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Prospective antimycotoxigenic action of wild *Opuntia ficus-indica* by-products

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Abstract: Numerous natural compounds perform pharmaceutical, antimycotic and antitoxic purposes in the body system. The aim was to evaluate new phytoconstituents that provide antimycotoxigenic properties against mycotoxins. Bioactive materials chosen were fruit peels and cladodes of wild *Opuntia ficus-indica* containing a measurable quantity of bioactive phytochemicals. The highest concentration of bioactive metabolites was recorded for protocatechuic and *t*-cinnamic acids. A reduction effect of bioactives was estimated against aflatoxins, ochratoxin A and zearalenone in a simulated body system. Antifungal activity was determined in liquid media to evaluate antimycotic properties. Lyophilised extracts caused an aflatoxin reduction in media by 14.65% to 23.77% for fruit peels and cladodes, respectively. It caused a decrease of 59% zearalenone and 51% of ochratoxin A in a in a simulated body fluid. The cladode extract manifested better antimycotic and antimycotoxigenic characteristics due to its bioactive contents. These results support a modern antimycotoxin trend of food preservation that has a considerable impact on food safety.

Keywords: mycotoxins; antimycotoxigenic; antifungal activity; phenolic acids; wild *Opuntia ficus-indica*

The *Opuntia ficus-indica* by-products (OPFBs) separated before preparation of the edible part include fruit peels (FPs), seed, stem and cladodes (CLs). The OPFBs accumulate the bioactives, mainly antioxidant pigments. The exploitation of OPFBs would in part solve the problem of waste materials by their utilization as a rich bioactive reservoir showing pharmacological activity against various chronic diseases (Osuna-Martínez et al. 2014). In a previous part, proximate analysis of by-products revealed high microelements, total phenolics and essential fatty acids. Extracts of CLs and FPs showed considerable antimicrobial activity against *Fusarium fungi* that suggest promising mycotoxin reductions (Abdel-Razek et al. 2019). In that study, relatively high contents of total phenolics and total flavonoids were previously reported for wild OPFBs. For this reason, the profile of phenolic fractions in the OPFB dry extracts was determined in the present study.

Mycotoxins are harmful compounds produced by toxigenic fungi on foods. They cause acute and chronic diseases concerning human and animal health. Aflatoxins (AFs) have been recognised as carcinogenic substances that induce mutagenic changes in tissues (Shahat et al. 2017). Mycotoxin biosynthesis could be reduced using active constituents. Bioactives, such as microbial metabolites, revealed a mycotoxin decrease during *in vitro*

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studies. Plant bioactives performed an antifungal activity, which leads to antimycotoxigenic properties (Shehata et al. 2018). It is important to search for new promising natural materials that reduce fungal growth and break down mycotoxins.

Natural bioactives may have the same impact on fungi as synthetic or pharmaceutical compounds. This impact has two aspects, which could occur individually or consecutively. Firstly, the antifungal or antimycotic effect when fungal growth is reduced. Secondly, the influence on mycotoxin secretion that is known as an antimycotoxigenic impact. Comparatively, the two impacts may occur parallelly or consecutively, resulting in a reduction of contamination levels. However, it is striking that the effect on the production of mycotoxins is not so obvious as the fungal growth reduction. It should be noted that reducing the fungal growth would be followed by a mycotoxin decrease.

Concerning the global trend of increasing the food safety of products, particularly natural components should be used. Implementation of natural extracts in pre- or post-harvesting stages was reported with positive impacts on mycotoxin reduction (Shehata et al. 2018; Abdel-Razek et al. 2019). The correct handling and more extensive use of OPFBs provide economical sources of bioactives with recognised antimycotoxigenic properties. Thence, the study evaluated antimycotoxigenic potentials of two types of OPFBs, as well as their detoxification activities against mycotoxins as a novel perspective for food safety.

MATERIAL AND METHODS

Preparation of raw material extracts. Full coloured red-purple *Opuntia* fruits and CLs were collected from an arid land around Alexandria, Egypt. The CLs and FPs were oven (JP Selecta s.a., Spain) dried at 40 °C for two days, then ground into fine powder, and stored (at 4 °C). The powder was extracted using ten times volume of 80% methanol. The flask was covered and shaken overnight using a shaking incubator (VWR® Incubating Orbital Shaker, Model 3500I, USA), then the extracts were filtered [PMillipore PVDF membrane (0.22 μm)USA]. The filtrate was evaporated in a vacuum rotary evaporator (SCILOGEX RE100-Pro, China) (40 °C), the concentrated solution was lyophilised using a Dura-Dry MP freeze-dryer system (USA).

Fungal strains. Four strains of identified toxigenic fungi of *Aspergillus* spp. were used for the antimycotic and antiaflatoxigenic experiments. These strains were *A. flavus* ITEM 698, *A. parasiticus* ITEM 11, *A. fumigatus* ATCC 1022 and *A. niger* ITEM 3856.

Determination of tocopherol and sterols. Tocopherol content in the extracts was determined according to the method described by Katsanidis & Addis (2001). A sample extract was injected into high-performance liquid chromatography (HPLC), the sample volume was adjusted to 20 μL, the isocratic system was applied with 1.0 mL min^{-1} flow and wavelength of 295 nm, using a UV detector (Shimadzu UV-1201, Japan). Sterol content in the dry extracts was determined according to the method described by Stuper-Szablewska et al. (2017).

Determination of phenolic acids and carotenoids. Phenolic compound content in dry extracts of the FPs or CLs was determined according to the method and conditions of Kurasiak-Popowska et al. (2016). Carotenoids were separated and their quantity in samples was evaluated by means of Acquity ultra-high performance liquid chromatography (Waters, USA) according to the method and conditions described by Kurasiak-Popowska et al. (2019). In brief, the analysis was performed using an Acquity H class UPLC system equipped with the Waters Acquity PDA detector (Waters, USA). Chromatographic separation was carried out on an Acquity UPLC® BEH C18 column (100 × 2.1 mm, 1.7 μm) (Waters, Ireland). The elution was achieved using the following mobile phases in the appropriate gradient: A: acetonitrile with 0.1% formic acid, B: 1% aqueous formic acid (pH = 2.0). The phenolic compound concentrations were determined at λ = 320 and 280 nm, and were identified based on a comparison of retention time of the analyte peak with retention time for standards and by adding a specific amount of the standard to the analysed samples and repeating this analysis. The limit of detection was 1 μg g^{-1} of the sample.

Antifungal evaluation in liquid medium. Antifungal activity of OPFBs was estimated using a liquid medium of yeast extract sucrose (YES). The impact of testing materials was expressed as a decrease of the mycelium weight of growing fungi. Briefly, in 500 mL conical flasks about 150 mL of YES was autoclaved, and then inoculated with the tested fungal strains (2.3×10^5). Strains were activated first in Czapek Dox Agar media, and then spores were transferred to Tween-water and used for inoculation of the YES liquid medium. Flasks were incubated at 25 °C for 5 days, and the obtained mycelia were then collected by filtration under controlled conditions using filter paper of known weight.

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The reduction of the mycelium weight of particular fungal species compared to the control represented the antifungal efficacy of applied material was calculated using the following equation:

$$ME = \left(W_c - \frac{W_t}{W_c} \right) \times 100\ (\%) \quad (1)$$

where: ME – the efficacy of the material to inhibit fungal growth (expressed as inhibition ratio); W_c – weight of dried mycelia obtained from the control medium; W_t – weight of dried mycelia obtained from a medium with the OPFB addition (1 mg 100 mL^{-1}).

Antitoxigenic properties of FP and CL dry extracts. Antiaflatoxigenic fungal properties of the OPFBs (FPs and CLs) were estimated for 10, 50, 100 mg of lyophilised extract per 1 mL of the medium according to the method described by Shehata et al. (2019).

Reduction of mycotoxin contents. The mycotoxin reduction rate was evaluated in a simulated body fluid by spiking a known amount of the particular toxin to 10 mL of phosphate buffer saline (PBS; pH 7.3). The reduction rate was determined against three types of mycotoxins including aflatoxins (AFs), ochratoxin A (OCA) and zearalenone (Zen). Spiked amounts were 500 ng L^{-1} for aflatoxins (B_1, B_2, G_1, and G_2), and 800 and 1 650 ng L^{-1} for OCA and Zen, respectively. The OPFB degradation efficiency in the PBS was estimated. The results were expressed as a reduction percentage of the initial concentration according to the following equation:

$$TC_R = \left(TC_c - \frac{TC_t}{TC_c} \right) \times 100\ (\%) \quad (2)$$

where: TC_R – the OPFB efficacy to reduce mycotoxin content (expressed as inhibition ratio); TC_c – the amount of the mycotoxin in the control fluid; TC_t – the amount of the mycotoxin in the OPFB treated fluid.

Statistical analysis. All analyses were carried out in triplicate and data are expressed as means ± SD. One-factor ANOVA was used to determine significances at $P = 0.05$ (SPSS 16 software).

RESULT AND DISCUSSION

Carotenoid content in OPFB dry extracts. The carotenoid content was estimated in the CLs and PLs extracts. Total carotenoid content (TCC) was 1.46 ± 0.21 and 2.46 ± 0.25 mg kg^{-1} DW for CLs and PLs, respectively. Beta-carotene was recorded as the major carotenoid in both extracts with the content of 1.42 ± 0.11 and 0.72 ± 0.05 mg kg^{-1} DW for CLs and PLs, respectively. Alpha-cryptoxanthin was the second major carotenoid evaluated (Table 1). Lutein and zeaxanthin were not detected in CLs, while in PLs their content was 0.15 and 0.21 mg 100 g^{-1} DW, respectively.

Tocopherol content in OPFB dry extracts. The highest tocopherol content (TTC) was recorded for the CL extract (1.29 ± 0.11 mg kg^{-1} DW) (Table 1). Among tocopherol isoforms, γ-tocopherol was dominant and its content reached 0.88 ± 0.02 mg kg^{-1} DW in the CL dry extract. Conversely, the FPs contained lower tocopherol amounts (0.142 ± 0.055 mg kg^{-1} DW). The relatively higher contents of tocopherol in CLs might be triggered by high polyunsaturated fatty acid (PUFA) contents as estimated in our previous investigation since tocopherol is recognised as an efficient lipophilic antioxidant (Abdel-Razek et al. 2019).

Sterol content in OPFB dry extracts. The predominant sterol found in the FP and CL extracts was β-sitosterol (159.3 ± 0.77 and 329.5 ± 0.96 mg kg^{-1} DW, respectively), and was followed by campesterol and lanosterol**.** While, Δ-5-avenasterol reached relatively lower contents in CLs (12.8 ± 0.91 mg kg^{-1} DW), it was not detected in FPs extract.

Ramadan & Morsel (2003) documented β-sitosterol as a major sterol present in the extract of most OPF parts,

Table 1. Tocopherol, carotenoid and sterol content in the *Opuntia* by-product dry extracts ($n = 3$; mean ± SD)

Compound	Peels (PLs) (mg 100g^{-1} DW)	Cladodes (CLs) (mg 100g^{-1} DW)
Carotenoids		
Lutein	0.15 ± 0.02	ND
Zeaxanthin	0.21 ± 0.06	ND
β-Carotene	0.72 ± 0.05	1.42 ± 0.11
α-Cryptoxanthin	0.38 ± 0.08	1.04 ± 0.14
TCC	1.46 ± 0.21	2.46 ± 0.25
Tocopherol		
α-Tocopherol	0.06 ± 0.05	0.31 ± 0.02
β-Tocopherol	ND	0.09 ± 0.07
γ-Tocopherol	0.067 ± 0.003	0.88 ± 0.02
δ-Tocopherol	0.015 ± 0.002	0.01 ± 0.001
TTC	0.142 ± 0.055	1.29 ± 0.11
Sterols		
Campesterol	2.3 ± 0.07	6.1 ± 0.05
β-sitosterol	159.3 ± 0.77	329.5 ± 0.96
Lanosterol	1.77 ± 0.22	0.56 ± 0.08
δ-5-avenasterol	ND	12.8 ± 0.91
TSC	163.37 ± 1.06	348.96 ± 2.00

ND – not detected; TCC – total carotenoid content; TTC – total tocopherol content; TSC – total sterol content

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with the content up to 21 g kg^{-1}. Campesterol is a fraction present in OPF pulp, seed and FPs. Generally, sterols have been reported having broad biological functions (Lanuzza et al. 2017).

Phenolic compounds in OPFB dry extracts. Protocatechuic acid (PCA), *t*-cinnamic acid, isorhamnetin, *p*-coumaric, chlorogenic and caffeic acids were recorded as the major phenolic compounds in the OPF cladodes (Table 2). These phenolics were also detected in FPs, however at relatively lower amounts. Generally, the phenolic acid profile of the two by-products (CL and FP dry extracts) was characterised by significant amounts of protocatechuic acid in CLs (148.6 ± 0.14 mg kg^{-1} DW) and FPs (73.28 ± 0.08 mg kg^{-1} DW), which was the most abundant phenolic compound. Caffeic, gallic, syringic, and vanillic acids were also present either in CL or FP extracts. These phenolic compounds were previously reported to show a strong antioxidant activity (Zielinski et al. 2014). Generally, the phenolic content of CLs showed higher values (twice on average) compared to FPs, which indicated a higher antioxidant potential of this extract.

Reduction of mycotoxin concentration by OPFB extracts. To spotlight towards the OPFB significance for antimycotic and antimycotoxigenic impacts, the efficiency of the OPFBs (1mg 100 mL^{-1} media) against toxigenic fungi was determined. The obtained results showed a strong inhibition against *Aspergillus* strains. Among the tested strains, the strongest inhibition was recorded for *A. flavus* and *A. parasiticus* reaching 80.5% and 81%, respectively, a reduction followed the CL addition (Figure 1). These ratios decreased to 70.7% and 67.2% using FPs in media for the same fungi, respectively. While the inhibition rate for *A. fumigates* and *A. niger* was lower for both types of OPFBs.

The results that showed a significant reduction of aflatoxin concentration ranged between 19 and 23% for CLs (1 mg 100 mL^{-1}) and 14 to 16% for FPs (1 mg 100 mL^{-1}) in the growth media of *A. parasiticus* ITEM 11, giving a strong evidence of their antimycotoxigenic impacts (Figure 2). These results were proved by the simulated body fluid experiment that was employed to predict the OPFB behaviour inside the living cells using phosphate buffer saline (PBS, pH 7.3). The OPFB behaviour against spiked mycotoxins showed a significant Zen reduction to 59% and OCA reduction to 41% besides the aflatoxin reduction rates (Figure 3). These results were in agreement with the *in vivo* study of Brahmi et al. (2011), who evaluated a chemopreventive impact of OPF against genotoxicity and aflatoxicosis of the AFs in rats.

Table 2. Phenolic compound content in *Opuntia ficus-indica* by-products extracts (n = 3; mean ± SD)

Compound	Peels (PLs) (mg kg^{-1})	Cladodes (CLs) (mg kg^{-1})
Apigenin	0.37 ± 0.008	0.5 ± 0.014
Catechin	ND	11.7 ± 0.01
Kaempferol	ND	0.076 ± 0.005
Naringenin	0.06 ± 0.013	0.25 ± 0.01
Quercetin	0.11 ± 0.012	0.65 ± 0.017
Isorhamnetin	13.08 ± 0.17	49.27 ± 0.11
4-hydroxybenzoic acid	1.6 ± 0.12	8.63 ± 0.18
Caffeic acid	9.86 ± 0.05	14.6 ± 0.12
Chlorogenic acid	16.54 ± 0.02	25.9 ± 0.27
Ferulic acid	7.83 ± 0.06	11.8 ± 0.03
Gallic acid	2.44 ± 0.009	6.45 ± 0.07
Coumaric acid	31.66 ± 0.018	48.1 ± 0.15
Protocatechuic acid	73.28 ± 0.88	148.6 ± 0.14
Sinapic acid	0.82 ± 0.009	1.5 ± 0.06
Syringic acid	5.67 ± 0.16	12.9 ± 0.13
Cinnamic acid	38.27 ± 0.14	78.6 ± 0.18
Vanillic acid	6.41 ± 0.09	12.4 ± 0.005
Vanillin	0.1 ± 0.006	0.5 ± 0.004
Gentisic acid	1.46 ± 0.05	2.39 ± 0.15
Myricetin	0.71 ± 0.005	1.73 ± 0.04
Pyrogallol	2.64 ± 0.08	4.93 ± 0.05
3,4-dihydroxybenzaldehyde	ND	0.087 ± 0.002
Chrysin	ND	0.18 ± 0.003

ND – not detected

Due to these results, the OPFBs have relatively high abilities for degrading mycotoxins in liquid systems, which are more pronounced in the case of CLs and associated with higher bioactives content contributing mainly to antioxidant properties (Abdel-Razek et al. 2019). Phenolic acids, inclusively protocatechuic acid, possess well-recognised antioxidant activity and generate a reducing effect against hazardous compounds. Further, the PCA showed an antibacterial, antioxidant and anticancer properties, and hepatoprotective activity versus oxidative cirrhosis induced in liver due to its anti-inflammatory and antioxidant actions (Chao & Yin 2009; Tanaka et al. 2011). Additionally, a protective effect against microbial contamination was related to its antimicrobial and food preservative characteristics (Kakkar & Bais 2014). Thus, this pointed to the application of PCA-rich extracts against carcinogens and pre-carcinogens such as mycotoxins

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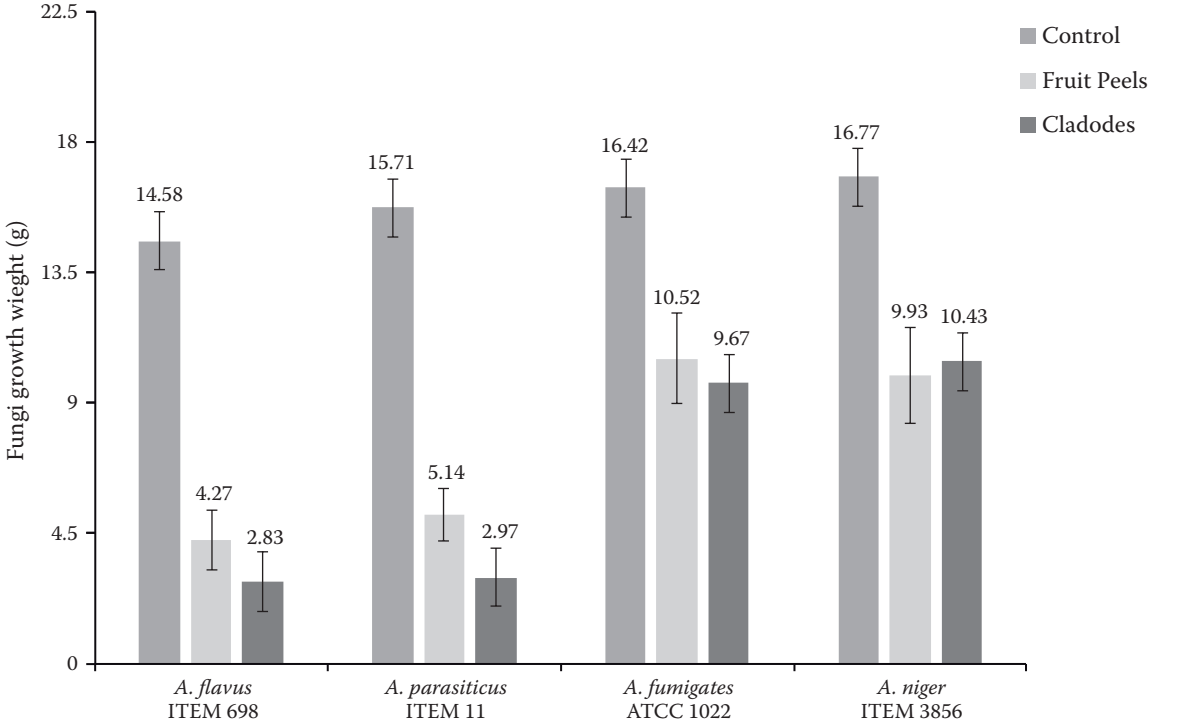

Figure 1. The impact of wild *Opuntia* by-product (OBPEs) extracts on aflatoxigenic fungi

The applied concentration of cladode and peel extract in media was 1 mg 100 mL^{-1} of media

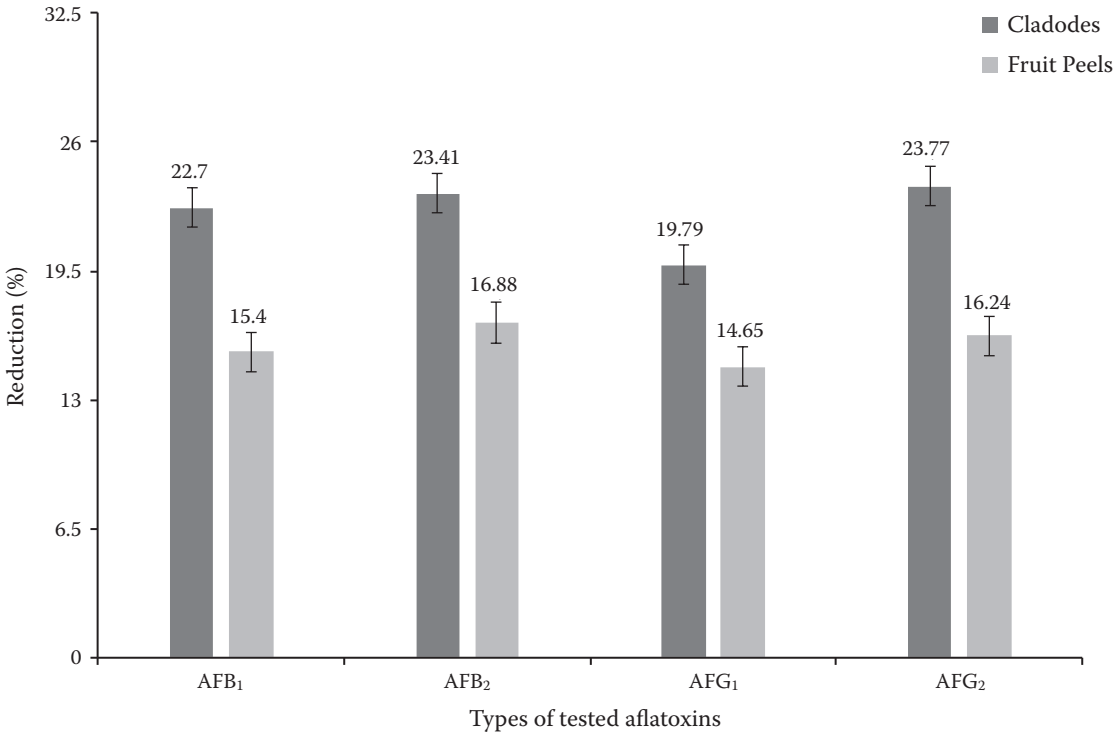

Figure 2. Reduction of aflatoxin concentration in liquid media by OPFB extracts

Aflatoxins were estimated in media inoculated by *A. parasiticus* compared to the control containing 500 ng mL^{-1} of each aflatoxin type; OPFBs – *Opuntia ficus-indica* by-products; AFB_1 – aflatoxin B_1; AFB_2 – aflatoxin B_2; AFG_1 – aflatoxin G_1; AFG_2 – aflatoxin G_2

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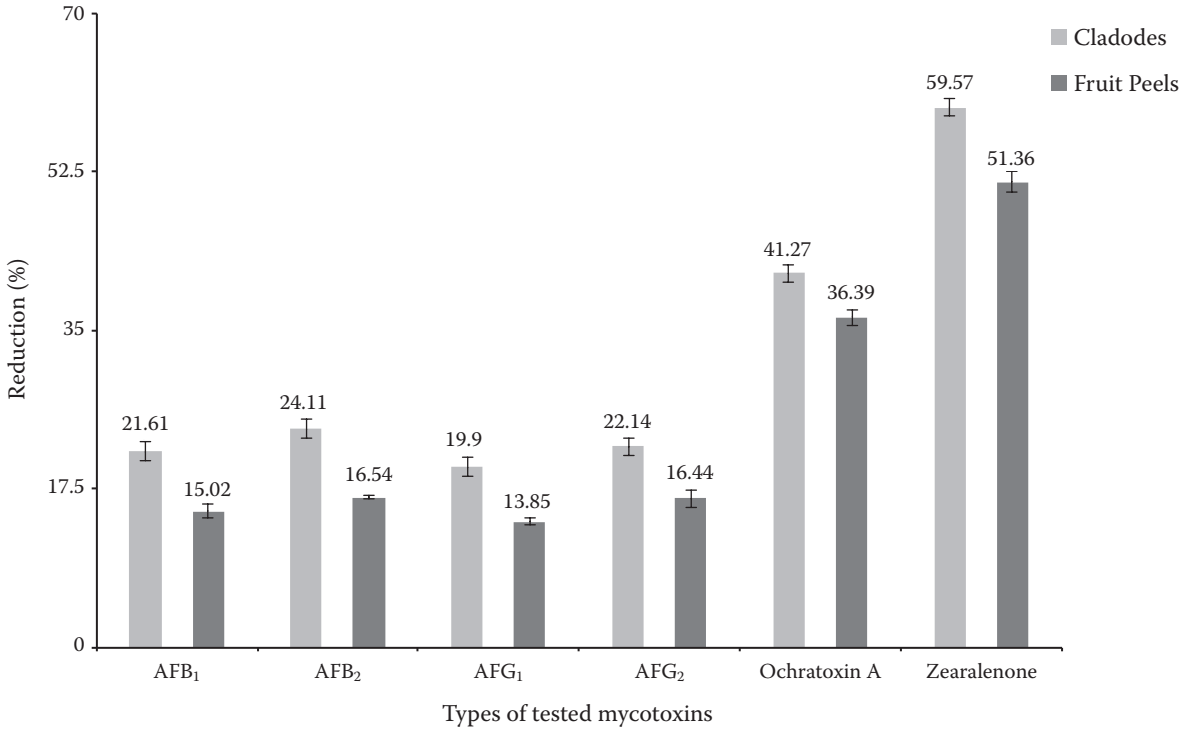

Figure 3. The OPFB extract efficacy to reduce mycotoxins in a stimulated body fluid

The stimulation was done by OPFBs 10 mg L^{-1} of PBS (24 h incubation); reduction was estimated compared to spiked mycotoxins in the control; OPFBs – the *Opuntia ficus-indica* by-products; PBS – phosphate buffer saline; AFB_1 – aflatoxin B_1; AFB_2 – aflatoxin B_2; AG_1 – aflatoxin G_1; AFG_2 – aflatoxin G_2

and particularly aflatoxins. The CLs extract was previously reported to have a protective effect against oxidative damage, which definitely correlated with the existence of various antioxidants including tocopherol, carotenoids, phenolic acids and flavonoids (Stintzing & Carle 2005). Recently, the OPF expressed a significant increment of antioxidant potency in plasma (20%) and in blood (5%) after consumption of meals with their supplements (Ávila-Nava et al. 2014). This could provide defence against aflatoxin oxidative stress impacts.

The supplementation of food and beverages using OPFB extracts provides a novel solution to the mycotoxin limitation. The addition of such by-products could increase both mycotoxin reduction in food materials and enhance antioxidant activity in living systems.

CONCLUSION

The wild types of plants have been recognised having significant contents of bioactive components. Phytoconstituents of the OPFBs play a significant pharmaceutical, antimycotoxigenic and antimycotic functions both *in vitro* and *in vivo* due to their antioxidant potential. These phytoconstituents are represented mainly by flavonoids, phenolic acids and sterols. The OPFBs were found to have an essential content of phenolic acids, particularly protocatechuic acid that holds an advantage of being a vital antioxidant. Other compounds that have antioxidant potency were also detected. Freeze-dried OPFB extracts exhibited good antimycotic and antimycotoxigenic properties including the ability of mycotoxin degradation in both liquid media and simulated body fluid system (mainly OCA and Zen in PBS buffer). This reflected their potential use against mycotoxin oxidative stress.

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