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BIOACTIVE SECONDARY METABOLITES PRODUCTION BY TERRESTRIAL *BACILLUS CIRCULANS* STRAINS

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ABSTRACT

Screening for antagonistic microorganisms is a promising tool for discovering new antimicrobial metabolites. In this study, two *Bacillus* sp. (HU6 and HU8) were isolated from soil and assessed for their antimicrobial activity by the agar-well diffusion method against different human pathogens. According to general characteristics, morphology, and physio-chemical properties of the isolates they were identified as *Bacillus circulans*. Both strains exhibited a broad spectrum of activity against Gram positive and Gram negative pathogenic bacteria. The highest antimicrobial activity was achieved from ethyl acetate extract of *B. circulans* HU6 against *E. coli* where the inhibition zone was 26.3 ± 0.82 mm. The bioactivity of the isolates extract was not affected when subjected to heat treatment or proteolytic digestion suggesting that the nature of the active metabolites was not proteinaceous. The GC/MS analysis of the extracts revealed the presence of 4 bioactive compounds: Pyrrolo[1,2-a]pyrazine-1,4-dione, Trans-13-octadecenoic acid, 2,5-piperazine dione,3,6-bis (2-methyl propyl), and cis-13-eicosenoic acid.

Keywords: Antimicrobial activity; *Bacillus circulans*; Ethyl-acetate extract; GC/MS; Soil bacteria

INTRODUCTION

The emergence of pathogenic bacteria represents a growing threat to human health and is given an impact attention to search for new drugs from different sources. Novel approaches for the development of new antibiotics such as combinatory chemistry tools have been pursued along with the discovery of new compounds from microorganisms isolated from extreme environments. For example, polypeptide antibiotics produced by *Bacillus* species are gaining much importance **(Usta and Demirkan 2013)**. It reported that *Bacillus subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans* and *B. Cereus* are the most widley studied *Bacillus* species for the production of antibiotics **(Kaspar et al. 2019)**. In a matter of fact, *Bacillus* sp. are known to produce many antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins **(Abriouel et al. 2011; Shahid et al. 2021)**. Moreover, *Bacillus* bacteriocins are increasingly becoming more important due to broad spectra of inhibition.

Arid environments in Jordan are wide and the prevailing climate is dry and hot during summer and very cold during winter with rain in the form of thunder showers. These environments favor the prevalence of stress surviving microorganisms. Little attention has been paid for the screening and isolation of new antibiotic producers from the arid regions of Jordan. **El-Banna et al. (2007)** succeeded to extract antimicrobial substances (of a high activity against Methicillin Resistant *S. aureus*) from different *Bacillus* sp. isolated from various Jordanian regions. Whereas, **Saadoun et al. (2008)** were able to isolate 161 different *Streptomyces* isolates from soil samples representing different habitats of north Jordan. These were then characterized and assessed for their antagonistic activity against four clinical multi-drug resistant *Pseudomonas aeruginosa* test pathogens. On the other hand, **Al-Saraireh et al. (2015)** isolated a *Bacillus* spp. from soil with an antibacterial activity against selected Gram positive pathogens. Recently, we succeeded to isolate 4 *Bacillus* spp. that have a broad spectrum of activity against Gram positive and negative pathogens **(Massadeh and Mahmoud 2019)**. Subsequently, **Al-Turk et al. (2020)** reported the production of 2 active compounds that were produced by 2 *strains of B. licheniformis* isolated from the same location. Therefore, this study aimed to further screen the area of the Hashemite University attempting to produce antimicrobial metabolites from new strains of *Bacillus* spp. isolated from soil samples.

MATERIALS AND METHODS

Isolation and identification of *Bacillus* strains

Strains of *Bacillus* spp. were isolated from soil samples collected from different regions around the Hashemite University Campus **(Massadeh and Mahmoud 2019)**. The isolates were identified to the genus level by observing their morphology and biochemical reactions according to the methods described by **Brawn (2004)** and **Garrity et al. (2001)**. Further identification of the isolates was performed using Microgen *Bacillus* ID kits (Microgen bioproducts, UK) provided with Microgen identification system software (MID-60) which are based on the fermentation of 12 different sugars and other advanced biochemical tests.

Cultivation of strains and extract preparation

Each isolate was cultivated in an Erlenmeyer flask containing nutrient broth (NB). The flasks were incubated at 30 ˚C in an incubator shaker (human lab, Korea) running at 100 rpm for 3 days. After incubation was completed, the cultures were centrifuged at 6000 rpm for 15 min (Wagtek centrifuge, UK) and the supernatant was preserved as cell free extract. The bioactive constituents of the extracts were recovered and concentrated by the addition of ethylacetate to the cell-free extract in 1:1 ratio and the mixture was agitated for 2 h at 100 rpm speed. Thereafter, the layer of ethylacetate was collected and dried using rotary evaporator. The recovered extract was resuspended in 50 mM sodium phosphate buffer of pH 6.8 **(Berić et al. 2013)**.

Screening for Antimicrobial activity

The antimicrobial activity of the isolates was tested by the agar well diffusion method against the following pathogens that were obtained from the Clinical department, Faculty of Allied Sciences; The Hashemite University: *Streptococcus pneumonia* ATCC 6303, *Staphylococcus aureus* ATCC 11632, *Proteus mirabilis* ATCC 12453, *Proteus vulgaris* ATCC 33420, *Klebsiella oxytoca* ATCC 13883, *K. pneumoniae* ATCC 10536, *Escherichia coli* ATCC 10145, *Pseudomonas aeruginosa* ATCC 29737, *Enterobacter aerogenes* ATCC 13047 and *Salmonella* sp. Group B ATCC 15611. For each pathogen, a suspension of 0.1 ml taken from a culture having an optical density (OD) value of 0.5 at 550 nm was transferred to a sterile bottle containing 20 ml of freshly prepared NA (cooled to a temperature of 40 °C). The inoculated NA medium was poured into a Petri-dish. After the agar was solidified, wells of 6 mm in diameter were punched in the agar plates. This was followed by the addition of 40 µl of the ethyl acetate extract representing each

isolate's extract filled into the wells of the agar plates directly. The petri-dishes were kept at 4°C for 2 h and then incubated at 37 ˚C for 24 h. The diameter of the inhibition zones was measured in mm. Streptomycin, amoxicillin and bacitracin were used as positive control while sterile sodium phosphate buffer was used as negative control **(Umer et al. 2013)**.

Time profile for antimicrobial activity of isolates

To assess the antimicrobial activity of *B. circulans* HU6 and *B. circulans* HU8 throughout their growth stages, each strain was cultivated in a shake culture as previously mentioned for 120 h. Every 24 h, a whole flask was taken to prepare a fresh ethylacetate extract to perform the antibacterial activity test against the test pathogens (*E. coli* and *S. pneumonia* in case of HU6; *S. aureus* and *P. aeruginosa* in case of HU8) in the agar well diffusion method. The OD_{550} of HU6 and HU8 cultures was recorded as an indicator of their growth **(Boottanun et al. 2017)**.

Extract concentration vs. pathogens growth

The effect of ethylacetate extract concentration of each isolated strain on pathogens growth was studied by adding different concentrations of ethyl acetate extract of each isolate (50 µl, 100 µl, 500 µl, and 1000 µl) to a 20 ml of nutrient broth pre-inoculated with 0.1 ml of pathogen's broth culture (OD_{550} = 0.5). Thereafter, the culture was incubated for 24 h at 37°C in incubator shaker running at 150 rpm. After incubation was completed, the optical density (OD_{550}) of this culture was recorded. An extract-free culture of each pathogen was employed as a control **(Sopirala et al. 2010)**.

Characterization of antimicrobial metabolites

Proteolytic digestion

The ethylacetate extract of both isolates was subjected to proteolytic enzymes susceptibility test using 100 mg/ml of proteinase K. the samples were incubated overnight at 56°C. After incubation, the samples were examined for antimicrobial activity by the agar well diffusion method and a crude extract sample without enzymatic treatment was employed as a negative control (Boottanun et al. 2017).

Heat stability test

The extracts of both isolates were subjected to heat at 80°C for 1 hr and then tested for antimicrobial activity against selected pathogens employing the agar well diffusion method **(Al-Turk et al. 2020)**.

Analysis of extracts by Gas chromatography/Mass spectrometry (GC-MS)

The ethylacetate extract of each isolate was introduced to GC-MS analysis for possible identification of the antimicrobial compounds present in each extract. This was achieved by injecting 1 µl sample into an Agilent DB-5 MS column (30 m × 0.25 mm) connected to Agilent 6890 GC system/5973 MS detector. Helium gas was used a carrier gas at a flowrate of 1 ml/min. a temperature gradient program was applied at 80 °C for 2 min followed by a temperature elevation to reach 500 °C. The resulting m/z peaks (mass to charge ratio) were compared with mass spectrum library of corresponding organic compounds by chemistation system **(Melo et al. 2014)**.

Statistical Analysis

The data was statistically analyzed using ANOVA (analysis of variance) and Tukey test was applied to test the significance at $P \leq 0.05$ (Tukey 1949). The significant differences among the values were expressed as letters. Standard errors among the replicates were represented by bars on the figures and ± in the tables.

RESULTS AND DISCUSSION

The area of the Hashemite University (HU) was chosen for the possible isolation of antibiotic producing *Bacillus* spp. due to its unique arid climate and closure to the main petrol refinery company and many heavy industries. The environmental conditions (temperature, drought salinity, etc.) plays a role in increasing the availability of antimicrobial metabolites from the inhabiting microorganisms. These stress conditions may affect the metabolism of these organisms causes enhancement in their defense mechanisms and enzymatic systems to adapt to such environments. **Lancini and Prrenti (1982)** claimed that the inhabitance must compete for water and essential nutrients required for microbial growth.

Isolation and identification of antagonistic strains

In a previous study attempted to screen the area of the Hashemite University (HU) for antibiotic producing microorganisms, we succeeded to isolate 3 *Bacillus* sp. namely: *B. stearothermophilus*, *B. firmus*, and *B. circulans*. The isolates were able to inhibit the growth of a wide range of pathogens **(Massadeh and Mahmoud 2019)**. Thereafter, we collected new soil samples and succeeded to isolate another 3 strains of *Bacillus* sp. namely: *B. subtilis* and two *B. licheniformis* **(Al-Turk et al. 2020)**. However, in this study we collected new samples from new regions around the HU campus for possible isolation and screening of new *Bacillus* sp. capable of producing antimicrobial metabolites.

Among the huge number of bacteria isolated from soil samples collected, 7 bacterial colonies showed antagonism against their accompanying microbial community in NA Petri-dishes. The isolates were purified by transferring the colonies into new NA plates. Several subcultures were performed to finally get pure colonies of each isolate. The preliminary characterization of the isolates showed that the isolates belongs to *Bacillus* genus since the cells were rod shaped, Gram positive, mobile, spore forming under aerobic conditions, and catalase positive. After conducting the in vitro primary screening for antimicrobial activity of each isolate against test pathogens, two isolates designated as *Bacillus* sp. HU6 and *Bacillus* sp. HU8 that exhibited a wide range of activity were taken for further identification using Microgen *Bacillus* ID kits and antimicrobial activity tests. The results of studying the morphoogical and physio-chemical features of the 2 isolates are presented in Table 1. Both isolates were very similar as they were able to utilize different carbon sources and able too grow at 50 °C. The major differences between the 2 isolates are represented by the optimum temperature and pH for growth (Table 1).

Table 1 Morphological and Biochemical activities of the Studied *B. circulans* strains

Indicators	Result	
	Bacillus sp. HU6	*Bacillus* sp. HU8
Gram´s reaction	+	+
Cell shape	rod	rod
Spore formation	+	+
Motility	+	+
Catalase production	+	+
Glucose fermantation / acid	+	+
Starch hydrolysis	+	+
Oxygen requirements	+	+
Benzidine reaction	+	+
Citrate Utilization	-	-
Voges-Proskauer Test	-	-
Growth at 50˚ C	+	+
Growth at 60˚ C	-	-
Optimun Temperature for growth	28°C	31°C
Optimum pH for growth	6.5-7	7-8

The analysis of the results using Microgen *Bacillus* identification system software (MID-60) revealed that the *Bacillus* isolates stay much closer to *B. circulans*. *Bacillus* sp. HU6 was identified as *B. circulans* by a 96% of probability and the most closeset species was *B. subtilis* by a 72% of probability. While *Bacillus* sp. HU8 was identified as *B. circulans* by a 94% of probability and the most closest species was *B. sphaericus* by a 51% of probability. Overall, *Bacillus* sp. HU6 and *Bacillus* sp. HU8 were identified and recorded as *B. circulans* HU6 and *B. circulans* HU8.

Antimicrobial activity of *B. circulans* HU6 and *B. circulans* HU8

The antimicrobial activity of *B. circulans* HU6 and *B. circulans* HU8 was studied against referenced 2 Gram-positive and 8 Gram negative bacteria (Table 2). The extracts of both strains showed the ability to inhibit the growth of many pathogens in the agar well diffusion method. The ethyl acetate extract of *B. circulans* HU6 has an inhibitory effect against all test pathogens except *E. aerogenes*, *P. vulgaris*, and *Salmonella* sp. While, *B. circulans* HU8 showed an inhibitory effect against all test pathogens except *K. pneumonia*, *P. vulgaris*, and *Salmonella* sp. The highest antimicrobial activity was achieved from the ethyl acetate extract of *B. circulans* HU6 isolate against *E. coli* where the inhibition zone was 26.3 ± 0.82 mm. On the otherside, the lowest antimicrobial activity achieved was from the ethyl acetate extract of *B. circulans* HU8 isolate 2 against *E. aerogenes* where the inhibition zone was 9.7± 2.08 mm. The antimicrobial action of the 2 strains was bactericidal as the zones of inhibition were very pronounced without visible growth of the test pathogens. As compared to the antibiotic disks used as a control in this experiment, the pathogens were sensitive to the extracts of the 2 strains employed as in most cases there was no significant difference ($P \leq 0.05$). These results are in agreement with many previous studies that claimed that bacteriocin-like substances of *Bacillus* spp. have a broad spectrum of inhibition of bacteria including *E. coli*, *P. aeruginosa*, etc. **(Boottanun et al. 2017; Guo et al. 2012; Motta et al. 2007; Lee et al. 2001)**.

Table 2 Antimicrobial activity of the *B. circulans* isolates (HU6 and HU8) against the test pathogens using the agar well ddiffusion method

Test Pathogen	* Zone of inhibition (mm)				
	B. circulans HU6	*B. circulans* HU8	Cl	C2	C3
S. aureus	21 ± 1.8a	24 ± 0.7a	18 ± 0.51a	20 ± 1.15a	23.66 ± 1.20a
S. pneumonia	23 ± 1.2a	24.5 ± 0.8a	19 ± 0.48a	20 ± 1.06a	23.77 ± 1.20a
E. coli	26.3 ± 0.82b	12.6 ± 1.5b	19 ± 0.57a	20 ± 1.16a	21.66 ± 1.20a
E. aerogenes	NI	9.70 ± 2.08b	18 ± 0.58a	20 ± 1.11a	22.72 ± 1.2a
K. oxytoca	18 ± 0.16a	25.5 ± 1.8b	18 ± 0.58a	20 ± 1.16a	22.76 ± 1.2a
K. pneumonia	10.6 ± 1.2b	NI	19 ± 0.48a	20 ± 1.06a	23.77 ± 1.2a
P. mirabilis	21.3 ± 1.4a	20.2 ± 0.8a	18 ± 0.58a	20 ± 1.16a	22.76 ± 1.2a
P. vulgaris	NI	NI	20 ± 0.68a	21± 1.13a	22.76 ± 1.2a
P. aeruginosa	22 ± 1.9a	17 ± 0.4b	20 ± 0.70a	21± 1.2a	22.76 ± 1.2a
Salmonella sp.	NI	NI	19 ± 0.57a	20 ± 1.16a	21.66 ± 1.20a

*Values represents the maens ± standerd deviation of the mean (SD) of triplicate measurments. P value < 0.05. Similar letters indicate insignificant difference. NI: No inhibition. C1, C2 and C3 are the positive control antibiotic disks (10 µg/disc) representing C1: Streptomycin, C2: Amoxicillin and C3: Bacteriocin.

Antimicrobial activity during growth

Figure 1 represents the antibacterial activity of *B. circulans* HU6 and *B. circulans* HU8 during their growth stages against the test pathogens mentioned. Both isolates entered stationary phase after 72 h as indicated by the OD_{550} values of their culture. Generally, the antagonistic activity of HU6 and HU8 was observed after being cultured for 24 or 48 h against test pathogens and increased with time to achieve the maximum activity after 96 h. These recorded activities were stable with a non-significant decrease until the end of cultivation. The fermentation time needed for the maximal yield of the antimicrobial substances production seems to be similar among the isolates, 72-96 h which is in agreement with our previously published data **(Massadeh and Mahmoud 2019)** employing *Bacillus* sp. isolated from the same area of study and the results of **Zheng and Slavik (1999)**, **Janisiewicz (1988)**, and **El-Banna and Winkelmann (1998)**. Furthermore, this result is in relevance to the secondary metabolites production that can be induced by many factors such as, stress, environmental factors, and cells communication as claimed by **Kleerebezem and Quadri (2001)**.

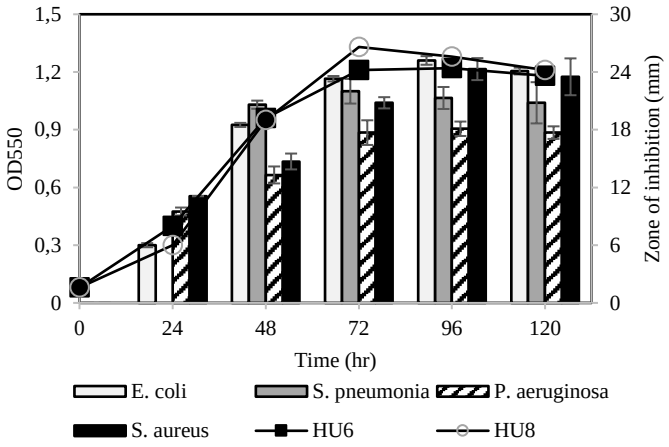


Figure 1 Bioactivity of *B. circulans* HU6 and HU8 throughout their growth stages. Growth of HU6 and HU8 is represented by the OD_{550} of their cultures (straight lines) while the bioactivity is displayed as zones of inhibition in mm against *E. coli* and *S. pneumonia* in case of HU6; *S. aureus* and *P. aeruginosa* in case of HU8. Values represent the means ± standard error of the mean (SE) of triplicate measurement.

Effect of extract concentration on pathogens growth

Ethyl acetate extracts of both *Bacillus circilans* strains (HU6 and HU8) at different concentrations (50-1000 µl/20 ml liquid culture) were examined against *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. pneumonia* grown in liquid media to evaluate its efficiency as a crude antibiotic. As a result, the growth of pathogens was significantly reduced by elevating the concentration of ethyl acetate extract in their culture (Figure 2). The OD_{550} of pathogens culture decreased to more than a half. Noticeably, when a 1000 µl of the ethyl acetate extract was used, the growth of pathogens ceased as the absorbance of the cultures was dropping by the end of incubation time especially in the culture of *E. coli*. These results are in agreement with our previously published findings where the inhibition of pathogens growth was noticed in case of using elevated concentrations of 4 *Bacillus* sp. extracts in their culture **(Massadeh and Mahmoud 2019)**. The inhibition activity was dose dependent as explained by **Boottanun et al. (2017)**, where they claimed that increasing the concentration of the secondary metabolites in the culture of pathogens tested could accelerate they death or cessation of growth.

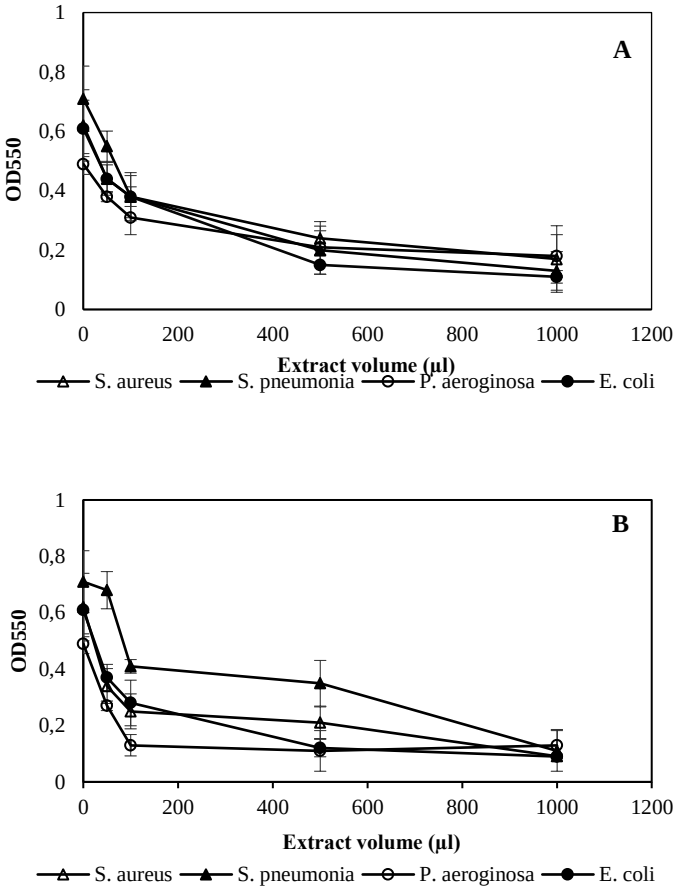


Figure 2 Antimicrobial effect of HU6 (A) and HU8 (B) extract concentration on pathogens growth in nutrient broth media after 24 hr of incubation as represented by the OD_{550} values. Values represent the means ± standard error of the mean (SE) of triplicate measurement.

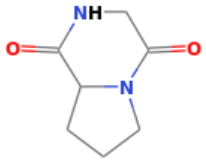
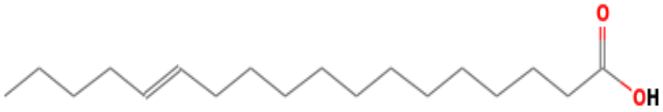
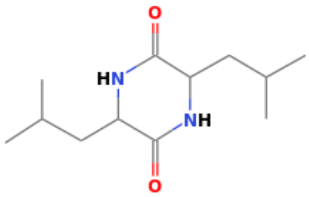
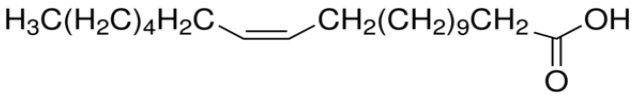
Partial characterization of the extracts metabolites

The ethyl acetate extracts of both *B. circulans* strains were subjected to heat treatment and proteolysis by enzymes. The antibacterial activity of the ethyl acetate extracts of both strains was not reduced even after heating the extracts at 80 C° for 1 h. **Berić et al. (2013)** and **Smitha and Bhat (2013)** reported the thermal stability of many antibacterial compounds isolated from *B. licheniformis* whereas **Maldonado et al. (2009)** claimed that most metabolites of *Bacillus* sp. were proved to be stable at different temperatures which is in agreement with our results. On the other hand, the bioactivity of both extracts was not affected when treated with Proteinase K suggesting that the inhibitory compounds constituting the extracts were not proteinaceous in nature. **Zhao et al. (2013)** reported that the compounds that still active after proteolysis are non-peptide compounds such as lipopeptides or polyketides and these compounds could resist a wide range of temperatures and escape proteolysis.

GC/MS analysis

The chemical analysis of both ethyl acetate extracts by GC/MS indicated the identification of 4 major antimicrobial compounds (Table 3): (1) Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro in both isolates extract with a retention time of 12.397 min. This cyclic peptide was produced by some *Streptomyces* strains and sponge-associated marine bacteria, in addition to some endophytes as well as *B. subtilis* and *B. licheniformis* **(Al-Turk et al. 2020)**. **Ser et al. (2015)** reported the isolation of *Streptomyces* strain MUSC149^T from mangrove soil with a strong antioxidant activity. The chemical analysis for this strain's extract revealed the identification of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro as the antioxidant agent. On the other hand, **Melo et al. (2014)**, identified the presence of this compound in the extract of an Antarctic endophytic fungus exhibiting a strong antibacterial activity against *E. coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Moreover, this compound was produced by a sponge associated bacteria (SAB) that was capable of inhibiting *Vibrio alginolyticus* **(Durai et al. 2013)**. (2) Trans-13-octadecenoic acid detected in both isolates extract with a retention time of 16.127 min. This Trans fatty acid was detected in the culture extract of *B. subtilis* and *B. licheniformis* **(Al-Turk et al. 2020)** and in tuber extracts of *Solena amplexicaulis* plant and has been reported as anti-inflammatory and anticancer compound **(Krishnamoorthy and Subramanium 2014)**. However, **Sunil et al. (2018)** identified this compound as an anti-inflammatory and antimicrobial compound. (3) 2,5-piperazine dione,3,6-bis (2-methyl propyl) which was detected in the extract of *B. circulans* HU8 with a retention time of 16.473. This compound was identified as an antifungal compound based on the study of **Yang and Chang (2010)**. They detected the availability of this compound in the culture of *Lactobacillus plantrum* AF1 and tested its activity against *Aspergillus flavus*. Furthermore, this antifungal compound was also extracted from the culture of *Streptomyces albus* **(Narasaiah et al. 2014)**. (4) cis-13-eicosenoic acid detected in the extract of *B. circulans* HU8 with a retention time of 16.126. This fatty acid belongs to biologically active fatty acids with relevant antibacterial, antifungal, and anticancer activities **(Rahman et al. 2015; Naeim et al. 2020; Xu et al. 2011). Kikukawa et al. (2014)** produced this fatty acid from *Mortierella* Fungi and assessed its biological activity. Furthermore, **Hameed et al. (2008)** extracted Cis-13-Eicosenoic acid and trans-13-Octadecenoic acid from *P. fluorescens* and studied the antifungal activity against *T. horzianum*.

Table 3 GC-MS analysis of the ethyl-acetate extract of the two *Bacillus circulans* strains

Compound	Structure	Retention time
Pyrrolo [1, 2-a] pyrazine-1,4-dione, hexahydro		12.397
Trans-13-octadecenoic acid		16.127
2,5-piperazine dione,3,6-bis (2-methyl propyl)		16.473
Cis-13-Eicosenoic acid		16.126

CONCLUSION

Two *B. circulans* strains (HU6 and HU8) were isolated from soil samples collected from the area of The Hashemite University campus. Both strains were able to produce secondary metabolites that inhibited the growth of Gram positive and negative pathogenic bacteria. The GC/MS analysis revealed the presence of 4 bioactive compounds that could be produced throughout the isolates growth phases.

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