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Antimicrobial Activity of Secondary Metabolites from a Soil *Bacillus* sp. 7B1 Isolated from South Al-Karak, Jordan

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Abstract

Soil is considered as a promising environment for discovering and isolating bacterial strains that are able to produce novel natural products. Therefore, a Gram positive *Bacillus* sp. 7B1 was isolated from a soil sample collected from south Al-Karak, Jordan. The bacterial isolate was cultivated, identified and its culture medium was extracted. The crude extract was purified using silica gel column chromatography. The antibacterial activity of the crude extract and the purified fractions was evaluated by agar diffusion test as well as by measuring the minimum inhibitory concentration in microbroth dilution assay. The produced crude extract was active only against the tested Gram positive bacteria, namely *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus*. Bioactivity-guided fractionations of the resulted crude extract led to the purification of compound ($\mathbf{C_2}$). In the present work, *Micrococcus luteus* was the most susceptible bacterial test strain to compound ($\mathbf{C_2}$) with a minimum inhibitory concentration of 25 µg/ml. Our findings highlighted the importance of soil bacterial isolates for production of compounds with interesting bioactivities that may contribute to the drug research field.

.**Keyword:** *Bacillus*, Antimicrobial, Partial purification, Al-Karak, Jordan.

1. Introduction

Microorganisms are able to synthesize secondary metabolites of various structures and, hence, bioactivities. Their production is regulated by nutrients, growth rate, enzyme inactivation and induction (Demain, 1998). They are produced to help the organism in competing successfully with other organisms in their natural habitat and to adapt with the changed environmental conditions (Teasdale *et al.*, 2008). These metabolites have played a key role in the discovery and development of many antibiotics (Motta *et al.*, 2004). Consequently, a large number of drugs approved for marketing nowadays are of microbial origin (Harvey, 2008; Butler *et al.*, 2014).

Despite the large number and diversity of these metabolites and due to the emergence of new infectious diseases and resistant pathogens that represent a serious problem for human life (Cragg *et al.*, 1997; El-Banna *et al.*, 2007; Disriac *et al.*, 2013), the need for new therapeutic agents from nature is still urgent. As less than 1% of soil bacterial species are currently known (Torsvik and Ovreas, 2002), soil represents a promising habitat for discovering and isolating new natural products (MacNeil, 2001).

Various reports confirmed the ability of *Bacillus* species to produce antimicrobial agents and compounds with potential biotechnological and pharmaceutical applications (Awais *et al.*, 2007; Hassan, 2014). In addition, numerous studies were conducted on the bioactivity of crude extracts produced by bacterial strains isolated from different habitats in Jordan. Soil *Streptomyces* species were able to inhibit the growth of multi-drug resistant *Pseudomonas aeruginosa* (Saadoun *et al.*, 2008); actinomycin C2 and actinomycin C3 were isolated from red soil *Actinomyces* strain and were reported to inhibit the growth of both *Micrococcus luteus* and *Staphylococcus aureus* (Falkinham *et al.*, 2009); El-Banna *et al.* (2007) showed that the substances produced by different *Bacillus* species isolated from various Jordanian sources have antibacterial activity against methicillin-resistant *S. aureus*.

In an ongoing work on natural products from microorganisms and due to our interest in isolating soil bacterial strains with the ability to produce biologically active metabolites, herein, we report the isolation, identification, cultivation and extraction of bioactive crude extract from a soil *Bacillus* sp. 7B1. Moreover, partial purification of the resulted crude extract using chromatographic methods is described.

2. Materials and Methods

2.1. Isolation and Identification of Bioactive Crude Extract Producing Strain

Bacillus sp. 7B1 was isolated from a soil sample collected from Al–Mazar (south of Al-Karak, Jordan). One gram of the sample was suspended in 10 ml sterile normal saline (0.85% NaCl) and serially diluted up to 10^{-6}. 50 µl from each dilution was spread on Nutrient Agar Media (Oxoid, UK) supplemented with 50 µg/ml of cyclohexamide and nystatin to inhibit the growth of fungi

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and yeasts. The agar plates were incubated at 27 °C and the isolate was streaked several times till a pure culture is obtained. 2 ml of *Bacillus* sp. 7B1 culture medium was stored in 80% glycerol (1:1) at – 20 °C for further use.

Bacillus sp. 7B1 was characterized morphologically (colony form and appearance, cell shape and size, etc.) and biochemically (Gram stain, oxidase, catalase, nitrate reduction, etc.) for 48-72 hours grown colonies on Nutrient Agar plates. The biochemical characteristics were determined using procedures described by Collins *et al.* (2004) and York *et al.* (2007). The production of acid from different carbohydrates was done as described by Helmke and Weyland (1984). Moreover, the identification of the isolate was confirmed using the commercial MICROGEN® BACILLUS-ID kit (Microgen bioproducts, UK) provided with Microgen Identification System Software (MID-60).

2.2. Bacterial Cultivation, Extraction and Purification of Produced Bioactive Crude Extract With Partial Characterization of The Active Compound

Bacillus sp. 7B1 was inoculated in 20 L of Luria-Bertani (LB) broth (0.5% tryptone, 0.5% yeast extract, 1% NaCl) (8 X 5 L Erlenmeyer flasks containing 2.5 L broth) with continuous shaking (130 rpm) at 27 °C. During the cultivation process, 50 ml daily samples were collected and used to measure the changes in the bacterial growth parameters and the increases in bioactivity. The growth was followed by measuring the increase in turbidity reading (OD_{600nm}) (UV. Spectrometer, Lambda 16, Perkin-Elmer, LANGEN) and changes in pH values while the bioactivity was measured by the agar diffusion test against *B. subtilis*. The cultivation was ended directly after the OD_{600nm} value decreased or the bioactivity reached its maximum. The cells were harvested by centrifugation (8000 rpm, 15 min, Sorvall ® RC-5B Refrigerated super speed, Dupont company/USA). The pH of the supernatant was adjusted to 8 and extracted with an equal volume of ethyl acetate. The organic phase was dried over sodium sulfate (anhydrous) and concentrated *in vacuo* at 45 °C using vacuum evaporator (Büchi Rotavapor R-215, Switzerland). The resulting crude extract was dissolved in methanol to 50 mg/ml and stored at 4 °C.

The resulting crude extract was applied onto a column of silica gel (Merck 60, 0.063-0.2 µm, Ø 3 cm x 14 cm). An elution was performed with organic solvents of increasing polarity starting from 100% cyclohexane through cyclohexane-ethyl acetate mixture, ethyl acetate-methanol mixture till 100% methanol as mobile phase and under atmospheric pressure. Each eluted fraction was collected and monitored by Thin Layer Chromatography (TLC) and used to measure its bioactivity by agar diffusion test. The fractions that exhibited bioactivity were further purified using size exclusion chromatography (Sephadex LH-20, Ø 3 cm x 33 cm) with 0.1% formic acid : methanol as eluent under atmospheric pressure. 10 ml fractions were collected and monitored by TLC. The fractions with bands of similar migration pattern on TLC were combined together and concentrated *in vacuo* using vacuum evaporator at 45 °C.

The resulting combined fractions were further purified by preparative TLC (PTLC) (20 cm x 20 cm glass plates coated with Merck 60 silica gel) using the Toluene : acetone : formic acid as mobile phase (30 : 70: 1). Each resulting band was scratched from the plate and its constituents were dissolved in acetone. The dissolved constituents were used to follow the bioactivity by agar diffusion test and the purity was tested by re-spotting them on TLC plates. The spotted compounds were detected using UV light at 254 nm and 366 nm. Moreover, developed spots were visualized with ninhydrin (0.1% spray-reagent of 2,2-dihydroxyin-1,3-dandion) with heating at 120 °C for detection of amino acids; bromocresol green (0.1 g bromocresol green in 500 ml ethanol and 5 ml 0.1 M NaOH) for detection of organic acid; and bromothymol blue (0.1% bromothymol blue in 10% aqueous ethanol) for detection of lipid and phospholipids (Touchstone, 1992).

2.3. In Vitro Antibacterial Activity Measurement

Antibacterial activities of the bacterial crude extract, partially purified fractions and the resulting pure compound, were determined by agar diffusion test; the minimum inhibitory concentration (MIC) was also measured by microbroth dilution assay using the standard methods mentioned in Clinical and Laboratory Standards Institute (CLSI, 2012) and as described by Al-Zereini (2014). Five bacterial test strains were included in the present study; three Gram positive bacteria [*Staphylococcus aureus* (ATCC 43300), *Bacillus subtilis* (ATCC 6633) and *Micrococcus luteus* (ATCC 10240)] and two Gram negative bacteria [*Proteus vulgaris* (ATCC 13048) and *Escherichia coli* (ATCC 25922)]. They were seeded on Muller Hinton Agar plates (Oxoid, UK).

500 µg of crude extract or 200 µg of the partially purified fractions was applied on a 6-mm blank filter disk and used in the agar diffusion test, while concentrations starting from 100 µg/ml of purified compound or positive control (Penicillin G) were used in microbroth dilution assay to measure the MIC. The biostatic or biocidal effect of the tested compounds was determined by re-streaking 10 µl from the culture in the first well where there is no obvious growth on a new Nutrient Agar plate. All assays were performed in triplicate and the average values are presented.

3. Results and Discussion

Throughout the present study, one bacterial isolate (*Bacillus* sp. 7B1) exhibited a promising bioactivity against the tested bacterial strains. *Bacillus* sp. 7B1 formed circular creamy yellow colonies on Nutrient Agar Medium. It is a Gram positive, 2-2.5 µm long and 1 µm wide bacilli (Figure 1), oxidase negative, catalase positive, hydrolyze starch and is able to reduce nitrate to nitrite. The biochemical characteristics are summarized in Table 1. It was identified with a 99% similarity to species belonging to the genus *Bacillus* by the Microgen Identification System Software (MID-60) supplied with the MICROGEN® BACILLUS-ID kit

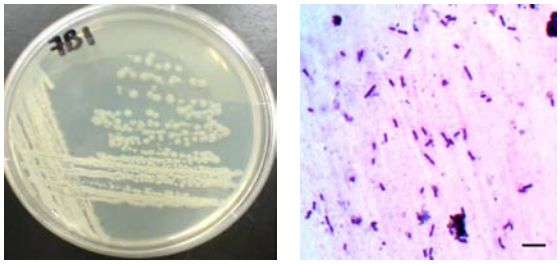

Figure 1. Microphotography of *Bacillus* sp.7B1. A) Colonies gown on Nutrient Agar Medium. B) Cell shape and Gram staining. Scale bar is 10 µm.

Table 1. Biochemical characteristic of bacterial isolate *Bacillus* sp.7B1

Test tests	Experimental result
Oxid/Ferm:[a]	
D-Glucose	+/+
Sucrose	+/+
Sorbitol	+/+
Maltose	+/+
D-Xylose	+/-
Organic compounds as N[b] and C[c] - source:	
L- Leucine	+
L- Phenylalanine	+
L- Histidine	+
L- Asparagine	+
L- Tyrosine	+
Glycine	+
Organic compounds as C-source:	
Sodium acetate	+
Sodium pyruvate	+
Hydrolysis of :	
Starch	+
Gelatine	-
DNA	-
Catalase	+
Oxidase	-
Nitrate reduction	+
H_2S – production	-

[a)] Oxidation/Fermentation [b)] Nitrogen source [c)] Carbon source

The cultivation of *Bacillus* sp. 7B1 was carried out for 196 hours, during which the pH value increased from 7.1 up to 8.5 at the end of cultivation period; the cells entered the stationary phase after 120-144 hours and the cultivation ended after the OD_{600nm} value started to decrease and the bioactivity of the crude extracts from daily samples reached their maximum. In contrary to typical idiolites (secondary metabolites that are produced during idiophase or stationary phase), the antibacterial activity of 500 µg/disc crude extract against *B. subtilis* could be detected early in the logarithmic growth phase (exponential phase), after 48 hours. The maximum activity was obtained at the beginning of the stationary growth phase, with no significant decrease during prolonged incubation (Figure 2).

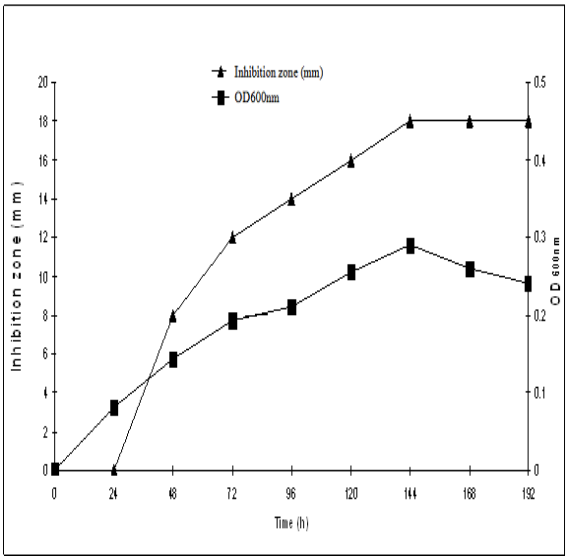

Figure 2. Cultivation parameters during growth of *Bacillus* sp.7B1 in LB medium. Changing in cell density (OD_{600nm}) and increase in inhibition zone diameter caused by 500 µg/disc of applied crude extract against *B. subtilis* are shown.

The crude extract, at 500 µg/disc, of strain 7B1 exhibited a promising anti-bacterial activity against the Gram positive test strains. It caused inhibition zones of 16, 18 and 26 mm against *S. aureus*, *B. subtilis* and *M. luteus*, respectively. Gram negative bacteria were resistant to the tested crude extract. This resistance may be attributed to the low permeability of the Gram negative bacteria outer membrane and the lipopolysaccharide barrier for the hydrophobic compounds (Delcoue, 2009; Wiener and Horanyi, 2011).

Several studies reported that *Bacillus* strains were able to produce a large number of antimicrobial peptides with different chemical structures, such as bacteriocins, iturin A and surfactin (Belhara *et al*., 2011). The production of these metabolites was reported to be a growth phase-dependent and produced, as typical secondary metabolites, during the stationary growth phase (Benitez *et al*., 2012). However, the early noticed production of the bioactive crude extract from *Bacillus* sp. 7B1 coincided with reported production kinetics observed for other antimicrobial peptides, namely subtilin, subtilosin and bacteriocin-like substances by different *Bacillus* species (Barboza-Corona *et al*., 2007; Stein, 2005). Nevertheless, the proteinaceuos nature of spotted compounds from *Bacillus* sp. 7B1 was not confirmed after spraying the TLC plates with ninhydrin reagent.

The extraction of culture fluid (19 L) with ethyl acetate yielded 859 mg of crude extract. Bioactivity-guided fractionations of the crude extract using silica gel column chromatography resulted in elution of eight fractions, three of which are polar antibacterial fractions (E-G) (Figure 3). Further purification of the combined bioactive fractions on sephadex LH-20 yielded eight sub-fractions. Only sub-fraction S_3 (100 mg) conferred antibacterial activity and caused an inhibition zone of 18 mm to *B. subtilis* at 200 µg/disc.

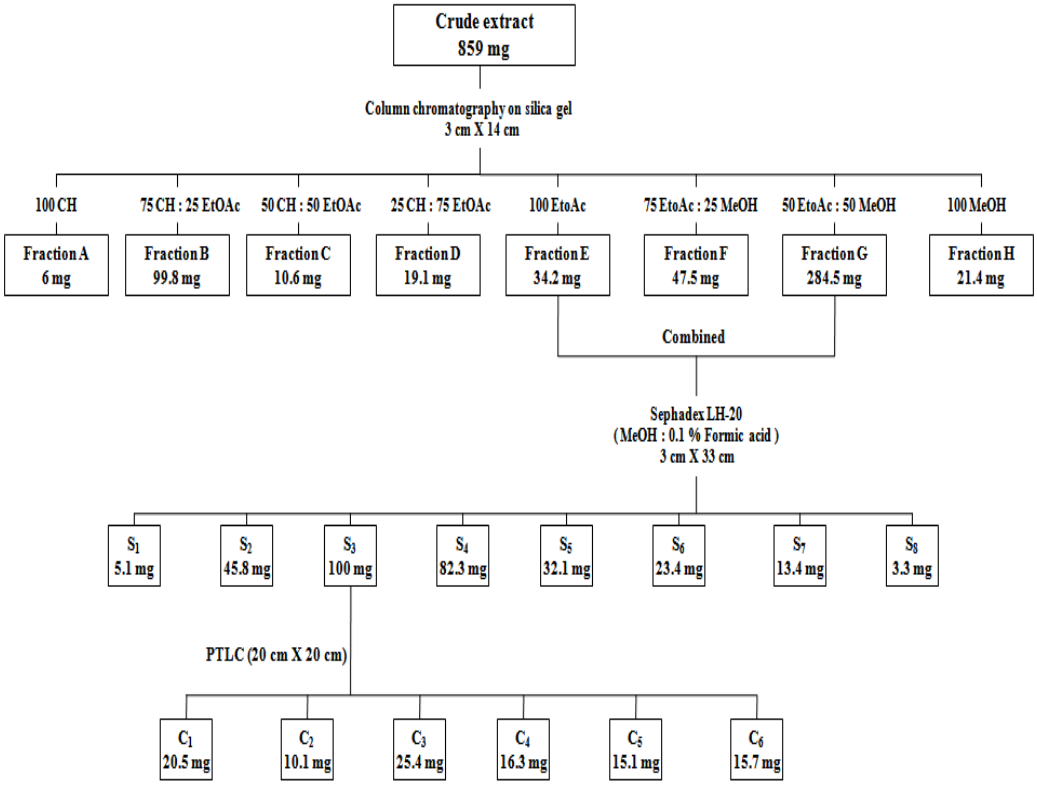

Figure 3. Purification scheme of crude extract resulted from *Bacillus* sp.7B1 cultivated in LB medium.

Generally, the percentage of the bioactive compound in the resulting fractions after each step of purification is increased and, thus, it exhibits an activity at a concentration less than that at the beginning of screening. Therefore, in the agar diffusion test, we started our screening with a higher concentration of crude extract (500 µg/disc) and after each step of purification, the fractions were examined at a concentration of 200 µg/disc.

The separation of sub-fraction S_3 constituents by preparative TLC resulted in the production of six bands (Figure 4), of which band number (2) (rate of flow, R_f: 0.25) was the only band that revealed an antibacterial activity in the agar diffusion test. Scratching of band number (2) from TLC plate, dissolving it in acetone followed by drying *in vacuo* using vacuum evaporator and re-spotting on a new TLC plate to examine its purity resulted in 10 mg of pure white colored compound ($\mathbf{C_2}$). 50 µg/disc of compound ($\mathbf{C_2}$) caused inhibition zones of 13, 15 and 17 mm against *S. aureus*, *B. subtilis* and *M. luteus* respectively. In microbroth dilution assay, the MIC value of compound ($\mathbf{C_2}$) was 25–100 µg/ml (Table 2). Compound ($\mathbf{C_2}$) had a bacteriostatic effect as it inhibited growth but did not kill the tested microorganisms. This may enable the human immune system to take its role and overwhelm the bacterial infections (Pankey *et al.*, 2004), especially those caused by Gram positive bacteria.

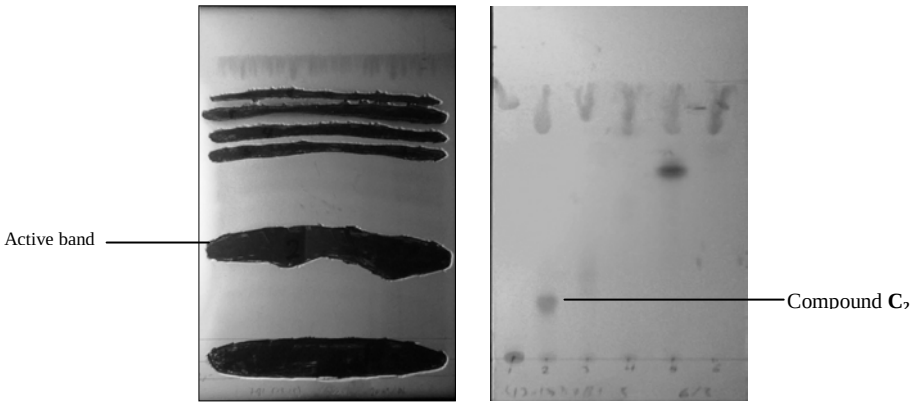

Figure 4. Thin layer chromatography of the partially purified and purified active compound obtained from *Bacillus* sp. 7B1. A) Migration profile of the partially purified S_3. B) The purified compound ($\mathbf{C_2}$). Bands on TLC plates were visualized by UV_{366nm}.

Table 2. Minimal inhibitory concentration (MIC) of the purified compound ($\mathbf{C_2}$) from *Bacillus*.sp.7B1 against susceptible bacterial strains

Tested microorganisms	MIC (µg/ml) *Bacillus* sp.7B1 (C_2)	Penicillin
M. luteus	25s	<8
B. subtilis	>100s	<8
S. aureus	100s	62.5s

s: bacteriostatic

In contrary to bacteriostatic antibiotics that slow or stop the bacterial growth, usually by inhibiting the protein synthesis without inducing the production of toxic radicals, bactericidal antibiotics promote the generation of hydroxyl radicals and, thus, leading to bacterial cell death (Bernatová *et al.*, 2013). Consequently, bactericidal agents induce ROS overproduction in mammalian cells causing an oxidative damage to DNA, proteins, and membrane lipids and mitochondrial dysfunction (Kalghatgi *et al.*, 2013). Furthermore, bactericidal agents may cause a sudden increase in the bacterial products and toxins in the human body which may potentially cause a harmful inflammation due to the cytokine production (Finberg *et al.*, 2004).

Herein, the bioactivity reported for the crude extract and the purified compound ($\mathbf{C_2}$) from *Bacillus* sp. 7B1 against the methicillin-resistant *S. aureus* is more than that obtained from similar strains isolated from Jordan. El-Banna *et al.* (2007) reported that the air flora *Bacillus megaterium* NB-3 and *Bacillus subtilis* NB-6 were inactive against some methicillin-resistant *Staphylococcus aureus* isolates and weakly active to other *S. aureus* isolates from meat samples collected from retail shops and slaughter houses located in Amman area, Jordan. Unfortunately, due to the low amounts of compound ($\mathbf{C_2}$) obtained from *Bacillus* sp. 7B1, it was not possible to perform additional physico-chemical characterizations nor structural elucidation. Therefore, a large scale of 100 L cultivation process is intended to be done in the future in order to gain adequate amount of $\mathbf{C_2}$ compound and elucidate its chemical structure.

Finally, several antibacterial agents were isolated and identified from several *Bacillus* species, among which were the *B. subtilis* group of bacteria including *B. amyloliquefaciens* (Wulff *et al.*, 2002; Al-Tarawneh, 2011; Cao *et al.*, 2011). Moreover, they are known as a source of antifungal compounds (Athukorala *et al.*, 2009) and the most promising candidates for microbial biocontrol agents (Arguelles-Arias *et al.*, 2009). Therefore, *Bacillus* species may be considered a promising source for isolation secondary metabolites with potential application in pharmaceutical and agricultural industry.

4. Conclusion

The present study pointed out the importance of soil bacterial isolates as a source of biologically active and potentially novel secondary metabolites. These metabolites are more effective against Gram positive bacteria than Gram negative strains. Moreover, members of the genus *Bacillus* are still a proliferative group of bacteria with species that are able to produce compounds that may contribute to pharmaceutical industry.

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