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Isolation and Diagnosis species related to *Stachybotrys* from Indoor Buildings in AL- Najaf Province and its ability to produce verrucarol toxin

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Abstract

Two species of *Stachybotrys* are isolated which are *S. chartarum* and *S. echinata*. It was observed that *S. chartarum* was more frequency than other fungi on wheat straw medium and reached to 82.5%.

Also *Stachybotrys* is growing on materials which rich in cellulose such as straw medium.*Stachybotrys* preferred to grow on the gypsum powder and reaches to 78.8% in these materials and components which supporting fungal growth.

HPLC results show that retention time or major peak of standard verrucarol toxin are located at 5.6 minutes from 4.5-6.5which identifies with toxin verrucarol produced from a filter extract of *S. chartarum* in the same area.

Keywords: *Stachybotryschartarum*, indoor Buildings,verrucarol toxinand . HPLC.

*Part of Ph.D Dissertation of the first auther.

Introduction

Different indoor locations such as furniture, carpet, wallpaper, bathrooms and potted plants may serve as amplification sites for the development of fungi (11 and 13). As good as, Fungal spores can be brought out into the indoor environments through natural open windows and doorways and mechanical ventilation systems (6 and 14).

Fungi such as *Stachybotrys* and *Wallemia* are usually found in the air samples from the living environments (14 and3).Many Fungi can be produced a large of secondary metabolites, some secondary metabolites pose potential health hazard to human, such toxic secondary metabolites are called mycotoxins(5).

The majority toxinsof *Stachybotrys* spp. are T-2 toxin, nivalenol, vomitoxin, verrucarol, trichoverrols, satratoxins, verrucarins and trichoverrins (8).

Materials and Methods

1- Indoor Fungi Samples Collection

This study has been done in different locations of AL- Najaf AL-Ashraf from Central City, AL-Kufa, AL-Qeswinia, AL-Nasar Quarter, AL-Rahma Quarter etc.. Samples were collected from different areas of buildings such asfamily houses, Schools building, Apartment building, stores etc... These samples were selected from different areas in each location that containing old places and more populated, like bathrooms, refrigerators, walls, carpets etc.

2-Standard toxin

Verrucarol toxin was obtained from the Dr. Halima Z. AL-Bahadli - in theplant protection-Faculty of Agriculture/ University of Baghdad.

3-Isolation and Diagnosis of fungi

Culture media were prepared and poured in petri dishes while the selective media considered as a traps media for fungus *Stachybotrys charatum* using sterilized filter papers, and wheat straw medium (W.St).In addition, gypsum medium was putting it in a place which covered by this

study. Dishes and traps were distributed in selected places that have been chosen as a four repeated sample for each location.

Later on, Petri dishes were opened in each place for 5 minutes, while the traps like gypsum were placed in a container containing water to keep it wet and left for one month. As well as these petri dishes were opened in refrigerators for 5 minutes, in the same time colonies of fungi were scraped from inside refrigerators using sharp tools. Followed these petri dishes were incubated at 25±2°Cfor a period of 3 days and then examined it. The media were used in this research are:-

A-Wheat Straw Medium (W.St)

This medium was prepared from 40 g of grinded wheat straw by electrical grinder, then soaked in distilled water with a volume of 500 ml for 24 hours. Followed by taking the mixture and boiling it for 15-20 mins in flask,. After boiling this mixture was filtered in another flask by a clean piece of a gauze to obtain the extraction (1). Followed by completing the volume to 1 L of distelled water. Then 17 $g.L^{-1}$ of agar was added to the medium followed by sterilized by autoclave. Same medium was prepared broth without adding agar. This medium was used for improving the growth of*Stachybotrys charatum.*

B- Gypsum powder Medium (G.P)

This medium was prepared from 40 g of gypsum followed by preparation steps as above mention above in (A). This medium was used for improving the growth of*S. charatum.*

The following criteria were taken in consideration in the growth identification:

A-Morphological features of growth colony which including color, texture, margin, colony reverse and pigments were produced.

B-Microscopic examination, observing fungi shapes and conidia and mycelium using light microscope and scanning electron microscope (SEM) as shown inDehooge*t al.*(4).

Fungal isolation, frequencywas calculated using the formula below:-

Frequency (%) = Number of genus or species fungal isolates X 100
Totally of all fungal isolates

3-Testing the ability of the isolated*S.charatum*fungus to produce secondary metabolites toxin

Picture (1) Colony of *S. chartarum* fungus growing on the wheat straw medium (25±2)^{0}C after 10 days.

A- Extraction

1- Pure isolated of the *S.charatum*was grown on W.St and incubated at 25C° for 14 days, followed, taking one disk from each pure culture in the center of Petri dishes containing W.St.

2- By taking a pure culture to detect produce toxic secondary metabolites of four repeated of each one. Followed by cutting of each petri dish by sterile knife and mixing it with 20 ml methanol.

3- The mixture was shaken for 15 minutes by a shaker apparatus.

4- The mixturewas filtered through a Whatman No. 1 paper.

5- Filtrate ration was puts in separate funnel.

6-Methanol was dried by reflex condenser.

7-Filtrate ration concentrated to reach about 1ml.

B- Detection

Thin layer chromatography (TLC) technique was used to detect *S.charatum*which produced secondary metabolites (15) according to the following procedures:-

1-Making straight light line on the TLC plate by adjusting 1.5 cm from the base plate.

2-Put 15 µl of extract of each*S.charatum*which isolated on the plate with a distance of 2 cm between two spots of each other.

3-Leaving spot to dry and then placed in the tank containing mixture of chloroform:methanol 95:5 (volume/volume) and then observed until the arrival of solution at a distance of about 2cm from upper edge of the plate.

4-Exiting the TLC plates for air drying for 5 minutes.

5-Examining TLC plate under UV light with wavelength 360nm to detect the presence of secondary metabolites by corresponding Rf and color for each compound appear on the plate.

C- Separation of secondary metabolites

1-Put 1000 µl from eachisolated*S.charatum*extracts on the plate as streak form.

2- Leaving stains to dry and then place in a tank contains a mixture of chloroform: methanol 95:5 (volume /volume) and observed until the arrival of solution at a distance of about 2 cm from the upper edge of the plate.

3- Exiting the TLC plate for air drying for 5 minutes.

4- Examining the plate under UV light with wavelength 360 nm and each compound determined by sterilize needle. After that scrape off the silica gel by blade and putting it in sterilized tube. This process was repeated until obtained suitable quantities of silica gel content for each compound.

5- Adding 2 ml methanol each one gram silica gel and centrifuging at 3500 rpm for 10 min.

6-Getting the chloroform and vaporize, then the sample of each compound was kept in the freezer.

Results and Discussion

1- Isolation fungi from indoorbuildings

Two speciesof *Stachybotrys* are isolated which are *S. chartarum* and *S. echinata* . It is observed that *S. chartarum* was more frequency than other fungi on wheat straw medium and reached about 82.5%. This is related to *Stachybotrys* is growing on materials which rich in cellulose such as straw medium (12).

Stachybotrys was preferred to grow on the gypsum powder and reached to 78.8% in these materials and components which supporting fungal growth.

2-Diagnosis of *Stachybotrys chartarum*

Stachybotrys chartarum isappeared ablack colonies on wheat straw agar medium at the temperature of 25±2^{0}C after 10 days of growth (picture 1). This result closed to that observed byAndersen*et al*.(2).

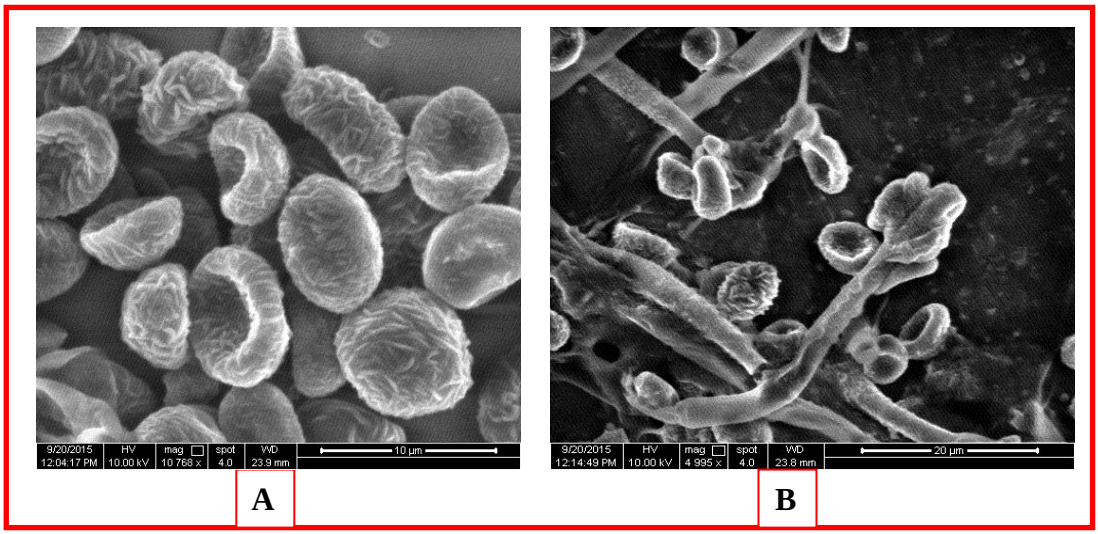
Picture (2) *S. chartarum* fungus growing on the wetchalks at (25±2)^{0}C after 30 days.

This result proved that *S. chartarum* is grown on wet chalks as a medium after one month in which it contains black spots, as shown in (picture 2) when it left chalks wet in containers. The gypsum was important factor to grow this fungus, especially in the walls in which *Stachybotrys* spp. started to grow even without inoculation by it. These results are closed to that obtainedbyMenetrez*et al*.(9).

Pictures (3) shows the SEM images of *S. chartarum* with different magnification, A: 10µm, B:50 µm demonstrated successful particle separation, conidia which clearly identified by the 5.0 µm pore size permitted capture most conidia. These result are closed to that obtained byNelson (10).

3-Separation and diagnosis of secondary metabolites

Picture (4) shows the result of chemical analysis of thin layer chromatography (TLC) in which the materials are extracted from the media cultivated of *S. chartarum* containing many toxins. TLC results producing verrucarol toxin which is compared with the standard toxin of verrucarol. It can be seen the same color and the same RF= 88%, which appeared in blue color spots (2).



Pictures (3) Scanning electron microscopy of *S. chartraum*.

A: Conidiospores 10µm; B: Conidiophores with conidia 20 µm.

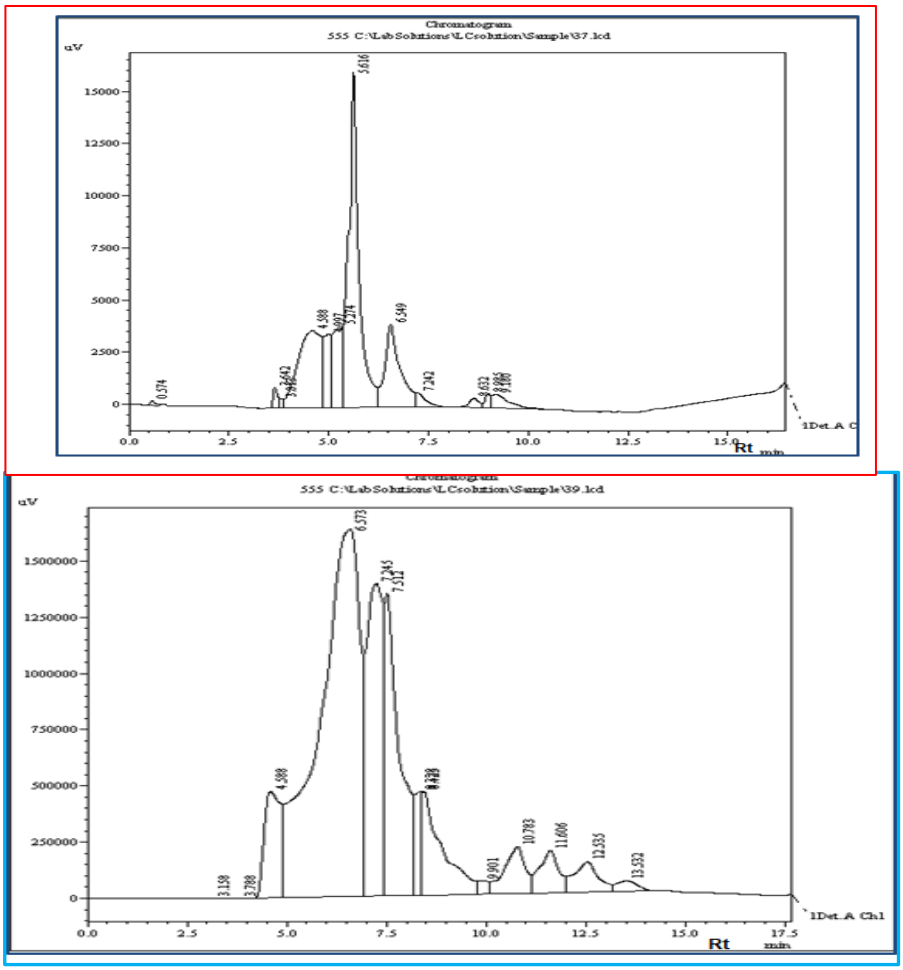


Figure (1) HPLC Chromatography analysis shown major peak of standard verrucarol toxin.

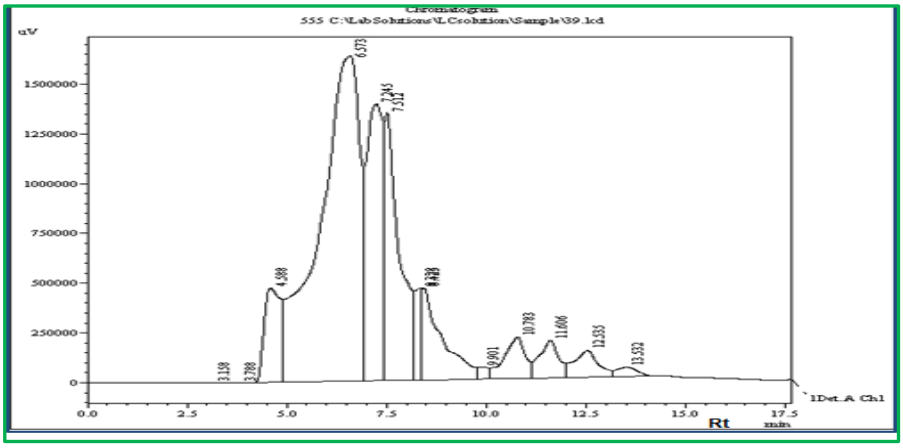
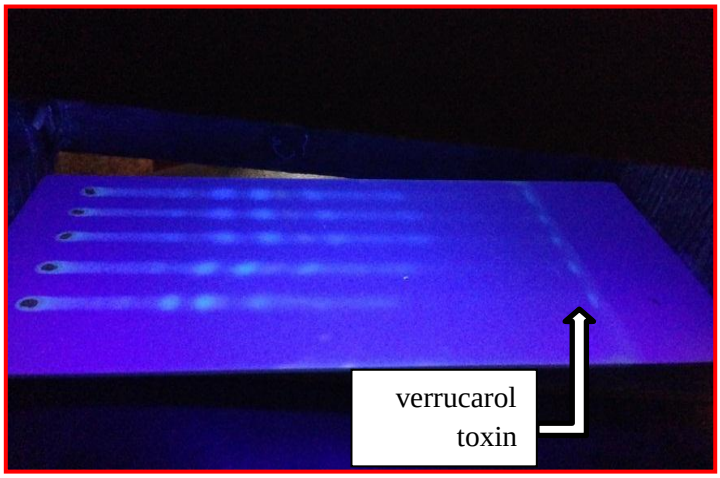


Figure (2) HPLC Chromatography analysis shown major peak of a filter extract of *S. chartarum* which produced verrucarol toxin.



Picture (4) Plates (TLC) shown the verrucarol toxin that had been separated from culture media of *S. chartarum*.

4-HPLC Study

S. chartarum is selected to produce macrocyclic trichothecenes by high performance liquid chromatography (HPLC) analysis. HPLC results showed that retention time or major peak of standard verrucarol toxin located at 5.6 minutes from 4.5-6.5 (figure 1), which identical with toxin verrucarol produced from a filter extract of *S. chartarum* in the same area(figure2).These results confirmed the presence of verrucarol toxin in the collected samples under study (7).

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عزل وتشخيص الانواع الفطرية التابعة لجنس *Stachybotrys* من المباني العامة في محافظة النجف وقابليتها على انتاج سم verrucarol

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المستخلص

تم عزل نوعين من *Stachybotrys* وهي *S.chartarum* و *S.echinata* لوحظ *S.chartarum* الاكثر ترددا من الفطريات الأخرى على وسط مخلفات الحنطة حيث وصل الى نحو 82.5٪.

Stachybotrys ينمو على المواد الغنية بالسليلوز مثل وسط تبن الحنطة، *Stachybotrys* يفضل النمو على باودر الجبس ووصل الى 78.8% في هذه المواد ،وهذه المكونات تدعم نمو الفطريات.

أظهرت نتائج HPLC ان زمن الاحتفاظ أو القمة الرئيسية في سم verrucarol القياسي وقع في زمن 5.6دقيقة من (4.5 -6.5) وهذا مماثل لسم verrucarol المنتج من المستخلص المرشح من الفطر *S. chartarum* والذي ظهر في نفس المنطقة.

الكلمات المفتاحية: جهاز HPLC، سموم toxin Verrucarol، المباني الخارجية، فطر *Stachybotry schartarum*

*جزء من اطروحة دكتوراه للباحث الاول.