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Research Article

Study of antibacterial activity and cytotoxicity of the bioactive compound of *Bacillus megaterium* L2 strains isolated from the oral cavity of hospital workers and visitors at Dental Health Centre, Babylon, Iraq

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Abstract
Because of the resistance of pathogenic bacteria to antibiotics, there is an urgent necessity to search for new antibiotics produced by *Bacillus spp*., which are characterized by their capability to produce secondary metabolites with high efficacy against numerous types of pathogenic bacteria. A total of 40 *Bacillus* isolates were isolated from the mouths of 150 volunteers from the Dental Health Center in Babylon and diagnosed based on phenotypic characteristics and biochemical and physiological reaction tests with a colorimetric reagent card using the VITEK2 analyzer. The active compounds were extracted from *Bacillus megaterium L2* and their antibacterial activity was tested against a group of gram-negative and gram-positive bacteria. The Minimum inhibitory concentration (MIC) of the extract was estimated, whereas 16 isolates showed high effectiveness against pathogenic bacteria, with the zone of inhibition ranging from 8-22 mm and the MIC ranging from 0.25–6.25 mg/ml. The active compounds were extracted, purified, and detected by Thin-layer chromatography (TLC), Infrared (IR) spectroscopy, and Ultraviolet (UV) spectroscopy. The cytotoxic activity of the extracts was studied using the MCF7 cell line. This showed that cytotoxicity effects on valid object count, nuclear morphology, and total nuclear intensity ranged from 17.245-441.24 and the cytotoxic effect on cell membrane permeability, mitochondrial membrane potential, and cytochrome C ranged from 49.04-601.79 Among the isolates, *Bacillus megaterium L2(B9)* was the best isolated strain of bacteria that was the most effective against anti-pathogenic bacterial strains- Gram positive (*Staphylococcus pyogenes* NCTC 8198 and *St. aureus* ATCC 29213) and gram negative (*Pseudomonas aeruginosa RW109, Escherichia coli O157, and Salmonella typhi* Ty2) and was non-toxic to human cells (MCF7).

Keywords: *Bacillus megaterium*, Cytotoxicity, Gram positive bacteria, Gram positive bacteria Infrared (IR) Spectroscopy, Ultraviolet (UV) Spectroscopy

INTRODUCTION

The emergence of antibiotic resistance among pathogenic bacteria is regarded as one of the world's most serious issues, posing a significant threat to the globe (Fernández-Ortuño *et al.*,2015; Yang *et al.*,2019). One of the primary causes of the emergence of antibiotic resistance is the usage of antibiotics without a doctor's prescription (Sample, 2018). As a result of utilizing these antibiotics without a doctor's prescription, 95 percent of Staphylococcus aureus is resistant to penicillin and 60 percent to methicillin (Innes *et al.*,2020)

Furthermore, the transmission of resistance genes across microorganisms increased in the spread of resistance among diverse specie (Edwards *et al.*, 2018). During the year, two million individuals are infected with various microbial diseases, and about 230,000 people die due to the phenomenon of antibiotic resistance in pathogenic bacteria (Centers for Disease Control and Prevention, 2015). According to World Health Organization (WHO 2011), the most widely used and effective antimicrobial agents are b-lactams, tetracyclines, polymyxins, polypeptides, aminoglycosides, and lincosamide . Eight hundred antibacterial and antimi-

crobial agents produced by *Bacillus spp*. (Saxena, 2019).

Some genus of Bacillus biosynthesizes antibiotics through a ribosomal or non-ribosomal mechanism. For example, gramicidin is produced by *Bacillus brevi* (Zhang *et al.*, 2020), gavaserin by *Bacillus polymyxa*, bacitracin by *Bcillus subtili* (Guevarra *et al.*, 2019), and subtilin by *Bacillus licheniformis* (Adhikari *et al.*, 2019).

Because the antibiotics produced by *Bacillus spp.* are very important, acquiring biological products that live in the oral cavity of humans may aid in discovering novel, effective, and efficient antibiotics and reducing the problem of antibiotic resistance. Because of the resistance of pathogenic bacteria to antibiotics, there was an urgent necessity to search for new antibiotics, so the research aimed to investigate *Bacillus spp* isolates that produce effective antibiotics.

MATERIALS AND METHODS

Ethical approval for research

Ethical approval was obtained from the relevant animal/ human ethics committee (Research Ethics Committee of the Dental Center in Babil Governorate, Iraq, Reference number: (00479-2018) to conduct the research using animals and/or involving humans.

Isolation of *Bacillus spp*.

Bacteria were isolated from the mouths of 150 volunteers of Hospital workers and Healthy visitors to the hospital for examination only who had not taken any antibiotics for four weeks. The volunteers were instructed not to eat, drink, smoke, or brush their teeth for two hours before taking the sample. Saliva was collected from subjects in Eppendorf tubes. After that, the samples were spread on agar media (MRS) under aerobic conditions and incubated at a temperature of 37 °C for a period of 72 hours. After the end of the incubation period, the growing colonies of developing *Bacillus spp*. from hospital workers and visitors were examined, which were different from each other. These colonies were activated in the preservation medium by adding 20% glycerol at 80 °C until use (Sarika *et al.,* 2012).

Characterization and identification of isolates

The isolates were identified morphologically and biochemically using Bergey's Manual of Systematic Bacteriology. The isolates were also characterized physiologically and biochemically using a Colorimetric reagent card from a VITEK 2 analyzer (BioMerieux, France) (Kamal *et al.,*2021).

Collection of test strains

Two gram-positive strains (*Staphylococcus aureus* ATCC 29213; *Streptococcus. pyogenes* NCTC 8198) and three gram-negative strains (*Escherichia coli O157; Pseudomonas aeruginosa RW109; Salmonella typhi* Ty2) were obtained from Imam Al-Sadiq Teaching Hospital in Hilla City, Babylon Governorate.

Evaluation of the antibacterial activity of *Bacillus megaterium* strains

The antibacterial activities of the extracts were measured by using agar well diffusion. A transfusion of bacteria was added to 5 ml of MSR broth and incubated at 37c for three days. Then 0.5 ml of the tester strain broth was added to the molten medium after it had cooled down. The plates were poured and left to dry and harden, and then to use a sterile cork borer. Four wells with a diameter of 8 mm were drilled on the plate and inoculated with 100 ml *Bacillus* strain extracts. Gentamycin (10 lg/ml) was used as a positive control. Plates were incubated at 37 °C for 24 hr. under aerobic conditions for bacteria (Li *et al.*, 2017). The extent of the extract's ability to inhibit pathogenic bacteria was measured based on the observation of the inhibition diameters and their diameters were measured in millimetres, where the test was carried out with three replications and the data are presented as Mean ± SD. When measuring the antibacterial ability, the minimum inhibitory concentrations (MIC) were determined using diffusion from tubes ((Li *et al.*, 2017).

Extraction, purification, and detection of the active antimicrobial compound from the selected *Bacillus spp.* isolates

The active substances against pathogenic bacteria were prepared by inoculating MRS broth with 16 selected isolates of *Bacillus spp.* and incubating under optimal and aerobic conditions. To extract the active substances, the bacterial culture was centrifuged at 10,000 rpm for a quarter of an hour at -4 °C and filtered through a 0.2 lm sterile nitrocellulose membrane filter (Whatman, Germany). Then, n-butanol was added to the filtrate (2:1 volume/volume), shaken with force, and left to separate. The filtrate represented by the supernatant was taken and concentrated in a vacuum rotary pump (Al-Saraireh *et al.*, 2015).

Chromatography of the antimicrobial compound

The extracted bioactive compound was analyzed by thin-layer chromatography (TLC) according to the method of Al-Saraireh *et al.* (2015). with slight modifications.

Identification of the active compound

Infrared (IR) spectroscopy

After extracting the active compounds (antibacterial) from the fermentation broth, they were mixed with pure salt potassium bromide to remove scattering effects from large crystals, and this powder mixture was mechanically pressed to form a thin layer through which

the Spectrometer's beam could pass (Al-Saraireh *et al.*, 2015).

Ultraviolet spectroscopy

After extracting the active compounds from the fermentation cultures, they are dissolved in n-butanol and measured by Ultraviolet (UV) spectrophotometer to know the λmax to give an idea of the lengths of absorption in the range from 200 nm to 800 nm (Muhammad, and Ahmed, 2015).

MCF-7 Cell lines

MCF-7 (Michigan Cancer Foundation-7) cell lines were derived from the pleural effusion of a 69-year-old female suffering from a breast adenocarcinoma. The cell lines were obtained from the Center of Biotechnological Research. No. of passage: 15. The cytotoxic effect of different compounds isolated from *Lantana camara* crude extracts was performed by using MTT ready-to-use kit (Intron Biotech) (Olson *et al.*, 2020).

Cytotoxicity antimicrobial components from the *B. megaterium* via High content screen on MCF-7

Six cellular variables were selected to study the cytotoxicity of the active compounds: nuclear density, nuclear morphology, cell number, cytochrome c, mitochondrial membrane potential and cell membrane permeability. Twenty-four hours later, it was determined at $37C^0$ with four different concentrations spectrum. The crude antimicrobial components (25, 50, 100, and 200 µg /ml) were obtained from the *B. megaterium*. Positive control (5.0 µM) of Paclitaxel was used on MCF-7 cell lines (Hassan *et al.*, 2015).

RESULTS AND DISCUSSION

Identification and characterization of *Bacillus* isolates

Bacillus isolates were identified based on morphological and biochemical properties. The VITEK2 analyzer found a 92 per cent match for the genus *Bacillus megaterium L2,* as shown in Table 1. The colony of *B. megaterium* strain was identified morphologically as a creamy yellow color and a diameter of 3 mm wavy on TSA medium, not pigmented, Gram-positive, motile, aerobically developing at a temperature of 40-45°C.

***Bacillus spp.* extracts and their antimicrobial activity**

The antibacterial activities of the active metabolites produced by 16 isolates of *Bacillus spp*. at optimized iconditions of incubation at 37 °C and pH 7.0 for four days are shown in Table 2.

Among all these isolates, *Bacillus megaterium L2* (B9) was the best isolate of bacteria that had the most effective anti-pathogenic bacteria against the strain- Gram-positive (*St. pyogenes* NCTC 8198 and *S. aureus* ATCC 29213) and Gram-negative (*P. aeruginosa RW109, E. coli O157, and S. typhi* Ty2). The zone of inhibition ranging from 6.63 - 21.60 mm was characterized and identified as *Bacillus megaterium* according to phenotypic characteristics and biochemical and physiological reaction tests with a Colorimetric reagent card using the VITEK2 analyzer.

The antibacterial extract extracted as secondary metabolites from *B. megaterium* showed the lowest MIC of 0.25, 0.5, and 1 lg/ml against *St. pyogenes, S. typhi, and P. aeruginosa, respectively.* In contrast , the highest MICs of 3.125 and 6.25 lg/ml were observed against *E. coli and S. aureus*, respectively, as shown in Table 3.

Characterization and purification of active metabolites

The extracted active compound appeared as a greenish solid on a TLC plate with an Rf of 7.8 cm, as shown in Fig. 1. The separation spot was then scratched and prepared for the identification processes. Ultraviolet (UV) spectroscopy was used to measure the λ max for antibiotics, which was 275.00 nm. This gives an idea about the types of these compounds (Fig. 2). Infrared Red (IR) spectroscopy was used to know the essential chemical functional groups present in produced antibiotics (Fig. 3).

Fig. 3 shows several absorption bands; each one refered to a specific functional chemical group, such as the Absorption band 3751.67 cm−1 refered to the O-H group (free). The absorption band at 3421.83 cm −1 - 3279.10 cm −1 refered to the O-H group (H-bonded) or N-H group (primary and secondary amines and amides, stretch). The absorption band at 2924.18 cm −1 - 2854.74 cm −1 refers to the C-H group (Aldehyde). The absorption band at 1674.26 cm −1 - 1637.62 cm −1 refered to the C-C group (Alkene) or (Amide). The absorption band at 1541.18 cm −1 - 1417.37 cm −1 refered to the (Aromatic) or Nitro (R-NO2) group. The absorption band at 1153.47 cm −1 - 1014.59 cm −1 refered to the C-O group (several chemicals) or C-N group (Amine). The absorption band at 918.15 cm −1 – 493.79 cm −1 refered to the C-H group (out of the plane bend).

Cytotoxicity of antimicrobial components from the *B. megaterium* via High content screen on MCF-7

The results presented in Fig. 4 summarize the extracellular crude extract concentrations and average intensities for each study**.** The positive control Paclitaxel used in the study was a tubulin target, which was considered as their mechanism of action. The Paclitaxel-treated cells have a force with the spindle assembly, cell division, and also chromosome segregation, which is contrary to colchicine, a drug that also targets tubulin, whereas Paclitaxel exactly stabilizes and guards micro-

Table 1. Determination of physicochemical tests of strain *B. megaterium* using API 50 BCL system , the VITEK 2 analyzer.

Biochemical test	Result	Biochemical test	Result
Esculine hydrolysis	++	Beta–Xylosidase	++
Beta–Galactosidase	--	D–Glucose	--
Ala–Phe–Pro arylamidase	++	Phosphorl choline	--
Ellman	++	D–Melezitose	--
D–Mannose	--	Methyl D – Xyloside	--
Beta–Mannosidase	++	Cyclodextrin	++
Inulin	--	L–Pyrrolydonyl arylamidase	--
Oleandomycin resistance	--	L–Lysine arylamidase	--
Tetrazolium red	--	Maltotriose	--
Leucine arylamidase	--	Palatinose	--
Alanine arylamidase	--	α – Glucosidase	++
Glycogen	--	Putrescine assimilation	--
Polmixin–B resistance	--	D – Tagatose	--
Phenylalanine	++	NaCl 6.5%	++
Tyrosine arylamidase	++	L– Asartate arylamidase	--
Myo inositol	++	Methyl α–D Glucopyranoside acidification	--
Glycine arylamidase	--	D–Galactose	--
L – Rhamnose	--	A–Mannosidase	--
N–Acetyl D–Glucosamine	--	β – Glucosidase	++
Pyruvate	--	D – Mannitol	++
D–Ribose	--	A–Galactosidase	++
Kanamycin resistance	--	β – N – Acetyl Glucosaminidase	--
D – Trehalose	--	L – Proline arylamidase	--

tubule against disassembly as described by Lee *et al*, (2016); Al Barzanchi and Sh. (2014).

Microscopic analysis of parameters and images of the studied samples using a Zeiss Axio Z1 fluorescence microscope with X1 CCD optical measurements and the results of the cytotoxicity assay are shown in Fig. 4 comparing between 200 µg/ml of extracellular crude extract and 5.0 µM of Paclitaxel on one side and another untreated cell line unite from the other side.

DISCUSSION

The results in Tables 1, 2, and 3 are consistent with what was done by Lee *et al.* (2016), who isolated a secondary bioactive metabolites compound from *Bacillus amyloliquefaciens* that can inhibit pathogenic bacteria and bacteria contaminating foods. Furthermore, Hu *et al.* (2021) isolated and purified active secondary metabolites as antimicrobials from *Bacillus atrophaeus.* Li *et al.* (2017) isolated and purified some effective metabolic substances as antibacterial from some *Bacillus spp.* present in the soil called *B. subtilis* and extracted some compounds that were effective against pathogenic bacteria.

The purification and characterization of the active compound from the isolate *B. megaterium L2* (B9) through

Fig. 1. *Separation of the crude antimicrobial components obtained from the B. megaterium supernatant on TLC plate*

Table 2. Antibacterial activity of *Bacillus* isolates (strains) against the tester strains.

Bacillus strains	Diameter of zone of inhibition (mm)				
	***St. aureus* ATCC 29213**	***St. pyogenes* NCTC 8198**	***E. coli O157***	***P. aeruginosa RW109***	***S. typhi* Ty2**
B1	8.78 ± 0.67	14.89 ± 1.75	8.99 ± 0.76	8.53 ± 0.76	8.25 ± 0.78
B2	8.25 ± 0.78	20.67 ± 1.14	8.66 ± 0.55	16.89 ± 1.64	8.95 ± 0.81
B3	10.33 ± 0.99	8.78 ± 0.67	9.98 ± 1.18	14.88 ± 1.75	8.63 ± 0.33
B4	9.63 ± 0.76	9.98 ± 1.18	8.33 ± 0.71	8.33 ± 0.71	8.77 ± 0.12
B5	8.99 ± 0.76	11.33 ± 0.92	16.85 ± 1.64	8.45 ± 0.81	7.63 ± 0.55
B6	8.66 ± 0.55	8.44 ± 0.81	8.69 ± 0.72	9.98 ± 1.18	8.11 ± 0.88
B7	9.98 ± 1.18	8.66 ± 0.22	8.78 ± 0.67	8.65 ± 0.70	8.13 ± 0.76
B8	8.60 ± 0.77	8.43 ± 0.71	10.33 ± 0.99	8.66 ± 0.44	10.63 ± 0.62
B9	17.87 ± 1.64	8.25 ± 0.78	19.30 ± 1.35	8.25 ± 0.78	21.60 ± 1.16
B10	13.63 ± 0.76	9.63 ± 0.70	8.43 ± 0.22	8.69 ± 0.76	8.33 ± 0.71
B11	8.23 ± 0.76	14.79 ± 1.75	9.63 ± 0.74	8.78 ± 0.67	9.98 ± 1.18
B12	8.93 ± 0.42	6.63 ± 0.16	6.63 ± 0.76	8.33 ± 0.76	8.66 ± 0.55
B13	9.78 ± 0.98	16.39 ± 1.64	18.33 ± 1.55	7.63 ± 0.76	10.33 ± 0.99
B14	5.70 ± 0.19	8.93 ± 0.76	11.63 ± 0.76	11.60 ± 0.70	8.66 ± 0.55
B15	8.53 ± 0.76	18.33 ± 1.55	10.63 ± 0.76	7.83 ± 0.70	8.78 ± 0.67
B16	8.33 ± 0.71	8.66 ± 0.55	8.83 ± 0.75	8.66 ± 0.55	8.53 ± 0.76

Ultraviolet (UV) spectroscopy (Fig. 2), Infrared Red (IR) spectrum (Fig. 3), and separation of the crude antimicrobial components obtained from the *B. megaterium* supernatant on TLC plate (Fig. 1). indicated the presence of a peptide component in the active compound. Compared to previous studies, Lin *et al.* (1994) observed strong bands indicating the presence of a peptide component depending on what was obtained through the FT-IR spectrum. In a study conducted by Kim *et al*. (2016), the FT-IR spectrum of purified bacitracin showed characteristic absorption valleys at 1540, 1650, and 3300 cm^{-1}, indicating that antibiotic contains peptide bonds. Scapini *et al. (* 2019) observed the absorption valley at 2936 cm1 resulting from CH stretching, indicating an aliphatic chain. The N-H bond deformation combined with the C-N molecule, so the absorption formed the peak at 1415 cm^{1}. Muhammad *et al.* (2016) isolated this molecule as an antibacterial polypeptide from the *B. brevis* MH9 and its structure was described by FT-IR spectrum to detect the absorption peaks, which were at the regions of 794 cm^{-1} (C=C), 3620 cm-1 (-OH).), 3490 cm-1 (H-O-H) , 1700 cm-1 (N-H),), 2350 cm-1 (-C=N), 1940 cm-1 (O-N-O), 2810 cm-1 (=C-H), 3430 cm-1 (ANACAHand in the region of

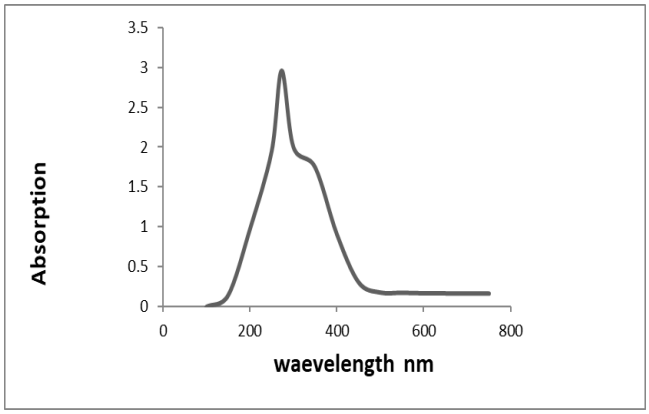

Fig. 2. *Ultraviolet spectrum for the crude antimicrobial components obtained from the B. megaterium*(Showing the λ max as 275.00 nm**).**

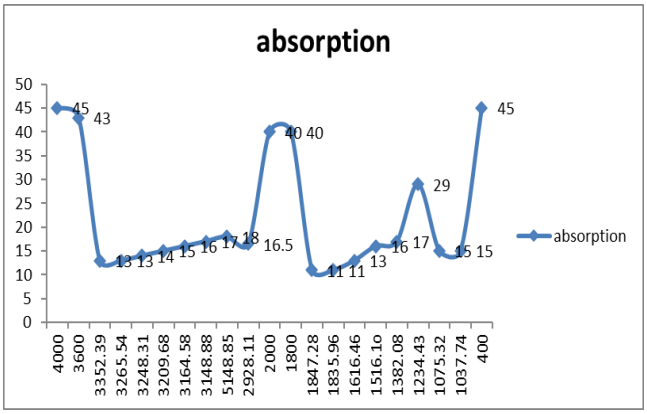

Fig. 3. *Infrared Red spectrum the crude antimicrobial components obtained from the B. megaterium*

Table 3. Minimum inhibitory concentration of antimicrobial compound produced by the *B. megaterium* against pathogenic bacteria

Bacterial strains	Minimum inhibitory concentration (mg/ml)
St. pyogenes NCTC 8198	0.25
S. typhi Ty2	0.5
P. aeruginosa RW109	1
E. coli O157	3.125
S. aureus ATCC 29213	6.25

1257 cm-1 (C-O). After extracting antibiotics from *Bacillus spp* fermentation cultures, the λ max for these with Ultraviolet at 275.00 nm. was consistent (Abbas *et al.*, 2017). Also, the present results agreed with Al Hafi *et al.* (2017), who measured the λ max for antimicrobial extracts and found the range of λ max as 215 to 320 nm.

The results of cytotoxicity antimicrobial components obtained from the *B. megaterium* via High Content screen on MCF-7 are presented in Table 4. This results summarizing the cytotoxicity effects and the statistical analysis of different concentrations of extract on mitochondrial membrane potential for MCF7 after 24h of incubation .The results agree with Ding *et al.* (2020) and Fira *et al.* (2018) who isolated this molecule as an antibacterial compound from *Bacillus megaterium* and *Bacillus subtils* . The results indicated that all concentrations showed activity against untreated samples, meaning that all used concentrations could penetrate the mitochondrial membrane and change the cancer cell intensity compared to the untreated ones. On the other hand, positive control can change the intensity of the cancer cell line (MCF-7). The targeted organelle for cell viability assay was the cytoplasm as described by Donato *et al.* (2018); Tolosa *et al.* (2015), who isolated antibacterial compound from *Bacillus polymyxa and Bacillus licheniformis*. When the Propidium iodide fluorescent probe was used, the color appeared red and cleared, as described by Kim and Jeon (2016); Sarika *et al.* (2012) isolated an antibacterial compound from *Bacillus polymyxa* and the color appeared red when the Propidium iodide fluorescent probe was used.

Conclusion

The bacteria *B. megaterium L2* (B9) present in the oral cavity of both the hospital workers and visitors, such as natural flora, can produce vital compounds that are killer to pathogenic Gram-positive bacteria (*S. aureus and St. pyogenes*) and Gram-negative bacteria (*E. coli, P. aeruginosa and S. typhi*), and are non-toxic to MCF cell lines. Thus, these bacteria can be used to produce antibiotics to solve the problem of antibiotic resistance.

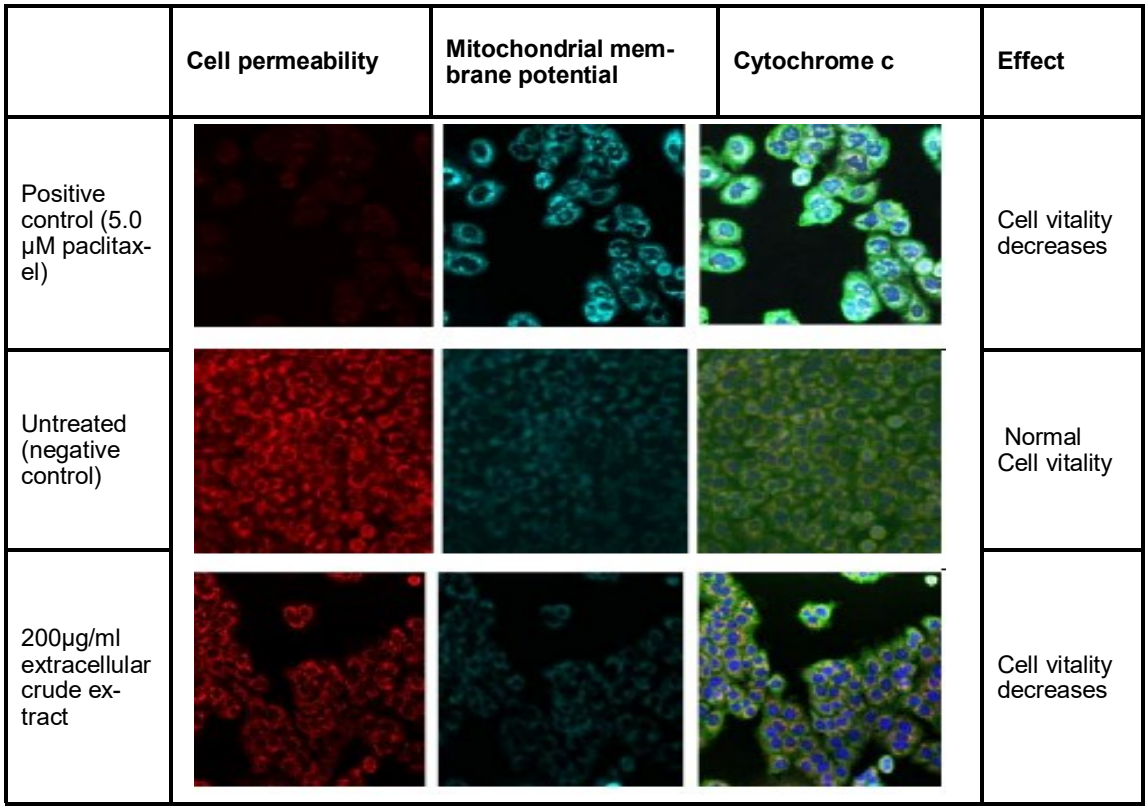

Fig. 4. *Showing entire cytotoxicity effect of B. megaterium L2* (B9) *the extracellular extract with positive and negative control on MCF-7.*

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Conflict of interest

The authors declare that they have no conflict of interest.

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