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# INDOOR FUNGI AND THEIR CILIOSTATIC METABOLITES

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**Abstract:** According to epidemiological studies, it is possible that some secondary metabolites of indoor airborne fungi could be responsible for health troubles which occupants suffer from. In our previous experiments, a model with tracheal rings of 1-day-old chicks *in vitro* was shown to be a very suitable method to study the ciliostatic chloroform-extractable endo- and/or exometabolites of filamentous fungi. In this study we isolated the filamentous fungi from walls of "mouldy" dwellings and schools (cultivation on dichloran 18% glycerol agar at 25 and 37°C for 10 d) in Slovakia. We studied the ciliostatic effect of the chloroform-extractable endo- and exometabolites of 96 representative isolates (stationary cultivation on the liquid medium with 2% of yeast extract and 10% of sucrose at 25°C for 10 days) on the cilia movement in tracheal organ cultures of 1-day-old chickens *in vitro* after 24, 48 and 72 hrs (incubation in the minimal essential medium according to Eagle with Earl´s salts and 20 µg of extract of metabolites dissolved in dimethylsulfoxide per 1 mL). Strains of *Penicillium* Link: Fr. sp., *Aspergillus versicolor* (Vuill.) Tiraboschi, *A. flavus* Link, *Cladosporium sphaerospermum* Penzig and *C. cladosporioides* (Fres.) de Vries were isolated most frequently. Two *A. flavus* isolates were able to produce aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ *in vitro* after cultivation on the liquid medium with 20% sucrose and 2% yeast extract. This is the first isolation of aflatoxigenic *A. flavus* strains from dwellings in Slovakia. All frequently isolated strains produced secondary metabolites with the strongest ciliostatic activity – their exo- and endometabolites stopped tracheal ciliary movement in chicks till 24 h. There are some toxic fungal metabolites in the indoor air not only with the ability to destroy ciliary movement in the upper airways *in vitro* but, probably, during long-lasting exposure to cause general intoxication of macroorganism via lung tissue.

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**Key words: indoor environment, filamentous fungi, ciliostatic metabolites, chicken's model, respiratory intoxication.**

## INTRODUCTION

There are many different germs of microscopic filamentous fungi and their toxic metabolites in the working and home environment. Aflatoxin $B_1$, ochratoxin A, zearalenone, secalonic acid D, deoxynivalenol, nivalenol, moniliformin, etc. have been detected in the working environment and some trichothecenes in dwellings [3, 5, 10, 15, 21] and in air conditioning systems [26].

People living in houses with mouldy walls and furnishings, damp indoor air and bad odours are not only frustrated because of subjective discomfort but, in some cases, their health has been seriously affected. Usually, the origin and real cause of their complaints are difficult to define. Children and adults living under such conditions suffer mostly from respiratory tract illnesses [16, 27].

The nasal, tracheal and bronchial epithelium with ciliated cells, mucous production and ciliary clearance provides the first contact between unfavourable environment and the human body. A model with the tracheal rings of 1-day-old chicks *in vitro* proved to be a very suitable method for the study of ciliostatic activities of fungal metabolites. Well known mycotoxins (e.g. sterigmatocystin, trichothecenes) as well as chloroform-extractable endo-

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and/or exometabolites of some fungal strains (*Alternaria* Nees sp., *Aspergillus versicolor, Penicillium chrysogenum* Thom, *Stachybotrys chartarum* (Ehrenb.) Hughes, *Trichoderma viride* Pers., *Ulocladium* Preuss sp. and others) were able to stop the ciliary movement *in vitro* in few hours or days in our previous experiments [6, 17, 18, 19, 20, 22].

In this study we isolated the filamentous fungi growing on the walls of mouldy dwellings and schools and studied the ciliostatic effect of their endo- and exometabolites extractable with chloroform on the cilia movement in tracheal organ cultures of the 1-day-old chicks *in vitro*.

## MATERIALS AND METHODS

**Fungi.** During 3 years scratch samples were taken from mouldy walls in 43 dwellings and 4 schools in Western and North Slovakia. The samples were examined in the mycological laboratory by direct microscopy and cultivated on the dichloran 18% glycerol agar (Hi Media Laboratories Pvt. Ltd., Bombay, India) – a general purpose medium with emphasis on enumeration of indoor fungi [28] at 25 and 37°C for 10 d.

Representatives of most frequent fungal isolates, total: 96, were inoculated on Sabouraud agar (Imuna Co., Šarišské Michaľany, Slovakia) slants and cultivated at 22–25°C for 8 days. The biomass from 3 tubes per each strain was replaced into 500 mL Erlenmeyer flasks with 200 mL of liquid medium (2% of yeast extract and 10% of sucrose). Such a system was stationary cultivated at 25°C for 10 d.

The isolates of *Aspergillus flavus* were tested for the ability to produce aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ *in vitro* according to Abarca *et al*. [1], i.e. after cultivation on the liquid medium with 20% sucrose and 2% yeast extract, pH 5.5 at 25°C for 14 days, filtration of the broth through Whatman No. 3 filter paper, 1 ml of the filtrate and standards of aflatoxins $B_1 + B_2$ and $G_1 + G_2$ (20 µl of each) were quantitatively spotted on TLC plates (10 × 10 cm), followed by developing in a solvent system (toluene/ethylacetate/formic acid, 6 : 3 : 1; v/v), air-drying and visualization under UV light (366 nm) when $B_1 + B_2$ were giving deep blue and $G_1 + G_2$ turquiose fluorescency.

**Metabolites.** Two types of chloroform extracts were used in the experiments:

1) Chloroform extracts of cultivation medium (exometabolites): After filtration of the biomass, the medium was twice extracted with 200 mL of chloroform, the pooled extracts were dried with anhydrous $Na_2SO_4$ and concentrated to dryness in a water bath.

2) Chloroform extracts of biomass (endometabolites): Biomass filter cakes were also extracted twice with 200 ml of chloroform in a blender (Stomacher Lab-Blender 400, Seward Medical UAC House, London, England) for 10 min and the dry extracts were obtained as mentioned above. Each residue was redissolved in dimethylsulfoxide ($Me_2SO$) to the concentration 1 mg/mL and kept at 4°C in a medicine flask with a teflon plug.

**Table 1.** Frequency of the occurrence of fungal species isolated from mouldy dwellings.

| Fungi | Samples with positive isolation (%) |
|---|---|
| *Absidia* sp. | 2 |
| *Alternaria* sp. | 9 |
| *Aspergillus* sp. | 9 |
| *A. flavus* | 30 (5[a]) |
| *A. glaucus* | 2 |
| *A. niger* | 5 |
| *A. ochraceus* | 12 |
| *A. restrictus* | 2 |
| *A. ustus* | 5 |
| *A. versicolor* | 33 |
| *Cladosporium* sp. | 9 |
| *C. cladosporioides* | 23 |
| *C. herbarum* | 9 |
| *C. sphaerospermum* | 30 |
| *Mortierella* sp. | 2 |
| *Neosartorya fischeri* | 5 |
| *Penicillium* sp. | 93 |
| *Phoma* sp. | 7 |
| *Ulocladium* sp. | 6 |
| *Wallemia sebi* | 2 |
| Yeasts | 5 |

Note: [a] - samples with *in vitro* aflatoxigenic *A. flavus* isolates.

**Toxicity *in vitro*.** The ciliostatic activity of the crude extracts of exo- and endometabolites was studied using chick tracheal organ cultures for the bioassay of mycotoxins [6, 14]. One-day-old chicks (State Research and Productional Co., Častá Hatchery, Slovakia) were decapitated, tracheas were removed within 3 min, washed twice in the minimal essential medium according to Eagle with Earl's salts – E-MEM (Institute for Sera and Vaccines, Prague, Czech Republic) and cut into 0.1 mm slices. Tracheal rings (20–30) were placed into a Petri dish (diameter 60 mm) with 2 mL of culture medium, i.e. E-MEM with 1% of 3% glutamine, 2.5% of 7.5% $NaHCO_3$ with phenol red (Institute for Sera and Vaccines), 10% of bovine fetal serum, 100 µg streptomycin, 100 U penicillin and 20 µg crude extract per 1 mL. Reference media were prepared with 1% $Me_2SO$ instead of extracts as well as controls with pure rich medium. The cultivation of organ models was carried out at 37°C and 5% $CO_2$.

The ciliary movement was observed on 5–7 tracheal rings microscopically (250 × magnification; Olympus BX50) after 0, 24, 48 and 72 h of incubation.

## RESULTS

After the mycological evaluation of 47 scratch indoor samples, the isolates belonging to 21 genera or species were obtained (Tab. 1). The most frequent were: *Penicillium* sp. (isolated from 93% of samples), *Aspergillus versicolor*

**Table 2**. Patterns of ciliostatic activity of representative fungal isolates from mouldy dwellings.

| Number of Fungal Isolates | | Ciliostatic Activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | Endometabolites | | | Exometabolites | | |
| Total | Strains | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| 33 | *Alternaria* sp. (2)<br>*Aspergillus flavus* (4 with 1[a])<br>*A. niger* (1)<br>*A. ochraceus* (1)<br>*A. restrictus* (1)<br>*A. ustus* (1)<br>*A. versicolor* (9)<br>*Cladosporium cladosporioides* (2)<br>*C. herbarum* (1)<br>*C. sphaerospermum* (2)<br>*Penicillium* sp. (7)<br>*Phoma* sp. (1)<br>*Wallemia sebi* (1) | + | + | + | + | + | + |
| 3 | *Alternaria* sp. (1)<br>*Aspergillus ustus* (1)<br>*Phoma* sp. (1) | + | + | + | – | + | + |
| 7 | *Alternaria* sp. (2)<br>*Aspergillus versicolor* (2)<br>*Penicillium* sp. (3) | + | + | + | – | – | + |
| 3 | *Aspergillus flavus* (1)<br>*A. versicolor* (1)<br>*Cladosporium cladosporioides* (1) | – | + | + | + | + | + |
| 3 | *Aspergillus flavus* (1[a])<br>*Penicillium* sp. (2) | – | + | + | – | + | + |
| 3 | *Cladosporium cladosporioides* (1)<br>*C. sphaerospermum* (1)<br>*Penicillium* sp. (1) | – | + | + | – | – | + |
| 2 | *Cladosporium cladosporioides* | – | – | + | + | + | + |
| 2 | *Aspergillus versicolor* (1)<br>*Penicillium* sp. (1) | – | – | + | – | + | + |
| 2 | *Penicillium* sp. | – | – | + | – | – | + |
| 7 | *Aspergillus flavus* (1)<br>*Cladosporium herbarum* (1)<br>*Penicillium* sp. (3)<br>*Phoma* sp. (1)<br>*Ulocladium* sp. (1) | – | – | – | + | + | + |
| 2 | *Cladosporium sphaerospermum* (1)<br>*Penicillium* sp. (1) | – | – | – | – | + | + |
| 3 | *Aspergillus flavus* (1)<br>*Cladosporium sphaerospermum* (1)<br>*Neosartorya fischeri* (1) | – | – | – | – | – | + |
| 5 | *Aspergillus ochraceus* (1)<br>*Cladosporium sphaerospermum* (1)<br>*Penicillium* sp. (3) | + | + | + | – | – | – |
| 1 | *Aspergillus glaucus* | – | – | + | – | – | – |
| 1 | *Penicillium* sp. | | nd | | – | + | + |
| 19 | *Aspergillus flavus* (1)<br>*Cladosporium cladosporioides* (1)<br>*C. sphaerospermum* (4)<br>*Penicillium* sp. (13) | – | – | – | – | – | – |

Notes: [a] - *in vitro* aflatoxigenic *A. flavus* isolates. + - fungal extracts stopped tracheal ciliary movement. – - ciliary movement was not inhibited by extracts. nd - not determinated.

(33%), *A. flavus* and *Cladosporium sphaerospermum* (30%) and *C. cladosporioides* (23%). The *A. flavus* isolates obtained from 5% of samples (houses in Western Slovakia) were able to produce aflatoxins *in vitro*. This is the first isolation of aflatoxin-producing *A. flavus* strains from dwellings in Slovakia. On the other hand, the isolates of *Absidia* Tiegh. sp., *Aspergillus glaucus* Link, *A. restrictus* G. Sm., *Mortierella* Coem. sp. and *Wallemia sebi* (Fr.) von Arx were found only rarely.

The ciliostatic activity of the exo- and endometabolites of 96 representants of the isolates is shown in Table 2. The most active chloroform-extractable metabolites, both

exo- and endometabolites, were produced by *Alternaria* sp. (2 isolates), *Aspergillus flavus* (4 isolates, including 1 aflatoxin-producing *in vitro*), *A. niger* van Tieghem, *A. ochraceus* Wilhelm, *A. restrictus* and *A. ustus* (Bain.) Thom and Church (1 isolate of each), *Cladosporium cladosporioides* and *C. sphaerospermum* (2 isolates of each), *C. herbarum* (Pers.) Link (1 isolate), *Penicillium* sp. (7 isolates), *Phoma* Sacc. sp. and *Wallemia sebi* (1 isolate of each). These metabolites stopped the ciliary movement in tracheal rings in 24 h.

The metabolites of 1 non-aflatoxigenic *Aspergillus flavus*, 4 *C. sphaerospermum* and 1 *C. cladosporioides*, and 13 *Penicillium* sp. isolates showed no ciliostatic activity under given experimental conditions at all.

One isolate of *Ulocladium* sp. tested produced high ciliostatically active exometabolites while its endometabolites did not stopped ciliary beating in the experiments conducted. Low ciliostatic activity – after 72 h was shown by exometabolites of an isolate of *Neosartorya fischeri* (Wehmer) Malloch and Cain although its endometabolites were not active. The *A. glaucus* isolate produced slightly active endometabolites that broke down ciliary movement and non-active exometabolites in 72 h. The metabolites of all other aspergilli, penicillia and cladosporia showed limited ciliostatic effect on chick's tracheal epithelium.

## DISCUSSION

The indoor environment of dwellings is colonized by a number of various species of microfungi. Their quantity in the air depends on the physiological properties of individual species, as well as on the type of activities of occupants [12, 28].

Vital and devitalized fungal propagules in the indoor air of houses are important autoallergens in some humans with genetic predisposition [4, 7, 8, 13, 25]. *Alternaria* sp. is considered to be the most potent fungal allergen [9].

There are health complication without a clear origin (bronchitis, sore throat, concentration difficulties, back-aches, irritation of eyes and mouth cavity, weakness feeling etc.) in a higher incidence in occupants of mouldy dwellings as well [23]. The mycotoxins and volatile products of microscopic fungi are new characteristics in damp house examination [11, 24, 29, 30, 31]. But almost nothing is known about the role of these compounds affecting the respiratory organs.

Ciliostatic activity of some pure mycotoxins as well as chloroform-extractable metabolites of some indoor fungi detected on 1-day-old chicks tracheal organ cultures has already been described by Jesenská and Bernát [6] and Piecková and Jesenská [20].

The aim of this study was to evaluate the ciliostatic activity of the endo- and exometabolites of microscopic filamentous fungi isolated from the walls of mould-affected dwellings and schools. They were colonized most frequently by the "first colonizers" (*Aspergillus* Fr.: Fr. sp. and *Penicillium* sp.) and the "second" ones (namely *Cladosporium* Link sp.). The third part (33%) of the investigated strains, particularly *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Phoma* sp. were able to produce compounds with very high ciliostatic toxicity. Similar results were obtained in our previous investigations on moulds on house walls and construction material [20, 22].

The most frequent species isolated are known producers of strong mycotoxins. *Alternaria* sp. can produce over 70 different myco- and phytotoxins. Sterigmatocystin is the most well-known mycotoxin of *Aspergillus versicolor* and was one of the most ciliostatically active compounds in our previous experiments [6]. Penicillia are also producers of a large variety of toxic metabolites. Although, mycotoxins are usually considered to be exometabolites, many of them (citreoviridin, cyclopiazonic acid, luteoskyrin, sterigmatocystin, verruculogen, etc.) can occur intracellularly [2]. It is possible that some mycotoxins were present in the crude extracts and were responsible for their ciliostatic effect observed in the experiment. A precious chemical analysis is needed to clarify this problem exactly.

There can be some toxic fungal metabolites in the indoor environment not only with ability to inhibit ciliary movement of upper airways *in vitro* but, probably, during long-lasting exposure to cause general intoxication of macroorganism via lung tissue and/or to facilitate bacterial or viral infection of its underlying epithelium.

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