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Antimicrobial activity of the fungus *Talaromyces funiculosus*

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Abstract

In this paper, we concentrated on the study of the ability of fungi to produce antimicrobials and the effect of some environmental parameters on it. For that reason, the fungus *Talaromyces funiculosus* was isolated from the soil and screened for its antimicrobials production against four pathogenic bacteria (*Bacillus subtilis, Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) by disk diffusion method with studying the effect of (pH, temperature and carbon source) on its growth as well as the production of antimicrobials. The results showed that the secondary metabolites of the fungus *Talaromyces funiculosus* have potent activity against gram-negative and positive bacteria. The results of the first week showed inhibition for all tested bacteria; the fungal extract after one week of incubation period will able to inhibit all tested bacteria; the largest inhibition zone was 15mm found against *P. aeruginosa* and 13mm against *B. subtilis* . In contrast, the fungal extract extracted after 2 and 3 weeks of incubation showed inhibition activity against only gram-positive bacteria. The largest inhibition zone of *B. subtilis* was found at 20mm in the second week, and the largest inhibition zone of *S. aureus* was found at 21mm in the third week. The results showed that the temperature 30 °C, the pH 6, and the carbon source maltose were the best for producing antimicrobials.

Keywords: *Talaromyces funiculosus*, Secondary metabolites, antimicrobial activity.

Introduction

Microorganisms play an important role in developing the chemistry of natural products and medical therapy. Fungi, one of the largest microorganisms, play a vital role in ecosystems and as one of the most important tools in biotechnology (Al-Daamy *et al*., 2018). The production of secondary metabolites such as antimicrobial agents consider one of the most important uses of fungi; these metabolites can be beneficial for the discovery of new compounds for drug discovery, and they have been considered to be a rich source of unique bioactive compounds since the discovery of penicillin in 1928 which was isolated from the fungus *Penicillium notatum* and introduced as the first antibiotic by Fleming (Abdel-Razek *et al*., 2020). Antimicrobials are substances that can inhibit the growth of microorganisms or kill them; they are widely used for treating bacterial infections in humans and animals (Serwecińska, 2020).

Antimicrobial resistance (AMR) is a natural mechanism used by microorganisms to protect themselves against the effect of antimicrobials. As a result of this adaptation, it becomes

stronger over time, which increases resistance and reduces the number of effective antimicrobials (Balabanova, 2020). AMR is a growing health problem worldwide due to the low rate of new antimicrobial discoveries compared to the rapid spread of antimicrobial-resistant pathogens. As a result of the constantly increasing resistance of microorganisms to antimicrobials, there has become an urgent need to discover or develop new types of antimicrobials that reduce the spread of diseases among humans. As new generations of antimicrobials allow shorter treatment and optimal effects on microbes, soil fungi have been recognized as a useful source of bioactive secondary metabolites (Muller *et al*., 2007; O'Rourke *et al*., 2020).

The current study aimed to isolate fungi from soil, having a great ability to produce antimicrobial bioactive compounds and screen their activity against pathogenic bacteria.

Materials and methods

Isolation of fungi

Six soil samples were collected from various areas in Basrah province. Fungi were isolated from soil samples by using the dilution method (Wicklow and Wittingham,1974), using two different types of media Potato carrot agar (PCA) and Malt extract agar (MEA). The preparation of the isolation media was done according to the direction of the manufacturing company (Hi media). Culture media were incubated at 25 ºc for one to two weeks.

Molecular diagnosis

Pure cultures from the isolated fungi were subcultured on PDA medium and allowed to grow for 5 days. DNA was extracted using Geneaid Mini g DNA Yeast kit for fungi and yeasts, according to the protocol attached with the Kit. The isolates were identified by amplifying the internal transcribed spacer (ITS) regions of the ribosomal DNA with a universal primer, a forward primer, ITS1, and a reverse primer, ITS4 (table1) (Friggens *et al*., 2017; Al-Dossary *et al*., 2020). The PCR product purification was carried out to recover isolates at Macrogen (Seoul, South Korea). The products were aligned and identified using the basic local alignment search tool (BLAST) program.

Table 1. Primer pairs sequences

Primer	Primer Sequences (5´-3´)	Length
ITS1	F-5-TCC GTA GGT GAA CCT GCG G-3	19 base
ITS4	R-5-TCC TCC GCT TAT TGA TAT GC-3	20 base

Tested bacteria

Two gram-positive bacteria: *Staphylococcus aureus* and *Bacillus subtilis*, and two gram-negative bacteria: *Pseudomonas aeruginosa* and *Escherichia coli* were maintained at Nutrient agar slants and stored at 4°C until use to test the effect of the of the fungal antimicrobial metabolites on it.

Preliminary Screening of isolated fungi for their antimicrobial activity

The fungal isolates' antagonistic potential was performed using a dual culture technique (Mishra *et al*., 2017) against two pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*). Five mm diameter mycelial disc was taken from the edge of each isolated

fungus, inoculated on one side of a Petri plate containing PDA, and incubated at 25°C for five days to grow. The nutrient broth was seeded with test bacteria at 37 °C for 24 h to prepare the bacterial suspension. Dilution was made for each bacterial type, then the opposite direction of the plate was wiped with diluted bacteria using a swab and left in the incubator for 24 hours at 37°C.

Preparation of the crude extracts

The fungal isolates which exhibited the highest antimicrobial activity in the preliminary screening study were further subjected to liquid surface fermentation and ethyl acetate extraction following the methodology described by Marcellano *et al*. (2017). one piece from the fungal colony (five mm in diameter) was inoculated into 100 mL Potato dextrose broth (PDB) , then they were incubated at 25°C for two weeks under stationary conditions in a rotary shaking incubator at 105 rpm. After two weeks, the cultures were filtered through sterile filter paper (whatman No.1); the antimicrobials were extracted from the liquid media three times using ethyl acetate solvent and left in the room to dry.

Screening for Antimicrobial Activity

The fungal extract was tested for its antimicrobial activity against four pathogenic bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Escherichia coli*(using the disk diffusion method). According to Rančić *et al*. (2006), Bacterial suspensions were prepared from 24 h old bacterial cultures. The bacteria were inoculated on the dried surface of a Mueller-Hinton agar medium (the dried crude extracts were dissolved by 0.1% dimethyl sulfoxide (DMSO). Sterile discs were placed in the extract to absorb the dissolved antimicrobial metabolites. Then it was transferred, placed on the petri dish, and wiped with the tested bacteria. DMSO and Rifampicin (5 mcg/disc) were used as negative and positive controls, respectively. All treatments were incubated at 37°C for 24 hours, and the resulting inhibition zones were measured.

Effect of temperature on bioactive metabolites production

Optimizing temperature for the production of antimicrobial metabolites was carried out at three different incubation temperatures 25, 30, and 35 °C. 100 ml of PDB was taken in 250 ml flasks; these flasks were autoclaved at 121°C for 15 minutes. Three replicates were used for each chosen temperature. One disk 5 mm diameter, was cut from four day old colony of the fungus and added to each flask. The inoculated flasks were incubated at 25°C for 14 days.

Effect of carbon sources on bioactive metabolites production

Optimizing the carbon source for antimicrobial metabolites production was carried out at two different incubation carbon sources, sucrose and maltose. 100 ml of PDB was taken in 250 ml flasks. These flasks were autoclaved at 121°C for 15 minutes, and three replicates were used for each chosen carbon. One disk of 5 mm diameter was cut from four day old colony of the fungus and add to each flask. The inoculated flasks were incubated at 25°C for 14 days.

Effect of pH on bioactive metabolites production

The pH of the fermentation broth was optimized for antimicrobial metabolites production by carrying out the fermentation at different pH values 5, 6 and 6.5 For each pH value. 100 ml of PDB was taken in 250 ml flasks. These flasks were autoclaved at 121°C for 15 minutes. Three replicates were used for each pH value. One disk of 5 mm diameter was cut from four days old colony of the fungus

and added to each flask. The inoculated flasks were incubated at 25°C for 14 days.

Production of antimicrobials under optimal conditions with different incubation periods

According to the previous experiments, the ability of the tested fungus to produce antimicrobials was tested under optimal conditions for its growth.

Statistical analysis

Minitab version 16 software was used to analyze the results using a one-way analysis of variance. Relative least significant difference values were calculated to determine significant differences between the fungal processes. A completely randomized design was used.

Results and discussion

Molecular identification

The analysis of the results of the nitrogen base sequences of the studied isolates through the Genebank database with translation elongation factor showed that the isolated fungi with 72% belonged to anamorphic fungi with eighteen species (Table 2). Followed by 16% ascomycetes fungi with four species, followed by the zygomycota, 8% with two species, and finally, the Basidiomycota, 4% with only one species. The appearance of the anamorphic fungi in high percentage may be due to the ability of these fungi to produce a large number of reproductive units and the secretion of different enzymes also, and they possess a great ability to tolerate the stress in the environment, all of these features and others made them one of the large groups of fungi in the environment. (Abdullah *et al*., 2000; Serna-Chavez *et al.*,2013; Al-Saadoon *et al*., 2014).

Table 2: The isolated fungi with their percentage of occurrence

No.	Fungal species	% of occurrence
1	*Alternaria alternata*	16.6
2	*A.chevalieri*	33.3
3	*A .fluvus*	66.6
4	*A. iranicus*	66.6
5	*A .niger*	100
6	*A. niveus*	83.3
7	*A . terreus*	50
8	*Aspergillus* sp.	66.6
9	*Chaetomium seminis-citrulli*	16.6
10	*Dimorphiseta acuta*	16.6
11	*Emericella* sp.	16.6
12	*Exserohilum* sp.	16.6
13	*Megasporoporia* sp.	16.6
14	*Microsphaeropsis arundinis*	50
15	*Mucor* sp.	33.3
16	*Paraconiothyrium estuarinum*	16.6
17	*Penicillium* sp. 1	100
18	*Penicillium* sp. 2	50
19	*Phoma* sp.	33.3
20	*Roussoella* sp.	16.6

21	*Ryzopus* sp.	16.6
22	*Talaromyces funiculosus*	33.3
23	*Trichocladium antarcticum*	33.3
24	*T. gilmaniellae*	33.3
25	*Trichoderma harzianum*	33.3

Preliminary Screening of isolated fungi for antimicrobial activity

The preliminary Screening of fungi for their bioactivity against two bacterial species *E. coli* and *S. aureus* revealed that all isolated fungi inhibited at least one pathogenic bacterium in the dual culture technique and created inhibition zones ranging from 4 to 20 mm (Table 3). *E. coli* was inhibited by most fungi. The highest antimicrobial activity was shown by *Aspergillus chevalieri,* which inhibited *S. aureus* by 20 mm (Table 3, Fig.1).

Antimicrobials are natural organic substances produced by microorganisms, including fungi. They are known to inhibit or kill Gram-positive and Gram-negative bacteria, so the survival of bacteria depends on their ability to adapt to the environment quickly. Some bacteria are naturally resistant to antimicrobials, as in the Gram-negative bacterium *Pseudomonas aeruginosa,* due to the properties of its cell wall; other bacteria show resistance to antimicrobials by changing their genes (Serwecińska,2020). This result is consistent with Muhsin and Mohammad (2012).

Table3: Preliminary Screening of isolated fungi against *Staphylococcus aureus* and *Escherichia coli*

No.	Fungal species	Inhibition zone(mm)	
		Staphylococcus aureus	*Escherichia coli*
1	*A.chevalieri*	20	0
2	*A. iranicus*	7	9
3	*A .niger*	0	4
4	*A. niveus*	4	4
5	*Aspergillus* sp.	0	4
6	*Chaetomium seminis-citrulli*	8	7
7	*Penicillium* sp. 1	7	6
8	*Penicillium* sp. 2	0	6
9	*Phoma* sp.	4	0
10	*Roussoella* sp.	0	16
11	*Talaromyces funiculosus*	0	12
12	*Trichocladium antarcticum*	14	0

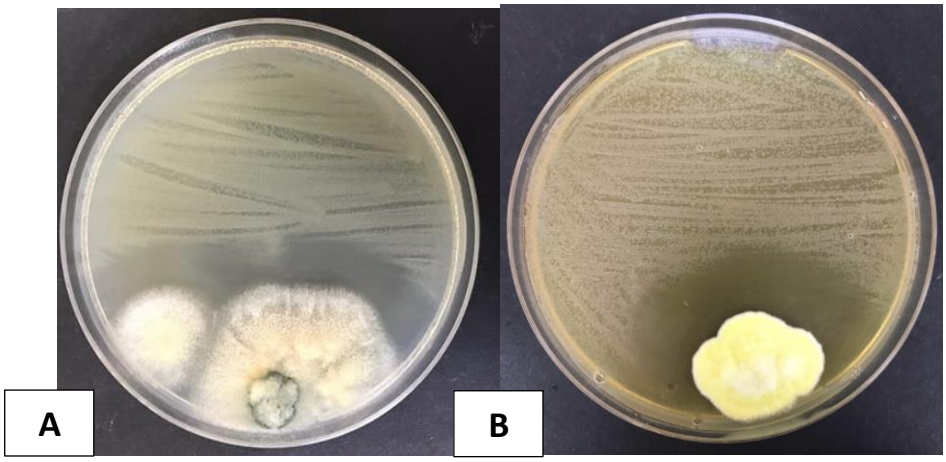

Fig.1: Preliminary test of A: *Penicillium* sp. 1 B: *Talaromyces funiculosus* against *Escherichia coli*

Evaluated the antibacterial activity of the fungal extracts

The extracts of the selected fungal isolates gave positive results in inhibiting the tested positive and negative bacteria. Still, in varying degrees for each extract between fungal species, the inhibition zone ranged between 6-16 mm on Gram negative bacteria, the fungus *Talaromyces funiculosus* showed the highest inhibition zone on E. *coli* by 16 mm, while the inhibition zone of positive bacteria ranged between 6-15 with the highest inhibition by *Penicillium* sp.1 extract on *Bacillus subtilis* 15mm (table 4).

In general, the liquid media enhanced the fungal growth and production of antimicrobial secondary metabolites as a bioactive compound for all fungi. The reason for the increase in the growth of the fungi may be due to the contact of the fungal mycelium with the nutrients in the medium, which led to their consumption and an increase in the activity and growth of the fungi compared to the initial test in the solid medium, and this is evident in the current results, as the extract of fungi inhibited all tested gram-positive and gram-negative bacteria (Marcellano *et al*., 2017).

Table 4: Primary antimicrobial activity of the fungal extract of selected fungi

Fungi	Inhibition zones (mm)			
	Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
Aspergillus iranicus	9	12	12	14
A .chevalieri	13	11	8	13
Chaetomium seminis-citrulli	9	9	14	13
Penicillium sp. 1	9	6	15	7
Roussoella sp.	7	11	8	9

Talaromyces funiculosus	9	16	13	10
Trichocladium antarcticum	10	10	6	10
Rifampicin	19	18	22	25
DMSO	0	0	0	0

Factors affecting the production of antimicrobial agents by *Talaromyces funiculosus*

Effect of temperature

The ability of *Talaromyces funiculosus* to produce antimicrobials was tested at Three temperatures 25,30 and 35°c. The inhibition zone ranged between 8-22 mm, and the highest inhibition was recorded at 30°c against Gram-positive bacteria *Bacillus subtilis* 22 mm and for Gram negative bacteria 11 mm against *Escherichia coli* and *pseudomonas aeruginosa* (table 5, fig.2).

Temperature directly affects the growth and overall metabolism of the microorganism through its effect on enzymes and protein production, and thus on the production of antimicrobials, and each microorganism has an optimal temperature for its growth at which it gives the best growth and the best productivity for all primary and secondary metabolic products (Pereira *et al*., 2013). The current study agrees with Pereira *et al*. (2013) that the temperature 30 ° C was the best for the fungus *Hypholoma fasciculare* to produce antimicrobial. In contrast, this study disagrees with Jafar *et al*., (2016) that the temperature 25 ° C was the best for the fungus *Trichoderma harzianum* to produce antimicrobial.

Table 5: The effect of different temperatures on antimicrobial produced by *Talaromyces funiculosus*

Fungus	Temperatures	Inhibition zones (mm)			
		Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
Talaromyces funiculosus	25	9	16	13	10
	30	11	11	22	16
	35	8	8	19	16
Rifampicin	Control +	19	18	22	25
DMSO	Control -	0	0	0	0

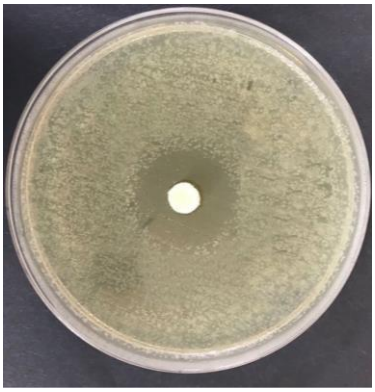

Fig.2: The effect of the fungal extract from the fungus *Talaromyces funiculosus* at 30 ° C on *Bacillus subtilis*

Effect of pH

Talaromyces funiculosus to grow and produce antimicrobials was tested at three degrees of pH, which are 5, 6 and 6.5 to find out the best pH in both growth and production of antimicrobials during the incubation period of 14 days. At pH 6 the antimicrobials inhibited all gram-negative and gram-positive bacteria, the highest inhibition of gram-negative bacteria was on *Escherichia coli* with an inhibition of 16 mm and the highest inhibition of gram-positive bacteria was on *Bacillus subtilis* with an inhibition of 13 mm, Whereas, at pH 5 and 6.5 the antimicrobials were inhibiting positive bacteria only. The values of inhibition of the fungus at different pH ranged from 8 to 13, and the highest inhibition was on *Bacillus subtilis* with an inhibition of 13 mm (table 6).

The pH is one of the important and determinant factors for the metabolism and production of enzymes and, consequently, the biosynthesis of antimicrobials. This was evident in the results, as the suitable pH stimulated the fungus to produce the antimicrobial (Jain and Pundir, 2011). This result is consistent with Merlin *et al*. (2013) who found that the best pH value for growth and production of antimicrobial for the fungus *Fusarium solani* was at pH 6.

Table 6: The effect of different pH on antimicrobial produced by *Talaromyces funiculosus*

Fungus	pH value	Inhibition zones (mm)			
		Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
Talaromyces funiculosus	5	0	0	13	11
	6	9	16	13	10
	6.5	0	0	11	8
Rifampicin	Control +	19	18	22	25
DMSO	Control -	0	0	0	0

Effect of carbon sources

Two carbon sources, sucrose, and maltose, were taken to optimize the carbon source for maximum antimicrobial production by the fungus *Talaromyces funiculosus*, the inhibition zones ranged between 7-15mm, and the largest inhibition zone was found in the presence of maltose by 15mm against positive bacteria *B. subtilis* and 11mm against negative bacteria *P. aeruginosa* (table 7).

The carbon source is one of the most important factors that nourish the fungal growth, so adding it to the media promotes growth, as the microorganism uses it first in the production of cells, enzymes, and basic primary metabolites, and then the rest of it is consumed in the production of secondary materials, including antimicrobials (Mc Quilken *et al*., 2002; Ruiz *et al*., 2010). This result is consistent with Jain & Gupta (2012), who studied the effect of different types of carbon sources on the growth and their effect on the production of antibiotics; maltose was the best for the growth and production of antibacterial by the fungus *Penicillium* sp.

Table 7: The effect of different carbon source on antimicrobial produced by *Talaromyces funiculosus*

Fungus	Carbon source	Inhibition zones (mm)			
		Pseudomonas aeruginosa	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
Talaromyces funiculosus	Maltose	11	7	15	10
	Sucrose	10	8	14	11
Rifampicin	Control +	19	18	22	25
DMSO	Control -	0	0	0	0

Production of antimicrobials under optimal conditions and different incubation periods

The ability of the fungus to produce the antimicrobials was tested when applying the optimum conditions selected from the previous experiments, and the effect of these conditions were measured at three periods 7, 14, and 21 days, as the results of the first week showed inhibition for all tested bacteria. The largest inhibition zone was found at 15mm against *P. aeruginosa* and 13mm against *B. subtilis* (table 8, Fig3).

The results of the 14 days test showed that the fungus extract was inhibiting only the positive bacteria and the highest inhibition was 20mm against *B. subtilis* (Table 8). At 21 days the fungal extract was also inhibited only positive bacteria, and the largest inhibition zone was on *S. aureus* with an inhibition zone 21 mm (Table 8).

The consumption of nutrients, especially the carbon source, led to facilitating and accelerating the growth of the fungus, reaching the stage of stability quickly, and producing antibiotics in a shorter period of 7 days, which were effective on gram-positive and gram-negative bacteria. Still, with the increase in their growth after 14 and 21 days the effect on negative bacteria disappeared, and the effect on the positive bacteria increased, and this is attributed to the cessation of production of some compounds that appeared at the first week and disappeared in the second and third week. And it could be an indication that the antagonist has

become more selective for the organisms it affects. It is possible that the crude extracts give more effective compounds once they are subjected to further purification, and this agreed with the researcher Bills *et al*. (2008); Takahashi *et al*. (2008) and Pelo *et al*. (2020).

Table 8: The effect of optimum conditions on antimicrobial produced by *Talaromyces funiculosus*

Fungus	Days	Inhibition zones (mm)			
Talaromyces funiculosus		*Pseudomonas aeruginosa*	*Escherichia coli*	*Bacillus subtilis*	*Staphylococcus aureus*
	7	15	10	13	10
	14	0	0	20	18
	21	0	0	20	21
Rifampicin	Control +	19	18	22	25
DMSO	Control -	0	0	0	0

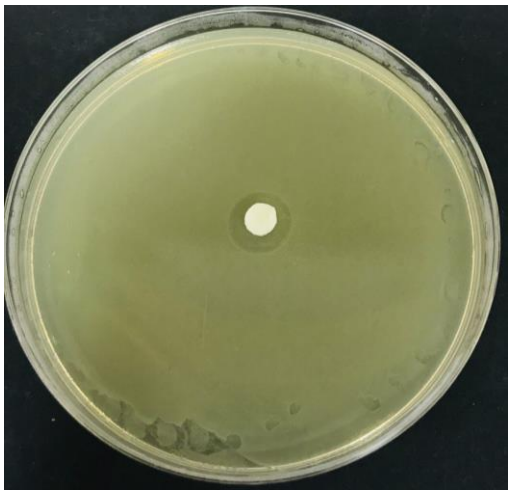

Fig3.: Effect of the fungal extract on *Escherichia coli*

Conclusion

Fungi are a remarkable source of antimicrobials production; in this study, the fungus *Talaromyces funiculosus* was able to produce antimicrobial agents that affect pathogenic gram-positive and negative bacteria. Also environmental factors like temperature, pH, and carbon source play an important role in the activation of the fungus to produce antimicrobial agents. Here in this study, it appear that the temperature of 30°c , pH 6 and the carbon source maltose were the best for the fungal growth and the production of the antimicrobial agents.

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النشاط الضد ميكروبي للفطر ***Talaromyces funiculosus***

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المستخلص

في هذا البحث تم التركيز على دراسة قابلية الفطريات على انتاج المواد الضد ميكروبية ودراسة تأثير بعض العوامل البيئية على هذه العملية. ولهذا الغرض عزل الفطر *Talaromyces funiculosus* من التربة ودرست قابليته على انتاج المضادات الميكروبية وتأثيرها على اربعة انواع من البكتريا الممرضة وهي , *Pseudomonas aeruginosa* *Escherichia coli*, *Bacillus subtilis* , *Staphylococcus aureus* باستخدام طريقة الانتشار بالأقراص ، اضافة الى دراسة تأثير بعض العوامل البيئية (الاس الهيدروجيني ودرجة الحرارة والمصدر الكاربوني) على النمو وانتاج المضادات الميكروبية . اظهرت النتائج ان منتجات الايض الثانوية للفطر *T. funiculosus* لها تأثير تثبيطي على البكتريا السالبة والموجبة لصبغة كرام ، اذ بينت نتائج الاسبوع الاول لمستخلص الفطر قابليته على تثبيط كل البكتريا المختبرة الموجبة والسالبة ، اذ كان اعلى تثبيط للبكتريا السالبة بمقدار 15 ملم على *P. aeruginosa* واعلى ثبيط على البكتريا الموجبة بمقدار 13 ملم على بكتريا *B. subtilis*. بينما أظهر المستخلص الفطري الذي تم استخلاصه بعد 2 و3 أسابيع من فترة الحضن نشاط تثبيطي ضد البكتيريا الموجبة لصبغة كرام فقط ، اذا كان اعلى تثبيط بمقدار 20 ملم على *B. subtilis* في الاسبوع الثاني بينما في الاسبوع الثالث اعلى تثبيط كان بمقدار 21 ملم على بكتريا *S. aureus* وبينت النتائج بأن درجة الحرارة 30 م والاس الهيدروجيني 6 والمصدر الكربوني المالتوز هي الافضل لتنشيط الفطر على انتاج المواد الضد ميكروبية.