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Original Article

FORMULATION AND EVALUATION OF WOUND HEALING ACTIVITY OF LINEZOLID TOPICAL PREPARATIONS ON DIABETIC RATS

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ABSTRACT

Objective: The aim of the study is to formulate and evaluate the topical preparations of antibacterial formulation for the treatment of diabetic wound infection.

Methods: Different types of topical formulations were prepared and evaluated for *in vitro* release. The prepared formulations were also tested for its antibacterial activity against the pathogens existing in diabetic wound infection. Based on *in vitro* drug release and antimicrobial activity, two formulations were selected as optimized formulations. Optimized formulations were tested for wound healing activity in diabetic rats.

Results: Based on *in vitro* drug release and antimicrobial activity two formulations (F8, F10) were selected as optimized formulations. FTIR studies of pure drug and optimized formulation shown absence of any incompatibility between drug and excipients. Optimized formulation shown good physicochemical properties and passed short-term stability study. F8 and F10 formulations were applied to untreated diabetic rats for diabetic wound infection, the rate of wound healing was quite faster. These results indicate that the linezolid semisolid dosage form could provide an adjunctive antimicrobial formulation for the management of diabetic wounds.

Conclusion: Further studies are required on chronic diabetic wounds with and without diabetic medications to confirm its effectiveness.

Keywords: *Staphylococcus aureus,* Linezolid, Semisolid dosage forms, Wound healing activity

INTRODUCTION

Diabetes mellitus is globally prevalent, diabetic wound and ulcer is one of its most severe and expensive complications. Diabetic wound and ulcer results from an intricate interaction of a number of risk factors. Patients with diabetic wound and ulcers often require amputations of the lower limbs and, in more than half the cases, infection is the preeminent factor [1]. Human skin comprises of protective layers and once the protective layer of skin is damaged, deep tissues are exposed to bacterial infection [2]. *Streptococcus aureus* is a bacterium which predominantly causes skin infection. Linezolid is the drug of choice for the treatment of the methicillin-resistant *Streptococcus aureus* (MRSA). Linezolid is the first synthetic antibiotic which belongs to a new class of antibiotics called the oxazolidinones. Linezolid inhibits protein synthesis by binding with the 50S ribosomal subunit thereby hindering the bacterial growth. Oral, as well as intravenous dosage forms of linezolid, are available for the treatment of MRSA. The present treatment for diabetic wound infections are mostly oral or IV antibiotic formulations. Failure in patients following and adhering to the right treatment leads to deteriorating the condition of diabetic wound infections.

Favorable results were noted by a number of studies using oral or intravenous linezolid for the treatment of soft tissue, bone, and joint infections. These factors make it a possible substitute for local antibiotic therapy in diabetic wound infections [3].

With these literature data, we have planned to formulate linezolid semisolids which may be beneficial and can be a possible adjuvant for local antibiotic therapy in diabetic patients from worsening of the diabetic wound and amputation of lower limbs. Semisolid dosage forms leads to more patient compliance along with existing oral and IV dosage form and hence patient may adhere to the treatment leading to better success in the treatment.

MATERIALS AND METHODS

Materials

Linezolid was procured from the Glenmark Generics Limited, Gujarat. Streptozotocin MP Biomedicals, LLc. All other excipients used were of analytical grade. All animal experimental procedures were approved by IAEC, Manipal (Reference No. IAEC/KMC/16/2014 dated January 27, 2014).

Drug-excipient compatibility studies

Drug–excipient compatibility studies were conducted for optimized formulation by Fourier Transmitter Infrared (FTIR) and Differential Scanning Calorimetry (DSC) [5].

Infrared spectroscopy

Infrared spectra were recorded using a Shimadzu FTIR 8300 spectrophotometer and the spectrum was recorded in the region of 4000 to 400 cm^{-1}.

Differential scanning calorimetry

Pure drug sample and optimized formulations were studied for DSC. DSC was performed using DSC-60, Shimadzu, Japan. The samples were placed in a sealed aluminum pan, before heating under nitrogen flow (30 ml/min) at a scanning rate of 5 °C/min from 30 °C to 300 °C.

Formulation and evaluation

Preparation of different semisolid formulations

Preparation of cream

Linezolid creams were prepared as per the composition is given in table 1. The required amount of lipids were weighed and kept for melting at 70 °C. Simultaneously, the aqueous phase was also kept for heating and was added slowly to lipid phase while mixing until it congealed. The drug was ground by using mortar and pestle. The powdered drug was levigated with the same volume of cream and mixed thoroughly to get a homogenized mixture.

Preparation of ointment

Linezolid ointments were prepared as per the composition is given in table 2 and table 3 by employing fusion method [6]. In this method the constituents of the base like stearyl alcohol, white soft paraffin, emulsifying wax and cetyl alcohol (solid ingredients) were placed together in the beaker and allowed to melt together at 70 °C. After melting, other ingredients were mixed and stirred gently during cooling stage. Formulation of ointment was done by including the active ingredient in the base by trituration using mortar and pestle.

Table 1: Composition of linezolid creams

Ingredients	Quantity in g for 10 grams formulation	
	F1	F2
Linezolid	0.1	0.1
Aqueous phase		
Benzoic acid	0.02	0.02
Sorbitol	0.3	0.8
Sodium lauryl sulphate	0.10	0.10
Tween 80	0.046	0.025
Sodium acetate buffer	3.0 ml	3.0 ml
Oil phase		
White bees wax	3.08	2.48
Cetyl alcohol	0.3	0.3
Mineral oil	2.9(3.41 ml)	3. (3.52 ml)
Span 60	0.154	0.175

Table 2: Composition of linezolid ointments F3, F4 and F5

Ingredients	Quantity in g for 10 grams formulation		
	F3	F4	F5
Linezolid	0.1	0.1	0.1
Emulsifying wax	0.8	-	3.0
White soft paraffin	8.5	2.4	5.0
Liquid paraffin	-	-	1.0
Cetyl alcohol	-	-	0.3
Stearyl alcohol	0.3	2.5	0.3
Benzoic acid	-	-	0.02
Methyl paraben	-	0.1	-
Polyethylene glycol-400	-	1.2(1.06 ml)	-
Cholesterol	0.3	-	-
Sodium acetate buffer	-	3.7 ml	-
Zinc stearate	-	-	0.28

Table 3: Composition of linezolid ointments F6, F7 and F8

Ingredients	Quantity in g for 10 grams formulation		
	F6	F7	F8
Linezolid	0.1	0.1	0.1
Polyethylene glycol-400	6.0	5.0	4.0
Polyethylene glycol-4000	2.0	3.0	2.0
Propylene glycol	1.9	1.88	2.05
Isopropyl myristate	-	-	0.8
Methyl paraben	-	0.02	0.02

Table 4: Composition of linezolid gel F9 and F10

Ingredients	Quantity in g for 10 grams formulation	
	F9	F10
Linezolid	0.1	0.1
HPMC ELV5	-	0.6
Carbopol 934	0.5	0.3
Triethanolamine	q. s.	q. s.
Propylene glycol	3.4	2.0
Glycerol	3.0	-
Sodium acetate buffer	3.0	7.0 ml

Preparation of gel

Linezolid gels were prepared as per composition is given in table 4 [7, 8]. The required amount of carbopol was weighed and soaked in 7.0 ml of buffer for 2h. Then hydroxypropyl methyl cellulose (HPMC ELV5) was dissolved in remaining buffer and added to carbopol 934 and the drug in propylene glycol also added to gel system. Triethanolamine was used to neutralize and adjust the pH of the gel system. The drug concentration in all formulations was kept constant at 1 % w/w. Propylene glycol was used as co-solvent and as a dispersion medium for the linezolid. Carbopol and HPMC at a ratio of 1:2 were prepared. HPMC was added to carbopol to improve the physical properties, viscosity, and yield of the gel product.

Physicochemical evaluation of the formulations

The spreadability, pH and viscosity of the prepared formulations were performed according to the standard procedures and the results were noted [9].

Spreadability

Spreadability of the optimized formulations was tested against standard weight applied on the glass sample plate and measuring the area of the sample. The spreadability (S) was calculated using the formula:

$S = \frac{m.l}{t}$ where S=spreadability, m=weight, l=spreaded area on the glass slide, t = time s.

pH

The pH of the formulations was recorded by making suspension of the formulations by dissolving 1 g of the formulation in 10 ml of distilled water. The pH was measured by digital pH meter

Viscosity

Viscosities of the optimized formulations were measured by using Brookfield Viscometer (LVDV-II) by using spindle number 27. The measurements were done at room temperature over the range of speed starting from 10, 20, 40, 60, 80 and 100 rpm.

Drug content estimation

1g of cream was weighed and dissolved in 100 ml of buffer and filtered through Whatman filter paper. From the filtrate 1 ml of sample was pipetted out and diluted to 10 ml with buffer to get a clear solution. Then the sample was analyzed in UV spectrophotometer, keeping the base solution without drug as blank and absorbance was noted.

Zone of inhibition studies

These studies were carried out by using pour plate method to identify the formulation activity against bacterial culture in required medium. 12h old cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* were used for this study by using nutrient agar medium [10, 11].

***In vitro* diffusion studies for formulations**

Diffusion cell was used for performing *in vitro* diffusion studies. 1g of the formulation was placed in the donor compartment, 30 ml of buffer was placed in the receptor compartment, and the two compartments were separated by using a sigma membrane.5 ml of sample was withdrawn at time intervals of 10, 20, 30, 40, 50 and 60 min. The temperature was maintained at 32 °C [12].

Animal studies

Male Sprague-Dawley rats (average body weight 280±40g) were opted to induce diabetes [13, 14].

Induction of diabetes

Rats were fasted for 12h and a single intraperitoneal injection of 40 mg/kg of streptozotocin was given for the induction of diabetes. Animals whose blood glucose level exceeded 200 mg/dl at 24h after treatment were considered diabetic [15, 16]

Wound model

Excision wound

The diabetic rats were anesthetized by injecting ketamine solution (0.3 ml) through I. P. Excision wound was imposed by cutting away 450 mm^2full thickness of a determined area of the depilated back of each diabetic rat. Epithelization period was noted as the number of days after wounding required for the scar to fall off leaving no raw wound behind [15].

Measurement of wound healing

Wound shrinkage rate was measured by planimetric measurement of the wound area every alternative day. Two Sprague-Dawley rats received marketed formulation containing framycetin sulphate 1% w/w as a reference and the remaining rats received optimized formulation of ointment and gel.

Stability studies

Stability studies of the optimized formulations were conducted. ICH real time stability studies at 25 °C/60% & 40 ° C/75% RH for a period of 1 mo. The samples were filled in a plastic box. The samples were withdrawn at the end and tested for appearance and drug content [16].

RESULTS AND DISCUSSION

Drug excipient compatibility studies

FTIR studies

FTIR spectra of pure drug and formulations are shown in fig. 1, 2 and 3. Most of the peaks are retained by the formulations. Some peaks have shown decreased intensity or broadening might be due to physical interactions with excipients which would not affect its release from the formulations.

Differential scanning calorimetry

DSC results also showed no interaction between drug and excipients.

Physicochemical evaluation of formulations

Spreadability test

Spreadability value for F8 and F10 is given in table 5.

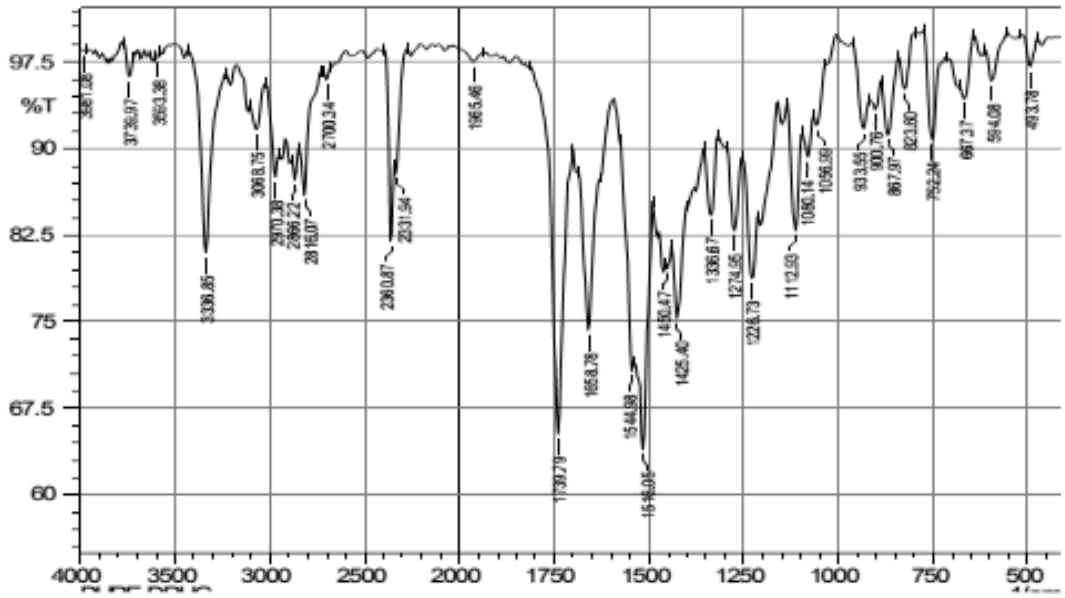

Fig. 1: Infrared spectroscopy of linezolid pure drug

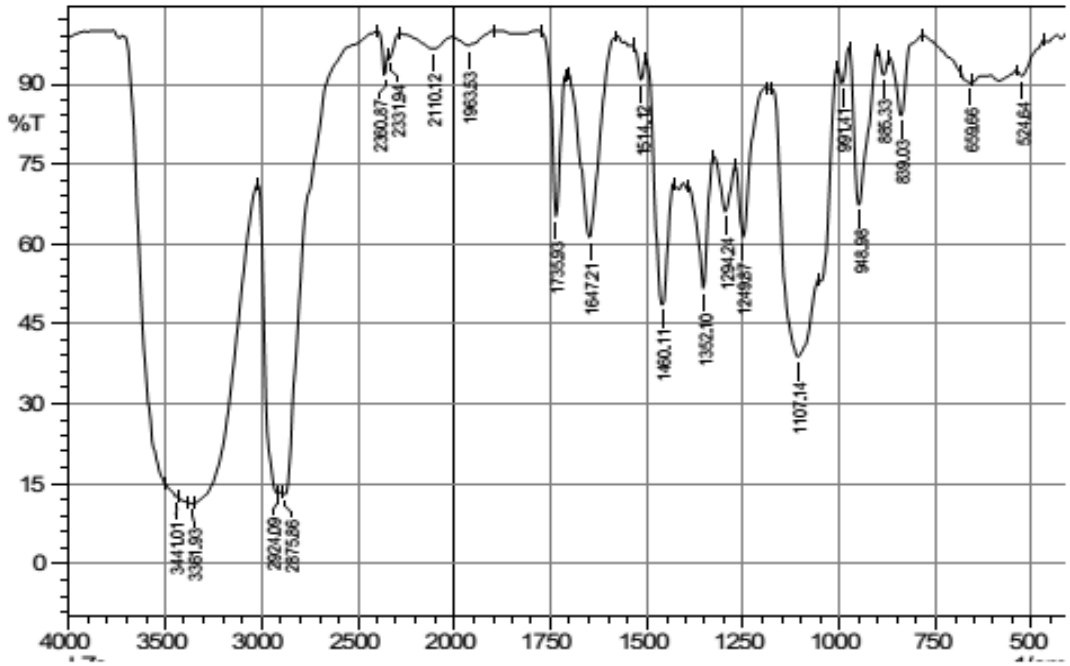

Fig. 2: Infrared spectroscopy of linezolid–ointment formulation

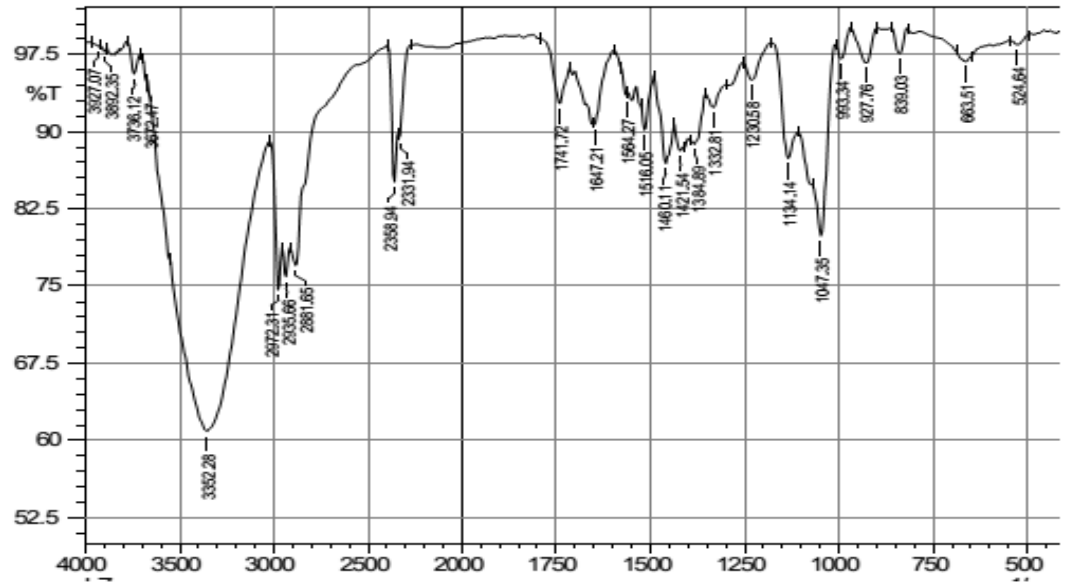

Fig. 3: Infrared spectroscopy of linezolid-gel formulation

Table 5: Spreadability value for F8 and F10

Formulation code	Average diameter(cm)	Radius (r)	Area=πr²	S=$\frac{m.l}{t}$ (g. cm²/s)
F8	4.13	2.06	13.34	44.47
F10	5.1	2.55	20.41	68.33

* n=3, Average of three determinations

F10 gel has shown more spreadability compared to F8 ointment formulation. The spreadability values indicate that the formulation can easily applied onto the skin.

pH

pH of all the formulations (F1-F10) are shown in table 6

Table 6: pH of all the formulations (F1–F10)

Formulation	pH
F1	6.1
F2	6.2
F3	6.6
F4	6.2
F5	6.0
F6	5.8
F7	5.9
F8	4.6
F9	5.0
F10	5.1

* n=3, Average of three determinations

Both F8 and F10 formulations showed optimum pH value, which was equal to the pH of skin at the site of the diabetic wound. So the drug diffusion will be more with this pH.

Viscosity

Viscosity at different shear rates for both formulations has shown in table 7.

Table 7: Viscosity values of F8 and F10 formulations

Revolutions per minute	Centipoise	
	F8	F10
10	87200	75200
20	61380	44467
40	46780	27133
60	21450	20067
80	16570	16117
100	14690	13420

* n=3, Average of three determinations

As shear and stress increases the viscosity of formulation decreases. A formulation containing PEG (F8) showed better viscosity and stability. Formulations containing carbopol: HPMC (1:2) (F10) gave a gel of highest viscosity structure and best drug diffusion. The long residence time of the gel combined with the ability of the gel to release the drug in the sustained matter will assist in enhancing bioavailability. Change in the ratio of the incorporation of the two polymers affects the rheological behavior and the release profile of the drug from the gel.

Drug content

Drug content of all the formulations are shown in table 9.

Table 9: Drug content of prepared different formulations

Formulation	Average drug content (mg/1g of the formulation)
F1	3.04±0.130
F2	5.19±0.500
F3	1.18±0.049
F4	9.21±0.140
F5	5.34±0.499
F6	9.34±0.100
F7	9.67±0.4
F8	10.13±0.39
F9	5.41±0.131
F10	9.19±0.138

* n=3, Average of three determinations

Not all the formulations showed theoretical concentration (1% w/w) of drug content. Only F8 showed theoretical value. F3 showed very less drug content, due to high forces of drug entrapment in the semisolid base.

Zone of inhibition

The zone of inhibition of pure drug against *Staphylococcus aureus* is given in fig. 4 and the zone of inhibition values of different formulations against *Streptococcus aureus* are given in table 8.

Fig. 4: Zone of inhibition of pure drug against *Staphylococcus aureus*

Table 8: Zone of inhibition of different formulations against *Staphylococcus aureus*

Formulation code	Zone of inhibition (mm)
Pure drug	26
F1	26
F2	28
F4	34
F5	30
F6	32
F7	36
F8	40
F9	30
F10	38

* n=3,±SD

***In vitro* diffusion studies**

For all the formulations three batches were taken for diffusion study (n = 3) and average value with standard deviation is reported in table 10.

Formulation F8 showed maximum drug release (24.97%) at 60thmin. F8 had optimum concentration of PEG 400(40%) and penetration enhancer's propylene glycol and isopropyl myristate. The zone of inhibition with *staphylococcus aureus* also found to be 40 mm which was highest compared to all other formulations. Formulation F10 showed maximum drug release (23.43%) at 60th min due to the presence of both carbopol and HPMC (1:2). Zone of inhibition was found to be 38 mm and pH of was 5.1, which is the pH at the diabetic wound site.

Animal studies

For 16 rats we induced diabetes by injecting streptozotocin 45 mg/kg body weight through Intraperitoneal route (I. P). From those only 7 rats showed required glucose (diabetic range) level and those animals were taken for further studies.

Blood glucose levels

Blood glucose levels of fasting 12 h rats were recorded by using accu-chek sensor glucometer and were noted as shown in table 11.

Wound area measurement

Initial wound area created was 450 mm^2. The wound area of individual rats was measured by tracing the wound on graph paper

and counting the squares (1 mm^2) in that area. The data of wound area measurement is shown in table 12**.** Results shows that order of wound healing was faster with formulation F10, F8 and reference formulation respectively. Fig. 5 depicts rat with the initial wound, fig. 6 depicts rat with a wound on fifteen days of treatment and fig. 8 depicts rat after seventeenth day of treatment F10 formulation.

Table 10: % Cumulative drug release of linezolid formulations

Media	Time in minutes	% Cumulative drug release									
		F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
	0	0	0	0	0	0	0	0	0	0	0
0.1M Sodium Acetate Buffer	10	1.50±0.07	1.01±0.13	0.24±0.13	1.54±0.13	1.17±0.10	0.36±0.27	3.83±0.56	5.06±0.67	2.05±0.20	4.83±0.41
	20	2.24±0.03	1.33±0.05	0.42±0.08	2.83±0.12	1.95±0.12	1.17±0.20	6.33±0.30	8.45±0.32	3.1763±0.08	8.36±1.13
	30	2.91±0.11	2.80±0.06	0.52±0.43	3.81±0.11	3.66±0.16	4.15±0.61	10.37±0.46	13.86±0.97	3.4152±0.09	14.77±1.26
	40	3.21±0.16	3.55±0.04	0.73±0.09	4.17±0.21	4.79±0.08	9.73±0.41	14.40±0.16	17.45±0.34	3.7082±0.08	18.58±0.64
	50	3.60±0.08	4.65±0.05	0.93±0.02	4.46±0.06	5.43±0.22	14.80±0.40	18.98±0.62	26.86±0.41	4.7918±0.131	19.77±0.16
	60	4.51±0.16	6.46±0.09	1.421±0.02	4.52±0.08	6.55±0.11	20.18±0.48	21.51±0.36	24.96±0.61	5.0674±0.06	23.43±0.36

* n=3,±SD

Table 11: Blood glucose levels

Rats with code and formulation	Blood glucose levels(mg/dl)
F8	268
F10	335
Reference formulation	214

Table 12: Wound area measurement

Rat with formulation code	Day	Initial wound area (mm^2)	Specific day wound area(mm^2)	% wound healing $(\frac{initial\ wound - specific\ day\ wound}{initial\ wound} \times 100)$
F 8	0		0	0
	5	450	314	30.2
	10		132	70.66
	15		22	95.11
	17		1	99.77
F 10	0		0	0
	5		275	38.88
	10		141	68.66
	15		16	96.44
	17		0	100
Reference formulation	0		0	0
	5		334	25.77
	10		185	58.88
	15		40	91.11
	17		23	94.88

* n=3, Average of three determinations, F 10 formulation showed better wound healing rates compared to F 8 and reference formulation.

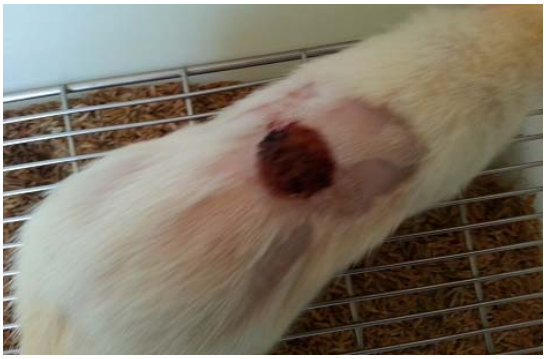

Fig. 5: Rat with initial wound

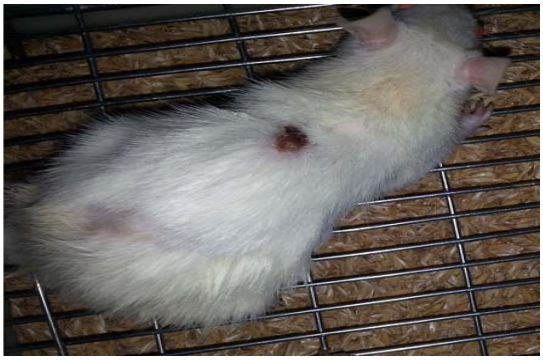

Fig. 6: Rat with wound after 15th day of treatment with F10

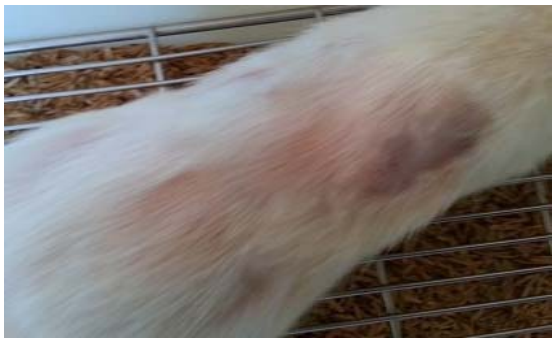

Fig. 7 Rat after 17th day of treatment

Stability studies

F8 formulation got liquefied at higher temperature and humidity. For F10 formulation evaporation of water was observed at higher temperature and humidity. There was no color change observed under higher temperature and humidity conditions. But both the formulations were stable at the low temperatures. This suggests that these formulations are to be stored in airtight container under cool conditions, protected from light. Further, long-term stability studies may provide precise required storage conditions.

CONCLUSION

These results indicate that linezolid semisolid dosage form could provide an adjunctive antimicrobial formulation for the management of diabetic wounds. Further studies are required on chronic diabetic wounds with and without diabetic medications to confirm its effectiveness.

CONFLICTS OF INTERESTS

No conflicts of interest

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