Research Article

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Archives in Cancer Research
ISSN 2254-6081

2021
Vol.9 No.3:006

Evaluation of Antimicrobial and Anticancer Activity of *Lactococcus garvieae* Derived Bacteriocin

Suneel Dodamani[1*] Basappa B Kaliwal[2], Shivani Tendulkar[1], Aishwarya Hattiholi[1]

[1]Department of Basic Science Research Center , University of KLE Academy of Higher Education and Research, Karnataka, India.

[2]Department of Biotechnology and Microbiology, University of Karnataka, Karnataka, India

[*]**Corresponding Author** : Suneel Dodamani , Department of Basic Science Research Center , University of KLE Academy of Higher Education and Research, Karnataka, India,Tel: 91-9901943638; E-mail: suneelddmn18@gmail.com

Received date: May 03, 2021; **Accepted date:** May 17, 2021; **Published date:** May 24, 2021

Citation: Dodamani S, Kaliwal BB, Tendulkar S, Hattiholi A (2021) Evaluation Of Antimicrobial And Anticancer Activity Of *Lactococcus garvieae* Derived Bacteriocin. Arch Can Res. Vol.9 No.3:002.

Abstract

Lactic acid bacteria (Lac B) are a prominent class of microorganisms that are capable of producing a variety of antimicrobial peptides or bacteriocins, which have inhibitory action on closely or far related organisms. In this study, we isolate and characterize bacteriocin from *Lactococcus garvieae* which, after dialysis showed significant activity against some bacterial and fungal strains, as well as anticancer activity against HT29 (Colorectal adenocarcinoma) cell lines.

Keywords: Lactic acid bacteria; Antimicrobial peptides; Bacteriocin; Anticancer activity

Introduction

Lactic acid bacteria (Lac B) are known to produce secondary metabolites like bacteriocins, that have bactericidal or bacteriostatic effects on closely related microorganisms. These antimicrobial peptides have been widely studied for their role in the food industry. They have been extensively used for fermentation as well as food preservation processes. One such bacteriocin is Nisin, produced by *Lactococcus lactis*. It is widely studied and has been approved by the US FDA for its commercial usage in the food industry. With bacteriocins being abundant and available from a large pool of bacteria, Klaenhammer has classified them into four major categories, based on similarities [1].

Evidence for the use of bacteriocin as a probiotic have been established and suggest the safety of their use in human health. Studies up to the genetic level of various bacterial and fungal species have been established to check the spectra of antimicrobial action of bacteriocins. Such in-depth study of bacteriocins is extremely helpful to entirely understand the peptide and its scope in human health. This also provides room for necessary re-engineering and modifications for efficient utilization as well as explore its gamut across industries. In this regard, Nisin has been studied for its properties in food industry, cosmetics, health etc. Nisin is also said to have enhanced innate immunity as reported by some studies. Many other bacteriocins isolated from various bacteria have shown antimicrobial properties similar to the mechanism of action of antibiotics. Microcin B17 from *E. coli* destructing DNA, colicins acting like RNases, Bac-GM17 from *Bacillus clausii* disrupting the integrity of bacterial membrane are some of the examples that show their strong resemblance to the action of antibiotics. This fact, adjunct to the anti-microbial and anti-fungal ability of bacteriocins makes it a potential broad-spectrum antibiotic and can also pose as an efficient alternative to the hurdles in the face of antibiotic resistance. In fact, to check the validity of the proposed alternative, bacteriocins have been studied in synergy with conventional antibiotics [2].

With sufficient study of these bacteriocins as antibiotics available, many scientific groups have taken the step further into cancer studies. Antimicrobial peptides like magainin have been studied for their cytotoxic capabilities. Their specificity towards cancer cell lines and the ability to overcoming the crisis of multi-drug resistance makes them efficient anticancer agents. Many *Lac B* species have been studied in this respect and justified the use of such organisms as probiotics. This anti-cancer aspect of *Lactococcus garvieae* derived bacteriocin has been studied in this paper, which opens new horizons for the widely available lactic acid bacteria [3].

Studies on *L. garvieae* isolated from clinical sample have shown activity against septum formation and the ones isolated from dairy source have shown inhibitory activity against *Staphylococcus aureus* . However, no existing studies were found regarding the scope of this bacteria in its broad-spectrum antimicrobial approach. We therefore study this gamut on selected bacterial and fungal strains, as well as the anticancer properties of the bacteriocin from *L. garvieae* of a dairy source. We look at the basic characterization of the bacteriocin with respect to its molecular weight through Tricine SDS-PAGE, protein estimation and its activity against the bacterial strains *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtillus, Bacilllus cerues, Escherichia coli; and fungal the strains Candida albicans, Aspergillus flavus, Aspergillus niger and Aspergillus fumigatus*; while FTIR analysis was done on the bacteriocin and bacteriocin-treated strains of *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus*. Further,

the anticancer activity of the bacteriocin was confirmed through MTT assay and DAPI staining [4].

Materials and Methods

Microbial culture

Cow milk procured from Vinay Kulkarni Dairy, Dharwad, India for culture. *Lactococcus garvieae* was isolated as the producer strain and further subjected to phenotype and genotype analysis reported in our previous study. The microbial culture was further subjected to extraction of bacteriocin [5].

Bacteriocin production and screening

A 24h-old culture of *Lactococcus garvieae* was inoculated (2%, v/v) into M17 broth (Sigma Aldrich) and incubated at 37°C for 25 hours, without agitation. The culture was then examined for bacterial growth at OD 600nm at regular intervals of 60 minutes. This was then subjected to bacteriocin screening, using the agar-spot test and well diffusion technique [6] .

The pH of the cell free supernatant was adjusted to 6.0 with 1 M NaOH to prevent the cells from the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU) per mL, one AU being the reciprocal of the highest dilution that showed a clear zone of growth inhibition [7].

Ammonium sulfate fractionation and dialysis

A 24h culture of the bacteriocin-producing Lac B strain was centrifuged (9000×g, 10 min, 4°C) and the peptide fraction was precipitated from the cell-free supernatant with 70% saturated ammonium sulphat. The suspension was desalted overnight at 4°C and agitated with a magnetic stirrer (Ika, RCT, Basic). Salted-out proteins were precipitated by centrifugation (10000×g, 20 min) and dissolved in a small volume of 10 mM phosphate buffer (pH 7.0). The suspension was desalted by dialysis with 10 mM phosphate buffer at 4°C for 12 h using benzoylated membranes (2 KDa; Sigma Aldrich Co, St Louis, USA). The samples were further subjected to protein estimation and bacteriocin assay [8].

Protein estimation

The protein concentrations in cell free extract were determined using the Bradford's protein estimation method. Using Bovine serum albumin (BSA) as the standard protein, all dilutions were prepared in ultra-pure water from a stock solution [9].

Molecular weight determination

The partially purified bacteriocin was examined by Tricine-SDS-PAGE (4% in the stacking gel, 10% in the spacer gel and 16.5% in the running gel) as described using Protein Marker I (14 – 116 kDa). After electrophoresis at 200V for 45 min, the gel was stained with Coomassie brilliant blue R (Sigma Aldrich Co, St Louis, USA) to visualize protein bands [10].

UV-Vis Spectroscopy studies

Ultraviolet and visible absorption spectra of the extracted protein were recorded on Shimadzu UV-102 spectrophotometer using distilled water as solvent. Absorption spectra were recorded on a Varian Cary 500 UV -VIS-NIR spectrophotometer.

FTIR studies

The bacteriocin was extracted by subjecting the bacterial culture to centrifugation at 12,000 rpm for 10 min and the resulting supernatant was lyophilized to concentrate the bacteriocin. The resulting bacteriocin was analyzed for the active groups by Fourier transform infra-red (FTIR) spectroscopy. The bacteriocin treated and untreated cells of indicator bacteria *Staphylococcus aureus, Salmonella typhi and Escherichia coli* were similarly subjected to FTIR analysis. The cells were pelleted and treated with 4600 AU ml^{-1} of bacteriocin and washed with ultra-pure water. The cells were further dried to remove moisture content and mixed with finely grounded potassium bromide (KBr). The IR spectra was recorded (in Drift mode) at absorbance between the wavelength 4000 and 400 cm^{-1} using a Thermo Nicolet 6700 spectrometer.

DNA extraction and quantification

DNA extraction was carried out as per the manufacturer's protocol (Qiagen India, mumbai) and was stored at -20°C until further use. The concentration of DNA was determined using nanodrop (NanoDrop 2000, Thermo Scientific).

PCR amplification

The plasmid DNA isolated from bacteria was subjected to Veriti, PCR (Invitrogen). The PCR reaction mixer contained 2.5 µl of 10X buffer, 1 µl of primer, 2.5 µl of 2.5 mM of each dNTP, 2.5 µl of taq DNA polymerase, 1 µl template DNA and 8.5µl nuclease free water. The PCR conditions were set at initial denaturation of 1min at 94 °C and final denaturation of 5 min at 94 °C for 35 cycles and annealing temperature at 62°C for 2 min and extension (72 °C, 1 cycle, 7 min). The reagents used were procured from GeNei, Bengaluru, India.

Primer sequence used:

F GGAATAAAGGAGCCGGAGTTAG (Sense)

R CGTCAGAGAAACGACTGGTAAT (AntiSense)

Gel electrophoresis

Gel electrophoresis was performed using 2.0 % agarose (Seakem, 50004 L) to analyze the size of amplified PCR product.

Determination of cytotoxic activity of bacteriocin

Reagents and materials

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics were from Hi-Media Laboratories

Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol were procured from E. Merck Ltd., Mumbai, India and 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was purchased from HiMedia, Mumbai.

Cell culture

Colorectal adenocarcinoma (HT-29) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 g/ml) and amphotericin B (5 g/ml) in humidity, at 5% CO2, 37C until confluent. The cells were dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in T25 culture flasks and further experiments were carried out in 96 micro-titre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of test samples

Test sample was diluted in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 50% (v/v) concentration and sterilized by filtration. Two-fold serial dilutions were prepared from stock to carry out cytotoxic studies.

Determination of cell viability

MTT protocol

The percentage growth inhibition was calculated using the following formula and CTC_{50} values were generated using graph pad prism 5.

% Growth inhibition = 100 – (Mean OD of individual test group X 100)

Mean OD of control group

Determination of anticancer property of bacteriocin by DAPI staining

To characterize cell-specific apoptotic process in HT-29 cells, analysis of chromatin condensation and nuclear fragmentation was done by DAPI staining using fluorescence microscopy. After treatment of 70–80% confluent HT-29 colorectal adenocarcinoma cells with varying concentration of sample for 24 h, cells were quickly washed with ice-cold PBS and fixed in ice-chilled acetone: methanol (1:1) mixture for 10 min at 4°C in the dark. The cells were then washed twice with ice-cold PBS and then incubated for 30 min with the DNA-specific fluorochrome, DAPI. The excess DAPI was removed with ice-cold PBS wash, and the cells were observed and photographed using fluorescence microscope.

Statistical analysis

All the experiments were performed in triplicates and results were expressed in terms of mean ± SD.

Results

Bacteriocin production

The bacteriocin production (data not shown) was assessed and the broth sample of *Lactococcus garvieae* was subjected to centrifugation. The resultant supernatant, Ammonium Sulphate Precipitated and dialyzed samples depicted bacteriocin activity of 8400 AU/ml, 10200 AU/ml and 12100 AU/ml respectively. The dialyzed protein was purified and further analysis of SDS-PAGE showed molecular weight of 12 kDa.

UV-VIS spectroscopy

The UV-VIS spectroscopy studies revealed the presence of bacteriocin as depicted by peaks at wavelengths 206, 226 and 270 nm along with the standard nisin, at wavelength 207 nm, thereby confirming bacteriocin activity in purified state in dialyzed and ammonium sulfate precipitated samples.

Antibacterial and antifungal study

Purified bacteriocin was used for checking its antimicrobial activity against MTCC strains and the result showed significant activity against all the gram-positive and gram-negative aerobic bacteria (Table 1). Among the anaerobic strains, activity was observed only in *Porphyromonas gingivalis*. The bacteriocin activity was also evaluated in combination with Tween 20, Tween 80 and EDTA against *Escherichia. coli, Staphylococcus. aureus, P. aeruginosa, Bacillus subtilis and Bacillus cereus.* Although no significant difference was seen in others, the combination of EDTA and bacteriocin showed increased activity against *E. coli*, while the presence of Tween 20 with bacteriocin showed enhanced activity against all the five bacterial strains. Further, different concentrations of the bacteriocin were tested against different fungal strains such as *C. albicans, A. flavus, A. niger and A. fumigatus*. While a distinct activity against *C. albicans* was observed, no significant activity was observed in the other fungal strains (Table 1).

Indicator organism	Characteristics	Activity of bacteriocin
Escherichia coli	Aerobic, Gram-negative	Yes
Staphylococcus aureus	Aerobic, Gram-positive	Yes
Pseudomonas aeruginosa	Aerobic, Gram-negative	Yes
Bacillus subtillis	Aerobic, Gram-positive	Yes
Bacillus cereus	Aerobic, Gram-positive	Yes
Porphyromonas gingivalis	Anaerobic, Gram-negative	Yes
Prevotella intermedia	Anaerobic, Gram-negative	No
Aggregatibacter actinomycetemcomittans	Anaerobic, Gram-negative	No
Candida albicans	Opportunistic pathogenic yeast	Yes

Aspergillus flavus	Saprophytic pathogenic fungus	No
Aspergillus fumigatus	Opportunistic conidial fungi	No
Aspergillus niger	Saprophytic, opportunistic fungi	No

Table 1: Bacteriocin activity on indicator strains.

FTIR analysis of bacteriocin

The FTIR spectrum was observed in the range of 1000-3500 cm^{-1}. Sharp peaks were observed at the frequencies 1630 and 3430 cm^{-1}. Smaller but distinct peaks were observed at the frequencies 1030, 1120, 1460 and 2970 cm^{-1}(**Figure 1**).

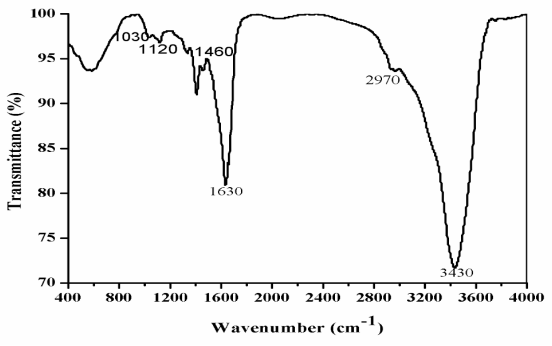

Figure 1: FTIR spectrum of *Lac B* derived bacteriocin.

FTIR analysis of bacteriocin activity

Small changes in frequency range 1000-1100 $cm^{-1}$are observed. Distinct shifts at 2923 and 2925 cm^{-1} are seen in the treated cells. Another such major peak is observed at the range 3400-3450 cm^{-1}. The peaks observed in the range 600-1450 cm^{-1} are significantly small, nevertheless show multiple shifts. The major peak shifts around 1540-1660 cm^{-1} are also notable.

Detection of genomic DNA and PCR amplification

Nanodrop concentration

The genomic DNA extracted, along with the amplified PCR product was detected in 2D- gel electrophoresis (**Figure 2**).

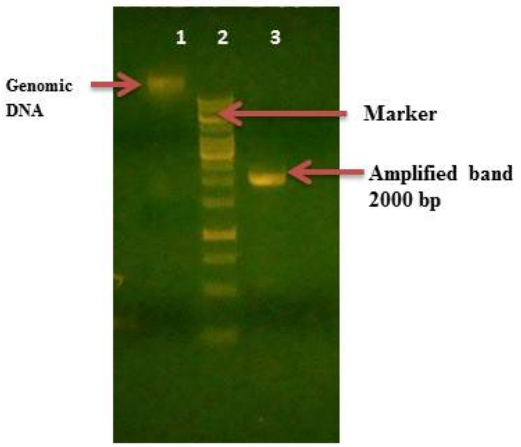

Figure 2: 2D- gel electrophoresis of (1) extracted genomic DNA, (2) DNA marker and (3) PCR product.

Cytotoxic activity of bacteriocin

The cytotoxicity of bacteriocin was calculated for its C50 value and the working dose was determined at >50 µg/ml. The bacteriocin that showed good cytotoxic effect at lower concentration in comparison to standard doxorubicin (2 µg/ml) was chosen further for apoptosis activity.

Apoptotic activity of the bacteriocin

The bacteriocin samples at 100 and 200 µg/ml were used for induction of apoptosis in HT-29 (colorectal adenocarcinoma). The bacteriocin showed apoptosis in cancer cells when tested by DAPI, a nuclear staining method (**Figure 3**).

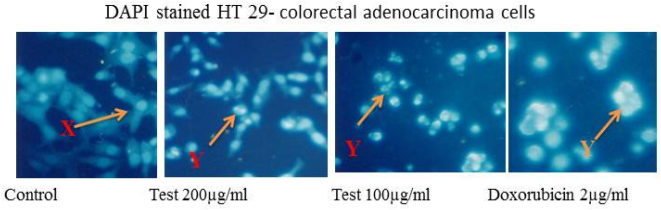

Figure 3: DAPI stained HT-29 colorectal adenocarcinoma cells.

Discussion

Bacteriocins from Lactic acid bacteria (*LacB*) have shown significant activity against closely related species and usually work by attacking the target membrane and damaging it *via* formation of poration complexes. These peptides do not harm the producing strains as they are protected by certain proteins providing them immunity against it.

Such mechanisms of cell damage and antimicrobial action have been studied on various bacterial (indicator) strains, thereby showing a broad-spectrum activity. Other modes of action include condensation of nuclear material, protein synthesis and nucleic acid biosynthesis inhibition, ribosome and ion loss etc.

Many studies have shown interest in the antibiotic potential of bacteriocins due to their mode of action, which closely resembles that of available antibiotics. *LacB* sourced bacteriocins have been extensively studied for it activity on various food borne pathogens.

Lactococcus garvieae was first reported to produce a bacteriocin named L1-5 by Villani et al. and was characterized and identified using PCR and other antimicrobial tests.

The *L. garvieae* derived bacteriocin from cow's milk has been characterized and the corresponding tests have been discussed in the previously reported paper.

In the current study, we look at the potential use of bacteriocin produced by this bacterium

against a wide range of strains for its antimicrobial and antifungal activity along with its apoptotic properties. This sort of study will help unveil the potentials of the bacteriocin as a broad-spectrum antibiotic and as an apoptosis inducer in anti-cancer studies, thereby providing a prospective alternative for chemical drugs and a solution for antibiotic resistance.

Identification and characterization of extracted bacteriocin from *Lac B*

The bacterial culture was first subjected centrifugation to extract the produced bacteriocin, that is usually present in the supernatant. It can be observed that in most of the studies, pH has been a factor of interest and it has been shown that the changes made in pH using NaOH do not significantly affect the activity of bacteriocin. However, this customizing is necessary to prevent any microbial inhibition caused due to the presence of lactic acid, as has been reported in many studies. The lactic acid produced by *LacB* acts as a permeabilizer and often has sublethal effect on bacterial cells as well as some bacteriocins.

The bacteriocin extracted was subjected to partial purification by ammonium sulphate precipitation and then dialysis. The bacteriocin was checked for its activity at every step of purification and showed the expected increasing trend, with the final dialyzed bacteriocin having activity of 12100 AU/ml. The activity was also confirmed with UV-Visible spectroscopy analysis. The bacteriocins fall in the molecular weight range of 5-60 KDa, The extracted product in this study was found to be a low molecular weight bacteriocin. The genomic DNA extraction and amplification with PCR technique confirms the identity of bacteriocin from *Lac B*.

Fourier transform Infrared spectroscopy has been used extensively to study protein structure of bacteriocin as well as the changes caused by the bacteriocin in the bacterial cells, to supplement the data on antimicrobial activity. The FTIR spectra of bacteriocin shows the trend observed in a secondary structure of peptides. The frequency range 1600-1690 cm^{-1} and 1480-1575 represents the C=O stretching of Amide I; and C-N stretching and N-H bending in Amide II, respectively. This trend can be seen, thereby confirming the presence of Amide I and II. The frequency of Amide I at 1630 cm^{-1} can also be due to the presence of β-sheets. The frequency range 2800-2900 cm^{-1} is said to be contributed by the C-H stretching of lipid acyl chains and this trend is visible in our study, at the frequency 2970 cm^{-1}. The peak at 3430 cm^{-1} may be due to the hydroxyl groups, suggesting the presence of primary and secondary amine groups or N-H stretching. The frequencies in the range 1000-1200 correspond to the phosphate groups suggesting the presence of phospholipids.

Lac B derived bacteriocin has potential antimicrobial activity

The antimicrobial and antifungal activity of the bacteriocin was checked with agar-diffusion assays. Significant activity with distinct clear zone formation was observed in all the aerobic bacteria, *P. gingivalis* (anaerobic) and *C. albicans* (fungi). The activity against aerobic bacteria was further checked in synergy with EDTA (2,2',2'',2'''-(Ethane-1,2-diyldinitrilo) tetraacetic acid), Tween 20 (Polysorbate 20) and Tween 80 (Polysorbate 80). The bacteriocins are said to have comparatively lesser effect on Gram-negative bacteria and are hence pre-treated with surfactants and detergents like EDTA. This helps the bacteriocin permeate the lipopolysaccharide containing cell membrane of the bacteria through the chelating action of EDTA on magnesium ions on the cell membran. This trend is observed in treated *E. coli* strains, wherein the activity of bacteriocin is higher in presence of EDTA, corresponding to reported studies. In case of Tween 20 and bacteriocin, increased activity was observed in all treated cells, except B. subtilis. This was however, not the case for Tween 80, as no significant activity change was observed. Although enhanced activity of bacteriocin in synergy with these compounds on gram-negative bacteria has been previously reported by many studied, contradictory results have also been obtained by others.

The bacteriocin action was further analyzed with the help of FTIR spectra of bacteriocin treated bacteria. For this, *E. coli, S. typhi and S. aureus* were used as indicator strains. The changes at frequency range 1000-1100 cm^{-1} corresponds to the deformation in carbohydrates. The peak shifts seen around the frequency 2923 cm^{-1} is more markedly seen for bacteriocin treated *S. aureus*. The peak around this frequency is said to be due to the anti-symmetric stretching of C-H in CH_2 bonds, in lipid acyl chains. This sort of vibration corresponds to the membrane fatty acids, and thus points towards changes brought in the membrane by the bacteriocin. The major peak shift in bacteriocin treated *S. aureus*, as compared to nisin treated strain, suggests the better performance of the bacteriocin than the latter. Although this comparison between the bacteriocin and nisin (positive control) follows through all the three strains, it is notable that this mode of action is very effective in gram-positive bacteria. As previously mentioned, bacteriocins have comparatively lesser effect on gram-negative strains due to their phospholipid rich membranes, and the FTIR spectra augments the evidence pointing to this. Similar trend is also observed for symmetric C=O vibrations around the frequency 1400 cm^{-1}, thereby suggesting antimicrobial activity of the bacteriocin. The frequency range 1535-1660 cm^{-1} representing the NH_2 bending and C=O, C=N stretching of amide I and II shows different effects on different strains, nevertheless decreasing the peak. The peak shifts in the range 600-1450 cm^{-1} relate to the phosphate groups, following the previously mentioned activity trend for *S. aureus.* The range 3200-3500 covers the O-H vibrations of polysaccharides.

Lac B has apoptotic effect on HT-29 cell lines

Antimicrobial peptides have been studied for their cytotoxic action in melanoma, leukemia, breast cancer cell lines etc. They act by inhibiting DNA synthesis and cell proliferation, without leaking the cytoplasmic components, while DNA fragmentation and nuclear condensation is induced by caspase-3. The activity of caspase-3 has been reported to be increased by the antimicrobial peptides along with induction of reactive oxygen species. Similarly, many mechanisms of cytotoxic action of *Lac B* have been studied . This study employs the commonly studied nuclear staining method by DAPI. The HT-29 (human colorectal

adenocarcinoma) cells showed shrinkage of cell plasma membrane without discharging the cellular components. The cytoplasm was observed to be dense with tightly packed organelles, thereby inducing apoptosis. These studies are redolent of the potential of the bacteriocin extracted from *L. garvieae*, as having anticancer activities. The cytotoxicity studied by MTT assay showed that 2 µg/ml was the sufficient dose of bacteriocin required to induce cell death. This suggests that the bacteriocin is efficient at a lower concentration.

The bacteriocin extracted from *Lactococcus garvieae* in this study, has been shown to have antimicrobial, antifungal and apoptotic activities. This corroboration however is not sufficient to prove the effectiveness of the bacteriocin as a probiotic, broad-spectrum antibiotic and anticancer drug. More organisms have to be tested to check the entire spectrum of the inhibition. These studies should also be supplemented with other compounds like EDTA and commercially used antibiotics to further check their synergistic effects. Since bacteriocins are peptides, they have a natural tendency to be digested by certain proteases. This aspect has to be explored to study the stability of these bacteriocins in biological systems. Another major aspect of concern in the antimicrobial studies is the development of resistance. Some bacterial strains have been reported to have developed resistance towards *Lac B* bacteriocins. These concerns need to be addressed to deploy these peptides as potential broad-spectrum antibiotics. With studies suggesting otherwise in the cancer scenario, more research has to be done on the multi-drug resistance restraining capacity of the bacteriocins.

Acknowledgement

The authors are thankful to the Post graduate Department of Studies in Biotechnology and Microbiology, Karnatak University Dharwad for providing all the necessary facilities, Department of Biotechnology, New Delhi for providing Bioinformatics lab facility, DBT-KUD-IPLS program (BT/PR14555/INF/22/126/2010) sanctioned by Department of Biotechnology, New Delhi and Purse program of Department of Science and technology, New Delhi.

Conflict of Interest

The authors declare no conflict of interest.

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