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The study of purified secondary metabolites extracts of *Bacillus subtilis* and its chemotaxis effect on biofilm-forming bacteria

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Abstract

Chemotaxis is the movement of a single cell organism as a reaction to a chemical stimulation in its surrounding environment. Biofilm-forming bacteria are the cause for numerous major health and environmental problems. Bacterial extracts were proven to induce negative chemotaxis reaction against biofilm-forming bacteria and biofilm development. Therefore, using and enhancing these extracts are considered as promising methods in pharmaceutical production and environmental science. In this study, twenty *Bacillus* isolates and five biofilm-forming bacteria (targeted bacteria) isolates from different water and sediments samples of different areas in Basra province were biochemically diagnosed. Secondary metabolites of *Bacillus* isolates were extracted and analysed. Total proteins in the extracts were determined using Biuret method and the highest two isolates (BS8) and (BS14) with 13.78 and 12.02 g/l protein, respectively were chosen for the experiment. GC-MS results showed the existence of compounds with proven high antimicrobial properties such as type D-amino acids, N-cyclopropyl carbonyl-, butyl and esters such as d- proline, N-methoxycarbonyl, and pentyl ester. Afterwards, the chemotaxis nature of the purified extracts was studied. The results showed that both extracts had a negative chemotaxis toward the targeted bacteria represented by transparent halos without bacterial growth around the spot where secondary metabolites extracts of *Bacillus subtilis* were placed. *K. kristinae* was the most affected species in regards of growth inhibition zone diameter with 23 and 24 mm for (BS8) and (BS14) extracts respectively, while *P. aeruginosa* was the least affected with 19 and 18 mm for (BS8) and (BS14) extracts respectively.

1. Introduction

Chemotaxis is the mechanism by which a single-celled organisms, bacteria and somatic cells direct their activity toward compounds in their own surrounding environment. Chemotaxis is considered positive when a movement towards the used compounds occurs, such as in the movement of bacteria towards amino acids, sugars, bilateral peptides, and purines, while it is considered negative when movement is in the opposite direction, as in bacteria away from antibiotics, toxins and some alcohols and fatty acids [1].

[2] stated that chemotaxis plays an important role in the development of biofilms as the bacteria produce chemical compounds that serve as an attraction agents between the bacteria and adhesion surface, while in the later stages of biofilm, other chemicals contribute to the destruction of the biofilm and thus moving away from the surface of adhesion [3]. Previously, [3][4][5] reported that bacteria with positive chemotaxis can travel to suitable sites for survival and growth in complex environments and that nutrient molecules (amino acids and sugars) are not all considered attractive, while on the other hand, not all attractants are good nutrients.

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Microbial secondary metabolites include a wide range of naturally produced compounds that perform a large number of vital activities. Secondary metabolites are not involved in growth, development, or reproduction; but support microbes living in its environment [6]. Secondary metabolites also play an important role in the physiological adaptation and many of them inhibit the growth of competing microbes [7].

Bacterial species belonging to the genus *Bacillus* can produce many secondary metabolites with antimicrobial effects towards other bacteria such as lipopeptides, polypeptides, macrolactones, fatty acids, polyketides, and isocumarines [8]. Therefore, these bacterial species were widely used in the medical and pharmaceutical industries for commercial production of antimicrobial substances [9][10] .

Several studies have been carried out to extract secondary metabolites of *Bacillus* species to investigate the inhibitory effect of these extracts against many plant and animal pathogens and also against biofilm-forming bacteria [11][12][13]. Most of these studies indicated that the secondary metabolites produced by *Bacillus* spp., such as lipopeptides, are low molecular weight substances with constant antagonistic efficacy within broad thermal ranges. In reality, surfactin, which is a bacterial lipopeptide, have detergent like characteristics toward biofilms by reducing the surface tensile strength between these biofilms and adhesion surface [14]. These properties draw researchers' attention to the possibility of purifying the active phase of bacterial extracts, and their uses as a bio-control agent against harmful biofilm-forming bacteria which are the cause of many environmental problems. Therefore, the aim of this study was to obtain local isolates belonging to *Bacillus* genus bacteria and to investigate the inhibitory effects of their purified secondary metabolites extracts against the growth of other targeted bacteria native to their environment.

2. Materials and Methods

2.1. Bacterial isolates and differential analysis

Fifty samples of water and sediment were collected for the period from January 2016 to April 2016 from different places in Basra province (Table 1). The samples were transferred to the laboratory for diagnosis, and then placed in a water bath at 80 °C to kill non-spore forming bacteria [15].

Table 1. Location of samples collection and bacterial count in water and sediment samples. (Colonies numbers were evaluated based on 10^{-6} and 10^{-4} dilutions for sediments and water samples, respectively)

Location	Total samples	Sediment samples		Water samples	
		Number	Cfu/10^{-6}	Number	Cfu/10^{-4}
fish aquaculture tanks at The Marine Science Center	4	2	35	2	22
Khor Al Zubair Port	10	5	19	5	12
Umm Qasr Port	17	8	27	9	15
Abu Flus Port	4	2	18	2	10
Abu Alkhasib	8	4	43	4	28
Fao Port	2	1	15	1	7
Siba	2	1	29	1	13
Well water (Zubair oil field area)	1			1	
Well water (PS1 oil field area)	1			1	
Well water (Artawi area)	1			1	

2.1.1. Sediment samples

Samples were collected (20 g per sample) using sterile dry polyethylene containers. 1 g of each sample was added to a test tube containing 9 ml of distilled water and series of decimal dilutions were created from each sample until $1x10^{-6}$ using distilled water [16]. $1x10^{-1}$, $1x10^{-3}$, $1x10^{-6}$ dilutions were filtered using 0.45 µm Millipore filter paper, the filter

papers were placed into Luria Bertani (LB) agar petri dishes and the plates were incubated at 35 °C for 18 hr. and diagnosed after incubation.

2.1.2. Water samples

250 ml water samples were collected using sterile Nalgen polycarbonate conical flasks (Merck, Germany). In sterile conditions, 1 ml of each sample was added to 9 ml of sterile distilled water and this step was repeated to achieve decimal dilution series (1×10^{-1} - 1×10^{-4}). After that, $1x10^{-3}$ and $1x10^{-4}$ dilutions were grown on the same environment for sediment samples and diagnosed [16].

2.1.3. Bacterial identification

Bacterial colonies of sediment and water samples were inspected under microscope with gram dye and gram-positive spore-forming bacilli were selected for later diagnosis by VITEK® 2 BCL card (bioMérieux, France). Furthermore, bacterial DNA was extracted using Wizard® Genomic DNA Purification Kit, and PCR amplification of the gyrase enzyme gene subunit A (*gyrA*) was carried out to distinguish *Bacillus* species [17]. The amplification of (1025 bp) amplicons of the *gyrA* subunit where used as a differential marker of *Bacillus* spp. using the following primers:

gyrA-f (5'-CAGTCAGGAAATGCGTACGTCCTT-3') and *gyrA*-r (5'-CAAGGTAATGCTCCAGGCATTGCT-3') [18].

Polymerase Chain Reaction (PCR) assay was performed using (Applied Biosystem, USA) thermocycler with GoTaq® G2 Green Master Mix (Promega, USA) and according to the manufacturer recommendations. The primers and components were mixed in the same 25 µl amplification reaction containing1µl primer pair mix, 2 µl DNA sample, 12.5 µl Green Master Mix, and 9.5 µl PCR grade water.

The amplification program consisted in a pre-denaturation phase at 94 °C for 2 min followed by 40 cycles: Denaturation 94°C for 30 sec, Annealing 51°C for 45 sec, Extension 72°C for 60 sec and Final Extension 72°C for 7 min. Finally, A 1% agarose gel electrophoresis for PCR reactions were used to distinguish between *B. subtilis* and other *Bacillus* spp. bacteria.

Biofilm-forming bacteria were isolated using the specific medium for each bacterial species which are Staphylococcus 110 agar; *Pseudomonas* isolation agar, Endo agar, Nutrient agar, and Mannitol salt agar then, were biochemically diagnosed using VITEK®GP & GN cards.

2.2. Secondary metabolites production, extraction, and purification

2.2.1. Secondary metabolites production

Secondary metabolites for *Bacillus* spp. were produced according to the method of [19]. Bacterial isolates were grown for 48 h at 30°C on a shaker at 120 rpm.

The produced secondary metabolites were extracted. According to [20], Ethyl acetate was added to the bacterial culture in 1: 1 ratio and the mixture was stirred on magnetic stirrer for six hours. After that, the samples were centrifuged at 5000 rpm for 10 minutes. Ethyl acetate layer was collected and dried in a rotary evaporator at 50 °C and the residual was dissolved in ethyl alcohol.

2.2.2. Thin layer chromatography (TLC)

A thin layer chromatography (TLC) assay was performed for the extracted metabolites using silica gel sheets (Silica gel 60 F 254 20 x 20 cm gel thickness: 0.25mm, Merck) with a moving phase consisting of (ethyl alcohol: acetic acid: distilled water) by ratios of (v: v: v 17:38:50) in accordance to [21]. Retention factor (Rf) was calculated using the following formula:

Rf = (distance travelled by the sample) / (distance travelled by solvent)

2.2.3. Total proteins

Total proteins were determined using BIOLABO Total Protein Biuret method kit (Biolabo, France) and following the instructions of the manufacturer. Absorptivity for the samples and standard was recorded using a UV-1800

Spectrophotometer (Shimadzu - Japan) at 550 nm and total proteins in the samples were estimated using the following equation:

Total proteins in the sample = (sample absorbance) / (standard sample absorbance) x standard concentration

Two samples with the highest total protein content were chosen for purification steps using Ion exchange chromatography (IEC) and gel filtration chromatography (GFC) according to [12].

2.2.4. Ion exchange chromatography (IEC)

Ion exchange chromatography was carried out according to [12]. (BS8) and (BS14) samples were added to the DEAE cellulose column (25x 3 cm) which was calibrated in phosphate buffer (100 mM, pH7.0). The column was then subjected to three volume wash with the same buffer so unbound proteins separate from the sample. Then, an increased volume of NaCl in the original phosphate buffer (until 100% NaCl) was added to separate the bound proteins from the column. Flow absorbance was continuously observed at 280 nm.

2.2.5. Gel filtration chromatography (GFC)

The bound part from the DEAE Cellulose column for each sample was collected and applied to a (Sephadex S-25) column eluted with the same previous phosphate buffer with a flow rate of 1 ml/min and calibrated with potassium dichromate and dextran blue. Absorbance was observed at 280 nm to record absorbance peak [12].

2.2.6. SDS-PAGE

SDS-PAGE for the purified extracts was performed according to [22]. After sample electrophoresis, the gel was stained with Coomassie Blue for a whole night and the dye was removed using a solution of (acetic acid: distilled water: methanol) (4:5:1 v:v:v).

2.3. (GC-MS) diagnosis of secondary metabolites

The active molecules in metabolites were determined using GC-MS [23]. An Agilent 78908/5977A GC-MS detector equipped with 30m × 250µm × 0.25µm Hp-5MS capillary column filled with adsorption phase of (5% phenyl, 95% dimethylpolysiloxane). Mass detector was operated on EI mode using helium as carrier gas at a flow rate of 1 ml/min. The injector was operated at 290 °C and the oven temperature was programmed as follows: the initial temperature was 70 °C for 2 minutes and then gradually increased by 16 °C/min until 250 °C. The compounds were diagnosed based on the comparison of their mass spectra with the 2014 NIST Library.

2.4. Chemotaxis between secondary metabolic extracts and target bacteria

The nature of the chemotaxis between purified secondary metabolites (BS8 and BS14) against targeted bacterial species was tested. The tested targeted bacteria were *Pseudomonas aeruginosa*, *Escherichia coli*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Staphylococcus sciuri*, and *Kocuria kristinae* by solid propagation method [24]. In brief, 0.1 ml of liquid bacterial cultures of the previously mentioned species were spread on agar based medium and incubated at 35 °C for 15 min. The medium were pierced with cork-borer and the formed holes were filled with 50 µl of the crude (without purification) or purified metabolite extract (after each purification step). Three replications were carried out for each extract X targeted bacteria combination. Petri dishes were incubated for 18 hours at 35 °C, and inhibition diameters (mm) were then measured using Vernier caliper.

2.5. Statistical analysis

To compare chemotactic effect means, collected data were subjected to analysis of Revised Least Significant Difference (RLSD) using IBM SPSS Statistic software 24 at a probability level of 0.05.

3. Results and discussion

3.1. Bacteria isolation and diagnosis

3.1.1. Isolation and identification of *Bacillus* spp.

The diagnosis using VITEK® 2 BCL card showed 20 bacterial isolates of the genus *Bacillus* spp. in the collected samples as shown in (Table 2), the diagnostic results showed that 15 isolates belonged to *Bacillus subtilis*, while 5 isolates were diagnosed as *Bacillus amyloliquefaciens*. Furthermore, PCR (*gyr*A) amplification results were positive in *B. subtilis* isolates and negative in *B. amyloliquefaciens* isolates (Fig. 1 A) which refers to the possibility of using this primer pair reported by [17] as an efficient marker to distinguish *B. subtilis* from *B. amyloliquefaciens*; however, this method might be limited to some *B. amyloliquefaciens* strains.

Table 2. Sources of the *Bacillus* spp. Isolates used in the experiment.

Isolate Number	Location	Sample Type	Bacterial Specie
BS1	fish aquaculture tanks at The Marine Science Center	Water Sample	*B. amyloliquefaciens*
BS2	fish aquaculture tanks at The Marine Science Center	Water Sample	*B. amyloliquefaciens*
BS3	Khor Al Zubair Port	Sediment Sample	*B. subtilis*
BS4	Umm Qasr Port	Sediment Sample	*B. subtilis*
BS5	Khor Al Zubair Port	Sediment Sample	*B. subtilis*
BS6	Khor Al Zubair Port	Sediment Sample	*B. subtilis*
BS7	Abu Alkhasib	Water Sample	*B. subtilis*
BS8	Umm Qasr Port	Sediment Sample	*B. subtilis*
BS9	Umm Qasr Port	Sediment Sample	*B. subtilis*
BS10	Fao Port	Sediment Sample	*B. subtilis*
BS11	Fao Port	Sediment Sample	*B. amyloliquefaciens*
BS12	Umm Qasr Port	Sediment Sample	*B. subtilis*
BS13	Umm Qasr Port	Sediment Sample	*B. amyloliquefaciens*
BS14	Umm Qasr Port	Sediment Sample	*B. subtilis*
BS15	Umm Qasr Port	Water Sample	*B. subtilis*
BS16	Umm Qasr Port	Water Sample	*B. subtilis*
BS17	Umm Qasr Port	Water Sample	*B. amyloliquefaciens*
BS18	Umm Qasr Port	Water Sample	*B. subtilis*
BS19	Umm Qasr Port	Water Sample	*B. subtilis*
BS20	Umm Qasr Port	Water Sample	*B. subtilis*

3.1.2. Isolation and identification of target bacteria (biofilm-forming bacteria)

Five biofilm-forming bacterial isolates were diagnosed and isolated using the VITEK GP diagnostic kit for gram-positive isolates and the VITEK GN card for gram-negative isolates respectively and by testing their ability to form biofilms by growth on the specific medium for each bacterial species. (Table 3) shows the results of the

Table 3. Sources of biofilm-forming bacterial species used as targeted bacteria in the study.

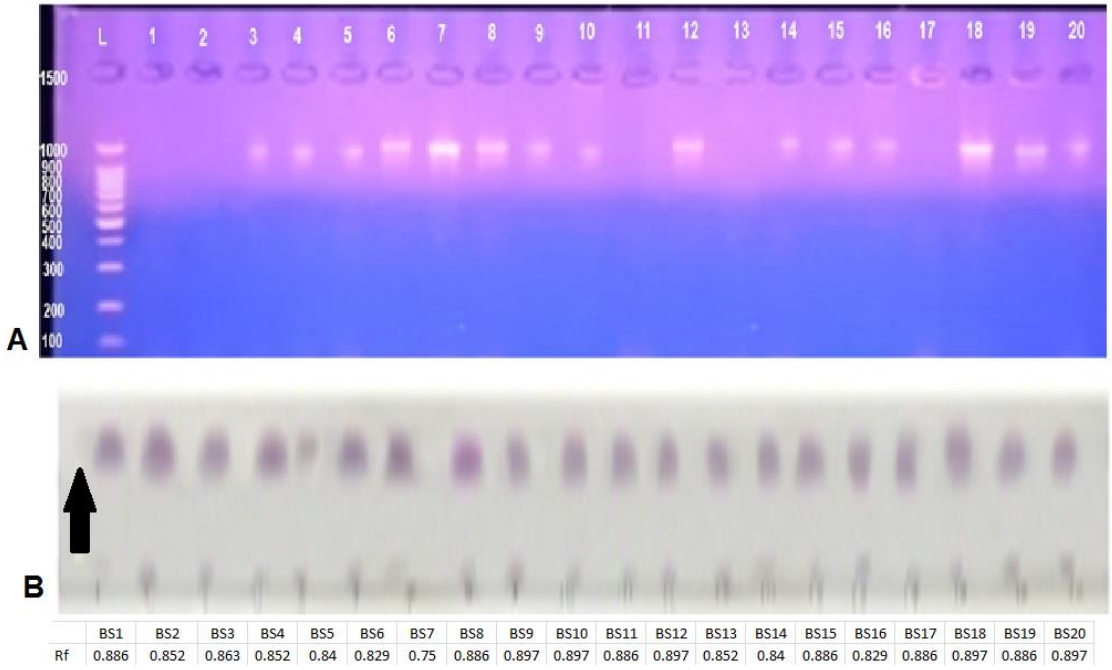
Location	Sample Type	Bacterial Specie
Umm Qasr Port	Sediment Sample	*Staphylococcus sciuri*
Umm Qasr Port	Sediment Sample	MRSA
Abu Alkhasib	Water Sample	*Pseudomonas aeruginosa*
Fao Port	Sediment Sample	*Escherichia coli*
Siba	Sediment Sample	*Kocuria kristinae*

diagnosis of target bacterial isolates which agrees with [25] results of these species being able to form biofilms in different environments.

3.2. Secondary metabolites production and extraction of *Bacillus* spp.

In our study, the selection of secondary metabolite production conditions, especially time, was based on the sheer number of previous studies [26][27][28]. All these works reported that the time required to grow *Bacillus* spp. species for the purpose of producing secondary metabolites ranged from 24-72 hours. [29] stated that it is 48 hours after growth when the bacterial culture entered the idiophase of stationary phase, and with the decrease of bacterial growth rate, secondary metabolite production begins. This production is usually interpreted as the result of main nutrients consumption such as carbon, nitrogen or phosphorus [30].

In fact, previous studies reported the use of several solvents to extract secondary metabolites from *Bacillus* bacteria such as N-hexane, dichloromethane, chloroform, methanol, dimethyl sulfoxide (DMSO), and ethyl acetate [23][29][31]. However, secondary metabolites extracted by ethyl acetate showed better antagonistic activity against target *Bacillus* species compared to metabolites extracted with other organic solvents. This is consistent with the findings in the current study where *Bacillus* secondary metabolites showed clear inhibitory activity against both negative and positive Gram target species.



	BS1	BS2	BS3	BS4	BS5	BS6	BS7	BS8	BS9	BS10	BS11	BS12	BS13	BS14	BS15	BS16	BS17	BS18	BS19	BS20
Rf	0.886	0.852	0.863	0.852	0.84	0.829	0.75	0.886	0.897	0.897	0.886	0.897	0.852	0.84	0.886	0.829	0.886	0.897	0.886	0.897

Figure 1. (A) Gel electrophoresis for the gene "*gyrA*" PCR amplification of bacterial isolates DNA, Positive amplifications of single (1025 bp) bands are noticed for *B. subtilis* isolates while negative results without amplifications belong to *B. amyloliquefaciens* isolates, in comparison to L: 1kb DNA ladder **(B) Rf results of TLC for secondary metabolites extracts of *Bacillus* spp.** The samples numbered 1-20 represent the 20 different bacterial isolates.

3.3. Secondary metabolites extracts inspection and purification

3.3.1. Thin layer chromatography (TLC)

The results of thin layer chromatography indicated the appearance of purple spots when using the ninhydrin reagent indicating that the secondary metabolites of *Bacillus* bacteria contained amino acids as shown in (Fig. 1 B) which also shows Rf values of *Bacillus* secondary metabolites extracts. These values were similar and ranged between (0.750 - 0.897) which is similar to surfactin Rf (0.7), and in accordance with [32] reports of similar Rf values due to the similarity in molecular weights of extracts.

3.3.2. Total protein content in secondary metabolites extracts

The results in (Table 4) indicated that the highest value of total proteins in the secondary metabolites extracts belonged to the isolate (BS8) with 13.78 g/l followed by isolate (BS14) with 12.02 g/l while the lowest value was found in (BS19) with 5.28 g/l. Based on these results and TLC results, both (BS8) and (BS14) extracts were chosen for purification and further chemotaxis inspection.

Table 4. Total proteins content in *Bacillus* secondary metabolites extracts.

Sample number	Absorbency at 550 nm	total protein (g/l)
blank	**0.133**	
standard	**0.843**	
BA1	1.483	10.55
BA2	1.376	9.79
BS3	1.511	10.75
BS4	1.387	9.87
BS5	1.345	9.57
BS6	1.523	10.83
BS7	1.496	10.64
BS8	1.937	13.78
BS9	1.189	8.46
BS10	1.376	9.79
BA11	1.253	8.91
BS12	0.937	6.67
BA13	1.388	9.88
BS14	1.69	12.02
BS15	1.207	8.59
BS16	0.904	6.43
BA17	1.15	8.18
BS18	0.894	36. 6
BS19	0.742	5.28
BS20	0.972	6.91

3.3.3. Ion Exchange Chromatography (IEC)

It is obvious that IEC for (BS8) and (BS14) samples resulted in two peaked graphs when absorbance collected at 280 nanometres (Fig. 2 A and B respectively). The first IEC peak refers to positive charged and neutral particles, including antimicrobial peptides, which are separated in the first phase, while the second peak refers to the negatively charged particles which are separated in the second phase [12]. IEC stage is crucial in extracts purification due to its high effectiveness in removing the pigments that contaminate the sample and to separate the sample effective phase from the non-effective phase [12][14][33].

3.3.4. Gel Filtration Chromatography (GFC)

GFC was carried out using Sephadex gel S-25, due to the desirable features of this type of gel such as high absorption and separation capacity in addition to its simple preparation procedure and the possibility of re-activation and reuse for several times. GFC for (BS8) and (BS14) samples resulted in a single peaked chart when observed in 280 nm (Fig. 2 C and D respectively) which is consistent with [12][34] results which also referred to the increased inhibitory activity of secondary metabolites GFC purified extracts against target *Bacillus* species compared to the crude extracts.

3.3.5. SDS-PAGE of secondary metabolite extracts

SDS-PAGE showed that there was one clear peptide band with molecular weights of 694 Dalton and 3779 for (BS14) and (BS8) purified extract respectively (Fig. 2 E), indicating the efficiency of the methods used in extracting and purifying secondary metabolites. Similar results were obtained by [14] who stated that peptides extracted from *Bacillus subtilis* appear aggregated in SDS-PAGE gel which is a common characteristic of peptides that possess tensile properties such as surfactin, which has high cleaning and inhibitory abilities against many bacterial species, which qualifies these peptides to be natural alternatives to chemicals used to treat a number of environmental problems, including biofilm-forming bacteria.

3.3.6. Gas chromatography mass spectroscopy (GC-MS)

GC-MS results showed that purified secondary metabolites extracts include amino acids and their derivatives, esters, hydrocarbons and other compounds (Tables 5 and 6), (TIC scans can be noted in Supplementary material, Supplementary Figs. 1 and 2) many of which have antimicrobial properties such as type D-amino acids [35], N-cyclopropyl carbonyl-[36], and esters (d-proline [37], N-methoxycarbonyl [38], and pentyl ester [39]). Our results are in agreement with study conducted by [40]. In fact, these bioactive extracts were previously described as promising biological control agents for numerous environmental problems caused by biofilm-forming bacteria [41]. Furthermore, type D-amino acids indirectly inhibit the formation of biofilms by interfering with protein synthesis [42]. Interestingly, GC-MS profile for the purified (BS8) extract was more diverse when compared to (BS14) purified extract. We suggest that many metabolites of (BS14) crude extracts were reduced by purification procedure [12] which might explain the few components remaining in GC-MS profile. Additionally, the significant difference between

(BS8) and (BS14) GC-MS profiles was previously reported between different bacterial strains of *Bacillus and other bacterial species* [43][44][45].

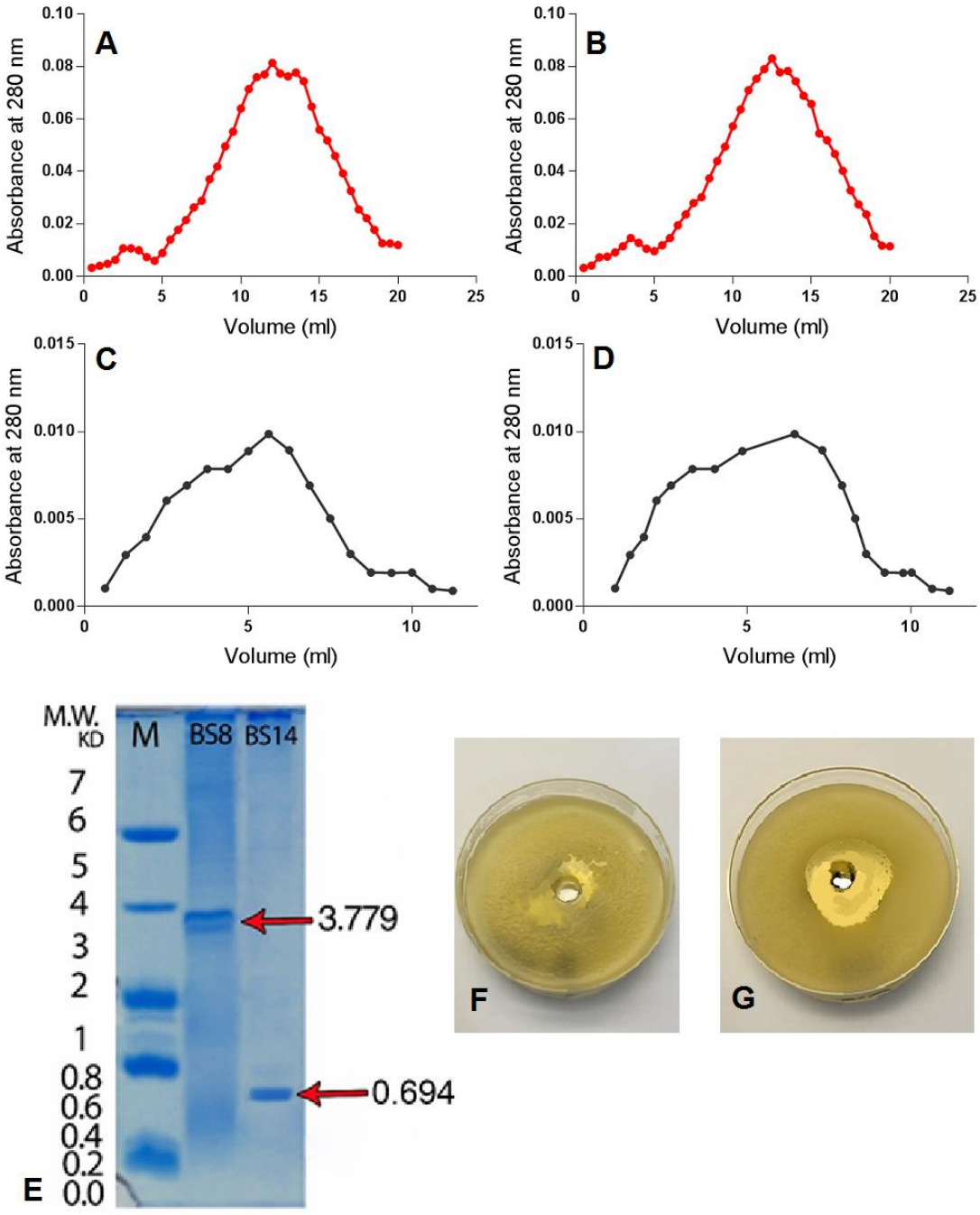


Figure 2. (A) and **(B)** The elution profile of proteins from the DEAE column eluted with100 mM phosphate buffer (pH 7.0) for (BS8) and (BS14) samples respectively. First peaks represent the unbound (effective) compounds, while the second peaks refer to the bound positively charged (un-effective) compounds. **(C)** and **(D)** The elution profiles of purified protein from the Sephadex S-200 column eluted with 100 mM phosphate buffer (pH7.0) for (BS8) and (BS14) samples respectively. **(E)** SDS-PAGE for the purified extracts compared with an aprotinin polypeptide marker with a molecular weight of 6.512 kDa. **(F)** and **(G)** Antimicrobial effect of the purified secondary metabolites extracts **(F)** BS14 extraction on *P. aeruginosa* **(G)** BS8 extract on *K. kristinae*

3.4. Chemotaxis between purified secondary metabolic extracts and target bacteria

The results showed a clear negative chemotactic effect of the purified secondary metabolites extracts of (BS8) and (BS14) against target bacteria. Transparent halos free of target bacterial growth were formed around the wells containing the secondary metabolites of the isolates. *K. kristinae* which was the most susceptible species against the purified extracts with 23 and 24 mm for (BS8) and (BS14) extracts respectively, while *P. aeruginosa* was the least susceptible with 19 and 18 mm for (BS8) and (BS14) extracts respectively (Table 7), (Fig. 2 F and G). Growth inhibition diameters results showed a significant increase in both extracts inhibitory effect after IEC purification and subsequently after GFC purification (Table 7), which indicates the increased efficiency of bacterial extracts using these purification methods. The reaction of targeted bacteria was previously identified as spatial redistribution where the bacteria tends to grow heavily in the places were secondary metabolites were not present [46].

Table 5. Bioactive compounds identified from GC–MS chromatogram of *B. subtilis* (BS8)

RT[a]	Name	Formula	Hits(DB)
13.644	2-Amino-2-methyl-1,3-propanediol	C4H11NO2	6
15.015	Phenol, 4-(2-aminoethyl)-	C8H11NO	10
15.471	1,3,5-Trioxane	C3H6O3	2
15.71	2H-Thiopyran, 5,6-dihydro-	C5H8S	5
15.902	Diethylene glycol, O,O-di(pivaloyl)-	C14H26O5	1
16.276	Pyrrole, 2-methyl-5-phenyl-	C11H11N	10
16.586	Phenethylamine, N-benzyl-p-chloro-	C15H16ClN	10
16.983	1-(1'-pyrrolidinyl)-2-butanone	C8H15NO	10
17.375	2-Cyclohexen-1-one, 3,5-dimethyl-	C8H12O	3
17.699	3-Ethoxy-4-methoxyphenol	C9H12O3	10
18.041	1H-Pyrazolo[3,4-d]pyrimidin-4-amine	C5H5N5	10
18.162	2-Cyclopenten-1-one, 2-hydroxy-3,4-dimethyl-	C7H10O2	10
18.328	Indolizine, 5-methyl-	C9H9N	10
18.569	Methyl-6-deoxy-6-fluoro-2,3,4-tri-O-methyl.beta.d-galactopyranoside	C10H19FO5	2
18.863	l-Norvaline, n-propargyloxycarbonyl-, nonylester	C18H31NO4	10
19.114	1-Propanone, 1-(5-methyl-2-thienyl)-	C8H10OS	1
19.228	Pentadecanoic acid, ethylester	C17H34O2	3
19.314	Ethyl 13-methyl-tetradecanoate	C17H34O2	10
19.855	l-Leucine, N-cyclopropylcarbonyl-, butyl	C14H25NO3	10
20.021	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	C11H18N2O2	10
20.165	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-	C14H22N2O2	2
20.234	d]pyrazineHeptanoic acid, 2-ethyl-	C9H18O2	10
20.417	Ethyl 9-hexadecenoate	C18H34O2	10
20.465	4-Fluorobenzylamine, N,N-diheptyl-	C21H36FN	4
20.628	Hexadecanoic acid, ethylester	C18H36O2	10
21.679	9-Octadecen-1-ol, (E)-	C18H36O	10
23.066	9-Octadecenoic acid, ethylester	C20H38O2	10
25.111	Phthalic acid, di(6-methylhept-2-yl) ester	C24H38O4	10
25.128	[1,2,4]Triazolo[1,5-a]pyrimidin-7-ol, 5-methyl-	C6H6N4O	5
27.46	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C14H16N2O2	2

a. RT stands for retention time for the component peak

Table 6. Bioactive compounds identified from GC–MS chromatogram of *B. subtilis* (BS14)

RT[a]	Name	Formula	Hits(DB)
10.843	d-Proline, N-methoxycarbonyl-, pentyl ester	C12H21NO4	10
20.362	6-Ethyl-4,5,7,8-tetrathiaundecane	C9H20S4	1

a. RT stands for retention time for the component peak

Table 7. Growth inhibition diameter induced by bacterial secondary metabolites crude extracts (without purification) and after Ion Exchange Chromatography (IEC) and Gel Filtration Chromatography (GFC) purification steps

Type	Sample	Growth inhibition diameters (mm)[a]				
		P. aeruginosa	*E. coli*	MRSA	*K. kristinae*	*S. sciuri*
Crude extract	BS8	8	9	11	11	12
	BS14	7	8	8	10	11
IEC	BS8	16	17	19	22	20
	BS14	14	16	20	22	19
GFC	BS8	19	19	22	23	21
	BS14	18	20	22	24	23

a. These numbers represent average read of three replications RLSD (0.05) = 0.46

4. Conclusions

The Iraqi marine environment represents a promising source of bacterial isolates producing various biologically active substances. The rapid growth of bacteria, the ease of growth requirements, and low production costs of bacterial secondary metabolites, might render them as an economic substitute for antibiotics to face the many environmental problems caused by bio-film forming bacteria. Furthermore, a significant increase in bacterial extracts inhibitory by the implementation of simple purification methods such as IEC and GFC.

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