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Full Length Research Paper

Mycotoxins profiling of the culture material of *Fusarium verticillioides* (Sacc.) Nirenberg culture (CABI-IMI 392668) isolated from rice in Niger State, Nigeria and its cytotoxic effects on human lymphocytes comparatively to those of some mycotoxin standards

Hussaini Anthony Makun[1]*, Michael Francis Dutton[2], Mulunda Mwanza[2] and Patrick Berka Njobeh[2]

[1]Department of Biochemistry, Federal University of Technology, P.M.B 65, Minna, Niger State, Nigeria.
[2]Food, Environment and Health Research Group, Faculty of Health Science, University of Johannesburg, Doornfontein Campus, 2028 Gauteng, P.O Box 17011, South Africa.

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An extract of *Fusarium verticillioides* (Sacc.) Nirenberg culture isolated from rice in Niger State, Nigeria, previously found to be acutely toxic to mice and chicks was established to contain 8.233 ppm of total fumonisins (FBs). The present work was undertaken to evaluate the *in vitro* cytotoxic effect to human lymphocytes of the same 4-year old extract via flow cytometry over a 3 h period in comparison with those of aflatoxin B_1 (AFB_1), ochratoxin A (OTA) and fumonisin B_1 (FB_1) and to further elucidate the mycotoxins in extract using thin layer (TLC), column (CC) and high performance liquid chromatographic (HPLC) techniques. The efficiencies of different solvents for extraction of FBs were also determined. A dose-dependent response was obtained for all toxins tested via cytotoxicity assay. At concentrations of 25, 50 and 100 µg/ml, OTA was more toxic than AFB_1 followed by the extract which was comparatively as toxic as FB_1. Cytotoxicity data also revealed that, apoptosis is the major form of cell death induced by the tested mycotoxins and extract. Fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) were found in the fungal extract at concentrations of 16.302, 6.423 and 2.456 ppm, respectively. The results also demonstrated that FBs are not extractible in hexane, benzene, diethyl ether and ethyl acetate, very low amount of the toxins can be recovered using dichloromethane (5 to 7%) and acetone (<10%), while methanol, acetonitrile and their aqueous solutions (32 to 87%) were found to be the best FBs extraction solvents.

Key words: Cytotoxicity, human lymphocytes, fumonisins, rice, Nigeria.

INTRODUCTION

A surveillance study for mycotoxigenic fungi contaminating field, marketed and stored rice (*Oryza sativa)* samples collected during the three seasons from Niger State, Nigeria, was previously conducted (Makun et al., 2007). In this regard, a total of 1062 fungi were isolated and identified from 196 mouldy rice samples and found to belong mainly to *Aspergillus, Penicillium, Fusarium, Mucor* and *Rhizopus* genera. Further interperitoneal screening of the metabolites of these fungal isolates for their toxic effects in white albino mice (5 to 6 weeks old), revealed that, *Fusarium verticillioides* (Sacc.) Nirenberg (HAM/Chibani/NG/01) caused lethality to mice at 40 mg/kg body weight and was therefore, regarded as one of the 13 most toxigenic fungi identified in rice in Niger State, Nigeria (Makun et al., 2009). The same culture material was subsequently interperitoneally administered to chicks (1-week old) and albino mice (5 to 6 weeks old)

*Corresponding author. E-mail: hussainimakun@yahoo.com. Tel: 08035882233.

and was found to be acutely toxic to the experimental animals affecting mainly the liver, kidney and gastro-intestinal tract (Makun et al., 2010). Oral administration of the petroleum ether residue (PER) of the extract at 833.33 and 2500mg/kg body weight, resulted in a mortality of 20% (1/5) and 10% (1/10) in mice and chicks, respectively. The intraperitoneal LD_{50} values of the same PER in both animals were found to range between 45.4 to 87.9 mg/kg body weight with mice being more susceptible and upon further analysis of PER via veratox competitive direct enzyme linked immunosorbent assay (CD-ELISA), total fumonisin (FBs) content was found to be 8.233 ppm. Fumonisins are not known to be acutely toxic *per se* at this level (Bondy et al.,1997; Sharma et al., 1997) and thus, it was inferred that there must be some other mycotoxins other than FB_1 in the crude extract, which likely acts synergistically with FB or other toxins present in order to elicit such acute toxic response. Further physico-chemical analysis to completely elucidate the toxins present in the extract was recommended and therefore, the present study was undertaken to accomplish this task.

The toxicity potential of the fungal extract on human lymphocytes was evaluated via flow cytometry and the mycotoxins were elucidated using thin layer and high performance liquid chromatographic techniques. Rice, the source of this fungus and its toxins is a staple food not only to Nigerians, but to other communities worldwide and so the importance of this investigation borders around food safety and public health.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade and the solvents used for HPLC were of HPLC grade and were all obtained from Merck or Sigma unless otherwise stated. Culture medium (RPMI-1640, Highveld Biologicals, South Africa) supplemented with 10% foetal calf serum (CCM) and overlaid on Histopaque 1077 (Sigma, address), SAX cartridge (ANATECH, Gauteng, South Africa), mycotoxin standards Zearalenone, deoxynivalenol and T-2 toxin standards were obtained from Sigma, St. Louis, USA. Fumonisin B_1, B_2 and B_3 were purchased from PROMEC, MRC, South Africa, apoptotic detection kits, FITC Annexin V apoptosis detection kit II, Lot. 35856 (BD Biosciences, San Diego, USA. www.bdbiosciences.com). The test kit contains propidium iodide staining solution for determination of necrosis, FITC Annexin stain for apoptotic assay and annexin binding buffer.

Equipments

Flow cytometer

BD FACSCalibur automated multicolor flow cytometer (BD Biosciences, San Jose, USA) at excitation at 488 nm, using a 639 nm band pass filter to collect the red propidium iodide fluorescence. The percentage of cells in each stage of the cell cycle was estimated using the BD FACSComp™ software, in conjunction with BD Calibrite™ beads, software.

HPLC

Shimadzu system (Kyoto, Japan), consisting of liquid chromatograph LC 20A fitted to degasser DGU 20A3, auto sampler (injection) SIL 20A, communications bus module CBM 20A, column oven CTO 20A, photodiode array detector SPD M20A and fluorescence detector RF 10AXL, all connected to a gigabyte computer with Intel Core DUO with Microsoft XP. It is fitted with symmetry column (250 x 4.6 mm i.d., 5 µm particle size) with a Waters Sentry™ guard column (Waters, Milford, USA).

Culturing of fungus on maize

Maize was used in place of rice because it is a preferred natural substrate for growth and mycotoxin production by *F. verticillioides* (Marasas, 2001). To prepare a maize medium, 500 g of maize grains was weighed; 100 ml of distilled water was added, mixed thoroughly in a 2.5 L Fernbach flask and left overnight for moisture equilibration. The maize substrate was then autoclaved for 20 mins at 120°C and 15 psi pressure. After cooling, it was aseptically inoculated with conidia or mycelium of 5 day-old pure culture of *F. verticillioides* grown on potatoes dextrose agar (PDA) slant tubes. 5 ml of triton X-100 (Searle Hopkin and Williams, Chalwell Health, Essex, England) treated sterile distilled water (one drop triton X-100 to 200 ml sterile distilled water) was added to each culture tube and the surface of the culture scrapped with a sterile inoculating loop. The suspension was then inoculated in a 2.5 L Fernbach flask containing the maize substrate and the flask incubated at 28°C as a static culture for 21 days.

Extraction of toxins

Extraction of toxins from the maize fungal culture material was performed in accordance with the method devised by Gbodi (1993) using dichloromethane (DCM) as the extracting solvent. In this case, 750 ml DCM was added into the Fernbach flask containing mouldy maize chopped into small fragments and blended for three minutes. The homogenate was filtered through a bed of hyflosupercell in Buchner funnel fitted with fast flow filter paper. The filtrate was again filtered through anhydrous sodium sulphate to remove excess moisture and clarify the extract. The clarified extract was evaporated to dryness at 50°C using rotatory vacuum evaporator. The oily viscous residue was added to chilled swirling petroleum ether (1:15 residue/ petroleum ether, v/v) and the mixture kept overnight in a deep freezer at -15°C for complete precipitation of the toxins. The crude toxin was recovered by filtration and dried in a fan oven at 50°C for 3 h.

A portion of the crude toxin obtained was defatted using a mixture of petroleum ether and hexane (1:15, v/v) and filtered through a fluted No 44 Whatman filter paper and supernatant allowed to dry on the filter paper at room temperature and stored in a bottle in the deep freezer for four years until used for this study.

Cytotoxicity assay

Culturing of human lymphocytes and exposure of the cultures to mycotoxins was according to the method of Maenetje et al. (2008). Accordingly, blood (12 ml) was obtained from a healthy human donor and put into heparinised tubes and the content mixed with an equal volume of culture medium supplemented with 10% foetal calf serum (CCM) and overlaid on Histopaque 1077 and centrifuged at

800 x g for 30 min (ethical clearance NO: AEC../2009. Academic Ethic Committee 2009, Faculty of Health Sciences, University of Johannesburg). The interface layer consisting of mononuclear cells was removed using a sterile pipette. The isolated lymphocytes were washed (3 times) with CCM (5 ml) with centrifugation for 10 min. The washed pelleted cells were then resuspended in CCM (10 ml) containing phytohaemagglutinin-p (1%, Sigma Ltd.) and cultured in a 96-well flat bottomed tissue plate (each well containing 100 µl cell suspension, having 106 cells/ml and 100 µl test solution) and incubated at 37°C in a 5% CO_2-buffered and humidified. Only blood sample with cell viability of above 97% was used for the assay and the cell viability of the donated blood was determined before use as follows; The cells were mixed with trypan blue, counted using a Neubauer haemocytometer and % viability was determined as: Cell/ml = n/v x dilution factor (5) x 10^4, Where: n = number of cells counted; v = area (number of big squares counted) x depth (0.1). Dilution factor = 2 (equal volume of cell suspension and trypan blue). % cell viability = (viable cell counted/total number of cells) x 100. The cells were incubated for 24 h.

For cytotoxicity assay, triplicate wells of cultured cells were treated with pure mycotoxin standards and crude extract of *F. verticillioides* diluted to concentrations of 25, 50 and 100 µg/ml. The mycotoxins standards used as references were aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1) and ochratoxin A (OTA). Controls consisted of cells treated with the carrier solvent in this case, dimethylsulphoxide (DMSO) for AFB_1 and OTA and water for FB_1 at volumes consistent with those used in delivering the mycotoxins and extracts to the cell cultures. The cell cultures were further incubated for 3 h in at 37°C in a CO_2 (5%)-buffered humidified incubator. Exposed cell cultures were then evaluated by flow cytometry.

The preparation of samples for flow cytometry and the operational conditions of the instrument during analysis were carried out according to the technical data sheet (2009) of FITC Annexin V apoptosis detection kit II, Lot. 35856 (BD Biosciences, San Diego, USA. www.bdbiosciences.com). The test kit contains propidium iodide staining solution for determination of necrosis, FITC annexin stain for apoptotic assay and annexin binding buffer. The cell cultures were washed three times with phosphate buffered saline (PBS) and in each case, taking out 100 µl of cells and mixing with equal amount of PBS, centrifuging for 5 min at 2500 rpm, the supernatant discarded and cells resuspended in 500 µl of 1× annexin buffer (600 µl annexin V binding buffer in 10 ml of water). Cells were then, stained for 15 min at room temperature with a solution containing 5 µl each of FITC annexin stain and propidium iodide. Measurement of percentages of cells in necrotic and apoptotic cycles were performed on the BD FACSCalibur automated multicolor flow cytometer at excitation wavelength of 488 nm, using a 639 nm band pass filter to collect the red propidium iodide fluorescence. The percentage of cells in each stage of the cell cycle was automatically generated by the BD FACSComp™ software, in conjunction with BD Calibrite™ beads, software of the flow cytometer.

Isolation and purification of mycotoxins from fungal extract

Mycotoxins were recovered from *F. verticillioides* extracts using solvents of varying polarity by column chromatographic (CC) technique employed by Gbodi (1993). To this effect, 1 g portion of crude extract was thoroughly mixed with 10 g of silica gel (Type 60 to 200 mesh) and 5 ml of acetone was added to the mixture, thoroughly mixed and acetone evaporated at 40°C in an oven. This dried toxin/silica gel mixture was applied to a silica gel column packed in hexane and eluted with 500 ml each of hexane, benzene, diethylether, dichloromethane, ethylacetate, acetone, methanol, aqueous methanol (methanol:water, 3:1, v/v), acetonitrile and aqueous acetonitrile (acetonitrile:water, 3.1, v/v) sequentially.

Aqueous solutions of methanol and acetonitrile were used because they are the most commonly used extraction solvents for trichothecenes and fumonisins (WHO, 1999; 2000). Fractions of 500 ml were collected and evaporated at 50°C on rotary evaporator and residues dried by passing through a stream of nitrogen gas and stored in vials.

Thin layer chromatography of fractions

Each of the CC fractions was subjected to a two-dimensional TLC method of Patterson and Roberts (1979) for the following *Fusarium* mycotoxins: deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA) and fumonisins (FBs). A Prepared silica gel G aluminium-backed TLC plates with added fluorescent indicator (Merck Art 5554) cut into 10 cm squares were used. The extracts were reconstituted with 200 µl of DCM, vortexed and 20 µl of extract solution was spotted about 10 mm from the edge of the plate. A similar procedure was followed for mycotoxin standards. The developing solvents for the 1st and 2nd runs for DON and T-2 were DCM/ethyl acetate/propan-2-ol (90:5:5, v/v/v) and toluene/ethyl acetate/formic acid (6:3:1, v/v/v), respectively. The trichothecenes plates were derivatized with chromotropic acid (Baxter et al., 1983) and after heating for 110°C were viewed under UV where they appeared as dark brown spots that fluoresce blue. For zearalenone analysis, plates were developed in DCM/acetone (9:1, v/v) and derivatized with cold dianisidine reagent prepared according to Malaiyandi et al. (1976). Fractions for FB analysis were reconstituted in methanol and the plates developed in DCM/methanol: acetic acid (80:20:2, v/v/v) and butanol/water: acetic acid (12:5:3, v/v/v). Fumonisins were visualized as purple spots on the dried plates after sprayed with p-anisaldehyde reagent, (prepared by mixing 70 ml, methanol, 5 ml sulphuric acid, 10 ml glacial acetic acid and 500 µl *p*-anisaldehyde) followed by heating in an oven for 3 min at 120°C. The retardation factors (RF_1 and RF_2) and colour of the individual spots on TLC were calculated and compared with those of standard mycotoxins to aid in the identification of mycotoxins present.

High performance liquid chromatography of fractions

The mycotoxins detected by TLC were quantified by the method employed by Chelule et al. (2000). The mycotoxin extracts reconstituted in 1 ml methanol were filtered through a 0.2 µm Millipore filter and filtrate used as analyte. The chromatographic separation of analytes and standards was performed by passing through the symmetry column. The operational oven temperature was 30°C. Residues for FB analysis were derivatized with *p*-hthaldialdehyde (OPA) prior to separation on a reversed-phase HPLC system. Fumonisins were detected by fluorescence detection at excitation and emission wavelengths of 335 and 440 nm, respectively (Chelule et al. (2000). The isocratic mobile phase made up of 0.1 M dehydrated sodium dihydrogen orthophosphate: methanol (80:20) (pH of 3.5 adjusted using orthophosphoric acid) was pumped at a flow rate of 1 ml/min. The injection volume used was 20 µl. Mycotoxins were quantified using peak area and external standard calibration.

Validation of methods

The detection limits of the TLC methods for DON, T-2 toxin, ZEA and FB_1, FB_2 and FB_3 were determined and found to be 100, 20, 20, 50, 50 and 100 µg/kg, respectively. Recoveries for FBs extraction in dichloromethane as employed here were performed by spiking mycotoxin-free rice samples with 500 µg/kg each of FB_1,

Table 1. Percentage of necrotic and apoptotic lymphocyte cells after exposure to mycotoxins and extract from *F. verticillioides* culture.

Treatment	25 µg/ml		50 µg/ml		100 µg/ml	
	Necrotic	Apoptotic	Necrotic	Apoptotic	Necrotic	Apoptotic
OTA	2.49 ± 0.71^{ab}	28.51 ± 2.26^{fgh}	3.58 ± 3.14^{l}	53.78 ± 2.42^{opq}	9.57 ± 1.09^{uwx}	$73.36 \pm 1.12^{\beta\alpha}$
AFB_1	3.92 ± 0.93^{cd}	21.84 ± 2.15^{fij}	8.64 ± 1.32^{m}	45.03 ± 0.72^{ors}	6.31 ± 0.87^{uyz}	$75.27 \pm 2.06^{\delta\varepsilon}$
FB_1	0.97 ± 0.07^{ace}	4.53 ± 0.73^{gik}	3.74 ± 0.15^{n}	9.42 ± 2.00^{prt}	$3.50 \pm 0.13^{wy\alpha}$	$33.65 \pm 2.18^{\beta\delta}$
Extract	1.35 ± 004^{bde}	2.07 ± 0.01^{hjk}	0.736 ± 0.01^{lmn}	30.24 ± 0.29^{qst}	$0.53 \pm 0.03^{xz\infty}$	$34.24 \pm 2.21^{\alpha\varepsilon}$

Data are expressed as mean ± standard deviation from triplicate measurements at each mycotoxin concentration. Values with same superscripts in the same column are significantly different ($P < 0.05$) from each other.

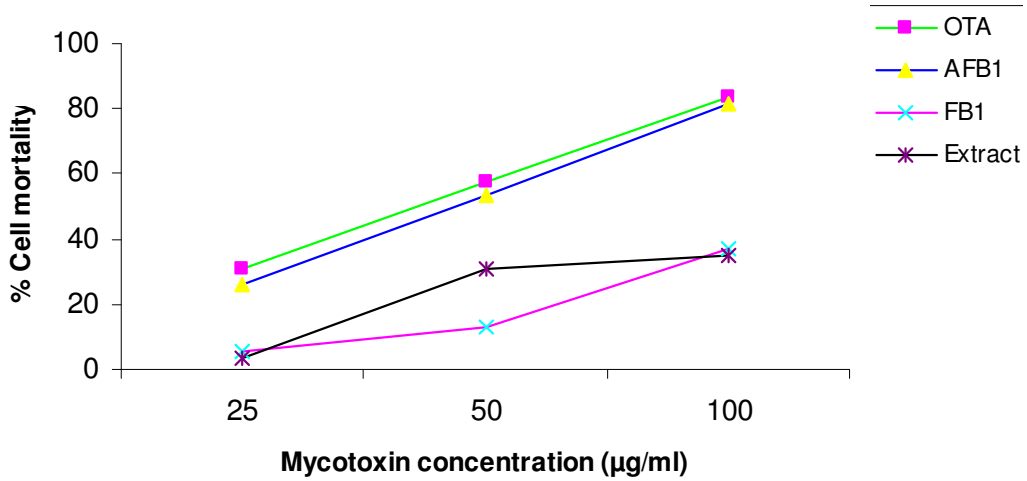

Figure 1. Effects of mycotoxin concentration on cell mortality of human lymphocytes.

FB_2 and FB_3 and recoveries rates found were in the range of 4.9 to 7.2%. The average recovery ± standard deviation for FB_1 was 5.7 ± 3.04%, while those of FB_2 and FB_3 were much lower and were 4.34 ± 2.92 and 4 2 ± 2.78%, respectively. The effectiveness of CC clean up with regards to recovering FB using different solvents was also assessed. In this case, it was found that FB_1 was not detected via HPLC when hexane, benzene, diethylether, ethylacetate, aqueous methanol and acetonitrile were used for CC, but in the dichloromethane, acetone, methanol and acetonitrile fractions, was recovered at rates ranging between 5 to 7, 7 to 10, 65 to 87 and 9 to 12%, respectively. FB_2 was found in only 2 fractions including aqueous methanol (32 to 48%) and acetonitrile (46 to 59%). Finally, it was found that aqueous acetonitrile was the only fraction containing FB_3 (17 to 32%). The HPLC limit of detection (LOD) of FB_1, FB_2 and FB_3 were 10, 20 and 15 µg/kg, respectively, while the linearity of HPLC calibration curves of the three FBs were all 0.9999. Data reported herein are not adjusted based on the recovery rates obtained.

Statistical analysis

Data on cytotoxicity assay was analyzed by using a one-way analysis of variance (ANOVA) following a pairwise multiple comparison procedures (Holm-Sidak method) on SigmaStat 3.5 for windows to derive mean values, which were compared by least significant difference (LSD) (Systat Inc., 2006). Mean values among treatments deemed to significantly differ if level of probability is ≤ 0.05. Linear regression equations were also calculated.

RESULTS

Cytotoxicity assay

The *in vitro* cytotoxic effect to human lymphocyte cells of a crude extract obtained from a culture of *F. verticillioides* was determined by flow cytometry and compared with that of AFB_1, OTA and FB_1 and the data presented in Table 1. The total cell death that is, the sum of percentage necrotic and apoptotic death was calculated and plotted graphically to show the effects of the different mycotoxins at various concentrations on cell mortality and this is presented in Figure 1. Same extract was further subjected to mycotoxin screening and quantification using CC, TLC and HPLC to further elucidate its mycotoxin contents.

Data obtained from cytotoxicity assay revealed that, the studied mycotoxin and fungal extract caused cell death to a greater extent via apoptosis than necrotic. This is

Table 2. Occurrence and concentrations of fumonisins in the column chromatographic fractions of the culture material of *F. verticillioides*.

Column chromatographic fraction	Positive sample by TLC			Positive sample by HPLC			Concentration (µg/kg) of mycotoxin		
	FB_1	FB_2	FB_3	FB_1	FB_2	FB_3	FB_1	FB_2	FB_3
Hexane	-	-	-	-	-	-	-	-	
Benzene	-	-	-	-	-	-	-		
Diethylether	-	-	-	-	-	-	-		
Ethylacetate	-	-	-	-	-	-	-	-	
Dichloromethane	+	-	-	+	-	-	1246.09		
Acetone	+	-	-	+	-	-	2820.29		
Methanol	+	-	-	+	-	-	10658.38		
Acetonitrile	+	+	-	+	+	-	1577.30	5963.00	
Aqueous methanol	-	+	-	-	+	-	-	460.00	
Aqueous acetonitrile	-	-	+	-	-	+	-	-	2456.00

noticeable as the mean ± standard deviation of the percentage death due to necrosis at the three levels of concentration (25, 50 and 100 µg/ml) used for all the tested toxins were significantly ($p < 0.01$) four to sixty times lower than the values obtained for cell mortality due to apoptosis (Table 1). The results also showed dose dependent necrotic and apoptotic death as cells in these two cell cycles increased with concentration for all the considered mycotoxins.

The sum of percentage cell mortality from necrosis and apoptosis at various concentrations for the tested toxins (Figure 1) revealed that, a decrease in cell viability was strongly influenced not only by the type of toxin, but also on the amount of the toxin the cells were exposed to; a significant dose dependent mycotoxin-induced cell mortality was observed. Accordingly, the cell mortality increased ($P<0.01$) significantly with increasing concentration levels of all the mycotoxins tested in the study (Table 1 and Figure 1). For instance, increasing the concentration of OTA from 25 to 100 µg/ml significantly ($p < 0.001$, R2 = 0.962) increased cell death from 31.0 to 82.9% ($y = 18.00 + 0.67x$) (y is the cell mortality and x is toxin concentration). Similarly, increasing the level of AFB_1 from 25 to 100 µg/ml resulted in a significant ($p < 0.001$, $R^2 = 0.94$) increase in percentage cell mortality from 25.77 to 81.58% ($y = 11.81 + 0.72x$) and a similar trend of 5.5 to 37.15% ($p < 0.0001$, $R^2 = 0.99$) was observed for FB_1 ($y = -6.50 + 0.43x$). The fungal extract also caused significant ($p = 0.001$, $R^2 = 0.68$) increase in cell mortality from 3.42 to 34.77% ($y = 1.52 + 0.37x$) as concentration was increased from 25 to 100 µg/ml. This means that OTA, AFB1, FB1 and *F. verticillioides* extract exposures to human lymphocytes resulted in a remarkable decrease in cell viability by 82.90, 81.59, 37.15 and 34.77%, respectively. The lowest cell viability value (17.10%) was recorded when cells were exposed to 100.0 µg/ml of OTA, while cells were most viable (96.58%) when exposed to 25 µg/ml of extract. Comparing the impact of concentration on cell death, significant ($p < 0.001$) cell mortality resulted at 25, 50 and 100 µg/ml of OTA and AFB_1. On the other hand, there was no significant death of cells at 25 and 50 µg/ml of FB_1 only at 100 µg/ml ($p < 0.001$). The extract caused significant ($p < 0.001$) cell mortality at 50 and 100 µg/ml, however, the values were not statistically different ($p < 0.001$). The cell mortality was influenced by the type of mycotoxins as the highest cell death was elicited by OTA (31.00, 57.36 and 82.90%) followed by AFB_1 (25.77, 53.67 and 81.58%), FB_1 (5.5, 13.16 and 37.15%) and extract (3.42, 30.97 and 34.77%) at 25, 50 and 100 µg/ml, respectively. While there were no significant differences between the values for OTA and AFB_1, and between FB_1 and extract, the cell death caused by OTA and AFB_1 were significantly higher than values for FB_1 and extract. Though the toxicities of FB_1 and the extract are similar, the later was three times significantly ($p < 0.001$) more toxic than the former at 50 µg/ml.

Mycotoxin profiling

Data on the mycotoxin profile of crude extract from *F. verticilliodes* culture as determined by TLC and HPLC techniques are shown in Table 2. Four *Fusarium* toxins; deoxynivalenol, T-2 toxin, zearalenone and FBs were analyzed for in all the CC fractions by TLC but while DON, ZEA and T-2 toxins were not detected in any of the fractions, FBs were found in the dichloromethane, acetone, methanol, acetonitrile, aqueous methanol and aqueous acetonitrile fractions. The detection of the FBs was confirmed by HPLC and their various concentrations determined by the same method. As found, data revealed the strain of *F. verticillioides* screened from rice in Niger State, Nigeria is toxigenic, producing the 3 predominant

FBs in nature including: FB_1, FB_2 and FB_3. Total FB (FB_1 + FB_2 + FB_3) content of the fungal extract found was 25.181 ppm.

As expressed previously (validation of methods section) with values, this study also reveals that, while FBs do not dissolve in hexane, benzene, diethylether and ethylacetate, very low solubility is observed in dichloromethane and acetone. On the other hand, the toxins are soluble in methanol, acetonitrile and their aqueous solutions. This implies that methanol, acetonitrile and their aqueous solutions are the best solvents for FBs assay.

DISCUSSION

F. verticillioides is a common fungal species mainly associated with maize spoilage worldwide but has also been found in other foods including rice (Dutton, 1996; Marasas, 2001; Reddy et al., 2008; Masheshwar et al., 2009). This fungus is reported to contain several products such as fumonisins (there are 18 of them with FB_1 and FB_2 being predominant), fusarins, moniliformin and beauvericin in its culture material (WHO, 2000). Though, fusarins, moniliformin and beauvericin were not determined here, the three fumonisins, especially FB_1 found in the culture material of *F. verticillioides* (Sacc.) Nirenberg (CABI-IMI 392668) has been linked to increased incidence of human oesophageal cancer in South Africa (Marasas et al., 1988) and China (Chu and Li, 1994; Wang et al., 1995) and equine leukoencephalomalacia and porcine pulmonary oedema (Marasas, 2001) should be of public health concern to the population of Nigeria.

One of the biological effects of mycotoxin intake by animals and humans is the elicitation of apoptosis. Several studies have been performed to assess the impact of mycotoxin on cell viability and proliferation of mammalian lymphocytes or cell lines. Such information for OTA (Lioi et al., 2004), FB_1 (Dombrink-Kurtzman et al., 1993) and AFB_1 (Meky et al., 2001) are documented. The tested fungal extract in this study were able to cause significant lymphocyte mortality. A direct relationship between the level of mycotoxin exposure and cell mortality of human lymphocytes was seen for the tested mycotoxins. The mechanism of mycotoxin actions at biomolecular levels may explain the dose-dependent apoptotic pattern of cell death induced by the studied mycotoxins. At the genomic level, AFB_1 is converted to its epoxide forming AFB_1-N7-guanine adducts that causes DNA strand breaks and point mutations (Smith and Moss, 1985). Under this pathological condition oxidative stress is elicited which activates the caspase-3 cysteine proteases that mediates the apoptotic cascade (Meki et al., 2001). It was also shown by Bennour et al. (2010) that, AFB_1 and OTA either in isolation or in combination, were involved in apoptotic process in cultured monkey kidney vero cells by causing increased DNA fragmentation with consequent activation of p53 tumour suppressor protein with further suppression of antiapoptotic factor bcl-2 production. OTA has also been established to have stimulatory effect on extracellular protein kinase and caspase or to induce unscheduled DNA synthesis that both cause apoptosis in human kidney cells (Hussein and Brasel, 2001). Caspase-dependent mitochondrial alterations and triggering of the activity of p53 are other mechanisms by which OTA induces apoptosis (Bouaziz et al., 2008). Unlike other mycotoxins, elicitation of apop-tosis by FB seems to be mainly non-genotoxic as FB_1 inhibits the enzyme, ceramide synthase thereby disrupting *de novo* sphingolipid biosynthesis and consequently, sphingolipid-mediated regulation of important cell functions including apoptosis and mitosis (Voss et al., 2002; Seefelder et al., 2003). In these studies, FB_1-induced apoptosis was also mediated by cytokine tumour necrosis factor (TNF) pathway that is involved in the regulation of apoptosis and cell replication similar in the case of sphingolipids. It must be emphasized here that AFB_1 and OTA can also induce apoptosis via non-genotoxic route by inhibiting macromolecular synthesis, which disrupts several lipids, protein or DNA-mediated cell function and regulations with consequent deregulation of processes including apoptosis (CAST, 2003). The finding that the tested toxins induce cell death mainly via apoptosis is in excellent agreement with those reported by Dombrink-Kurtzman et al., (1993), Lida et al. (1998), Wang et al. (1999) and Lioi et al. (2004).

The ensuing lymphocytes cell mortality provoked by all the toxins tested (in addition to the extract) in this study implies that they may cause negative immunomodulation (Sharma, 1993). And because immune cell mortality is the initial step of immunosuppression (Forsell et al., 1994) leading to secondary infection, the tested toxins are likely to be immunotoxic to humans. Cell mortality due to mycotoxin exposure as observed here may also lead to carcinogenesis in some cases (Dragon et al., 2001). AFB_1, OTA and FB_1 (IARC, 1993) are classified as possible human carcinogens. The extract could therefore be classified as a possible carcinogen since it has been shown to contain FB_1 and other fumonisins. Although, similar effects with regards to causing a reduction in cell viability were noted, significant variation among treatments was well established. Ochratoxin A was found to be the most toxic of all of the tested mycotoxins. Toxic effects of mycotoxins vary according to the type of mycotoxin (Al-azzawi et al., 1978) with one of them being more toxic than others as observed in the works of Lioi et al. (2004) and De Lorenzi et al. (2005). A similar trend was noted in this study. The lower toxicity of FB_1 or that of the fungal extract than observed for OTA and AFB_1 is consistent with the findings of Creppy et al. (2004) and Wangikar et al. (2005). Such variation may be attributed to the rate of biouptake of mycotoxins by cells (permeability of toxin across lipophilic cell membrane) enhanced by hydophobicity of the compound as indicated by Yiannikouris and Jouany (2002). This implies that,

non-polar substances (AFB_1 and OTA) can easily transverse lipophilic cell membranes and elicit their toxic potentials in the cell and are therefore, likely to be more toxic than the less permeable polar compounds (FB_1 and extract). Variation in toxicity among mycotoxins could also be dependent on other toxicological properties (ability to cause instant damage of biomolecules) of the toxin in question and variation in susceptibility of cell type and animal species to the toxin (Smith and Moss, 1985; Piva and Fabio, 1999).

The FBs content (25.181 ppm) of the fungal extract as found herein is significantly higher than that obtained in the previous study (8.233 ppm) (Makun et al., 2010) for the same extract. This remarkable variation is because while FBs concentrations were determined in all column chromatographic fractions here, the toxins were previously determined only in the aqueous methanol fractions, which have been identified in this study and elsewhere (WHO, 2000) not to elute completely all FBs present. The existence of 4 free carboxyl groups and an amine group in FBs structures makes them very polar and thus, soluble in water and other polar solvents but not soluble in non-polar solvents (Dutton, 1996). The solvents used in this study have varying polarities and can be arranged in order of increasing polarity based on their dielectric constants as non polar (hexane: 1.88; benzene: 2.3 and diethylether: 4.3; ethylacetate: 6.02; dichloromethane: 9.1) and polar (acetone: 21; methanol: 33; acetonitrile: 37.5 and water: 80) (Lowery and Richardson, 1987). The polarity of an aqueous solvent will be expected to range between 33 and 80. This was the sequence used in the CC elution protocol for FB. Since FBs are very polar, their insolubility in hexane, benzene, diethylether and ethylacetate and increasing solubility in dichloromethane, acetone, methanol, acetonitrile, aqueous methanol and aqueous acetonitrile as established in this investigation is normal. The observed solubility of FB in methanol, acetonitrile and their aqueous forms is consistent (WHO, 2000). The findings reported herein that methanol and acetonitrile are respectively, the best solvents for extraction of FB_1 and FB_2 is confirmed previously by D'Arco et al. (2008). However, a search on the literature failed to identify reports on the solubility of FBs in acetone and dichloromethane and since these solvents have some degree of polarity, the recorded low solubility of FBs in these solvents is expected particularly that there was high concentration of the toxins in the extract and elution that was conducted under limited pressure-gravity (D'Arco et al., 2008). It is important to note that, dichloromethane and acetone were used earlier than the very polar solvents in the elution protocol.

The demonstrated cytotoxic effects and mycotoxin profile (FB_1, FB_2 and FB_3) of the extract of the culture material of *F. verticillioides*, a fungal contaminant of a Nigerian staple, rice, points to the need for regular screening of foodstuffs for mycotoxins in the country with a view to eliminating them from the dietary system. A complete elucidation of mycotoxins present in the fungal extract in order to confirm the presence or absence of moniliformin, fusarins and beauverin which are usual components of culture material of *F. verticillioides* and which together with the FBs exhibit toxic synergistic effects (WHO, 2000) that could account for the previously observed acute effects in chicks and mice (Makun et al., 2010) is recommended.

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