CHAPTER-5

RESULT & DISCUSSION-II
5. Formulation, characterization and evaluation

This chapter is based on utilization of earlier studies in development and formulation of gel library with characterization in order to get best formulation and then, further *in vitro* and *in vivo* evaluation of best selected formulation.

5.1 Physicochemical characterization

5.2 *In vitro* antifungal activity

5.3 Morphological study

5.4 Stability studies

5.5 Skin irritation and *in vivo* toxicity study

5.6 Confocal laser scanning microscopy

5.7 *In vivo* antifungal study (mouse model)

5.1 Physicochemical characterization

5.1.1 Preliminary characterization of gel formulation library

After preparation, the total number of formulations was subjected to preliminary physicochemical examination like pH, viscosity, drug release and spreadability as presented in Table 5.1. All the gels were found with white opaque to translucent appearance. No visible precipitation was observed in all the formulations, whilst smooth and homogeneous texture was obtained. Given that pH of skin can vary according to age which might affects the permeation rate of the drug, the pH was found close to neutral value i.e. 7.0 which can provide better bioadhesive property. Considering pH determination of all 27 formulations, the values were found to lie within a range of 6.8 ± 0.2 – 7.3 ± 0.1, thus lying in the normal pH range of skin. The viscosity values of all the gels were found in the range of 46719 ± 19 to 47839 ± 27 centipoise (cP). Interestingly, the increase of rotation speed did not significantly change the viscosity of the gels, revealing the formation of stable gel structure. This might be because carbopol 940 forms a physically bonded network in which movement of the dispersion medium is restricted by intercalating three dimensional network of solvated particles. Also, this polymer consists of twisted strands often tied together by stronger types of Vander Waals Forces to form stable network throughout the system. From a patient compliance perspective,
spreadability is a pivot for topical gel formulation. The formulations were found to exhibit good spreadability by weight (in a range of $11.00 \pm 0.32 - 15.53 \pm 0.12 \text{ g.cm/s}$).

Chemical interaction for CLZ and carbopol 940 was carried out via FTIR analysis. Initially the substantial peaks were characterized and analyzed for individual compound, in addition to that, FTIR was obtained for the admixture of CLZ and polymer used in this present study. Comparative FTIR revealed the absence of any kind of chemical interaction, which was attributed that CLZ and carbopol 940 are compatible within the system. All of the characteristic peaks remained unaffected in the obtained spectrum of CLZ + carbopol 940 admixture sample (Figure 5.1).

5.1.2 In vitro drug release and mathematic modeling

The ability of gel formulations to deliver CLZ was examined by determining the drug release rate. In vitro release study was conducted in optimized ratio of phosphate buffer (pH 7.4) and

Table 5.1: Physicochemical characterization data representing pH, viscosity, drug release, and spreadability.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Codes</th>
<th>pH</th>
<th>Viscosity (cP)</th>
<th>Cumulative % drug release (6h)</th>
<th>Spreadability (g.cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CA–1S</td>
<td>6.9</td>
<td>46884 ± 24</td>
<td>82.44 ± 0.52</td>
<td>12.53 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>CA–2S</td>
<td>6.8</td>
<td>46719 ± 19</td>
<td>87.23 ± 0.36</td>
<td>14.75 ± 0.21</td>
</tr>
<tr>
<td>3</td>
<td>CA–3S</td>
<td>7.1</td>
<td>47839 ± 27</td>
<td>94.33 ± 0.60</td>
<td>14.00 ± 0.32</td>
</tr>
<tr>
<td>4</td>
<td>CT–1S</td>
<td>6.9</td>
<td>46738 ± 25</td>
<td>82.74 ± 0.26</td>
<td>12.75 ± 0.13</td>
</tr>
<tr>
<td>5</td>
<td>CT–2S</td>
<td>7.2</td>
<td>46814 ± 18</td>
<td>87.63 ± 0.43</td>
<td>13.75 ± 0.17</td>
</tr>
<tr>
<td>6</td>
<td>CT–3S</td>
<td>6.8</td>
<td>47473 ± 31</td>
<td>94.63 ± 0.31</td>
<td>13.15 ± 0.22</td>
</tr>
<tr>
<td>7</td>
<td>CAT–1S</td>
<td>6.8</td>
<td>47249 ± 22</td>
<td>83.82 ± 0.44</td>
<td>14.33 ± 0.16</td>
</tr>
<tr>
<td>8</td>
<td>CAT–2S</td>
<td>7.0</td>
<td>47399 ± 20</td>
<td>88.40 ± 0.29</td>
<td>13.33 ± 0.23</td>
</tr>
<tr>
<td>9</td>
<td>CAT–3S</td>
<td>7.2</td>
<td>47392 ± 37</td>
<td>95.68 ± 0.50</td>
<td>14.85 ± 0.34</td>
</tr>
<tr>
<td>10</td>
<td>CA–1C</td>
<td>6.9</td>
<td>46984 ± 30</td>
<td>79.02 ± 0.34</td>
<td>13.53 ± 0.12</td>
</tr>
<tr>
<td>11</td>
<td>CA–2C</td>
<td>7.3</td>
<td>47008 ± 29</td>
<td>81.33 ± 0.42</td>
<td>14.33 ± 0.14</td>
</tr>
<tr>
<td>12</td>
<td>CA–3C</td>
<td>6.8</td>
<td>46953 ± 18</td>
<td>83.95 ± 0.54</td>
<td>14.03 ± 0.25</td>
</tr>
<tr>
<td>13</td>
<td>CT–1C</td>
<td>7.0</td>
<td>46896 ± 36</td>
<td>79.49 ± 0.57</td>
<td>13.52 ± 0.19</td>
</tr>
<tr>
<td>14</td>
<td>CT–2C</td>
<td>7.2</td>
<td>47255 ± 28</td>
<td>81.82 ± 0.31</td>
<td>13.64 ± 0.11</td>
</tr>
<tr>
<td>15</td>
<td>CT–3C</td>
<td>6.9</td>
<td>46949 ± 31</td>
<td>84.47 ± 0.30</td>
<td>12.53 ± 0.18</td>
</tr>
<tr>
<td>16</td>
<td>CAT–1C</td>
<td>7.1</td>
<td>47391 ± 40</td>
<td>81.05 ± 0.48</td>
<td>14.15 ± 0.27</td>
</tr>
<tr>
<td>17</td>
<td>CAT–2C</td>
<td>6.9</td>
<td>47195 ± 27</td>
<td>82.21 ± 0.35</td>
<td>14.53 ± 0.20</td>
</tr>
<tr>
<td>18</td>
<td>CAT–3C</td>
<td>6.8</td>
<td>46849 ± 35</td>
<td>85.18 ± 0.43</td>
<td>13.75 ± 0.17</td>
</tr>
<tr>
<td>19</td>
<td>CA–1X</td>
<td>7.3</td>
<td>46988 ± 36</td>
<td>85.92 ± 0.26</td>
<td>13.53 ± 0.29</td>
</tr>
<tr>
<td>20</td>
<td>CA–2X</td>
<td>7.0</td>
<td>47105 ± 75</td>
<td>88.87 ± 0.35</td>
<td>13.33 ± 0.33</td>
</tr>
<tr>
<td>21</td>
<td>CA–3X</td>
<td>7.2</td>
<td>46966 ± 31</td>
<td>93.38 ± 0.25</td>
<td>13.53 ± 0.16</td>
</tr>
<tr>
<td>22</td>
<td>CT–1X</td>
<td>6.9</td>
<td>47227 ± 29</td>
<td>86.14 ± 0.36</td>
<td>13.85 ± 0.23</td>
</tr>
<tr>
<td>23</td>
<td>CT–2X</td>
<td>7.1</td>
<td>47310 ± 34</td>
<td>89.07 ± 0.29</td>
<td>14.00 ± 0.17</td>
</tr>
<tr>
<td>24</td>
<td>CT–3X</td>
<td>6.8</td>
<td>46794 ± 30</td>
<td>94.00 ± 0.42</td>
<td>13.75 ± 0.26</td>
</tr>
<tr>
<td>25</td>
<td>CAT–1X</td>
<td>6.9</td>
<td>47399 ± 23</td>
<td>87.05 ± 0.52</td>
<td>14.15 ± 0.18</td>
</tr>
<tr>
<td>26</td>
<td>CAT–2X</td>
<td>7.3</td>
<td>47118 ± 28</td>
<td>89.50 ± 0.46</td>
<td>13.45 ± 0.21</td>
</tr>
<tr>
<td>27</td>
<td>CAT–3X</td>
<td>7.1</td>
<td>47284 ± 27</td>
<td>94.98 ± 0.23</td>
<td>13.70 ± 0.33</td>
</tr>
</tbody>
</table>

CA = CLZ+BHA; CT = CLZ+BHT; CAT = CLZ+BHA+BHT; S = SDS; C = CTAB; X = TX100
methanol (6.4). Figure 5.2, shows the cumulative percent release of reduced best three formulations containing antioxidants’ micellar system with different surfactant i.e. SDS, CTAB and TX100. For all the formulations, the release data has been presented in Table 5.1 and release kinetics for best three in Table 5.2 whereas for all 27 formulations, has been provided in APPENDIX – C. The study was initially optimized and then conducted for 6 h to gain better comparison. In comparison to plain drug, CAT–3S and CAT–3X showed higher release with initial slight burst then controlled, which might be because of the present additives providing additional driving force and afterward controlled by polymeric network structure of gel. It is important to note down that higher localized release is required in treatment of superficial localized infections.

In order to describe the drug release profiles from the gel, the in vitro release data were fitted into mathematic models and analyzed. The in vitro release data were fitted into Zero order, First order, Higuchi and Korsmeyer–Peppas kinetic equations. It was found that all the formulations had a good fit to the zero order equation, likely, $R^2 = 0.956$ for CAT–3S, $R^2 = 0.997$ for CAT–3C, and $R^2 = 0.9995$ for CAT–3X, respectively. Interestingly, CAT–3S was found to have maximum $R^2$ in case of Higuchi as well as Korsmeyer–peppas model.
Korsmeyer–peppas model suggested that the release followed diffusion controlled mechanism \((n = 0.3)\). Results showed release exponent \(n\) values of about 0.3 attributing that drug release is driven by diffusion transport, following Fick’s law of diffusion, in other words, drug release is concentration dependent. This kind of obtained release is known to reduce the induction of fungi tolerance to the antifungal drug.

**Table 5.2: In vitro** release rate profile with model kinetics for best three formulations.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zero order</th>
<th>First order</th>
<th>Higuchi</th>
<th>Korsmeyer–Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R^2)</td>
<td>(K)</td>
<td>(R^2)</td>
<td>(R^2)</td>
</tr>
<tr>
<td>CAT–3S</td>
<td>0.956</td>
<td>0.231</td>
<td>0.506</td>
<td>0.001</td>
</tr>
<tr>
<td>CAT–3C</td>
<td>0.997</td>
<td>0.227</td>
<td>0.693</td>
<td>0.001</td>
</tr>
<tr>
<td>CAT–3X</td>
<td>0.995</td>
<td>0.252</td>
<td>0.653</td>
<td>0.001</td>
</tr>
</tbody>
</table>

CAT= CLZ+BHA+BHT; S= SDS; C= CTAB; X= TX100

**Figure 5.2:** Plot representing cumulative % drug release as function of time.

**5.2 In vitro antifungal activity**

The *in vitro* antifungal activity of optimized best three formulations viz., CAT–3S, CAT–3C and CAT–3X was assessed. The MIC (\(\mu g/ml\)) values were obtained and presented in Table
5.3–5.5. Lower the MIC values are indicative of higher antifungal activity. The antifungal study was conducted on the clinically collected samples. Most of the obtained clinical isolates were FLZ resistant *C. albicans* as shown in Table 5.3–5.5. For this reason MIC for FLZ in addition to 30 % v/v CLZ EtOH solution was obtained to gain better interpretation. In addition, to assess the interaction of BHA+BHT and CLZ within selected three formulations, 30% v/v EtOH CLZ solution was used. We hypothesized that hydroethanolic solution used for micellar solution preparation and afterward dispersed into the gel base probably could influence the antifungal activity. EtOH itself is known to have antimicrobial property so, surely could have impact on antifungal profile of the respective formulations. Interestingly, when examined EtOH did not responded below MIC 1024, however, was found evident to promote antifungal property of plain CLZ in combination. In comparison from the MIC values CAT–3S showed promising activity against the clinical isolates. In particular, the calculated MIC values for CAT–3S was found to lie within the range of 0.25 – 8.0 (µg/ml) against FLZ / MLZ resistant and FLZ susceptible *C. albicans* isolates. Moreover the activity was promising against *C. tropicalis* isolates. Surprisingly, all the three formulations were not found as active against *C. glabrata* clinical isolates.

Given the fact that results suggested an interaction between CLZ and micellar encapsulated BHA+BHT, the FIC was calculated to investigate whether the combination was synergistic. This was carried by FICI approach. Here, FICI represents the sum of FICs of each compound tested/ presented within the system (formulation), where, FIC is determined for each compound by dividing the MIC of each compound when used in combination by the MIC of each compound when used alone.

Considering that FICI value among three screened formulations, CAT–3S was found with maximum number of synergism against 30 clinical isolates (Table 5.3). With regard to the FICI calculated values, CAT–3S was found to be most promising against FLZ resistant PDI/MDL54 clinical isolate with FICI = 0.13, therefore, the latter studies were performed against this clinical isolate. Results found in this *in vitro* experiment suggested a clear and decisive role of the antioxidants as well as the micellar system. Moreover, MIC values revealed promising inhibition of the growth of *Candida* species at the concentration lower than the drug (CLZ). Therefore, *in vitro* antifungal activity showed CAT–3S was the best among others which was thereafter accounted for further analysis and studies.
Table 5.3: Antifungal activity (MIC µg/ml) of CAT–3S formulation against various *Candida* species clinical isolates.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Clinical Isolates</th>
<th>Type</th>
<th>Species</th>
<th>CAT–3S</th>
<th>BHA</th>
<th>BHT</th>
<th>FLZ</th>
<th>CLZ</th>
<th>FIG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PGI/DML14</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>64</td>
<td>&gt;512</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>PGI/DML34</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>1.0</td>
<td>&gt;512</td>
<td>178</td>
<td>&gt;512</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>PGI/DML41</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>0.25</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>1.0</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>PGI/DML54</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>128</td>
<td>&gt;512</td>
<td>4.0</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>PGI/DML61</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>8.0</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>128</td>
<td>32</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>PGI/DML85</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>8.0</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>32</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>PGI/DML43A</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>2.0</td>
<td>256</td>
<td>256</td>
<td>64</td>
<td>8.0</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>PGI/DML77A</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>2.0</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>16</td>
<td>0.1</td>
</tr>
<tr>
<td>9</td>
<td>PGI/DML94A</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>1.0</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>PGI/DML106A</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>0.50</td>
<td>128</td>
<td>32</td>
<td>16</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
<td>PGI/DML05C</td>
<td>R</td>
<td><em>C. albicans</em></td>
<td>4.0</td>
<td>&gt;512</td>
<td>128</td>
<td>64</td>
<td>8.0</td>
<td>0.5</td>
</tr>
<tr>
<td>12</td>
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<td>R</td>
<td><em>C. albicans</em></td>
<td>1.0</td>
<td>256</td>
<td>64</td>
<td>&gt;512</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
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<td>R</td>
<td><em>C. albicans</em></td>
<td>0.25</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td>14</td>
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<td>R</td>
<td><em>C. albicans</em></td>
<td>1.0</td>
<td>&gt;512</td>
<td>128</td>
<td>&gt;512</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
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<td>R</td>
<td><em>C. albicans</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>64</td>
<td>&gt;512</td>
<td>4.0</td>
<td>0.1</td>
</tr>
<tr>
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<td>PGI/DSS101</td>
<td>S</td>
<td><em>C. albicans</em></td>
<td>0.25</td>
<td>256</td>
<td>128</td>
<td>128</td>
<td>4.0</td>
<td>0.0</td>
</tr>
<tr>
<td>17</td>
<td>PGI/DSS114</td>
<td>S</td>
<td><em>C. albicans</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>128</td>
<td>256</td>
<td>8.0</td>
<td>0.0</td>
</tr>
<tr>
<td>18</td>
<td>PGI/DSS123</td>
<td>S</td>
<td><em>C. albicans</em></td>
<td>4.0</td>
<td>128</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>16</td>
<td>0.3</td>
</tr>
<tr>
<td>19</td>
<td>IGM/CM1021</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>128</td>
<td>64</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
<td>IGM/CM1025</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>0.50</td>
<td>&gt;512</td>
<td>32</td>
<td>&gt;512</td>
<td>4.0</td>
<td>0.1</td>
</tr>
<tr>
<td>21</td>
<td>IGM/CM1044</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>2.0</td>
<td>&gt;512</td>
<td>128</td>
<td>32</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td>IGM/CM2010</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>1.0</td>
<td>256</td>
<td>&gt;512</td>
<td>128</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>23</td>
<td>IGM/CM2004</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>4.0</td>
<td>&gt;512</td>
<td>64</td>
<td>64</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>24</td>
<td>IGM/CM2001</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>8.0</td>
<td>128</td>
<td>&gt;512</td>
<td>256</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>25</td>
<td>IGM/CM2033</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>2.0</td>
<td>&gt;512</td>
<td>128</td>
<td>64</td>
<td>8.0</td>
<td>0.2</td>
</tr>
<tr>
<td>26</td>
<td>IGM/CM4A05</td>
<td>S</td>
<td><em>C. tropicalis</em></td>
<td>4.0</td>
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<td>32</td>
<td>256</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>27</td>
<td>IGM/CM1070</td>
<td>S</td>
<td><em>C. glabrata</em></td>
<td>4.0</td>
<td>256</td>
<td>64</td>
<td>16</td>
<td>0.5</td>
<td>8.0</td>
</tr>
<tr>
<td>28</td>
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<td>S</td>
<td><em>C. glabrata</em></td>
<td>8.0</td>
<td>&gt;512</td>
<td>32</td>
<td>128</td>
<td>2.0</td>
<td>4.2</td>
</tr>
<tr>
<td>29</td>
<td>IGM/CM4A12</td>
<td>S</td>
<td><em>C. glabrata</em></td>
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Table 5.4: Antifungal activity (MIC µg/ml) of CAT–3C formulation against various *Candida* species clinical isolates.

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Table 5.5: Antifungal activity (MIC μg/ml) of CAT–3X formulation against various Candida species clinical isolates.

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5.3 Morphological study
To gain more insight on the best scrutinized formulation i.e. CAT–3S, the morphological studies were performed. From the SEM images as presented in Figure 5.3(a,b,c), it was well observed that CAT–3S was homogeneous with no signs of precipitation. Milky white appearance with a colloidal system characteristic was visualized. However, nano – micellar bodies were also visualized with uniform distribution within the gel matrix system. In addition, the image was also taken after duration of 1 month (Figure 5.3b) and lately ~ 9 months (Figure 5.3c) in order to visualize any morphological changes within the formulation, suggesting stability of CAT–3S for a period of 9 month cycle. On the other hand, micellar structures were also characterized via SEM and TEM. Figure 5.4(a,b,c), depicting spherical micellar formation with no structural transition, which can occur sometime due to presence of EtOH caused by compensation between electrostatic interaction and hydrophobic hydration. From TEM images (Figure 5.4b,c) and physicochemical analysis, the size was found to be ~ 118 nm, with polydispersity index 0.17 ± 0.04 and zeta potential – 18.34 ± 2.43 mV suggesting narrow distribution and good stability of the micellar bodies within the system. In addition, it should be noted that carbomer molecule are negative charged with tightly coiled structure therefore provided greater stability, which was well observed in the formulation.

The mechanistic antifungal activity of CAT–3S was estimated using C. albicans (PGI/DML41) cell viability assay via TEM. To gain information with respect to contact activity and cytological damage caused by CAT–3S, the higher concentration i.e. 10 μg/ml was intentionally selected to avoid the budding of cells and cell adherence that are unaffected by the exposure. From the images (Figure 5.5a,b,c), the morphological changes of C. albicans induced by treatment were clearly revealed by TEM. Figure 5.5a of untreated C. albicans was well defined, intact shapes with smooth surface. After 15 min of treatment, a slight but considerable alteration was observed on fungal cell wall, whereas after 2 h and 6 h, well defined ultrastructural changes were noticed. Morphological cell wall deformation (peeling/exfoliation) followed by shrinkage and complete cell damage was observed after 6 h (Figure 5.5d).

However, CLZ which is an imidazole derivative and known to act on fungal cell wall or membrane and binds to the heme part of cell wall leading to inhibition of ergosterol principally sterol in membrane and then destroys the integrity of the fungal membrane. In addition BHA and BHT do possess heme chelating property, so it is a speculation which
Figure 5.3: Scanning electron microscopy images of formulation (CAT-3S); (a) after preparation (b) after 1 month.

Figure 5.4: (a) Scanning electron microscopy image of prepared micelles, (b) and (c) transmission electron microscopy images of micelles dispersed within the formulation CAT-3S.
might be possible that they might have acted in a same manner and provided synergism in order to get better antifungal drug action.

Figure 5.5: TEM images of an unexposed (control) cell and CAT–3S (10 μg/ml) treated cell of C. albicans.

5.4 Stability studies

5.4.1 Photo–Stability study

In general, there are no such scientific reports suggesting that CLZ is photolabile, the United States Pharmacopoeia and very few articles from Santos et al. [44] recommends that photo protection of the drug during storage. In the present study, an examination was made of the ability of potential oxidation inhibitors (BHA/BHT) in prevention of CLZ photodegradation under UVC radiation. After the exposure of 14 h, the samples were analyzed for amount of remaining CLZ (% age). The presented Figure 5.6 shows the photodegradation of methanolic solution of CLZ and CAT–3S in comparison to the dark control of the CLZ. From the results, the CLZ concentration of dark control was found almost 100%, whereas, methanolic CLZ
solution showed significant degradation of 76%. In comparison to dark control, the formulation (CAT-3S) resist with degradation of 22%. The result showed that antioxidants present in CAT-3S led to an increase of approximately 3.5 times of exposed methanolic solution of CLZ. The enhanced photostability is well attributed to the standard antioxidants. It can be explained as antioxidants might have prevented the degradation caused by UV exposure by absorbing the UV radiations or by trapping the free radicals generated during the process of oxidation.

![Bar Chart]

**Figure 5.6:** Plot representing clotrimazole (CLZ) content after 14 h exposure to UVC radiations.

### 5.4.2 Physical stability

In 9 month stability cycle at room temperature and light protected, no changes were observed in appearance, with no color change and without any kind of precipitation (Figure 5.3c). Whist in 60 days, marginal decrease was found in cumulative % drug release and pH. In addition, viscosity was also found to decrease marginally which might be because of EtOH presence in the formulation, thus, affecting the spreadability. With regard to BHA+BHT loaded micellar particle size and zeta potential, they were reasonably steady with slight increase in size and decrease in charge (Figure 5.7). In addition, polydispersity index was also found with marginal decrease.
Figure 5.7: pH, particle size (nm), polydispersity and zeta potential (mV) of micellar structure, after preparation and after 60 days.

5.5 Skin irritation and *in vivo* toxicity study

Conventional therapy is associated with visual noticeable skin irritation check. If observed, it strongly restricts the applicability and acceptability of topical formulation by the patients. Ideally, the developed formulation should not cause any kind of irritation marks. In present investigation, skin irritation studies suggested that CAT–3S exhibited considerably no irritation. The primary irritation index (PII) was found to be 0.00, reflecting no irritation within the limited duration of studied time. In addition, no erythema or edema was observed on the abraded rat skin when compared with control (without treatment).

However, going beyond the conventional therapy evaluation and gain much clear perspective, we intended to examine the *in vivo* toxicity in major organs. The photomicrographs of skin histological sections of treated and untreated animals are shown in Figure 5.8. H and E stained sections of control skin sample showed epidermis consisting of a cornified squamous layer and underlying germinative layer. No inflammatory infiltrate, granulomatous evidence of malignancy was visualized. The formulation CAT–3S did not show sign of inflammation such as inflammatory infiltrate or edema. Compared to the control,
no histopathological changes were visualized in other major organs (liver, kidney, and intestine) in treated animals. These results revealed that the developed surfactant aided antioxidants within CLZ gel system is safe for topical delivery.

![Histological images of major organs](image)

**Figure 5.8:** Histological images of major organs (A) untreated and (B) formulation (CAT–3S) treated, suggesting no toxicity, Scale 50μm.

### 5.6 Confocal laser scanning microscopy

Rhodamine B is an amphoteric dye, although usually listed as basic as it has an overall positive charge. On the other hand, carbopol polymer is anionic in nature, thus generates negative charges along their backbone and has the efficiency to bind with positively charged moiety via ion–ion interactions [45]. Employing CLSM of rat skin, the penetration of the dye within gel was investigated in order to assess the penetration range. The results of the study demonstrated that the penetration and accumulation within epidermis section of skin (Figure 5.9). It can be concluded that system having surfactant aided antioxidant micellar system provides an extra driving force to the molecules present in carbopol gel base, allowing better penetration by destabilizing the membrane whereas, ethanolic solution of drug was found to accumulate in the stratum corneum only. This study suggesting the relevance of residence time for drug on infected site offered by three dimensional polymeric gel system.
Figure 5.9: Confocal laser scanning micrograph of rat skin (A) treatment with hydroalcoholic solution of Rhodamine B, and (B) CAT–3S (Rhodamine B in polymeric system), Scale 50μm.

5.7 In vivo antifungal study (mouse model)

The in vivo antifungal activity of plain CLZ formulation and developed formulation CAT–3S (1 mg/cm² and 5 mg/cm²) was determined by challenging the animals with FLZ resistant C. albicans (PGI/DML54) on 8 days mouse model. From Figure 5.10, it was found that infection in all animals was well established.

The efficacy of the formulation CAT–3S was assessed on the basis of viable CFU count at different time intervals after treatment. Results revealed that CAT 3S possessed significant therapeutic efficacy, as compared to plain drug. After three days both CAT–3S and plain CLZ formulation significantly reduced the growth of C. albicans. Interestingly, at dose level of 1 mg/cm² plain CLZ formulation exhibits somewhat higher efficacy than CAT–3S with 2.14 log₁₀ reduction in viable CFU (**p<0.01; Figure ) of C. albicans when compared with 48 h control. At the same time, formulation CAT–3S at dose level of 5 mg/cm² produced 2.28 log₁₀ reduction in viable CFU (**p<0.01; Figure ) of C. albicans when compared with 48 h control. However, this positive effect of plain CLZ formulation did not maintained throughout the experiment as increment in the viable CFUs was observed on day 5, 6, 7, and 8. In contrast to this, CAT–3S constantly reduced the burden of viable CFUs of infecting
organism and appreciated the longer term reduction of fungal infection in skin. In particular, CAT–3S induced 3.26, 3.58, 4.20, and 4.65 \( \log_{10} \) reduction in viable CFUs of \( C. \ albicans \) on day 5, 6, 7, and 8 respectively when compared with 48 h control. Similarly, formulation at 5 mg/cm\(^2\) decreased the burden of \( C. \ albicans \) by 4.52, 4.81, 5.38, and 5.73 \( \log_{10} \) when growth was observed after 5, 6, 7, and 8 days respectively. It was further interesting to note that on day 8 numbers of viable CFUs was increased to 7.11 \( \log_{10} \).

**Figure 5.10:** \textit{In vivo} antifungal activity representing the infection burden in 8 days mouse model.

As it can be clearly stated that the animals treated with CAT–3S, demonstrated low fungal burden in skin with a colony count significantly less abundant than those treated with plain CLZ formulation. This impact of CAT–3S can be explained in term of presence of BHA and BHT within SDS hydroethanolic micellar system in bioadhesive gel, providing longer residence time, higher bioavailability with synergistic effect offered by antioxidants.
References


