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Novel Natural and Synthetic Anticandidal Therapeutic Peptides to Combat Drug-Resistant Infections †

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Abstract: *Candida* species are considered as common flora of the healthy human mycobiome and occur on skin, mucosal surfaces of gastrointestinal and genitourinary tracks. Pathogenic *Candida* spp., reported to cause skin, vaginal and oral infections. Extensive use of antifungal agents has increased the drug resistance among pathogenic strains of *Candida*. To this effect, recently naturally occurring AMPs and synthetically modified peptides, are effectively being used as promising antifungal agents. Short peptides display better permeability to cross the yeast membrane, thus short antifungal peptide were designed using sequences from APD database. Natural peptides are potential source of antifungal agents. Considering above facts, we studied anticandidal potential of synthetic and natural peptides.

Keywords: synthetic; anticandidal; therapeutic peptides

1. Introduction

The increasing drug resistance seen in fungal pathogenic strains in recent years, specifically in clinical strains, requires immediate attention so that alternative antifungal agents can be developed. Among the fungal genera, members of the genus *Candida* are the most common causal organisms of human infection. They usually reside as a commensal in the genitourinary and gastrointestinal tracts as well as in the oral and conjunctival flora [1], causing both superficial and invasive infections under immunocompromised conditions. Superficial infection are known as candidiasis, which affects the mucous membranes or skin and is usually treated with topical antifungal drugs, with a low success rate. However, invasive fungal infections have recently been reported to be life-threatening due to inefficient prognostic methods and unsuitable antifungal therapies. Only three classes of conventional antifungal drugs, viz. fluconazole, caspofungin and amphotericin B, are used extensively for candidiasis treatment [2]. However, there are *Candida* strains which have been reported to be increasingly resistant to these antibiotics [3,4]. This increased occurrence of drug-resistant candidiasis desperately requires alternative antifungal agents so the resistance problem can be overcome.

Nowadays, various natural and synthetic antimicrobial peptides (AMPs) have been reported to inhibit *Candida* spp. and are considered promising alternative candidates for the treatment of drug-resistant *Candida* spp. Infections [5–8]. Naturally occurring AMPs generally consist of 10–50 amino acids with different structural groups, including sheet, helix, extended and looped structures [9]. Though some peptides, such as melittin and protegrin, exhibit potent activity, they also possess toxic effects on mammalian cells. Hence, researchers have also focused on synthetic antimicrobial peptides with enhancement activity, reduced cytotoxicity and resistance to protease enzymes. The main objective of the present study was to use available AMP sequences to construct short peptides with anticandidal properties and resistance to environmental degradation. Effective peptide modifications were included to enhance antimicrobial potency against *Candida* spp. and

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low toxicity towards mammalian cells. The minimum inhibitory concentration of peptides towards pathogenic *Candida* species and their biofilms as well as their hemolysis ability was studied.

2. Methodology

2.1. Peptide Design and Synthesis

Peptides were designed using peptide sequences from the APD database [10]. The antifungal activity predictive tool Antifp [11] was used to predict their antifungal potential in terms of protein binding potential, amphipathicity and charge. Peptide helical wheel projections were analyzed using the bioinformatics program Netwheels [12] (Castro et al., 2018). Peptides were synthesized by CSIR-IMTECH, Chandigarh, in solid phase using the Fmoc methodology. All peptides were >90% pure (as determined by RP-HPLC) and had the expected molecular weight.

2.2. Test Organisms

The reference *Candida* strains used in this study were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India, and clinical isolates were provided by the National Culture Collection of Pathogenic Fungi (NCCPF), Postgraduate Institute of Medical Education and Research, Chandigarh, India. The test strains used were *Candida albicans* (MTCC 184), *C. albicans* (MTCC 227) and one clinical isolate of *C. albicans* (400054). All strains were maintained on yeast malt agar (YMA), subcultured regularly (every 30 days or so) and stored at 4 °C. Glycerol stocks were preserved at −80 °C. The anticandidal activity of peptides was analyzed using agar well diffusion assay.

2.3. Biofilm Formation

A test strain biofilm was formed on pre-sterilized 96-well flat-bottom polystyrene microtiter plates in triplicate. The overnight grown culture OD_{600} was adjusted to 0.4 and 10 µL of cell suspension was inoculated in 190 µL YM broth in each well. The microtiter plate was incubated for 24–48 h at 37 °C. After incubation, the medium was removed by inverting the plate and any other planktonic cells present were removed by gentle flush with sterile distilled water. Then, 200 µL of crystal violet solution (0.2%) was added to all wells. After 15 min, the excess crystal violet was removed and plates were washed twice and air-dried. Finally, the cell-bound crystal violet was dissolved in 70% ethanol. Biofilm growth was monitored in terms of OD_{595} nm using an ELISA microplate reader (Thermo, Waltham, MA, USA). Antifungal agents, including fluconazole and amphotericin B, were used as controls in this study.

2.4. Purification and Characterization

For the characterization of the antimicrobial peptide produced by the *Bacillus* sp. strain, the SVDS-15 culture was grown in 1000 mL of NB for 24 h on a rotary shaker at 30 °C. Subsequently, cells were separated by centrifuge and the peptide was subsequently eluted in methanol. The peptide was redissolved in Milli-Q water and subjected to gel filtration (G50 Sephadex). Further purification was achieved using HPLC (1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) with a semi-preparative C18 column (Pursuit 10C18 250 × 21.2 mm) with acetonitrile and aqueous trifluoroacetic acid as a solvent system. The molecular mass of the HPLC-purified peptide was analyzed using matrix-assisted laser desorption ionization (MALDI) [13]. A de novo sequence was generated manually using a fragmentation pattern.

2.5. Determination of Antimicrobial Activity and MIC Values of Peptides

The MICs of purified peptides were evaluated by using a microtiter plate dilution assay. Test strains were grown to mid-log phase (5×10^5 CFU/mL) in a 96-well plate with different concentrations of peptides for 24–48 h at 37 °C (final volume of 200 µL).

OD_{600} was measured after 24–48 h using an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The lowest concentration that inhibited the growth of the test strain and did not show any increase in absorption after 48 h was considered as the MIC. Antifungal agents such as fluconazole and amphotericin B were included in this study as controls.

2.6. Hemolysis and Time-Kill Assay

Hemolysis assay was performed using rabbit blood (New Zealand white). For the experiment, the blood was centrifuged and the cells were washed and resuspended in phosphate-buffered saline (PBS). Different concentrations of peptide (50, 100 and 200 µg/mL) were mixed with blood cells and adjusted to a concentration of 2 × 108 cells/mL in PBS. Triton X-100 was used as a positive control. Centrifuge tubes were incubated in a CO_2 incubator at 37 °C and readings were recorded at different time intervals (1 h, 12 h and 24 h).

A time-kill assay of the purified peptide was performed [14]. *C. albicans* (MTCC 183) culture of 0.2 OD600 was centrifuged, washed and resuspended in PBS. The culture was treated with 5X concentration of AMP. After treatment, pellet cells were dried, coated with gold and observed under a scanning electron microscope (ZEISS, Jena, Germany) [15].

2.7. Emulgel Formulation and Skin Irritation Studies

For emulgel formulation, a gel base was prepared using carbopol 934 (Hi-media, Thane, India). The oil-phase emulsion contained cetomacrogol 1000, white soft paraffin, cetostearyl alcohol, light liquid paraffin and propylene glycol. The oil phase and an aqueous phase containing 0.5% *w*/*v* of purified peptide were mixed together for emulgel preparation [14]. The viscosity of the gel, its extrudability, appearance and pH were determined. The antimicrobial activity of the peptide was performed in vitro before performing skin irritation studies on mice.

For skin irritation studies of the SVDS-15 formulation, BALB/c female mice (eight weeks old) were used [16]. A plain gel and a 20% SLS solution were used as negative and positive controls, respectively. The SVDS-15 formulation was topically applied to a hairless skin area of the mice (approximately 1 cm^2). The experiment results were recorded at 24, 48 and 72 h.

3. Results and Discussion

3.1. Design and Synthesis of Peptides

In this study, the amino acid sequences of peptides were used as a framework to design short antimicrobial peptides. The effect of positively charged residue distribution on the biological viability of the antimicrobial peptide was studied by replacing several amino acid residues with Lys (K) to increase a net positive charge of the peptide. Short peptides containing 10–17 amino acids in their composition are known to display better permeability to cross the yeast membrane [17]. The designed peptides varied in total net positive charge, which ranged from +2 to +5. The peptide sequences and their characteristics, such as hydrophobicity, amphipathicity, hydrophilicity and charge were analyzed; details are listed in Table 1.

Table 1. Physicochemical properties of designed peptides (predicted using Antifp software [11] (http://webs.iiitd.edu.in/raghava/antifp)).

Peptide	Peptide Sequence	Hydrophobicity	Amphipathicity	Hydrophilicity	Charge	Mol. Wt.	Structure
SK01	MACVNQCPKAIDRFIVK	−0.12	0.65	−0.06	2	1937	Defensin-like beta
SK02	KQVYKACMNGKHLYC	−0.21	0.91	−0.19	3.5	1786	Defensin-like beta
SK03	GIRWLVYRLRKV	−0.25	0.92	−0.17	4	1559	Helical
SK04	HGLENKMYRHV	−0.31	0.94	0.12	2	1384	Helical
SK05	ATCHCSIHVSK	−0.09	0.6	−0.33	2	1186	Helical

Table 1. *Cont.*

Peptide	Peptide Sequence	Hydrophobicity	Amphipathicity	Hydrophilicity	Charge	Mol. Wt.	Structure
SK06	CMNGTQVYCR	−0.22	0.37	−0.41	1	1175	Helical
SK07	KILKVARAWLAK	−0.13	1.12	0.02	4	1396.7	Helical
SK08	Fmoc-KILKVARAWLAK	−0.13	1.12	0.02	4	1655.4	Helical
Pep1	VKILAVALKWRAKR	−0.2	1.14	0.31	5	1652.3	Helical
Pep2	VIHKRHDGVKRI	−0.38	1.26	0.62	4	1457.9	Helical

Further, the amphipathic orientation of the amino acids present in these synthetic peptides was determined using helical wheel projection, which allowed for the understanding of possible in silico interactions with the membrane.

3.2. Growth and Biofilm Optimization

All *Candida* strains were grown in YM broth containing varying concentrations of glucose (0.2–1%) and incubated for 24–48 h at 37 °C for biofilm studies. After incubation, the plates were stained with crystal violet. All strains grew well in YM broth containing glucose and formed excellent biofilms, with ODs ranging from 0.8 to 1.5. Glucose presence affected the biofilm formation of different MTCC strains as well as of clinical strains. Maximum biofilm formation was achieved by using 0.5% glucose in YM broth. *C. albicans* strains consistently formed more biofilm than other species.

3.3. Effect of Synthetic Peptides on Growth and Biofilm

In order to determine the effect of the synthetic peptides on and biofilm growth and inhibition, *Candida* strains with the ability to form biofilms were treated with increasing concentrations of peptides (50–500 µg/mL). SK01, SK03 and SK07 showed an inhibition of biofilms formed by MTCC and clinical strains. Increasing concentrations of the peptide resulted in an increased disruption of biofilms and complete biofilm inhibition was observed with concentrations ranging from 200 to 800 µg/mL. Synthetic peptide SK08 and pep2 showed good anticandidal activity against various test strains. The MIC values against *Candida albicans* (MTCC 184), *C. albicans* (MTCC 227) and *C. albicans* (400054) were 200, 400 and 1000 µg/mL, respectively. Recently, three synthetic peptides (PNR20, PNR20–1 and 35,409) have been reported to exhibit antifungal activity against various *Candida* spp. [18] (Torres et al., 2023). Synthetic peptides KU2 and KU3 showed MIC values ranging from 8 to 16 mg/L [6].

3.4. Characterization of SVDS-15 Peptide

The antimicrobial peptide-producing *Bacillus* sp. strain SVDS-15 was selected for this study as it showed strong antimicrobial activity against all *Candida* strains. Considering the anticandidal potential of the peptide, further purification was carried out from CFB using a combination of chromatographic techniques. The SVDS-15 peptide showed very low MIC values against the test strains. Its MIC values against *Candida albicans* (MTCC 184), *C. albicans* (MTCC 227) and *Candida* (400054) were 12, 20 and 30 µg/mL, respectively. MALDI mass spectrometry of HPLC-purified SVDS-15 peptide revealed that it has a molecular mass as 1296 Da and belongs to the loloatin antimicrobial class (Figure 1). Loloatins have been reported to exhibit in vitro antimicrobial activity against methicillin-resistant *Staphyloccoccus aureus*, vancomycin-resistant enterococci and drug-resistant *Streptococcus pneumoniae* [19]. Moreover, the purified SVDS-15 peptide was found to be non-hemolytic in nature. Consequently, it could be used as a potent therapeutic agent. The peptide was found to be non-hemolytic in nature as it did not cause the lysis of blood cells (Figure 2).

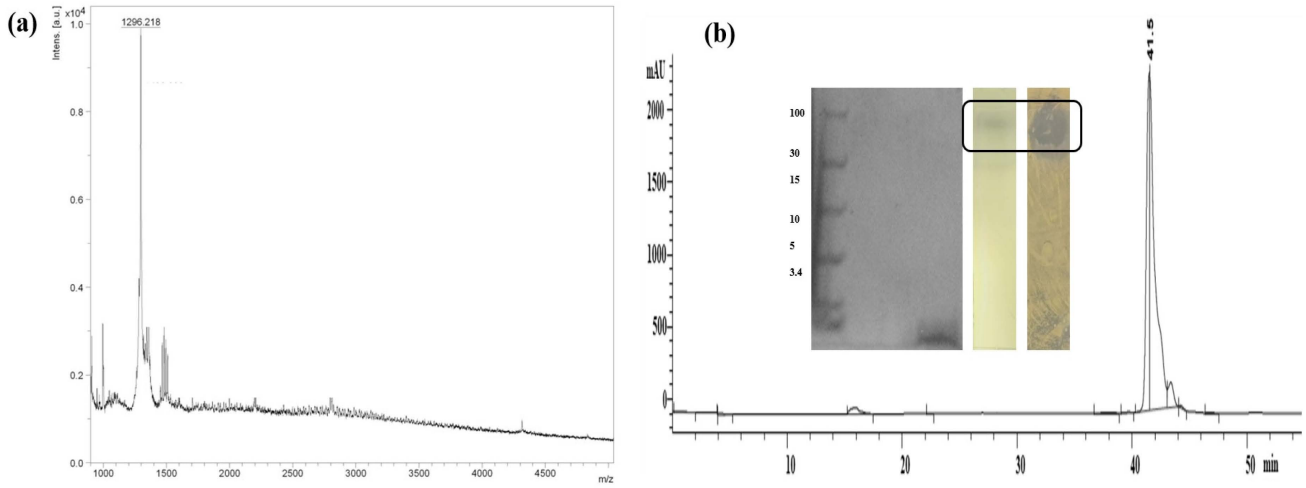


Figure 1. (**a**) MALDI mass spectrometry of SVDS-15; (**b**) RP-HPLC profile of SVDS-15 peptide inset showing tricine–SDS-PAGE of peptide, TLC stained with phosphomolybdic acid and bioautography demonstrating a clear inhibition zone.

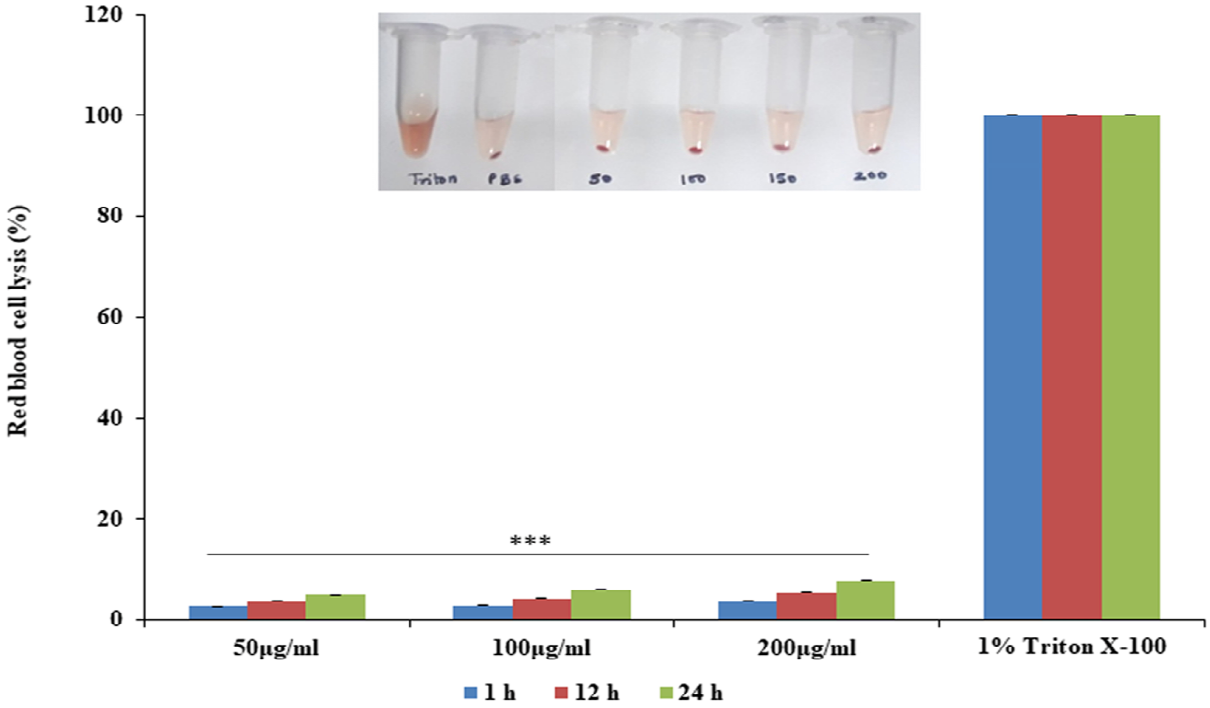


Figure 2. Hemolysis assay of SVDS-15 (*** $p < 0.0005$).

3.5. Scanning Electron Microscopy of SVDS-15 Peptide

SEM images of *C. albicans* (MTCC 183) cells treated with SVDS-15 revealed that the peptide disintegrated the cell membranes of cells and caused the dispersion of intracellular contents. However, the control cell membrane was intact and the cytoplasm was homogenous after peptide treatment (Figure 3). Similar observations of cell membrane lysis and release of cell content by peptide treatment has been reported earlier [14,20].

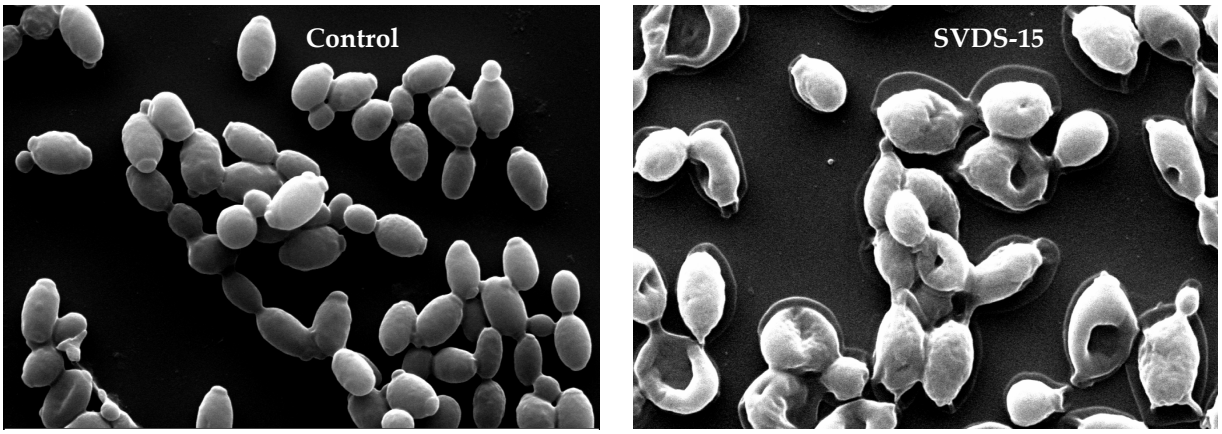


Figure 3. Scanning electron microscopy images of *C. albicans* MTCC 183 (magnification 10,000×) after treatment with SVDS-15 peptide (5×).

3.6. Emulgel Formulation of Peptides

The optimized peptide emulgel contained cetomacrogol 1000 (2.5%), propylene glycol (15.0%), white soft paraffin (10.0%), cetostearyl alcohol (7.0%), liquid paraffin (2.5%), carbopol 934 (0.5%), isopropyl alcohol (1.5%) vitamin E TPGS (5.0%) and water (55.5%). The emulgel formulations showed in vitro antimicrobial activity against *C. albicans* MTCC 183 (Figure 4). The emulgel formulation was creamy and opaque in appearance, with a pH of 6.8 ± 0.2 and good extrudability. Individual component testing was also performed and they did not exhibit any activity against *C. albicans* (MTCC 183) cells. Emulgel formulations of antimicrobial peptides have been successfully prepared and reported [14,21,22]. The viscosity of the emulgels was found to be 1451 and 1522cP for A52 and SVDS-15, respectively.

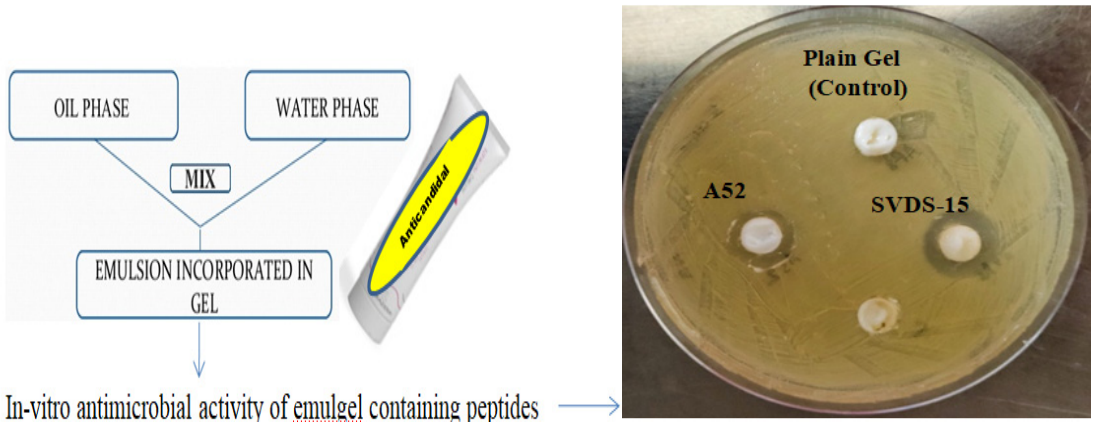
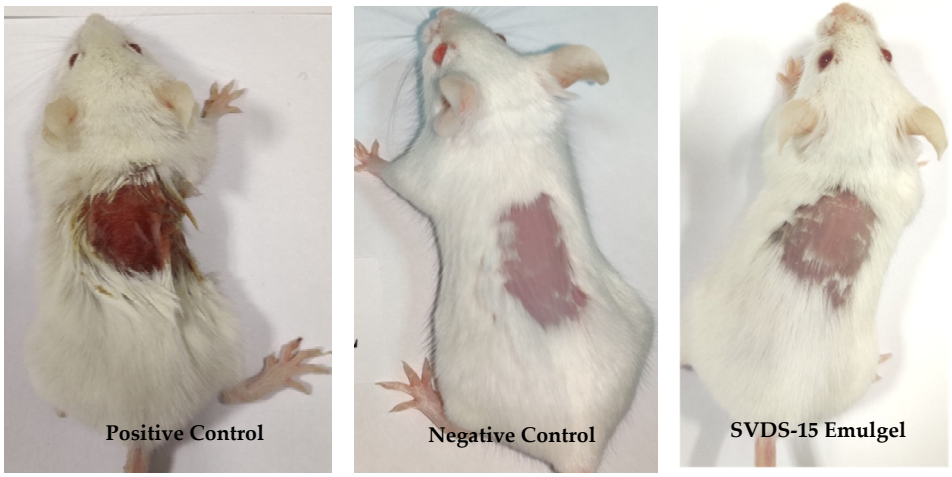


Figure 4. Preparation of emulgel and in vitro assessment of its antimicrobial activity against *C. albicans* (MTCC 183).

3.7. Skin Irritation Study of Emulgels

The skin irritancy results of the emulgel formulation were determined using the Draize test and the results showed that the emulgel formulation did not exhibit any skin irritation, even at 72 h after application. However, the positive (SLS treated) control caused hardening, redness and dryness in mouse skin (Figure 5). Many natural peptides which show no toxicity towards animals have been reported in previous studies [20,23]. The results of the skin irritation study showed that our emulgel formulations can be safely applied on the skin.



(**a**) (**b**) (**c**)

Figure 5. Skin irritation study results at 72 h: (**a**) positive control, 20% SLS treated; (**b**) negative control, plain gel; and (**c**) SVDS emulgel.

4. Conclusions

The increasing drug resistance of pathogenic fungal strains, specifically of *Candida* strains, requires immediate attention for the development of alternative antifungal agents. The main objective of this study was to develop potent antifungal agents using synthetic as well as natural peptides. The SVDS-15 peptide was successfully purified and its emulgel was developed for topical application. Synthetic (SK08 and pep2) and natural (SVDS-15) peptides could be used as potent therapeutic agents against various resistant *Candida* strains.

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Institutional Review Board Statement: Institutional Animal Ethics Committee for the Purpose of Control and Supervision of Experiments on Animals has reviewed and approved study. The approval number of the experiment is 18/08.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing is not applicable to this article.

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Conflicts of Interest: The author declare no conflict of interest.

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