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# Extractions of *Aspergillus flavus* Link ex Gray and *Cladosporium cladosporioides* (Fresen.) de Vries from Allergenic Microfungi and Application of Toxicity Tests

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**Abstract:** *Aspergillus flavus* Link ex Gray and *Cladosporium cladosporioides* (Fresen.) de Vries, isolated from the soil of Florya Atatürk Forest, were extracted and some toxicity tests were carried out in order to determine their toxic effects on mice for the first time in Turkey. The method of extraction complied with the one described in the literature. During the process, Coca's solution was used as an extractive agent. Sterile filtration was employed in the sterilization process. The stocks were diluted to 1/10 of their concentrations before the toxicity tests were carried out. It was found that the *A. flavus* Link ex Gray and *C. cladosporioides* (Fresen.) de Vries extracts were sterile and that the experiments performed on 10 mice proved to be not toxic.

**Key Words:** Allergy, *Aspergillus flavus* and *Cladosporium cladosporioides* extracts, toxicity tests

## Allerjenik Mikrofunguslardan *Aspergillus flavus* Link ex Gray ve *Cladosporium cladosporioides* (Fresen.) de Vries'in Ekstrelerinin Hazırlanması ve Toksisite Testi Uygulamaları

**Özet:** *Aspergillus flavus* Link ex Gray ve *Cladosporium cladosporioides* (Fresen.) de Vries Türkiye'de ilk defa Florya Atatürk Ormanı toprağından izole edilmiş, ekstreleri hazırlanmış ve toksik etkilerini tayin etmek için fareler üzerinde toksisite testi uygulamaları yapılmıştır. Ekstraksiyonlar literatürdeki metoda uygun olarak hazırlanmıştır. Bu maksatla ekstraktif olarak Coca solüsyonu, sterilizasyon için steril filtrasyon tekniği kullanılmıştır. Stok çözeltiler toksisite testi uygulamalarından önce 1/10 oranında seyreltilmiştir. Hazırlanan *A. flavus* Link ex Gray ve *C. cladosporioides* (Fresen.) de Vries ekstrelerinin steril olduğu ve 10 fare üzerindeki deneyde toksik olmadığı bulunmuştur.

**Anahtar Sözcükler:** Allerji, *Aspergillus flavus* ve *Cladosporium cladosporioides* ekstreleri, toksisite testleri

## Introduction

Hypersensitivity or allergies are of great importance for physicians. In 1906, von Pirquet coined the term "allergy" to describe a reaction other than the normal one. This state was described as an ability in the body developing as a result of a first contact with organic or inorganic substances, which at the second contact, causes a reaction of different characteristics, intensity and timing. Nowadays, our understanding of allergies allows us to define them as specific hypersensitivities to antigen damaging tissues. Furthermore, allergen is the term used for antigens or haptens causing an allergy. We can observe allergens in several forms ranging from foreign proteins to simple substances such as quinine and chrome (1).

Sifting, extraction, clarification, sterilization, sterility test and standardization are the processes that we implicate for extracting allergenic substances to be used in intra-cutaneous or intra-dermal tests (2). In the process of extraction, Coca's solution is used (2).

Being sterile and free from pyrogen is an obligation for stock. In the process of filtration, a membrane filter with a pore width of 0.80, 0.45 and 0.20 µm is used. It is suggested that the pH of the extraction be 8.2.

Contact allergies often stem from proteins (antigen, allergen). Allergies are divided into 4 groups: Type I, early or immediate allergy; Type II, allergy damaging cells (allergy to medicine); Type III, allergy caused by antigen-antibody compounds; and Type IV, late reaction allergy, contact allergy or contact dermatitis. The difference between type I and type IV is that the former has a high molecular structure whereas the latter has a low molecular weight of 100-1000 (2). *Alternaria, Aspergillus, Cladosporium, Fusarium, Helminthosporium, Mucor, Penicillium, Phoma, Rhizopus* and *Trichothecium* are various microfungi, some of which are found in soil and known to cause allergies, and so are *A. flavus* Link ex Gray and *C. cladosporioides* (Fresen.) de Vries (3). In this study, results obtained from research performed on *A. flavus* Link ex Gray and *C. cladosporioides* (Fresen.) de Vries, their extractions and toxicological applications are presented.

## Materials and Methods

### a) Soil sample, isolation and identification of *A. flavus* and *C. cladosporioides*

In May 2000, a soil profile in Florya Atatürk Forest was opened and the surface was cleaned up vertically. The sample was then taken from a depth of 10 cm under aseptic conditions. Afterwards, it was mixed and left to dry at room temperature. In the process of isolating microfungi from the soil sample, the Soil Dilution Plate Method was employed (4). The dried sample was added to some sterile distilled water to form a 1/10 suspension. The suspension was mixed for 30 minutes in a mechanical mixer (5). More sterile distilled water was added to the liquid to form 1/100, 1/1000 and 1/10000 suspensions. Of these, the most apposite for use were the 1/1000 and 1/10000 ones, the latter of which was preferred in our study (6). Before the organic matters and soil particles were deposited (7), 1 ml of the latter suspension was cultivated on a medium of Peptone Dextrose Agar (8) with a sterile pipette (9). In order to prevent bacteria and *Actinomycetes* from producing, 30 mg/l streptomycin was added to the medium along with the same amount of rose bengal in order to limit the size of colonies (8). Of the microfungi colonies which formed after an incubation period of 7-10 days at 25°C, microfungi were isolated and cultivated in Malt Extract Agar and Czapex Dox Agar media (10). Following another 7-10 day incubation period at 25°C, the preparations were dyed with picric acid and identified by means of lactophenol solution. In the preparations extra-thin lamellae were used and the identifications of *A. flavus* and *C. cladosporioides* were carried out according to the literature (11,12).

### b) Coca's solution

Coca's solution was used so that the agent in *A. flavus* and *C. cladosporioides* would pass into the extracted material. Coca's solution consists of NaCl, phenol, $NaHCO_3$ and distilled water (2).

### c) Extraction

Nine millilitres of Coca's solution was added to 1g of *A. flavus* and *C. cladosporioides*. The mixtures were mixed for 24 hours at 4°C in a magnetic mixer. They were then centrifuged for 10 minutes at 2500 rpm. Following this process, the extracts were centrifuged twice more for the same period of time (2).

### d) Filtration

The extracts were first filtered through a rough filter wetted with Coca's solution. Later they were filtered through S&S black bandaged paper and sterilized in a laminar cabinet using the Sterile Filtration Technique. At this stage the extracts were filtered in a Sartorius sterile filtration injector through membrane filters of 0.80 µm, 0.45 µm and 0.20 µm pore diameters.

### e) The dilution of pure extract before controls

Extracts which belong to a particular species and which are obtained by means of sterile filtration are called pure extracts (2). Aytuğ et al. (2) have stated that extracts should not be used in the diagnosis and treatment of allergies, in sterility and toxicity controls or in skin tests unless diluted as much as necessary. This is why the pure extracts used in this study were diluted.

Special Diluent Solution I:

0.9% NaCl + 0.5% Phenol + Distilled water → 1000 ml

Special Diluent Solution II:

Special Diluent Solution I + Glycerine (50:50)

For Sterility and Toxicity Test:

Special Diluent Solution I + Special Diluent Solution II + Pure extract (9:1)

For sterility and toxicity tests 1% extracts containing 5% glycerine were used.

f) Sterility Test*

For these tests to be conducted, anaerobic and aerobic media were used; 2-3 drops of extracts in thioglycollatte were examined for 14 days at 35°C and in Sabouraud Dextrose Agar for the same period of time at 25°C (13).

g) Toxicity Test**

Laboratory animals used: 10 mice (Balb/c strain)

Weight of mice:

1st experiment group (5 mice used for the injection of *A. flavus* extract): 20.1, 22.3, 23.4, 24.3, 26 g (mean 23.22 g)

1st control group (5 mice for control): 20.1, 22.3, 23.4, 24.3, 26 g (mean 23.22 g)

2nd experiment group (5 mice used for the injection of *C. cladosporioides* extract): 20.8, 21.6, 23.8, 25.2, 26.2 g (mean 23.52 g)

2nd control group (5 mice for control): 20.8, 21.6, 23.8, 25.2, 26.2 g (mean 23.52 g).

Each mouse was injected with 0.5 ml diluted extracts subcutaneously in their abdomen. The mice were then followed and their diet was not changed. On the 8th day they were reweighed (2).

## Results and Discussion

It is stated in the literature that *A. flavus* and *C. cladosporioides* are isolated from soil and that they are allergenic (3). As evidenced by the reproduction of no single microorganism in the sterility test, the extracts were sterile. That no mortalities were observed among the 10 mice during the toxicity test is enough proof that the extracts were not toxic. Eight days after the injection of *A. flavus*, it was observed that the weight of the 5 mice in the 1st experiment group increased by 9 g in total (mean 1.8 g), (Table 1a). The same increment was observed in the 5 mice in the 1st control group after the same period of time (Table 1b). Likewise, 8 days after the injection of *C. cladosporioides*, the 5 mice in the 2nd experiment group increased a total of 10.2 g (mean 2.04 g) in weight (Table 2a). The same increment in weight was observed in the 2nd control group (Table 2b), which is in accordance with the literature (2).

Table 1 a. First and last weight of the five mice subjected to toxicity test in the 1st experiment group

| Weight of mice | 1st group | |
|---|---|---|
| | Total weight | Mean weight |
| Weight of mice before injection | 116.1 g | 23.22 g |
| Weight of mice eight days after the injection of *A. flavus* extract | 125.1 g | 25.02 g |

Table 1 b. First and last weight of the five mice in the 1st control group

| Weight of mice | 1st control group | |
|---|---|---|
| | Total weight | Mean weight |
| Weight of mice before controls | 116.1 g | 23.22 g |
| Weight of mice after eight days | 125.1 g | 25.02 g |

Table 2 a. First and last weight of the five mice subjected to toxicity test in the 2nd experiment group

| Weight of mice | 2nd group | |
|---|---|---|
| | Total weight | Mean weight |
| Weight of mice before injection | 117.6 g | 23.52 g |
| Weight of mice eight days after the injection of *C. cladosporioides* extract | 127.8 g | 25.56 g |

Table 2 b. First and last weight of the five mice in the 2nd control group

| Weight of mice | 2nd control group | |
|---|---|---|
| | Total weight | Mean weight |
| Weight of mice before controls | 117.6 g | 23.52 g |
| Weight of mice after eight days | 127.8 g | 25.56 g |

Important studies on allergies have been carried out by Davies (14), Agarwal and Shivpuri (15), Aytuğ (16), Cosentino et al. (17), Rosas et al. (18) and Çolakoğlu (19-21).

In conclusion, we believe that using extracts from microfungi of Turkish origin in medical microbiology will help the economy of the nation in a short period.

* Sterility tests were carried out in GATA Haydarpaşa Educational Hospital, Microbiology Department, Istanbul, Turkey

** Toxicological tests were carried out in Marmara University, Medical Faculty Experimental Research and Animal Laboratory, Istanbul, Turkey

This is the first study carried out on mycology with the aim of producing extracts. We therefore hope that it will be a guide for other researchers studying both the process of extraction and toxicological applications.

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