

EXTRACTIONS OF *RHIZOPUS NIGRICANS* EHRENBERG AND *PENICILLIUM EXPANSUM* (LINK) THOM FROM ALLERGENIC MICROFUNGI AND APPLICATION OF TOXICITY TESTS

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Özet

Bu çalışmada *Rhizopus nigricans* Ehrenberg ve *Penicillium expansum* (Link) Thom Türkiye’de ilk defa Fatih Ormanı toprağından izole edilmiş, ekstraları 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 ve sterilizasyon için steril filtrasyon tekniğı kullanılmıştır. Stok çözeltiler toksisite testi uygulamalarından önce 1/10 oranında seyreltilmiştir. Hazırlanan *R.nigricans* Ehrenberg ve *P.expansum* (Link) Thom ekstralarının steril olduğu ve 10 fare üzerindeki deneyde toksik olmadığı bulunmuştur.

Anahtar Kelimeler: Allerji, *Rhizopus nigricans* ve *Penicillium expansum* ekstraları, toksisite testleri

Abstract

For the first time in Turkey, a study has been done including isolating *Rhizopus nigricans* Ehrenberg and *Penicillium expansum* (Link) Thom from the soil of Fatih Forest, extracting that *Rhizopus nigricans* Ehrenberg and *Penicillium expansum* (Link) Thom, and carrying out some toxicity tests in order to determine their toxic effects on mice. Extractions were prepared in accordance with the method in literature. For this purpose, as an extractive Coca’s solution and for sterilization, sterile filtration technique were used. Before the application of toxicity tests, the stocks were diluted to 1/10 of their concentrations. The results were that *R.nigricans* Ehrenberg and *P.expansum* (Link) Thom extracts were sterile and they were not toxic according to the tests applied 10 mice.

Key Words: Allergy, *Rhizopus nigricans* and *Penicillium expansum* extracts, toxicity tests

Introduction

It is so certain that hypersensitivity or allergy is important for physicians. Von Pirquet used the term allergy to define a reaction other than normal in 1906, and this state was described as an ability in the body which develops as a result of a first contact with organic or inorganic substances and which, at the second contact, causes a reaction of different characteristic, intensity, and timing. With the help of achievements in microbiology, today we are able to define it as a specific hypersensitivity against an antigen damaging tissues. The antigens or haptens causing allergy are called as allergen. As simple substances like quinine and chrome, or as foreign proteins, allergens are found in various forms (1).

The process in the usual method for extracting allergenic substances to be used in intra-cutaneous or intra-dermal tests is sifting, extraction, clarification, sterilization, sterility test and standardization (2). Coca’s solution is used in the process of extraction.

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

Contact allergy often stems 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 allergy. *R.nigricans* Ehrenberg and *P.expansum* (Link) Thom are also the species isolated from soil and cause allergy (3). In this research, results of studies on toxicity applications and extract constitution of *R.nigricans* Ehrenberg and *P.expansum* (Link) Thom from allergenic microfungi are given.

Materials And Methods

a) Soil sample, isolation and identification of *R.nigricans* and *P.expansum*

In September 2000, a soil profile in Fatih 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 in room temperature. In the process of isolating microfungi from the soil sample, "Soil Dilution Plate Method" was employed (4). The dried sample was added into 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 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 production of bacteria and *Actinomycetes* 30 mg/l streptomycin was added into the medium along with the same amount of rose bengal aimed at limiting 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 the Potato Dextrose Agar and Czapek Dox Agar media (10). Following another 7-10 days incubation period at 25 °C, the preparations were dyed with picric acid and identified by means of lactophenol solution.

In the preparations extra-minces lamellae were used and the identifications of *R.nigricans* and *P.expansum* were carried out according to Zycha and Siepmann (11) and Raper et al. (12).

b) Extraction

Coca's solution was used as an extraction material for the active substance in *R.nigricans* and *P.expansum* to pass into extraction material. Coca's solution consists of NaCl, phenol, NaHCO₃ and distilled water (2). 9 ml of Coca's solution was added into 1g of *R.nigricans* and *P.expansum*. 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).

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

c) Dilution of pure extract

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 diagnosis and treatment of allergy, in sterility and toxicity controls and in skin tests unless diluted as much as necessary. This is why the pure extracts used in this study were diluted. The following solutions were used in the experiments.

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 II + Pure extract (9:1)

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

d) Sterility Test**

**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

Sterility tests were conducted in both anaerobic and aerobic media. 2-3 drops of extracts in Thioglycollate were examined for 14 days at 35 °C and in Malt Extract Broth for the same period of time at 25 °C (13).

e) Toxicity Test***

First of all mice in experiment and control groups were weighed in toxicity test. Each mouse was injected 0.5 ml diluted extracts subcutaneously in their abdomen in experiment groups. No application on mice was done in control groups. Mice were then followed and their diet was not changed. On the 8th day they were reweighed (2).

Laboratory animals were used in toxicity test: 10 mice (Balb/c) strain

Initial weights of mice:

1st experiment group (5 mice used for the injection of *R.nigricans* extract):20.5, 20.8, 21.0, 22.2, 23.0 g (mean 21.5 g)

1st control group (5 mice for control):20.5, 20.8, 21.0, 22.2, 23.0 g (mean 21.5 g)

2nd experiment group (5 mice used for the injection of *P.expansum* extract):21.0, 20.6, 20.9, 22.0, 22.1 g (mean 21.32 g)

2nd control group (5 mice used for control):21.0, 20.6, 20.9, 22.0, 22.1 g (mean 21.32 g)

Results And Discussion

The identification of *R.nigricans* was carried out according to reference (11): The characteristic features are the presence of rhizoids at the base of sporangiophores (2-3 mm in length, which may grow in clusters),and stoloniferous habit. An aerial hypha grows out and where it touches on the substratum it bears rhizoids and sporangiophores. Growth in this manner is repeated. Measure of sporangium is 160-350 µ and it is globose. The colour of sporangium is initially bright and white colour, but later turns into black colour. Diameter of columella is 80-100 µ and it is semiglobose. Spores are 10-12x7-8 µ. They are oval and dark brown. The identification of *P.expansum* was carried out according to reference (12): Colonies on Czapek Dox Agar growing rapidly, attaining a diameter of 4.5 to 5 cm in 10 days incubation period at 25 °C. Conidiophores are 200-400 µ in length, mostly 3.0 to 3.5 µ in diameter. Penicilli asymmetric, commonly once or twice branched. Metulae usually borne in verticils of 3 to 6 and measuring about 12 to 15 µ by 2.5 to 3.0 µ. Sterigmata usually ranging from 9 to 12 µ by 2 to 2.5 µ. Conidia elliptical, measuring 3.0-3.5 µ in diameter. The isolation of *R. nigricans* and *P.expansum* from soil and their allergenic feature is in accordance with the literature (3). The non-reproduction of microorganisms during sterilization test shows us that extracts are sterile. There were no mortalities after the experiment done on 10 mice in toxicity test; therefore, extracts were not toxic. 8 days after the injection of *R.nigricans*, it was observed that the weight of the 5 mice in the 1st experiment group increased by 11.2 g totally (mean 2.24 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).

| Weight of mice | 1 st group | |
|---|-----------------------|-------------|
| | Total weight | Mean weight |
| Weight of mice before injection | 107.5 g | 21.5 g |
| Weight of mice 8 days after the injection of <i>R.nigricans</i> extract | 118.7 g | 23.74 g |

Table 1 a. Initial and last weight of the 5 mice subjected to *R.nigricans* toxicity test in the 1st experiment group.

| Weight of mice | 1 st control group | |
|--------------------------------|-------------------------------|-------------|
| | Total weight | Mean weight |
| Weight of mice before controls | 107.5 g | 21.5 g |
| Weight of mice after 8 days | 118.7 g | 23.74 g |

Table 1 b. Initial and last weight of the 5 mice in the 1st control group.

Likewise, 8 days after the injection of *P.expansum*, the 5 mice in the 2nd experiment group increased 10.5 g totally (mean 2.1 g) in weight (Table 2a). The same increment in weight was observed also in the 2nd control group (Table 2b), which is in accordance with the literature (2). There are similarities between this study and literature 2. These can be explained as in below. Aytuğ et al. (2) reported that the pollen extracts were prepared according to the method described in literature (3). For this purpose Coca's solution for extraction and sterile filtration technique for the sterilization were used. The toxicity tests were applied to the mice. The results were that pollen extracts were not toxic according to the tests applied 5 mice. There are important studies on allergy subject by Mohovic et al. (14), Gerritsen et al. (15), Iversen and Pedersen (16), Rosas et al. (17) and Chih-Shan et al. (18).

| Weight of mice | 2 nd group | |
|--|-----------------------|-------------|
| | Total weight | Mean weight |
| Weight of mice before injection | 106.6 g | 21.32 g |
| Weight of mice 8 days after the injection of <i>P.expansum</i> extract | 117.1 g | 23.42 g |

Table 2 a. Initial and last weight of the 5 mice subjected to *P .expansum* toxicity test in the 2nd experiment group.

| Weight of mice | 2 nd control group | |
|--------------------------------|-------------------------------|-------------|
| | Total weight | Mean weight |
| Weight of mice before controls | 106.6 g | 21.3 2 g |
| Weight of mice after 8 days | 117.1 g | 23.42 g |

Table 2 b. Initial and last weight of the 5 mice in the 2nd control group.

From the point of results, we certainly defend that the use of extracts from microfungi of Turkish origin in medical microbiology carries great importance and beneficities for the economy of nation. Aiming to produce extracts, this is a preliminary research in its kind in Turkey in the field of mycology. We, therefore, hope that it will be a guide for other researchers in both the process of extracting and its toxicological applications.

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