

Original Research Article

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## Studies on Cytotoxic Effects of Ochratoxin A Extracts on Human Cell Lines (Hfl-1 and Hep-2)

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### ABSTRACT

Ochratoxin A (OTA), the mycotoxin produced by species of *Aspergilli*, specifically *Aspergillus carbonarius* and *Aspergillus ochraceus*, and *Penicillium verrucosum* have harmful effects on humans, animals, and crops that result in illnesses and economic losses. Therefore, this study has been undertaken the cytotoxic effects of ochratoxin A of the four moulds extracts which has been used in this study in two human cell lines (Hfl-1 and Hep-2). Control experiment (uncontaminated solid substrate extract) showed negligible cytotoxic effects on both cell lines after 72 hr exposure. All mould (OTA) extracts examined showed a cytotoxic effect on both cell lines, although it is possible that other compounds in the extracts may have contributed to the cytotoxic effects. Effect of the toxin extracts on the cell lines increased with incubation time. *Aspergillus ochraceus* J002 and *A. ochraceus* strain CBS 588.68 showed higher cytotoxic effects on Hfl-1 than *A. carbonarius* J001 and *P. verrucosum* strain IMI 285522 whereas on Hep-2, *A. carbonarius* J001 and *A. ochraceus* strain CBS 588.68 showed higher cytotoxic effects than *A. ochraceus* J002 and *P. verrucosum* IMI 285522. IC<sub>50</sub> ranged between 1.37 to 38.87 using Hfl-1 cells and 1.94 to 50.0 using Hep-2 cells. IC<sub>50</sub> values by *Aspergillus ochraceus* strain CBS 588.68 was not determined after 72hr, this may have been due to higher concentration of OTA in the extract than suspected.

#### Keywords

Ochratoxin A  
*Aspergillus carbonarius*,  
*Aspergillus ochraceus*,  
*Penicillium verrucosum*.

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### Introduction

Contamination of foodstuff with mycotoxins such as ochratoxins is a major matter of concern for human and animal health. Ochratoxin A (OTA) is a nephrotoxic and carcinogenic mycotoxin mainly produced by *Penicillium verrucosum* in temperate and cold climates and by *Aspergillus ochraceus*

and related species belonging to section Circumdati in warmer and tropical climates. *Aspergillus carbonarius* and some species belonging to *Aspergillus niger* aggregate have been reported as OTA producers (Dalcero *et al.*, 2002; Magnoli *et al.*, 2003; Battilani *et al.*, 2003; Abarca *et al.*, 2004; Samson *et al.*, 2004). Ochratoxin A has been

detected worldwide in a wide range of food products, including cereals (Magnoli *et al.*, 2006a), poultry feeds (Rosa *et al.*, 2006), feedstuffs (Magnoli *et al.*, 2005), green coffee beans (Pardo *et al.*, 2004), dried vine fruits (Magnoli *et al.*, 2004), peanuts (Magnoli *et al.*, 2006b). Ochratoxin A is the major compound and the most toxic form, followed by ochratoxin B (OTB), which differs from OTA only by the substitution of chlorine by hydrogen in the isocoumarin moiety. Ochratoxin A is a structural analogue of phenylalanine, and contains chlorinated dihydroisocoumarine moiety. It inhibits protein synthesis (Creppy *et al.*, 1983), and impairs blood coagulation (Gupta *et al.*, 1979), glucose metabolism (Pitout, 1986), and induces oxidative damage by enhancing lipid peroxidation (Omar *et al.*, 1991).

Ochratoxin A is strongly cytotoxic, inducing tubule - interstitial nephropathy in rats and in other animal species (Boorman, 1989; Krogh *et al.*, 1988). Ochratoxin A dependent induction of apoptosis is reported *in vivo* (kidney of rats and mice) and *in vitro* (Gekle *et al.*, 2000; Luhe *et al.*, 2003). Persistent cell proliferation has also been described in the kidney as a consequence of cell death. Both carcinogenicity and cytotoxicity of OTA have been discussed in relation to free radical formation leading to oxidative cell damage (Schaaf, 2002).

The cytotoxicity of ochratoxin A has been investigated by several authors (Bondy and Armstrong, 1998; Dietrich *et al.*, 2001). The carcinogenicity of OTA in rats and mice is well established, OTA induces renal tumours in rats of both sexes and in male mice (Bendele *et al.*, 1985; Boorman, 1989). In rats, male animals have been found to be considerably more sensitive than females. In the rat kidney, tumour induction is seen at a dose level (70µg/kg body weight) (Boorman, 1989).

The objective of this study was to investigate the cytotoxic effects of ochratoxin A extracted from 4 ochratoxigenic moulds *in vitro* using two human cell lines, Hfl-1 and Hep-2.

## Materials and Methods

### Ochratoxigenic moulds and ochratoxin A preparation

Fungal genera used in this experiment were *Aspergillus ochraceus* strain CBS 588.68 (CBS Holland) and *Penicillium verrucosum* strain IMI 285522 (CABI, UK), and two fungal species were isolated from cereal samples obtained from the south of Libya and named according to their morphological appearance according to Samson *et al.*, 2002 as *Aspergillus carbonarius* isolate and *A. ochraceus* isolate. All fungal strains were grown on solid substrate at 25°C for 7 days. After incubation, OTA was extracted with methanol for 30 min. Extracts were filtered using Whatman filter paper NO. 1 and the filtrates were evaporated under a stream of nitrogen. The residues (crude OTA extract) stored at 4°C until needed.

### Chemicals

Ochratoxin A standard solution (R-Biopharm, Rhone Ltd, Glasgow, Scotland). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl) and 2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole, UK) were used for cytotoxicity assay. The MTT method is based on the respiratory activity of the cell principally mitochondrial succinate-tetrazolium reductase system, which converts the yellow tetrazolium salt to a blue formazan dye. The amount of formazan produced is proportional to the amount of living cells in the culture. Sterile phosphate buffer saline (PBS) (Cambrex Bio Science,

Wokingham, UK) was used for cell lines washing.

### **Cell Lines maintenance and OTA Extract application**

HeLa- derived larynx epithelium (Hep-2) cell line and Human foetal lung fibroblasts (Hfl-1) cell line were purchased from the European Collection of Cell Culture (ECCC, Salisbury, UK). The Hep-2 cell line was grown and maintained in minimal essential medium (MEM) (BioWhittaker, Berkshire, UK) containing Earls's salts and glutamine, whereas Hfl-1 cell lines fibroblasts was cultured and grown in Ham's F12 medium (BioWhittaker, Berkshire, UK). Both media were supplemented with 1% (v/v) nonessential amino acids (Bio Whittaker BE1-114E), 100 IU ml<sup>-1</sup> penicillin (Sigma, P-0906), 100µg ml<sup>-1</sup> streptomycin, (Sigma, P- 0906), Amphotericin B (0.25µg/ml, Bio Whittaker BE17-836E) and foetal bovine serum (FBS, 10%, Bio Whittaker) at the attainment of 80-90% confluence determined by microscopy (Olympus CK2), 4ml Trypsin/EDTA solution (0.05%-0.02%, v/v) (Gibco, Paisley, UK) was added for cell detachment, and the cells washed twice with sterile PBS (Sigma, UK) to ensure trypsin/EDTA removal. It was then transferred into a fresh media and incubated at 37°C, 5% CO<sub>2</sub> incubator until needed.

Five milliliter of fresh medium were used to reconstitute OTA and a serial dilution was made in triplicate using 100µl of toxin extracts in 96 microtiter plates (TPP, Trasadingen, Switzerland). One hundred microlitres (100µl) of cell suspension was added into each well and the background was made by distributing 200 µl of growth medium into 2 rows of eight wells and a negative toxin was made by distributing 100µl of the cell line. The plates were then incubated in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) at

37°C for 24, 48 and 72hr. Cell suspension was made as described by Finlay *et al.*, (1999).

### **Evaluation of Cytotoxicity of Fungal extracts**

An evaluation of the cytotoxicity of ochratoxin A extract was carried out using (MTT) assay. The procedure described by Betancur-Galvis *et al.*, (1999) was employed in this experiment. After incubation of the cell lines with the OTA extracts, the supernatants were removed and 80µl of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide was added to each well and incubated in CO<sub>2</sub> incubator (5% CO<sub>2</sub>) at 37°C for 4 hours, the dye was removed and then 100µl of Dimethylsulfoxide (DMSO) was added to dissolve any intracellular formazan crystal and followed by agitation for 15 minutes on a rotary shaker. Absorbance was measured in a plate reader (Dynex MR11, Worthing, UK) at 570nm. Absorbance of untreated cells served as control and background absorbance from wells containing no cells as a blank. The percentage of cytotoxicity was calculated using the formula,

$$\% \text{ cell survival} = \frac{A - B}{A} \times 100$$

Where, *A* is the mean optical density of control wells, and *B* is the optical density of wells with moulds extract.

*IC*<sub>50</sub> concentration was calculated from the linear Interpolation of the 2 test points that bracket 50% inhibition, one point lower and one point higher (MCL-5 Metabo, Woburn Massachusetts 2005) using the formula,

$$IC_{50} = \frac{(50\% - low\%) \times (C_h - C_l)}{High\% - Low\%} + C_l$$

Where,  $C_h$ = High concentration,  $C_l$ = low concentration, *High%*= High percentage, *Low%*= low percentage.

The  $IC_{50}$  value stands for a 50% inhibition of cell growth or metabolic cell activity.

## Results and Discussion

Four ochratoxigenic moulds were grown on solid substrate at 25°C for 7 days. After incubation, OTA was extracted with methanol and the toxin extract of each mould was examined for their cytotoxicity effects on 2 human cell lines (Hfl-1 and Hep-2).  $IC_{50}$  values (concentration of toxin extract which result in 50% cell death) of each extract were determined on both cell lines. Tables 1 and table 2 showed the  $IC_{50}$  values obtained for each mould extract at different incubation time. Ochratoxin A extracted from *Aspergillus carbonarius* isolate showed a cytotoxic effect on both cell lines during the period of incubation (Table 3). The cytotoxicity of OTA extracted from *A. carbonarius* isolate was high on Hep-2 compared to that on Hfl-1, and resulted in 98% cell death after 24h, 99% cell death after 48h, and 99% cell death after 72h when 100µl of the OTA extract was added (OTA concentration was 180 ppb). On Hfl-1, the percentage cell death at the same toxin concentration was 83% after 24h, 91% after 48h and 95% after 72h.

The toxic effect of OTA extracted from *A. ochraceus* isolate was determined during 72h of incubation. Table 4 show the percentage cell death of both cell lines when 40 ppb of the toxin extract was used. On Hep-2, the percentage cell death obtained after 24h was 97%, whereas 98% cell death was achieved after 48h and 99% of cells death was achieved after 72h. For Hfl-1 cell lines, the percentage of cell death was 84% obtained after 24h, and 87% after 48h and it

was 97% after 72h. *Aspergillus ochraceus* strain CBS 588.68 showed cytotoxic effects on both cell lines (Table 5). The average percentage of cell death on Hep-2 cell lines with toxin extract concentration of 60ppb was 97% after 24h, whereas the percentage of cell death was 98% after 48h, and 99% after 72h. On Hfl-1 cell lines; 74% of the cell death was obtained after 24h and 92% was obtained after 48h; and 98% of cell death was exhibited after 72h..

The cytotoxicity of OTA produced by *Penicillium verrucosum* strain IMI 285522 was determined in both cell lines. Table 6 show the cytotoxic effect of OTA extract on Hep-2 and on Hfl-1 cell lines. The percentage of cell death on Hep-2 obtained when a toxin concentration of 50ppb was added were 96%, 97% and 99% after 24h, 48h and 72h respectively, whereas on Hfl-1 cell line, the percentage of cell death was 87% after 24h, 95% after 48h and 99% after 72h.

The crude toxin extracts showed cytotoxic effects on both Hep-2 and Hfl-1; the effects increased with incubation time for both cell lines. *Aspergillus ochraceus* isolate and *A. ochraceus* strain CBS 588.68 showed higher cytotoxic effects on Hfl-1 than the other moulds, whereas on Hep-2 *A. carbonarius* isolate and *A. ochraceus* CBS588.68 showed higher cytotoxic effects than *A. ochraceus* isolate and *P. verrucosum* strain IMI 285522

The cytotoxicity of ochratoxin A has been investigated by several authors (Bondy and Armstrong, 1998; Dietrich *et al.*, 2001). The carcinogenicity of OTA in rats and mice is well established, OTA induces renal tumours in rats of both sexes and in male mice (Bendele *et al.*, 1985; Boorman, 1989). In rats, male animals have been found to be considerably more sensitive than females.

**Table.1** Mould extract concentration needed to obtain 50% inhibition of Hfl-cell lines metabolism

Fungi	Incubation time (hr)		
	24	48	72
	Concentration (µl/ml)		
<i>A. carbonarius</i> isolate	11.98	9.17	1.40
<i>A. ochraceus</i> isolate	17.59	4.05	6.23
<i>A. ochraceus</i> CBS 588.68	24.10	11.83	ND
<i>P. verrucosum</i> IMI 285522	50.0	13.31	1.94
Negative control	ND	ND	ND

ND= not detected.

**Table.2** Mould extract concentration needed to obtain 50% inhibition of Hep-2 cell lines metabolism

Fungi	Incubation Time (hr)		
	24	48	72
	Concentration (µl/ml)		
<i>A. carbonarius</i> isolate	12.46	9.17	1.37
<i>A. ochraceus</i> isolate	17.59	8.11	6.23
<i>A. ochraceus</i> CBS 588.68	17.50	11.83	ND
<i>P. verrucosum</i> IMI 285522	38.87	13.31	1.69
negative control	ND	ND	ND

ND= not detected.

**Table.3** The cytotoxic effect of OTA (180ppb) extract produced by an *A. carbonarius* isolate on Hep-2 and Hfl-1 cell lines at different incubation periods

OTA concentration (ppb)	Incubation time (hr)					
	24		48		72	
	Hep-2	Hfl-1	Hep-2	Hfl-1	Hep-2	Hfl-1
180	98.15	82.89	99.18	91.34	99.91	95.26
90	88.35	81.57	98.50	88.45	98.74	89.05
45	83.01	68.42	86.50	78.87	98.50	84.91
22.5	51.56	54.60	59.37	70.44	61.77	78.69
11.25	32.67	33.55	41.74	58.80	54.09	71.00
6.63	20.59	18.42	35.97	48.20	52.23	64.79
3.31	15.19	15.13	31.89	36.28	51.63	52.36
1.65	05.39	9.80	25.40	24.17	46.41	64.44

**Table.4** The cytotoxic effect of OTA (40ppb) extract produced by an *A. ochraceus* isolate on Hep-2 and Hfl-1 cell lines at different incubation periods.

OTA concentration (ppb)	Incubation time (hr)					
	24		48		72	
	Hep-2	Hfl-1	Hep-2	Hfl-1	Hep-2	Hfl-1
40	96.79	87.76	98.29	84.21	98.88	97.94
20	96.95	73.43	98.13	81.57	98.15	97.92
10	60.23	68.95	71.31	75.00	97.83	96.74
5	43.18	60.52	51.36	65.97	90.53	92.60
2.5	36.18	46.05	38.54	50.14	88.30	82.54
1.25	26.42	30.26	34.85	44.77	82.48	73.07
0.63	20.31	20.39	29.16	44.71	82.33	71.89
0.31	18.32	17.10	28.20	28.65	77.34	69.23

**Table.5** The cytotoxic effect of OTA (60ppb) extract produced by an *A. ochraceus* strain CBS 588.68 on Hep-2 and Hfl-1 cell lines at different incubation periods

OTA concentration (ppb)	Incubation time (hr)					
	24		48		72	
	Hep-2	Hfl-1	Hep-2	Hfl-1	Hep-2	Hfl-1
60	96.79	74.34	98.29	92.00	98.88	98.81
30	96.95	67.76	98.13	89.55	98.15	93.78
15	60.23	57.89	71.31	77.05	97.83	83.13
7.5	43.18	54.60	51.36	71.89	90.53	73.43
3.75	36.18	33.55	38.54	58.57	88.3	69.85
1.87	26.42	27.63	34.85	43.19	82.48	65.34
0.94	20.31	15.13	29.16	41.42	82.33	62.38
0.47	18.32	05.26	28.20	39.34	77.34	60.59

**Table.6** The cytotoxic effect of OTA (50ppb) extract produced by *P. verrucosum* strain IMI 285522 on Hep-2 and Hfl-1 cell lines at different incubation periods

OTA concentration (ppb)	Incubation time (hr)					
	24		48		72	
	Hep-2	Hfl-1	Hep-2	Hfl-1	Hep-2	Hfl-1
50	96.59	86.86	97.90	94.73	99.62	99.11
25	50.00	81.04	70.35	87.50	98.50	97.63
12.5	40.62	65.07	65.06	76.30	90.61	80.76
6.25	23.72	46.71	48.95	58.28	87.55	63.28
3.125	18.03	32.89	44.23	53.25	73.84	57.31
1.56	10.79	23.02	36.13	47.46	66.54	50.88
0.78	10.70	09.86	30.40	40.97	48.43	48.50
0.39	6.81	04.60	29.40	30.60	44.41	38.04



In the rat kidney, tumour induction is seen at a dose level (70µg/kg body weight) (Boorman, 1989). Ochratoxin A is not mutagenic and only weak genotoxic effects have been observed in some mammalian cell systems (Bendele *et al.*, 1985; Doop *et al.*, 1999; Ehrlich *et al.*, 2002; Follmann and Lucas, 2003; Zepnik *et al.*, 2001). Experiments using radio- labeled OTA indicate that OTA does not form covalent DNA adducts in detectable concentration (Gautier *et al.*, 2001; Gross-Steinmeyey *et al.*, 2002; Mally *et al.*, 2004). However OTA is only slowly eliminated in rats and tissue-specific retention may play an important role in OTA toxicity (Zepnik *et al.*, 2003).

Ochratoxin A has been found in human blood samples, and it was found more frequently at high average concentration in blood samples obtained from people living in regions where a fatal human kidney disease occurs (Perkova-Bocharova *et al.*, 1988). As cereals are widely used in animal feeds in many parts, OTA may come through animal products in human diet and absorbed from the gastrointestinal tract. Distribution in a number of species is via blood, mainly to the kidneys, lower concentrations being found in liver, muscle and fat. Transfer to milk has been demonstrated in rats, and humans, but little transferred to the milk of ruminants owing to metabolism of OTA by the rumen microflora (Creppy, 2002). The 4,5-(dimethylthiazol-2-yl) and 2,5-diphenyltetrazolium bromide (MTT) assay have been used by many authors for cytotoxicity screening and was established as accurate, rapid and reproducible for assaying the viability of various cell lines on exposure to mycotoxins (Hanelt *et al.*, 1994; Widestrand *et al.*, 2003). Other colorimetric assays including bromodeoxyuridine uptake (Eriksen *et al.*, 2004; Widestrand *et al.*, 1999), trypan blue exclusion assay (Robb *et*

*al.*, 1990) and lactate dehydrogenase (Widestrand *et al.*, 1999) have been used, however, the MTT assay is the most widely employed among the other techniques.

Since crude toxin extracts were used for cytotoxicity assay, it is likely that the cytotoxic effects shown could have been caused by the toxin alone, the toxin in combination with other extracted compounds or even the latter. Although the solvent was evaporated and the extract reconstituted for the assay the role of other metabolites could not be ruled out. It is important that future work on the fungal isolates obtained in this study investigates the effect of purified enzyme extracts on different human cell lines. The significance of this study is that the secondary metabolites of the fungal isolates from Libya could have cytotoxic effects on mammalian cells.

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