SECTION A: EXPERIMENTAL: NIMBATIKTAM (Oral Formulation)

4.1. QUALITY CONTROL AND STANDARDIZATION OF NIMBATIKTAM

4.1.1. Organoleptic observations
Color, odor and taste of the drug were studied as per protocol

4.1.2. Loss on drying
The powdered drug sample (10 g) was placed on a tarred evaporating dish and dried at 105°C for six hours and weighed. The drying was continued until two successive reading matches each other or the difference between two successive weighing was not more than 0.25% of constant weight.

4.1.3. Total ash
The ground drug (1.0 g) was incinerated in a silica crucible at a temperature not exceeding 450°C until free from carbon. It was then cooled and weighed to get the total ash content.

4.1.4. Acid insoluble ash
Total ash found was boiled with 25 mL of dilute HCl (6N) for five minutes. The insoluble matter collected on an ashless filter paper, washed with hot water and ignited at a temperature not exceeding 450°C to a constant weight. After that weight was taken and the acid insoluble ash was calculated.

4.1.5. pH of 1% solution
One gram of accurately weighed drug was dissolved in water (100 mL) and filtered. pH of the filtrate was checked with a standardized glass electrode.

4.1.6. pH of 10% solution
Ten gram of the accurately weighed drug was dissolved in water (100 mL) and filtered. pH of the filtrate was checked with a standardized glass electrode.

4.1.7. Bulk density
Weighed quantity of drug was kept in 50 mL measuring cylinder and volume of the drug was measured. The bulk density was calculated in g mL⁻¹.
4.1.8. Tapped density

Ten grams of test sample was introduced into a dry 100 mL graduated cylinder (readable to 1.0 mL) without compacting and the unsettled apparent volume was noted as \( V_o \). The cylinder was mechanically tapped 500 times initially and the tapped volume was measured, \( (V_a) \) to the nearest graduated unit. Repeated the tapping an additional 750 times and the tapped volume \( (V_b) \), was measured to the nearest graduated unit. When the difference between the two volumes was less only 1%, than \( V_b \) was taken as the final tapped volume, \( (V_f) \). The tapped density was calculated in g mL\(^{-1}\).

4.1.9. Extractive value (water soluble)

Exactly 4.0 g of coarsely powdered air-dried material was placed in a glass-stopper conical flask. To this, added 100 mL of water and then weighed to obtain the total weight including the flask. It was attached to a reflux condenser and boiled gently for one hour; cooled and weighed. The original total weight of the flask was readjusted with the solvent. Shaked well and filtered rapidly through a dry filter. The 25 mL of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath. The filtrate was dried at 105°C for six hours, cooled in desiccators for 30 minutes, and then weighed without delay. Content of extractable matter was calculated as % w/w.

4.1.10. Extractive value (alcohol soluble)

Drug was dissolved in methanol and methanol soluble extractive was calculated as above and represented as % w/w.

*The results of all the physicochemical standardization parameters have been documented in Table 5.1.*

4.1.11. Phytochemical investigations

The extracts and formulations were screened for the detection of various classes of chemical constituents like alkaloids, glycosides, tannins, sugars & carbohydrates, saponins, proteins & amino acids, resins, lipids and fats etc.

4.1.11.1. Tests for alkaloids

The test for alkaloids was done with following reagents:
a. Dragendorff's reagent

It was prepared by mixing solution A (17 gm of bismuth subnitrate + 200 gm tartaric acid + 800 mL distilled water) and solution B (160 gm potassium iodide + 4 (x) mL distilled water) in 1:1, v/v proportion. From this solution, working standard was prepared by taking 50 mL of this solution and adding 100 gm of tartaric acid and making upto 500 mL with distilled water. The above Dragendorff's reagent was sprayed on whatmann (No. 1) filter paper and the paper was dried. The test filtrate after basification with dilute ammonia was extracted with chloroform and the chloroform extract was applied on the filter paper, impregnated with Dragendorff's reagent, with the help of a capillary tube. Development of an orange red colour on the paper indicated the presence of alkaloids.

b. Mayer's reagent (Potassium mercuric iodide reagent)

The Mayer's reagent was prepared as follows: 1.36 gm of mercuric chloride was dissolved in 60 mL of distilled water. The solution was mixed and diluted up to 100 mL with distilled water. To a little of the test filtrate, taken in a watch glass, a few drops of the above reagent was added. Formation of cream colored precipitate showed the presence of alkaloids.

c. Wagner's reagent (Iodine-potassium iodide)

Approximately 1.27 g of iodine and 2.0 g of potassium iodide were dissolved in 5.0 mL of water and the solution was diluted upto 100 mL with water. When few drops of this reagent were added to the test filtrate, a brown flocculent precipitate was formed indicating the presence of alkaloids.

d. Hager's reagent

A saturated aqueous solution of picric acid was employed for this test. When the test filtrate was treated with this reagent, an orange yellow precipitate was obtained, indicating the presence of alkaloids.

e. Mayne's reagent (Potassium-cadmium iodide)

Approximately 20 mg of potassium iodide was dissolved in 20 mL of water and the solution was added to 10 g of cadmium iodide, dissolved in 50 mL of water. This reagent with test filtrate gave a yellow precipitate, which was amorphous at first and
then turned crystalline. The crystalline matter was soluble in excess of the reagent and in ethanol, indicating the presence of alkaloids

f. Tannic acid reagent

A freshly prepared 5% aqueous solution of tannic acid was used for this test. To a little of the test filtrate, the above reagent was added. Formation of precipitate (soluble in ammonia and dilute acids) gave the indication of presence of alkaloids.

4.1.1.2. Test for glycosides

About 2.0 mL of alcoholic extract was taken and subjected to following tests:

a. Killer-Killani Test

One mL of glacial acetic acid containing traces of ferric chloride and one mL of concentrated sulphuric acid were added to the extract and observed for formation of reddish brown color at the junction of two layers and the upper layer turned bluish green in presence of glycoside.

b. Borntrager’s test

One mL of benzene and 0.5 mL of dilute ammonia solution were added to the ethanolic extract and observed for formation of reddish pink color.

c. Legal test

Concentrated ethanol extract was made alkaline with few drops of 10% sodium hydroxide solution and then freshly prepared sodium nitroprusside solution was added to the solution and observed for the formation of blue color.

d. Baljet Test

To the concentrated ethanolic extract, sodium picrate reagent was added and observed for the formation of orange and yellow color.

4.1.1.3. Tests for tannins

The test residue of each extract was taken separately in water, warmed and filtered. Tests were carried out with the filtrate using following reagents:

a. Ferric chloride reagent

A 5% w/v solution of ferric chloride in 90% alcohol was prepared. Few drops of this solution were added to a little of the above filtrate. Presence of dark green or deep
blue colour was observed.

b. Lead acetate test

Few drops of 10% w/v solution of basic lead acetate in distilled water was added to the test filtrate. Presence of precipitate was observed.

c. Potassium dichromate test

On addition of few drops of solution of potassium dichromate in a test filtrate, presence of dark colour was observed.

d. Gelatin solution test

Gelatin solution (1% w/v in water) was prepared in 10% Sodium Chloride solution. A little of this solution was added to the filtrate. Presence of white precipitate was observed.

e. Bromine water test

Bromine solution was added to the test filtrate. Decolourization of bromine water was observed.

4.1.11.4. Tests for sugars and carbohydrates

a. Molisch's test

The Molisch's reagent was prepared by dissolving 10 g of α-naphthol in 100 mL of 95% alcohol. A few mg of the test residue was placed in a test-tube containing 0.5 mL of water, and it was mixed with two drops of Molisch's reagent. To this solution, added one mL of concentrated sulphuric acid from the side of the inclined test-tube, so that the acid formed a layer beneath the aqueous solution without mixing. A red brown ring appears at the junction of two liquids.

a. Fehling's solution test

The Fehling's solution was prepared as follows:

Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper sulphate</td>
<td>34.64 g</td>
</tr>
<tr>
<td>Sulphuric acid</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 mL</td>
</tr>
</tbody>
</table>
Sodium B

Sodium potassium tartrate - 176 g
Sodium hydroxide - 77 g
Distilled water to - 500 mL

The two solutions were mixed in equal volumes immediately before use.

A little of the test residue was dissolved in water, and a few mL of the Fehling’s solution was added to it. This mixture was then warmed. Presence of red precipitate of cuprous oxide was observed.

4.1.11.5. Tests for saponins

a. Foam test

Few mg of the test residue was taken in a test tube and shaken vigorously with a small amount of sodium bicarbonate and water. A stable, characteristic honeycomb like froth was observed for presence of saponin.

b. Haemolysis test

Little of the test residue was dissolved in normal saline in such a way that five mL of the solution represented one gm of the crude drug. In a series of five test-tubes, doses of 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL and 1.0 mL were added and volume was made upto one mL in each case with normal saline. One mL of diluted blood (0.5 mL of rabbit’s blood diluted to 25 mL with normal saline) was added to each tube and changes observed. The haemolysis of blood indicate presence of saponins.

4.1.11.6. Tests for proteins and amino acids

a. Biuret test

Few mg of the residue was taken in water and one mL of 4% sodium hydroxide solution was added to it. This was followed by a drop of 1% solution of copper sulphate. Formation of violet or pink color was observed for the presence of protein.

b. Xanthoproteic test

Little residue was taken with two mL of water and 0.5 mL of concentrated nitric acid was added to it. Formation of yellow color was observed for the presence of protein.

c. Millon's test (Mercuric nitrate solution)

Millon’s reagent was prepared by dissolving 3.0 mL of mercury in 27 mL of fuming
nitric acid, keeping the mixture well cooled; this solution was then diluted with equal quantity of distilled water. Aqueous solution of the residue was taken and to it, 2.0 to 3.0 mL of Million's reagent was added. Formation of white precipitate was observed for the presence of protein.

d. Ninhydrin test

The Ninhydrin reagent is 0.1% w/v solution of ninhydrin in n-butanol. Little of this reagent was added to the test extract. Formation of violet or purple color was observed for the presence of amino acids.

4.1.11.7. Test for resins

(a) Distilled water (5.0 mL) was added to the extract and observed for turbidity.

(b) A mixture of extract in acetone (3.0 mL) and HCl (3.0 mL) was heated on a water bath for 30 minutes and observed for pink color.

4.1.11.8. Test for lipids/fats

Small quantity of powdered drug was rubbed on a paper and observed for a permanent translucent stain.

4.1.11.9. Test for phenolic compounds

a. Ferric chloride solution

The extract was taken in water and warmed; to this 2.0 mL of Ferric chloride solution was added and observed for the formation of green and blue color.

b Lead acetate solution

To the extract Lead acetate solution was added and observed for the formation of black precipitate.

4.1.11.10 Tests for Flavanoids (Shinoda test)

Small quantity to test residue was dissolved in 5.0 mL ethanol (95%, v/v) treated with few drops of concentrated hydrochloric acid and 0.5 g of Magnesium metal. The pink, crimson or magenta colour was developed within a minute or two, if flavonoids were present.

The results of all the phytochemical investigations were summarized in Table 5.2.
4.1.12. Determination of microbial contamination

4.1.12.1. Total fungal count

Media used

Potato Dextrose Agar (PDA)

Composition of Media:

<table>
<thead>
<tr>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato Dextrose Agar (PDA)</td>
<td>39 g</td>
</tr>
<tr>
<td>Distilled water up to</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>6.3-6.5</td>
</tr>
</tbody>
</table>

Procedure

The different dilution of drug was made in sterile distilled water aseptically and poured into three petriplates containing solidified and sterile PDA media. The media containing different diluted drug sample were incubated for 48 h in BOD incubator at 22 °C temperature and colonies were counted. Results are shown in Figure 5.1 and summarised in Table 5.3.

4.1.12.2. Total bacterial count

Media used

Casein soyabean digest agar medium

Composition of Media:

<table>
<thead>
<tr>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic digest of casein</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Papaic digest of soyabean meal</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water up to</td>
<td>1000 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>

Procedure

The different dilution of drug was made in sterile distilled water aseptically and poured into three petriplates containing solidified and sterile Casein soyabean agar media. The media containing different diluted drug sample were incubated for 18 h in BOD incubator at 37°C temperature and colonies were counted. Results are shown in Figure 5.2 and summarised in Table 5.4.
4.2. CHEMICAL TOXICITY EVALUATION OF NIMBATIKTAM

4.2.1. Analysis of heavy metals in Nimbatiktam using Atomic absorption spectroscopy (AAS)

Heavy metal analysis was carried out as per the method described by Horwitz, 1970 and Chu et al., 2010. The ground drug (1.0 g) was incinerated in a silica crucible at a temperature 600°C until ash is formed. Ash was taken in the reaction vessels and dissolved in 6 mL of concentrated HCl followed by 2 mL of concentrated HNO₃ and left to stand overnight. The suspension obtained was heated slowly for 2 h under reflux condenser for complete digestion. The volume was made up to 100 mL with distilled water. This sample was analyzed by atomic absorption spectrometer and content was evaluated forming regression equation obtained from standard plots for lead, cadmium, mercury, and arsenic.

4.2.2. Analysis of aflatoxins in Nimbatiktam using HPLC

The aflatoxin in herbal drugs was analyzed by the HPLC fluorescence method as discussed by Horwitz, 1970 and Tagami et al., 2007.

a) Extraction of aflatoxins: The Association of Analytical Chemist (AOAC) official method of analysis was followed. In this method, 15 g of Nimbatiktam was dissolved in a mixture of 100 mL methanol and 25 mL of 0.1M HCl followed by partitioned with 10% NaCl (in water, w/v) and hexane. In aqueous layer added dichloromethane (DCM). Process was repeated for 2-3 times and the collected DCM extract was evaporated up to 2-3 mL. This extract was passed through silica column followed by washing of the column with a mixture of benzene: acetic acid (9:1, v/v) and ether: hexane (3:1, v/v). Finally aflatoxin was eluted with 100 mL of DCM: acetone (90:10, v/v). The eluted aflatoxin was concentrated up to 5.0 mL and was dried over nitrogen.

b) Derivatization of extracted aflatoxins: Dried extract was taken and added 200 µL of hexane and 50 µL of trifluoroacetic acid. It was mixed on vortex mixer exactly for 30 seconds, stand for five minutes and 1.95 mL of water: acetonitrile (9:1, v/v) mixture was added. This sample was used for HPLC analysis after filtration with 0.22 µm filter.
c) **Derivatization of standards**: Taken known concentrations (20 ppb, 40 ppb, and 80 ppb) of standard aflatoxin B1, G1, B2 and G2 and were derivatized in the same manner as for sample.

c) **HPLC analysis for aflatoxins**: Twenty microlitre of derivatized sample (both extract and standards) were injected into HPLC column (C18; 15 cm x 4.6 mm) at a flow rate of 1.0 mL/min using water: acetonitrile: methanol (70:17:17, v/v) as mobile phase and fluorescent detector. The peaks of aflatoxin in formulations were compared with peak of standards (B1, G1, B2 and G2) and were analyzed. The HPLC chromatogram of standard aflatoxins is shown at Figure 5.7, whereas HPLC chromatograms of aflatoxins obtained from Nimbatiktam are shown at Figure 5.8. Results of aflatoxins analysis in Nimbatiktam have been summarized in Table 5.6.

4.2.3. **Analysis of pesticides in Nimbatiktam using GC-MS**

The pesticide analysis of herbal drugs was carried out using GC-MS method as discussed by Horwitz, 1970 and Weaver and Trucksess, 2010. Accurately weighed, 50 g of sample was dissolved into methanol and added one gm of sodium oxalate. It was taken into separating funnel and added diethyl ether (50 mL) and petroleum ether (50 mL). It was shaken for one minute. Organic layer was transferred into another separating funnel and added 600 mL of water with saturated solution of sodium chloride. Aqueous layer was discarded and this process was repeated for 2-3 times. Organic layer was then passed through sodium sulphate and evaporated to 2-5 mL. This concentrated solution was again mixed with acetonitrile (30 mL) and petroleum ether (30 mL) and it was eluted with diethyl ether over silica column. The ether elute was concentrated to five mL in rotavapor and analysed by GC-MS. GC-MS analysis was performed using Agilent system (7890A series, Germany), operated by GC Chemstation software and equipped with a split/splitless injector. All pesticides were resolved in a HP-5 (30m x 0.32mm, 0.25 μm film thickness) column. The GC oven programme was as follows: 60 °C hold 2 min, rate 30 °C/min to 200 °C, rate 3 °C/min to 230 °C, rate 4 °C/min to 300 °C, with a total acquisition program of 34.17 min. Helium was used as a carrier gas with a column head pressure of 9.92 psi. Detector temperature was set at 300 °C. Injector was operating in the splitless mode. Mass spectra were acquired in EI mode (70 eV); in m/z range 30–600. Results of pesticide analysis in Nimbatiktam have been summarized in Table 5.7.
4.3. LONG TERM ORAL TOXICITY STUDIES OF NIMBATIKTAM IN WISTAR RATS

The long term oral toxicity studies of Nimbatiktam was carried out as per the OECD guideline (OECD, 408).

4.3.1. Selection of animal species

The preferred rodent species of the rat was the Wistar rat. Healthy young adult animals of commonly used laboratory strains were used. Females were nulliparous and non-pregnant. At the commencement of the dosing the age of animals were 08 weeks old and their weight was fall in an interval within ± 20 % of the mean initial weight as tabulated in Table No. 5.10.

4.3.2. Housing and feeding conditions

Adult Wistar Albino rats weighing 115-160 g were procured from Central animal house facility Jamia Hamdard. The animals were housed in polypropylene cages and maintained at 28 ± 2°C, 70 ± 5% RH and at 12 hr light/dark photoperiod. They were fed with standard rat pellet diet and water ad libitum during the experiment.

4.3.3. Preparation of animals

The animals were randomly selected, marked to permit individual identification, and kept in their cages for seven days prior to dosing for toxicities studies to allow for acclimatization.

4.3.4. Assignment of animals

Each animal was assigned a unique identification number by making over them.

4.3.5. Vehicle and volume

For dose preparation, Nimbatiktam (200, 400 and 800 mg/kg body weight) was suspended in water (using tween 80, as suspending agent) so that volume of the single dose did not exceed two mL/animal. The formulation was homogenous and used daily as a fresh suspension.

4.3.6. Administration of doses

The animals were dosed with the Nimbatiktam and it was administered daily for 90 days using gavage. The protocol followed has been given in Table 4.1 and dose regime in Table 4.2.
Table 4.1 Study protocol for oral toxicity studies of Nimbatiktam

<table>
<thead>
<tr>
<th>Name of the study</th>
<th>90 days oral toxicity study of Nimbatiktam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test material</td>
<td>Nimbatiktam (Powder)</td>
</tr>
<tr>
<td>Animal model</td>
<td>Albino wistar rats</td>
</tr>
<tr>
<td>Age</td>
<td>8 – 12 weeks</td>
</tr>
<tr>
<td>Weight</td>
<td>180 gms (Average)</td>
</tr>
<tr>
<td>Animal procured from</td>
<td>Central animal facility, Jamia Hamdard (Protocol Number: 173/CPCSEA)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male and female</td>
</tr>
<tr>
<td>Number of animals per group</td>
<td>10 in each group</td>
</tr>
<tr>
<td>Groups</td>
<td>8 groups (4 male and 4 female groups)</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Intra-gastric administration</td>
</tr>
<tr>
<td>Dose volume (per animal)</td>
<td>2 mL/Animal</td>
</tr>
<tr>
<td>Number of administration</td>
<td>Daily single dose</td>
</tr>
<tr>
<td>Concentration of dose</td>
<td>200, 400, 800 mg/kg BW</td>
</tr>
<tr>
<td>Vehicle for administration</td>
<td>Water (double distilled)</td>
</tr>
<tr>
<td>Study duration</td>
<td>90 days</td>
</tr>
</tbody>
</table>

Table 4.2 Dose regime of Nimbatiktam for oral toxicity studies

<table>
<thead>
<tr>
<th>S.No</th>
<th>Groups and sex</th>
<th>Dose mg/kg body weight</th>
<th>Number of animal used</th>
<th>Volume of drug (mL/Animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Low dose male</td>
<td>200</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Low dose female</td>
<td>200</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>Medium dose male</td>
<td>400</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>Medium dose female</td>
<td>400</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>5.</td>
<td>High dose male</td>
<td>800</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td>High dose female</td>
<td>800</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>7.</td>
<td>Control male</td>
<td>Control</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>8.</td>
<td>Control female</td>
<td>Control</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

4.3.7. Observations

General clinical observations were made at least once a day at the same time up to 90 days. All animals were inspected for signs of morbidity and mortality at least twice daily, usually at the beginning and end of each day. Once prior to the first exposure and once a week thereafter, detailed clinical observations were made in all animals. These observations were made outside the home cage in a standard arena and at similar time on each occasion. Signs noted including changes in skin, fur, eyes (dullness, opacities), occurrence of secretions and excretions and autonomic activity (e.g., pupil size, and unusual respiratory pattern). Changes in gait, condition of teeth
and breathing abnormalities has also been recorded. Results are summarized in Table 5.8 and Table 5.9.

4.3.8. Body weight

Individual weight of animals were determined before Nimbatiktam was administered and at least weekly, thereafter measurement of food consumption was also made. Changes in weight were calculated and recorded (Table 5.10). At the end of the study, surviving animals were weighed and then humanly killed.

4.3.9. Haematology and clinical biochemistry

At the end of the study, blood samples were collected from the overnight fasted animals just prior to killing the animals and the following haematological examinations were made.

- Haematocrit
- Haemoglobin concentration
- Erythrocyte count
- Total and differential leukocyte count
- Platelet count and
- Blood clotting time/potential

The results of haematological changes for male and female rats were summarized in Table 5.11.

Biochemical parameters for Liver function test and Kidney function test were carried out in the blood samples collected at the end of the study. Liver function tests include determination of alanine aminotransferase, aspartate aminotransferase (Reitman and Frankel, 1957) and alkaline phosphatise (Walter and Schutt, 1974) in serum. Kidney function tests include determination of creatinine (Bartels et al., 1972), sodium, potassium and blood urea nitrogen (Stevens et al., 2006).

The results for liver function test for male and female rats have been given in Table 5.12. Whereas for kidney function test in Table 5.13.

4.3.10. Gross necropsy

All animals in the study were subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, spleen, were trimmed of any adherent tissue, and their wet weight taken as soon as possible after dissection to avoid drying. These tissues (liver, kidneys, spleen,
stomach and lungs) were fixed in 10% formalin, routinely processed and embedded in paraffin wax.

4.3.11. Histopathology

Full histopathology was carried out on the preserved organs control, medium dose, low dose and high dose groups. Blocks were made and sections were taken on slides. These slides were processed for double staining and permanent slides were prepared by following method (Table 4.3).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Solutions</th>
<th>Time</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deparaffinization</td>
<td>Xylene</td>
<td>2 minutes</td>
<td>Remove paraffin</td>
</tr>
<tr>
<td></td>
<td>Absolute alcohol</td>
<td>2 minutes</td>
<td>Replace xylene with paraffin</td>
</tr>
<tr>
<td>Hydration</td>
<td>95% alcohol</td>
<td>2 minutes</td>
<td>Replace alcohol with water</td>
</tr>
<tr>
<td></td>
<td>80% alcohol</td>
<td>2 minutes</td>
<td>Replace alcohol with water</td>
</tr>
<tr>
<td>Washing</td>
<td>Water</td>
<td>2 minutes</td>
<td>Adds water</td>
</tr>
<tr>
<td>Staining</td>
<td>Harris’s Haematoxylin</td>
<td>1-6 minutes</td>
<td>Nuclei are stained blue</td>
</tr>
<tr>
<td>Bluening</td>
<td>Tap water</td>
<td>Several changes (5-15 minutes)</td>
<td>Blue nuclei</td>
</tr>
<tr>
<td>Dehydration</td>
<td>70% alcohol</td>
<td>2 minutes</td>
<td>Replace water with alcohol</td>
</tr>
<tr>
<td></td>
<td>80% alcohol</td>
<td>2 minutes</td>
<td>Replace water with alcohol</td>
</tr>
<tr>
<td></td>
<td>95% alcohol</td>
<td>2 minutes</td>
<td>Replace water with alcohol</td>
</tr>
<tr>
<td>Counter stain</td>
<td>0.5% eosin-Y in 95% alcohol</td>
<td>20-60 seconds</td>
<td>Stains cytoplasm pink</td>
</tr>
<tr>
<td>Dehydration</td>
<td>95% alcohol (twice)</td>
<td>1-2 dips</td>
<td>Dehydrate and also</td>
</tr>
<tr>
<td></td>
<td>Xylene - I</td>
<td>2 minutes</td>
<td>Removes excess eosin</td>
</tr>
<tr>
<td>Clearing</td>
<td>Xylene - II</td>
<td>2 minutes</td>
<td>Replace alcohol with xylene and clear tissues</td>
</tr>
<tr>
<td>Mounting</td>
<td>Done with DPX* (Mixture of distyrene, a plasticizer and xylene)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A colourless synthetic resin mounting media which replaces Xylene-Balsam. It preserves the stain and dries quickly.
4.4. ASSAY FOR CONSTITUENTS (FLAVONOID AND PHENOLIC) OF NIMBATIKTAM

The total flavonoid content of Nimbatiktam was carried out using spectrophotometric method described by Qayyoom et al., 2009.

4.4.1. Determination of total flavonoid content in Nimbatiktam using UV spectrophotometer

**Reagents**

Aluminium chloride (AlCl₃) (0.1 gm/mL), Sodium acetate (CH₃COONa) (1 M), different dilutions of rutin standard (10 - 100 µg/mL).

**Method**

The 10 mg/mL solution of Nimbatiktam was prepared in methanol and 0.5 mL was added to test tube containing 1.5 mL methanol. Further, 0.1 mL of AlCl₃ and 0.1 mL of CH₃COONa were added, followed by addition of 2.8 mL distilled water. The mixture was kept for 30 minutes at room temperature and absorbance was taken at 415 nm. Similar procedure was followed for standard rutin and control (omitting substance).

After taking the absorbance of standard dilutions as mentioned above, calibration curve was plotted (Figure 5.14). Flavanoid content in Nimbatiktam was calculated by using standard calibration curve and results were summarised in Table 5.14.

4.4.2. Determination of total phenolic content in Nimbatiktam using UV spectrophotometer

The total Flavonoid content of Nimbatiktam was by using UV-spectroscopic method described by Qayyoom et al., 2009.

**Reagents**

Folin-ciocalteu reagent (FCR) (10% in distilled water), Sodium carbonate (Na₂CO₃) (1 M in distilled water) and Gallic acid (standard) 1.0 mg/mL solution in methanol. Different dilutions of standard gallic acid (25 - 300 µg/mL) in methanol.

**Method**

Half mL of sample (10 mg/mL Nimbatiktam in methanol) was added in test tube containing 5 mL of FCR. The mixture was shaked and 4 mL of sodium carbonate
solution was added. The absorbance was read at 765 nm after 15 minutes. Similar procedure was followed for samples and blank (omitting substance).

The calibration curve was plotted (Figure 5.15) and total phenolic content in Nimbhitktam was calculated by using regression equation and results were recorded in Table 5.15.
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4.5. HPTLC ANALYTICAL METHOD DEVELOPMENT FOR ANALYSIS OF NIMBATIKTAM IN FORMULATION USING HPTLC

4.5.1. HPTLC instrumentation and general condition

The samples were spotted in the form of band of six mm with a Camag microliter syringe on precoated Silica gel aluminium plate 60F 254 (20 cm x 10 cm with 0.2 mm thickness, E. Merk, Germany) using Camag Linomat V (Switzerland) sample applicator. A constant application rate of 120 nL/sec was employed and space between two bands was 6.6 mm. Linear ascending development was carried out in twin trough glass chamber, saturated with mobile phase. The optimized chamber saturation time for mobile phase was 15 minutes at room temperature. The length of the chromatogram run was 80 cm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air drier. Scanning was performed using Camag TLC scanner III in the absorbance mode at 265 nm. The source of radiation utilized was deuterium and tungsten lamp. The slit dimension was kept at 6 X 0.45 mm and 20 mm/s scanning speed was employed.

4.5.2. Method development/optimisation of Nimbatiktam

The Nimbatiktam was dissolved in methanol to get a concentration of 2.0 mg/mL. It was applied in duplicate on TLC plate (20 cm x 10 cm) for finger printing using chloroform: ethyl acetate containing 1% acetic acid 8.0:2.0 (v/v) as mobile phase after a proper selection and optimization. The developed chromatogram was then scanned in multi wavelength scanner in the range of 250-365 nm at an interval of five nm, to find out the best suitable wavelength (the wavelength of at which maximum number of spots with maximum area) was observed for finger printing of Nimbatiktam. Further, it was scanned on the selected wavelength of 265 nm in single wavelength mode and the data obtained was analyzed by using WINCATS software (Figure 5.16-5.17). The two major peaks in densitometric analysis showed better linearity over the concentration range of 2-100 µg. These chromatograms were selected as standard 1 and standard 2 (Figure 5.18). The peaks of standards represent the respective concentrations of Nimbatiktam.

4.5.3. Preparation of calibration curves

Different volumes of Nimbatiktam stock solution (2.0 mg/mL), 1.0, 2.0, 4.0, 5.0, 10.0, 20.0, 40.0 and 50.0 µL were spotted in triplicate on TLC plate to obtain
concentration of 2.0, 4.0, 8.0, 10.0, 20.0, 40.0, 80.0 and 100.0 µg/spot. The data of peak area of substance 1 and substance 2 Vs drug concentration were treated by linear least-square regression by WINCATS software (Table 5.17-5.18).

4.5.4. Method validation

The developed chromatographic method was validated as per the ICH guidelines for the accuracy, limit of detection, limit of quantification and precision, similar to the other methods reported by laboratory (Ansari et al., 2005; Ahmad et al., 2008 and Singh et al., 2011).

Accuracy as recovery

The accuracy of the method was determined by recovery studies. For this, preanalyzed samples were spiked with standard in three different concentration levels i.e. 80, 100 and 120% and the mixtures were reanalyzed by the method proposed (Table 5.19).

Limit of detection and Limit of quantification

The limits of detection and limits of quantification were determined by blank determination method using signal-to-noise ratio.

Precision

The precision of the method was checked by intermediate precision intra-day, inter-day, inter-system and inter-analyst precisions were carried out. Inter-day and intra-day precisions were done by preparing and applying six different concentrations of samples in the same day and in three different days, respectively. Inter-system and inter-analyst precisions done by repeating same procedure with using different system and by different persons, respectively. Results are documented in Table 5.20.

4.5.5. Assay of neem oil extract in formulations

The developed method was applied for analysis of prepared tablet formulation. The tablet formulations were dissolved in methanol to get 2.0 mg/mL equivalent of neem oil extract and filtered through 0.45 µm membrane filter. Four microlitres of same was applied in duplicate on TLC plate (20 cm x 10 cm) for quantification. The neem oil extract was quantified with respect to substance 1 and substance 2 by WINCATS software using regression equation obtained from calibration curve and the mean of duplicate samples were calculated. Results are given in 5.21.
4.6. DETERMINATION OF IMMUNOMODULATORY ACTIVITY OF NIMBATIKTAM

4.6.1. Requirements

a. Male mice
b. Sheep red blood cells (SRBC)
c. Nimbatiktam
d. Cyclophosphamide (Immunomodulating agent)
e. Disposable syringe (1 mL) 27½ gauge needles
f. Bovine serum albumin (BSA) saline (0.1 % in saline)
g. 96 wells titration plates
h. Auto analyzer to SRBC
i. Micropipettes
j. Micro tips with stands
k. Tip discard box
l. Troughs for SRBC and normal saline

4.6.2. Preparation of Alsever's reagent

Alsever’s reagent provides isotonicity and nourishment to SRBCs. Its composition is as follow:

- Sodium chloride: 420 mg
- Sodium citrate: 800 mg
- Dextrose: 2.5 g
- Distilled water up to: 100 mL

Mixed thoroughly and stored at 4°C.

4.6.3. Processing of antigens for sensitization

Fresh SRBC were collected aseptically from the jugular vein of sheep and stored in cold sterile Alsever’s reagent. Further SRBC are washed three times with pyrogen free sterile saline (1 % NaCl, w/v) and adjusted to the concentration of $5 \times 10^9$ cells per mL for immunization and challenge at the required time schedule.
4.6.4. DTH (Delayed type hypersensitivity) by foot thickness measurement

The method described by Doherty was followed for study (Doherty, 1981). Group of six mice each were immunized with by injecting 0.2 mL of $5 \times 10^9$ SRBC/mL, intraperitonially (i.p.) after diluting in normal saline. Test material was administered two hours after SRBC injection and once daily on consecutive days. Six days later the thickness of the left hind foot was measured with plethysmometer and was considered as control. The mice were then challenged by injecting the same amount of SRBC intradermally into the right hind foot paw. The foot thickness was measured again after 24 h.

Mice ($n=6$) in groups (I to VI) were immunized on day zero by injecting 200 µL of $5 \times 10^9$ SRBC/mL, intraperitonially (i.p.). Test drugs (Group III–VI) were administered for seven days including the day of immunization; however cyclophosphamide was administered once at zero day (Group II–VI). On day 7, the animals from groups I–VI were challenged intradermally (Table 4.4). After 24 hrs the foot paw thickness was measured by a plethysmometer. Group I was served as control.

![Figure 4.1 Development of DTH (Delayed Type Hypersensitivity) model](image)

Table 4.4 Dosing and challenge schedule protocol for SRBC induced delayed type hypersensitivity (DTH) study of Nimbatiktam

<table>
<thead>
<tr>
<th>Gr.</th>
<th>Group Name</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Control + 1% sodium carboxy methyl cellulose (sensitized)</td>
<td>Normal feed and normal saline</td>
<td>7 Days</td>
</tr>
<tr>
<td>II.</td>
<td>Cyclophosphamide (CP)</td>
<td>CP with dose of 50 mg/kg b.w.</td>
<td>Once, at Day 0</td>
</tr>
<tr>
<td>III.</td>
<td>Nimbatiktam</td>
<td>Nimbatiktam (200mg/kg BW)</td>
<td>7 Days</td>
</tr>
<tr>
<td>IV.</td>
<td>Nimbatiktam with CP</td>
<td>Nimbatiktam 200 mg/kg b.w. + CP (once)</td>
<td>7 Days, CP at Day 0</td>
</tr>
<tr>
<td>V.</td>
<td>Nimbatiktam</td>
<td>Nimbatiktam (400mg/kg BW)</td>
<td>7 Days</td>
</tr>
<tr>
<td>VI.</td>
<td>Nimbatiktam with CP</td>
<td>Nimbatiktam 400 mg/kg b.w. + CP (once)</td>
<td>7 Days, CP at Day 0</td>
</tr>
</tbody>
</table>

Results of DTH response of Nimbatiktam are summarised in Table 5.21.
4.6.5. Humoral response study by haemagglutination antibody titre using mice sera (in vivo)

Groups of six mice, each were immunized by injecting 200 μL (0.2 mL) of $5 \times 10^9$/mL intraperitonially (i.p.) on day zero and challenged seven days later by injecting an equal volume of SRBC intraperitoneally. Blood samples were collected on day +7 (before challenge) for primary antibody titre and on day +14 (7 days after challenge) for secondary antibody titre, haemagglutination antibody titre was determined following the micro titration technique described by Nelson and Mildenhall, 1967 (Nelson and Mildenhall, 1967). The value of the highest serum dilution causing visible haemagglutination was taken as titre. BSA saline was served as control. In micro titration plate, 50 μL of diluted serum sample (in 0.1 % normal saline, 1:1 v/v) was taken. Further, 50 μL volume of normal saline containing 0.1% BSA saline was added followed by addition of 1% SRBC suspension in BSA saline. After mixing the samples containing erythrocytes, it was settled at room temperature for about 90 minutes until control wells showed an equivocally negative pattern (small button). The test material at respective doses was administered orally from the day of immunization for fourteen consecutive days.

Mice (n=6) in groups (I to VI) were immunized on day 0 by injecting 200 μL of $5 \times 10^9$/mL i.p. Test drugs (I) was administered for the seven days including the day of immunization. Cyclophosphamide as standard immunosuppressant was administered once only on day zero of the experiment on +7 day, blood was collected from the retro orbital plexus from group I to VI for the primary antibody titres following which groups were challenged (200 μL) of $5 \times 10^9$ SRBC /mL (i.p). On day +14, blood samples were collected from the retro orbital plexus from all the groups for the secondary antibody titres (Table 5.22).

![Figure 4.2 Development of humoral response model](image-url)
Table 4.5 Dosing and challenge schedule protocol for SRBC induced humoral antibody (HA) titre study of Nimbatiktam

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Group Name</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (sensitized) 1% sodium carboxy methyl cellulose</td>
<td>Normal feed and normal saline</td>
<td>14 Days</td>
</tr>
<tr>
<td>II</td>
<td>Cyclophosphamide (CP)</td>
<td>CP with dose of 50 mg/kg b.w.</td>
<td>Once, at Day 0</td>
</tr>
<tr>
<td>III</td>
<td>Nimbatiktam 200 mg/kg b.w.)</td>
<td>Nimbatiktam (400 mg/kg b.w.)</td>
<td>14 Days</td>
</tr>
<tr>
<td>IV</td>
<td>Nimbatiktam 200 mg/kg b.w. + CP</td>
<td>Nimbatiktam (400 mg/kg b.w.) + CP (once)</td>
<td>14 Days, CP at Day 0</td>
</tr>
<tr>
<td>V</td>
<td>Nimbatiktam 400 mg/kg b.w.)</td>
<td>Nimbatiktam (400 mg/kg b.w.)</td>
<td>14 Days</td>
</tr>
<tr>
<td>VI</td>
<td>Nimbatiktam 400 mg/kg b.w. + CP</td>
<td>Nimbatiktam (400 mg/kg b.w.) + CP (once)</td>
<td>14 Days, CP at Day 0</td>
</tr>
</tbody>
</table>

Results of Humoral response of Nimbatiktam are documented in Table 5.22.
4.7. PHARMACOKINETIC STUDIES ON NIMBATIKTAM USING SALANNIN AND AZADIRACHTIN AS MARKER COMPOUNDS IN RAT PLASMA BY UPLC-MS/MS METHOD

4.7.1. Chemicals and reagents

Salannin (C$_{34}$H$_{44}$O$_{9}$; assigned purity >99.5%; M.P. 248°C) and Azadirachtin (C$_{34}$H$_{44}$O$_{9}$; assigned purity >95.0%; M.P. 159°C) was purchased from LGC promochem Pvt Ltd. India. LC-MS grade acetonitrile (Assigned purity 99.9%; Lot No; 90525) was purchased from Sigma-Aldrich, Germany. Ethyl acetate and dichloromethane were purchased from Merck Specialties Pvt. Ltd. (E. Merck, New Delhi, India). MS grade ammonium acetate and ammonium formate were obtained from Fluka analytical, Sigma-Aldrich, Netherland. Formic acid (assigned purity >98%; Lot No: 1439632) was commercially obtained from Fluka analytical, Sigma-Aldrich, Germany. Water used in the entire analysis was prepared in-house with Milli-Q water purification system procured from Millipore (Millipore Corporation, USA). Other chemicals used were of analytical grade from commercial sources.

4.7.2. UPLC conditions

UPLC was performed with a Waters ACQUITY UPLC™ system (Serial No# F09 UPB 920M; Model Code# UPB; Waters Corp., MA, USA) equipped with a binary solvent system, an auto-sampler, column manager and a MS detector autotunable (Serial No# JAA 272; Synapt; Waters, Manchester, UK). Chromatographic separation was performed on a Waters ACQUITY UPLC™ BEH C8 (100.0 mm x 2.1 mm; 1.7 µm) column at 40 ± 5 °C. The mobile phase for UPLC analysis consisted of acetonitrile-1.0 mM ammonium fomate (for Salannin and Azadirachtin), which was degassed. The flow rate of the mobile phase was kept at 0.25 mL min$^{-1}$. A fixed amount of 10 µL of sample solution was injected in each run. The total chromatographic run time was 2.0 min. The column and auto-sampler were maintained at 40 ± 5 and 4 ± 2 °C, respectively and the pressure of the system was 15000 psi.

4.7.3. Q-TOF-MS conditions

Mass spectrometry was performed on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) mass spectrometer. The nebulisation gas was set to 500 L h$^{-1}$, the cone gas set to 50 L h$^{-1}$ and the source temperature set to 100 °C. The
capillary voltage and sample cone voltage were set to 3.0 kV and 40 V, respectively. The Q-TOF Premier™ was operated in V mode with resolution over 8500 mass with 1.0 min scan time, and 0.02 s inter-scan delay. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the MassLynx V 4.1 software incorporated in the instrument. Argon was employed as the collision gas at a pressure of 5.3 x 10⁻⁵ Torr. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions of m/z 619.37→459.29 for salannin and m/z 743.16→725.21 for azadirachtin, respectively with a scan time of 2.0 min and 0.02 s inter-scan per transition. The optimum values for compound-dependent parameters like trap collision energy (Trap CE) and transfer collision energy (Tran CE) were set to 13.2 and 11.9 V, respectively for fragmentation information.

4.7.4. Calibration standards and quality control (QC) sample preparation

Calibration curve standards consisting of a set of eight non-zero concentrations (A–H) were prepared by 5% methanolic analytes spiking in blank rat plasma (50 μL aqueous aliquots to 950 μL blank rat plasma) yielding concentration range from 1–1000 ng and 11.12–5719.95 ng for salannin and azadirachtin, respectively. QC samples were prepared at four levels; LLOQQC (lower limit of quantification QC), LQC (Low QC), MQC (middle QC) and HQC (high QC) samples. The concentrations of LLOQQC, LQC, MQC, and HQC samples of salannin were 1, 5, 400 and 800 ng mL⁻¹, respectively. The concentration of LLOQQC, LQC, MQC, and HQC samples of azadirachtin were 11.12, 205.91, 915.19 and 2287.98 ng mL⁻¹, respectively. All the solutions were maintained at 2-8°C before use.

4.7.5. Bioanalytical method validation of salannin and azadirachtin

The method validation of salannin and azadirachtin in Wistar rat plasma was performed according to USFDA guidelines. The linearity of the method was determined by analysis of eight standards plots containing non-zero concentrations. Peak area ratios of analytes were utilized for the construction of calibration curves, using weighted (1/x²) linear least squares regression of the plasma concentrations and the measured peak area ratios (Figure 5.22 and 5.23 for salannin and azadirachtin, respectively). The LLOQQC was determined based on the signal-to-noise ratio of 10:1. For determining the intra-day accuracy and precision, replicate analysis of plasma samples of salannin was performed on the same day. The run
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consisted of a calibration curve and six replicates of LLOQ, LQC, MQC and HQC samples. The inter-day accuracy and precision were assessed by analysis of five precision and accuracy batches on three consecutive validation days. The six replicates were run for LLOQQC, LQC, MQC and HQC samples. The results for precision and accuracy batches of salannin and azadirachtin were summarized in Table 5.23 and Table 5.24, respectively.

4.7.6. Ex- vivo stability studies of salannin and azadirachtin

The stability of salannin and azadirachtin in Wistar rat plasma was evaluated by analyzing six replicates of plasma samples of LLOQQC, LQC, MQC and HQC which were exposed to different conditions (time and temperature). Percentage stability was determined as:

\[
\% \text{ Stability} = \left( \frac{\text{mean corrected response of stability stock}}{\text{mean response of comparison stock}} \right) \times 100.
\]

4.7.6.1. Freeze thaw stability

The freeze/thaw stability in plasma was evaluated for three consecutive freeze–thaw cycles from -20°C to room temperature. Six replicates of LLOQQC, LQC, MQC and HQC were analyzed after undergoing three freeze-thaw cycles.

4.7.6.2. Bench top stability

Bench top stability was determined for 24 h storage at room temperature, using six sets each of LLOQQC, LQC, MQC and HQC. The QC samples were quantified against the freshly spiked calibration curve standards.

4.7.6.3. Autosampler stability

In order to assess autosampler stability, six sets of LLOQQC, LQC, MQC and HQC samples were kept in an autosampler in polypropylene container programmed at 10°C and were analyzed after 110.67 hours along with freshly spiked samples, and the concentration was calculated against the freshly spiked calibration standards.

4.7.6.4. Injector stability

Short-term stability was determined after the exposure (of processed samples) at 10°C for 24 h in autosampler using six sets each of LLOQQC, LQC, MQC and HQC. After specified storage conditions, samples were processed and analyzed. The
analytes are considered to be stable when the precisions are below 15% and the accuracies are in the range of 85–115%, respectively for both levels.

4.7.6.5. Long term stability

The long-term stability was assessed after storage of the standard spiked plasma samples at deep freeze (−80°C) for one month. Six replicates of LQC and HQC were used for analysis.

The result of stability studies for salannin and azadirachtin in blank plasma samples were documented in Table 5.25 and 5.26, respectively.

4.7.7. Pharmacokinetic investigation in vivo

Male Wistar rats (n=6; 200–250 g, 6–8 weeks old) was taken from Central Animal House, Jamia Hamdard and kept in an environmentally controlled room (temperature: 25±2 °C, humidity: 60±5%, 12 h dark–light cycle) for 10 days before the experiments. Animals were fed on standard pelleted diet (Ashirwad Industries, Chandigarh, India) and water was provided ad libitum. The rats were fasted overnight before the day of the experiment. The animal protocol used in this study was approved by Jamia Hamdard Institutional Animal Ethics Committee (Protocol Number: 173/CPCSEA). Rats were fasted for 12 h with free access to water prior to the pharmacokinetic investigation. The bioanalytical method was implicated for quantitative estimation of salannin and azadirachtin in Wistar rat’s plasma after a single oral dose of formulation (8 mg kg⁻¹). Blood samples were collected in preheparinised glass tubes at different time intervals post-dosing (0, 0.5, 1, 1.5, 2, 4, 6, 12 and 16h). Blood samples were centrifuged (6000×g; 10 min; 4°C) to separate plasma fractions. The collected plasma samples were preserved for investigation at −80 °C, and thawed before analysis.

4.7.8. Sample preparation protocol for analysis of salannin and azadirachtin in rat plasma

4.7.8.1. Salannin

All the solutions (Calibration curve standards, QC samples and unknown plasma samples) were withdrawn from storage equipment area and allowed to thaw in wet ice bath and thereafter vortexes to ensure complete mixing of contents. Three hundred microlitre aliquot of each samples were taken into borosilicate glass tube.
Further, 200 μL of formic acid (5% w/v) was added and vortexed (300×g; 5 min). Four mL of ethyl acetate was added to the solution, then vortexed and kept on an ice bath for 5 min. The supernatant (2 mL) was transferred to borosil glass tubes and it was evaporated to dryness at 40 °C under a hot stream of nitrogen (30 psi; 5 min). The sample was reconstituted in 500 μL mobile phase solution (acetonitrile: ammonium formate buffer; 20: 80, v/v) and after vortexing for 10s at 300×g, the solution was transferred into the clean autosampler vials and 10 μL was injected into UPLC/QTOF-MS/MS system for analysis. All the sample processing was carried under low light condition.

### 4.7.8.2. Azadirachtin

All the solutions (Calibration curve standards, QC samples and unknown plasma samples) were withdrawn from storage equipment area and allowed to thaw in wet ice bath and thereafter vortexed to ensure complete mixing of contents. Three hundred microlitre aliquot of each samples were taken into borosilicate glass tube. Further 200 μL of orthophosphoric acid (5% w/v) was added and vortexed (300×g; 5 min). Four mL of tertiary butyl methyl ether (TBME) was added to the solution, then vortexed and kept on an ice bath for 5 min. The supernatant (2 mL) was transferred to borosil glass tubes and it was evaporated to dryness at 40 °C under a hot stream of nitrogen (30 psi; 5 min). The sample was reconstituted in 500 μL mobile phase solution (acetonitrile: ammonium acetate buffer; 20: 80, v/v) and after vortexing for 10s at 300×g, the solution was transferred into the clean autosampler vials and 10 μL was injected into UPLC/QTOF-MS/MS system for analysis.

### 4.7.9. Analysis of pharmacokinetic parameters of salannin and azadirachtin in Nimbatiktam

Concentration–time curves were established for each analyte and used for the determination of pharmacokinetic parameters such as peak plasma concentration \( C_{\text{max}} \), peak time \( T_{\text{max}} \), extent of absorption (AUC) and half-life \( T_{0.5} \), by a noncompartmental analysis using PK Solutions Version 2.0 (Summit Research Services, USA).

The peak plasma concentration-time graph of salannin and azadirachtin has been depicted as Figure 5.24 and Figure 5.25, respectively. The results of pharmacokinetic parameters are documented in Table 5.27 and Table 5.28 for salannin and azadirachtin, respectively.
4.8. COLUMN CHROMATOGRAPHIC ISOLATION, CHARACTERISATION, AND IDENTIFICATION CONSTITUENTS OF NIMBATIKTAM

4.8.1. Apparatus, chemicals and instruments

- All the chemicals and reagents were obtained from S.D. fine Chemicals and were of analytical (AR) grade.
- Sodium sulphate was used as drying agent for various solvents used to run the column.
- Silica gel (Qualigens), 60-120 mesh for column packing and silica gels G (Qualigens) for TLC, were used for chromatographic isolation; spots were visualized by exposure to iodine vapours and UV radiation.
- All the weighing were done on a single pan mettler balance.
- Melting points were determined on Perfit melting point apparatus.
- Ultraviolet spectra were recorded on Shimadzu spectrophotometer in methanol.
- Infrared spectra were recorded on Bio-Red FTIR Spectrophotometer using KBr pellets; \( v_{\text{max}} \) values are given in cm\(^{-1}\).
- \(^1\)H NMR spectra were screened on Bruker spectrospin 300 MHz instrument using CDCl\(_3\) as solvent and TMS as an internal standard. Chemical shift values are given in ppm scale and coupling constants (J) in Hz.
- \(^13\)C NMR spectra were recorded on Bruker Spectrospin 100 MHz in 5 mm spinning tubes at 27 °C.
- Mass spectra were scanned by effecting ESI ionization at 70 eV on a JEOL-JMS-DX 303 instrument equipped with direct inlet probe system.

4.8.2. Preparation of slurry

Nimbatiktam (87 g) was taken in a china dish and heated continuously on a water bath by gradually adding methanol in small portions with constant stirring, till the desired consistency was obtained. Silica gel (220 g) column grade was added with continuous mixing until the whole methanolic extract get adsorbed on silica gel. It was air-dried and finally passed through sieve # 8 to get free floor uniform particle size.
4.8.4. Homogeneity of fractions
The fractions collected were subjected to thin layer chromatography (TLC) to check the homogeneity of various fractions. Chromatographically identical fractions (having same $R_f$ values) were combined together and concentrated. They were then crystallized with suitable solvent system.

4.8.5. Isolation of phytoconstituents
The extract was dissolved in a minimum amount of methanol and adsorbed on silica gel (60-120 mesh) for preparation of slurry. The air-dried slurry was chromatographed over the silica gel column packed in petroleum ether. The column was eluted with petroleum ether, chloroform, methanol and their mixtures in order of increasing polarity to isolate the compounds.

4.8.6. Identification and characterization of phytoconstituents
The isolated compounds were identified by using various sophisticated techniques like UV spectroscopy, IR spectroscopy, NMR and Mass spectroscopy.
SECTION B: EXPERIMENTAL: LAJJALU KERAM AND 777 OIL
(Dermal Formulations)

4.1. QUALITY CONTROL AND STANDARDISATION OF LAJJALU KERAM AND 777 OIL

4.1.1. Organoleptic property
Color, odor and taste of the drug were studied, as per protocol.

4.1.2. Density
Ten mL of drug was taken in pycnometer and it is weighed on electronic balance. Density was calculated in wt/mL.

4.1.3. Viscosity
Ten mL of sample was taken and poured into Ostwald viscometer. The viscosity was calculated with respect to water.

4.1.4. Refractive index
One drop of sample was analyzed by Abbe’s refractometer and average refractive index for Lajjalu Keram, and 777 oil was calculated, respectively.

4.1.5. Determination of rancidity
One mL of sample oil (Lajjalu Keram and 777 oil) was mixed with 1 mL of concentrate hydrochloric acid in a test tube followed by addition of 1.0 mL of 1% solution of phloroglucinol in diethyl ether. It was thoroughly mixed and color changed was observed.

4.1.6. Acid value
Five mL of sample (Lajjalu Keram and 777 oil) was dissolved in 50 mL of a mixture of equal volumes of alcohol and ether (previously neutralized with 0.1 N sodium hydroxide using phenolphthalein as an indicator) contained in a flask. The flask was connected with a suitable condenser and warmed slowly, with frequent shaking, until it dissolves. Added one mL of phenolphthalein, and titrated with 0.1 N sodium hydroxide until it remains faintly pink after shaking for 30 seconds.

The acid value was calculated by using following formula.
Acid Value = (5.61 X N)/W

Where,
N= Volume of KOH consumed in mL
W= Wt of sample in gram

4.1.7. Saponification value

Substance was dissolved in a tarred, 250 mL flask, and 25.0 mL of 0.5 N alcoholic potassium hydroxide was added. The flask was heated on a steam bath, under a suitable condenser to maintain reflux for 30 minutes, frequently rotating the contents. Then added 1 mL of phenolphthalein, and titrated the excess potassium hydroxide with 0.5 N hydrochloric acid. A blank determination was also performed under the same conditions.

The saponification value was calculated by using following formula.

\[
\text{Saponification value} = \frac{(B-A) \times 28.05}{W}
\]

Where,
B= Volume (in mL) of HCl consumed by Blank KOH
A= Volume (in mL) of HCl consumed by Sample + KOH
W = Wt of sample in gram

4.1.8. Ester value

The Ester value was determined by taking difference in calculated saponification value and acid value.

\[
\text{Ester Value} = \text{Saponification value} - \text{Acid value}
\]

The results of quality control parameters for Lajjalu Keram and 777 oil was summarised in Table 5.30 and 5.31, respectively.

4.1.9. Phytochemical investigations

Lajjalu Keram and 777 oil were screened for presence/absence of various classes of chemical constituents as per the protocols for oral formulation (Experimental Section A Part 4.1.11.). The results were summarized in Table 5.32 and 5.33 for Lajjalu Keram and 777 oil, respectively.
4.1.10. Determination of microbial contamination of Lajjalulu Keram and 777 oil

4.1.10.1. Total fungal count

Media used

Potato dextrose agar (PDA)

Composition of media

- Potato dextrose agar (PDA): 39 g
- Distilled water up to: 1000 mL
- pH: 6.3-6.5

Procedure

The different dilution of Lajjalulu Keram and 777 oil were made in sterile distilled water aseptically and poured into petriplates containing solidified and sterile PDA media. The media containing different diluted drug samples were incubated for 48 h in BOD incubator at 22°C temperature and colonies were counted. Results are shown in Figure 5.28 and summarised in Table 5.34.

4.1.10.2. Total bacterial count

Media used

Casein soyabean digest agar medium

Composition of media

- Pancreatic digest of casein: 15.0 g
- Papaic digest of soyabean meal: 5.0 g
- Sodium chloride: 5.0 g
- Agar: 15.0 g
- Distilled water up to: 1000 mL
- pH: 7.3 ± 0.2

Procedure

The different dilution of Lajjalulu Keram and 777 oil was made in sterile distilled water aseptically and poured into three petriplates containing solidified and sterile Casein soyabean agar media. The media containing different diluted drug samples were incubated for 18 h in BOD incubator at 37°C temperature and colonies were counted. Results are shown in Figure 5.29 and summarised in Table 5.35.
4.2. CHEMICAL TOXICITY EVALUATION OF LAJJALU KERAM AND 777 OIL

4.2.1. Analysis of heavy metals in Lajjalu Keram and 777 oil using Atomic absorption spectroscopy (AAS)

Heavy metal analysis was carried out as per the method described by Horwitz, 1970 and Chu et al., 2010. The ground drug (1.0 g) was incinerated in a silica crucible at a temperature 600°C. Residue was taken in the reaction vessels and dissolved in 6 mL of concentrated HCl followed by 2 mL of concentrated HNO₃ and left to stand overnight. The suspension obtained was heated slowly for 2 h under reflux condenser for complete digestion. The volume was made up to 100 mL with distilled water. This sample was analyzed by atomic absorption spectrometer and content was evaluated forming regression equation obtained from standard plot for lead, cadmium, mercury, and arsenic.

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

The aflatoxin in herbal drugs was analyzed by the HPLC fluorescence method as discussed by Horwitz, 1970 and Tagami et al., 2007.

a) Extraction of aflatoxins: The Association of Analytical Chemist (AOAC) official method of analysis was followed. In this method, 15 g each of Lajjalu Keram and 777 oil was dissolved in 100 mL methanol (containing 25 % 0.1M HCl) and partitioned with 10 % NaCl (in water w/v) and hexane. In aqueous layer added dichloromethane (DCM). Process was repeated for 2-3 times and the collected DCM extract was evaporated up to 2-3 mL. This extract was passed through silica column followed by washing of the column with a mixture of benzene: acetic acid (9:1, v/v) and ether: hexane (3:1, v/v). Finally aflatoxin was eluted with 100 mL of DCM: acetone (90:10, v/v). The eluted aflatoxin was concentrated upto 5.0 mL and was dried over nitrogen.

b) Derivatization of extracted aflatoxins: Dried extract was taken and added 200 µL of hexane and 50 µL of trifluoroacetic acid. It was mixed on vortex mixer exactly for 30 seconds, stand for five minutes and 1.95 mL of water: acetonitrile (9:1, v/v) mixture was added. This sample was used for HPLC analysis after filtration with 0.22 µm filter.
c) Derivatization of standards: Taken known concentrations (20 ppb, 40 ppb, and 80 ppb) of standard aflatoxin B1, G1, B2 and G2 and were derivatized in the same manner as for sample.

c) HPLC analysis for aflatoxins: Twenty microlitre of derivatized sample (Both extract and standards) were injected into HPLC column (C18; 15 cm x 4.6 mm) at a flow rate of 1.0 mL/min using water: acetonitrile: methanol (70:17:17, v/v) as mobile phase and fluorescent detector. The peaks of aflatoxin in formulations were compared with peak of standards (B1, G1, B2 and G2) and were analyzed. The HPLC chromatogram of standard aflatoxins are shown at Figure 5.7, whereas HPLC chromatogram of aflatoxins obtained from Lajjalu Keram and 777 oil are shown in Figure 5.30 and 5.31, respectively. Results of aflatoxin analysis in Lajjalu Keram and 777 oil are summarized in Table 5.37.

4.2.3. Analysis of pesticides in Lajjalu Keram and 777 oil using GC-MS

The pesticide analysis of herbal drugs was carried out using GC-MS method as discussed by Horwitz, 1970 and Weaver and Truckess, 2010. Accurately weighed, 50 g of sample was dissolved into methanol and added one gm of sodium oxalate. It was taken into separating funnel and added diethyl ether (50 mL) and petroleum ether (50 mL). It was shaken for one minute. Organic layer was transferred into another separating funnel and added 600 mL of water with saturated solution of sodium chloride solution. Aqueous layer was discarded and this process was repeated for 2-3 times. Organic layer was then passed through sodium sulphate and evaporated to 2-5 mL. This concentrated solution was again mixed with acetonitrile (30 mL) and petroleum ether (30 mL) and it was eluted with diethyl ether over silica column. The ether elute was concentrated to five mL in rotavapor and analysed by GC-MS. GC-MS analysis was performed using Agilent system (7890A series, Germany), operated by GC Chemstation software and equipped with a split/splitless injector. All pesticides were resolved in a HP-5 (30m x 0.32mm, 0.25 μm film thickness) column. The GC oven programme was as follows: 60 °C hold 2 min, rate 30 °C/min to 200 °C, rate 3 °C/min to 230 °C, rate 4 °C/min to 300 °C, with a total acquisition program of 34.17 min. Helium was used as a carrier gas with a column head pressure of 9.92 psi. Detector temperature was set at 300 °C. Injector was operating in the splitless mode. Mass spectra were acquired in EI mode (70 eV); in m/z range 30–600. Results of pesticide analysis in Lajjalu Keram and 777 oil have been summarized in Table 5.38.
4.3. ACUTE DERMAL TOXICITY STUDIES OF LAJJALU KERAM AND 777 OIL ON INTACT SKIN OF WISTAR RAT

4.3.1. Selection of animal species

The eight week old adult wistar rats (115-160 gm) with healthy intact skin was taken having weight which was fall in an interval within ± 20 % of the mean initial weight.

4.3.2. Number and sex

Five animals from each group (male and female) were used at each dose level i.e. 1000, 2000, 5000 mg/kg BW.

4.3.3. Housing and feeding condition

The animals were caged individually. The temperature of the experimental animal room was kept at 22 ± 3°C and the relative humidity was maintained at 30-70 per cent. Artificial lighting was made with a sequence of 12 h light and 12 h dark. A conventional laboratory diet was used for feeding. Unlimited supply of drinking water was provided.

4.3.4. Grouping of animals

Healthy young adult animals were acclimatized to the laboratory conditions for five days prior to the test. The animals were randomized and assigned identification mark to the treatment and control groups. Four groups were prepared, approximately 24 h before the test, fur was removed from the dorsal area of the trunk of the test animals by shaving. Not less than 10 % of the body surface area was cleared for the application of the test substance.

4.3.5. Application of drug

The drug dose was applied to each group of shaved animals as shown in Table 4.6. The study protocol for acute dermal toxicity studies of Lajjalu Keram and 777 oil are summarised in Table 4.7.
Table 4.6 Groups and treatment for acute dermal toxicity studies of Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>Group (5Male + 5 Female in each group)</th>
<th>Treatment (Single application on first day and observation on 14th day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (Control)</td>
<td>No drug was applied</td>
</tr>
<tr>
<td>Group II (Lajjalu Keram low dose Male)</td>
<td>The group of animals were applied with Lajjalu Keram in low dose (1000 mg/kg body weight)</td>
</tr>
<tr>
<td>Group III (Lajjalu Keram medium dose)</td>
<td>The group of animals were applied with Lajjalu Keram in medium dose (2000 mg/kg body weight)</td>
</tr>
<tr>
<td>Group IV (Lajjalu Keram high dose)</td>
<td>The group of animals were applied with Lajjalu Keram in high doses (5000 mg/kg body weight)</td>
</tr>
<tr>
<td>Group V (777 oil low dose Male)</td>
<td>The group of animals were applied with 777 oil in low dose (1000 mg/kg body weight)</td>
</tr>
<tr>
<td>Group VI (777 oil medium dose)</td>
<td>The group of animals were applied with 777 oil in medium dose (2000 mg/kg body weight)</td>
</tr>
<tr>
<td>Group VII (777 oil high dose)</td>
<td>The group of animals were applied with 777 oil in high doses (5000 mg/kg body weight)</td>
</tr>
</tbody>
</table>

Table 4.7 Study protocol for acute dermal toxicity studies of Lajjalu Keram and 777 oil

<table>
<thead>
<tr>
<th>Name of the study</th>
<th>Acute dermal toxicity study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test material</td>
<td>Lajjalu Keram and 777 oil</td>
</tr>
<tr>
<td>Animal model</td>
<td>Wistar rats</td>
</tr>
<tr>
<td>Age</td>
<td>8 - 12 weeks</td>
</tr>
<tr>
<td>Weight</td>
<td>180 gm (Average)</td>
</tr>
<tr>
<td>Animal procured from</td>
<td>Central animal facility, Jamia Hamdard (173/CPCSEA)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male and Female</td>
</tr>
<tr>
<td>Number of animal per group</td>
<td>5 per group</td>
</tr>
<tr>
<td>Groups</td>
<td>4 groups</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Dermal application</td>
</tr>
<tr>
<td>Number of administration</td>
<td>Single application on first day and observation upto 14th day</td>
</tr>
<tr>
<td>Concentration of dose</td>
<td>1000, 2000, 5000 mg per kg body weight</td>
</tr>
<tr>
<td>Study duration</td>
<td>Observation for 14 days</td>
</tr>
<tr>
<td>Parameters observed</td>
<td>Cage side observations, weekly body weight and daily mortality record etc.</td>
</tr>
</tbody>
</table>

4.3.6. Observations
General clinical observations were made once daily at the same time up to 14 days and recorded. All animals were inspected for signs of morbidity and mortality at least twice daily, usually at the beginning and end of each day. Once prior to the first exposure and once a week thereafter, detailed clinical observations were made in all animals. These observations were made outside the home cage in a standard area and at similar time on each occasion. Signs noted including changes in skin, fur, eyes (dullness, opacities), occurrence of secretions and excretions and autonomic activity (e.g., pupil size, and unusual respiratory pattern). Changes in gait, condition of teeth and breathing abnormalities has also been recorded. Subcutaneous swelling and abdominal distension were also observed. Results were summarized in Table 5.39 and mortality records have been given in Table 5.40.

4.3.7. Body weight observation

Individual weights of animals were determined before the test substance is applied on skin and at least weekly thereafter. Changes in weight were calculated and recorded (Table 5.42-5.44). Toxicity sign was also observed and results were summarised in Table 5.45. At the end of the test, surviving animals are weighed and then humanely killed.

4.3.8. Histopathology of skin

Histopathology of skin was carried out of control and high dose groups. The observations were shown in Figure 5.32 and results are summarized in Table 5.46.
4.4. ESTIMATION OF CONSTITUENTS (PHENOLICS, FLAVONOIDS AND DETERMINATION OF TOTAL FATTY ACIDS) OF LAJJALU KERAM AND 777 OIL

The total flavonoid and phenolic content of Lajjalu Keram and 777 oil was carried out using spectrophotometric method described by Qayyoom et al., 2009. The GC-FID analysis was done by modified method as described by Yu et al., 2006.

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

Reagents

Aluminium chloride (AlCl₃) (0.1 gm/mL), Sodium acetate (CH₃COONa) (1 M), different dilutions of rutin standard (10 - 100 µg/mL).

Method

The 10 mg/mL solution each of Lajjalu Keram and 777 oil was prepared in methanol and 0.5 mL was added to test tube containing 1.5 mL methanol. Further, 0.1 mL of AlCl₃ and 0.1 mL of CH₃COONa were added, followed by addition of 2.8 mL distilled water. The mixture was kept for 30 minutes at room temperature and absorbance was taken at 415 nm. Similar procedure was followed for standard rutin and control (omitting substance).

After taking the absorbance of standard dilutions as mentioned above, calibration curve was plotted (Figure 5.14). Flavanoid content in Lajjalu Keram and 777 oil were calculated by using standard calibration curve and results were summarised in Table 5.47.

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

Reagents

Folin-ciocalteu reagent (FCR) (10% in distilled water), Sodium carbonate (Na₂CO₃) (1 M in distilled water) and Gallic acid (standard) 1.0 mg/mL solution in methanol. Different dilutions of standard gallic acid (25 - 300 µg/mL) in methanol.

Method

Half mL of sample (10 mg/mL) each of Lajjalu Keram and 777 was added in test
tube containing 5 mL of FCR. The mixture was shaked and 4 mL of sodium carbonate solution was added. The absorbance was read at 765 nm after 15 minutes. Similar procedure was followed for samples and blank (omitting substance).

The calibration curve was plotted (Figure 5.15) and total phenolic content in Lajjalu Keram was calculated by using regression equation and results were recorded in Table 5.48.

4.4.3. Estimation of fatty acid constituents in Lajjalu Keram and 777 oil using GC/FID analysis

Instrumentation

The GC analysis of Lajjalu Keram and 777 oil was performed on Perkin Elmer Clarus 500™ equipped with autosampler using Supelco wax 10 column (30 X 0.25 mm; film thickness 0.25 μM). The carrier gas used was hydrogen at 10 psi flow pressure; oven temperature was programmed at 130°C held for 5 minutes and raised at 4°C/min to a final temperature at 240°C and held for 12.5 minutes. The injector temperature was 260°C and injection volume was 1.5 μL. Detector used was flame ionization detector (FID) and detector temperature was set at 290°C.

Identification of fatty acid

The fatty acid methyl ester was identified by GC-FID by comparison of their retention time with those of reference standard available in the laboratory and analyzed under same condition. The chromatograms of GC/FID analysis of Lajjalu Keram and 777 oil were shown in Figure 5.33 and Figure 5.34, respectively. The fatty acid composition was expressed as percentage of total fatty acid methyl ester in the oil. The composition of different type of fatty acid present in Lajjalu Keram were presented in Table 5.51. whereas composition of different type of unsaturated fatty acid present in Lajjalu Keram were tabulated in Table 5.52. The composition of different type of fatty acid and unsaturated fatty acid present in 777 oil was also observed and summarised in Table 5.53 and Table 5.54, respectively.
4.5. **ANALYTICAL METHOD DEVELOPMENT (HPLC QUANTIFICATION) OF MIMOSINE IN LAJJALU KERAM**

The HPLC analysis of mimosine in Lajjalu Keram was carried out using newly developed validated and published method by us (Musthaba *et al.*, 2011a).

4.5.1. Chemicals

HPLC grade Water was purchased from Merck, Mumbai, India. Mimosine was purchased from CDH, New Delhi, India. Other chemicals and reagents were of AR grade.

4.5.2. Chromatographic condition

The method was conducted using a reversed-phase technique. Chromatography was performed, under ambient conditions, with Shimadzu HPLC equipment comprising quaternary LC-10A VP pumps, a variable-wavelength programmable UV-visible detector, SPD-10AVP column oven, and a SCL 10AVP system controller. Samples (20 μL) were injected by means of a Rheodyne injector fitted with a 20 μL loop. The instrumentation was controlled by use of Class-VP 5.032 software. Compounds were separated on a 250 × 4.6 mm, 5-μm particle, C18 reversed phase column (Phenomenex). The mobile phase was water: orthophosphoric acid (98.8:0.2, v/v) filtered through a 0.22 μm membrane filter (Millipore) and sonicated before use; pH 3.0 was adjusted with orthophosphoric acid; mimosine was eluted isocratically with a flow rate of 1.0 mL min⁻¹. The eluate mimosine was monitored by UV detector at wavelength of 284 nm.

4.5.3. Preparation of stock solution

A standard solution of mimosine was prepared by dissolving appropriate amount of the compound in water to give a final concentration of 500 μg/mL. Standard solutions of mimosine 5-500 μg/mL were prepared by subsequent dilution and an aliquot (20 μL) of the diluted solution was injected into column. Each solution was chromatographed in triplicate. The corresponding peak areas were plotted against the concentration of the compound injected (Table 5.53).

4.5.4. Preparation of sample solution

Two grams of Lajjalu Keram was transferred in volumetric flask (25 mL) containing 15 mL water and sonicated for 10 minutes at room temperature. The resultant
solution was filtered through 0.22 μm membrane filter, which was used for the analysis after making the volume up to 25 mL with distilled water.

4.5.5. Method Validation

The developed chromatographic method was validated as per ICH guidelines and several other methods reported by laboratory (Baboota et al., 2007; Alam et al., 2009 and Kamal et al., 2011) for the linearity, accuracy, precision, limit of detection, limit of quantification and robustness.

**Linearity**

A stock solution of mimosine (500 μg mL⁻¹) was prepared by dissolving 5 mg drug in 10 mL methanol and solutions of different concentration (5-500 μg mL⁻¹) are prepared in aliquot dilution for construction of calibration plots from the stock solution. The prepared dilutions were injected in series, and concentrations were plotted against peak area obtained (Table 5.53).

**Accuracy**

Accuracy was determined by the standard addition method. Previously analyzed samples of mimosine were spiked with 0, 50, 100, and 150% extra mimosine standard and the mixtures were reanalyzed by the proposed method. The experiment was performed in triplicate. Recovery (%), RSD (%), and standard error of mean (SEM) were calculated for each concentration (Table 5.54).

**Precision**

Precision was determined as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. For both intra-day and inter-day variation, solutions of mimosine at four different concentrations (100, 200, 300, and 500 μg mL⁻¹) were determined in triplicate (Table 5.55).

**Limit of Detection (LOD) and limit of quantification (LOQ)**

In the present chromatographic condition the limits of detection and the limits of quantification were determined by blank determination method using s/n (signal-to-noise ratio). The signal-to-noise was calculated using LC Solution software.
Robustness

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of mimosine. Robustness was determined by changing the flow rate of mobile phase to 0.8 and 1.2 mL min\(^{-1}\) and the wavelength of detection to 283 and 285 nm (Table 5.56).

4.5.6. Analysis of mimosine in Lajjalu Keram

Analysis of mimosine in Lajjalu Keram was carried out by the optimized and validated HPLC method and the values of chromatographic parameters were comparable with the standard.
4.6. ANALYTICAL METHOD DEVELOPMENT (HPLC QUANTIFICATION) OF RUTIN IN 777 OIL

The quantitative determination of rutin was done by newly developed, validated and published HPLC method by us (Musthapa et al., 2011b).

4.6.1. Chemicals

HPLC grade methanol, HPLC water was purchased from Merck, India. Rutin was purchased from Sigma, India. Other chemicals and reagents were of AR grade.

4.6.2. Chromatographic condition

The method was conducted using a reversed-phase technique. Chromatography was performed, under ambient conditions, with Shimadzu HPLC equipment comprising quaternary LC-10A VP pumps, a variable-wavelength programmable UV–visible detector, SPD-10AVP column oven, and a SCL 10AVP system controller. Samples (20 µL) were injected by means of a Rheodyne injector fitted with a 20 µL loop. The instrumentation was controlled by use of Class-VP 5.032 software. Compounds were separated on a 250 × 4.6 mm, 5 µm particle, C\textsubscript{18} reversed phase column (Phenomenex). The mobile phase was methanol: water (60: 40, v/v). PH adjusted to 3.0 with ortho phosphoric acid (60 : 40). The mobile phase was sonicated and filtered through a 0.22 µm membrane filter (Millipore). Rutin was eluted isocratically with a flow rate of 1.0 mL/min. The eluate rutin was analyzed by UV detector at wavelength of 360 nm.

4.6.3. Preparation of stock solution

A standard solution of rutin was prepared by dissolving an appropriate amount of the compound in methanol to give a final concentration of 1000 µg/mL. Standard solutions of rutin 1-1000 µg/mL were prepared by subsequent dilutions and an aliquot (20 µL) of the diluted solution was injected into column. Each solution was chromatographed in triplicate. The corresponding peak areas were plotted against the concentration of the compound injected (Table 5.57).

4.6.4. Preparation of sample solution

Two grams of 777 oil was transferred in a volumetric flask (25 mL) containing 15 mL methanol and sonicated for 10 minutes at room temperature. The resultant
solution was filtered through 0.22 μm membrane filter which was used for the liquid chromatographic (LC) analysis after making the volume up to 25 mL with methanol.

4.6.5. Method validation

The developed chromatographic method was validated as per ICH guidelines and several other methods reported by laboratory (Zafar et al., 2005 and Ahmad et al., 2011) for the linearity, accuracy, precision, limit of detection, limit of quantification and robustness.

*Linearity*

A stock solution of rutin (1000 μg mL⁻¹) was prepared by dissolving 10 mg drug in 10 mL methanol and solutions of different concentration (1-1000 μg mL⁻¹) are prepared in aliquot dilutions for construction of calibration plots from the stock solution. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area (Table 5.57).

*Accuracy as recovery*

Accuracy was determined by the standard addition method. Previously analyzed samples of rutin were spiked with 0, 50, 100, and 150% extra rutin standard and the mixtures were re-analyzed by the proposed method. The experiment was performed in triplicate. Recovery (%), %RSD and standard error of mean (SEM) were calculated for each concentration. The results obtained are summarised in Table 5.58.

*Precision*

Precision was determined as both repeatability and intermediate precision, in accordance with the ICH recommendations. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. For both intra-day and inter-day variations, solutions of rutin at four different concentrations were determined in triplicate. The results are given in Table 5.59.

*Detection (LOD) and quantification (LOQ) limits*

In the present chromatographic condition, the limits of detection and the limits of quantification were determined by blank determination method using s/n (signal to
noise ratio) of three and ten, respectively. The ratio of signal-to-noise was calculated using LC Solution software.

**Robustness**

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions on the determination of rutin. Robustness was determined by changing the mobile phase flow rate to 0.8 and 1.20 mL min\(^{-1}\) and the concentration of methanol in the mobile phase to 57 and 63%. The results are documented in Table 5.60.

4.6.6. Analysis of rutin in 777 oil

The analysis of rutin in 777 oil, was carried out by the optimized and validated HPLC method and the values of chromatographic parameters were found close to the standards.
4.7. DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF LAJJALU KERAM AND 777 OIL (DERMAL FORMULATION)

Anti-inflammatory activity of Lajjalu Keram and 777 oil were carried out against carrageenan induced rat paw edema as per the method described by Balian using digital plethysmometer (Balian et al., 2006).

4.7.1 Animals

Adult male Wistar rats of 12 weeks age (150-250 g) were used for the study. Animals were procured from and kept at central animal house facility of the Hamdard University. Animals were housed at 22 ± 3 °C and 30-70% relative humidity with a sequence of 12 h light and 12 h dark. Animals were allowed free access to standard pellet diet and water ad libitum.

4.7.2 Procedure

Animals were randomly divided into four groups (n=6) as control, Lajjalu Keram, 777 oil and Volini gel® (Ranbaxy). Respective formulations were applied to the right hind paw of different groups by rubbing 50 times on the plantar region with the index finger. Control group was untreated. After 30 minutes of the application of different formulations 0.1 mL of 1% w/v homogenous suspension of carrageenan in distilled water was injected in the sub-plantar region of right hind paw of all the four groups including control. Paw volume was recorded immediately before carrageenan injection using a digital plethysmometer and regarded initial paw volume. Further, paw volume was recorded at duration of 1, 2, 4, 6, 12 and 24 h after carrageenan injection. Percentage inhibition of edema was calculated by using the following formula:

\[
\text{% Inhibition at X hours} = \frac{\text{Final volume of Paw (X hours)} - \text{Initial Paw volume}}{\text{Initial paw volume}} \times 100
\]
SECTION C: EVALUATION OF SHELF LIFE OF NIMBATIKTAM, LAJJALU KERAM AND 777 OIL

The shelf life of Nimbatiktam, Lajjalu Keram and 777 oil were evaluated as per ICH guideline (ICHQ1AR2C, 2003).

The shelf life of Nimbatiktam was analysed by evaluating the organoleptic character, pH, extractive value, HPTLC fingerprint and assay of Nimbatiktam by HPTLC analysis at different time intervals. Assay of three batches of Nimbatiktam were examined immediately after production (0 months), 3 months and after six months of storage of drug on shelf at room temperature using the analysis methods of drugs as given in experimental section 4.1 (A) (Table 5.62).

Shelf life of Lajjalu Keram and 777 oil were determined by assay of mimosine (in Lajjalu Keram) and Rutin (in 777 oil) using newly developed and validated HPLC method. Other organoleptic properties (Physical appearance, colour, odour and taste), acid value, viscosity and GC fingerprint were also analysed at different time intervals up to six months after storing the drug on shelf at room temperature (Table 5.63).

The assay data was further evaluated using Sigmaplot™ 11, (Cranes software International, Bangalore, India) and percentage drug remaining was plotted against time in months. The shelf life was determined using this plot as the time at which the 95% one side confidence limits for the mean curve intersects the acceptance criteria of 90% drug remaining.