Extraction and Characterization of Antibacterial Compound from *Aspergillus niger*

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Abstract

To investigate the antagonistic properties of the fungal isolates (*Aspergillus niger, Penicillium sp, Alternaria sp*) obtained from inflamed patients eyes against pathogenic bacteria(*Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus sp)* isolated from keratitic patient, agar well method was used, in which the antibacterial activity of fungal isolates was examined by testing the effect of their culture filtrates on growth of the bacterial pathogens. For this purpose (52) corneal scraping samples were taken from patients of microbial keratitis referred to Ibn Al-Haetham Eye Teaching Hospital in Baghdad. Results showed that 6 (21.42%) were positive for fungal occurrence after culturing on the related selective media, the fungal isolates were belonged to *A. niger* 3 (10.71%), *Penicillium* sp 2 (7.14%), *Alternaria* sp 1 (3.57%). Results revealed that *A.niger* possessed the highest inhibitory effect against *P.aeruginosa, S.aureus, S.epidermidis*, *Bacillus* sp with inhibition zones of (15, 25, 30, 32) mm in diameter, respectively, followed by *Penicillium* sp. Adversely, while no inhibitory effect was recorded by the filtrate of *Alternaria* sp used. Among on bacteria *Bacillus* sp was the most effective and sensitive isolate.The suspected antibacterial compound produced by *A.niger* was characterized and partially purified by using infrared (IR), UV absorbance and thin layer chromatography (TLC). Results show that the compound appeared as a single dark spot on the TLC plate under UV light that had R_f value of 0.45. From IR and UV analysis it was confirmed that the compound was aromatic- nitrogenous in nature. From such achievement, it can be said that the active antibacterial compound might be tensidol which is an alkaloid or a new derivative of alkaloid.

Keywords: Antibacterial substance, *Aspergillus niger*, antagonistic effect, secondary metabolite.

Introduction

Secondary metabolites are small molecules that are not directly involved in metabolism and growth of the organism. Both plants and fungi are known for producing a large number of chemically diverse secondary metabolites. While the role of some of these metabolites makes sense biologically as inferring an advantage to the producer, e.g. antibiotics, virulence factors, siderophores and pigments, the benefit of others is less obvious or unknown. The general belief is that the secondary metabolites must contribute to the survival of the producer in its environment where it competes with other organisms [1]. Where as the ability to produce individual secondary metabolites is a species specific. The actual production of secondary metabolites has, in broad terms, been reported to be affected by the developmental stage of the fungus (i.e. conidiation) and intrinsic and extrinsic factors of the environment as substrate, composition, pH, water activity, temperature, light and oxygen availability [2, 3].

The identification of microorganisms that produce bioactive compounds is of great interest in the development of new molecules to fight against many pathogens. Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substances, insecticides, compounds that promote or inhibit growth, attractor, repellent etc. Secondary metabolites produced from fungi vary in production, function and specifity to a particular fungus. These metabolites are being exploited in different fields of medicine and industry [4]. *Aspergillus niger* is a versatile filamentous fungus found in the environment all over the world in soil and on decaying plant material, and it has been reported to grow on a large number of foods and feeds [5].

Particularly *Aspergillus niger* is one of the best pharmaceutical friendly organism that produces a lot of industrially important

enzymes as well as some other products. Related to antimicrobial activity, it shows high potency in producing antimicrobial compounds such as tensyuic acid, nigerazine B, tensidol A, ochratoxin [6]. During growth of the organism in culture media, the strains are used to secrete some compounds (secondary metabolites) in environment which can be utilized as antimicrobial substances [7]. According to what have been mentioned above and because the very limited studies about extraction of antibacterial compound from fungi this study was aimed to extract and characterize the antibacterial compound produced from *A. niger.*

Materials and Methods

Isolation and Identification of fungal isolates:

Samples were taken from patients suffering microbial keratitis in Ibn Al-Hiatham Eye Teaching hospital in Baghdad for a period of nine months from November to July (2010). All samples were collected by medically qualified personnel, upon completion of the ocular examination and after instillation of topical anesthetic. A sterile syringe needle was used to scrape the area of infection, then the scrapings were inoculated onto sabouraud agar and incubated at 28 ˚C for 7 days. After incubation a positive cultures were further analyzed by transferring a loopfull of the fungal isolate onto a glass slide, then lactophenol–cotton blue stain was added before examined under 40× magnification power of the compound light microscope for hyphal and spore morphology.

Detecting antagonism of keratitic bacterial and fungal causatives. [7,8]

In order to examine the antagonistic effect of the fungal species isolated from inflamed eye against the pathogenic bacteria, the agar well method was used. The fungal isolates (*A.niger, Penicillium sp, Alternaria sp*) were cultured on a sabroaud agar for 7 days at 30˚C, then a 5mm block of mature growth on the agar plate was inoculated in 100 ml of sabrouad broth in a 500 ml Erlenmeyer flask, then incubated at 30ºC in a rotary shaker at (120 rpm) for 7 days. After incubation, the obtained mycelia were separated by centrifuging at 6000 rpm for 15 min. The supernatant was passed through a Millipore filter (0.22 µm) to get a spore free culture filtrate. An actively growing test bacterial isolate (1×10^8) was spread on the nutrient agar plate; Four wells were made in the plate, 2 cm away from the center. The culture filtrate (antibacterial compound) in a quantity of (100µl) was pipetted in each well, while one well was filled with only sabroaud broth medium as a control. The plates were incubated at 37 ˚C for (24-48) hrs. Antimicrobial activity was expressed as diameter (mm) of the inhibitory zone. For each test, two replicates were performed.

Isolation and purification of antibacterial compound [7]

A modified Jackson medium was inoculated with an agar block of actively growing culture of *A. niger* and incubated at 30˚C for 7 days. It was then centrifuged at 6000 rpm for 15 min, and the supernatant was passed through a Millipore filter (0.22 µm porosity) to get a spore free filtrate.

For extraction of antibacterial compounds, the filtrate was treated with an equal volume of ethyl acetate. Then, the mixture was shaken in a separating funnel. The organic layer was separated and collected. The solvent was removed in vaccum using a rotary vaccum evaporator. The residue after being collected and weighed, was purified by preparative TLC on a slica gel plate, which was carried out with crude extract that eluted by hexane and ethyl acetate solvent mixture in a ratio of 8:2. After that, the relative mobility of each spot was determined through the relative flow (R_f). While antibacterial activity of the extracted residue was determined by using agar well method.

Characterization of the partially purified antibacterial compounds:

In an attempt to characterize the partially purified antibacterial compound, the following tests were performed:

Determination of the R_f value:

The first type of the tests used to characterize the partially purified antibacterial compound is to determine the R_f value of this compound and the R_f value was measured at the same condition that was used in this study (using silica gel thin layer chromatographic plate (TLC) and using hexane- ethyl acetate mixture as a mobile phase).

Determination of UV absorbance:

In order to identify the antibacterial compound produced by *A. niger*, the partially purified antibacterial compound was subjected to UV absorption spectrum to detect the absorption bands in the sample.

FTIR analysis:

Analysis by FTIR was used to characterize the functional group present in the sample then from the results, the chemical structure of the sample can be proposed. This analysis was performed at the Department of chemistry, Al-Nahrain University.

Results and Discussion

Isolations and identifications of fungi causing keratitis:

After culturing the 52 speciems of keratitis patients attended Ibn Al-Haitham Eye Teaching Hospital in Baghdad on sabaroud agar for 7 days at 28°C, fungi were identified according to their morphology and spores. Results indicated that fungal infection represents (21.42%) of the total specimens.

Upon culturing on sabauroud agar, colonies of *Alternaria* sp (3.57%) were dark greenish surface with gray periphery, black on reverse side, while the microscopic features hyphae distinctly septate and yellow brown in color. Macroconidia are dark brown, multicelled, with septa both transverse and longitudinal, drumstick shaped, arranged in tandem long chains.

While the cultural characteristics for the colonies of *A. niger* which represent (10.71%), initially covered with a white, fluffy, aerial mycelium and as colony matures, their surface covered with black spores, the reverse side of the colony remains a light tan colour and the microscopic characteristics, hyphae are hyaline and distinctly septate. Conidiophores are long.

The fungal isolate that its colony was initially white and fluffy then turning shades of green. The microscopic feature hyphae are hyaline and septate. Conidiophores give rise to branching phialides forming a brush, conidia are borne in long chains from sterigmata, the tip of which are blunt and appear cut off at right angles, such characteristics come in accordance with those belonged to *Penicillium sp* (7.14%).

From the results mentioned it can be concluded that *A. niger* was the most prevalence fungus isolated from the mycotic keratitis patients. This was agreed with Al-shakarchi [9], found that out of 396 cases enrolled. Positive fungi growth was obtained in 74 cases (18.7%), the most common fungi isolated were *Aspergillus* spp. 42 (56.8%) followed by *Fusarium* spp. 20 (27%), *Pencillium* spp. 4 (5.4%), *Scopulariopsis* spp. 2 (2.7%), *Geotrichum* spp. 1 (1.4%), *Alternaria* spp. 1 (1.4%) and *Candida* spp. 4 (5.4%).

Al-shakarchi *et al*. [10] studied the management of fungal keratitis in Iraq with the eye drop amphotercin B 0.1%. they found that from 129 patients of suppurative keratitis, 22 patients had culture proven fungal keratitis (17%). in which the most common fungi isolated were *Aspergillus sp* (10 cases) followed by *Fusarium* sp (8 cases), *Scopulariopsis* spp (2 cases), *Pencillium* sp and *Candida* sp (one case for each of them).

Al-shakarchi *et al*., [11], they identified the prevelance of fungal keratitis in Iraq and identify types of fungi responsible for corneal ulceration. Out of 100 patients were included in the study. fungal growth were obtained from 16 cases and the most common fungus isolated was *Aspergillus* sp (9 cases) followed by *Fusarium* sp and *C. albicans* (three cases for each of them), *Scopulariopsis* sp was isolated from one case.

Sherwal and Verma, [12], found that fungal infection represented 32.50%. Among them *Aspergillus* species (56.42%) was the most common fungus isolated followed by *Curvurlaria* (17.95%), *Cladosporium* (7.70%), *Candida* species (5.13%), *Fusarium* (5.13%), *Alternaria* (5.13%), *Penicillium* (2.57%). They also mentioned that the fungal infection is a life threatening condition, which needs early diagnosis and treatment to save the patients' eye.

Chowdhary and Singh [13], found that from a group study of 485 cases, 191(39%) were diagnosed as mycotic (fungal) keratitis. Microscopic examination of KOH mounts and Gram-stained smears revealed presence of fungi elements in the corneal scrapings of (62.3%) and (60%) of the subsequently fungal culture-positive cases, respectively. Men

(68%) were more affected by fungal keratitis than women (32%). They found also that the *A. niger* was the most common fungus isolated, followed by *Curvularia* species in the culture-proven cases of fungal keratitis.

Antagonism effect of fungal isolate:

To examine the antagonistic properties of the fungal isolates (*A. niger, Penicillium sp, Alternaria sp*) obtained from the inflamed eye against pathogenic bacteria (*P.aeruginosa, S.aureus, S.epidermidis, Bacillus sp*), agar well method [14] was used. Antibacterial activity of the fungal isolates was examined by testing the effect of fungal culture filtrates on the growth of bacterial pathogens. A portion of 100µl of the filtrate was pippeted in each well made in the nutrient agar precultured with the pathogenic bacteria, then incubated at 37 ˚C for 24hrs before inhibition zone was measured.

Results display in Table (1) and Figs. (1, 2, 3, 4) showed that the filtrates of *A.niger* gave the highest inhibitory effect when compared with the filtrates of (*Penicillium* sp and *Alternaria* sp), *Bacillus*1 sp was determined as the most sensitive tested bacteria followed by *S. epidermidis1*, *S. aureus2*, *P. aeruginosa* 1 with (32, 30, 25, 15) mm inhibition zones, respectively.

Antibacterial activity of *Penicillium* sp was found to be less than that of *A.niger* when its inhibition zone was (18, 20, 19, 10) mm against (*Bacillus1 sp, S. epidermidis1, S. aureus2, P. aeruginosa*1), respectively. On the other hand, filtrate of *Alternaria* sp shows no inhibition activity when tested against (*S. epidermidis, S. aureus, P.aeruginosa* and *Bacillus sp).*

Table (1)
Inhibitory effect of fungal isolates against pathogenic bacteria after 24 hrs of incubations period.

Bacterial isolate	***Inhibition zone diameter (mm)****		
	A. niger	***Pencillium sp***	***Alternaria***
*P.aeruginosa*1	15	10	–
P.aeruginosa 2	10	7	–
*S.aureus*1	22	18	–
*S.aureus*2	25	19	–
*S.epidermidis*1	30	20	–
*S.epidermidis*2	25	15	–
*Bacillus sp*1	32	18	–
*Bacillus sp*2	29	15	–

****Diameter was calculated after subtracting the diameter of the well (5 mm).***

Natarajan *et al.*,[7] found that the compound isolated from broth extract of *A.niger* showed a high degree of antibacterial activity against *S. aureus, B. subtilis, E.coli, Salmonella typhi, P. aeurogenosa, and Proteus vulgaris* with inhibition zones (16, 19, 13, 14, 14, 11) mm, respectively.

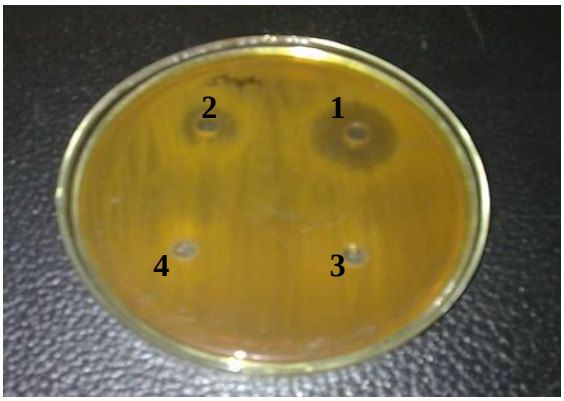


Fig. (1) Antibacterial activity of fungal isolates against Staphylococcus aureus grown on BHI agar incubated at 37˚C for 24hrs. 1: Filtrate of Aspergillus niger 2: Filtrate of Penicillium sp 3: Filtrate of Alternaria sp 4: Control.

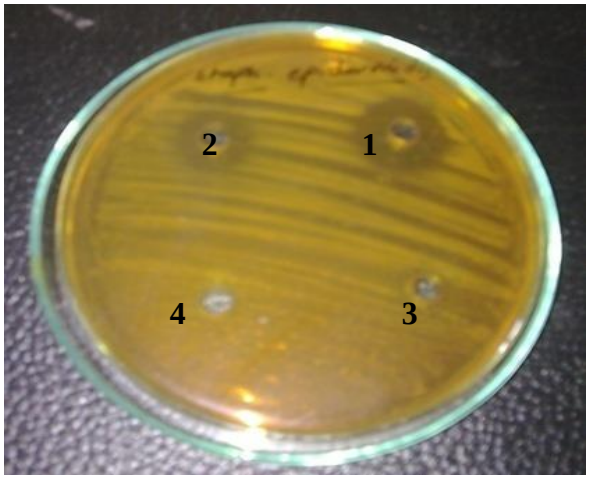


Fig. (2) Antibacterial activity of fungal isolates filtrate against Staphylococcus epidermidis isolated from inflamed eye grown on BHI agar incubated at 37°C for 24hrs.
1: Filtrate of Aspergillus niger.
2: Filtrate of Penicillium sp.
3: Filtrate of Alternaria sp.
4: Control.

Fig. (3) Antibacterial activity of the fungal isolates against Bacillus sp isolated from inflamed eye grown on nutrient agar incubated at 37°C for 24hrs.
1: Filtrate of Aspergillus niger.
2: Filtrate of Penicillium sp.
3: Filtrate of Alternaria sp.
4: Control.

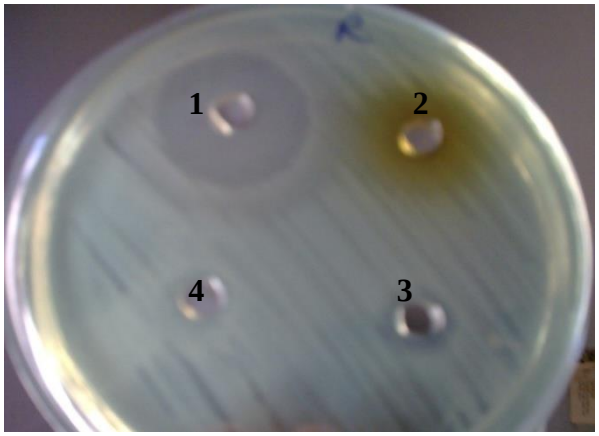


Fig. (4) Antibacterial activity of fungal isolates on P.aeruginosa isolated from inflamed eye grown on nutrient agar incubated at 37°C for 24hrs.
1: Filtrate of Aspergillus niger.
2: Filtrate of Penicillium sp.
3: Filtrate of Alternaria sp.
4: Control.

Fawzy *et* al. [15] studied the antimicrobial activity of extracellular and intracellular extracts of *A.niger* against 10 different bacterial isolates comprising of both Gram negative and Gram positive organisms. They found that the extracellular extracts of fungi were found to posses activity against the Gram-negative bacteria only, while the intracellular extracts didn't do so.

In the study of Fukuda *et al*., [8] an alkaloid (tensidol A and B) was isolated from fungi, found to have potential miconazole activity against *C. albicans*.

Characterization of the antibacterial compound:

To characterize the partially purified antibacterial compounds, the results of the three tests used can be disscussed as follows:-

a- Thin layer chromatography (TLC) method:

When the partially purified antibacterial compound was spotted on silica-gel TLC plate and examines under UV transilluminator for characterization and purification, the result showed that the active antibacterial compound had a single dark fluorescent spot under UV light and had an R_F value of (0.45).

b- Ultraviolet (UV) absorption:

The UV absorption spectrum for the partially purified antibacterial compound

shows two peak at 225nm and 275nm (Fig.(5)). These bands may be attributed to $\pi - \pi *$ transition n- π transition, respectively.

Such result is close to that reported by Natarajan *et al.,* [7] who mentioned that the UV spectrum for the component isolated from *A. niger* predicted the ɹ max at 280.

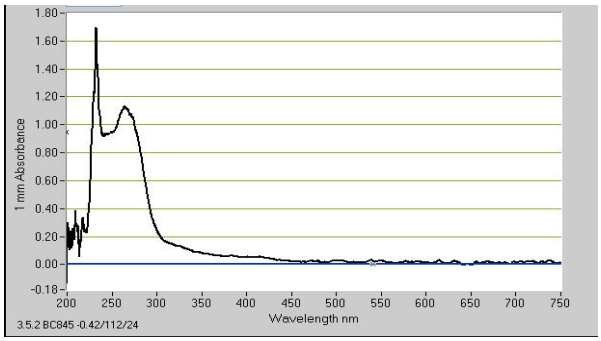


Fig. (5) Ultraviolet spectrum of the partially purified antibacterial compound from Aspergillus niger.

c- IR analysis:

The partially purified antibacterial compound isolated from *A.niger* was subjected to IR analysis, to detect the functional chemical group which may lead us to propose a possible chemical structure for the compound. According to Socrates [16], the suspected organic compound was characterized by the IR spectrum. As shown in Fig.(6) the IR results of the partially purified compound (the main absorption bands) shows the presence of different bands corresponding to the following functional group present in the molecular structure: stretching band at 3367.5 cm^1 for N-H, stretching band at 2925.8 cm^1 for C-H, 1647.1 cm^1 for C=C, 1232.4 cm^1 for C-N (cyclic β lactams), 1550 for N-H bending, 1379 for O-H bending, 1740 for C=O, 1460 for C-H bending. Such characteristics are close to those IR analysis of (tensidol) extracted from the broth extract of *A.niger* and found to have antibacterial activity by Natarajan *et al*., [7].

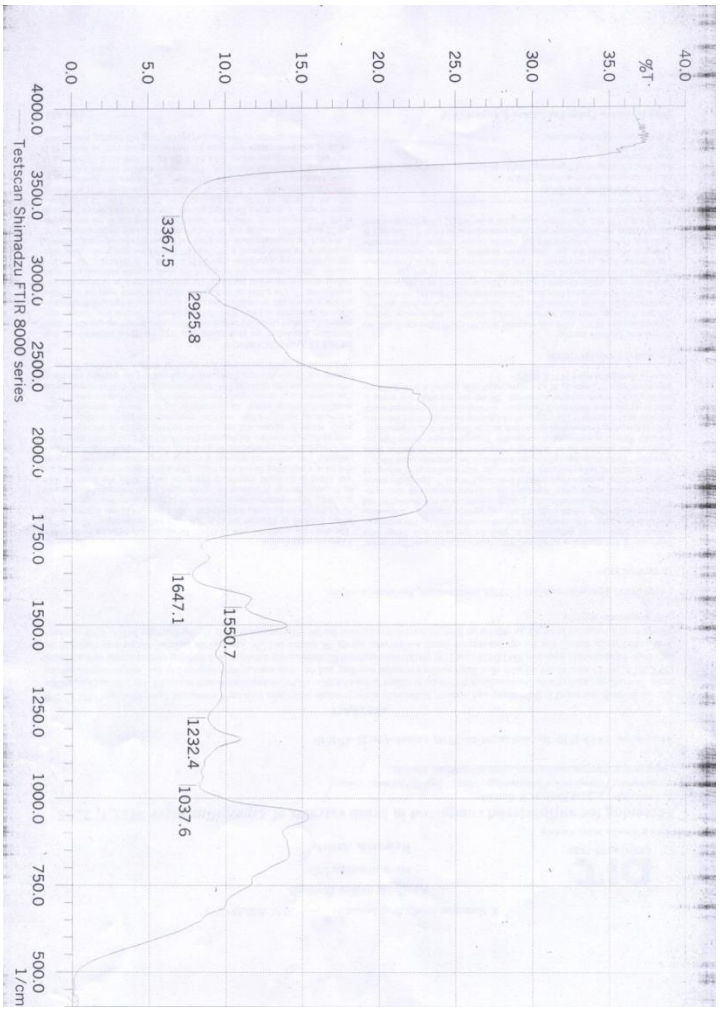
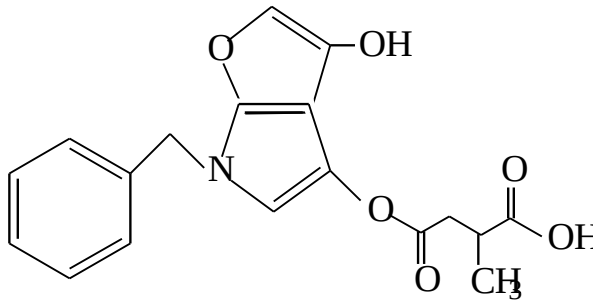


Fig.(6) Infrared spectrum of the partially purified antibacterial compound from A. niger.

The chemical structure for the partially purified antibacterial compound can be proposed from the IR results as follows:



Conclusion

The fungal species *A. niger* has the ability to produce antibacterial compound which it might be tensidol or a new derivatives of alkaloid.

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الخلاصة

استخدمت طريقة الحفر (Agar well method) للتحري عن الصفات المستضدة بين عزلات الفطريات (*Aspergillus niger, Penicillium sp, Alternaria*) التي تم الحصول عليها من عينات التهابات العيون وعزلات البكتريا المرضية المأخوذة من مرضى التهابات القرنية المراجعين لمستشفى ابن الهيثم التعليمي للعيون في بغداد. ولهذا الغرض فقد تم جمع (52) عينة كشطات اخذت من مرضى التهاب قرنية العين المراجعين لمستشفى ابن الهيثم التعليمي للعيون في بغداد. اشارت النتائج الى ان 6 (21.42%) منها موجبة لتواجد الفطريات بعد زرعها على الاوساط الزرعية الاختيارية حيث كانت ممثلة بالانواع والنسب المئوية الاتية: *Aspergillus niger* (10.71%), *Penicillium* sp (7.14%), *Alternaria* sp (3.57%). وتم التحري عن الفعالية المضادة للبكتريا المرضية والتي اظهرتها رواشح المزارع الفطرية واوضحت النتائج الى ان عفن الـ *A.niger* امتلك اعلى فعالية مضادة للبكتريا *P.aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis*, Bacillus sp عندما بلغت اقطار مناطق منع النمو (15, 25, 30, 32) ملم على التوالي. تبعه في ذلك عفن ال *Penicillium*. و على العكس من ذلك، فلم تشمل اي فعالية تثبيطية لرواشح فطر الـ *Alternaria* المستخدم. وكانت عزلة بكتريا *Bacillus* هي الاكثر تاثرا وحساسية من بين العزلات البكتيرية التي تم الحصول عليها. تم توصيف وتنقية الجزيئية للمركب المنتج من قبل عفن الـ *A.niger* والذي تعود له الفعالية التثبيطية للبكتريا المرضية وذلك باستخدام الـ Infrared (IR), Ultraviolet (UV), thin layer chromatography (TLC) . اشارت النتائج الى ظهور هذا المركب كبقعة مظلمه واحدة على صفيحة الـ TLC تحت الاشعة الفوق البنفسجية وامتلاكه لقيمة Rf 0.45 وامكن التاكد بكون هذا المركب ذات صفة طيارة ونايتروجينية في طبيعته و ذلك بعد اجراء اختبارات الاشعة الفوق الحمراء (IR) والفوق البنفسجية (UV). من خلال ما تحقق يمكن القول بان المركب المضاد البكتيري الفعال بانه (Tensidol) احد المركبات الالكلويدية او احد مشتقاتها.