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Antibacterial effect of *Bacillus subtilis* extract on the growth of pathogenic bacteria and analyzed by GC-MS

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Abstract:

The antibacterial effect of three selected isolates of *Bacillus subtilis* (Sar1, Sar2, Sar3) was tested by the agar diffusion method, and the results showed that there was no inhibitory effect of these bacteria against the growth of pathogenic bacteria; *Staphylococcus aureus, Staphylococcus haemolyticus Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*, while the undiluted and half-diluted (200, 100, 50) mg/ml extracts, of the three cultures which were tested by the Agar Well Diffusion method, had varying inhibitory effects towards the growth of these five pathogenic bacteria and more effect on gram-positive compared to its effect on gram-negative bacteria.

Through GC-MS detection of secondary metabolites of *Bacillus subtilis* (Sar1) extract, which were selected because they are identical to *Bacillus subtilis* bacteria contained in the standard GeneBank with a percentage of (95)%, it was found that they consist of (33) chemical substances according to the number of peaks produced. After injecting the extract into the GC device, and when this information was entered into the MS device, it was diagnosed by its names with the calculation of its molecular weight and the amount of area it occupies as a percentage within the used extract understudy.

Key words: Antibacterial, *Bacillus subtilis*, GC-MS

التاثير المضاد لمستخلص *Bacillus subtilis* على نمو البكتيريا المرضية ، وتحليله بجهاز GC-MS

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الخلاصة:

تم اختبار التأثير المضاد لثلاثة عزلات مختارة من بكتيريا *Bacillus subtilis* (Sar3,Sar2, Sar1) بطريقة الانتشار بأقراص الاكار Agar Diffusion method واظهرت النتائج عدم حدوث أي تاثير تثبيطي لهذه البكتريا على نمو الانواع البكتيرية المرضية *Stphylococcus aureus* ، *Stphylococcus haemolyticus* ، *Eschericha coli* ، *Klebsiella pneumoniae* و *Pseudomonas aeruginosa*، بينما كان لمستخلصات المزارع البكتيرية الثلاثة المخففة (200، 100، 50) ملغم /مل وغير المخففة,

بطريقة الانتشار في حفر الاكار Agar Well Diffusion method فعالية تثبيطية متفاوتة تجاه نمو البكتريا المرضية بانواعها الخمسة واكثر تاثيرا على البكتيريا الموجبة لصبغة كرام قياسا بتاثيره الاقل على البكتيريا السالبة لصبغة كرام.

من خلال الكشف بجهاز GC-MS عن مواد الايض الثانوي لمستخلص بكتريا(Sar1) *Bacillus subtilis* والتي تم اختيارها لكونها مطابقة وبنسبة (95)% لبكتريا *Bacillus subtilis* الموجودة في بنك الجينات القياسية ، تبين انها مكونة من (33) مادة كميمائية حسب عدد القمم الناتجة بعد حقن المستخلص في جهاز GC، وعندما ادخلت هذه المعلومات في جهاز MS تم تشخيصها باسمائها مع حساب الوزن الجزيئي لها وكمية ما تشغله من مساحة كنسبة مئوية ضمن المستخلص المستخدم قيد الدراسة.

الكلمات المفتاحية:المضادات البكتيرية، *Bacillus subtilis*، GC-MS .

1.Introduction:

Bacillus subtilis has been extensively studied to identify new forms of antibacterial compounds that can be produced to combat multidrug-resistant microorganisms (MDR) [1]. These alternatives include but are not limited to, the so-called Bacteriocins, which are non-invasive chemicals, harmful to public health, prevents food spoilage, foodborne diseases and thus maintains food quality and consumer health [2]. In this regard, Caulier *et al*, [3], mentioned that *B. subtilis* produces three types of antimicrobial compounds: Bacillaene, Macrolactin, and Difficidin, which are known for their anti-growth activity of gram-negative and gram-positive bacteria through their inhibition of protein synthesis, and other species of *Bacillus* genus that produce Biosurfactants are biologically active compounds and are used as antimicrobials or as inhibitors of genes coding for biofilm formation in *S. aureus* [4].

Gas Chromatography-Mass Spectrometry (GC-MS) is one of the modern diagnostic methods used to detect the components of secondary metabolites, which are produced in large quantities and usually excreted outside the cells, as it works to analyze the active compounds present in them. It is a complex technique that separates the mixture of compounds into their components with determining the mass spectrum of the compound [5]. In Gas Chromatography (GC), the mobile phase is an inert gas such as helium, which carries the sample mixture through what is indicated as the stationary phase because it works on the principle that the mixture will separate into individual substances when heated and then transferred with the heated gases through a column containing an inert gas (such as helium) so that the separated materials will then flow from the shaft hole to (MS) Mass Spectrometry, [6].

The current study aims to:

1- Detect the antibacterial effect of isolated bacteria and their extracts on the growth of different types of pathogenic bacteria by an ex vivo study.

2- Determine *B. subtilis* (Sar1) extract components using gas chromatography-mass spectrometry (GC-MS).

2.Research Method:

Isolation and identification of *Bacillus subtilis*:

Bacillus subtilis (Sar1,Sar2,Sar3) was isolated from soil and diagnosed by conventional and molecular methods in a previous study [7].

Antibacterial activity of *B. subtilis* and their extracts on the growth of some pathogenic bacteria:

In this study, the revelated ability of three diagnosed types of *B. subtilis* (Sar1, Sar2, Sar3) bacteria and their extracts to inhibit the growth of pathogenic bacteria :*Staphylococcus aureus, Staphylococcus haemolyticus, Escherichia coli, Klebsiella pneumonia*, and *Pseudomonas aeruginosa* was isolated from

different disease cases, and diagnosed by postgraduate students in the Biology Department/Education for Pure Sciences College/Mosul University.

Agar Disc-Diffusion method:

The nutrient agar medium was inoculated with the three types of identified bacteria, and incubated at (37)˚C for (24) hours.

Liquid cultures of pathogenic bacteria used in this study were prepared and incubated at (37)˚C for (24) hours, and the suspension was measured with a Spectrophotometer at an absorbance of (600) nm and compared with 0.5 MacFarland turbidity standered, which is equivalent to ($1.5\ X10^8$) CFU/ml, transferred from it (0.1) ml and spread on the surface of the nutrient agar medium, the petri dishes were left at laboratory temperature until their surface was dried.

According to what was reported by Egorov [8], discs (6) mm from the cultures of the three bacterial isolates (Sar1, Sar2, Sar3) were made with sterilized cork borer by alcoholic flame and distributed on the surface of the nutrient agar inoculated with pathogenic bacteria (*Staphylococcus aureus, Staphylococcus haemolyticus, Escherichia coli, Klebsiella pneumonia,* and *Pseudomonas aeruginosa*)at a rate of (5) disc/petri dish and (3) replicates/pathogenic bacteria. The result was read, after incubating it at (37)˚C for (24) hours by measuring the diameter of the inhibition zone around each disc in mm.

Agar Wells Diffusion method:

The Agar Wells Diffusion method was used, according to Schwalbe *et al*., [9]with some modifications, by preparing extracts from the three liquid cultures of Sar1, Sar2, Sar3, each separately, grown on nutrient broth and incubating them at (37)˚C for (7) days, then centrifuging the bacterial suspension at a speed of (5000) RPM for (15) minutes. The supernatant was taken and filtered with Millipore filters with pores diameter (0.22) µm.

Liquid cultures of the pathogenic bacteria used in the study were prepared and transferred onto the surface of the nutrient agar in the same as previously mentioned manner, after which (3) wells were made inside the petri dish using a sterile cork borer with a diameter of (6) mm and it was filled with about (100) microliters of the liquid bacterial culture extract for the three isolates understudy, taking into account the work of three replicates/treatment, the diameter of the inhibition zone was measured in mm around each hole after incubating it at (37)˚C for (24) hours.

These extracts were also dried using an oven at (40)˚C for (2) days, then dissolved (1) g of it in (5) ml of sterile distilled water, and thus the concentration was obtained (200) mg/ml, then this extract was sterilized by membrane filters, half-dilutions of (100,50) mg/ml were prepared from it, and the three concentrations were distributed over the wells in the same way as before, after spreading the pathogenic bacteria on the surface of the nutrient agar medium.

Gas chromatography Mass Spectrometry (GC-MS):

The dried extract belonging to one of the selected isolates (sar1) was sent to the College of Agriculture at the University of Basra to diagnose it's content of chemical compounds using a gas chromatograph connected to a mass spectrometer type GC-MS QP210 ULTRA, which is supplied by Shimadzu Japanese company, to separate organic and inorganic compounds, based on a special library equipped with a database of thousands of compounds that are continuously renewed every three months.

Chemical compounds were identified based on their retention time in the capillary column of the GC and then computer matching to the mass spectra, using the NSTA08 library database and using the GC-MS Solution software**.**

Results and Discussion:

The results of the test of the inhibitory activity of *B. subtilis* by the Agar Diffusion method showed that there was no inhibitory effect of this bacteria against the five pathogenic bacterial species, Figure (1)

shows that *B. subtilis* (Sar1) agar disc have no effect in inhibiting the growth of *Pseudomonas aeruginosa*.

The lack of effect by *B. subtilis* bacteria can be explained in that the production of antimicrobials or antibiotics takes place in the stage of nutrient deficiency as a reaction by bacterial cells [10] and the bacteria used in the agar disc did not exceed the incubation period (24) hours, also, these results were in contradiction to the results obtained by the researcher Hassan [11] who noted the inhibitory effect of *B. subtilis* agar disc on the growth of pathogenic bacteria, the difference in results may be attributed to the difference in the medium used.

Figure 1. No effect of *B. subtilis* (Sar1) agar discs in inhibiting the growth of *Pseudomonas aeruginosa*.

On the other hand, the results of testing the efficacy of diluted and undiluted extracts using the Agar-Well Diffusion method showed a different effect according to the type of pathogenic bacteria as shown in Table (1) and Figure (2).

In general, it is noted from Table (1) that the antimicrobial effect of diluted extract at a concentration of 200 mg/ml was more effective than undiluted on the growth of gram-positive bacteria compared to their effect on gram-negative bacteria, and as shown in Figure (2) by it's effect on *S.aureus* and *S.haemolyticus*.

According to these results, the concentration of (200 and 100) mg/ml is considered the minimum inhibitory concentration (MIC) for the growth of gram-negative (*E.coil*, *K.pneumonia)* and gram-positive (*S.aureu*s, *S.haemolyticus*), respectivily, while the researcher Sulaiman [12] found that MIC represented by the concentration (200) mg/ml vegetable oils which separated from Flax, Castor, Fenugreek, and Cress plants, inhibiting the growth of gram-negative and gram-positive bacteria such as *E.coli, , Pseudo. aeruginosa, Staph aureus* and *Str. pyogenes.*

Table 1. Antimicrobial effect of *B. subtilis* extract on the growth of pathogenic bacteria by an agar wells diffusion method.

Pathogenic bacteria	Undilute Extract			Dilute Extract (mg/ml)								
				200			100			50		
	Sa r1	Sa r2	Sa r3	Sa r1	Sa r2	Sa r3	Sa r1	Sa r2	Sa r3	Sa r1	Sa r2	Sa r3
S.aureus	+	+	+	++	++	+	+	+	+	+	–	–
S.haemolyticus	+	+	+	++	++	+	+	+	+	–	–	–
E.coil	–	–	–	+	+	+	–	–	–	–	–	–
K.pneumonia	–	–	–	+	–	–	–	–	–	–	–	–
P.aeruginosa	–	–	–	–	–	–	–	–	–	–	–	–

(-): No effect, (+):The diameter of the inhibition zone is (8-10) mm, (++):The diameter of the inhibition zone is (10-15) mm, [13].

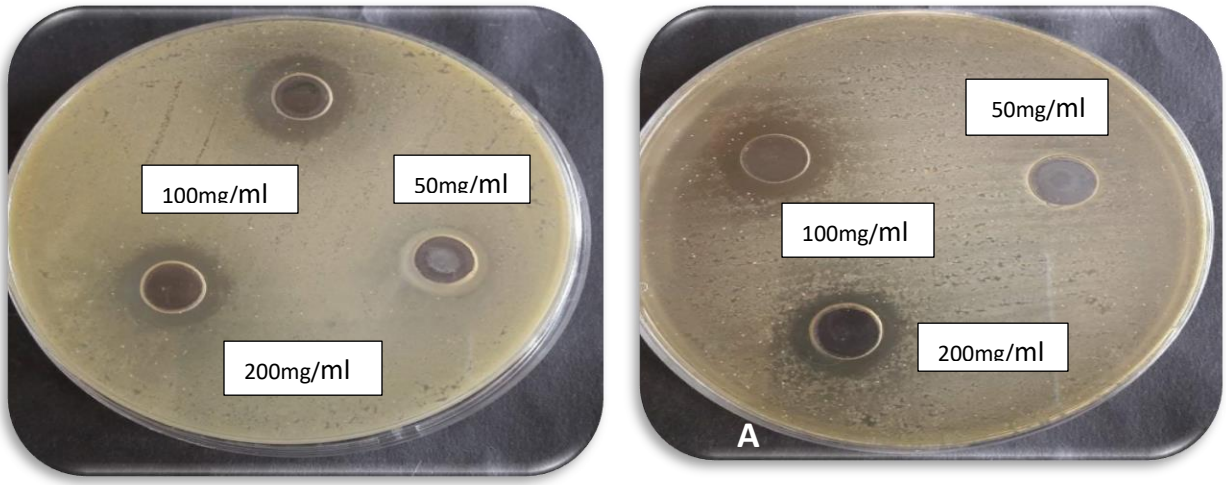


Figure 2. Inhibition zone of pathogenic bacteria A(*S.aureus*) and B (*S.haemolyticus*) with *B.subtilis*(Sar3) extract by Agar-Well Diffusion method.

This effect may be due to the bacteria's production of the antibiotic Bacillysoin, which is characterized by it's high inhibitory activity against *S.aureus* bacteria exclusively [14], as well as its production of Bacteriocin, which has an inhibitory effect against a group of pathogenic microorganisms [15],[16], in addition to its production of peptide antibiotics such as Glycopeptide antibiotics known to have limited inhibition effects on the growth of MRSA and *Clostridium difficile* [17],[18].

The difference in the composition of the cell wall had a significant effect on increasing the effect of extract on gram-positive bacteria, compared to its lowest effect on gram-negative bacteria. The reason for this is due to the possession the last bacteria of the outer layers, consisting of lipo-polysaccharides, that are missing in gram-positive bacteria[19], which is acts as a permeability barrier that prevents the entry of antimicrobial substances (including the extracts understudy) into the bacterial cell, especially the bacteria *Pseudomonas aeruginosa* that has a high ability to resist due to the high efficiency of its efflux pumps and it's low outer membrane permeability [20], while the Peptidoglycan layer and Teichoic acid that make up the wall of the positive bacteria do not prevent the permeability of antimicrobial

substances [21], because it's cell wall represents a direct contact area with the outer environment [22], in addition to the presence of differences between the antimicrobial compounds produced by these bacteria, some of which have a wide spectrum of activity and the other has a narrow spectrum [3].

The growth of pathogenic bacteria *Vibrio* spp was also controlled by extract of unpathogenic *Bacillus subtilis* using wells method under laboratory conditions [23]. The results of this study were similar to what was indicated by Yahya *et al*., [24] in the lack of effect of filtrate of *Bacillus* sp. on the growth of gram-negative bacteria *P.aeruginosa, K.pneumonia, E.coli* and *Proteus mirabilis*, and it's clear effect on the growth of gram-positive bacteria *S.aureus, Candida albicans*, in terms of the diameter of the inhibition zone, which ranged between (12-30)mm. On the other hand, *Bacillus subtilis* and its metabolites have been used as probiotics by achieving a natural balance between beneficial and harmful bacteria, especially in the event of an increase in the number of disease-causing bacteria, including *S.aureus* and *E.coli* in vivo experiments [25].

It was found through the detection of secondary metabolites in the extract of *Bacillus subtilis* (Sar1), which were selected because they are identical to the bacteria *Bacillus subtilis* contained in the standard GeneBank with a percentage of (95%, that it is composed of (33) chemical substances according to the number of peaks produced after injecting the extract in the GC apparatus (Figure 3). When this information was entered into the MS apparatus, it was diagnosed by its names with the calculation of its molecular weight and the amount of area it occupies as a percentage within the extract under study (Table -2) and (Figure -4) note that three subjects were selected (Pentanoic acid, 4-methyl-), (2-Pyrrolidinone, 4-methyl) and (Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro) based on the large size of the area occupied by the extract (15.87), (13.79), and (9.09)%, respectively.

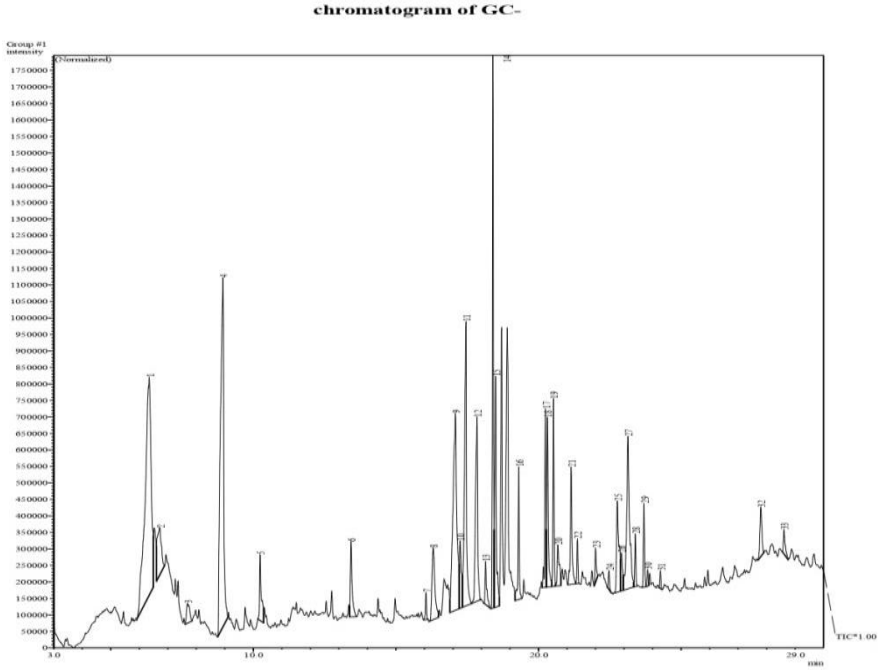


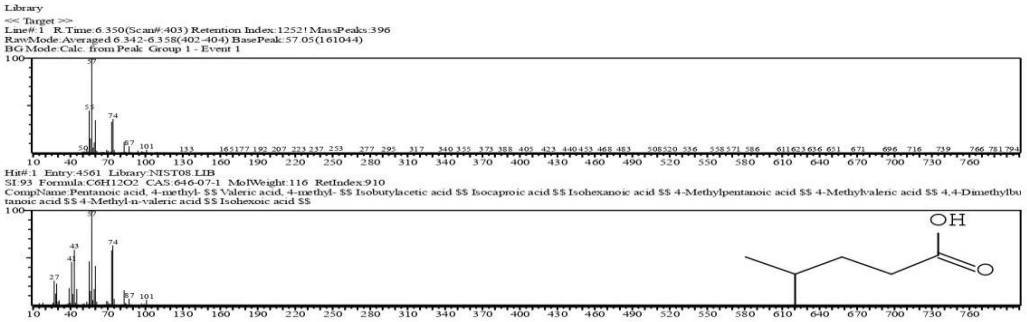
Figure 3. The curve of chemicals separated from the extract of *B. subtilis*(Sar1).

Table 2. Chemical compounds from the extract of *B. subtilis* (Sar1).

Peak Report TIC

Peak#	R.Time	Area%	Name
1	6.349	15.87	Pentanoic acid, 4-methyl-
2	6.725	2.42	4-Penten-1-ol, propanoate
3	7.693	0.73	N-(3-Methylbutyl)acetamide
4	8.936	13.79	2-Pyrrolidinone, 4-methyl-
5	10.237	1.40	5H-1-Pyrindine
6	13.424	1.57	Acetamide, N-(2-phenylethyl)-
7	16.056	0.33	Tridecanoic acid, 12-methyl-, methyl ester
8	16.308	2.53	3-Pyrrolidin-2-yl-propionic acid
9	17.084	9.09	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
10	17.254	1.59	Pentadecanoic acid, methyl ester
11	17.455	7.03	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
12	17.842	4.61	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
13	18.145	0.80	9-Hexadecenoic acid, methyl ester, (Z)-
14	18.401	6.73	Hexadecanoic acid, methyl ester
15	18.496	4.46	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
16	19.304	1.95	Methyl 9,10-methylene-hexadecanoate
17	20.242	2.53	9-Octadecenoic acid (Z)-, methyl ester
18	20.311	2.89	cis-13-Octadecenoic acid, methyl ester
19	20.523	2.33	Octadecanoic acid, methyl ester
20	20.678	1.01	5,9-Tetradecadiyne
21	21.142	2.34	Dodecanamide
22	21.365	0.55	Methyl 9,10-methylene-octadecanoate
23	21.999	0.52	9-Octadecenamide, (Z)-
24	22.469	0.25	Methyl 18-methylnonadecanoate
25	22.762	2.67	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.al
26	22.908	0.80	9-Octadecenamide, (Z)-
27	23.136	5.29	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-
28	23.393	0.69	Isosteviol methyl ester
29	23.697	1.12	Isosteviol methyl ester
30	23.826	0.22	Kaur-16-en-18-oic acid, 13-hydroxy-, methyl ester, (4.alpha.)-(.+/-.)-
31	24.272	0.22	Docosanoic acid, methyl ester
32	27.794	1.14	.gamma.-Sitosterol
33	28.609	0.50	Stigmast-5-en-3-ol, oleate
		100.00	

A



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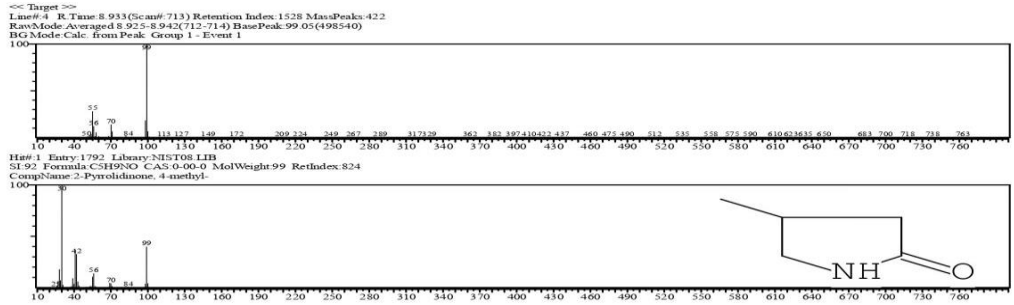

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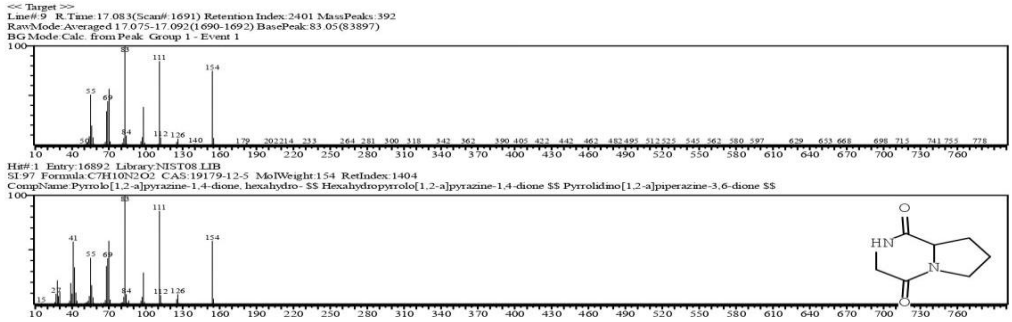


Figure 4. The Curve of The Three Compounds and Their Chemical Composition, A: Pantanoic acid 4-methyl, B: 2-pyrrolidinone 4-methyl, C: Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro.

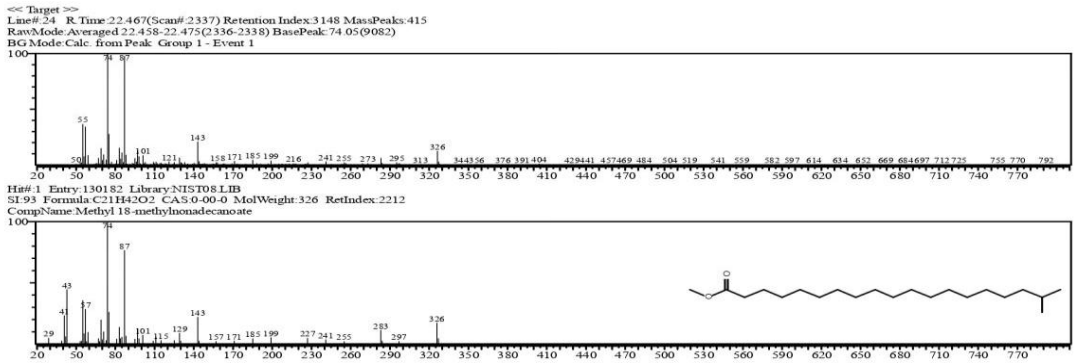
Vitro antibacterial assay showed that all the prepared compounds, including Pentanoic acid, had good antibacterial activity against many gram-positive bacteria (including Multi Drug Resistance "MDR"-clinical isolates) with minimum inhibitory concentration (MIC) values in the range (2-4) µg/ml [26], Pentanoic acid,4-methyl- is one of the metabolites of substances N-Acyl-homoserine lactones (AHL), which were detected by GC-MS and secreted by the pathogenic bacteria *Pectobacterium carotovorum* subsp. *carotovoru*, as auto-inducer (AI) for quorum sensing (QS) in difficult environmental conditions experienced by bacteria. This indicates the possibility of using these products (Pentanoic acid, 4-methyl-) in the biological control of these pathogenic bacteria to reduce its pathological effect [27].

Phaechamud *et al.,* [28], indicated that the antagonistic activity of 2-Pyrrolidinone, 4-methyl- increases with increasing concentration, as the value of the inhibition zone reached (2 and 1) mm in each of *E.coli* and *S. aureus,* respectively. On the other hand, GC-MS analysis confirmed the presence of 2-Pyrrolidinone, 4-methyl in metabolites of filtrate of *Bacillus subtilis* isolated from the area surrounding the roots of plants, and it was found that it can act as plant defense factors by enhancing its growth, productivity, and endurance to resist saline environment conditions [29].

The compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro (Table -2 and Figure4) is also produced by various species of the genus *Streptomyces*, and it has been shown to have antitumor and antioxidant activity [30],[31], by reducing the number of free radicals, and thus to the prevention of chronic diseases [32]. On the other hand, this compound was isolated from filtrates (56) isolates of *Bacillus tequilensis* MSI45, diagnosed by the 16S rRNA gene, and found it has effective antimicrobial and antioxidant activity against multidrug-resistant *S. aureus* infections [33].

In addition to what was mentioned above, we chose three other substances as they constitute the least area among the components of the studied filtrate, (Table -2) and (Figure -5). No other studies were found that succeeded in separating the two substances (0.22)%, Kaur-16-en-18-oic acid, 13-hydroxy-, methyl ester, (4.alpha.)-(.+/-.)- and Docosanoic acid, methyl ester from any source, while the Methyl 18-methylnonadecanoate, which constitutes an area (0.25)% within the bacterial filtrate, it was found from the published data of one of the studies [34], that this substance was separated from the extract of the seeds of the plant *Gundelia tournefortii* with nine other substances and it was found to have medical importance as an anti-inflammatory, preventings cancer and liver disease, antihistamine, anti-acne, and has other anti-insect properties. while pointing Adesanwo *et al*., [35] that the percentage occupied by this compound from the alcoholic extract of the leaves of the *Melanthera scandent* plant was (0.42)%, with an increase of (40.5)% over our results.

A



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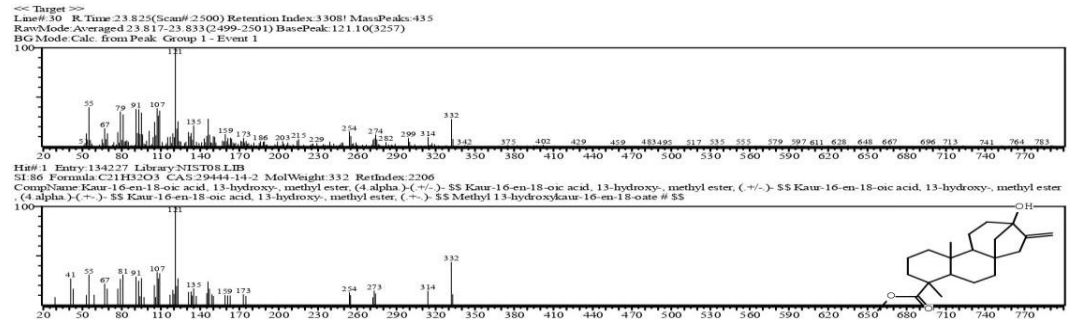

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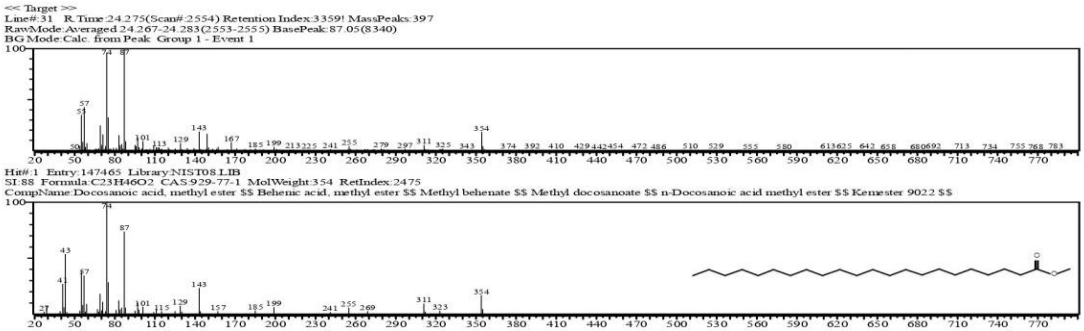

Figure 5. The curve of the three compounds and their chemical composition, A: Methyl 18-methylnonadecanoate, B: Kauran-19-oic acid, methyl ester Methyl kauran-18-oate, C: Docosanoic acid, methyl ester.

4.Conclusion:

1-*B. subtilis* filtrates have an antimicrobial effect but in a narrow spectrum.

2-The possibility of controlling the growth of pathogenic bacteria, especially *Staphylococcus aureus*, by *B. subtilis* filtrate, and gives a promising alternative to using it instead of antibiotics.

3- It was found that the production of secondary metabolites occurs after the starvation of cells and entering the stage of sporulation.

4-It was found that *B. subtilis* can produce secondary metabolites in different numbers and types in terms of their separation by GC-MS.

5-This study is considered the first in separating the constituent compounds of *B. subtilis* filtrate.

5.Acknowledgements:

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