

Proceeding Paper

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

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[†] Presented at the 3rd International Electronic Conference on Antibiotics (ECA 2023), 1–15 Dec 2023; Available online: <https://eca2023.sciforum.net/>.

Abstract: A single paragraph of about 100 words to give a brief introduction to your work.

Keywords: keyword 1; keyword 2; keyword 3 (List three to ten pertinent keywords specific to the article yet reasonably common within the subject discipline.)

1. Introduction

In recent years, increasing drug resistance in fungal pathogenic strains specifically, clinical strains require immediate attention to develop alternative antifungal agents. Among the fungal genera, members of the genus *Candida* are most common causal organisms of human infections. It usually resides as a commensal in the genitourinary and gastrointestinal tracts, and also occur as oral and conjunctival flora (Naglik et al., 2011) causing both superficial and invasive infections under immunocompromised condition. Superficial infections are known as candidiasis that affects mucous membranes or skin and usually treated with topical antifungal drugs with low successes rate. However, invasive fungal infections are reported recently 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 (Lum et al., 2016). However, there are *Candida* strains which have been reported to be increasing resistant to these antibiotics (Eksi et al., 2013; Ademe and Girme, 2022). This increased occurrence of drug resistant candidiasis desperately requires alternative antifungal agents to overcome the resistance problem.

Nowadays various natural and synthetic antimicrobial peptides (AMPs) have been reported to inhibition *Candida* spp. and they are considered to be promising alternative candidates to treat drug resistant *Candida* spp. infections. (Singh et al., 2014; Lum et al., 2015; Raman et al., 2015; Lyu et al., 2016). Naturally occurring AMPs are generally consist of 10–50 amino acids with different structural groups, including sheet, helix, extended, and looped structures (Zasloff, 2002). Though few peptides such as melittin and protegrin exhibit potent activity, they also possess toxic effects on mammalian cells. Hence, researchers are also focusing on synthetic antimicrobial peptides with enhancement activity, reduced cytotoxicity and resistance to protease enzymes. The main objective of present study was to use available AMP sequences to construct short peptides with anticandidal property, resistance to environmental degradation. Effective peptide modifications was included to enhance antimicrobial potency against *Candida* spp. and low toxicity towards mammalian cells. Minimum inhibitory concentration of peptides towards pathogenic *Candida* species and their biofilms, hemolysis ability was studied.

Citation: Sharma, D. Novel Natural and Synthetic Anticandidal Therapeutic Peptides to Combat Drug Resistant Infections. *Med. Sci. Forum* **2023**, *24*, x. <https://doi.org/10.3390/xxxxx>

Academic Editor(s): Name

Published: 1 December 2023



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2. Methodology

2.1. Peptide Designing and Synthesis

Peptides were designed using peptide sequences from APD database (Wang and Wang et al., 2004). Antifungal activity predictive tool Antifp (Raghav et al., 2018) was used to predict their antifungal potential in terms of protein binding potential, amphipathicity and charge. Peptides helical wheel projections were analyzed using bioinformatics program Netwheels (Castro et al., 2018). Peptides were synthesized by CSIR-IMTECH, Chandigarh on solid phase using the Fmoc methodology (Behrendt et al., 2016). All peptides were >90% pure (as determined by RP-HPLC) and had the expected mol weight

2.2. Test Organisms

The reference *Candida* strains used in the study were procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India and clinical isolates were provided by National Culture Collection of Pathogenic Fungi (NCCPF), Postgraduate Institute of Medical Education and Research, Chandigarh, India. 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) and subcultured regularly (every 30 days or so) to store at 4 °C as well as glycerol stocks were made to preserve as -80 °C stocks. Anticandidal activity of peptides were analyzed using agar well diffusion assay.

2.3. Biofilm Formation

Test strains biofilm was formed on pre-sterilized 96 well flat bottom polystyrene micro-titre plates in triplicates. The overnight grown culture OD₆₀₀ was adjusted to 0.4 and 10 µL of cell suspension was inoculated in 190 µL YM broth in each well. The microtitre plate was incubated for 24–48 h at 37 °C. After the incubation medium was removed by inverting the plate and any other planktonic cells present were removed by gentle flush with sterile distilled water. Then, 200µL 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 OD₅₉₅ nm using ELISA microplate reader (Thermo, USA). Antifungal agents including fluconazole and amphotericin B were used as controls in this study.

2.4. Purification and Characterization

For the characterization of antimicrobial peptide produced by *Bacillus* sp. strain 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 centrifuged and peptide was subsequently eluted in methanol. 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 solvent system. Molecular mass of HPLC purified peptide was analyzed using Matrix-Assisted Laser Desorption Ionization (MALDI). (Singh et al., 2014). The de novo sequence was generated using fragmentation pattern manually.

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 the peptides for 24–48 h at 37 °C (final volume of 200µL). OD₆₀₀ was measured after 24–48 h using ELISA microplate reader (Thermo, USA). The lowest

concentration that inhibited growth of test strain and did not show any increase in absorption after 48 h was considered as 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 experiment blood was centrifuged, cells were washed and resuspended in phosphate-buffered saline (PBS). Different concentrations of peptide (50, 100 and 200 µg/mL) was mixed with blood cells and adjusted to the concentration of 2×10^8 cells/mL in PBS. Triton X-100 was used as positive control. Centrifuge tubes were incubated in a CO₂ incubator at 37 °C and readings were recorded at different time intervals (1 h, 12 h and 24 h).

Time kill assay of the purified peptide was performed (Sharma et al., 2020). *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 and coated with gold and observed under scanning electron microscope (ZEISS, Jena, Germany). (Raje et al., 2006).

2.7. Emulgel Formulation and Skin Irritation Studies

For emulgel formulation, gel base was prepared using carbopol carbopol 934 (Hi-media, India). Oil phase emulsion contained cetomacrogol 1000, white soft paraffin, ceto-tearyl alcohol, light liquid paraffin and propylene glycol. Oil phase and aqueous phase containing 0.5% *w/v* of purified peptide was mixed together for emulgel preparation (Sharma et al., 2020). Viscosity of the gel, its extrudability, appearance and pH was determined. Antimicrobial activity of peptide was performed *in vitro*, before performing skin irritation studies on mice.

For skin irritation studies of SVDS-15 formulation, BALB/c female mice (eight week old) were used (Draize et al., 1944). Plain gel and 20% SLS solution were used as negative and positive controls, respectively. SVDS-15 formulation was topically applied to the hairless skin area of the mice (approximately 1 cm²). 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. Effect of positively charged residue distribution on the biological viability of the antimicrobial peptide was studied, replacing few amino acid residues with Lys (K) to increase the net positive charge of the peptide. Short peptides containing 10–17 amino acids in their composition are known to displayed better permeability to cross the yeast membrane (Gong and Karlsson, 2017). Designed peptides varied in total net positive charge which ranged from +2 to +5. Peptide sequences and characteristics such as hydrophobicity, amphipathicity, hydrophilicity and charge were analyzed and details are listed in Table 1.

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

Table 1. Physicochemical properties of designed peptides (predicted using Antifp software; Raghav et al., 2018).

Peptide	Peptide Sequence	Hydrophobicity	Amphipathicity	Hydrophilicity	Charge	Mol. Wt.	Structure
SK01	MACVNQCPKAIDRFIVK	-0.12	0.65	-0.06	2	1937	defensin-like beta
SK02	KQVYKACMNGKHLVC	-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
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

3.2. Growth and Biofilm Optimization

All *Candida* strains were grown in YM broth containing varying concentration 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 biofilm with O.D ranging from 0.8–1.5. Glucose presence affected the biofilm formation of different MTCC strains as well as 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 growth and biofilm inhibition, *Candida* strains with ability to form biofilm was treated with increasing concentrations of peptides (50–500 µg/mL). SK01 SK03 and SK07 showed inhibition of biofilms formed by MTCC and clinical strains. Increasing concentrations of the peptide resulted in increased disruption of biofilms and complete biofilm inhibition was observed with concentration of ragging from 200–800 µg/mL. Synthetic peptide SK08 and pep2 showed good anticandidal activity against various test strains. MIC value 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 has been reported to exhibit antifungal activity against various *Candida* spp. (Torres et al., 2023). Synthetic peptide KU2 and KU3 showed MIC values ranging from 8–16 mg/L (Lum et al., 2015).

3.4. Characterization of SVDS-15 Peptide

Antimicrobial peptide producing *Bacillus* sp. strain SVDS-15 was selected for the 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 combination of chromatographic techniques. SVDS-15 peptide showed very low SVDS-15 peptide showed low MIC values against test strains. MIC value 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 it belong to the antimicrobial class loloatins (Figure 1). The loloatins have been reported to exhibit in vitro antimicrobial activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and drug-resistant *Streptococcus pneumoniae* (Gerard et al., 1999, Singh et al., 2014).

Moreover, purified SVDS-15 peptide was found to be non-haemolytic in nature. Consequently, it could be used in as a potent therapeutic agent. The peptide was found to be non-haemolytic in nature as it did not caused lysis of blood cells (Figure 2).

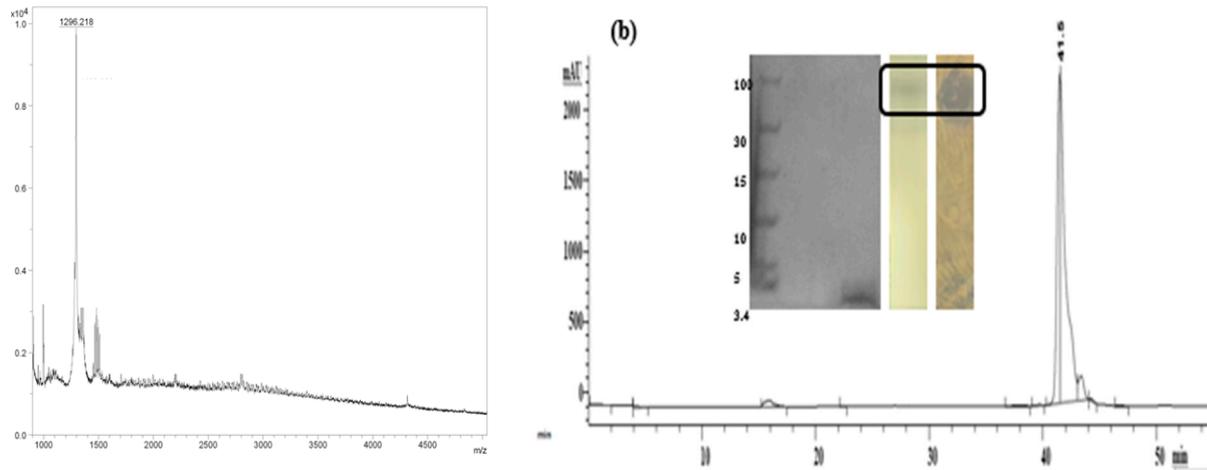


Figure 1. (a) MALDI mass spectrometry of SVDS-15 (b) RP-HPLC profile of SVDS-15 peptide inset showing Tricine–SDS–PAGE of peptide, TLC stained with phosphomolybdic acid and bioautography demonstrating a clear inhibition zone.

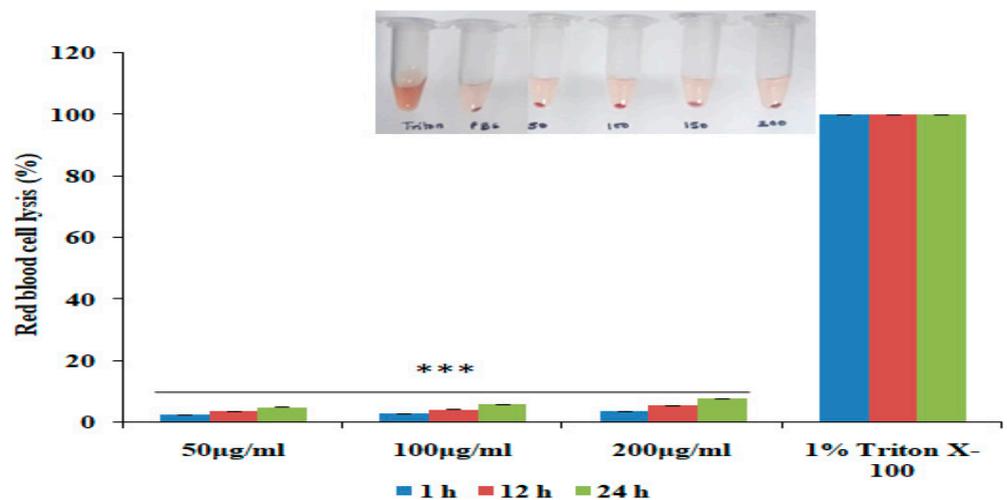


Figure 2. Hemolysis assay of SVDS-15.

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 cell membrane of cells and caused indispersion of intracellular contents. However, control cells membrane was intact and cytoplasm was homogenous after peptide treatment (Figure 3). Similar observations of cell membrane lysis and release of cell content by peptides treatment has been reported earlier (Miller and Zachary, 2017; Singh et al. 2022).

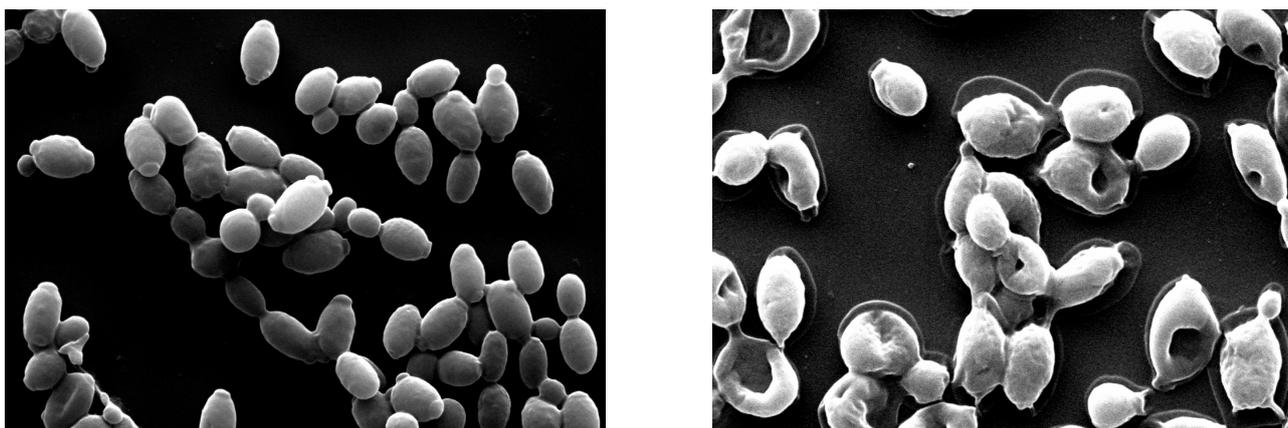


Figure 3. Scanning electron microscopy images of *C. albicans* MTCC 183 (magnification 10,000×) after treatment with SVDS-15 peptide (5X).

3.6. Emulgel Formulation of Peptides

Optimized peptide emulgel contains 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). Emulgel formulation was creamish and opaque in appearance, having pH 6.8 ± 0.2 and good extrudability. Individual components testing was also performed and they did not exhibited any activity against *C. albicans* (MTCC 183) cells. Emulgel formulations of antimicrobial peptides has been successfully prepared and reported (Neff et al., 2020; Sharma et al., 2020). The viscosity of emulgels were found to be 1451 and 1522cP for A52 and SVDS-15, respectively.

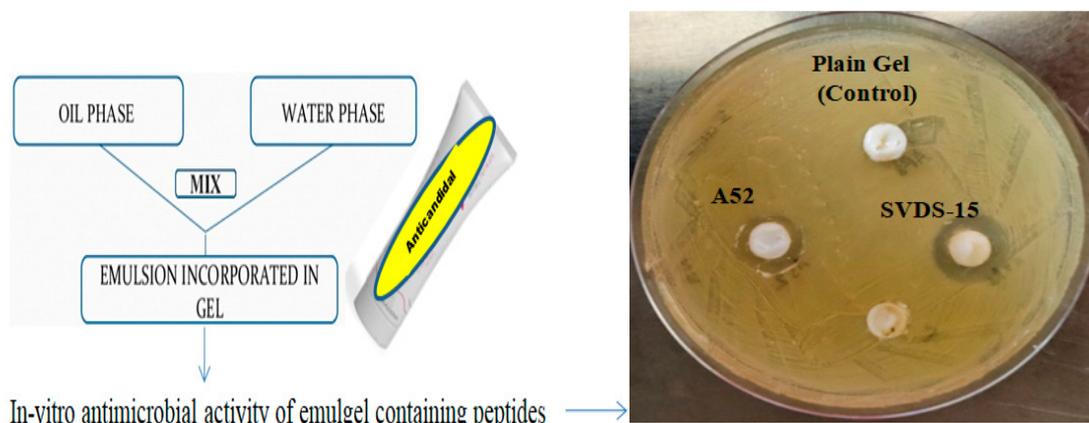


Figure 4. Preparation of emulgel and its in vitro assessment of antimicrobial activity against *C. albicans* (MTCC 183).

3.7. Skin Irritation Study of Emulgels

Results of skin irritancy of emulgel formulations were determined using Draize test and results showed that emulgel formulation did not exhibited any skin irritation even at 72 h after application. However, the positive control (SLS treated) showed hardening, redness and dryness in mice skin (Figure 5). Many natural peptides have been reported which shows no toxicity towards animals studies (Gordya et al., 2017; Singh et al. 2022). The results of skin irritation study showed that emulgel formulations can be safely applied on the skin.

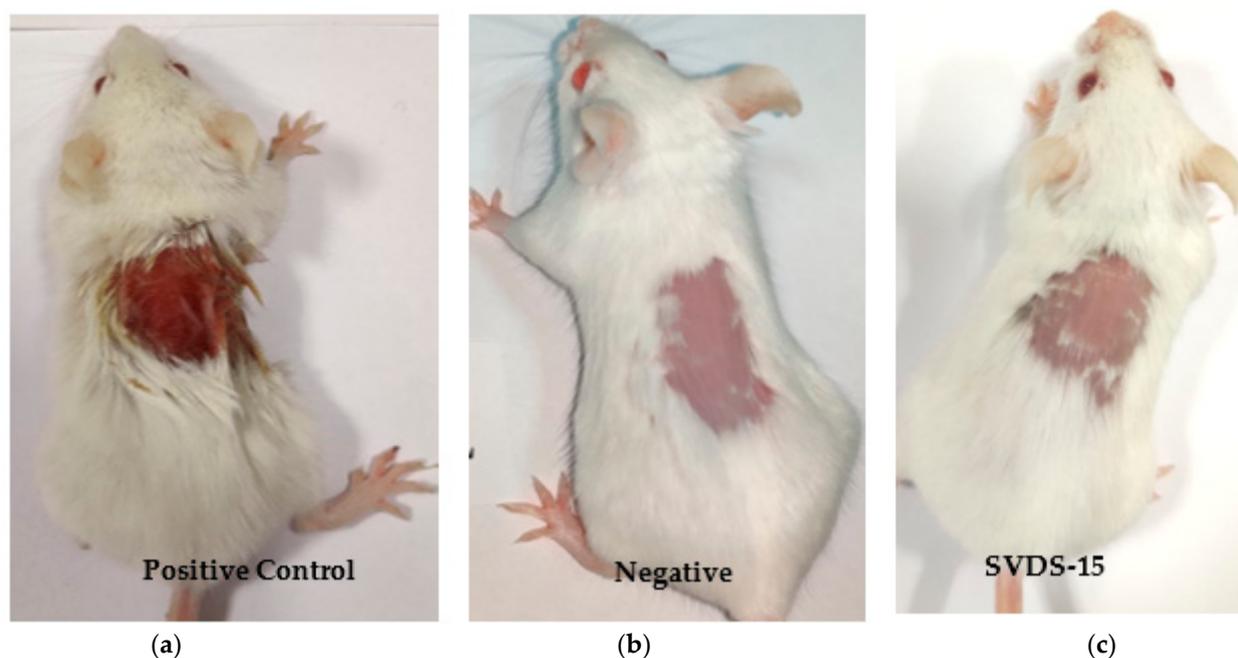


Figure 5. Skin irritation study results at 72 h (a) positive control, 20% SLS treated, (b) negative control, plain gel, and (c) SVDS emulgel.

4. Conclusions

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