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# ANTIMICROBIAL ACTIVITY OF NOVEL PEPTIDE COMPOUND PRODUCED BY *BACILLUS LICHENIFORMIS* ISOLATED FROM IRAQI SOIL ON UTI BACTERIA

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## Abstract

The study were performed to detection the inhibitory effect of antimicrobial compound produced from the isolate *Bacillus licheniformis (BL1)* isolated from rhizospheric reign of plant Wheat, and studied its effect on some pathogenic bacteria isolated from urinary tract infection (UTI) from local hospitals in Baghdad city, Iraq. These isolates were ( *Escherichia coli, Klebsilla pneumonia, Pseudomonas aeruginosa* and *Proteus mirabilis, Acinetobacter baumanii, Serratia marcescens, Streptococcus agalactiae, Staphylococcus aureus* and *Staphylococcus epidermidis* ), the identification of all isolates were done using different cultural characteristics, physiological and biochemical tests, confirmed the identification with VITEK 2 system. the isolates of UTI were tested for antibiotics susceptibility test against teen various antibiotics, results showed the highest resistance were recorded to Nalidixic acid 91j and for Tetracycline 82j, while lowest resistance was 9j to Amikacin. The antimicrobial activity of the isolate *Bacillus licheniformis (BL1)* was detected by using agar wells diffusion method, appeared good activity against pathogenic bacteria, Extraction partial purified of active compound with ethyl acetate from BL1 and exposure the crud extracted to TLC thin layer chromatography, Rf-value obtained was 0.785, then exposure to HPLC high performance liquid chromatography on wave length 345 nm. various different peaks appeared on different retention time (embeds 5.5 to 30 min), the chemical structures for the extracted compound analysis with FTIR Fourier transformed infrared. The antimicrobial activity for the crude extracted and partial purified were detected and the results showed that crude extracted of BL1 had higher activity than the supernatant compared with the activity of partial purified compound .

***Key words :*** UTI bacteria; *Bacillus licheniformis; Antimicrobial activity.*

## Introduction

Bacillus is one of the most important and characterized genus of Gram-positive bacteria. The interest in this genus is due to the ability to form endospores, and to produce chemical metabolites of interest in the agronomic, pharmaceutical and industrial fields of life . Bacillus is a complex genus at the genotypic, phenotypic, metabolic, taxonomic, and ecologic level , making them to be very versatile in different ambient, especially in soil (Amin *et al.*,2015).

Treatment of infectious disease with multidrug resistant strain of bacteria are becoming a significant problematic in the whole world. Resistance of Antibiotic becoming the most important challenge posed to the health professionals and resulting in higher medical costs, and increased mortality., the WHO commending to using safe and effective medicines in treatment of infectious diseases (WHO, 2014).

Sorted new antibiotic from natural environ is becoming progressively important for the pharmaceutical industry as pathogenic bacteria and i are mooted becoming resistant to generally used therapeutic agent. from several hundred antibiotics that have been produced and purified, only a few have been appropriately non-toxic of use in

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medication. Those are small group of microorganisms belonging to the genera Streptomyces, Penicillium, Bacillus, Micromonospora and Cephalosporium (Chandra, *et al.*,2015) Approximately 800 different peptide antibiotics which are potently against pathogenic bacteria were produced by *Bacillus* spp. (Saxena, S., 2015). Several species of Bacillus biosynthesize the peptide antibiotics Such a *B. brevis, B. polymyxa, B. licheniformis.*

Recently, many investigation have been carried out to isolate different strains of terrestrial Bacillus and identify their inhibitory metabolic compounds (Khokhaw *et al.,* 2013 ). The aim of the current study was to isolate *B. licheniformis* from rhizospheric plant root in Iraqi soil and investigate antimicrobial activity against some isolates of MDR UTI bacterial isolates using crud and partial purification metabolic compound of *B. licheniformis* .

## Materials and Methods

### Bacillus Isolates

Soil samples were taken from rhizosphere areas of different plants from Baghdad city/Iraqi soil. Using the method of serial dilution to isolate *Bacillus licheniformis* the isolates was acquainting utilization different cultural characteristics, physiological and biochemical assayed as delineative in literatures (Brekeley *et al.,* 1984 and Brown, 2005). The identification of isolates were confirmed using VITEK 2 system.

### Isolation and Identification of UTI bacteria

Bacteria were isolated from urine specimen collected from local hospitals in Baghdad city, Identification were done according to the morphological and biochemical test (Hoiby *et al.,* 2015). VITEK 2 system were used to confirmed the identification of all isolates.

### Composing the filtrates of *B. licheniformis*

The first step in screening the isolates of Bacillus which have the ability to produced metabolite with antimicrobial activity were done by inoculating in nutrient broth and incubation period at 30°C for three days in an orbital shaker at 180 rpm, then centrifuged at 6000 rpm for 20 minutes, and filtration with Millipore filter papers size 0.22 µm, antimicrobial activity was determined using Agar well diffusion method, about 100 µl of *B. licheniformis* filtrate were loaded in to the wells of 5mm were made in Muller Hinton agar plates inoculated with the UTI bacterial isolates separately, negative control ( fill with distilled water only ). The plates were incubated at 37°C / 24 hours and the inhibitory zones were measured in mm. (Mussa and Baqer, 2017).

### Extraction the active compound produced by *B. licheniformis* isolates

The isolate *B. licheniformis* BL1 which showed higher activity against UTI were extracted by growing in nutrient broth in 37°C for twenty four hours. The filtrates of isolate were blend with volume of ethyl acetate as solvent in separation funnel and then mixed gently. The organic phase was collected, and dried at room temperature. The yield from extract was dissolved in ethanol in order to calibration of antimicrobial activisms (Abdu-Allah. and Mussa A. H 2015).

### Purification the antibacterial compound

The extracted bioactive compound was blazers by thin layer chromatography (TLC) according to the method recorded in (Batrakov *et al.,* 2003 ),the samples were spotted on silica gel plates (Merck, KGaA, Germany; 60 F254, 0.25 mm) 1.0 cm above the bottom of the plates. Afterwards, the plates were articulated in a chromatography jar containing mixture of chloroform and methanol in ratio (85:15). The spots were visualized by using diazotized sulphanilic acid under UV at 254 nm in an UV illuminator. $R_f$ values of the spots were measured. Then the spot Scrubbed by the spatula and dissolved in ethyl acetate solvent finally dry in a desiccated vacuum at 40°C and then dissolved in methanol. (Mussa and. Ziayt 2018).).

### High performance liquid chromatography (HPLC)

Active compound were purified partially investigated on preparative HPLC column and the chromatogram. The mobile phase comprised of 63% (methanol: acetonitrile) and 37% (buffer: water). Detector at 345 nm. flows rate: 1.0 milliliter in minute. Sample size was : 10 µl. The sample was loaded on a HPLC column (Shimadzu LC-8A, Kyoto, Japan) C18 reversed-phase column (Zorbax SB-C18, 5.0µm, 4,6 mm*250 mm, Rockland Technologies Ind., Newport, DE, U.S.A.),. the retention times of compound were determined which is specific value for each compound. (Al-Thubiani et al , 2018)

### Fourier transform Infrared (FTIR)

Active compound extracted from *B. licheniformis (BL1* ) was carry out using Fourier transform infrared (FTIR). The spectra were scanned in the range from 400-4000 $cm^{-1}$). Using potassium bromide pellet technique by (bauker Tensor 27 with ART unit, Shimadzu Japan). (Kummer *et al.,* 2009).

### Antibacterial activity of *B. licheniformis* compound on uropathogenic bacteria

**A.** Effect of crude compound extracted on uropathogenic bacteria: agar well diffusion method

utilized to distinguish the action of crude extracted from *B. licheniformis* BL1 against uropathogenic bacteria.

**B.** Effect of purified compound on uropathgenic isolates**:** It was made as described previously in **A** but instead of crude compound, the wells filled with 100µl of purified compound in different concentration( 50,100,200) µg/ml. MIC of compound were determined (Suleman and Mussa, 2018).

## Results and Discussion

As a result of the identification tests, three isolates of *B. licheniformis* isolated from rhizospheric root of wheat plant, their antimicrobial activity against the bacteria causing UTI (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Proteus mirabilis, Acinetobacter baumannii, Serratia marcescnes, Morganella morganii, Enterobacter cloacae, Streptococcus agalactiae, Staphylococcus aureus and Staphylococcus epidermidis*), was investigated. Antibiotics susceptibility for UTI Isolates were tested. The results obtained according to Clinical and Laboratory Standards Institute (CLSI, 2017). Using Different types of standard antibiotic discs and the results summarized in Fig. 1.

The tested bacterial isolates showed 91% resistance to Nalidixic acid, while the percentage of resistance to Tetracycline 82% and (Nitrofurantion, Amoxicillin clavulanic acid, Trimethoprin) were 64%, resistance and the Ampicillin salbactam 45% resistance to the (Norfloxacin, Levofloxacin, ciprofloxacin) showed 36%, finally Amikacin 9% resistance. The results showed that most of UTI isolates appeared MDR and this results agreement with numerous others research which focusing to the important of MDR bacteria and it roles in increasing risk factor of the UTI.( Khawcharoenporn *et al.*, 2013) found in his study that only *Serratia spp*., *Providencia spp*. Appeared MDR among many bacteria causative agent of UTI, While (Cohen-Nahum *et al.*, 2010) noticed that prevalence of MDR *Proteus mirabiles* strains has increased in the last few years and the genes coding for resistance was extended spectrum beta lactamase (ESBL) and this type of resistance were found to be predominant risk factors for MDR in *E.coli* and *Klebsiella spp*. (Hyle *et al.*,2005) and Abo-Ksour *et al.*, 2017).

Resistance % (Murugan *et al.*, 2012) found in his study that all common isolates bacteria causing UTI including *Staphylococcus spp*., *Streptococcus spp*., *Enterobacter spp., Klebsiella spp.* and *E. coli* appeared MDR.

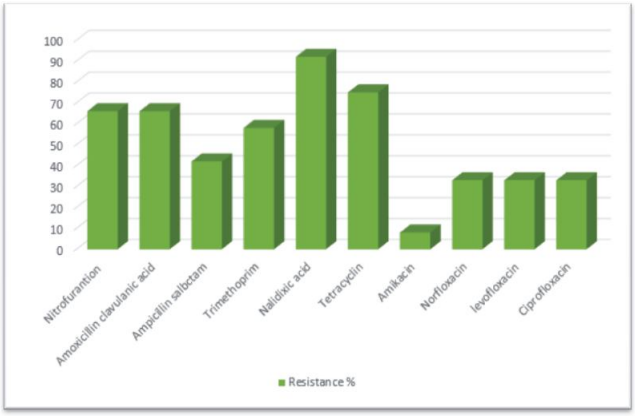


**Fig. 1:** The antibiotic s susceptibility test of UTI isolates.

### Investigation of antimicrobial activity of *B. licheniformis* filtrates

The study were design to examined the antimicrobial activity by using filtrates of *B. licheniformis* isolates. The obtained results appeared. That isolates of *B. licheniformis (BL1*, BL2, BL3) showed antimicrobial activity against UTI bacteria (Table 1).

The isolate (BL1) showed higher activity compare with other isolates (BL2, BL3), the maximum activity was reported against *Staphylococcus aureus*. The widest inhibition zone diameter was (18 mm) and the minimum activity was (8 mm) against *Enterobacter cloacae*.

The results obtained was similar to various other researches, (Alvarez-Ordóñez *et al*., 2014) mentioned that *B. licheniformis* could prevented the growth of pathogenic strains of bacteria. and (Yilmaz *et al*., 2006) ) ensured The inhibitory effect of *B. licheniformis* was recorded against some genus like *Staph. aureus, Strep. pyogenes* and yeasts included *C. albicans, Cryptococcus neoformans*. Many researchers mentioned the secondary metabolites secretion from *Bacillus* spp. appeared higher effectiveness against gram positive bacteria compare to gram negative bacteria and this is could be due to permeable of outer membrane and LPS barrier for the metabolites (Delcoue, 2009) and (Wiener and Horanyi, 2011).

### Extraction and Purification of the bioactive compound from the *B. licheniformis* BL1

The extraction of bioactive compound with ethyl acetate as organic solvent, were done, The organic phase was separated with separated funnel and dried, then re suspended with methanol. the compound obtained was considered as crude bioactive compound.

### Purification of bioactive compound through TLC

The bioactive compound extracted with ethyl acetate

**Table 1:** Antimicrobial activity of *B. licheniformis* filtrate on UTI isolates, zone of inhibition were measured in mm.

| Bacterial isolates | Zone of inhibition(mm) | | |
|---|---|---|---|
| | BL1 | BL2 | BL3 |
| *Klebsiella pneumoniae* | 12 | 11 | 8 |
| *Escherichia coli* | 8 | 8 | 0 |
| *Pseudomonas aeruginosa* | 9 | 7 | 0 |
| *Serratia marcescens* | 12 | 10 | 8 |
| *Enterobacter cloacae* | 8 | 0 | 0 |
| *Acinetobacter spp* | 9 | 8 | 0 |
| *Proteus mirabilis* | 13 | 8 | 6 |
| *Morganella morganii* | 11 | 10 | 0 |
| *Staphylococcus aureus* | 18 | 12 | 11 |
| *Streptococcus agalactiae* | 15 | 14 | 9 |

exposure to TLC and the separation spot with $R_f$ 0.785, Fig. 2 Detection of compound was prepared through TLC based on mobility of the compound on the silica plate that is measure in expression of retardation factor. The separation spot was appeared in silica layer as, yellow molecule aromatic polyketide antibiotic can soluble in chloroform, dichloromethane, water, and can increase solubility when increasing temperature and that is characterization agreement with the character pointed by Al-Thubiani *et al.,* (2018). Many researches used TLC

**Fig. 2:** Disaffiliated the bioactive compound elicit from *B. licheniformis* BL1 on TLC.

as identification technique of antifungal and antibacterial compounds produced by producing soil bacteria, and as initial step for isolation and analysis of antibacterial substance produced by different genus (Mussa and Baqer, 2017).

**Identification of active compound by HPLC**

The spots appeared on TLC plate with $R_f$ = 0.785 were scarped as mentioned previously and loaded on a HPLC column. 10 µL of sample was loaded on a HPLC (Shimadzu LC-8A, Kyoto, Japan). The chromatogram were shown in Fig. 3 methanol: acetonitrile was used as the mobile phase, the figure showed presence different various peaks with different retention time which indicated presence more than one active compound. The RT between (5 to 33 min ) as appeared in the figure, the presence of the peaks with RT 18 min it belong to the Bacitracin, and this was similar to the result obtained by others researchers using the same condition and solvent. (Al-Thubiani *et al.,* 2018) in his report indicated that the peaks he obtained in RT 18 min, which belong to

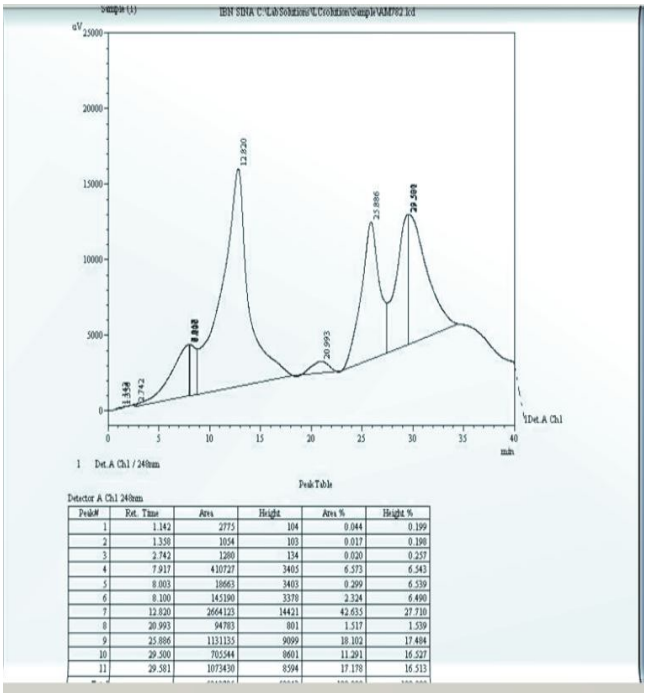


**Fig. 3:** HPLC chromatography showing different peaks on different RT of bioactive compound production from *B. licheniformis* BL1.

bacitracin antibiotic when using the same condition and solvents in HPLC as we do in the current study .farther steps in purification and characteristics each compound found in HPLC chromatogram is needed as mentioned by (Dhiravisitd *et al*., 2018).

**Identification of compound by FTIR**

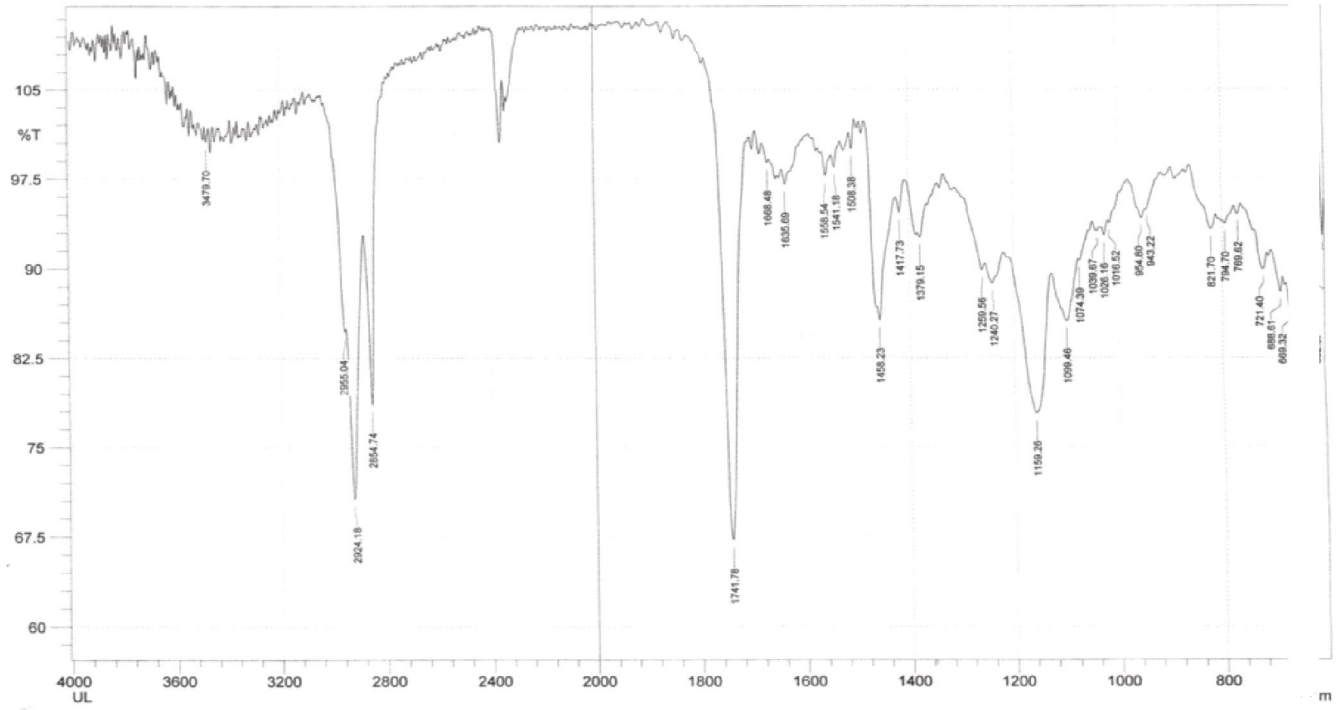


**Fig. 4:** The (FTIR) Fourier Transform Infrared spectrum of antimicrobial compound produced by *B. licheniformis*, isolate BL1.

The results of (FTIR) of *B. licheniformis* isolate BL1 appeared in fig. 4 exhibited absorption at 1743 cm> $^{1}$ corresponding to carboxyl group. Valley at 1626 cm> $^{1}$ indicating Gausain amid bound CO-N bond (amide I band in helical protein). Absorption at 3119 cm> $^{1}$ revered to (hydrogen bound OH group) and Valley at 927 cm> $^{1}$ corresponding to C-$NH_2$. Valley at 1400cm> $^{1}$ revered to (COO) stretching indicated aliphatic chine. While Valley at 2865 cm> $^{1}$ shows C-H stretch . All these valleys indicated the compound contains peptide bonds and peptide nature of compound.

***Licheniformis*, isolate BL1**

The results obtained in present study were agreements with others studies focusing on the chemical structural of bioactive compound production by *Bacillus spp* (Bechard *et al.*, 1998) found in his research the FT-IR spectrometry of purified bacitracin appeared a propriety of peptide bonds according to found absorption bundle at 1540, 1650 and 3300 cm_1. and presence the absorption bundle at 1740 cm_1 which referred to the a lactone ring, and the C-H stretching appeared in absorption (2950, 2850, 1460 and 1400 cm_1) referred the presence of an aliphatic chine. (Al-Thubiani *et al.*, 2018), he obtained the same absorption, and suggested the nature of antimicrobial compound could be Bacitracin like structure of cyclic polypeptide, the chemical structural of bacitracin (C66H103N17O16S) is a polypeptide antibiotic could production by the genus *B. subtilis* and *B. licheniformis*, (Guo*et al.*, 2012) and (Kim, and Jeon, 2016)

**Antibacterial activity of *B. licheniformis* compound on uropathogenic bacteria**

The results of antimicrobial activity of crude and purified compound were summarized in table 2. The partial purified compound shows high antimicrobial activity against all tested microorganisms. The concentration 200 µg/ml of purified compound was found to have higher activity for isolate BS1. The inhibition zone on *Staph. aureus* 34, 32, 30, mm in concentration 200, 100, 50, µg/ml respectively and for *E.coli* the inhibition zone was 20, 10, 10 mm, respectively. the activity of crude in some genus could be higher than extracted compound and that attributed to presence others compound in crude and the activity could returned to it. The effect of these active compound produced by *Bacillus* spp. on different bacteria and fungi could be bacteriostatic which stop or slower the reproduction of bacteria, by preventing the protein synthesis, or could be bactericidal antibiotic which booster the procreating of hydroxyl radicals which lead to bacterial decease. (Bernatova *et al.*, 2013) mentioned in his research that active metabolite which extracted and purified from *B. subtilis* had the ability to stop protein synthesis in pathogenic bacteria, (Al-Saraireh, *et al*., 2015) reported the active compound produced from different species of Bacillus could prevention the growth of *Staph. aureus, Micrococcus lates,* and this activity

**Table 2:** Inhibition Zone (mm) of crude and purified compound (three concentration) on UTI.

| Zone of inhibition of purified compound | | | Zone of inhibition(mm) | Bacterial isolates |
|---|---|---|---|---|
| 200 µg/ml | 100 µg/ml | 50 µg/ml | Crude compound | |
| 12 | 0 | 0 | 16 | *Klebsiella pneumonia* |
| 20 | 10 | 10 | 10 | *Escherichia coli* |
| 12 | 10 | 10 | 15 | *Pseudomonas aeruginosa* |
| 20 | 17 | 12 | 12 | *Serratia marcescens* |
| 14 | 12 | 0 | 8 | *Enterobacter cloacae* |
| 20 | 12 | 0 | 12 | *Acinetobacter spp* |
| 17 | 15 | 12 | 15 | *Proteus mirabilis* |
| 14 | 12 | 10 | 12 | *Morganella morganii* |
| 34 | 32 | 30 | 30 | *Staphylococcus aureus* |
| 24 | 20 | 15 | 34 | *Streptococcus agalactiae* |

is higher than supernatant, while (Sirtori *et al*., 2008) mentioned that the crude extracted from *Bacillus spp.* appeared the hyperactivity against *Listeria monocytogens* and *Enterococcus fecalis*. The effectiveness of *Bacillus* spp. to have inhibitory activity against the pathogenic bacteria attributed to the ability of produce various metabolites which have these effect .this study pointed out the importance of soil bacterial isolates to be source of novel active metabolites and it is more effects in MDR UTI bacteria with broad spectrum metabolite.

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