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Purification, Characterization, Identification, and Anticancer Activity of Bacteriocin from *Leuconostoc carnosum*

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ABSTRACT

Cancer is the second most common cause of mortality in most developed countries, behind heart disease. Cancer is defined as the aberrant proliferation of cells. It is also the most feared disease. By 2025, it is anticipated that there will be more than 20 million new instances of cancer worldwide. Chemotherapy is still the most widely utilized therapeutic option among the several alternatives offered to patients. Currently available anticancer medications are either entirely manufactured molecules or substances extracted from plants and their derivatives. Evidence exists that conventional cancer treatments have a number of unfavorable side effects. It is well known that cancer cells can get resistant to the treatments that are currently accessible. Effective cancer treatment is still hard to come by, even with the latest developments in tumor therapy.A new treatment consisting of pure bacteriocin from *Leuconostoc carnosum* was effect on cancer cell. Different concentrations (1000, 500, 250, 125, 62.5, 31.25) µg /ml of bacteriocin purified from Leuconostoc carnosum which isolated from broccoli were applied on the A549 lung cell line. A significant cytotoxicity ratio of bacteriocin was 67.57828 % caused by the highest used concentration 1000µg/mL towards A549 lung cell line compared to Normal Human Fibroblast cells which were resist to the same concentration of it with maximum cytotoxicity 5.870841 %. The IC50 for A549 lung cell line was 164.8µg/ml while IC50 for NHF cell line was 111.9. A highest activity of bacteriocin against cancer cells was established at1000µg/mL.

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1. Introduction

Globally, cancer is one of the main causes of morbidity and death among the non-communicable diseases. The second most frequent of cancer in both men and women is lung cancer. Two histologic types for the majority of lung cancer cases: small cell lung cancer 13% and non–small cell lung cancer 84% [1]. While radon exposure is a major contributor to lung cancer that is not tied to smoking, smoking is directly associated to 80% to 90% of lung cancer deaths [2]. Because most anticancer drugs now used in clinical settings have harmful side effects, their use is restricted. Natural sources such as plants, animals, and microorganism are able to produce bioactive chemicals that have the potential to be medicinally significant. Natural products have been essential for the treatment and prevention of human diseases since ancient times [3]. The lactic acid bacteria has properties in anti-cancer and it one of the research concerns. During fermentation, lactic acid bacteria produce not only a wide range of active substances such as organic acids and reductases but they can also make bacteriocins, which have antibacterial properties. Thus far, there has been reduction the danger of cancer by preventing the growth of pathogens [4]. Bacteriocins are ribosomally- created peptides cationic that are generated by nearly every type of bacteria. Due to their cationic peptide composition, bacteriocins preferentially bind to the negatively charged cell membranes of cancerous cells in contrast to the neutrally charged cell membranes of normal cells [5].

Leuconostoc is a genus that belongs to the Lactobacillaceae family. Every species in this genus is heterofermentative, meaning that it produces lactic acid, CO2, ethanol, and/or acetic acid by the metabolism of carbohydrates via the pentose phosphate and phosphoketolase route [6]. *Leuconostoc carnosum* has been identified from processed vegetables and is commonly found in the microbiota of foods, primarily meat-based products. It has the ability to create bacteriocins, which helps preserve feed [7, 8]. Natural materials are becoming a promising source for novel therapeutic medications, particularly those with anticancer properties. The goal of this research is to use a natural material with no side effect as alternative of conventional chemical material in cancer therapy.

2. METHOD

2.1 Isolation of Lactic acid bacteria produced for bacteriocin

Between November 2022 and January 2023, fresh vegetables (broccoli and cauliflower) were randomly gathered from Baghdad marketplaces in Iraq. Lactic acid bacteria (LAB) were isolated on de Man, Rogosa, and Sharp broth (MRS). MRS medium containing lactic acid bacteria was incubated for 24 hours at 30°C. Next, centrifuging the cultures for 30 minutes at 4°C / 4000 rpm yielded cell-free supernatant (CFS). It was sterilized by passing it through a Millipore filter with a 0.22 µm pore size and using 1N NaOH to get the pH down to 6.0. The agar well diffusion method was used to assess the bacteriocin that lactic acid bacteria produce's capacity to inhibit several pathogenic bacteria (*S. aureus, S. agalactiae, E. coli, and P. aeruginosa*). Adjusting the indicator bacteria to the 0.5 McFarland standard and cultured on Mueller Hinton agar medium. Next, create a well in it with a 6 mm diameter and fill it with 100 microliter of CFS [9].16s rRNA using polymerase chain reaction was employed to identify the bacterial isolate that produced the biggest inhibitory zone.Bacteriocin was isolated by precipitating the supernatant with a specified weight of ammonium sulfate [10], followed by ion exchange (DEAE-Cellulose) [11, 12]. Lastly, Sephacryle S-200 gel filtering was employed [13].

2.2 Application of bacteriocin on cell line

Cell Line: In this investigation, the cell lines utilized were Adenocarcinomic human alveolar basal epithelial cells were isolated from malignant lung tissue of a 58-year-old male patient to create the A549 lung cell line which was first developed in 1972 by Giard, D. J. et al. The cells are employed as models for the study of lung cancer inside Iraq and in the creation of medication treatments [14, 15]. Additionally, Cell Technologies Lab (Harithiya) provided NHF cells.

Cell culture conditions: (Modified Eagle Medium) was used to maintain the A549 lung cell line and the normal human derived adipose tissue NHF cells. Using Trypsin-EDTA, cells were passaged twice a week at 50% confluence, reseeded, and incubated at 37°C [16, 17].

2.3 Cell cytotoxicity assay

The MTT cell viability test was used to detect the cytotoxic effect of different concentrations of pure carnosin on the proliferation of adherent cells of the A549 lung cell line and NHF cells in 96-well microtiter plates [18]. MTT stain (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) was used in this assay in the following manner:

1. Cell preparation: Both types of cell lines suspension were made by discarding the previous media and treating the cells for no more than ten minutes with 2-3ml of trypsin-EDTA. The result cell suspension was reseeded to the new flask ((25ml) contained 20 ml of MEM medium for both cell lines) , it was incubated for 1-3 min at 37 °C in presence of 5% CO2 in air depending on the kind of cell line. Following the addition of 200 µl of cell suspensions ($1x10^4$ cell/ml) in growth medium was added to each well, the plate was covered with self-adhesive film and incubated for 24 hours at 37ºC with 5% carbon dioxide added, until the cell reached confluent monolayer (vary depending on the type of cell line) [19] .
2. Carnosin treatment: Sterilization of Purified carnosin by filtering through a 0.22 µm Millipore filter and diluted with serum free medium with varying concentrations of carnosin, (1000, 500, 250, 125, 62.5, 31.25) µg /ml under aseptic conditions. After removing the medium, each well received an individual addition of 200 µl of carnosin at different concentrations. A triplicate was done for each treatment. The control of cancer cell lines was treated with 200 µl of serum free medium without bacteriocin and the plate sealed with adhesive film and incubated at 37ºC with 5% CO2 for a full 72 hours.
3. Applying the MTT stain: After 72h of treatment, cell viability was measured by removing the carnosin and medium from plate as well as unattached (dead) cells were removed by washing with PBS. Then, 28µl of MTT dye (yellow solution 2 mg/ml) was added to each well and incubated for 1.5 hrs at 37°C. The MTT

dye solution was disposed of and the crystals that were still in each well dissolved with 130 microliters of 1% DEMSO. Then, these wells were incubated with shaking for 15 min at 37 °C to solubilize the MTT-purple formazan crystals [20].

4. Readings: The absorbency was determined using an ELISA microplate spectrophotometer set to 492 nm wave length. The assay was run in triplicate. The following formula was used to determine the inhibition rate of cell growth (the percentage of cytotoxicity),[21]:-
   IR% = (OD Control – OD treat) / OD Control × 100
   IR%: The inhibition rate as a percentage, C: The absorbance of control (optical density at 492nm), T: The absorbance of the test of each concentration (optical density 492nm).
5. MTT assay is relying on the metabolic reduction of colourless tetrazolium salt by mitochondrial enzyme activity in viable cells. The half maximal inhibitory concentration or IC50 was determined by using a linear regression of the concentration against the cytotoxicity [22]
6. Fifty microliter of Crystal Violet stain was added to the plates and incubated for 15 minutes at 37^{o}C, the stain was carefully washed with tap water until the dye was removed. The cell was examined with an inverted microscope at a magnification of 100x and photographed on digital camera [23,24].

2.4 Statistical Analysis

GraphPad Prism 6's unpaired t-test was used to statistically assess the collected data [25].A mean ± standard deviation of three measurements was used to present the results [26]

3. RESULTS and DISCUSSION

From broccoli and cauliflower, 180 lactic acid bacterial isolates were obtained. To find the antibacterial activity against indicator bacteria, the CFS of it was examined. The isolate from broccoli (LabBr no. 28) had the highest activity against all indicator bacteria out of the 20 isolates that showed antibacterial activity. This isolate, *Leuconostoc carnosum*, was selected to be the bacteriocin producer in this investigation after identification by 16s rRNA.

According to the bacteriocin purification data, ammonium sulfate saturation range for *L. carnosum* bacteriocin precipitation was between 40 and 60%, with an activity of 640 AU/ml. The bacteriocin was eluted at 0.25-0.5M of NaCl using ion exchange chromatography with 1280 AU/ml of bacteriocin activity. After the fractions corresponding to the activity of bacteriocin were combined and concentrated, it was applied in Sephacryl S-200, and only one absorption peak—25600 AU/mg—was found to correspond to the particular activity of the purified bacteriocin.

3.1 Application of bacteriocin on cell line

The cytotoxic effect different concentrations (1000, 500, 250, 125, 62.5, 31.25) µg /ml of purified bacteriocin on A549 lung cell line and normal human derived adipose tissue NHF cells was assessed in this study using MTT assay. After 72 hours the impacts were observed over the cell viability. The results appeared that the cytotoxic ratio was 67.57828 % at the highest concentration of bacteriocin towards A549 lung cell line. The lowest cytotoxic ratio was 12.80222 % at the lowest concentration of it, as seen in figure 1, whereas NHF cells, which were resistant to bacteriocin, had maximal cytotoxicity 5.870841 % at concentrations of 1000µg/mL , as shown in figure 2.

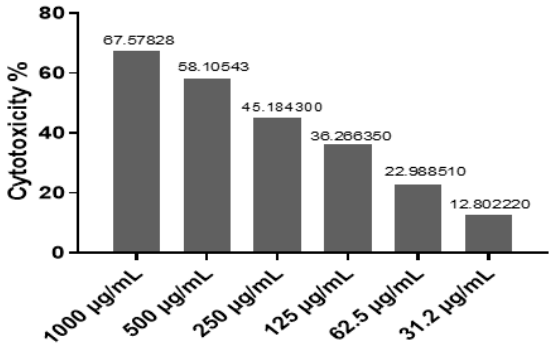

Figure1. Cytotoxic activity of purified bacteriocin on A549 lung cell line

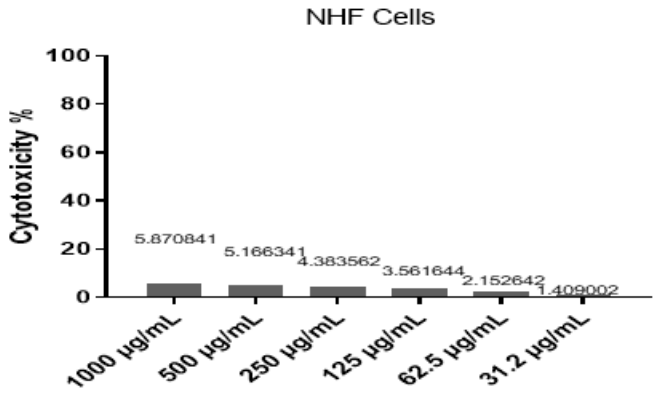

Figure 2. Cytotoxic activity of purified bacteriocin on NHF cells

The bacteriocin proved efficient in killing cancer cells at half the inhibitor concentration after being incubated for 72 hours at 37ºC. The IC50 for the A549 lung cell line was 164.8µg/ml and IC50 for the NHFcell line was 111.9 as seen in (Figure 3 and 4). The IC50 value was represented the ability of a substance to inhibit a specific biological or biochemical function. In pharmacological research, IC50 is frequently employed as a measure of antagonist drug potency to determine the impact of the material on the target.

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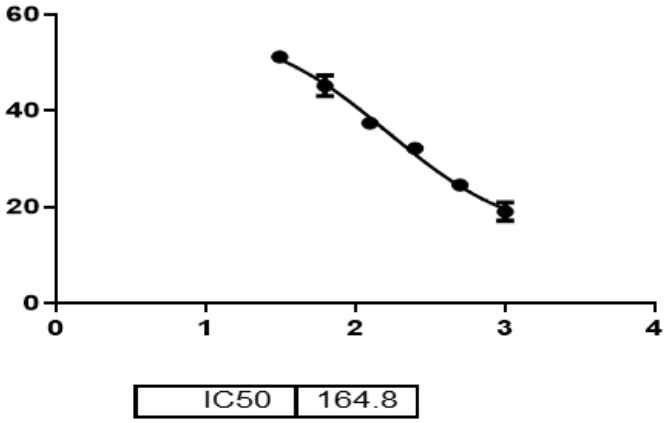


Figure 3. Half maximal inhibitory concentration IC50 effect of bacteriocin on A549 lung cell line

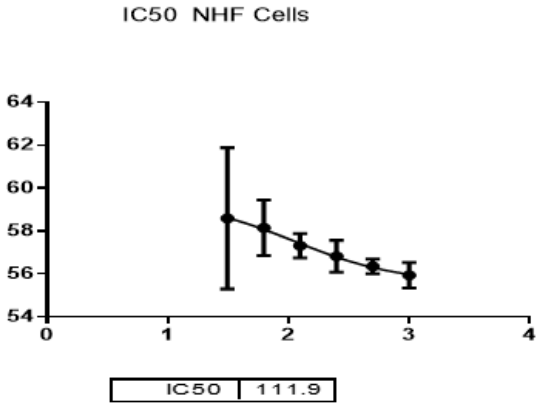
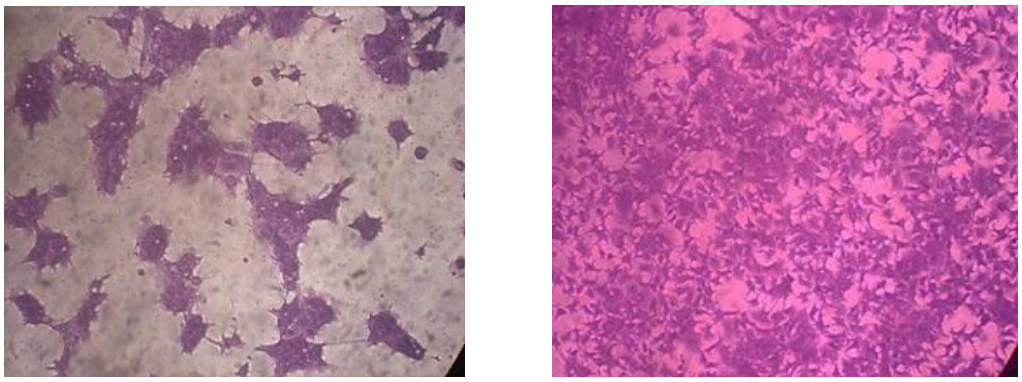


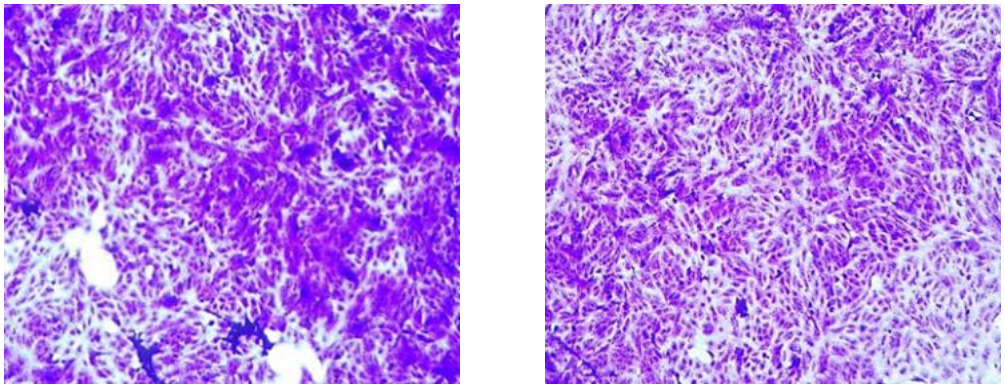
Figure 4. Half maximal inhibitory concentration IC50 effect of bacteriocin on NHFcells

Cytopathic effects were observed in A549 lung cell line after treatment with bacteriocin. The cells' altered morphology such as (cell shrinkage, membrane blebbing, or formation of apoptotic bodies) could be an indication of cell death and decrease in cell density compared to control wells as seen in figure 5. Bacteriocin have the effective role in treating cancer through its inhibitory effect and fatal toxicity on lung cancer cells line compared to effect of it on NHF cells line that showed in figure 6.



A. Treated B. control

Figure 5. Cytotoxic effects of bacteriocin on A549 lung cell line after 72hr.



A. Treated B. Control

Figure 6. Cytotoxic effects of bacteriocin on NHF cells after 72hr.

The bacteriocin's cytotoxicity and its capacity to attack cancerous cells differently may rely on its structural attributes like that quantity of amino acids with a positive charge, hydrophobicity and capacity to produce oligomerization or amphipathic complexes, similar to other antimicrobial peptides [27]. Since bacteriocins are cationic peptides by nature, they preferentially bind to the negatively charged cancer cell membrane as compared to normal cell membranes which are neutral in charge. The cancer cell membrane has higher fluidity which facilitates membrane instability. Additionally, the cancer cells membranes have a greater quantity of microvilli than normal cells which enhances the cancer cells' surface area and leading to bind of a greater quantity of antimicrobial peptides to the membrane of cancer cells than normal cells [28, 29].

LAB prevents the growth of tumors By a variety of methods, such as anti-proliferative activity, activation of apoptosis or depolarization of the cell membrane resulting in permeability alterations, cell cycle arrest, as well as anti-mutagenic, and anti-inflammatory properties. Necrosis and apoptosis can be induced by a small number of them [30]. In colorectal cancer cells, *L. mesenteroides* mediates its pro-apoptotic and anti-proliferative activities [31]. Additionally, the post biotic and bacterial extracts of *L.pseudomesenteroides* were effect on the L-929, HT-29, and Caco-2 cell lines' biochemical activities and viability [32].

The combined action of *Weisella cibaria* and nanoparticles decreased the cancer line viability rate after 72-hour period [33]. The growth of human lung cancer cell lines A-549 was suppressed by the pediocin generated by *P. acidilactici* and there combinant Cloning of pediocin in Pichia pastoris [34]. Many anticancer drugs function by subjecting tumor cells to oxidative stress, which is believed to be the main source of the most macromolecular alterations in the cell. have the potential to harm DNA, membrane lipids, and proteins [35].

4. Conclusions

Our findings demonstrated that the highest activity of bacteriocin against A549 lung cell line was at highest concentration whereas the NHF cell line was resisted to it at the same concentration. The IC50 for A549 lung cell line was at low concentration.

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