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ANTIBIOTIC EXTRACTION AS A RECENT BIOCONTROL METHOD FOR *ASPERGILLUS NIGER* AND *ASPERGILLUS FLAVUS FUNGI* IN ANCIENT EGYPTIAN MURAL PAINTINGS

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ABSTRACT

Biodeterioration of mural paintings by *Aspergillus niger* and *Aspergillus flavus Fungi* has been proved in different mural paintings in Egypt nowadays. Several researches have studied the effect of fungi on mural paintings, the mechanism of interaction and methods of control. But none of these researches gives us the solution without causing a side effect. In this paper, for the first time, a recent treatment by antibiotic *"6 penthyl α pyrone phenol"* was applied as a successful technique for elimination of *Aspergillus niger* and *Aspergillus flavus*. On the other hand, it is favorable for cleaning Surfaces of Murals executed by tembera technique from the fungi metabolism which caused a black pigments on surfaces.

KEYWORDS: Biodeterioration, Antibiotic, Mural paintings, Biocontrol, Nfer Bau Ptah Tomb

1. INTRODUCTION

Biodeterioration of mural paintings is one of the most observed phenomena in the recent years. This biodeterioration is caused by the attack of microrganisms like fungi which thrive and feed on the murals (Allsopp, 2004). Several researches studied the physical, chemical, and aesthetic damage caused by fungi in mural paintings (Abd El-Hady, 1993; David, 1998; Florian, 2004; Alarcon,2009). Numerous techniques have been developed to deal with this problem.

The traditional methodology depends on using scalpels, brushes, and scrapers for treatment (Richardson, 1967; Vanevo, 1988; Ashurst, 1990), but biocides were always used in chemical treatment (Hempel, 1978; Allsopp, 1983; Richardson; 1988, Warscheid; 2000). Both mechanical and chemical treatments deal with the superficial mycelium on the mural's surface only without the spores and fruity body which usually existed inside the pores of the murals which help re-growth in the suitable environmental conditions (Warscheid, 2000).

The irradiation methodology, for controlling fungi in murals, usually uses Gamma rays which is impractical for tombs and temples because of the need of high doses to kill the fungi which requires gamma irradiation of activity about 10^6 Curi. This source needs several tons shielding materials and it is impossible and impractical to be moved away from its installation (Florian, 2004; Justa, 1992).

Methods of biological treatment have a negative side effect more than the positive effect; they depend on using fungi or bacteria as organisms not as enzymes or antibiotics.

In this paper, we extracted the antibiotics from Trichoderma harzianum and tested them for controlling the growth of fungi of Aspergillus niger and Aspergillus flavus and metabolism.

2. MATERIALS AND METHODS

2.1. Media used

2.1.1 Cultivation media

Malt Extract Agar (MEA) according to (Smith and Onion, 1983)

Ingredients in g/1;
Malt extract 20
Peptone 10
Glucose 20
Agar 20
Distilled water 1Litter

The PH medium was adjusted at 5.5. This medium was used for the cultivation of Trichoderma sp.

2.2. Secondary metabolite production medium

Yeast Extract Sucrose medium (YES) according to Scott, 1970

The medium was used to enhance the production of extra cellular secondary metabolites. It contains yeast extract, 20g/l ; Sucrose 150g/l and distilled water 1Litter.

2.1.2 Preparation of fungal broth

Spores were scrapped from the mycelium after 7 days of growth at 28°C on (MEA) medium and suspended in sterile distilled water. Liquids of 2ml of spore suspension were used to inoculate 250ml. Erlenmeyer flasks were used each containing 100ml sterile (YES) medium. The ten inoculated flasks were incubated at 28°C for 21 days. The mycelium was then harvested by filtration. The filtrate was then used for extraction of extra cellular secondary metabolites (Scott, 1970).

2.3. Preparation of concentrated fungal filtrate

Yeast extract sucrose broth of Trichoderma sp. (1000ml) was concentrated using a speed vacuum device (Max dry plus) to a total volume of 25 ml. The concentration was tested for the fungal activity.

2.4. Extractions of secondary metabolites

Yeast extract sucrose broth (25ml) obtained from concentrated fungal culture was mixed with (100ml) chloroform / methanol (2:1,v/v). The mixture was shaken vigorously in separated funnel and left to settle down forming a dense lower organic layer containing the secondary metabolite. Extracted metabolites were then concentrated by using a speed vacuum device to a volume of 2ml (Robbers, 1996).

2.5. Purification of the secondary metabolite of Trichoderma sp.

Column packing and equilibration

Two millimeters of extracted metabolites of previous method was subjected to separation using column chromatography (1.5 cm diameter and 50 cm long) puked by silica gel (G100) after activated at 80°c for 30min, then subjected to elution with chloroform: methanol (2:1 v/v) and then 1 ml fractions were collected separately (Robbers,1996; Youssry,1998). All fractions, 26 fractions, were tested against Aspergillus niger and Aspergillus flavus to select a fraction with antifungal activity.

3. RESULTS

3.1. Antifungal abilities of Trichoderma sp.

The main point of the research is to know the best antifungal for controlling the Aspergillus niger and Aspergillus flavus growth. We found 26 fractions of the Trichoderma all of them were tested against the mentioned fungi.

26 Petri dishes were used each containing (YES) and 0.5 ml of each fraction (one Petri for every fraction) and a disc of Aspergillus flavus. The same operation was done with Aspergillus niger then incubated in incubator for 7 days for growth at 28°C, Fig.1.

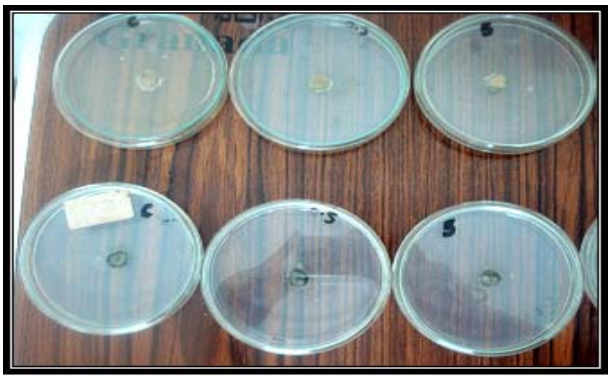


Fig. 1 The Petri dishes containing the (0.5 ml) of the fractions, the (YES) and a disc of the mentioned fungi before incubation.

After the incubation period ended, the fungi in all Petri dishes had grown except the Petri containing fraction number 21 which was completely clean without any conidia (mycelium) or spores, (Fig.2).

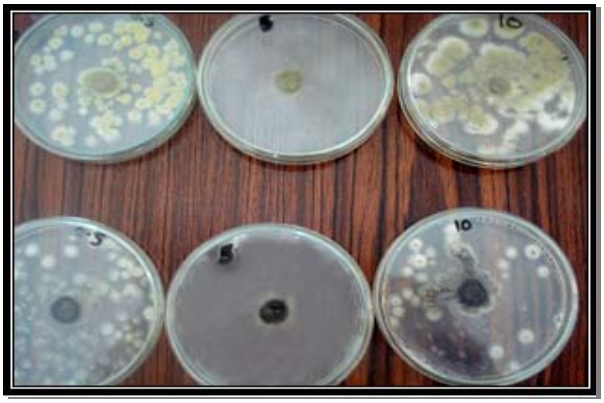


Fig. 2 The Petri dishes containing the (0.5 ml) of the fractions, the (YES) and a disc of the mentioned fungi after incubation.

3.2. Characterization of the purified active fraction

In order to determine the chemical structure of the active fraction compound, the following spectra measurements were carried out.

Mass spectroscopy was done using DI-50 unit of Shimadzu GCMS-QP5050A at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University, and the result was shown in Fig. 3.

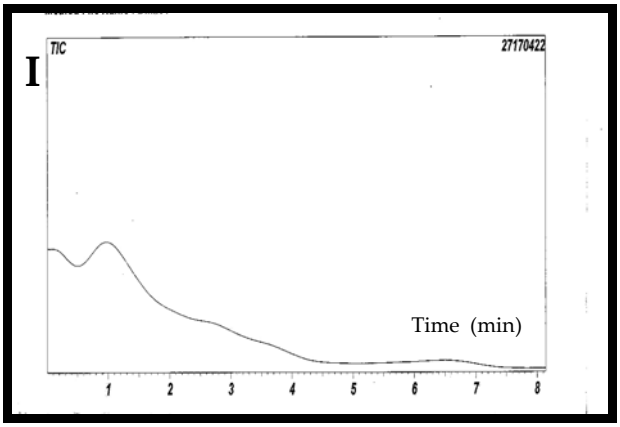


Fig. 3 The total (TIC) of the Antibiotic of the activated fractions.

3.2.1 Mass Spectroscopy analysis

The data from Fig. 3 shows that the active fraction is completely pure and that is clear from the curve which shows only one peak after 1 min. In addition to the characteristic mass number of the effected fraction shown in Fig. 4.

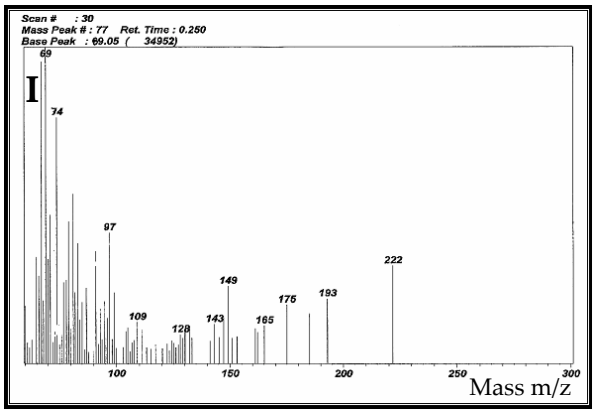


Fig.4 The mass spectrum of the effected fraction and the activated mass numbers of it

The data shows that the activation mass number of the fraction is 222.

3.2.2 Infrared Spectroscopy Analysis

The infrared absorption spectrum of the purified fraction was estimated at the Micro Analytical Center - Faculty of Science, Cairo University, as shown in the type of IR. Fig. 5.

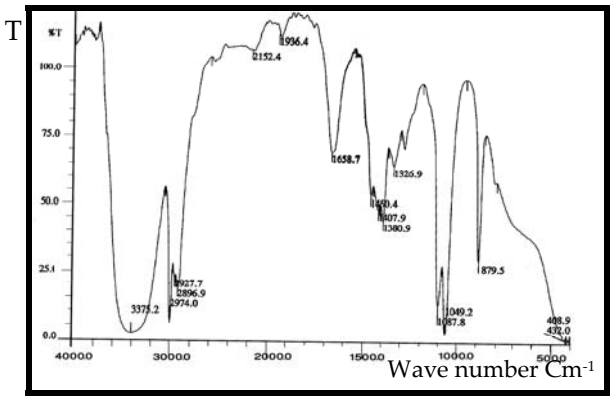


Fig. 5 Infrared spectrum of the activation fraction.

Fig. 5 Shows the functional groups of the fraction which could be explained in Table 1.

Table 1 The absorbed bands of the Functional groups as resulted in the analysis of the activated fraction.

Wave number cm $^{-1}$	Functional groups
3375.2	O-H
2927.7 2896.9 2974.0	C-H
2152.4 1936.4	C≡N
1658.7	C=O
1450.4 1407.9	Aromatic bands
1380.9 1326.9	C-H
1049.2 1087.8	C-O-
879.5 408.9 432.0	-O-C-O-

3.2.3 Nuclear Magnetic Resonance (NMR)

The proton (1H) NMR spectrum was estimated at the Micro Analytical Center – Faculty of Science – Cairo University, which presented the following data in Fig.6

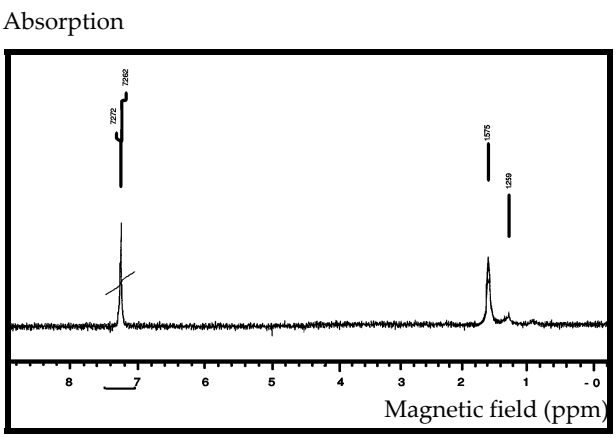
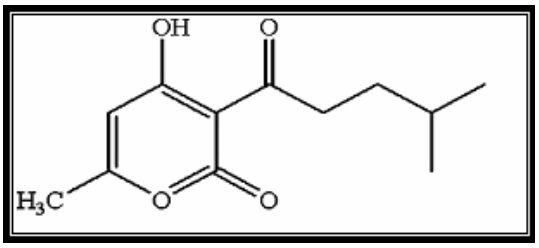


Fig. 6 NMR spectrum shows the Aromatic Functional groups in the activated fraction.

The NMR indicated that there is a Phenol cycle (7272) and a Penthyle cycle (1575) in the chemical composition of the fraction. After all of these analyses were studied biochemically, they confessed that the fraction is an antibiotic with a chemical composition (6 Penthyle α Pyrone Phenol).



The chemical structure of the antibiotic.

3.3. The Application of the Antibiotic on the Deteriorated prepared Mural Painting models

The main point of this research is to treat the deteriorated mural paintings by Fungi without causing any side effect. The following eight models of mural painting were prepared by

tempra technique. Four models were deteriorated by *Aspergillus niger* and the other were deteriorated by *Aspergillus flavus* and incubated for 7 days at 28°C. After incubation period, the samples were treated by the Antibiotic and lifted in open area for 30 days without any protection and after 30 days the murals were undeteriorated and on other hand there is no effect on the pigments as shown in Figs. (7, 8).

(a) (b)

Fig. (7) Deteriorated mural models by *Aspergillus niger* (a) before treatments (b) after treatment and leaving for 30 days

(a) (b)

Fig. 8 Deteriorated mural models by *Aspergillus flavus* (a) before treatments (b) after treatment and leaving for 30 days

3.4. The Application of the Antibiotic on the Deteriorated Mural Paintings of Nfer Bau Ptah Tomb, Giza, Egypt

The antibiotic "6 penthyl α pyrone phenol" which proved to be successful in the experimental study as mentioned above, was used for treating the Deteriorated Mural Paintings in Nfer Bau Ptah Tomb as shown in Fig 9.

A B C

Fig. (9) Mural Paintings of Nfer Bau Ptah Tomb, Giza, Egypt. before treatment (A & B) and after treatment (C).

This antibiotic gave a perfect result for the elimination of *Aspergillus niger* and *Aspergillus flavus* without causing any damage. On the other hand, it is successful for cleaning the mural surfaces from the fungi Metabolism. The final process was sterilization of the tomb by

isopropyl alcohol (CH_3-CH(OH)-CH_3) to avoid any new contamination with fungi spores in the future.

4. DISCUSSION & CONCLUSION

In the current study, the extraction of antibiotic 6 Penthyle α Pyrone Phenol from trichoderma harzianum for controlling *Aspergillus niger* and *Aspergillus flavus* fungi was applied for the 1st time for treating ancient Egyptian mural paintings. This new method excludes the disadvantages of using fungi or bacteria themselves as biocontrol methods. It excludes the probability of infection by new microbes and production of stains on the surface which change the color of the mural paintings. Furthermore, it has several advantages than the traditional treatments, (mechanical, chemical, and irradiation). It prevents the dispersion of micro-organisms spores in the air around mural paintings that can be caused by mechanical methods. It is very simple, easy to apply, on the contrary of irradiation treatment which needs a lot of lead tons for protection and very hard to be applied in the archaeological field. It is very safe, it inhibits the growth of fungi not only at the surface of mural paintings, but also inside their pores due to the death of infection spores. In addition, it has no effect on the colors of the mural painting pigments, durable, non toxic and economic.

The use of the recent technique of treating the two Fungi *Aspergillus niger* and *Aspergillus flavus* contaminated Egyptian mural paintings by the antibiotic, is promising and seems very applicable. It has the advantage of being non toxic, non expensive, practical, durable, and rapid, in addition to having no effect on the colors of paintings.

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