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MYCOBIOTA OF SOME MEDICINAL PLANTS AND THEIR TOXIGENIC POTENTIAL

MYKOCENÓZA NIEKTORÝCH LIEČIVÝCH RASTLÍN A ICH TOXICKÝ POTENCIÁL

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70 samples of thyme and 30 samples of mint from Vayk region of Armenia were examined for the contamination by filamentous fungi and aflatoxins. 38 species of microfungi, which belong to 9 genus and two classes – *Zygomycetes Hyphomycetes*, *have been* isolated and identified. High frequency of occurrence for the following species from genus *Aspergillus*: *A. flavus, A. niger, A. nomius, A. fumigatus, A. foetidus* is noticed. Most frequently *P. aurantio-groseum, P. radulatum, P. brevi-compactum* species from genus *Penicillium* has been isolated in thyme and mint samples. Ability of 68 strains from section *Flavi*, against biosynthesis of aflatoxin B_1, is studied, which have been isolated from thyme at different stages of processing and packing of medicinal tea. 17 strains, which belong to *A. flavus, A. nomius, A. parasiticus, A. caleatus* species, produced aflatoxin B_1 in range from 5 to 150 ng/100 ml. The majority of strains from the mentioned species did not lose viability at low water activity (a_w = <0.400). In 19 samples of ready product from thyme, with high degree of contamination by species from section *A. Flavi*, aflatoxins have not been found out.

Key words: thyme, mint, filamentous fungi, mycotoxins, aflatoxin B_1, water activity, strains, contamination

Medicinal plants are widely used as raw material for pharmaceutical preparations and as a supplement for dietetic products, specifically for "self medications". These plants are normally carrying a great number of bacteria and molds, often from soil origin (Weisser et al., 1971). In spite of their origin, natural drugs should not be viewed by simple tools of folk medicine since they are a class of pharmaceutical products and should meet the requirements of quality safety and efficacy (Calixto, 2000). Studies of Hitokoto et al shown, that *Aspergillus* and *Penicillium* species were predominant in herbal drug, but microfungi from *Rhizopus, Mucor, Cladosporium* and *Aureobasidium* genus were also found in a few samples. Current practices of harvesting, handling and production frequently cause additional contamination (Hitokoto et al., 1976). Fungi are a normal component of food microflora and may be responsible for spoilage and production of mycotoxins (Aziz et al., 1998; Chourasia, 1995). At present, some investigators (Kallings et al.,1966; Udagawa et al., 1996) have reported fungal population of the medicinal plants. *Aspergillus flavus, A. candidus, A. niger, A. luchuensis, A. ochraceus, A. nidulans, F. moniliforme, F. oxysporum, Alternaria alternata, Curvularia* spp., *Chaetomium* sp., *Penicillium citrinum* and *Rhizopus stolonifer* were reported as the most common fungi isolated from drug plants (Ayres et al., 1980; Aziz and Youssef, 1991; Misra, 1981; Roy et al., 1988; Takatori et al., 1977). Results for assessment of toxigenic fungi on Argentinean medicinal herbs shown, that 52% out of 152 samples were contaminated with species from genus *Aspergillus*: 27% belonging to the Flavi section and 25% to the Circumdati section (Rizzo et al., 2004).

The fungal contaminates has been reported to affect the chemical composition of the raw materials and thereby, decreases the medicinal potency of the herbal drugs (Roy, 2003). The concern with the quality of the natural products is due to the potential fungal contamination and the risk of the presence of mycotoxins (Bugno et al., 2006). Mycotoxins are produced by fungi on plants in the field before harvest or later after harvest during long storage under favourable conditions (Gedek, 1985).

Aflatoxins are secondary metabolites produced by filamentous fungi *Aspergillus flavus* and *A. parasiticus* (Reddy et al., 2001). According to results of analysis of Hitokoto et al. (1978), Romagnoli et al. (2007), Abou Donia (2008) aflatoxins and others mycotoxins are not often found out in herbal raw materials and in ready to use herbal plants. However, according to Aziz et al. (1998) from 84 analyzed samples 17 were contaminated with aflatoxin B_1 and three samples – with ochratoxin A. Three samples of peppermint and chamomile, and one sample of lime tree and carob tree contained aflatoxin B_1. The highest level was 160 $\mu g\ kg^{-1}$ found in the fennel. In previous study, Rani and Singh (1990) found that 89% of samples of fennel, coriander, cumin, and ammi were contaminated with aflatoxin B_1 at the levels 3 000 ppb, 1640 ppb, 1580 ppb, and 2550 ppb, respectively. In addition, Roy et al. (1988) and Roy and Chourasia (1990) determined that the seeds of *Piper nigrum* and *Mucuna prurians*, and the barks of *Acacia catechu, Coriandrum sativum* and *Elettaria cardamomun* were contaminated with aflatoxin B_1 at levels below 20 $\mu g/kg$. Gautam (2009) was determinates different mycotoxins in 12.5% of powdered samples of medicinal herbs.

Furthermore, previous works show that aflatoxins levels are not reduced by domestic cooking with either microwave or conventional gas oven heating (Midio et al., 2001) and that do not decompose at the temperature of boiling water during the preparation of the drink (Feuell, 1996).

This work was performed to determine the incidence of potential toxigenic fungi and their mycotoxins on dired medicinal herbs, belonging to two species, which are used as raw material for tea and drugs.

Material and methods

Mycological analysis

After surface disinfection samples were washed with fresh distillate water. The analyses were carried out with direct

plating and dilution plating methods. For plating method food particles were placed directly on solidified agar media. For dilution 1 : 10, 10 g of sample was dissolved in 90 ml sterilized water and mixed for 15 minutes (Pitt and Hocking, 1997). For isolation of filamentous fungi CYA (Chapek-Yeast Agar medium, HiMedia Ltd.), GYA (Glucose-Yeast Agar medium, HiMedia Ltd.), and MEA (Malt-Extract Agar medium, HiMedia Ltd.) were used. The plates were incubated at 28 °C for 7 days (NF ISO 7954-88). After incubation the colony forming unit (cfu) was accounted according to NF ISO 7698-91, and frequency of occurrence was detected (El-Kady et al., 1982).

The growing fungi were identified morphologically based on macro- and microscopic characteristics using following manual: Raper and Fennell (1977), Pitt (1979), Samson et al. (1995), Samson et al. (2007).

Mycotoxin production of the isolates

Production of aflatoxin was chemically determined with representative isolates of *Aspergillus flavus, A. nomius, A. parasiticus, A. coelatus* respectively. 100 ml of yeast extract sucrose medium (2% yeast extract and 5% sucrose) were inoculated with about 10 spores per ml and incubated at 28 °C for 14 days. After incubation, the contents of each flask were mixed with 120 ml of chloroform: water (100 : 10, v/v) and were shaken vigorously. The extracts were sequentially filtered through anhydrous sodium sulfate. The chloroform extracts were collected for dryness.

Detection of natural occurrence of mycotoxin in the medicinal plants

Natural occurrence of aflatoxin, was chemically examined in 25 g of each sample of herbal plants. Samples were extracted for detection of aflatoxins B_1 by thin layer chromatography (TLC) according to the method described in (Manuel of Methods.1998).

Measurement of water activity of the medicinal plants

Determination of a_w was spent with AquaLab (Decagon Devices, Pullman, WA, USA).

Results and discussion

There was studied mycotoxicological safety of 70 samples of thyme (*Thymus kotschynus*) and 30 samples of mint (Mint longifolius) collected from Vayk region of Armenia.

In Armenia there are five species of thyme among which the greatest distribution has *Thymus kotschynus* (*Boiss grow. et Hohen*). The investigated samples of mint were selected from natural populations of the same region.

As a result of mycological analysis of medicinal plants 38 species of filamentous fungi was isolated and identified. The predominant mycoflora obtained was distributed in nine genera and two classes – *Zygomycetes* and *Hyphomycetes* (Table 1). Species belonging to *Aspergillus* (15 spp.) and *Penicillum* (10 spp.) genera were most prevalent among all isolated species and were found in 39.4% and 26.3% of the analyzed samples. Other species of moulds were isolated from medicinal plant samples, such as, *Alternaria, Trichoderma, Trichotecium, Mucor* spp., *Rhizopus* and *Cladosporium* spp. Species belonging to *Aspergillus* genera (40.2%) were most prevalent among all isolated species. Species of *Aspergillus*, which can produce aflatoxins are very common on analyzing samples of thyme and mint. 15 species from *Aspergillus* genera were isolated, 5 of them (55.7%) belonged to section *Flavi*. For species of *A. flavus* and *A. nomius* from the specified (mentioned) section high frequency of occurrence in samples of thyme is noticed. Previous work (Grigoryan et al., 2007) has shown a similar contamination. In 35% of the analyzed samples of thyme monopole development of *A. flavus* and *A. nomius* species was observed.

Most often in samples of mint and thyme there are species from section *Nigri*, among which *A. carbonarius* species, known as the active producer of ochratoxin A, is found out in 30% of samples of thyme. *A. niger* and *A. flavus* were the most frequent *Aspergillus* species yielded in all examined medicinal plant samples in this investigation. This was in accordance with the results of Roy and Chourasia (1990), Rezakova and Kubatova (2005), who stated that *A. flavus* was the main contaminant of different herbal drug samples. Species of microfungi with rare frequency of occurrence concern *A. flavipes* and *A. tamarii.*

From leaves of mint most frequently were isolated species, potential producers of citrinine and citreoviridine, *P. citreo-viride* and *P. citrinum*. Field fungi were observed to invade developing on the raw materal and dry processing, major field fungi genera from *Dematiacea* family are: *Alternaria, Stemphyllium* and *Cladosporium*.

Fungi of the genera *Aspergillus* and *Penicillium*, largely distributed in the Armenian ecosystem, are known to contain strains, that produce mycotoxins (Osipyan et al., 2001).

The composition and frequency of occurrence of filamentous fungi, contaminating of samples of thyme and mint is presented in Table 2.

Table 1 The classification of isolated fungi from studied samples

Class	Order	Family	Genera	Quality of species
Zygomycetes	*Mucorales*	*Mucoraceae*	*Mucor*	2
			Rhizopus	1
Hyphomycetes	*Hyphomycetales*	*Moniliaceae*	*Aspergillus*	15
			Penicillium	10
			Trichoderma	1
			Trichotecium	1
		Tuberculariaceae	*Fusarium*	1
		Dematiaceae	*Alternaria*	4
			Stemphyllium	3

Table 2 Composition and frequency of occurrence of fungi, contamination of thyme and mint

Species of fungi	Thyme, *n* = 70	Mint, *n* = 35	Species of fungi	Thyme, *n* = 70	Mint, *n* = 35
Mucor albo-ater Naumov	1.4	–	*P. citrinum* Thom	–	57.1
M. mucedo Fres.	28.5	–	*P. clavigerum* Demelius	–	22.8
Rhizopus nigricans Ehrenb	–	17.1	*P. frequentans* Westl.	5.7	–
Aspergillus carbonarius Thom	15	–	*P. lividum* Westl.	10	–
A. flavus Link	90	40	*P. purpurogenum* Stoll.	–	25.7
A. parasiticus	27	–	*P. radulatum* Smith	2.8	31.4
A. fumigatus Fres	40	50	*P. rubrum* Stoll.	–	14.3
A. nidulans (Eidam)Wint	15	18	*P. brevi-compactum*	5.7	20.0
A. niger v. Teigh	80	90	*P. aurantio-griseum*	4.3	11.4
A. tamari Kita	–	4.2	*Trichotecium roseum*	–	5.7
A. terreus Thom	10	–	*Trichoderma viride* Pers. et Fr.	–	17.1
A. versicolor (Vuill)Tiraboschi	–	13.3	*Alternaria alternata* Nees et Neerg	10	25.7
A. oryzae	–	13	*Al. cheiranthi*	–	2.8
A. nomius	60	–	*Al. grisea* Szilvinyi	2.8	–
A. caelatus	12	–	*Al. tenuis* Nees	1.4	8.5
A. foetidus	50	60	*Stemphyllium botryozum* Wallr	–	5.7
A. japonicus	10	8	*St. ilicis* Tengwall	–	2.8
A. flavipes	1.5	5.7	*St verruculosum* Saccardo	–	2.8
Penicillium chizogenum Thom	–	34.3	*Fusarium monilifirme* Sheld	–	5.7

*High occurrence – more than 50%. Moderate occurrence – between 25 – 50%. Low occurrence – between 11 – 33%. Rare occurrence – less than 11%

Table 3 The Influence of temperature treatment and a_w on contamination level of thyme by fungi from section *A. Flavi*

Samples of thyme*	a_w	T °C and time in min	Level of contamination by fungi from *A. Flavi* section in cfu/g
After harvesting 1	0.737	20 – 24	1.5×10^3
2	0.732	22 – 24	2×10^2
During draing 3	0.660	25 – 28	4×10^2
4	0.637	„ – „	9×10^3
5	0.580	28 – 30	7×10^4
6	0.460	„ – „	5×10^5
During pakcaging 7	0.472	22 – 25	3×10^5
Additional termal treatment 8	0.381	60; 30	10^5
8	0.360	60; 40	3×10^3
9	0.358	60; 60	6×10^2
10	0.345	90; 20	90
11	0.330	90; 30	80
12	0.300	90; 60	40

* from each bath there were taken five samples

For growth of filamentous fungi and mycotoxin production some factors such as temperature, water activity (a_w) and others are considered. Therefore, influence of mentioned factors on contamination level of thyme by fungi was studied. In samples of herbal plants with high water activity (> 0.600), there was occurrence of fungi from following genera; *Trichoderma, Alternaria, Trichotecium, Penicillium.* Fungi of genus *Aspergillus* are isolated form samples with low water activity (<0.400).

Both ground and whole leaves of thyme before packing have been subjected to additional short-term thermal processing, for the purpose of inhibition growth of fungi from section *Flavi*. Results have shown the following dynamics of decrease in values of water activity and degree of contamination of experimental samples of thyme (Table 3). The received results show the high ability of adaptation of some strains from section *Flavi – A. flavus* and *A. nomius.*

It is studied toxigenic potential of 68 strains, which belong to species from *Flavi* section, which are potential producers of aflatoxins. In fungal extracts of investigated strains it is found out aflatoxin B_1 from 5 to 150 ng on 100 ml liquid media.

The analysis shown in Table 4 indicated that 27.6 % of *A. flavus* presented the ability to produce aflatoxin B_1 or aflatoxins B1 and B_2; 10 % of *A. parasiticus* and 38.4 % of *A. nomius* presented ability to produce aflatoxins B_1. The

Table 4 Toxigenic potential of strains from section *Flavi*, determination in samples of thyme

Species from section *A. Flavi*	Number of cases of isolation of fungi species*	Number of cases of determination of aflaxogenic strains	Content of aflatoxin B_1/100ml medium
A. flavus	70/**63***	40/**11**	5 – 80
A. parasiticus	70/**23**	10/**1**	0 – 25
A. nomius	70/**60**	13/**5**	20 – 150
A. caelatus	70/**30**	5/**0**	– *

* not detected

Table 5 Incidence (analyzes /positive samples) and range of aflatoxins levels in thyme

Samples of thyme	Incidence			Range of AFs levels in ng/kg		
	No	%	AFB_1	AFB_2	AFG_1	AFG_2
Lives before drying *n* = 5	0/5	0.0	ND	ND	ND	ND
Stem before drying *n* = 5	0/5	0.0	ND	ND	ND	ND
After 20 days *n* = 4	0/4	0.0	ND	ND	ND	ND
Ready dry thyme *n* = 5	0/5	0.0	ND	ND	ND	ND

ND – not detected

results of studies of Rizzo (2004) shown, that *A. flavus* and *A. parasiticus* were the predominant species isolated from medicinal herbs and 50% of 40 isolates were toxigenic.

Correlation between the incidences of contamination of samples by microfungi was not found out, as well as the presence of toxigenic strains of filamentous fungi. The interrelation between level of water activity and the content of toxigenic strains of microfungi in samples is not revealed as well. In case of monopol contamination of medicinal herbs by species, potential producers of aflatoxins, from analyzed samples more often were allocated aflatoxigenic strains.

It was spent mycotoxicological analysis of 19 samples of thyme, with high level contamination by fungal species, for determination of aflatoxin B_1 (Table 5). In one of samples aflatoxin B_1 it is not found out. The obtained data confirms results presented in works of following authors Hitikotto (1978), Romagnoli et al. (2007), Abou Donia (2008). So, it seems that not all *A. flavus* strains are aflatoxigenic on this kind of matrix (Elshafie et al., 2002; Romagnoli et al., 2007). The reason could be that these kinds of substrate are not favorable for aflatoxins formation, because they contain essential oil and other biologically active compounds. The content of thymol, in thyme essential oils from thyme leaves, was from 21 – 38 %, depend on harvest period (Ebrahimi et al., 2008). Level of contamination of thyme done not depend on content of thymol in essential oils. Samples of dry leaves of thyme, with high thymol content (38%) are contaminated by filamentous fungi, including aflatoxigenic strains.

From these our present investigation, it is conceivable that fungi from section *Flavi* are moulds that should be considered in relation to fungal contamination and mycotoxin production. It was concluded that medicinal plants may be products of high risk and therefore, more studies are necessary to find methods of decontamination.

Conclusions

As a result of this and many other reports it is obvious that some medicinal plants are a favourable substrate for fungi, particularly for potential producers of mycotoxins such as aflatoxins (*A. flavus* and *A. parasiticus*). Six species of potential producers of mycotoxins, *A.flavus, A. parasiticus, A. nomius, A. oryzae, A. coelatus, A. tamarii* were isolated. In connection with climate change in recent years in Armenia the contamination level of medicinal plants by potential aflatoxigenic fungi from section *Flavi* has been increased. Potential producer of aflatoxin species such as *A. flavus* and *A. nomius* have occurred more often in thyme and mint. Contamination of herbal plants by these species of toxigenic fungi has also risen within recent years, which is an actual and serious problem in Armenia. The presence of a wide range of storage fungi indicates that considerable improvements could be made during post-harvest storage.

Therefore, fungal contamination of medicinal plants, especially raw materials, should be prevented as much as practicable during harvest and processing.

Súhrn

V práci sa analyzovalo 70 vzoriek tymiánu a 30 vzoriek mäty z regiónu Vayk v Arménsku na kontamináciu vláknitých húb a aflatoxínov. Bolo izolovaných a identifikovaných 38 druhov mikroskopických húb patriacich do 9 rodov a dvoch tried – *Zygomycetes Hyphomycetes*. Z rodu *Aspergillus* bola zistená vysoká frekvencia výskytu druhov: *A. flavus, A. niger, A. nomius, A. fumigatus, A. foetidus*. Vo vzorkách tymiánu a mäty boli z rodu *Penicillium* najčastejšie izolované druhy *P. aurantio-groseum, P. radulatum, P. brevi-compactum.* Bola študovaná schopnosť 68 kmeňov zo sekcie *Flavi*, na biosyntézu aflatoxínu B_1, ktorý bol izolovaný z tymiánu v rôznych štádiách spracovania a balenia liečivých čajov. Zo 17 kmeňov patriacich k *A. flavus, A. nomius, A. parasiticus, A. caleatus* produkovalo aflatoxín B_1 v intervale od 5 do 150 ng/100 ml. Väčšina kmeňov zo spomínaných druhov nestratila životaschopnosť pri nízkej vodnej aktivite (a_w = < 0,400). V 19 vzorkách hotového produktu tymiánu s vysokou kontamináciu druhou zo sekcie *A. Flavi* aflatoxín nájdený nebol.

Kľúčové slová: tymián, mäta, vláknité huby, mykotoxín, aflatoxín B_1, vodná aktivita, kmene, kontaminácia

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