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Cytotoxicity and antimicrobial activities of the novel lactic acid bacterium, *Lactobacillus brevis* A13, isolated from the gut of the polychaete, *Perinereis nuntia*

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ABSTRACT

The present study was implemented to evaluate the cytotoxicity and antimicrobial activities of the novel lactic acid bacterium, *Lactobacillus brevis* A13, isolated from the gut of the polychaete worm, *Perinereis nuntia*. The results indicated that *L. brevis* A13 acquired a broad antimicrobial spectrum against the tested bacterial pathogens with multiple antibiotic resistance (MAR) index value of 0.57. Pure bacteriocin had a molecular weight of approximately 4.0 kDa and was designated as "Brevicin-13". Furthermore, brevicin-13 had a bactericidal nature, causing disruption and deformation of the cell membrane with cavities observed on the surface of *Klebsiella pneumoniae* ATCC 13883 cells. Compared to untreated cancer cells, brevicin-13 exhibited very strong cytotoxic activity, with inhibition rates of 93%, 95% and 91% against three cancer cell lines; colon cancer (Caco-2), liver cancer (HepG2) and breast cancer (Mcf-7), respectively. The inhibition was clearly shown as anti-proliferative activity and detrimental effects of the cell nucleus due to the induction of apoptosis upon exposure to brevicin-13.

INTRODUCTION

Marine life is fascinating and comprised an enormously complex life forms existing in unique communities **(Egan *et al*., 2008)**. The association between different marine invertebrates and microbes has evolved vast number of defensive strategies, enabling them to combat in an extraordinary competitive environmental conditions **(Bright & Bulgheresi, 2010; Hamid *et al*., 2013)**. Bioactive compounds produced by polychaete-associated bacteria are considered the potential pool of eco-friendly metabolites which could be advantageously applied in numerous industrial and pharmaceutical products **(Rajasree *et al*., 2014; Datta *et al*., 2015; Satheesh *et al*., 2016; Rizzo & Lo Giudice, 2018)**. Previous studies have significantly focused on the myriad of novel bioactive metabolites produced by epibiotic bacterial communities associated with polychaetes **(Campbell & Cary, 2001; Alain *et al*., 2002; Cambon-Bonavita *et al*., 2002; Shankar *et al*., 2015)**. However, the emerging investigations from bacterial communities

associated with the gut passage of polychaetes are still scarcely considered **(Andresen & Kristensen, 2002; Woulds *et al*., 2014)**.

Lactic acid bacteria (LAB) are a group of Gram-positive, facultative anaerobic bacteria commonly found as natural inhabitants of animals and humans' gastrointestinal tract (GIT) microflora, serving as the first defense barrier against invading pathogenic bacteria **(Desriac *et al*., 2010)**. LAB comprise several genera such as *Lactococcus*, *Enterococcus*, *Pediococcus*, *Weissella*, *Carnobacterium*, *Marinilactibacillus* and *Lactobacillus* **(Saranraj *et al*., 2013)**. LAB have the "Generally Recognized as Safe" (GRAS) status granted by the US Food and Drug Administration (FDA). They have intensively been studied as potential probiotic candidates used in medicines for both human and livestock industries **(Liu & Pan, 2010; Liévin-Le Moal & Servin, 2014; Chandra, 2016; Sadhu & Ganguly, 2017)**.

Bacteriocins are ribosomally-synthesized bacteriostatic or bactericidal proteins and peptides produced by either Gram-negative or Gram-positive bacteria, where LAB are the most important producers and have gained great momentum **(Kumar *et al*., 2012)**. Bacteriocins are divided into 3 main classes; Class **I** "lantibiotics", Class **II** "non-lantibiotics" and Class **III** "large heat-labile bacteriocins" **(De Vuyst & Leroy, 2007)**. Many investigations were carried out for the potential use of LAB bacteriocins as alternative solutions to various human health problems such as cancer, oral-care, vaginal infections, contraception and skincare **(Perez *et al*., 2015; Sivaraj *et al*., 2018)**.

Hence in the current study, an attempt was achieved to investigate the bioactive potential of a newly identified LAB (*Lactobacillus brevis* A13) from the gut of the polychaete, *Perinereis nuntia*, and evaluate its antimicrobial and cytotoxicity activity against three cancer cell lines; colon cancer (Caco-2), liver cancer (HepG2) and breast cancer (Mcf-7).

MATERIALS AND METHODS

Sample collection and treatment

Fresh samples of the polychaete, *Perinereis nuntia*, were collected with sterile forceps from the sandy bottom of the Suez Canal coast in Ismailia City, Egypt, and immediately transferred to the laboratory. The worms were anaesthetized by adding few drops of 75% ethanol and gently rinsed with sterile seawater to remove all traces of sand and debris **(Neave *et al*., 2012)**. Guts of some worms were carefully removed, washed with sterile saline solution (0.85% NaCl) to remove any extraneous particles and placed in sterile microcentrifuge tubes. The collected gut tissue was homogenized with sterile saline solution, transferred into de Man Rogosa and Sharpe (MRS) broth medium (Difco, USA) and incubated under anaerobic condition at 30°C for 72hrs. Aliquots (100 µl) of broth culture were evenly spread on MRS agar plates containing 1% $CaCO_3$ and incubated anaerobically at 30°C for 48hrs. The developed colonies, forming clear zones of acid formation with catalase negative activity, were picked up and transferred into

MRS broth for regular use or preserved in 30% glycerol at -80°C for long-term storage **(Hamdan *et al*., 2016)**.

Antimicrobial assay of selected LAB

The isolated LAB were inoculated in MRS broth, incubated at 30°C for 48hrs, and cells were collected by centrifugation (6,000 ×*g* for 15 min, 4°C). Cell-free supernatants were adjusted to pH 7.0 using 1N NaOH, sterilized using 0.45 µm filters (Sartorius, Germany) and considered as "crude extracts" to be furtherly in studies **(Hamid *et al*., 2013)**.

The antibacterial property of all crude extracts of the LAB isolates were tested against six bacterial pathogens, including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14025 and *Streptococcus iniae* CIP 105804, using the agar well diffusion method of **Nami *et al*. (2014**). Wells (5 mm diameter) were punched out, using sterile cork borers, in Muller Hinton agar plates inoculated with overnight cultures of the target pathogens. Then, an amount of 100 µl of the filtered cell-free supernatants were placed in each well and plates were incubated at 37ºC for 24hrs. The diameter of inhibition zones around each well was measured in millimeter(s) (mm) scale.

Identification of LAB

The LAB isolate, which exhibited the highest level of antimicrobial activity, was subjected to identification by both morphological and biochemical characterisation. The potential isolate was initially examined for cell shape, and sugar fermentation pattern was conducted using the API 50 CHL system (BioMérieux, Marcy I'Etoile, France) in accordance with the manufacturer's instructions **(Ameen *et al*., 2020)**. Furthermore, the selected LAB isolate was identified by 16S rRNA gene sequencing following the method of **Elkhateeb *et al*. (2018)**. The obtained sequences were compared to those in the NCBI database using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antibiotic susceptibility

Twenty different commercial antibiotics (Oxoid, UK) were used to evaluate the antibiotic resistance of the selected LAB isolate using the disc diffusion method **(Song *et al*., 2018)**. Antibiotic discs were placed on the surface of MRS agar plates, seeded with 100 µl of the LAB culture broth, and plates were incubated at 30°C for 24hrs. Inhibition zone around each disc was measured, and multiple antibiotic resistance (MAR) index was calculated according to the following equation **(Jain *et al*., 2012)**:

$$\text{MAR index} = \frac{a}{b}$$

Where; "a" is the sum of resistant antibiotics, and "b" is the total number of antibiotics used.

Biosafety assessment

In order to evaluate the safety of the LAB isolate, hemolysis test was performed as described in the study of **Bennani *et al*. (2017)**. An overnight culture of LAB isolate was

inoculated on blood agar medium, incubated anaerobically at 30°C for 24hrs and visually examined for signs of hemolysis. The hemolytic reaction of the isolate was recorded in one of three forms; a clear zone of complete blood hydrolysis around the bacterial colony (β-hemolysis), green-hued zones of partial blood hydrolysis around the colony (α-hemolysis) or the absence of hemolysis (γ-hemolysis).

Extraction and purification of the antimicrobial compound

The candidate LAB isolate was inoculated in MRS broth and incubated at 30ºC for 24hrs. Ammonium sulphate (60-80%) was added to the neutralized culture supernatant with continuous stirring overnight at 4ºC. Protein pellets were collected by centrifugation at 8,000 ×*g* for 20 min, re-dissolved in 0.1 M potassium phosphate buffer (pH 7.2) and dialyzed for 12hrs at 4ºC, using cellulose acetate dialysis membrane (1000 Da cut-off, Sigma-Aldrich, Germany) **(Rajaram *et al.*, 2010)**. The crude protein extract was freeze-dried for further purification.

Purification of lyophilized protein was carried out by RP-HPLC (Agilent 1200 series Technologies Inc., USA) using C18 reverse-phase column, following the method of **Zhang *et al.* (2018)**. Eluted fractions were pooled, evaporated and screened for antibacterial activity by the spot-on-lawn assay described in the work of **Oliveira *et al.* (2017)**. Briefly, 10 µl of each protein fraction was spotted onto a double-layered Muller Hinton agar plates seeded with 1% of *Klebsiella pneumonia* ATCC 13883 as an upper layer overlaid on Muller Hinton medium supplemented with 1.2% agar as a bottom layer. The plates were incubated at 30ºC for 24hrs, and the antimicrobial activity around each protein spot was expressed in arbitrary unit (AU/ml) using the following formula **(Hamdan *et al.*, 2016)**:

$$\text{Arbitary Unit (AU/ml)} = \frac{\text{Zone of inhibition (mm)}}{\text{Volume of the sample loaded}} \text{ x } 1000$$

Measurement of molecular mass and protein concentration

During each purification step, protein concentration was measured in accordance to the method of Bradford (1976). The purity and molecular weight of the active fraction were determined by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by **Ge *et al.* (2016)**. The gel was stained with Coomassie brilliant blue R-250 (Sigma-Aldrich, Germany) and the molecular weight of the protein band was compared with standard markers (10-200 kDa).

Amino acid sequence analysis

The purified protein was excised from the gel followed by amino acid sequence analysis using automated Edman degradation (Shimadzu Corp) **(Phumisantiphong *et al.*, 2017)**. The similarity search of the peptide sequence was conducted using BLAST software in the NCBI database.

Mode of action determination

The mode of action of the purified antibacterial compound, either bacteriostatic or bactericidal, was investigated using scanning electron microscopy (SEM) following

Sharma *et al*. (2018). Actively growing cells of the indicator bacterium, *Klebsiella pneumoniae* ATCC 13883, was treated with 50 µl of the pure active compound and incubated at 30°C for 24hrs. Furthermore, indicator bacterium without the addition of the active compound was used as a negative control. Cell pellets of both treated and non-treated samples were fixed overnight by 2.5% glutaraldehyde at 4°C and were further processed according to **Jiang *et al*. (2017)**. Morphological changes were visualized under SEM (JEOL JSM-IT200 Series, Japan).

In vitro cytotoxicity activity

The cytotoxic activities of the pure antibacterial compound were in vitro evaluated on 3 human cancer cell lines; human epithelial colorectal adenocarcinoma cells (Caco-2 cells), human liver hepatocellular carcinoma cells (HepG2 cells) and human breast carcinoma cells (Mcf-7 cells) using microculture tetrazolium (MTT) assay **(El-Naggar *et al*., 2016)**. Cells (1.0×10^5) were treated with different concentrations of the pure compound (10 μg/ml to 10 mg/ml) in a 96-well microplate and incubated at 37°C for different incubation periods (12, 24 and 48hrs). Then, an amount of 20 µl of MTT solution (5 mg/ml) was added into each well and incubated for 4hrs at 37°C followed by the addition of 200 µl of Dimethyl sulfoxide (DMSO) to thoroughly dissolve the purple MTT-formazan crystals. The absorbance of each well was measured at 560 nm using a microtiter plate reader (ELISA reader). The percentage of cell viability in each well was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Ac - At}}{\text{Ac}} \text{ x } 100$$

Where; "Ac" is the absorbance of the untreated cells, and "At" is the absorbance of the treated cells.

RESULTS

Screening for antimicrobial-producing LAB

Ten LAB isolates were successfully isolated from the gut of the polychaete worms. The crude cell-free extract of strain A13 exhibited a broad antimicrobial spectrum against different bacterial pathogens (Table 1). Therefore, the potential LAB isolate "A13" was subjected to biochemical and molecular characterization.

Table 1. Antimicrobial activities of LAB isolate A13 against indicator bacterial strains

Target Pathogen	Inhibition zone (mm)
Escherichia coli ATCC 25922	30
Staphylococcus aureus ATCC 25923	25
Klebsiella pneumonia ATCC 13883	22
Pseudomonas aeruginosa ATCC 27853	30
Salmonella typhimurium ATCC 14025	35
Streptococcus iniae CIP 105804	25

Identification of A13 isolate

The selected strain A13 was Gram positive, non-spore forming rods and was further identified as *Lactobacillus brevis* using API 50 CHL sugar fermentation profile (Data not shown). Moreover, the identity of the LAB isolate was confirmed by the 16S rRNA gene sequencing, which revealed 99% similarity with the corresponding 16S rRNA gene sequences of *Lactobacillus brevis* strain SKB1023 (accession number MH844893.1) retrieved from the Gen Bank databases. Consequently, the selected LAB isolate was identified as *Lactobacillus brevis* A13 and deposited in the NCBI database under the accession number of LC484968.

Antibiotic resistance pattern

The results showed that *L. brevis* A13 acquired resistance against a broad spectrum of antibiotics, such as β-lactam antibiotics (ampicillin/sulbactam, piperacillin/tazobactam, cefuroxime, ceftazidime), broad-spectrum antibiotics (cefotaxime), fluoroquinolone antibiotics (norfloxacin, ciprofloxacin) and aminoglycoside antibiotics (tobramycin) with MAR index value of 0.57 (Table 2).

Hemolytic activity of *L. brevis* A13

Lactobacillus brevis A13 demonstrated γ-hemolytic (non-hemolytic) activity when grown on blood agar medium.

Table 2. Antibiotic resistance profile of *Lactobacillus brevis* A13

Antibiotic	Code	Inhibition zone (mm)
Amikacin (30 µg)	AK	10
Ampicillin/Sulbactam (20 µg)	SAM	0
Cefotaxime (30 µg)	CTX	0
Ceftazidime (30 µg)	CAZ	0
Cefuroxime (30 µg)	CXM	0
Ciprofloxacin (5 µg)	CIP	0
Erythromycin (15 µg)	E	18
Gentamicin (10 µg)	GM	11
Imipenem (10 µg)	IPM	38
Neomycin (30 µg)	N	11
Norfloxacin (10 µg)	NOR	0
Piperacillin/Tazobactam (110 µg)	TZP	0
Rifampicin (30 µg)	RD	23
Tobramycin (10 µg)	TOB	0

Purification and structural analysis of the active bacteriocin

The crude protein extract produced by *L. brevis* A13 was subjected to purification via two steps: ammonium sulphate precipitation followed by fractionation by RP-HPLC (Fig. 1). One fraction corresponding to peak number 5 at the retention time of 11 min showed distinct antibacterial activity, whereas other fractions had almost no antimicrobial activity (Data not shown). Moreover, the specific activity of the pure antimicrobial fraction was 324 AU/mg protein with 65-fold increase and 3.5% yield compared to the

cell-free supernatant (Table 3). Pure bacteriocin showed a single band on SDS-PAGE electrophoresis, with a molecular weight of approximately 4.0 kDa compared to the corresponding protein marker (Fig. 2). From the N-terminal of the active bacteriocin band, 25 amino acids were sequenced (Gly-Lys-Pro-Ala-Trp-Cys-Trp-Tyr-Thr-Leu-Gly-Ser-Leu-Cys-Cys-Ala-Gly-Tyr-Asp-Ser-Gly-Thr-Cys-Asp-Tyr). Alignment of the amino acid sequences by BLAST program in Gen Bank revealed areas of homology to glycocin F and the bacteriocin was designated as "brevicin-13".

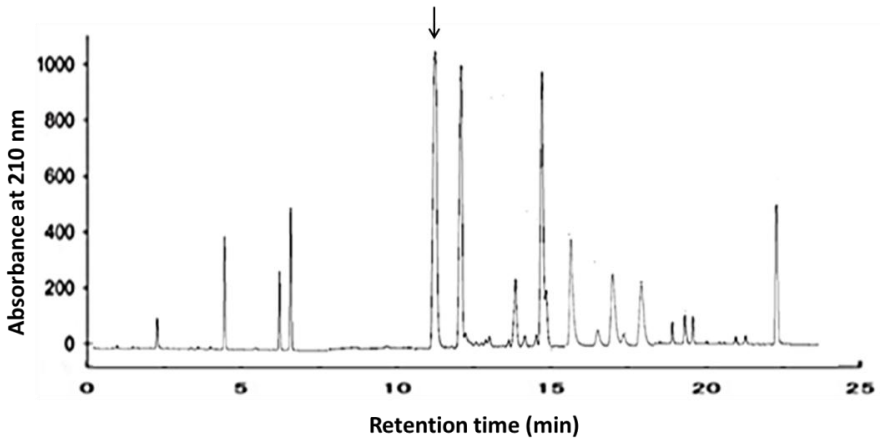

Fig. 1. RP-HPLC peaks for the precipitated crude protein from *L. brevis* A13
❖ Arrow shows the active band showing antimicrobial activity

Table 3. Purification of the bacteriocin produced by *L. brevis* A13

Sample	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Total protein (mg/ml)	Specific activity (AU/mg)	Purification (fold)	Yield (%)
Cell-free supernatant	1000	2200	2200000	420	5	1	100
Ammonium sulphate	20	3500	70000	180	19.5	4	43
RP-HPLC	5	4800	24000	14.8	324	65	3.5

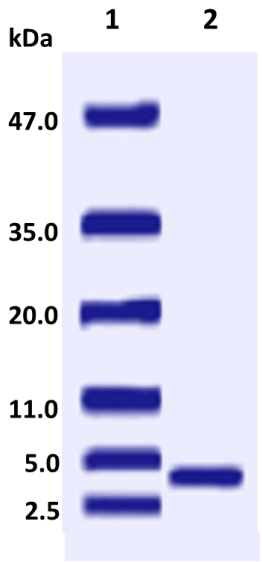

Fig. 2 SDS-PAGE of bacteriocin produced by *L. brevis* A13

lane **1**: protein marker; *lane* **2:** pure bacteriocin

Mode of action of brevicin-13

An attempt was initiated to gain insight into the effect of brevicin-13 on *Klebsiella pneumoniae* ATCC 13883 using SEM. Compared to untreated cells, the damage of *K. pneumoniae*-treated cells was observed with wrinkled, rough and irregularly-shaped cell surface (Fig. 3).

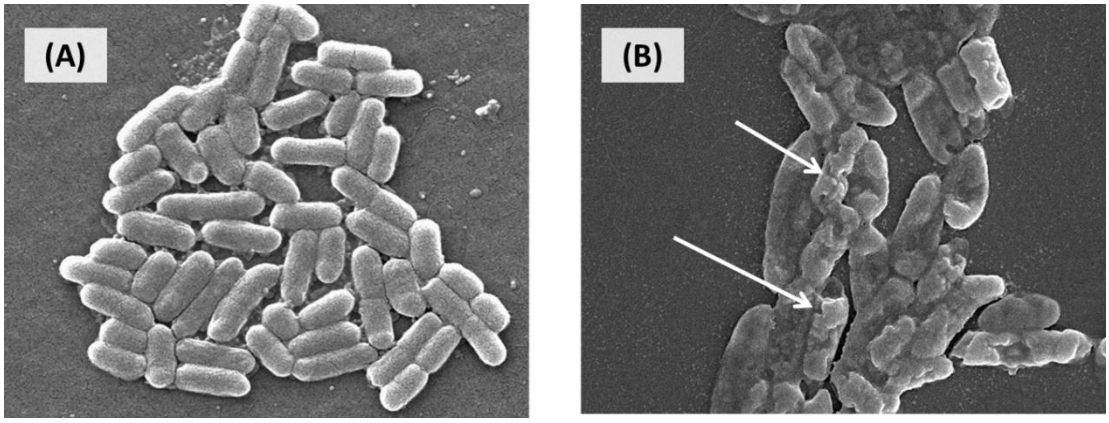

Fig. 3. SEM micrographs showing the bactericidal effect of brevicin-13 on *Klebsiella pneumoniae* cells **(A)** untreated cells, **(B)** bacteriocin-treated cells

❖ Arrows show affected bacteriocin-treated cells

Cytotoxicity activity of brevicin-13

The cytotoxic potential of brevicin-13 against various cancer cells, Caco-2, Mcf-7 and HepG2, showed anti-proliferative activity and detrimental effects of the cell nucleus due to the induction of apoptosis upon exposure to brevicin-13 (Fig. 4). Furthermore, the IC_{50} concentration inhibiting 50% of the cell growth was calculated for each cancer cell line. After 48hrs of incubation, the results showed that brevicin-13 exhibited IC_{50} values of 20 μg/ml, 10 μg/ml and 15 μg/ml against Caco-2, Mcf-7 and HepG2 cells, respectively (Table 3). In addition, brevicin-13 (50 μg/ml) showed the strongest cytotoxic activity, with inhibition rates of 93%, 95% and 91% against Caco-2, Mcf-7 and HepG2 cell lines, respectively, compared to untreated cancer cells.

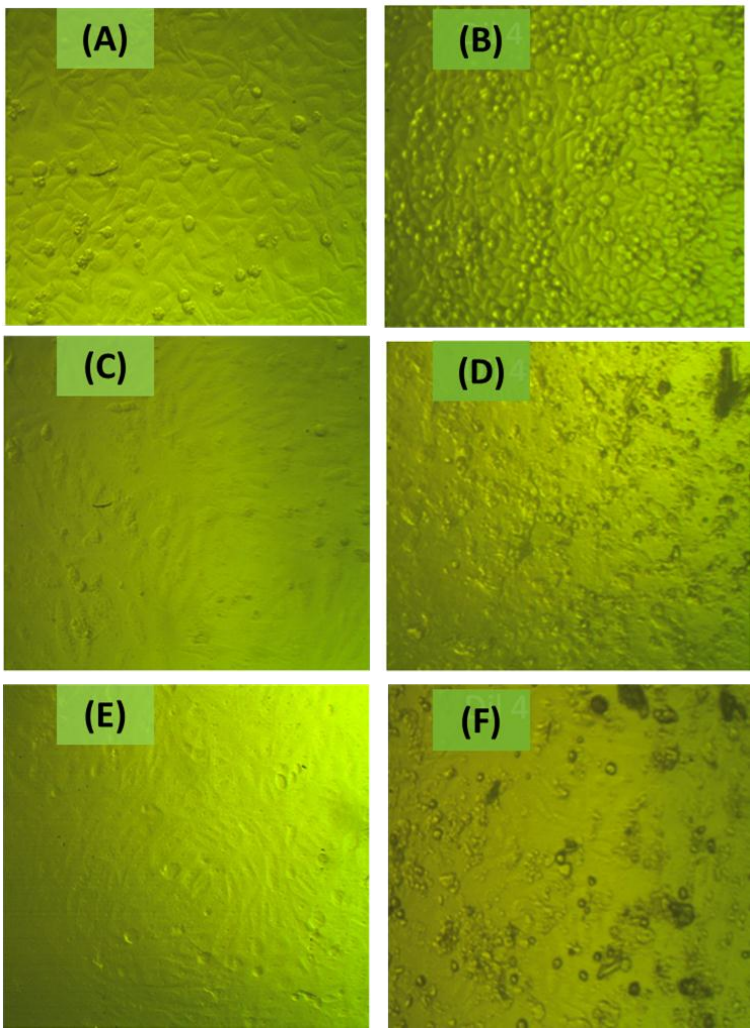

Fig. 4. Effect of brevicin-13 exposure on the viability of various cancer cell lines **(A)** untreated Caco-2 cells, **(B)** treated Caco-2 cells, **(C)** untreated Mcf-7 cells, **(D)** treated Mcf-7 cells, **(E)** untreated HepG2 cells and **(F)** treated HepG2 cells

Table 4. Toxicity (%) and IC_{50} of brevicin-13 against three cancer cell lines

Cancer Cell line	**Toxicity (%)**	**IC_{50} (µg/ml)**
Caco-2	93	20
Mcf-7	95	10
HepG2	91	15

Where; **Caco-2** (colorectal adenocarcinoma cells), **Mcf-7** (breast carcinoma cells), **HepG2** (hepatocellular carcinoma cells) and **IC_{50}** (the inhibition concentration of 50% of cell growth).

DISCUSSION

The antibacterial activity of LAB against both Gram-positive and Gram-negative bacteria is considered an attractive feature, enabling them to possess the potential application in the food industry and the medical sector **(Juodeikiene *et al*., 2012)**. Moreover, **Rahmeh *et al*. (2019)** related such antimicrobial activity of LAB to the production of active metabolites during the entire growth such as lactic acid, hydrogen peroxide and bacteriocins. Therefore, in the present study, the potential LAB isolate "*Lactobacillus brevis* A13" was selected as a prospective strain for further studies.

Tambekar and Bhutada (2010) deduced that LAB strains with high MAR index values (>0.2) are originated from high risk environmental sources of excessive use of antibiotics. Additionally, resistance to antibiotics indicates that *Lactobacillus brevis* A13 would be able to withstand the undesirable high concentrations of antibiotics occasionally present in the environment **(Ahmed *et al*., 2017)**.

Moreover, **Tallapragada *et al*. (2018)** postulated that blood hemolysis is a virulence factor among pathogenic microorganisms and the absence of hemolytic activity is the first criterion and the safety prerequisite for the selection of a potential LAB strain.

The crude protein extract produced by *L. brevis* A13 was subjected to two-step purification protocol; ammonium sulphate precipitation followed by fractionation by RP-HPLC. The increased biological activity of the purified bacteriocin could be attributed to the elimination of inhibitory compounds during the purification process **(Batdorj *et al*., 2006)**.

The low molecular weight (<5kDa) of the bacteriocin produced by *L. brevis* A13 suggests that it might belong to class II non-lantibiotics, which included small thermostable peptides acting on membrane structures **(Cotter *et al*., 2013)**. Several studies assessed that most bacteriocins from LAB belong to class II with low molecular mass **(Gautam & Sharma, 2009; Amortegui *et al*., 2014; Elyass *et al*., 2017**).

Alignment of the amino acid sequences, performed by BLAST program database, revealed no similar homology with other deposited proteins, and thus this novel bacteriocin was designated as "brevicin-13". Furthermore, the results indicate that brevicin-13 had a bactericidal nature that is a characteristic phenomenon caused by most bacteriocins produced by LAB **(Zhan *et al*., 2016; Dey *et al*., 2019)**. Similarly, the bacteriocin, pentocin JL-1, isolated from *Lactobacillus pentosus* caused disruption and deformation of the cell membrane of methicillin-resistant *Staphylococcus aureus* cells **(Jiang *et al*., 2017)**. Addingly, **Kodali *et al*. (2013)** reported that many bacteriocins destroy the integrity of the cytoplasmic membrane of the target organism via the formation of cytoplasmic membrane pores; thus increasing the permeability of the cytoplasmic membrane and causing leakage of low molecular mass metabolites or dissipating the proton motive force and causing cell death through the cessation of all reactions that require energy.

The cytotoxic effect of some bacteriocins on the proliferation of mammalian cancer cells has been intensively studied **(Er *et al*., 2015; López-Cuellar *et al*., 2016; Delesa, 2017)**. Inhibition of colon cancer by LAB takes place by different mechanisms, including altering the physiological conditions inside the colon, producing anti-mutagenic and/or anti-carcinogenic compounds and enhancing the host's immune response **(Kumar *et al*., 2012)**. Moreover, **De Giani *et al*. (2019)** reported that the exact mechanism of cytotoxic action of bacteriocins is not fully understood and requires explanation. Nevertheless, it was reported that bacteriocins can kill target bacteria by membrane permeabilization or by binding to a specific membrane protein called "bacteriocin receptor", where the interaction between the bacteriocin and the receptor protein leads to membrane leakage and cell death.

Therefore, the current study revealed that brevicin-13 produced by *Lactobacillus brevis* A13, isolated from the polychaete *Perinereis nuntia*, exhibited significant *in vitro* antimicrobial activities and cytotoxic property against the proliferation of three cancer cell lines (Caco-2, Mcf-7 and HepG2). Nonetheless, further *in vivo* assessment is highly recommended to investigate its potential health benefits to humans.

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