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Research Paper

THE COMBINATION EFFECT OF PURE ACIDOCIN AND BIOFILM EXTRACT AGAINST *Staphylococcus aureus IN VITRO*

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Abstract

Twenty five *Lactobacillus acidophilus* isolates from healthy cows' milk and isolates taken from crops of chickens, both of them produced the Acidocinmeasured bydifferent zones of inhibition using the well diffusion methods in Muller Hinton agar media against *Staphylococcus aureus* isolates that produced biofilm (3mm)thickness. One of the *Lactobacillus acidophilus* isolates (which was isolated from the previous experiment), causes highest inhibition zone against *Staphylococcus aureus* isolates by a crud acidocin extracted with specific activity211.764 AU/ml and 0.850 mg/ml protein concentration, after that the acidocin was purified by gel chromatography filtration method,the result was high specific activity of 575.296 AU/ml with 0.591 mg/ml protein concentrated and then found the molecular weight was (39,445 KD).

Key words: *Staphylococcus aureus*, biofilm, *Lactobacillus acidophilus*, acidocin, antibacterial activity.

INTRODUCTION

Lactobacilli play an important role in suppressing undesirable intestinal microflora, organic acids and hydrogen peroxide produced by *Lactobacillus acidophilus* have demonstrated broad – spectrum inhibition, previous studies suggested that bacteriocin either mediate or facilitate inhibitory activity ,the crude lactocidin produced by *Lactobacillus acidophilus* demonstrated antimicrobial effect against *Staphylococcus aureus*criteria for bacteriocin identification include a little potential for broad – spectrum inhibition restricted to closely related species, a bactericidal mode of action , andproteinaceous nature [1].

The ability of probiotic bacteria to stimulate the immune system is an additional reason for supporting their use as alternatives to antibiotics for improving animal health and protection against infectious agents. In spite of the interest in the use of probiotics in commercial poultry production, to date there is little information available on the mechanisms through which probiotic bacteria affect the chicken immune response [2]. *Lactobacillus acidophilus* has been demonstrated to prevent and alleviate disturbances and to normalize the cytokine profile which might be of an advantage for patients suffering from infection [3].

Dairy LAB manufacture extracellular polysaccharides (EPS) , in nature bacterial EPS fulfills a variety of diverse functions including cell protection , adhesion of bacteria to solid surfaces , and participate in cell – cell interaction, incorporation of EPS or EPS-Producing (EPS+) culture in dairy foods can provide viscosifying , stabilizing and water – binding function , EPS also

contributes to the mouth –feel, texture , and taste perception of fermented dairy products , EPS may even play a role in the probiotic activity of certain LAB milk fermented with EPS+ dairy LAB generally develops a ropy or viscous texture and EPS+ strain of LAB are widely used in yogurt manufacture to enhance viscosity and reduce synergetic [4].

The sequence of the acidocin B biosynthetic gene cluster was also determined and showed high nucleotide sequence similarity to that of gassericin A, the nuclear magnetic resonance (NMR) solution structure of acidocin B in sodium dodecyl sulfate micelles was elucidated, revealing that it is composed of four-helices of similar length that are folded to form a compact, globular bundle with a central pore. This is a three-dimensional structure for a member of subgroup II circular bacteriocins, which are classified based on their isoelectric points of 7 or lower, comparison of acidocin B with carnocyclin A, a subgroup I circular bacteriocin with four helices and a pI of 10, revealed differences in the overall folding, the observed variations could be attributed to inherent diversity in their physical properties, which also required the use of different solvent systems for three-dimensional structural elucidation [3].

Because the studies of the biofilm and Acidocin productions in Iraq are limited therefore this study aims for detecting the biofilm production and the Antimicrobial effect of purified acidocin also the acidocin combined with biofilm against *Staphylococcus aureus*isolated from Mastitis in cows in *vitro & vivo.*

MATERIALS AND METHODS

Twenty five (25) milk samples, and twenty five (25) chicken crop swab samples were collected from field of Veterinary Medicine College University of Baghdad , Abu Ghraib, field of College of Agriculture-University of Baghdad , Radwanyya fields, and somePopular markets in Baghdad. The crop is an enlargement or out pouching of the esophagus proximal to the proventriculus or glandular stomach, the crop is generally regarded as a food storage area when the stomach is full, were collected by sterile swab and immediately transferred to laboratory and cultured in 10 ml MRS broth media in order to isolate and Diagnosis *Lactobacillus acidophilus*.The ability of *Lb. acidophilus* isolates to produce acidocin. Three screening techniques were used as described by lewus and Montville [5].Acidocin activity assay according to Parente*et al*. [6] and Pilasombut*et al*.[7].

Biofilm extract antigen (BiA)

Staphylococcus aureus which produce biofilm was cultured on Tryptic soya broth over night at 37 °C, then the biofilm was removed from the surface of the broth and from the wall of the tube kindly by disposable sterilized inoculation loop and accumulate it in a sterile test tube, then dried at 40°C for 48 hours, and the total protein concentration of this extract antigen which was measured according to Biuret procedure was 18 mg/ml and it was diluted to become 4.5 mg / ml.[8].

Determination of Acidocin Protein Concentration: It was determined according to Bradford (1976).and Aciocin activity determined according to Parente*et al*. (1995) and Pilasombut*et al*. (2005).The Arbitrary Unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the test isolate.

Purification of acidocin: The acidocin purified according to Powell *et al*.,2007 by using the cell –free supernatant (Crude acidocin extract) which heated to 80^{o}C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15 minutes. The supernatant than mixed with ann-butanol at a ratio 1:1. The mixture was centrifuged at 4000 rpm for 10 minutes to achieve phase separation. The organic phase was evaporated at 80°C by rotary evaporator, then the sediment was re-suspended in 20 mM sodium citrate buffer (pH5) and referred to as partial purified acidocin (PPA). Gel filtration chromatography by sepharose 6B was used as a second step for acidocinpurification.

Effect of Crude acidocin on Cell Lysis and Viability

The effect of crude acidocin on indicator bacteria was studied using the following procedure:

Culture *of Staphylococcus aureus* was used to inoculate BHI broth to obtain 5 ×10^{5} CFU/ml. Crud acidocin solution was added to culture of indicator bacteria to obtain final concentrations

(6.35, 3.15) mg/ml of acidocin and the culture were incubated at 37°C for 24 hours. After incubation period, the number of viable bacteria was determined by dilution and plating on nutrient agar plates. At the same time, the turbidities of culture was measured spectrophotometrically at 600nm (Parente and Hill,1992b; Kelly *et al.,* 1996). The inhibition rate was calculated as :

$$\text{Inhibition rate}(\%) = \frac{\text{No.cells in zero time - No. cells in required time}}{\text{No. of cells in zero time}} \times 100$$

(Kandela,2006).

The effect of Acidocin on cell permeability

Ten ml of 18 hour old culture of *Staphylococcus aureus* was harvested by centrifugation at 3000 rpm for 15 minutes, the cells washed twice with sterile buffer (pH 6.5), then re-suspended in 10 ml of the same buffer . Pure Acidocin was added 3.15 mg/ml to the washed cells at a ratio of 0.1:1.0. After 1 hour of incubation at 37°C, the cells were harvested by centrifugation at 3000 rpm for 15 minutes. DNA concentration was determined by optical density reading at 260 nm. Cells suspended in buffer (pH 6.5), without Acidocin and in the same buffer containing acidocin, but without cells, served as controls (Todorov*et al* ., 2006).

RESULTS AND DISCUSSION

Identification of *Lactobacillus acidophilus*

After the isolation, purification and biochemical diagnostic tests for bacteria *Lactobacillus acidophilus*, this test to confirm our diagnosis, after incubation the isolates at 37 °C for 24 hours, a sterile cotton swab was used or inoculating loop, suspend sufficient growth from the agar plate culture in RapID™ inoculation fluid (2 ml) to achieve a visual turbidity equal to a 2 McFarland turbidity standard or equivalent. Suspension should be mixed thoroughly and vortexed if required.

Using a pipette, gently transfer the entire contents of the inoculation fluid tube into the upper right hand corner of the panel. After adding the test suspension incubates RapID™ ONE panels at 35-37°C into incubator for 4 hours.

RapID™ ONE panels contain 18 reaction cavities that provide 19 test scores test cavity 18 is bifunctional, containing two separate tests in reagent providing the first test result, and then the same cavity is scored again after the addition of reagent to provide the second test result.

Test cavities 15 through 17 require RapID™ ONE reagent and are designated with a box drown around bifunctional test 18 which uses RapID™ Spot Indole reagent, requiring test. Figure (1a, b, c).

Figure (1 a): The test before incubation

Figure(1 b): Test after incubation and before adding the reagents

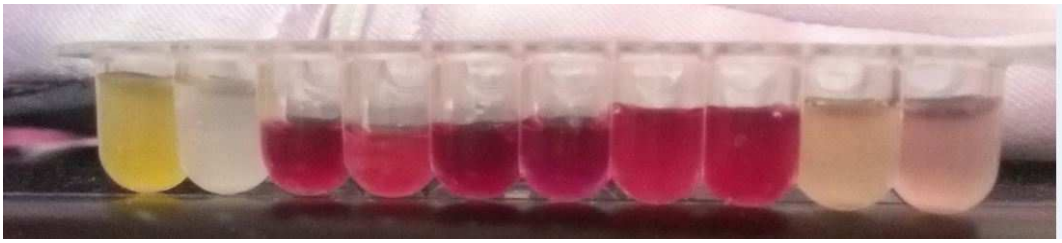

Figure (1 c): Test after adding the reagents.

Detection of Acidocin production

Used three strains of *lactobacillus acidophilus* one of them isolated from milk samples of healthy cow, others crop of chickens and standard strain, showed the differences inhibitory spectra of *lactobacillus acidophilus* against *Staphylococcus aureus* isolates that producing biofilm, all bacteria gave positive result for degree of inhibition (moderate inhibition zone or strong inhibition zone) against 3 species of *lactobacillus acidophilus* ,this result harmonize with Abo- Amer,(2006) who showed that *L. acidophilus* have antimicrobial effect against *Staphylococcus aureus.*

The inhibitory activity of isolates were determined by several methods such as flip-streak methods, spot on the lown methods and agar well diffusion methods, however, agar well diffusion assay demonstrated more apparently positive result compared with the previously mentioned, this result is consistent with (Kumar *et al* ., 2010).

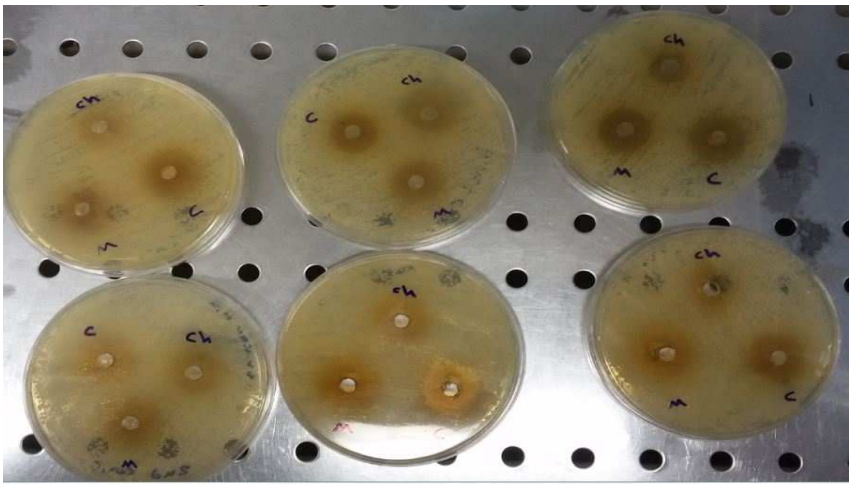

Figure (2a) : Antibacterial activity of *lactobacillus acidophilus* against *S.aureus* by well diffusion methods. * 1= *L.acidophilus* (milk) ,2= *L.acidophilus* (chicken) and 3= *L.acidophilus* (standard)*.

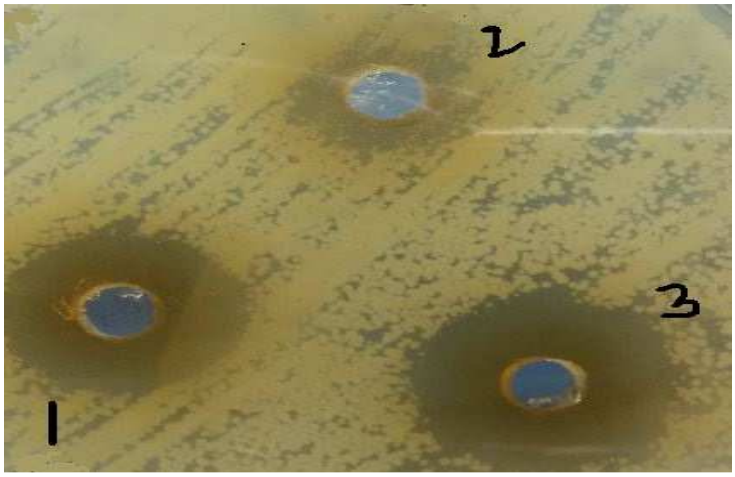

Figure (2 b): antibacterial activity of *lactobacillus acidophilus* against *S.aureus* by well diffusion methods. * 1= *L.acidophilus* (milk) ,2= *L.acidophilus* (chicken) and 3= *L.acidophilus* (standard)*.

Local isolation of *lactobacillus acidophilus* have been selected derived from milk were extracted acidocin as a probiotic which gave the high level inhibitory diameter by well diffusion assay test , 180 AU/ml., and specific activity 211.764 AU/mg , and yield 100% ,then a certain bacteria were cultured on selective media. The Results showed that the best pH was 5.5 in MRS medium enhanced the development of bacterial growth and secretion of Acidocin, beside the bacteriocin characterized showed an antimicrobial activity at the acidic pH more than the basic pH (Al-Jumaily*et al* ., 2014).

The best period time for the growth bacteria to secrete the acidocin was 24 hours, decreasing of activity after prolonged incubation of producer strain has been occur as a result of extracellular proteases , protein aggregation or re-adsorption to the producer cell surface (Hernandez *et al* ., 2005).The specific activity of acidocin recorded as a high level in growth in anaerobic conditions it was the favored for acidocin production than aerobic conditions this an agreement with (Al-Jumaily*et al* ., 2014).

The partial purification of acidocin was performed by n- butanol in ratio 1 : 1 , acidocin was removed from the aqueous phase and could be recovered from the organic phase ,this suggest that at least part of the acidocin molecule has a hydrophobic character and shares this property with other bacteriocin (Sanni*et al* ., 2003). The activity of acidocin was 660AU/ml, specific activity was 929.577 AU/mg, protein concentration 0.71, purification fold 4.389 and 36.66%yield as shown in table (4-12) . The extraction of acidocin using n-butanol in a 1:1 ratio was reported by (Al-jumaily*et al* ., 2015).

Purification of acidocin by Gel Filtration Chromatography

After partial purified acidocin (PPA) were applied on sepharose6B column (2 × 96) cm dimensions, the column had been equilibrated with 20 mM sodium citrate buffer (pH 5) . Each eluted 5 ml fraction read at 280 nm and the curve was plotted between the absorbance and fraction number which gave three protein peaks and one major peak locked between tube (30-43).

The maximum activity of Acidocin was observed in the fraction (33). The specific activity from that fraction was 575.296 AU/mg protein, and protein concentration 0.591 mg/ ml., as shown in figure (4-9)

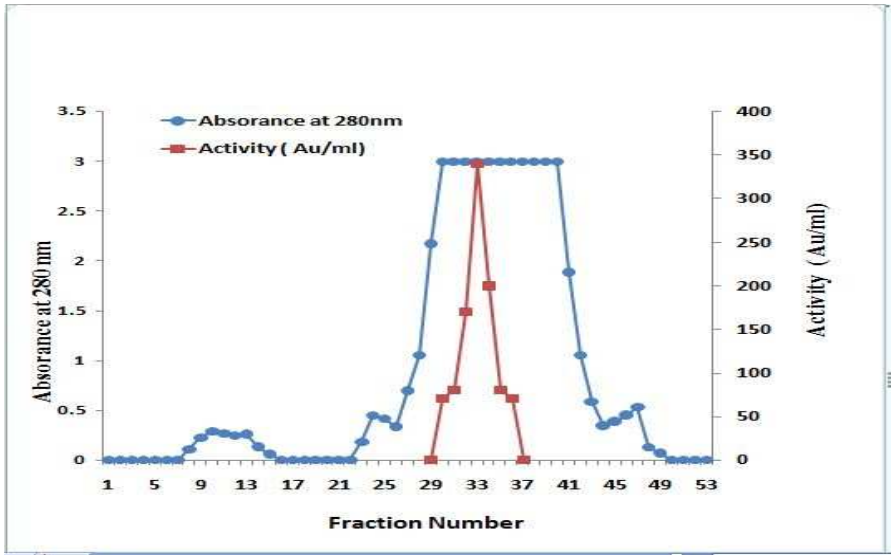

Figure (4-10) purification of acidocin by gel filtration chromatography showing the high Specific activity 575.296AU/mg protein in fraction number 33 & the Absorbance at 280 nm.

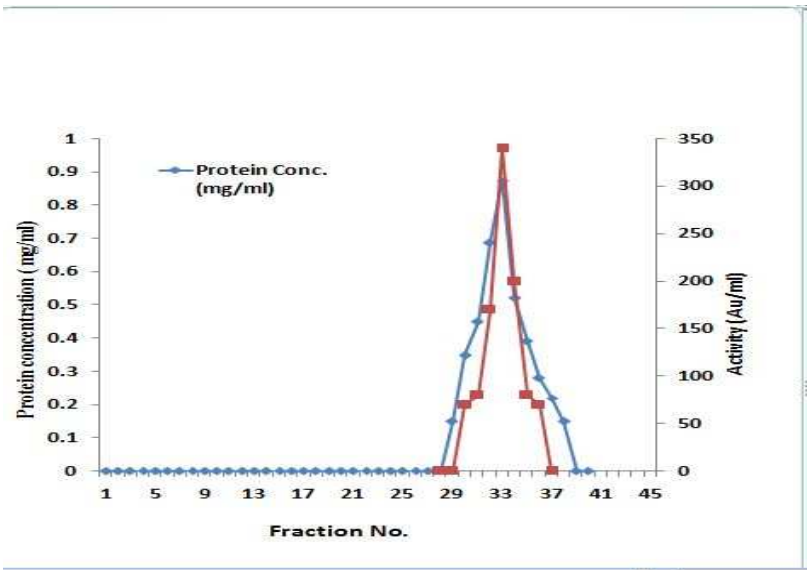

Figure (4-11) showed the relationship between protein concentration with acidocin effectiveness production by *L. acidophilus*

The strong hydrophobicity is characteristic property of the class II peptide bacteriocins, for instance the proportion of hydrophobic amino acid in the *Lactobacillus acidophilus* group bacteriocin, namely acidophilin 801, gassericin A, lactobin A and lactin F, amounts to 50.8, 45.7, 38.0 and 29% respectively (Tahara and Kanatani, 1996).

This strong hydrophobic character is one of the main reasons why bacteriocin purification is tedious and cumbersome. (Ahmed *et al* .2010).

In vitro Antibacterial activity of Acidocin

The acidocin extract gave a high zone inhibitory effective against bacteria *Staphylococcus aureus* that producing (3) mm biofilm thickness, the crude acidocin extraction also crud after heating 80 °C, showed in well diffusion (6 mm), activity as 180 AU/ml, while the crude acidocin after heating 80°and and mixed with n- butanol by shaker 24 hours then separated the organic

phase showed specific activity 929.57 AU/mg protein and after purification acidocin by gel filtration chromatography gave 575.296 AU/ml protein.

This result agreement with Hassan *et al*.(2013) which found that all tested metabolites of *lactobacillus acidophilus* showed significant bacterial activity, as indicated by zone of inhibition in culture plates, exhibited the highest zone of inhibition against *S.aureus* and *E.coli* in both growth media, metabolites of *lactobacillus acidoplilus* significantly inhibited the growth of pathogenic bacteria used, and can be used as potential antibiotic or probiotic agents.

The researchers found that *lactobacillus acidophilus* supernatants succeeded in eliminating wound infection caused by *S. aureus*, and the ability of acid supernatants of *Lactobacillus acidophilus* isolated from a vinegar were tested against methicilline resistant *Staphylococcus aureus* (MRSA) with lipolytic activity isolated from acne pimple, gave a significant effect of cell free supernatant on MRSA isolates, furthermore , subinhibitory concentrations of acid supernatant found to be very effective in inhibiting the production of lipase from biofilm and planktonic cells on MRSA isolates (Harith& Hala,2011).

The acidocin of *lactobacillus acidophilus* has antibacterial activity against the growth of different gram positive and gram negative bacteria (Al-Gharbawee,2012).A probiotic food supplement derived from *L. acidophilus*n.v. Er 317/404 is widely used in Armenia and neighboring countries as breast milk substitute (added as a supplement to dairy milk) and to treat gastrointestinal problems including various forms of dysbacteriosis, in addition this supplement is used to alleviate symptoms of inflammatory bowel diseases and acute infections, during and especially after taking antibiotics, as an immune stimulator in virus and somatic diseases, clinical trials of probiotic food supplement have been undertaken in Armenia, Russia, Ukrania and the Baltic contries. (Akopyan,2004).

Effect of acidocin on *S.aureus* isolates producing different thickness of biofilm

Figure (1) shown therewere significant differences of the rate of inhibition zone and activity in different thickness of biofilm by crude acidocin. The results revel that the biofilm thickness (0.2,0.3 and 0.9 mm) showed significant increment in the value $p<0.05$ in rate of inhibition zone by crude acidocin as(48.33 ± 3.15) (48 ± 4) and (48 ± 2) respectively, as compared with other biofilm thickness. The rate of inhibitor zone have less values in the thickness of the biofilm(0.4, 0.6, 0.7,0.8, 1, 1.2, and1.4 mm).

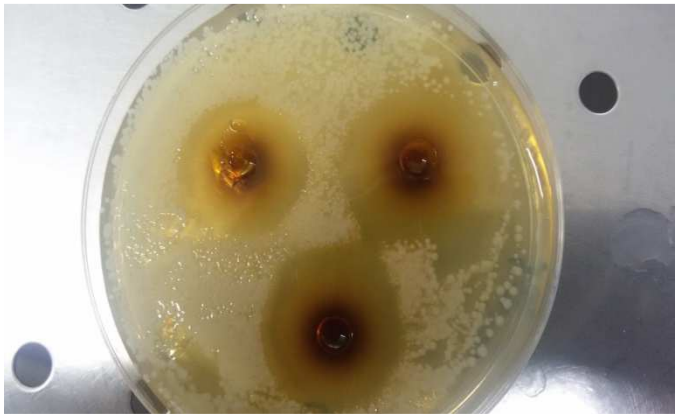

Figure (1): Antimicrobial effect of crude acidocin against *Staphylococcus aureus* isolates.

In the other side, the results appeared no significant increment in the lower values in rate of inhibition zone by crud acidocin (26 ±0.57) and (18 ±0.25) of biofilm thickness 2.8 mm and the lowest value of rate of inhibition zone by crude acidocin (18±0.25) and activity (180±2.58) in 3mmthickness biofilm(Table 1).

Table(1): The activity of crud acidocin extract by different inhibition zone in well diffusion assay against the growth of *S. aureus* (5×10^5 CFU/ml) secreted various thickness of biofilm .

Characters / Thickness of biofilm(mm)	Rate of inhibition zone by crude acidocin (mm)	Activity AU/ml
0.2	48.33 ± 3.15A	483.33 ± 31.58A
0.3	48 ± 4A	480 ± 40A
0.4	39.5 ± 2.06B	395 ± 20.61B
0.5	38 ±0.57BC	380 ± 5.77B
0.6	39 ±3.7B	390 ± 37.85B
0.7	39 ± 1.0B	390 ± 10B
0.8	37 ± 2.14B	370 ± 21.44B
0.9	48 ± 2A	480 ± 20A
1	41 ±3.0B	410 ± 30B
1.2	41 ±1.0B	420 ± 5.0B
1.4	38.66 ±1.76B	386.66 ± 17.63B
1.8	36 ± 0.57C	360 ± 5.77C
2	24 ± 2E	240 ± 20D
2.2	32 ±2.0D	320 ± 20C
2.4	26 ± 0.57E	260 ± 5.77D
2.6	22 ±1.15E	220 ± 11.54D
2.8	26 ±0.57E	260 ± 5.77D
3	18 ±0.25F	180 ± 2.58E

Different Capital letter denote significant($p<0.05$) difference among rate of inhibition zone and activity.

This means that the more biofilm layer increases bacterial resistance to the acidocin as a probiotic extraction , this result agreement with (Joseph *et al* .,2011) when he appeared that the chronic nature of many diseases is attributed to the formation of bacterial biofilms which are recalcitrant to traditional antibiotic therapy, the role of the extracellular matrix is multifaceted,including facilitating nutrient acquisition, and offers significant protection against environmental stresses .

Staphylococcus aureus biofilms dramatically increase the frequency of plasmid transfer events by both conjugation and mobilization, thereby promoting horizontal spread of antibacterial resistance determinants, this phenomenon probably results, in part, from the close cell-to-cell contact occurring in the biofilm and the fact that the biofilm matrix may act tostabilize contacts between neighboring bacteria, together with the fact that staphylococci resident in biofilms show elevated mutation frequencies to antibiotic resistance ,this observation identifies biofilms as a privileged environment for the emergence and spread of antibacterial resistance in*S. aureus* (Victoria *et al*., 2013).

Effect of Acidocin on Viability and Lysis of *S.aureus* isolates produced biofilm layer 3 mm.

The effectiveness of acidocin extracted was tested against isolates of *S. aureus* (5×10^5 CFU/ml) that produced 3 mm thickness of biofilm at different periods of time, in addition 0.1 ml of 25 , 12.5 , 6.25 and 3.125 mg/ml of pure acidocin, to the 10 ml growth of *S. aureus* the result showed antibacterial effect with a reduction of cells count of the treated sample after a ½ hour, 1 hour, 1 ½ hour, 2 hours, 2½ hours, 3 hours,4 hour, and 18 hours table (2)

The most important thinks on these results, Acidocin demonstrated a bacteriolytic mode of action as immediate decrease of bacterial count by Mils andMisra technique from cultured of *S. aureus* that treated with 0.1 ml by different concentration of pure acidocin, indicate cell lyses, this results was agreement with Al- Gharbawee,(2012) showed that the acidocin produced from *L. acidophilus* was exhibited bactericidal mode of action with cell lysis.

These concentrations of pure acidocin gave inhibition zone 34mm, against *S. aureus* isolates that produced biofilm thickness 3 mm, measured by well diffusion methods after incubated 18 hours at 37°C., so the result depended these concentrations of acidocin to determines the dose of injection when we treated the nursing mice as a laboratory animal design.

Acidocin effectiveness as a bactericidal on sensitive microorganisms by, inhibiting the transport of amino acid and causing the leakage of essential compounds by the formation of pores in the cytoplasmic membrane (Ahmed *et al* ., 2010).

Table (2): The effectiveness of different concentration with different time by pure acidocin to the logarithm growth of *S. aureus* that produced (3 mm)thickness of biofilm layer .

Pure acidocin conc.mg/ml **Time (hour)**	**25**	**12.5**	**6.25**	**3.125**
	Rate of bacterial count Log	**Rate of bacterial count Log**	**Rate of bacterial count Log**	**Rate of bacterial count Log**
½	**4.5300±0.04813 Ac**	**4.5580±0.03355 Ac**	**4.7297±0.04437 Aa**	**4.6807±0.05571 Ab**
1	**4.2540±0.06935 Bc**	**4.4543±0.01105 Bb**	**4.4417±0.19877 Bb**	**4.5983±0.06902 Ba**
1½	**3.5870±0.05292 Cc**	**3.6617±0.10790 Cc**	**3.8183±0.02924 Cb**	**3.9233±0.03118 Ca**
2	**3.0637±0.06363 Dd**	**3.4233±0.10898 Dc**	**3.5650±0.08073 Dd**	**3.7840±0.07697 Da**
2½	**2.6947±0.01291 Ec**	**2.7277±0.09122 Eb**	**2.7557±0.07163 Eb**	**2.9123±0.02136 Ea**
3	**1.9910±0.00520 Fc**	**2.2523±0.02717 Fb**	**2.4013±0.00953 Fa**	**2.4580±0.00608 Fa**
4	**0.5923±0.06398 Gd**	**0.8937±0.06426 Gc**	**1.0777±0.02080 Gb**	**1.1700±0.05090 Ga**
18	**-**	**-**	**-**	**-**

The table shown there were significant differences in pure acidocin concentration 25 mg/ml among the time in which the concentration in a half hour have superior value as compare with other times , and the lowest one in the time four hours .

Also the table shown high significant differences of pure acidocin concentration (12.5, 6.25 , 3.125) mg/ml in a half hour, while lower significant differences in four hour.

The best action of pure acidocin concentration was 6.25mg/ml, in half hour and in three hour , while the acidocin concentration 3.125 mg/ml was the best at the time one hour , one and a half, two , two and a half, three, and four hours.

The best action of acidocin was in concentration 6.25mg/ml , in a half hour and in three hour while the concentration 3.125mg/ml., was superior action in one ,one and a half, two, two and a half ,three, and four hours.

The result showed the significant activities of pure acidocin concentrations against S.aureus produced biofilm thickness 3mm resulted a strong decrease in rate of bacterial count, the most effective in low concentration was 3.125 mg/ml after 4 hours that complete killing of S. aureus cells.This results agreement with Hassan et al , (2012) when the acidocin was effected to Enteropathogenic*E.coli* .andwith Ali,(2010) when the bacteriocin (plantaracin) was exhibit bactericidal mode of action with cell lysis. Bactericidal activity of bacteriocins may be accompanied by lysis of sensitive cells (bacteriolyticbacteriocins) (Essam, *et al* ., 2013 c)

The effect of pure Acidocin on cell permeability

The effect of Acidocin on cell permeability of *S. aureus* isolate 5×10^5 CFU/ml^{-1} that produced biofilm 3 mm thickness, measured by used Spectrophotometer at 260 nm absorbance after added 100 µl of acidocin to 1 ml of *S.aureus* growth, the results showed the lyses due to Acidocin activity 25 mg/ml. The results shown the treated of *S.aureus* isolates with acidocin gave reading 2.640 , untreated 0.461, and acidocin only 0.492, indicated the effected treatment by acidocin that rupture the cell membrane and made DNA leaked from cell of *S. aureus* bacteria due to the cell lysis table (3).

Table (3): Showing the level of DNA recorded after treatment the *S. aureus* isolate that produced biofilm 3 mm thickness with pure acidocin.

Treatment	Absorbance at 260 nm
	S. aureus
Treated cell	2.640
Untreated cells	0.461
Acidocin (no cells)	0.492

These results gave an idea or conclusions that cationic antimicrobial peptides disrupt bacterial membranes, thus allowing free exchange of intracellular and extracellular contents of the target cells (Zhao *et al* ., 2006, Heunis*et al.,* 2011).

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