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Occurrence and Exposure Assessment of Aflatoxin B_1 and Ochratoxin A in Pearl Millet (*Pennisetum glaucum* L.) from Tunisia

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HIGHLIGHTS

- Aflatoxin B_1 (AFB_1) and Ochratoxin A (OTA) were respectively detected in 32 and 28% millet samples from Tunisia.
- Approximately, 28 and 24% samples were above standard limits for AFB_1 and OTA, respectively.
- The estimated daily intake of OTA and AFB_1 were 3.76 and 3.89 ng/kg b.w. per day, respectively.

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Acronyms and abbreviations
AFB_1=Aflatoxin B_1
HPLC=High Performance Liquid Chromatography
OTA=Ochratoxin A

ABSTRACT

Background: Ochratoxin A (OTA) and Aflatoxin B_1 (AFB_1) are toxic secondary metabolites produced by certain mold species. In this primarily survey, we examined the OTA and AFB_1 contamination of pearl millet grains distributed in Tunisia.

Methods: Twenty-five pearl millet (*Pennisetum glaucum* L.) samples from different regions of Tunisia were analyzed by High Performance Liquid Chromatography coupled with fluorescence detector in order to evaluate the contamination with of AFB_1 and OTA. *Statistical tests* were performed with XLSTAT 2018.

Results: AFB_1 and OTA were detected in 32 and 28% millet samples, respectively. Mean amounts of these mycotoxins in the contaminated samples were of 24.54±17.54 µg/kg for OTA and 22.72±23.09 µg/kg for AFB_1. Approximately, 28 and 24% of analyzed samples were found above the European Union limits for AFB_1 and OTA, respectively. The estimated daily intake of OTA and AFB_1 were 3.76 and 3.89 ng/kg b.w. per day, respectively. No significantly (p>0.05) difference in OTA and AFB_1 contamination rate was found between samples taken from different regions.

Conclusion: Consumption of millet in Tunisia might be an important contributing factor to the risk of dietary exposure to OTA and AFB_1.

Introduction

Pearl millet (*Pennisetum glaucum* L.) is a major cereal food grain in Africa and India, and cultured as a forage crop in the United States, Australia, and South America (Jurjevic et al., 2007; McBenedict et al., 2016). Pearl millet grains have elevated nutritional values, due to the high amino and fatty acids, essential minerals, and vitamins contents (Manwaring et al., 2016). Moreover, the plant is well adapted to low rainfall and high temperature which allows it to acclimate well to the hot and dry regions where other cereal crops such as wheat or maize would not survive (Jurjevic et al., 2007). Pearl millet is a main staple food, along with sorghum, in a large region of Africa (Ben Romdhane et al., 2019; Loumerem et al., 2008).

Mycotoxins are toxic secondary metabolites synthesized by diverse fungal species growing on several agri-

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cultural products and processed food in the field or during transportation and storage. The Ochratoxin A (OTA) is mainly produced by *Penicillium verrucosum* and *Aspergillus ochraceus*. It possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties (Amézqueta et al., 2009; Bui-Klimke and Wu, 2015). Aflatoxin B_1 (AFB_1) is a mycotoxin synthesized mostly by *Aspergillus flavus* and *Aspergillus parasiticus* species and is the most potent natural carcinogen (Marchese et al., 2018; Rushing and Selim, 2019).

OTA and AFB_1 have been found in several commodities as diverse as spices, rice, barley, wheat, sorghum, cottonseed, nut crops, soybean, and milk (Bui-Klimke and Wu, 2015; Marchese et al., 2018). Pearl millet grains are generally considered as not good substrates for mycotoxin contamination when compared to groundnut and cereals like corn and sorghum (Chintapalli et al., 2006). But the earlier studies indicated that pearl millet grains are subject to mould contamination with dominance of *A. flavus* and *A. parasiticus* (Raghavender et al., 2007). Thus, many studies have shown the contamination of millet by mycotoxins from India and Sub-Saharan African countries (Ayalew et al., 2006; Chala et al., 2014; Raghavender et al., 2007). The European Commission (2002) has established maximum permitted levels for mycotoxins of major concern in unprocessed cereals other than maize, with 2 µg/kg being the maximum permitted level for AFB_1 and 5 µg/kg for OTA.

In North African countries, including Tunisia, in spite of the importance of pearl millet as a food grain, information concerning mycotoxin contamination is lacking. We surveyed the OTA and AFB_1 contamination of pearl millet grains obtained from different farmers and grain shops in Tunisia.

Materials and methods

Millet samples

Twenty-five pearl millet (*Pennisetum glaucum* L.) samples destined for human consumption were collected in January and February 2014 from retail shops (n=13) and farmers (n=12) across Tunisia. Farmer's samples were collected from the most known regions for millet culture located in the north (n=5), center (n=4), and south (n=3) of Tunisia representing different bioclimatic zones. Sub-humid (Nabeul) or semi-arid superior (Tunis) for northern regions, arid superior (Kairouan) or semi-arid inferior (Mahdia) for central regions, and arid inferior for southern regions (Medenine). Laboratory samples of 0.5 kg were ground to fine powder in a stainless steel blender to obtain consistent homogeneity. Grain samples were placed in air tight sterile plastic bags and stored at 4 °C.

OTA and AFB1 analysis

OTA and AFB_1 analysis were performed according to the European commission's regulations No. 657/2002 for official control of mycotoxins in foodstuffs (European Commission, 2002). For AFB_1 and OTA and analysis, the extraction and cleanup of the samples were performed using AflaStar™ and OchraStar™ immunoaffinity columns, respectively, according to the manufacturer's instructions.

Twenty-five g of finely grounded millet was added to 100 ml mixture of methanol:water (60:40, v/v) for AFB_1 and methanol:water (80:20, v/v) for OTA; then, blended at high speed for 3 min. The samples were centrifuged at 5000 rpm during 10 min and filtered through Whatman No.4 filter paper. Eight ml of the supernatant were collected and diluted with 16 and 24 ml of phosphate buffer saline solution for AFB_1 and OTA analysis, respectively. The diluted extract was loaded into the immunoaffinity column and eluted at 1-2 drops/s. Then, the column was washed with 20 ml of deionized water and the bound mycotoxins were eluted with 2 ml (2×1 ml) of methanol High Performance Liquid Chromatography (HPLC) grade or a solution of methanol:acetic acid (98:2, v/v), respectively. The methanolic extract was dried with a SpeedVac Concentrator, re-suspended in 0.5 ml of HPLC grade methanol and injected in the Liquid Chromatography (LC) apparatus. The OTA and AFB_1 detection and quantification were made by HPLC (Knauer, Germany) equipped with a C18 column (Waters Spherisorb 5 mm, ODS2, 4.6x250 mm). The OTA and AFB_1 detection were performed with fluorescence detection (Waters 474, Milford, Massachusetts, USA). For AFB_1 detection a post-column derivatization with Iodine was performed. The mobile phase was constituted with acetonitrile:water: acetic acid (57:41:2) and acetonitrile:water:methanol (50:20:30) for OTA and AFB_1 analysis, respectively, with a flow rate of 1.0 ml/min (injection volume 25 ml). The OTA and AFB_1 quantification were accomplished with Empower (Waters, Milford) software, based on a calibration curve set up by five AFB_1 and OTA standard solutions (5, 10, 50, 100, and 500 ng/ml in HPLC-grade methanol). The detection limits (LOD) were 0.3 ng OTA/g and 0.06 ng AFB_1/g.

Statistical analysis

For statistical analysis, the normality of the variable was evaluated with the Kolmogorov-Smirnov test. Mann-Whitney U test for two independent samples, were used to evaluate possible level differences among groups of samples (farmers and retail shop). A nonparametric Kruskal-wallis test was also used to evaluate possible level differences among regions. Non-parametric statistical tests were performed with XLSTAT 2018.

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Results and discussion

Occurrence of AFB_1 and OTA

The levels of AFB_1 and OTA in sampled millet grains are given in Table 1. A total of 13 out of 25 (52%) analyzed samples were contaminated with the studied mycotoxins. The levels of contamination of millet were in the ranges 3.5-53.52 µg/kg for OTA and 0.87-60.12 µg/kg for AFB_1. The average contamination of millet with both mycotoxins was very high, with mean amounts of positives samples of 24.54±17.54 µg/kg for OTA and 22.72±23.09 µg/kg for AFB_1. The median levels of OTA and AFB_1 concentrations were 24.83 and 15.20 µg/kg, respectively. Considering the samples with no detected mycotoxins, the mean contamination of millet was of 6.8±14.2 µg/kg for OTA and 7.2 ±16.5 µg/kg for AFB_1.

The co-occurrence of the two carcinogenic mycotoxins was found in two samples, one sample taken from a retail shop (Tunis/North) and one sample collected from a farmer (Medenine/South). The highest amounts of two mycotoxins were registered for the same sample, sourced from a farm located in the southern region of Tunisia (El Jorf/Medenine). The second most contaminated sample with OTA originates from the same region (El Jorf/Medenine). It is worth to notice that 3 of the 4 positives farmer's samples come from Medenine region in the south of Tunisia characterized by an arid climate. However, the statistical Kruskal-wallis test showed no significant (p>0.05) difference in OTA and AFB_1 contamination between samples taken from different regions.

Otherwise, the statistical Mann-Whitney test showed no significant differences in both OTA and AFB_1 contamination between samples taken from farmers and retail shops (p>0.05). Aflatoxins and ochratoxins are usually considered as storage mycotoxins and their contamination usually increases in physical damaged grains and in poor *storage* conditions. AFB_1 levels in Tunisian millet were comparable to AFB_1 contamination level of 15% found by Raghavender et al. (2007) in stored seed pearl millet samples from India with a concentration range of 20 to 230 µg/kg. Sirma et al. (2016) reported that 10% of millet samples from Kenya contaminated above the limit of 5 µg/kg with concentrations up to 1 658.2 µg/kg. To note that even if the rate of AFB_1 contamination was higher in Tunisian millet, the maximal concentrations in Kenyan and Indian grains were superior to those found in our samples. Furthermore, contrariwise to our results, a Nigerian study on the fonio millet showed very low levels of AFB_1 up to 1.4 µg/kg, despite a high prevalence of equal to 81% (Ezekiel et al., 2012). The same for Ethiopian finger millet which was reported by Chala et al. (2014) as less affected with aflatoxins contamination comparing to sorghum.

Moreover, this study showed that Tunisian millet is more contaminated by OTA than millet from different African and Asian countries. Indeed, to date, OTA was not found in the majority of researches about millet. This could be due to the Mediterranean climate of Tunisia which could favor the development of fungal species producing ochratoxin A. The only study to report contamination of millet with OTA was realized in Uganda (Echodu et al., 2019). The authors compared the ochratoxins contamination of several products and they found that sorghum had the highest mean concentration of pooled total ochratoxins (3.8 µg/kg), followed by sesame (1.4 µg/kg), millet (1.1 µg/kg), and maize (0.4 µg/kg); The range of the measured total ochratoxins was 0-3.2 µg/kg for millet, which was much lower than that found in Tunisian millet samples with a range of 3.5-53.52 µg/kg.

Table 1: Amounts (µg/kg) of OTA and AFB_1 in contaminated pearl millet samples from Tunisia

Code	Origin	Location/Region	Mycotoxin amount * (µg/kg)	
			OTA	AFB_1
M3	Retail shop	Kairouan/Center	24.83	<LD
M5	Retail shop	Nabeul/North	<LD **	56.91
M6	Retail shop	Nabeul/North	<LD	0.87
M7	Retail shop	Nabeul/North	25.79	<LD
M8	Retail shop	Tunis /North	9.65	<LD
M10	Farmer	Mahdia/Center	<LD	14.09
M11	Retail shop	Tunis /North	<LD	20.97
M14	Retail shop	Tunis /North	14.48	<LD
M18	Farmer	Medenine/South	<LD	10.18
M19	Farmer	Medenine/South	40.03	<LD
M20	Farmer	Medenine/South	53.52	60.12
M21	Retail shop	Nabeul/North	<LD	2.37
M22	Retail shop	Tunis /North	3.5	16.3

*Maximum permitted levels for OTA and AFB_1 are 5 and 2 µg/kg, respectively.
**LD= Limit of Detection (0.3 µg/kg for OTA; 0.06 µg/kg for AFB_1)

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Daily intake of AFB_1 and OTA

In Tunisia, millet consumption has risen significantly during the last decades from 2.40 kg/person/year in 1985 to 6.1 kg/person/year in 2015. This consumption is high in comparison with other derived cereals products such as rice (2.7 kg/person/year in 2015) and barely products (3.2 kg/person/year in 2015) which make millet the second consumed cereal after wheat in Tunisia (Khaldi and Saaidia, 2016). According to present study, the average contaminations of pearl millet grains with OTA and AFB1 were 13.21 and 13.98 µg/kg, respectively. For a Tunisian adult (60 kg b.w.), the estimated daily intake of OTA and AFB_1 were 3.76 and 3.89 ng/kg b.w. per day, respectively. Theses value represents 21.91% of the provisional tolerable daily intake (17.1 ng/kg b.w. per day) established for OTA.

In comparison, the human risk assessment of exposure to total ochratoxin and aflatoxin via consumption of millet in northern Uganda was estimated to 4.1×10^{-5} and 5.4×10^{-4} ng/kg b.w. per day for an adult (73 kg), respectively (Echodu et al., 2019). These values are very low compared to found in our study. Higher values were found by Kilonzo et al. (2014) who has assessed the exposure of households to aflatoxins through consumption of maize and maize products in Kenya. The mean dietary exposure to aflatoxin in maize kernels was reported as 292±1 567 ng/kg b.w. per day, while the mean dietary exposure to aflatoxin in maize meal was detected as 59±62 ng/kg b.w. per day. Thus, in Kenya, the maize represents an important source of human exposure to aflatoxins.

Otherwise, Kortei et al. (2019) estimated a daily intake of total aflatoxins for Ghanaian adults via consumption of rice, cereal based foods, and pasta at levels of 13, 271, and 3.9 ng/kg b.w. per day, respectively. The last value was similar to the estimated daily intake of AFB_1 from millet consumption in Tunisia. Thus, the millet despite that it does not represent a main cereal in the consumption of the Tunisian, it contributes largely in the total daily intake of OTA and AFB_1.

Conclusion

The results showed that consumption of millet in Tunisia might be an important contributing factor to the risk of dietary exposure to OTA and AFB_1. Based on these preliminary results, a full survey on mycotoxin contamination of pearl millet, during field and storage conditions, from different agro-climatic regions of Tunisia should be carried out in the future.

Author contributions

S.L. conducted the experimental work and wrote the manuscript. Z.H. did the HPLC laboratory analysis of the samples. A.G. revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

All the authors declared that there is no conflict of interest in the study.

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