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MYCOBIOTA OF SPICES AND AROMATIC HERBS

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ABSTRACT

A total of 67 samples of spices and herbs were tested for mould contamination. From 50.7% of samples, moulds were not isolated. The most dominant genera were *Aspergillus* and *Penicillium*. Potential producers of mycotoxins *Aspergillus* spp. and *Penicillium* spp. were tested for the ability to produce some mycotoxins. Isolates of potentially toxinogenic species were found to produce various mycotoxins, namely alfatoxin B_1 (*Aspergillus flavus*), cyclopiazonic acid (*Aspergillus flavus*), sterigmatocystin (*Emericella nidulans*), roquefortine C (*Penicillium allii, P. chrysogenum, P. crustosum, P. expansum*), penitrem A (*P. crustosum*) and patulin (*P. expansum*). Some of the tested isolates produce two mycotoxins: *A. flavus* (aflatoxin B_1 and cyclopiazonic acid), *P. crustosum* (roquefortine C and patulin) and *P. expansum* (roquefortine C and patulin). None of the tested isolates of *Aspergillus* section *Nigri* screened, appeared to produce ochratoxin A. Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A. Aflatoxin B_1 was found in 5 (45.5%) out of 11 samples analysed with levels ranging from 0.14 to 2.9 $\mu g.kg^{-1}$. In one sample we detected aflatoxin G_1. Ochratoxin A was found in 3 samples (27.3%), with levels ranging from 2.2 to 5.19 $\mu g.kg^{-1}$. No sample was contaminated by aflatoxins or ochratoxin A above the maximum admitted threshold established by the European legislation.

Keywords: mycobiota; spices; aflatoxin; ochratoxin A

INTRODUCTION

Spices have been used for flavour, colours, aroma and preservation of food or beverages for thousands years **(Ozbey and Kabak, 2012)**. Because of their processing and environmental conditions, spices can be heavily contaminated with toxigenic fungi and mycotoxins. Mycotoxins are secondary metabolites produced naturally by filamentous fungi, which are considered toxic substances when present in food for human and food for animals **(da Rocha et al., 2014)**. For spices there are two groups of mycotoxins of concern, aflatoxins and ochratoxin A **(Ozbey and Kabak, 2012)**. Aflatoxins are produced by fungi that belong to *Aspergillus* genus and especially by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* **(Cary and Ehrlich, 2006, Marín et al., 2009)**. Ochratoxin A is a secondary metabolite produced by filamentous fungi of the genera *Aspergillus* and *Penicillium* present in a wide range of foodstuffs. The most relevant ochratoxin A producing species are *Penicillium verrucosum*, *Aspergillus ochraceus*, *Aspergillus niger* and *Aspergillus carbonarius* due to their prevalence in foodstuffs (cereals, grapes, coffee, etc.) and the number of strains are able to produce ochratoxin A **(Amézqueta et al., 2012, Luque et al., 2013, Rodríguez et al., 2011)**. Prevention of microbial contamination in dried herbs and spices lies in the application of good hygiene practices during growing, harvesting and processing from farm to fork **(Sagoo et al., 2009)**.

The aim of the study was the determination of potentially toxigenic filamentous fungi from genera *Aspergillus* and *Penicillium* from spices and herbs. A special emphasis was laid on the ability of isolated *Aspergillus* and *Penicillium* species to produce some significant toxic extrolites - mycotoxins.

MATERIAL AND METHODOLOGY

Samples

Totally 67 samples of spices and herbs from different countries (Table 1) were analysed. The samples (approximately 100 g) were collected from the storage rooms of food factory.

Mycological analysis

Dilute plate technique was used for isolation of fungi from the samples according to **Samson et al. (2002)**. Sample in weigh of 20 g was mixed with 180 ml of saline solution (0.85% sodium chloride) with 0.05% Tween 80 in homogenizer. Then 0.1 ml of appropriate dilution made up to 10^{-2} was applied on DRBC (Dichloran Rose Bengal Chloramphenicol agar). After 5 to 7 days of incubation at 25 ± 1°C, in dark resulting colonies were transferred onto appropriate identification media.

The identification of *Aspergillus* species. Conidial suspensions were inoculated at three equidistant points both on Czapek-yeast Extract agar (CYA), Czapek-yeast with 20% Sucrose (CY20S) and malt extract agar (MEA) and incubated in the dark at 25 ± 1 °C, 7 days. Species identification was done according to **Klich (2002), Pitt and Hocking (2009), Samson et al. (2002, 2010)** and **Samson and Varga (2007)**.

The identification of *Penicillium* species. The penicillia were inoculated at three equidistant points both on Czapek-yeast Extract agar (CYA), Malt Extract agar (MEA) and Creatine Sucrose agar (CREA) and incubated in dark at 25 °C. Sub-cultivation on CYA at 37 °C was

Table 1 Sample of spices and herbs

Sample	Country of origin	Number of samples	Sample	Country of origin	Number of sample
basil	Egypt	2	**crushed bay leaves**	Turkey	1
crushed white pepper	Vietnam	3	**crushed rosemary**	Morocco	2
marjoram	Egypt	2	**crushed thyme**	Poland	2
garlic powder	China	2	**crushed ginger**	Nigeria	5
onion powder	France	2	**nutmeg**	Indonesia	3
crushed black pepper	Vietnam	2	**paprika (spicy)**	Spain	8
granulated onion	France	2	**paprika**	Hungary	7
granulated garlic	China	1	**chive**	China	1
curry	Spain	1	**leaves of parsley**	Slovakia	1
crushed marjoram	Egypt	3	**dill**	Poland	2
cumin powder	Finland	1	**crushed green pepper**	India	2
salvia	Albania	2	**leaves of celery**	Slovakia	2
crushed chillies	China	6	**savory**	Hungary	2

used as well. Species identification was done after 7 days according to **Pitt aHocking (2009), Samson et al., (2002, 2010)** and **Frisvad a Samson (2004)**.

The identification of *Fusarium* species. Potato Dextrose agar (PDA) was used for observation of colony characteristics. "Synthetischer Nährstoffarmer agar" (SNA) was used for micromorphological features. Cultures were incubated at the room temperature and natural light. Species identification was done after 10 days according to **Leslie a Summerell (2006), Nelson et al. (1983), Nirenberg (1981) Pitt a Hocking (2009) and Samson et al. (2002, 2010)**.

Mycotoxins screening by a modified agar plug method

The ability of selected potentially toxigenic isolates to produce relevant mycotoxins in *in vitro* conditions were screened by the means of thin layer chromatography (TLC) according to **Samson et al. (2002)** modified by **Labuda a Tančinová (2006)**.

The cultivation for screening of extracellular metabolites (aflatoxin B_1, aflatoxin G_1, citrinin, patulin, ochratoxin A) were carried out on YES (Yeast Sucrose agar) and for intracellular (cyclopiazonic acid, penitrem A, roquefortin C, sterigmatocystin) on CYA (Czapek-yeast Extract agar); conditions of cultivation in dark at 25 °C, 14 days. In each tested isolate, 3 pieces of mycelium together with cultivation medium of approximately 5 x 5 mm area were cut from colonies and extracted in 1000 ml of chloroform:methanol (2:1, v/v) on vortex for 2 minutes. Then 20 µl of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v/). The visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5% methylbenzothiazolone hydrochloride in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot; penitrem A after spraying with 20% $AlCl_3$ in 60% ethanol, heated at 130 °C for 8 min and then detectable as a dark green to black spot on daylight; roquefortin C after spraying with $Ce(SO_4)_2$ x 4 H_2O visible as an orange spot. Directly under UV light (365 nm) were visualised following mycotoxins: aflatoxin B_1 (blue spot), aflatoxin G_1 (green), citrinin (yellow-green), ochratoxin A (bluish-green), sterigmatocystin (reddish).

The determination of mycotoxins in sample

In the 11 samples were determined following mycotoxins: aflatoxin B_1, B_2, G_1, G_2 and ochratoxin A. Analyses were performed by HPLC method (high-pressure liquid chromatography) in an external accredited laboratory.

RESULTS AND DISCUSSION

In the current study from 50.7% of the samples, moulds were not isolated (basil, crushed black pepper, granulated garlic, curry, cumin powder, salvia, crushed chillies, crushed bay leaves, paprika (spicy), dill, crushed green pepper, savory). These findings are similar to data reported by **Witkowska et al. (2011)**, where in 50% of samples of commercial herbs and spices were detected moulds. The fungal species recovered from the samples are listed in Table 2. Species of 11 genera were isolated and identified. The *Aspergillus* and *Penicillium* were the most common genera. **Hashem and Alamri (2010)** from 15 spices isolated as the most common genera *Aspergillus, Penicillium* and *Rhizopus*. *Rhizopus* (*Rhizopus stolonifer*) was isolated from granulated onion, only. **Hammami et al. (2014), Kong et al. (2014), Yogendrarajah et al. (2014)** and other authors reported *Aspergillus* and *Penicillium* as predominant fungi in spices. With regard to *Aspergillus* genus, the following species were isolated: *A. flavus, A. fumigatus* and *Aspergillus* section *Nigri*. *A. flavus* is a very important producer of aflatoxins and is frequently occurring in food commodities **(Luo et al., 2012)**. Species of *Aspergillus* section *Nigri* are important producers of ochratoxin A.

In the *Aspergillus* section *Nigri*, *A. niger* and *A. carbonarius* produce ochratoxin A **(Almela et al., 2007)**. The isolates of this section were the most frequent in our study.

Table 2 Mycobiota isolated from the samples of spices and aromatic herbs

Sample	Isolated species (group)
crushed white pepper	*Aspergillus* section *Nigri*, *Emericella nidulans*, *Eurotium* sp. *Penicillium crustosum*, *Penicillium expansum*, *Penicillium chrysogenum*
marjoram	*Aspergillus* section *Nigri*, Penicillium expansum
garlic powder	*Aspergillus* section *Nigri*, *Penicillium allii*, *Penicillium chrysogenum*
onion powder	*Aspergillus flavus*, *Aspergillus* section *Nigri*, *Penicillium glabrum*, *Penicillium chrysogenum*
granulated onion	*Aspergillus* section *Nigri*, *Penicillium* sp., *Rhizopus stolonifer*
crushed marjoram	*Aspergillus* section *Nigri*, *Aspergillus fumigatus*, *Cladosporium* sp., *Penicillium atramentosum*, *Penicillium solitum*
crushed ginger	*Aspergillus flavus*, *Aspergillus* section *Nigri*, *Paecilomyces* sp.
nutmeg	*Paecilomyces* sp.
paprika	*Penicillium chrysogenum*
chive	*Fusarium proliferatum*
leaves of parsley	*Alternaria* sp., *Geotrichum candidum*
leaves of celery	*Cladosporium herbarum*, *Geotrichum candidum*

Table 3 Potential ability of moulds isolated from spices and aromatic herbs to produce relevant mycotoxins in *in vitro* conditions, tested by TLC method

Tested isolates	Source of isolates	OTA	AFB1	AFG1	CPA	STER	RC	PA	PAT
Aspergillus section *Nigri*	crushed white pepper	0*/1**	-	-	-	-	-	-	-
	marjoram	0/1	-	-	-	-	-	-	-
	garlic powder	0/2	-	-	-	-	-	-	-
	granulated onion	0/1	-	-	-	-	-	-	-
	crushed ginger	0/1	-	-	-	-	-	-	-
Aspergillus flavus	granulated onion		1/1	0/1	1/1	-	-	-	-
	crushed ginger		0/1	0/1	1/1	-	-	-	-
Emericella nidulans	crushed white pepper	-	-	-	-	1/1	-	-	-
Penicillium allii	garlic powder	-	-	-	-	-	2/2	-	-
Penicillium chrysogenum	crushed white pepper	-	-	-	-	-	3/3	-	-
	garlic powder	-	-	-	-	-	2/2	-	-
	paprika	-	-	-	-	-	1/1	-	-
Penicillium crustosum	crushed white pepper	-	-	-	-	-	1/1	1/1	-
Penicillium expansum	crushed white pepper	-	-	-	-	-	1/1	-	1/1
	marjoram	-	-	-	-	-	1/1	-	1/1

** number of tested isolates, * number of isolates with ability to produce mycotoxin, OTA - ochratoxin A, AFB1 - aflatoxin B_1, AFG1 - aflatoxin G1, CPA - cyclopiazonic acid, STER - sterigmatocystin, RC - roquefortine C, PA - penitrem A, PAT - patulin, TLC - thin layer chromatography

Kong et al. (2014) isolated *Aspergillus* section *Nigri* as the most frequent in spices in China markets. Isolated pencillia were *Pencillium allii*, *P. atramentosum*, *P. crustosum*, *P. chrysogenum*, *P. expansum*, *P. glabrum*, *P. solitum* (in alphabetical order). **Hammami et al. (2014)** detected *P. aurantiogriseum*, *P. charlesii*, *P. verruculosum*, *P. citrinum*, *P. commune*, *P. griseofulvum*, *P. melanoconidium*. **Yogendrarajah et al. (2014)** in their study identified *Penicillium* only as genus.

Apart from the two regulated mycotoxins in European Union (aflatoxins and ochratoxin A), we determined the ability of the isolates obtained from the analysed samples, to produce other mycotoxins (cyclopiazonic acid, patulin, penitrem A, roquefortine C and sterigmatocystin).

Table 4 Contamination of spices with aflatoxins and ochratoxin A

Sample	Mycotoxins (µg.kg^{-1})				
	AFB1	**AFB2**	**AFG1**	**AFG2**	**OTA**
crushed white pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed chillies	**0.14**	<0.10	<0.10	<0.10	**5.19**
nutmeg	**0.14**	<0.10	<0.10	<0.10	<0.20
paprika (spicy)	<0.10	<0.10	<0.10	<0.10	**2.2**
paprika	**0.11**	<0.10	<0.10	<0.10	**2.35**
crushed green pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed black pepper	<0.10	<0.10	<0.10	<0.10	<0.20
crushed ginger	**2.9**	<0.10	**3.2**	<0.10	<0.20
crushed ginger	<0.10	<0.10	<0.10	<0.10	<0.20
nutmeg	**0.55**	<0.10	<0.10	<0.10	<0.20
paprika (spicy)	<0.10	<0.10	<0.10	<0.10	<0.20

AFB1 - aflatoxin B_1, AFB2 - aflatoxin B_2, AFG1 - aflatoxin G_1, AFG2 - aflatoxin G_2, OTA - ochratoxin A

The ability to produce relevant mycotoxins are shown in Table 3. The isolates of potentially toxinogenic species were found to produce various mycotoxins, namely aflatoxin B_1 (*Aspergillus flavus*), cyclopiazonic acid (*Aspergillus flavus*), sterigmatocystin (*Emericella nidulans*), roquefortine C (*Penicillium allii, P. chrysogenum, P. crustosum, P. expansum*), penitrem A (*P. crustosum*) and patulin (*P. expansum*). Some of the tested isolates produce two mycotoxins: *A. flavus* (aflatoxin B_1 and cyclopiazonic acid), *P. crustosum* (roquefortine C and patulin) and *P. expansum* (roquefortine C and patulin). None of the tested isolates *Aspergillus* section *Nigri* screened appeared to produce ochratoxin A.

Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A (Table 4). Aflatoxin B_1 was found in 5 (45.5%) out of 11 samples analysed with levels ranging from 0.14 to 2.9 µg.kg^{-1}. No sample was contaminated by aflatoxin B_1 above the maximum admitted threshold established by the European legislation **(Commission regulation, 2010b)**. In one sample we detected aflatoxin G_1. Ochratoxin A was found in 3 of samples (27.3%), with levels ranging from 2.2 to 5.1 µg.kg^{-1}. No sample was contaminated by ochratoxin A above the maximum admitted threshold established by the European legislation **(Commission regulation, 2010a)**. **Prelle et al. (2014)** showed that 15.4% and 23.8% of samples were contaminated with aflatoxins and ochratoxin A, respectively. In our study, 2.3% of spice samples contaminated by ochratoxin A get over the threshold admitted by European Regulation. **Zhao et al. (2013)** presented that about 11% of the 480 Chinese spices samples tested contained detectible levels of aflatoxin B_1, with the highest concentrations found in chili, prickly ash and pepper. **Zinedine et al. (2006) reported** the higher level of aflatoxin B_1 contamination in red paprika (9.68 µg.kg^{-1}). The analysis of the spice samples contamination (in Morocco) with aflatoxin B_1 revealed that paprika is frequently contaminated, since 95% were contaminated with that mycotoxin and 40% of samples exceeded European regulation for that contaminant **(Mahgubi et al., 2013)**. Co-occurrence of aflatoxin B_1 and ochratoxin A in samples of crushed chillies and paprika was detected in our study. **Ozbey a Kabak (2012)** reported co-occurrence of these mycotoxins in 62.5% of red chilli flake, 40.9% of red chilli powder and 4.3% pepper of powder samples.

CONCLUSION

From 50.7% of samples, moulds were not isolated. The most dominant genera were *Aspergillus* and *Penicillium.* The isolates of potentially toxinogenic species were found to produce various mycotoxins (alfatoxin B_1, cyclopiazonic acid, sterigmatocystin, roquefortine C, penitrem A and patulin). None of the tested isolates *Aspergillus* section *Nigri* screened appeared to produce ochratoxin A. Totally 11 samples were analysed for the presence of aflatoxins and ochratoxin A. Aflatoxin B_1 was found in 45.5% out of 11 samples analysed. Ochratoxin A was found in 27.3% of samples.

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