ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY JANUARY 2017 ISBN 1595-689X VOL18 No. 1
AJCEM/1701 http://www.ajol.info/journals/ajcem
 http://dx.doi.org/10.4314/ajcem.v18i1.1
AFR. J. CLN. EXPER. MICROBIOL. 18 (1): 1- 10

IN VITRO AND *IN VIVO* ANTIMICROBIAL ACTIVITY OF PARTIALLY PURIFIED ENTEROCIN PRODUCED BY *ENTEROCOCCUS FAECALIS* AND ITS APPLICATION IN WOUND HEALING

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ABSTRACT

Background: The recent global upsurge in antibiotic resistance among bacteria associated with wounds has contributed to high treatment failures in wound management. Enterocin are produced by enterococci and has been reported to inhibit the growth of many bacteria including those associated with wound infections.
Objectives: In this study, antibacterial and physico-chemical properties of partially purified enterocin (PPE) from *Enterococcus faecalis* was determined. Also, the possible application of the enterocin in wound management was evaluated.
Materials and Methods: Eight different enterocin were tested and that with highest antibacterial (E3) was partially purified using standard methods. The molecular weight of the PPE was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis after which the *in vitro* anti-*Staphylococcus aureus* potential of the PPE was determined.
Results: Enterocin (E3) was effective against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter cloaca*, *Listeria monocytogenes* and *Proteus vulgaris*. The activity of the E3 was very prominent at pH of 4 and 8. The molecular size of the isolated enterocin was 5.5 KDa. The photomicrograph of the skin tissue of the skin treated with partially purified enterocin for day 7showed epidermis covered by atrophic stratified squamous epithelium. A synergistic interaction was noticed when Eusol was used with the enterocin.
Conclusions: From this study, enterocin from *E. faecalis* has a low molecular weight and inhibited bacteria isolates from wound and also aids physiological healing of wound.The antibacterial potency of this bacteriocin indicates that it is an alternative therapeutic agent that can be employed in wound care and management.

Key Words: Enterocin, *Enterococcus faecalis*, wounds, bacteriocin, *Staphylococcus aureus*, skin.

L'ACTIVITE ANTIMICROBIENNE IN VITRO ET IN VIVO DE L'ENTEROCINE PARTIELLEMENT PURIFIEE PRODUITE PAR L'ENTEROCOCCUS FEACALIS ET SON APPLICATION DANS LA GUERISON DES PLAIES.

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RESUME

CONTEXTE: La récente recrudescence mondiale de la résistance aux antibiotiques parmi les bactériesassociées à des plaies est responsable pour l'échec de traitement élevé dans la gestion des plaies. Les Enterocin sont produits par les enterocoques et on a rapporté qu'elles inhibent la croissance de nombreuses bactéries, y compris celles associées à l'infection de plaies.

But: Dans cette étude, les propriétésantibactériennes et physico – chimiques de l'enterocin partiellement purifié (PPE) de *l'Enterococcusfaecalis*ont été déterminées. On a également évalue l'application possible de l'enterocin dans la gestion des plaies.

Matériels et Méthodes: Huit enterocindifférents ont été testés et l'enterocin plus élevéantibactérien (E3)a été purifié partiellement en utilisant les méthodes standards. Le poids moléculaire du PPE a été déterminé par électrophorèse sur gel de polyacrylamide de dodécylsulfate de sodium après que le potentiel *in vitro* anti – *Staphylococcus aureus* du PPE a été déterminé.

Résultats: Enterocin (E3) a été efficace contre *Staphylococcus aureus, Klebdiella pneumoniae, Enterobactercloaca, Listeria monocytogenes* et *Proteusvulgaris*. L'activitéde E3 était trèséminente à pH de 4 et 8. La taillemoléculaire d'enterocin isolé était 5,5 KDa. La photomicrographie du tissu cutané de la peau traitée avec de l'enterocin partiellement purifié pour lr jour 7 montre l'épiderme recouvert d'un épithéliumpavimenteux stratifié atrophique. Une interaction synergique a été observéelorsqu'Eusol a été utilisé avec l'enterocin.

Conclusion: De cette étude, l'enterocinde *E. faecalis* isolé de chien a un faible poids moléculaire et inhibe les isolats bactériens de plaies et aide également la guérison physiologique de la plaie. La forceantibactérienne de cette bactériocine indique qu'il s'agit d'un agent thérapeutique alternatif qu'on peut êtreemployé dans le traitement et la gestion des plaies.

Mot clés: Enterocin, *Enterococcusfaecalis*, plaies, bactériocine, *Staphylococcus aureus*, la peau.

INTRODUCTION

During the course of bacterial growth, wide range of antimicrobial metabolites such as organic acids, ethanol, diacetyl, hydrogen peroxide, antibiotics, bacteriocins and other compounds have been recognized. Bacteriocins are antimicrobial peptides or precursor peptides synthesized ribosomally and are produced by bacteria. They have inhibitoryeffect on both closely and distantly related bacteria but not on the producers (1,2).

Bacteriocins are proteins or complexed proteins biologically active with antimicrobial action against other bacteria, especially closely related species (3,4). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract. This creates the possibility of improving their characteristics to enhance their activity and spectra of action (5). Bacteriocinhave been extensively employed in the food industry since they inhibit the growth of most bacteria that spoil and contaminate food (1,6,7).

Bacteriocins are generally low molecular weight proteins that attack the target cells by binding to cell surface receptors. Their mechanisms of action vary and include pore formation of the cell wall or cytoplasmic membrane, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (8-10). Bacteriocins produce localized holes in cell wall and cellular membrane with the leakage of macromolecules such as proteins into external medium and cause death of pathogenic organisms. At lower concentration,bacteriocin modifies the ion permeability of the cells, dissipating both components of proton motive force (11,12). Enterocins are enterococcalbacteriocins which belong to class II bacteriocins, they are non-modified antimicrobial peptides (13,14).

Wound is a breach formed in the normal continuum of the cellular and molecular structure of the body, thereby creating a disruption in the anatomic and as well as in their functional continuity. Wound healing or wound repair is an intricate process in which the skin, organ or tissue repairs itself after injury (15,16).Wound is a serious public health problem globally. The management of people with wounds is a major challenge, in addition to their profound effect on the quality of life of patients with wounds. An understanding of the wound healing process is needed to successfully manage wounds and the identification and prevention of factors that may delay or interrupt wound healing. Early wound treatment will reduce public health expenditure and prevent impairment of the quality of life of affected patients (17).

Impaired skin integrity leads to the development of a wound that could involve different tissues, from the epidermis to deeper layers such as muscles. After injury, the blood clots form a scab that protects the injured area (18). A wound is a tissue damage which could be caused by mechanical force or chemical substances and it could involve more than one tissue or organ.

The cause of injury could be accidental, for example penetrating plants, animal bites or deliberate, like surgical interventions and gun shots (19). The discontinuity of the skin at such sites gives opportunity to microorganisms and other foreign bodies to explore the tissues (20) or the internal organ, therefore prompt attention to wounds is vital for timely healing.

Bacterial contamination, colonization and infection of wounds by single or mixed populations have been reported to lead to treatment failure. Pressure of antibiotics on bacterial associated with wounds has also been reported (18,21). Enterocin has been reported to be effective against both Gram-negative and Gram-positive bacteria. Most wound healing regimen performs two major roles: prevention of invasion of microorganism and/or elimination of colonized microbes and also facilitation or regeneration of damaged tissues (22). The possibility of application of enterocin in wound management has not been reported, hence the aims of this study.

MATERIALS AND METHODS

Sources of *Enterococcus faecalis*

Eight strains of *E. faecalis* were collected from the stock culture of the Department of Microbiology, Ekiti State University, Ado-Ekiti. The isolates were sub-cultured on Bile aeculin (Oxoid) and incubated at 37 °C for 24 h. All the isolates produced black halo on the agar. Gram staining, motility and oxidase tests were conducted on the isolates.

Preparation of Cell Free Supernatant (CFS)

The strains were cultured in Mann Rogosa Sharpe (MRS) broth and incubated for 24 h at 37 °C. The broth was centrifuged at 10,000 *g* for 10 min at 4°C. To ensure sterility, the supernatant was further filter through 0.45-µm pore size filters (Carl Schleicher &Schüll). The pH of the CFS was adjusted to 6.2 . A 1 N NaOH and 130 U/mL of catalase (Sigma Chemical Co., St. Louis, MO, USA) was also added to eliminate the activity of organic acids and hydrogen peroxides respectively in the supernatants.

Source of Test Bacteria and Determination of Antibacterial Activity of CFS

Nine different test bacteria comprises of 3 Gram positive and six Gram negative were collected from the stock culture of the Department of Microbiology Ekiti State University, Ado-Ekiti, Nigeria. The isolates were sub-cultured on different selective media and incubated at 37 °C for 24 h. The test bacteria include the following: *Bacillus subtilis*, *Enterobacter cloaca*, *Escherichia coli*, *Klebsiella pneumonia*, *Listeria monocytogenes*, *Proteus vulgaris*, *Salmonella* Typhi, *Serratia marcescens* and *Staphylococcus aureus***.** Each of the test organisms was grown at 37 °C in Mueller-Hilton broth (Oxoid) for 18 h and adjusted to an optical density of 0.5 McFarland Standard. The standardized culture was seeded on the surface of sterile Mueller-Hilton Agar (Oxoid). Agar well-diffusion test was used to determine the activity of the CFS on the isolates. The plates were incubated at 37 °C at 24h after which the zone of inhibition was measured to the nearest milliliter as described by Davoodabi *et al.* (23).

Determination of Rate of Kill of *Staphylococcus aureus*

The rate of killing of *S. aureus* by the crude enterocin was determined using spectrophotometric method. The crude enterocin was incorporated into 10 mL Nutrient broth in test tubes. Negative control tube has no enterocin. A 100 µL of standardized inoculums of *S. aureus* was inoculatedinto 10 mL of both test and control tubes. The tubes were incubated at 37 °C on an orbital shaker at 120 rpm. A aliquot was removed from the culture medium at 0, 4 and 8 h for the determination of the optical density at 560nm.

Partial Purification of Enterocin by Ammonium Sulphate Precipitation

Different concentrations (60%,70%, 80% and 90%) of ammonium sulphate was added to 50 ml of CSFto different levels of saturation with constant stirring and the solution was kept overnight at 4°C. The protein precipitate was pelleted by centrifugation at 10,000 × *g* for 20 min and dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 5.0). The supernatant was transferred to a clean sterile container.The fraction that showed the highest inhibitory activity of the indicator organisms (*S. aureus*) activity was used for further analysis.

Determination of Protein Content of the Enterocin Produced

Bradford method of protein quantification was used to assay for protein content of the enterocin that has the best inhibitory activity on the isolates. A 0.4 ml Bradford reagent was added to 1.6 ml of the enterocin to make up to 2 ml total volume. Optical density (OD) of the resulting solution was thereafter taken at 595 nm after 5 min. The optical density of each of the samples was calculated from the equation of the bestfit linear regression line obtained from the graph of the bovine serum albumin (BSA) standard curve.

Determination of Molecular Weight of the Partially Purified Enterocin

The molecular size of the partially purified enterocin harvested was analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Coomasie blue (Sigma) was used for staining. For the estimation of enterocin size, the molecular weight marker for SDS-PAGE with six distinctly separated bands and known molecular weights.

Evaluation of *In vitro* Activity of Partially Purified Enterocin

Soft MRS agar (0.7%, w/v) containing indicator organisms (from the stock culture of Department of Microbiology, Ekiti State University, Ado-Ekiti), was overlaid onto Mueller Hinton plates. Wells were made in the lawn of hardened soft agars. Aliquots (50 µl) of supernatant of overnight cultures (16 h) were poured in the wells. The plates were incubated overnight at 37 °C. A clear zone of inhibition around the well was taken for enterocin production.

Determination of Stability of Enterocin

The sensitivity of the of partially purified enterocin from *E. faecalis* to different pH was estimated by adjusting the pH of the cell free supernatant to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1M NaOH or 1M

HCl and testing against the indicator strains after 30 minutes and 2 hours of incubation. The sensitivity to heat was also tested by heating cell free supernatant samples to 30, 40, 50, 60, 70, 80, 90 and 100 ^{o}C and assay the residual activity after 5, 10, 15, 20, 30 and 40 min of incubation.

The stability of the enterocin to some chemicals was tested on cell-free supernatant treated for 2 hours with 0.1 mg/ml and 1ml final concentration of the following chemical substance sodium chloride, magnesium chloride, sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid (EDTA) and urea, while the untreated enterocin was used as control.After the treatment the activity of the enterocin was determined on *Staphylococcus aureus* using micro dilution method of CLSI (24), 200µl of the treated enterocin was introduced into each well of the microtitre plate and one loopful of the test organism *Staphylococcus aureus* was dispensed into each of the wells, covered and incubated for 37 ^{o}C for 24 hours. After incubation, the plates were examined for growth. One activity unit (AU) of enterocin was defined as the reciprocal of the last serial dilution demonstrating inhibitory activity.

Animal Experiment

Animal Care and Management

The study was carried out on thirty healthy Wistar rats weighing between 150-200 g. They were obtained from the Animal house of the College of Medicine, Ekiti State University, Ado-Ekiti and acclimatized therein for a week. The rats were housed under standard laboratory conditions of natural light/dark cycle at room temperature and humidity; fed on standard rat pellets (Ladokun Feeds, Ibadan, Nigeria) and given water *ad libitum*. The rats were assigned randomly into 5 groups of 6 rats each and housed in individual compartment of plastic cages. All animals were handled in accordance with the Guidelines for animal research as detailed in the National Research Council's Guide for the Care and Use of Laboratory Animals (25).

Induction of Experimental Skin Lesion and Animal Treatment

All animals were selected on the basis of non-presence of any pre-existing skin lesion and grouped as shown in Table 1. For surgical proceedings, the animals were weighed and anesthetized by intramuscular administration of 10% ketamine hydrochloride (Rotex Medica®, 0.1 ml/kg body weight) and diazepam (0.1 ml/kg body weight). Each animal was then shaved on the right dorso-lateral aspect of the thoracic wall by drawing an imaginary line caudally from the lower margin of the ear. Antisepsis of the area was performed with 4% alcohol based iodine soaked in gauze. In the center of the shaved area, a surgical skin lesion of 2cm by 2cm area of skin was measured and excised by exposing the dorsal muscle fascia with the aid of a surgical scalpel. Care was taken to remove the *Panniculus carnosus*. For pain control, animals received aspirin (100mg/kg weight) diluted in distilled water, until euthanasia.

The wounds were then dressed with gauze soaked with the appropriate agent for Groups A, B, C, D and E and then secured with gauze taped circumferentially round the animals. The wound of group A animals was treated with distilled water, Group B was treated with eusol, *Staphylococcus aureus* was inoculated on the wounds in Group D rats and then PPE was used for the treatment 24 hours after confirmation of inoculation of *Staphylococcus aureus*, PPE and Eusol was used to treat the wounds in Group E rats while Group C rats was treated with PPE alone. Wound dressing was done every two days. For the Group D rats, pus, swelling and redness of the skin were observed due to the treatment with *Staphylococcus aureus*.

Measurement of Wound Area and Percentage of Wound Closure

For biometric analysis, images of the skin wounds on the day of surgery and before each wound dressing was captured with a Digital camera (14.1 megapixels), and positioned at a distance of 15 cm. The 'Image J' program was applied to digital images to calculate the diameter of the wound area. Percentage of wound closure was calculated using the following formula: [(Area of 1 Day – Area of X Days)/Area of 1Day] × 100%.

Histological Examination

Under ether anaesthesia, 3 animals from each group were sacrificed at days 7 and 14. Specimens of the wound area was removed, fixed in 10% Neutral buffered formalin, for slide preparation according to routine procedures. Slices of 5µm were stained with Hematoxylin-Eosinfor demonstration of general skin architecture. Photomicrography of the tissue was carried out by examination under Leica DM750 research microscope with a digital camera attached. Digital photomicrographs of the tissue sections were taken at various magnifications.

Statistical Analysis

The values were analyzed using Statistical Package for the Social Science (SPSS) version 14. The results were subjected to Analyses of variance test and the post hoc (multiple comparisons) test was done by

Dunnett's test. The significance level was fixed at p=0.05.

RESULTS

All the strains of *Enterococcus faecalis* screened grew on Bile Aesculin Agar. And the spectrum of activity of the cell free supernatants of the isolates showed varying results. Only enterocin produced by *Enterococcus faecalis* E3 (Enterocin E3) was effective against *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter cloaca*, *Listeria monocytogenes*, and *Proteus vulgaris* as shown in Table 2. The enterocin had the highest zone of inhibition on *Staphylococcus aureus* (176.79 mm^2) followed by *Enterobacter cloaca* and *Proteus vulgaris* with areas of inhibition of 165.20mm^2 and 154.00 mm^2.

TABLE 1: THE GROUPING OF ALBINO RATS IN WOUND HEALING EXPERIMENT

Group	Description	Treatment	Bacterial Challenge
A	Control	Not Eusol nor PPE treated	None
B	Eusol	Eusol treated	None
C	PPE	Partially purified enterocin treated	None
D	PPE + Staph	Partially purified enterocin treated	*Staphylococcus aureus*
E	PPE and Eusol	Partially purified enterocin and Eusol treated	None

TABLE 2: AREA OF INHIBITION OF ENTEROCINS PRODUCED BY *ENTEROCOCCUS FAECALIS* STRAINS AGAINST SELECTED BACTERIA (MM^2)

	Enterocins							
Organisms	E1	E2	E3	E4	E5	E6	E7	E8
S. aureus	0.79	0.00	176.79	3.14	3.14	78.57	0.79	3.14
K. pneumoniae	0.79	0.00	113.14	3.14	38.50	3.14	12.57	3.14
E. cloaca	3.14	0.79	165.20	19.64	7.07	19.64	113.14	3.14
E. coli	7.07	12.57	3.14	0.79	19.64	452.58	0.79	0.79
L. monocytogenes	0.79	38.50	113.14	0.00	0.79	12.57	50.29	19.64
P. vulgaris	19.64	3.14	154.00	0.00	113.14	132.79	63.64	7.07
S. marcescens	7.07	0.00	19.64	0.00	0.79	3.14	3.14	0.79
Salmonella Typhi	3.14	7.07	3.14	7.07	38.50	0.00	28.29	0.00
B. subtilis	0.79	0.79	7.07	3.14	63.64	0.00	0.00	19.64

The properties of partially purified enterocin E3 is shown in Table 3. The enterocin activity of the purified enterocin was 625AU/ml. The protein concentration of the purified enterocin is 14.18mg/ml, the total activity of the purified enterocin is 31250 AU/ml, total protein of the purified enterocin is 709 mg/ml, specific activity of the purified enterocin is 44.07AU/mg, yield of the purified enterocin is 10.01 and the fold of the purified enterocin is 12.68.As shown in Table 4, the activity of enterocin was tested over a temperature range of 40 to 100 ^oC. At each temperature the enterocin E3 was exposed for a period of 30 through 180 minutes. At temperature of 40 to 80 ^oC the enterocin was still active at 180 minutes of exposure. The evidence of activity was not noticed when the enterocin was exposed for 60 mins at 90 ^oC. At 100 ^oC there was no activity of the enterocin on the test organism (*Staphylococcus aureus*). As shown in Table 7, the *in vitro* activity of enterocin E3 on the test organism (*Staphylococcus aureus*).

With increased time of exposure to enterocin, the turbidity of the culture of the test organism decreased showing increased lysis (activity of enterocin). After 5 hours of exposure of the test organism to enterocin, the turbidity of the broth decreased by 34.88%. The inhibition of the enterocin was lower than the

gentamicin. At the probability level of 0.05, there was significant difference between the activity of enterocin and the control (normal saline) so also was gentamicin and the control. There was no significant difference between the activity of enterocin and gentamicin.

TABLE 3: PROPERTIES OF PURIFIED ENTEROCIN FROM *E. FAECALIS*PRIMARILY ISOLATED FROM DOGS

Properties	Crude extract	Ammonium sulphate precipitation
Volume (ml)	2000	50
Enterocin Activity (AU/ml)	156	625
Protein Concentration (mg/ml)	44.88	14.18
Total Activity (AU)	312000	31250
Total Protein (mg)	89760	709
Specific Activity (AU/mg)	3.47	44.07
Yield (%)	100	10.01
Fold	1	12.68

TABLE 5: EFFECT OF CHLOROFORM ON THE ACTIVITY OF PARTIALLY PURIFIED ENTERIOCIN E3

Concentration (%v/v)	Time (h)				
	1	2	3	4	5
25	+	+	+	+	+
50	+	+	+	-	-
75	+	-	-	-	-
98	-	-	-	-	-

+ = growth, - = no growth

TABLE 6: EFFECT OF METAL ION ON THE ACTIVITY OF PARTIALLY PURIFIED ENTERIOCIN E3

Metal ions	Concentration of metal ions (mM)			
	10	15	20	25
Na^{+}	+	+	+	+
Al^{3+}	+	+	-	-
Cu^{2+}	+	-	-	-
Zn^{2+}	+	+	+	-
Mn^{2+}	+	+	-	-
Fe^{2+}	+	-	-	-
Co^{2+}	-	-	-	-
Pb^{2+}	-	-	-	-

+ = growth, - = no growth

The macroscopic observation of wound healing in the skin of the experimental rats in different treatment groups is shown in Plate 1. The reactions of the six animals whose wounds were infected by *S. aureus* were shown in Table 8. Except the animal D1 all other animals developed pus with D2 and D6 shown copious pus formation. All the animals except D5 and D6 showed sign of swelling around the wound while all the animals showed sign of redness around the wound except D6.

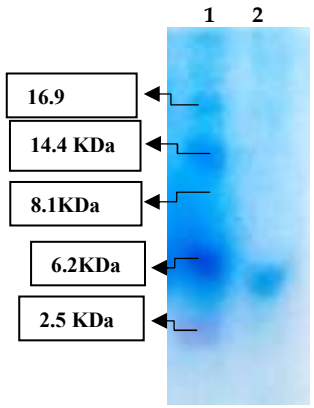


Figure 1: Coomassie blue-stained membrane enterocin E3 produced by *Enterococcus faecalis* E3 determined by SDS-PAGE gel electrophoresis. Lane 1, molecular weight markers; and lane 2, enteriocin (with molecular size of about 5 KDa).

As shown in Plate 2, the photomicrograph of the skin in group A for day 7 is showing tissue covered by stratified squamous epithelium. The sub-epithelial layer is made up of relatively normal looking skin adnexal structures.

TABLE 7: *IN VITRO* ACTIVITY OF PARTIALLY PURIFIED ENTEROCIN ON *S. AUREUS*

Time	Enterocin	Normal Saline	Gentamicin
0	0.49±0.02	0.46±0.04	0.50±0.04
1	0.32±0.06	0.49±0.05	0.31±0.06
2	0.25±0.01	0.58±0.12	0.21±0.02
3	0.21±0.09	0.81±0.22	0.18±0.05
4	0.30±0.04	1.03±0.42	0.20±0.02
5	0.34±0.06	1.93±0.13	0.03±0.01

The values obtained from the measurement of the wound area and the percentage of the wound closure is in Table 9.

A fairly thickened fibro collagenous tissue (day 14)as shown in the photomicrograph of the skin tissue was covered by an atrophic stratified squamous keratinizing epithelium. Some skin adnexal structures are seen but randomly arranged in a loose connective tissue. The papillary dermis is not properly delineated hence the boundary between the epidermis and the dermis is not well defined.

TABLE 8: REACTIONS OF WOUNDS INFECTED WITH *STAPHYLOCOCCUS AUREUS*

Animal	Reactions of the animals		
	Pus	Swollen	Redness
D1	-	+	+
D2	++	+	+
D3	+	+	+
D4	+	+	+
D5	+	-	+
D6	++	-	-

Key: - = no reaction, + = mild reaction, ++ = Severe reaction

The photomicrograph of the skin in group B for day 7 is displaying marked acanthosis, hyperkeratosis, para keratosis and papillomatosis in areas. The epidermis shows undue epithelial proliferation and projection with prominent granular layer. There are some skin adnexal structures seen. For day 14, the photomicrograph is displaying acanthosis, hyperkeratosis and papillomatosis in areas. The epidermis shows undue epithelial proliferation and projection. The granular layer is prominent. There are some skin adnexal structures seen.

TABLE 9: CLOSURE IN INDUCED WOUND IN EXPERIMENTAL RATS TREATED WITH ENTEROCIN

Treatment group	Wound diameter (cm)					
	Days					
	1	7	14	1	7	14
Group A	6.462±1.096	3.984±1.048	2.138±0.017	0	38.35	66.91
Group B	4.953±0.799	1.988±0.712	0.023±0.004	0	59.84	99.54
Group C	6.440±1.154	1.968±0.523	0.076±0.011	0	69.44	98.82
Group D	5.990±1.428	1.350±0.465	0.067±0.009	0	77.46	98.88
Group E	5.305±0.755	1.865±0.538	0.068±0.009	0	64.84	98.72

The photomicrograph of the skin tissue in group C on day 7 is showing the epidermis covered by atrophic stratified squamous epithelium. A few skin adnexal structures and blood vessels are seen. There is evidence of healing. On day 14, the photomicrograph of the skin tissue is showing healed epidermis. The epidermis and the dermis are well delineated and the skin adnexal structures are conspicuously seen with blood vessels of various calibres.

The photomicrograph of the skin tissue in group D on day 7 is displaying mild papillomatosis and hypergranulosis. The sub-epithelial layer shows paucity of skin adnexal structures and marked clearance of inflammatory activity. However, there is mild infiltration of the stroma by chronic inflammatory cells with numerous proliferation of blood vessels of different calibres. For day 14, the photomicrograph is showing skin tissue with restoration of the architecture and scanty infiltration of the tissue by lymphocytes.

The photomicrograph of the skin tissue in group E on day 7 is showing skin tissue with the epidermis displaying elongation of the rete peaks and moderate band-like infiltration of the papillary dermis. There is also severe loss of the skin adnexal structures. On day 14, the photomicrograph is showing moderate presence of chronic inflammatory cells in the sub-epithelial layers.

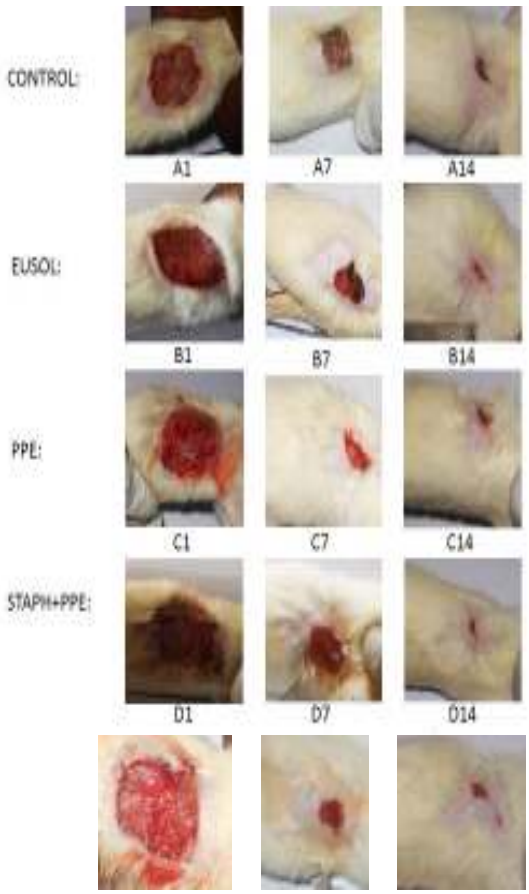
DISCUSSION

Enterocin-producing enterococci have been isolated from different sources and several strains of the species have been reported to produce this class of bacteriocins (26). Enterocins are heat stable and have low molecular weight (26). For this research the bacteriocin-producing enterococci used was isolated from rectal swab of dogs. It has been well established that some enterocins produced by enterococci have the ability to inhibit the growth of the some microorganisms including *Staphylococcus aureus* (27). This study confirms that enterocin E3 exhibited the inhibitory activity against some microorganisms including *Staphylococcus aureus*.

The purification of enterocin was done by using ammonium sulphate precipitation method since ammonium sulphate precipitation is the most commonly used method to purify proteins from culture broths (28). After the purification of the enterocin, the volume of the enterocin was reduced but the activity was increased; the yield of the purified enterocin was reduced but the fold was increased. This is similar to the report of Ohmomo *et al.* (29), David *et al.* (27).

Enterocins have been reported to be of low molecular weight (26). Majority of the enterocin that have been characterized so far, have molecular weight under 10 KDa (30). Enterocin E3 purified in this study also had this characteristic as it has a molecular weight of about 5 KDa. Jennes *et al*. (31) and Ohmomo *et al*. (29) reported enterocin with amolecular size of 3.4 KDa and 2.5 KDa respectively.From the result of the effect of temperature on the activity of the partially purified enterocin on Table 4, enterocin E3 is heat stable which complies with the report that enterocins are heat stable (26). The activity of enterocin E3 was very prominent at the pH range of 4-8. On exposure to lysozyme and lipase,its activity was not inhibited. This is similar to the report of Nemade and Musaddiq (10).

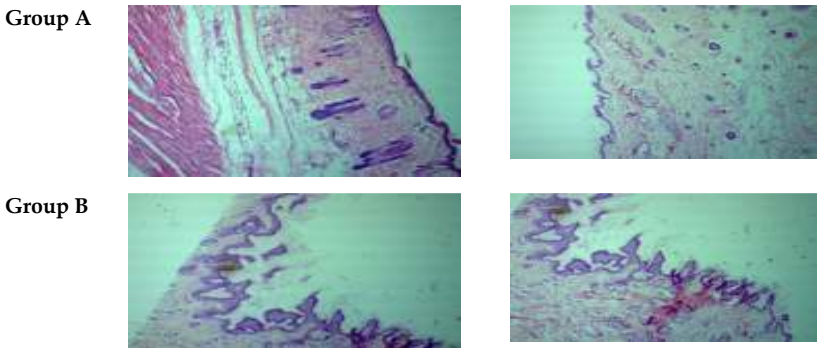
Histological observations showed that re-epithelialization tended to be greater in the PPE-treated wound than in the non-treated (control group). Many rete ridges were observed in the non-treated control group, but very few in the treated groups. Parkand Barbul (32) showed that histological analysis of a well-treated wound contained a large amount of fibroblast proliferation, collagen synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing. Thus, PPE might be useful as an agent for wound dressing. Results obtained from our study agree with the work of Lancaster *et al.* (33) who reported that colistin was very effective in cancer management. They also suggested that probiotics reduced the period of inflammatory phase of wound healing as wound contraction and cell regeneration were compatible with each other.



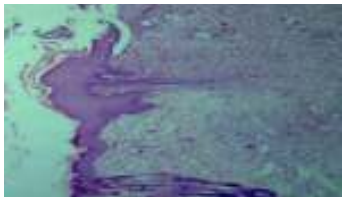
Also, in the non-treated group, the skin adnexal structures seen are randomly arranged in a loose connective tissue and the papillary dermis was not properly delineated, thus the boundary between the epidermis and the dermis were not well defined. These effects were not observed in the PPE treated group showing its efficiency in wound healing. In the Eusol treated group, on the 7th day of the experiment, marked acanthosis, hyperkeratosis, parakeratosis and papillomatosis was displayed in areas.The epidermis shows undue epithelial proliferation and projection with prominent granular layer. These features were still persistent on the 14th day of the experiment. The overall impression is consistent with pseudo-epitheliomatous hyperplasia. It is important to note that the histological features in the wound treated with eusol for 7 days are less prominent, when compared with the wound treated for 14 days. This implies that the longer the period of dressing with eusol the like hood of more undue proliferation of the epithelial cells in the epidermis.

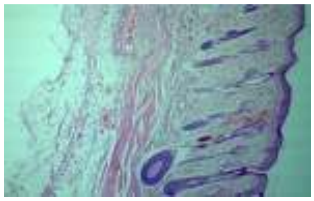
Plate 1: Macroscopic observations of wound healing in the skin of rats treated with Eusol, PPE, infected with *Staphylococcus aureus* and treated with PPE compared to control group at days 3 (A1, B1, C1, D1, E1), 7 (A7, B7, C7, D7,E2), 14 (A14, B14, C14, D14, E14) after excision.

Plate 2: Histological photomicrograph of the skin of the experimental animals. Group A=wounds without treatment, Group B=wounds with eusol, Group C= wounds treated with PPE, Group D= wounds colonized with *Staphylococcus aureus* and treated with PPE, Group E= wounds treated with eusol and PPE



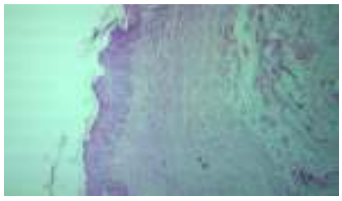
Group C





Group D

Group E



process and at day 14, there was a mild infiltration of the stroma by chronic inflammatory cells with numerous proliferation of blood vessels of different calibres. These histological features are suggestive of good therapeutic effect of PPE on the *Staphylococcus aureus*. This is in agreement with the report of Oscariz and Pisabarro (34) who reported that enterocins are active against Gram-positive food-borne pathogens such as *Staphylococcus aureus*.

In conclusion, the histological appearance seen in the PPE treated group may be suggestive of a good healing process and also a synergistic interaction was between eusol and partially purified enterocin.

Hence, the fewer the days eusol is used the better the outcome of the wound. Infections such as *Staphylococcus aureus* can dramatically slow the process of healing by prolonging the phases of wound healing.

In the *Stappylococcus aureus*-infected group, treated with PPE, the overall histologic changes on day 7 simulates granulation tissue which is a healing

Acknowledgments

The authors acknowledged the technical assistance of Dr. Bamidele Femi, Department of Biochemistry, Federal University of Technology, Akure, Nigeria, and the Technical staff of the Departments of Microbiology, and Anatomy of Ekiti State University, Ado-Ekiti, Nigeria.

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