Incidence of Aflatoxigenic Fungi in Peanuts (*Arachis hypogea* L.) from Markets in Slovakia

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Abstract

A total of 10 samples of peanuts (*Arachis hypogea* L.) were collected from the different markets in Slovakia in 2013 and 2014. Mycological analysis was carried out for the detection of fungi using standard media with focus on genera *Aspergillus*. The exogenous mycobiota was determined by the method of direct placing of peanuts samples on agar plates. The description of micro- and macromorphological features was used for identification of *Aspergillus* strains. The potentially toxigenic isolates were tested on their ability to produce aflatoxins (AFB_1 and AFB_2) *in vitro* conditions by TLC method. *Aspergillus* section *Nigri* were isolated from the surface of the peanuts, which seems to be the most wide-spread in peanuts grains from markets in Slovakia, because was detected more frequently than other species (60 strains). All of the potential aflatoxigenic fungi, which were obtained from 10 samples of peanuts (39 strains), were analyzed for determination of AFB_1 and AFB_2 production. The aflatoxins were produce by all of the 13 strains (100%) of *A. parasiticus* and 10 strains (38.46%) of *A. flavus* isolated from peanuts. Therefore, study concluded that peanuts seem to be risk products.

Keywords: *Aflatoxins*, *Aspergillus parasiticus*, mycotoxin, peanuts.

1. Introduction

Peanut (*Arachis hypogaea* L.) is cultivated in many countries and China is the major producer with production of 14.60 million tons [1]. Peanuts are used in the fabrication of sweets, candies and pastes and mainly as a raw material in oil production. About 60% of the world production of peanut kernels is destined to the extraction of oil, with peanut oil being the fifth most consumed type of oil [2]. Peanuts are considered to be a high–risk product for contamination with aflatoxins (AFs) since they are frequently contaminated with fungi, particularly *Aspergillus flavus* and *Aspergillus parasiticus*, and because of long peanut drying time and occurrence of rainy periods after uprooting [3]. The genus *Aspergillus* is ubiquitous in nature and distributed worldwide. It is among the most studied of all fungal genera due to their economic impact as being both industrially important bio–producer of certain enzymes and a causative agent in food spoilage [4]. Mostly, members of the *Aspergillus* section *flavi* includes three species (*Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*) producing aflatoxins, highly toxic and carcinogenic compounds of concern in food safety. *A. flavus* also produces other mycotoxins such as cyclopiazonic acid (CPA), and indole-tetramic acid [5]. CPA occurs naturally in corn (Gallagher et al., 1978) and peanuts [6,7]. as co–contaminant with aflatoxins. Chemically, aflatoxins possess a polycyclic structure derived from a coumarin nucleus attached to a bifuran system on one side and either to a pentenone (series B aflatoxins) or a six-membered lactone (series G aflatoxins) on the other side. Eighteen different compounds are currently known; however, aflatoxins B_1, B_2, G_1 and G_2 are the most common in nature [8] and are named according to the fluorescence they emit when exposed to

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ultraviolet light (B = blue and G = green). Among all classes of aflatoxins, aflatoxin B_1 (AFB_1) is the most toxic form for mammals and presents hepatotoxic, teratogenic and mutagenic properties, causing damages such as toxic hepatitis, hemorrhage, edema, immunosuppression and hepatic carcinoma [9]. It has been classified as a class 1 human carcinogen by the International Agency for Research on Cancer [10]. Optimum conditions for aflatoxin production by these species is 33 °C and 0.99 a_W, while for the growth is 35 °C and 0.95 a_W [11]. Peanut kernels are usually stored at relatively low moisture content (less than 9.5%). If the a_W (or equilibrium relative humidity; ERH) of the kernels remains below 0.60, moulds are unable to grow and the stored kernels will be stable. However, if temperature and moisture gradient develop in the storage by the insects and rodents biological activity, higher moisture of localized pockets may develop, opening the way for mould germination, growth and consequently aflatoxin production [12]. Aflatoxins, especially AFB_1, directly affect the quality of peanuts and their derivatives used for animal and human food consumption. After the ingestion, these toxins are absorbed in the gastrointestinal tract, and are biotransformed in the liver by microsomal enzymes of cytochrome P 450 system, creating the active form of AFB_1 (AFB_1 epoxide), which is able to affect the metabolism of nucleic acids, as DNA and RNA, and protein synthesis. Covalent binding of aflatoxin results in a decrease in both DNA and RNA synthesis rates in the liver [13]. The objective of our study was to monitor the occurrence of mycobiota in peanut samples collected from different Slovak markets, with focus on genera *Aspergillus*. Special emphasis was laid on the ability of some potentially toxigenic aspergily to produce some selected mycotoxins (aflatoxin (AFB1, AFB2) and cyclopiazonic acid (CPA).

2. Materials and methods

Mycological analysis of peanuts samples

In this study, we analysed 10 samples of peanuts (*Arachis hypogea* L.). All samples were collected from different markets in Slovakia (Table 1). Peanuts were sampled generally in good condition without a visible damage. Peanuts were separated from hulls and brown skin. A total of 50 peanuts (6–7 healthy peanut kernels) were plated on Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) (MERCK, Germany) from each sample. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C. In this way was determined an exogenous mycobiota.

Table 1. Peanut samples used in the study from different markets in Slovakia

Sample	*Markets*	*Country of origin*
1	Hyper Tesco	USA
2	Tesco	USA
3	Billa	China
4	Albert, Brno	China
5	Tesco	China
6	Albert, Brno	USA
7	Billa, Taliansko	USA
8	Kolumbia	Columbia
9	Supermarket KON-RAD	China
10	Kaufland	China

Identification of *Aspergillus* species

Aspergillus strains were isolated and cultivated on CYA (Czapek yeast agar, [14]), CYA20S (Czapek yeast agar with 20% Sucrose, [14]) and MEA (Malt Extract agar, [14]). Genus *Aspergillus* was identified at species level based on morphological characters according to the manuals of [14–17].

Mycotoxins screening by modified agar plug method

Toxinogenity of selected isolates was screened in *in vitro* conditions by means of the thin layer chromatography (TLC) according to [18], modified by [19]. Only isolates identified as *A. parasiticus* and *A. flavus* were screened. Extracellular metabolites–AFB_1 and AFG_1 were carried out on YES agar and intracellural cyclopiazonic acid on CYA agar. Three small pieces (each 5x5 mm) were cut from the colony growing on CYA and placed into 1.5 ml Eppendorf vials. Then 500 µl of extraction solvent

(chloroform:methanol, 2:1, v/v) was added to vials containing the agar plugs and shaken on a vortex for at least 2 minutes. Afterwards, extracts (30 µl) were applied as spots to the TLC plate (Silicagel 60, Merck, Germany) 1 cm apart. Consequently, the spots were dried and plates were developed in a toluene:ethylacetate:formic acid (5:4:1, v/v/v) solvent system that gave an average R_f value of 0.3 for AFB_1, 0.2 for AFG_1 and 0.58 – 0.90 for CPA.

Mycotoxin visualization

Mycotoxin visualization of AFB_1 and AFG_1 were detected under UV–light (365 nm) directly as a colored spot: AFB_1 (blue spot) and AFG_1 (green spot). CPA was visualized by spraying with Ehrlich reagent [19,20] and after drying detected as a violet tailing–spot in daylight.

3. Results and discussion

In this study, the incidence of the aflatoxigenic fungi in peanuts from different markets in Slovakia was investigated. Fungi of the genus *Aspergillus* were found in all peanut samples. This study include 99 strains isolated from 10 peanut samples (Figure 1) where *A. niger* section *Nigri* was predominant in all samples (60 strains). Among these fungi, section *Flavi*, which takes its name after infamous member, *Aspergillus flavus*, and the black aspergilli, *Aspergillus niger* have been frequently seen in peanuts as dominant colonists [4]. The samples were contaminated by potentially toxicogenic species *A. parasiticus* and *A. flavus*. The presence of *A. parasiticus* was detected in five samples (13 strains). *A. flavus* was found also in five samples, but with a higher frequency (26 strains). Strains *A. flavus* and *A. parasiticus* were the most important for this study because of their known toxigenic potential. In study of Passone et al. (2009) [21] was *A. flavus* the most frequently isolated species from peanuts. Atayde et al. (2012) [22] also studied mycobiota and occurrence of aflatoxins in peanuts and they recorded that *Aspergillus flavus* was one of the most frequent species from the genus *Aspergillus* (13.4%), too. In another study *A. flavus* was found in 58.3% in the groundnut samples [23]. Peanuts are considered to be one of the most susceptible food material for fungal growth and aflatoxin production, several researches have investigated it for the presence of aflatoxin, particularly AFB_1 [24–26].

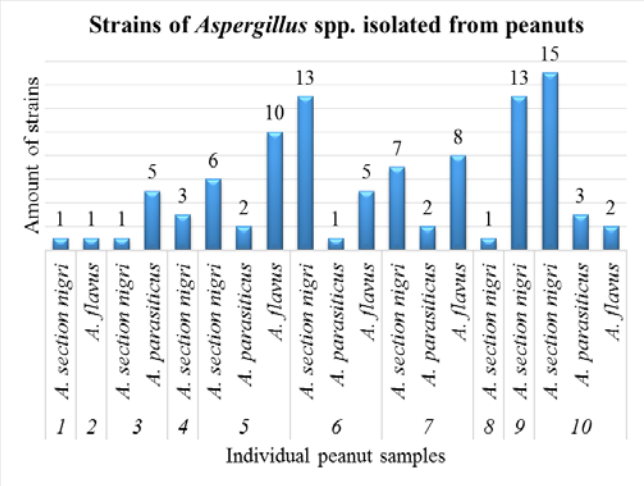

Figure 1. Occurence of *Aspergillus* spp. in individual peanut samples

In the present study, thin layer chromatography (TLC) was used to determinate aflatoxins (AFB_1 and AFB_2) and cyclopiazonic acid (CPA) produced by strains of the genus *Aspergillus* isolated from peanut samples. Results are presented in Table 2.

Table 2. Mycotoxins production by the genus *Aspergillus* isolated from peanut samples

Samples	*Aspergillus flavus*		*Aspergillus parasiticus*	
	AFB_1	**CPA**	**AFB_1**	**AFG_1**
2	$1^1/1^2$	$1^1/1^2$		
3			$5^1/5^2$	$5^1/5^2$
5	$10^1/1^2$	$10^1/1^2$	$2^1/2^2$	$2^1/2^2$
6	$5^1/5^2$	$5^1/5^2$	$1^1/1^2$	$1^1/1^2$
7	$8^1/1^2$	$8^1/1^2$	$2^1/2^2$	$2^1/2^2$
10	$2^1/2^2$	$2^1/1^2$	$3^1/3^2$	$3^1/3^2$

AFB_1–aflatoxin B_1, AFG_1–aflatoxin G_1, CPA–cyclopiazonic acid, ^{1}–number of screened strains, ^{2}–number of positive isolates

Isolates of *A. parasiticus* are typically aflatoxigenic, producing both types, B_1 and G_1 aflatoxins but not CPA. Other authors have reported that nontoxigenic isolates of *A. parasiticus* are extremely rare [27–29]. In this study, all isolated strains of *A. parasiticus* (13 strains) from 5 samples resulted in AFB_1 and AFG_1 producers (100%) (Table 2). Production of AFB_1 by *A. flavus* strains was reported by 10 (38.46%) from all 26 isolated strains. However, *A. flavus* is not only producing aflatoxin B_1, it is able to produce cyclopiazonic acid, too. Natural co–

occurrence of aflatoxins and CPA had been reported in peanuts [7]. Our results showed that CPA was produce by 9 (34.62%) of the 26 strains of *A. flavus*. Both mycotoxins are dangerous and according to Vaadome et al. (2006) [30] the presence of both toxins in food and feed may result in additive or synergistic toxic effect. Mutegi et al. (2009) [26] in western Kenya reported a possible increase in aflatoxin contamination of peanut products at market level. Studies in other countries have also reported a high level of aflatoxin contamination of peanuts and peanut products at market level [31–32]. As a factor which was previously discussed, that would lead to increase of aflatoxins in post–harvest is the storage time [33]. This study also showed a high occurrence of aflatoxin and CPA contamination of peanuts. Several other studies have documented a high fungal and aflatoxin prevalence and incidence in peanuts from market and their products [32,34,35]. The maximum permissible aflatoxin level in peanuts distributed in Slovakia is 2,0 μg/kg for AFB_1 and 4,0 μg/kg for account of AFG_1, AFB_1, AFG_2 and AFB_2 for groundnuts (peanuts) and other oilseeds and processed products made of them intend for a direct human consumption or use as a food ingredient except crude vegetable oils destined for refining and refined vegetable oils [36]. In our study, CPA was also detected but there is no Slovak legislation for CPA in food. On the other hand, TLC method used in our study is a qualitative method and if there is the same difference, it will not be detectable.

4. Conclusions

This study revealed occurrence of aflatoxigenic fungi in peanut samples collected from the different markets in Slovakia, and their ability to produce aflatoxins and CPA. Result showed that from 10 samples of peanuts were isolated 99 strains of fungi, 13 strains of them was identified as *A. parasiticus* and 26 strains as *A. flavus*. Both, *A. flavus* (10 strains–38.46%) and *A. parasiticus* (13 strains–100%) produced aflatoxin AFB_1, in addition strains of *A. parasiticus* produced AFG_1. Moreover, *A. flavus* (7 strains–26.92%) also produced CPA. Aflatoxins and CPA contamination of peanuts is the most prominent economic problem for the industry and dangerous for human and animal health. This study clearly confirms that peanuts are a significant source of important toxinogenic fungi. Therefore, it is very important to check the country of which are the peanuts imported, but also storage conditions of peanuts in Slovakia, in order to prevent potential contamination.

Acknowledgements

This work was co–funded by VEGA 1/0611/14 and European Community under project no 26220220180: Building Research Centre Agrobiotech.

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