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Investigation of the Antiviral Bioactivity of *Lactobacillus bulgaricus* 761N Extracellular Extract against Hepatitis C Virus (HCV)

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ABSTRACT
This study was designated to investigate the antiviral activity of the extracellular extract of *Lactobacillus bulgaricus* 761N against Hepatitis C virus. The cytotoxicity of the extracellular extract of *L. bulgaricus* 761N assay was conducted on two cell lines involving the Human Liver Hepatocellular Carcinoma (HepG2) cell line and the human Peripheral Blood Mononuclear Cells (PBMC). The antiviral capacity of the extracellular extract of the candidate strain against Hepatitis C Virus (HCV) was tested using real time quantitative polymerase chain reaction (RTqPCR). The pre-treatment trial was found to be of a higher a ntiviral activity than the post-treatment trial. It was found that it possesses also a moderate antioxidant activity that was observed to be higher on HepG2 cell line than on PBMC. Purification by the fractionation process using size exclusion chromatography revealed the presence of two fractions (8 and 15) that were confirmed by SDS-PAGE and were of molecular weights 14.4 and 45 kDa, respectively. Fraction 8 was found to be of a higher antiviral bioactivity in case of pretreatment trial than fraction 15. In case of post-treatment trial, fraction 8 was also observed to be of a higher antiviral capacity than fraction 15.

Key words: Antioxidant, bacteriocin, hepatitis C virus, lactic acid bacteria, RTqPCR

INTRODUCTION

The word 'probiotic' comes from the Greek language 'pro bios' which means 'for life' opposed to 'antibiotics' which means 'against life'. The history of probiotics began with the history of man by consuming fermented foods, it is well known that the Greeks and Romans consumed much of the fermented foods (Gismondo *et al*., 1999; Guarner *et al*., 2005). Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (*Lactobacillus* spp.). Therefore, these bacteria affect the gut micro flora positively and decrease the microbial toxic activity (Gismondo *et al*., 1999; Cakir, 2003; Chuayana *et al*., 2003). Probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. Probiotics were found to have antimicrobial capacity owing to the production of a wide variety of bacteriocins. During the last few years, a large number of new bacteriocins produced by Lactic Acid Bacteria (LAB) have been identified and characterized. LAB-bacteriocins involve a heterogeneous group of physicochemically diverse ribosomally-synthesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum against Gram-positive bacteria (Cintas *et al*., 2001). However, antiviral activity of lactic acid bacteria is less mentioned in a few literatures against a limited number of viruses as influenza virus (Serkedjieva *et al*., 2000) and herpes simplex virus (Wachsman *et al*., 1999). This circumstance

prompted us to engage in search for unprecedented Hepatitis C virus invasion inhibitors from LAB bacteriocins.

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma (Alter, 2007; Asselah *et al*., 2010). The number of people worldwide infected by HCV is estimated to be over 170 million (WHO., 2009). HCV remains endemic in many countries; Egypt reported the highest prevalence of HCV worldwide, ranging from 6% to more than 40% among different regions and demographic groups (Lehman and Wilson, 2009). There is no vaccine effective for HCV at present (Jawaid and Khuwaja, 2008). Currently, the standard therapy for HCV is pegylated interferon (PEG-INF) with Ribavirin. This therapy achieves 50% Sustained Virological Response (SVR) (Schvarcz *et al*., 1995; Reichard *et al*., 1998). As pegylated interferon is expensive, standard interferon is still the main therapy for HCV treatment in under developed countries. On the other hand, studies have shown that pegylated IFN and RBV therapy have severe side effects like hematological complications. New therapeutic approaches are under study like interferon related systems, modified forms of Ribavirin, internal ribosome entry site (HCV IRES) inhibitors, NS3 and NS5a inhibitors, novel immunomodulators and specifically targeted anti-viral therapy for hepatitis C compounds (Mumir *et al*., 2010).

Bacteriocins may be a relevant solution for treatment of HCV upon the fact of their multiple modes of action; however, this point wasn't presented to research over the last decades. Bacteriocins were observed to carry out various antiviral mechanisms as decreasing the expression process of viral essential genes as those needed for viral coat protein synthesis (Wachsman *et al*., 1999), blocking the host cell receptor required for viral attachment acting like nucleoside analog (Berkhout *et al*., 1997), reduction of expression of receptors needed for viral infection and reduction of infectious virus yield (Serkedjieva *et al*., 2000). So, the present study aimed to investigate the antiviral activity of the extracellular extract of *Lactobacillus bulgaricus* 761N against Hepatitis C virus.

MATERIALS AND METHODS

Cytotoxicity assay: For the determination of the concentration of treatments that does not exert a toxic effect on HepG2, the cytotoxicity assay was performed. A cell suspension of 6×10^4 cell mL^{-1} was collected and seeded in 96-well plates (100 µL cell suspension per well). The plates were incubated at 37°C in humidified 5% CO_2 for 24 h. After obtaining a semi confluent cell layer, the exhausted old media were discarded and100 µL of different treatment concentrations (previously prepared in RPMI media) or RPMI 1640 medium (as a negative control) were added. The cell plates were incubated at the same growth conditions for 3 days. After 3 days, the culture medium was discarded, 100 µL of neutral red stain (100 µg mL^{-1}) was added to each well and incubated for 3 h at 37°C in humidified 5% CO_2 (Borenfreund and Puerner, 1985).

Antiviral assay: The antiviral assay was performed according to the method of El-Fakharany *et al*. (2008) with some modifications:

- **Pre-treatment assay:** HepG2 cells were grown in 6 well plates for 24 h. Before virus inoculation, non-cytotoxic concentration of the treatment was added to the cells and incubated for 90 min. Then the treatment was removed and the cells were washed twice with PBS. Hepatitis C virus (HCV) was inoculated onto the cells for 90 min. The virus was removed and the cells were washed twice with PBS then Dulbecco's Modified Eagl's Medium (DMEM) was added supplemented with 10% Fetal Calf Serum (FCS) to the cells. The cells were then incubated for 96 h at 37°C and 5% CO_2
- **Post treatment assay:** HepG2 cells were grown in 6 well plates for 24 h. Cells were inoculated with HCV onto near confluent HepG2 cell monolayers for 1 h then the cells were washed twice with PBS. Treatment was added to the cells that are then incubated for 96 h at 37°C and 5% CO_2. The reduction in viral load due to the treatment was determined from results of RTqPCR

RNA extraction from HepG2 cells: Total RNA was extracted using QIA amp RNA mini kit (Qiagen, USA).

Reverse transcription-nested PCR of genomic and anti-genomic RNA strands of HCV: Reverse transcription-nested PCR was carried out according to Lohr *et al*. (1995) with few modifications. Primer sequences used were as follows: 1CH: 5'-ggtgcacggtctacgagacctc-3', 2CH: 5'-aactactgtcttcacgcagaa-3', P2: 5'-tgctcatggtgcacggtcta-3', D2: 5'-actcggctagcagtctcgcg-3' and F2: 5'-gtgcagcctccaggaccc-3'. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency, specific control assays and rigorous standardization of the reaction were employed: (1) cDNA synthesis without RNA templates to exclude product contamination, (2) cDNA synthesis without RTase to exclude *Taq* polymerase RTase activity and (3) cDNA synthesis and PCR step done with only the reverse or forward primer to confirm no contamination from mixed primers. These controls were consistently negative.

Strand-specific RT-qPCR: The real time quantitative (RTq) PCR was done to the final PCR product based on the SYBR Green I dye and Light Cycler fluorimeter using a standard HCV infected serum samples.

Antioxidant activity of crude treatment: The total intracellular ROS generated during HCV infection was detected in HepG2 and PBCs, respectively using 2, 7`-dichlorofluorescein diacetate (DFCH-DA) method. All cell samples were analyzed using a BD FACS Calibur flow cytometer with Cell Quest software. Cells were excited at 485 nm and DCF fluorescence was read on FL1 (530 nm) in log scale with FL1 gain set to 443. The inhibition percentage of ROS production was calculated by the equation:

$$(F0\text{-}Ft/F0)\times 100$$

where, F0 fluorescence of the control sample and Ft fluorescence of the treated sample.

Purification of the crude treatment: Skim milk medium containing the extracellular fraction (crude enzyme that produce oligobioactive peptides) was subjected to ultrafiltration (150 kDa, VRR 5-20). The retentate was discarded while the permeate was subjected to ultrafiltration (10 kDa, VRR 5). The hydrolysate contains the bioactive peptides according to Konrad and Kleinschmidt (2008), hydrolysate was subjected to several processing steps. First crude enzyme was inactivated by heating at 80°C for 20 min. Second separation of hydrolysate by centrifugation at 10,000 rpm for 30 min. Third desalting (Demineralization) was achieved by using a negatively charged Sephadex gel G50. Forth fractionation of peptide fraction was done by using size exclusion chromatography using Sephadex G200. The purification process was followed by bioactivity testing and confirmed by performing SDS-PAGE.

Statistical analysis: Statistical analysis of data is represented by the mean of triplicate groups±standard deviation. One way analysis of variance (ANOVA) and two way analysis of variance were used according to Bishop (1983). Probability test (p) was carried out to show the significance degree, $p \leq 0.0001$ highly significant, $p \leq 0.005$ significant and $p > 0.005$ non significant. LSD (at $\alpha = 0.05$) is the least significant differences.

RESULTS

Cytotoxicity assay: The extracellular extract of *Lactobacillus bulgaricus* 761N was observed to possess a low cytotoxicity on HepG2 cell line. The 16% concentration of the extracellular extract of *Lactobacillus bulgaricus* 761N supplemented with the cell culture media would be the selected concentration for both antioxidant and antiviral activity assessment.

Antiviral assay: By the use of reverse-phase contrast microscopy, the morphology of cells were investigated and illustrated in Fig. 1. The negative control (Fig. 1a) showed a confluent monolayer of HepG2 cells. In pre-treatment trial where the treatment is added to cells prior to exposure to viral infection (Fig. 1b), vaculation of some cells was detected, besides the appearance of multinucleated cells. Both features indicated that cells suffered from stress caused by the viable viral load. These features increased obviously in case of post treatment trial where the cells are subjected to viral

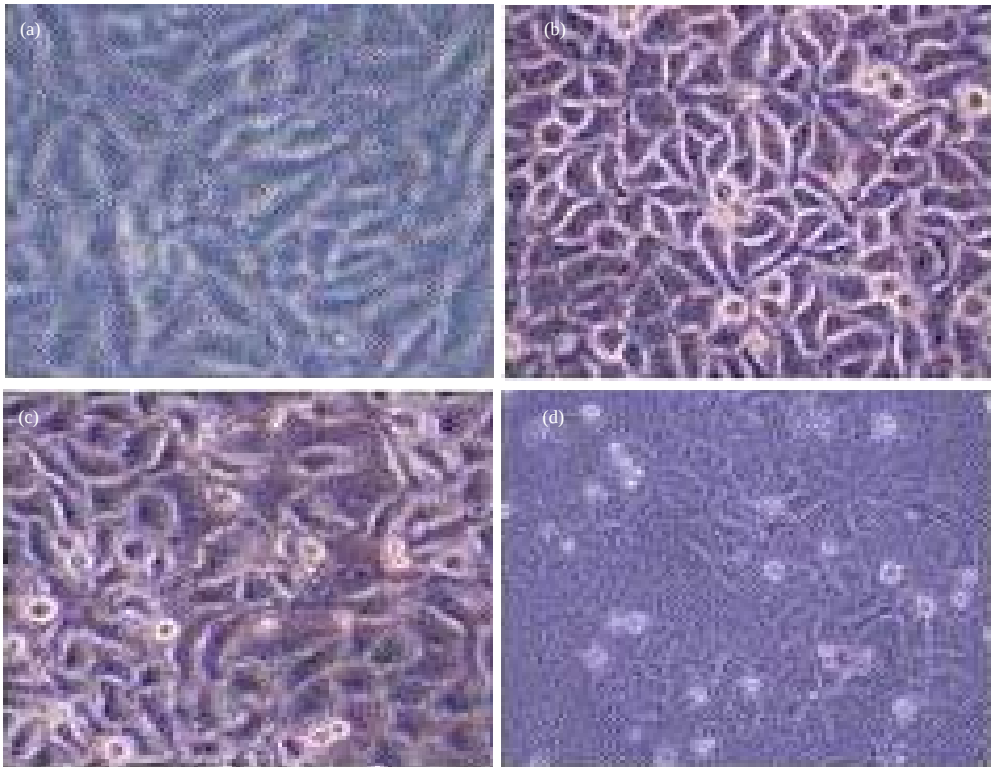

Fig. 1(a-d): HepG2 cell morphology, (a) Negative control, (b) Pretreatment trial, (c) Post treatment status and (d) Positive control

infection prior to addition of the treatment (Fig. 1c). However, in case of the positive control (Fig. 1d) the cells appeared degenerated as indicated by cell membrane lysis.

The viral load of HCV was assessed as a result of HCV amplification by RT-PCR, nested-PCR followed by real time PCR which provides the viral load in the form of copy per milliliter. Assessing the antiviral activity of *L. bulgaricus* 761N extracellular extract was performed by estimation of the percent reduction in the viral load as shown in Table 1. Both treatment trials have a highly significant different influence on the initial viral load ($p<0.0001$). Pretreatment trial has achieved more reduction of the initial viral load than that achieved in the case of post-treatment trial.

Agarose gel electrophoresis: The molecular weight of the amplified sequence of HCV is at 174 bp. In case of positive control (Fig. 2, lane 2) the band was clearly obvious, began to be thinner after undergoing post-treatment trial (Fig. 2, lane 3) and was so faint after undergoing the pretreatment trial (Fig. 2, lane 4) which refer to reduction of viral load meaning that the initial viral load represented by the positive control was reduced after achieving the post-treatment trial and more better reduction in case of pretreatment trial.

Table 1: Antiviral activity of *L. bulgaricus* extracellular extract on HCV

Item	Viral load (copy/mL)	Reduction (%)
Positive control	9531311	0.00
Post-treatment	2773936±277.39	70.9±0.0001[b]
Pre-treatment	1078462±431.38	88.69±0.0004[a]
F value	706.81	
LSD	0.0182	

a, b: Symbols to express significant difference in the statistical analysis

Antioxidant activity of crude treatment: The Reactive Oxygen Species (ROS) produced in HCV-infected HepG2 (Fig. 3) was higher indicating a higher level of stress,

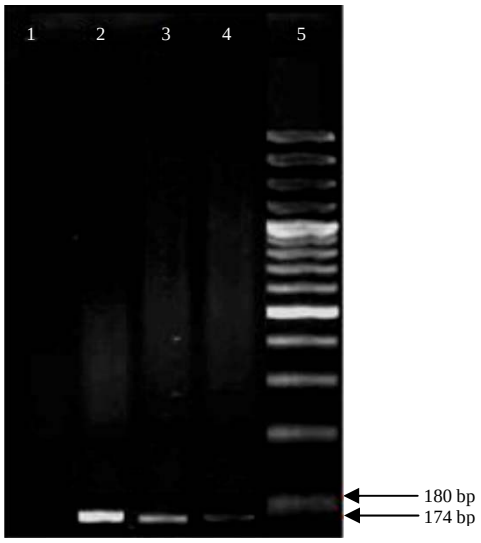

Fig. 2: RtqPCR product, Lane 1: Negative control, Lane 2: Positive control, Lane 3: Posttreatment trial, Lane 4: Pretreatment trial, Lane 5: DNA marker

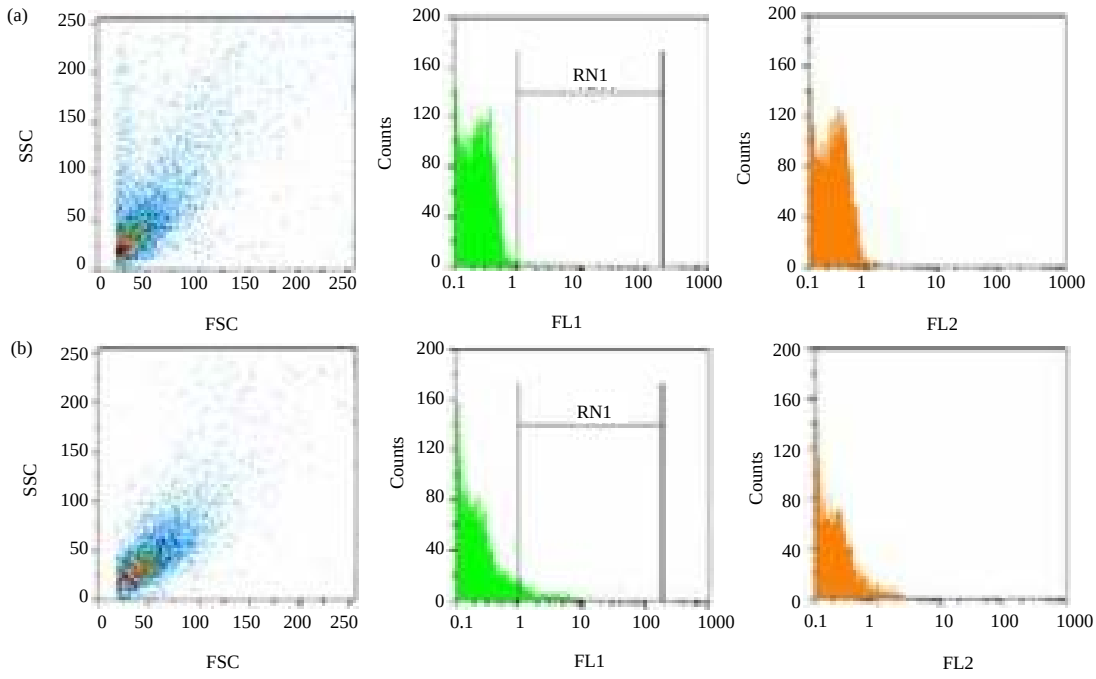

Fig. 3(a-c): Continue

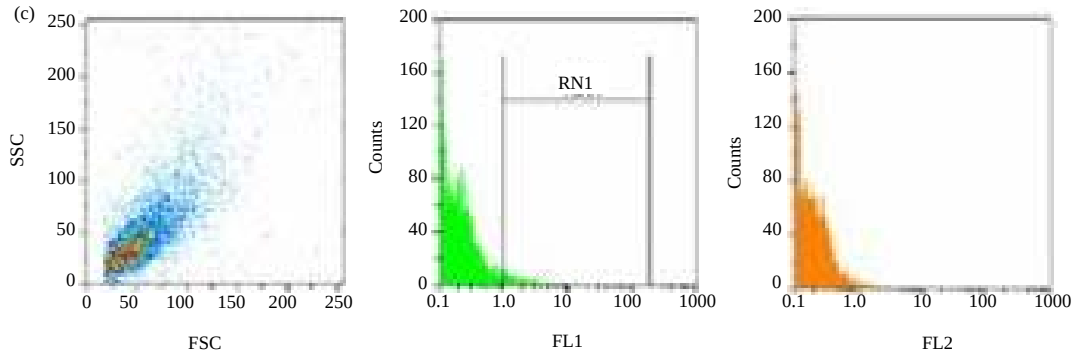

Fig. 3(a-c): Antioxidant activity of crude treatment on HepG2 cells infected with HCV using flow cytometer, (a) Negative control, (b) Infected cells and (c) Traeted cells

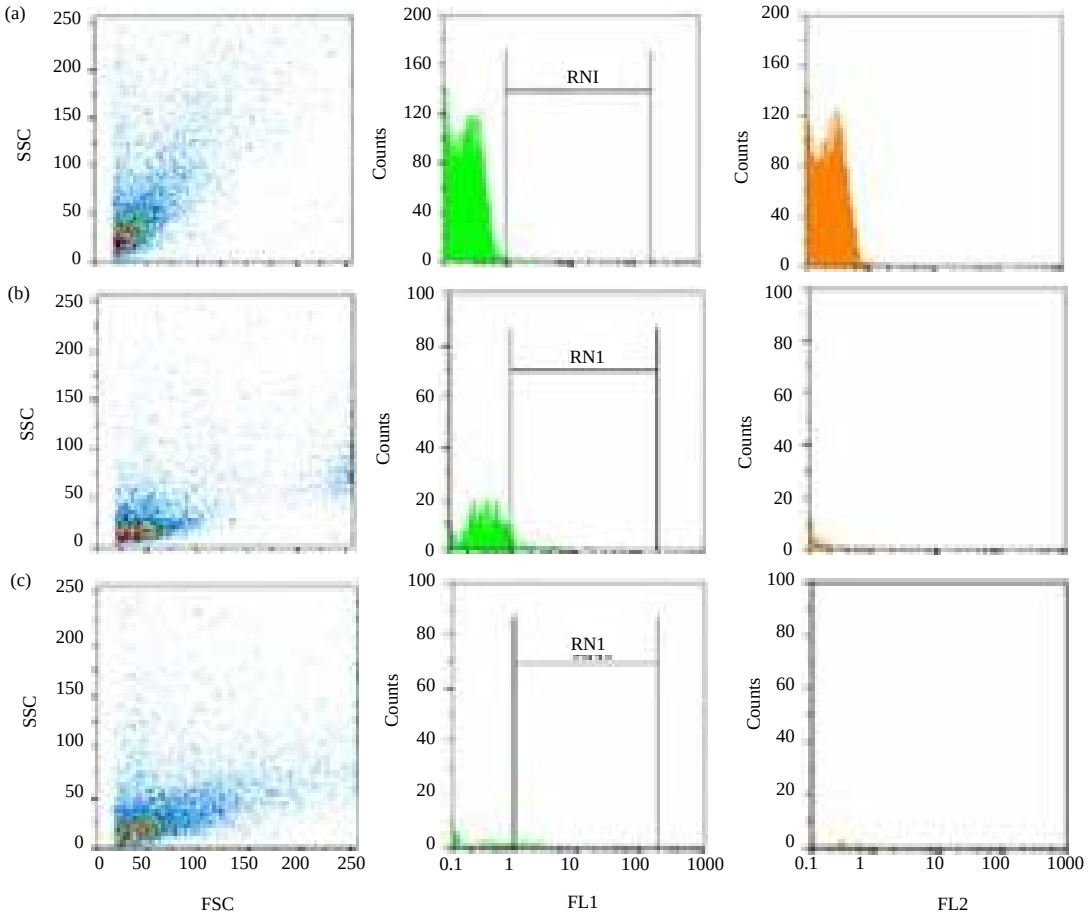

Fig. 4(a-c): Antioxidant activity of crude treatment on PBMC infected with HCV using flow cytometer, (a) Negative control, (b) Infected cells and (c) Traeted cells

wereas the uninfected HepG2 cells (Fig. 3a) had the lowest percent of fluorescent gated due to absence of stress factor (i.e., no exposure to viral infection). In comparison, the *L. bulgaricus* 761N-treated HepG2 cells (Fig. 3c) showed an intermediate level of fluorescence of 1.62 and hence an intermediate percent of ROS reduction. This is an indication of the effectiveness of *L. bulgaricus* 761N extracellular extract on the elevation of stress induced by HCV infection (Fig. 4). However, the ROS production in HCV-infected PBMC (Fig. 4b) was higher indicating a higher level of stress, unlike

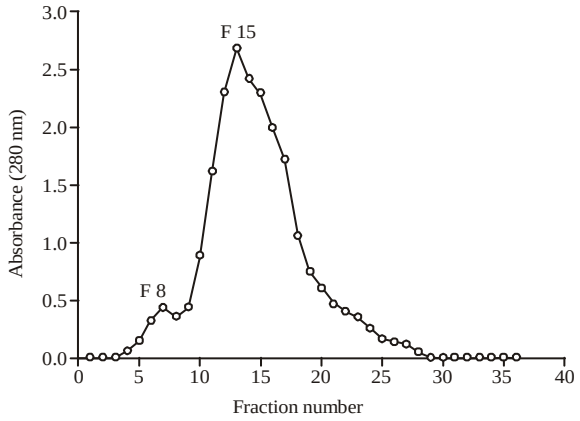

Fig. 5: Bacteriocin fractionation by size exclusion chromatography

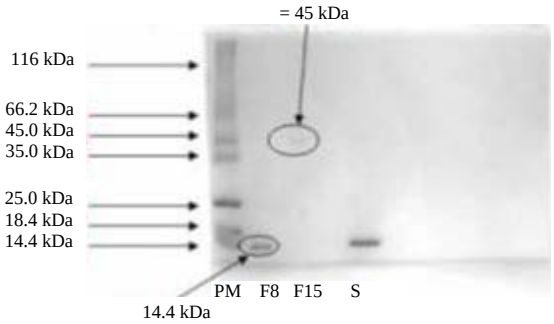

Fig. 6: SDS PAGE of the semi-purified fractions, PM: Protein marker, F8: Fraction No. 8 and F15: Fraction No. 15 resulting from bacteriocns fractionation by size exclusionm column chromatography, St: Standard (alpha-lactalbumin (Sigma aldrich) 14.4 kDa)

the uninfected PBMC cells (Fig. 4a) that were not subjected to viral stress. It was observed that *L. bulgaricus* 761N-treated PBMC cells (Fig. 4c) showed an intermediate level of fluorescence of 0.42, i.e., an intermediate percent of ROS reduction. It was also observed that the free radicals scavenging capacity of *L. bulgaricus* 761N extracellular extract was higher in case of HepG2 cell line with a 57.78% reduction of free radicals than in case of PBMC with a 54.35% reduction of free radicals.

Purification of the crude extract: Two fractions were obtained following the fractionation of the semi-purified extracellular extract of *L. bulgaricus* 761N using size exclusion chromatography which are fraction No. 8 (F8) and fraction No. 15 (F15) as shown in Fig. 5. The two fractions F8 and F15 obtained from fractionation process (Fig. 5) were subjected to SDS-PAGE and it was found that they are of molecular weight 14.4 and 45 kDa, respectively as shown in Fig. 6.

Table 2: Antiviral activity of the semi-purified fractions represented by % reduction of the initial viral load

Item	Viral load (copy mL^{-1})	Reduction of the viral load (%)
Positive control	54932409	0.00
Pretreatment F8	4327±4.33	99.992±0.001[a]
Posttreatment F8	28856±28.86	99.947±0.001[b]
Pretreatment F15	75796±75.8	99.862±0.001[c]
Posttreatment F15	23938983±4787.8	56.42±0.0002[d]
F value	1.826×10^7	
LSD	0.0002	

Data is Mean±SD, [a,b,c,d]Significant difference in the statistical analysis

Antiviral activity of semi-purified fractions: Both treatment trials for each fraction have a highly significant different influence on the initial viral load ($p<0.0001$). Pretreatment trial using the semi-purified fraction No. 8 possessed the highest antiviral bioactivity as shown in Table 2, followed by post-treatment trial using the same fraction and pretreatment trial using the semi-purified fraction No. 15. However, the least antiviral capacity was observed when conducting the post-treatment trial using the latter fraction.

It was found that F value of treatment trials after purification process (1.826×10^7) is higher than that of crude extract (706.81) meaning that treatment using semi-purified fractions with the convenient trial has a more significant influence on the initial viral load than that using the crude extract.

DISCUSSION

Bacteriocins obtained from LAB, has been mentioned in many literatures to have a very low cytotoxicity that agree with the current research findings. El-Adawi *et al.* (2012), observed that extracellular extract of several LAB were safe on PBMC (ranging from 10-20%). On other word, it had no TC50 even in the maximum concentrations. Another study by El-Fakharany *et al.* (2008) tested the Low molecular weight bioactive proteins, termed as lactoferrin, for its cytotoxicity on both HepG2 and PBMC. It was found that viability of both cell lines were unaffected. Those findings agreed with the present study results on which the low molecular weight bioactive peptides contained in the extracellular extract exert a low cytotoxicity.

Antioxidant activity was observed among the subcellular fractions of LAB in many literatures. The current study focused on the antioxidant capacity of the extracellular fraction that has proven to possess free radical scavenging capacity. The current results agreed with the previous results obtained by Kullisaar *et al.* (2002) which showed the antioxidative potential of lactobacilli which expressed manganese superoxide dismutase capable of eliminating hydroxyl radicals. Kaushik *et al.* (2009) reported the potentiality of Lp9 isolate of *L. plantarum*has antioxidative activity in the extracellular matrix. El-Adawi *et al.* (2012) measured the antioxidative activity of the whole cell, intracellular and extracellular extract of LAB; those measurements were based on four models involving

hydroxyl radical, superoxide, hydrogen peroxide and 2, 2-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity. El-Adawi *et al.* (2012) has observed that LAB possessed the highest antioxidant activity in the extracellular extract for all tested LAB strains.

Little attention has been paid to the antiviral activity of bacteriocins in literatures which might be due to the obvious impact on pathogenic bacteria. This made them more specialized as antimicrobial preservatives. Anti-influenza virus activity of a Bacteriocin produced by *Lactobacillus delbrueckii* was conducted by Serkedjieva *et al.* (2000). The study on the anti-influenza virus effect was conducted with respect to the specificity and selectivity of viral inhibition. Delcroix and Riley (2010) have reported that traditional antiviral therapy has relied on small molecules such as protease inhibitors or nucleotide analogues to inhibit viral enzymes. In the last decades, proteins and nucleic acid molecules have shown very promising antiviral properties. Earlier trials in the late 1980s had observed that certain naturally occurring short peptide sequences have the ability to enter cells when added to culture media. These findings have been approved in the present proposed method for introduction of the treatment to the HCV infected cells and also, that the low-molecular-weight peptides are those responsible for the antiviral activity. These results might lead to a novel strategy to efficiently overcome the impermeable cell barrier mentioned by Delcroix and Riley (2010). These overall observations render an impact for a dual role for these low molecular weight bioactive peptides involving both a high cell penetrating capability that could suppress the viral replication and the more recent their ability to act as delivery systems for their ability to promote intracellular uptake of conjugated cargoes forming chimaeric cell-penetrating peptides (CPPs) (Gupta *et al.*, 2005).

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