REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 23, No. 4 (2022)
http://www.veterinaria.org
Article Received: 10 August 2022; Revised: 01 November 2022; Accepted: 20 November 2022

Isolation and Evaluation of Antibacterial Agents Produced by Soil Bacillus SP. and Study Some of their Immunological Parameters

Mohammad J. Al-Jassani[*1], Mohammed Abdulkadhim Sayah[2], Maytham T. Qasim[3], Abed J. Kadhim[4], Entsar Hachim Muhammad[5]

[1] Department of Forensic Science, College of Science / Al-Karkh University of Science/Iraq
pcr2000@yahoo.com
[2] Al-Manara College For Medical Sciences/ (Maysan)/Iraq
[3] Department of Anesthesia, College of Health and Medical Technology, Al-Ayen University, Thi-Qar, Iraq
[4] Al-Nisour University College/Iraq
[5] Department of Medical Laboratory Technics, AlNoor University College, Nineveh, Iraq

Abstract
A total of 120 soil samples were gathered from various locations in Iraq's Babylon province. Thirty Bacillus isolates were collected, purified, and identified based on morphological and biochemical characteristics. Antimicrobial activity was assessed on all isolates against Gram-positive and negative pathogenic bacteria through primary screening. The results obtained that 10 isolated have such potential. Secondary metabolic products were extracted from extracellular and tested for their activity against pathogenic bacteria using the well diffusion method in secondary screening. Three isolates (MA3, HI-11 & AM-15) had diameters more than 1 mm were considered to be potential isolates and were subjected for immune study. Antigens derived from secondary metabolic products (antibacterial agents) were generated and tested in local rabbits using the DTH-skin test and differential white blood cells. Six animals of both sexes were randomly assigned to one of two antigen groups in the first group (3 rabbits). After 14 days, a booster dose in the same amount was administered. The second group, consisting of three animals, was used as a control group. No significant differences were found in the DTH-skin test ($P< 0.05$) were recorded between the concentrations 15mg/ml and 7.5mg/ml after 24, 48, and 72 hrs., but there was a significant differences ($P<0.05$) between these concentrations and 7.5 mg/ml and 3.75 mg/ml and control site. There was no significant difference between all types of cells (neutrophils, lymphocytes, monocytes, and eosinophils) of immunized and control groups.

Keywords: *Bacillus*, DTH- skin test, antimicrobial activity, neutrophils, lymphocytes

INTRODUCTION

Bacillus is a genus of Gram-positive bacteria and a member of the phylum Rutium, is a bacilli ranging from (0.5 x 1.2 µm) to (2.5 x 10 µm), aerobic or facultatively anaerobic and derives its energy from respiration or fermentation, these bacteria can produce vesicles It is allowed to withstand unfavorable environmental conditions (Eppinger et al., 2011).

Bacillus is a diverse group of procaryotes forming spores at some stage of their growth and producing different types of antibiotics for medical and agriculture applications (Ahmed, 2007, Jassim and Al-Amery, 2019). *B. subtilis*, *B. megaterium*, has been often employed in biochemical research due to its vast metabolic capability and physical properties favorable to biotechnology applications. Bacillus can also produce vitamin B12 via an oxygen-free adenosylcobalamin route. (Hitchins et al., 1968; Elmerich and Aubert, 1971; AlThubiani et al., 2018; Mannaa and Kim, 2018; Eppinger et al., 2011; Al-Bdairi et. al. 2022; Alkhafaje et. al. 2022).

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The antibacterial activity of crude or purified extracts from diverse sources has been thoroughly examined, but there have been few researches on the purification of antimicrobial compounds from *Bacillus* bacteria and their antibacterial activity.
The goal of this work was to isolate and evaluate antibacterial bioactive chemicals from *Bacillus* against pathogenic bacteria, which could aid as an alternative antibiotic and in the development of new antibiotics.

MATERIALS AND METHODS

Collection of Samples: The soils were obtained at random from various locations in Babylon province. These samples were stored in sterile, dry polyethylene sacs at room temperature until they were needed.

Isolation of Bacillus bacteria: Soil samples were treated with calcium carbonate for 24 hours before being serially diluted to a concentration of 10^{-6}. The pour plate method was used to cultivate one milliliter of the diluted soil. (Pridham *et al*, 1975). The bacteria plates were incubated at 37ºC for 48 hours. On the same media, the colonies were subcultured many times to obtain a pure culture.

Diagnosis of isolates: Microscopic and biochemical tests were performed to diagnose bacterial isolates based on the classification of (Don, *et al*, 2005)

Screening of the antimicrobial agent:
Each of the *Bacillus* spp. isolates were inoculated in a 500ml flask containing 250ml of ISP-2 broth (pH 7.2) then incubated at 28°C for 2 days. The fermented broth was filtered using What man No.1 filter paper then centrifuged at 6000rpm for 15min. The supernatant was extracted with an equal volume (1:1 v/v) of ethyl acetate with vigorous shaking for one hour. The ethyl acetate phase was separated and evaporated in a water bath at 80°C. The residue obtained was weighed (Atta, 2010) and used for antibacterial analyses by the agar well diffusion method (Pridham *et al* 1975) against standard pathogenic organisms (*Streptococcus pyogenes, Staphylococcus albus, Staphylococcus aureus*, *Klebsiella pneumonia, Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa and Aeromonas hydrophila*).

Antigen preparation: Antigen was prepared by using formaldehyde buffer saline 1% (KWCA) 10and used in the immunization of rabbits and antimicrobial agent antigen that used in delayed Type hypersensitivity (DTH)- skin test (Al-Sultany and Jassim, 2016).

Immunization of rabbits
Six local breed rabbits of both sexes (about 1.5 Kg. B.Wt.) were used and divided randomly into two groups: the first group (3 animals) were immunized with 1ml of *Bacillus* secondary metabolisms extract that was injected subcutaneously, and a booster dose of 1ml was injected subcutaneously 14 days later. The second group (3 animals) control was injected

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subcutaneously with 1ml of PBS (pH 7.2) and considered as a control (Al-Sultany and Jassim, 2016).

Immunological tests

Delayed-Type Hype sensitivity (DTH)-skin test was done for all immunized rabbits on day 20 of immunization .as suggested by Hudson, and Hay 1980 with some modification, Using different protein concentrations of sonicated (KWCS) antigen of MA3 isolate concentrated antigen used in a dose 15 mg /ml, 7.5 mg/ml and 3.75 mg /ml and PBS(PH 7.2) as a control region by intradermal injection of immunized animals Blood samples in day 20 of immunization 2ml of blood were collected from animals into EDTA containing containers and blood smear were prepared by staining Giemsa stain to estimate the differential white blood cell.

Blood samples

On day 20 of immunization 2ml of blood were collected from animals into EDTA-containing containers and blood smears were prepared by staining Giemsa stain to estimate the differential white blood cell (Coles 1986).

RESULTS AND DISCUSSION:

Sample Collection

All ten isolates were identified in this investigation based on their physical characteristics and biochemical assays. The results revealed that all of these isolates belong to the bacillus species, and they were assigned a unique code (table 1). The 10 *Bacillus* isolates showed antimicrobial activity against some Gram-positive and some Gram-negative bacteria as shown in figure (1) this was reported earlier by (Immanual *et al*, 2006).

Table 1: Sampling sites

Site	Code
Al-Mahaweel	MA3
Al-Kothar	KO-7
Al-Hilla	HI-11
Al-Kothar	KO13
Al-Amam	AM-15
Al-Hamza	HA-28
Al-Hamza	HA-37
Al-Kasim	KA-41
Al-Mashrwia	MA-42
Al-Mashrwia	MA-CL43

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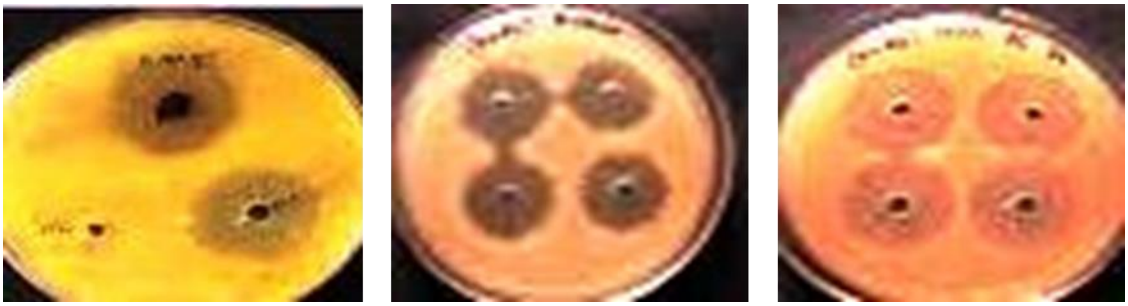

Figure 1: The inhibition zone of the active isolates' culture filtrate extract against the tested microorganisms.

Characterization of active Isolates: For partial identification of isolates, morphological and biochemical characterization of probable isolates were explored. For identification based on the results of several biochemical assays, Bergey's Manual of Systematic Bacteriology (2nd edition) was utilized as a reference. isolates were Gram-positive and were identified as belonging to the genus Bacillus (Narjes, 2021). Table (2) lists the morphological characteristics of more activity isolates

Table2: Morphological characteristics of the active isolates

Characteristics	**Isolates**		
	MA3	HI-11	AM-15
Gram stain	+	+	+
Shape of colony	Round	Round	Round
Elevation	flat	Convex	Convex
Size	Large	Large	small
Margine	Even	Even	Even
Texture	Smooth	Smooth	Smooth

Delayed-Type Hypersensitivity (DTH)-skin test

The results in table (3) clarified the diameter of erythema (mm) after 24, 48, 72 hrs.

Table 3: Means of skin erythema (mm) of immunized rabbits by MA3 secondary metabolisms extract (antimicrobial agent) isolated

Concentration (mg/ml)	MEAN± S.E (mm)		
	24hrs	48 hrs	72 hrs
15	8.32±0.04	6.76±0.43	2.76±0.2
7.5	6.02±0.20	4.00±0.20	2.94±0.32
3.75	3.31±0.10	3.27±0.22	1.04±0.2
Control site PBS (PH 7.2)	1.1±0.02	1.1±0.02	1.00±0.00

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Delayed-Type Hypersensitivity response is the principle pattern of cell-mediated immunization protocols which elicited cell immune response in mediated immune response, that induced by CD4 and CD8 T cell17. The induration of the skin at the site of injection may be due to the accumulation of activated macrophages and other nonspecific inflammatory cells in the dermis and between muscle fibers (Tizard. 2009). No significant differences ($P< 0.05$) in the DTH skin test were recorded between the concentrations 15 mg/ml and 7.5 mg/ml after 24, 48, and 72 hrs., but there was a significant differences $P<0.05$) between these concentrations and 7.5 mg/ml and 3.75 mg/ml and control. The results are in agreement with (Konna et al, 2001).

Differential white blood cells count: There were no significant differences ($P<0.05$) between all types of cells (neutrophils, lymphocytes, monocytes, and eosinophils) between the immunized group and control group (table 4).

Table4: Differential white blood cells count in the immunized group and control

Groups	Types of cells				
	Neutrophils %	Lymphocytes	Monocytes %	Eosinophils %	Basophils %
Immunized group	23.61±4.22	40.10±3.40	3.93±0.15	4.0 5±0.94.	0.30±0.2
Control group	32.54±2.62	35.46±2.16	3.86±0.15	0.39± 0.35	0.15±0.1

The increase in the number of lymphocytes without significant differences in the immunized group compared with the control group may be due to regeneration of lymphocytes from the bone marrow and migrated to the site of injection20and this agreement with (Juyapal, 2007; Al-Sultany and Jassim 2016).

CONCLUSION

Three isolates (MA3, HI-11 & AM-15) had given good antibacterial activity with no significant differences ($P< 0.05$) in the DTH skin test were recorded between the concentrations 15 mg/ml and 7.5 mg/ml after 24, 48, and 72 hrs., but there was a significant differences $P<0.05$) between these concentrations and 7.5 mg/ml and 3.75 mg/ml and control site. There was no significant difference between all types of cells (neutrophils, lymphocytes, monocytes, and eosinophils) of immunized and control groups.

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