CHAPTER 5

RESULTS AND DISCUSSION
SECTION A: RESULT AND DISCUSSION - NIMBATIKTAM

5.1. Physicochemical standardization of Nimbatiktam

Physico-chemical evaluation such as organoleptic characters, loss on drying (LOD), ash value, acid insoluble ash, bulk density, extractive value, presence of heavy metals and aflatoxins was done by the WHO/AYUSH guidelines (WHO, 1998b) as mentioned in sub section 4.1 of experimental section A. The evaluation was carried out for three batches of formulations supplied and prepared by CCRAS (AYUSH) and results are summarized as follows.

Table 5.1 Summary of physicochemical standardization of Nimbatiktam

<table>
<thead>
<tr>
<th>No</th>
<th>Parameter</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
<th>Average ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Organoleptic characters</td>
<td>Yellow crystalline powder with bitter taste and pungent odour</td>
<td>Yellow crystalline powder with bitter taste and pungent odour</td>
<td>Brownish Yellow crystalline powder with bitter taste and pungent odour</td>
<td>Brownish Yellow crystalline powder with bitter taste and pungent odour</td>
</tr>
<tr>
<td>2</td>
<td>LOD at 105°C (% w/w)</td>
<td>1.36</td>
<td>1.80</td>
<td>1.78</td>
<td>1.65 ± 0.25</td>
</tr>
<tr>
<td>3</td>
<td>Total ash (% w/w)</td>
<td>0.34</td>
<td>0.32</td>
<td>0.37</td>
<td>0.34 ±0.02</td>
</tr>
<tr>
<td>4</td>
<td>Acid insoluble ash</td>
<td>0.08</td>
<td>0.10</td>
<td>0.06</td>
<td>0.08 ±0.02</td>
</tr>
<tr>
<td>5</td>
<td>10% solution (pH)</td>
<td>4.95</td>
<td>6.3</td>
<td>6.7</td>
<td>5.98 ±0.92</td>
</tr>
<tr>
<td>6</td>
<td>1% solution (pH)</td>
<td>6.17</td>
<td>7.0</td>
<td>7.2</td>
<td>6.79 ±0.55</td>
</tr>
<tr>
<td>7</td>
<td>Bulk density (gm/cc)</td>
<td>0.32</td>
<td>0.42</td>
<td>0.35</td>
<td>0.36 ±0.05</td>
</tr>
<tr>
<td>8</td>
<td>Tap density (gm/cc)</td>
<td>0.57</td>
<td>0.73</td>
<td>0.60</td>
<td>0.63 ±0.08</td>
</tr>
<tr>
<td>9</td>
<td>Water soluble extractives (% w/w)</td>
<td>25.5</td>
<td>26.73</td>
<td>26.00</td>
<td>26.08 ±0.62</td>
</tr>
<tr>
<td>10</td>
<td>Alcohol soluble extractives (% w/w)</td>
<td>99.3</td>
<td>99.8</td>
<td>99.5</td>
<td>99.53 ±0.25</td>
</tr>
</tbody>
</table>

Discussion

The quality control of botanical products is prime requirement of AYUSH for any product to be registered for production/sale for human use in India/abroad. The physicochemical parameters of three batches of Nimbatiktam was carried out to set a standard limit of these parameters in prepared formulations that can be used by different manufacturers for quality control of Nimbatiktam.
5.2. Phytochemical investigations of Nimbatiktam

Phytochemical investigations were performed by chemical tests including tests for alkaloids, glycosides, tannins, sugar & carbohydrates, saponins, proteins & amino acids, resins, lipids/fats, phenolic compounds and for flavonoids as discussed in section 4.1.11 (A). The results of same have been summarised in Table 5.2.

Table 5.2 Phytochemical analysis of Nimbatiktam

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Phytochemical parameters</th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NIMBA 1</td>
<td>NIMBA 2</td>
<td>NIMBA 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mfg Date:</td>
<td>Mfg Date:</td>
<td>Mfg Date:</td>
</tr>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Dragendorf’s test</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>ii.</td>
<td>Mayer’s test</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>iii.</td>
<td>Tannic Acid test</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Glycosides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Killer killiani test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ii.</td>
<td>Born trager test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Tannins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Molish test</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ii.</td>
<td>Fehling solution test</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>iii.</td>
<td>Barfoed’s test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Saponins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Foam test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ii.</td>
<td>Haemolysis test</td>
<td>--</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Proteins and amino acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Ninhydrin test</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>ii.</td>
<td>Xanthoproteic test</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>iii.</td>
<td>Millon’s test</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>7.</td>
<td>Resins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Staining test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>9.</td>
<td>Phenolic compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Ferric Chloride test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Lead Acetate test</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>10.</td>
<td>Flavonoids</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

The chemical tests revealed the presence of alkaloids, tannins, saponins, proteins & amino acids, lipid/fats, phenolic compounds and flavonoids in Nimbatiktam.
5.3. Determination of microbial contamination of Nimbatiktam

The results of the total microbial load of Nimbatiktam are presented in Figure 5.1 and Figure 5.2. No significant microbial load was recorded in both the fungal and bacterial counts (Tables 5.3 and Table 5.4).

**Determination of total fungal count**

![Potato dextrose agar plates showing total fungal counts of Nimbatiktam](image)

*Figure 5.1 Potato dextrose agar plates showing total fungal counts of Nimbatiktam*

**Calculation**

\[
\text{Total fungal count} = \frac{\text{No. of CFU} \times \text{Dilution factor}}{\text{Wt. of the sample}}
\]

**Table 5.3 Total fungal count of Nimbatiktam after 48 h at 25 °C in BOD incubator**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Drug</th>
<th>Dilution factor</th>
<th>No. of Colonies</th>
<th>Total bacterial count/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nimbatiktam 1:10</td>
<td>10</td>
<td>02</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>Nimbatiktam 1:100</td>
<td>100</td>
<td>Nil</td>
<td>---</td>
</tr>
</tbody>
</table>

**Determination of total bacterial count**

![Soyabeen casein agar plates showing total bacteria counts of Nimbatiktam](image)

*Figure 5.2 Soyabeen casein agar plates showing total bacteria counts of Nimbatiktam*
Calculation

Total bacterial count = No. of CFU x Dilution factor/ Wt. of the sample

Table 5.4 Total bacterial count of Nimbatkam after 12 h at 37 °C in BOD incubator

<table>
<thead>
<tr>
<th>S. No</th>
<th>Dilution factor</th>
<th>No. of Colonies</th>
<th>Total bacterial count/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10</td>
<td>Nil</td>
<td>-----</td>
</tr>
<tr>
<td>2.</td>
<td>100</td>
<td>Nil</td>
<td>-----</td>
</tr>
</tbody>
</table>

Discussion

Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria and fungi. As anticipated, this microbiological milieu depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations. Risk assessment of the microbial load of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point (HACCP) schemes.

In this study Nimbatkam was found to have negligible amount of total fungal load and absence of bacterial load.
5.4. Chemical Toxicity Evaluation (Heavy metals, aflatoxins and pesticides)

5.4.1. Test for heavy/toxic metals

The heavy metal analysis in Nimbatiktam was carried out using atomic absorption spectrophotometer as per the method (Horwitz, 1970 and Chu et al., 2010). Calibration curves for each heavy metal were set to ensure the accuracy of the Atomic absorption spectrophotometer. Standards with the varying concentration were set for the calibration of the atomic absorption spectrophotometer. The standard plot for Cadmium, Lead, Arsenic and Mercury were prepared as shown in Figures 5.3, 5.4, 5.5 and 5.6, respectively.

The samples of Nimbatiktam were analyzed and presence/absence of heavy metals were calculated from regression equation obtained from the standard plots, Results of analysis have been summarized in Table 5.5.

![Graph showing standard calibration plot for Cadmium at 228 nm using atomic absorption spectrophotometer.]

Figure 5.3 Standard calibration plot for Cadmium at 228 nm using
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Figure 5.4 Standard calibration plot for Lead at 283 nm using AAS

\[ y = 0.012x + 0.017 \]
\[ R^2 = 0.999 \]

Figure 5.5 Standard calibration plot for Arsenic at 193 nm using AAS

\[ y = 0.007x + 0.053 \]
\[ R^2 = 0.996 \]

Table 5.5 Summary of heavy metal analysis in Nimbatiktam (AAS) using AOAC

<table>
<thead>
<tr>
<th>Heavy Metals</th>
<th>Equation of calibration</th>
<th>( r^2 )</th>
<th>Absorbance B-1 (n=3)</th>
<th>Absorbance B-2 (n=3)</th>
<th>Absorbance B-3 (n=3)</th>
<th>Mean ± SD</th>
<th>Content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium (ppm)</td>
<td>( y = 0.327x + 0.040 )</td>
<td>0.985</td>
<td>0.053</td>
<td>0.053</td>
<td>0.053</td>
<td>0.053 ± 0.0001</td>
<td>0.040</td>
</tr>
<tr>
<td>Lead (ppm)</td>
<td>( y = 0.012x + 0.017 )</td>
<td>0.999</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.000</td>
</tr>
<tr>
<td>Arsenic (ppb)</td>
<td>( y = 0.007x + 0.053 )</td>
<td>0.996</td>
<td>0.075</td>
<td>0.073</td>
<td>0.076</td>
<td>0.075 ± 0.0015</td>
<td>3.095</td>
</tr>
<tr>
<td>Mercury (ppb)</td>
<td>( y = 0.022x + 0.176 )</td>
<td>0.984</td>
<td>0.181</td>
<td>0.181</td>
<td>0.179</td>
<td>0.180 ± 0.0012</td>
<td>0.197</td>
</tr>
</tbody>
</table>
Discussion

The present study is apparently the first heavy metal analysis of Nimbatiktam. The study revealed that these formulations contained high levels of Arsenic as compared to Cadmium and Mercury. The amount of Lead was found absent.

An excessive level of Cd in the body has been shown to result in kidney and liver damages as well as deformation of bone structures (Abbas et al., 2008). These manifestations of Cadmium toxicity can only be detected if renal Cadmium concentration of more than 50 µg/g tissue. Since, in these formulations as the concentrations of Cadmium are very less so it can be concluded that the drug is safe for human use.

Chronic arsenic toxicity (arsenicosis) due to drinking of arsenic contaminated ground water is a major environmental health hazard throughout the world including India. Pigmentation and keratosis are the specific skin lesions characteristics of arsenic toxicity. It also produces various systemic manifestations over and above skin lesions, important ones being chronic lung disease like chronic bronchitis, chronic obstructive pulmonary disease and bronchiectasis, liver disease like non-cirrhotic portal fibrosis and other diseases like polyneuropathy, peripheral vascular disease, hypertension and ischeamic heart disease, diabetes mellitus, non-pitting oedema of feet/hands, weakness and anaemia (Mazumder, 2008).
The target organs for Lead toxicity are kidney and nervous system. The Food and agricultural organization/World Health Organization (1993) has established a PTWI (Provisional tolerable weekly intake) of 25 μg of Lead/kg body weight for humans. In formulations concentrations of Lead is absent so the Nimbatiktam can be consider as safe drug.

5.4.2. Analysis of aflatoxins

The analyses of aflatoxin (AF) were done by method of AOAC (Horwitz, 1970 and Tagami et al., 2007). All data were obtained without any interference in the analysis. Aflatoxin analysis of Nimbatiktam is not simple because of the highly coloured fatty materials that are co-extracted with AF. The silica gel columns provided a quick and simple solution to sample clean up. After clean-up, it solves the problems of interference. Figure 5.7 shows HPLC chromatograms of mixed standards aflatoxins of B1, B2, G1 and G2 (80 ppb each) by HPLC using water: acetonitrile : methanol (70:17:17, v/v/v) as mobile phase and florescent as detector at 365 nm (excitation) and 425 nm (emission). Figure 5.8 shows the HPLC chromatograms of aflatoxins extracted from Nimbatiktam using same method as for standard aflatoxin batch -1 analysis. Results are summarized in Table 5.6.

Results indicated that the little higher level of G1 was found in Nimbatiktam while other all aflatoxin was below permissible limit. The all aflatoxins in subsequent batches were found below permissible limit.

![Figure 5.7 HPLC chromatograms of aflatoxins (mix standard of 80 ppb each of B1, B2, G1 and G2) by HPLC using water: acetonitrile: methanol (70:17:17, v/v/v) as mobile phase with florescent detector at 365 nm (excitation) and 425 nm (emission) (excitation) and 425 nm (emission)](image-url)
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Figure 5.8 HPLC chromatograms of Nimbatiktam showing aflatoxins by HPLC using water: acetonitrile: methanol (70:17:17, v/v/v) as mobile phase with fluorescent detector at 365 nm (excitation) and 425 nm (emission)

Table 5.6 Summary of aflatoxin content in Nimbatiktam by HPLC using AOAC

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Aflatoxin</th>
<th>Detection Limit</th>
<th>Nimbatiktam (In ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Batch 1</td>
</tr>
<tr>
<td>1</td>
<td>B1</td>
<td>0.3 ppb</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td>2</td>
<td>B2</td>
<td>0.3 ppb</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td>3</td>
<td>G1</td>
<td>0.3 ppb</td>
<td>0.765</td>
</tr>
<tr>
<td>4</td>
<td>G2</td>
<td>0.3 ppb</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>

Discussion

Aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts (Kneifel et al., 2002). Aflatoxin-producing fungi sometimes build up during storage (De Smet et al., 1992). Procedures for the determination of aflatoxin contamination in herbal drugs are published by the WHO (WHO 2000), Journal of AOAC and other literatures. In the present study various types of major aflatoxins (B1, B2, G1 and G2) was determined by the method by AOAC after using a suitable clean-up procedure, Results indicated the presence of B1, B2 and G1, while G2 was found to be absent.

The batch 1 of Nimbatiktam was showing little high AF G1 content may be due to contaminated raw material i.e. contaminated neem seed or its oil used for extraction of Nimbatiktam. Later on B-2 or B-3 it was found out but below permissible limit.
5.4.3. Pesticide analysis

The GC/MS analyses of pesticide were done as per the method described by as discussed by Horwitz, 1970 and Weaver et al., 2010. The retention times of the pesticides were measured using individual standard solutions at concentrations of 5.0 μg mL⁻¹. The GC-MS instrument was operated in full scan mode, varying the oven temperature and the carrier gas flow rate. The most representative (most intense) ions were selected for quantification of the pesticides in the Nimbatiktam samples.

Results indicated the absence of all types of pesticides in the formulations as mentioned in Table 5.7.

Table 5.7 Residual pesticides in Nimbatiktam by GC/MS using AOAC method

<table>
<thead>
<tr>
<th>Type S. No</th>
<th>Parameters</th>
<th>Detection Limit</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Batch 1</td>
<td>Batch 2</td>
</tr>
<tr>
<td>1.</td>
<td>4,4-DDE</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>2.</td>
<td>2,4-DDD</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>3.</td>
<td>2,4-DDT</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>4.</td>
<td>4,4-DDD</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>5.</td>
<td>4,4-DDT</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>6.</td>
<td>Methoxychlor</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>7.</td>
<td>Aldrin</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>8.</td>
<td>Dieldrin</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>9.</td>
<td>2,4-DDE</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>10.</td>
<td>Heptachlor</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>11.</td>
<td>Heptachlor Epoxide Isom B</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>12.</td>
<td>Alpha-HCH</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>13.</td>
<td>Beta-HCH</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>14.</td>
<td>Lindane</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>15.</td>
<td>Delta BHC</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>16.</td>
<td>2,4-D</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>17.</td>
<td>Dichlorvos</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>18.</td>
<td>Chlorpyriphos</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>19.</td>
<td>Butachlor</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>20.</td>
<td>Malaxon</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>21.</td>
<td>Malathion</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>22.</td>
<td>Methyl Parathion</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>23.</td>
<td>Endosulfan I</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>24.</td>
<td>Endosulfan II</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>25.</td>
<td>Ethion</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>26.</td>
<td>Monocrotophos</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>27.</td>
<td>Endosulfan Sul</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>28.</td>
<td>Diazinon</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>29.</td>
<td>Deltamethrin</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
<tr>
<td>30.</td>
<td>Phosalone</td>
<td>0.1 ppb</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>
Discussion

Exposure to pesticides causes a range of human health problems. It is estimated that nearly 10,000 deaths annually are due to use of chemical pesticide worldwide, with about three-fourths of these occurring in developing countries (Horrigan et al., 2002). Study correlates the pesticide exposure to chronic disease like diabetes, hypertension, ophthalmic disorders etc. Asthma, a chronic disease, was prevalent among the people (2.2%), which are associated with pesticides exposure (Hoppin et al., 2002).

So there are chances of presence of pesticide in herbs used for medicinal purposes. Different international agencies, world health organization (WHO), food and agriculture organization (FAO), the US environment protection agency (USEPA)/Food and Drug Administration Act (FDA) stipulated different limits for different pesticides.

In the present study, there was absence of all type of pesticides (organo phosphates and organo chlorides) which was analyzed GC/MS.
5.5. Long term oral toxicity of Nimbatiktam

The long term oral toxicity of Nimbatiktam was carried out as per OECD guidelines, (OECD, 408) for 90 days. Following observations were made for determining the long term toxicity of the animals

a. Cage side observation for all animals
b. Mortality record of the animals
c. Body weight record
d. Haematological observations
e. Liver function test
f. Kidney function test
g. Histopathological observations of kidney, liver, spleen, lungs and stomach

The cage side observation of animals subjected to toxicity were carried out on several parameters on each day upto 90 days as given in Table 5.8, which didn’t shown any abnormality for full time of study. Out of 60 rats used, none of them showed mortality upto 90 days (Table 5.9). The body weight record of wistar rats (Table 5.10) didn’t shown any irregularity in all treatment groups. Similarly thirteen week treatment of wistar rats with different dosage of Nimbatiktam didn’t show any significant difference in haematological (Table 5.11) parameters as well as liver function test (Table 5.12) and kidney function test (Table 5.13). Statistical analysis of the experimental data was performed by applying student \( t \)-test using GraphPad InStat 3.0 software (GraphPad Software, La Jolla, CA, USA).

Table 5.8 Cage side observation for all animals subjected to long term oral toxicity studies

<table>
<thead>
<tr>
<th>S. No</th>
<th>Evaluation parameters</th>
<th>Cage side observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Condition of the fur</td>
<td>Normal</td>
</tr>
<tr>
<td>2.</td>
<td>Skin</td>
<td>Normal</td>
</tr>
<tr>
<td>3.</td>
<td>Subcutaneous swellings</td>
<td>Nil</td>
</tr>
<tr>
<td>4.</td>
<td>Abdominal distension</td>
<td>Nil</td>
</tr>
<tr>
<td>5.</td>
<td>Eyes – dullness</td>
<td>Nil</td>
</tr>
<tr>
<td>6.</td>
<td>Eyes – opacities</td>
<td>Nil</td>
</tr>
<tr>
<td>7.</td>
<td>Pupil diameter</td>
<td>Normal</td>
</tr>
<tr>
<td>8.</td>
<td>Color and consistency of the faeces</td>
<td>Normal</td>
</tr>
<tr>
<td>9.</td>
<td>Condition of teeth</td>
<td>Normal</td>
</tr>
<tr>
<td>10.</td>
<td>Breathing abnormalities</td>
<td>Nil</td>
</tr>
<tr>
<td>11.</td>
<td>Gait</td>
<td>Normal</td>
</tr>
</tbody>
</table>
### Table 5.9 Mortality record of animals of various doses group treated with Nimbatiktam

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Male (n=10)</th>
<th>Female (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200 mg/kg BW</td>
<td>400 mg/kg BW</td>
</tr>
<tr>
<td>Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mortality</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

### Table 5.10 Body weight record for male and female rats orally treated with Nimbatiktam for 13 weeks

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Body weight of male animals (g) (n=10), Mean±SD</th>
<th>Body weight of female animals (g) (n=10), Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 200 mg 400 mg 800 mg</td>
<td>Control 200 mg 400 mg 800 mg</td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Weeks</td>
<td>119±1.97 129±5.95 131±9.94 128±9.19</td>
<td>153.5±3.34 142.5±7.91 149±8.76 150±9.43</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>164±5.78 185±14.34 173±17.67 193±11.6</td>
<td>189±13.70 186±13.5 189±13.70 193±13.37</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>216±17.13 241.5±17.65 234±15.06 253±18.29</td>
<td>216±14.30 208±16.19 205±21.73 213±9.49</td>
</tr>
<tr>
<td>13 Weeks</td>
<td>257.5±21.25 293±25.84 282±15.49 313±11.60</td>
<td>229±17.92 224±21.19 230±33.59 235±29.15</td>
</tr>
</tbody>
</table>

Doses are given in mg/kg/day
Table 5.11 Haematological data of male and female rats orally treated with Nimbatiktam for 13 weeks

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Male. Mean±SD, (n=10)</th>
<th>Female. (n=10), Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters</strong></td>
<td>Control</td>
<td>200 mg</td>
</tr>
<tr>
<td>RBC (mill/C.mm)</td>
<td>8.23±0.91</td>
<td>8.58±0.6</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.43±1.17</td>
<td>14.06±1.65</td>
</tr>
<tr>
<td>Hemoglobin (%)</td>
<td>63.28±8.68</td>
<td>68.63±6.79</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>83.6±1.17</td>
<td>85.48±6.50</td>
</tr>
<tr>
<td>MCV</td>
<td>18.67±2.10</td>
<td>18.04±1.15</td>
</tr>
<tr>
<td>MCH%</td>
<td>24.85±3.75</td>
<td>25.01±4.52</td>
</tr>
<tr>
<td>Total Leucocytes</td>
<td>6736±1844</td>
<td>6880±1131</td>
</tr>
<tr>
<td>Platelet *10^6/mm³</td>
<td>877.3±220.4</td>
<td>889±129</td>
</tr>
</tbody>
</table>

Doses is given in mg/kg/day

Table 5.12 Liver function test for male and female rats orally treated with Nimbatiktam for 90 days

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Male. Mean±SD, (n=10)</th>
<th>Female. Mean±SD, (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters</strong></td>
<td>Control</td>
<td>200 mg</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.18±0.10</td>
<td>0.19±0.07</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>180.70±11.11</td>
<td>189.40±12.7</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>48.20±10.11</td>
<td>50.90±12.7</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>205.80±53.1</td>
<td>200.8±45.7</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>7.21±0.59</td>
<td>7.06±0.21</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>1.75±0.38</td>
<td>1.29±0.17</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>5.38±0.60</td>
<td>5.44±0.33</td>
</tr>
<tr>
<td>Alb:Glob Ratio</td>
<td>0.33±0.09</td>
<td>0.24±0.03</td>
</tr>
</tbody>
</table>

Doses is given in mg/kg/day
### Table 5.13 Kidney function test of male and female rats orally treated with Nimbatiktam for 90 days

<table>
<thead>
<tr>
<th>Treatment groups →</th>
<th>Male, Mean±SD, (n=10)</th>
<th>Female, Mean±SD, (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td>Control</td>
<td>200 mg</td>
</tr>
<tr>
<td>Blood urea (mg/dL)</td>
<td>32.4 ± 8.42</td>
<td>30.6 ± 7.63</td>
</tr>
<tr>
<td>Creatinine (mg %)</td>
<td>0.9 ± 0.21</td>
<td>0.9 ± 0.20</td>
</tr>
<tr>
<td>Serum uric acid (mg/100mL)</td>
<td>2.6 ± 1.23</td>
<td>2.0 ± 0.85</td>
</tr>
<tr>
<td>Sodium (meq/L)</td>
<td>145.7 ± 6.37</td>
<td>144.6 ± 7.71</td>
</tr>
<tr>
<td>Potassium (meq/L)</td>
<td>5.7 ± 0.75</td>
<td>4.5 ± 0.80</td>
</tr>
<tr>
<td>Total Protein (g/dL)</td>
<td>7.3 ± 0.65</td>
<td>7.0 ± 1.15</td>
</tr>
</tbody>
</table>

Values are mean (SD), n=10. Dosage is given in mg/kg/day

### Table 5.13A Weight of the organs for male and female rats orally treated with Nimbatiktam for 90 days

<table>
<thead>
<tr>
<th>Organs</th>
<th>Control</th>
<th>200 mg</th>
<th>400 mg</th>
<th>800 mg</th>
<th>Control</th>
<th>200 mg</th>
<th>400 mg</th>
<th>800 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>0.31±0.06</td>
<td>0.81±0.05</td>
<td>0.81±0.06</td>
<td>0.88±0.08</td>
<td>0.71±0.03</td>
<td>0.73±0.04</td>
<td>0.73±0.07</td>
<td>0.89±0.10</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.67±0.04</td>
<td>0.67±0.05</td>
<td>0.68±0.05</td>
<td>0.76±0.04</td>
<td>0.59±0.06</td>
<td>0.60±0.04</td>
<td>0.62±0.04</td>
<td>0.77±0.06</td>
</tr>
<tr>
<td>Liver</td>
<td>7.88±0.47</td>
<td>8.05±0.49</td>
<td>7.85±0.47</td>
<td>8.14±0.49</td>
<td>7.82±0.41</td>
<td>7.92±0.44</td>
<td>7.65±0.43</td>
<td>7.97±2.51</td>
</tr>
</tbody>
</table>

*Values are in Mean ±S.D; Values are no significantly different from control, weight in gm, n=10*
Histopathological observations of different organs examined for oral toxicity studies of nimbatiktam

Histopathology of Kidney

Histopathological examination of kidney tissue showed normal histological appearance of renal parenchyma, glomerular tubule in all treatment groups including control (Figure 5.9).

**Figure 5.9** Photomicrograph of sections of kidneys of different treatment groups showing glomerulus (G) and tubules (T) at low resolution (100X) and high resolution (400X)

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Low dose female group (100X); F. Low dose female group (400X); G. Low dose male group (100X); H. Low dose male group (400X); I. High dose female group (100X); J. High dose female group (400X); K. High dose male group (100X); L. High dose male group (400X)
Chapter 5

Histopathology of Liver

Histopathological examination of liver tissue showed normal histological appearance of portal triad (PT), central vein (CV) (100X), bile duct (BD) and portal vein (PV) (400X) in all treatment group including control (Figure 5.10).

Figure 5.10 Photomicrograph of sections of liver tissue of different treatment groups showing portal triad (PT), central vein (CV) (100X), bile duct (BD) and portal vein (PV) (400X)

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Low dose female group (100X); F. Low dose female group (400X); G. Low dose male group (100X); H. Low dose male group (400X); I. High dose female group (100X); J. High dose female group (400X); K. High dose male group (100X); L. High dose male group (400X)
Histopathology of Lung

The histopathological examination of lung tissues showed normal histological appearance of lung bronchiole and alveoli in photomicrographs of all treatment groups including control (Figure 5.11).

![Photomicrograph of sections of lungs of different treatment groups showing normal histological appearance of lung bronchiolo and alveoli in all treatment groups including control](image)

**Figure 5.11** Photomicrograph of sections of lungs of different treatment groups showing normal histological appearance of lung bronchiole and alveoli in all treatment groups including control

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Low dose female group (100X); F. Low dose female group (400X); G. Low dose male group (100X); H. Low dose male group (400X); I. High dose female group (100X); J. High dose female group (400X); K. High dose male group (100X); L. High dose male group (400X)
Histopathology of Spleen

The histopathological examination of spleen tissues showed normal histological appearance of splenic sinuses (Sin), central artery (Art) and lymphocytes in photomicrographs of all treatment groups including control (Figure 5.12).

Figure 5.12 Photomicrograph of sections of spleen tissues showed normal histological appearance of splenic sinuses (Sin) of red pulp of spleen, central artery (Art) and lymphocytes stored in white pulp of spleen in photomicrographs of all treatment groups including control

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Low dose female group (100X); F. Low dose female group (400X); G. Low dose male group (100X); H. Low dose male group (400X); I. High dose female group (100X); J. High dose female group (400X); K. High dose male group (100X); L. High dose male group (400X)
Histopathology of Stomach

The histopathological examination of stomach tissues showed normal histological appearance of epithelium (E) and mucosal layer (M) at low resolution (A, 100X) and high resolution (B, 400X) in photomicrographs of all treatment groups including control (Figure 5.13).

Figure 5.13 Photomicrograph of sections of skin tissues showed normal histological appearance of squamous epithelium (E) and muscularis mucosal layer (M) at low resolution (A, 100X) and high resolution (B, 400X) in photomicrographs of all treatment groups including control.

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Low dose female group (100X); F. Low dose female group (400X); G. Low dose male group (100X); H. Low dose male group (400X); I. High dose female group (100X); J. High dose female group (400X); K. High dose male group (100X); L. High dose male group (400X)
Discussion

Male and female rats in all three dose groups were similar to the control group in weight gain, food intake, water intake and behavioural activity over the study period. There were no markable abnormal changes observed throughout the study period. Haematology and blood chemistry, in rats at all three Nimbatiktam doses showed insignificant difference from control groups. Only slight elevation of RBC, platelet and haemoglobin was observed in high dose treated group. The elevation was higher in female group than male group. The functioning of major organs (kidney, liver, lungs, spleen and stomach) was also found similar to control group. Minor exceptions were elevated blood urea values at high doses, and increased total protein in the high dose treated group. Histopathological examination of the kidney, liver, lung, spleen and stomach tissues did not reveal any change.

Oral nimbatiktam was ingested at doses up to 800 mg/kg per day in the rat for up to 3 months resulted in normal growth with no changes in haematologic or hepatic parameters, and only minor alterations in renal and blood chemistry parameters. There were no evidence of abnormal histology. These data suggest the long-term daily oral consumption of Nimbatiktam is safe in rats. Thus, Nimbatiktam may potentially be safe for clinical use as an antipsoriatic agent.
5.6. Assay for constituents (Flavonoids and Phenolics)

5.6.1. Determination of total flavonoid content of Nimbatiktam

Total flavonoid content of Nimbatiktam was determined as per the method described by Qayyoom et al., 2009 using spectrophotometer at 415 nm wavelength. The colorimetric method was carried out for all three batches using standard calibration plot of rutin in the range of 10 - 100 µg/mL, (Figure 5.14) with regression equation $y = 0.009x + 0.100$ and correlation coefficient $r^2 = 0.993$. The average content of total flavonoids of triplicate analysis of each batch and their average are given in Table 5.14, which were found as 0.019 % w/w Nimbatiktam.

![Figure 5.14 Standard plot of rutin for estimation of total flavonoidal content by using UV spectrophotometer](image)

**Table 5.14 Total flavonoid content of Nimbatiktam**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Batches</th>
<th>Mean of flavonoidal content (µg/g) (n=3)</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Batch 1</td>
<td>200.169</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Batch 2</td>
<td>199.492</td>
<td>199.887 ± 0.479</td>
</tr>
<tr>
<td>3</td>
<td>Batch 3</td>
<td>200.231</td>
<td></td>
</tr>
</tbody>
</table>
5.6.2. Determination of Total Phenolic Content:

Total phenolic content of Nimbatiktam was determined as per the method described by Qayyoom et al., 2009 using spectrophotometer at 765 nm wavelength. The colorimetric method was carried out for all three batches using standard calibration plot of rutin in the range of 25 - 300 μg/mL, (Figure 5.15) with regression equation \( y = 0.010x + 0.097 \) and correlation coefficient \( r^2 = 0.995 \). The average content of total flavonoids of triplicate analysis of each batch and their average are given in Table 5.15, which were found as 0.040 % w/w Nimbatiktam.

![Graph showing the standard plot of gallic acid for estimation of total phenolic contents by using UV spectrophotometer](image)

**Figure 5.15 Standard plot of gallic acid for estimation of total phenolic contents by using UV spectrophotometer**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Batches</th>
<th>Mean of phenolic content (μg/g) (n=3)</th>
<th>Average ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Batch 1</td>
<td>400.186</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Batch 2</td>
<td>406.122</td>
<td>404.279 ± 3.551</td>
</tr>
<tr>
<td>3</td>
<td>Batch 3</td>
<td>406.530</td>
<td></td>
</tr>
</tbody>
</table>
5.7. Analytical method development for analysis of Nimbatiktam in formulation

Selection and optimization of mobile phase

Mobile phase containing different solvent composition were tried for selection of mobile phase. Finally, chloroform: ethyl acetate mixture in different proportion was investigated for their suitability as mobile phase. A ratio of 8:2 (v/v) resulted in good resolution of neem oil extract constituents but with broadening of peak. Eventually, chloroform: ethyl acetate containing 1% acetic acid (8:2 v/v) was found to give a sharp well resolved and well defined peaks in Nimbatiktam. Hence, this mobile phase was selected for further studies.

Finger printing of neem oil extract

The chromatograms scanned at different wavelength showed that the peaks were sharp and well defined with maximum area at wavelength 265 nm hence it was selected for fingerprint analysis. The fingerprinting analysis showed 10 distinct peaks at different R_f values i.e. at 0.13, 0.19, 0.26, 0.33, 0.38, 0.48, 0.55, 0.63, 0.69 and at 0.78 (Figure 5.16 and Figure 5.17), which were tested for linearity (Table 5.16)

Figure 5.16 HPTLC finger printing of Nimbatiktam (4μg) at 265 nm showing peaks of all 10 spots
Table 5.16 Rf value and linearity of all peaks obtained in HPTLC analysis of Nimbatiktam

<table>
<thead>
<tr>
<th>S.No</th>
<th>Rf of Peaks</th>
<th>Linearity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.13</td>
<td>Non Linear</td>
</tr>
<tr>
<td>2</td>
<td>0.19</td>
<td>Non Linear</td>
</tr>
<tr>
<td>3</td>
<td>0.26</td>
<td>Non Linear</td>
</tr>
<tr>
<td>4</td>
<td>0.33*</td>
<td>Linear</td>
</tr>
<tr>
<td>5</td>
<td>0.38</td>
<td>Non Linear</td>
</tr>
<tr>
<td>6</td>
<td>0.48</td>
<td>Non Linear</td>
</tr>
<tr>
<td>7</td>
<td>0.55**</td>
<td>Linear</td>
</tr>
<tr>
<td>8</td>
<td>0.63</td>
<td>Non Linear</td>
</tr>
<tr>
<td>9</td>
<td>0.69</td>
<td>Non Linear</td>
</tr>
<tr>
<td>10</td>
<td>0.78</td>
<td>Non Linear</td>
</tr>
</tbody>
</table>

*Substance 1 ** Substance 2

**Calibration**

Out of ten distinct peaks at different Rf values obtained, only two peaks (Figure 5.18) at Rf 0.33 and 0.55 (substance 1 and substance 2) showed good linearity at their λmax 265 nm (Figure 5.19) by increasing concentration 4 µg to100 µg. The results of concentration and respective area and height as obtained in densitometry analysis are shown in Table 5.17. The correlation coefficient $r^2$ with respect to peak area was found 0.993 for substance 1 and 0.993 for substance 2, (Table 5.18). The two peaks thus obtained with different concentration of Nimbatiktam were calibrated for its linearity which was used for quantification of Nimbatiktam in different formulation.
Figure 5.18 HPTLC chromatogram showing peak of substance 1 and substance 2 (at Rf 0.33 and 0.55) in Nimbatiktam at 265nm (4 µg)

Figure 5.19 Super imposed UV spectra of substance 1 (Rf 0.33) & substance 2 (Rf 0.55) showing λmax at 265 nm

Table 5.17 Standard calibration curve of two linear peaks obtained in HPTLC analysis of Nimbatiktam (λmax 265 nm)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg)</th>
<th>Area of Peak (Area ± SD)</th>
<th>Height of Peak (Height ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Substance 1</td>
<td>Substance 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>299.9 ± 19.0</td>
<td>712.7 ± 1.0</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>299.9 ± 19.0</td>
<td>712.7 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>481.6 ± 8.3</td>
<td>1337.2 ± 155.4</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>586.3 ± 18.3</td>
<td>1492.2 ± 176.0</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>828.1 ± 98.5</td>
<td>1680.3 ± 195.0</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>1343.0 ± 33.1</td>
<td>2783.0 ± 151.5</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>2768.0 ± 19.3</td>
<td>5227.0 ± 123.7</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>3666.3 ± 321.0</td>
<td>6181.3 ± 855.1</td>
</tr>
</tbody>
</table>
Table 5.18 Linear regression data for calibration plot (area) of two linear peaks obtained in HPTLC analysis of Nimbatiktam

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Substance 1</th>
<th>Substance 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/spot)</td>
<td>4-100</td>
<td>4-100</td>
</tr>
<tr>
<td>Regression equation</td>
<td>$Y = 33.735x + 162.07$</td>
<td>$Y = 55.058x + 712.66$</td>
</tr>
<tr>
<td>Correlation coefficient ± SD</td>
<td>0.993 ± 0.008</td>
<td>0.993 ± 0.011</td>
</tr>
<tr>
<td>Slope ± SD</td>
<td>33.735±0.22</td>
<td>55.058± 0.15</td>
</tr>
<tr>
<td>Intercept ± SD</td>
<td>162.07±0.50</td>
<td>712.66±0.44</td>
</tr>
</tbody>
</table>

Accuracy as recovery

The recovery of the method was determined by spiking a previously analyzed test solution of formulation with additional Nimbatiktam, which was found to be 97.4 - 98.66% with respect to substance 1 and 98.6-99.7% with respect to substance 2. The value of recovery and % RSD as depicted in Table 5.19 shows that the method is accurate.

Table 5.19 Recovery studies of two linear peaks obtained in HPTLC analysis of Nimbatiktam

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th>Substance 1</th>
<th>Substance 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Percentage of standard spiked</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>2.</td>
<td>Amount of standard spiked(µg)</td>
<td>48</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>204</td>
</tr>
<tr>
<td>3.</td>
<td>Amount recovered (µg) Mean±SD</td>
<td>47.36±0.11</td>
<td>135.6±0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62.18±0.12</td>
<td>168.5±0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70.12±0.29</td>
<td>201.2±1.67</td>
</tr>
<tr>
<td>4.</td>
<td>Percentage recovery</td>
<td>98.66</td>
<td>99.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>103.66</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.4</td>
<td>98.6</td>
</tr>
</tbody>
</table>

Precision

Inter-day, Intra-day, Inter-analyst and Inter-system precisions were carried out by applying six samples. Assay for each analysis was done and %RSD was calculated, which were found below 3% (Table 5.20). The low value of RSD (%) indicates the precision of the method.
Table 5.20: Precisions studies of substance 1 and substance 2 obtained in HPTLC analysis of Nimbatiktam in different condition

<table>
<thead>
<tr>
<th>Precisions</th>
<th>% RSD (Area)</th>
<th>% RSD (Rf)</th>
<th>% RSD (Assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>substance 1</td>
<td>substance 1</td>
<td>substance 1</td>
</tr>
<tr>
<td>Inter day</td>
<td>2.22</td>
<td>1.76</td>
<td>0.52</td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>Intra day</td>
<td>2.65</td>
<td>2.11</td>
<td>0.67</td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>Inter analyst</td>
<td>2.90</td>
<td>2.91</td>
<td>0.99</td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Inter system</td>
<td>2.44</td>
<td>2.75</td>
<td>1.0</td>
</tr>
<tr>
<td>precision</td>
<td></td>
<td></td>
<td>1.52</td>
</tr>
</tbody>
</table>

Limit of detection and Limit of Quantification

The LOD and LOQ of the method, determined by the signal to noise ratio and found 1 μg and 4μg, respectively with respect to both the substances, which indicates the method can be used for detection and quantification of Nimbatiktam/neem oil over a very wide range of concentrations.

Discussion

The proposed method is very simple and economic. It can be applied for quantitative estimation of Nimbatiktam/neem oil in formulations. The present method provide a way for standardization and quantity control of herbal formulation in which the chemical markers yet, have not been identified. The peaks of each spot obtained in finger printing of neem oil extract at 265 nm serves as different component separately. Hence, each peak can be used a separate marker for quantification purpose. The calibration plot prepared for those peaks showed good linearity in wide range of concentration (substance 1 Rf 0.33 and substance 2 Rf 0.55) out of total 10 peaks. The λmax 265 nm was selected because of the sharpest, highest and maximum number of peaks obtained at this wavelength.
5.8. Immunomodulatory activity of Nimbatiktam

The immunomodulatory activity of Nimbatiktam was carried out as per the protocol of given in section 4.6 (A) for delayed type hypersensitivity (Doherty, 1981) and for humoral response against haemoagglutination antibody titre (Nelson and Mildenhall, 1967).

Results of DTH response for different groups have summarized in Table 5.21. Control group showed high paw swelling where as cyclophosphamide showed immune suppressant effect as evident from significant change in reduction in paw volume (***p< 0.001 vs control). However paw volume in Nimbatiktam group (III & IV) was significantly higher as compared to cyclophospham ide (***p< 0.001 vs cyclophosphamide). Similar results were obtained with Nimbatiktam group 400 mg/kg as per se.

Table 5.21 DTH response of Nimbatiktam against SRBC

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (mg/kg)</th>
<th>DTH Response Paw swelling (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Sensitized control (1% sodium carboxy methyl cellulose)</td>
<td>0.658 ± 0.07</td>
</tr>
<tr>
<td>II.</td>
<td>Cyclophosphamide</td>
<td>0.317 ± 0.05***</td>
</tr>
<tr>
<td>III.</td>
<td>Nimbatiktam (200mg/kg BW)</td>
<td>0.490 ± 0.04**</td>
</tr>
<tr>
<td>IV.</td>
<td>Nimbatiktam 200 mg/kg b.w.) + CP (once)</td>
<td>0.405 ± 0.03**</td>
</tr>
<tr>
<td>V.</td>
<td>Nimbatiktam (400mg/kg BW)</td>
<td>0.442 ± 0.047*</td>
</tr>
<tr>
<td>VI.</td>
<td>Nimbatiktam 400 mg/kg b.w.) + CP (once)</td>
<td>0.369 ± 0.028**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, *p< 0.005 vs Control, **p< 0.01 vs Control, ***p< 0.001 vs control compared to control group animals, (n=6)
### Table 5.22 Humoral response of Nimbatiktam against SRBC

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment (mg/kg)</th>
<th>Haemagglutination with Primary antibody titre at 7th day</th>
<th>Haemagglutination with secondary antibody titre at 14th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Sensitized control (1% sodium carboxy methyl cellulose)</td>
<td>7.27 ± 0.04</td>
<td>7.32 ± 0.05</td>
</tr>
<tr>
<td>II.</td>
<td>Cyclophosphamide</td>
<td>4.82 ± 0.06</td>
<td>4.95 ± 0.14</td>
</tr>
<tr>
<td>III.</td>
<td>Nimbatiktam 200 mg/kg b.w.)</td>
<td>5.86 ± 0.05</td>
<td>6.50 ± 0.04*</td>
</tr>
<tr>
<td>IV.</td>
<td>Nimbatiktam 200 mg/kg b.w.) + CP</td>
<td>4.92 ± 0.08</td>
<td>5.34 ± 0.07**</td>
</tr>
<tr>
<td>V.</td>
<td>Nimbatiktam 400 mg/kg b.w.)</td>
<td>5.34 ± 0.06</td>
<td>5.29 ± 0.05**</td>
</tr>
<tr>
<td>VI.</td>
<td>Nimbatiktam 400 mg/kg b.w.) + CP</td>
<td>5.16 ± 0.13</td>
<td>5.18 ± 0.03*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, *p < 0.005 vs Control, **p < 0.01 vs Control, compared to control group animals, (n=6)

### Discussion

The augmentation of humoral response by Nimbatiktam as evident from an enhancement of antibody responsiveness to SRBC in mice as a consequence of both pre and post immunization treatment, indicates the enhanced responsiveness of macrophages and B lymphocytes subsets involved in antibody synthesis. Therefore the inhibitory effect of Nimbatiktam on DTH response could be due to their influence on the biological mediators. Decreased DTH reactivity may further be explained by simultaneous presence of high titres of antibodies, promoting the elimination of antigen.
5.9. Method development and validation for pharmacokinetic studies of salannin and azadirachtin

5.9.1. Selection of column and optimization of chromatographic condition

The objective of this study was to chromatographically quantify salannin and azadirachtin in rat plasma. Two analytical columns, namely, Chirobiotic V2 (25 cm × 4.6 mm, 5 μm) and UPLC™ BEH C8 (100.0 mm × 2.1 mm; 1.7 μm), were shortlisted during method development. Baseline chromatographic resolution could not be achieved on the Chirobiotic V2 column with a mobile phase ammonium formate buffer (10mM, pH 4.00): Acetonitrile (70: 30, v/v). A good resolution was observed on the UPLC™ BEH C8 column containing mobile phase acetonitrile - 1.0 mM ammonium formate for salannin and azadirachtin. In order to undertake successful quantifications, tuning parameters for ESI⁺ were optimized for the protonated precursor and product ions of analytes. Salannin and azadirachtin was found to have retention time of 1.26 ± 0.23, and 1.12 ± 0.04 min respectively under the chromatographic conditions described. Figure 5.20 shows chromatograms of standard salannin at 250 ng/mL, whereas Fig 5.21 shows azadirachtin.

Figure 5.20 UPLC MS Chromatogram showing salannin in blank plasma at RT 1.26 min
5.9.2. Linearity, Limit of Quantification, Accuracy and Precision

Linearity of salannin was established over a concentration range of 1.0 ng/mL-1000 ng/mL, in spiked human plasma with linear coefficient of regression ($r^2 > 0.997$) using least squares linear regression model, Fig 5.22. Similarly, linearity of azadirachtin was established over a concentration range of 1.2 ng/mL-5719.95 ng/mL ($r^2 > 0.996$), in spiked human plasma Fig 5.23. The limits of quantification in the present method were 1.00 ng/mL both for salannin and azadirachtin.

Figure 5.22 Representative calibration curve of salannin in blank plasma using UPLC/MS
Three precision and accuracy batches were run to check intra and interday precision and accuracy. The results for precision and accuracy are summarized in Table 5.23 (salannin) and Table 5.24 (azadirachtin). The analysis of different QC samples of salannin shows precision in the range of 0.230 to 4.522 % RSD, whereas accuracy from 99.18-101.75 %. Similarly analysis of azadirachtin QC samples showed precision in the range of 0.111-2.105, whereas accuracy ranged from 99.85-103.5 %.

Table 5.23 Intra and inter-day precision and accuracy for determination of salannin in rat plasma by LC-MS/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nominal</th>
<th>Intra-day precision (n=6)</th>
<th>Inter-day precision (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±S.D (ng/mL)</td>
<td>%CV</td>
</tr>
<tr>
<td>LLOQC</td>
<td>1</td>
<td>1.00 ±0.041</td>
<td>4.111</td>
</tr>
<tr>
<td>LQC</td>
<td>5</td>
<td>4.96 ±0.137</td>
<td>2.767</td>
</tr>
<tr>
<td>MQC</td>
<td>400</td>
<td>400.66 ± 2.557</td>
<td>0.638</td>
</tr>
<tr>
<td>HQC</td>
<td>800</td>
<td>797.52 ± 3.021</td>
<td>0.379</td>
</tr>
</tbody>
</table>

Table 5.24 Intra and inter-day precision and accuracy for determination of azadirachtin in rat plasma by LC-MS/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nominal</th>
<th>Intra-day precision (n=6)</th>
<th>Inter-day precision (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±S.D (ng/mL)</td>
<td>%CV</td>
</tr>
<tr>
<td>LLOQC</td>
<td>11.12</td>
<td>10.21 ±0.21</td>
<td>2.105</td>
</tr>
<tr>
<td>LQC</td>
<td>205.91</td>
<td>206.51±1.58</td>
<td>0.765</td>
</tr>
<tr>
<td>MQC</td>
<td>915.19</td>
<td>914.72±1.01</td>
<td>0.111</td>
</tr>
<tr>
<td>HQC</td>
<td>2287.98</td>
<td>2286.16±6.20</td>
<td>0.271</td>
</tr>
</tbody>
</table>
5.9.3. Ex vivo stability studies of salannin and azadirachtin

The results of freeze thaw stability, bench top stability, autosampler stability, and long term stability are summarized in Table 5.25 (salannin) and Table 5.26 (azadirachtin). Freeze-thaw stability was assessed by assaying six replicates of QC samples at low concentrations (LQC), medium concentrations (MQC), and high concentrations (HQC) previously frozen and thawed at room temperature over three cycles. The comparison was made to freshly spiked calibration standards. Salannin and azadirachtin was proved to be stable in biological samples for three freeze and thaw cycles. Bench top stability for salannin and azadirachtin in rat plasma was established for 9.0 h for which samples were left on bench at room temperature and then processed with freshly spiked sample before analysis. In order to assess autosampler stability, six sets of low, medium and high QC samples were kept in an autosampler in polypropylene container programmed at 10 °C and were analyzed after 110.67 hours along with freshly spiked low QC (LQC), medium QC (MQC) and high QC (HQC) samples, and the concentration was calculated against the freshly spiked calibration standards. Moreover, the results of frozen storage on stability indicated that both of the drugs were stable for at least 30 days when kept frozen below −50°C. The salannin and azadirachtin were found to be stable as the precisions of all the stability indicators was below 15% and the accuracies was in the range of 85–115% (Table 5.25 and Table 5.26).

Table 5.25 Freeze thaw stability, bench top stability, autosampler stability, and long term stability data of salannin in rat plasma

<table>
<thead>
<tr>
<th>Storage Pd and condition</th>
<th>Sample Type</th>
<th>Nominal Concen (mg/mL)</th>
<th>Mean (ng/mL)</th>
<th>RSD (%)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze Thaw cycles</td>
<td>LQC 5</td>
<td>4.32</td>
<td>11.901</td>
<td>86.493</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>399.26</td>
<td>0.467</td>
<td>99.816</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>799.61</td>
<td>0.554</td>
<td>99.951</td>
<td></td>
</tr>
<tr>
<td>Bench Top Stability</td>
<td>LQC 5</td>
<td>5.06</td>
<td>6.760</td>
<td>101.290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>399.26</td>
<td>0.467</td>
<td>99.816</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>799.69</td>
<td>1.010</td>
<td>99.961</td>
<td></td>
</tr>
<tr>
<td>Autosampler stability</td>
<td>LQC 5</td>
<td>5.02</td>
<td>3.191</td>
<td>100.437</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>403.61</td>
<td>3.049</td>
<td>100.901</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>798.44</td>
<td>0.391</td>
<td>99.805</td>
<td></td>
</tr>
<tr>
<td>Injector stability</td>
<td>LQC 5</td>
<td>5.13</td>
<td>6.157</td>
<td>102.687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>411.35</td>
<td>5.496</td>
<td>102.836</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>800.01</td>
<td>0.551</td>
<td>100.002</td>
<td></td>
</tr>
<tr>
<td>Long Term stability</td>
<td>LQC 5</td>
<td>4.96</td>
<td>2.767</td>
<td>99.183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>400.66</td>
<td>6.638</td>
<td>100.165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>801.00</td>
<td>0.484</td>
<td>100.126</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.26 Freeze thaw stability, bench top stability, autosampler stability, and long term stability data of azadirachtin in rat plasma

<table>
<thead>
<tr>
<th>Storage Pd and condition</th>
<th>Sample Type</th>
<th>Nominal Conc (ng/mL)</th>
<th>Mean</th>
<th>RSD (%)</th>
<th>% Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze thaw cycles</td>
<td>LQC 5</td>
<td>4.32</td>
<td>11.901</td>
<td>86.493</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>399.26</td>
<td>0.467</td>
<td>99.816</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>799.61</td>
<td>0.554</td>
<td>99.951</td>
<td></td>
</tr>
<tr>
<td>Bench top Stability</td>
<td>LQC 5</td>
<td>5.06</td>
<td>6.760</td>
<td>101.290</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>399.26</td>
<td>0.467</td>
<td>99.816</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>799.69</td>
<td>1.010</td>
<td>99.961</td>
<td></td>
</tr>
<tr>
<td>Autosampler stability</td>
<td>LQC 5</td>
<td>5.02</td>
<td>3.191</td>
<td>100.437</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>403.61</td>
<td>3.049</td>
<td>100.901</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>798.44</td>
<td>0.391</td>
<td>99.805</td>
<td></td>
</tr>
<tr>
<td>Injector stability</td>
<td>LQC 5</td>
<td>5.13</td>
<td>6.157</td>
<td>102.687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>411.35</td>
<td>5.496</td>
<td>102.836</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>800.01</td>
<td>0.551</td>
<td>100.002</td>
<td></td>
</tr>
<tr>
<td>Long term stability</td>
<td>LQC 5</td>
<td>4.96</td>
<td>2.767</td>
<td>99.183</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MQC 400</td>
<td>400.66</td>
<td>0.638</td>
<td>100.165</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HQC 800</td>
<td>801.00</td>
<td>0.484</td>
<td>100.126</td>
<td></td>
</tr>
</tbody>
</table>

5.9.4. Pharmacokinetic Study in male wistar rats

These methods were successfully applied to an open label, balanced, randomized, single dose, crossover, bioequivalence study of salannin and azadirachtin involving male wistar rats following oral administration of 8 mg of herbal antipsoriatic formulation under fasting condition. Venous blood samples were collected in K3 EDTA tubes predose at 0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 12.0, and 24.0 h after dosing. Venous blood samples were collected at 0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 12.0, and 24.0 h after dosing. The peak plasma concentration (Cmax) and time (Tmax) to reach Cmax were read directly from the data. The total areas under the plasma concentration-time curve from time zero to infinity (AUC0→∞) and from time zero to the last measurable concentration (AUC0→t) were also calculated by a noncompartmental analysis using PK Solutions Version 2.0 (Summit Research Services, USA). Mean plasma concentration–time of salannin and azadirachtin were shown Figure 5.24 and Figure 5.25 respectively. The mean estimates of pharmacokinetic parameters derived from the plasma concentration profiles are summarized in Table 5.27 (salannin) and Table 5.28 (azadirachtin).
Figure 5.24 Linear plot of mean plasma salannin concentration (ng/mL) versus time (h) in male wistar rats

Table 5.27 Pharmacokinetic parameters (mean ± SD) of salannin in oral herbal formulations, based on its plasma concentrations in rat plasma

<table>
<thead>
<tr>
<th>Drug</th>
<th>$t_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>$\text{AUC}_{0-24}$ (h ng/mL)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salannin</td>
<td>1.5 ± 0.000</td>
<td>716.8 ± 0.004</td>
<td>1617.865 ± 0.018</td>
<td>4.41 ± 0.017</td>
</tr>
</tbody>
</table>

Figure 5.25 Linear plot of mean plasma azadirachtin concentration (ng/mL) versus time (h) in male wistar rats
Table 5.28 Pharmacokinetic parameters (mean ± SD) of azadirachtin in oral herbal formulations, based on its plasma concentrations in rat plasma

<table>
<thead>
<tr>
<th>Drug</th>
<th>$t_{\text{max}}$ (hrs)</th>
<th>$C_{\text{max}}$ (ng/mL)</th>
<th>AUC$_{0-24}$ (h ng/mL)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azadirachtin</td>
<td>1.5 ± 0.000</td>
<td>454.3 ± 0.001</td>
<td>1462.4 ± 0.019</td>
<td>6.78 ± 0.010</td>
</tr>
</tbody>
</table>

5.9.5. Conclusion

A simple, fast and accurate validated LC-MS/MS method was developed for the analysis of salannin and azadirachtin in plasma. Waters Acquity UPLCTM BEH C8 column was found to be selective for the separation of salannin and azadirachtin from plasma. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability-indicating and was used successfully for a pharmacokinetic study of salannin and azadirachtin in herbal oral antipsoriatic formulation.
5. 10. Isolation of constituents by column chromatography

5.10.1. Nimbin

Elution of the column with chloroform - methanol (99:1) afforded colourless crystals of 1, recrystallized from acetone, 58 mg (0.06 % yield), Rf 0.3 (chloroform - methanol, 19:1). m.p: 203-205 °C; UV \( \lambda_{\text{max}} \) (MeOH): 213, 230 nm; IR \( \nu_{\text{max}} \) (KBr): 2952, 2845, 1735, 1721, 1436, 1376, 1239, 1155, 1052, 872 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 7.41 (1H, d, \( J = 2.4 \) Hz, H-21), 7.38 (1H, d, \( J = 1.5 \) Hz, H-23), 6.40 (1H, dd, \( J = 1.5, 2.4 \) Hz, H-22), 5.89 (1H, d, \( J = 10.2 \) Hz, H-3), 5.55 (1H, d, \( J = 10.2 \) Hz, H-2), 4.95 (1H, dd, \( J = 6.3, 4.5 \) Hz, H-15a), 4.82 (1H, dd, \( J = 6.0, 5.1 \) Hz, H-6β), 3.69 (1H, d, \( J = 5.1 \) Hz, H-7β), 3.62 (3H, brs, COOCH\(_3\)), 3.25 (3H, brs, COOCH\(_3\)), 2.93 (1H, d, \( J = 8.1, 6.3 \) Hz, H-17β), 2.25 (1H, d, \( J = 6.0 \) Hz, H-5α), 2.11 (1H, dd, \( J = 4.5, 5.7 \) Hz, H-9α), 1.95 (1H, dd, \( J = 5.7, 1.8 \) Hz, H2-11a), 2.09 (1H, dd, \( J = 4.5, 3.0 \) Hz, H2-11b), 1.92 (3H, brs, COOCH\(_3\)), 1.67 (3H, brs, Me-18), 1.34 (1H, dd, \( J = 12.9, 6.3 \) Hz, H2-16a), 1.24 (1H, dd, \( J = 7.5, 8.1 \) Hz, H2-16 b), 1.27 (3H, brs, Me-29), 1.15 (3H, brs, Me-19), 0.97 (3H, brs, Me-30); \(^{13}\)C NMR (CDCl\(_3\)): Table 5.29 +ve ESI MS \( m/z \) (rel. int.): 542 [M + H]\(^+\) (C\(_{30}\)H\(_{38}\)O\(_9\)) (52.8).

5.10.2. 1,2-Dioleoyl glyceryl Phosphate

Elution of the column with chloroform : methanol (49:1) furnished pale yellow crystals of 2, recrystallized from chloroform - methanol (1:1), 206 mg (0.23 % yield); Rf 0.4 (chloroform - methanol, 7:3); m.p. 90-92 °C; UV \( \lambda_{\text{max}} \) (MeOH): 220 nm (log \( \varepsilon \) 4.2); IR \( \nu_{\text{max}} \) (KBr): 3452, 2932, 2848, 1735, 1635, 1438, 1377, 1257, 1159, 1050, 872 cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 5.31 (2H, m, H-9', H-9''), 5.01 (2H, m, H-10', H-10''), 4.15 (1H, m, H-2), 3.67 (1H, d, \( J = 11.1 \) Hz, H2-1a), 3.62 (1H, d, \( J = 11.1 \) Hz, H2-1b), 3.62 (1H, d, \( J = 7.2 \) Hz, H2-3a), 3.59 (1H, d, \( J = 7.2 \) Hz, H2-3b), 2.31 (2H, m, H2-2'), 2.19 (2H, m, H2-2''), 2.02 (2H, m, H2-8'), 1.97 (2H, m, H2-8''), 1.93 (2H, m, H2-11'), 1.84 (2H, m, H2-11''), 1.65 (2H, m, CH2), 1.57 (2H, m, CH2), 1.36 (4H, m, 2 x CH2), 1.30 (16H, brs, 8 x CH2), 1.26 (14H, brs, 7 x CH2), 1.21 (4H, brs, 2 x CH2), 1.17 (2H, brs, CH2), 1.01 (3H, t, \( J = 5.7 \) Hz, Me-18'), 0.86 (3H, t, \( J = 6.1 \) Hz, Me-18''); \(^{13}\)C NMR (MeOD): 8170.37 (C-1', C-1''), 137.74 (C-9', C-9''), 129.03 (C-10', C-10''), 82.49 (C-3), 69.98 (C-1), 52.52 (C-2'), 52.50 (C-2''), 44.02 (CH3), 42.56 (CH2), 39.72 (CH2), 39.70 (CH2), 39.68 (CH2), 37.58 (CH2), 33.95 (2 x CH2), 29.49 (2 x CH2), 28.99 (5 x CH2), 26.93 (2 x CH2), 24.72 (CH2), 20.87 (4 x CH2), 16.71
Compound 1 was obtained as colourless crystals from chloroform-methanol (99:1) eluents. It responded positively to limonoid tests. The UV spectrum exhibited an absorption maximum at 213, 230 nm and the IR spectrum showed absorption bands at 1735 cm⁻¹ (cabomethoxy), 1671-1721 cm⁻¹ (carbonyls of αβ-unsaturated ketone and ester), 1376 cm⁻¹ (geminal methyls), β-substituted furan at 1510 and 872 cm⁻¹. It has 4 degrees of double bond equivalents, two of them in furan ring. The ¹H NMR data were indicative of the limonoid nature of 1 with the presence of five quaternary methyl singlets at δ 0.97, 1.15, 1.24, 1.67, and 1.95. A pair of doublets at δ 5.89 (J = 10.2 Hz, H-2, H-3) and 6.40 (1H, dd, J = 1.5, 2.4 Hz, H-22) could be assigned to the olefinic protons, δ 3.62 (3H, brs, COOCH₃) and δ 3.25 (3H, brs, COOCH₃) accounted for dimethyl ester. The ¹H-NMR spectrum further showed the presence of signals at δ 4.82 (1H, dd, J = 6.0, 5.1 Hz), 3.69 (1H, d, J = 5.1 Hz) and 4.95 (1H, dd, J = 6.3, 4.5 Hz) attributable to H-6β, H-7β and H-15α, respectively. It showed two proton doublet at δ 7.41 and 7.38 assigned to vinylic protons H-21, H-22, respectively. A three proton broad singlet δ 1.92 was attributed to acetyl proton. The chemical shift and coupling constant showed that C-6 carries acetate moiety while C-4 and C-11 have carbomethoxy moiety. The ¹³C NMR spectrum of 1 showed important signal for acetyl carbons at δ 46.08 and 27.56, vinylic carbons at δ 129.02 (C-2), 146.53 (C-3), 125.93 (C-20), 142.91 (C-21), 110.58 (C-22) and 138.77 (C-23) and methene carbons at 71.31 (C-6), 39.93 (C-9), 87.91 (C-15) and 49.40 (C-17) (Table 5.29). On the basis of ESI MS and ¹³C NMR spectra the molecular weight of 1 was established at m/z 542 consistent of a molecular formula of C₃₀H₃₈O₉. On the basis of above discussion the structure of compound 1 was identified as (4a,5a,6a,7a,15b,17a)-6-(Acetyloxy)-7,15:21,23-diepoxy-4,8-dimethyl-1-oxo-18,24-dinor-11,12-secochola-2,13,20,22-tetraene-4,11-dicarboxylic acid dimethyl ester. This is a known limonoid reported as nimbin (Sengupta et al., 1960; Harris et al., 1968).
Compound 2 was obtained as pale yellow crystals from chloroform-methanol (1:1) eluants. It produced effervescence with Sodium bicarbonate solution. Its IR Spectrum exhibited characteristic absorption bands for hydroxyl groups at (3452 cm⁻¹), carboxylic ester groups (1735 cm⁻¹), unsaturation (1635 cm⁻¹) and aliphatic chain (1159 cm⁻¹). The mass spectrum of 2 showed a molecular ion peak at \( m/z \) 718 [M + H₂O]⁺ corresponding to a molecular formula of a fatty acid (C₃₉H₇₃O₈P.H₂O). It indicated two double bond equivalents; each of them was adjusted in the vinylic linkage. The H NMR spectrum of 2 showed two proton multiplet at δ 5.31 and δ 5.01 assigned to vinylic H-9, H-9' and H-10, H-10' respectively. A three proton triplet at δ 1.01 (J = 5.7 Hz) and δ 0.36 (J = 6.1 Hz) accounted for methyl protons. The ¹³C NMR spectrum of 2 displayed signals for carboxylic ester carbons at δ 170.37 (C-1', C-1''), vinylic carbons at δ 137.74 (C-9', C-9'') and δ 129.03 (C-10', C-10''), methyl carbons at δ 16.7 (C-18') and 14.3 (C-18'') respectively. On the basis of spectral data analysis and chemical reactions the structure of 2 has been identified as 1,2-dioleoyl glyceryl phosphate.

![Figure 5.26 Chemical structure of Nimbin isolated from Nimbatiktam](image)

**Figure 5.26 Chemical structure of Nimbin isolated from Nimbatiktam**

Jamia Hamdard

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Table 5.29 $^{13}$C NMR data of compound 1 isolated from Nimbatiktam by column chromatography

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta c$</th>
<th>Position</th>
<th>$\delta c$</th>
<th>Position</th>
<th>$\delta c$</th>
<th>Position</th>
<th>$\delta c$</th>
<th>$\delta c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>201.35</td>
<td>8</td>
<td>47.95</td>
<td>15</td>
<td>87.91</td>
<td>22</td>
<td>110.58</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>129.02</td>
<td>9</td>
<td>39.93</td>
<td>16</td>
<td>34.21</td>
<td>23</td>
<td>138.77</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>146.53</td>
<td>10</td>
<td>42.68</td>
<td>17</td>
<td>49.40</td>
<td>28</td>
<td>173.64</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>46.08</td>
<td>11</td>
<td>27.56</td>
<td>18</td>
<td>16.87</td>
<td>29</td>
<td>15.08</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>49.05</td>
<td>12</td>
<td>172.76</td>
<td>19</td>
<td>19.57</td>
<td>Ac</td>
<td>170.13, 21.25</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>71.31</td>
<td>13</td>
<td>134.87</td>
<td>20</td>
<td>125.93</td>
<td>OCH$_3$</td>
<td>51.45</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>72.59</td>
<td>14</td>
<td>156.72</td>
<td>21</td>
<td>142.91</td>
<td>OCH$_3$</td>
<td>53.04</td>
<td></td>
</tr>
</tbody>
</table>
5.1. Quality control and standardization of Lajjalu Keram and 777 oil

Physicochemical evaluation such as organoleptic characters, rancidity, density, refractive index, viscosity, acid value, saponification value and ester value was done by the same procedure as mentioned in experiment section 4.1 (A). Each evaluation was carried out in three batches and results are summarized as follows (Table 5.30 and Table 5.31).

Table 5.30 Summary of physicochemical standardization of Lajjalu Keram

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>Batch I</th>
<th>Batch II</th>
<th>Batch III</th>
<th>Mean + S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Colour</td>
<td>Greenish</td>
<td>Greenish</td>
<td>Greenish</td>
<td>Greenish</td>
</tr>
<tr>
<td>2.</td>
<td>Odour</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
</tr>
<tr>
<td>3.</td>
<td>Taste</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
</tr>
<tr>
<td>4.</td>
<td>Rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
</tr>
<tr>
<td>5.</td>
<td>Density (g/mL)</td>
<td>0.913</td>
<td>0.922</td>
<td>0.915</td>
<td>0.917 ±0.005</td>
</tr>
<tr>
<td>6.</td>
<td>Refractive index</td>
<td>1.4525</td>
<td>1.4535</td>
<td>1.4550</td>
<td>1.454 ±0.001</td>
</tr>
<tr>
<td>7.</td>
<td>Viscosity (cps)</td>
<td>56.19</td>
<td>55.36</td>
<td>54.25</td>
<td>55.267 ±0.973</td>
</tr>
<tr>
<td>8.</td>
<td>Acid value</td>
<td>1.26</td>
<td>1.32</td>
<td>1.22</td>
<td>1.267 ±0.050</td>
</tr>
<tr>
<td>9.</td>
<td>Saponification value</td>
<td>30.26</td>
<td>27.89</td>
<td>32.36</td>
<td>30.170 ±2.236</td>
</tr>
</tbody>
</table>

Table 5.31 Summary of physicochemical standardization of 777 Oil

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter</th>
<th>Batch I</th>
<th>Batch II</th>
<th>Batch III</th>
<th>Mean + S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Colour</td>
<td>Magenta</td>
<td>Magenta</td>
<td>Magenta</td>
<td>Magenta</td>
</tr>
<tr>
<td>2.</td>
<td>Odour</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
</tr>
<tr>
<td>3.</td>
<td>Taste</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
</tr>
<tr>
<td>4.</td>
<td>Rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
<td>No rancidity</td>
</tr>
<tr>
<td>5.</td>
<td>Density (g/mL)</td>
<td>0.901 gm/cc</td>
<td>1.025</td>
<td>0.928</td>
<td>0.951 ±0.065</td>
</tr>
<tr>
<td>6.</td>
<td>Refractive index</td>
<td>1.450</td>
<td>1.368</td>
<td>1.390</td>
<td>1.403 ±0.042</td>
</tr>
<tr>
<td>7.</td>
<td>Viscosity (cps)</td>
<td>45.71</td>
<td>50.35</td>
<td>51.48</td>
<td>49.18 ±3.058</td>
</tr>
<tr>
<td>8.</td>
<td>Acid value</td>
<td>16.71</td>
<td>19.56</td>
<td>18.45</td>
<td>18.24 ±1.437</td>
</tr>
<tr>
<td>9.</td>
<td>Saponification value</td>
<td>261.28</td>
<td>258.36</td>
<td>260.25</td>
<td>259.96 ±1.481</td>
</tr>
<tr>
<td>10.</td>
<td>Ester value</td>
<td>244.56</td>
<td>238.80</td>
<td>242.80</td>
<td>242.05 ±2.952</td>
</tr>
</tbody>
</table>

Discussion

The results of physicochemical standardization of Lajjalu Keram and 777 oil can be used for quality control of these two formulations.
5.2. Phytochemical investigations of Lajjalu Keram and 777 oil

Phytochemical investigations of Lajjalu Keram and 777 oil were performed by chemical tests including tests for alkaloids, glycosides, tannins, sugar & carbohydrates, saponins, proteins & amino acids, resins, lipids/fats, phenolic compounds and for flavonoids. Results of Phytochemical analysis of Lajjalu Keram and 777 oil are are given in Table 5.32 and 5.33 respectively.

Table 5.32 Phytochemical constituents of Lajjalu Keram

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Batch I</th>
<th>Batch II</th>
<th>Batch III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Dragedorf's test</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Mayer's test</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>iii.</td>
<td>Tannic acid test</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>2.</td>
<td>Glycosides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Killer Killiani test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ii.</td>
<td>Born trager test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Tannis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Molish Test</td>
<td>+++</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Fehling solution test</td>
<td>--</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iii.</td>
<td>Barfoed test</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Saponnis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Foam test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ii.</td>
<td>Haemolysis test</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Proteins and amino acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Ninhydrin test</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ii.</td>
<td>Xanthoproteic test</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iii.</td>
<td>Millon's test</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Resins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>Lipid/fat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Staining test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>9.</td>
<td>Phenolic compounds</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Ferric chloride test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Lead acetate test</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>10.</td>
<td>Flavonoids</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Batch I</th>
<th>Batch II</th>
<th>Batch III</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>i.</td>
<td>Dragedorf's test</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Mayer's test</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>iii.</td>
<td>Tannic acid test</td>
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<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>2.</td>
<td>Glycosides</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Killer Killiani test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ii.</td>
<td>Born trager test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Tannis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Molish Test</td>
<td>+++</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Fehling solution test</td>
<td>--</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iii.</td>
<td>Barfoed test</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
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<td>5.</td>
<td>Saponnis</td>
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<td></td>
<td></td>
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<tr>
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<td>Foam test</td>
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</tr>
<tr>
<td>ii.</td>
<td>Haemolysis test</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Proteins and amino acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.</td>
<td>Ninhydrin test</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ii.</td>
<td>Xanthoproteic test</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>iii.</td>
<td>Millon's test</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
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<td>7.</td>
<td>Resins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>Lipid/fat</td>
<td></td>
<td></td>
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<tr>
<td>i.</td>
<td>Staining test</td>
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<td>++</td>
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<td>++</td>
</tr>
<tr>
<td>ii.</td>
<td>Lead acetate test</td>
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<td>++</td>
<td>++</td>
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<tr>
<td>10.</td>
<td>Flavonoids</td>
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### Table 5.33 Phytoclimical constituents of 777 oil

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
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<th>Batch II</th>
<th>Batch III</th>
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<tr>
<td>1.</td>
<td>Alkaloids</td>
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<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>ii. Mayer’s test</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>iii. Tannic acid test</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>2.</td>
<td>Glycosides</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>i. Killer killiani test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>ii. Born trager test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
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<td>Tannins</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride test</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Lead acetate test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4.</td>
<td>Carbohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i. Molish test</td>
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<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>ii. Fehling solution test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>iii. Barfoed test</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
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<td>Saponins</td>
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<tr>
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<td>i. Foam test</td>
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<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>ii. Haemolysis test</td>
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<td></td>
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</tr>
<tr>
<td>6.</td>
<td>Proteins and amino acid</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>i. Ninhydrin test</td>
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<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ii. Xanthoproteic test</td>
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<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>iii. Millon’s test</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Resins</td>
<td>--</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>8.</td>
<td>Lipid/fat</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>i Staining test</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>9.</td>
<td>Phenolic compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>i. Ferric Chloride test</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ii. Lead acetate test</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>10.</td>
<td>Flavonoids</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Discussion**

The chemical tests revealed that the presence of alkaloids, saponin, lipids, phenolics and flavonoidal compounds in both Lajgalu Keram and 777 oil. Tannins and carbohydrate was found to be present only in Lajgalu Keram while glycosides and resins were absent in both of the formulations.
5.3. Determination of microbial contamination of Lajjalu Keram and 777 oil

**Total fungal count**

Potato dextrose agar medium was used for total fungal count, which on incubation at 37 °C for 48 h with Lajjalu Keram in 1:10 and 1:100 dilution showed 2.8 and 14.0 \( \times 10^3 \) CFU/g, respectively (Table 5.34 and Figure 5.28). The 777 oil on similar treatment didn’t showed any CFU/g and whole of sample was found sterile.

![Figure 5.28 Potato Dextrose Agar plates showing total fungal counts of Lajjalu Keram and 777 oil](image)

**Calculation**

Total fungal count = No. of CFU x Dilution factor/ Wt. of the sample

**Table 5.34 Total fungal count of Lajjalu Keram and 777 oil after 48 h at 25 °C in BOD incubator**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Dilution factor</th>
<th>Drug</th>
<th>No. of Colonies</th>
<th>Total fungal count/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Lajjalu Keram</td>
<td>28</td>
<td>2800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>Lajjalu Keram</td>
<td>14</td>
<td>1400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>NIL</td>
<td>NIL</td>
</tr>
</tbody>
</table>
Total bacterial count

Lajjalu Keram and 777 oil on 18 h incubation in BOD at 37 °C in casein soyabean agar media didn’t show any colony in 1:10 and 1:100 dilutions as evident from Fig 5.29 and Table 5.35.

![Figure 5.29 Soyabean casein agar plates showing total bacterial count of Lajjalu Keram and 777 oil](image)

Calculation

Total bacterial count = No. of CFU x Dilution factor/ Wt of the sample

Table 5.35 Total bacterial count of Lajjalu Keram and 777 oil after 18 h at 37 °C in BOD incubator

<table>
<thead>
<tr>
<th>S.No</th>
<th>Dilution factor</th>
<th>Drug</th>
<th>No. of Colonies</th>
<th>Total fungal count/gm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Lajjalu Keram</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>Lajjalu Keram</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>NIL</td>
<td>NIL</td>
</tr>
</tbody>
</table>

Discussion

In this study absence of total bacterial count has been observed in both Lajjalu Keram and 777 oil. However there was presence of colonies forming units (CFU) in Lajjalu Keram in the diluted solution (1:10; 1:100) of Lajjalu Keram incubated with the fungus media. 777 oil didn’t showed any fungal growth.
5.4. Chemical toxicity evaluation of Lajjalu Keram and 777 oil

5.4.1. Analysis of heavy metals in Lajjalu Keram and 777 oil using Atomic Absorption Spectroscopy (AAS)

The heavy metals contents in Lajjalu Keram and 777 oil was analysed using the method as described for Nimbatiktam 4.2.1 (A) and the calibration plots and regression equation used were as per the Nimbatiktam. The content of heavy metals of all batches of Lajjalu Keram and 777 oil are summarised in Table 5.36.

Table 5.36 Summary of presence of heavy metal contents in Lajjalu Keram and 777 oil (AAS)

<table>
<thead>
<tr>
<th>S. No</th>
<th>Heavy Metals</th>
<th>Equation of calibration</th>
<th>Drug</th>
<th>B-1 (n=3)</th>
<th>B-2 (n=3)</th>
<th>B-3 (n=3)</th>
<th>Mean ± SD</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cadmium (ppm)</td>
<td>( y = 0.327x + 0.040 ) (( r^2=0.985 ))</td>
<td>LK*</td>
<td>0.0541</td>
<td>0.0543</td>
<td>0.0539</td>
<td>0.054</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 'O'**</td>
<td>0.0541</td>
<td>0.0547</td>
<td>0.0543</td>
<td>0.054</td>
<td>0.044</td>
</tr>
<tr>
<td>2</td>
<td>Lead (ppm)</td>
<td>( y = 0.012x + 0.017 ) (( r^2=0.999 ))</td>
<td>LK*</td>
<td>0.017</td>
<td>0.017</td>
<td>0.017</td>
<td>0.017</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 'O'**</td>
<td>0.019</td>
<td>0.016</td>
<td>0.018</td>
<td>0.017</td>
<td>0.056</td>
</tr>
<tr>
<td>3</td>
<td>Arsenic (ppb)</td>
<td>( y = 0.007x + 0.053 ) (( r^2=0.996 ))</td>
<td>LK*</td>
<td>0.105</td>
<td>0.104</td>
<td>0.105</td>
<td>0.105</td>
<td>7.392</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 'O'**</td>
<td>0.159</td>
<td>0.160</td>
<td>0.157</td>
<td>0.159</td>
<td>15.095</td>
</tr>
<tr>
<td>4</td>
<td>Mercury (ppb)</td>
<td>( y = 0.022x + 0.176 ) (( r^2=0.984 ))</td>
<td>LK*</td>
<td>0.174</td>
<td>0.176</td>
<td>0.177</td>
<td>0.176</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 'O'**</td>
<td>0.173</td>
<td>0.178</td>
<td>0.177</td>
<td>0.176</td>
<td>0.000</td>
</tr>
</tbody>
</table>

*LK = Lajjalu Keram; **7 'O' = 777 oil;

Discussion

The study revealed that the formulations contained little high levels of cadmium as compared to lead and arsenic, whereas mercury was found nil in Lajjalu Keram and negligible in 777 oil.

An excessive level of Cd in the body has been shown to result in kidney and liver damages as well as deformation of bone structures (Abbas et al., 2008). These manifestations of toxicity can only be detected if renal cadmium concentration of more than 50 µg/g tissue. Since in these formulations as the concentrations of cadmium are very less so it can be concluded that the drug is safe for use.

The target organs for lead toxicity are kidney and nervous system. The Food and Agricultural Organization/World Health Organization (1993) has established a PTWI (Provisional Tolerable Weekly Intake) of 25 µg lead/kg body weight for humans. The
mean blood lead level intake of adults is in the range of 20-514 μg/day. The formulations tested here have very less concentrations of lead is too less to reach the toxic level of blood concentration.

5.4.2. Analysis of aflatoxins in Lajjalu Keram and 777 oil using HPLC

The analyses of aflatoxins (AF) in Lajjalu Keram and 777 oil were done by method of AOAC as described in Nimbatiktam 4.2.2 (A). All data were obtained without any interference in the analysis. Figure 5.30 and Figure 5.31 shows the HPLC chromatograms of aflatoxins extracted from Lajjalu Keram and 777 oil, respectively using same method as for standard aflatoxin analysis. All four aflatoxin (B1, B2, G1 and G2) has been observed in Lajjalu Keram while in 777 oil only two (G1 and G2) has been detected. The aflatoxins in both formulations were found in the permissible limit (Table 5.37).

Figure 5.30 HPLC chromatogram of Lajjalu Keram showing very small amount of aflatoxins in fluorescence detector at 365 nm (excitation) and 425 nm (emission)

Figure 5.31 HPLC chromatogram of 777 oil showing absence of aflatoxins in fluorescence detector at 365 nm (excitation) and 425 nm (emission)
Table 5.37 Summary of test of aflatoxin in Lajjalu Keram and 777 oil using HPLC

<table>
<thead>
<tr>
<th>S. No</th>
<th>Aflatoxin</th>
<th>Detection Limit</th>
<th>Formulations</th>
<th>Results of three batches</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B1</td>
<td>0.3 ppb</td>
<td>Lajjalu Keram</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>777 oil</td>
<td>Not Detected</td>
</tr>
<tr>
<td>2</td>
<td>B2</td>
<td>0.3 ppb</td>
<td>Lajjalu Keram</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>777 oil</td>
<td>Not Detected</td>
</tr>
<tr>
<td>3</td>
<td>G1</td>
<td>0.3 ppb</td>
<td>Lajjalu Keram</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>777 oil</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td>4</td>
<td>G2</td>
<td>0.3 ppb</td>
<td>Lajjalu Keram</td>
<td>Less than 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>777 oil</td>
<td>Less than 0.3</td>
</tr>
</tbody>
</table>

In the present investigation different types of aflatoxins (B1, B2, G1 and G2) were determined by the method of AOAC after using a suitable clean-up procedure. Results indicated all four aflatoxins in all three batches of both the formulations were found within the permissible limit.

5.4.3. Results of pesticides determination of Lajjalu Keram and 777 oil

The analysis of pesticides in Lajjalu Keram and 777 oil was done as per the method (Weaver et al., 2010) as discussed in subsection 4.2.3 (A) of the retention times of the pesticides were measured using individual standard solutions at concentrations of 5.0 µg mL$^{-1}$. The GC-MS instrument was operated in full scan mode, varying the oven temperature and the carrier gas flow rate. The most representative (most intense) ions were selected for quantification of the pesticides in the Lajjalu Keram and 777 oil. Results indicated the absence of all types of pesticides in both formulations as mentioned below (Table 5.38).
Table 5.38 Residual pesticides in Lajjalu Keram and 777 oil by GC/MS using AOAC method

<table>
<thead>
<tr>
<th>Type</th>
<th>S. No</th>
<th>Parameters</th>
<th>Results in all batches</th>
<th>Detection Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lajjalu Keram</td>
<td>777 oil</td>
</tr>
<tr>
<td>Organochlorides</td>
<td>31.</td>
<td>4-4-DDE</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>32.</td>
<td>2,4-DDD</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>33.</td>
<td>2,4-DDT</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>34.</td>
<td>4,4-DDD</td>
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<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>35.</td>
<td>4,4-DDT</td>
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<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>36.</td>
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<td>Not Detected</td>
</tr>
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<td></td>
<td>37.</td>
<td>Aldrin</td>
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<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>38.</td>
<td>Dieldrin</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>39.</td>
<td>2,4-DDE</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
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<td>Not Detected</td>
</tr>
<tr>
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<td>41.</td>
<td>Heptachlor Epoxide Isom B</td>
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<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>42.</td>
<td>Alpha-HCH</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>43.</td>
<td>Beta-HCH</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>44.</td>
<td>Lindane</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>45.</td>
<td>Delta BHC</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>46.</td>
<td>2,4-D</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>47.</td>
<td>Dichlorvos</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>48.</td>
<td>Chlorpyriphos</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>49.</td>
<td>Butachlor</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>50.</td>
<td>Malathion</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>51.</td>
<td>Malathion</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>52.</td>
<td>Methyl Parathion</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>53.</td>
<td>Endosulfan I</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>54.</td>
<td>Endosulfan II</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>55.</td>
<td>Ethion</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>56.</td>
<td>Monocrotophos</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>57.</td>
<td>Endosulfan Sul</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>58.</td>
<td>Diazinon</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>59.</td>
<td>Deltamethrin</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
<tr>
<td></td>
<td>60.</td>
<td>Phosalone</td>
<td>Not Detected</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>
5.5. Acute dermal toxicity studies Lajjalu Keram and 777 oil on intact skin of male Wistar rat

The acute dermal toxicity of Lajjalu Keram and 777 oil was carried out as per OECD guidelines (OECD, 402) described in subsection 4.3 (B).

Dermal toxicity study of Lajjalu Keram and 777 oil with 1000, 2000 and 5000 mg/kg body weight was conducted in male and female Wistar rats. The cage side observation was made on each day (upto 14th day) same time after single dermal application of formulations. Several parameters like skin swelling, fur, eyes, colour of faeces, condition of teeth, breathing abnormalities was observed which were found normal in each group of treatment of Lajjalu Keram and 777 oil (Table 5.39).

Table 5.39 Cage side observations for all animals subjected to acute dermal toxicity studies on Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>Cage side observations</th>
<th>Lajjalu Keram</th>
<th>777 oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Condition of the fur</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>2.</td>
<td>Skin</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>3.</td>
<td>Subcutaneous swellings</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>4.</td>
<td>Abdominal distension</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>5.</td>
<td>Eyes – dullness</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>6.</td>
<td>Eyes – opacities</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>7.</td>
<td>Pupil diameter</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>8.</td>
<td>Color and consistency of the faeces</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>9.</td>
<td>Condition of teeth</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>10.</td>
<td>Breathing abnormalities</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>11.</td>
<td>Gait</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

The mortality record of dermal application of both the drugs did not show any mortality upto 14th day (Table 5.40).
Table 5.40 Mortality record of animals of various doses group treated with Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>Days</th>
<th>1000 mg/kg BW</th>
<th>2000 mg/kg BW</th>
<th>5000 mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>LK*</td>
<td>'7' O**</td>
<td>LK*</td>
</tr>
<tr>
<td>1st hour</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4th hour</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16th hour</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24th hour</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mortality: 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5

*LK=Lajjalu Keram; **'7'O=777 oil

The body weight records of control group male and female rats was observed on 0, 7th and 14th days which didn’t show any significant variation among animals (Table 5.41).

Table 5.41 Body weight records of control animals of male and female rats for two weeks

<table>
<thead>
<tr>
<th>Dose</th>
<th>Control Male (g)</th>
<th>Control Female (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>1    2    3    4    5</td>
<td>1    2    3    4    5</td>
</tr>
<tr>
<td>Day 0</td>
<td>165  160 165 170 165</td>
<td>175 180 180 175 180</td>
</tr>
<tr>
<td>Day 7</td>
<td>170 165 170 175 170</td>
<td>180 185 185 180 180</td>
</tr>
<tr>
<td>Day 14</td>
<td>175 170 170 170 175</td>
<td>180 185 190 185 180</td>
</tr>
</tbody>
</table>

Body weight records of male and female rats treated with 1000 mg/kg BW of Lajjalu Keram and 777 oil (single dermal application on first day) didn’t show any significant variation as evident from Table 5.42.
Table 5.42 Body weight records of male and female rats treated with dermal application of Lajjalu Keram and 777 oil (1000 mg/kg BW) for two weeks

<table>
<thead>
<tr>
<th>Dose</th>
<th>1000 mg/kg Body weight (Male)</th>
<th>1000 mg/kg Body weight (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>1</td>
</tr>
<tr>
<td>Day 0</td>
<td>Lajjalu Keram</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>165</td>
</tr>
<tr>
<td>Day 7</td>
<td>Lajjalu Keram</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>170</td>
</tr>
<tr>
<td>Day 14</td>
<td>Lajjalu Keram</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>175</td>
</tr>
</tbody>
</table>

Body weight records of male and female rats treated with 2000 mg/kg BW of Lajjalu Keram and 777 oil (single dermal application on first day) also didn’t show any significant variation as evident from Table 5.43.

Table 5.43 Body weight records of male and female rats treated with dermal application of Lajjalu Keram and 777 oil (2000 mg/kg BW) for two weeks

<table>
<thead>
<tr>
<th>Dose</th>
<th>2000 mg/kg Body weight (Male)</th>
<th>2000 mg/kg Body weight (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>1</td>
</tr>
<tr>
<td>Day 0</td>
<td>Lajjalu Keram</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>168</td>
</tr>
<tr>
<td>Day 7</td>
<td>Lajjalu Keram</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>172</td>
</tr>
<tr>
<td>Day 14</td>
<td>Lajjalu Keram</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>165</td>
</tr>
</tbody>
</table>

Body weight records of male and female rats treated with high amount of acute dose i.e. 5000 mg/kg BW of Lajjalu Keram and 777 oil (single dermal application on first day) also didn’t show any significant variation as evident from Table 5.44.

Table 5.44 Body weight records of male and female rats treated with dermal application of Lajjalu Keram and 777 oil (5000 mg/kg BW) for two weeks

<table>
<thead>
<tr>
<th>Dose</th>
<th>5000 mg/kg Body weight (Male)</th>
<th>5000 mg/kg Body weight (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>1</td>
</tr>
<tr>
<td>Day 0</td>
<td>Lajjalu Keram</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>165</td>
</tr>
<tr>
<td>Day 7</td>
<td>Lajjalu Keram</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>170</td>
</tr>
<tr>
<td>Day 14</td>
<td>Lajjalu Keram</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>175</td>
</tr>
</tbody>
</table>

The visual toxicity/abnormal behaviour of male and female rats were carried out every day at same time in control group and treated groups which didn’t showed any toxicity or abnormal behaviour upto the observation period (Table 5.45)
Table 5.45 Determination of visual toxicity/abnormal behaviour of animals of various doses group treated with Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>Group (treatment)</th>
<th>Drug</th>
<th>Days (n*/s)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Control</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>Lajjalu Keram</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>Lajjalu Keram</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>Lajjalu Keram</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

* Number of animals showing toxicity signs / Number of animals dosed

The histopathological observations of skin part on which the drug was applied were carried out after removing it on 14th day and observing their sections at 100X and 400X. The photomicrographs of dermis of control group were similar to those of treated group and they didn't show any abnormality in sections (Table 5.46), same is evident from Figure 5.32.

Table 5.46 Histopathological observation of skins of all the groups treated for dermal toxicity study of Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation</th>
<th>Lesion in skin membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>I</td>
<td>Control</td>
<td>NAD*</td>
</tr>
<tr>
<td>II</td>
<td>Lajjalu Keram</td>
<td>NAD*</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>NAD*</td>
</tr>
<tr>
<td>III</td>
<td>Lajjalu Keram</td>
<td>NAD*</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>NAD*</td>
</tr>
<tr>
<td>IV</td>
<td>Lajjalu Keram</td>
<td>NAD*</td>
</tr>
<tr>
<td></td>
<td>777 oil</td>
<td>NAD*</td>
</tr>
</tbody>
</table>

* Nothing Abnormal Detected
Histopathological observations of dermal toxicity studies of Lajjalu Keram and 777 oil

Histopathological examination of skin tissues showed normal histological appearance of epidermis and dermis in all treatment groups including control (Figure 5.32).

Figure 5.32 Photomicrograph of sections of skin of different groups showing a normal histological appearance of epidermis and dermis at low resolution (A, 100X) and high resolution (B, 400X)

A. Control female group (100X); B. Control female group (400X); C. Control male group (100X); D. Control male group (400X); E. Female rat group treated with Lajjalu Keram (100X); F. Female rat group treated with Lajjalu Keram (400X); G. Male rat group treated with Lajjalu Keram (100X); H. Male rat group treated with Lajjalu Keram (400X); I. Female rat group treated with 777 oil (100X); J. Female rat group treated with 777 oil (400X); K. Male rat group treated with 777 oil (100X); L. Male rat group treated with 777 oil (400X)
Chapter 5

Result and Discussion

There was no mortality observed in the treated group and control group of animals. Similarly no visible signs of toxicity, such as changes in respiration, circulatory, autonomic and central nervous system, behavioural pattern were observed in study. Physical and behavioural activity was also found normal in all the animals. No reaction was observed on the test substance applied area of the skin. Similarly, no signs and the toxicity were observed in control animals. Compared with control, animals belonging to the test substance treated group (both sexes) did not show a significance change in body weight gain on day 7 and 14. Necropsy examination did not reveal any abnormal lesion in any group.

From the results observed in the study no mortality was observed in the study under the conditions of test. It was concluded that the dermal LD50 of Lajjalu Keram and 777 oil for wistar rats are more than 5000 mg/kg. Based on acute toxicity studies, both the formulations were well tolerated upto 5000 mg/Kg single application.
5.6. Estimation of constituents of Lajjalu Keram and 777 oil

5.6.1. Determination of total flavonoid content in Lajjalu Keram and 777 oil using UV spectrophotometer

Total flavonoid contents in Lajjalu Keram and 777 oil was determined by slightly modified method reported by Qayyoom et al., 2009 as discussed in subsection 4.4 (B) experimental. The total flavonoid contents in the three batches of both of formulations were calculated from the standard plot (Figure 5.14) and the results documented in Table 5.47.

Table 5.47 Result of total flavonoid content in Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>S. No</th>
<th>Batches</th>
<th>Mean of flavonoidal content (µg/g)(n=3)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lajjalu Keram</td>
<td>777 oil</td>
</tr>
<tr>
<td>1.</td>
<td>Batch 1</td>
<td>231.020</td>
<td>283.265</td>
</tr>
<tr>
<td>2.</td>
<td>Batch 2</td>
<td>230.612</td>
<td>282.857</td>
</tr>
<tr>
<td>3.</td>
<td>Batch 3</td>
<td>230.000</td>
<td>282.449</td>
</tr>
</tbody>
</table>

Discussion

The total flavonoids in Lajjalu Keram were found as 0.023 % w/w whereas in 777 oil, it was found as 0.282 %.

5.6.2. Determination of total phenolic content in Lajjalu Keram and 777 oil using UV spectrophotometer

Total Phenolic constituents of Lajjalu Keram and 777 oil were determined by Folin-Ciocalteu reagent in alkaline medium as described by Qayyoom et al., 2009. Different concentrations of gallic acid were prepared in 80% methanol and considered as standard. The absorbance of samples and standard was measured at 760 nm. A graph (concentration Vs absorbance) was plotted with known concentration of gallic acid standard (Figure 5.15). The total phenolic contents in Lajjalu Keram and 777 oil were calculated from the standard plot and the results are documented in Table 5.48.
Table 5.48 Result of total phenolic content in Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>S. No</th>
<th>Batches</th>
<th>Mean of phenolic content (µg/g) (n=3)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lajjalu Keram</td>
<td>777 oil</td>
</tr>
<tr>
<td>1</td>
<td>Batch 1</td>
<td>339.831</td>
<td>486.78</td>
</tr>
<tr>
<td>2</td>
<td>Batch 2</td>
<td>337.288</td>
<td>486.271</td>
</tr>
<tr>
<td>3</td>
<td>Batch 3</td>
<td>342.373</td>
<td>486.61</td>
</tr>
</tbody>
</table>

Discussion

The contents of total phenolics were found as 0.034 % w/w in Lajjalu Keram where as 0.048 % in 777 oil. Polyphenols due to their hydroxyl group, has shown strong scavenging ability (Hatano et al., 1989). It is also considered that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in human when ingested upto one gram daily (Patnayak et al., 2012). The inhibitory effects on mutagenesis can be correlated its effects on psoriatic patients.

In the present study the result showed that 777 oil was found to have comparatively higher amount of phenolic compounds than Lajjalu Keram. It indicates the better efficacy of 777 oils in psoriatic management. It also explains the comparatively better anti-inflammatory action of 777 oil than Lajjalu Keram which has been explained in the biological activity section of this drug.
5.6.3. Estimation of fatty acid constituents in Lajjalu Keram and 777 oil using GC/FID analysis

The GC/FID fingerprints for fatty acid composition of Lajjalu Keram and 777 oil were carried out by derivatizing it in the forms of fatty acid methyl esters (FAMES). There are thirteen fatty acids detected in Lajjalu Keram (Figure 5.33) while sixteen were detected in 777 oil (Figure 5.34). All fatty acids were confirmed by comparison with the standard of methyl esters of respecting fatty acids. Lajjalu Keram showed presence of thirteen fatty acids, out of which seven were saturated fatty acids (95.2%) and rest unsaturated fatty acids (3.27%) (Table 5.49). Among unsaturated fatty acid maximum amount was of three types of dienoic fatty acid (2.53%) (Table 5.50). 777 oil showed presence of sixteen fatty acids, out of which seven were saturated fatty acids (68.37%) and rest unsaturated fatty acids (3.27%) (Table 5.51). Among unsaturated fatty acid maximum amount of three types of dienoic fatty acid (24.05%) was found (Table 5.52) with Lajjalu Keram.

![Figure 5.33 GC-FID chromatogram showing different fatty acids and their contents in Lajjalu Keram](image-url)
Table 5.49 Composition of different type of fatty acid present in Lajjalu Keram

<table>
<thead>
<tr>
<th>Fatty Acid composition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturated fatty acid (7)</td>
<td>95.2%</td>
</tr>
<tr>
<td>Total unsaturated fatty acid (6)</td>
<td>3.27 %</td>
</tr>
<tr>
<td>Total fatty acid (13)</td>
<td>98.47%</td>
</tr>
</tbody>
</table>

Table 5.50 Composition of different type of unsaturated fatty acid present in Lajjalu Keram

<table>
<thead>
<tr>
<th>Type of unsaturated fatty acids</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoenoic fatty acid (1)</td>
<td>0.11%</td>
</tr>
<tr>
<td>Dienoic fatty acid (3)</td>
<td>2.53%</td>
</tr>
<tr>
<td>Pentaenoic fatty acid (1)</td>
<td>0.203</td>
</tr>
<tr>
<td>Hexaenoic Fatty acid (1)</td>
<td>0.383</td>
</tr>
</tbody>
</table>

Figure 5.34 GC-FID chromatogram showing different fatty acids and their contents in 777 oil

Table 5.51 Composition of different type of fatty acid present in 777 oil

<table>
<thead>
<tr>
<th>Fatty Acid composition</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturated fatty acid (7)</td>
<td>68.37 %</td>
</tr>
<tr>
<td>Total unsaturated fatty acid (9)</td>
<td>24.05 %</td>
</tr>
<tr>
<td>Total fatty acid (16)</td>
<td>92.42 %</td>
</tr>
</tbody>
</table>
Table 5.52 Composition of different type of unsaturated fatty acid present in 777 oil

<table>
<thead>
<tr>
<th>Type of unsaturated fatty acids</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoenoic fatty acid (3)</td>
<td>5.90 %</td>
</tr>
<tr>
<td>Dieneic fatty acid (3)</td>
<td>16.55 %</td>
</tr>
<tr>
<td>Trienoic fatty acid (1)</td>
<td>0.20 %</td>
</tr>
<tr>
<td>Pentaenoic fatty acid (1)</td>
<td>0.43 %</td>
</tr>
<tr>
<td>Hexaenoic Fatty acid (1)</td>
<td>0.97 %</td>
</tr>
</tbody>
</table>

Discussion

Fatty acids (FAs) occur commonly as esters of glycerols i.e. triacyl glycerols in natural fats of animal and plant origin. In case of psoriasis, increased concentrations of free arachidonic acid (AA) and its proinflammatory metabolites have been observed. Replacement of arachidonic acid by alternative precursor polyunsaturated fatty acids (PUFA), which can be metabolized via the same enzymatic pathways as AA, might be a therapeutic option in psoriasis. The presence of PUFA in the Lajjalu Keram and 777 oil correlates the rationale of use of Lajjalu Keram and 777 oil in management of psoriasis.
5.7. Analytical method development (HPLC quantification) of mimosine in Lajjalu Keram

*Optimization of chromatographic condition*

Various combinations of water, methanol and acetonitrile were tested for optimization of the chromatographic conditions. The buffers such as ammonia, phosphate and acetate at varying condition were also tested. The mobile phase water: orthophosphoric acid (98.8: 0.2 v/v); at 25 °C (column temperature) has given sharp, well defined peak of mimosine and drug as well as (Figure 5.35) with a low RT 2.61 ± 0.01 min.

![Figure 5.35 HPLC Chromatogram of mimosine (black) and sample (red) (RT 2.61 min, Conc 500 μg/mL λ= 284 nm); mobile phase water: orthophosphoric acid (98.8: 0.2 v/v)](image)

*Validation of the method*

The proposed method was validated as per ICH guidelines and similar methods as reported by laboratory (Baboota *et al.*, 2007; Alam *et al.*, 2009 and Karna! *et al.*, 2011).

*Linearity*

The proposed method was found linear in the rage of 5-500 μg/mL with regression equation $y = 4766.8x - 17726$ and regression coefficient $r^2 = 0.9998$ (Table 5.53).
Table 5.53 Calibration curve of mimosine by HPLC

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Mean area ± SD</th>
<th>RSD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>249967±5348.33</td>
<td>2.14</td>
<td>3087.95</td>
</tr>
<tr>
<td>10</td>
<td>486229±12850.29</td>
<td>2.64</td>
<td>7419.33</td>
</tr>
<tr>
<td>20</td>
<td>967968±10398.95</td>
<td>1.07</td>
<td>6004.02</td>
</tr>
<tr>
<td>40</td>
<td>1935225±33520.41</td>
<td>1.73</td>
<td>19353.59</td>
</tr>
<tr>
<td>50</td>
<td>2290557±33993.49</td>
<td>1.48</td>
<td>19626.73</td>
</tr>
<tr>
<td>100</td>
<td>4801614±67247.08</td>
<td>1.40</td>
<td>38826.26</td>
</tr>
<tr>
<td>200</td>
<td>9318811±48178.19</td>
<td>0.51</td>
<td>27816.51</td>
</tr>
<tr>
<td>300</td>
<td>14318753±88619.41</td>
<td>0.62</td>
<td>51165.94</td>
</tr>
<tr>
<td>500</td>
<td>23864591±202400.38</td>
<td>0.85</td>
<td>116859.34</td>
</tr>
</tbody>
</table>

Accuracy as Recovery

The proposed method afforded a good recovery of 99.24–101.73% after spiking the additional standard drug solution to the previously analyzed test solution (0, 50, 100 and 150%). The values of the recovery (%), % RSD and SEM have been shown in Table 5.54, which indicated the accuracy of the proposed method.

Table 5.54 Accuracy studies of mimosine quantification in Lajjalu Keram by HPLC (n=3)

<table>
<thead>
<tr>
<th>Amount of drug added to analyte (%)</th>
<th>Theoretical content (µg/mL)</th>
<th>Area± SD</th>
<th>RSD (%)</th>
<th>Concentration found (µg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>273114.33±1117.11</td>
<td>0.41</td>
<td>70.17</td>
<td>100.24</td>
</tr>
<tr>
<td>50</td>
<td>105</td>
<td>405631.67±6249.77</td>
<td>1.54</td>
<td>104.21</td>
<td>99.24</td>
</tr>
<tr>
<td>100</td>
<td>140</td>
<td>546897.33±2578.75</td>
<td>0.47</td>
<td>140.51</td>
<td>100.36</td>
</tr>
<tr>
<td>150</td>
<td>175</td>
<td>692986.33±5636.48</td>
<td>0.81</td>
<td>178.04</td>
<td>101.73</td>
</tr>
</tbody>
</table>

Precision

The precision was considered at two levels, the repeatability and the intermediate precision. The repeatability of the sample application was determined as the intraday variation, whereas the intermediate precision was determined by carrying out the inter-day variation for the determination of mimosine at three different concentration levels of 100, 200, 300, and 500 µg/mL in triplicates. The results of the repeatability and intermediate precision were expressed in terms of the %RSD and are shown in Table 5.55; the low values of the %RSD indicated the repeatability of the proposed method.
Table 5.55 Precision studies of mimosine quantification in Lajjalu Keram by HPLC

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>Repeatability (Intra-day precision)</th>
<th>Intermediate precision (Inter-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area ± SD (n = 3)</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>100</td>
<td>4801614±67247.081</td>
<td>1.40</td>
</tr>
<tr>
<td>200</td>
<td>9318811±48178.189</td>
<td>0.51</td>
</tr>
<tr>
<td>300</td>
<td>14318753±88619.413</td>
<td>0.62</td>
</tr>
</tbody>
</table>

Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ of the method were determined by the signal to noise ratio and were found to be 10.3 and 35.6 ng/mL, respectively, which indicated that the proposed method can be used for the detection and quantification of mimosine at a very low concentrations.

Robustness of the method

There was no significant change observed in the retention time of mimosine by changing the composition of the wavelength and flow rate at 500 µg/mL. The low value of the %RSD indicated the robustness of the method as shown in Tables 5.56.

Table 5.56 Robustness of mimosine quantification in Lajjalu Keram by HPLC

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Wavelength</th>
<th>Mean area ± SD (n = 3)</th>
<th>SEM</th>
<th>% RSD</th>
<th>Mean Rt ± SD(n = 3)</th>
<th>SEM</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
<td>24590706±333444.36</td>
<td>192519.84</td>
<td>1.36</td>
<td>2.70±0.02</td>
<td>0.01</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Used</td>
<td>284</td>
<td>24028905±89661.51</td>
<td>5176.62</td>
<td>0.37</td>
<td>2.62±0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>HPLC</td>
<td></td>
<td>285</td>
<td>24545094±104588.09</td>
<td>60385.73</td>
<td>0.43</td>
<td>2.59±0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>pH</td>
<td>0.8</td>
<td>24085334±386844.48</td>
<td>223351.32</td>
<td>1.61</td>
<td>2.60±0.02</td>
<td>0.01</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>23898756±451667.69</td>
<td>260778.11</td>
<td>1.89</td>
<td>2.65±0.04</td>
<td>0.02</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>24605375±268611.18</td>
<td>155087.28</td>
<td>1.09</td>
<td>2.64±0.05</td>
<td>0.03</td>
<td>1.79</td>
</tr>
</tbody>
</table>
Analysis of mimosine in Lajjalu Keram

The developed and validated method was applied for analysis of mimosine in Lajjalu Keram. A single HPLC peak was observed at the same retention time from the oil formulation shown in Figure 5.35. There was no interaction between the mimosine and the other components present in the oil. The mimosine content was found to be 0.0070% w/w with a % RSD of 0.41. The low RSD value indicated the suitability of this method for the routine determination of mimosine in Lajjalu Keram.

Conclusion

The HPLC method to quantify the marker compound mimosine in the anti-psoriatic herbal preparation “Lajjalu Keram” was found accurate, precise and reproducible. The method was most significant for routine quality control analysis of “Lajjalu Keram” because of wide range of linearity, simple mobile phase, UV detection, lack of extraction procedure, low RT and use of no internal standard.

The proposed method is the first attempt of HPLC analysis for quality control of Lajjalu Keram, an ayurvedic, antipsoriatic herbal formulation.
5.8. Analytical method development (HPLC quantification) of rutin in 777 oil

Optimization of chromatographic condition

To optimize the chromatographic conditions, various combinations of water, methanol, acetonitrile, and ethanol were tested. Various buffers such as ammonia, phosphate, and acetate were also tested. The method was also optimised by varying the pH. Finally, the best chromatographic separation were obtained at ratio of methanol-water (60:40, v/v) as mobile phase; adjusted to pH 3.0 by orthophosphoric acid, which produces a good separation with compact resolved peak of rutin at RT 0.38 (Figure 5.36) in standards as well as sample (Figure 5.37).

Figure 5.36 HPLC chromatogram obtained from rutin in methanol-water 60:40 (% v/v), RT 3.8 min; at λ = 360 nm

Figure 5.37 HPLC chromatogram obtained from 777 oil in methanol-water 60:40 (% v/v), RT 3.8 min at λ = 360 nm
Validation of the Method

The proposed HPLC method was validated as per ICH guidelines similar to the other methods reported by laboratory (Zafar et al., 2005 and Ahmad et al., 2011).

Linearity

The linearity range of rutin solutions was obtained up to 1.0–1000.0 μg/mL (Table 5.57) with a regression coefficient of 0.9998 and regression equation \( y = 32390 \times - 33639 \), which was used for calculation of content of rutin in sample.

Table 5.57 Calibration curve data of Rutin by HPLC

<table>
<thead>
<tr>
<th>Concentration (μg mL(^{-1}))</th>
<th>Mean area ± SD ((n = 3))</th>
<th>RSD</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31414 ± 368.43</td>
<td>1.17</td>
<td>212.72</td>
</tr>
<tr>
<td>5</td>
<td>153958 ± 4117.07</td>
<td>2.67</td>
<td>2377.07</td>
</tr>
<tr>
<td>25</td>
<td>794074 ± 4229.59</td>
<td>0.53</td>
<td>2442.03</td>
</tr>
<tr>
<td>50</td>
<td>1558230 ± 26758.99</td>
<td>1.72</td>
<td>15449.76</td>
</tr>
<tr>
<td>100</td>
<td>3348692 ± 18014.76</td>
<td>0.54</td>
<td>10401.13</td>
</tr>
<tr>
<td>200</td>
<td>6263393 ± 9860.37</td>
<td>0.16</td>
<td>5693.06</td>
</tr>
<tr>
<td>300</td>
<td>9564093 ± 28039.29</td>
<td>0.29</td>
<td>16188.97</td>
</tr>
<tr>
<td>400</td>
<td>12738214 ± 101131.7</td>
<td>0.79</td>
<td>58390.12</td>
</tr>
<tr>
<td>500</td>
<td>16482468 ± 260772.6</td>
<td>1.58</td>
<td>150561.56</td>
</tr>
<tr>
<td>1000</td>
<td>32328201 ± 155527.7</td>
<td>0.48</td>
<td>89796.60</td>
</tr>
</tbody>
</table>

Accuracy

The proposed method afforded a recovery of 98.91–101.16% after spiking the additional standard drug solution to the previously analyzed samples of leaf extract solution. The values of the recovery in terms of %, RSD (%) and SEM (Table 5.58) indicated the accuracy of the proposed method.

Table 5.58 Accuracy studies of rutin quantification in 777 oil by HPLC

<table>
<thead>
<tr>
<th>Amount of drug added to analyte (%)</th>
<th>Theoretical content (ng/mL)</th>
<th>Area ± SD ((n = 3))</th>
<th>RSD (%)</th>
<th>SEM</th>
<th>Concentration found (ng/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
<td>4645.66±46.07</td>
<td>0.99</td>
<td>26.60</td>
<td>179.02</td>
<td>99.45</td>
</tr>
<tr>
<td>50</td>
<td>270</td>
<td>7082.33±98.01</td>
<td>1.38</td>
<td>56.59</td>
<td>267.08</td>
<td>98.918</td>
</tr>
<tr>
<td>100</td>
<td>360</td>
<td>9398.33±69.43</td>
<td>0.74</td>
<td>40.09</td>
<td>362.23</td>
<td>100.62</td>
</tr>
<tr>
<td>150</td>
<td>450</td>
<td>11869±17.78</td>
<td>0.15</td>
<td>10.26</td>
<td>455.23</td>
<td>101.16</td>
</tr>
</tbody>
</table>
Precision

The precision was considered at two levels of the ICH suggestions, that is, the repeatability and the intermediate precision. The repeatability of the sample application was determined as an intra-day variation, whereas the intermediate precision was determined by carrying out the inter-day variation for the determination of rutin at four different concentration levels of 5, 25, 50, and 100 µg/mL. The results of the repeatability and intermediate precision were expressed in terms of the RSD (%) (Table 5.59). The low values of the RSD (%) indicated the repeatability of the proposed method.

Table 5.59 Precision studies of rutin quantification in 777 oil by HPLC

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Repeatability (intra-day precision)</th>
<th>Intermediate precision (inter-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area ± SD (n = 3)</td>
<td>SEM</td>
</tr>
<tr>
<td>5</td>
<td>153958 ± 4117.08</td>
<td>2377.07</td>
</tr>
<tr>
<td>25</td>
<td>794074 ± 4229.59</td>
<td>2442.03</td>
</tr>
<tr>
<td>50</td>
<td>1558230 ± 26758.99</td>
<td>15449.76</td>
</tr>
<tr>
<td>100</td>
<td>3348692 ± 18014.76</td>
<td>10401.130.537964</td>
</tr>
</tbody>
</table>

Limit of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ of the method were determined by the signal to noise ratio method and were found to be 0.027 and 0.095 µg/mL, respectively, which indicated that the proposed method can be used for the detection and quantification of rutin in low concentrations.
Robustness of the Method

There was no significant change in the retention time of rutin by changing the composition of the mobile phase and flow rate at 5 μg/mL concentration. The low value of the RSD (%) indicated the robustness of the method as shown in Table 5.60.

Table 5.60 Robustness studies of rutin quantification in 777 oil by HPLC

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Mobile phase composition (Methanol:water)</th>
<th>Mean area ± SD (n = 3)</th>
<th>SEM</th>
<th>% RSD</th>
<th>Mean Rt ± SD (n = 3)</th>
<th>SEM</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent composition</td>
<td>57:43</td>
<td>153324 ± 851.97</td>
<td>491.90</td>
<td>0.55</td>
<td>3.73 ± 0.02</td>
<td>0.01</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>63:37</td>
<td>153907.33 ± 588.42</td>
<td>339.74</td>
<td>0.38</td>
<td>3.616 ± 0.029</td>
<td>0.02</td>
<td>0.79</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8</td>
<td>153363.33 ± 576.19</td>
<td>332.68</td>
<td>0.37</td>
<td>3.86 ± 0.037</td>
<td>0.02</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>152623 ± 604.81</td>
<td>349.20</td>
<td>0.39</td>
<td>3.83 ± 0.04</td>
<td>0.02</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Analysis of Rutin in ‘777’ oil

The analysis of rutin in 777 oil was carried out by the optimized HPLC method and the values of chromatographic parameters were found identical to the standard ones. Rutin in 777 oil was analyzed by HPLC under identical conditions and the chromatogram is recorded (Figure 5.37) indicating a good peak of rutin. This figure also shows extra peaks that may signify the other ingredients present in the ‘777’ oil. The rutin content was found to be 0.018% w/w with a RSD (%) of 0.60. The low RSD value indicated the suitability of this method for the routine determination of rutin in this formulation.

Conclusion

An accurate, precise, and reproducible HPLC method for quantification of marker compound rutin in ‘777’ oil was developed and validated. Solubility of rutin was tested and it was found that it is soluble upto 0.41% in coconut oil. The method was found most significant for routine quality control analysis of ‘777’ oil because of wide range of linearity, simple mobile phase, UV detection, lack of extraction procedure, low retention time, and use of no internal standard. The rutin analysis in 777 oil is the first HPLC quality control method of 777 oil.

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5.9. Determination of anti-inflammatory activity of Lajjalu Keram and 777 oil (dermal formulation)

Results of carrageenan induced inflammation in the right hind paw of the wistar rats revealed a significant oedema (Table 5.61). Lajjalu Keram and 777 oil extract were applied half an hour before injecting carrageenan to the plantar surface of hind paw by gently rubbing fifty times with index finger and paw volume was measured by dipping in mercury. A reduction in the size of the oedema in the hind paw was recorded after applying formulations. After comparing with toxic control of carrageenan, Lajjalu Keram showed a maximum inhibition of 72.11% (Table 5.61) while in case of 777 oil it was found a little higher i.e. 75.47% at 24 h. Standard volini gel showed highest inhibition of paw edema viz. 87.76% at 24 h. Results indicate good anti-inflammatory activity of Lajjalu Keram and 777 oil which is less than but comparable to standard volini gel (Figure 5.38).

Table 5.61 Percentage inhibition of edema due to 777 oil on carrageenan induced rat paw edema

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Control</th>
<th>Lajjalu Keram</th>
<th>777 oil</th>
<th>Standard Volini</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.No</td>
<td>Time in hours</td>
<td>% Edema</td>
<td>% Edema</td>
<td>% Inhibition</td>
</tr>
<tr>
<td>1.</td>
<td>0</td>
<td>26.42</td>
<td>24.77</td>
<td>6.23</td>
</tr>
<tr>
<td>2.</td>
<td>1</td>
<td>69.18</td>
<td>44.44</td>
<td>35.76</td>
</tr>
<tr>
<td>3.</td>
<td>2</td>
<td>125.16</td>
<td>54.79</td>
<td>56.22</td>
</tr>
<tr>
<td>4.</td>
<td>4</td>
<td>153.46</td>
<td>53.50</td>
<td>65.14</td>
</tr>
<tr>
<td>5.</td>
<td>6</td>
<td>127.04</td>
<td>38.82</td>
<td>69.44</td>
</tr>
<tr>
<td>6.</td>
<td>12</td>
<td>81.76</td>
<td>21.16</td>
<td>70.45</td>
</tr>
<tr>
<td>7.</td>
<td>24</td>
<td>66.04</td>
<td>18.42</td>
<td>72.11</td>
</tr>
</tbody>
</table>
Figure 5.38 Percentage inhibition of edema by Lajjalu, 777 oil and Volini ® at different time intervals

Discussion:

Carrageenan, a sulphated polygalactose having macrophage toxic properties, accelerates development of edema in the paw of the rat after the injection in paw. The edema is due to release of histamine, serotonin and prostaglandin like substances. Lajjalu Keram and 777 oil recorded significant anti-inflammatory activity in carrageenan induced in animal model. From the above result the anti-inflammatory activity of the 777 oil was found to be more effective than Lajjalu Keram.
C. EVALUATION OF SHELF LIFE OF NIMBATIKTAM, LAJJALU KERAM AND 777 OIL

Results of stability studies on Nimbatiktam, indicated that there was insignificant difference compared to fresh sample in terms of physical appearance, extractive values, fingerprint analysis and assay as given in Table 5.62.

Similarly, results of stability studies on Lajjalu Keram and 777 oil didn’t show any significant difference in comparison to fresh sample in terms of physical appearance, pH, viscosity, GC/FID, mimosine content (Lajjalu Keram) and rutin content (777 oil) (Table 5.63).

Table 5.62 Results of stability studies of Nimbatiktam at 0, 3 and 6 months duration for determination of shelf life

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th>0 Month</th>
<th>3 Month</th>
<th>6 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Physical appearance</td>
<td>Crystalline powder</td>
<td>Crystalline powder</td>
<td>Crystalline powder</td>
</tr>
<tr>
<td>2</td>
<td>Color</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
</tr>
<tr>
<td>3</td>
<td>Odor</td>
<td>Pungent</td>
<td>Pungent</td>
<td>Pungent</td>
</tr>
<tr>
<td>4</td>
<td>Taste</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
</tr>
<tr>
<td>5</td>
<td>pH (1% solution)</td>
<td>6.30 ± 0.11</td>
<td>6.48 ± 0.23</td>
<td>6.59 ± 0.29</td>
</tr>
<tr>
<td>6</td>
<td>Extractive value (Water)</td>
<td>26.50 ± 1.36</td>
<td>25.73 ± 0.98</td>
<td>23.50 ± 0.95</td>
</tr>
<tr>
<td>7</td>
<td>Extractive value (Alcohol)</td>
<td>99.53±0.25</td>
<td>99.63±0.37</td>
<td>99.12±0.67</td>
</tr>
<tr>
<td>8</td>
<td>HPTLC fingerprint</td>
<td>Total 10 spots</td>
<td>Total 10 spots</td>
<td>Total 10 spots</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Rf 0.13, 0.19,</td>
<td>(Rf 0.11, 0.19,</td>
<td>(Rf 0.13, 0.19,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.26, 0.33, 0.38,</td>
<td>0.26, 0.32, 0.38,</td>
<td>0.28, 0.33, 0.38,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.48, 0.55, 0.63,</td>
<td>0.48, 0.51, 0.63,</td>
<td>0.48, 0.55, 0.62,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.69, 0.78)</td>
<td>0.69, 0.78)</td>
<td>0.69, 0.78)</td>
</tr>
<tr>
<td>9</td>
<td>Assay</td>
<td>99.11 ±0.68</td>
<td>98.96 ± 0.47</td>
<td>98.76 ± 0.38</td>
</tr>
</tbody>
</table>

Table 5.63 Results of stability studies of Lajjalu Keram and 777 oil at 0, 3 and 6 months for determination of shelf life

<table>
<thead>
<tr>
<th>S.No</th>
<th>Tests</th>
<th>Fromulations</th>
<th>0 Month</th>
<th>3 Month</th>
<th>6 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Physical appearance</td>
<td>Lajjalu Keram</td>
<td>Hazy liquid</td>
<td>Hazy liquid</td>
<td>Hazy liquid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>Clear liquid</td>
<td>Clear liquid</td>
<td>Clear liquid</td>
</tr>
<tr>
<td>2</td>
<td>Colour</td>
<td>Lajjalu Keram</td>
<td>Greenish</td>
<td>Greenish</td>
<td>Greenish</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>Magenta</td>
<td>Magenta</td>
<td>Magenta</td>
</tr>
<tr>
<td>3</td>
<td>Odour</td>
<td>Lajjalu Keram</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
<td>Smell of coconut oil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>Characteristic</td>
<td>Characteristic</td>
<td>Characteristic</td>
</tr>
<tr>
<td>4</td>
<td>Taste</td>
<td>Lajjalu Keram</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>Bitter</td>
<td>Bitter</td>
<td>Bitter</td>
</tr>
<tr>
<td>5</td>
<td>Acid Value</td>
<td>Lajjalu Keram</td>
<td>1.87 ± 0.23</td>
<td>1.56 ± 0.26</td>
<td>1.13 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>16.35 ± 0.84</td>
<td>15.59 ± 0.86</td>
<td>13.20 ± 0.06</td>
</tr>
<tr>
<td>6</td>
<td>Viscosity</td>
<td>Lajjalu Keram</td>
<td>56.28 ± 1.35</td>
<td>55.28 ± 1.89</td>
<td>56.28 ± 1.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>777 oil</td>
<td>45.71 ± 1.27</td>
<td>42.35 ± 1.27</td>
<td>47.64 ± 1.27</td>
</tr>
<tr>
<td>7</td>
<td>GC-FID</td>
<td>Lajjalu Keram</td>
<td>13 Fatty acids</td>
<td>13 Fatty acids</td>
<td>12 fatty acids</td>
</tr>
</tbody>
</table>
Assay of marker

<table>
<thead>
<tr>
<th></th>
<th>777 oil</th>
<th>16 Fatty acids</th>
<th>14 Fatty acids</th>
<th>15 Fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lajjalu Keram (Mimosine)</td>
<td>98.36 ± 1.96</td>
<td>98.16 ± 1.65</td>
<td>97.95 ± 1.30</td>
<td></td>
</tr>
<tr>
<td>777 oil (Rutin)</td>
<td>98.39 ± 0.50</td>
<td>98.17 ± 0.30</td>
<td>98.01 ± 0.48</td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.39 Percentage drug constituents of Nimbatiktam remaining vs time (Months) plot for shelf life determination of 777 oil
Figure 5.40 Percentage drug (Mimosine) remaining vs time (Months) plot for shelf life determination of Lajjalu Keram

Figure 5.41 Percentage drug (Rutin) remaining vs time (Months) plot for shelf life determination of 777 oil
Discussion

Till date no specific guidelines are available regarding the shelf life of the pure ayurvedic formulations from any government organization except a Gazette notification issued in 2009, in which the AYUSH has been directed to display the common date of expiry of the Ayurveda, Siddha and Unani (ASU) drugs. In this context AYUSH has proposed a generalised shelf life of common ayurvedic formulations like for churna (powder drugs) as 2 years, Gutika (pills) as 3 years etc (Gupta et al., 2011).

The evaluated shelf life can be used as exact date of shelf life for these formulations. In this experiment the 90% of the Nimbatikta was found to be remained for 52.8 months, whereas in case of Lajjalu Keram and 777 oil, same were found to be remained for 44.2 and 44.7 months respectively as per mean curve of sigma plot in Figure 5.39, 5.40 and 5.41. However since Shelf life of maximum 2 years can be assessed based on this plot. So it can be concluded that these formulations have shelf life up to two years.