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ORIGINAL ARTICLE

Effect of Purified Bacteriocin from *Acinetobacter baumannii* on some Pathogenic and Environmental Isolates and Its Inhibitory Effect on Hemolysin Production from *S. aureus*

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KEYWORDS

Purified bacteriocin; *Acinetobacter baumannii*; Hemolysin production; *S. aureus*

ABSTRACT: From 122 isolates 63 isolates were diagnosed as Acinetobacter baumannii. The highest percentage of isolation was 37 (58.7%) from wounds and the lower percentage was 7 (11.1%) from burns. The antimicrobial susceptibility test was performed the highest resistance percentage was Cephalothin at 95.2 %, while the low percent was gentamycin was 47.6 %. The minimal inhibitory concentrations (MICs) for imipenem were tested the MIC values for imipenem resistant isolates ranged from 16 µg ml^{-1} to 64 µg ml^{-1}. Bacteriocins proteinaceous or peptidic toxins produced by bacteria offer promising potential as substitutes or conjugates to current therapeutic compounds. Three methods were used to detect bacteriocin production Acinetobacter baumannii, Agar well diffusion method, cup assay, and Disk method. From 63 *Acinetobacter baumannii* isolates 27 (42.85%) isolates were able to produce bacteriocin. Agar well diffusion method was the best for detection and the best isolate was (No. 48) for bacteriocin production for all methods. Bacteriocin purified by two steps method ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G150 column and characterized. The specific activity was 1541U mg protein with 6.3 bacteriocin folds and 64% recovery yield. The molecular weight of bacteriocin was (14000) Da. Bacteriocin activity was stable at pH values (3-9). Also, bacteriocin showed high thermostability at different temperatures (20-80)°C for (30) min. The antimicrobial activity of crude and purified *Acinetobacter baumannii* bacteriocin from (No. 48) isolate was maximum antibacterial activity against *E. coli* , *S. aureus* (purified 30 mm, crude 22 mm for both) and *K. pneumonia* isolates (purified 25 mm, crude 20 mm).

INTRODUCTION

The diagnostic properties of *Acinetobacter baumannii* are gram-negative, according to aeration properties considered strict aerobic coccobacillus alos this bacterium is non-motile, on the other hand, their occurrence in the environment frequently found in soil, water, sewage it belongs to the family Neisseriaceae [1]. Baumann scientist is the first one who isolated *Acinetobacter baumannii* in 1968, it is almost considered a new pathogen, and as many researchers suggested the first appearance of this pathogen was in Iraq, specifically in military treatment facilities during the Iraq War, and was called "Iraqibacter" [2, 3]. *Acinetobacter baumannii* have able to produce different types of virulence factors for protection, one of the most important is bacteriocins, the chemical nature of bacteriocins is considered peptidic toxins produced by bacteria, and offer promising potential as substitutes or conjugates to current therapeutic compounds, like this compounds related to the ability of *A. baumannii* to spread, as well as its capacity to survive on the most ecological surface. The

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resistance to wide ranges of antibiotics likes almost all available families of modern antibiotics; *A. baumannii* nosocomial strains have this high resistance [4]. *Acinetobacter baumannii* is also related to their ability to form biofilms, which are assemblages of surface microbial cells that are enclosed in an extracellular polymeric matrix [5]. In recent years *A. baumannii* commonly has found in Intensive Care Units and this is related to their rapid resistance to most antibiotics, this bacteria causes open-wounds-contamination, also catheters-contamination, as well as ventilation tubes, on the other hand, can cause fatal meningitis and *pneumonia* [6]. The aim of this study was to detect bacteriocin production from *Acinetobacter baumannii* and its antimicrobial activity against resistant indicator bacterial isolate, extract and purification of bacteriocin, and study under minimum inhibitory concentrations of imipenem the effectiveness of bacteriocin production and effect of purified bacteriocin on *S. aureus* hemolysin production.

MATERIALS AND METHODS

Specimens-collection

122 *Acinetobacter baumannii* isolates were collected from Ibn-Albalady hospital, Al-Shahid Dhari hospital for burning and teaching laboratories of Medical City hospital during period 1 September 2020 to 20 December 2020, from different clinical sources were identified by culturing on blood, MacConkey agar and confirm by biochemical test and VITEK 2 system.

Indicator Bacterial Isolates

*E. coli (*environmental isolate) and *S. aureus, K. pneumoniae* (pathogenic isolates) were obtained from Ibn-Albalady hospital, Al-Shahid Dhari hospital for burning and teaching laboratories of Medical City. All isolates were identified in the mentioned hospitals and then re-identified by the VITEK 2 system [7].

Antibiotic susceptibility test

Kirby-Bauer method was useed to carry out the antibiotics susceptibility test for 12 different antibiotics: cephalothin, gentamycin, rifampicin, amikacin, imipenem, trimethoprim, amoxicillin-clavulanic acid, ciprofloxacin, ceftriaxone, chloramphenicol, cefotaxime, and tetracycline. Inhibition zones were measured according to Clinical Laboratories Standards Institute CLSI (2017) [8].

Minimum Inhibitory Concentrations (MICs)

MIC for Imipenem was determined using the agar dilution method the results were interpreted after 18-24 hours of incubation at 37^{o}C according to the Clinical Laboratories Standards Institute CLSI (2017) [8].

Bacteriocin detection

To screen *Acinetobacter baumannii* isolates for their ability to produce bacteriocin. Three methods were used for detection, agar well diffusion assay was described by Lewus, 1991 Cup [9], disk method by Al-Qassab and Al-Khafagi, (1992) [10] and disk Assay method by Tagg and McGiven (1971) [11].

Bacteriocin activity assay

Bacteriocin Activity Assay According to Parente *et al.*, (1995) [12] and Pilasombut et al., (2005) [13].

Extraction and purification bacteriocin

Crude extracted bacteriocin according to Chhibber & Vadehra (1986) [14] and purified Gel filtration chromatography according to Sure et al., (2016) [15] in each step protein concentration was determined by the Lowrey method [16].

Characterization of bacteriocin

A- Determination of the molecular weight

The molecular weight of the Bacteriocin was estimated by gel filtration chromatography Sephadex G150 by Andrews, (1964).

B- Effect of pH

To determine the effect of pH on bacteriocin purified. The bacteriocin was adjusted to various pH values in the range of (3 to 9). The pH-adjusted bacteriocin. Samples were incubated at 37°C for 30 min and then neutralized to pH 7 and tested for bacteriocin activity by the agar

well diffusion method [17].

C- Effect of temperature

It was assayed by treating bacteriocin purified with (20, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 80) °C respectively. Bacteriocin activity was assayed after (30) minutes at each of these temperatures [18].

Antibacterial activity of crude and purified bacteriocin on pathogenic isolates

The agar well diffusion method was used to detect the antibacterial activity of crude and purified bacteriocin produced by *Acinetobacter baumannii* against pathogenic isolates [19].

Sub minimum inhibitory concentrations on imipenem

The isolate was grown in brain-heart infusion broth and was treated with sub-MIC of imipenem. After 16 hours, the inoculated medium was added to 50 ml of brain-heart infusion broth, and incubated mitomycin C was added at a concentration of 0.5 µg/ml and the flask was returned to the shaking incubator for another 8 hours, the culture was centrifuged with a refrigerated centrifuge at a temperature of 4°C at a speed of 10,000 rpm for a period of 15 minutes, the supernatant was taken and test for bacteriocin production capacity under the influence of antibiotics.

Antibacterial activity of bacteriocin on (S. aureus) hemolysin production

Co-Culture method

The co-Culture technique was used to determine of antibacterial effect of purified bacteriocin on *S. aureus* hemolysin production. The bacterial culture of *S. aureus* was grown in nutrient broth at a ratio of 1:1 (bacterial broth: purified bacteriocin from each serial diluting (100, 75,50, 25) µg ml^{-1}, the control medium contained nutrient broth only. Co-cultures and control were incubated at 37°C for 24 h. After the incubation streaked directly on blood agar which was used for bacterial hemolysin production [20].

RESULTS AND DISCUSSION

Distribution of *Acinetobacter baumannii*

From 122 isolates sixty-three isolates of *Acinetobacter baumannii* were diagnosed, distributed as 37(58.7) isolates from wounds 19 (30.2 %) isolates from urine, and 7(11.1 %) from burns, a significant difference was noticed at $P < 0.001$ ($\chi^2 = 21.7$) Table 1. Research in 2013 [21] found *A. baumannii* 27(23.48%) in wounds specimens, 8(6.95%) in urine specimens and a low percentage were in burns specimens which accomplished 6(5.22%). our study agreed with the study in 2006 [22] which found the maximum percentage of *A. baumannii* isolation (44.11%) was from wounds followed by urine (32.35%).

Table 1. The distribution of *Acinetobacter baumannii* according to type of specimen.

Clinical specimens	Number of Specimens Positive to *Acinetobacter baumannii*	Percentage
Wounds	37	58.7
Urine	19	30.2
Burns	7	11.1
Total	63	100
χ^2	21.7**	
	P < 0.001	

According to Gender and Age of *Acinetobacter baumannii* distribution as Table 2 shows that the incidence was higher (significant difference at P<0.05) between 40 (63.5%) males and 23 (36.5%) females, this incidence may be attributed to the fact that the immunity among males less than among females). The current results agreed with some studies [23, 24] that concluded a high number of bacterial isolates were found in males than in females.

Table 2. Distribution of *Acinetobacter baumannii* according to gender and age.

Age (Years)		Male	%	Female	%	Total	%	χ^2	df (n-1)	*P*-value
1	< 10	2	5	1	4.34	3	4.8	0.33	1	0.56 NS
2	11-20	5	12.5	3	13	8	12.7	0.5	1	0.48 NS
3	21-30	10	25	7	30.4	17	27	0.529	1	0.46 NS
4	31-40	8	20	5	21.7	13	20.6	0.692	1	0.4 NS
5	41-50	6	15	3	13	9	14.3	1	1	0.31 NS
6	51-60	4	10	2	8.6	8	9.5	0.66	1	0.41 NS
7	61-70	3	7.5	1	4.3	4	6.3	1	1	0.31 NS
8	71-80	2	5	1	4.3	3	4.8	0.33	1	0.56 NS
χ^2		11.6		11.435		22.46			----------	
df (n-1)		7		7		7				
***P*-value**		0.115 NS		0.121 NS		0.002**				
Total		40	63.5	23	36.5	63	100	4.587*	1	0.032*

* χ^2: Chi-square. * df: Degree of Freedom. * *P*: Probability.

Antibiotic susceptibility test

The antimicrobial susceptibility test was performed for all bacterial isolates to 12 different antibiotics as shown in Figure 1 the highest resistance percentage was for Cephalothin (95.2 %), while the low percentage was for gentamycin (47.6 %). The resistance level to rifampicin (88.9 %), amoxicillin-clavulanic acid (82.5 %), chloramphenicol (77.8 %), tetracycline (65.1 %) and to ciprofloxacin, amikacin, cefotaxime, trimethoprim, ceftriaxone and imipenem, was 60.3 %, 58.7 %, 57.1 %, 55.6 %, 54 % and 49.2 % respectively.

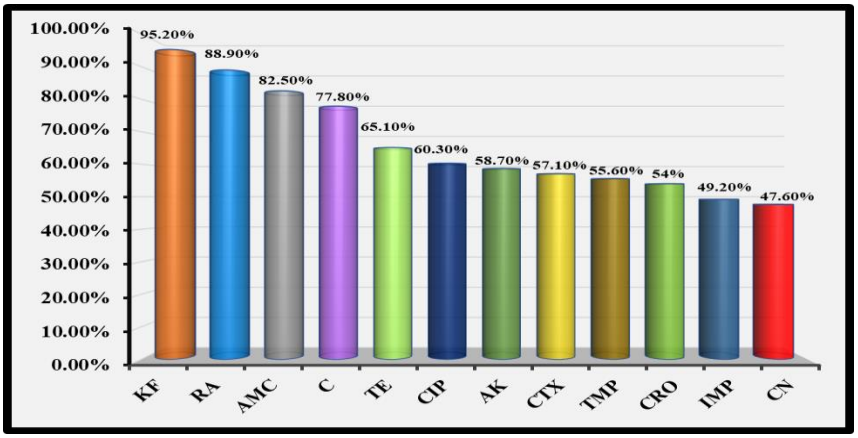

Figure 1. Antibiotic resistance of *Acinetobacter baumannii*.
Abbreviations: RA: Rifampicin, KF: Cephalothin, TE: Tetracycline, CTX: Cefotaxime, C: Chloramphenicol, CRO: Ceftriaxone, CIP: Ciprofloxacin, CN: Gentamycin, AMC: Amoxicillin-Clavulanic acid, TMP: Trimethoprim, IMP: Imipenem, AK: Amikacin.

Current results were agreed with a local study in 2015 [25] which concluded that low resistance appeared in imipenem at 53 %, and rifampicin at 100 % compared with the current result imipenem was 49.2, rifampicin at 88.9 %.

Minimum Inhibitory Concentrations (MICs)

MIC for Imipenem was determined using the standard agar dilution method. The breakpoint for imipenem ≤16g ml^{-1} according to CLSI (2017) [8], the MIC values for imipenem resistant isolates for 28 (44.4%) ranged from 16 µg ml^{-1} to 64 µg ml^{-1} agreed with Zhu et al., (2018) who found MIC was ranged from 16-64 µg ml^{-1}.

Detection of bacteriocin production

Three methods were used to detect isolates of

Acinetobacter baumannii bacteriocin production. 27 (42.85%) able to produce bacteriocin, The Wells method was the best method for production of bacteriocin 19 (3.15%), then Disks method 7 (11.11%) finally cup assay 4 (6.4%) and No.48 was the best isolate for bacteriocin production to all method this result agrees with the study in 2015 25 which found that three isolates from 15 have the ability to produce bacteriocin.

Acinetobacter baumannii-Bacteriocin effect:

The effect of Acinetobacter baumannii-bacteriocin on *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumonia*, was determined maximum antibacterial activity against *E. coli* (25) mm, *Klebsiella pneumonia* (20) mm and *Staphylococcus aureus* 20 mm Figure 2. The impact of bacteriocin in bacterial cells can be either fatal bactericidal [27, 28] or bacteriostatic [29], and similar mechanisms of action of bacteriocins on its impact on the target cell with the mechanisms of action of antibiotics it has been acting on cell wall or plasma membrane [30].

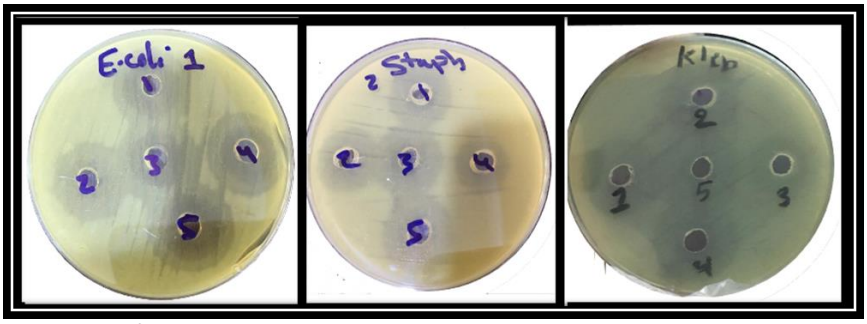

Figure 2. Antibacterial activity of *Acinetobacter baumannii*-Bacteriocin on *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia*.

The current result agreed with the study in 2015 [25] which concluded there was a positive effect on bacterial growth by Acinetobacter baumannii-bacteriocin, the bacterial species that tested were *E. coli* , *S. aureus*, *K. pneumonia*, addition to Pseudomonas aeruginosa and Proteus mirabilis.

Purification

Bacteriocin activity was purified up to 6.3 purification folds' chromatographic technique; the overall yield and activity are summarized in Table 3. The bacteriocin crude was subjected to ammonium sulfate precipitation with a 70% saturation ratio. This technique is useful to quickly remove large amounts of contaminant proteins that were commonly used in precipitation, it was highly solubility, very pure, low cost, and had no effect on protein Bhatt [31]. It was found that this ratio gave specific activity of 285Unit/mg proteins. This result indicated that there was an increase in the specific activity compared with that of the crude extract (242 Unit/mg proteins). The final stage is purification.

Table 3. Steps of purification of bacteriocin produced by *Acinetobacter baumannii*.

Purification step	Volume (ml)	Activity (U ml^{-1})	Protein concentration (mg ml^{-1})	Specific activity (U mg^{-1})	Total activity (U)	Purification (folds)	Yield (%)
Crude extract	75	80	0.33	242	6000	1	100
Ammonium sulphate precipitation 70%	45	114	0.4	285	5130	1.17	85.5
Dialysis	30	160	0.23	695.6	4800	2.8	80
Sephadex G150	21	185	0.12	1541	3885	6.3	64

Gel Filtration chromatography

In the gel Filtration Chromatography, bacteriocin, the maximum activity of bacteriocin was observed in the fractions (30-32), Figure 3 the enzyme activity (185 unit ml^{-1}) with specific activity (1541 unit mg^{-1}) with a yield of an enzyme (64 %).

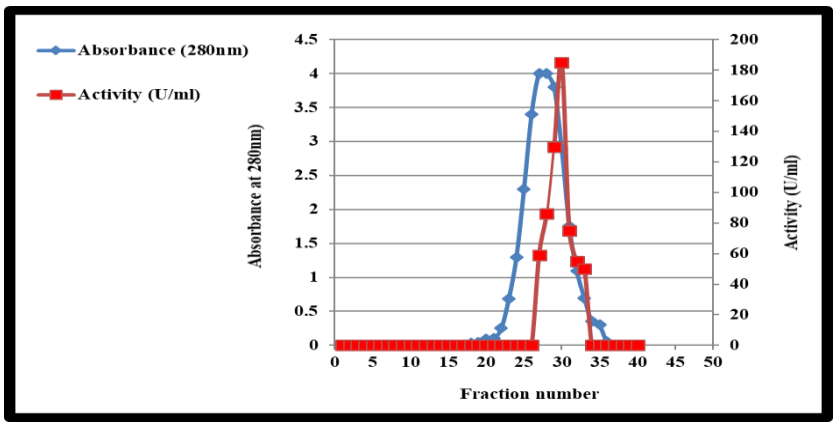

Figure 3. The Gel-filtration Chromatography of the bacteriocin was performed using Sephadex G-150 80×1.5cm column equilibrated with 0.05M phosphate buffer pH=7.5

Characterization of bacteriocin

A-Determination of Molecular Weight

When used gel filtration chromatography, the molecular weight of bacteriocin was (14) KDa by a comparison of the standard marker proteins [32]. A study by Abdulsada in 2021 concluded that m. weight of bacteriocin was 8.3 KDa, while Rubaye, (2019) [33] concluded that m. weight of bacteriocin was 13.500 dalton.

The molecular weight of bacteriocin produced by *Acinetobacter baumannii* is shown in Table 4 and Figure 4.

Table 4. Antibacterial activity of Bacteriocin from *A.Baumannii* on *E. coli, S. aureus and K. pneumonia* isolates *in-vitro*.

Tested Bacteria	Inhibition Zone (mm)		
	Crude Bacteriocin 32 µg ml^{-1}	Purified Bacteriocin 32 µg ml^{-1}	Control D.W
E. coli	22	30	0
S. aureus	22	30	0
K. pneumonia	20	25	0

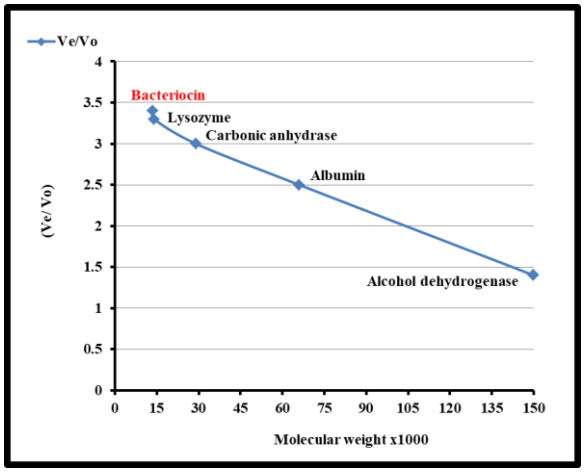

Figure 4. Molecular weight of bacteriocin produced by *Acinetobacter baumannii*.

B- pH Stability for bacteriocin

The activity of bacteriocin was stable at pH values (3 to 9) but its activity was decreased at (pH 2-10) and lost at (pH11-12), (Figure 5) [34]. These results disagreed with Abdulsada, 2021 [35] who concluded that bacteriocin (klebocin produced by *Klebsiella pneumonia*) was stable at pH 4-8, decreased at pH 3 and 9 and lost at pH = 2, 10, and 11.

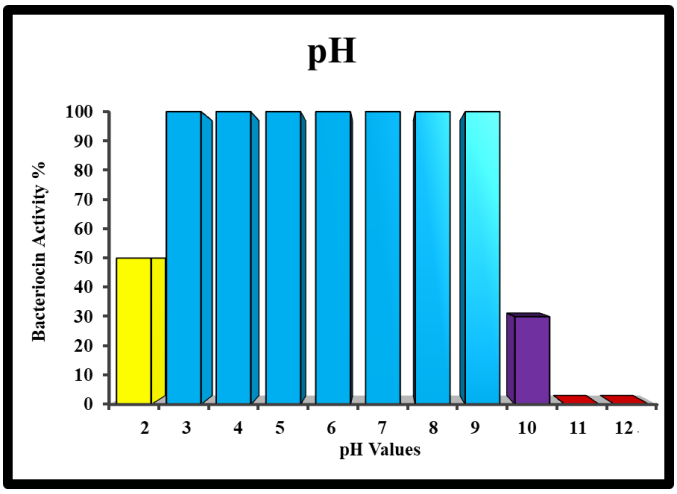

Figure 5. Stability of bacteriocin produced by *Acinetobacter baumannii* at different pH values.

C- Thermostability for bacteriocin

Bacteriocin showed high thermostability at different temperatures (20-80)°C for (30) min, activity was decreased after 30 min to 90 and lost at 100°C, and the bacteriocin released by Gram-negative bacteria has shown stability at temperatures as high as 80°C for 30 minutes (Figure 6) [36, 37]. The current results disagreed with the study in 2016 [38] which showed that bacteriocin activities at 100°C for 5 and 30 min and by autoclaving at 121°C for 15 min still remained at 100 % stability.

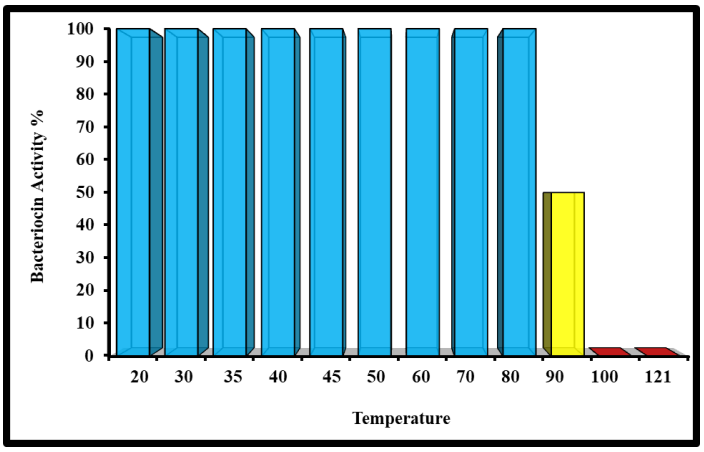

Figure 6. Residual activity of purified bacteriocin at different temperatures for 30 minutes.

Antibacterial activity of crude and purified bacteriocin

In this study crude and purified *Acinetobacter baumannii* from (No. 48) isolate bacteriocin recorded maximum antibacterial activity against *E. coli* , *S. aureus* (purified 30 mm, crude 22 mm for both), and *K. pneumonia* isolates (purified 25 mm, crude 20 mm), respectively (Table 4), with high significant antibacterial activity contrast with control, purified bacteriocin was significantly higher than crude bacteriocin Figure 7, these results agreed with Abdulsada, 2021 [35] who concluded that inhibition zone around different tested bacteria by purified bacteriocin higher than crude and partial purified bacteriocin with significant difference noticed between crude, partial purified and purified bacteriocin. Also agreed with the study in 2019 [33] which concluded that the mean inhibition zone of purified bacteriocin (Salivaricin from L. salivarius) was 27.6 higher than crude which was 15.33.

Sub minimum inhibitory concentrations of imipenem on bacteriocin production

The effect of sub-minimum inhibitory concentrations of imipenem on the effectiveness of bacteriocin was tested result shows the loss of their ability of bacteriocin production Figure 8.

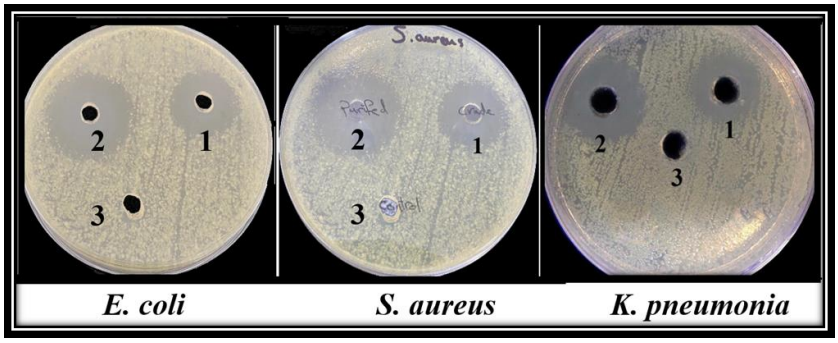

Figure 7. Antibacterial Activity of Crude and Purified bacteriocin against *E. coli*, *S. aureus* and *K. pneumonia* 1: Crude bacteriocin. 2: Purified bacteriocin. 3: Control.

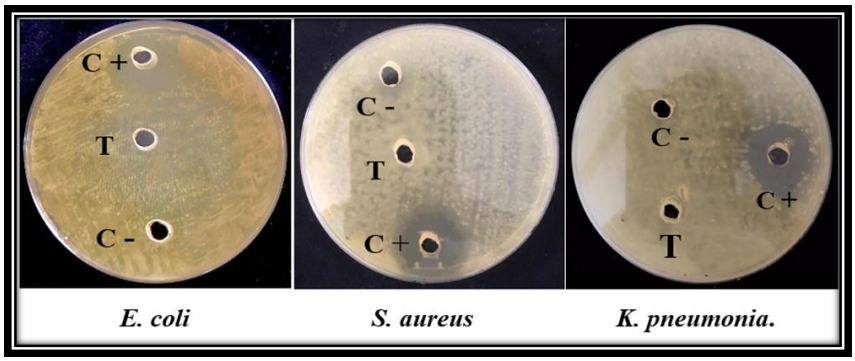

Figure 8. Effect of Sub minimum inhibitory concentrations (MIC) of imipenem on bacteriocin production. (T): bacterial growth with imipenem. (C+): Control bacterial growth without imipenem. (C-): broth only.

Inhibitory effect of purified Bacteriocin on S. aureus

hemolysin production

The result shows the inhibitory effect of purified Bacteriocin on hemolysin production in *S. aureus*, Figure 9 shows a loss of their ability to produce hemolysin at concentrations of 100, 75 µg ml^{-1} while no effect at 50 and 25 µg ml^{-1}.

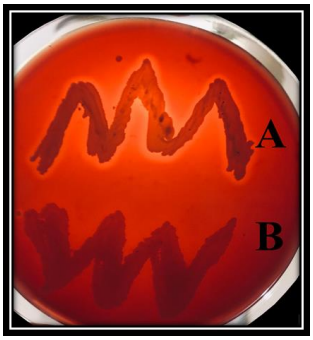

Figure 9. Inhibitory effect of purified bacteriocin against *S. aureus* by Co-Culture Method. A- Growth of *S. aureus* without bacteriocin (Hemolysin-Production). B- Growth of *S. aureus* with bacteriocin (Inhibition of Hemolysin-Production at 100µ gm^{-1}).

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Not applicable.

Conflict of interest

The authors declared no conflicts of interest.

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