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Dermal application of lactic acid based cream of a non pathogenic *Kocuria marina* (BMKO1) strain against *Epidermophyton floccosum* (MTCC 613) symptomatic excision mice model

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ABSTRACT

The aim of this study was to evaluate the antifungal efficacy of *Kocuria marina* (BMKo1) derived Lactic acid against *Epidermophyton floccosum* (MTCC-613) infections induced on male Swiss Albino mice model (*Mus musculus*). For this purpose, the isolated strain was subjected to 'flask fermentation' and the Lactic acid produced as fermentation product, was quantified and analysed. Prior to preclinical test, healthy mice models of approximately 8 weeks old and 25-30 gm (weight) were subjected to intra-dermal administration for a period of 15 days to test for toxicity. Mortality, clinical signs, body weight changes were continually monitored. Then the mouse models were inoculated with 100 µl/ml (V/V) of *E. floccosum* (MTCC-613) spore suspensions following 'Excision model'. After induction of the infection, the symptomatic mice groups were subjected to topical application of *Kocuria* lactic acid cream based formulation at a concentration of 1µl/ml (V/V). The naked eye observations were made on the infected lesions till the absolute deduction of infection of excised skin surfaces. The degrees of deduction of infection were converted into scores and the percentages (%) of deduction of infection were calculated and the average value was derived. There were inclusion of positive control (Fluconazole) and negative control (group with infection induced excision, but without any drug application) mice groups for the sake of comparison. Further, with absolute deduction of infection score observed in mice group, applied with *Kocuria* derived Lactic acid was akin to Fluconazole activity. However, the infection induced mice group was found to be with substantial increase of degree of infection. This study have curtain raised about the anti *Epidermophyton* infection activity of a cream based Cell free Lactic acid derived from a non pathogenic strain of *Kocuria marina* on mouse models.

Keywords: *Kocuria marina*, *Epidermophyton floccosum*, Lactic acid

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INTRODUCTION

Anthrophillic dermatomycoses have a pivotal role to cause the dermal infections. Among them, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Epidermophyton floccosum* are reported as invasive agents causing acute as well as chronic infections [1, 2, 3]. As the invasiveness is an inherent characteristic of fungal strains, most of the prescribed drugs fail to inhibit the fungal growth inside the host tissue. Besides, many of marketed drugs are observed to be poorly delivered in the host body, thus making the fungal strain to evade the mechanism of action of drug. Strategies should be formulated to improve the bioavailability of the usual poorly absorbed drugs [4]. More so, majority of the failures in new drug development have been attributed to poor water solubility of the drug [5].

Hence, there is a need to develop antifungal agents from natural which can have the sufficient druggability [6]. In drug development processes, both synthetic as well as natural products [7] are in practice because natural products are the single most important resource for drug leads (www.imm.ac.cn).

In the context of development of drugs from natural sources, *in vitro* as well as *in vivo* studies are essential to launch a prodrug [8] in pharmaceutical industry. The *in vivo* studies specifically on animal models are important strategies to evaluate the drug delivery system as animal models are essential tools for biological research[9].

This present pursuit aims at evaluating Lactic acid extracted from an Actinomycetal strain namely *Kocuria marina* (Gene bank Accession No. MH752204) against the dermatomycotic infected (*Epidermophyton floccosum*, MTCC-613) symptomatic mice models. This is important to mention that Lactic acids, being weak organic acids [10] have immense importance in antifungal efficacy studies.

MATERIALS AND METHODS

Lactic acid Extraction

A strain of Actinobacteria namely *Kocuria marina* (BMKo1) was isolated and identified from a local nosocomial environment. The details of isolation and identification procedure are not included in this communication. The pure cultures of *K. marina* was subjected to fermentation study with different carbohydrates, namely, Glucose, Lactose, Sucrose, Dextrose and Mannitol. For this purpose, O/N ($10^{5cells/ml}$) *K. marina* culture was inoculated to a series of fermentative carbohydrates broth and slants containing 1% carbohydrates and Phenol red. The tubes were incubated at 37^0 C for a period of 24 hrs. Observation regarding fermentation (if any) was based on change in coloration of the medium both in broth and slant indicated by indicator Phenol red. Phenol red gives yellow coloration in acidic condition and remains red in neutral pH.

After initial screening with different carbohydrate broths, Glucose broth was selected for lactic acid production by fermentation. Therefore, Nutrient Broth was prepared with 1% glucose and phenol red was added as the indicator. Then O/N ($10^{5cells/ml}$) nosocomial isolate was inoculated to the fermentative sugar broth. The inoculated Glucose broths were allowed to incubate at 37^0C for 48hrs. After complete fermentation (complete color change from Red to Yellow), the broth was subjected to centrifugation (3000 rpm for 15 min in R4 Remi Centrifuge). The pellet was discarded and the supernatant was collected which was considered as the crude drug containing Lactic acid. The lactic acid produced by the nosocomial strain was catalogued as NLA (Nosocomial Lactic acid).

Quantification of lactic acid

The amount of Lactic acid produced by the Nosocomial strain into the medium was quantified by Titration method [11] of the cell free extract against 0.1M NaOH with Phenolphthalein as indicator. Therefore, 2 ml of NLA was mixed with distilled water in a conical flask to make the volume up to 10 ml. At the same time 2 drops of Phenolphthalein indicator was added to it. After that NaOH was taken in the burette and titration was carried out. The initial and end point reading were noted and finally the quantity of acid was calculated.

Preparation of pharmaceutical formulation

The test lactic acid was prepared in a cream based pharmaceutical formulation [12] for animal study. For this purpose, Bees wax and wool fat were melted together. Then liquid paraffin was added as the base material to it with gentle heating. Aqueous based purified lactic acid in 1:1 concentration, warmed at same temperature as that of the base mixture was added to it and mixed thoroughly. The mixture was then stirred continuously till the mixture is cooled down. The stable mixture was stored (at room temperature) in a sterile vial for future.

Toxicity test for the Lactic acid (LA) based cream

The toxicity test for Lactic acid based cream was performed in the Dept. Of Pharmacology, VIMSAR, Burla, India with prior supervision of Pharmocologist of the said Institute. The crude sterile lactic acid was extracted from the strain of *Kocuria marina* (BMKo1). After quantitative estimation the lactic acid was made into a pharmaceutical cream formulation. The prepared cream was evaluated for various properties such as compatibility, drug content, viscosity, *in vitro* skin permeation etc.[13]. Three numbers of mice were taken as test mice members for the toxicity. The pelvic areas ($1cm^3$) of rats were shaved using sterile razors. The Lactic acid based formulation was applied topically on the shaved skin surfaces of test rat group for a period of 15 days. The control group was kept unapplied. Mortality, clinical symptoms, body weight changes were continually monitored regularly by following standard *in vivo* study parameters.

In vivo effect of Lactic acid based cream on symptomatic Excision Mice model

The *in vivo* antidermatophytic activity was carried out in the Despande Lab. Pvt. Ltd, Bhopal, India. The test male Swiss albino rats were categorized into 4 groups, where one group (Group I) was asymptomatic and other 3 groups were symptomatic. The details in groupings of test mice are given in below figure 1.

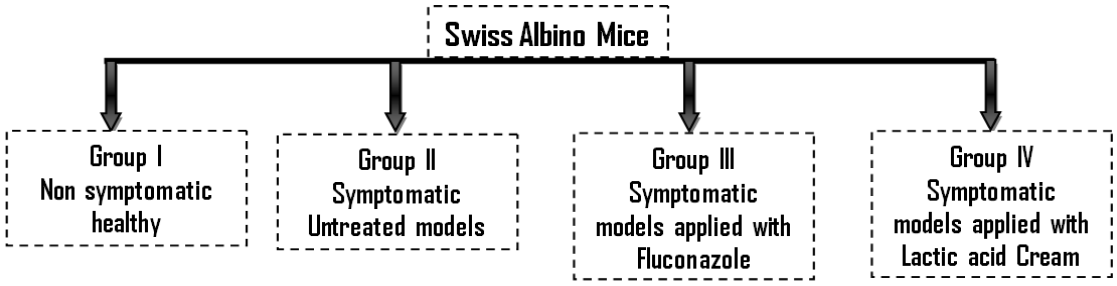


Figure 1: Grouping of Mice models

Group I included the non symptomatic healthy mice members, Group II included mice members left untreated, Group III considered the symptomatic mice members treated with Fluconazole, and Group IV included the symptomatic mice members applied with LA based formulation.

Group I mice members were not given any immunosupression as well as kept untreated, whereas group II, III, and IV were injected subcutaneously with the immunosupressant (500 mg, w/v of Estradiol Valerate) and were observed for a time period of 3 days. Then the flanks of mice were shaved with electric razor and the exposed area lightly abraded with a sterile scalpel. The *Epidermophyton floccosum* (MTCC-613) had been availed from IMTECH, Chandigarh. Conidia were obtained from 7 days old culture of test fungus by washing the surface of the media with sterile distilled water and a suspension (approx. 10 spores / µl) was prepared. All the four groups of mice were kept in separate cages for regular observation. Further, the lesions were visually examined daily to determine the severity and recovery of lesions and after a period of 7 days, the intensity of infection was measured in scores. On seventh day of infection the lesions were scored as follows: 0 (absence of lesion), 1 (appearance of erythema at infected site or new hair growth on the bald exposed area), 2 (moderate erythema spreading over entire infected site), 3 (intense erythema with abrasions, swelling and scaling), 4 (severely erythematous lesion with crusting spreading over the entire

exposed area). The average lesion scores were calculated for each group by dividing the sum of the lesion scored by the number of animals in the group. Moreover, the infected site of each animal was carefully examined daily throughout the treatment period to determine % recovery of infected site and treatment scores were given to mice groups as follows: 0 (not cured), 1 (25% cured), 2 (50% cured), 3 (75% cured), 4 (100% cured). The average scores were calculated.

RESULTS & DISCUSSION

The observations regarding different carbohydrate utilization by the test isolates have been given below.

Table 1: Carbohydrate utilization by nosocomial isolates

Carbohydrate Type	Glucose	Sucrose	Dextrose	Lactose	Mannitol
Inference	+	+	+	+	+

(NB: + ve indicating of Fermentative)

From the results displayed in Table 1, it is observed that, the test strain was able to utilize the entire test Carbohydrates like Glucose, Sucrose, Dextrose, Lactose and even Mannitol.

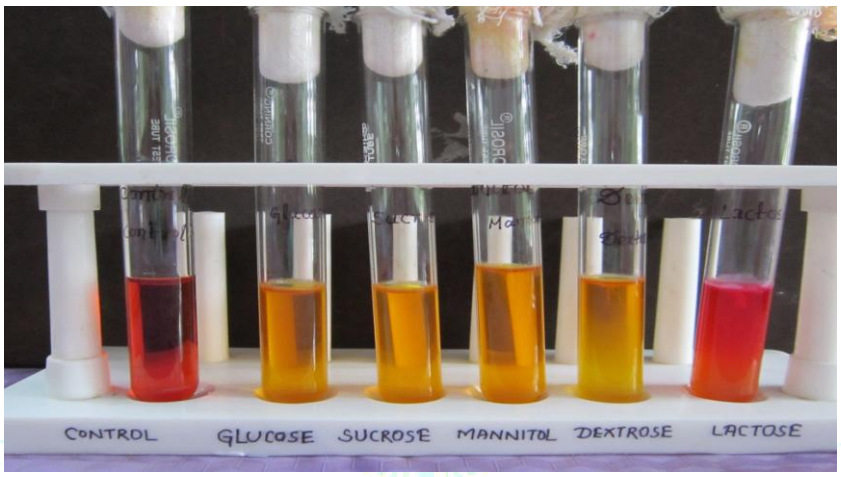


Figure 2: Depicting Carbohydrate fermentation of *K.marina*

It was observed that both the broth and slants meant for fermentation test were changed into yellow due to acid production (Figure 2).

Quantification of lactic acid

The titration experiment carried out to determine the quantity of Lactic acid produced by the bacterial cells indicated that the total volume of Sodium hydroxide (NaOH) consumed was 0.32 ml. Therefore, 0.32 / 1000×0.1 moles / l or 0.00003 moles / litre NaOH was required to titrate 10^5 CFU/ml of the bacterial cell free extract containing the fermented product, i.e., Lactic acid.

The quantification of produced Lactic acid / Number of bacterial cell has been done as follows, based on the Bronsted-Lowry theory:

As the number of moles of NaOH= Number of moles of acid; 1 mole of LA reacts with 1 mole of NaOH.

So, 0.00003 mole of NaOH reacted with 0.00003 moles of LA.

As the amount of crude cell free extract taken for titration was 2 ml,

2ml of cell free Lactic acid = 0.002 litre of Cell free Lactic acid, i.e. 0.00003 moles of NaoH / 0.002 litre Cell free Lactic acid = 0.015 moles / litre Cell free Lactic acid. Therefore, the 0.015 moles / liter cell free lactic acid was produced from 10^2 CFU/ liter of bacterial cells in the medium containing only 1% Glucose following 18 hrs of incubation. The results of the titration have been exhibited in Fig 3.

A B C

Figure 3: Titration of cell free Lactic acid produced by *K. marina;* a: Flask containing cell free LA (yellow color), b: After titrated with NaoH color was Red, and c: Excess of NaOH (PH ≥7 color was deep pink)

Toxicity test for the LA based formulation

After 15 days of observation on LA based formulation applied on the surfaces of mice models, it was found that the mice had developed immediate hypersensitivity (HS) reaction (Fig.4.a) which was observed as Rubor (Redness). However, the inflammation started to disappear after 72 hrs (Graph 1). There was no trace of HS after 15 days of regular application of lactic acid based formulated cream. Also there were no unscheduled diseases or deaths in any model during the study period. The rats belonging to both groups were healthy and active. Initial development of HS is quite acceptable because, the HS is demonstration of a type of vertebrate defence mechanism. Mention may be made here that this toxicity testing of Kocuria derived LA on animal models helped to calculate the no observed adverse effect level (NOAEL) dose as this test is a prerequisite in clinical trials.

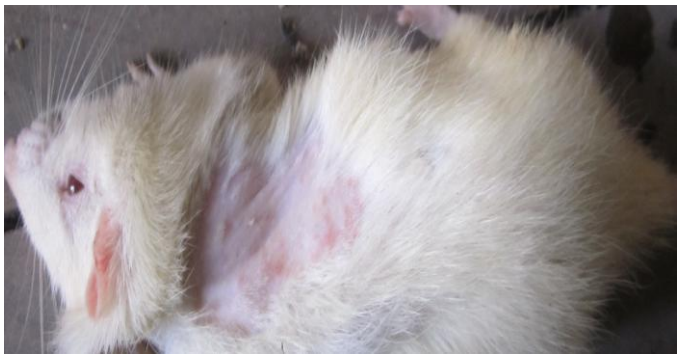


Figure 4.a: Skin surface of the representative mice model of Test group on day 1 showing immediate Hypersensitivity reaction

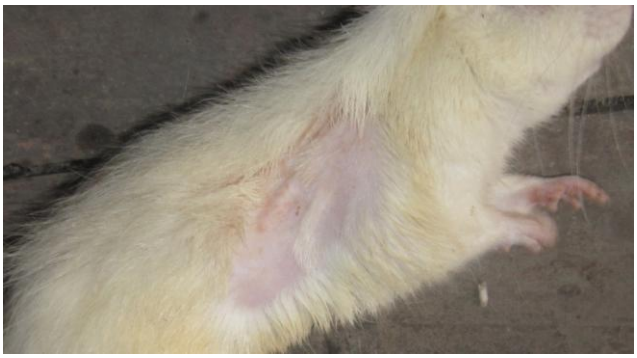


Figure 4.c: Skin surface of the representative Mice of Test group on day 15 showing disappearance immediate Hypersensitivity reaction (HS)

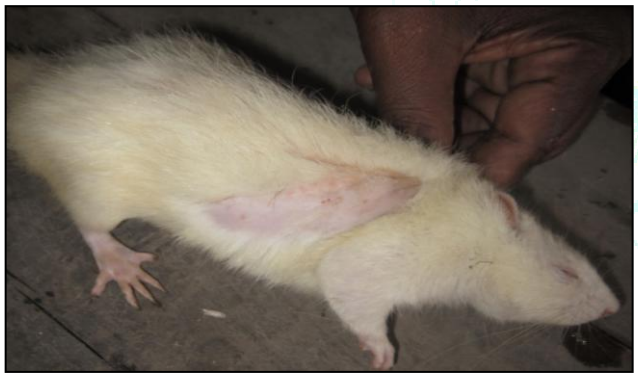


Figure 4.b: Skin surface of the representative mice model of Control group on day 1

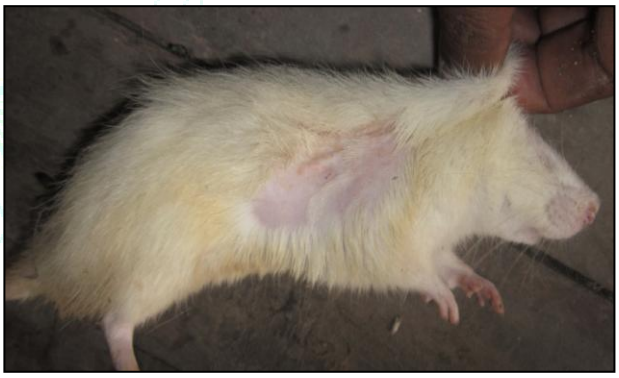
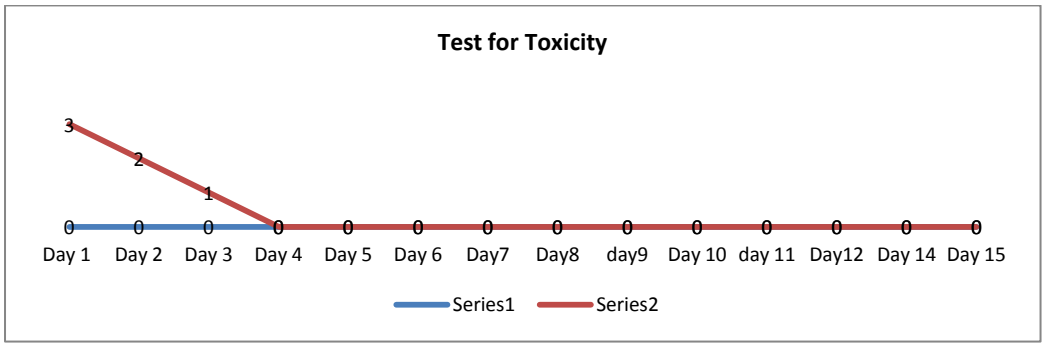


Figure 4.d: Skin surface of the representative Mice of Control group on day 15



Graph 1: Depletion of immediate HS in test Mice model

The appearance of redness on skin surface of test mice model on application of LA based formulation on 1st day could be due to local inflammation but not leading any cellulites on subsequent days give a clear proposition about the non toxicity of the said Lactic acid cream formulation. The fermentative product of a nosocomial Actinobacteria BMKo-1 was observed to be nontoxic on mice models when applied topically.

***In vivo* effect of *Epidermophyton floccosum* (MTCC 613) infection induced on mice model**

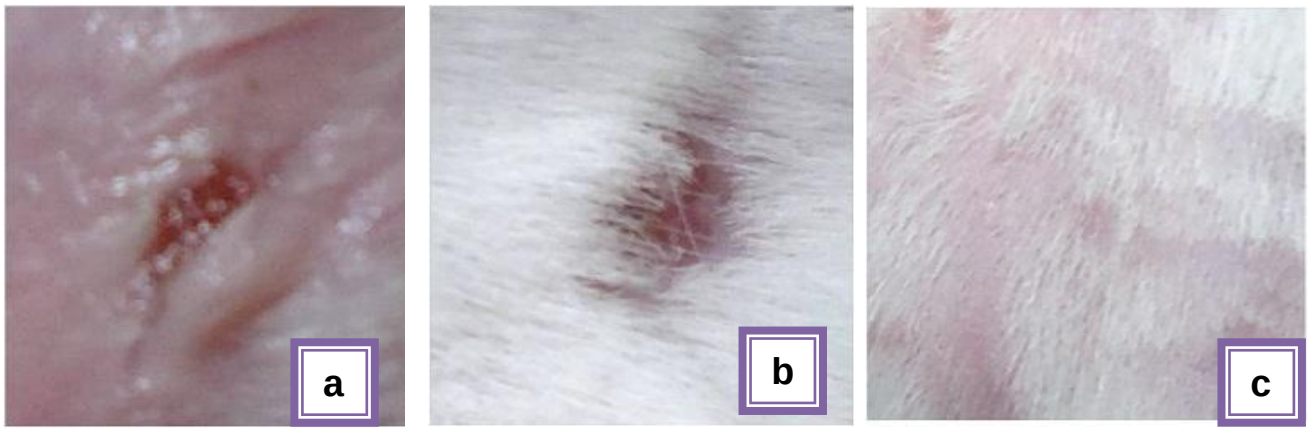
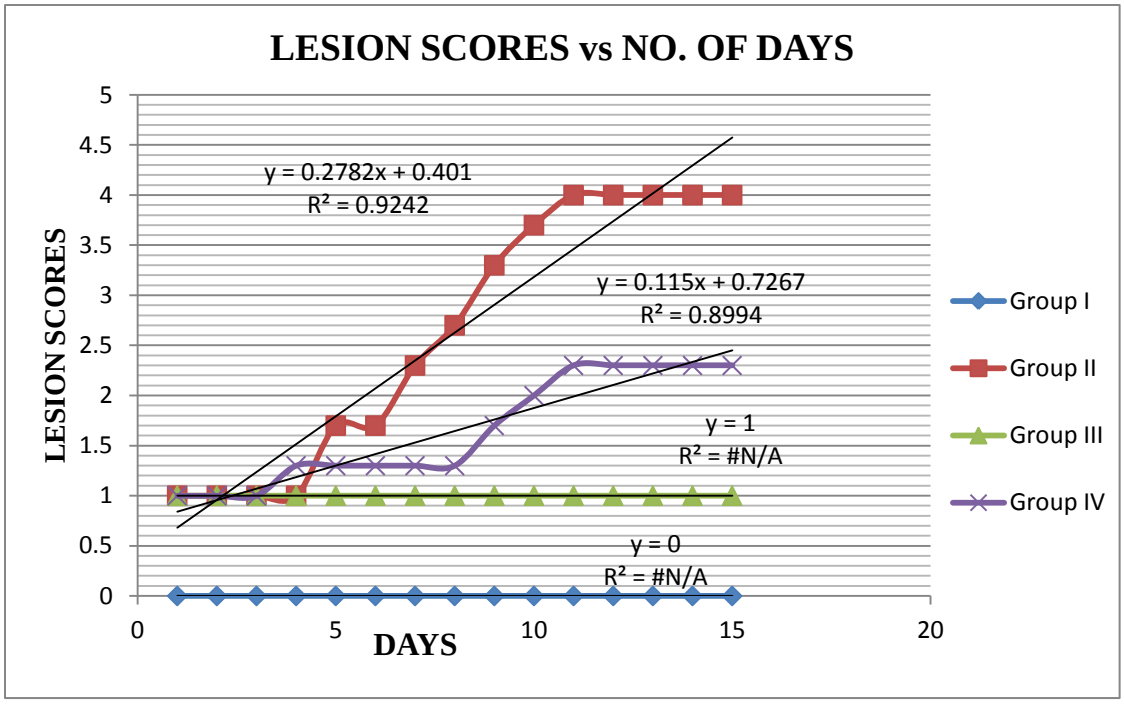


Figure 5: Representative mice skin surfaces induced with *Epidermophyton* infection a: Infected but restricted from any dermal application, b: applied with LA based formulation and c: Applied with the standard drug Fluconazole

Table 2: Lesion Scores of the 4 animal groups

LESION SCORES																
	Non symptomatic Healthy (I)				Symptomatic and Untreated (II)				Treated with Fluconazole (III)				Treated with LA formulation (IV)			
DAYS	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg
1	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0	1	1	1	1.0
2	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0	1	1	1	1.0
3	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0	1	1	1	1.0
4	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0	1	2	1	1.3
5	0	0	0	0.0	2	1	2	1.7	1	1	1	1.0	1	2	1	1.3
6	0	0	0	0.0	2	1	2	1.7	1	1	1	1.0	1	2	1	1.3
7	0	0	0	0.0	3	2	2	2.3	1	1	1	1.0	1	2	1	1.3
8	0	0	0	0.0	3	3	2	2.7	1	1	1	1.0	1	2	1	1.3
9	0	0	0	0.0	4	3	3	3.3	1	1	1	1.0	2	2	1	1.7
10	0	0	0	0.0	4	3	4	3.7	1	1	1	1.0	2	2	2	2.0
11	0	0	0	0.0	4	4	4	4	1	1	1	1.0	2	2	3	2.3
12	0	0	0	0.0	4	4	4	4	1	1	1	1.0	2	2	3	2.3
13	0	0	0	0.0	4	4	4	4	1	1	1	1.0	2	2	3	2.3
14	0	0	0	0.0	4	4	4	4	1	1	1	1.0	2	2	3	2.3
15	0	0	0	0.0	4	4	4	4	1	1	1	1.0	2	2	3	2.3

NB: Average lesion score: 0 (absence of lesion), 1 (appearance of erythema at infected site or new hair growth on the bald exposed area), 2 (moderate erythema spreading over entire infected site), 3 (intense erythema with abrasions, swelling and scaling), 4 (severely erythematous lesion with crusting spreading over the entire exposed area).

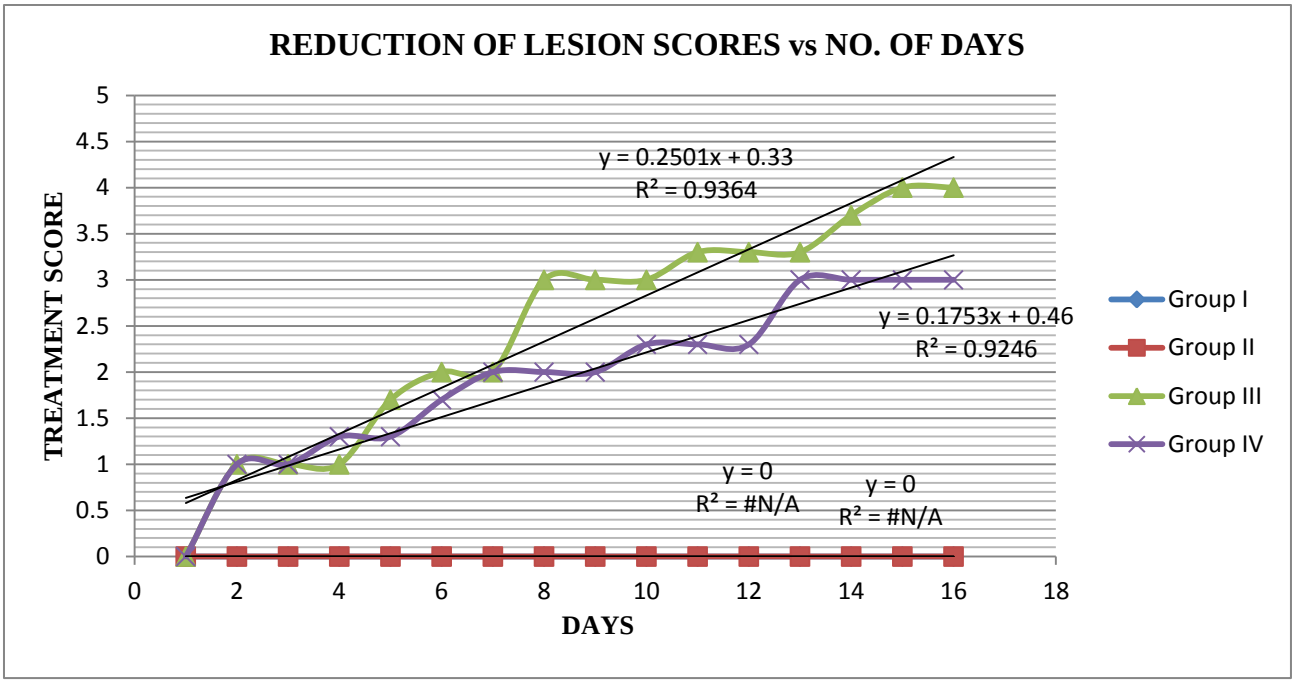


Graph 2: Regression analysis of lesion scores with no. of days

Table 3: Reduction of Infection scores of mice group after treatment

TREATMENT SCORES																
	Non symptomatic, Healthy (I)				Symptomatic and Untreated (II)				Treated with Fluconazole (III)				Treated with LA formulation (IV)			
DAYS	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
1	0	0	0	0.0	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0
2	0	0	0	0.0	0	0	0	0.0	1	1	1	1.0	1	1	1	1.0
3	0	0	0	0.0	0	0	0	0.0	1	1	1	1.0	1	1	2	1.3
4	0	0	0	0.0	0	0	0	0.0	1	2	2	1.7	1	1	2	1.3
5	0	0	0	0.0	0	0	0	0.0	2	2	2	2.0	1	2	2	1.7
6	0	0	0	0.0	0	0	0	0.0	2	2	2	2.0	2	2	2	2.0
7	0	0	0	0.0	0	0	0	0.0	3	3	3	3.0	2	2	2	2.0
8	0	0	0	0.0	0	0	0	0.0	3	3	3	3.0	2	2	2	2.0
9	0	0	0	0.0	0	0	0	0.0	3	3	3	3.0	2	2	2	2.3
10	0	0	0	0.0	0	0	0	0.0	4	3	3	3.3	2	2	2	2.3
11	0	0	0	0.0	0	0	0	0.0	4	3	3	3.3	2	2	2	2.3
12	0	0	0	0.0	0	0	0	0.0	4	3	3	3.3	3	3	3	3.0
13	0	0	0	0.0	0	0	0	0.0	4	3	4	3.7	3	3	3	3.0
14	0	0	0	0.0	0	0	0	0.0	4	1	4	4.0	3	3	3	3.0
15	0	0	0	0.0	0	0	0	0.0	4	1	4	4.0	3	3	3	3.0

N.B. Treatment scores. 0 (not cured), 1 (25% cured), 2(50% cured), 3 (75% cured), 4(100% cured)



Graph 3: Regression analysis of scores of reduction of infection with no. of days

In the tests comprising *in vivo* application of Lactic acid based formulation on the test mice groups the observed results are reported in Table 2 & 3; Whereas the visual observation of test mice models are given in Figure 5. From the table it is observed that Group II symptomatic mice members remain uncured (with 0 scores). While Group IV symptomatic mice treated with LA based formulation were observed with 75% cure (Score 3) in the day 12th. Although there was 75% cure was observed with group III mice members applied with Fluconazole at day 7. In addition, the statistical regression analysis substantiated the fact that there was a positive correlation between the reduction intensity of infection with advancement of number of days.

The results observed here have inferred about the *in vivo* antidermatomycotic activity of lactic acid based formulation on test mice models. The effectiveness was at par with the antimycotic activity of Fluconazole. This is further to mention that lactic acid which is a natural producing metabolite from an actinomycetal strain could able to cure the induced dermatophytic infection efficiently without imposing any toxic effect on the test mice models. All the four groups of the mice members were found to be healthy during the whole test period.

CONCLUSION

From this it is suggested that the Lactic acid (015 moles / L while titrating against NaOH, from 10^{2} CFU/ L) based formulation had anti infective property with least toxicity, when tested against symptomatic mice members. The anti infective property was quite comparable with activity of standard drug. As it is relevant to mention that the results given here is unbiased as the whole set of experiments were carried out in Despande Lab Pvt. Ltd. following the 'blind test' rule. Hence this formulation has can be implemented as an excipient or drug active moiety in the antidermatomycotic drug designing processes. In other words, this study have curtain raised about the anti *Epidermophyton* infection activity of a cream based Lactic acid derived from a non pathogenic strain of *Kocuria marina*. The result of this analysis has revealed an appreciable amount of druggability of a Lactic acid produced from an orphan nosocomial strain in the name of bio prospective activity by using the existing biological resources.

Conflict of Interest: There is no conflict of interest.

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