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Assessment of toxigenic fungi on Argentinean medicinal herbs

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Abstract

This work was performed to determine the incidence of toxigenic fungi and their mycotoxins on 152 dried medicinal and aromatic herbs, belonging to 56 species, which are used as raw material for drugs.

International methodologies for fungal enumeration and identification were applied as well as TLC and HPLC techniques for toxins detection. The 52% out of 152 samples were contaminated with species from *Aspergillus* genus, 27% belonging to the *Flavi* section and 25% to the *Circumdati* section. The 16% of the total samples was contaminated with species from *Fusarium* genus.

Aspergillus flavus and *A. parasiticus* (*Flavi section*), were the predominant species isolated, 50% out of 40 isolates were toxigenic. Aflatoxin concentrations ranged from 10 to 2000 ng/g. Only 26% of isolates from the *Circumdati* section (*A. alliaceus, A. ochraceus* and *A. sclerotiorum*) produced ochratoxin A in low concentrations between 0.12 and 9 ng/g. From a total of 29 strains of *Fusarium spp.*, 27.5% were *Fusarium verticillioides* and *F. proliferatum*, which produced fumonisin B1and fumonisin B2 ranged from 20 to 22000 μg/g and from 5 to 3000 μg/g respectively. The remaining species, *F. equiseti*, *F. oxysporum*, *F. semitectum*, *F. compactum*, *F. sambucinum* and *F. solani* were able to produce neither group A and B trichothecenes nor zearalenone.

The incidence of *A. ochraceus* and *Fusarium spp*. and their toxigenic capacities on medicinal herbs were studied for the first time in Argentina. It would be important to look for natural contamination to define acceptability limits which allow the control of sanitary quality of medicinal herbs used as phytotherapic medicines in several countries.
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Introduction

Taking into account previous surveys on herbal drugs in Argentina (Rizzo et al., 1998a, Rizzo et al., 1998b; Rizzo and Chiale, 1999), regulations on fungal counts and aflatoxins occurrence were established, at present as law in force (Resolucion 144/98, 1998; Disposición 2671, 1999; Disposición 2673, 1999). These preliminary surveys of stored dried raw materials revealed fungal contamination with other toxycogenic species than *Aspergillus flavus* and *A. parasiticus* (Flavi Section). Strains belonging to *Aspergillus* genus from the *Circumdati* section and species of *Fusarium genus*, were found. All of them are able to produce toxic metabolites for human health (Pittet, 1998).

With respect to aflatoxins, secondary metabolites produced by *A. flavus* and *A. parasiticus*, a strong association has been proved between estimated aflatoxins intake and incidence of hepatocelullar carcinoma. Due to these evidences aflatoxins have been classified in *Group 1* 'as carcinogenic to humans' by International Agency for Research on Cancer (IARC, 1993a).

Species of the *Circumdati* section (*A. ochraceus, A. melleus, A. alliaceus, A. petrakii, A. ostianus, A. sclerotiorum, A. sulphureus, A. auricomus* and *A. albertenses*) as well as *Penicillium verrucosum* are able to produce Ochratoxin A (OTA). This nephrotoxic metabolite, related to both Balkan endemic nephropathy and urothelial urinary tract tumors, has been included in *Group 2B* as 'possibly carcinogenic to humans' by IARC (Castegnaro and McGregor, 1998).

Meanwhile, strains of *Fusarium verticillioides, F. subglutinans* and *F. proliferatum* may produce several mycotoxins, such as fumonisins FB1 and FB2. They have been statistical associated with a high incidence of esophageal and primary liver cancer on humans in several countries. On the basis of available toxicological evidence, the toxins derived from *F. verticillioides* have been classified in *Group 2B* as 'possibly carcinogenic to humans' (IARC, 1993b).

Moreover, other species of *Fusarium* genus as *F. graminearum, F. culmorum, F. equiseti, F. compactum* could produce trichothecenes and zearalenone. These toxins have been related with citotoxic (Ueno, 1983) and hyperestrogenic activities (Shier, 1998) respectively. Consequently, their possible presence on herbs must be considered taking into account the consumer protection.

The aim of this study attempted to determine the incidence and quimiotaxonomy of toxicogenic strains of *Aspergillus, Penicillium* and *Fusarium* in medicinal herbs, used as raw material, to elaborate phytotherapic drugs in Argentina.

Materials and methods

Samples: A total of 152 samples of dried raw material (300 g each), belonging to 56 species of medicinal herbs, collected from 13 regulated enterprises, were analyzed (Table 1). Sampling were made using USP technique (USP 23, 1995). Information about geographic origin of samples were required and registered. The herb samples were maintained in zipper top paper bags to prevent humidity changes till the analysis time.

Humidity determination: 10 g of each sample were ground and analyzed in ULTRA-X with IR lamp to determine directly the percentage of humidity by weight loss due to water evaporation.

Fungal count, isolation and identification: Decimal dilution of samples were made in sterile 0.1% peptone solution and 1 ml of each was placed into Dichloran Rose Bengal Chloranphenicol Agar (DRBCA) plates by duplicate. The colonies were enumerated after 7 days at 27 °C (Pitt et al., 1992). The results by dilution plating were expressed as colony forming units per gram of sample (cfu/g).

Fungal contaminants, especially species of *Aspergillus, Penicillium* and *Fusarium* potentially toxicogenics were identified, using specific media and proper guides (Klich and Pitt, 1988; Nelson et al., 1983; Booth., 1971; Samson and van Reenen-Hoekstra, 1988). Isolates from *Flavi* and *Circumdati* sections and *P. verrucosum* were maintained on Potato Dextrose Agar (Merck) and isolates of *Fusarium spp*. on Spezieller Nährstoffarmer Agar (SNA) slants. All of them were kept at 1–4 °C.

Toxicogenic capacity: For testing related mycotoxins production, strains of *A. flavus, A. parasiticus* and *Fusarium spp*. were growing on sterile rice for 7 days and 28 days, respectively. Species from *Circumdati* section were growing on both sterile wheat and rice for 11 days. All of the inoculated media were incubated at 25 °C. After incubation, all of cultures were treated at 60 °C overnight to dry the mycelial mat and then they were finely ground to perform the chemical analysis.

Detection of Aflatoxins (AfB1, AfB2, AfG1, AfG2) and Zearalenone (ZEN): A modified method (Shotwell et al., 1966) using thin-layer chromatography (TLC) with two developing solvent systems in the same direction was applied. Anhydrous ethyl ether:hexane (3:2) for clean-up and chloroform:acetone (9:1) for detection were employed. Only

Table 1. Species of medicinal and aromatic herbs analyzed

Scientific name	Popular name	Scientific name	Popular name
Aconus calamus L.	Cálamo	*Erythrina crista-galli* L.	Ceibo
Aesculus hippocastanum L.	Castaño de Indias	*Euphorbia serpens* H.B.K.	Yerba Meona
Aloysia triphylla L'Hérit.	Cedrón	*Fucus vesiculosus* L.	Fucus
Alternanthera pungens	Yerba del Pollo	*Fumaria officinalis* L.	Fumaria
Arctostaphylos uva ursi	Uva ursi	*Gentiana gliesii*	Nencia
Archangélica officinalis	Angélica	*Geoffroea*	Chañar
Atropa belladonna L.	Belladona	*Hamamelis virginiana* L.	Hamamelis
Baccharis articulata Lam.	Carqueja	*Haplopappus rigidus* Phil.	Baila Bien
Blepharocalyx tweediei	Anacachuita	*Hipericum perferatum* L.	Hipérico
Boldea boldus Looser	Boldo	*Illisium verum* Hooker Phil.	Anís estrellado
Bulnesia retama	Retamilla	*Lavandula officinalis* Cnaix	Lavanda
Carduus marianus L.	Cardo mariano	*Lipidium sativum* L.	Mastuerzo
Cassia angustifolia Vahl.	Sen	*Lippia turbinata* Griseb.	Poleo
Cecropia adenopus Mart.	Ambay	*Marrubium vulgaris* L.	Marrubio
Citrus aurantium L.	Cascara de naranja	*Melissa officinalis* L.	Melisa
Cnicus benedictus L.	Cardo santo	*Mentha piperita* L.	Menta piperita
Coreandrum sativum L.	Coreandro	*Minthostachys mollis*	Peperina
Crataegus oxyacantha L.	Crataegus	*Passiflora coerulea* L.	Pasionaria
Cynara scolymus L.	Alcachofa	*Piper angustifolium* R.et P.	Mático
Cynodon dactylon	Gramilla	*Rhamnus purshiana* DC.	Cascara sagrada
Chamomilla recutita L.	Manzanilla romana	*Ruta graveolens* L.	Ruda
Chelidonium majus L.	Celidonia	*Satureja parvifolia* Phil.	Muña Muña
Chenopodium	Paico	*Smilax campestris* Griseb	Zarzaparrilla
Dryopteris filix (L.) Schott.	Helecho	*Tilia spp.*	Tilo
Echium plantagineum L.	Borraja de Campo	*Usnea barbata* (L.)Vigg.	Yerba de la Piedra
Ephedra triandra (Tul.)J.H.	Tramontana	*Valeriana officinalis* L.	Valeriana
Equisetum giganteum L.	Cola de Caballo	*Viscum album* L.	Muerdago
Erithraea chilensis Pers.	Canchalagua	*Xanthium spinosum* L.	Cepa Caballo

bi-dimensional TLC was performed for very dirty extracts using the same solvent systems mentioned above. For confirmation, spray reagents of 30% sulfuric acid solution for aflatoxins and bis-diazotized benzidine for ZEN (Malaiyandi et al., 1976) were applied.

Detection of Ochratoxin A (OTA): was performed using chloroform:aqueous fosforic acid 0.1 M (60:5) for extraction, immunoaffinity column VICAM® for clean-up and high performance liquid chromatography (HPLC) for detection (Solfrizzo et al., 1998; Vicam, 1999).

Detection of Fumonisins (FB_1,FB_2): was performed using acetonitrile:methanol:water (25:25:50) as extraction solvent, immunoaffinity column VICAM® for clean-up (Solfrizzo et al., 2000) and HPLC for detection (AOAC, 2000b).

Detection of Trichotecenes: were determined by a modified Trucksess method (AOAC, 2000a; Rizzo et al., 1995) with acetonitrile:ethylacetate:water (50:41:9) as extraction solvent, cleanup column packed with charcoal:alumina:celite (0.7:0.5:0.3) and TLC for detection. This method allows the detection of Group A (T-2, neosolaniol) and Group B (DON). Trichotecenes were confirmed using spray of 25% sulfuric acid solution for Group A and aluminum chloride solution for Group B (Romer, 1986).

Results

From 152 samples studied 27% was contaminated with *Flavi* section, 25% with *Circumdati* section and 16% with *Fusarium spp*. Considering the 56 species of medicinal plants involved (Table 1), 34 (61%) were contaminated with either of them (Table 2).

The mean moisture content of the samples was 11.4%, ranged from 6.2% (*Xanthium spinosum* L.) to 19.6% (*Fucus vesiculosus* L.).

Generally, fungal count of samples fulfil the established limit including in application's requirements for approval raw material (fungal count $\leqslant 10^4$ cfu/g). Only 11 samples out of 152 (7.2%) attained 10^5 cfu/g. The most frequent contaminants were species from the *Nigri* section belonging to *Aspergillus* genus. Either *A. carbonarius, A.*

Table 2. Medicinal plants contaminated with *A. flavus*, *A. ochraceus* and *Fusarium spp.*

Scientific names (popular names)	*A. flavus* group	*A. ochraceus* group	*Fusarium spp.*
Aloysia triphylla L'Hérit. (Cedrón)	*A. flavus, A. sojae*	*A. ochr group*	N
Alternanthera pungens H.B.K (Yerba de pollo)	*A. flavus*	*A. sclerotiorum* *A. ochr group*	*F. equiseti,* *F. oxysporum* *F. semitectum*
Arctostaphylos uva ursi (Uva ursi)	*A. flavus*	N	N
Baccharis articulata Lam. (Carqueja)	*A. parasiticus*	*A. ochr group*	N
Blepharocalyx tweediei Hook.et Arn. (Anacachuita)	*A. flavus*	N	N
Boldea boldus Looser (Boldo)	N	*A. alliaceus ,* *A. ochr group*	*F. oxysporum*
Cassia angustifolia Vahl. (Sen)	*A. flavus*	N	N
Cecropia adenopus Mart. (Ambay)	*A. parasiticus* *A. flavus*	*A. ochr group*	*F. oxysporum*
Citrus aurantium L. (Cascara de naranja amarga)	*A. flavus*	N	N
Cnicus benedictus L. (Cardo Santo)	*A. parasiticus,* *A. flavus*	N	N
Coreandrum sativum L. (Coriandro)	*A. flavus*	N	N
Crataegus oxyacantha L. (Crataegus)	*A. flavus*	N	N
Cynara scolymus L. (Alcachofa)	N	*A. ochr group*	*F. oxysporum*
Cynodon dactylon (L.)Peerson (Gramilla)	*A. flavus*	*A. ochr group*	*F. equiseti.*
Chamomilla recutita (L.) Rau (Manzanilla)	*A. flavus*	N	*F. compactum*
Chenopodium ambrosioides L. (Paico)	N	*A. ochr group*	*F. compactum*
Echium plantagineum L. (Borraja)	*A. flavus*	*A. ochr group*	N
Equisetum giganteum L. (Cola de Caballo)	*A. flavus* *A. sojae*	*A. alliaceus,* *A. ochraceus, A. sulphureus*	*F. verticillioides* *F. equiseti*
Erithraea chilensis Pers. (Canchalagua)	N	N	*F. verticillioides*
Euphorbia serpens H.B.K. (Yerba meona)	*A. parasiticus*	N	N
Gentiana gliesii (Nencia)	N	*A. alliaceus*	N
Hamamelis virginiana L. (Hamamelis)	*A. flavus*	N	N
Lipidium sativum L. (Mastuerzo)	N	N	*F. solani*
Lippia turbinata Griseb. (Poleo)	*A. flavus*	*A. ochr group*	N
Marrubium vulgaris L. (Marrubio)	*A. parasiticus,* *A. sojae,* *A. flavus*	*A. ochr group*	*F. equiseti*
Melissa officinalis L. (Melisa)	*A. flavus, A. sojae*	N	*F. verticillioides,* *F. equiseti*
Mentha piperita L. (Menta)	*A. flavus*	N	*F. equiseti*
Minthostachys mollis (Kunth) Gris. (Peperina)	*A. parasiticus,* *A. sojae* *A. flavus*	*A. alliaceus* *A. ochr group*	*F. compactum* *F. verticillioides* *F. semitectum*
Passiflora coerulea L. (Pasionaria)	*A. flavus*	*A. ochraceus*	*F. verticillioides* *F. subglutinans* *F. semitectum*
Ruta graveolens L. (Ruda)	*A. flavus*	N	N
Smilax campestris Griseb (Zarzaparrilla)	N	*A. alliaceus*	N
Tilia spp. (Tilo)	*A. flavus*	*A. ochraceus*	*F. verticillioides* *F. equiseti*
Valeriana officinalis L. (Valeriana)	*A. flavus*	N	*F. semitectum* *F. equiseti* *F. proliferatum*
Viscum album L. (Muerdago)	*A. flavus*	N	N

N: non-detected.

awamorii, A. sclerotium, A. japonicus or *A. niger* were present in the 89% of the samples studied.

Strains of *A. flavus, A. pararasiticus* and *A. sojae* were detected on 27 species of plants (Table 2). From 40 strains of *A. flavus* and *A. parasiticus* isolated, 50% produced aflatoxins. *A. flavus* isolated from *Coreandrum sativum* L., *Mentha piperita* L., *Viscum album* L., *Passiflora coerulea* L., *Arctostaphylos uva ursi, Valeriana officinalis* L. and *Lippia turbinata* Griseb. produced the lowest range of AfB1 and AfB2 (10–100 μg/kg); strains from *Aloysia triphylla* L'Hérit., *Cynodon dactylon* (L.) Peerson, *Tilia spp*. and *Melissa officinalis* L. produced a moderated concentration of AfB1 and AfB2 (100–1000 μg/kg). The highest concentration of AfB1 and AfB2 (1000–2000 μg/kg) were obtained by strains isolated from *Cecropia adenopus* Mart., *Cnicus benedictus* L., *Ruta graveolens* L. *and Marrubium vulgaris* L. Besides, *A. parasiticus* isolated from *Minthostachys mollis* (Kunth) Gris. and *Baccharis articulata* Lam. produced the highest concentrations of AfB1, AfB2, AfG1 and AfG2 (1000–2000 μg/kg). On the other hand the isolated from *Euphorbia serpens* H.B.K. produced very low amount of them (10–100 μg/kg).

Circumdati section strains were detected on 35 samples belonged to 17 species of medicinal plants., *A. ochraceus, A. alliaceus, A. sulphureus* and *A. sclerotiorum* were identified from 26 isolates (Table 2) and the 26.9% of them produced OTA. The amount of OTA detected on both rice and wheat media was very low, ranged from 0.12 to 9 μg/kg (Table 3). Non-toxigenic *P. verrucosum* was only found on *Tilia spp* and *Xanthium spinosum* L.

Strains of *Fusarium* were detected on 17 species of medicinal plants (Table 2). From 29 isolates, *F. verticillioides, F. proliferatum, F. equiseti, F. oxysporum, F. semitectum, F. compactum, F. sambucinum* and *F. solani* were identified. All of *F. verticillioides* and *F. proliferatum* isolates produced fumonisins FB1 and FB2. Concentrations of fumonisins detected were from 250 to 25000 μg/g. The FB2/FB1 mean $\pm$ SD was 0.30 ± 0.19 including the atypical value of 0.04 (*F. verticillioides* INM519) (Table 4). None of the remainder *Fusarium* strains were able to produce T-2, neosolaniol, DON or/ and ZEN.

Discussion

In this new survey an important number of toxicogenic fungi were isolated. They belong to xerophylic *Aspergillus spp*, mainly from the *Flavi* and *Circumdati* sections, and less frequently to species of *Fusarium*, which need a higher moisture content. Nevertheless, the diversity of *Fusarium* species found on Argentinean dried medicinal herbs was higher than reports published by authors from other countries (Aziz et al., 1998; Chourasia, 1995).

Species of *Aspergillus*, which produce aflatoxins and OTA are very common on these particular products. Previous works (Rizzo et al., 1998a, b; MacDonald and Castle, 1996; Halt, 1998) have shown a similar contamination.

Relation among toxigenic fungi, fungal count and moisture content were not found. The presence of toxigenic fungi may depend on both, extrinsic (environmental and geographic conditions) and intrinsic factors (constituents of each species of herb) (Efuntoye, 1996). For instance, samples from different regions, belonging to the same species of medicinal herbs, such as *Melissa officinalis* L. and *Mentha piperita* L., have shown differences in mycobiota populations (influence of geographic conditions). Moreover, same specific herbs coming from different regions, such as *Piper angustifolium* R.et P. and *Illisium verum* Hooker Phil. as well as *Lavandula officinalis* Cnaix, have had the lowest fungal count (<10 ufc/g) and shown absence of toxicogenic fungi (influence of intrinsic factors). Similar behavior they have had in the previous sampling mentioned above.

Table 3. Production of OTA by *A. ochraceus* group on sterile wheat and rice

Plant species source	Fungal species	Ochratoxin A ng/g	
		Wheat medium	Rice medium
Equisetum giganteum L	*A. alliaceus* INM130	7.5	n.d.
	A. ochraceus INM122	0.20	n.d.
Minthostachys mollis (Kunth) Gris	*A. alliaceus* INM124	6	8
Alternanthera pungens H.B.K	*A. sclerotiorum* INM132	9	3.4
Passiflora coerulea L.	*A. ochraceus* INM125	0.12	n.d.
	A. ochraceus INM128	0.23	n.d.
Boldea boldus Looser	*A. alliaceus* INM127	0.37	n.d.

n.d. = non-detected.

Table 4. Production of Fumonisins FB1 and FB2 on sterile rice

Plant species	Fungal species	Fumonisin μg/g (ppm)		
		FB1 FB2	FB1 + FB2	FB2/FB1
Melissa officinalis L.	*F. verticillioides INM519*	FB1:4000 FB2:150	4150	0.04
Passiflora coerulea L.	*F. verticillioides INM520*	FB1: 200 FB2: 130	330	0.65
Valeriana officinalis L.	*F. proliferatum INM302*	FB1:22000 FB2:3000	25000	0.14
Minthostachys mollis (Kunth)Gris.	*F. verticillioides INM304*	FB1:4000 FB2:700	4700	0.18
Equisetum giganteum L	*F. verticillioides INM308*	FB1:2500 FB2:1000	3500	0.40
	F. verticillioides INM301	FB1:200 FB2:50	250	0.25
Erithraea chilensis	*F. verticillioides INM306*	FB1:2200 FB2:800	3000	0.36
Tilia spp.	*F. verticillioides INM311*	FB1:900 FB2:350	1250	0.39

Percentages of toxigenic *A. flavus* isolated from different substrates, such as corn, poultry feeds, etc. (Chulze et al., 1989; Dalcero et al., 1998), were similar (around 50%). Consequently, it is possible to consider that the subtracts do not affect the ratio between toxicogenic and non-toxicogenic *A. flavus* strains in our country.

OTA production by strains from the *Circumdati* section was very low in both media assayed , in agreement with other findings (Skrinjar and Dimic, 1992). Although better results were obtained on wheat, HPLC method had to be applied to determine their toxigenic capacities.

Taking into account that the number of reports dealing with the production of OTA by members from the *Nigri* section has been increasing (Abarca et al., 1994, 2001; Dalcero et al., 2002) and knowing that these strains are the most frequent contaminants, it would be necessary to study them as producers in the future surveys.

Strains of *F. verticillioides* and *F. proliferatum* isolated had a very high toxigenic capacitiy (ppm) (Table 4). Data of FB2/FB1 ratio were similar with those related to natural fumonisins found in commercial corn hybrids in Argentina (Ramirez et al., 1996). These results allow us to infer that *Fusarium spp* on medicinal plants have the same quimiotoxicological behavior as the contaminants of Argentinean corn (Chulze et al., 1996).

Fungi that produce OTA and Fumonisins were registered in medicinal herbs for the first time in our country.

Because of toxigenic species from *Flavi* and *Circumdati* sections and strains of *Fusarium spp.* were detected together, more than one related toxin could be present as natural contaminants in herbs (Table 2).

Acceptability limits must be established for phytotherapic drugs consumed as alternative medicines in several countries. Besides, detection methods have to be validated.

One of the most important task should be to control each herb before being channeled to the drug industry which allow them to assure the quality of end products.

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