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Research Article

In vitro Assessment the Potential Antioxidant and Antitumor Activities of *Bifidobacterium* Derived Bacteriocins

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ABSTRACT

Nowadays there are increasing interest in using microbial bioactive peptides as therapeutic agents or as adjuvant to increase the effectiveness of available therapies, a promising approach in this line is using of probiotics secreted peptides. *Bifidobacterium* is one of the favorite patented probiotics genera and most of human enteric *Bifidobacterium* secretes bacteriocins peptides to the surroundings. Bifidobacterial – associated bacteriocins are classified as GRAS peptides, and recently were attracted attention that become a widespread research topic in various fields including cancer drug discovery development. This study was conducted to seek for antioxidant and antitumor activities of bifidobacterial – derived bacteriocins. Two bacteriocins, Bifidin B1 and Bifidin B2, were partially purified from enteric *Bifidibacterium longum* Bl and *Bifidobacterium bifidum* B2, respectively. Bifidins were physiochemically characterized in respective of thermal, pH, and storage stability. Their proteinaceous nature was confirmed. Potential antioxidant activity in terms of free radical scavenging activity was evaluated, both Bifidins exhibited antioxidant activity, the highest percentage scavenging activity against DPPH was 70.55±0.2673 %, recorded for Bifidin B1, and was followed by 68.1 ±1.753% scavenging capacity for Bifidin B2, while both of Bifidins B1 and B2 had almost close values for scavenging of superoxide anion radicals, 66 ± 1.970 % and 65.64 ± 1.343%, respectively. Bifidins demonstrated potential antitumor activity on two human cancer cell lines, MCF-7 and Skov-3, however, the antiproliferative activities does not exceed 60.8%. Bifidins B1 and B2 showed highest cytotoxicity against Skov-3 cells rather than MCF-7 cells, with IC_{50} values of 28.9± 8.76 µg/ml, and 29.87± 9.13 µg/ml, respectively.

Keywords: Probiotic, Bacterial secreted peptides, Bacteriocins, Bifidibacterium, Antioxidant activity, Antitumor activity.

INTRODUCTION

Probiotics are live microorganisms which when administrated in adequate amount confer a health benefit on the host[1]. Among the probiotic's genera, *Bifidobacterium* are one of the favorite patented probiotics[2], *Befidobacterium* are Gram –positive, with high GC%, anaerobic, branched rod-shaped bacterium, and are a natural part of the bacterial flora in human body[3]. *Befidobacterium* members are the most abundant bacteria in human colon, that constitute more than 3% of total microbial flora population of human gut, and act as health-promoting microorganism[4]. Since its first recognition and isolation by Henry Tissier 1899[5], a widening evidence accumulating to support their prophylactic and therapeutic efficacy of many disorders in human and animals[6]. Several mechanisms have been proposed to explain the *Bifidobacterium* efficacy in curing and preventing infections, one of these mechanisms include production of antimicrobial proteinaceous substances, bacteriocins and bacteriocin-like peptides[7]. Bacteriocins are ribosomally-synthesized cationic peptides produced by almost all group of bacteria. Although Gram-negative bacteria can produce bacteriocins, but the vast majority of characterized bacteriocins so far are produced by Gram-positive bacteria particularly lactic acid producing bacteria (LAB)[8]. The classification of bacteriocins has been revised from time to time, the latest classification arranges bacteriocins into two major categories, based on their structural and physiochemical properties[9]: Class I (Lantobiotics): are small (<5 kDa) heat-stable post-translationally modified peptides, containing polycyclic thioether amino acids and the unsaturated residues. Lantobiotic are further classified into two types depending on the difference in charge[10]. Class II, or non-lantobiotic bacteriocins, are small (<10 kDa) heat stable peptides, do not undergoes extensive post-translational modification. They have amphiphilic helical structure, which allows them to insert into the membrane of the target cells, leading to depolarization and death. This group further subdivided into four subclasses, class IIa-class IId[11]. The most extensively studied LAB bacteriocin, Nisin A and its variant, are the main representatives of lantobiotics. Nisin A produced by *Lactobacillus. lactis* subsp. lactis, was first purified bacteriocin, and approved in 1988 as a generally recognized as safe (GRAS) by the FDA due to its low

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Table 1: Effect of storage temperature for a month on Bifidins B1 and B2 activity/stability.

	Temperature °C		
	25	4	-18
Bifidin B1	-	±	+
Bifidin B2	-	±	+

(+ active, ± moderately active, - inactive)

Table2: IC_{50} values calculated at 72h of Bifidins B1 and B2 on cancer cell lines.

Cell lines	Bifidin B1	Bifidin B2
MCF7 µg/ml	50.21± 7.5 µg/ml	59. 26± 6.68
Skov-3 µg/ml	28.97± 8.76 µg/ml	29.87 ± 9.13

Data are presented as the mean ± SD, (n=3)

toxicity in humans[12]. Bacteriocins display broad-spectrum activity against bacteria based on electrostatic interactions with negatively charged lipids or lipoteichoic acid on the bacterial surface[13]. The antioxidant and antineoplastic activities have been also explored on mammalian cancer cells soon after its discovery in the late 1970s[14]. With the growing the popularity of peptide therapeutics, the scientific community started exploring bacteriocins as novel therapeutic agents against cancer cells, and some bacteriocins have shown selective cytotoxicity against neoplastic mammalian cell lines as compared to normal cells. This makes them promising candidates to be effective therapeutic agent[15]. One advantage of most bacteriocins are exhibiting their activity at nanomolar concentrations, as opposed to the antimicrobial peptides produced by eukaryotic cells, which normally have 10^2 - 10^4 fold lower activities[16] Among well-known bacteriocins, azurin from *Pseudomonas aeruginosa*, is a member of the cupredoxin family of redox proteins, which becomes a potential anticancer drug because of some of its unique properties. Azurin can preferentially penetrate human cancer cells and exerts cytostatic and apoptotic effects with no apparent activity on normal cells[17]. *Bifidobacteriam* derived bacteriocins was first reported in 1980 and was characterized by display broad-spectrum activity against various pathogenic bacteria[18]. Although the anti – tumor activity of *Bifidobacterium* is not studied in details, recently it has been shed light on potentially antioxidant and antitumor activities[19]. The exact mechanism of cancer cells specificity has not completely clear. In general, the hydrophobicity of bacteriocins peptides and hydrophobic interaction is suggested to play a role for membrane permeabilization and in antitumor activity[20]. Peptides with higher hydrophobicity are enter deeper into the hydrophobic core of the cell membrane, causing stronger activity of disrupting the cancer cell membrane[21].

There are only few published documents of *Bifidobacterium* derived bacteriocins that possess antioxidant and antitumor properties, the present study therefore, aimed screening for potential antioxidant and antitumor activities of bacteriocins obtained from locally isolated *Bifidobacterium*.

MATERIALS AND METHODS

Microorganisms and growth conditions.

Bifidobacterium longum Bl (Bl) and *Bifidobacterium bifidum* B2 (B2) were used in the study for extraction of bacteriocins, both were previously isolated from human infants' feces, and suggested to be promising probiotic strains with regard bacteriocins production and antilisterial activity[22]. They were refreshed from 30% glycerol cultures stocks at -80°C on de - Man, Rogosa and Sharpe (MRS) agar medium (Himedia, India), supplemented with 0.05% L- cysteine (MRS-C), cultures were incubated at 37°C under anaerobic conditions (anaerobic jar supplied with Gas Pak, BioMerieux, France) for 48h. several subculture steps were carried out to obtain pure cultures of isolates. Diagnosed culture of *Listeria monocytogenes* (Lm) was kindly provided by Biology Dept. College of science/ Baghdad university/ Iraq, was used as the indicator organism. Lm was propagated on brain heart infusion (BHI) agar medium. The antimicrobial/antilisterial activity of bacteriocins was checked by agar-well diffusion assay[23], briefly, BHI agar was seeded with fresh overnight culture of Lm at a final concentration 10^6 CFU/ml, poured into sterile Petri dishes and allowed to solidify at room temperature, wells 5 mm were cut off in agar using sterilized cork borer, the wells were filled with 80µL of cell free bifidobacterial supernatants and allowed to diffused into agar for 3h at 4°C, plates were incubated at 37°C for 48h, the formed inhibition zones were recorded in mm after subtraction of 5 (well diameter).

Extraction and partial purification of Bacteriocins

Bacteriocins were extracted according to Mostafa and colleagues with some modification[24]. Cell free supernatants (crude) were obtained from fresh 24h old broth cultures of B1 and B2 by centrifugation (10,000×g for 15min. at 4°C). Supernatants were filter sterilized (0.45µm pore size filter, BioMerieux, France), the obtained filtrates were adjusted to pH 7, and H_2O_2 was excluded by treatment with catalase (100U/ml). The obtained crudes were precipitated to 20 - 80% by solid ammonium sulfate salt at 4°C, the precipitate at different saturations were collected and dissolved separately in 0.02 M phosphate buffer saline (PBS) pH 7, dialyzed overnight against same buffer at 4°C. Extraction was carried out by stirring with equal volume of methanol (50% v/v) for 3h. Organic fraction was then recovered, methanol evaporated completely at 55°C and the residues was dissolved in PBS pH 7 and then lyophilized. Protein concentration was determined at each step, using bovine serum albumin (BSA) as a standard[25], along with checking antiliteral activity, by agar-well diffusion method. The resulted bacteriocins were designated, as Bifidin B1 that was obtained from *Bifidobacterium longum* Bl, and Bifidin B2 from *Bifidobacterium bifidum* B2.

Physicochemical characterization of Bifidins

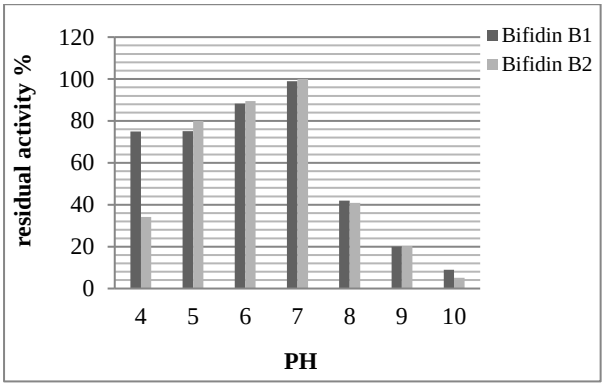

Figure 1: pH stability of Bifidins B1 and B2 at different pH values (4 – 10), results are mean ±SD, (n=3)

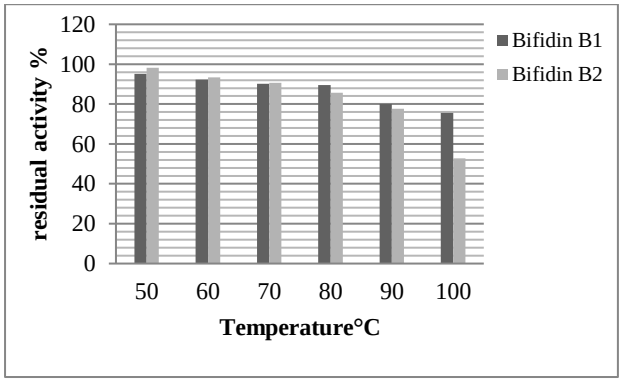

Figure 2: Thermal stability of Bifidins B1 and B2 at different temperature values (50 – 100) °C, results are mean ±SD, (n=3)

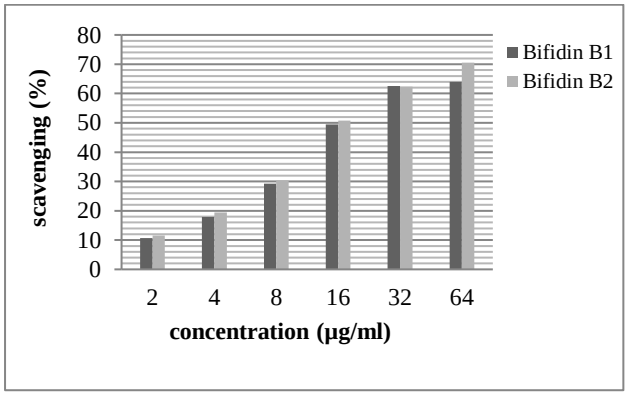

Figure 3: DPPH radical scavenging activity of Bifidin B1 and Bifidin B2, each value represents means ± SD, (n=3).

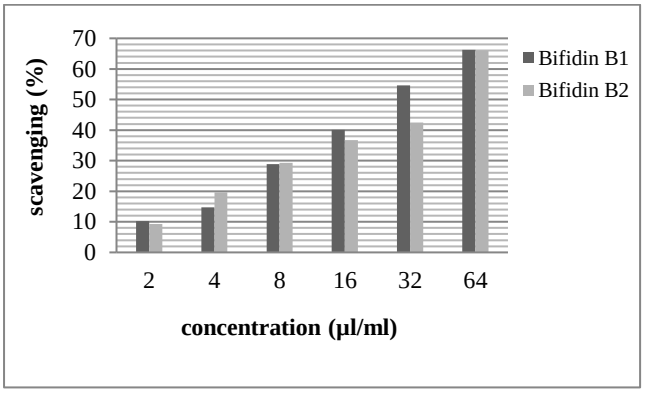

Figure 4: Superoxide anion radical scavenging activity of Bifidin B1 and Bifidin B2, each value represents means ± SD, (n=3).

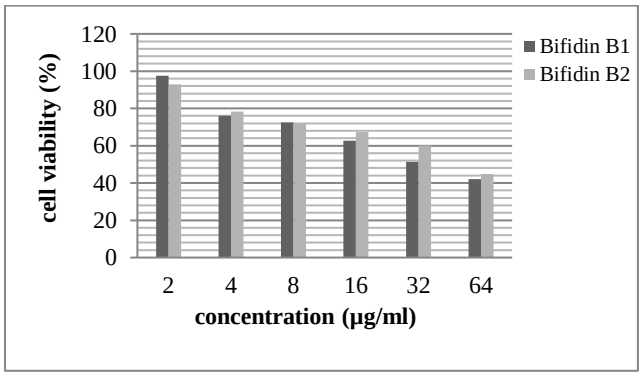

Figure 5: Cytotoxicity of Bifidins B1 and B2 against MCF-7 cell line, cells were treated for 72h. with different concentration (2-64µg/ml) of B1 and B2 separately. Data are expressed as mean viability ±SD, (n=3).

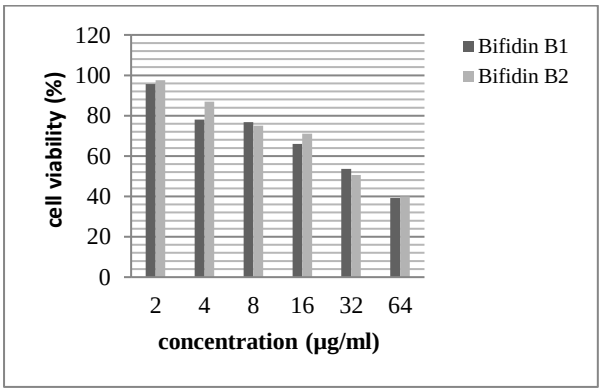

Figure 6: Cytotoxicity of Bifidins B1 and B2 against Skov-3 cell line, cells were treated for 72h. with different concentration (2- 64 µg/ml) of each B1 and B2 separately. Data are expressed as mean viability ±SD, (n=3).

The obtained Bifidins were characterized with respect to: pH and thermal stability, sensitivity to lysis enzymes, and stability during storage[26].

pH stability; 500µl of Bifidins B1 and B2, was adjusted individually to 4 – 10, and incubated for 2h at 37°C. The residual antilisterial activity was checked by agar well – diffusion method, result was represented as percentage of remaining activity compared to control (native untreated Bifidin).

Thermal stability; 500µl of Bifidins were separately exposed to temperatures 50, 60, 70, 80, 90, and 100°C in water bath for 30 minutes, the heat treated Bifidins samples residual activity was assayed as mentioned above. Result was represented as percentage of residual activity compared to control (heat-untreated samples).

Sensitivity to lysis enzymes; 500µl of both Bifidins were individually treated with enzymes; Protienase K, trypsin, pepsin, and amylase 0.1mg/ml (Sigma,USA). The

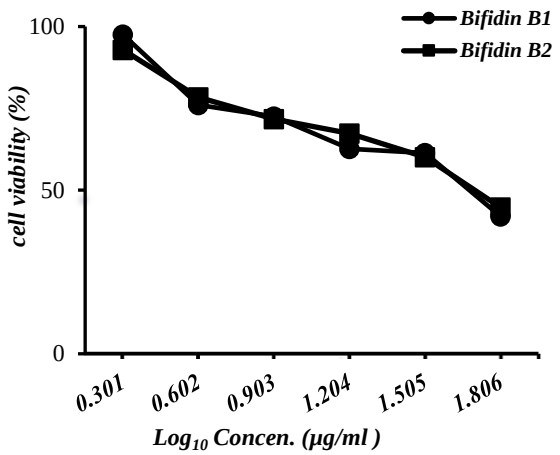

Figure 7: Estimation the IC_{50} value of MCF-7 cell line, cells were treated for 72hr with different concentration (2-64µg/ml) of each Bifidins, B1 and B2. Data are presented as mean ± SD, (n=3)

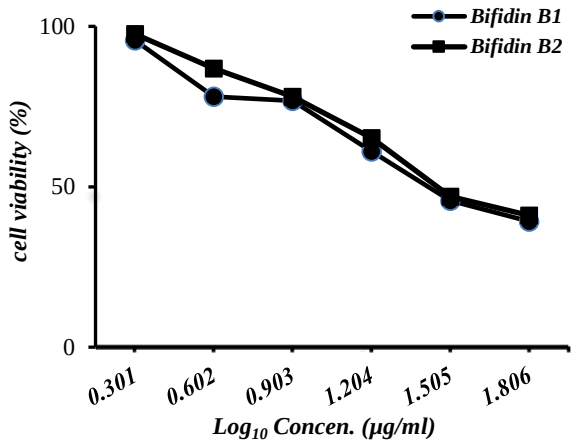

Figure 8: Estimation IC_{50} value of Skov-3 cell line, cells were treated for 72h. with different concentration (2-64µg/ml) of each Bifidins, B1 and B2. Dataare presented as mean ± SD, (n=3)

enzyme treated samples were incubated for 3h. at 37°C, except for samples containing trypsin were incubated at 25°C, the residual activity was assayed by agar-well diffusion method.

Stability during storage; Bifidins stored at three different temperature (25, 4, and -18 °C) for one month, and the antilisterial activity was checked by previously described method.

Antioxidant activity assay (In Vitro)

The antioxidant activity was investigated in terms of free radical scavenging activity by two methods:

Scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH)

The efficiency for scavenging DPPH was evaluated quantitatively for Bifidins by their ability to reduce and decolorize the purple – colored of DPPH solution to yellow[27]. In brief, 100µL of various 2-fold concentration (2 up to 64 µg/ml) in PBS of both Bifidins were separately mixed with equal volume of freshly prepared DPPH (60µM, in absolute methanol) radical solution, the mixtures were vortexed and allowed to stand for 30 min in dark at 25 °C. The absorbance of resulting solution was measured at 517nm against blank (methanol), L-ascorbic acid (0.1%) was used as reference antioxidant (positive control), and control was set up by mixing 100µL of PBS with 100µl of DPPH. Assay were performed in triplicate. The percentage of scavenging activity against DPPH was calculated, based on control reading by following formula:

$\% = [A_0 - A_1/A_0] \times 100$

Where A_0 is the absorbance of control, A_1 is the absorbance of sample.

Superoxide anion radical scavenging activity (SOSA).

SOSA was determined in accordance to the procedure of Liu and colleagues[28]. Superoxide radicals were generated from phenazine methosulphate/ nicotinamide adenine dinucleotide (PMS/NADH), that reduce nitro blue tetrazolium (NBT) into purple-colored formazan. 100 µl of both NBT (50µM) and NADH (70µM) were mixed together, and 100 µl of various concentration (2 – 64 µg/ml) of Bifidins were individually added to mixture, the reaction was initiated by adding 100 µl of PMS (10 µM) per tube, tubes incubated at 25°C for 5 min. The absorbance of resulted solution was taken at 560 nm, decreased absorbance of the reaction mixture was indicated higher radical scavenging activity. 0.1% L-ascorbic acid used as measuring standard, control was set up by mixing of all used reagents with 100µl of PBS. The percentage of scavenging activity against superoxide anion was calculated using the same followed formula for scavenging of DPPH.

In Vitro evaluation the cytotoxic activity against tumor cell lines

Cell lines and growth conditions

Two Human tumor cell lines, ovarian carcinoma (Skov-3) and Human Breast cancer (MCF-7), were used in the assessment of potential antitumor activity of Bifidins. Normal cell line, Rat Embryo Fibroblast (REF), was used as a predictive model of normal cells. All cell lines were obtained from cell culture research laboratory, Iraqi Biotechnology Company, iRAQBiotech (Baghdad, Iraq). The cancer cell lines were maintained in (DMEM) Dulbecco's Minimum Essential Medium (Sigma-Aldrich, Germany), supplemented with 1% penicillin /streptomycin and 10% fetal calf serum (FCS), REF cell line was maintained in RPMI-1640 medium (Sigma-Aldrich, Germany), supplemented with 1% penicillin/streptomycin and 10% FCS, all cell cultures incubated in 95% humidified atmosphere with 5% CO_2 at 37°C.

Cells viability assay

The potential cytotoxicity of Bifidins against cell lines were evaluated by Methyl Thiazolyl Tetrazolium (MTT) assay. MTT solution (0.2mg/ml) was prepared in PBS. The solution was filtered through 0.2 µm syringe filter to remove any blue formazan product, and then stored in sterile dark screw-capped bottles at 4°C. The solution was used within no longer than two weeks of preparation.

Cytotoxicity assay was conducted on 96-well microtiter plates according to Al-Shammari and colleagues[29]. All cell lines cells were prepared at concentration 1×10^4 cells/ml, 200µl of cells suspension in growth medium were seeded per well. The plates were sealed with a self-adhesive film, lid placed on and preincubated in 95% humidified atmosphere with 5% CO_2 at 37°C. After 24h or confluent monolayer was achieved, when the cells were in exponential growth, the medium was removed and various serial 2-fold concentrations (2 - 64 µg/ml) of Bifidins were individually seeded to the wells, control cells treated with serum free media only, and blank wells was contained medium no cells, the plates were incubated in 95% humidified atmosphere, with 5% CO_2 at 37°C for 72h. Cell viability was measured, by replacing the medium, by 20 µl of MTT solution to each well, and reincubated for 1.5h under same conditions, thereafter the MTT solution was carefully aspirated, the crystals remaining in the wells were solubilized by the adding 50µL of dimethyl sulfoxide (DMSO), followed incubation for 15 minutes at 37°C with shaking. The absorbency was read in an ELISA microplate reader (Biochrom, UK) at 584 nm, (reference wavelength=630nm). The experiment was carried out in triplicates and percentage of cell viability was calculated by the following formula:

cell viability % =

[(AT-AB) / (AC-AB)] × 100

Where AT represent the absorbance of treated well(samples), AB represent the absorbance of the blank, and AC represent the absorbance of control.

A graph was plotted between the percentage cell viability and concentration. Dose-response curve of % cell viability versus $\log_{10}$ concentration were constructed and IC_{50} values were determined from plots by interpolation[30]

Statistical analysis

one – way analysis variance (ANOVA) and t – test was used for data analysis. All results are expressed as mean ±SD. $P<0.05$ were considered statistically significant.

RESULTS

Both of Bifidins B1 and B2 were recovered most of their antilisterial activity in the pellet at 80% saturation with ammonium sulfate, and the activity was maintained during methanolic extraction, activity was assessed with checking of antilisterial activity by agar – well diffusion assay (in terms of inhibition zone diameter), and protein concentration (µg/ml) reduction.

Physicochemical characterization of Bifidins

Both of Bifidin B1 and B2 remained stable and exhibited antilisterial activity in wide pH range from acidic to neutral (4 – 7). Bifidin B1 was exhibited relatively more expanded pH stability image, since it remained active over the pH 4 – 7, while the Bifid in B2 was stable at pH 5 – 7, and both were lost most of their antilisterial activity at pH over 8 (fig.1). Both Bifidins were found to be heat stable, the Bifidin B1 considered to be more heat stable, as it retained 75.6% of its activity after heating at 100°C for 30 minutes compared with control, while at the same temperature Bifidin B2 retained only 56.7 % of its activity (fig-2). The proteinaceous nature of Bifidins was confirmed by testing their sensitivity with proteolytic enzymes. Bifidins B1 and B2 completely lost their antilisterial activity after treatment with Proteinase K, trypsin and pepsin, while Bifidins activity appeared unrelated to amylase, as their activity were not lost. Bifidins activity were relatively stable under cold storage conditions, the antilisterial activity become lower but effectiveness not completely lost by long term storage in cold, both Bifidins revealed almost half activity after storage for month at 4°C, while they maintained almost full stability in storage for a month at temperature -18°C. No antilisterial activity was detected in storage at room temperature 25°C (Table-1).

Anti-oxidant activity

The potential antioxidant activity of Bifidins was evaluated against two different free radicals, DPPH and superoxide anion, due to there is no universal method to evaluate antioxidant activity, thus it is necessary to use different methods to properly evaluate the antioxidant capacity[31]. Interestingly both Bifidins were exhibited potential antioxidant activity in a concentration-dependent manner against both used free radicals, with no significant (P.>.0.05) difference of scavenging activities between two evaluated Bifidins, and between two dependent assays. The highest percentage scavenging activity against DPPH was 70.55±0.267 %, recorded for Bifidin B2 at concentration 64(µg/ml), it was followed by 68.1 ±1.753% scavenging capacity for Bifidin B1 at same concentration, compared with ascorbic acid as a reference antioxidant (fig.3). It was clear from a view of Bifidins reducing capacity assay against superoxide anion, that both Bifidins B1 and B2 had almost close scavenging values, 66 ±1.970% and 65.94 ±1.343%, respectively at the highest used concentration (fig.4).

Antitumor activity assay

The potential antitumor activity, cytotoxicity and viability of living cells was evaluated with MTT assay, which based on the ability of mitochondrial succinate dehydrogenase of viable cell to reduce MTT into a purple colored formazan, thus color formation serves as a useful and convenient marker of only the viable cells[32]. Two human tumor cell lines Skov-3 and MCF7 were used as *in vitro* model, along with REF normal cell line (for safety checking). The experimental results demonstrate that the viability of Bifidins treated cancer cells was significantly decreased in concentration response manner, with non - worthy cytotoxic effects on REF cells (9.10% maximum viability inhibition). However, cell viability inhibition does not exceed 60.8%, in both used cancer cell lines (fig.5,6). Bifidin B1 exhibited significant ($P<0.05$) cytotoxic effects against both of MCF7 and Skov-3 cells, and Skov-3 was the most sensitive to B1 and B2 with IC_{50} values of 28.97±8.76 µg/ml and 29.87±9.13µg/ml, respectively (fig.7, 8), and table.2.

DISCUSSION

Probiotic bacteriocins are of interest in medicine because they are made by nonpathogenic microorganisms that normally colonize the human gut. The antimicrobial

properties of bacteriocins has been known since their discovery and has been studied and applied extensively. Nevertheless, there was insufficient studies on the antitumor properties of bacteriocins and its antitumor behavior are poorly understood. In this study we tried to shed some light on the antioxidant and antitumor activities of bacteriocins extracted from locally isolated enteric *Bifidobacterium*, as this bacterium and its secreted products recognized as GRAS.

Bacteriocins stability is an important concern when used as therapeutic agent, the Bifidins under study pronounced wide pH range stability, and showed heat stability that stay relatively active after exposing to 100°C for 30 minutes. The heat and pH stability are an advantage and maybe of value parameters, because many pharmaceutical processing involves harsh steps, such as heating and exposing to extreme pH values. The pH and thermal stability of bacterial bacteriocins has been attributed to the unusual amino acids residues in bacteriocins structures, which provide strength to tolerate extreme circumstance[33]. Many studied bacteriocins are known for their activity over a wide pH range[34], similar phenomenon of wide pH stability has also been demonstrated for the bacteriocins; plantaricin, bulgaricin, and lactobulgaricin, produced by human enteric *Lactobacillus*[35]. LAB associated bacteriocins are inherently tolerant to high thermal stress[36]. Heat stability of bifidobacterial bacteriocins have been previously confirmed[37]. The obtained Bifidins have probably a proteinaceous nature, and insensitivity to amylase indicating that carbohydrates moieties were not required for antibacterial activity[38]. This was in accordance with[39], they published, in that *Bifidobacterium* compete with surrounding bacteria by secreting antagonistic proteinaceous compounds with different molecular mass affected microbial cells, and the proteinaceous nature of LAB associated bacteriocins was earlier reported. The stability of current bacteriocins during prolonged storage makes them superior as pharmaceutical products, and indicating that the cold temperature may be the most appropriate preservation technique. The obtained Bifidins met the criteria for being considered a bacteriocin, they were sensitive to proteases, insensitive to high temperature for 30 minutes, more stable in acidic and neutral pH[40].

Reactive Oxygen species (ROS) are formed in our body due to exogenous and endogenous factors and are found to be responsible for many diseases when produced in excess, antioxidant compounds have an important role in sequestering ROS from body that can decrease the risk of multiple chronic degenerative diseases, for instance cancer, Alzheimer, cataract, and coronary heart disease[41]. Nowadays alternatives of natural antioxidant compounds from bio-resources are sought and developed to obtain compound which is specific, better activity, and safer[42]. In this respect the use of probiotic and its secreted peptides is a potentially promising tool to present antioxidant activity and reduce damaged caused by oxidation. The experimental data in this work revealed that Bifidins exhibited reducing ability against the used free radicals (DPPH and superoxide anion), and this serves as a significant indicator of its potential antioxidant capacity. Antioxidant interact with free radicals, transfer either electrons or hydrogen atom, thus neutralized its free radical character[43]. The reducing ability of both Bifidins were increased linearly with increasing of concentration (fig 3 and 4). Evidence has showed that probiotic bacteria present significant antioxidant abilities both *in vivo* and *in vitro*[44]. Many studies have recently concerned the antioxidant capacity of probiotics culture supernatant, intact cell, bacteriocins, and surfactants[45,46]. Studies have demonstrated probiotics and secreted bacteriocins in strain- dependent feature could exert antioxidant activity in different ways[47]. The precise mechanisms of oxidation-resistant ability of probiotics are not properly elucidated[48]. However, the modulation of redox status of the host by probiotics and their products take place via several proposal mechanisms, such as, chelating ability, probiotics secreted peptides chelates metal ion. For instance, *Lactobacillus casei* KCTC 3260 cell – free supernatant was found to possess a high antioxidant ability by chelating both of Fe^{+2} and Cu^{2+} [49], and the intracellular cell -free extract of *Lactobacillus halveticus* CD6 demonstrated chelating ability for Fe^{2+} [50]. The chelating capacity of probiotics may be due to the physiological chelates that exist in the cell – free extracts of probiotics[51]. Probiotics prevents lipids peroxidation, and free oxygen radical production[52,53], due to their ability to create the low redox potential needed for their optimal growth rate and peptides production[54]. The improvement of oxidative status, by increases the levels of antioxidant metabolites of the host, like enhancement the levels of plasma thiamine and folate, has been emphasized in rats model feed various species of *Lactobacillus* and *Bifidobacterium* intact cells and cell free supernatants[55]. The oxidation-resistant ability of human enteric *Bifidobacterium* was realized since decade[56]. The culture supernatant, and intracellular cell free extract of *Bifidobacterium animalis* 01 were found to effectively scavenge hydroxyl radicals and superoxide anion *in vitro* while enhancing the antioxidant activities of mice[57].

It is become clear that any compound with strong antioxidant property will also have potential antitumor activity because of the role of free radicals in the development of cancer[58]. Probiotics have been shown to influence all cell types and path ways implicated in metastasis. *In vitro* and *in vivo* data from experimental studies have been shown probiotics strains suppress viability of selected tumor cells, besides the anti-metastatic behavior of probiotics are different in distinct species[59]. The MTT assay demonstrated that the Bifidins B1 and B2 significantly inhibited the viability of MCF -7 and Skov-3, with low toxicity towered REF cells, suggesting a possible selectivity for tumor cells and low toxicity against non-tumor cells. Moreover, both Bifidins B1 and B2 were exhibited significant antitumor potential against Skov-3 cells (28.97±8.76µg/ml and 29.87±9.13µg/ml, respectively), and this fall within the accepting limits of promising for crud, according to the

American National Cancer Institute (NCI), the criteria of cytotoxic activity for crude extract is IC_{50} < 30 µg/ml[60]. The result of this study is in accordance with previous study was used to assessed the survival of three cancer cell lines, Burkitt lymphoma (LBR2), breast adenocarcinoma cells (MDAMB- 231), and Human pancreatic cancer (PANC-1) under influence of *Bifidobacterium breve* culture supernatant, that revealed reduction in cells viability by 60% for 48hr exposures, in concomitant, 25% increasing in cells membranes permeability for albumin was observed[61]. The cell-free supernatant of *Bifidobacterium adolescentis* BCRC 14606 showed the strongest inhibitory effect (64.5%) on MAD-MB-231 compared to *Lactobacillus paracasei* NTU 101 and *Lactobacillus. plantarum* NTU 102[62]. The *Lactobacillus plantarum* 5BL culture obtained supernatant elicits greater cytotoxicity against Hela and HT-29 cells than against MCF-7[63]. There is no complete picture of bacteriocins mechanisms of effects on cancerous cell lines, may not be attributable to a single mechanism but rather to a combination of events not yet fully understood[64]. However, Antitumor activities of bacteriocins have been demonstrated via membranolytic, apoptotic, and necrotic mechanisms[65,66]. In general, bacteriocins have fast acting mechanism, which form pores in the target membrane, even at extremely low concentration[20]. Depending on cationic nature and the hydrophobicity of bacteriocins peptides and hydrophobic interaction, is suggested to play a crucial role for membrane permeabilization and in antineoplastic activity[21]. Most of analyzed bacterial bacteriocins, at low concentration induces apoptosis of cancer cells, while at high concentration it caused necrotic death[67]. Morphological and biochemical modifications of transformed cell membranes may play a role in selective targeting of cancer cells by bacteriocins. Cancer cells generally incorporate phosphatidylserine (PS), in the outer leaflet of the plasma membrane, PS is usually found on the inner leaflet of the cytoplasmic membrane of normal mammalian cells. However, it can be transferred to the outer leaflet of the plasma membrane of cells undergoing apoptosis, which disrupts the asymmetry observed for normal mammalian cell membranes[68,69]. Cancer cell membranes display other properties that may facilitate killing by bacteriocins compared to normal cell membranes. Some transformed cells may incorporate lower levels of cholesterol in their membranes, enhancing fluidity. Cell membranes of human leukemia and lung cancers display increased fluidity due to a lower level of cholesterol in their membranes compared to membranes of normal leukocytes and pulmonary cells, this increase in membrane fluidity may potentiate lytic effects bacteriocins and other peptides[70,71]. In line with apoptotic and necrotic actions, microcin (bacteriocin produced by *Klebsiella pneumoniae* RYC492), cytotoxicity on three cancer cell lines was studied in details, it induced cells apoptosis and confines with; cell shrinkage, fragmentation of DNA, decline of potential of mitochondrial membrane and also release of calcium ions from intracellular stores[66]. Plantaricin A (a bacteriocin of *Lactobacillus plantarum* C11), It caused 75% loss in the cell viability of human T cell leukaemia (Jurkat), the cytotoxicity against Jurkat cells were observed as a fragmentation of cell nuclei and plasma membrane, and had also impact on increasing of intracellular concentration of caspase-3[72]. The presented anticancer activity of Niacin is related to induction of apoptosis, stopping of cell cycle and reduction of HNSCC cell proliferation, in part, through cation transport regulator homolog 1(CHAC1), a proapoptotic cation transport regulator and through a concomitant CHAC1-independent influx of extracellular calcium[73]. The anti-proliferative assay of cell free supernatants from three strains of human breast milk Lactobacilli, *L. casei* SR1, *L. casei* SR2, and *L. paracasei* on cervix cancer cell line (Hela cells) have been categorized as upregulation the expression of apoptotic genes BAX, BAD, caspase3, caspase8, and caspase9 and downregulation the expression of BCl-2 gene[74]. On the other hand[75] 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.

CONCLUSIONS

Based on the results obtained in this study, it can be concluded that the befidobacterial derived bacteriocins possess significant potential antioxidant and antitumor activities to warrant further extensive searching work in view of complete purification of Bifidicins, characterization of bioactive molecules, and their precise mechanism of action. And exploration for clinical application.

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