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Inhibitory Effect of Egyptian Garlic Extract on Penicillic Acid Production

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THE INHIBITORY effects of aqueous Egyptian garlic extract on growth and penicillic acid production of *Penicillium hirsutum* were established. Minimal inhibitory concentration (MIC) of the aqueous garlic extract was determined by the agar diffusion assay, which was 30 mg/ml. Growth of the fungus in broth containing higher concentrations of garlic extract (18 and 24 mg/ml) showed that sporulation was completely inhibited after 7 days of incubation or became very slight after 10 days at these mentioned concentrations. The increase in garlic concentration caused a gradual increase in the average values of mycelial dry weights reaching a maximum at 24 mg/ml. In the contrary, the increase in garlic extract concentration induced a reduction in the levels of penicillic acid production. The amount of penicillic acid in presence of 24 mg/ml of garlic was approximately 44% that of control culture filtrate after 10 days of incubation, however penicillic acid was not detected completely at the same garlic extract concentration after 7 days of incubation. This study was also extended to analyze and evaluate the percentage of the main components in garlic extract that may be responsible for these inhibitory effects, applying GC-MS chromatographic analysis. This is the first report on the inhibition of penicillic acid production by a natural substance as garlic extract.

Keywords: Garlic, Mycotoxins, Penicillic acid, Antifungal activity, *Penicillium hirsutum*.

Over the years much efforts have been devoted to the search for new antifungal materials from natural sources for food preservation (Onyeagba *et al.,* 2004 and Haciseferogullari *et al.,* 2005). Among the natural fungicidal substances, garlic extract has been found to be active in various trials (Yoshida *et al.*,1987; Tariq & Magee, 1990; Ghahfarokhi *et al.,* 2003 and Irkin & Korukluoglu, 2007). Moreover, aflatoxin production by *Aspergillus parasiticus* and keratinolytic activity of *Trichophyton mentagrophytes*, were reported to be inhibited by the use of garlic (Graham & Graham, 1987 and Ghahfarokhi *et al.,* 2003).

Among the toxigenic fungi, *P.hirsutum*, is a frequent contaminant of different food products, flower and vegetable bulbs and seems to be common, widespread species occurring in storage and is known to produce a variety of secondary toxic metabolites (Frisvad, 1981; Frisvad & Samson, 2004; Satio *et al.,* 2004 and Overy *et al.,* 2005) including penicillic acid (Frisvad & Filtenborg, 1983; Ezzat *et al.,* 2007

and Ismaiel, 2007). The hazardous effects and carcinogenic nature of penicillic acid made it a cause of concern (Palmgren & Ciegler, 1983; Wannemacher *et al.,* 1991; Macri *et al.,* 2002 and He *et al.,* 2004). The potential risk of penicillic acid for human health was suggested when penicillic acid had been isolated from agricultural products such as poultry feed, blue eye diseased corn, commercial corn, dried beans and tobacco products (Kurtzman & Ciegler, 1970; Pero *et al.,* 1972 and Thrope & Johnson, 1974) and from mold-fermented sausage (Ciegler *et al.,* 1972).

In spite of reports on the antifungal activity of garlic (Graham & Graham, 1987; Ghahfarokhi *et al.,* 2003 and Irkin & Korukluoglu, 2007) and inhibition of penicillic acid production by many chemicals (Lari & Thomas, 1980; Gourama & Bullerman, 1988 and Garza *et al.,* 1993), no data have appeared on the effect of garlic on the production of this toxin.

Material and Methods

Materials

Garlic bulbs

Fresh garlic (*Allium sativum* L.): bulbs were purchased from a local supermarket in Zagazig town, Egypt.

Microorganism

P. hirsutum has been isolated from a local sample of paddy rice grains and was found to produce the mycotoxin, penicillic acid. It was identified to the species level according to Frisvad & Samson (2004).

Culture media

Czapek-Dox`s agar supplemented with 0.5% (w/v) yeast extract (Kurtzman & Ciegler, 1970) used for maintaining and culturing of the fungus during the determination of the antifungal activity tests and the MIC of garlic extract.

Modified Raulin-Thom broth, containing 0.07% (w/v) Na_2HPO_4 in equimolecular weight to the original phosphorus source $(NH_4)_2HPO_4$ in the Raulin-Thom broth (Bentley & Keil, 1962 and Lindenfelser & Ciegler, 1977), was the most preferable during a previous screening study for selection of the optimum fermentation medium for maximum penicillic acid production by *P. hirsutum* (Ismaiel, 2007).

Methods

Preparation of the aqueous garlic extracts

This was adopted according to Curtis *et al.* (2004) and Bakri & Douglas (2005). Garlic bulbs were peeled and washed several times. The fresh garlic cloves (255 g) were blended in 255 ml distilled water, and homogenized in a domestic juicer (Braun Combimax 700 Vital, Germany) for 3 min at average speed to extract the juice. The juice was then centrifuged at 3000 rpm for 10 min. The precipitated material was weighted and the supernatant was filtered through Whatman no.1 filter paper and the resulting filtrate was then filter sterilized by

passing through a 0.45 µm cellulose nitrate membrane filter. By subtracting the weight of the insoluble material from the weight of the original cloves, the final concentration of the garlic extract in solution was determined. The garlic extract was used immediately or stored at 4ºC until used.

Antifungal activity tests

MIC determination : Sensitivity of the fungus to garlic extract concentrations (30, 60, 120, 240, 360, 480, 540 and 600 mg/ml) was determined by inoculating sterile Petri-dishes with 0.2 ml fungal suspension contain approximately 10^6 spores/ml. Twenty five milliters of agar medium was poured in each dish. Wells were punched in the agar and each filled with 50 µl of each tested garlic extract concentration. The plates were then incubated at 4ºC for 2 hr then incubated for 4 days at 30ºC and the resulted inhibition zones around the wells were then measured. The well containing the highest dilution (lowest concentration of garlic) that still showed a zone of inhibition around, it was seemed to be the MIC.

Effect of garlic extract on mycelial dry weights and penicillic acid levels of P. hirsutum : The modified Raulin-Thom broth was adjusted to pH 3, using varying amounts of 1N of both HCl and NaOH, was transferred to 250 ml Erlenmeyer flasks at 50 ml aliquots and sterilized at 121ºC for 20 min. Different concentrations of aqueous garlic extracts (0.0, 0.60, 1.20, 3.00, 6.00, 12.0, 18.0, 24.0, and 30.0 mg/ml culture medium) were separately added to the culture medium. The flasks were inoculated with 6.0 mm of 7-days old *P. hirsutum* culture under aseptic conditions and incubated for 10 days at 25ºC which are the optimum conditions for maximum penicillic acid production by *P. hirsutum* (Ismaiel, 2007). The fungal cultures were observed visually after 2, 4, 7 and 10 days.

Mycelial dry weights

At the end of the incubation period, the culture flasks were filtered through pre-weighted Whatman no.1 filter papers, oven-dried at 80ºC till a constant weight.

Determination of penicillic acid

The amounts of penicillic acid in the culture filtrates were determined quantitatively by the colorimetric method described by Bentley & Keil (1962) and Wirth & Klosek (1972) with some modifications. A known volume of the culture filtrate was extracted with equal volume of ethyl acetate, shaked for about 10 min and allowed to stand for 10 min. The ethyl acetate layer was then separated, dried and evaporated in a water bath at 60ºC. The dried ethyl acetate extract was then dissolved in a known volume of hot water. After cooling, a 3-fold excess of concentrated ammonia was added. The resultant reddish purple complex was allowed to develop for 20 min and the absorbance was measured at 545 nm with a spectrophotometer (Spectronic Molton Roy Co., 20 D). Meanwhile, penicillic acid production by *P.hirsutum* was determined qualitatively after spotting on TLC plates with standard penicillic acid (Sigma Chemical Co., Louis St., USA) according to the method described by Ciegler & Kurtzman (1970) and Aziz & Moussa (2002).

Statistical analysis

All experiments were conducted in triplicates and the mean ± standard deviation (SD) of these triplicates was calculated. The results of antifungal activity tests were analyzed by ANOVA (at 0.05 and 0.01 levels)

Chemical analysis of garlic extract

A suspension of acetone garlic extract was prepared for the GC-MS analysis, that was carried out in Mass Spectrometry Unit, Central Scientific Services Lab, National Research Centre, Dokki, Cairo, Egypt. 1.0 µl of the suspension was injected into GC, model Thermo Trace GC 2000 equipped with MS equipment, model Finnigan SSQ 7000 instrument. The MS is coupled with a DBWAX capillary column (30 cm x 0.25 mm internal diameter, film thickness 0.25 µm). The electron impact was 70 ev. The injector temperature was programmed at 220ºC and the column was held at 40ºC for 3 min and then programmed to 250ºC at 5ºC/2 min. The components were identified by comparison of retention indices and mass spectra with those of the standards in the library.

Results and Discussion

In the present work, a preliminary test was conducted to elucidate the antifungal effect of different concentrations of the aqueous Egyptian garlic extract towards *P. hirsutum* by the agar cup-plate method (Table 1). The size of the inhibition halo was clearly proportional to the concentration of garlic extract applied to each well. It has been found that the MIC of aqueous garlic extract necessary for growth inhibition of *P. hirsutum*, was 30 mg/ml. Various values for MIC of aqueous garlic extract were reported by other authors. Bakri & Douglas (2005) recorded low value of 8.9 mg/ml against *Candida albicans*, while, Irkin & Korukluoglu (2007) found high MIC of 325 mg/ml against *Aspergillus niger*. The susceptibility of many fungi to the antifungal activity of garlic extract was decided by many authors (*Trichosporon* and *Rhodotorula,* Tansey & Appleton, 1975; *Torulopsis, Trichophyton* and *Cryptococcus,* Fromtling & Bulmer, 1978 and *Aspergillus,* Hitokoto *et al.,* 1980).

The relationship between spore production by *P. hirsutum* in Raulin-Thom broth containing varying concentrations of garlic is summarized in Table 2. After 2 days of incubation, sporulation was very slight at 0.6 mg/ml and inhibited completely at the higher concentrations. After 4 days of incubation, it was slight at 1.2 mg/ml and inhibited at 3 to 24 mg/ml. After the extended incubation periods (7-10 days), sporulation became intense at the lower concentrations (0.6 to 3 mg/ml), completely inhibited after 7 days of incubation at 18 to 24 mg/ml and may be also inhibited or very slight at these higher concentrations after 10 days of incubation. Graham & Graham (1987) showed that the higher concentration of garlic extract (0.25-0.4%) inhibited the sporulation of *A. parasiticus*. The inhibition of fungal spores germination may be attributed to the presence of allicin and ajoene (a derivative of allicin) and some essential oils as main components of garlic extract (Naganawa *et al.,* 1996; Yamada & Azuma, 1997 and Benkeblia, 2004).

TABLE 1. Relation between concentration of aqueous garlic extract (mg/ml) and the inhibition zones diameters (mm) of the tested *P. hirsutum*.

Concentration of garlic extract (mg/ml)	Diameter of inhibition zones (mm)
30	7.66 ± 1.15
60	8.66 ± 1.52
120	11.66 ± 1.15
240	14.0 ± 0.00
360	18.0 ± 1.00
480	18.6 ± 0.57
540	19.0 ± 0.00
600	21.0 ± 1.00

MIC = 30 mg/ml. The results of this Table were analyzed statistically by ANOVA by comparison of the concentrations of aqueous garlic extract (mg/ml) at the mean values of different diameters of inhibition zones. At 0.05 level, significant values of inhibition zone diameters were obtained in comparison of the concentration either at 30 or 60 mg/ml with others tested concentrations ($P<0.05$). Also, significant values were obtained in comparison of the concentration at 120 mg/ml with all tested concentrations. The concentration at 240 mg/ml showed significant values with the concentrations at 360, 480, 540 and 600 mg/ml. Though, the concentration at 360 mg/ml showed significant values in comparison with the concentration at 600 mg/ml but no significant values were obtained, as compared with the concentrations either at 480 or 540 mg/ml ($P > 0.05$). At the concentration of 480 mg/ml, a significance was obtained, as compared with the concentration at 600 mg/ml ($P=0.023$) however, no significance was obtained, as compared with the concentration at 540 mg/ml ($P = 0.724$) . At the concentration of 540 mg/ml, a significance was obtained, as compared with the concentration at 600 mg/ml ($P = 0.047$). At 0.01 level, high significant values of inhibition zone diameters were obtained in comparison of the concentration either at 30 or 60 mg/ml with others tested concentrations ($P < 0.01$). High significant values were obtained in comparison of the concentration at 120 mg/ml with the concentrations at 360, 480, 540 and 600 mg/ml. The concentration at 240 mg/ml showed high significant values, as compared with the concentrations at 360, 480, 540 and 600 mg/ml. High significant values were obtained in comparison of the concentration at 360 mg/ml with the concentration at 600 mg/ml ($P < 0.01$).

TABLE 2. Influence of varying concentrations of aqueous garlic extract on spores production by *P. hirsutum* in Raulin-Thom broth incubated at 25ºC for different days .

Concentrations of garlic extract (mg/ml)	Spores production (a)			
	Days of incubation			
	2	4	7	10
0.00	+	+ +	+ + + +	+ + + +
0.60	+	+ +	+ + + +	+ + + +
1.20	-	+	+ + +	+ + +
3.00	-	-	+ + +	+ + +
6.00	-	-	+ +	+ +
12.0	-	-	-(+)	+
18.0	-	-	-	-(+)
24.0	-	-	-	-(+)

+ = very slight, + + = slight, + + + = heavy, + + + + = very heavy.

Figure 1 summarizes the effect of different concentrations of garlic extract (the sublethal concentrations less than 30 mg/ml) on mycelial dry weights and penicillic acid production by *P. hirsutum*. It was obvious that the average values of mycelial dry weights slightly increased with the increase of garlic concentrations reaching a maximum at 24 mg/ml. Inhibition of mycelial production was essentially complete at a garlic concentration of 30 mg/ml. Similar results were obtained by Graham & Graham (1987), who showed that apparent stimulation of mycelial production at the lower garlic concentrations was due to the presence of small amounts of nutrients such as minerals and vitamins in the added garlic. The stimulation of mycelial growth at the concentrations less than 30 mg/ml of garlic extract may be due to the presence of lower amounts of allicin and sulfide compounds in the extract and were insufficient to inhibit or decrease the mycelial growth. These components in garlic extracts are responsible for antifungal activity (Bianchi *et al.*, 1997 and Ankri & Mirelman, 1999). Removal of allicin from the reaction by solvent extraction decreased the antifungal activity of garlic extract (Hughes & Lawson, 1991). The contrary was took place for penicillic acid production where the increase in the garlic concentration causes a decrease in the levels of penicillic acid. The amount of penicillic acid produced in the presence of 24 mg/ml of garlic was approximately 44% of that produced in the culture control filtrate (free from any garlic extract) after 10 days of incubation. It is worthy to mention that penicillic acid was completely, undetected at a garlic concentration of 24mg/ml after 7 days of incubation. Moreover, the fungal mycelial growth and toxin production were inhibited by garlic concentration of 30 mg/ml. Garlic extracts were reported to have an inhibitory effect on aflatoxins production by *A. parasiticus* (Graham & Graham, 1987 and Lawson, 1996) and keratinase activity in *T. mentagrophytes* (Ghahfarokhi *et al.*, 2003). The antifungal mode of garlic extracts action were recognized from their capability to decrease oxygen uptake (Szymona, 1952), inhibit the synthesis of lipids, proteins and nucleic acids (Adetumbi *et al.*, 1986), damage membranes (Ghannoum, 1988), collapse the fungal hyphae, increase the number and size of the vacuoles in cells and increase the cell wall thickening (Bianchi *et al.*, 1997).

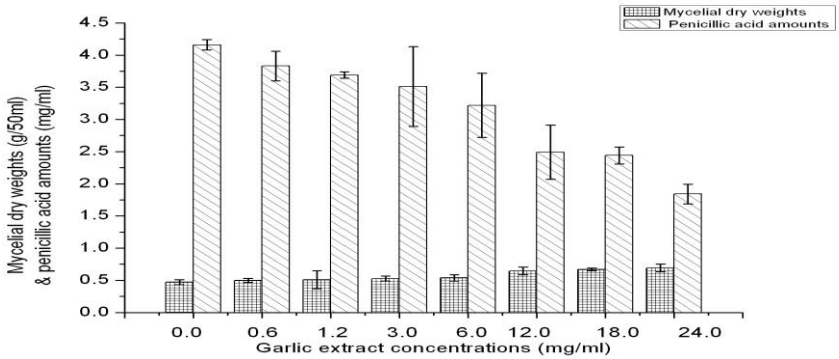

Fig. 1. Influence of varying concentrations of aqueous garlic extract on mycelium dry weights (g/50 ml) and penicillic acid levels (mg/ml) of *P. hirsutum* in Raulin-Thom broth incubated at 25ºC for 10 days.

The main components of acetone garlic extract were determined using GC-MS chromatogram analysis as represented in Table 3 and electronically photographed as shown in Fig. 2. It is interest to mention that the garlic extract consists mainly of about 17 components. The major components were quinolinium 1-ethyl-iodide (CAS) (quinoline ethiodide), 60.83% ; 8, 9, 10, 11- tetrahydrocyclonona [de] naphthalene -7, 12-dione, 5.88%; trisulfide, methyl-2-propenyl (CAS) (methylallyl trisulfide), 4.88% ; benzene, (1.3-daimethyl-3-butenyl)-(CAS) (1-pentene, 2-methyl-4-phenyl), 4.54%. The results also indicated that the garlic extract contains less percentages of 3, 4-dihydro-3-vinyl-1.2 dithiin, 3.61%; 1.2- divinylbenzene, 3.24% and disulfide, di-2-propenyl (CAS) [1.2-bis (allyl) disulfane], 2.18%. In this connection, the volatile compounds of suspension of micronized garlic powder were determined using GC-MS analysis and mainly consisted of linear chain of aldehydes, allyl disulfides and disulfides (Bianchi *et al.,* 1997). A concentrated garlic extract containing 34% allicin, 44% total thiosulfinates and 20% vinyldithiins possessed potent *in vitro* fungistatic and fungicidal activity against three different isolates of *C. neoformans* (Davis *et al.,* 1994). Allicin (diallyl thiosulfinate), is formed from allin by the action of allinase and gets metabolized rapidly into diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, *S*-allyl mercaptocysteine, *S*-allyl cysteine and dithiines (Welch *et al.,* 1992; Sundaram & Milner, 1993; Sigounas *et al.,* 1997; Dirsch *et al.,* 1998 and Hirsch *et al.,* 2000). Barone & Tansey (1977) suggested that the sulfur-reduced compounds present in garlic might act by binding with sulfhydrlic groups of essential amino acids, proteins and enzymes. This study confirms this hypothesis, since the analysis of the garlic extract revealed the presence of some thioalkylating agents that may responsible for the antifungal activity. Most of the biological effects of allicin including its antimicrobial activity can be related to its strong SH-modifying capacity and antioxidant properties (Koch & Lawson, 1996 and Lawson, 1998).

In conclusion, the results obtained indicate that, garlic extract had inhibitory effects on growth, spore formation and penicillic acid production by *P. hirsutum*. The inhibitory activities of garlic extract were manifested by determination of the main active components applying GC-MS chromatogram analysis which revealed that garlic extract consists of 17 components. The major components were quinoline ethiodide; 8, 9, 10, 11- tetrahydrocyclonona [de] naphthalene -7, 12-dione; methylallyl trisulfide; 1-pentene,2-methyl-4-phenyl; 3,4-dihydro-3-vinyl-1.2dithiin; 1.2-divinylbenzene and 1.2-bis(allyl)disulfane. This study suggests the suitability of garlic extract as a natural and safe additive to food preparations, offering a protection against intoxication from penicillic acid.

TABLE 3. Main components of acetone garlic extract.

Retention time (min)	Compounds	Area (%)
11.35	2-(3-hydroxy-1-oxopropyl)-1.1-1-trimethyl-hydrazinium hydroxide inner salt	2.20
15.54	Phenyl acetylene-2-d	0.33
16.41	1-hexanol, 2-ethyl-(CAS)	1.79
18.10	Disulfide, di-2-propenyl(CAS)	2.18
19.42	4-methoxy-1-(2-oxobut-3-enyl) azetidin-2-one	1.12
20.07	Trisulfide, methyl-2-propenyl (CAS)	4.88
21.90	3.4-Dihydro-3-vinyl-1.2 dithiin	3.61
22.65	[4S-(2E, 4R*, 5S*, 6R*, 7E)] -10-(benzoyloxy)-5-hydroxy- 4.6-dimethoxy-2.8 dimethyl-2.7-decadienoic acid methyl ester	3.84
24.69	8-(Trimethylsilyl)-6-octenteneitrile	2.92
37.32	Benzene, [(Fluoromethyl) thiol]-(CAS)	0.73
39.43	8, 9, 10, 11- tetrahydrocyclonona [de] naphthalene- 7.12-dione	5.88
40.01	Tetradecaoic acid (CAS)	0.44
41.16	Quinolinium, 1-ethyl-, iodide (CAS)	28.69
41.52	Quinolinium, 1-ethyl-, iodide (CAS)	24.46
43.32	Quinolinium, 1-ethyl-, iodide (CAS)	7.68
43.96	1,2-Divinyl benzene	3.24
44.65	Hexadecanoic acid (CAS)	0.48
45.80	Benzene, (1,3- dimethyl-3-butenyl)-(CAS)	4.54
46.22	(E)- 3,3-diphenyl-4-hexenoic acid	0.97
Total		99.98%

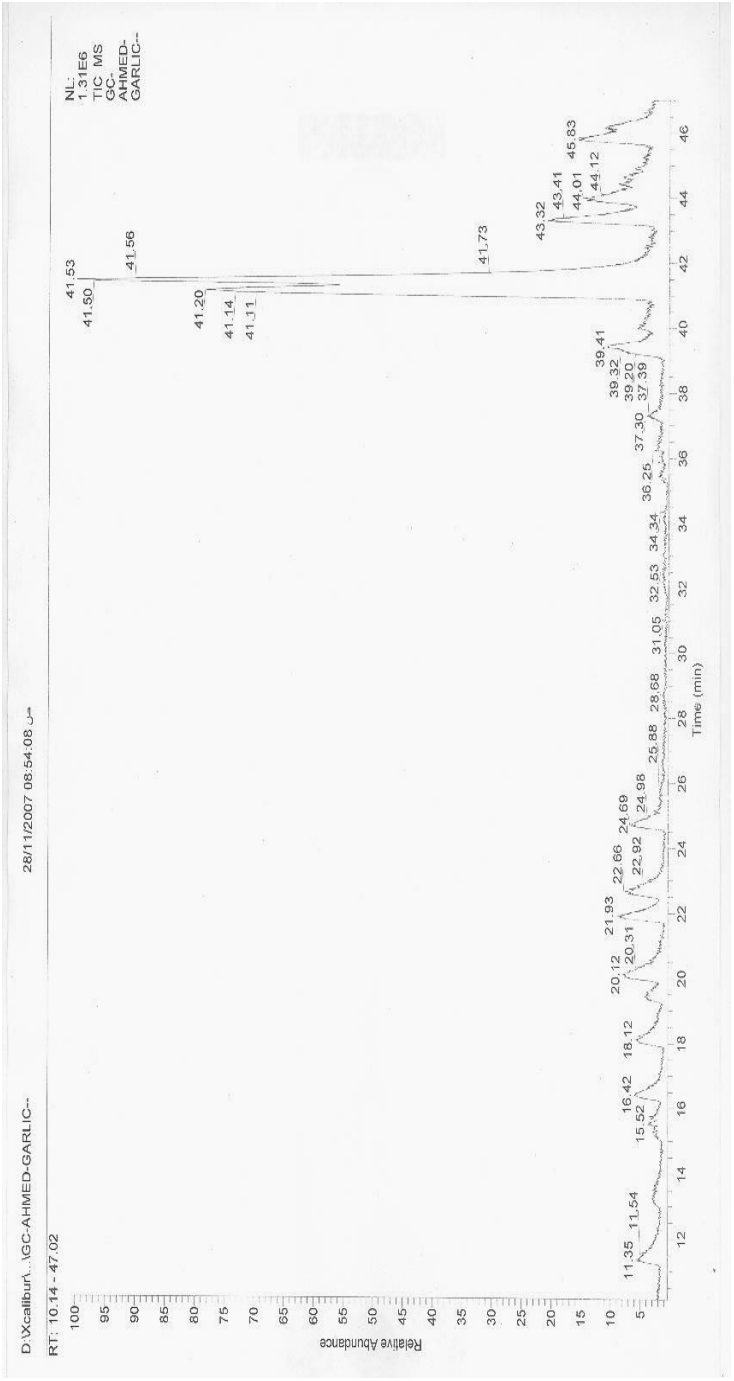

Fig. 2. GC-MS chromatogram of acetone garlic extract.

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التأثير المثبط لمستخلص الثوم المصرى على إنتاج السم الفطرى حمض البنسيليك

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فى هذا البحث ، تمت دراسة التأثير الضدى لمحلول مستخلص الثوم المائى على نمو فطرة *البنسيليوم هيرسيوتم* وإنتاجه من السم الفطرى حمض البنسيليك و إتضح أن اقل تركيز مثبط لنمو الفطرة هو ٣٠ ملليجرام لكل ملليلتر وذلك بعد انتشار مستخلص الثـوم داخـل الوسط الصلب. وقد كان لمستخلص الثوم عند التركيزين ١٨ و ٢٤ ملليجرام لكل ملليلتر تأثيرا مثبطا على إنتـاج جـراثيم الفطرة وذلك بعد ٧ أيام من التحضين بينما لوحظ أن إنتاج الفطرة للجـراثيم يكون ضئيلاً جدا بعد ١٠ أيام من التحضين عند هذين التركيزين. إتضح أيضا انه بزيادة تركيزات مستخلص الثوم يزداد الوزن الجاف للفطرة تدريجيا حتى الوصول إلى اعلى معدل عند التركيز ٢٤ ملليجرام لكل ملليلتر. وعلى النقيض ، انخفضت معدلات إنتاج حمض البنسيليك السـام بزيـادة تركيـزات مستخلص الثـوم فقد أدى التركيـز ٢٤ ملليجرام لكل ملليلتر إلى انخفاض فى كمية حمض البنسيليك المنتجة بنسبة ٤٤٪ مقارنة بالكمية المنتجة من الحمض فى رشيح المزرعة للفطرة النامية على وسط سائل غير معامل بمستخلص الثوم وذلك بعد ١٠ أيام من التحضين ، بينما فقدت الفطرة قدرتها تماما على إنتاج حمض البنسيليك أثناء نموها على وسط سائل يحتوى على نفس التركيز من مستخلص الثوم بعد التحضين لمدة ٧ أيام.

إمتد هذا البحث إلى عمل دراسة تحليلية لتعيين وتقييم نسب المركبات الأساسية المتواجدة فى مستخلص الثوم والتى يمكن أن يكون لها هذا التأثير الضدى على نمو فطرة *البنسيليوم هيرسيوتم* وذلك بإستخدام تقنية كروماتوغرافيا الغاز و طيف الكتلة.