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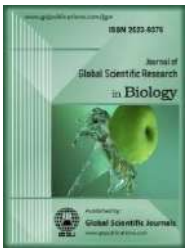

A Novel in Vitro Evidence on Anticancer Effect of Local Isolate Enterococcus Faecalis Bacteriocin

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ABSTRACT

Bacteriocin was purified from Enterococcus faecalis (En. faecalis) and its cytotoxic effect on the breast cancer cell line (MCF-7) and the liver cancer cell line (HepG2) was evaluated based on the comparison with the standard human cell line (WRL68). This was conducted using estimating the percentage of cell viability after in vitro treatment with bacteriocin for 24 hours, at 37° C and 5% CO2 using the MTT test. The results showed a toxic effect on the cell growth of MCF-7, and the inhibition rate was 60.9% at a concentration of 400 µg/ml, and 54.3% for HepG2 at the same attention. The study confirms the ability of bacteriocin to reduce the percentage of viability of the two cancerous lines (MCF-7, HepG2), with the difference in the inhibition rate according to the type of the cell line and the concentration. It was concluded that the bacteriocin of En. faecalis could be employed as a promising anticancer agent for this type of cells.

1. Introduction

Bacteriocins are known as biologically active proteinous toxins or antimicrobial peptides. They are antibiotic-like substances that are synthesized by ribosomally and are produce by some types of bacterial cells as extracellular substances. Bacteriocins have inhibitory or lethal effects against bacterial strains that are related closely or not closely with the Bacteriocin-producing bacteria and its lethal action is attributed to the association with sensitive cell recipients [1-3]. Several recent studies have been conducted with the aim of purifying and identifying bacteriocins and their applications in several aspects, the most important of which is the therapeutic targets [4-8].

En. faecalis has been described as a gram-positive coccus, catalase-negative, belonging to the Enterococcacea family. It's one of the Lactic acid bacteria (LAB) and the strains of this bacterium can produce substances that are characterized by antimicrobial activity, which is similar to the action of antibiotics, except that they are much more specialized and they are known as bacteriocins [9, 10]. In [11], they indicated that the bacteriocins produced by enterococci are in the form of heterogeneous structures with a difference in their mechanism of action.

Generally, lactic acid bacteria produce many types of bacteriocins with different sizes, properties, structures and inhibitory action spectrums that

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were recently classified into three classes (class I, II and III) depending on their peptides biosynthesis [12] while they were previously classified by [13] and [14] into four classes and subclasses (class I, IA, IB, class II, IIa, IIb, IIc, IId, class III, class IV).

En.faecalis is a member of oral and intestinal microbiota that could play a role in the aetiology of oral and colorectal cancers [15]. On the other hand, [16] referred that *En.faecalis* bacteriocin is characterized by anticancer effects against various cancer cells such as bladder, breast and colon cancers cells.

There are several studies produced bacteria and they investigated their effect as a potential anti-cancer agents. Our study aimed to evaluate the biotherapeutics effect of bacteriocin that was extracted from a locally isolated strain of *En.faecalis* through *in vitro* screening study of two types of cancer cell lines namely; liver cancer line (HepG2) and breast cancer line (MCF-7) compared to the normal cell line (WRL68) as control.

2. Materials and Methods

A local isolate of *En.faecalis* obtained from the Department of Biology/ College of Science/ University of Mosul was used in this study. This bacterium was stored in 20% glycerol at -72℃ and refreshed in brain heart infusion broth [17]. Subculturing steps were carried out to ensure its purity using the VITEK2 system [18].

Screening and Extracting of Bacteriocin:

Agar well diffusion method:

The method of [19, 20] was employed to investigate the production of bacteriocin from *En. faecalis* and the test was performed as follows:

1- *En. faecalis* was cultured in 1 ml of brain heart infusion (BHI) broth and with shacking incubated for 24 hours at 37 °C.
2- The absorbance was measured at the wavelength of 600 nm in a Spectrophotometer to obtain a reading of (0.6 - 0.5).
3- One ml of bacterial culture was precipitated using cooled centrifuge at 7000xg for 30 minutes at 4 °C.
4- An amount of 100 µL of the supernatant was taken and placed by a micro pipette in a previously prepared well in the center of BHI agar.
5- *Staphylococcus aureus* and *Escherichia coli* isolates were used as sensitive types to bacteriocin.
6- The results were recorded as transparent areas around the well emerged and these were photographed.

Extraction of Bacteriocin:

Several steps were performed to extract the bacteriocin from *E. faecalis* after making sure that *E. faecalis* was able to produce it. The steps involved:

A. Preparation of bacterial suspension: 25ml of sterile BHI medium was inoculated with 1 ml of *En.faecalis* and incubated at 37 C° for 18- 24 hrs., using a shaker incubator at 180 xg.

B. Preparation of cell free culture supernatant (CFCS): To prepare CFCS, the growing *En.faecalis* cells in the previously prepared suspension were precipitated using a cool centrifuge at 7000 xg for 30 min. and 4℃. The supernatant was filtered using a 0.22 Millipore microfiltration membrane [21, 22].

Purification of Bacteriocin

Three steps were used to purify bacteriocin of *En.faecalis*. The precipitation process with ammonium sulfate was the first step of purification. Ammonium sulfate was gradually added to the previously prepared CFCS with a degree of saturation (70%) and stirred for 20 minutes at 4℃. Then the solution was left for 42 hours at the same temperature (4℃) until completely precipitated. After that, it was centrifuged at 6000 xg for 30 min at 4℃. Tris HCl buffer 0.05 M at pH 7.0 was added to the precipitate. The second step is dialysis, which was performed to get rid of salts in the solutions, which are of small molecular weights by using dialysis bags (7 KDa Cut off), which allow the passage of small particles only [23].

This process was carried out by placing the precipitate obtained in the previous step in the dialysis bags, which are tightly attached to the bottom and tying them with a thread from the top

with a tight tie. These bags were dipped into a volumetric container that contains distilled water and the process was conducted at 4℃ with continuous stirring. The membrane screening process continued for 24 hours, taking into consideration the alternation of water every six hours to get rid of sulfate ions [24]. the supernatant in the dialysis bags is applied to the separation column by the third step gel filtration technique and sephadex G 100 was prepared as recommended by (Pharmacia fine chemical company). It was suspended in (0.05 M Tris-HCL buffer pH 7.0),the air was removed and filled in a glass column (1.5 × 20 cm) and then it was equilibrated with the same buffer solution. The proteolytic solution obtained from the previous step was applied and after that , the outlet portion of the gel filtration was withdrawn at a flow rate (20 ml/ hr., 3 ml/ fraction) using the same buffering solution for equilibration. The absorption of each fraction was measured at 280 nm [25, 26] and the purified active fractions were subjected to a subsequent experiments in the current study.

Determining the bacteriocin molecular weight

Gel filtration was used to determine the bacteriocin molecular weight of *En.faecalis* by what was mentioned in [27, 28] as follows:

Sephadex (G- 150) column (2 × 40 cm) was washed with (0.05M) phosphate buffer (pH 8.0). Using the blue dextran (2000 KDa), the void volume (vo) was determined in the column. Two ml of 2 mg/ ml of blue dextran solution was passed through Sephadex and eluted with the same buffer. The fractions (5 ml/ tube) were collected and the absorbance at 600 nm was read. The void volume was determined by calculating the volume of the fractions from the beginning of the peak and then the estimation of the elution volume (ve) was conducted after preparing the standard curve using standard proteins (Alcohol-dehydrogenase 150000 D, Albumin 60000 D, Carbonic anhydrase 29000 D and Lysozyme 14000 D) and the purified bacteriocin was passed singly through Sephadex and eluted with the same buffer.

The absorbance at 280 nm for each protein was read and the elution volume (Ve) was calculated. The relationship between (Ve/Vo) and the molecular weight for the standard proteins were plotted to obtain the standard curve. The bacteriocin molecular weight was estimated from the (Ve/Vo) value for bacteriocin on the standard curve.

MTT assay

The cytotoxic effect of the bacteriocin extracted from *E.faecalis* on two tumor cell lines [hepatocellular cancer (HepG2) and breast cancer (MCF-7)] and WRL68 normal cell line (All cell lines were supplied by the Center for Natural Product Research and Drug Discovery in Malaysia) were estimated using the MTT assay. In this assay, purple formazan was formed due to the yellow dye reduction by the mitochondrial enzyme. The purple colour of formazan formation is an indicator of mitochondria's normal functioning, which is relevant to the viability of the cell [29]. Moreover, the method of [30] was employed to grow cells lines as follows:

1- The cells of each cell line were dissolved separately using a water bath at (37) °C.
2- The cells of each line were placed in a 25 cm^3 animal cell culture container (falcon), comprising the culture medium Roswell Park Memorial Institute (RPMI-1640) and 10% cow-calf serum.
3- The culture vessels, including the cellular suspension and the medium, were incubated in 5% CO2 incubator at (37) °C for 24 hours.
4- After incubation, and after ensuring the growth of the contamination-free culture, a secondary culture was performed.
5- The cells were examined using an inverted microscope to ensure their viability, contamination-free and their growth to the required number (approximately 500-800 thousand cells / ml).
6- The cells were transferred to the growth chamber and the culture medium was discarded.
7- The cells were washed with (PBS) solution and disposed of and the process was repeated twice for (10) minutes each time.
8- A sufficient amount of the enzyme trypsin / EDTA was added to the cells and incubated for (30-60) seconds at (37) °C. They were observed until transformed from a monocyte layer to a single cell. Then the enzyme action was halted by adding a new development medium that contains the serum.

9- The cells were collected in centrifuge tubes and placed in a device with (2000 rpm) speed for 10 minutes at room temperature to precipitate the cells and to dispose of the trypsin and the medium used. The supernatant was removed and the cells were suspended in a fresh medium containing 10% serum.

10- The cell number was counted by taking a certain volume of the cell suspension plus the same volume of Trypan Blue, to determine the number of cells and their vitality percentage using the Haemocytometer chip, as in the equation below:

$C = N \times 10^4 \times F / ml$

where:

C = the number of cells in one ml of solution
N = the dilution factor

F = the number of cells on slide 10^4 = the slide dimensions

11- Finally, the cell suspension was distributed in new containers and incubated in a 5% CO_2 incubator at (37) C° for (24) hours. The cells were distributed in the vessels with the required concentration.

The cells with the concentrations (1×10^4 to 1×10^6 cell/ml) were cultured in a 96-well plate to a final volume of 200µl culture medium per well and each plate was incubated at 37℃ and 5% CO_2 for 24 hours. The medium was removed and 200 µl of the concentrations of bacteriocin (6.2, 12.5, 25, 50, 100, 200, 400µg/ml) were added (three wells per concentration). The plates were incubated at 37 C° and 5% CO_2 for 24 hours. Then 10 µl of MTT solution was added to each well and after that, the plates were incubated in the same conditions for 4 hours. Furthermore,100 µl of DMSO (Dimethyl sulfoxide) as the solubilizing solution was added to each well and incubated for 5 minutes. Absorbance was measured at a wavelength of 570 nm by an ELIZA reader [31]. Moreover, statistical analysis was performed on the optical density readings to determine the IC_{50} for each cell line.

3. Results and Discussion

The results of the investigating bacteriocin production from *En.faecalis* showed the emergence of inhibition zones around the well of bacteriocin-sensitive isolates (*S.aureus, E. coli*) as shown in figures 1 A and B.

The summary of the purification steps for the bacteriocin is demonstrated in table (1). There was a gradual decrease in the protein concentration with the subsequent increase in the specific activity.

After purifying the bacteriocin by gel filtration chromatography using Sephadex G-100, results showed that the highest bioactivity of the collected portion was found in the fractions 17,18 and 19 as demonstrated in figure 2.

This finding conforms with the observations of [32], who indicated that a single peak of protein was observed when the bacteriocin was purified by gel filtration. Moreover, from the results of determining the molecular weight of *En.faecalis* bacteriocin as shown in Figure 3, it is evident that the approximate value of the molecular weight of the purified bacteriocin is 27 KDa.

In the study of [32], it was shown that the molecular weight of bacteriocin was 34 KDa based on SDS- PAGE. The differences in the molecular weight could be attributed to the difference on bacterial strains and the methods used for determining the molecular weight. Several studies demonstrated that *En.faecalis* bacteriocin is included in type III bacteriocins, which were characterized by large and heat- labile antimicrobial proteins [8, 13, 32-34].

The MTT cytotoxicity assay was used to determine the toxic effect of the purified bacteriocin from *En. faecalis* on two types of cells, namely; breast cancer cells (MCF-7) and liver cancer cells (HepG2). This test depends on the transformation of the MTT [3-(4,5- Dimethyl thiazol-2-yl)2,5diphenyl tetrazolium bromide] to insoluble purple formazan by succinate dehydrogenase enzyme that is located in the mitochondria. This change is regarded as an indicator of the normal functions of the mitochondria and this reflects the cell viability [29].

When the cell lines (MCF-7, HepG2, WRL68) were exposed to concentrations ranging between (6.2 – 400 µg/ ml) of *En.faecalis* bacteriocin for 24 hours at 37 °C, the extent of the toxic effect was evaluated by estimating the percentage of the

growth inhibition compared to the control. Results showed that the purified bacteriocin demonstrates high efficiency against breast cancer cells (MCF-7), as it resulted in cytostatic at rates of (60.9%, 56.9%,33.6%,23.1%, 16.8%4.4%and 4%) at concentrations of (400µg/ml,200,100, 50,25, 12.5,6.2) respectively. On the other hand, there is no high cytotoxicity effect on the normal cell line (WRL68) and the inhibition ratio ranged between (4.2- 37.4%) at concentrations of (6.2- 400µg/ml) as in table 2.

In addition to that, the results showed a significant difference P≤0.0001 when calculating IC_{50} after treating the MCF-7(98.95 µg/ml) with bacteriocin compared with the normal cell line WRL68(268.0 µg/ml) as shown in figure 4.

Throughout studying the bacteriocin effect on the liver cancer cell line, results showed that it has less effectiveness compared with the previous line where the cells were inhibited by percentages of (54.3%, 49%, 38.6%,26.4%, 16.1 %, 3.1%, and 2.1%) at concentrations (400 µg/ml,200,100, 50,25 12.5,6.2) respectively. The normal cell line WRL68 gave inhibition rates ranging between (4.9 % - 27.9%) at concentrations (6.2 – 400 µg/ ml) as it is shown in table 3.

After treatment of bacteriocins with cancer cells HepG2 (54.13 µg/ml) and normal cells (123.9 µg/ml) a significant difference at P≤0.0001 was shown in figure 5 below:

From the former results, it is evident that the effectiveness of *En.faecalis* bacteriocins varied by the type of cancer cell line and the concentration of the bacteriocin used. So, it is noticed that it gave the highest inhibition rate for breast cancer cells MCF-7, which was 60.9% at the concentration of 400 µg/ ml, followed by the liver cancer cells, with an inhibition rate of 54.3% at the same concentration. From the results above, it is clear that there is a direct relationship between bacteriocin concentration and anti-cancer activity.

The results conform with the results of [15], which is the only study on this topic that tested the *E. faecalis* bacteriocin on four cancer cell lines using MTT assay and found that the rate of inhibition increased gradually with increasing the dose. The vitality of the cells (AGS, MCF-7, Hela, HT-99) decreased with increasing the concentration after incubation for 24 hours, but there is no evidence about the effect on normal cells (HUVEC). Accordingly, it was concluded that the bacteriocins could be used as pharmaceutical compounds with good therapeutic properties because they are not toxic to normal mammalian cells and further verification is necessary, which is required for physical, chemical, structural and functional properties of the secreted anticancer substances before they are used as anticancer medications. Therefore, from the current study, it was concluded that the bacteriocin from *En. faecalis* could be employed as a potential anticancer agent**.**

4. Acknowledgment

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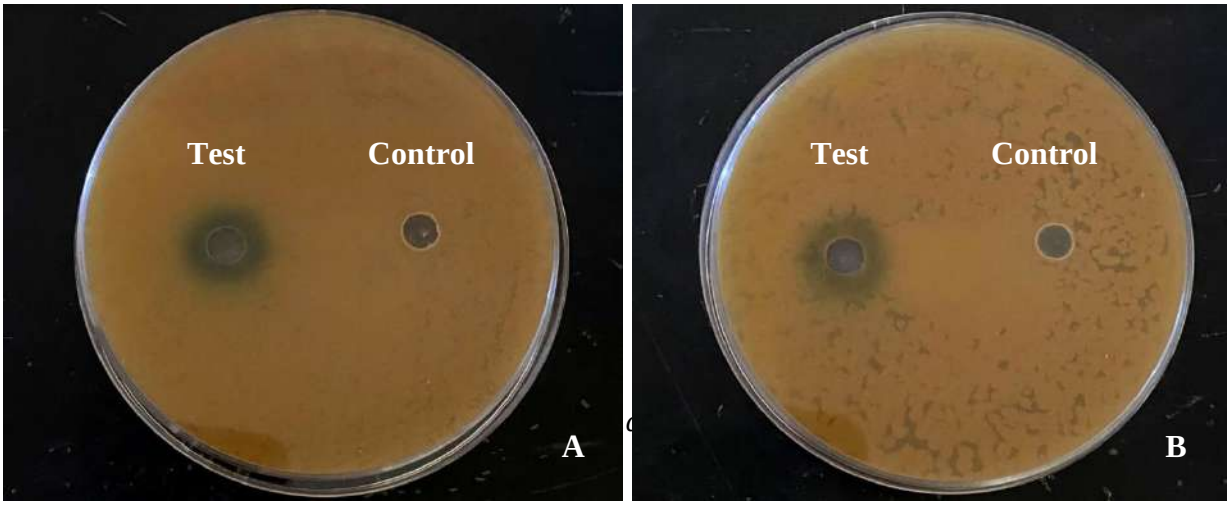


Figure 1: Screening of *En. faecalis* for bacteriocin production by agar well diffusion method

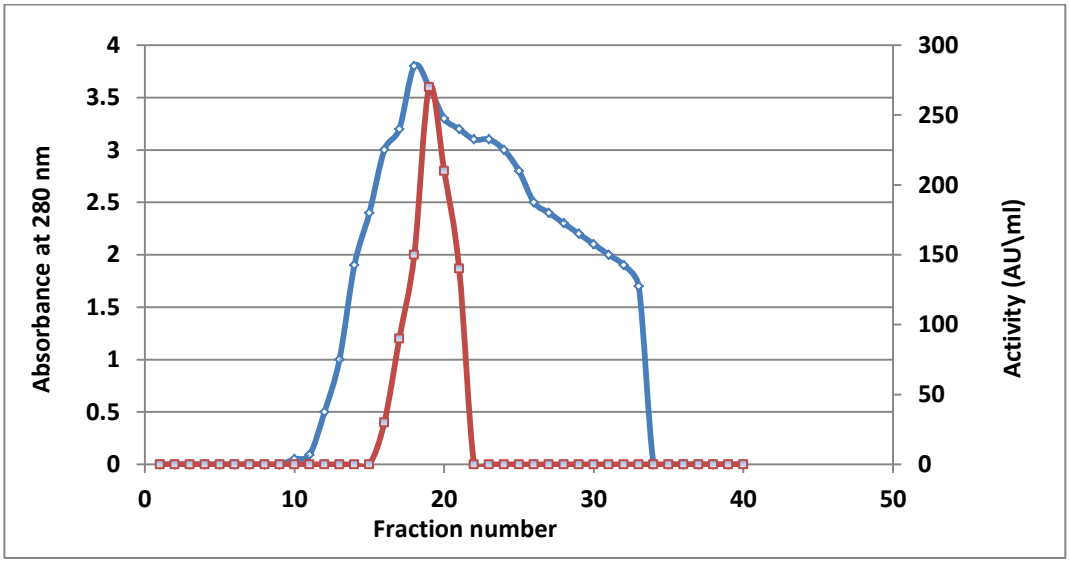


Figure 2: Purification of bacteriocin (*En.faecalis*) by gel filtration chromatography

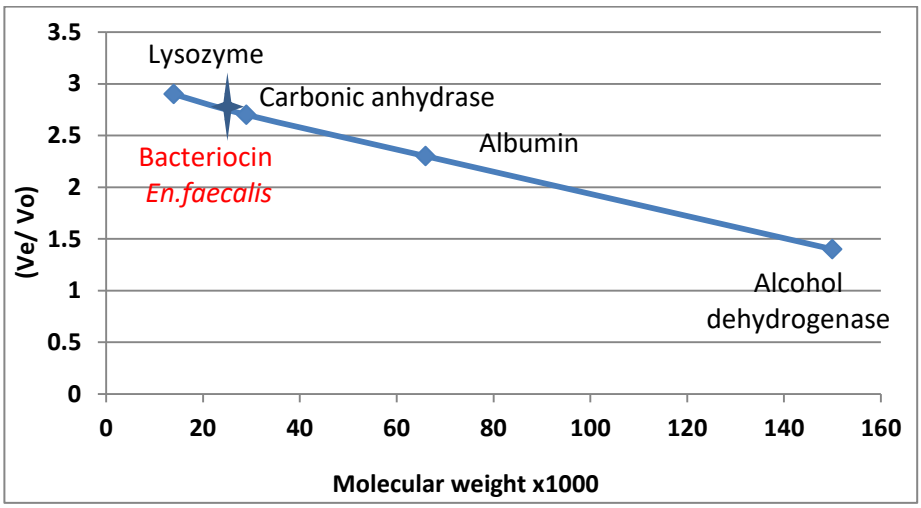


Figure 3: Molecular weight of bacteriocin produced by *Enterococcus faecalis*

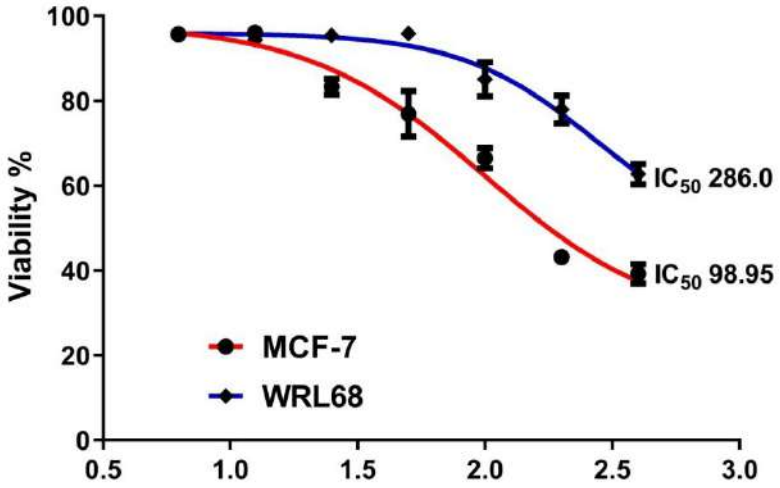
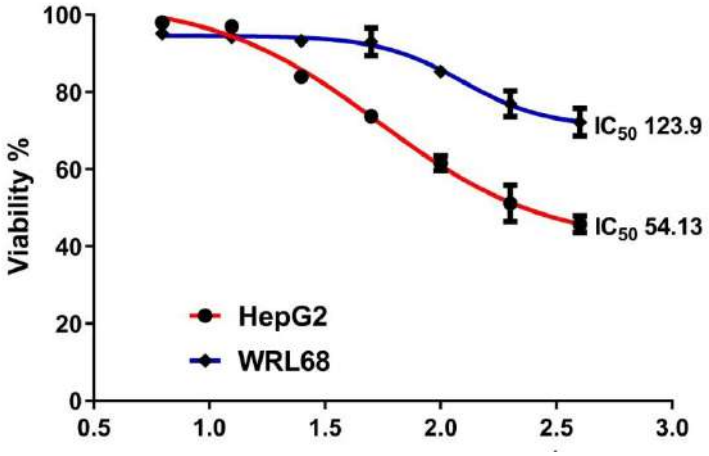


Figure 4: Effect of *En. faecalis* bacteriocin Cytotoxicity on MCF-7 compared to normal cells



Log Concentration g/ml^{-1}

Figure 5: Cytotoxicity of *En. faecalis* bacteriocin on HepG2 compared to the normal cells

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Table 1. Purification steps of bacteriocin produced by *En. faecalis*

Purification step	Volume (ml)	Activity (AU/ml)	Protein concentration (mg/ml)	Specific activity (AU/mg protein)	Total activity (AU)	Purification (folds)	Yield (%)
Crude extract (CFCS)	50	155	0.4	387.5	7750	1	100
Ammonium sulfate precipitate (70%)	30	210	0.3	700	6300	1.8	81.2
Gel filtration on chromatography Sephadex G-100	18	270	0.1	2700	4860	6.9	62.7

a. Activity units (AU/ml)= Reciprocal of the highest dilution × 1000 / volume of bacteriocin added.
b. Protein concentration = Bradford *et al*., 1979.
c. Specific activity (AU/mg): represents bacteriocin (*En. faecalis*) activity divided by protein concentration
d. Total activity (AU): represents activity (AU/ml) × Volume (ml).
e. Purification fold: represents specific activity of purified fraction divided by specific activity of crude extract.
f. Yield (%): represents (total activity of purified fraction divided by total activity of crude extract) ×100.

Table 2: The effect of bacteriocin extracted from *En. faecalis* on breast cancer cell line MCF-7 compared to the normal cell line WRL68 using the MTT test

Cell line	Viability% Mean ± SD						
	concentration µg/ml						
	400	200	100	50	25	12.5	6.2
MCF-7	39.1± 2.3	43.1± 0.6	66.4± 2.3	76.9± 5.3	83.2± 1.8	95.6± 0.8	96.0± 0.2
WRL68	62.6± 2.3	77.9± 3.3	85.6± 4.0	94.3± 0.3	95.3± 0.9	95.6± 1.8	95.8± 0.4

Table 3 : The effect of bacteriocin extracted from *En. faecalis* on HepG2 cell line compared to the normal cell line WRL68 when using MTT test

Cell line	Viability% Mean ± SD						
	concentration µg/ml						
	400	200	100	50	25	12.5	6.5
HepG2	±45.7 2.1	±51.0 4.7	± 61.4 1.9	±73.6 1.7	±83.9 1.71	±96.9 1.70	±97.9 0.7
WRL68	±72.1 3.5	±76.8 3.3	± 85.2 1.6	±92.9 3.3	±93.1 1.6	±94.1 1.1	±95.1 0.5