RESEARCH PAPER

Synergistic Antifungal Liposomal Gel Containing Lawsone and Amphotericin B: In Vitro and In Vivo Efficacy Against Candida albicans and Rhizopus oryzae

Mr. Vinayak R. Bodhankar[1*], Dr. Vivek Daniel[2]
[1*] Research Scholar, Oriental University, Indore (Corresponding Author).
Email: vinayakbodhankar1996@gmail.com
[2] Professor, Faculty of Pharmacy, Oriental University, Indore

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ABSTRACT
Candida albicans and Rhizopus oryzae dermal fungal infections continue to pose a clinical challenge due to increasing resistance to antifungals, systemic toxicity, and reduced dermal drug penetration. Liposomal drug delivery systems enhance bioavailability, skin permeation, targeted delivery, and combination treatment provides synergistic effects. This research aimed to formulate a liposomal gel containing lawsone and amphotericin B, and to evaluate its synergistic antifungal activity through in vitro assays and in vivo studies using female Wistar rats with experimentally induced candidiasis. The thin-film hydration method was used to prepare liposomes, which were then placed in a gel matrix composed of Carbopol 934. Clinical parameters (erythema, scaling, and lesion size) were monitored, followed by histopathological evaluation using hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) staining to monitor tissue changes. The in vitro findings supported the high effectiveness of the combination gel, which was shown by the larger zones of inhibition and a much lower minimum inhibitory concentration than monotherapy and marketed formulations, reducing by as much as four-fold MIC. Fractional inhibitory concentration index (FICI) revealed a high level of synergism with Candida albicans (0.245) and Rhizopus oryzae (0.375), as FICI values of 0.5 or less confirm synergy. In vivo study showed extensive clinical improvement ($p < 0.05$), and close-to-normal tissue architecture and low levels of fungus in the combination-treated groups. Overall, the outcomes highlight the improved antifungal potential of the combinational liposomal gel, suggesting its promise as an effective topical therapy for dermal fungal infections.
Keywords: Lawsone; Amphotericin B; Liposomal gel; Synergism; antifungal; Candida albicans; Rhizopus oryzae; etc.
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1. Introduction

Fungal pathogens create significant health challenges throughout the world, both in milder cutaneous mycoses and more severe and lethal systemic infections. Candida albicans is still considered one of the most common fungal strains that cause mucocutaneous and systemic candidiasis, predominantly in patients with compromised immune systems.On the same note, the main causative species of mucormycosis, a fast-progressing fungal infection with high mortality and morbidity are species of Rhizopus, namely Rhizopus oryzae [1,2].The emergence of COVID-19-related mucormycosis further emphasized the necessity of effective antifungal treatment of resistant and destructive fungal pathogens [1].Amphotericin B (AmB) remains considered a gold-standard broad-spectrum antifungal agent in mucormycosis and severe candidiasis.Its clinical use is

limited due to the adverse effects associated with its administration through infusion, dose-dependent nephrotoxicity, and limited water solubility [3].The development of liposomal amphotericin B has greatly increased its therapeutic index by decreasing systemic toxicity and preserving antifungal value [3].Also, lipid-based topical drug delivery systems, such as liposomes and ethosomes, have exhibited enhanced skin penetration, controlled release, localized drug retention, and thus enhancing outcomes in cutaneous fungal infections [4-7].Amphotericin B liposomal have shown good results in dermal candidiasis models with reduced systemic exposure and targeted delivery [5,6].Nevertheless, limitations like the emergence of resistance, biofilm-related tolerance and tissue diffusion still hinder the effectiveness of monotherapy.

Dual drug antifungal therapy has been receiving growing attention as a strategy to enhance therapeutic effect, reduce the dose of drugs and slow down the spread of resistance.A lot of research has shown synergistic effects between amphotericin B and natural bioactive compounds leading to increased antifungal activity against Candida albicans [8-10].In vitro interaction such as the measurement of a fractional inhibitory concentration index offers a rational approach to discovering synergistic combinations [11].Such synergistic approaches are mainly relevant in biofilm-creating Candida strains, where resistance to conventional antifungals is usually detected [9].

Antifungal phytoconstituents are important medicinal herbs. Lawsonia inermis (henna) is a conventionally used medicinal herb for its antifungal and antimicrobial properties.Its main active constituent, lawsone (2-hydroxy-1,4-naphthoquinone), has been shown to have significant antifungal activity against dermatophytes, Candida species, and other pathogenic fungi [12–15].The occurrence of naphthoquinones and its analogous bioactive molecules that cause antifungal activities as reported by the phytochemical investigation [13,14].The effectiveness of henna extracts and preparations has also been proven in in vitro and in vivo models of fungal infections such as candidiasis by experimental research [16,17]. Lawsone, like many phytoconstituents, has limitations including a low water solubility, reduced stability, and reduced skin penetration in mono-use.

Recent advancement in lipid-based drug delivery systems and nanotechnology present viable alternatives to these shortcomings.Liposomes increase drug solubility, stability, permeability, and controlled release while reducing systemic toxicity [4,18]. Insertion of phytochemicals into nanocarrier systems has been revealed to enhance their bioavailability and therapeutic efficiencies. [18]. A liposomal gel encapsulating lawsone and amphotericin B may therefore provide a synergistic antifungal approach, combining the potent fungicidal potential of amphotericin B with the complementary antifungal and potential biofilm-disrupting behavior of lawsone.

Despite reported antifungal activities of liposomal amphotericin B and lawsone separately, a limited study has investigated their combined delivery within a single topical liposomal gel system, pointing to both *Candida albicans* and *Rhizopus* species. Considering the growing occurrence of resistant Candida strains and mucormycosis caused by *Rhizopus* spp. [2], The development of a more effective and synergistic topical antifungal formulation is highly warranted.

Therefore, the present research aims to develop and characterise a synergistic antifungal liposomal gel containing lawsone and amphotericin B and to evaluate its antifungal potential in both in vitro and in vivo against *Candida albicans* and *Rhizopus* species. This therapeutic approach may offer an innovative and effective substitute for the management of challenging severe fungal infections.

2. Materials and Methods

2.1 Materials

Lawsone (2-hydroxy-1,4-naphthoquinone) was procured from Yucca Enterprises, Mumbai, India. Amphotericin B, soya lecithin, cholesterol, Carbopol 934, triethanolamine, propylene glycol, glycerol, and other chemicals were obtained from Swapnroop Drugs and Pharmaceuticals, Aurangabad, India. Analytical grade solvents, including chloroform and methanol, were also sourced from Swapnroop Drugs and Pharmaceuticals. Phosphate-buffered saline (PBS, pH 7.4) was prepared in-house using standard laboratory procedures. Candida albicans and Rhizopus oryzae strains were acquired from recognised microbiological culture collections for use in antifungal assays.

2.2 Formulation of Liposomal Antifungal Gel

2.2.1 Preparation of liposomes

Preparation of liposome follows the film hydration methods, containing Lawsone and Amphotericin B in an organic solvent. Soya Lecithin (400 mg) and Cholesterol (120 mg) were transformed using the thin film hydration method. The organic phase mixture

containing Chloroform and methanol (1:1) ratio was transferred into a dry round-bottom flask. Dissolve phospholipids, cholesterol and the drug. The solution was allowed to evaporate using a rotary flash evaporator, at reduced pressure for 30 – 60 min. An organic thin film was formed on the inner side of round bottom flask after solvent evaporation. The hydration was performed by transferring 1x phosphate-buffered saline solution (20 ml) into the RBF. The RBF was allowed to rotate at 60 rpm for 1 h. Transfer the suspension into a beaker and allowed to ultrasonicate for 10 min with 10 sec On-OFF cycle until a homogeneous mixture is formed. The homogenous mixture was allowed to cool for 12 h at ambient temperature and stored in 2 – 8°C till further use.

2.2.2 Preparation of liposomal gel

The Liposomal gel was prepared using Carbopol 934 and triethanolamine for pH adjustment. The Liposome containing Lawsone and Amphotericin B, containing 1% equivalent suspension, was taken into a test tube (5 ml each). The 1% Carbopol 934 was dissolved in distilled water and stirred till the homogenous mixture formed. The hydration time of Carbopol gel was 24 h, and kept aside for 24 h for complete swelling. The drug-containing liposomal suspension was added slowly into the Carbopol gel with continuous stirring on the magnetic stirrer (8 h). The propylene glycol (6.2%) and glycerol (1%) were added to the gel base, followed by the addition of triethanolamine to adjust the pH to 7.

2.3 In Vitro Antifungal Studies

For the in-vitro evaluation of antifungal activity, *Candida albicans* and Rhizopus oryzae were employed as the test organisms. Sabouraud dextrose agar (20 mL) was dispensed into sterile Petri plates and subsequently inoculated with 100 µL of the prepared fungal suspensions.

2.3.1 Determination of Zone of Inhibition (ZOI)

After inoculation, plates were left to dry at room temperature of about 15 minutes.Wells of 8 mm diameter were then carefully created in the agar medium, and each well was filled with 100 mg of the respective gel formulations:

1. Gel containing Lawsone
2. Gel containingAmphotericin
3. Combination gel containing Lawsone and Amphotericin B
4. Commercial formulation (Amphotericin B)

The plates inoculated were incubated at 25^0 C for 48 hours.Following the incubation period, antifungal effect was determined by measuring the diameter of the inhibition zone around the wells.The results were recorded as the mean dimensions of the growth inhibition zones for each formulation.

2.3.2 Determination of Minimum Inhibitory Concentration (MIC)

Following inoculation, the plates were allowed to dry at room temperature for approximately 15 minutes. Two-fold serial dilutions of each drug were prepared (320-20 µg/mL). For combination testing, Lawsone and Amphotericin B were combined in fixed ratios (1:1).Each well was filled with different concentrations of formulations. Incubation conditions were the same as agar diffusion.MIC was recorded as the lowest concentration, showing no visible growth.

2.4 In Vivo Antifungal Studies

2.4.1 Test Materials

Two topical gel formulations were evaluated in the present study: Sample A (marketed antifungal gel) and Sample B (prepared formulation, i.e. liposomal gel containing lawsone and amphotericin B). Both formulations were semisolid gels intended for dermal application. A standardised suspension of *Candida albicans* (1×10^6 CFU/mL) was used to induce cutaneous fungal infection.

2.4.2 Experimental Animals

Female Wistar rats (*Rattus norvegicus*) weighing 170–200 g were used for the study. Animals were procured from Crystal Biological Solutions. Animals were maintained under standard laboratory conditions: temperature 22 ± 2 °C, relative humidity 55 ± 5%, and a 12 h light/dark cycle. Rats were housed in polypropylene cages (four animals per cage) with stainless-steel tops and clean paddy husk bedding. Commercial pelleted feed (VRK Pvt. Ltd., Pune) and potable RO-treated water were provided ad libitum. Before experimentation, animals were acclimatised to laboratory conditions over a period of 7 days after which they were monitored on a daily basis regarding clinical abnormalities.Only animals with desired body weight and health requirements were taken.

2.4.3 Ethical Approval and Animal Welfare

All experimental studies were conducted under the provisions of the Committee for the Control and Supervision of Experiments on Animals (CCSEA), Government of India, as reported in The Gazette of India (December 15, 1998), and in accordance with ISO 10993-2: Biological Evaluation of Medical Devices - Animal Welfare Requirements.

The study protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (Proposal No. CCSEA/PCBS/2025-26/P-03).

2.4.4 Study Design

A total of 20 rats were randomly allocated into four groups:

- Normal control (n = 2)
- Group I – Disease control (n = 6)
- Group II – Infection + Sample A (n = 6)
- Group III – Infection + Sample B (n = 6)

The total duration of the experimental model was 14 days. A similar grouping strategy for evaluating topical antifungal formulations in experimental candidiasis has been previously reported (Qushawy et al., 2018; Abdellatif et al., 2017).

2.4.5 Development of Cutaneous Candidiasis

A standardized suspension of *Candida albicans* (1×10^6 CFU/mL) was prepared. The dorsal region of each rat was shaved aseptically 24 h prior to inoculation. Cutaneous infection was induced by intradermal injection of 0.3 mL of the fungal suspension into the centre of the shaved dorsal area. Slight oedema formed at the injection site was gently dispersed. Visible fungal infection developed within 72 h post-inoculation. To prevent licking or cross-contamination, infected animals were housed individually after infection induction. The normal control group received 0.3 mL sterile saline intradermally instead of the fungal suspension. (Asghar et al., 2024; Abdellatif et al., 2017).

2.4.6 Treatment Protocol

For treated groups (Group II and Group III), topical dosing was initiated 72 h after fungal inoculation. A standard dermal application rate of 2 mg/cm² was adopted to ensure uniform and reproducible topical exposure. The infected surface area (cm²) of each animal was measured prior to dosing. The total quantity of gel applied per application was calculated using the formula:

$$Dose(mg)=Infectedarea(cm^2)\times 2mg/cm^2$$

The calculated quantity was accurately weighed and applied uniformly over the infected region.

- **Group II:** Received Sample A twice daily.
- **Group III:** Received Sample B twice daily.

Treatment was continued for 14 consecutive days.

2.4.7 Evaluation Parameters

2.4.7.1 Clinical Assessment

The semi-quantitative scoring system (0-3 scale) was used to assess the severity of infection basing on erythema, scaling, and lesion size.The scores were measured at scheduled periods, during the study period.

2.4.7.2Histopathological Examination (Hematoxylin and Eosin Staining, n = 8)

By the conclusion of the experiment, 2 animals in each group and 2 healthy animals were euthanised, and skin tissue samples of the affected part were taken out.Tissues were fixed using neutral buffered formalin 10% and subjected to standard procedures and embedded in paraffin.Cross-sections of 3-5 000 m were sectioned with a microtome (Leica Biosystems, Germany).Sections were deparaffinized in xylene and rehydrated with decreasing alcohols up to distilled water.A stain of Hematoxylin was used to stain the nucleus and bluing was done under running tap water.Cytoplasmic elements and extracellular matrix were then stained with eosin.Sections were dried, stained with xylene and mounted with DPX.A light microscope (Motic Microscopes, USA) was used to microscopically examine epidermal thickness, inflammatory cell infiltration, and tissue architecture.

2.4.7.3Histopathological Examination (Periodic Acid–Schiff Staining, n = 8)

To visualise fungi, Periodic Acid Schiff (PAS) staining was performed on additional sections of the tissues. Sections were deparaffinized and rehydrated. The oxidation was done in the presence of 0.5 percent periodic acid solution for 5 min.After rinsing with distilled water, sections were treated with Schiff's reagent for 15 min. Slides were washed under lukewarm running tap water for 5 min to develop a characteristic dark pink colouration indicative of fungal elements.The counterstaining was done in Mayer hematoxylinfor 1 min then washed.Sections were dried, cleaned, and mounted with synthetic mounting medium.The magenta stains characteristics were used to identify the fungal hyphae and the yeast cells under the microscope.

3. Results

3.1 In Vitro Antifungal Activity

3.1.1 Zone of Inhibition

Table 1. Observed Zone of Inhibition (ZOI) of various antifungal gel preparations against Candida albicans, and Rhizopusoryzae (in mm).

	Lawsone	**Amphotericn B**	**Lawsone + Amphotericin B**	**Marketed**
Candida	2.5	2.9	7.4	5.3
	2.7	3.2	7.6	5.6
	2.4	3.4	7.3	5.8
Mean ± SD	2.6 ± 0.15	3.16 ± 0.25	7.43 ± 0.15	5.56 ± 0.25
Rhizopus	2.3	4.2	8.2	6.5
	2.8	4.7	8.6	7.1
	2.9	4	8.9	6.8
Mean ± SD	2.6 ± 0.32	4.3 ± 0.36	8.56 ± 0.35	6.8 ± 0.3

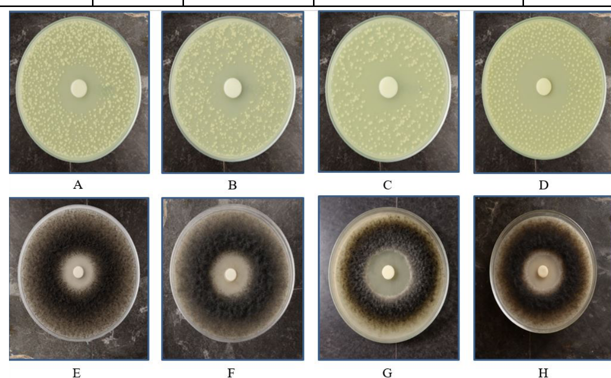

Figure 1. Representative agar well diffusion plates with antifungal activity of various formulations against stated fungal pathogens.(A) Lawsone gel against Candida albicans, (B) Amphotericin B gel against Candida albicans, (C) Combination gel containing lawsone and amphotericin B against Candida albicans, (D) Marketed formulation against Candida albicans, (E) Lawsone gel against Rhizopus oryzae, (F) Amphotericin B gel against Rhizopus oryzae, (G) Combination gel against Rhizopus oryzae, and (H) Marketed formulation against Rhizopus oryzae. Experiments were performed thrice.

One-way ANOVA followed by Tukey-Kramer post-hoc test showed statistically significant difference ($p < 0.05$) between all the treatment groups. The Lawsone + Amphotericin B combination had a much greater zone of inhibition than Lawsone alone, Amphotericin B alone and the commercially available formulation against Candida and Rhizopus species.

3.1.2 Minimum Inhibitory Concentration (MIC)

Table 2. Zone of inhibition (ZOI) of Lawsone, Amphotericin B, Combination Formulation, and Marketed Product at different concentrations against *Candida albicans.*

Concentration(µg/mL)	**Lawsone**	**Amphotericn B**	**Lawsone + Amphotericin B**	**Marketed**
20	0	0	7.4	0
40	0	0	7.6	5.6
80	2.4	2.9	7.8	5.8
160	2.5	4.2	8.2	6.5
320	2.8	4.7	8.6	7.1

Calculated MIC values:

Table 3: MIC values of Lawsone, Amphotericin B, their combination and marketed product against *Candida albicans*

Formulation	**MIC (µg/mL)**	**Justification**
Lawsone	80 µg/mL	First visible ZOI at 80µg/mL
Amphotericin B	80 µg/mL	First visible ZOI at 80µg/mL
Lawsone + Amphotericin B	20 µg/mL	ZOI present at lowest dose
Marketed	40 µg/mL	First visible ZOI at 40µg/mL

The combination formulation exhibited the lowest MIC value (20 µg/mL) against Candida albicans and this means it is more potent in antifungal activity. In comparison, the individual drugs exhibited higher MIC values (80 µg/mL), while the marketed formulation showed an intermediate MIC of 40 µg/mL. The reduction from 80 µg/mL to 20 µg/mL represents a 4-fold decrease relative to the individual agents and a 2-fold decrease compared to the marketed product.

3.1.3 Fractional Inhibitory Concentration Index (FICI)

Since the total MIC of the combination = 20 µg/mL, and it is a 1:1 combination.

MIC of Lawsone in combination = 10 µg/mL

MIC of Amphotericin B in combination = 10 µg/mL

$$FICI = \frac{\text{MIC of A in combination}}{\boldsymbol{MICofAalone}} + \frac{\text{MIC of B in combination}}{\boldsymbol{MICofBalone}}$$

$$FICI = \frac{\mathbf{10}}{\mathbf{80}} + \frac{\mathbf{10}}{\mathbf{80}}$$

= 0.125 + 0.125

= 0.245

Table 4. Zone of inhibition (ZOI) of Lawsone, Amphotericin B, Combination Formulation, and Marketed Product at different concentrations against *Rhizopus oryzae.*

Concentration(µg/mL)	Lawsone	Amphotericn B	Lawsone + Amphotericin B	Marketed
20	0	0	0	0
40	0	0	7.4	0
80	0	5.2	8.1	5.9
160	2.3	5.8	8.7	6.4
320	3.2	6.4	9.2	7.4

Calculated MIC values:

Table 5: MIC values of Lawsone, Amphotericin B, their combination and marketed product against *Rhizopusoryzae.*

Formulation	MIC (µg/mL)	Justification
Lawsone	160 µg/mL	First visible ZOI at 160µg/mL
Amphotericin B	80 µg/mL	First visible ZOI at 80µg/mL
Lawsone + Amphotericin B	40 µg/mL	ZOI present at lowest dose
Marketed	80 µg/mL	First visible ZOI at 40µg/mL

Against *Rhizopus*, the combination formulation demonstrated the lowest MIC value (40 µg/mL), indicating superior antifungal activity compared to the individual agents and the marketed product. Lawsone and Amphotericin B alone exhibited MIC values of 160 µg/mL and 80 µg/mL, respectively, while the marketed formulation showed an intermediate MIC of 80 µg/mL. The reduction from 160 µg/mL to 40 µg/mL represents a 4-fold decrease relative to Lawsone, and the decrease from 80 µg/mL to 40 µg/mL reflects a 2-fold reduction compared to both Amphotericin B and the marketed formulation.

Since the total MIC of the combination = 40 µg/mL, and it is a 1:1 combination.

MIC of Lawsone in combination = 20 µg/mL

MIC of Amphotericin B in combination = 20 µg/mL

$$\mathbf{FICI} = \frac{\text{MIC of A in combination}}{\boldsymbol{MICofAalone}} + \frac{\text{MIC of B in combination}}{\boldsymbol{MICofBalone}}$$

$$\mathbf{FICI} = \frac{20}{160} + \frac{20}{80}$$

= 0.125 + 0.25

= 0.375

The combination of Lawsone and Amphotericin B was shown to have a synergistic interaction against Candida albicans, and Rhizopus oryzae giving a fractional inhibitory concentration index (FICI) of 0.245 and 0.375 respectively. This significant reduction in MIC shows the increased antifungal activity of the combination system, which could be explained by a synergistic interaction between Lawsone and Amphotericin B, which allows the effective inhibition of stated fungi with reduced concentrations.

3.2 In Vivo Antifungal Activity

1. Clinical scoring: erythema, scaling, lesion size (0–4 scale)

Scoring Criteria (Reference Scale provided in Table 6): The 0-4 grading scale was used for five pathological parameters.

Table 6: Reference scale for clinical scoring

Grade	Interpretation
0	No histopathological change (normal tissue)
1	Minimal change (slight thickening, focal or mild alteration)
2	Mild change (focal moderate change without architecture loss)
3	Moderate change (diffuse involvement, structural alteration)
4	Severe change (extensive tissue alteration, destruction)

Table 7: Clinical scoring parameters for erythema, scaling, and lesion size on Day 7 and Day 14 across different treatment groups.

Erythema (Day 7)			**Erythema (Day 14)**		
Disease Control	**Sample A**	**Sample B**	**Disease Control**	**Sample A**	**Sample B**
3	**2**	**1**	**4**	**3**	**1**
4	**3**	**2**	**4**	**3**	**2**
3	**2**	**1**	**3**	**3**	**1**
4	**3**	**1**	**4**	**3**	**1**
3	**2**	**1**	**3**	**2**	**1**
4	**3**	**2**	**4**	**3**	**2**
Scaling (Day 7)			**Scaling (Day 14)**		
Disease Control	**Sample A**	**Sample B**	**Disease Control**	**Sample A**	**Sample B**
4	**2**	**1**	**4**	**3**	**2**
4	**3**	**1**	**3**	**2**	**2**
3	**3**	**1**	**3**	**3**	**2**
3	**2**	**2**	**4**	**3**	**1**

4	**3**	**3**	**4**	**3**	**1**
3	**2**	**1**	**4**	**3**	**1**
Lesion size (Day 7)			**Lesion size (Day 14)**		
Disease Control	**Sample A**	**Sample B**	**Disease Control**	**Sample A**	**Sample B**
3	**2**	**1**	**4**	**3**	**2**
3	**3**	**1**	**3**	**2**	**1**
4	**3**	**1**	**4**	**3**	**2**
4	**2**	**2**	**4**	**2**	**1**
3	**2**	**2**	**3**	**3**	**1**
3	**3**	**2**	**4**	**2**	**1**

The one-way ANOVA showed statistically significant difference between the experimental groups in all of the clinical parameters measured, such as erythema, lesion size, and scaling, on the Day 7 and Day 14 ($p < 0.05$). Post hoc analysis (Tukey-Kramer) supported significant intergroup differences ($p < 0.05$) further.

2. Histopathological Examination (H&E staining, n=8)

Parameters Assessed

1. Epidermal Hyperplasia
2. Stratum Corneum Disruption
3. Collagen Disorganisation
4. Hyperkeratosis
5. Inflammatory Cell Infiltration

Histopathology Scoring (0–4 Scale)

Table 8: Histopathological parameters chosen for analysis in skin tissue samples

Sr. No.	**Groups**	**Epidermal Hyperplasia**	**SC Disruption**	**Collagen Disorganization**	**Hyperkeratosis**	**Inflammation**	**Total Score**
1	Normal	0	0	0	0	0	0
2	Disease Control	3	3	2	3	2	13
3	Sample A	1	1	1	1	0	4
4	Sample B	0	1	1	1	0	3

1. Normal Control: Histological analysis of normal skin showed that there was a well-structured epidermis, and its stratification was intact and that the stratum corneum was uniformly compact. The collagen fibres were tightly packed in the dermis without any architectural distortion. The follicles of the hair were well preserved structurally and distributed all around the dermis. There was no inflammatory cell infiltration, oedema or tissue injury, which means that the skin morphology was normal.

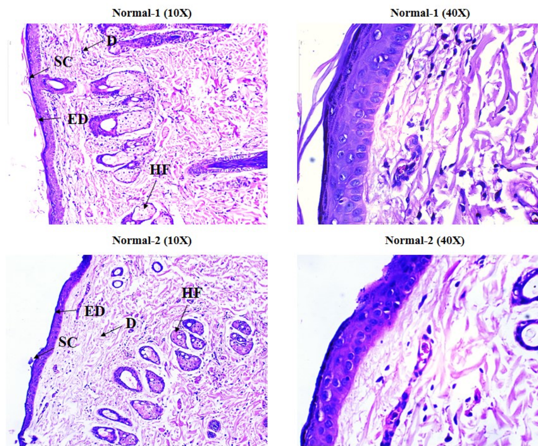

Figure 2. Histopathology of Healthy (Normal Skin Tissue) (Hematoxylin & Eosin Staining), showing Epidermis and Dermis is intact.

2. Disease Control (Candida albicans–Infected): Tissue samples of the Candida albicans-infected untreated group exhibited significant pathological changes that were indicative of fungal invasion and the resultant inflammation. The epidermis exhibited abnormal hyperplasia, disruption of normal stratification, and focal loss of stratum corneum, which is indicative of fungus-induced tissue damage. There was prominent hyperkeratosis and disorientation of dermal collagen bundles accompanied by expanded interstitial spaces. Inflammatory cell infiltration was moderate to dense, consisting mainly of mononuclear cells, which has been shown to be an active inflammatory response to fungal invasion. The results

prove the effective development of cutaneous candidiasis.

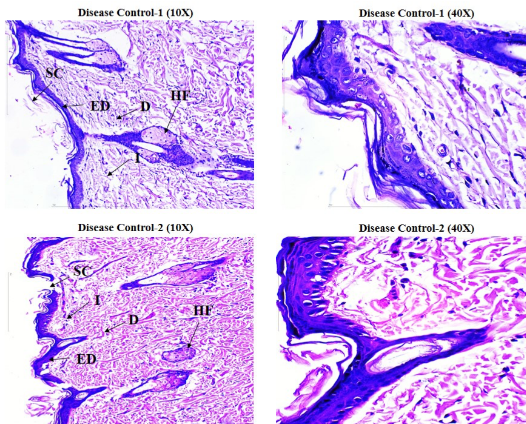

Figure 3. Histopathology of Control Group that is cutaneous candidiasis with Epidermal Hyperplasia, Hyperkeratosis and Inflammatory Cell Infiltration (H&E Staining).

3. Sample A–Treated Group: Skin sections from Sample A-treated animals exhibited partial epidermal and dermal architecture restoration in skin sections. Mild epidermal and stratum corneum thickening was also seen, but the general integrity of the structure was also intact as compared to the disease control. Sample A-1 had little hyperkeratosis and a small amount of epidermal thickening remaining. Sample A-2, in its turn, displayed almost normal morphology, where epidermal layers were intact, hair follicles were intact, and no signs of inflammatory infiltrates were present. The results suggest that Drug A had a moderate antifungal and tissue-protective effect, which minimized the severity of fungal-induced skin damage.

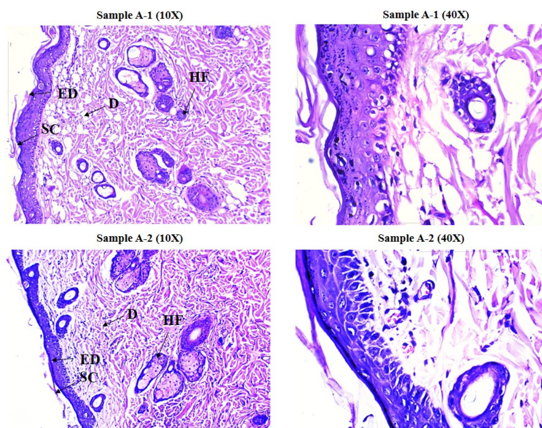

Figure 4. Skin tissue treated with Sample A (Marketed Antifungal Gel) histopathology, with some Epidermal and Dermal architecture (H&E Staining) recovered.

4. Sample B–Treated Group: Sample B-treated skin showed significant improvement as compared to the disease control.Sample B-1 had mild hyperkeratosis only, and the dermal collagen alignment was preserved.Sample B-2 did not exhibit any pathological changes, similar to the normal control group.The epidermis was normal in terms of its thickness and stratification, the stratum corneum seemed to be intact and there was no inflammatory cell infiltration.

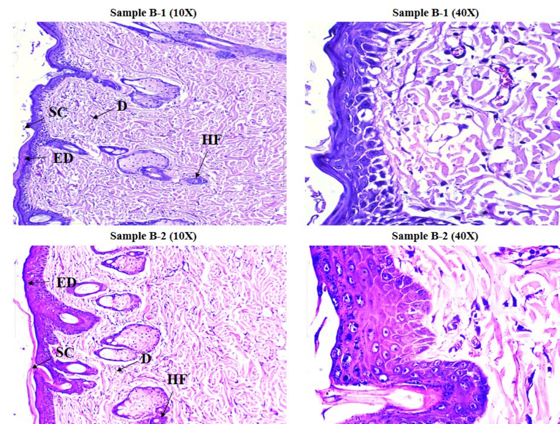

Figure 5. Histopathology of skin tissue untreated with Sample B (Liposomal Gel with Lawsone and Amphotericin B) exhibiting close to normal morphology and a decrease in inflammation (H&E Staining).

3. Histopathological Examination (Periodic Acid Schiff's staining, n=8)

1. Normal Control: Skin has normal epidermal architecture with clear stratification and normal keratinisation.PAS staining reveals absence of magenta-positive fungal elements in the stratum corneum, epidermis or the adnexal structures.Dermal connective tissue is seen to be compact with normal fibroblast and maintained adnexal units, and there is no cellular infiltration.The examined sections do not reveal any pathological alterations, oedema, or structural degeneration.

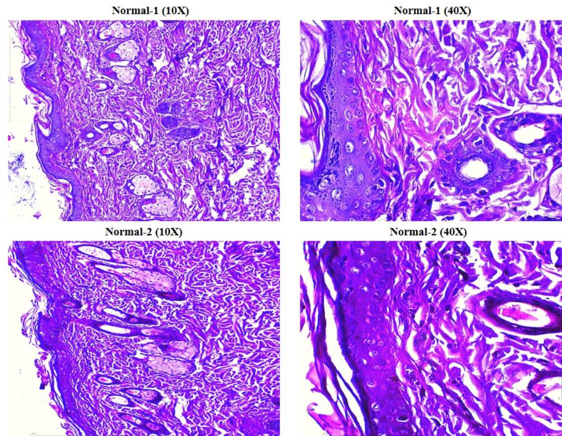

Figure 6. Periodic Acid-Schiff (PAS) Staining of Healthy Skin with no fungal elements.

2. Disease Control (Candida albicans-Infected): The epidermis has surface disruption and hyperkeratosis and focal parakeratosis which is related to active fungal colonisation. The staining of PAS shows magenta-positive yeast cells and branching hyphal elements, mostly concentrated in the skin keratinised layers and around the follicular orifices.There are moderate to significant infiltrates of inflammatory cells in the dermal layers, congestion of the vessels, and interstitial oedema.

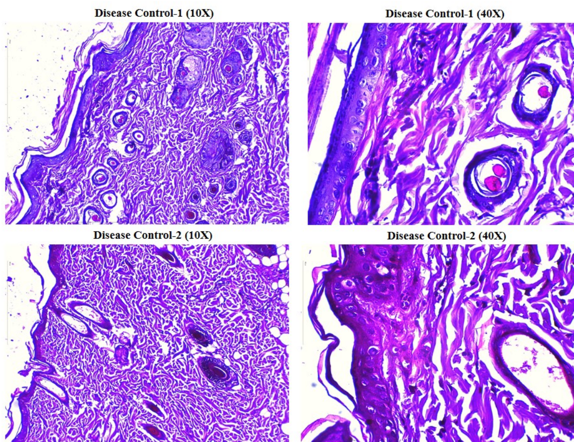

Figure 7. PAS staining to show the presence of Fungal Hyphae and Yeast Cells in the Disease Control Group.

3. Sample A - Treated Group: In sections, there is restoration of epidermal continuity to some degree, with enhanced keratin layer integrity.PAS staining exhibits less presence of fungal structures, isolated yeast cell and low amounts of hypathal forms confined to the superficial stratum corneum. Dermal architecture seems well preserved, with an insignificant inflammatory cell infiltration localised primarily in the adnexal structures.

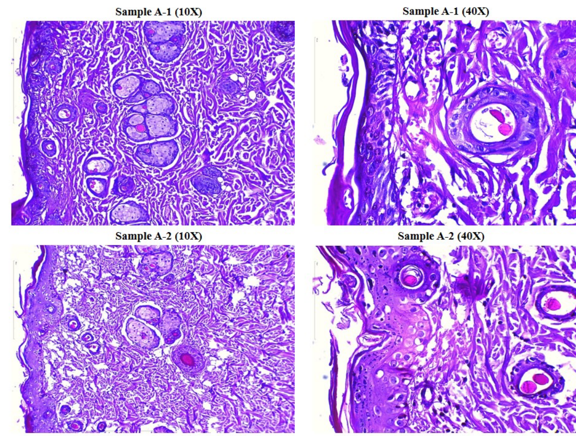

Figure 8. PAS Skin tissue Staining of Sample A-Treated Group, which had reduced fungal colonization.

4. Sample B - Treated Group: Epidermal stratification is very close to normal, and there is little interference with the surface keratinization and well-preserved morphology of the basal cells.PAS staining reveals extremely limited sparse fungal components limited to the epidermal surface, with no observable branching hyphae or follicular colonization.There is little or no inflammatory reaction in dermal tissue and the alignment of collagen and the vascular channels remains intact.No active propagation of fungi is observed in the tissue.

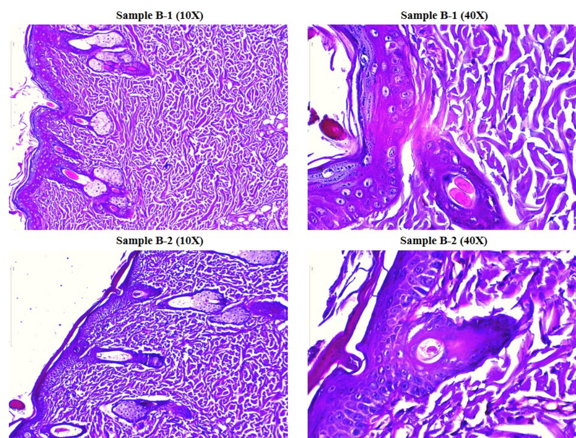

Figure 9.PAS staining of skin tissue of Sample B-treated group showing minimal fungal elements and no active infection.

4. Discussion

The present study determined the antifungal activity of a new liposomal gel formulation, Lawsone and Amphotericin B, on Candida albicans and Rhizopus oryzae, by using detailed in vitro and in vivo analyses.The findings showed that there are high levels of antifungal synergies and improved treatment

outcomes as compared to single drugs and a commercially available formulation.The in vitro findings indicate the combination formulation has a much better antifungal activity against Candida albicans and Rhizopus oryzae than single agents and the available commercial product.Against C. albicans, the combination had the lowest MIC (20 µg/mL), which is a 4-fold decrease compared to Lawsone and Amphotericin B alone (80 µg/mL) and a 2-fold decrease compared to the formulation available commercially (40 µg/mL).Likewise against Rhizopus oryzae, the combination exhibited a greater efficacy at an MIC of 40 µg/mL, a 4-fold drop compared to Lawsone (160 µg/mL) and two-fold drop as compared to Amphotericin B and the formulation available commercially (80 µg/mL). FICI values of 0.245 and 0.375 confirmed that there is a synergistic effect between Lawsone and Amphotericin B. The synergism was probably due to the complementary effect of Lawsone, a derivative of naphthoquinone with reported antifungal activities mediated by generation of reactive oxygen species and perturbation of membrane integrity, and Amphotericin B, which binds ergosterol and disrupts fungal cell membrane integrity.

The in vivo efficacy evaluation in a rat model of cutaneous candidiasis validated the in vitro results.Clinical parameter evaluation such as erythema, scaling, and lesion size showed a substantial improvement in rats with the liposomal combination gel (Sample B) and statistically significant improvement ($p < 0.05$) over disease control and marketed formulation (Sample A).It is worth noting that the group receiving the liposomal formulation (Sample B) had a much higher reduction in erythema, size and scaling of lesions when compared to the disease induced control as well as marketed formulation (Sample A).These improvements were corroborated by histopathological analysis. Hematoxylin and eosin (H&E) stained sections of the combination-treated group exhibited a restoration of normal epidermal stratification, low levels of hyperkeratosis, a well-preserved dermal collagen architecture, and the lack of significant inflammatory infiltrates.On the other end, there was intense epidermal hyperplasia, collagen disorganisation, and inflammatory cell infiltration of disease control animals which is in line with active fungal infection and tissue damage.Periodic Acid-Schiff (PAS) staining was used which gave a clear visual indication of fungal load in the tissues.Combination-treated group exhibited significant decrease in magenta-positive fungi with scattered yeast cells that were localised to the epidermal surface layers and no branching hyphae that depict reduced fungi viability and colonisation. This was considerably different to the disease controls which showed a lot of fungal structures that invaded epidermal and follicular areas supporting the antifungal activity of the liposomal gel.

This synergistic liposomal gel has a superior efficacy and safety profile, warranting its consideration as a promising therapeutic contender in the management of cutaneous fungal infection caused by Candida albicans and Rhizopus spp. Its dual-drug strategy has considerable benefits in reducing the drug dosage, alleviating resistance, and improving clinical outcomes.Further research including pharmacokinetic profiling, long-term toxicity and extended antifungal spectrum analysis is required to enable clinical translation[64-70].

5. Conclusion

The current study has been able to substantiate the fact that a synergistic liposomal gel co-encapsulating Lawsone and Amphotericin B is a rational and effective approach to topical treatment of cutaneous fungal infections caused by Candida albicans and Rhizopus oryzae. High in vitro antifungal activity was demonstrated by the formulation with significantly larger zones of inhibition, reduction in MIC values up to four-fold, and FICI values of 0.245 and 0.375 indicating true pharmacodynamic synergism (FICI $\leq$ 0.5).The results suggest that the combination system boosts the antifungal efficacy and allows significant dose reduction of Amphotericin B.Notably, the synergy in vivo was supported by the in vitro results.The liposomal combination gel resulted in statistically significant differences ($p < 0.05$) in erythema, scaling, and lesion size scores compared to disease control and marketed treatment groups. Histopathological analysis with the help of H&E staining revealed the almost perfect epidermal and dermal structure restoration with minimum inflammatory infiltration, and PAS staining revealed a significant decrease in fungal load and no active invasion of hyphae.Collectively, this study introduces a novel dual-mechanism, liposome-based topical antifungal platform that combines membrane-targeting and oxidative stress–mediated fungal inhibition.This formulation is promising as a candidate in translational development, owing to its synergistic interaction, improved therapeutic performance, and

favourable histological recovery. Future investigations focusing on long-term safety, stability profiling, and expanded antifungal spectrum assessment are required to advance this innovative approach toward clinical application.

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