Evaluation of Antimicrobial and Antioxidant activity of secondary metabolites isolated from endophytic bacterium *Bacillus megaterium* isolated from wheat root in Iraq

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Received: 5 September 2015 **Accepted: 19 May 2015**

Abstract

The present study was carried out to find out the antimicrobial activity of endophytic bacteria isolated from wheat plant root by surface sterilization method. Bacterial strain was identified as *Bacillus megaterium.* Secondary metabolites was carried out by ethyl acetate solvent .Secondary metabolites were demonstrated for antimicrobial activity against Gram-:Negative Bacteria namely; *Escherichia coli ,Salmonella typhi* and *Pseudomonas aeruginosa* which range between (16.5-31.0)mm and range between(29.5-33.0)mm against Gram-Positive Bacteria and antifungal activates tested against yeasts test namely; *Candida albicans* with inhibition zone 16.0mm and 12.0mm against *Candida kruzi*. Minimum inhibitory concentrations were in the range between (6.25- 12.25) and (3.12-25.0) µg/ ml against Gram-Negative and Gram-Positive bacteria respectively and(50)µg/ml against yeasts test and minimal bactericidal concentrations ranged from (12.5 to 25.0) µg/ ml and (6.12-50)µg/ ml against Gram-Negative and Gram- -Positive bacteria respectively and minimal fungicidal concentrations (100) µg/ ml against yeasts. The antioxidant activity was analyzed using2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays have shown high rates of inhibition .A verification of non-toxicity of the bacterial secondary metabolites against human blood revealed a negative test. The metabolite produced by the endophytic bacteria could be an alternative source of antimicrobial and antioxidants.

Keywords: Antimicrobial activity, Antioxidant activity, Endophytic bacteria, bacteria.

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تقييم الفعالية الضد ميكروبية والضدتأكسدية للايض الثانوي المعزول من بكتريا *Bacillus megaterium* المعزولة من النسيج الداخلي لجذور نبات الحنطة في العراق

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الخلاصة

تضمنت الدراسة الحالية عزل وتشخيص بكتريا *Bacillus megaterium*. *من النسيج الداخلي لجذور نبات الحنطة المحلية باستخدام تقنية تعقيم السطح* surface sterilization method وتنقيتها وتنميتها في وسط Luria broth وقد استخدم المذيب اثيل استيت ethyl acetate للحصول على الايض الثانوي البكتيري والذي اختبرت فعاليته الضد ميكروبية تجاه ثلاثة عزلات بكتيريه سالبه لصبغة غرام تضمنت *Escherichia coli , Salmonella typhi ,Pseudomonas aeruginosa* وبأقطار تثبيط تراوحت بين(16.5-31.0)ملم وتجاه ثلاثة عزلات بكتيرية موجبة لصبغة غرام تضمنت *Streptococcus pneumonia, Staphylococcus aureus* و*Bacillus cereus* وبأقطار تثبيط تراوحت بين(29.5-33.0)ملم وكذلك اختبرت الفعالية الضد فطريه تجاه نوعين من الخميرة تضمنت *Candida albicans* وبقطر تثبيط 16.0ملم وتجاه *Candida kruzi* وبقطر تثبيط 12.5 ملم باستخدام تقنيه الانتشار بالأقراص .وتراوحت قيم التركيز المثبط الأدنى بين (12.25 -6.25) و(3.12-25.0)مايكروغرام/ مل ضد العزلات البكتيرية السالبة والموجبة لصبغة غرام على التوالي ، أما ضد الخمائر فكانت 50 مايكرو كرام/ مل. كما اختبر التركيز القاتل الأدنى اذ تراوحت بين (12.5-25.0) و (6.12-50) مايكرو غرام/ مل ضد العزلات البكتيرية السالبة والموجبة لصبغة غرام على التوالي، أما ضد الخمائر فكانت 100 مايكرو غرام/ مل. كما قيمت الفعالية الضد تاكسدية باستخدام 2, 2-diphenyl-1- picrylhydrazyl (DPPH) وقد أظهرت نسب تثبيط عالية. كما اختبرت السمية الخلوية للايض الثانوي البكتيري والذي لم يظهر أي سمية تجاه كريات الدم الحمر عند التراكيز المختبرة أن الايض المنتج من بكتريا Endophytic يمكن أن تكون مصدرا بديلا جيدا للمضادات الميكروبية والتاكسدية الصناعية.

الكلمات المفتاحية: الفعالية الضد ميكروبية، الفعالية الضد تأكسدية، بكتريا النابوت الداخلي، البكتريا.

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Introduction

Plant-associated microorganisms fulfill important functions for plant growth and health. On the one hand, plants protect themselves by producing some compounds called secondary metabolites against pathogenic microbes. Some endophytic microorganisms produce antibiotics or growth-stimulating factor to benefit plant. Plant endophytes are microorganisms that live in the internal organs or cell gaps of healthy plants. Many factors, such as soil conditions and phytopathogen populations, influence the population structures of endophytic bacteria (1 ,2) . Endophytic bacteria have been isolated from the interior of the stems and roots of many plants, such as ginseng, cotton, sweet corn, canola, wheat, and others (3 ,4). Microbes that inhabit asymptomatically in the living tissues of plants without causing any substantive negative effect are known as endophytic microbes (5). Each plant species that exists on earth is considered to be a host for one or more endophytes. Diversity also associated with the colonizing bacterial taxa (6). Many bioactive substances that endophyte produce were relatively new to us. Therefore there is a huge potential to screen novel, highly active and low toxicity antimicrobial compounds from endophytic microorganisms (7) Metabolites isolated from the endophytes are good sources of novel secondary metabolic products having diverse structural groups and showing antibacterial, antifungal, anticancer, antiviral, antioxidant, insecticide, antidiabetic and immunosuppressive activities(8,9).Research on antibiotics and other microbial natural products is pivotal in the global fight against the growing problem of antibiotic resistance. It is necessary to find new antibiotics to tackle this problem (10, 11).

Materials and Methods

Plant selection and location

Wheat plant was collected from different parts of South of Iraq from Missan city during the year 2015. The roots of plants were washed with tap water and processed for the isolation of endophytic bacteria.

Media preparation and growth conditions of microorganism

The bacterial medium used in the isolation of endophytic bacteria contained the following: tryptone, 20 g/L; yeast extract, 5 g/L; trace salt (1 mL/L: FeSO4·7H2O, 0.01 g/L; MnCl2·4H2O, 0.01 g/L; ZnSO4·7H2O, 0.01 g/L); glucose, 5 g/L; agar, 15 g/L; at pH 7.3.

Isolation of endophytic bacteria

Endophytic bacteria were isolated from Wheat plant roots from three different locations. The roots of Wheat plant were surface sterilized with 99% ethanol for 60 s followed by 3.125% sodium hypochlorite for 6 min, washed in 99% ethanol for 30 s, and finally rinsed in sterile water. The surface-sterilized roots were then aseptically sectioned into 1 cm fragments, distributed onto the isolation media, and incubated at 28°C for 2 to 15 days. The only bacterial colonies that developed in the media were separately transferred to fresh media to obtain a pure culture (12).

Preparation of bacterial extract

Endophytic bacterium was inoculated into 100 ml of sterile nutrient broth and kept at 37±2°C for 24 hrs. With continuous shaking. Then 20 ml of grown culture was transferred into 1000 ml of nutrient broth after incubated at 37°C for 5 days under continuous shaking at 120 rpm/min .Mass cultivated cultures were centrifuged and the supernatant was mixed with equal volume of ethyl acetate (1:1) in a separating funnel and after vigorous shaking, the organic material was collected and subjected for evaporation.

Assay of Antimicrobial Activity

Test bacteria

Bacterial pathogens used in the study were obtained from the Culture Collection from AL-Sadder Hospital in Misan city of Iraq during the year 2015. Include three gram negative bacteria include *Escherichia coli , Salmonella typhi ,Pseudomonas aeruginosa* and three gram positive bacteria include *Staphylococcus aureus, Streptococcus pneumonia and Bacillus cereus* . The bacteria were maintained in Nutrient agar (NA) and Sabourauds dextrose agar (SDA) slants are used for the maintenance of yeasts.

Antimicrobial Activity

Filter paper discs (0.6 mm) after being sterilized by autoclave were socked in bacterial crude extract solution for 5 min., filter paper discs with extract were placed on the surface of Muller-Hinton agar medium in Petri-dishes Plates streaked with 0.2 ml of microbial suspension. Plates were incubated at 37 ◦C for 24 hr for bacteria and 48 hr for yeasts an appearance of inhibition zones(mm) around the filter paper disc indicating the bioactivity of crude metabolites of the tested bacterial isolates (13). The diameters of the clear zones were measured and compared with control agar plates containing discs with solvent only (ethyl acetate) as control, triplicates were made.

Minimal inhibitory concentration and minimal bactericidal concentration and Minimal fungicidal concentration test.

The minimal inhibitory concentration (MIC), minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC) values were determined by the standard serial dilution assay (14). Bacterial extract isolate was selected for this test. The inhibitory test was carried out on Muller-Hinton agar medium for bacteria and Sabourauds dextrose agar for yeasts.

Antioxidant activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was measured according to (15). 1 ml of bacterial extract was mixed with 0.5 ml of 0.2 mM methanolic DPPH solution. The reaction was allowed to stand at room temperature in the dark for 30 min and the absorbance was recorded at 517 nm against a blank(methanol solution).Dilutions were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90 and 1.95 µg/ml. Tests were carried out in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

Scavenging effect (%) = [(A0-A1)/A0] × 100, where A0 and A1 are the absorbance of the control and the sample, respectively.

Toxicity test

Cytotoxicity of the bacterial secondary metabolites was examined by using human Red Blood Cells (RBC) following a previously described method (16).

Statistical Analysis

Data were analyzed using Analysis of Variance (ANOVA) between any pair of variables.

Results and Discussion

The growth of one endophytic bacterium named as isolate *B. megaterium* was observed from the cut edge of the surface sterilized root of wheat after 24 hrs on the nutrient agar plates. Considerable growth was observed after 24 hrs and this strain was isolated, grown and subsequently pure culture was maintained on nutrient agar slant as well as in 10% glycerol at 4°C. The bacterium was observed to be Gram positive and rod shaped Endospore formation. Based on the results obtained in (Table1) the isolate was identified as *B.megaterium*.

Table1.Biochemical testes of *Bacillus megaterium*.

Biochemical tests	Results
Catalase	+ve
Oxidase	+ve
Indole	-ve
MR/VP	+ve/-ve
Citrate utilization	-ve
Nitrate reaction	-ve
Urease production	-ve
Triple sugar ion agar	H_2S -ve , acid slant

The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of antibiotics resistance by plant and human pathogens (17).Endophytes are chemical synthesizer inside plants (18) in additional, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms (19).The antimicrobial activity of the endophytic bacterium *B.megaterium* was observed against all the tests bacteria. inhibition zones ranged between 16.5-31.0 mm against Gram-Negative Bacteria and ranged between 29.5 33.0 mm against Gram-Positive Bacteria

also observed against testes fungi with inhibition zones ranged between 12.5-16.0 mm(Table2) (Figs.1). Endophytes are reported as novel source of antimicrobial compounds (20). Endophytic microorganisms are excellent sources of bioactive natural products that can be use to satisfy demand of pharmaceutical and medical industries), since a single endophyte may be able to produce a variety of bioactive metabolites (21). MIC and MBC of extract ranged from 3.12-50 µg/ml against bacterial isolates and MIC and MFC ranged between 50.0-100 µg/ml against test yeasts . the lowest concentration without visible growth was defined as the MIC. (Table.2 , Fig. 1).

Table 2. Growth inhibition zones and MIC and MBC exhibited by the bacterial extract against microbial strain

Gram-Negative Bacteria	**Inhibition zones (mm)**	**MIC(µg/ml)**	**MBC(µg/ml)**
E. coli	30.0	6.25	12.5
Sal. typhi	31.0	6.25	12.5
Ps.aeruginosa	16.5	12.5	25.0
Gram-Positive Bacteria			
S. aureus	30.5	25.0	50.0
Strep. pneumoniae	29.5	6.25	12.5
B. cereus	33.0	3.12	6.12
Yeasts			MFC**(µg/ml)**
C.albicans	16.0	50	100
C.kruzi	12.5	50	100

Numbers represent average of three replicates $P \leq 0.05$

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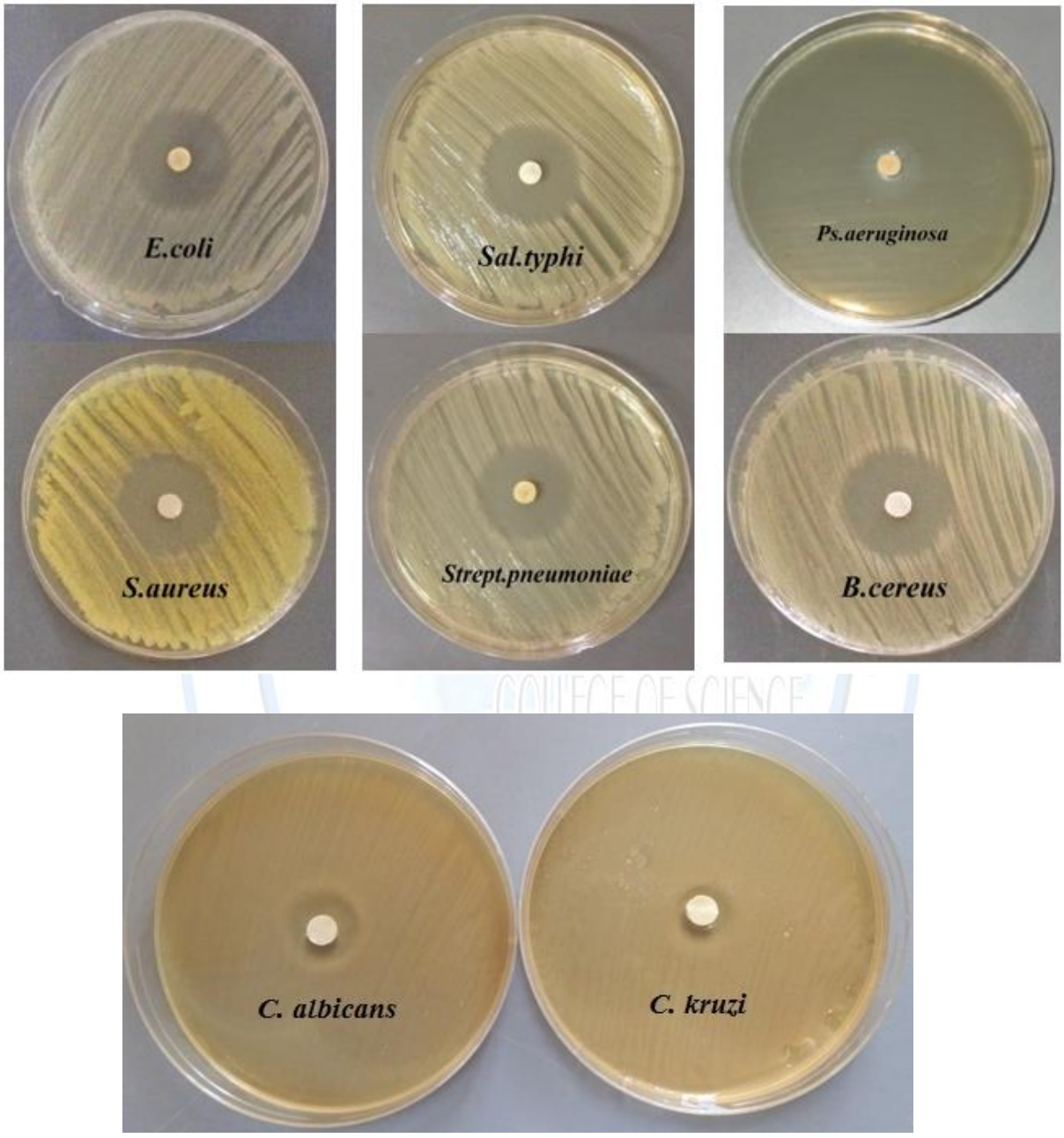

Fig. 1. Inhibition zones exhibited by endophytic bacterium extract against microbial strains

A verification of non toxicity of the bacterial extract against human blood revealed a negative test. Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initiation or propagation of oxidizing chain reactions. DPPH radical scavenging assay is a swift and sensitive method for the antioxidant activity. to determination of free radical scavenging activity using the stable 2, 2- diphenyl-1-picryl-hydrazyl radical (DPPH) has received the utmost attention owing to the ease of use and its convenience (Moreno *et al*., 1998). It was observed that the scavenging activity of secondary metabolites from *B .megaterium* at all concentrations from 1.95 to 1000µg/ml is rather strong (20-80%).The extract improved 80% inhibition at higher concentrations, indicating lesser antioxidant capacity than positive control Figure (2) .

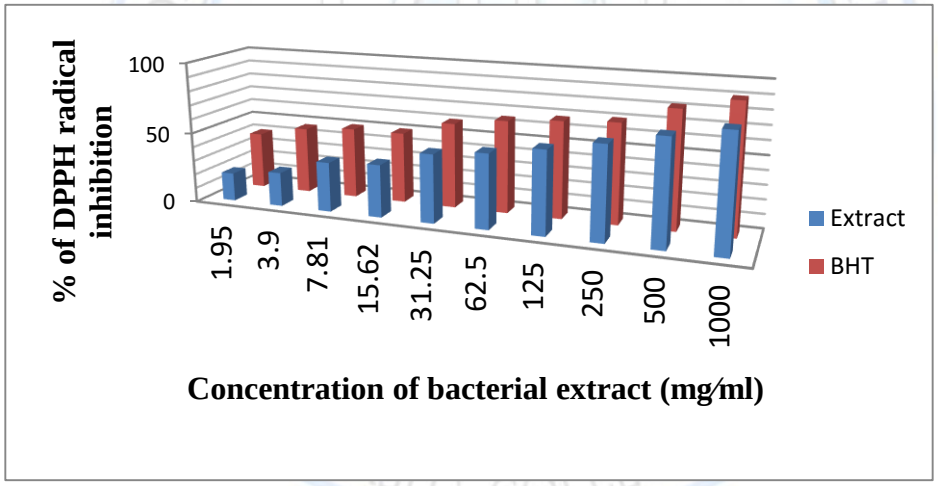

Figure 2:Antioxidant (DPPH scavenging) activity of investigated bacterial extract presented as percentage of DPPH radicals inhibition

Conclusion

Endophytic bacteria have proven to be rich sources of novel natural compounds with a wide spectrum of biological activities. This study revealed that this endophytic bacterium was showing significant antibacterial, antifungal and antioxidant activity.

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