequently interfere with binding of primers or amplification step.[51] Increases in band intensity and appearance of new PCR products have been attributed to conformational changes in DNA,[52] which might improve the access of primer(s) to the binding site(s). Furthermore, enhancement and reduction of signal intensity of an amplified DNA fragment might be related to localized over- or under-amplification of that gene locus in the genome, which could result from changes at the chromosome level.

Instability in template DNA was observed in all treatments which may be due to DNA damage. Although RAPD appears to be instrumental in observing definitive changes, it requires enough time and sufficient theoretical knowledge for initial standardization to obtain reproducible and unambiguous results. Interpretation of molecular events responsible for differences observed in the RAPD pattern is not easy since different DNA alterations may induce similar types of changes. The RAPD is known to produce non-reproducible bands, but once established and standardized, there are certain additional benefits to using this method for early genotoxicity studies other than being fast.

Differences in sensitivity were observed, depending on the primer sequence. This observation suggests the mode of action of FB_1 and/or AFB_1. The five primers used showed a greater alteration after the treatment and the appearance of new bands in all the extracts-treated groups were produced from those primers. The mechanism by which these toxins affect the sequence of DNA has been extensively supported in the literature.[53] Some of the AFB_1 adducts have been shown to be capable of inducing base substitution, frameshifts, insertions and deletions at specific loci of the DNA. For example, AFB_1 adduct induces G > T transversion at specific loci within p53.[54,55] The resulting alterations in DNA can induce changes in the DNA sequence at specific places generating different annealing primer-template sites.[56] This is probably the reason why altered bands were always the same in most of the concentrations and the exposure periods in both the qualitative and the quantitative analysis.

Generation of new annealing primer-template sites would be in accordance with the presence of new bands in the amplification profiles. The nature of the RAPD reaction, where the final products are the result of an exponential multiplication of the most abundant and stable fragments co-amplified in the first cycles is the cause of the differences in the concordance among replicates. In other words, it is necessary that new annealing sites appear in a high proportion of the cell population to get a high reproducibility. The first new bands appeared at the high concentration of FB_1 and/or AFB_1 ($D\text{-}7_{1466}$, $D\text{-}7_{525}$) suggested that the proportion of cells with a new annealing primer-template was increased at the greater concentration. The RAPD assay is able to detect mutation only if they occur in at least 2% of the DNA.[56] A concentration-dependent effect was observed when the same chemical and the same cells were used. Similar results were previously demonstrated a dose-dependent effect of the genotoxic action of mycotoxin when measured by micronuclei induction.[57]

The combined use of *in vitro* systems and the RAPD technique permits detection of alterations in DNA caused by multiple mechanisms with a sufficient degree of sensitivity. Alterations were detected in an unspecific form by losses and/or gains of bands and variations in the amplification intensity. Nevertheless, when the objective is to establish the existence of DNA damage, that is, for hazard identification in risk assessment studies, the presence in the fingerprint of any of these abnormalities would be enough to identify a genotoxic effect. For example, the presence of one or both of the two new bands in DNA extracts of cells treated either with a chemical or with an environmental sample can be considered as a suitable genotoxicity biomarker of chronic exposure.

The protective effects of *A. hybridus* extract against FB_1 showed that the extract was more effective at its greater dose than at the lesser dose. In addition, the ability of the extract to eliminate the cytotoxic effects induced by AFB_1 appeared less effective compared with that induced by FB_1. The difference between AFB_1 and FB_1-induced cytotoxic effects may be due to the stronger oxidative stress caused by AFB_1 even at a lower concentration than FB_1. Several studies showed the benefits of antioxidant compounds in the diet against the toxicity of mycotoxins.[58] The inhibition of DNA and protein synthesis induced by AFB_1 and FB_1 were decreased by pre-treatment of the CaCo-2 and Hep G2 cell lines with the antioxidant cyaniding-3-*0*-β-glucopyranoside.[58] The ability of *A. hybridus* extract to inhibit the cytotoxic effects induced by the mixture of AFB_1 and FB_1 was more pronounced at the higher concentration of the extract (40 μg/mL) than the lower concentration (20 μg/mL). Moreover, this protective effect was smaller in the case of the mycotoxin mixture compared to that of FB_1 only. These results were similar to those reported by Guerra *et al*.[59] who suggested that the inhibitory action of

cyaniding-3-*O*-β-glucopyranoside on AFB_1 and OTA-induced toxicity is likely to be attributed to its antioxidant power.

Previous studies showed that the aqueous extract of *A. hybridus* has a significant immune-stimulating effect and its stem extract has been credited with antimalarial activity and these effects are attributed to the presence of amaranthine, isoamaranthine, hydroxycinnamates, quercetin, kaempferol glycosides, amaranthoside, amaricin, and stigmasterol glycoside.[60] These authors concluded that the hepatoprotective activity of *A. hybridus* might be due to antioxidant defence factors and phenolics might be the main constituents responsible for the activity. Isolated polyhydroxylated nerolidols which have antiradical and reducing capacities and could act as antilipoperoxidants.[61]

In conclusion, *A. hybridus* extract has a high content of total phenolic, total carotenoids, β-carotene, folic acid, linolenic, linoleic, palmitic, calcium, magnesium, iron, zinc, and selenium. AFB_1 or FB_1 alone or in combination induced toxic effects on rat hepatoma cells. However, a mixture of the two mycotoxins was most potent. The H4IIE-*luc* cells showed a weak antagonistic effect when exposed to the mixture of the two mycotixins as compared to the single toxin exposures. The viability of cells was decreased by increasing concentrations of mycotoxins. Moreover, the binary mycotoxin mixture posed a significant threat to the treated hepatoma cell line as indicated by the absence/appearance of new bands beside the severe DNA damage. Combined treatment with *A. hybridus* extract and mycotoxins resulted in significant improvement in cell viability accompanied with a significant decrease in DNA damage and genotoxic effects. This improvement was more pronounced in the individual toxin-treated cells and a dose dependent manner of the extract.

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Conflict of interest

There is no conflict of interest.

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