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TOXIC METABOLITES PRODUCED BY ASPERGILLUS EGYPTIACUS

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Abstract. The toxicity of a crude chloroform extract of *A. egyptiacus*, partially purified on silica gel column, was examined by intraperitoneal injection of sublethal dose to mice. Respiration slowed down and became mainly thoracic. After dissection widespread hemorrhages of the lung were observed. Histological examination of the lung showed the congestion, dilatation of the alveoli and inflammation of the pleural membrane. The liver, when stained with H & E, showed a dark area, mostly around the central veins, where the cytoplasm was mostly acidophilic. The hepatic cells stained with PAS revealed no reserved carbohydrate material. Activity of the toxin against the growth of some microorganisms (bacteria and fungi) was examined.

Introduction

Mycotoxin is a general term used to describe fungal metabolites, highly toxic to animals and man. There are numerous reports relating specific fungi to outbreaks of mycotoxicoses in farm and laboratory animals.[1-3] A general review of the problem of mycotoxicosis has shown that the aspergilli produce a multitude of toxins.[4-9]

In an earlier paper[11] the authors reported that A. *egyptiacus* produced mycotoxin(s) when grown on de Iongh's medium. 1,000-fold dilution of chloroform extract of this fungus induced 0% hatchability of chicken embryos. Inhibition of *Bacillus megaterium* growth by the toxic extract was induced.

The present investigation was undertaken for studying the histological and histochemical effects in mice induced by *A. egyptiacus* metabolite(s) as well as their inhibitory effects on some fungi and bacteria. Purification and characterization of the principal toxin(s) produced by this isolate are in progress.

Materials and Methods

Organism. The isolate of *A. egyptiacus* Moubasher Moustafa from *Aspergillus versicolor* group was isolated from Egyptian soil,[10] and a culture of this species is kept in the Culture Collection of C. M. I. under No. C. M. I. 14145.

Cultivation. The cultivation was made in 6 (2-l) Erlenemeyer flasks each containing 500 ml of de Iongh's medium previously used by the authors.[12] The flasks were sterilized at 1½ atm. for 20 min and inoculated with a spore suspension of the test isolate and incubated as surface cultures at 28° for two weeks.

Extraction. At the end of the incubation period the contents of the culture flasks (medium + mycelium) were homogenized for 5 min in a high speed blender (16,000 rpm) with twice its volume of chloroform. The extraction procedure was repeated three times. The combined chloroform extracts were washed with distilled water, dried over anhydrous sodium sulphate, filtered, and then concentrated to near dryness.

Column chromatography. The crude extract was dissolved in *n*-hexane applied to 4 × 60 cm column packed with silica gel (60-120-mesh) and eluted with 2 l of each of the solvent systems according to the following sequence: *n*-hexane, diethyl ether, chloroform, chloroform : methanol 97 : 3, chloroform : methanol 5:1 and methanol.

Assay of the column fractions. Column fractions were screened by bioassay using the agar diffusion method against *B. megaterium*, which proved to be sensitive to the crude extract of the test isolate.[11] *B. megaterium* was cultivated in petri dishes on PDA medium. Filter discs (6 mm dia) (Whatman No. 1) were soaked with the different fractions of the eluant. After evaporation of the solvent, the discs were applied to the plates. Control discs were soaked with chloroform. The plates were incubated at 35° for 24 hr.

Test for growth inhibition of some bacteria and fungi by partially purified extract of A. egyptiacus. The test organisms listed in the Table were used in this study. The procedure was the same as previously used in case of *B. megaterium* assay using the PDA medium for bacteria and malt extract agar (4% malt extract, 0.5% peptone and 2% agar) for fungi.

Lethal dose (LD_{50}). Two-week old white male mice (15-20 g) were used to determine the lethal dose (LD_{50}). The animals were injected intraperitoneally with different doses of the partially purified extract dissolved in propylene glycol (0.2 ml). Control animals were injected with 0.2 ml propylene glycol only.

TABLE. GROWTH INHIBITION OF SOME MICROORGANISMS BY THE TOXIC METABOLITE(S) PRODUCED BY A. EGYPTIACUS

Organism	Inhibition	Organism	Inhibition
Fungi :			
Aspergillus niger	0	*Cunninghamella echinulata*	+ + +
Aspergillus flavus	0	*Cladosporium herbarum*	+
Aspergillus parasiticus	0	*Penicillium duclauxi*	0
Aspergillus carneus	+	*Neurospora crassa*	+
Aspergillus egyptiacus	+ + +	**Bacteria :**	
Aspergillus terricola	0	*Bacillus megaterium*	+ + +
Mucor racemosus	+ +	*Bacillus cereus*	+ +
Mucor hiemalis	+ + +	*Bacillus subtilis*	+ +
Mucor oryzae	+ +	*Pseudomonas aeuriginosa*	0
Rhizopus nigricans	+ +	*Serratia sp.*	+
Rhizopus oryzae	+ +	*Sarcina sp.*	0
Cunninghamella elegans	+ + +	*Nocardia sp.*	0

+ + + High inhibition.
+ + Moderate inhibition.
+ Low inhibition.
0 No inhibition.

Histological and histochemical studies. Each mouse received, by intraperitoneal injection, three sub-lethal doses, one every 48 hr. Twenty-four hr after the last dose, the animals were sacrificed. Slices of liver, lung, kidney, skin and adrenal gland were fixed in acetic acid-formalin (AAF). The fixed tissues were washed in running water, dehydrated in ascending grades of alcohol, clarified in methyl benzoate and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin (H & E) for general histology, toluidine blue and Feulgen reaction for RNA and DNA respectively, periodic acid Schiff's (PAS) reagent for carbohydrates and bromophenol blue for general proteins.

Results and Discussion

11.25 g of a thick brown oily material were collected after chloroform extraction and evaporation. After fractionation, the active compound(s) were eluted by chloroform: methanol 97:3 fractions. These fractions were combined and evaporated under vacuum and a yellow solid substance (1.9 g) was obtained.

LD_{50} of the fraction eluant. When the test animal was injected with the toxic fraction (0.9 mg/0.2 ml propylene glycol), which is equivalent to 45 mg/kg, it became inactive and less sensitive. The posterior limbs appeared very weak; this made its movement very sluggish. The breath increased at the beginning, then slowed down and became mainly thoracic. When the animal was dissected, the lung showed many spots of bleeding but the other organs appeared normal.

Histological and histochemical studies. Histological examination of the different organs revealed that the lung and the liver are the sites of action of the toxic substance produced by *A. egyptiacus*.

The Lung

1. *Haematoxylin and eosin* (*Plate I, Fig.* 2 and 4) The alveoli of the treated lung appeared wider than the control. Their lining epithelia were thinner with pale pinkish cytoplasm. Most of the nuclei of these cells appeared thin and deeply stained blue (piknotic nuclei). An inflammatory fluid (serous), stained pale violet, exuded in between the alveolar walls forming oedema of the lung. Also, the pleural membrane stained intensely with eosin, forming a fibrous network which indicates a case of sero-fibrinous inflammation. The congestion of the lung was evident. Also bleeding in some areas was observed. Red blood cells were present in the lumen of the alveoli and bronchioles. The blood capillaries were much dilated. The number of lymphocytes was markedly increased. Patches of pigment granules in between the alveoli were noticeable.

2. *Periodic acid Schiff's* (*PAS*), (*Plate I, Fig.* 6). The wall of the alveoli appeared less positive than normal. The inflammed area of pleura appeared in the form of pink network of fibrin. The serous exudate in between the alveolar walls was stained pink.

3. *Bromophenol blue.* The fibrin of the inflamed pleura was stained blue whereas the serous exudate appeared light blue.

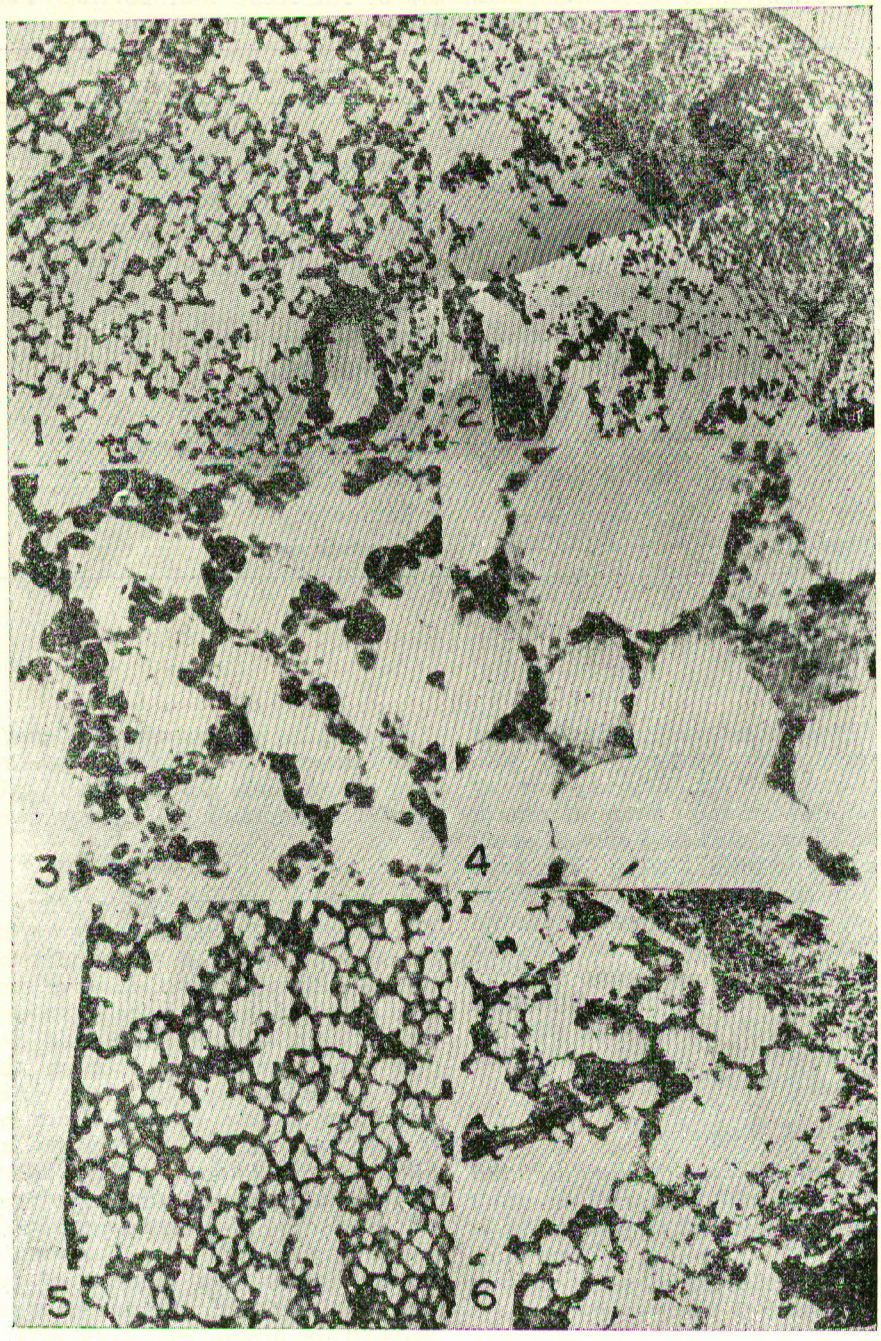

Fig. 1. Section of the control lung (H & E×125)

Fig. 2. Section of the lung of the treated animal. Notice the sero-fibrinous inflammation of pleura (H & E×125).

Fig. 3. High-power view of the control lung to show the epithelia of the alveolar walls (H & E 360).

Fig. 4. High-power view of section from the lung of animal to show dilation of the alveolar sacs and their treated lining epithelia thinner than the normal (H & E×360).

Fig. 5. Section of the control lung (PAS×125).

Fig. 6. Section from the lung of treated animal to show the inflammation of the pleura (PAS×125).

4. *Toluidine blue.* The cytoplasm of the alveolar lining cells was almost negative to the stain. But most of their nuclei appeared dark blue as in the H & E section.

5. *Feulgen reaction.* No significant changes appeared in the DNA content of the nuclei.

The Liver

1. *Haematoxylin and eosin* (*Plate II, Fig.* 2). Sections stained with haematoxylin and eosin revealed granules. The nuclei of some of these cells appeared normal, except for a few which were small in size and were stained dark blue. The blood sinusoids were wider than normal.

2. *Periodic acid Schiff's* (*PAS*), (*Plate II, Fig.* 4). The cytoplasm of the hepatic cells, unlike the control, showed no reserve carbohydrate material and it was moderately stained pink in a homogeneous manner. Very few hepatic cells contained few and dark positive granules.

3. *Toluidine blue.* The cytoplasm of most of the

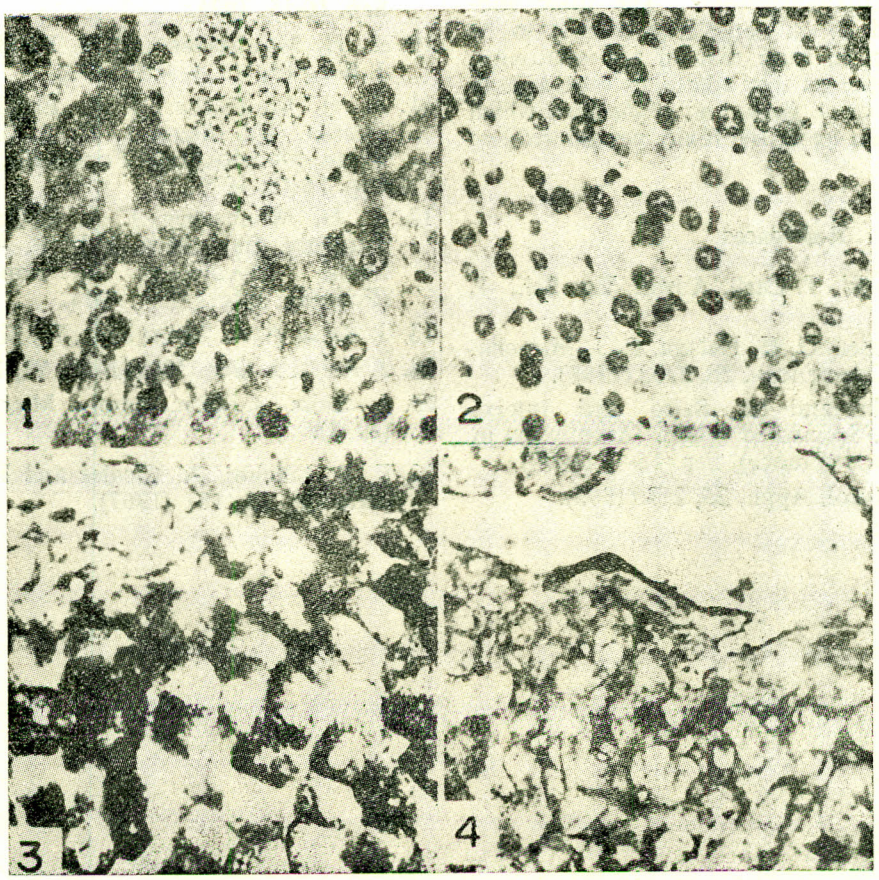

Fig. 1. Section of the control liver (H & E×360).
Fig. 2. Section of liver from the treated animal showing the dark cells surrounding the central veins and the paler ones at the periphery of the hepatic lobules. Notice the dilatation of blood sinusoids (H & E×360).
Fig. 3. Section of the control liver (PAS×360).
Fig. 4. Section of liver from treated animal showing the depletion of carbohydrate material (PAS×360).

that some areas were stained darker than others. Generally, these dark areas were mostly found around the central veins, with the lighter ones at the periphery of the hepatic lobules. The cytoplasm of the cells in the dark areas was mostly acidophilic, and contained very fine basophilic granules, homogenously distributed in it. The nuclei of these cells were mostly rounded in shape, appeared swollen and contained dark chromatin granules. The cytoplasm of the cells of the lighter areas contained no hepatic cells was stained blue, with basophilic granules homogeneously distributed in it. The nuclei of these cells were slightly larger than normal and contained dark blue chromatin granules. In some areas, the hepatic cells appeared lighter in colour, and most contained rounded darkly stained nuclei like in H & E sections. Some of these cells contained small and lightly coloured nuclei.

4. *Feulgen reaction.* The reaction showed almost the picture of the nuclei in H & E.

Influence of the toxic metabolite(s) on growth of some bacteria and fungi. The Table shows the effect of partially purified extract of *A. egyptiacus* on the growth of some bacteria and fungi. It was noted that the toxic fraction of *A. egyptiacus* extract inhibited the growth of all fungi tested except *A. terricola*, *A. parasiticus*, *A. flavus*, *A. niger* and *P. duclauxi*. The phycomycetes, especially *Cunninghamella echinulata*, *C. elegans*, *Mucor heimalis* as well as *A. carneus*, were more sensitive than the other fungi. The sensitivity of phycomycetes was also observed under the influence of aflatoxins,[13] rubratoxin B[14], patulin and diacetoxyscirpenol.[15] The toxic substance(s) produced by *A. egyptiacus* seem(s) to be different in its action than that of the aflatoxins. Lillehoj *et al.*[16] reported that aflatoxins were able to inhibit the growth of *A. flavus* and *P. duclauxi* which is not the case when using the toxic metabolite(s) of *A. egyptiacus*.

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