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OCCURRENCE OF TOXIGENIC FUNGI IN HERBAL DRUGS

Adriana Bugno[1]*; Adriana Aparecida Buzzo Almodovar[1]; Tatiana Caldas Pereira[1]; Terezinha de Jesus Andreoli Pinto[2]; Myrna Sabino[1]

[1]Instituto Adolfo Lutz, São Paulo, SP, Brasil; [2]Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, SP, Brasil

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ABSTRACT

The increase in the consumption of natural drugs have made their use a Public Health problem due to the possibility of access to products without adequate conditions of use. The concern with the quality of the natural products is due to the potential fungal contamination and the risk of the presence of mycotoxins. Ninety-one samples of medicinal plants were evaluated for the fungal contamination and the mycotoxigenic potential of *Aspergillus* and *Penicillium* isolated from the samples. Results indicated that predominant mycoflora was distributed in 10 genera. From these, 89.9% of the isolates corresponded to genera *Aspergillus* and *Penicillium*, which are extremely important from the mycotoxicological standpoint. 21.97% of the *Aspergillus* and *Penicillium* isolates proved to have the ability for producing aflatoxins (42.9%), ochratoxin A (22.4%) and citrinine (34.7%). The presence of toxigenic moulds represents a potential risk of mycotoxin contamination and considering the worldwide increased use of herbal products as alternative medicines, it is necessary setting standards for toxigenic moulds in crude herbal drugs in order to reduce the risks for consumers' health.

Key words: herbal drugs, medicinal plants, toxigenic moulds, mycotoxins

INTRODUCTION

Plants have been used in the prevention, treatment and cure of disorders and diseases since ancient times. In spite of their origin, natural drugs should not be viewed as simple tools of folk medicine since they are a class of pharmaceutical products and should meet the requirements of quality, safety and efficacy (3,4).

The advancements of synthetic medicine overshadowed the traditional herbal medicine for over 50 years. However, in the last years there was a progressive increase in the demand of herbs and preparations of botanical origin as alternative or complementary medicine due to economical, social and cultural factors (3,4). The increasing popularity of natural drugs made their use a Public Health problem due to the lack of effective surveillance of the use, efficacy, toxicity and quality of these natural products. The premise that traditional use of these medicinal products for generations establishes their safety does not necessarily attest to their safety and efficacy. Indeed, the adverse effects of long-term herbal use, adulteration with toxic compounds and contamination by pathogenic microbials or natural toxins like mycotoxins have been reported for herbal products and medicinal plants (1,2,7-11,13,16-22,25).

The concern over quality of these products is mainly due to their potential contamination, considering their natural origin. Practices used in harvesting, handling, storage, production and distribution make medicinal plants subject to contamination by various fungi, which may be responsible for spoilage and production of mycotoxins (1,11,13,25). Fungi of the genera *Aspergillus* and *Penicillium*, largely distributed in the Brazilian ecosystem, are known to contain strains that produce mycotoxins.

In spite of the extensive research on the occurrence of mycotoxins in foods, there are some reports available on the

*Corresponding Author. Mailing address: Instituto Adolfo Lutz, Av. Dr. Arnaldo, 355, Cerqueira Cesar. 01246-902, São Paulo, SP, Brasil. Tel.: (+5511) 3068-2863, Fax: (+5511) 3068-2926. E-mail: adrbugno@ial.sp.gov.br

incidence of toxigenic mycoflora and mycotoxins in medicinal plants and phytotherapic compounds worldwide (1,2,5,7-11, 13,16-22,25).

Considering the little information on the toxigenic moulds in medicinal plants in Brazil, the objectives of the present study were to evaluate the predominant mycoflora and the extent of fungal contamination in medicinal plants and investigate the strains of fungi isolated for their ability to produce mycotoxins, such as aflatoxins, ochratoxin A and citrinine. The data obtained will be value as an indicator of the potencial for mycotoxin production.

MATERIALS AND METHODS

Sampling

Ninety-one samples of medicinal herbs, composed by 65 different plant species, were evaluated in order to assess the predominant mycoflora and the extent of fungal contamination. The products were chosen on the basis of their commercial availability and popularity of use and were obtained from four different suppliers in São Paulo (Table 1).

Evaluation of fungal contamination

Ten gram of each sample were mechanically homogenized in 90.0 mL of buffered peptone water (MERCK) for 2 minutes. Tenfold serial dilutions were performed up to 10^{-6}, in buffered peptone water (MERCK). Enumeration of fungi was performed by pour plating method (26), using Sabouraud Agar with chloramphenicol (DIFCO). Plates were incubated upside down at 26 ± 1ºC for 7 days. After incubation, the fungal colonies were counted, recorded and the number of colony-forming units (CFU) per gram were calculated.

Mould colonies representative of all morphologically different types present were inoculated onto Potato Dextrose Agar (MERCK) and incubated at 26 ± 1ºC for 10 days. Identification was performed by cultural and morphological characteristics and followed the taxonomic schemes of Raper and Fennel (15) for the genus *Aspergillus* and Pitt (14) for the genus *Penicillium*.

Evaluation of toxigenic potential

All 223 isolates of *Aspergillus* and *Penicillium* were screened for the ability to produce aflatoxins and ochratoxin A by the inoculation in Coconut Agar Medium (6,12) at pH 7.0 ± 0.1, and the ability to produce citrinine by the inoculation in Coconut Agar Medium (6,12) at pH 5.0 ± 0.1. All plates were incubated at 26 ± 1ºC, for 10 days. After the incubation, the colony and the culture medium around it were transferred to glass flasks, weighted and macerated in chloroform (MERCK), at a ratio of 3 mL/g. The macerate produced was filtered in filter paper, and the filtrate obtained was evaporated to dryness on a water bath. Mycotoxins were qualitatively detected by thin-

Table 1. Herbal drugs analyzed.

Common name	Scientific name
Absinthe	*Artemisia absinthium*
Abutua	*Chondrodendron tomentosum*
Agoniada	Plumeria lancifolia
Altea	*Althaea officinalis*
Angélica	*Angelica archangela*
Anise	*Pimpinella anisum*
Artichoke[(b)]	*Cynara scolymus*
Baccharis[(a)]	*Baccharis gaudichaudiana*
Boldo[(a)]	*Peumus boldus*
Burdock	*Arctium lappa*
Caaroba	*Jacarandá caroba*
Cáscara sagrada[(b)]	*Rhamnus purshiana*
Catuaba[(a)]	*Trichilia catigua*
Centaury	*Centaurium erythraea*
Chá-de-bugre	*Cordia ecalculata*
Chamomile[(b)]	*Matricaria recutita*
Chapéu-de-couro[(a)]	*Echinodorus macrophyllus*
Chinese rhubarb	*Rheum palmatum*
Cipó-prata	*Banistera argyrophylla*
Colomba	*Jateorhiza palmata*
Condurango	*Marsedenia condurango*
Congorosa	*Maytenus ilicifolia*
Corn silk	*Zea mays*
Escamônea	*Convolvulus scammonia*
European Elder[(b)]	*Sambucus nigra*
Fennel	*Foeniculum vulgare*
Frangula	*Rhamnus frangula*
Fucus[(b)]	*Fucus vesiculosus*
Germander	*Teucrium chamaedrys*
Ginkgo[(c)]	*Ginkgo biloba*
Green tea	*Camelia sinensis*
Guaraná[(b)]	*Paullinia cupana*
Holy thistle	*Carduus benedictus*
Horse chesnut	*Aesculus hippocastanum*
Horsetail[(a)]	*Equisetum arvense*
Hyssop	*Hyssopus officinalis*
Ipê-roxo	*Tabebuia avellanedae*
Jaborandi	*Pilocarpus microphylus*
Jalap[(a)]	*Phytolacca americana*
Jasmime	*Jasminum officinalis*
Jurubeba	*Solanum paniculatum*
Krameria	*Krameria triandra*
Lavander[(a)]	*Lavandula officinalis*
Lemongrass	*Cymbopogon citratus*
Linden	*Tília cordata*
Macela	*Achyrocline satureoides*
Malva	*Malva sylvestris*
Melissa[(a)]	*Melissa officinalis*
Muirapuama	*Ptychopetalum olacoides*

Mullein	*Verbascum densiflorum*
Oak	*Quecus robur*
Paraguay tea	*Illex paraguariensis*
Pfaffia	*Pfaffia paniculata*
Quassia[(a)]	*Quassia amara*
Quebra-pedra	*Phyllanthus niruri*
St. John's wort	*Hypericum perforatum*
Senna[(a)]	*Cassia senna*
Stevia[(a)]	*Stevia rebaudiana*
Sucupira	*Bowdichia* spp
Tonka beans	*Dipteryx odorata*
Urucum	*Bixa orellana*
Uva-ursi	*Arctostaphylus uva-ursi*
Valerian	*Valeriana officinallis*
Yarrow	*Achilea millefolium*
Yellow chinchona	*Chinchona calisaya*

(a) 2 samples; (b) 3 samples; (c) 4 samples.

layer chromatography (TLC), as described by Soares and Rodriguez-Amaya (24). The residue obtained above, which was resuspended in 1.0 mL of chloroform (MERCK), and mycotoxin standards (SIGMA) were spotted onto TLC plates (20 x 20 cm MERCK aluminium sheets, coated with 0.25-mm layer thickness of silica gel G). The chromatogram was developed at room temperature, in unsaturated chamber containing a solvent system composed of a mixture of toluene, ethyl acetate and formic acid (50:40:10). Visualization was performed under UV light at 365 nm.

The chemical confirmation of mycotoxin identity was performed by adequate techniques. The presence of aflatoxins was confirmed by derivatization with trifluoroacetic acid (23) and by spraying the developed plates with aqueous solution of sulfuric acid 50% (5,16,18). The presence of ochratoxin was confirmed by two-dimensional chromatography and by the exposure of plates to NH_3 vapor (23). The presence of citrinine was confirmed by exposure to NH_3 vapor (18).

RESULTS AND DISCUSSION

The risk of the presence of microorganisms in a pharmaceutical product depends on this finality of the use, its nature and its potential damage that may be caused to the consumers. Considering natural flora, current production conditions and the need to warrant the quality and the safety of these products, monographs of the US Pharmacopoeia (26) for products that contain raw material of natural origin establish a maximum fungal contamination limit of 2×10^2 CFU/g of the product.

Table 2 presents the frequency of distribution of the 91 samples according to the fungi counts obtained.

Table 2. Distribution of the herbal drugs samples according to the counts of fungi.

Enumeration limits (CFU/g)	Number of samples
$0 \leq 2 \times 10^1$	11 (12.09%)
$2 \times 10^1 \leq 2 \times 10^2$	30 (32.97%)
$2 \times 10^2 \leq 2 \times 10^3$	27 (29.67%)
$2 \times 10^3 \leq 2 \times 10^4$	14 (15.38%)
$2 \times 10^4 \leq 2 \times 10^5$	6 (6.59%)
$2 \times 10^5 \leq 2 \times 10^6$	3 (3.30%)

The results showed that 54.9% of the samples exceeded the limit determined by the US Pharmacopoeia (26) and these results are in agreement with those of previous studies (16-18,21,25). *Cymbopogon citratus* (3.98×10^5 cfu/g), *Hypericum perforatum* (3.30×10^5 cfu/g), *Equisetum arvense* (2.58×10^5 cfu/g), *Trichilia catigua* (6.09×10^4 cfu/g), *Baccharis gaudichaudiana* (4.56×10^4 cfu/g), *Echinodorus macrophyllus* (4.12×10^4 cfu/g), *Phytolacca americana* (2.92×10^4 cfu/g), *Achyrocline satureoides* (2.85×10^4 cfu/g) and *Phyllanthus niruri* (1.95×10^4 cfu/g) were the most contaminated samples. According to plant part used, the highest counts of fungal isolates were observed in leaves and aerial parts (50.0%) followed by flowers (16.0%), rhizomes and roots (12.0%), barks (12.0%) and seeds (10.0%).

Although high fungal loads may be accepted due to the natural origin of those products, they indicate the potential for spoilage and mycotoxigenesis (13).

The predominant mycoflora obtained was distributed in 10 genera (Table 3). The genus *Aspergillus* was the most dominant genus recovered (179 isolates) followed by *Penicillium* (44 isolates) and these two genera were found in 90.1% and 39.6% of the samples analyzed. All these results are in agreement with that reported by others (1,2,8-11,13,18-22). The presence of a wide range of storage fungi indicates that considerable improvements could be made during post-harvest storage.

Strains of *Aspergillus flavus*, *Aspergillus niger* and *Penicillium citrinum* were the most dominant and frequently isolated (23.39%, 20.97% and 12.50%, respectively), followed by *Aspergillus ochraceus* (10.48%), *Penicillium chrysogenum* (5.24%) and *Aspergillus parasiticus* (4.44%). These results approximate with previous reports that showed *Aspergillus flavus*, in particular, was the main contaminant of different herbal and spices samples (1,2,8-11,13,16-21).

Most of the identified moulds have been reported to have ability to produce mycotoxins. The 223 isolates of *Aspergillus* and *Penicillium* were evaluated for their ability to produce aflatoxins, ochratoxin A and citrinine. The number of isolates, toxigenic isolates and types of mycotoxins they were able to produce are presented in Fig. 1. Fourty-nine of these isolates

Table 3. Distribution of the fungi detected in samples of herbal drugs.

Fungi isolated	Number of isolates
Alternaria	1 (0.40%)
Aspergillus flavus	58 (23.39%)
Aspergillus fumigatus	8 (3.23%)
Aspergillus niger	52 (20.97%)
Aspergillus ochraceus	26 (10.48%)
Aspergillus parasiticus	11 (4.43%)
Other *Aspergillus* spp	24 (9.68%)
Chaetomium	2 (0.81%)
Cladosporium	4 (1.61%)
Mucor	4 (1.61%)
Paellomyces	1 (0.40%)
Penicillium chrysogenum	13 (5.24%)
Penicillium citrinum	31 (12.50%)
Phoma	2 (0.81%)
Rhizopus	9 (3.63%)
Trichoderma	2 (0.81%)

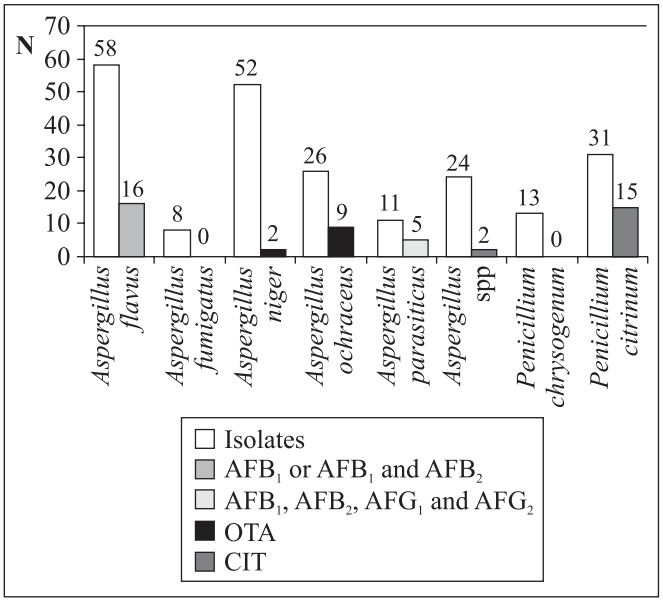

Figure 1. Distribution of toxigenic isolates detected in herbal drugs according to the type of mycotoxins produced.

(21.97%) were found to produce mycotoxins: 42.9% were found to be aflatoxigenic strains, 22.4% ochratoxigenic strains and 34.7% citrinine-producing strains.

The analysis of Fig. 1 indicated that 27.6% *Aspergillus flavus* presented the ability to produce aflatoxin B_1 or aflatoxins B_1 and B_2; 45.5% *Aspergillus parasiticus* presented ability to produce aflatoxins B_1, B_2, G_1 and G_2; 34.6% *Aspergillus ochraceus* and 3.8% *Aspergillus niger*, the ability to produce ochratoxin A; 48.4% *Penicillium citrinum* and 8.3% of other *Aspergillus* spp, the ability to produce citrinine.

Although this study did not attempt to examine crude herbal drugs for the presence of mycotoxins, the results showed there is a potential risk for mycotoxins contamination, especially during prolonged storage in poorly conditions without temperature and moisture control that usually render medicinal plants more susceptible to moulds growth and mycotoxins production.

CONCLUSION

In the present study, 54.9% of the medicinal plants analyzed did not comply with the maximum acceptable limit for fungal contamination. Among fungi isolated, the presence of the genera *Aspergillus* and *Penicillium* was greater than other genera. 21.97% of the *Aspergillus* and *Peniciillium* isolates presented the ability to produce mycotoxins, such as aflatoxins, ochratoxin A and citrinine. Although the presence of toxigenic moulds in a product did not imply in mycotoxins detection, their presence represents a potential risk of contamination with mycotoxins. Considering the worldwide increased use of herbal products as alternative medicines and the risk of purchase and use of natural products contaminated with moulds and mycotoxins, it is necessary setting appropriate standards for toxigenic moulds and mycotoxins in crude herbal drugs and medicinal plants in order to reduce the risks for consumers' health.

RESUMO

Ocorrência de fungos toxigênicos em drogas vegetais

O aumento no consumo de produtos naturais transformou seu uso em um problema de Saúde Pública devido a possibilidade do acesso a produtos sem adequadas condições de uso. A preocupação com a qualidade dos produtos naturais é devida à potencialidade de contaminação por fungos e ao risco da presença de micotoxinas. Noventa e uma amostras de plantas medicinais foram avaliadas quanto à contaminação fungica e ao potencial micotoxigênico de *Aspergillus* e *Penicillium* isolados nestas amostras. Os resultados indicaram que a micoflora predominante esteve distribuída entre 10 gêneros. Entretanto, 89,9% dos isolados corresponderam aos gêneros *Aspergillus* e *Penicillium*, extremamente importantes do ponto de vista micotoxicológico. Verificou-se que 21,97% dos isolados de *Aspergillus* e *Penicillium* demonstraram capacidade para produzir aflatoxinas (42,9%), ocratoxina A (22,4%) e citrinina (34,7%). A presença de fungos toxigênicos

representa risco potencial de contaminação com micotoxinas e considerando o aumento no consumo de produtos de origem vegetal como alternativa terapêutica, é necessário estabelecer padrões para a presença de fungos toxigênicos em drogas vegetais a fim de reduzir os riscos à saúde do consumidor.

Palavras-chave: drogas vegetais, plantas medicinais, fungos toxigênicos, micotoxinas

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