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Original Article

ANTI-PROLIFERATIVE ACTIVITIES OF PURIFIED BACTERIOCIN FROM *ENTEROCOCCUS MUNDTII* STRAIN C4L10 ISOLATED FROM THE CAECUM OF MALAYSIAN NON-BROILER CHICKEN ON CANCER CELL LINES

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ABSTRACT

Objective: This study aim at experimenting the effects of the bacteriocin from *Ent. mundtii* strain C4L10, an apoptogenic properties, that have the ability to inhibit bacteria at the same time the tendency to induce anti-proliferative characteristics on cancer cell lines.

Methods: A purified bacteriocin from *Ent. mundtii* C4L10 strain (accession number KC731423) isolated from Malaysian non-broiler chicken was screened for its anti-proliferative activities against four cancer cell lines; breast cancer (MCF7), lung cancer (H1299), colon cancer (HCT116) and oral cancer (HSC3) by an *in vitro* MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], colorimetric assay. The half maximal inhibition, IC_{50} values was determined by non-linear regression analysis using the Prism software (Graphpad).

Results: The susceptibility of cancer cell lines to the bacteriocin extract was in the order: oral cancer HSC3, breast cancer MCF7, lung cancer, H1299 and colon cancer HCT116 with the IC_{50} values, 9.00 µg/ml, 11.51 µg/ml, 15.25 µg/ml, and 20.57 µg/ml, respectively. The approximate molecular weight for this purified bacteriocin was 10 kDa, as observed by SDS-PAGE.

Conclusion: Bacteriocin from *Ent mundtii* strain C4L10 could be employed as potential antimicrobial agent and for anti-proliferative agent in cancer control.

Keyword: Cancer, Enterococcus, Bacteriocin, Anti-proliferative, MTT.

INTRODUCTION

Cancer arises from an accumulation of mutations in oncogenes, tumor suppressor genes and genes that maintain the genomic integrity of the cell [1]. Oncogenes lead to increased net growth rates of the cell when activated by a point mutation, and/or fold over expressed [1]. Cancer is a genetic disease [2], although, environmental and other non-genetic factors participate in the different stages of tumorigenesis. It is widely accepted that cancer arises due to mutation in cancer-susceptibility genes. These genes belong to one of three classes [2, 3]: gatekeepers, caretakers and landscapers. Gatekeepers directly regulate growth and differentiation in the pathways of the cell and comprise oncogenes and tumor suppressor genes. Caretakers, in contrast, promote tumorigenesis indirectly [4, 5].

They function in maintaining the genomic integrity of the cell. Mutation of caretakers can lead to genetic instability, and the cell rapidly accumulates changes in other genes that directly control cell birth and death. Landscaper defects do not directly affect cellular growth, but generate an abnormal stromal environment that contributes to the neoplastic transformation of cells [6]. Cancer, malignant tumors or neoplasms is a large group of diseases that can affect any part of the body. The most important feature of cancer is that it leads to rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs.

A process referred to as metastasis a major cause of death from cancer. Among all cancers, Oral cancer has one of the most deadly and it cause of more than 90% oral malignancies resulting to oral squamous cell carcinoma (OSCC) [7]. The next leading cause of death among cancer is the colon cancer and this cancer is associated with high-fat or low-fiber in diet [8, 9]. Tumor growth has been attributed to changes in the structure of the glycan residues in the glycoprotein and glycolipid group located on the cell surface [10, 11]. These abnormal glycosylations coming from dysfunction of glycosyl transferases and (or) glycosidases, often lead to a shortening of the glycan chains or an over-expression of structures on the cells which are normally absent or discrete [10].

Bacteriocins are of importance in medicine because of their production from non-pathogenic bacteria that are normal flora of the human body. Bacteriocin was first reported in the year 1925 through the observation of Gratia that *Escherichia coli* φ caused the inhibition of *E. coli* V. He then called this inhibitory agent colicine since it killed *E. coli*, and then later (renamed colicin). Depletion of these harmless bacteria resulted from the use of antibiotic gives way to opportunistic pathogenic bacteria to invade the human body.

Despite the fact that bacteriocins interact only with sensitive strains among bacteria, there were reports showing that they could have toxic effect on mammalian cells as a result of the presence of a number of receptors for various substances on the cells [12, 13]. The most studied bacteriocin is nisin, which has received wide acceptance for several years in preventing bacterial growth in foods, and it was recently been tested for prevention of growth of cancer cells.

Antimicrobial peptides have been investigated as a therapeutic agent, because of their ability to perform many biological functions, notably among them are inhibition of membrane protein synthesis, DNA synthesis, antiviral properties, and apoptosis or cytotoxicity of tumor cells [14-16]. Because of these properties, antimicrobial peptides have been investigated as potential therapeutic drugs [17]. Many studies on anti-proliferative activities of bacteriocin were mainly focused on gram negative bacteria. This report is one of the very few studies on bacteriocin from gram positive Enterococcus strain as anti-proliferative potential agent. This study aim at experimenting the effects of the bacteriocins from strain C4L10, particularly apoptogenic-bacteriocins having the ability to kill other bacteria at the same time showing the tendency to induce anti-proliferative characteristics on human cell lines.

MATERIALS AND METHODS

Previously isolated *Ent. mundtii* C4L10 (accession number KC731423) from the caecum of a non-broiler chicken in Kuantan, Malaysia was stored under 50% glycerol at -80°C and resuscitated on MRS agar [18]. Several sub-culturing steps were then carried out to obtain a pure culture of the isolate.

Extraction of bacteriocin

Bacteriocin extraction was done using the three Phase Partitioning (TPP) protocol and cytotoxicity was determined by MTT assay on the cell lines. This extraction method involved the collection of pellets from the supernatant after centrifugation of the culture broth. Depending on the starting volume of the broth culture, for every 10 mL of the broth, 200 µl of 80% ammonium sulphates and 200 µl of 100% tertiary butanol (v/v) were added to the bacterial palette. The mixture was vortexed for 1.0 min, and left to settle in order to achieve complete phase separation after which it was centrifuged at 6,000 × g for 3 min. The upper layer and the interfacial phase were discarded. Same amount of t-butanol used at the initial stage was added to the lower aqueous phase containing the protein of interest. The mixture was allowed to settle followed by centrifugation. Discarding the upper layer, the interfacial phase was collected and subjected to chloroform/methanol precipitation method for purification, removal of salts and concentration of the protein of interest.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The characterization of the bacteriocin (peptides/proteins) was performed by SDS-PAGE [19]. The SDS-PAGE was carried out for the extracted protein samples possessing antimicrobial activity. The experiment was carried out on Mini-PROTEIN, mini vertical electrophoresis apparatus (Bio-Rad, UK) using the 12% gel. The samples were prepared by mixing the protein at 2:1 ratio with 2xSDS-sample buffer. The gel was stained with coomassie brilliant blue, along with the marker proteins (Fermentas, Lithuania) [20]. The apparent molecular mass of the sample was calculated by comparison with the mobility of the standard markers.

MTT Bioassay

The cytotoxic effect of the bacteriocin extract from strain *Enterococcus mundtii* C4L10 against breast cancer (MCF7), lung cancer (H1299), colon cancer (HCT116) and oral cancer (HSC3) were evaluated using MTT bioassay. MTT assay detects the blue formazan product produced as a result of the reduction of the yellow dye MTT by mitochondrial succinate dehydrogenase. The production of the blue formzan product is an indication of a normal functioning mitochondria which is related to the cell viability [21].

All cell lines were kindly provided by Kulliyyah of Dentistry, International Islamic University Malaysia which was initially obtained from Section of Molecular Craniofacial Embryology, Tokyo Medical and Dental University, Japan. The cells were grown in culture medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin mixture. The cultured cell was checked for their confluency using the inverted microscope. The medium in the flask or dish was removed completely by suction and washed twice by using PBS to remove any leftover medium. 1.0 ml of trypsin was added until it completely covered the monolayer. The cells were kept in the incubator for 1—2 min to accelerate cell detachment. Detached cells were observed and re-suspended to avoid cell clamping. The warmed medium was then added into the new flask/dish and the cell suspension was re-suspended to homogenize before it is distributed. Cell viability and cell counting were determined by using the trypan blue exclusion test using a regular microscope.

The cells were seeded in 96-well microtitreplate (100 µl/well) with concentration of 4×10^4cells/cm^2. At 40-50% confluency, the cultivated cells were exposed to various concentrations of the bacteriocin extract (21.60, 10.80, 5.40 and 2.70 µg/mL) prepared in 1% DMEM and incubated for 24 hr (5% CO_2, 37°C). Three wells containing the same amount of the DMEM and having no bacteriocins extract were used as control. About 30 µl MTT reagent (2 mg/mL in PBS) was then added to the wells except for the cell-free blank control. Cells were maintained in 37°C at 5% CO_2 and in complete humidity for 3 hr. Subsequently, the MTT solution was replaced with 100 µl of DMSO. The optical density of the wells was measured at two wavelengths 570 nm and 650 nm by means of a spectrophotometric plate reader (Sunrise Tecan, Austria). The percentage cell viability was determined based on the formula: % Viability = (optical density of sample/optical density of control) ×100.

RESULTS

Bacteriocin purification

A summary of the purification steps for the bacteriocin is presented on table 1. As could be noticed in this table, there was a gradual decrease in the amount of the protein with a subsequent increase in the specific activity with purification factor of 9.3. The TPP technique used in this study to partially purify the bacteriocin of *Ent. mundtii* from MRS broth yielded 7.0 mg of protein with a protein content of 1.4 mg (specific activity 35.7 A. U. of bacteriocin per mg of protein).

TPP purified fraction produced a 10 kDa molecular weight band, as shown by SDS-PAGE analysis (fig. 1).

Table 1: Purification table of *strain* C4L10 Bacteriocin

Sample	Volume (ml)	Activity[1] (AU/ml)	Total activity	Protein (mg/ml)	Total [5] protein (mg)	Specific Activity[2] (AU/mg)	Yield[3] (%)	Purification Factor[4]
Crude Sample	2	400	800	130	26	3.846	100	1
Tpp (80% $NH_4(SO_4)_2$ conc.+100% Tert. butanol)	0.2	1200	160	70	1.4	35.7	50	9.28

[1]Activity unit (AU/ml) = Reciprocal of the highest dilution x1000/volume of bacteriocin added, [2]Specific activity (AU/mg) = Total activity of the subsequent purification step/Total protein of the same step,

[3]Recovery (%) = Total activity of subsequent step x 100/Total activity of crude preparation, [4]Purification fold = Specific activity of subsequent step/ Specific activity of crude preparation.

Antipoliferative activity of bacteriocins

Cell lines were incubated for 24 hr with varying doses of the bacteriocin ranging from 2.68, 5.35, 10.69 and 21.39 µg/mL, and then cell viability was determined by the MTT assay. A dose-dependent decrease in the number of viable cells was observed in all the cell lines.

Bacteriocin extracts from strain C4L10 seemed to inhibit the viable cells maximally in a concentration dependent manner. Despite the fact that the bacteriocin extract produced cytotoxic effect on all the cell lines, the highest cytotoxic effect was however recorded by oral cancer cells followed by breast cancer cell lines, while the least sensitive was colon cancer.

The plot on fig. 2 was generated using Graphpad Prism software, along with the calculated IC_{50} values for the four cell lines. A non-linear regression analysis was performed, and a sigmoidal dose-response curve (variable slope) was fitted. Logarithmic base 10 (Log_{10}) of bacteriocin concentration was plotted against % cell viability. Based on Graphpad estimation, (fig. 1), the log IC_{50} were 1.061, 1.183, 1.313 and 0.954 for MCF7, H1299, HCT116 and HSC3 cell lines, respectively.

These were equivalent to IC_{50} of 11.51 µg/mL, 15.25 µg/mL, 20.57 µg/mL and 9.009 µg/mL for MCF-7, H1299, HCT116 and HSC-3 respectively. Their respective R-Square (R^2) values were MCF7 (0.9169), H1299 (0.6773), HCT116 (0.1104) and HSC3 (0.4457). The P-value for all were at 0.5 and the Standard Error (Std Error) for log IC_{50} for MCF-7, HI299, HCT116 and HSC3 were 0.06741, 0.1083, 0.1588 and 0.01575, respectively. This IC_{50} was within the accepted limit for promising crude extract for further purification since threshold level should be lower than 30 µg/mL (threshold according to the American National Cancer Institute) [22].

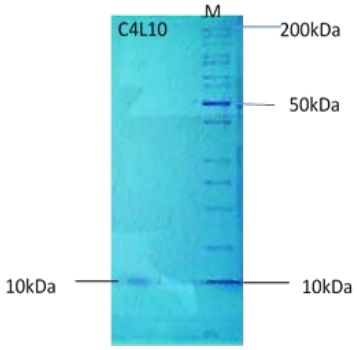

Fig. 1: The SDS-PAGE result showed a single 10 kDa band of protein produced after TPP extraction method on 12% polyacrylamide gel. Lane M contained protein marker (Page Ruler 200-10kd Ladder Fermentas)

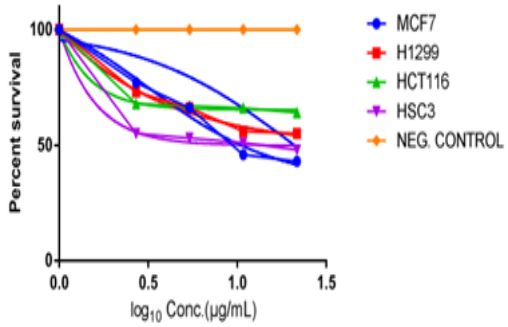

Fig. 2: Estimation of the half maximal inhibitory (IC_{50}) of Breast cancer MCF7, Lung cancer H1299, Colon cancer HCT116 and Oral cancer HSC3 cell lines. Non linear regression analysis was performed using Graph Pad software

The dose response plot was drawn using GraphPad Prism (fig. 2), along with the calculated IC_{50} values for the 4 cell lines. A non-linear regression analysis was performed, and a sigmoidal dose-response curve (variable slope) is fitted. Log_{10} bacteriocin concentration was plotted on the X axis and % cell viability are plotted on the Y axis. The upper asymptote was used to calculate the IC_{50} value. Statistical differences among fractions were determined by one way ANOVA using GraphPad Prism 5 (GraphPad Software Inc., San Diego, USA). Differences were considered significant at $p < 0.05$.

Fig. 3 showed the effect of bacteriocin extracts from strain C4L10 on the viability of cancer cell line viewed by an inverted microscope with and without bacterocin treatment. The non viable cells are seen floating.

Fig. 3: Effects of purified bacteriocin from strain C4L10 on the viability of cell line. Photomicroscopic picture of bacteriocin effect on cell line (plate A) and of negative control, (plate B), after 24 hrs of incubation at 5% CO_2 and observed at 20 X magnification

DISCUSSION

Small size bacteriocins were commonly isolated from many Enterococcus strains. Other studies [23] showed that *Enterococcus faecium* (EF2019, EF1819, EF2119, EF1839, EF529, EF24/10) isolated from rabbits feces produced bacteriocin of molecular mass ranging from 3 to 10 kDa, and 17.5kD protein molecular mass was produced by the *Enterococcus faecium* BC25 isolated from the rumen of a cow [24]. In contrast to the findings of [25] who identified the bacteriocin like substances from *Ent. faecium* D081821 and D081833, to be 3 kDa in size.

Despite the anti-bacterial properties of different types bacteriocin are well described, the anti-proliferative properties of bacteriocin on cancer cell is poorly understood. The anti-proliferative properties of this bacteriocin could be partly attributed to the presence of glycosyl transferases in its conserved domain. Glycosyl transferases, is a member of a large family enzymes known to carry-out biosynthesis of glycoconjugates, oligosaccharides, and polysaccharides [26]. The bactericidal action of a bacteriocin against sensitive bacteria is primarily defined by the specific receptors on the cell envelope [27, 28]. In line with this, the sensitivity of tumor cells to bacteriocin may be attributed to the accumulation of glycopeptides and/or glycolipids on cell membranes of these cells, resulting in more affinity for the bacteriocin when compared with normal cells. Therefore, the binding of the bacteriocin to these constituents, create lethal events that could lead to cell death. In another study, it was noticed that, in a virally transformed tumor cells, there was a general increase in the level of the neutral glycopeptides and glycolipids in cell membrane when compared to that of a parental normal cells which did not undergo transformation [29]. These trends have been noticed in spontaneous tumor cells as well as transformed cells due to virus [30]. Furthermore, the cytotoxic activity could be due to the presence in the bacteriocin extract of active products that could probably have anti-growth effects.

This therapeutic approach takes advantage from the known tendency of transformed cells to express selective carbohydrate motifs otherwise hidden in normal cells [31]. On the other hand, [32-34] hypothesized that the ability of a bacteriocins to have a toxic effect on tumor cells will likely depend on the phase of the cell cycle rather than on the presence of precise surface receptors with greater attraction for bacteriocins in tumor cells. Efforts were made in glycoconjugate construction for the creation and evaluation of vaccines based on carbohydrate cancer-associated antigens. [35]. The elucidation of the mechanisms involved in the action of the bacteriocin of the strain C4L10 and its evaluation as a possible anticancer drug warrants further investigation. Thus, the bacteriocin of this strain could be a source for new lead structures in drug design to combat cancer.

CONCLUSION

The result obtained in this study showed that bacterioicin of a molecular weight of approximately 10 kDa was isolated from *Ent. Mundtii* strain C4L10. This strain previously isolated from the caecum of Malaysian non-broiler chicken, was not only shown to

produce bacteriocin active against *Staphylococcus aureus*, it also showed anti-proliferative properties against human cancer cell lines. Based on MTT test, different cell lines responded differently to bacteriocin treatment. From this observation, it can also be concluded that oral cancer cell lines is most sensitive to this bacteriocins followed by the breast cancer cell lines as observed from the cytotoxic index IC_{50}. Colon cancer cell line was however shown to be less susceptible to bacteriocin treatment. The elucidation of the mechanisms involved in the action of the bacteriocin of the strain C4L10 and its evaluation as a possible anticancer drug warrants further investigation. From the results, it can be concluded that *Ent. mundtii* C4L10 is a potential isolate capable of producing bacteriocin with an antitumour properties. To the best of our knowledge, this is the first report on the bacteriocin from gram positive *Ent. mundtii* strain showing anti-tumor properties.

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CONFLICT OF INTERESTS

Declared None

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