OPTIMIZATION OF COMPATIBLE SOLUTE PRODUCING HALOPHILIC BACTERIA FOR OCCUPATIONAL SKIN INFECTIONS AMONG SALTPAN WORKERS AND ITS EFFICACY AS A TOPICAL FORMULATION

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Abstract **:** The saltpan workers are working in an environment which are highly saline. The environmental humidity, pH, and deposition of salt in palm, in between fingers, legs and hands promote skin diseases like impetigo, folliculitis, furuncles and carbuncles. In the present study, samples collected from different infected parts of saltpan workers of Thoothukudi District showed aggregate of different pathogens in which *Staphylococcus* sp., is of increased concentration followed by *Streptococcus* sp., *Pseudomonas aeruginosa* and *Escherichia coli*. Furthermore, saline soil sample was collected from the coastal regions of Thoothukudi District, plated in MM63 medium and observed for the growth of halophilic isolates. Anti-microbial activity was performed using the intracellular solutes against different test organisms and the arbitrary unit was determined against *Staphylococcus* sp.,. A topical formulation was prepared using different solvent extract of intracellular solutes isolated from halophilic bacteria, identified as *Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri* on performing 16S rRNA gene sequencing. The effect of topical treatment was accomplished by animal experimental studies. The intracellular compounds showing prophylactic activity against pathogens were identified by GC-MS analysis. Comparatively, the formulation prepared from *Pseudomonas stutzeri* was as effective as standard topical ointment.

Key words **– Skin diseases, halophilic bacteria, arbitrary unit, wound healing and anti-inflammatory**

I. INTRODUCTION

Tuticorin and Nagapattinam are the two major salt producing Districts, accounting for about 80% of the state's salt production. In terms of working conditions, the processes followed in Tamil Nadu are highly primitive and involves intensive manual labour, in very hot and windy conditions for most part of the day. The saltpan environment consists of fine dust particles that are inevitable during salt producing operations. The wet condition prevailing in the saltpan environment all through the day is the main causative factor for the large scale occurrence of dermatitis among salt workers. The workplace exposure to some physical, chemical or biologic hazard has been a necessary contributing factor in the development of occupational disease. The extreme environment not only affects the saltpan workers but also the native population of microbes on the external surface of the workers causing occupational dermatitis. Further related factors like stress, age, weight, fitness, medical condition and acclimatization to the heat are responsible for skin diseases such as solar keratosis, squamous cell cancer, basalioma, telangiectasia, photoaging etc.,.Therefore it is imperative to understand the etiology of the skin infections like folliculitis, cellulitis, furuncle, carbuncle, impetigo, ecthyma, paronychia etc., associated with saltpan workers. However, some microorganisms Staphylococcoi, Micrococci, Corynebacteria and Brevibacteria, usually have the ability to form compatible solutes in order to protect against drying out or high salt concentration and thus contributing to the formation of an intact skin barrier. The compatible solutes, which are also, referred to as stress protection substances, found in microorganisms living in extreme conditions, are low molecular compounds found in cytoplasm. In the present study the population of microbe is studied from infected regions of the saltpan workers, which when identified showed concentration of four organisms viz., *Escherichia coli*, *Pseudomonas aerugenosa*, *Streptococcus* sp., and *Staphylococcus aureus.* Among the organisms studied, the presence of *Streptococcus* sp., and *Staphylococcus aureus* were well marked in causing skin infections. Hence the study focused to relay on *Staphylococcus aureus*, which was responsible for many potential skin infections like Impetigo, folliculities, furuncle, carbuncle, acute paronychia, botryomycosis etc.,The present study was conducted in an attempt to provide a counter- measure for wound infections. The invention relates to formulations that contain an osmolyte originating from halophilic microorganisms (*Oceanobacillus oncorhynchi* & *Pseudomonas stutzeri*) and unsaturated fatty acids, with the osmolyte being present in an amount ranging between 1% and 50 Wt. % based on the total weight of the formulation. The formulation was tested for wound healing activity by performing excision wound model in Wistar albino rats. In recent years, several studies have been carried out on isolation of compatible solutes to explicate their potential in wound management.

II. MATERIALS AND METHODS

Specimen collection from saltpan workers

For microbial study, a total of 10 infected persons were selected in the study area. Using sterilized swabs, samples were taken from the hand, below ear lobe and the foot of the saltpan workers. The sterile swab was gently rotated on the surface of the above mentioned infected regions. The samples were transported from the field to the laboratory by using saline media. The samples were swabbed on the nutrient agar plates and incubated at 37^0C for 24 hours. After 24 hours, the Petriplates with

bacterial colonies were observed and subjected to morphological and biochemical tests. The genus of the organisms was identified from the above test results using standard methods.

Sampling area

In this study, saline soil samples were collected from solar salt pans of Thoothukudi District, Tamil Nadu, India. The collected saline soil sample was placed in a sterile container and immediately transported to the laboratory for further analysis.

Isolation of halophilic bacteria from the saline soil sample

The sample was serially diluted in the range of 10^{-1} to 10^{-7} in a series of test tube. The samples were spread plated in the Mineral Salt MM63 medium with 12g/100 ml concentration of NaCl. The pH of the medium was adjusted to 8–9. The plates were incubated at 30°C and the colonial appearance was examined after incubation for 3-7 days.

Morphological and biochemical identification

The isolated bacterial were subjected to morphological and biochemical confirmations (Sawale *et al*., 2013).

Extraction of Intracellular solutes

The extraction of cytoplasmic solutes was performed as described by Cònovas et al.1997 with the following minor modifications: The halophilic isolates were grown in 200 ml of minimal MM63 broth until late-exponential phase (OD_{600} = 1–1.2). Cells were collected by centrifugation and washed with the same medium without any carbon source. To extract the cytoplasmic compatible solutes, pellets were resuspended in 10 ml of double-distilled water and, after 10-min incubation at room temperature, cell debris was removed by centrifugation. Supernatants were extracted twice with chloroform and freeze-dried. Cell extracts were resuspended in 0.5 ml of D_2O and mixed with three different solvents namely, dimethyl sulfoxide, ethyl acetate and phenol.

Antimicrobial screening:

The supernatant extract of each suspension were assessed for antibacterial property against bioassay strains of bacteria viz., *E.coli*, *Staphylococcus aureus*, *Vibrio* sp., *Pseudomonas* sp., and *Klebsiella* sp., (Pathogens were isolated from the wound samples of infected patients and identified by morphological and biochemical test) .To perform the test, bioassay strain was cultivated on Mueller Hinton agar and wells were made in plate agar using sterile cork borer. Then, 50 µl and 100 µl of each supernatant were added to each well and the plates were incubated at 35°C for 24h. Afterward, the exhibition of a clear zone of growth inhibition was observed, measured and quantified which were considered antimicrobial activity.

Quantification of antibacterial activity:

Zones of inhibition against various test organisms were measured from the above anti – bacterial activity in mms and data was computed using the reported quantification procedure (Velho- Pereira and Kamat, 2011) to obtain:

Percent area specific differential antibiotic activity score

(PASDASS) = [AWG/TSA] x 100

where AWG is the area on the plate without growth of test pathogen [area of zone of inhibition-area of the plug (28.26 mm^2)], TSA is the total swabbed area of the pathogen on the plate (6358.5 mm^2).

Percent overall inhibition efficiency score (POIES), was calculated using the following equation:

POIES= (TNIS/TNTS) x 100

Where TNIS is total number of inhibited species and TNTS is total number of test species. The ideal score for multispecific inhibition would be 100.

Arbitrary unit (au) of bioactive compound

The arbitrary Unit of the bioactive compound produced by halophilic bacteria isolates were determined by serially diluting the bacterial culture (10^{-2} , 10^{-4}, 10^{-8}, 10^{-16}, 10^{-32}, 10^{-36}, 10^{-40} till 10^{-52}) then adding 100 µl of each dilution into the wells of seeded Muller Hinton agar by *Staphylococcus aureus* . The plates incubated at 35°C for 24 hrs and the Arbitrary Unit of each bioactive compound was determined by the reciprocal of the highest dilution exhibiting the antimicrobial effect.

Invivo Studies

All the experiment were performed with the approval of the protocol by the Institutional Animal Ethics Committee (IACE) at Department of Pharmocology, S.B.College of Pharmacy Sivakasi Approximately (100-200g) weighing adult albino rats were maintained in a room temperature of 22 + 2 ^{0} C for 12 hours light / dark cycle and relative humidity of 60 ± 5 % and were given uniform pelleted diet and water libtum. Eight hours each experiment. Animals were given water only in order to avoid food interference with suitable absorption.

Wound Healing Activity

One model for in vivo wound healing activity was tried in the present study excision wound models. The animals were randomly allocated into four group animals each. Group I was assigned as a control, Group II was assigned as standard(Povidine–Iodine ointment), Group III received from the halophilic isolates (GD30 and DM27) in solvent extracts viz., DMSO and Ethyl acetate(5%), Group IV received from the halophilic isolates (GD30 and DM27) in solvent extracts viz., DMSO and Ethyl acetate (10%).

Excision Wound Model

The excision wound model was made by excising the full skin approximately 500 m^2 on the back of the animal under ether anesthesia. Wound contraction was assessed by tracking the wound area on polythene paper first and subsequently transferred to 1 mm^2 graph sheet from which the wound surface area was evaluated on day 10 and 20. The evaluated surface area was then employed for wound contraction (taking the initial wound 500 m^2 as 100 %).Remaining excision wound area is measured in mm^2.

Anti- inflammatory activity by carrageenan induced paw edema

Sprague-Dawley or Wistar rats of either sex weighing between 100-150 g are used. Animals have fasted over night and have divided into control, standard and different test groups each consisting of six animals. The test compounds and the standard drugs are administrated by oral or intraperitoneal route. Thirty minutes later the rats are challenged by a s.c.injection of 0.05 ml of 1 % solution of carrageenan on the planter surface of the left hind paw. The paw is marked with ink at the level of lateral malleolus and immersed in the column of a digital plethysmometer for measuring the paw volume. The paw volume

is measured immediately after the carrageenan injection and then at 2, 3 and 4 hrs intervals. The peak effect of carrageenan usually occurs at 3 h after the injection. The efficacy of different drug has tested on its ability to inhibit paw edema compared to control group. The experimental results has expressed as statistical comparisons of Mean ± SEM carried out by one way analysis of variance (ANOVA)

GC – MS Analysis

GC-MS analysis of ethyl acetate fractions was performed on a Thermo GC- Trace Ultra Version:5.0 equipped with a DB-5 MS capillary non polar column (30 m x 0.25 mm id, film thickness 0.25 µm) in Analyzer Solution Research Association, Coimbatore, Tamil Nadu (India). The oven temperature was set at 70- 260 ˚C and electron ionization at 70 eV. Helium used as the carrier gas at a flow rate of 1 mL/min. Scanning range was 50- 650 amu. Interpretation of mass spectrum of the unknown part has compared with the spectrum of known components stored in the the database of National Institute Standard and Technology (NIST) and WILEY library. The name, molecular weight, nature and activity of the components of the test materials have ascertained.

PREPARATION OF SKIN OINTMENT.

Type of preparation: Absorption ointment base

Procedure: Hard paraffin (50 g) and cetostearyl alcohol (50g) was gently heated on water-bath. Wool fat (50 g) and white soft paraffin (850 g) were mixed and stirred until all the ingredients were melted. The extracted intracellular solute from the halophilic isolate DM27 (based on the results of animal experimental studies) was added and mixed well. Then the suspension was decanted, stirred until cold and packed in a sterilized container.

16S rRNA Gene Sequencing

The halophilic isolates were identified based on 16S rRNA gene sequencing. Briefly, the gDNA was extracted that served as a template for the 16S rRNA gene amplification using universal forward primer (27F) (5'-AGAGTTTGATCMTGGCTCAGTAC-3') and reverse primer (1492R) (5'- GGYTACCTTGTTACGACTT-3') (Biozone (India) Pvt. Ltd.) via standard polymerase chain reaction (PCR) protocol. The amplified gene product (1 Kb) was checked on a 1.5% agarose gel against a 1 Kb DNA ladder. The BLASTn search program was employed to find nucleotide sequence homology.

III. RESULTS AND DISCUSSION

Four different organisms were isolated from infected parts of salt pan workers, namely *Escherichia coli*, *Pseudomonas aerugenosa*, *Streptococcus sp.*, *Staphylococcus aureus* that were identified by performing biochemical tests. Thirty four colonies with two different morphology in Mineral Salt (MM63) medium were chosen for bacterial isolation. The selected white colored colony was named as GD30 and the Reddish brown colony was named as DM27. Different sizes and shapes of colonies were observed after the incubation period of 96 hours. The isolated pure cultures were subjected to various biochemical characterization and the results were indicated in Table 1. The cell free supernatant extracts were obtained by the above mentioned solvent extraction method and it is been used for determining anti-microbial activity. Furthermore, the results of the present study were supported by the results obtained by Delgado-et al., 2012 who has selected the isolates based on their colony morphology. Several biochemical tests were performed to differentiate the isolates. The isolates were maintained by sub- culturing at regular intervals of 20 days and stored at 4˚C until further use.

The same appreciable result was recorded in the previous study against different test pathogens by Kannahi and Eshwari.,2016. Percent area specific differential antibiotic activity score and Percent overall inhibition efficiency score was calculated against the test organisms shown in Table 2. The arbitrary unit of the halophilic isolates (GD30 and DM27) was determined against *Staphylococcus aureus*. The inhibition effect of the test organism was found till dilution factor 10^{-48} for the isolate GD30 and 10^{-52} for the isolate DM27. Hence the arbitrary unit of the halophilic isolate is 48 AU and 52 AU respectively. Similarly, a study by Majid BaseriSalehi and Nima Bahador.,2014 demonstrated the inhibitory effect till 10^{-128} dilution by *Bacillus licheniformis*.

The intracellular solute extract of GD30 and DM27 exhibited significant wound healing activity as compared to control in excision wound model. Remaining excision wound area was measured in mm^2.It was observed that the wound contracting ability of both 10% (w/v) extract treated group shows almost equal activity to standard topical ointment and is plotted in graph (Fig.1). The significant wound healing started from the sixth day onwards. The wound closure time was lesser, as well as the percentage of wound contraction was more with the 10% (w/v) ethyl acetate supernatant extract treated groups. The epithelization of wound with 10% (w/v) ethyl acetate extract treated group was found to be earlier as compared to 10% (w/v) DMSO extract. In the 10% (w/v) of DM27 extract treated rat the wounds were completely healed (epithelization period) in 18 ± 2 days whereas in the control animals it took more than 22 ± 2 days.

Inflammation is a response of living tissues to injuries that involves mediator release, cell migration, activation of various enzymes, migration of cells, breakdown of tissues and repair (Katzung 2004). The standard experimental model of acute inflammation is the carrageenan induced hind paw edema (Turner, 1965). Carrageenan induced paw edema takes place in three phases, the first phase involves the release of serotonin and histamine from mast cells, in second phase has provided by kinins and the third phase has mediated by prostaglandins, cycloxygenase and lipoxygenase products (Vinegar et al., 1969). The effect of DMSO and ethyl acetate supernatant extracts of halophilic isolates on carrageenan induced rat paw edema at one hour interval study was compared to that control for the evaluation of anti- inflammatory activity on the basis of mean ± standard deviation inhibition of paw edema volume. The experiment showed the extracts exhibited statistically significant ($p<0.001$) inhibition of paw volume in a dose dependent manner. Significant observation was observed with DMSO and ethyl acetate extract doses till fourth hour. However, maximum inhibition of paw edema was observed in ethyl acetate and the results are compared with Diclofenac sodium 20 mg/ kg body weight (Fig.2).

GC-MS analysis of the halophilic isolates showed characteristic properties of anti-microbial effect. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and nature of the compound has presented in the Table 3. A PCR product of around 750bp was detected in the isolates GD30 and DM27. The amplicons of each bacterial isolates was sequenced and they were subjected to BLAST analysis for sequence similarity.16 S rRNA sequence was analysed to ensure the accurate taxonomic position of the Halophilic isolates (GD30 and DM27) reported in this study. G+C analysis

also revealed the diversity and ultimately the specificity of species at the genotypic level. On the basis of phenotypic characteristics and the comparison of partial 16 S rRNA gene sequence, the isolates were identified as *Oceanobacillus oncorhynchi and Pseudomonas stutzeri* (Table 4). Previous studies also reported the morphological, biochemical and 16S rRNA analysis of halophilic bacteria like *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera isolated from salt pans (Kumar *et al*., 2012).

It was also found to possess anti-inflammatory, antimicrobial and antioxidant properties that rationalized its more supportive and significant role as ideal wound healing drug, hence a topical formulation was prepared by using 10% (w/v) ethyl acetate supernatant extract of the strain DM27 ie., *Pseudomonas stutzeri.*

Future research is to employ a mouse surgical wound model infected with *Staphylococcus aureus* and *Streptococcus pyogenes*, the major causative agents of skin infections, to promote a promising and a prominent immunomodulating agent using compatible solutes from halophilic bacteria.

IV. CONCLUSION

The potential of the strains *Oceanobacillus oncorhynchi* and *Pseudomonas stutzeri* as a promising resource for antimicrobial compounds is fairly recent with no reports on the structure elucidation of the antimicrobial compound and scarce reports on its antimicrobial activity. Recently, new bioactive metabolites that are derived from only a small fraction of the investigated halophilic bacteria, have revealed the huge production of new bioactive chemicals entities and thus may have potential use in medicine. In conclusion, the results of the present study revealed that the ethyl alcoholic supernatant extract ointment of *Pseudomonas stutzeri* promotes healing process and it could be effectively used as a wound healing agent. The present study also indicates the efficacy of bioactive compound as an efficient therapeutic agent in acute anti-inflammatory conditions. However, crude extracts have themselves wound healing action. Further trials in humans are required to determine the efficacy of compatible solutes and to establish what, if any, adverse effects are observed and efforts shall be taken to develop the commercial preparation as topical formulation.

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Table 1: Morphological and Biochemical tests of the isolated organisms

Strains named	**Colony colour**	**Gram's stain**	**Cell shape**	**MR**	**VP**	**Motility**	**Catalase**	**Oxidase**	**Urease**	**Identified Genus**
GD30	White	+	Rod	+	-	+	+	+	+	*Bacillus* sp.,
DM27	Reddish brown	-	Rod	+	-	+	+	+	+	*Pseudomonas* sp.,
LW1	Yellow	+	Cocci	+	-	+	+	-	-	*Staphylococcus* sp.,
LW2	Greyish white	-	Rod	+	-	+	-	-	-	*Escherichia* sp.,
LW3	Bluish green	-	Rods	-	-	+	+	+	-	*Pseudomonas* sp.,
LW4	Yellow	+	Cocci	+	-	-	-	-	-	*Streptococcus* sp.,

Table: 2 Quantification of antibacterial activity of halophilic isolates (GD30 and DM27) in DMSO, Ethyl Acetate, and Phenol solvent extract

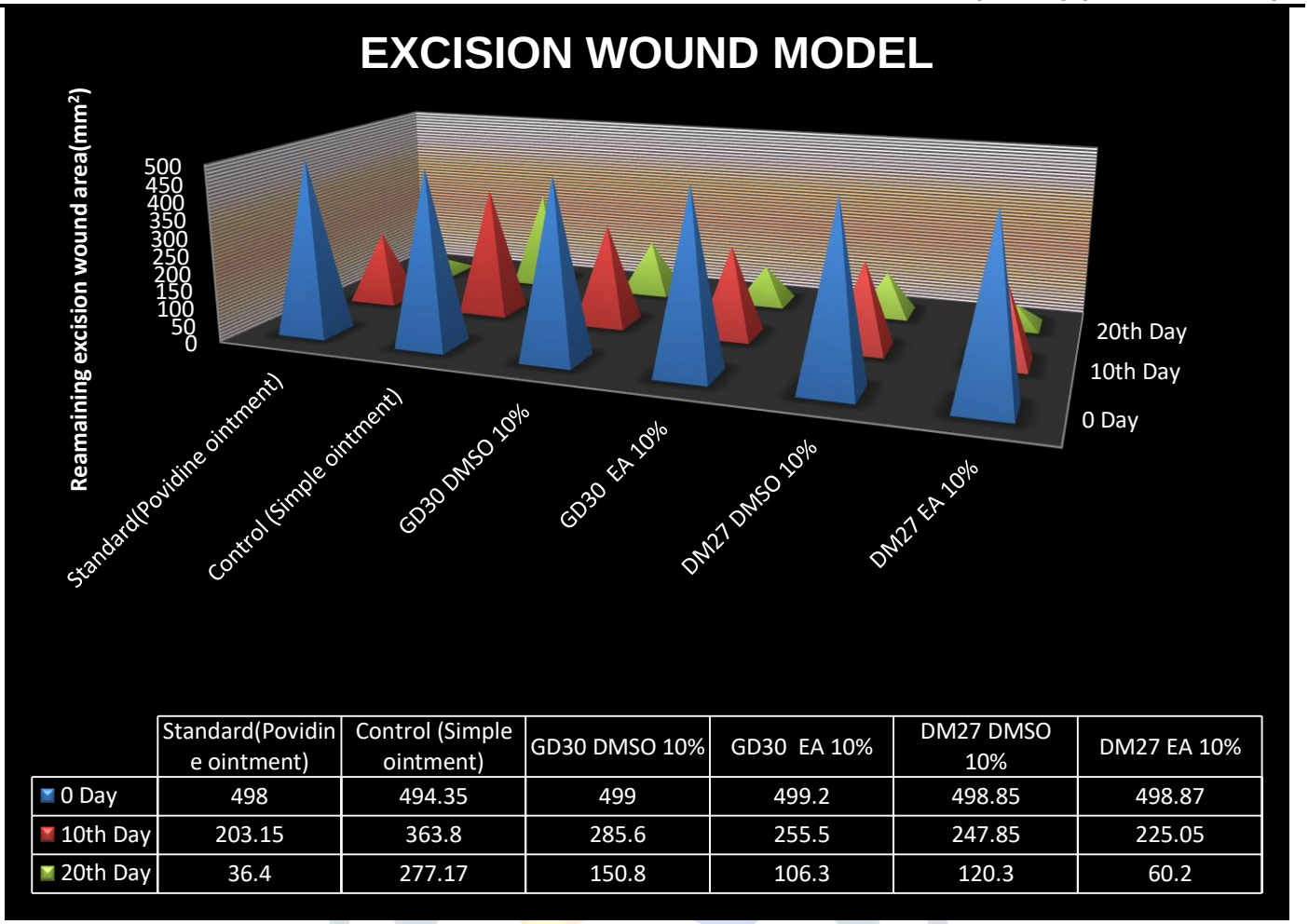
Solvent Extract	Test Organism	GD3007 Zone of Inhibition in mm				DM0207 Zone of Inhibition in mm			
		(PASDASS)= [AWG/TSA] x 100 (%)		POIES= (TNIS/TNTS)x 100 (%)		(PASDASS)= [AWG/TSA] x 100		POIES= (TNIS/TNTS) x 100	
		50 µl	100 µl	50 µl	100 µl	50 µl	100 µl	50 µl	100 µl
DMSO	*Escherichia coli*	-	11.9	40	80	6.8	7.9	60	60
	Staphylococcus aureus	8.8	10.8			-	-		
	Vibrio sp.,	-	-			-	-		
	Pseudomonas sp.,	8.8	10.8			7.9	16.8		
	Klebsiella sp.,	-	9.8			8.8	9.8		
ETHYL ACETATE	*Escherichia coli*	10.8	10.8	80	100	7.9	10.8	80	80
	Staphylococcus aureus	7.9	8.8			-	-		
	Vibrio sp.,	8.8	12.8			8.8	10.8		
	Pseudomonas sp.,	-	8.8			8.8	15.8		
	Klebsiella sp.,	8.8	10.8			10.8	13.8		
PHENOL	*Escherichia coli*	30.8	29.8	100	100	29.8	34.8	100	100
	Staphylococcus aureus	27.8	29.8			32.8	35.8		
	Vibrio sp.,	24.8	28.8			29.8	32.8		
	Pseudomonas sp.,	25.8	30.8			34.8	39.8		
	Klebsiella sp.,	26.8	34.8			34.8	36.8		

Table 3 List of components identified from the halophilic isolates using GC-MS

Strain	RT	Name	Molecular Weight	Molecular Formula	Activity
GD30	4.98	2,3,4 Trimethyl heptanes	142.286	$C_{10}H_{22}$	Antibacterial, antipyretic, analgesic, anti-inflammatory.
	14.07	3,3,5 Trimethyl heptanes	142.86	$C_{10}H_{22}$	Antibacterial, antipyretic, analgesic, anti-inflammatory,
	15.83	Cetane	226.41	$C_{16}H_{34}$	Antibacterial, antipyretic, analgesic, anti-inflammatory,
	17.41	Isohexadecane	226.44	$C_{16}H_{34}$	Antibacterial, antipyretic, analgesic, anti-inflammatory,
DM27	4.98	2,3,4 Trimethyl heptanes	184.14	$C_8H_8O_5$	Antibacterial, antipyretic, analgesic, anti-inflammatory.
	14.13	n-heptacosane	1701.20	$C_{76}H_{52}O_{46}$	Antioxidant activity, antimicrobial activity

Table 4 Identification of isolated halophilic strains based on 16S rRNA gene sequence and their accession numbers (BLAST similarity search results)

Strain ID	Strain name / Genus	Number of nucleotides of 16S rRNA gene	Accession number of 16SrRNA gene	Closely related taxa	Sequence similarity (%) of 16SrRNA gene
GD30	*Oceanobacillus*	1497	LT221188	*Oceanobacillus oncorhynchi*	92%
DM27	*Pseudomonas*	1150	ABI26690	*Pseudomonas stutzeri*	90%



	Standard(Povidine ointment)	Control (Simple ointment)	GD30 DMSO 10%	GD30 EA 10%	DM27 DMSO 10%	DM27 EA 10%
0 Day	498	494.35	499	499.2	498.85	498.87
10th Day	203.15	363.8	285.6	255.5	247.85	225.05
20th Day	36.4	277.17	150.8	106.3	120.3	60.2

Figure 1 Wound Healing activity of the halophilic isolates GD30 and DM27 (Day 0- Day 20)

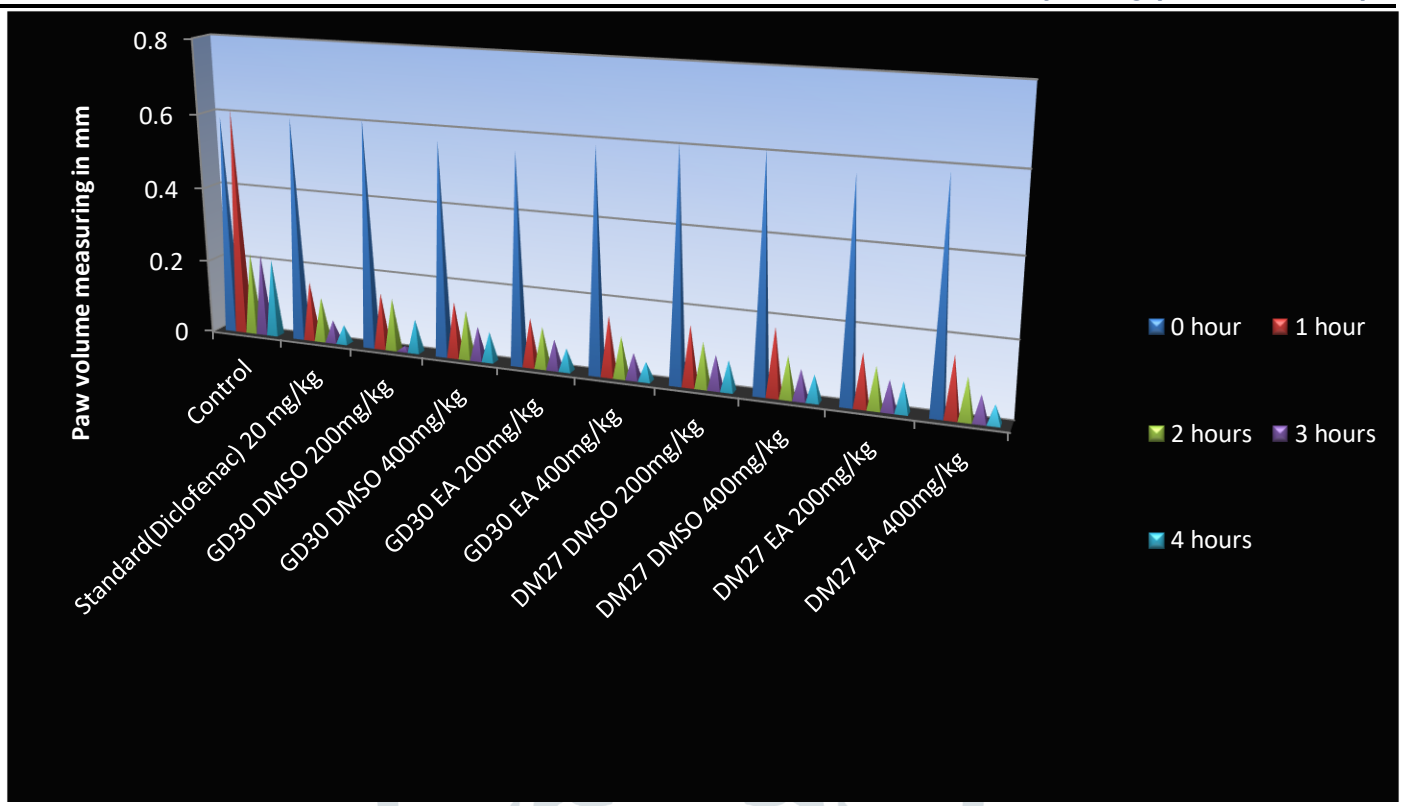


Figure 2 Anti inflammatory of halophilic isolates GD30 and DM27