

Original Research Article

***In vitro* analysis of efficacy of chlorination in detoxifying ochratoxin A from freshly shelled sweet corn kernels spiked with toxigenic *Aspergillus ochraceus* strain**

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A B S T R A C T

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Ochratoxin A (OTA) is a possible carcinogen produced by *Aspergillus* and *Penicillium* species in agricultural commodities like coffee and corn, and has been reported to be quite stable to several detoxifying treatments. Chlorination with sodium hypochlorite (NaOCl), a widely used disinfectant and sanitizer in food industry, was evaluated for its efficacy to inactivate OTA. Sweet corn spiked with highly toxigenic *Aspergillus ochraceus* strain (OTA yield: 0.35 mg/kg) was found to be degraded upon NaOCl treatment (200 ppm for 1h) to below detectable level as assayed by TLC and HPLC analyses. The OTA extracted from treated samples displayed reduced cytotoxicity to human intestinal epithelial (Int-407) cells, when analyzed by microscopic examination and MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] assay. When similar chlorination treatment was given to unspiked samples, their nutritional and sensory attributes were found to be retained. Thus, the findings endorse use of chlorination in food commodities for ensuring their safety.

Introduction

Ochratoxin A (OTA) is one of the important mycotoxins produced in foods by different fungal species such as *Aspergillus ochraceus*, *A. carbonarius*, *A. westerdijkiae*, *A. steynii*, *A. niger*, and *Penicillium verrucosum* (Khoury and Atoui, 2010), and has been reported to be quite stable to several detoxifying agents (Batista *et al.*, 2009). OTA contains a polyketide moiety, dihydroisocoumarin coupled via a peptide linkage to phenylalanine. It is known to have nephrotoxic, immunotoxic, teratogenic and

carcinogenic effects, and hence has been classified as possible carcinogen (Group 2B) for humans by International Agency for Research on Cancer (Merwe, 1965; IARC, 1993; WHO, 2001). The major OTA contaminated food commodities include cereal grains (wheat, sorghum and corn), coffee beans, wine grape and dried grape (Azizi *et al.*, 2012). Cereals contribute to daily OTA ingestion to a greater extent (Miraglia and Brera, 2002). Due to high moisture content sweet corn is highly

susceptible to mold growth and OTA contamination (Shotwell *et al.*, 1969). About 70-100% of corn and its byproducts were found to be contaminated with OTA (Legarda and Burdaspal, 2001; Juan *et al.*, 2008). In one report, OTA in corn samples has been reported even up to 0.23 mg/kg (Rosa *et al.*, 2009).

The OTA detoxification strategies involve physical, chemical or microbiological treatments to reduce or eliminate OTA by degradation, modification, or adsorption (Amézqueta *et al.*, 2009; Abrunhosa *et al.*, 2010). Some of the adsorbents include activated charcoal, cholestyramine, sodium and calcium aluminum silicates (mainly zeolites) and bentonite. The modifying/detoxifying agents include alkaline hydrogen peroxide, ozone, sodium hydroxide, monomethylamine, and ammonium and calcium hydroxides. In ready-to-eat products like bread, preservatives such as potassium sorbate and calcium propionate were found to prevent mold growth and OTA formation (Arroyo *et al.*, 2005). Decontamination of pre-formed OTA in the food commodities is a major challenge and still there is a lack of well established process to detoxify OTA while retaining its nutritional and sensory qualities (Hajare *et al.*, 2005; Kumar *et al.*, 2012a). Chlorine-based sanitizers such as calcium or sodium hypochlorite (200 ppm) and aqueous chlorine dioxide (5 ppm) have been widely used in food industries (Horvitz and Cantalejo, 2012; Goodburn and Wallace, 2013). For ensuring microbiological safety in sprouts, which have been reported to be associated with many outbreaks and illnesses, US FDA (2013) has even recommended a very high concentration (20,000 ppm) of hypochlorite treatment.

In the present study, chlorination using sodium hypochlorite was evaluated for its

efficacy to detoxify OTA from sweet corn kernels spiked with toxigenic *A. ochraceus* spores by performing MTT [3-(4,5-dimethylthiazole - 2yl) - 2,5-diphenyl tetrazolium bromide], an *in vitro* cytotoxicity assay in human intestinal epithelial (Int-407) cells and microscopic examinations. Also, nutritional and sensory attributes of unspiked sweet corn samples subjected to chlorination was performed. The findings reveal the suitability of chlorination wash of commodities prone to be contaminated with ochratoxigenic strains to ensure their safety.

Materials and Methods

Spiking of sweet corn with *A. ochraceus* spores

A highly toxigenic *A. ochraceus* strain (MTCC 4643) procured from Microbial Type Culture Collection (MTCC, Chandigarh, India) was grown in yeast extract sucrose (YES) agar slant [5 g/l yeast extract, 30 g/l glucose, 50 mg/l each of adenine, histidine, leucine, uracil and lysine, and 17 g/l agar] for 3 days and spores were harvested using 0.01% Tween 80 in water. The spore count was determined by serial dilution and spread plating on YES agar plates as discussed earlier (Kumar *et al.*, 2012a). The spores ($\sim 10^4$) were spiked on the freshly shelled sweet corn kernels (10 g), incubated for 3 or 7 days at 99% RH (relative humidity) in ambient temperature ($26 \pm 2^\circ\text{C}$).

NaOCl treatment of spiked kernels

The optimal NaOCl concentration was selected based upon a pilot study, where its efficacy to reduce standard OTA (0.25 $\mu\text{g}/500 \mu\text{l}$) was tested at various concentrations of NaOCl (25-200 ppm) by TLC analysis. As 200 ppm of NaOCl was

found to degrade OTA to below detectable level, for further detail studies NaOCl was used at this concentration. When kernels were dipped in NaOCl solution the active moiety i.e. OCl⁻ and HOCl were found to decay with time, hence the treatment time was fixed for 1 h, beyond which these moieties were undetectable.

Extraction of OTA from *A. ochraceus* infected sweet corn samples

Toxin was extracted using a modified procedure of Association of Analytical Chemists (AOAC, 2000). The infected samples after NaOCl treatment (10 ml) were ground together to fine paste, homogenized in 30 ml of sodium bicarbonate solution (3%), centrifuged at 10,000 g for 20 min and filtered through Whatman No. 1 paper. The filtrate was extracted in a separating funnel having 30 ml of chloroform and 3 ml of phosphoric acid (0.1 M) for 20 min. The upper aqueous phase was collected and passed through a pre-equilibrated [2 ml each of methanol, distilled water, and 3% sodium bicarbonate solution, sequentially] C-18 Sep-Pak cartridge (Waters Corporation, Milford, Mass., U.S.A.).

The column was washed with 2 ml of phosphoric acid (0.1 M) and later with same volume of distilled water. OTA was finally eluted with 8 ml of ethyl acetate-methanol-acetic acid solution (94.5:5:0.5) into a test tube containing distilled water (2 ml). The suspension was mixed by vortexing for 15 s, allowed for separation for 3 min and the upper solvent phase containing OTA was withdrawn and dried under nitrogen gas. The pellet was dissolved in methanol and analyzed by TLC and HPLC. Potable water washing after chlorination of infected kernels may lead to loss of toxin, hence not performed with the samples meant for OTA analyses.

Analysis of OTA using thin layer chromatography (TLC)

An aliquot (25 µl) of OTA was spotted on TLC plate (silica gel G; 0.25 mm), developed in toluene-ethyl acetate-formic acid (90%) (6:3:1), visualized under ultraviolet (366 nm) light and quantified using HPTLC documentation system (CAMAG, Muttenz, Switzerland) (Golinsky and Grabarkiewicz-Szczesna, 1984).

Analysis of OTA using high performance liquid chromatography (HPLC)

An aliquote (10 µl) of OTA was analysed by a reverse-phase HPLC (UltiMate 3000, Dionex, Sunnyvale, Calif., U.S.A.; C18 column: 250 × 4.6 mm, Acclaim 120 and pore size 5 µm; detector: variable wavelength detector, and software: Chromeleon 6.8) using an isocratic solvent system [acetonitrile/water/acetic acid (50:48:2, v/v/v)] at the flow rate of 1.0 ml/min (Ghali *et al.*, 2009). The OTA was detected at its absorption maxima of 333 nm using UV detector and quantified using a calibration curve prepared with standard OTA (2.5-50 µg/ml) (Pohland *et al.*, 1992).

Assay of OTA toxicity

It was performed by MTT [3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide] dye conversion assay in human intestinal (embryonic jejunum and ileum) epithelial (Int-407) cells (Kumar *et al.*, 2012a). MTT (yellow colored dye) when enters into the mitochondria gets reduced to an insoluble formazan (blue colored) product (λ_{max} : 550 nm) by mitochondrial succinate dehydrogenase in a metabolically active cells. In brief, cells (~5 × 10³) were seeded in 96 well microtitre plate containing Dulbecco's modified Eagle's medium

(DMEM) supplemented with calf bovine serum (10%) and antibiotics [penicillin (100 U/ml equivalent to 62.5 µg/ml), streptomycin (10 µg/ml)]. Calf bovine serum was pre-heated at 56°C for 30 min to inactivate various inhibitors of cell growth before addition into the media. Cells were incubated in a humidified incubator [CO₂ atmosphere (5%) and air (95%)] at 37°C for 24 h for attachment and OTA (1.25 to 5 µg/200 µl) extracted from NaOCl treated and untreated corn samples was added and the cells were further incubated for another 24 h. Then MTT dye was added and incubated with the cells (~8 x 10³) for 4 h. Precipitated formazan was dissolved overnight at 37°C in 10% SDS in 0.01N HCl and quantified by measuring at 550 nm using a scanning plate reader (Bio-Tek Instruments, Vt., U.S.A.). The morphological changes in these cells were visualized using an inverted microscope, equipped with CCD camera (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany).

Determination of residual chlorine level in sweet corn samples

It was performed using N, N-diethyl-p-phenylenediamine (DPD)-ferrous titrimetric method (Otson and Williams, 1980; Harp, 2002). The DPD amine is oxidized by chlorine (hypochlorite ion or hypochlorous acid) to products including a semi-quinoid cationic red colored compound known as a Würster dye which is a relatively stable free radical species. This is titrated with ferrous reducing agent to the colorless end point. In brief, sweet corn (100 g) was dipped in 100 ml of NaOCl (200 ppm) up to 1 h at 26±2°C, washed twice with potable water and ground in 100 ml of milli Q water, centrifuged and supernatant was analyzed for chlorine content by mixing with 5 ml of phosphate buffer (0.17 M Na₂HPO₄, 0.34 M KH₂ PO₄ and 2.7 mM EDTA), 5 ml of DPD

indicator solution (36 mM H₂SO₄, 3.8 mM DPD and 0.68 mM EDTA), potassium iodide (6 mM) and titrating with freshly prepared ferrous ammonium sulfate (FAS; 0.00282 N) until the red color completely disappeared. The volume (ml) of FAS titrant required to get colorless end point is equivalent to chlorine (mg/l or ppm).

Analyses of physical and nutritional properties of NaOCl washed but unspiked sweet corn kernels

Unspiked sweet corn samples were treated with NaOCl, washed with potable water (1:2; w/v) twice for 5 min each to remove residual chlorine, and later air dried for 2 h. Moisture content (%) and water activity of samples were determined by infrared drying using a moisture analyzer (Sartorius MA 100, Chicago, U.S.A.) and water activity meter (AqualabCX2T, Decagon Devices Inc., Wash., U.S.A.) which works on chilled-mirror dew point technique, respectively (Kumar *et al.*, 2012a). Nutritional parameters were analyzed in terms of energy value, content of carbohydrate, dietary fiber, protein, fat [saturated fat, mono unsaturated fatty acids (MUFA), poly unsaturated fatty acids (PUFA) and trans fat] and vitamin C.

Dietary fiber content was determined using BIS (Bureau of Indian Standard) method (IS 11062: 1984) that is based on enzymatic-gravimetric method of AOAC official method 991.43 (1992). Protein content was determined using BIS method (IS 7219: 1973) based on Kjeldahl method. From nitrogen content, protein content was calculated by applying conversion factor (N factor) of 5.83. Total fat content and fatty acid composition was determined by gas chromatography analysis (Agilent Technologies GC- FID, Santa Clara CA, U.S.A.) according to the AOAC official

method 996.06 (2007). Total vitamin C content was determined by the BIS method (IS 5838: 1970) based on titrimetric process using dye 2,6 dichlorophenol indophenols (DCPIP). Energy content was determined by standard calculation based on the protein, sugar, fat and carbohydrate content of the sample. Carbohydrate content was determined by deducting the percentage values of moisture, ash, protein, and fat.

Sensory evaluation

Unspiked sweet corn samples (250 g) upon NaOCl treatment and potable water washing was steamed for 40 min in a pressure cooker and sensory attributes were evaluated by 50 trained panelists (25 men and 25 women) in a Taste Panel Laboratory in individually partitioned compartments (Meilgaard *et al.*, 1999; Kumar *et al.*, 2007). The quality attributes including appearance, color, texture, odor, taste, and overall acceptability were evaluated on a 9-point hedonic scale.

Statistical analyses

The experiments were performed in three independent sets in three replicates. Statistical analysis using one-way ANOVA ($\alpha = 0.05$) was performed using software BioStat 2009 Professional 5.8.0.0 (AnalystSoft Inc., Vancouver, BC, Canada) and results were expressed as mean and standard deviations.

Results and Discussion

Mycotoxins have always remained a problem in food safety and global food trade (Ramos *et al.*, 1998; Garcia *et al.*, 2011). About 25% of world grain crops are annually affected by fungal invasion and mycotoxin contamination (Mannon and Johnson, 1985). Due to high toxicity and carcinogenicity of mycotoxins including

OTA, international regulations have set their limits in food stuffs for both human and animal consumption (FAO, 2004). The permissible limits of OTA in foods are as follows, for cereal grains and coffee beans: <5 $\mu\text{g}/\text{kg}$; grape juice, wine and beer: <2-3 $\mu\text{g}/\text{kg}$; and dried fruits: <0.2 $\mu\text{g}/\text{kg}$ (EC 105/2010).

These limits also have implications on their international trade. In the current study NaOCl, an approved disinfectant and sanitizer was tested for its efficacy to inactivate OTA. NaOCl has been reported to be useful for various food applications such as treatment of Indian mango before subjecting to gamma irradiation as a phytosanitary treatment for exporting to USA, processing of litchi for hygienization and shelf life extension, or US FDA approval for the treatment of sprouts (US FDA, 2013; Kumar *et al.*, 2012b; APEDA, 2007). OTA extracted from *A. ochraceus* spiked sweet corn samples not subjected to NaOCl wash has been abbreviated as OTA_U and that from NaOCl washed samples as OTA_T. Henceforth, this terminology has been used in the manuscript wherever required.

NaOCl treatment degraded OTA in *A. ochraceus* spiked sweet corn

Aqueous suspension of OTA (0.25 $\mu\text{g}/500 \mu\text{l}$) (Fig. 1, L1) was found to be degraded to below detectable level at 200 ppm of NaOCl treatment when analyzed on TLC plates (Fig. 1, L5). Below this NaOCl concentration complete degradation of OTA was not observed (Fig. 1, L2-L4). Therefore, NaOCl at 200 ppm was used for assaying the efficacy of chlorination on the inactivation of OTA from spiked sweet corn samples. Level of OTA_U was found to be ~10 mg/kg after 7 days of incubation (Fig. 1, L6; Fig. 2B) which got reduced by 66±7%

when subjected to NaOCl wash (Fig. 1, L7; Fig. 2C). At lower concentration of OTA (0.35 mg/kg) (Fig. 1, L8), NaOCl treatment was found to be very effective and it reduced the OTA concentration to below detectable level (Fig. 1, L9). Thus, the treatment efficacy of chlorination for destroying OTA in the food was found to depend on OTA concentration. Similar findings have been reported earlier from this laboratory pertaining to the efficacy of gamma radiation treatment on OTA degradation (Kumar *et al.*, 2012a).

NaOCl treatment also resulted in reduced cytotoxicity

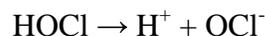
The normal adherent morphology of Int-407 cells was found to be significantly changed to non-adherent upon treatment with OTA_U (Fig. 3A-C). Such morphological change has been reported earlier as an indication of cytotoxic effect (Basu *et al.*, 1999; Herbst-Kralovetz *et al.*, 2013). However, in case of OTA_T, the adherent cell morphology remained unchanged and found to be similar to the control cells (Fig. 3D-G).

The above findings were also validated by MTT assay (Fig. 3H). The viability of Int-407 cells was found to be reduced by ~4, 18 and 42% at the OTA concentration of 1.25, 2.5 and 5 µg/200 µl of reaction mixture with OTA_U samples, respectively (Fig. 3H), whereas, in case of OTA_T, the viability was reduced marginally by 9% even at the highest OTA concentration used in this experiment i.e. 5 µg/200 µl (Fig. 3H).

At lower OTA concentrations (1.25 and 2.5 µg/200 µl), the toxicity was negligible (Fig. 3H). The detoxification of OTA by NaOCl could be primarily due to the loss of structural integrity possibly by opening of OTA ring structure (Bozořglu, 2009).

Potable water washing left no residual chlorine in sweet corn treated with NaOCl

The sodium hypochlorite reacts with water as follows:



The two chemical species, hypochlorous acid (HOCl) and hypochlorite ion (OCl⁻) are commonly referred as “free available” chlorine and act as oxidizing agents. NaOCl treated sweet corn samples when washed twice with potable water, the residual chlorine was found to be below detectable level (< 3 ppm) (data not shown). Similar observation has been earlier reported in case of NaOCl treated and potable water washed litchi fruits also from this laboratory (Kumar *et al.*, 2012b). Generally, it has been suggested to wash the food samples after chlorination or any chemical process to reduce the residue level (McGlynn, 2004).

Physical, nutritional and sensory qualities of NaOCl treated unspiked sweet corn samples

In the fresh (untreated) corn samples moisture content and water activity was found to be ~73% and 0.93, respectively. Nutritionally, sweet corn is as good as other staple cereals such as rice or wheat (USDA, 2002). In the untreated corn samples content (g/100 g) of carbohydrate, dietary fiber, protein, and fat was found to be ~16, 12, 3, and 0.2, respectively (Table 1). The vitamin C content and energy per 100 g was ~10 mg and 80 kcal., respectively (Table 1). Insignificant changes were observed in the physical (data not shown) and nutritional properties of treated corn samples (Table 1).

Fig.1 Thin-Layer Chromatography (TLC) of ochratoxin A (OTA; 0.25 µg/500 ml) treated with L1: Water (control), L2-L5: NaOCl [L2 (25 ppm), L3 (50 ppm), L4 (100 ppm), L5 (200 ppm)]; *A. ochraceus* spiked sweet corn [OTA (10 mg/kg) in sweet corn] treated with L6: Water (control), L7: NaOCl (200 ppm); *A. ochraceus* spiked sweet corn [OTA (0.35 mg/kg) in sweet corn] treated with L8: Water (control), L9: NaOCl (200 ppm)

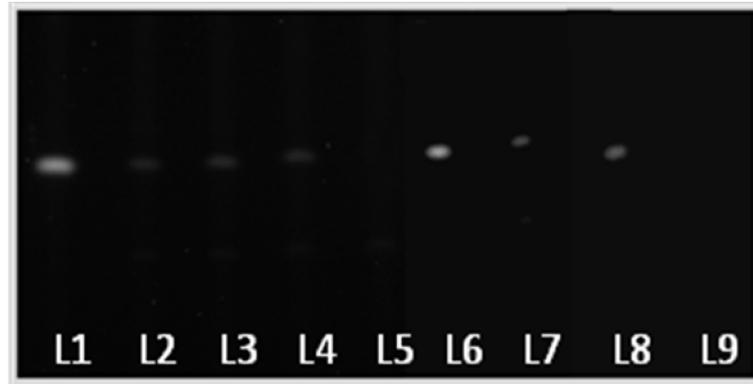
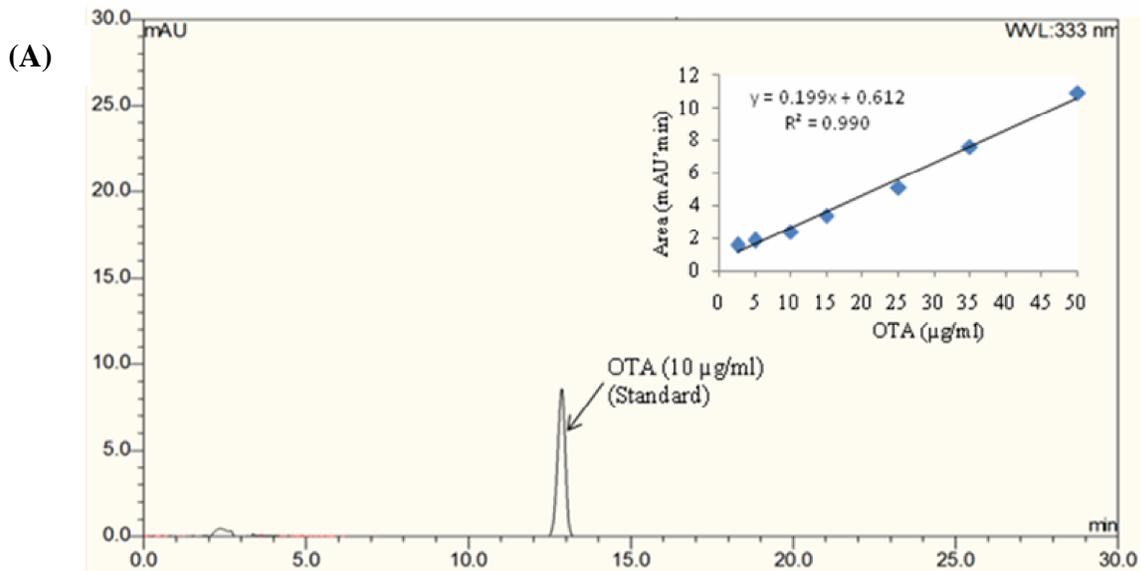
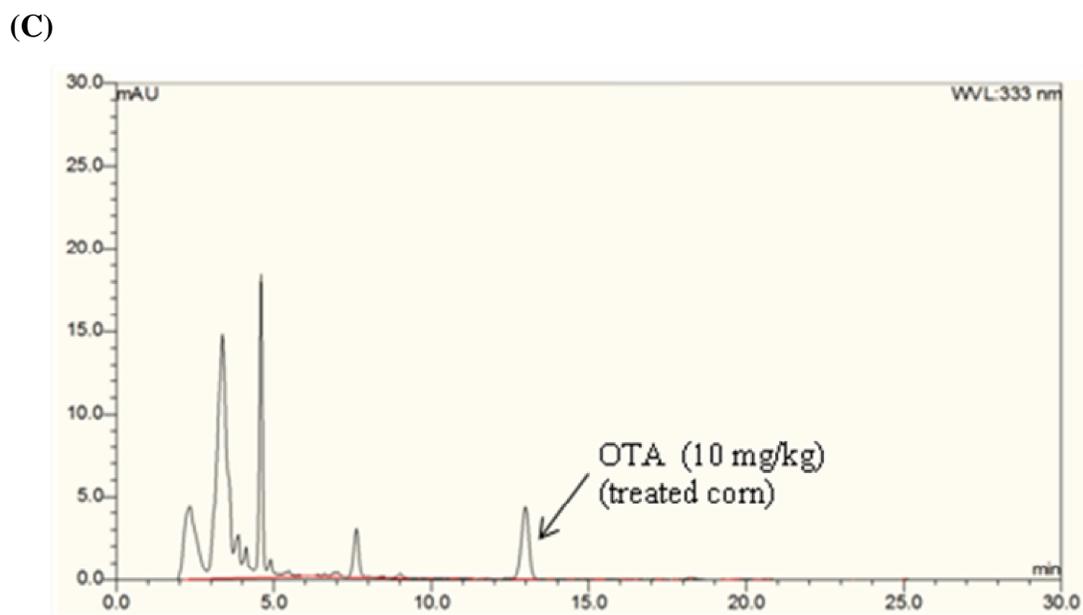
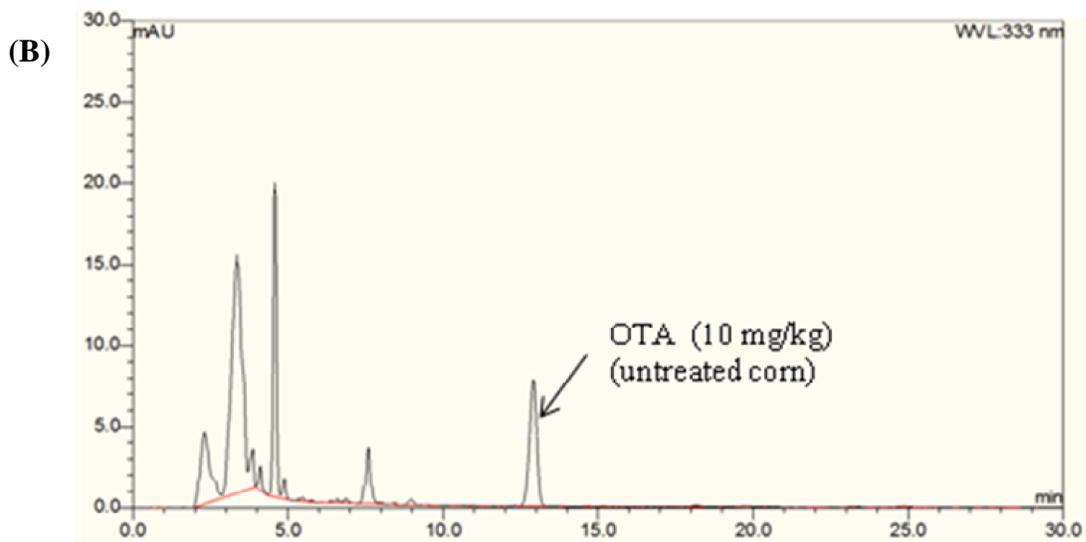


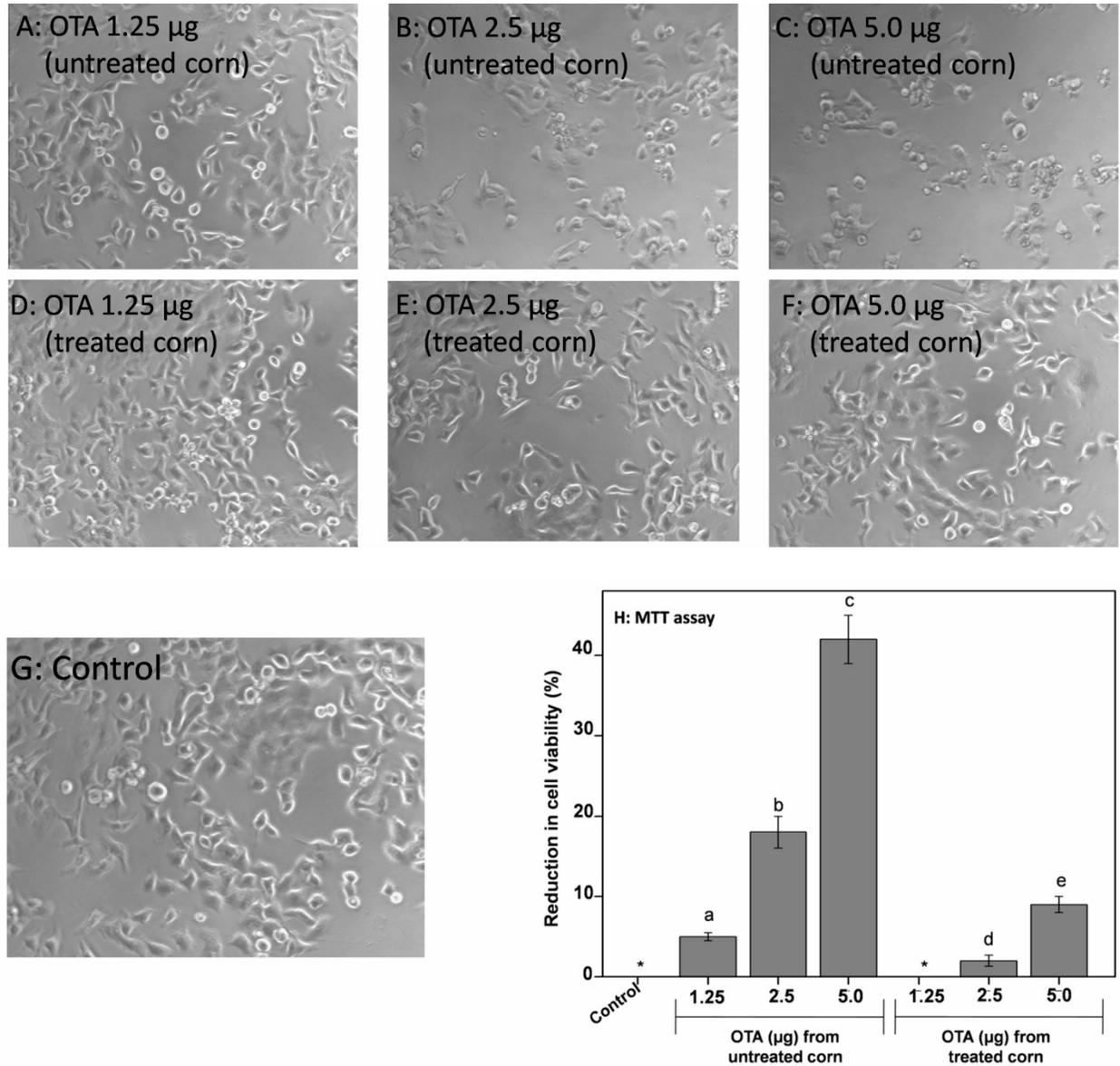
Fig.2 High Performance Liquid Chromatography (HPLC) showing reduction of ochratoxin A (OTA) from *A. ochraceus* spiked sweet corn upon NaOCl (200 ppm) treatment (A) Standard OTA (10 µg/ml) (calibration curve with standard OTA 2.5-50 µg/ml, shown in inset); (B) OTA (10 mg/kg) in sweet corn; (C) OTA (10 mg/kg) in sweet corn treated with NaOCl





Note: No OTA was detected upon NaOCl treatment in *A. ochraceus* spiked sweet corn having OTA of 0.35 mg/kg.

Fig.3 Toxicity analysis of OTA extracted from *A. ochraceous* spiked sweet corn in Int-407 cells in 200 µl reaction mixture A-C: Untreated corn, D-F: NaOCl (200 ppm) treated corn by microscopic observation and MTT assay[#] (H)



Note: Sweet corn without OTA served as control (G).

‘*’ Indicates no effect on cell viability.

Different letters on the top of the bars indicate significant difference among means at $P \leq 0.05$.

Table.1 Nutritional and sensory quality of control and sodium hypochlorite treated corn

Nutritional parameters (per 100 g fresh weight)	Control corn	Treated corn
Energy (k.cal)	80±2 ^a	82± ^a
Carbohydrate (g)	16±3 ^a	17±2 ^a
Dietary fibre (g)	11.6±2.3 ^a	8.2±3.5 ^a
Protein (g)	3.1±0.3 ^a	2.9±0.3 ^a
Fat (g)	0.2±0.03 ^a	0.2±0.04 ^a
Saturated fatty acid (mg)	0.1±0.02 ^a	0.1±0.02 ^a
Monounsaturated fatty acid (mg)	0.05±0.01 ^a	0.06±0.02 ^a
Polyunsaturated fatty acid (mg)	0.03±0.01 ^a	0.01±0.01 ^a
Trans fatty acid (mg)	0.01±0.003 ^a	0.01±0.002 ^a
Vit. C (mg)	9.7±3.3 ^a	11.5±3.0 ^a
Sensory parameters (9-point hedonic scale*)	Control Corn	Treated Corn
Appearance	8.0±0.9 ^a	7.5±1.3 ^a
Color	8.1±1.0 ^a	7.3±1.5 ^a
Aroma	7.9±1.1 ^a	7.3±1.5 ^a
Texture	6.8±1.4 ^a	7.8±0.8 ^a
Flavor	7.4±0.9 ^a	7.1±1.4 ^a
Taste	7.3±1.0 ^a	7.6±1.1 ^a
After taste	7.3±1.2 ^a	7.8±1.0 ^a
Overall acceptability	7.5±1.1 ^a	7.5±1.2 ^a

* 9 = like extremely, 8 = like strongly, 7 = like very well, 6 = like fairly well, 5 = like moderately, 4 = like slightly, 3 = dislike slightly, 2 = dislike moderately and 1 = dislike extremely. Same letters row wise indicate no significant difference among means at $p \leq 0.05$

Also, organoleptically, treated samples were well received by the taste panelists and overall acceptability ranged between 7 and 8 (i.e. 'like very well' to 'like strongly') similar to the fresh control samples (Table 1).

Thus, the study provides a solution for detoxification of OTA from cereals like sweet corn through chlorination treatment, whose inclusion in the processing chain can reduce the risk to human health posed by the OTA contamination. The NaOCl being a cheap and easily available chemical also ensures economic feasibility of the process.

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