CHAPTER 4
MATERIAL AND
METHODS
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4.1. Standardisation of Method

The following methods were optimized for testing of antithrombotic and thrombolytic activity.

Method-1

1. 5 test tubes were taken and labeled 1 to 5.
2. In each test tube, 2 ml non oxalated blood (of rabbit) was taken.
3. 0.5 ml of Streptokinase (5000 units/ml) was added in tube no. 2 and 3.
4. All test tubes were incubated at 37°C.
5. After some time when clot was formed, 0.5ml of Streptokinase was added to tube no. 4 and 5.
6. The time of clot formation and clot dissolution was noted.
7. Tube no. 1 used as blank.

Method-2

1. 5 test tubes were taken and labeled 1 to 5.
2. In each test tube, 2 ml non oxalated blood (of rabbit) was taken.
3. Streptokinase (5000 units/ml) was added as follows-
   3.1. 0.25ml in tube no. 2.
   3.2. 0.5ml in tube no. 3.
   3.3. 1.0ml in tube no. 4.
4. All test tubes were incubated at 37°C.
5. After some time when clot was formed, 0.5ml of Streptokinase was added to tube no. 5.
6. The time of clot formation and clot dissolution was noted.
7. Tube no. 1 used as blank.

**Method-3**

1. 5 test tubes were taken and labeled 1 to 5.
2. In each test tube, 2 ml non oxalated blood (of rabbit) was taken.
3. All test tubes were incubated at 37°C.
4. After some time when clot was formed, Streptokinase (5000 units/ml) was added as follows-
   4.1. 0.1 ml in tube no. 2.
   4.2. 0.2 ml in tube no. 3.
   4.3. 0.3 ml in tube no. 4.
   4.4. 0.4 ml in tube no. 5.
5. The time of clot formation and clot dissolution was noted.
6. Tube no. 1 used as blank.
4.1.1 Observations:

- SK- Streptokinase
- NS- Normal saline

Table 4.1: For Method 1-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:32am</td>
<td>----</td>
<td>1.5ml</td>
<td>----</td>
<td>10:40am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:32am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>10:32am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>10:52am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>10:52am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>10:52am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>11:02am</td>
<td>11:00am</td>
<td>11:15am</td>
</tr>
<tr>
<td>5.</td>
<td>11:00am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>11:09am</td>
<td>11:08am</td>
<td>11:18am</td>
</tr>
</tbody>
</table>

- 2 ml non oxalated blood (of rabbit) was taken in each tube.

Table 4.2: For Method 2-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>11:31am</td>
<td>----</td>
<td>1.5ml</td>
<td>----</td>
<td>11:34am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>11:31am</td>
<td>0.25ml</td>
<td>1.25ml</td>
<td>11:31am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>11:24am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>11:24am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>11:24am</td>
<td>1.0ml</td>
<td>0.5ml</td>
<td>11:24am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>5.</td>
<td>11:30am</td>
<td>0.5ml</td>
<td>1.0ml</td>
<td>11:38am</td>
<td>11:36am</td>
<td>11:45am</td>
</tr>
</tbody>
</table>

- 2 ml non oxalated blood (of rabbit) was taken in each tube.
Table 4.3: For Method 3-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>11:03am</td>
<td>----</td>
<td>1.5ml</td>
<td>----</td>
<td>11:07am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>11:03am</td>
<td>0.1ml</td>
<td>1.4ml</td>
<td>11:08am</td>
<td>11:07am</td>
<td>Not</td>
</tr>
<tr>
<td>3.</td>
<td>11:09am</td>
<td>0.2ml</td>
<td>1.3ml</td>
<td>11:15am</td>
<td>11:14am</td>
<td>11:29am</td>
</tr>
<tr>
<td>4.</td>
<td>11:09am</td>
<td>0.3ml</td>
<td>1.2ml</td>
<td>11:14am</td>
<td>11:13am</td>
<td>11:24am</td>
</tr>
<tr>
<td>5.</td>
<td>11:15am</td>
<td>0.4ml</td>
<td>1.1ml</td>
<td>11:19am</td>
<td>11:18am</td>
<td>11:26am</td>
</tr>
</tbody>
</table>

- 2 ml non oxalated blood (of rabbit) was taken in each tube.

Table 4.4: For Method 1 (With 1 ml of blood)-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:35am</td>
<td>----</td>
<td>1.0ml</td>
<td>----</td>
<td>10:42am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:35am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:35am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>10:35am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:35am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>10:35am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:41am</td>
<td>10:40am</td>
<td>10:47am</td>
</tr>
<tr>
<td>5.</td>
<td>10:41am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:47am</td>
<td>10:46am</td>
<td>10:53am</td>
</tr>
</tbody>
</table>

- 1 ml non oxalated blood (of rabbit) was taken in each tube.
Table 4.5: For Method 2 (With 1 ml of blood)-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:12am</td>
<td>----</td>
<td>1.0ml</td>
<td>----</td>
<td>10:17am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:12am</td>
<td>0.25ml</td>
<td>0.75ml</td>
<td>10:12am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>10:12am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:12am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>10:18am</td>
<td>1.0ml</td>
<td>0.0ml</td>
<td>10:18am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>5.</td>
<td>10:18am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:23am</td>
<td>10:22am</td>
<td>10:32am</td>
</tr>
</tbody>
</table>

- 1 ml non oxalated blood (of rabbit) was taken in each tube.

Table 4.6: For Method 3 (With 1 ml of blood)-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:41am</td>
<td>----</td>
<td>1.0ml</td>
<td>----</td>
<td>10:47am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:41am</td>
<td>0.1ml</td>
<td>0.9ml</td>
<td>10:47am</td>
<td>10:46am</td>
<td>11:07am</td>
</tr>
<tr>
<td>3.</td>
<td>10:41am</td>
<td>0.2ml</td>
<td>0.8ml</td>
<td>10:48am</td>
<td>10:47am</td>
<td>11:00am</td>
</tr>
<tr>
<td>4.</td>
<td>10:41am</td>
<td>0.3ml</td>
<td>0.7ml</td>
<td>10:48am</td>
<td>10:47am</td>
<td>11:57am</td>
</tr>
<tr>
<td>5.</td>
<td>10:41am</td>
<td>0.4ml</td>
<td>0.6ml</td>
<td>10:47am</td>
<td>10:46am</td>
<td>11:54am</td>
</tr>
</tbody>
</table>

- 1 ml non oxalated blood (of rabbit) was taken in each tube.
Table 4.7: Repeat of Method 1 (With 1 ml of blood)-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:17am</td>
<td>----</td>
<td>1.0ml</td>
<td>----</td>
<td>10:22am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:17am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:17am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>10:24am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:24am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>10:24am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:29am</td>
<td>10:28am</td>
<td>10:36am</td>
</tr>
<tr>
<td>5.</td>
<td>10:24am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:30am</td>
<td>10:29am</td>
<td>10:36am</td>
</tr>
</tbody>
</table>

- 1 ml non oxalated blood (of rabbit) was taken in each tube.

Table 4.8: Repeat of Method 2 (With 1 ml of blood)-

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Time of blood taken</th>
<th>Vol. of SK taken</th>
<th>Vol. of NS added</th>
<th>Time of SK added</th>
<th>Clot formed</th>
<th>Clot dissolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10:55am</td>
<td>----</td>
<td>1.0ml</td>
<td>----</td>
<td>10:59am</td>
<td>----</td>
</tr>
<tr>
<td>2.</td>
<td>10:55am</td>
<td>0.25ml</td>
<td>0.75ml</td>
<td>10:55am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>3.</td>
<td>10:55am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>10:55am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>4.</td>
<td>10:55am</td>
<td>1.0ml</td>
<td>0.0ml</td>
<td>10:55am</td>
<td>----</td>
<td>NA</td>
</tr>
<tr>
<td>5.</td>
<td>11:01am</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>11:07am</td>
<td>11:06am</td>
<td>11:16am</td>
</tr>
</tbody>
</table>

- 1 ml non oxalated blood (of rabbit) was taken in each tube.
4.2 Preparation of plant extract

The following plants were chosen for the proposed research work-

- *Nepeta hindostana* (whole plant)
- *Nigella sativa* (seeds)
- *Terminalia belerica* (fruits)

4.2.1 Collection, identification and standardization

Selected plants were collected from various sources and authenticated taxonomically at the National botanical Research Institute (Lucknow, India). They were washed with distilled water to remove dirt and soil and shade dried.

4.2.2 Extraction

The dried plant materials (desired portion of plant) were powdered and passed through a 10-mesh sieve. The coarsely powdered materials were extracted with different solvents (distilled water, methanol and ethanol). The extracts were filtered, pooled and concentrated at reduced temperature. The drug extracts were suspended in normal saline. The standardization of extracts was followed by HPTLC.
4.2.3 Method of extraction

1. *Terminalia belerica*, *Nepeta hindostana*, *Nigella sativa* were shade dried and powdered.

2. Extraction was carried out by maceration.

3. For aqueous extraction, 50g of dried powder was taken in 500 ml conical flask having 250ml of distilled water, put 2-4 drops of chloroform, covered by aluminium foil and kept on shaker for 48 hrs.

4. For methanolic extraction, 50g of dried powder was taken in 500 ml conical flask having 250ml of methanol, covered by aluminium foil and kept on shaker for 72 hrs.

5. For ethanolic extraction, 50g of dried powder was taken in 500 ml conical flask having 250ml of ethanol, covered by aluminium foil and kept on shaker for 72 hrs.

6. After maceration, mixture was filtered and filtrate was used for further experiment.

7. Filtrates were concentrated over water bath to form semisolid.

8. The semisolid products were weighed and stored in small plastic bottles/MCTs.
Table 4.9- Extract yields in different solvents:

<table>
<thead>
<tr>
<th>S.No</th>
<th>Plant (50 g)</th>
<th>Solvent</th>
<th>Extract weight (in grams)</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Terminalia belerica</em></td>
<td>Distilled water</td>
<td>4.69</td>
<td>9.38</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>Methanol</td>
<td>2.81</td>
<td>5.62</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>Ethanol</td>
<td>3.12</td>
<td>6.24</td>
</tr>
<tr>
<td>4.</td>
<td><em>Nepeta hindostana</em></td>
<td>Distilled water</td>
<td>3.45</td>
<td>6.90</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>Methanol</td>
<td>1.76</td>
<td>3.52</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>Ethanol</td>
<td>2.73</td>
<td>5.46</td>
</tr>
<tr>
<td>7.</td>
<td><em>Nigella sativa</em></td>
<td>Distilled water</td>
<td>1.13</td>
<td>2.26</td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>Methanol</td>
<td>0.48</td>
<td>0.96</td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>Ethanol</td>
<td>0.55</td>
<td>1.10</td>
</tr>
</tbody>
</table>
4.3 Dilution of extracts

Two dilutions were used for anti thrombotic and thrombolytic activity, 5 times and 10 times. Dilutions were made as follows-

4.3.1 Extract diluted 10 times
- 0.5g extract was dissolved in 5ml of normal saline.

4.3.2 Extract diluted 5 times
- 0.5g extract of *Terminalia belerica* and *Nepeta hindostana* were dissolved in 2.5ml of normal saline
- 0.2g extract of *Nigella sativa* was dissolved in 1.0 ml of normal saline
4.4 Protocols for Testing of activity:

4.4.1 For anti-thrombotic activity

1. Six Microcentrifuge Tubes (MCTs) were taken and labeled 1 to 6.
2. In each tube 0.5 ml non oxalated blood (of rabbit) was taken.
3. 0.2 ml of Streptokinase (5000 units/ml) was added in tube no. 2.
4. 0.1 ml of diluted test solution was added in tube no. 3.
5. 0.2 ml of diluted test solution was added in tube no. 4.
6. 0.3 ml of diluted test solution was added in tube no. 5.
7. 0.5 ml of diluted test solution was added in tube no. 6.
8. Volume of each MCT was made up to 1ml by normal saline.
9. All test tubes were incubated at 37°C.
10. The time of clot formation was noted.
11. Monitoring was done for 90 minutes.
12. Tube no. 1 was used as blank.
4.4.2 For thrombolytic activity

1. Six test tubes were taken and labeled 1 to 6.
2. In each test tube 0.5 ml non oxalated blood (of rabbit) was taken.
3. All test tubes were incubated at 37°C and wait until clot was formed.
4. The time of clot formation was noted.
5. 0.5 ml of Streptokinase (5000 units/ml) was added in tube no. 2.
6. 0.1 ml of diluted test solution was added in tube no. 3.
7. 0.2 ml of diluted test solution was added in tube no. 4.
8. 0.3 ml of diluted test solution was added in tube no. 5.
9. 0.5 ml of diluted test solution was added in tube no. 6.
10. Volume of each test tube was made up to 1 ml by normal saline.
11. All tubes were kept on incubator shaker at 37°C.
12. The time of clot dissolution was noted.
13. Monitoring was done for 90 minutes.
14. Tube no. 1 used as blank.
**Fig. no. 4.2: Clot lysis:** Clot lysis can be clearly observed. Tube no.1 is negative control to which NS was added, no clot lysis was observed in tube no.1. Tube no.2 is positive control to which 0.5ml (5K IU) SK was added. Clear clot lysis can be seen tube no.2. Tube no.3 to 6 have different concentrations of test solutions (of *Terminalia belerica*) with increasing order.
4.4.3 For antiplatelet activity

The antiplatelet activity was tested for all plant extracts by spectrophotometric methods. [1]
Method of anti platelet activity was standardized by taking aspirin as standard and collagen as platelet aggregation inducer [1]. First the optimum dose of aspirin was determined and then the test samples were tested by same procedure at various concentrations.

4.4.3.1 Dilution of extracts

Two dilutions were used for antiplatelet activity, 50 times and 100 times. Dilutions were made as follows-

1- Extract was diluted 100 times
   - 1mg dissolved in 100ml of normal saline.

2- Extract was diluted 50 times
   - 1mg dissolved in 50ml of normal saline.

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4.4.3.2 For determination of standard dose of aspirin

1. Rabbit whole blood (9 ml) was used.
2. Anticoagulant (1 ml 1.9% tri sodium citrate) was added.
3. Centrifuged at 300g (1500 rpm) for 15 mins.
4. Platelet rich plasma (PRP) was taken (supernatant).
5. The PRP concentration was adjusted to get OD 1.0 (at 400 nm \( \lambda \))
   by addition of normal saline.
6. 10 test tubes were taken and marked 1 to 10.
7. 1.0 ml of PRP was taken in each tube.
8. Various doses of aspirin were added in increasing order.
9. Tube 1 was taken as control and no aspirin was added.
10. All test tubes were incubated for 3 mins at 37°C.
11. Now 0.2 ml of collagen (0.2 mg/dl) was added in each tube.
12. Aggregation was induced under continuous stirring at 1000 rpm
    for 3 mins.
13. The aggregation was monitored under spectrophotometer at 400 nm.
15. Normal saline was used as blank and for volume makeup.
Table 4.10: Determination of standard dose of aspirin

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Vol. of Aspirin soln. (0.9mg/dl)</th>
<th>N.S. (ml)</th>
<th>O.D. *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>--</td>
<td>0.8ml</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.1 ml</td>
<td>0.7ml</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.2 ml</td>
<td>0.6ml</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.3 ml</td>
<td>0.5ml</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.4 ml</td>
<td>0.4ml</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>6</td>
<td>0.5 ml</td>
<td>0.3ml</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>7</td>
<td>0.6 ml</td>
<td>0.2ml</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>8</td>
<td>0.7 ml</td>
<td>0.1ml</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>9</td>
<td>0.8 ml</td>
<td>0.0ml</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td>10</td>
<td>0.9 ml</td>
<td>0.0ml</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>11</td>
<td>1.0 ml</td>
<td>0.0ml</td>
<td>0.4±0.2</td>
</tr>
</tbody>
</table>

* n=5, mean±SD

- 1 ml of diluted PRP was taken in each tube
- 0.2ml of collagen (0.2mg/dl) was added in each tube.
For determination of standard dose of aspirin

![Graph showing optical density vs volume of aspirin solution (0.9mg/dl) in ml.](image)

**Fig. 4.2:** The maximum activity was obtained at 0.4ml volume (at given conc.) hence this concentration was taken as standard.
4.4.3.3 For the activity of test samples

1. Rabbit whole blood (9 ml) was used.
2. Anticoagulant (1ml 1.9% tri sodium citrate) was added.
3. Centrifuged at 300g (1500 rpm) for 15 mins.
4. Platelet rich plasma (PRP) was taken (supernatant).
5. The PRP concentration was adjusted to get OD 1.0 at 400 nm $\lambda$.
6. 6 test tubes were taken and marked 1 to 6.
7. 1.0 ml of PRP was taken in each tube.
8. 0.4 ml of aspirin was added in tube 2.
9. Tube 1 was taken as control.
10. In tube 3to 6, test samples were added in increasing order.
11. Volume of each tube was made up to 2ml by normal saline.
12. All test tubes were incubated for 3mins at 37°C.
13. Now 0.2ml of collagen (0.2mg/dl) was added in each tube.
14. Aggregation was induced under continuous stirring at 1000 rpm for 3 mins.
15. The aggregation was monitored under spectrophotometer ad 400 nm.
16. Normal saline was used as blank and for volume makeup.
4.5 TLC

Thin layer chromatography was performed on aluminum TLC plates (approx 10 cm × 3 cm) precoated with 200-μm layers of silica gel. Samples were applied as spot of 2mm diameter and the separation was performed in a glass chamber previously saturated with mobile phase for 15 min at room temperature (25±2°C). The plates were dried at room temperature in air and derivatized with anisaldehyde-sulphuric acid reagent and warmed (at 75°C for 5 min) to identify compact bands.

4.5.1 Mobile phase:

- For *Terminalia belerica*:-
  Ethyl acetate + n-Hexane (9.5 : 0.5, v/v/) + 1 drop of Formic acid

- For *Nepeta hindostana*:-
  Toluene + Ethyl acetate + GAA (8.5 : 1.0 : 0.8, v/v/v)

- For *Nigella sativa*:-
  Toluene + Ethyl acetate + GAA (8.0 : 1.5 : 0.5, v/v/v)
4.6 HPTLC

4.6.1 HPTLC-UV$_{254\text{nm}}$ fingerprinting and image analysis (a qualitative HPTLC fingerprint):

*Terminalia belerica* and *Nepeta hindostana* were air-dried and pulverized. The powdered material was extracted by maceration. Thereafter methanolic extracts of both plant samples were concentrated under reduced pressure and finally vacuum dried. The protocol for preparing sample solutions was optimized for high quality fingerprinting and also to extract the marker compounds efficiently. The plates were developed and scanned as discussed above. The peak areas were recorded.

4.6.2 Chromatography

Chromatography was performed, as described previously on 20 cm × 10 cm aluminum Lichrosphere HPTLC plates precoated with 200-μm layers of silica gel 60F$_{254}$ (E. Merck, Darmstadt, Germany). Samples were applied as bands 6 mm wide and 10 mm apart by means of Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-μL syringe. The toluene–methanol–formic acid (7.0:2.7:0.3, v/v/v) as mobile phase was performed in a 20 cm × 10 cm twin-trough glass chamber (Camag) previously saturated with mobile phase for 15 min at
room temperature (25±2°C) and relative humidity 60±5%. The plates were dried at room temperature in air and derivatized with anisaldehyde-sulphuric acid reagent and warmed (at 75°C for 5 min) to identify compact bands. Densitometric analysis was performed at 254 nm in reflectance mode with a Camag TLC scanner III operated by WinCATS software (Version 1.2.0). The slit dimensions were 5 mm × 0.45 mm and the scanning speed of 20 mm s⁻¹.

- **Solvent system (Mobile Phase):**
  Toluene–methanol–formic acid (7.0:2.7:0.3, v/v/v)

- **Stationary Phase:**
  Precoated 200-μm layers of silica gel 60F₂₅₄
4.7 Fractionation

Fractionation of plant extracts were done by TLC chromatography.

4.7.1 TLC of plant extracts

Chromatography was performed as described previously on glass plates (approx 20 cm × 8 cm) coated with layer of silica gel. Samples were applied as bands of 6 mm wide. The separation was performed in 2 glass chambers previously saturated with mobile phase for 15 min at room temperature (25±2°C). The plates were dried at room temperature in air and derivatized with anisaldehyde-sulphuric acid reagent and warmed (at 75°C for 5 min) to identify compact bands.

Mobile phase:

- For *Terminalia belerica*:-
  Ethyl acetate + n-Hexane (9.5 : 0.5, v/v) + 1 drop of Formic acid

- For *Nepeta hindostana*:-
  Toluene + Ethyl acetate + GAA (8.5 : 1.0 : 0.8, v/v/v)

4.7.2 Collection of Sample

1. The separated bands of sample were taken by scrapping each band separately and numbered them.
2. Each fraction was dissolved in 2 ml of solvent (i.e. normal saline for aqueous and methanol for alcoholic).
3. The sample were then filtered under vacuum and taken for the activity testing.
4.7.3 Activity Tests of Fractions:

4.7.3.1 For antiplatelet activity

1. Rabbit whole blood (9 ml) was used.
2. Anticoagulant (1ml 1.9% tri sodium citrate) was added.
3. Centrifuged at 300g (1500 rpm) for 15 mins.
4. Platelet rich plasma (PRP) was taken (supernatant).
5. The PRP concentration was adjusted to get OD 1.0 at 400 nm λ.
6. Various test tubes were taken according to number of samples and marked.
7. 1.0 ml of PRP was taken in each tube.
8. 0.4 ml of aspirin was added in tube #2 (standard dose determined earlier).
9. Tube 1 was taken as control.
10. In tube 3 to onward, 0.5ml of test sample was added.
11. Volume of each tube was made up to 2ml by normal saline.
12. All test tubes were incubated for 3mins at 37°C.
13. Now 0.2ml of collagen (0.1mg/ml) was added in each tube.
14. Aggregation induced under continuous stirring at 1000 rpm for 3 mins.
15. The aggregation was monitored under spectrophotometer at 400 nm.
16. Normal saline and methanol were used as blank.
4.7.3.2 For thrombolytic activity

1. Various test tubes were taken according to no. of samples and marked.
2. In each test tube 0.5 ml non oxalated blood (of rabbit) was taken.
3. All test tubes were incubated at 37°C and wait until clot was formed.
4. The time of clot formation was noted.
5. 0.5 ml of normal saline or methanol was added in tube no. 1 & used as blank.
6. 0.5 ml of Streptokinase (5000 units/ml) was added in tube no. 2.
7. 0.5 ml of test solution was added in tube no. 3 and further.
8. All tubes were kept on incubator shaker at 37°C.
9. The time of clot dissolution was noted.
10. Monitoring was done for 90 minutes.
4.8 Column Chromatography

4.8.1 Preparation of slurry
The concentrated extracts of the drug were taken in a China dish and heated continuously on a water bath by gradually adding methanol in small portions with constant stirring, till desired consistency was obtained. A weighed quantity of silica gel for column chromatography was then added slowly with continuous mixing with a steel spatula until the whole methanolic solution of plant extract adsorbed on silica gel particles. It was dried in the air; the larger lumps were broken by rubbing between hands and finally passed through a sieve (No. 8) to get uniform particle size.

4.8.2 Packing of column & Isolation of phytoconstituents
A column of 3.0 feet, height and 16 mm internal diameter was taken, cleaned properly and dried. The lower end of the column was plugged with non-absorbent cotton wool. The column was clamped and fitted in a vertical position on a stand. The column was then half filled with petroleum ether (b.p. 60-80 °C). Silica gel (for column, 60-120 mesh) was then poured in small portions and allowed to settle down and the dried plant extract slurry was loaded over the column and then eluted successively with different solvents, in their order of increasing polarity. The developments and elution of the column were carried out with successive series of different solvents in various combinations, such as petroleum ether (100), petroleum ether: chloroform (75:25, 50:50, 25:75,), chloroform (100), chloroform: methanol (99:1, 98:2, 97:3, 95:5, 90:10, 80:20) and methanol to isolate the compounds.
4.8.3 Homogeneity of the fractions

The fractions collected were subjected to thin layer chromatography (TLC) to check homogeneity of various fractions. Chromatographically identical fractions (having same R_f values) were combined together and concentrated. Fractions were then crystallized with suitable solvent system.

Fig. 4.3: Column Chromatography
REFERENCES-


