RESEARCH ARTICLE

Antibacterial, Antibiofilm and Anticancer Activities of Ethyl Acetate Extract of *Bacillus spp*.

Haneen A. I.[1], Neihaya H. Z.[1] Ali Alhammar[2]

[1]*Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq*
[2]*Biotechnology Research Center, AL-Nahrain University, Baghdad, Iraq*

Received: 29th August, 2021, Revised: 18th October, 2021, Accepted: 3rd November, 2021, Available Online: 25th December, 2021

ABSTRACT

Bacillus spp. can produce a wide range of metabolites with important biological activities and extensively used in many applications**.** About 40 soil samples were collected from different gardens in Baghdad city, and only 52 bacterial isolates were identified. Thirty-eight (73.1 %) of isolates were *Bacillus spp***.** which were**:** *Bacillus cereus* (21; 55.3%), *Bacillus subtilis* (11; 28.9%), and *Bacillus amyloliquefaciens* (6; 15.8%)**.** B1 and B16 isolates belong to *B. cereus* and *B. subtilis*, respectively, were selected to perform the subsequent tests. Biofilm formation varied among the tested pathogenic bacteria. Fourier transform infrared spectroscopy (FTIR) analysis of *B1* and *B*16 ethyl acetate extracts showed functional groups of secondary metabolites with major peaks belong to COOH, C-H, O-H CO-N, and N-H stretches. While, gas chromatography–mass spectrometry (GC-MS) analysis of *B*1 and *B*16 extracts revealed multiple active compounds (cyclopentan, hexane, phenol, 2,2-methylenebis, L-proline, propanoic acid, oxalic acid-isobutyl pentyl ester, pyrrolo pyridazine and octadecenoic acid). Interestingly, B16 and B1 extracts showed high activity against *Staphylococcus aureus* and *S. pyogens*, and moderate activity against *Klebsiella spp*., *Escherichia coli,* and *Serratia marcescens* with no effect on *Pseudomonas aeruginosa*. Antibiofilm activity of B16 and B1 extracts showed high inhibition against *Streptococcus pyogenes* and moderate on *S. aureus, E. coli* and *K. spp.*, while low effect on *S. marcescs* and no effect on *P. aeruginosa*. Anticancer activity was also observed by exposing ethyl acetate extract, produced by *B*1 and *B16* against breast cancer cell line MCF7 at different concentrations.

Keywords: Antibacterial, Antibiofilm, Anticancer activity, *Bacillus spp.,* Ethyl acetate extract.

International Journal of Drug Delivery Technology (2021); DOI: 10.25258/ijddt.11.4.24

How to cite this article: Haneen AI, Neihaya HZ, Alhammar A. Antibacterial, Antibiofilm and Anticancer Activities of Ethyl Acetate Extract of *Bacillus spp*. International Journal of Drug Delivery Technology. 2021;11(4):1262-1268.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Bacillus genus are commonly found in soil and represent a wide range of physiological abilities which allow them to grow in every environment and compete desirably with other organisms within same environment because it can form extremely resistant spores and produce metabolites with antagonistic effects on other microorganisms.[1] *B. subtilis* is the most commercially important bacteria, used for the formation of a range of metabolites (amino acids, vitamins and antibiotics) and industrial enzymes.[2] *B. cereus* is probiotic bacteria and its consumption helps the host to keep intestinal microbial balance, improve bowel regularity, reduce the number of pathogens, restore normal intestinal microbiota and reduce the level of carcinogens.[3]

B. subtilis have higher concentrations of acetic acid, 3-methyl-butanoic acid ethyl ester, 2-methyl-propanoic acid ethyl ester, 3-methyl-1-butanol, and benzyl alcohol, 2-methyl-butanoic acid, 3-methyl-butanoic acid, 2-methyl-propanoic acid and compounds such as hexanal,2-pentylfuran, 1-hexanol, produced by all the *Bacillus* strains.[4] *B. cereus* produced the highest level of nitrogen-containing compounds. The condiment fermented with *B. licheniformis* had the highest level of ketones, usually termed with butter-like aroma.[5] However, all *Bacillus* strains formed aceto-phenone is characterized by sweet and floral odours.[6]

The compounds include 2-butanone-4-hydroxy-3-methyl, cycloserine, isobutyl acetate, ethanol 2-dodecyloxy, phenol, 2,4-bis (1,1-dimethylethyl) heptadecane, 2, 6, 10, 15-tetramethyl, hentriacontane, oxalic acid, 6-ethyloct-3-Yl Hep, Sulfurous Acid and others have one or more activity as antimicrobial, anticancer, antibacterial, antifungal, antitumor, antioxidant, anti-inflammatory, and antiviral.[7] Cancer is a most dangerous disease that threatens human life in various parts of the world, considered the second causes of death in the world after heart disease. Breast cancer is the second cancer effect on people worldwide and the most common cancer among females.[8] So, the study aimed to extract and determine the active compounds from *Bacillus* spp. isolated from soil, and study the effect of their extract against some pathogens, and determining their antibiofilm and anticancer properties.

**Author for Correspondence:* *neihaya_2008@yahoo.com*

MATERIALS AND METHODS

Collection of Samples

Approximately 4 gram of soil (from 40 samples) were collected from gardens in Baghdad city at a depth of 5 to 10 cm under soil surface with sterile spatula and put in sterile polythene bags, all of them were transferred to the laboratory, and kept at 4ºC.[9]

Bacterial Isolation

Serial dilutions were made by mixing 1 gram of the soil sample with 9 mL of distilled water to make 10^{-1} and so on till 10^{-6} dilution. Subsequently, 0.1 mL was taken from each dilution, and streaked on Nutrient agar (Oxide- England) and Tryptic soy agar (Oxide-England), then incubated at 37°C for 24 hours.[10]

Bacillus spp. Diagnosis

It's primarily depended on cultural characteristics, microscopic examination, and biochemical tests.[11] Then it's confirmed by VITEK 2 System (Gallenkamp-England).

Preparation of Cell Free Supernatant

Bacterial isolates (B1 and B16) cultured in Nutrient broth and incubated for 24 hours at 37ºC under shaking at 150 rpm. Then, cell free supernatant (CFS) was obtained by centrifugation at 10,000 rpm for 15 minutes at 4ºC according to other researches[12] with some modification.

Pathogenic Bacteria

Tested pathogenic bacteria were obtained from laboratories of the post-graduate in Biology Department, College of Science, Mustansiriyah University, Iraq. Bacterial isolates were: *S. aureus, S. pyogenes, K.* spp., *P. aeruginosa, E. coli,* and *Serratia marcescens.*

Biofilm Formation

Biofilm quantification was determined by crystal violet assay.[13] Briefly, fresh culture was suspended in 5 mL of NaCl solution, and 20 µL of this solution were transported to each well of the microplate. Then, 180 µL of Brain Heart Infusion Broth (Hi–media, India) with 2% sucrose added to each well and incubated at 37ºC for 24 hours. After incubation, the plate was washed with 0.9% (w/v) NaCl for three times, and 200 µL of absolute ethyl alcohol were added for 20 minutes. The plate was dried at room temperature for 30 minutes, then 200 µL of 0.5% crystal violet were added for 15 minutes. After eliminating the dye solution and washing with sterile distilled water, the attached dye was solubilized with 0.5% (v/v) ethanol and O.D was determined by using a microtiter plate reader at 560 nm (BioTek- USA). The biofilm formation was considered for $OD_{560} \geq 0.1$, on a scale of weak formation ($0.1 \leq OD_{560} > 0.2$) and medium formation ($0.2 \leq OD_{560} > 0.5$).[14]

Extraction of Metabolites from *Bacillus* spp. by Using Ethyl Acetate

Cell free supernatant (CFS) of isolates were mixed with ethyl acetate at volume (50:50) and stirred with a magnetic stirrer for 6 hours. The upper organic layer was isolated with a separating funnel and centrifuged at 5000 rpm for 10 minutes. The ethyl acetate layer transmitted into a clean flask and the extract was pooled and dried at room temperature. The yield from the extract was resolved in ethanol 95% for antibacterial test.[1]

Fourier Transform Infrared (FTIR) Analysis

The extract was dried, and then determined in infrared apparatus (Perkin-Elmer) to demonstrate the surface functional groups. The samples with KBr pellets were made in 1 mm thickness and 10 to 13 mm diameter. The samples were measured in the spectral range of 4000 to 400 cm^{-1}.[15].

Gas Chromatography-Mass Spectrum (GC-MS) Analysis

To determine the active compounds from bacterial extract, samples was applied using GC-MS apparatus (Shimadzu Thermo- India) with column TG-SQC; 15 m x 0.25 mm × 0.25 µm. Helium was used as the transporter gas and the detector temperature was 280°C. Active compounds were recognized by comparing their mass with standards of National Institute of Standards and Technology (NIST) library.[16]

Antibacterial Activity of Ethyl Acetate Extract

Agar well diffusion assay was used for this test as mentioned in biofilm formation except 100 µL of extract was loaded into the wells, and blank well was filled with ethanol 95% as negative control. The inhibitory zones (mm) were measured after completing the incubation period.[17]

Antibiofilm Activity of the Extracts

Biofilm inhibition assay as mentioned in other researches,[18] except adding 100 µL of the extract to each well, and sterile distilled water were used as negative control. The absorbance of the plates were measured at 450 nm in an ELISA reader (Human, Germany). The amount of inhibition and disruption were calculated using the following equation:[19]

$$\% \text{ inhibition} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Anticancer Activity of the Extract *In-vitro*

Preparation and Maintenance the Cell Lines

According to some research,[20] the breast cancer (MCF-7) cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), (100 U) 20 µg/mL penicillin, and 100 µg/mL streptomycin. Incubation was conducted at 37°C with an atmosphere of 5% CO2. Normal breast (MCF-7) cells were cultured in RPMI 1964. The cells were monitored daily to form a confluent monolayer. Sub culturing was established by discarding the growth incubated cells. Followed by washing the cells with sterile PBS under a septic conditions. Trypsin-versine solution (2–3 mL) was added to the confluent monolayer and incubated at 37ºC for 2 to 3 minutes until they became rounded and separated into single cells with gentle shaking. Then 20 mL of the culture medium supplemented with 10% fetal calf serum were added to the flask in order to make cell suspension.

Anticancer Assay

The breast cancer cell-line (MCF7) was routinely cultured in 25 mL flask and incubated under standard conditions

(37ºC in a humid chamber supplemented with 5% CO_2). Cytotoxicity effect of various concentrations of the extract on the proliferation of the adherent cells was studied in 96-well Microtiter.[21] The extract was sterilized by filtration through 0.22 µm membrane filter and diluted starting with (1000, 100, 10, and 1.0) µg/mL under aseptic conditions. The cell suspension of the cancer cell line was prepared as mentioned above. A cell suspension of 200 µL containing 1×10^5 cell/mL was added to each well and incubated at 37ºC for 24 hours until the cells reached 60 to 70% confluence. The following day, the medium was removed and 200 µL of various concentrations of crude extract were added to the cells with the use of 3 well replicates for each concentration. The control involved cancer cells without extract treatment instead were treated with 200 µL of serum free medium. While reaching to the end of incubation time, the extract and medium were removed from the plate and washed with PBS to eliminate dead cells. MTT solution was made by dissolving 5 mg MTT crystals in 1-mL of PBS solution. The MTT solution (10 µL) was added to each well of 96-well plate, then incubated for 4 hours at 37ºC. After that all the solution added to the 96-well plate, including media was removed. Then, 50 µL of di-methyl-sulf-oxide (DMSO) was added onto each well and were shaken for around 5 minutes till colorless DMSO solution becomes purple. After full dye's solubilization, the colored solution's absorbance, derived from living cells was measured at 570 nm with the help of ELISA reader (Expert Plus reader; Asys Hitech GmbH, Eugendorf, Austria). The viability percentage was calculated using the formula below:

$$Viability\ \% = \frac{A\ test - A\ blank}{A\ control - A\ blank} \times 100$$

where A represents absorbance.

Statistical Analysis

The obtained data was run through analysis of variance (ANOVA) test, followed by student's t-test to compare various groups. Results were expressed as mean + standard error of the mean and the $p>0.05$ values were considered statistically non-significant. While *p-values* <0.05 and <0.01, 0.001, 0.0001 were considered significantly and highly significantly different, respectively. Lysergic acid diethylamide (LSD) was conducted by Statistical Product and Service Solutions (SPSS).[22]

RESULTS AND DISCUSSION

Isolation and Identification of Bacteria

Among 52 bacterial isolates from soil, only 38 isolates (73.1%) belong to *Bacillus spp.* distributed as *B. cereus* 21 (55.3%), followed by *B. subtilis* 11 (28.9%), and *B. amyloliquefaciens* was 6 (15.8%) (Figure 1). The identification of *Bacillus spp.* was done by cultural characteristics, microscopic identification, and biochemical tests, and then confirmed by using the VITEK 2. *Bacillus* is the most abundant genus in the rhizosphere of soil, as a soil bacteria that aggressively colonizes plant roots, and benefits plants by providing natural growth promotion.[23]

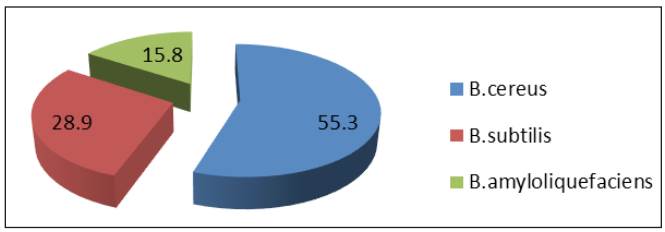

Figure 1: The percentage of *Bacillus* sp. isolated from soil

Biofilm Formation Assay by Tested Bacteria

Biofilm formation was evaluated using crystal violet. Biofilm formation differs between the tested pathogenic bacteria, and *S. pyogenes* and *P. aeruginosa* had high values, while *Klebsilla* spp, *E. coli* and *S. aureus* had moderate biofilm formation values. *S. marcescens* showed weak biofilm formation value (Table 1). Crystal violet binds negatively charged molecules on the cell surface, polysaccharides, and nucleic acids. In result, it allowed measuring the whole biofilm.[24]

Extraction of Metabolites by Ethyl acetate

The extract of *B. subtilis* and *B. cereus* isolates (B1 and B16) showed three layers, which are from top to bottom: transparent, turbid and yellow layer with precipitation cells (Figure 2). The organic phase was collected and dried, then used for further experiments such as FTIR, GC-Mass and physiological activities.

FTIR Analysis

Functional groups of secondary metabolites produced by *B. cereus* and *B. subtilis* (B1 and B2 isolates) extracts were identified by The FTIR as summarized in (Table 2).

Table 1: Biofilm formation by pathogenic bacteria as measured by crystal violet

Pathogenic bacteria	*Biofilm formation*
S. aureus	++*
S. pyogenes	+++
E. coli	++
P. aeruginosa	+++
Klebsiella spp.	++
S. marcescens	+

* +++ =High biofilm, ++ =Moderate,+=Weak

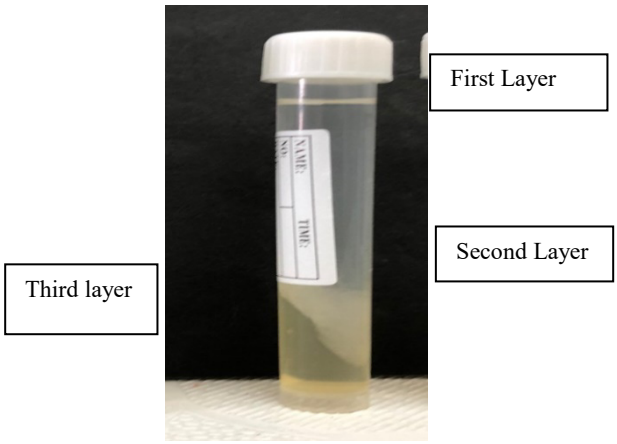

Figure 2: Crude extract with ethyl acetate solvent

The major peaks were 1660.77 to 1344.43 cm^{-1} belong to COOH stretch, 2916.47 to 2955.04 cm^{-1} to C-H, (3223.16-3306.10) cm^{-1} to O-H stretch, 1653.05 cm^{-1} to CO-N stretch, (1516.10–1545.03) cm^{-1} to N-H combined bond with (C-H) to form (amid II band) stretch were indicated to presence peptide component, (2850.88- 2956.97) cm^{-1} to CH as shown in (Figure 3 and 4). These results for both *Bacillus* spp. were similar to findings by (25, 26, and 7).

GCMS Analysis

GC-MS analysis showed active compounds from ethyl acetate extract of *B. subtilis* and *B. cereus* as shown in Figure 5 and 6. These compounds were cyclopentan, 1,3-dimethyl-cis ,Hexan, 3-methyl, Phenol, 2,2-methylenebis, L-Proline,N-Valerly-, pentadecyl ester, Propanoic acid, 2-methyl, Nonane, 2-methyl, Oxalic acid-isobutyl pentyl ester, Methyl ricinoleate, 1-Trifluorosilyltridecane and two major bioactive compounds available in the extracellular non-protein fraction were, fatty acid ester namely Octadecenoic acid, methyl ester could be determined in phosphatidylcholin (lecithin) isolated from the cell membrane of the Gram-positive bacterium *B. subtilis* (Table 3 and 4). This bacterium produces a cyclopeptide surfactin that is one of the most potent natural antibiotics known have antimicrobial and anti-carcinogenic activity, and pyrrolo (1,2- a) pyridazine was found to have cytotoxicity against human cancer cell line.[27] Some previous researches[28] have reported thareported that Phenol, 2,2-methylenebis have antifungal, antimicrobial and antioxidant effects. The Oxalic acid has antimicrobial and anti-inflammatory properties.[29]

Antibacterial Activity of Ethyl Acetate Extract

The activity of the *B. subtilis* (B16) and *B.cereus* (B1) extracts measured on some pathogenic bacteria and showed high activity against Gram positive bacteria (*S. aureus* and *S. pyogens*) (Figure 7), and moderate activity against Gram negative bacteria *(K. spp*., *E. coli,* and *S. marcescens*), and no activity against *P. aeruginosa* (Table 5). *B. spp.* is considered as extremely useful microorganisms for producing antibacterial agents.[17] Several studies reported that secondary metabolites produced from *B. spp.* were more effective against Gram positive bacteria. The resistance of Gram negative bacteria to these secondary metabolites can be subjected to the low permeability of the outer membrane and the lipopolysaccharide barrier for these organisms (Table 6).[30]

Table 2: Functional groups for B. cereus and B. subtilis analyzed by FTIR

B. cereus			*B. subtilis*		
Peak value	*Assignment and intensity*	*Functional groups*	*Peak value*	*Assignment and intensity*	*Functional groups*
1456.3	C-C stretch	Alkenes	1454.38	C-C stretch	Alkenes
1516.10	N-H bend	Amines	1516.10	N-H bend	Amines
1730.21	C=O stretch	Carboxylic acid	1730.21	C=O stretch	Carboxylic acid
3223.16	O-H stretch	Alcohol, phenols	3225.09	O-H stretch	Alcohol, phenols

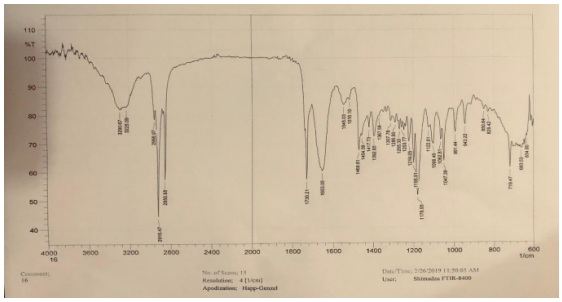

Figure 3: FTIR spectrum of *B. subtilis* (B16) extract

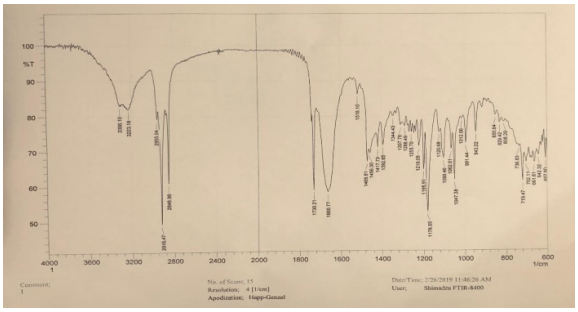

Figure 4: FTIR spectrum of *B. cereus* (B1) extract

Figure 5: GC-MS chromatogram of *B. subtilis* ethyl-acetate extract

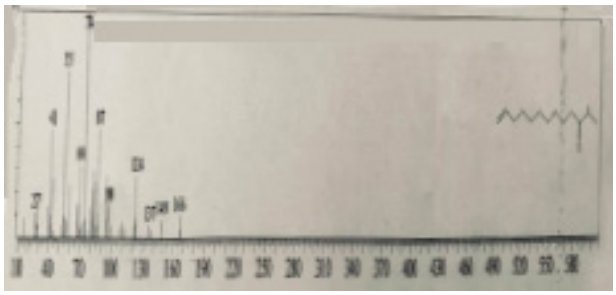

Figure 6: GC-MS chromatogram of *B. subtilis* thyl-acetate extract

Table 3: Major compounds identified from *B. subtilis* (B16) extract by GCMS

S. NO	*-p*	*Name of the compound*	*Molecular weight*	*Molecular formula*
1	2.06	Cyclopentan, 1,3-dimethyl-cis	98	C_7H_{14}
2	2.10	Hexan, 3-methyl	100	C_7H_{16}
3	19.10	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methy lpropyl)	210	$C_{11}H_{18}N_2O_2$
4	22.50	13-Octadecanoic acid, methyl ester	296	$C_{19}H_{36}O_2$
5	23.72	Phenol, 2,2-methylenebis	340	$C_{23}H_{32}O_2$
6	19.07	L-Proline,N-Valerly-,pentadecyl ester	409	$C_{25}H_{47}NO_3$
7	23.70	Propanoic acid, 2-methyl	410	$C_{27}H_{38}O_3$
8	6.28	Nonane, 2-methyl	142	$C_{10}H_{22}$

Table 4: Major compounds identified from *B. cereus* (B1) extract by GCMS

S. NO	*RT(min)*	*Name of the compound*	*Molecular weight*	*Molecular formula*
1	2.03	Cyclopentan, 1,3-dimethyl-cis	98	C_7H_{14}
2	2.10	Hexan, 3-methyl	100	C_7H_{16}
3	19.10	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methy lpropyl)	210	$C_{11}H_{18}N_2O_2$
4	22.50	Trans-13-Octadecanoic acid,methyl ester	296	$C_{19}H_{36}O_2$
5	23.72	Phenol, 2,2-methylenebis	340	$C_{23}H_{32}O_2$
6	19.07	L-Proline,N-Valerly-,pentadecyl ester	409	$C_{25}H_{47}NO_3$
7	6.28	Oxalic acid, isobutyl pentyl ester	216	$C_{11}H_{20}O_4$
8	23.70	Propanoic acid, 2-methyl	410	$C_{27}H_{38}O_3$
9	22.50	Methyl ricinoleate	312	$C_{19}H_{36}O_3$
10	7.99	1-Trifluorosilyltridecane	268	$C_{13}H_{27}F_3Si$

Table 5: Activity of *B. cereus* and *B. subtilis* extract on pathogenic bacteria

Pathogenic bacteria	*Extract of B. cereus*	*Extract of B. subtilis*	*Antibiotic control (mm)*	
	Inhibition zones (mm)			
S.aureus	23	22	CTX_{30}:14	FEP_{30}:15
S.pyogenes	18	17	IMP_{10}:14	ATM_{30}: -
E.coli	13	9	AK_{30}:12	FEP_{30}:11
P. aeruginosa	_	_	IMP_{10}:10	AK_{30}:11
Klebsiella spp.	12	14	CTX_{30}:14	IMP_{10}:11
S. marcescens	12	13	IMP_{10}:11	FEP_{30}:12

Some researches[31] suggested that the outer membrane of Gram-negative bacteria keep on its inner membrane, and so higher doses of proteins are required for inhibition of their growth. The variation in antimicrobial activity has been attributed to the genetic profile of different bacterial strains.[32]

Antibiofilm Activity of the Extract

The ratio of biofilm inhibition of ethyl acetate extract differs between the pathogenic bacteria as shown here. High inhibition was showed on *S.aureus, S. pyogens*, and *E.coli,* while moderate inhibition against *P. aeruginosa,* and *Klebsiella spp.*, but showed low effect on biofilm formation by *S. marcescens* (Table 6).

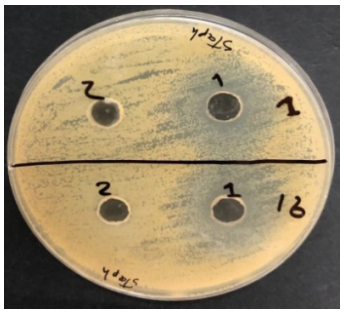

Figure 7: Antibacterial activity of *Bacillus* isolates (1 and 16) extracts against: 1- *S. aureus*, 2-control

Table 6: The ratio of antibiofilm effect of *B. cereus* and *B. subtilis* extracts

	Antibiofilm %	
Pathogenic bacteria	*Extract of B. cereus*	*Extract of B. subtilis*
S. aureus	70.2	66.7
S. pyogens	55.5	59.3
E. coli	53.8	46.2
P. aeruginosa	45.8	37.5
Klebsiella spp.	31.6	36.8
S. marcescens	7.4	2.9

Table 7: Activity of *B. cereus* and *B. subtilis* extracts on the breast cancer cell line

	Concentration (µg/mL)								
Extract type / *% cell survival*	*1 µg* *Mean ± SE*		*10 µg* *Mean ± SE*		*100 µg* *Mean ± SE*		*1000 µg* *Mean ± SE*		*F ratio* *(p value)*
Extract of *Bacillus cereus*	A 75.7	1.47	74.9	6.6	A 48.8	6.4	B 84.5	19.12	2.11 (0.178)
Extract of *Bacillus subtilis*	86.4	4.2	88.6	15.5	A 63.8	4.9	B 91.9	7.8	1.78 (0.2)
F ratio (p value)	3.6(0.04)*		1.8(0.2)		11.2(0.001)**		5.45(0.01)**		—

A, B, C: LSD for column and a ,b ,c: LSD for rows. *: Significant; **: High significant; Similar letters mean no significant differences between the comparative mean.

The reduction in cell metabolism happens because most antimicrobial peptides work by interacting with the bacterial cell surface, followed by disruption of cellular integrity.[33]

Anticancer Effect of Ethyl Acetate Extract on Breast Cancer Cell Line

B.cereus and *B. subtilis* extracts were used to evaluate their anti-cancer activity against the breast cancer cell line MCF7. Interestingly, and the extract at concentration 100µg/mL was effective against MCF7 cells for each *B.cereus* (48.8%) and *B. subtilis* (63.8%), moreover,1000 µg/mL concentration inhibited growth up to 84.5% and 91.9%. respectively (Table 7). Results of (Table 7) reported that *B. subtilis* was found to express eicosane, pentacosane, phthalic acid etc. These compounds all together were cytotoxic against MCF-7 at 150 µg/mL and 300 µg/mL respectively in MTT assay, and *B. cereus* with free extracts exerted significant cytotoxicity on human cervical cancer cell line (HeLa) and breast cancer cell line (MCF-7) with IC50 of 150 µg/mL and 300 µg/mL respectively in MTT assay showing reduced growth and apoptosis.[34]

CONCLUSIONS

B. cereus constitutes the highest percentage among the *Bacillus spp.* isolated from soil, followed by *B. subtilis*. FTIR and GCMS analysis of ethyl acetate extract showed different types of functional groups of secondary metabolites produced by *B. cereus* and *B. subtilis.* The extracts were more active against Gram positive than Gram negative pathogenic bacteria. Also, these extracts had the highest inhibition on breast cancer cell line MCF7. Hence, it is possible to apply these active compounds for antibacterial, antifungal, and antiviral activities in different fields, along with their anticancer effects against different types of cancer.

ACKNOWLEDGMENT

The authors would like to thank Al-Mustansiriyah University (www.uomustansiriyah.edu.iq), and Biotechnology research center at Al-Nahrain University (http://brc.nahrainuniv.edu.iq/), Baghdad, Iraq for the support of this work.

REFERENCES

1. Amin M, Rakhisi Z, Ahmady A (2015) Isolation and Identification of *Bacillus* Species From Soil and Evaluation of Their Antibacterial Properties. Clin Microb Infec, 2015;2(1):1-4.
2. Harwood C, *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. Trends Biotechnol, 1992;10: 247-256.
3. Reid G, Jass J, Sebulsky MT, McCormick JK, Potential uses of probiotics in clinical practice. Clin. Microbiol. Rev. 2003;16(4): 658-672.
4. Sugawara E, Ito T, Odagiri S, Kubota K, Kobayashi A, Comparison of Compositions of Odor Components of Natto and Cooked Soybeans. Agr Biol Chem, 1985;49(2):311-317.
5. Owens JD, Allagheny N, Kipping G, Ames JM, Formation of Volatile Compounds during *Bacillus subtilis* fermentation of Soya Beans. J. Sci. Food Agric., 1997;74(1):132-140.
6. Jirovetz L, Smith D, Buchbauer G, Aroma Compound Analysis of Eruca sativa (Brassicaceae) SPME Headspace Leaf Samples Using GC, GC–MS, and Olfactometry. J. Agric. Food Chem. 2002;50(16):4643-4646.

7. Seerangaraj V, KannanSuruli VU, Meganathan B, Seerangaraj V, Selvam S, Selvaraj J, Isolation and Characterization of Bioactive Compounds from *Bacillus cereus* and *Bacillus subtilis* from Oreochromis mossambicus and Labeo rohita. Int. J. Pharm. Sci., 2017;43(2):71-77.
8. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. J. Thorac. Dis. 2017;9(3):448.
9. Avşar C, Koyuncu H, Aras ES. Isolation and molecular characterization of Bacillus spp. isolated from soil for production of industrial enzymes. Biological and Chemical Research. 2017;3(9):72-86.
10. Singh P, Sharma R, Shukla AK, Singh R, Isolation of *Bacillus spp*. from Soil for Antimicrobial Production and Antibiotic Resistance. Adv Biotech. Micro, 2018;8(4):1-5.
11. MacFaddin JF. Biochemical tests for identification of medical bacteria. 2000;912.
12. Suganthi C, Mageswari A, Karthikeyan S, Anbalagan M, Sivakumar A, Gothandam KM. Screening and optimization of protease production from a halotolerant Bacillus licheniformis isolated from saltern sediments. J Genet Eng Biotechnol 2013;11(1):47-52.
13. Haneen A, Neihaya H, The Biological Activity of Protein Extracts of *Bacillus* spp. isolated from Soil against some pathogenic bacteria. Mustansiriyah J. of Science, 2019;30(4):29-38.
14. Neihaya HZ, Zaman HH. Investigating the effect of biosynthesized silver nanoparticles as antibiofilm on bacterial clinical isolates. Microb. Pathog. 2018;116:200-208.
15. Kummer A, Saini P, Shrivastara JN, Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. *Ind* . J. Expres, 2009;47:57-62.
16. Adeleye IA, Omadime ME, Daniels FV. Antimicrobial activity of essential oil and extracts of Gongronema latifolium Decne on bacterial isolates from blood stream of HIV infected patients. J. Pharmacol. Toxicol. 2011;6(3):312-320.
17. Amin A, Khan M , Ehsanullah M, Haroon U, Azam F, Hameed A, Production of peptide antibiotics by *Bacillus spp.* GU 057 indigenously isolated from saline soil. Braz. J. Microbiol. 2012;43(4):1340–1346.
18. Costa GA, Rossatto FP, Medeiros AW, Correa AF, Brandlli A, Frazzon AG, and Damotta AS, Evaluation antibacterial and antibiofilm activity of the antimicrobial peptide P34 against *Staphylococcus aureus* and *Enterococcus faecalis* Anais da Academia Brasileira de Ciências, 2018;90(1):73-84.
19. Stepanović S, Vuković D, Hola V, Bonaventura GD, Djukić S, Ćirković I, Ruzicka F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Apmis. 2007;115(8):891-899.
20. Freshney RI. Culture of animal cells: a manual of basic technique and specialized applications. John Wiley & Sons; 2015 Dec 23.
21. Betancur- Galvis LA, Morales GE, Forero JE, Roldan J, Cytotoxic and Antiviral Activities of Colombian Medicinal Plant Extracts of the Euphorbia genus. Mem. Inst. Oswaldo. Cruz, 2002;94:541-546.
22. SAS , Statistical Analysis System, User's Guide. Statistical, Version 9th ed. SAS, Inst. Inc. Cary. N.C, USA (2012).
23. Gutiérrez MF, Probanza A, Ramos B, Colón JJ, Lucas García JA, Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR). J. Plant Nutr. 2003;26(5):1101-1115.
24. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH, Kjelleberg S, Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae.* Proc Nat Acad Sci, 2005;102:16819-16824.
25. Salem SH, El-Sheikh HH, Naguib MM, Heikal YA, Potential antimicrobial activity of the antagonistic Bacillus strains to *Saccharomyces cerevisiae*. J. innov. pharm., 2015;2(4):632-645.
26. Parthiban K, Vignesh V, Thirumurugan R, Characterization and in vitro studies on anticancer activity of exopolymer of *Bacillus thuringens* S13. African Journal of Biotechnology, 2014;13(21): 2137-2144.
27. Nanthini M, Chamundeeswari M, Seethalakshmi R, Rachel LF, Sreekumar G, Cytotoxic activity of probiotic *Bacillus subtilis* SK09 against human colon cancer cell line (HT-29). Int. Res. J. Pharm., 2017;8(4).
28. Rangel Sánchez G, Avocado roots treated with salicylic acid produce phenol-2,4-bis (1,1-dimethylethyl), a compound with antifungal activity. J .Plant Physiol, 2014;171(3-4):189-198.
29. Premlata S, Krishan KM, Padma K, Gas Chromatography-Mass Spectrometric Analysis of acetone Extract of *Cenchrus Ciliaris* (Dhaman Grass). IJSN, 2015;6(4):652-661.
30. Wiener MC, Horanyi PS. How hydrophobic molecules traverse the outer membranes of Gram-negative bacteria. Proceedings of the National Academy of Sciences. 2011 Jul 5;108(27):10929-10030.
31. Prudêncio CV, Dos Santos MT, Vanetti MC. Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology. Journal of food science and technology. 2015 Sep;52(9):5408-5417.
32. Sharma G, Dang S, Gupta S, Gabrani R. Antibacterial activity, cytotoxicity, and the mechanism of action of bacteriocin from Bacillus subtilis GAS101. Med Princ Pract. 2018;27(2): 186-192.
33. Nawrocki KL, Crispell EK, McBride SM. Antimicrobial peptide resistance mechanisms of gram-positive bacteria. Antibiotics. 2014;3(4):461-492.
34. Nemudzivhadi V, Masoko P. In vitro assessment of cytotoxicity, antioxidant, and anti-inflammatory activities of Ricinus communis (Euphorbiaceae) leaf extracts. Evidence-Based Complementary and Alternative Medicine. 2014 Jan 1;2014.