Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Sciences
Biotechnology Department

Study the effect of epidermin produced by local isolate of *S. epidermidis* on antibacterial activity of some agents used in wounds and burns treatment.

A thesis
Submitted to the College of Science of AL-Nahrain University
As partial fulfillment of the requirements for the degree of Master of Science in Biotechnology.

By

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B. Sc. Biotechnology (2003)

Al-Nahrain University

Sha'aban **1427**

August **2006**

Abbreviation

Abbreviation	Mean
HAI	**Hospital Acquired Infection**
MRSA	**Methicillin Resistant *S.aureus***
VRE	**Vancomycin Resistant enterococci**
BHI	**Brain Heart Infusion**
UTI	**Urinary Tract Infection**
CFU	**Colony Forming Unit**
CONS	**Coagulase Negative Staphylococci**
EDTA	**Ethylene Diamine Tetraacidic acid**
SDS	**Sodium Dodecy Sulfate**
D.W.	**Distilled Water**
Tris base	**Tris (hydroxymethyl) aminomethane base**
S.	**Staphylococcus**
E	**Erythromycin**
C	**Chloramphenicol**
TE	**Tetracycline**
CN	**Gentamicin**
S	**Streptomycin**

mm	**Millimeter**
Kb	**Kilobase**
Hrs.	**Hours**
µg	**Microgram**
mM	**Millimolar**
mg	**Milligram**
BSA	**Bovine Serum Albumin**
PB	**Phosphate buffer**
E.	**Escherichia**

Acknowledgments

At the beginning, thanks to **Great Merciful God** who gave me health, faith, strength and patience, and facilitated the ways for me to accomplish this work.

I would like to express my deep gratitude, thanks and appreciations to my supervisors **Dr. Muneera Chaloob Al-Ibadi** and **Dr. Ayad Mohammed Ali Al-Ubaidy** for their continuouses guidance, support and advices through whole period of research. This work would never have been done.

Thanks due to my parents for their support and to my brother and sister.

My gratitude to my colleagues and friends in the department of Biotechnology, college of science, Oday Adnan , Hassan Abdulhadi , Saif Riadh, Solaf Jawhar , Sura Ali , Mayassa Fadhil, Hiba Abdulkader , Muntaha Abdulrazaq , Worood Kamil, and Mr. Ali Al-Samak. And a word of thank to staff of department of Biotechnology in Al-Nahrain university for their appreciable help Miss Shayma'a , Miss Oroba , Miss Tania, Mr.Yasin and Mr. Hamid for their help and valuable advices.

Special thank to Bassam for his constant support throughout this work.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

دراسة تاثير الابيديرمين المنتج من عزله محليه لبكتريا *epidermidis*.

S على الفعالية المضادة للبكتريا لبعض المواد المستخدمه في علاج الجروح

و الحروق .

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

فرح هيثم عبد المجيد جليل

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٣

شعبان ١٤٢٧

أب

٢٠٠٦

الخلاصة

تضمنت هذه الدراسة عدة جوانب متعلقة بإنتاج مادة الابيدرمين من بكتريا *Staphylococcus epidermidis* , وتنقيتها ودراسة تأثيرها التأزري بوجود بعض المواد المستخدمة في علاج الجروح والحروق كالأتي:

- عزل وتشخيص عزلات *S. epidermidis* من الجروح والحروق واختيار قابليتها في إنتاج الابيدرمين تم جمع ٧٠ نموذج (مسحة) من جلد المصابين بجروح أو حروق من عدة مستشفيات في مدينة بغداد، خلال الفترة ما بين تشرين الأول ٢٠٠٤ و شباط ٢٠٠٥.
شخصت خمس عزلات منها على إنها *S. epidermidis* واجري اختبار كفاءة هذه العزلات لأنتاج الابيدرمين من خلال تحديد قابلية هذه العزلات في تثبيط نمو عزلات الاختيار البكتيرية *Staphylococcus aureus, Escherichia coli* باستخدام طريقة التنقيط بالا كار، إضافة إلى قياس فعالية الحيوية. وجد إن جميع هذه العزلات كانت منتجة للابيدرمين و أظهرت فعالية مضادة لنمو البكتريا *S. aureus* ولكنها لم تؤثر في بكتريا E. coli كما اختلفت هذه العزلات في فعاليتها الحيوية، حيث كانت العزلة F *S epidermidis* هي الأكفأ.

-استخلاص وتنقية الابيدرمين للعزلة F *S. epidermidis* ، وبعد تنقيتها في وسط شبه صلب والقيد المركزي المبرد، الترسيب بكبريتات البوتاسيوم ، الديلزة و كهروماتوغرافيا الترشيح الهلامي بعمود Sephadex G-75. وتم حساب الفعالية الحيوية وتقدير البروتين لهذه المادة خلال خطوات التنقية. إذ تم الحصول على ابيدرمين منقى و بتركيز ٠,٢ ملغرام/ملليلتر و بفعالية ٥٠ وحدة فعالية/ملليلتر.

-التأثير التأزري للابيدرمين مع بعض المواد المستخدمة في علاج الجروح والحروق في تثبيط النمو البكتيري. استخدم لهذا الغرض كل من العقارات(والمواد الفعالة لها):
Samacyclin (Tetracycline HCL 3%)، Nifusin (Nitrofurazon)
Silverin (Silver sulphadiazine 1%) ،حامض ألخليك (٦%) و الايودين (١٠%).
حيث حدد تأثير هذه المواد كلا على حدة(بتركيز ١٠٠%) و بوجودها مع الابيدرمين(بتركيز ٥٠%) (حجم/حجم)، من خلال التأثير المثبط لبكتريا *S. aureus* ، بطريقة الانتشار في الاكار أظهرت النتائج إن Samacyclin هو الأكفأ في تثبيط البكتريا وحل ثانيا Nifusin ثم Silverin تلاه حامض الخليك فيما اظهر الايودين التلأثير الأقل.

-تم تحديد المحتوى البلازميدي لعزلات *S. epidermidis* إذ أظهرت النتائج احتواء جميع العزلات على حزم بلازميدية صغيرة وذات حجوم متقاربة. كما حددت صفة إنتاج الابيدرمين كصفة بلازميدية عند إجراء التحييد باستخدام ethidium bromide و لوحظ إن البلازمد المسؤول عن إنتاج الابيدرمين يحمل جينات المقاومة للمضادات الحيوية.

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1-3 Staphylococci:

Staphylococcus is a one genus of Gram-positive cocci which is of great medical importance. Staphylococci was first seen by Kock in 1878 cultivated in liquid medium by Pastenr in 1880 and named so by Ogston in 1881. The name Staphylococcus was derived from Greek noun Staphyle (a bunch of grapes) and Coccus (a grain or beery).

Staphylococci are spherical, Gram- positive, non motile, non spore forming, occasionally capsulate, most are catalase positive and micrococci are usually oxidase positive. Staphylococci ferment glucose and facultative anaerobic (Collee *et al.*, 1996).

Staphylococci are found primarily on the skin and in the mucous membranes of humans and warm- blooded animals, and aggregate into small, grape like clumps related infections are one of the most common causes of nosocamia hospital acquired infection (HAI), yet they are increasingly difficult to treat due to the rate of which the bacteria acquire antibiotic resistance (Willett , 1980).

Ninety percent of Staphylococci strains are penicillin resistant leaving, only methicillin and vancomycin to treat the majority of infections. However, with increase numbers reports of methiclillin resistant *Staphylococcus aureas* (MRSA) and vancomycin resistant Entercocci (VRE) chemists are faced with daunting task of generating antibiotics with novel modes of action, and doctors with the task of curing seemingly incurable infections (Foster, 2005).

1-4 Classification:

Staphylococcal colonies are usually not difficult to recognize when grown on an agar medium. They are relatively large, reaching 2 millimeter to 3 mm after 24 hours. If incubated at 35 °C, up to 7 mm in 48 to 72 hrs.

Currently three species of *Staphylococcus* are recognized these are:

- *Staphylococcus aureus*.
- *Staphylococcus saprophyticus*.
- *Staphylococcus epidermidis*.

1-4-1 *Staphylococcus aureus*

Is the only member of the family Micrococcaceae that produces coagulase. A positive coagulase test alone is used in many laboratories to presumptively identify this species. In addition, many strains of *S. aureus* also produce β-hemolysis on blood agar. Coagulase production and β-hemolytic are two characteristics that link Staphylococci with virulence, although the former appears to be more reliable. The ability of most strain of *S. aureus* to ferment mannitol and to reduce telluride to free tellurium are two other characteristics that are used by many microbiologists in differentiating *S. aureus* from *S. epidermidis*. In addition most strains of *S. aureus* produce deoxyribonuclease (DNase), an enzyme that has been used in the past by microbiologists to identify *S. aureus* and to predict virulence. The DNase reliable test is not as reliable as the coagulase test for either of these determinations and is not recommended as a substitute (Koneman *et al.*, 1983 and Collee *et al.*, 1996).

1-4-2 *Staphylococcus saprophyticus:*

S. saprophyticus is a coagulase – negative species of staphylococcus which is often in urinary tract infection (UTI).Along with several other staphylococcus species *S. saprophyticus* is resistant to the antibiotic novobiocin, a characteristic that used in laboratory identification. The organism is rarely found in healthy humans but is commonly isolated from animals and their carcasses. It is implicated in 10-20% of urinary tract infection UTI. In females between the ages of (17-27) it is the second most common causes of urinary tract infections UTI. It may also reside in the urinary tract and bladder of sexually active females.

S. saprophyticus is phosphatase – negative, urease and lipase positive (Internet#I).

1-4-3 *Staphylococcus epidermidis:*

S. epidermidis is a common member of the normal florae of skin and mucous membranes. Its large numbers and ubiquitous distribution make it one of the most commonly isolated organisms in the clinical laboratory. While at one time the appearance of *S. epidermidis* in clinical material could be dismissed as contamination, it is now one of the most important agents of (HAI). Immunosuppresed or neutropenic patients are particularly at risk, as are individuals with indwelling catheters or prosthetic devices. It can also cause endocardiftis in individuals with previous heart valve damage. The hydrophobic nature of the organism's cell surface facilitates its adherence to synthetic devices as well as damaged heart valves. Initial colonization a copious amount of extracellular polysaccharide or slime is synthesized, forming a protective biofilm around the colony. Because many isolates are multiply antibiotic

resistant, these infections are very serious and can even be fatal (Internet #I).

1-4-3-1 Diseases caused by *S.epidermidis*

Catheter infections		
erythema	pus	suppuration
inflammation		
Prosthetic implant infection		
fever osteomyelitis	pain	inflammation
Wound infections		
inflammation suppuration	erythema pain	pus
Cystitis UTI- catheterized patients		
pain on urination inflammation	dysuria chills	frequent urination suprapubic pain
Septicemia		
fever hypotension dyspnea	chills tachycardia acute confusion	headache tachypnea bacteremia
Endocarditic		
fever malaise anorexia swelling splinter hemorrhages	chills fatigue weight loss splenomegaly osler nodes (red nodules on fingertips)	night sweats weakness joint pain systemic emboli heart murmur

Endopthalmitis		
pain	edema	blurred vision

1-4-3-2 Sites and sources of *S. epidermidis*

skin, normal flora	mucous membranes, normal flora	nasopharynx, normal flora
oropharynx, normal flora	conjunctiva, normal flora	GI tract, normal flora
wounds, pathogen	heart valves, pathogen	catheters, pathogen
prostheses, pathogen		

1-4-3-3 Diagnostic factors of *S. epidermidis*

growth on blood agar	gamma hemolysis	catalase positive
coagulase negative	novobiocin sens.	

1-4-3-4 Virulence factors of *S. epidermidis*

adhesion	capsule	slime
biofilm		

1-4-3-5 Treatment and prevention of *S. epidermidis*

removal of catheters	removal of prosthetic devices	vancomycin
trimethoprim-sulfamethoxazole		

(Internet # II).

The ability of *S. epidermidis* strains to grow in the presence of human transferrin and varying amounts of ferric iron was studied. At initial bacterial densities up to 10^4 CFU/ ml, none of the three strains grew when transferrin iron saturation was below the full saturation point, whereas the bacteria grew consistently when transferrin was fully iron – saturated and there was no-transferrin bound iron in the medium.

Precultivation of the bacteria under iron – restricted conditions to induce siderophore production did not abolish the growth dependence of partially saturated transferrin. The results indicate that at low bacterial densities, *S. epidermidis* can not utilizes transferrin bound iron for growth and that its proliferation is depended on non- transferrin bound iron (Matinaho *et al.,* 2001).

S. epidermidis is a major causative agent of septic infections in neutropenic patient receiving high-dose chemotherapy and other immunocompromised patients. High dose chemotherapy is associated with high serum iron concentrations, which exceed the iron – binding capacity of the major iron carrier protein, transferrin, in the host. This results in the appearance of non-transferrin bound iron in the circulation of the patients. As iron is an essential growth factor for bacteria, it has been suggest hyperferremia, together with the lack of neutrophilic granulocytes, predisposes these patients to infections with opportunistic bacteria (Matinaho *et al.*, 2001).

1-5 Medical importance of *S. epidermidis:*

S. epidermidis coagulase-negative staphylococci (CONS) which were earlier thought to be more commensally of the human skin and mucosa have emerged as major pathogens in many systemic. One important properly of this organism which is responsible for its persistence and /or opportunistic invasion in the tissues is its ability to produce slime (Gray *et al.,* 1982; Quie and Belani, 1987).

Slime not only helps the organism in colonization of the host tissue, but also protects it from phagocytes and from the action of antibiotics (Peters *et al.*, 1982 ; Quie and Belani, 1987; Heilmann *et al.,* 1996; Nayak and Satpathy, 2000).

On the contrast, staphylococci produce several types of antibacterial substances which differ with respect to their chemical nature and mode of action. A part from lytic enzymes like lysozyme and lysostaphin, there exist two main groups bacteriocins (staphylococcins) and low molecular weight antibiotic-like substances (Sahl and Brandis, 1981; Ottenwalder *et al.,* 1995).

The spectrum of inhibitory activity of the agents were shown to be similar to that of bacteriocins produced by other Gram-positive bacteria in that it included strains a wide variety of other Gram-positive genera (Navaraton *et al.,* 1998).

Among the most thoroughly studied staphylococcal bacteriocins in recent years are the *S. epidermidis* products epidermin, Pep5, epilancin K7 and epicidin 280 (Nascimento *et al*., 2006).

. All of these are classified as lantibiotics (i.e, low molecular weight, posttranslational modified peptides containing the distinctive amino acids Lanthionine and/or β-methyl Lanthionine and sometimes dehydroamino acids) (Sahl and Brandis, 1981; Vandekamp *et al.,* 1995; Blaesse *et al.,* 2000).

As a consequence of these modifications, the processed, biologically active lantibiotics are relatively short, flexible and thermally stable molecules. Several other bacteriocins isolated from *S. epidermidis* have subsequently been shown to be either epidermin or epidermin variants (Sahl, 1994).

1-6 Bacteriocins of Gram-positive bacteria:

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar bacterial strains. They are phenomenological analogous to yeast and paramecium killing factors, and are structurally, functionally and ecologically diverse. Bacteriocins were first discovered

by Gratia in 1925. He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage, all within a span of a few years. He called his first discovery a colicine because it killed *E. coli*. Bacteriocins are categorized in several ways, including producing strain, common resistance mechanisms, and mechanism at killing. There are several large categories of bacteriocin which are only phenomenological related. These include the bacteriocins of Gram-positive bacteria, the colicins, the microcins, and the bacteriocins of *Archaea* (Internet #1).

Several recent monographs and reviews have focused on characteristics of subcategories of the bacteriocins-like agents produced by Gram-positive bacteria (Harris *et al.* , 1989 ; Jung , 1991a ; Jung and Sahl , 1991 ; Kolter and Moreno, 1992 ; Ray and Daeschel , 1992 ;Bierbaum and Sahl , 1993).

It particular emphasis some of the better characteristics ribosomally synthesized, cationic, low molecular-weight, heat stable bacteriocins produced by gram-positive bacteria. (Jack *et al.*, 1995)

The bacteriocin loses activity, when exposures to the temperature 120 °C at 15 min when the pH of bacteriocin is acidic or basic that is lose.

In recognition of discovery that antibiotic subset of the colicin type may also be produced by noncoliform bacteria, the more general term “bacteria”. Bacteriocins were specifically defined as protein antibiotics of the colicin type, i.e., molecules characterized by lethal biosynthesis, predominant intraspecies killing activity, and adsorption to specific receptor on the surface of bacteriocins-sensitive cells. Further distinguishing features of the colicins included their relatively high-molecular weight and the plasmid association of their genetic determinants (Jacob *et al.*, 1953).

As a result initially of the influence and efforts of the scientist Frederieq, knowledge of the colicins advanced at a great rate and more than 20 different types were identified on the basis of their actions against a set of specific colicin-resistant (generally receptor-deficient) mutants. Frederieq also observed that certain strains of staphylococci produced substances, named by "staphylococcins" that were inhibitory to the growth of other staphylococci and some other Gram-positive bacteria but not gram-negative bacteria. However, attempts to categorize the staphylococcins in manner similar to the receptor based colicin classification were not successful (Gagliano and Hinsdill, 1970; Jetten and Vogle, 1972).

S. epidermidis ranks among the most important nosocomial pathogens, mainly because of its ability to colonized indwelling medical devices by forming a biofilm (Gotz *et al.,* 2000 and Gotz and Peters, 2000).

In addition, many antibiotics lose their effectiveness against *S. epidermidis* in the biofilm environment because of the impenetrable slime capsule (Slach and Nichols, 1982 and Farber *et al.,* 1990).

Virulence factors in *S. epidermidis*. An agar deletion mutant has recently been constructed and characterized, but no other global regulator has been studied so far. (Van Eiff *et al.,* 2000).

1.7 Classification of bacteriocin:

Bacteriocin could be classified into four types that depended to the chemical nature and molecular weights (Jack *et al.,* 1995):

1.7.1 Lantibiotics:

lantibiotics synthesized by Gram-positive bacteria (Staphylococci, Streptococci, Bacilli) are a special group of polycyclic polypeptides

constituted of protein, amino acids and unusual posttranslational modified amino acids (i.e. dehydroalanine, dehydrobutirine, lanthionine, B-methyllanthionine) , these last ones exhibiting interchain thioether bridges and ring structures. Some lantibioties are already used in medical therapy (nisin, gallidermin, epidermin) as well as for food preservation (nisin). Their special structures and synthesis have focused substantial scientific interest, the lantibiotics proving to be ideal experimental models not only for microbiology, but also for biochemistry, molecular biology, gene technology and protein engineering in the purpose to get more insights in the fundamental aspects of the cell biology (Israil, 1992 and Klaenhammes, 1993).

The molecular weight is very low < 10,000 dalton. There subgroup have been defined on the basis of their distinctive ring structures (Internet #I).

Lantibiotic are containing modified amino acids: in particular, the thiorether amino acid lanthionine and 3-methyllanthionine and the dehydroamino acids 2, 3-didhydroatanine and 2, 3 didhydrobutyrine. Lantibiotics are ribosomally synthesized as precursor peptides, consisting of a leader sequence and a propeptide part (Schnell *et al.*, 1988).

Precursors peptides are converted into the mature peptide by posttranslational modification followed by processing and export from the producing cell (Sahl and Bierbaum, 1998).

The cationic lantibiotic peP_5 is produced by *S.* epidermin strains (Sahl and Bierbaum, 1981) and is classified along with nisin, subtitin and epidermin as a pore-forming type **a** lantibiotic (Sahl and Bierbaum, 1998; Hoffmann *et al.*, 2004). The epidermin gene for peptide synthesis *Epi A* was previously characterized (Schnell *et al.*, 1988), maturation *Epi B, C* and *D* (Kupke *et al.*, 1995; Kupke and Gotz, 1996; Peschel *et al.*, 1996), processing *Epi P* (Geibler *et al*., 1996), and regulation *Epi Q* (Peschel, 1993) in the heterologous cloning host *S. carnosus*. The gene *Epi F, E*

and *Q* encode the subunits of ATP-binding cassette ABC exporter that confers on the producer immunity to epidermin by expelling the antimicrobial peptides from the cytoplasm membrane (Otto *et al.,* 1998 and Peschel and Gotz, 1996).

The epidermin transporter gene *Epi T* has been shown to be defective, since it is disrupted by a deletion causing a frameshift. The *Gdm T* gene of the closely related lantibiotic gallidermin from *S. gallinarum*, however, is intact and mediated a considerable increase of epidermin processing strains. This effect was dependent on the presence of the adjacent gene, *Gdm H*, indicating that *Gdm H* acts as an accessory factor for the ATP-binding cassette transporter *Gdm T* (Peschel *et al.,* 1997), *Gdm H* encode a membrane protein without similarity to proteins of known function. Homologous genes are lacking in all lantibiotic gene clusters except those of the epidermin and gallidermin determinants (Hille *et al.,* 2001).

Type A comprises screw- shaped, amphipathic molecular with masses of (2.164 to 3.488) dalton and with two to seven net positive charges, and these active peptides with the N-terminal consensus sequence –Try- Gly-Asn-Gly-Val-Xaa-Cys-(Abee, 1995).

Type B consists of more globular molecular with molecular masses of 1.959 to 2.041 dalton and with either no net charge or anet negative charge these protein complexes requiring two different peptides for activity. Most bacteriocins are biologically active single chin peptides. Some are only active as partners with a second peptide (Juan *et al.*, 2001).

Epidermin is member of a family of lantibiotics that bind to cell wall precursor lipid component of target bacteria and disrupt cell wall production. The duramycin family of lantibiotics binds phosphoethanolamine in the members of its target cells and seems to disrupt several physiological functions (Internet #I).

1.7.2 Non-Lanthionine or non lantibiotics:

Containing peptides, into three subclass on the basis of either their distinctive N-terminal sequence, their formation of biocomponent pores, or the presence of a functional sulfhydryl grouping and the molecular weight is low that become more evident(Abee, 1995) .

1.7.3 Bacteriocins with high molecular- weights and heat labile: that include many bacteriolytic extra cellular enzyme (hemolysins and muramidase), that may mimic the physiological activities of bacteriocins. The molecular weight are more > 200 compounds with a single SH residue that should be in a reduced form for the antibiotic to be active thiolbiotic.

1.7.4 Complex bacteriocins: that contains essential lipid or carbohydrate moieties in addition to protein, without cysteine residue (Jack *et al.*, 1995).

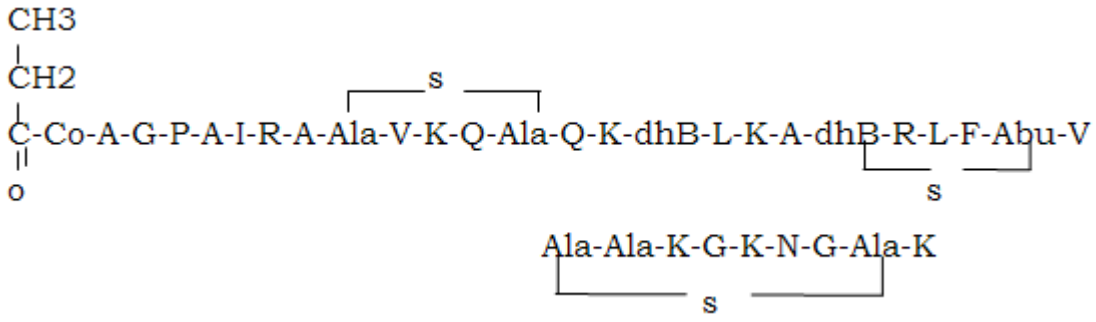
1.8 Type of staphylococcin:

There are many types of staphylococcin, such as Pep5, staphylococcin 1580 and epidermin (Sahl and Brandis, 1981).

Staphylococcin are producing by different of molecular weight. Some there are high (100.000-500.000) dalton,such as: staphylococcin C55,staphylococcin 414 and staphylococcin 462 (produced by *S. aureus*)other of staphylococcins are small of molecular weights (10.000-25.000)dalton (table1-1) (Tagg *et al.*,1976 and Sahl and Brandis., 1981).

Staphylococcin like peptide peP5 produced by *S.epidermidis* 5, it a small and strongly basic peptide positive charge, with a bacterial activity on members of the family Micrococcaceae (Sahl and Brandis, 1981).

It has been shown to cause a raid efflux of low-molecular weight (6000) dalton, compounds such as K+ ions, amino acids and ATP from the cytoplasm of sensitive cell (Sahl and Brandis, 1981; Ralph *et al*., 1995).



Abbreviations
Ala-S-Ala, lanthionine
Abu-S-Ala, 3-methyllanthionine

Figure (1-1): Pep5 positions of lanthionine and B-methyllanthionine formation through thioether linkage in several lanthionine

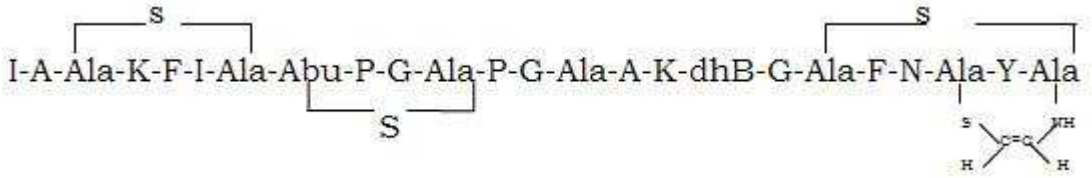
Pep5 have been shown to induce autolysis, of susceptible staphylococcal cells resulting in massive cell wall degradation, particularly in the area of the septa between dividing daughter cell , Pep5 contain from 8 amino acids positive charge, and 3 amino from lanthionine. The Pep5 biosynthetic gene cluster is encoded on the 18 kb plasmid (PED503) of *S.epidermidis* fig.(1-1) (Meyer *et al*., 1995; Pag *et al*., 1999; Hoffmann *et al*., 2004).

The staphylococcin 1580 produced by *S.epiermidis* **,**complex of chemical compounds (41.8% protein. 34% carbohydrates and 21.9% peptide).The molecular weight between(150.000-400.000) dalton ,and it's part by uses of urea 6 M or 5.1% SDS to small unit with molecular weights (10.000-25.000) dalton (Jetten and Vogels,1972).

Staphylococcin 1850 is effect in different types of Gram-positive bacteria special of *Staphylococci* spp.

Amino acids analysis, mass determination (2.165 dalton) and N-terminal sequencing (Lle-Ala-Xaa-Lys-Phe-Lle-Xaa-Pro-Gly-Ala-Lys-block), demonstrated that staphylococcin 1850 is identical to epidermin, a lanthionine containing antibiotic peptide (lantibiotic) fig.(1-2) (Sahl, 1993).

Epidermin produced by *S.epidermidis* is 21 amino acids peptide amide antibiotic, with high antimicrobial activity against pathogenic Gram-positive bacteria such as: *Proionbacterium acnes*, *streptococci* and hence is of therapeutic value in topical treatment of acne in humans and used to treat staphylococcal infection in mice (Schnell *et al*., 1988).



Abbreviations
Ala-S-Ala, lanthionine
Abu-S-Ala, 3-methyllanthionine

Figure (1-2): Epidermin positions of lanthionine and B-methyllanthionine formation through thioether linkage in several lanthionine.

Most of bacteriocins are produce from staphylococci are , heat constant that resist heat degree of 121°C with for 15 min. and contain 5% from serum (Mituis and Mizuno,1969 ;John *et al*.,1976).

Staphylococcin are maintaining their activities at pH (3.5-8.5), they are protein nature and effect with enzyme lysis proteins such as: trypsin,

pronease and chemotrypsine (Jetten and Vogle, 1972 and Ralph *et al.*, 1995).

Some staphylococcins are reducing their activity, when treated with non lysis protein enzyme such as: glycolytic, lipolytic those are improving their activity part of bacteriocins are different of chemical nature (Jack *et al*., 1995).

Table 1-1 Physic-chemical characteristic of Staphylococci bacteriocins

References	Enzyme		pH	Heat	Molecular weight	Bacteria	Bacteriocin
	Enzyme resistance	Enzyme sensitive					
Sahl, Brandis (1981)	-	Pronase Trypsin	8,5-4	Resist 100°C for 15 min.	1 x 10^5 more than	*S. aureus*	Staphyloc-occin C55
Hale and Hinsdill, 1973 Sahl, Brandis, 1981	Ficin	Trypsin Pronase	8	Resist 70°C more time.	1.25 x 10^4	*S. aureus*	Staphyloc-occin 414
Hale and Hinsdill, 1973 Sahl, Brandis, 1981	Lipase	-	-	Resist 70° C of 24 hr. sensitive 70°C for 3 hr.	9 x 10^3	*S. aureus*	Staphyloc occin 462
Sahl, 1986 Marianne *et al.*,	Lysozym-e	Trypsin Pronase	8,5-3,5	Resist 120°C for	2 x 10^4	*S. epiderm-*	Staphyloc-occin 1580

1988	Lysostap -hin	chymotr -ypsine		15 min.		*idis*	
Sahl, Brandis (1981)	-	Pronase Trypsin chemotr -ypsine	8-2	Resist 80°C of 1 hr. sensitive 121°C for 15 min.	6×10^3	*S. epiderm- idis*	Staphyloc- occin Pep 5

- No effect

Epidermin: is a tetra cyclic peptide produced and secreted by *S. epidermidis*. It is a member of the lantibiotic family, a group of mostly plasmid – encoded, ribosomally synthesized and post transitionally modified antimicrobial peptides that are secreted by *S.epidermidis* mainly act against Gram-positive bacteria. Lantibiotics interact with lipid- bound peptido-glycan precursors and form transient, potential-dependent pores in cytoplasm membranes of bacteria (Schnell *et al.*, 1988; Bruno and Montville., 1993; Breukin *et al.*, 1999).

They are characterized by the presence of the thioether amino acids lanthionine and 3-methyllanthionine (lanthionine – containing antibiotic peptides) and additionally contain α- β - didehydro amino acids (Sahl and Bierbaum, 1998).

The biosynthesis of epidermin from the precursor peptide *Epi A* involves a series of post-transnational modification reactions. In particular, the formation of the unsaturated amino acid S-[(Z)-2- amino vinyl] –D– cysteine (Allgaier *et al.*, 1986).

From ser 19 and the C-terminal cysteine residue cys 22 of *Epi A* has been studied in detail. The reaction involves addition of a (Z) - enethiol group

to the didehyfroalanine residue generated from Ser 19 by dehydration. Flavoprotein *Epi D* catalyses the formation of the reactive (Z) - enethiol group by oxidative decarboxylation of the C-terminal cysteine residue (Kupke *et al.,* 1994; Kupke and Gotz, 1997).

Epidermin (Epi) and Gallidermin (Gdm) are highly homologous tetra cyclic polypeptide antibiotic, but differ by only a single amino acid in the N-terminus (I-6 in Epi, L-6 in Gdm) (Ottenwalder *et al.,* 1995).

Gdm and Epi *e*xhibit activity against a variety pathogenic Gram-positive bacterium, such as staphylococci, streptococci and propionibacterium acens (Kellner *et al.,* 1988).

The maturation of Epi is terminated by extra cellular cleavage of the leader peptide by *Epi P*. (Geibler *et al.,* 1996).

The Epi structural gene (*Epi A*) and the genes encoding the modifying enzyme (*Epi B, Epi C*) are located in an operon on 54-Kilobase (kb) plasmid of *S. epidermidis* (Schnell *et al.,* 1992 and Augustin et *al.,* 1992).

Transcription of this operon is positively regulated by the regulator protein *Epi Q*, whose gene *Epi Q* is in orientation opposite that of the *Epi A*BCD operon and is contrans-cribed with *Epi P*, the gene that encodes the processing protease (Peschel *et al.,* 1993).

In contrast, *Gdm A* is chromosomally encoded in *S. gallinarum*. The structural genes *Gdm A* and *Epi A* exhibit a level of nucleotide sequence identity of 85% (Schnell *et al.,* 1989).

The characterization of a DNA region that mediates immunity and increased epidermin production, located upstream of the structural gene *Epi A*. the sequence of a 2.6 kb DNA fragment revealed three operon reading frames, *Epi F, E* and *G*, which may form an operon. *Epi F* is a hydrophilic protein with conserved ATP- binding sites, which *Epi E* and *G* have six alternating hydrophobic regions and very likely constitute the integral membrane domains. When *Epi F* was overproduced, it was at

least partially associated with the cytoplasm membrane. A potential mechanism for how *Epi FEG* mediates immunity is discussed (Augustin *et al*., 1992; Peschel and Gotz, 1996).

1-9 Biosynthesis and molecular genetic structure of epidermin:

Epidermin was characterized by the presence of the thioethre amino acid, lanthionine and 3-methyllanthionine (lanthionine – containing antibiotic peptides), and addionally contain α – β – dihydroamino acid (Fig. 1– 3) (Kellner *et al.,* 1989 ; Ottenwalder *et al*., 1995; Sahl and Bierbaum, 1998 and Pag and Sahl, 2002).

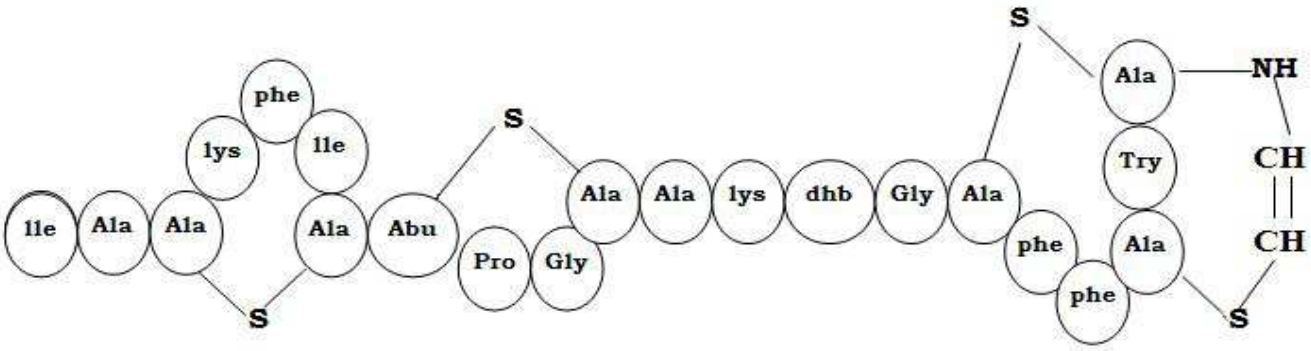


Figure (1-3) Epidermin structure (Pag and Sahl, 2002)

The biosynthesis of all lantibiotics proceeds from structural genes, which code for prepeptides which enzymatically modified to give the mature peptides. The genes involved in biosynthesis , processing , export…..etc , are found in gene clusters adjacent to the structural genes and code for transporters , immunity functions , regulatory proteins and the modification enzymes *Lan B*, *Lan C* and *Lan D* , which catalyze the biosynthesis of the rare amino acids. *Lan B* and *Lan C* are responsible for the dehydrobutyrine and subsequent addition of cysteine SH, group to the

dehydroamino acids, which results in the thioether ring (Bierbaum *et al.*, 1996).

In the biosynthesis of epidermin a further modification enzyme, *Lan D* is involved *(Epi D*), which is catalyzes the oxidative decarboxylation of the C-terminal Cys –residue to an S- aminovinal-D-cysteine (Kuple *et al*., 1992 and Kuple *et al*., 1996).

The gene cluster for the production of epidermin (Epi) is located on the 54-kb plasmid of *S.epidermidis*. The gene cluster consists of at least 5 operons *Epi ABCD, Epi Q, Epi T , Epi H , Epi FEG* (Augustin *et al* ., 1992 ; Schnell *et al* ., 1992 ;Peschel and Gotz , 1996 and Peschel *et al* ., 1997).

Epi A is contrascribed with *Epi B, C* and *D,* which are involved in the posttranslational modification of epidermin. The flavin mononucleotide containing enzyme *Epi D*, which is unquiet the epidermin system, has been recently shown to catalyze the oxidative decarbxylation of the C – terminus of pre-epidermin (Kupke *et al*., 1992; Schell *et al*., 1992 and Kupke *et al*., 1993).

Analysis of the genetic control of the epidermin operon has shown that *Epi A* is preceded by a promoter and followed by a terminator, while *Epi B*, lacking its own promoter, is contranscribed from the *Epi A* promoter. This suggests that the terminators is "leaky" and regulates down stream transcription (Augustin *et al*., 1992; Schnell *et al*., 1992; Peschel *et al*., 1999; and Kies *et al*., 2001).

Overall , two major transcripts have been found , one corresponding to *Epi A* and the other probably corresponding to full – length transcription of *Epi ABCD* (Peschel and Gotz , 1996).

The role of *Epi Q* in the biosynthesis of the lantibiotic epidermin was essential for epidermin production. It was shown that *Epi Q* controls epidermin production, by transcription activating the *Epi A* promoter,

used for transcription of most of the epidermin biosynthetic genes. Additional copies of *Epi Q* increased epidermin production in the epidermin production wild – type's strain of *S.epidermidis* (Hille *et al.*, 2001).

The epidermin transporter gene *Epi T* has been shown to be defective, since it is disrupted by a deletion causing a fame shift. The *Gdm T* gene of the closely related lantibiotic gallidermin from *S.gallinarum*, however, is intact and mediated a considerable increase of epidermin production, when cloned in epidermin producing strains. This effect was dependent on the presence of the adjacent gene, *Gdm H*, indicating that *Gdm H* acts as an accessory factor for the ATP binding cassette transporter *Gdm T* (Peschel *et al*., 1997 and Hille *et al*., 2001).

Epi H also contributes to immunity to epidermin. *Epi H* alone affected susceptibility to epidermin only moderately, but it let a multiplication of the immunity level mediated by the *FEG* immunity gene when cloned together with *Epi T* gene, suggesting a synergistic activity of the *H* and *FEG* system (Weil *et al*., 1990; Hille *et al.*, 2001 and Haffmann, 2004).

Epi D is uniquely found in the epidermin gene cluster – *Epi Q* and *Epi P* , are also located down stream from *Epi A* , but are in the opposite orientation to *Epi A* , *Epi B* , *Epi C* and *Epi D* , *Epi Q* shares homology with contain regulatory genes such as : *Pho B* in *Escherichia coli* , while *Epi P* is homologous to several serine protease (Augustin *et al.* , 1991 ; Schnell *et al.* , 1991 ; Augustin *et al.* , 1992).

The similarities have led to suggestions, that *Epi Q* may play a regulatory role in epidermin biosynthesis and, that *Epi P* may be involved in the cleavage of the 30 – amino acid leader from the epidermin molecule (Augustin *et al*., 1992; Peschel *et al.*, 1999 and Kies *et al*., 2001).

The plasmid – encoded epidermin biosynthesis gene, *Epi D* of *S. epidermidis* was expressed in *E. coli* by using both the *Mal E* fusion

system and T7 RNA polymerase promoter system (Kupke *et al*., 1992 and Kupke *et al*., 1995).

12N-terminal residues are highly related to nisin and subtilin, whereas the C-terminus is completely different, and contains the unsaturated S-amino vinyl – D-cysteine. The natural variants gallidermin, staphylococcin T, [Vall, leu6] - epidermin and mutacin B-Ny 266 as well as mutacin I and III. Show the same bridging pattern as epidermin. (Kellner *et al.,* 1988 ; Mota-Meira *et al.,* 1997 ; Furmane *et al.,* 1999).

Although 16 of the 22 amino acids of mutacin 1140 are identical to *epidermin*, the proposed secondary structure of mutacin 1140 different significantly in the N-terminal half (Hillman *et al.,* 1998).

A novel feature of the proposed mutacin 1140 structure is that the Cys-derived part of the diamino acid, while the Thr-derived part is located toward the C-terminus, which has not been previously observed for type **A** lantibiotic (Jack *et al*., 1995).

1-10 Mode of action of epidermin:

The low molecular – weights bacteriocins of the Gram – positive bacteria demonstrate bactericidal activity, which is directed principally against certain other Gram – positive bacteria (Tagg and Wannarnarker , 1976 and Kalchaya *et al.* , 1992).

The lantibiotic epidermin has, like nisin, been shown to have a concentration – dependent mode of action, which is also affected by physiological conditions such as: ionic strength, temperature and pH, as well as by the growth phase of the target cells (Sahl and Brandis, 1980).

Epidermin is bactericidal to sensitive cells of many Gram- positive bacteria and stable staphylococcal L–forms. The bacteriocin inhibited

simultaneously the synthesis of : deoxyribonucleic acid (DNA) , ribonucleic acid (RNA) , protein and polysaccharides , leading to speculation that treated cells no longer have sufficient energy to carry biosynthetic processes , and that the energy – transducing cytoplasm membrane may be the primary biochemical target (Sahl , 1985 ; Sahl *et al.* , 1985 and Sahl , 1991).

Furthermore, epidermin inhibited the transport of glucose, glutamic acid, rubidium ions and O – nitrophenyl – β – galctoside and exhibited no phospholipase activity (Jetten and Vogels, 1972).

Epidermin , seem to affect the membrane permeability barrier by forming water – filled membrane channels or pores , probably by a barrel – stave mechanism (Benz *et al.* , 1991 and Klaenhammer , 1993).

In addition to their membrane pore – forming capabilities, both epidermin and nisin have been shown to induce autolysis in many types of Gram – positive bacteria such as: *Staphylococcus simulans* (Benz *et al*., 1991 and Bierbaum and Sahl, 1993).

1-11 Mode of action of modified and unmodified bacteriocins from Gram-Positive bacteria.

The antibiotic activity of bacteriocins from Gram-positive bacteria, whether they are modified (class I) bacteriocins, lantibiotics or unmodified (class II), is based on interaction with the bacteria membrane.

However, recently work has demonstrated that for many bacteriocins, generalized membrane disruption models as elaborated for amphiphilic peptides (e.g. thyroidal pore or carpet model) cannot adequately describe the bactericidal action. Rather, specific targets seem

to be involved in pore formation and other activities. For the nisin and epidermin family of lantibiotics, the membrane bound cell wall precursor lipid II has recently been identified as a target. The duramycin family of lantibiotics binds specifically to phosphoethanolamine which results in inhibition of phospholipase A_2 and various other cellular functions.

Most of class II bacteriocins dissipate the proton motive force (PMF) of the target cell, via pore formation. The subclass (IIa) bacteriocin activity likely depends on a mannose permease of the phosphotransferase system (PTS) as a specific target. The subclass (IIb) bacteriocin two component also induce dissipation of the (PMF) by formation cation – or anion- specific pores; specific target have net yet been identified. Finely, the subclass (IIc) comprises miscellaneous peptides with various modes of action such as membrane permeabilization specific inhibition of sputum formation and pheromone activity (Kordel *et al.*, 1989 and Hechard and Sahl, 2002).

1-12 Molecular and genetic structure of epidermin:

Various Staphylococci produce small, highly modified peptides that exert antibiotic activity mainly against gram-positive bacteria. These lantibiotics, which have lanthionine as a characteristic component, are ribosomally synthesized as prepeptide and posttranslational converted into the mature peptides, the structure and prepeptide sequences of the following lantibiotics from various Staphylococcal strains have been determined (Gotz *et al.,* 1990) epidermin (Allgaier *et al.,* 1988 and Schnell *et al.,* 1988), gallidermin (Kellner *et al.,* 1988 and Schnell *et al.,* 1989), and Pep5 (Kaletta *et al.,* 1989 and Kellner *et al.,* 1989).

Additional lantibiotics are produced by a variety of microorganism, such as lactococci, bacilli and actinomycetes (Jung, 1991).

Epidermin is ribosomally synthesized, as shown by the cloning and sequencing of the structural gene, *Epi A* (Schnell *et al.,* 1988). Recently, it was shown that *Epi A* is the first gene of an operon comprising *Epi A, Epi B, Epi C* and *Epi D*. Two additional genes, *Epi Q* and *Epi P*, are transcribed in the opposite direction (Schnell *et al.*, 1992)

All genes are located on the 54-Kb plasmid of *S. epidermidis*. The involvement of the gene products in epidermin biosynthesis was verified by complementation analysis of *S. epidermidis* mutants (Augustin and Gotz, 1990 and Augestin *et al.*, 1992).

The characterization of a DNA region that mediates immunity and increased epidermin production, located upstream of the structural gene *Epi A*. the sequence of a 2.5-Kb DNA fragment revealed three operon reading frames *Epi F, E* and *Epi G*, which may form on operon. In the cloning host *S. carnosus*, the three gene mediated an increased tolerance to epidermin, and the highest level of immunity (Seven fold) was achieved with *S. carnosus* carrying *Epi FEG* and *Epi Q*. the promoter of the first gene, *Epi F*, responded to the activator protein *Epi Q* and a contained palindrome sequence similar to the *Epi Q* binding site of the *Epi A* promoter, which is also activated by *Epi Q*. Inactivation of *Epi F, E* or *G* resulted in the complete loss of the immunity phenotype. An epidermin sensitive *S. epidermidis* mutant was complemented by a DNA fragment containing all three genes. When the *Epi FEG* genes were cloned together with plasmid, containing the biosynthetic genes *Epi ABCDQP*, the level of epidermin production was approximately fivefold higher. The proteins *Epi F, E* and *G* are similar in deduced sequence and proposed structure to the components of various ABC transporter systems. *Epi F* is a hydrophilic protein with conserved ATP binding sites,

while *Epi E* and *G* have six alternating hydrophilic reasons and very likely constitute the integral membrane domains. When *Epi F* was overproduced in *S. carnosus*, it was at least partially associated with the cytoplasm membrane. A potential mechanism for how *Epi FEG* mediates immunity is discussed (Peschel and Gotz, 1996).

Epidermin is a well- characterized member of the lantibiotics, a group of peptide antibiotics that are distinguished by the presence of the rare amino acid lanthionine (Jung, 1991 and Sahl *et al.*, 1995).

The bactericidal action is mainly caused by pore formation in the cytoplasm membrane (Sahl, 1991). Epidermin, like all other lantibiotics found so far, is ribosomally synthesized. A 14-Kb DNA fragment, sufficient for low-level epidermin production, has been subcloned in *S. carnosus* (Augustin *et al.*, 1992).

The nucleotide sequence revealed the presence of seven genes that are organized in three transcriptional units (Schnell *et al.*, 1992).

Epi A is contranscribed with *Epi B, C* and *D*, which are involved in the posttranslational modification of epidermin. The flavin mononucleotide-containing enzyme *Epi D*, which is unique to the epidermin system, has been recently shown to catalyze the oxidative decaboxylation of the C-terminus of pre-epidermin (Kupke *et al.*, 1992 and Kupke *et al.*, 1994). *Epi B* and *Epi C* are proposed to catalyze the dehydration of serine and threonine residues and the formation of thioether bonds. Genes related to *Epi B* and *C* is involved in the biosynthesis of the lantibiotics nisin (Engelke *et al.*, 1992 and Kuipers *et al.*, 1993).

1-13 Separation and Purification of bacteriocins:

Many method of bacteriocin separation had been used from the bacteria produced it, such as precipitation by ammonium sulphate that separated from different types of bacteriocins that produced by staphylococcin at

concentration between 55-85% that used separated to the staphylococcin 1580 and epidermin and used to separate bacteriocin that produced from *S.aureus, S. simulaus* and *S. saprophytics* (Dajani and Wannaraker, 1973; Sebastiano *et al.*, 1982; Raplh *et al.*, 1995).

The other methods that separated is freezing and thawing after growth strain that produced it in semisolid media that used to produced bacteriocin from *S. epidermidis* and the ultrafiltration in strain that produced from *S. aureus* that used filter paper 0.45 Mm diameter, and the adsorption method that used ionic exchange that uses ionic exchange amberlite IRC-50 for isolated bacteriocin produced by many strain from staphylococcin (Jetten and Vogels, 1972; Jetten and Vogels, 1973 and Sebastiano *et al.*, 1982).

The Staphylococcin 414 produced from *S. aureus* 414 that released by using mechanical destroy method, and the staphylococcin 462 that produced from *S. aureus* 462 that not release from cell by mechanical destroy method, but release from treatment urea 7M. Staphylococcin 1580 that produced from *S. epidermidis* by treatment of 5% sodium chloride, and extraction of bacteriocins from *S. aureus* by treatment of acids (Gagkiano and Hinsdill, 1970; Jetten *et al.*, 1972 and Hale and Hinstill, 1973).

After separated bacteriocins that purified at used many and various method for purpose produced pure and active (Dajani and Wannamaker, 1969).

Bacteriocins that produced from staphylococci differ in their nature chemical and physical structures for it's. Many methods that purified, but most consist on concentrated of bacteriocin crude by precipitated at used acid, salt and ethanol or mixed from different solvent and after that used differs methods purification from chromatographic, ionic exchange, centrifugation and high pressure liquid chromatography (HPLC)… etc.

Most bacteriocins from type's lantibiotics are used purified by HPLC (Sahl and Brandis, 1981 and Sahl, 1994).

The methods that used in purification of bacteriocin production from staphylococci that supply on initial steps for purified, but the essential method that used HPLC (Sahl, 1985; Sahl *et al.*, 1985; Allgaier *et al.*, 1991 and Sahl, 1994).

The purification of staphylococci 414 by used centrifugation and gel-filtration chromatography by sephadex G-200, and purified staphylococci 1580 the same method, but addition that used of gel-filtration chromatography by used sephadex G-50. The epidermin is used same method and column chromatography and same column is sephadex G-50 or G-75 on produced high purity (Gagliano and Hinsdill, 1970 and Jetten and Vogels, 1972a).

While the purified of staphylococci 462 by used sephadex G-200 after electrophoresis that used Gel more acryclamids that contain SDS (SDS-PAGE) SDS- Poly- acryclamids Gel electrophoresis that the end step (Hale and Hinsdill, 1973).

The staphylococci C_{55} that used precipitation by 85% ammonium sulphate after that uses dialysis pH 7.2 and use Gel- chromatography by sephadex G-100 (Dajani and Wannaraker, 1973).

A significant improvement in the yield of the lantibiotics epidermin and gallidermin has been achieved by used of a two compartment fermented system. In this system, inner and outer chambers are separated by low-molecular weight cutoff dialysis membrane, which allows nutrient influx into the inner compartment in which the cells are growing and efflux of the bacteriocin into the outer chamber, from which the bacteriocins are subsequently purified. Both cells and high molecular weight protein are thus excluded from the starting material used for purification. With application of optimized media and strain selection, gallidermin recovery

was increased from less than 10 to as much as 720 mg/L, making purification large quantities of these bacteriocins possible (Ungermann *et al.*, 1991; Allgaier *et al.*, 1991).

The method is based on observations that the fully processed bacteriocin molecules:

I) Are excreted by the producer cell.

II) Are cationic.

III) Adsorb to the cell surface of the producer strains (and other Gram-positive bacteria).

IV) Adsorb in a pH dependent manner, high 90% at about pH 6 and low 1% at about pH 2.

V) Adsorb efficiently to heat- killed cells. As a result, a large- scale partial purification method was developed for three non lanthionine. Containing bacteriocins pediocin (ACH), Leuconocin (Lem 1) and sakacin **A** and the lantibiotic nisin (Van de Ven *et al.*, 1991).

Epidermin, which is produced and secreted into the medium by *S. epidermidis*, can be purified by reversed- phase high performance liquid chromatography (Fiedler *et al.*, 1985).

An antibacterial agent by *S. epidermidis* was isolated from culture Supernatants; its spectrum of activity is restricted to staphylococci micrococcus. The epidermin was purified to homogeneity by column chromatography on servachrome XAD-2; CM- sephadex C-25, sephadex G-50, and sephadex G-75. Chemical analysis showed it to be a peptide with a molecular weight of about 3000 dalton. Purified material was rapidly inactivated by trypsin, chymotrypsine and pronase and it was relatively heat- stable (Sahl and Brandis, 1981).

First attempts to purity the anti-staphylococcal of *S. epidermidis* by means of $(NH_4)_2SO_4$ Ammonium sulphate precipitation, DEAE- cellulose chromatography, isoelectric focusing and sephadex gel filtration were

unsatisfactory. The reason for this was the low concentration of epidermin in the culture supernatant (1-2 mg/ml) in relation to the high content of peptide which are components of the medium. However, we able to devise a very effective on the basis of two characteristic properties of epidermin found later during chemical analysis (Barrow, 1963 and Sahl and Brandis, 1981).

About 6% of the tested strains of *S. aureus* produced antagonistic substance against staphylococcal indicator strains. The production was low in liquid cultures and could not be induced by ultraviolet irradiation or by treatment with mitomycin C. the antagonistic substance could be classified into at least five groups on the basis of their properties and cross-resistance pattern. One groups consisted of lytic enzymes and the four others of staphylococcin. One of the four types of staphylococcin was active against *S. aureus* only, and the three other types had a broader inhibitory spectrum against Gram-positive organisms but not against Gram-negative bacteria. A relationship was found between some groups of producing strains and their phage type. The ability to produce staphylococcin was eliminated spontaneously upon storage and more rapidly by treatment with ethidium bromide, SDS, acridine orange and growth at 42°C. The resistance to several inorganic salts was Co-eliminated. No co-elimination of penicillinase production was observed. Selective effects during elimination were ruled out, and the results suggest that the genes for staphylococcin production are plasmid-borne determinants (Jetten and Vogels, 1972 and Jetten and Vogels, 1973).

1-14 Application of epidermin

Pep5 and epidermin bacteriocins were tested on clinical strains of *S.epidermidis* and *S. aureus* isolated from catheter-related infections. These bacteriocins were inhibitory to several isolates at a concentration of

640 activity units/ ml. The ability of bacteriocins in inhibiting adhesion of *S. epidermidis* to silicone catheters was evaluated. When Pep5 and epidermin were added to in vitro catheter colonization experiments, there was a significant decrease in the cell number of *S. epidermidis* adhered to silicone catheters. Bacteriocins used to decrease bacterial attachment to medical devices may represent a novel strategy to control catheter-related infections (Fontana *et al*., 2006)

Some lantibiotics are already used in medical therapy (nisin, gallidermin, epidermin) as well as for food preservation (nisin). Their special structures and synthesis have focused substantial scientific interest, the lantibiotics proving to be ideal experimental models not only for microbiology but also for biochemistry, molecular biology, gene technology and protein engineering in the purpose to get more insights in the fundamental aspects of the cell biology (Israil, 1992).

3-1 Isolation and identification of *S.epidermidis:*

Seventy skin samples (swab) were collected from patients in Al-Yarmook hospital, Al-Kadhymia hospital and Al- Wasti hospital during the period from 10/2004 to ٢/2005. Fifty samples were *S.epidermidis*, only five strains were produced epidermin by antibacterial agents test. Only one isolate which produced the highest level of epidermin was used in this study.

3-1-1 Morphology and culture characteristic:

Fifty isolates were subjected to the morphological and cultural characteristic study. Results showed that all these isolates shown a creamy yellow pigment colonies, which were 2-3 mm in diameter, on BHI agar at 37°C for 24hrs.Moreover, microscopically examination demonstrated grape like clusters of cells with Gram-positive bacteria, cocci, non-motile and non spore former.

S.epidermidis was grown in mannitol salt agar, which established from *S.aureus* by non ferment mannitol. They prefer aerobic environment, temperature of growth 37°C, range of pH (6.5 – 7.5), in the blood agar that not produced of hemolytic.

3-1-2 Biochemical and physiological characteristic:

Results in table (3-1) showed the biochemical and physiological tests performed on *S.epidermidis*. Results indicated that these isolates were negative to coagulase, oxidase but positive to catalase. Furthermore, it was unable to ferment mannitol aerobically, unable to produce DNase, and unable to produce hemolysin.

Morphological and biochemical characterization agreed with the data stated by Holt *et al*., (1994) and Atlas *et al*., (1995).

This isolates of *Staphylococcus* sp. belong to the species *Staphylococcus epidermidis*.

In addition to the above tests, biochemical identification is also done by API Staph system, which confirmed the previous conventional identification.

Table (3-1) Biochemical and physiological characteristics of *S.epidermidis:*

Bacterial isolates	Test	Result
Fifty *S. epidermidis*	Coagulase	-ve
	Catalase	+ve
	Oxidase	-ve
	Aerobic mannitol fermentation	Non-fermenter
	DNase production	-ve
	Haemolysis on blood agar	non-hemolytic

3-2 Antibiotic susceptibility of bacterial isolates

The disk diffusion method was used to determine the sensitivity of *S.epidermidis* isolates to several antibiotics, that antibiotic resistance among *S.epidermidis* isolates varied according to the nature of the isolates or antibiotics.

Result showed all *S.epidermidis* isolates were sensitive to Erythromycin, Tetracycline; Chloramphenicol while it's was resistance to Gentamicin and Streptomycin table (3-2).

Table (3-2) Antibiotic resistance of *S. epidermidis* isolates*:*

***S.epidermidis* Isolates (Fifty samples)**	**Antibiotics**				
	Erythromycin (E)	Streptomycin (S)	Gentamicin (CM)	Tetracycline (TE)	Chloramphenicol (C)
	S	R	R	S	S

3-3 Antibacterial activity of *S.epidermidis*:

S.aureus isolate was inhibited by the crude extract produced by *S.epidermidis* isolates. *E.coli* isolate was not inhibited. This confirms by the results obtained by (Jetten and Vogle, 1972a; John *et al*., 1976).
Epidermin don't have the same lytic activity like lysostaphin, and doesn't have the same spectrum of activity. Like other bacteriocins from Gram-positive microorganisms, it doesn't inhibit any Gram-negative bacteria, but dose inhibit several other genera of Gram-postive (Gaglino and Hinsdill, 1970; Patin and Richard, 1995).
Studies of the activity spectra of different bacteriocins against strains of the homologous and of heterologous species have aided differentiation between bacteriocins and have to lead the establishment of bacteriocin

typing procedures for use in epidemiological studies as specific marker properties of bacteria (Koninsk, 1982; Ralph *et al*.; 1995).

3-4 Determination of protein concentration:

Protein concentration was determined in the crude (supernatant filtrate), purification steps were carried out using the method of Bradford (1976), with Coomassie brilliant blue-250 using BSA fig. (3-1)

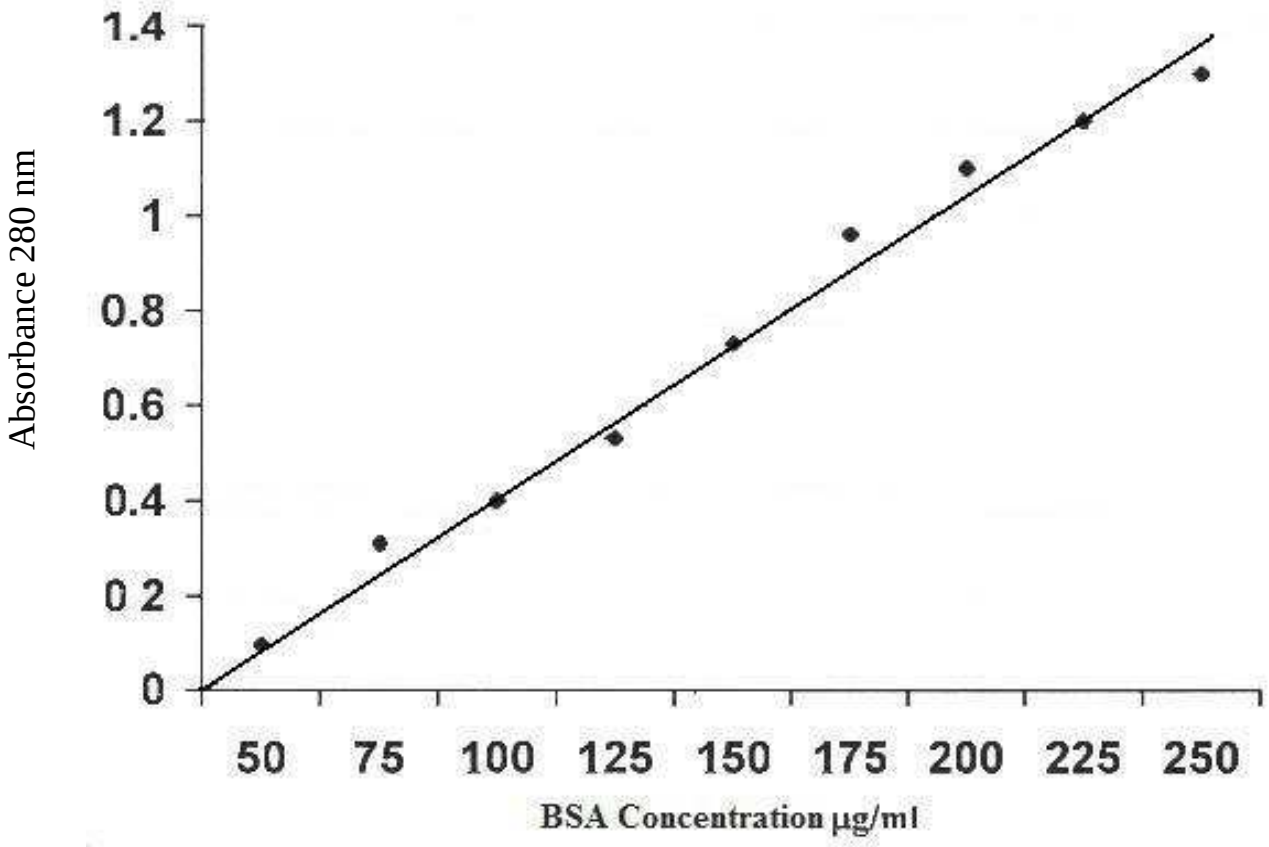


Figure (3-1): Standard curve of protein (BSA) concentration determination.

The epidermin concentration before purification is 120µg/ml, while after purification 160µg/ml.

3-5 Epidermin purification:

The purification procedure is summarized in table (3-3) and fig. (3-2) the crude supernatant was heated at 70°C for 15 min. before precipitation with ammonium sulfate to sterilize the supernatant and to inactivate any heat labile enzymes that might reduce the recovery of antibacterial substance during purification (Al- Ibadi *et al*., 2004).

The first step in epidermin purification was precipitation with ammonium sulphate. This step eliminated most of the contaminating proteins and removed water to obtain a partially purified epidermin. Gradual saturation of ammonium sulphate was used to define which saturation ratio was optimum for the precipitation of epidermin. Results showed that the epidermin activity was at it highest level at saturation ratio of (0-55%) which resulted after dialysis step against several changes 0.01M phosphate buffer pH 7.0 from the above result it can be noted that most of the epidermin. Protein precipitation using ammonium sulphate depends on the salting out phenomenon. Since ammonium sulphate has the ability to neutralize charges at the surface of the protein and to disrupt the water layer surrounding the protein, it will eventually cause a decrease in the solubility of the protein which in turn will lead to the precipitation of the protein by the effect of salt (England and Seifter, 1990).

Ammonium sulphate are widely used because of its availability, high solubility and low cost as well as other important feature of ammonium sulphate that it causes no damages to proteins (Volesky and Loung, 1985).

After being precipitation, the epidermin extract was dialyzed against distilled water for three continuous days with three successive changes.

The second step of purification was achieved by gel filtration through sephadex G-75.

The result shown in fig. (3-3) indicates the presence of two major peaks. Number of fraction (1-16) was pooled to represented the first peak; however other fraction (١٩-3٦) represented the second peak. Estimation of the epidermin activity in all eluted fractions showed that the first peak contains no epidermin activity, while epidermin activity was found in the second peak. The fraction of the second peak were then all collected and concentrated with sucrose.

Our method of purification seems to have accomplished in the final product, epidermin activity could be identified in the final product, and this result was previously reported by (Sahl and Brandis, 1981 and Al-Ibadi *et al*., 2000).

The final specific activity of pure epidermin was about 250-fold greater than that in the culture supernatant with a final recovery of 15%, these results were similar to those reported by (Patin and Richard, 1995) fig(3-2).

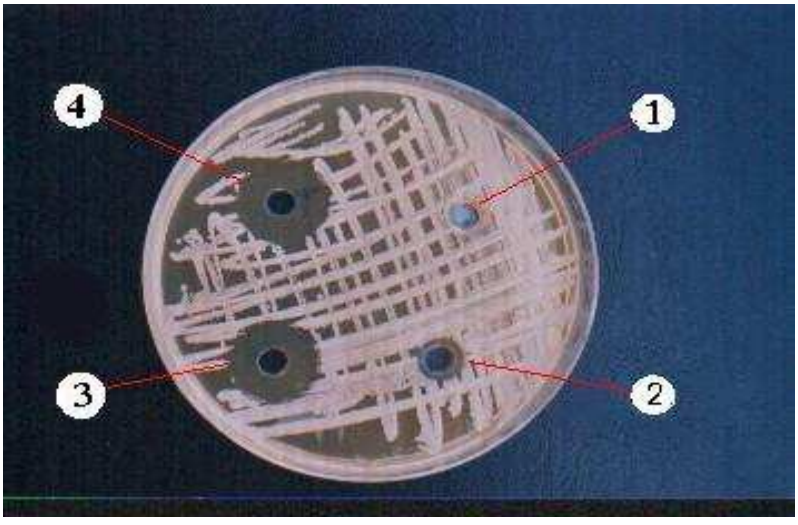


Figure (3-2): Epidermin activity during purification steps against *S.aureus*.

1- **Saline (negative control).**

2- **Crude epidermin.**

3- **Pure epidermin (dialysis step).**

4- **Pure epidermin (gel filtration).**

Table (3-3): Yield and specific activity of the epidermin at various stages of the purification procedures.

Stage of purification	Volume (ml)	Protein (mg/ml)	Activity* (Au/ml)	Total activity	Specific activity	Recovery (%)	Fold of purification
1 Culture supernatant fluid (crude)	500	16	20	10000	1.25	100	1
2 Ammonium sulphate precipitation	70	3.5	40	2800	1.33	28	~2
3 Dialysis against phosphate buffer	50	0.8	40	2000	50	20	40
4 Sephadex G-65 column	30	0.2	50	1500	250	15	200

*Antibacterial activity against *S.aureus*

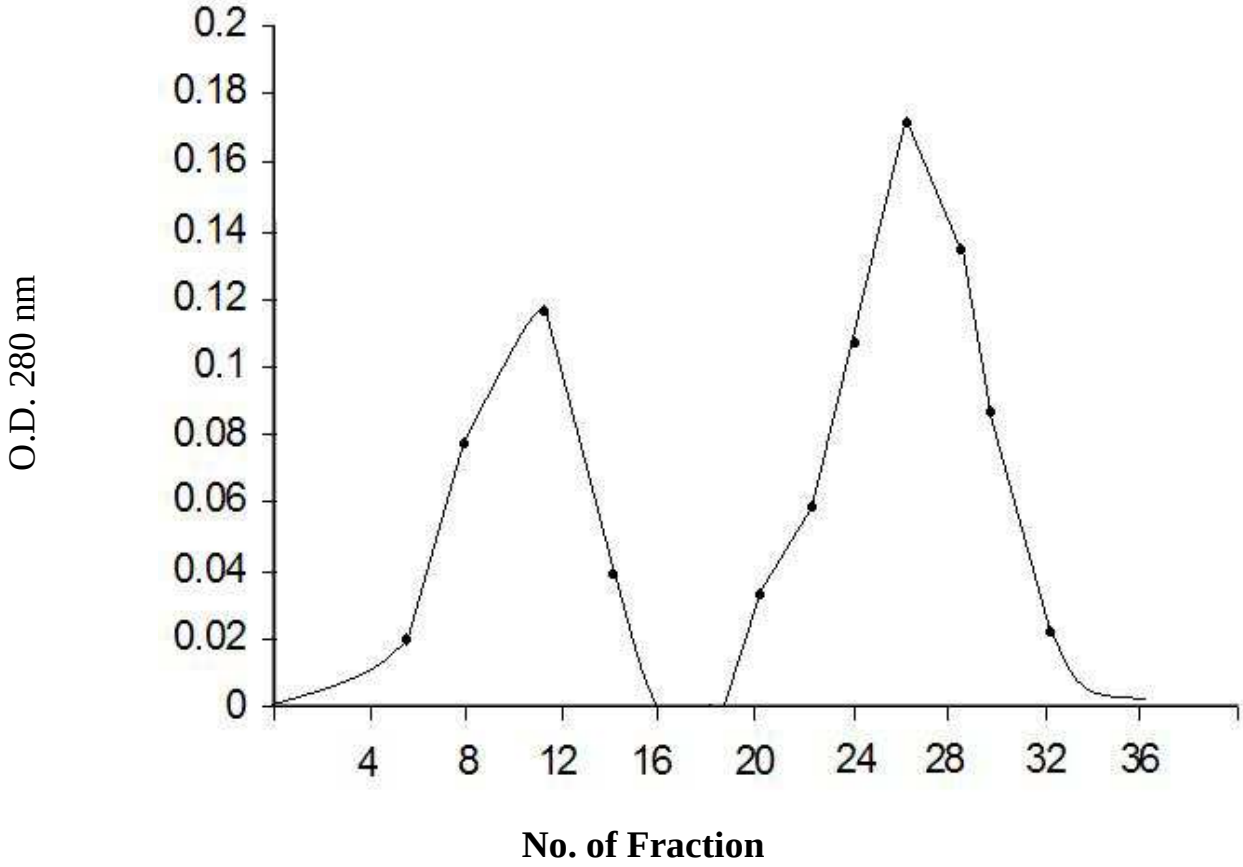


Figure (3-3): Gel filtaration chromatography on sephadex G-75 column (1.6x70) cm. Elution was achieved with 0.05M phosphate buffer pH 6.8-7, flow rate of 3ml/fraction (0.5ml/min).

3-6 Determination the effect of purified epidermin with some drugs and agents that used in treatment of wounds and burns:

Table (3-4) showed that the in vitro effect of purified epidermin on the antibacterial activity of some drugs and agents that used in treatment of wounds and burns.

Results showed, samacyclin was the most effective agent inhibiting, *S.aureus*. Nitfusin was the second, silverin, acetic acid 6% and iodine 10% were the least inhibitory respectively agents *S.aureus*. While epidermin was exhibit activity to those of acetic acid 6%.

Samacyclin has the greater inhibitory effect against microorganisms, because it has better aqueous solubility and skin permeability (Lee and Huang, 1995).

Silverin has been commonly used as a topical antimicrobial agent after burn injury (Fox *et al.*, 1990).Silverin activity due to the active compound (silver sulphadiazine).

Nitrofurazone is a synthetic antibacterial agent that is routinely used in the topical treatment of burn and wounds. It is active against a variety of Gram-positive and negative bacteria.

Table (3-4): Activity of full-strength and diluted agents with epidermin * against *S.aureus.* **

Agents (active compound)	Inhibition zone diameter (mm)of:	
	(100%) agent	(V/V) agent+epidermin
1- Samacyclin (Tetracycline HCL 3%)	35 39	37 40
2-Nifusin (Nitrofurazon)	20 25	26 27
3-Silverin (Silver sulphadiazine 1%)	15 18	23 26
4-Acetic acid 6%	14	14
5-Iodine 10%	9	12

*Inhibition zone diameter of pure epidermin =19 mm

** *S.aureus* = $1.5x10^8$ CFU/ml (colony forming unit).

The reason for the antibacterial activity of acetic acid may be due to the used in the concentration 6%.

Our results showed: Siliverin, Nifusin and theirs active compound diluted with epidermin produced mean zones larger than that produced by undiluted agents or as an epidermin only fig. (3-4).

This may be due to the ability of absorption of Silverin and Nifusin by agar (in vitro) or by skin or eschar (in vivo) during the treatment, and their ability had increased by using the epidermin compounds.

Samacyclin and acetic acid 6%, diluted with epidermin produced approximation zones them that produced by undiluted agents. This result probably due to the chemical structure and contents of these agents such as acids which interact with other compounds (protein and polypeptide) of epidermin and inhibited there's activity. The activity loss of bacteriocins (epidermin) upon treatment with acidic and alkaline pH values is a vane is likely to be due to denaturation of the protein or to loss of cofactor (Patin and Richard, 1995; Al-Ibadi *et al*., 2000).

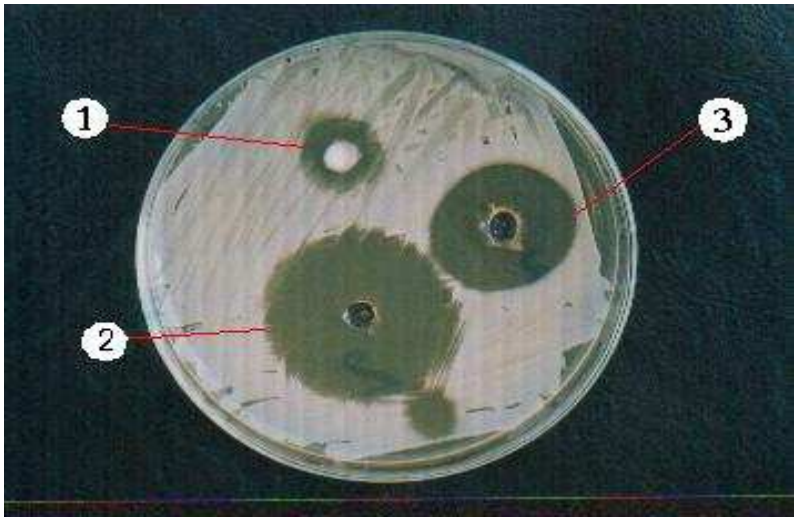


Figure (3-4): Synergistic effect of epidermin with agents (Silverin) against *S.aureus*.

1- Silverin

2- Epidermin +Silverin

3- Epidermin

Iodine 10% with epidermin was exhibited increase inhibition zone size.

Any antimicrobial agents used for burn or wound treatment should be tested in some manner for its ability to penetrate the skin or eschar.

Bacteriocins of staphylococci are bacteriocidal against Gram-positive bacteria, that include some associatation with wound and burns infections.

Staphylococcins have some limitation, which reduce their effectiveness as drugs treatment for wounds and burns infections. First, they are not effective against Gram-negative bacteria. Second, they are not effective against all Gram-positive bacteria and even in sensitive Gram-positive strains; there are a sensitive variant cell which can multiply in the presence of a bacteriocin. A previous paper described that bacteriocins can be combined to have a better antibacterial effect against Gram-positive bacteria (Mary *et al*., 1993).

Results of our study reports that epidermin and some agents (that used in wound and burns treatment), can be combined to have a better activity against *S.aureus.* The variants of activity may be due to the difference adsorption both epidermin and agents.

Agent may be associated with the mechanism by which epidermin enters the cell following binding to the cell surface. It has been proposed that following of binding a bacteriocin to the surface, the barrier functions of the cell well of sensitive cells are impaired. This disruption of cell wall functions allow other molecules (agents) to pass through the wall, come in contact with the cytoplasm membrane and destabilization its function (Ray, 1992).

The result presented here indicated that epidermin in combination with some agents will have a greater antibacterial activity against *S.aureus* and probably against other bacteria. This consideration will be important in the treatment of wounds and burns infection.

3-7 Plasmid isolation:

In order to determine the plasmid profile of *S.epidermidis* and *S. epidermidis* F isolates used two methods, the alkaline lysis and boiling method by (Maniatis *et al*., 1982), and second the salting out method described by same

Author which give accepted results.

Results were shown in the fig. (3-5) indicated that the isolates containing a small plasmid bands approximately in the same size compared with each other, and with pBR322 plasmid. As shown in fig. (3- 5) the plasmid bands were common in the isolates of clinical. (Prescott *et al.* , 1990).

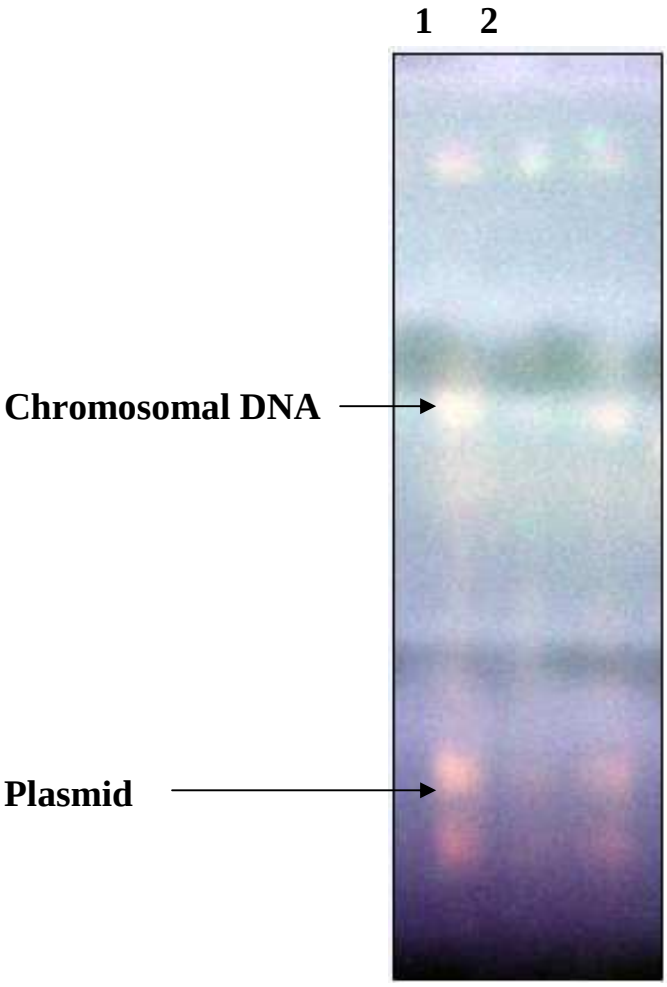


Figure (3-5): Gel electrophoresis pattern of isolates plasmid profile from *S.epidermidis* and *S. epidermidis* F migrated on agarose gel (0.7%) in TBE buffer at 5V/cm for 2 hr.
(1) Plasmid contained of crude isolate of *S.epidermidis F*
(2) Plasmid contained of isolate *S.epidermidis F*

3-8 The relationship between plasmid content of *S. epidermidis* and virulence factors:

Curing of plasmid by SDS and ethidium bromide:

Plasmids curing of bacterial isolates were used to determine whether the genes responsible for virulence factors [bacteriocins (epidermin) and antibiotic resistance] are located on the plasmid or not. For that, many attempts were made in order to cure *S.epidermidis* F plasmid (s) ethidium bromide. Isolate selected to perform curing experiment for their multiresistance to antibiotics.

S.epidermidis F isolate was selected to perform curing experiment, in which this isolate was epidermin production resistant for 2 antibiotics from 5 ones were tested (table 3-٢).

Results showed in table (3-5) indicated that the highest concentration of ethidium bromide that allows the bacterial growth, were 50 µg / ml. From this concentration appropriate dilutions were made and spread on BHI agar, and then 100 colonies were tested on a selective media containing a specific antibiotic (Gentamicin and Streptomycin) in order to determine the cured isolate, which cannot growth on this antibiotic. Also used the antimicrobial test to detect the production of epidermin.

Table (3- 5): Effect of ethidium bromide on the growth of *S.epidermidis* F isolate.

Bacterial isolate	Ethidium bromide concentrations (µg/ml)										
	0	20	50	100	200	250	300	400	800	1600	3200
	+	+	+ -	-	-	-	-	-	-	-	-

(+): Growth

(–): No growth.

(+ -) allow bacterial growth.

Number and percentage of cured strains obtained from treatment with ethidium bromide. This isolate was lost the resistance to, gentamycin, and streptomycin and epidermin productions.

These results indicate that Streptomycin and Gentamicin resistance genes and epidermin production gene are carried on same plasmids (one plasmids), because all the cured cells were lost both of the two antibiotics resistance and epidermin production.

Result in above declared that the plasmid code for 3 genes (epidermin, Streptomycin and Gentamicin).

The above results indicated that ethidium bromide is a powerful agent in eliminating antibiotic resistance plasmids and this agree with the data mentioned by other researcher (Bouanchaud *et al*., 1969).

Ethidium bromide has also been used to demonstrate the plasmid nature of the gene for bacteriocin synthesis (Jetten and Vogle, 1973)

Epidermin is produced by *S.epidermidis* which harbors the 54-kb plasmid, pTu32. The plasmid contains not only the epidermin structural

gene EpiA, but also flaking DNA region which is necessary for epidermin biosynthesis (Augustin *et al.*, 1992, Schnell *et al*., 1992).

Curing percentage (45%) was good and this may be attributed to several factors. Lerman, (1963) referred that the efficiency of curing generally varies from less than 0.1% to more than 99% depending upon the agent involved, the bacterial strain, and the conditions used and he assumed that curing activity is generally related to the ability of these compounds to intercalate into the DNA molecule.

2-1 Equipments and apparatus:

The following equipments and apparatus were used during this study:

Equipments	Company (Origin)
Autoclave	Tomy (Japan)
Balance	Ohans (France)
Compound Light Microscope	Olympus (Japan)
Refrigerated centrifuge	MSE (U.K.)
Distillator	GFL (Germany)
Oven	Sanyo (Japan)
Hot plate with magnetic stirrer	Gallenkamp (England)
Incubator	Termaks (U.K.)
Micropipettes	Witey (Germany)
Sensitive balance	Delta Range (Switzerland)
Vortex	Buchi (Swissrain)
Spectrophotometer	Miltonroy (USA)
Lyophilizor	Labconco (England)
pH meter	Mettler Toledo (U.K.)
Water Bath	Termaks (U.K)
Minimal electophoresis apparatus	Bio Red(Italy)

2-2 Chemicals:

The following chemicals were used during this study:

Material	Company (Origin)
Chloroform, NaOH, $BaCl_2$, H_2SO_4, Bovin Serum Albumin (BSA); Ethanol 95%, Crystel violet, Iodine, Safranin, Phosphoric acid 85%, Na_2HPO_4, NaH_2PO_4.	BDH- England
Glycerol	Difco – USA
Sodium dodoecyl sulphate (SDS), Coomassie brillant blue G-250, Ethylen diamine tetra acetic acid (EDTA), Ammonium sulphate, Tris (hydroxyl methyl).	Fluka – Switzerland
Agar	Himedia – India
Boric acid	Riedel – Dehaeny- Germany
Ethidium bromide, Agarose	Sigma – USA
Ethanol	Iraq

2-3 Antibiotics:

The following antibiotic discs were used in this study:

Antibiotic	Disc potency (µg)	Company (Origin)
Chloramphenicol (C)	30	Al-Razzi (Iraq)
Erythromycin(E)	15	Al-Razzi (Iraq)
Gentamicin(CN)	10	Al-Razzi (Iraq)
Streptomycin(S)	10	Al-Razzi (Iraq)
Tetracycline(TE)	30	Al-Razzi (Iraq)

2-4 Bacterial Isolates:

Bacterial isolates	Source
Staphylococcus epidermidis	Central Hospital of Childhood, Al-Kadhymia Hospital, Al-Wasitee Hospital and Al-Yarmook Hospital
Staphylococcus aureus	Central Hospital of Childhood
Escherichia coli	Central Hospital of Childhood

2-5 Drugs used in wound and burn treatment and mixing with epidermin:

Drugs/chemicals	Active substances	Source
Acetic acid 6%	_	Iraq
Iodine 10%	_	Iraq
Nifucin	Nitrofurazon	GDR
Samacyclin	Tetracycline HCL 3%	Samarra/Iraq
Silverin	Silver sulphadiazine 1%	Jordan
Soframycin	Framycetin Sulphate + Garamicidin	Ireland

2-6 Media (Ready made culture media):

These media were prepared as recommended by the manufacturing companies; pH was adjusted to 7.0, autoclaved at 121°C for 15 minutes.

2-6-1 Brain Heart Infusion Agar (Difco USA).

2-6-2 Brain Heart Infusion Broth (Difco USA).

2-6-3 Nutrient Broth (Biolif – Italy).

2-6-4 Nutrient Agar (Oxoid).

2-6-5 Tryptic Soy Broth (Oxoid).

2-6-6 Mannitol Salt Agar (Biolife)

2-7 Prepared cultured media

2-7-1 Blood agar media (Atlas *et al.*, 1995)

Blood agar medium composed of blood agar base 40 g dissolved in 950 ml of distilled water, pH was adjusted to 7.0 and sterilized by autoclaving at 121°C for 15 min. ,cooled to 45°C ,then 50 ml human blood was added and mixed well. This medium was used for the detection of hemolysis type produced by *Staphylococcus* sp.

2-8 Stains:

- **Gram's Stain (Atlas *et al.*, 1995).**

It's composing of 4 regents:

- A primary stain – Crystal Violet.
- A mordant – Gram's Iodine Solution.
- A decolorizing agent – an organic solvent (alcohol 95%).
- A secondary stain or counter stain – Safranin.

This stain was prepared dissolving 2 g of crystal violet in 20 ml of 95% ethanol and the final volume was completed to 100 ml with distilled water and filtered before use.

- **Safranin Counter Stain (Atlas *et al.*, 1995).**

This stain was prepared by dissolving 0.25 g of safranin *O* in 10 ml of 95% ethanol and the final volume was completed to 110 ml with D.W. allow to stand several days and filter before use.

- **Coomassie Brilliant blue G-250 (Bradford, 1976).**

This stain was prepared by added 0.01 g from stain to the 5 ml of ethanol 95% and 10 ml from phosphoric acid that concentration of(85%) and final volume was completed to 100 ml with D.W.

2-9 Buffers and Solution:

2-9-1 Phosphate buffer 0.01M pH 6.8-7.0

Na_2HPO_4 = 9.52 g, $Na_2H_2PO_4$ = 6.00 g

Dissolved in 1000 ml and Sterilized by autoclaving at 121°C for 15 min. and prepared 0.05M phosphate buffer pH 6.8

2-9-2 TE buffer (Maniatis *et al.*, 1982)

Comprised of 1 mM EDTA and 10 mM Tris-HCL, pH was adjusted to 8.0 then sterilized by autoclave at 121°C for 15min.

2-9-3 Tris-HCL (15 m M, pH 7.0)

It was prepared by dissolved 0.181 g Tris-HCL in 100 ml distilled water, then pH was adjusted to 7.0 and sterilized by autoclaving at 121°C for 15 min.

2-9-4 SET buffer (Maniatis *et al.*, 1982)

Comprised of 75 mM NaCl and 25 mM EDTA, and 20 mM Tris-HCL, pH was adjusted to 8.0, and then sterilized by autoclave at 121°C for 15min.

2-9-5 NaCl solution (5 M) in distilled water (Tobias and kieser, 1995).

2-9-6 **Sodium Dedecyl Sulphate solution (SDS)** (Tobias and kieser, 1995).

It was freshly prepared by dissolving 10 g SDS in 100 ml sterilized D.W.

2-9-7 Lysozyme solution (Tobias and kieser, 1995).

This was prepared by dissolving 50 mg lysozyme in 1 ml of sterile D.W., this prepared freshly.

2-9-8 Tris-Borate- EDTA (5X) (TBE) pH 8.0 (Maniatis *et al.,* 1982)

Tris- base	54 g
Boric acid	27.5 g
EDTA (0.5 M)	20 ml
D.W. to 1000 ml.	

2-9-9 **Ethidium Bromide Solution (10 mg/ml)** (Bounchaud *et al.,* 1969)

It was Prepared by dissolving 0.2 g of ethidium bromide in 20 ml distilled water and stirred on magnetic stirrer for few hours to ensure that the

ethidium bromide has been dissolved then it was filtered, and stored in a dark bottle at 4°C.

2-9-10 Bovine Serum Albumin (BSA) stock solution:

Bovine Serum Albumin (BSA) stock solution was prepared by dissolved 10 mg of (BSA) in 5 ml of D.W.

2-10 Reagent

2-10-1 Catalase reagent (Atlas *et al*., 1995)

This reagent composed of 3% of hydrogen peroxide.

2-10-2 Oxidase reagent (Atlas *et al*., 1995)

One gram of tetramethyl-*p*-phynlene-diamine dihydrochloride was dissolved in 100 ml of D.W. and kept in dark bottle in refrigerator.

2-11 Api Staph kit (Api Bio Merieux,Lyon,France)

Api Staph kit consists of:

A- Galleries: The gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients

B- Api Staph reagents

VP1, VP2, N1T1, N1T2, ZYMA, ZYMB.

-VP1 (40% potassium hydroxide).

-VP2 (5% alpha-naphthol).

-N1T1 (sulfonilic acid).

-N1T2 (N, N-dimethyl-naphthylamine).

-ZYMA (Tris-hydromethyl-aminomethane).

-ZYMB (Fast blue BB).

Methods:

2-12 Collection of sample

Seventy skin samples (swab) were collected from patients with burn and wound infections of Al-Wasitee hospital, Al-Kadkimia hospital and central of childhood hospital, during the period from October/2004 to Febreuary/2005,

2-13 Maintenance of bacterial isolates:

Maintenance of bacterial isolates was performed according to (Maniatis *et al.*, 1982 and Atlas *et al.*, 1995) as following:

2-13-1 Short- term storage

Colonies of bacteria were maintained for periods of few weeks on the surface of BHI agar media, the plates were tightly wrapped in parafilm and stored at 4°C.

2-13-2 Medium – term storage:

Bacterial isolates were maintained by streaking on slants of tryptic soy agar or nutrient agar medium for periods of few months. Such medium was prepared in screw – capped vials containing 10-15 ml of the medium. The isolate was streaked on these slant media and incubated at 37^{o}C for 24hr., after that, the slants were taken and wrapped with parafilm and stored at 4^{o}C.

2-13-3 Long – term storage:

Bacteria can be stored for one year in either nutrient broth or brain heart infusion broth medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacteria in a small screw capped vials with final volume of 10 ml and stored at –20^{o}C.

2.14 Measurement of bacterial growth:

Growth of bacterial was monitored by MacFarland tube No. 5 turbidity standard (which prepared by adding 0.6 ml of 0.048 M $BaCL_2$ [1.175% w/v $BaCl_2$ $2H_2O$] to 99.5 ml of 0.36 N H_2SO_4). And which is equivalent to bacterial concentration for inoculums 1.5 x 10^8 organisms/ml.

2-15 Identification of bacterial isolates (Holt *et al*., 1994; Atlas *et al*., 1995).

2-15-1 Morphological and cultural characteristics

Morphology of colonies was studied on Brain Heart Infusion BHI agar. Color, shape, size and edge of colonies were recorded after 24 hrs. of incubation at 37°C.

Gram's stain

A single colony was transferred by a loop to a clean slide.

The smear was stained with crystal violet, treated with iodine, decolorized with 95% alcohol and counterstained with safranin, then examined by a microscope.

2-15-2 Biochemical and physiological characteristics:

2-15-2-1 Catalase production test (Maza *et al*., 1997)

This test was performed by adding (2-3) drops of hydrogen peroxide (H_2O_2) 3% on a single colony grown on BHI agar plate. Appearance of bobbles was regarded as positive result.

2-15-2-2 Oxidase production test (Harely and Prescott, 1996)

Filter paper was saturated with substrate (tetramethyl-P-phenylene-diamine-dihydrochloride), colony of bacterial isolate to be test was rubbed on the filter paper with the sterile wooden applicator stick. Immediate color changes to deep blue indicate a positive result.

2-15-2-3 Coagulase production test

Plasma diluted 1:10 in a normal saline (0.85% NaCl) was prepared and placed in a small tube. The bacterial isolate under test was inoculated into the tube, by adding 0.1 ml of overnight culture. The tube was incubate at 37°C and examined for coagulation after 1, 3 and 6 hrs. The tube that showed negative result was left at room temperature overnight and reexamined. The conversion of the plasma into soft gels was observed by tilting the tube to a horizontal position.

2-15-2-4 DNase Production Test (Colle *et al.*, 1996)

DNase agar plate containing toluidine blue (100mg/ml) indicator, was inoculated with the bacterial culture, and then incubated at 37°C for 24-48 hrs. Development of a rose pink halo surrounding the areas of bacterial growth indicated the hydrolysis of DNA.

2-15-2-5 Hemolysis patterns on blood agar

A single colony of overnight growth culture was streaked on blood. The type of hemolysis produced by growing colonies was observed and recorded.

2-15-2-6 Mannitol fermentation test

The bacterial isolate was streaked on the plate of mannitol salt agar and incubate overnight, fermentation of mannitol from red to yellow indicate producing of acid from mannitol.

2.16 Identification by using Api staph (Overman *et al*., 1985):

2.16.1 Preparation of galleries

Five ml of tap water dispensed into incubation try provide a humid atmosphere during incubation.

2.16.2 Preparation of bacterial suspension

By using sterilized loop, a well-isolated colony from plating medium was picked. The inoculum was emulsified in 5ml suspending medium (Api Staph. Media) by rubbing against the slide of the tube and mixed thoroughly with the water.

The biochemical reaction performed by the Api Staph. And their interoperations are listed in below:

Test	Substrate	Reaction/Enzymes	Results	
			Negative	Positive
0	No substrate	Negative control	Red	___
GLU FRU MNE MAL LAC TRE MAN XLT MEL	D-Glucose D-Fructose D-Mannose Maltose Lactose D-Trehalose D-Mannitol Xylitol D-Melibiose	(positive control) Acidification due to carbohydrate utilization	Red	Yellow
NIT	Potassium nitrate	Reduction of nitrate to nitrite	NTT 1 +NIT 2/10 min.	
			Colorless-light pink	Red
PAL	ß-naphtyl-acid phosphate	Alkaline phosphatase	ZYM A + ZYM B /10 min.	
			Yellow	Violet
VP	Sodium pyruvate	Acetal-methyl-carbinol production	VP 1+VP 2 /10 min.	
			Colorless	Violet - pink
RAF XYL SAC MDG NAG	Raffinose Xylose Sucrose α- methyl-D-glycoside N-acetyl-glucosamine	Acidification due to carbohydrate utilization	Red	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Orange-Red
URE	Urea	Urease	Yellow	Red-Violet

2.17 Antibiotic Sensitivity Test (Atlas *et al.*, 1995):

A sterile cotton swab was dipped into the inoculums of freshly culture 18 hrs. $1.5x10^{8}$ cell/ml of *S.epidermidis* isolates and the entire surface of the mullar hinton agar plates was swabbed three times by rotating the plate approximately 60 mm between streaking to ensure even distribution, then the discs of antibiotics were applied and incubated at 37^{o}C for 24hr. The diameter of the zone of inhibition (clear area around disks) indicates the sensitivity of bacteria to that antibiotic, and the results were compared with NCCLs (2002).

2.18 Antibacterial activity of *S.epidermidis*: (Nathan *et al.*, 1978)

Crude culture of *S. epidermidis* isolates were tested for inhibitory activity towards the target isolates (*S.aureus* and *E.coli*), following the deferred antagonism procedure (agar spot test).

Overnight culture of *S. epidermidis* isolates were spotted onto the surface of agar plates BHA, and incubated for 24hrs. at 37°C to allow colony development.

Approximately 0.1ml of 1.5x10 cells/ml of the target isolates (*S. aureus* and *E. coli*), were incubated onto 7ml of an appropriate soft agar (0.7% w/v), and poured over the plate onto which the producer *S. epidermidis* had been grown. The plates were checked for inhibitory zones (inhibition was scored positive if the zone was wider than 2mm).

2.18.1 Titer of protein determination:

The titer of epidermin activity was determinate by using the serial two fold dilutions of samples (crude and pure bacteriocin). The titer of epidermin activity was defined as the reciprocal of the dilution after the

last serial dilution giving a zone of inhibition and expressed as activity units (Au) per milliliter (Patin and Richard, 1995).

2.19 Extraction of epidermin from *S.epidermidis* (Jetten and Vogle, 1972)

According to the results of inhibitory activity, this isolates were selected which had higher epidermin, one of them which had higher activity was and used for this study called *S. epidermidis* F. *S.epidermidis* F was grown in semi solid medium consisting of 0.4% agar and trypticase soy broth, then incubated at 37^{o}C for 48hrs. After that place at –20^{o}C for 4hr. then dissolved collected medium and centrifuged at 18000 rpm for 10 min. After cooled centrifugation, the supernatant were taken which is crude staphylococcin (epidermin).

2.20 Determination of protein concentration (Internet#4):

Determination of Protein concentration was performed to (crude and pure samples) as originally described by Bradford (1976). And as following:

1- Several dilution of standard protein (BSA) was prepared from BSA stock solution in the same buffer and according to volumes.
2- Added 0.5 ml from sample and 0.5 ml BSA to each tubes, after that it's added 4.5ml from coomassie brilliant blue G-250(0.01%) to each dilution and left to stand for 2min. at room temperature.
3- The absorbance at 580 nm was measured: the blank was prepared from 0.45 ml of the buffer and 4.5 ml of the dye reagent.

4- A standard curve was plotted between the amounts of protein corresponding absorbance of the standard protein. The protein concentration of unknown samples was calculated from the standard curve. This determination was used for all purification steps.

2.21 Purification of epidermin (Jetten and Vogle, 1972; Valisena *et al.*, 1982; Sebastiano *et al.*, 1982 and AL-Ibadi *et al.*, 2000):

Absorbance 280

The crude of epidermin (containing antibacterial activity) was treated with (NH4)2So4 [(0-55%) saturated solution pH7.0]. The resuspended ammonium sulfate precipitate was transferred into dialysis tube and dialysis at 4°C against several changes 0.01M phosphate buffer pH 7.0, and antibacterial activity was determined (by using agar diffusion method described by Nathan *et al.*, 1978).

The partial purified of epidermin was lyophilized by the lyophilizing system.

The third step of purification was done by gel filtration chromatography on sephadex G-75 column. [Sephadex G-75 resin after activation was packed in a column (1.6x70) cm and equilibrated with 0.05M phosphate buffer pH 6.8]. About 5ml of the suspension lyophilized sample (4mg/ml) was applied to the column. Elution was performed with 0.05M phosphate buffer pH 6.8 at flow rate 3ml/fraction (0.5ml/min.).

The fraction were read at 280 nm and fractions assayed for inhibitory activity. The position fractions were collected and concentrated with sucrose. This purified substance was stored at -20°C.

2.22 Determination of the antibacterial of purified epidermin with drugs used treatment of wounds and burns (Nathan *et al*., 1978):

Both the activity of drugs (that used in treatment of wounds and burns), and the effect of purified epidermin with these drugs were determined by using modified agar diffusion method.

Commercially available 60mm Petri dishes containing BHA were inculated with (0.1ml of 1.5x10 CFU/ml) target isolate of *S. aureus* by using sterile swabs. Three evenly spaced holes 3mm in diameter were made in the agar of each plate with sterile cork borer. To identify the intrinsic drugs activity of the diluents, two control wells were filled with epidermin, drug alone (100µl) and saline respectively. Test well contained 50% of epidermin +50% of drug. An equal volume of each agent was expressed into each well (two replica plates were prepared for each agent).Test plates were then incubated at 37°C for 24hrs. and zones of inhibition were measured using a ruler in millimeter inhibition zone. A clear area test well indicates that the agent had retained its antibacterial activity. This method was repeated to each drugs and chemical (Silverin, Samacyclin, Nifusin, Iodine 10% and acitic acid 6%).and there's active compound (Silver sulphadiazine 1%, Tetracycline HCL 3% and Nitrofurazon) respectively.

2.23 DNA Extraction:

DNA extraction was done by salting out method which described by Pospiech and Neumann (1995)

-Culture of *S.epidermidis* F and *S.epidermids* grown in a flask contained 100 ml BHI broth, number of bacteria was determined by using

MacFarland tube ($1.5x10^8$) which was pelleted from 20 ml by centrifugation at 6000 rpm for 15 min.

-The pellet washed with 3 ml of SET buffer and resuspended the cells with 3 ml SET buffer, then freshly prepared lysozyme (final concentration 1 mg/ml) was added, and incubated at 37°C for 30 min.

-Added 2 ml of 10% SDS, mixed by inversion, and then incubated at room temperature for 15 min.

-Added 2 ml of NaCL 5M, mix thoroughly by inversion.

-An equal volume of chloroform was added, mixed by inversion by for 15 min then centrifuged (6000 rpm at 4°C) for 20 min.

-The aqueous phase (upper) was transferred to another sterile tube, and 0.6 ml of DNA volumes of isopropanol was added, mixed by inversion, and kept at room temperature for 5 min.

-Cooled centrifuged at 13000 rpm for 15 min at 4°C.

-The isopropanol was discarded and the precipitated DNA dissolved in 1 ml TBE buffer and stored at –20°C.

2.24 Agarose Gel electrophoresis (Maniatis *et al*., 1982)

Agarose gels (0.7%) were run horizontally in Tris- borate EDTA buffer (TBE 1X). Samples of DNA were mixed with 1/10 volume of the loading buffer and added to the wells in the gel. Generally, gels were run for 2-3 hrs. at 5 v/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gels were stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by U.V. illumination at 302 nm on an U.V. transilluminator. Gels were distained in distilled water for 30-60 min to get red of background before photographs were taken.

2.25 Curing of Plasmid DNA

By using Ethidium Bromide:

Curing experiment was performed on epidermin production *S.epidermidis* F by using of ethidium bromide in these experiments according to Trevors (1986) and as follows:

Cells of the selected isolated were grown in 5 ml of BHI broth to mid log phase ($O.D_{600}$ ~ 0.4-0.5). A 0.1 ml of each culture were inoculated in a series of 5 ml fresh BHI broth tubes containing various concentration of ethidium bromide (0, 10, 20, 50, 100, 200, 250, 300, 400, 800, 1600 and 3200 µg/ml). All tubes were incubated at 37^{o}c for 24-48 hrs. The growth density of the different tubes was measured visually and compared with the control to determine the effect of each concentration as curing agent. The lowest concentration of curing agent that inhibited the growth of the bacterial isolated was considered as the Minimum Inhibitory Concentration (MIC).

Samples were taken form tubes containing the highest concentration of ethidium bromide that still allows bacterial growth and diluted appropriately. Then 0.1 ml samples from suspected dilutions were spread on brain heart infusion agar plates and incubated overnight at 37^{o}C to score the survived colonies.

The survivors were analyzed for the presence or absence of antibiotic resistance and antagonism, as a result of eliminating the plasmid by selecting 100 colonies of from bacterial that produce epidermin. These colonies were cheked for its ability epidermin and antibiotic sensitivity. If colonies were inhibited the growth of bacterial and lose resistance to this antibiotic, it means that cells of this colony are cured cell.

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
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Date:
(Chairman)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member/Supervisor)

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Scientific Degree:
Date:
(Member/Supervisor)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr.Laith Abdul Al-Aziz Al-Ani**.

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

Supervisor Certification

I certify that this was prepared under my supervision in Al-Nahrain University-College of Science as a partial fulfillment of degree of master of Science in Biotechnology.

Signature:
Supervisor:
Date:

Signature:
Supervisor:
Date:

In review of the available recommendation I forward this thesis for debate by examining committee.

Signature:

Name:

Chairman of Biotechnology Department

Date:

Conclusions:

1. Fifty isolates of *S. epidermidis* were produced epidermin, and showed antibacterial activity against *S. aureus*, *E. coli* was not inhibited. S.epidermidis F isolate was the most efficient activity.

2. Epidermin of *S. epidermids* F isolate was extracted and purified. The purified epidermin was obtained a concentration 0.2 mg/ml. with activity 50 Au/ml.

3. Purified epidermin showed synergistic effect with some drugs and agents that used in treatment of wounds and burns.

4. Plasmid profile was determined for all *S. epidermidis* isolates. Results showed gene encoding for epidermin are carried on plasmid, and this gene codes for antibiotics resistance gene also.

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Chapter Three: Results & Discussion

الاهداء

الى روح المصطفى والرسول الاعظم(ص)

الى سندي في الحياة ، الى الذي منحني الثقه بالنفس وحثني على المثابره.

والدي

الى نبع الحنان ، الى فخري واعتزازي ، الى التي كانت عونا ورفيقا لي.

والدتي

الى ذخري ، الى الذي ناصرني واخذ بيدي لاكون فاتحه لمستقبله الزاهر.

اخي

الى صديقتي ورفيقة طفولتي وصباي ، نبراسي في دروب العلم.

اختي

اقدم ثمرة جهدي المتواضع هذا

فرح

Introduction

Staphylococcus epidermidis is a Gram-positive spherical pathogenic bacteria that occur in microscopic clusters resembling grapes and are common in medical device-associated, *S.epidermidis* is an inhabitant of the skin and is non hemolytic. They are facultative anaerobes that grow by aerobic respiration or by fermentation (Foster, 2005).

S.epidermidis bacteria is responsible for a growing number of infections among hospital patients whose immune systems are weakened. Such infections often start at skin wounds caused by catheters. Little is known about mechanisms of pathogenesis of *S. epidermidis* infections. A characteristic of many pathogenic strains of *S. epidermidis* is the production of a slime resulting in biofilm formation. The slime is predominantly a secreted teichoic acid, normally found in the cell wall of the *staphylococci*. This ability to form a biofilm on the surface of a prosthetic device is probably a significant determinant of virulence for these bacteria (Internet # VI).

S. epidermidis is very likely to contaminate patient-care equipment and environmental surfaces, possibly explaining the high incidence of *S. epidermidis* in hospital settings. While there is extensive information concerning *S. aureus* virulence factors, there is relatively little known about *S. epidermidis* mode of action. Infection is treated primarily with vancomycin or rifampin (Foster, 2005)

Some lantibiotics produced by *S. epidermidis* Which a group are of ribosomally synthesised, post-translationally modified peptides containing

unusual amino acids, such as dehydrated and lanthionine residues. This group of bacteriocins has attracted much attention in recent years due to the success of the well characterized lantibiotic, nisin, as a food preservative. Numerous other lantibiotics have since been identified and can be divided into two groups on the basis of their structures, designated type-**A** and type-**B**. To date, many of these lantibiotics have undergone extensive characterization resulting in an advanced understanding of them at both the structural and mechanistic level. (McAuliffe *et al.*, 2001)

Epidermin is produced by *S. epidermidis* which harbors the 54-kb plasmid. The plasmid contains not only the epidermin structural gene *Epi A*, but also a flanking DNA region which is necessary for epidermin biosynthesis. The DNA sequence of this region revealed, in addition to *Epi A*, five additional open reading frames, *Epi B, C, D, Q* and *P* (Schnell *et al.*, 1992).

The antimicrobial peptide epidermin is distinguished by thioether amino acids such as *mso*-lanthionine, 3-methyllanthionine, and 2-aminovinylcysteine. The enzyme *Epi B*, encoded on a plasmid of the producing strain *S. epidermidis*, is very likely involved in the formation of these unusual amino acids. The function of serine protease *Epi P* in epidermin biosynthesis was investigated. Epidermin is synthesized as a 52-amino-acid precursor peptide, *Epi A*, which is posttranslationally modified and processed to the mature 22-amino-acid peptide antibiotic (Peschel *et al*., 1996).

1-2 Aim of the study

Attempt to increase the capability of some materials which used in treatments of wounds and burns by mixing with the epidermin that produced from bacteria S.epidermidis.

Epidermin also had the antimicrobial activity against some pathogenic bacteria that causes infections, so it’s possible to use epidermin or adding to some antimicrobial drugs used for treatment of infections.

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رَبِّه ِ قُلْ هَلْ يَسْتَوِي الَّذِينَ يَعْلَمُونَ وَالَّذِينَ لاَ يَعْلَمُونَ إِنَّمَا يَتَذَكَّرُ أُوْلُوا الأَلْبَابِ(٩)

صَدَقَ اللّهُ العَظيمِ

سورة الزمر أية (٩)

Summary

This study was included many steps, about epidermin of *Staphylococcus epidermidis*: extraction, purification and synergistic effect of pure epidermin with some drugs and agents that used in treatment of wounds and burns.

- Seventy skin samples (swabs) were cultured from many Hospitals in Baghdad, during the period from October/2004 to February/2005. Only five isolates were identified as *S.epidermidis*, the ability of these isolates to produce epidermin was determinated by growth inhibition against (*Staphylococcus aureus* and *Escherichia coli*) using agar spot method and epidermin activity was determinated. All isolates of *S.epidermidis* have the ability to produced epidermin and shown antibacterial activity toward *S.aureus,* whereas *E. coli* was not inhibited. *S. epidermidis F* isolate have the highest activity.

- Extraction and purification of *S.epidermidis F* epidermin, after growth in semi solid media by: cooling centrifugation, ammonium sulfate precipitation, dialysis and gel filtration chromatography on sephadex G-75 column. Biological activity and protein concentration were determined during all steps of purification. Purified epidermin was obtained at a concentration 0.2 mg/ml with activity 50 Au/ml.

- Synergestic effect of pure epidermin with some agents that used in treatment of wounds and burns. Studied for this purpose drugs and agents was used (with active compounds): Samacyclin (Tetracyclin HCL 3%),Nifusin(Nitrofurazon), Silverin (Silver sulphadiazine 1%), Acetic acid 6% and Iodine 10%. The effect of these agents (alone) with

concentration 100% and with epidermin 50% (v/v), were determined by using agar diffusion method against *S.aureus*. Results showed, Samacyclin was the most effective agents, Nifusin was the second, and Silverin, acetic acid 6% and iodine 10% were the least effective agents.

- Plasmid profile was determined for all *S.epidermidis* isolates. Results showed: The isolates containing a small plasmid bands approximately in the same size compared with each other. Curing results (by using ethidium bromide), showed that epidermin production was carried on plasmid not on chromosome, and the plasmid code for antibiotic resistance gene also.