5. MATERIALS AND METHODS

Neerazhivu choornam is a Siddha antidiabetic solid dosage form popularly used by the traditional Siddha practitioner.

On ethno medical survey among the Siddha physicians the rare & original Tamil poem describing the usefulness of Neerazhivu choornam in the management of diabetes mellitus has been documented here for the first time. This poem has been passed by word of mouth from generation to generation.29

Figure 1. The original Siddha(Tamil) poem describing Neerzhivu choornam

Collection and Authentication:

The ingredients of Neerazhivu choornam were purchased from reputed local raw material traders and the raw materials were authenticated at Captain Srinivasa Murti Research Institute for Ayurveda, A.A.Hospital campus, Arumbakkam, Chennai by organoleptic and microscopic evaluation. The samples are cleaned and shade dried, organoleptic characters of individual raw
drugs were recorded, powdered and passed through sieve no. 80, labeled and stored in an air tight glass container in a cool place.

Table :2 Preparation of Neerzhivu choornam

<table>
<thead>
<tr>
<th>S.No</th>
<th>Vernacular name of the ingredients</th>
<th>Parts used</th>
<th>Biological source &amp; Family</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kadukkaithol</td>
<td>Skin</td>
<td>Terminalia chebula (Combretaceae)</td>
<td>2 part</td>
</tr>
<tr>
<td>2</td>
<td>Kariveppilai</td>
<td>Leaves</td>
<td>Murraya koenigii (Rutaceae)</td>
<td>2 part</td>
</tr>
<tr>
<td>3</td>
<td>Nellivatral</td>
<td>Fruits</td>
<td>Emblica officinalis (Euphorbiaceae)</td>
<td>2 part</td>
</tr>
<tr>
<td>4</td>
<td>Navalkottai</td>
<td>Seeds</td>
<td>Syzygium cumini (Myrtaceae)</td>
<td>1 part</td>
</tr>
<tr>
<td>5</td>
<td>Seenthil</td>
<td>Stem</td>
<td>Tinospora cordifolia (Menispermaceae)</td>
<td>1 part</td>
</tr>
<tr>
<td>6</td>
<td>Keezhkkainelli</td>
<td>Whole plant</td>
<td>Phyllanthus fraternus (Euphorbiaceae)</td>
<td>1 part</td>
</tr>
<tr>
<td>7</td>
<td>Koraikizhangu</td>
<td>Rhizomes</td>
<td>Cyperus rotandus (Cyperaceae)</td>
<td>1 part</td>
</tr>
</tbody>
</table>
Methods of preparation:\n
Kadukkaithol, kariveppilai, nellivatral (each 2 parts), naval kottai, seenthil, keezhkkainelli, koraikizhangu (each one parts) are separately collected, cleaned, size reduced individually and passed completely through number 80 sieve. Then the ingredients are mixed well in a mortar to get uniform mixer and stored in a closed glass container in a cool and dark place.

**Dosage:** 1gm to 2gm two times a day orally with vehicle- milk as per Siddha literature.

**Standardization of Neerazhivu Choornam.**

The prepared in-house Neerazhivu choornam has been standardized as per WHO guidelines under the various headings using standard protocol obtained from various Pharmacopoeia, Herbal Pharmacopoeia, Herbal compendium, reference books and official agency guidelines.

**5.1 PHARMACOGNOSTICAL EVALUATION**

a. Organoleptic evaluation

b. Powder Microscopical studies

c. Ash values, Extractive Values, Loss on drying (Proximate analysis)

d. Determination of pesticide residues

- Determination of specific organochlorine, organophosphorus pyrethroid insecticide residues and dithio corbamates
- Organochlorine and Pyrethroid insecticides
- Organophosphorus insecticides
e. Determination of heavy metal contamination
   - Limit test for total toxic metals as lead
   - Detection of cadmium, copper, iron, lead, nickel and zinc

f. Determination of microbial contamination

g. TLC & HPTLC studies

**Organoleptic evaluation**

The color, odour, taste, texture (grittiness), particle size and solubility in water and alcohol were noted for the in-house prepared Neerizhivu Choornam as organoleptic qualitative parameters to identify the choornam and documented as preliminary standards for the Neerizhivu choornam.

**Powder microscopical studies**

The materials used in the Neerizhivu Choornam preparation were dried for a minimum of the 15 days under shade, powdered and passed through sieves with aperture size of 180µm and 125µm separately to obtain fine and very fine powders, respectively were subjected to microscopical examination. The specimens were treated with the following reagents in order to evaluate components of diagnostic value: 50% glycerin as temporary mountant; 2% Phloroglucinol in a mixture of 90% ethanol and conc. HCl (1:1) for lignin; 5% alcoholic ferric chloride for phenolic compounds; 2% iodine solution for starch grains; 0.08% ruthenium red in 10% lead acetate for mucilage and Sudan III red for oil globules.
Determination of Ash values

The residue remaining after incineration is the ash content of the drug, which simply represents inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it as a form of adulteration. For determining ash, the powdered drug is incinerated so as to burn out all organic matter.

Ash values are used to determine the quality and purity of a crude drug, especially in the powdered form. Ash contains inorganic radicals like phosphates, carbonates and silicates of sodium, potassium, magnesium, calcium etc.

The object of ashing vegetable drugs is to remove all traces of organic matter which may otherwise interfere in an analytical determination.

The ash is determined by three different methods which measure total ash, acid-insoluble ash and water soluble ash. Acid-insoluble ash value of a crude drug is always less than total ash value of the same drug.

The total ash method is designed to measure the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plant tissue itself, and “non-physiological ash”, which is the residue of the extraneous matter (sand, soil) adhering to the plant surface.

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.
*Water soluble ash* is the difference in weight between the total ash and the residue after treatment of the total ash with water.

**Procedure:**

**Total ash**

Placed about 2 to 4 gram of the ground air-dried material, accurately weighed, in a previously ignited and tarred silica crucible. Spread the material in an even layer and ignited it by gradually increasing the heat to 500-600°C, until it is white, indicating the absence of carbon. Cooled in a desiccator and weighed. If carbon-free ash cannot be obtained in this manner, cooled the crucible and moisten the residue with about 2ml of water or a saturated solution of ammonium nitrate. Dried on a water bath, then on a hot-plate and ignited to constant weight. Allow the residue to cool in a suitable desiccators for 30 minutes and then weighed without delay. Calculated the content of total ash in mg per gram of air-dried material. Percentage of total ash was calculated by using the formula-

\[
\% \text{ of total ash} = \left( \frac{\text{weight of ash obtained}}{\text{weight of air dried drug taken}} \right) \times 100
\]

**Acid insoluble ash**

To the crucible containing the total ash, added 25 ml of hydrochloric acid (70g/l) TS, covered with a watch-glass and boiled gently for 5 minutes. Rinsed the watch-glass with 5ml of hot water and added this liquid to the crucible. Collected the insoluble matter on an ash less filter-paper and washed with hot water until the filtrate is neutral. Transferred the filter-paper containing the insoluble matter to the original crucible, dried on a hot-plate and ignited to constant weight. Allowed the residue to cool in a suitable desiccator for 30 minutes and then
weighed without delay. Calculated the content of acid-insoluble ash in mg per gram of air-dried material.

**Water-soluble ash**

To the crucible containing the total ash, added 25 ml of water and boiled for 5 minutes. Collected the insoluble matter in a sintered-glass crucible or on an ashless filter-paper. Washed with hot water and ignited in a crucible for 15 minutes at a temperature not exceeding 450°C. Subtracted the weight of this residue in mg from the weight of total ash. Calculated the content of water-soluble ash in mg per gram of air-dried material.

**Determination of Extractive Values**

Extraction is the process of isolation of soluble material from an insoluble residue, which may be liquid or solid, by treatment with the solvent. The solvent used for extraction is known as *menstrum* and the inert insoluble material that remains after extraction is called *marc*. Extractable matter represents the amount of active ingredients extracted with solvents from the given amount of medicinal plant material.

Extractive values are employed for materials for which as yet no suitable chemical or biological assay exists. It is useful for the evaluation of a crude drug. It gives an idea about the nature of the chemical constituents present in a crude drug. It is useful for the estimation of specific constituents, soluble in that particular solvent used for extraction.

WHO recommends hot extraction and cold maceration methods for extractable matters.
Water soluble extractive

This method is applied to drug which contain water-soluble active constituents, such as tannins, sugar, plant acid mucilage, glycoside, etc.

Alcohol soluble extractive

Alcohol is an ideal solvent for extraction of various chemical like tannin, resin etc. therefore this method is frequently employed to determine the extractive value for standard drug. Generally 95% alcohol is used to determine the alcohol soluble extractive values. In some cases dilute alcohol may also be used depending upon the solubility of the drug.

Procedure

Hot extraction

Place about 4.0 gram of coarsely powdered air-dried material, accurately weighed, in a glass-stoppered conical flask. Add 100 ml of water and weighed to obtain the total weight including the flask. Shaked well and allowed to stand for 1 hour. Attached a reflux condenser to the flask and boiled gently for 1 hour, cooled and weighed. Readjust to the original total weight with the solvent specified in the test procedure for the plant material concerned. Shaked well and filtered rapidly through a dry filter. Transfered 25 ml of this filtrate to a tared flat-bottomed dish and evaporated to dryness on a water bath. Dried at 105°C for 6 hours, cooled in a desiccator for 30 minutes, then weighed without delay. Calculated the content of extractable matter in mg per gram of air dried material.
Cold Maceration

Placed about 4.0 gram of coarsely powdered air-dried material, accurately weighed, in a glass-stoppered conical flask. Macerated with 100 ml of the solvent specified for the plant material concerned for 6 hours, shaking frequently and then allowed to stand for 18 hours. Filtered rapidly taking care not to lose any solvent, transferred 25 ml of the filtrate to a tarred flat-bottomed dish and evaporated to dryness on water-bath. Dried at 105ºC for 6 hours; cooled in a desiccator for 30 minutes and weighed without delay. Calculated the content of extractable matter in mg per gram of air-dried material.

For ethanol-soluble extractable matter, used the concentration of 90 % ethanol, for water-soluble extractable matter, used water as the solvent.

Determination of Loss on drying67

Placed about 2-5 gram of the prepared air-dried material accurately weighed, in a previously dried and tared flat weighing bottle. Dried the sample in an oven at 100-105ºC. Dried until two consecutive weighing do not differ by more than 5 mg. Calculated the loss of weight in mg per gram of air-dried material.

Determination of Pesticide residues68-69:

Pesticides are simple substances or mixtures used to eliminate undesirable vegetable and animal life in agricultural and urban ecosystems. Owing to the great varaiablity in plant chemical composition that results from factors to which plants are exposed during their growth, storage and the different stages of manipulation, characterization and standardization of phytopharmaceuticals are necessary. Medicinal plant materials are liable to contain pesticides residues, which accumulate from agricultural practices, such as spraying, treatment
of soil during cultivation and administration of fumigants during storage. Pest control is used for the production of growing plants, plant products in warehouses.

Pesticides can be classified according to their chemical composition, function and mode of action in organisms. Chemically the compounds can be divided into three groups; Biological, Inorganic, and Organic Pesticide. Mainly the organochlorine, organophosphorus, carbonate and triazine compounds which is the largest and has pronounced physiological activity. Therefore it is necessary of performing the determination of pesticides in accordance with the procedure outlined by national/international agencies.

Pesticides are the only toxic substances released intentionally into our environment to kill living things. This includes substances that kill weeds (herbicides), insects (insecticides), fungus (fungicides), rodents (rodenticides), and others. Pesticides can cause many types of cancer in humans. Some of the most prevalent forms include leukemia, non-Hodgkins lymphoma, brain, bone, breast, ovarian, prostate, testicular and liver cancers. Acute dangers - such as nerve, skin, and eye irritation and damage, headaches, dizziness, nausea, fatigue, and systemic poisoning - can sometimes be dramatic, and even occasionally fatal.

**Table 3. Pesticides with acceptance limit**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Substance</th>
<th>Limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alachlor</td>
<td>0.02</td>
</tr>
<tr>
<td>2.</td>
<td>Aldrin and dieldrin (sum of)</td>
<td>0.05</td>
</tr>
<tr>
<td>3.</td>
<td>Anilophos</td>
<td>0.1</td>
</tr>
<tr>
<td>4.</td>
<td>Atrazine</td>
<td>0.1</td>
</tr>
<tr>
<td>5.</td>
<td>Azinphos-methyl</td>
<td>1.0</td>
</tr>
<tr>
<td>6.</td>
<td>Bromopropylate</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Concentration</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>7</td>
<td>Chlordane (sum of cis-, trans – and Oxythlordane)</td>
<td>0.05</td>
</tr>
<tr>
<td>8</td>
<td>Chlorfenvinphos</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>Chlorpyrifos</td>
<td>0.2</td>
</tr>
<tr>
<td>10</td>
<td>Chlorpyrifos-methyl</td>
<td>0.1</td>
</tr>
<tr>
<td>11</td>
<td>Cypermethrin (and isomers)</td>
<td>1.0</td>
</tr>
<tr>
<td>12</td>
<td>DDT (sum of p,p’-DDT, o,p’-DTT, p,p’-DDE and p,p’-TDE)</td>
<td>1.0</td>
</tr>
<tr>
<td>13</td>
<td>Deltamethrin</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>Diazinon</td>
<td>0.5</td>
</tr>
<tr>
<td>15</td>
<td>2,4-Dichlorophenoxy acetic acid</td>
<td>0.05</td>
</tr>
<tr>
<td>16</td>
<td>Dichlorvos</td>
<td>1.0</td>
</tr>
<tr>
<td>17</td>
<td>Dithiocarbamates (as CS₂)</td>
<td>2.0</td>
</tr>
<tr>
<td>18</td>
<td>Endosulfan (sum of isomers and Endosulfan sulphate)</td>
<td>3.0</td>
</tr>
<tr>
<td>19</td>
<td>Endrin</td>
<td>0.05</td>
</tr>
<tr>
<td>20</td>
<td>Ethion</td>
<td>2.0</td>
</tr>
<tr>
<td>21</td>
<td>Fenitrothion</td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td>Fenvalerate</td>
<td>1.5</td>
</tr>
<tr>
<td>23</td>
<td>Fonofos</td>
<td>0.05</td>
</tr>
<tr>
<td>24</td>
<td>Heptachlor (sum of Heptachlor and Heptachlorepoxide)</td>
<td>0.05</td>
</tr>
<tr>
<td>25</td>
<td>Hexachlorrobenzene</td>
<td>0.1</td>
</tr>
<tr>
<td>26</td>
<td>Hexachlorocyclohexane isomers (other than )</td>
<td>0.3</td>
</tr>
<tr>
<td>27</td>
<td>Isoproturon</td>
<td>0.05</td>
</tr>
<tr>
<td>28</td>
<td>Lindane ( -Hexachlorocyclohexane)</td>
<td>0.6</td>
</tr>
<tr>
<td>29</td>
<td>Malathion</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>Methidathion</td>
<td>0.2</td>
</tr>
<tr>
<td>31</td>
<td>Metoxuron</td>
<td>0.05</td>
</tr>
<tr>
<td>32</td>
<td>Metribuzin</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Pesticide</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>33</td>
<td>Parathion</td>
<td>0.5</td>
</tr>
<tr>
<td>34</td>
<td>Parathion-methyl</td>
<td>0.2</td>
</tr>
<tr>
<td>35</td>
<td>Permethrin</td>
<td>1.0</td>
</tr>
<tr>
<td>36</td>
<td>Phosalone</td>
<td>0.1</td>
</tr>
<tr>
<td>37</td>
<td>Piperonyl butoxide</td>
<td>3.0</td>
</tr>
<tr>
<td>38</td>
<td>Pirimiphos-methyl</td>
<td>4.0</td>
</tr>
<tr>
<td>39</td>
<td>Profenophos</td>
<td>0.05</td>
</tr>
<tr>
<td>40</td>
<td>Pyrethrins (sum of)</td>
<td>3.0</td>
</tr>
<tr>
<td>41</td>
<td>Quinalphos</td>
<td>0.05</td>
</tr>
<tr>
<td>42</td>
<td>Quintozene (sum of qintozone, pentachloroaniline and methyl pentachlorophenyl sulphide)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Pesticide residues:**

**Note:**

i) **Reagents:** All reagents and solvents are free from any contaminants, especially pesticides, which might interfere with the analysis. It is often necessary to use special quality solvents or if this is not possible, solvents that have recently been re-distilled in an apparatus made entirely of glass. In any case, suitable blank tests have been carried out.

ii) **Apparatus:** Cleaned the apparatus and especially glassware to ensure that they are free from pesticides, for example, soaked for at least 16 hours in a solution of phosphate-free detergent, rinsed with large quantities of distilled water and washed with acetone and hexane or heptane.

iii) **Kept the samples (before analysis) at a temperature below 0ºC, protected from light.**
Extraction (Solution A):

Instrument:

Rotary evaporator

Reagents:

Acetone HPLC grade (distilled)

Carbophenothion

Toluene HPLC grade (distilled)

Procedure:

1. To 10 g of the test sample (accurately weighed), coarsely powdered, added 100 mL of acetone and allow to stand for 20 minutes.

2. Added 0.200 mL of a solution containing 50 µg/mL of carbophenothion in toluene.

3. Homogenised using a high-speed blender for 3 minutes. Filtered and washed the filter cake with two quantities, each of 25 mL, of acetone.

4. Combined the filtrate and the washings and heated using a rotary evaporator at a temperature not exceeding 40°C until the solvent had almost completely evaporated.

5. To the residue added a few mL of toluene and heated again until the acetone is completely removed.

6. Dissolved the residue in 8 mL of toluene. Filtered through a membrane filter (45 µm), rinsed the flask and the filter with toluene and diluted to 10 mL with the same solvent (solution A).

Purification:

Organochlorine, organophosphorus and pyrethroid insecticides (by size exclusion chromatography):
Instrument:

Size exclusion chromatography with accessories as mentioned under chromatographic system

Reagents:

Methyl red
Oracet blue 2R
Toluene HPLC grade (distilled)

Preparation of reagents:

0.5 g/L solution of methyl red in toluene:
Dissolved 50 mg of Methyl red in sufficient toluene to produce 100 mL.

0.5 g/L solution of oracet blue 2R in toluene:
Dissolved 50 mg of oracet blue 2R in sufficient toluene to produce 100 mL.

Chromatographic system:

Column : A stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with styrene-divinylbenzene copolymer (5 µm) (TSK GEL-SCX).

Flow rate : 1.5 mL/min.

Mobile phase : Toluene

Performance of the column:

1. Injected 100 µl of a solution containing 0.5 g/L of methyl red and 0.5 g/L of oracet blue 2R in toluene and proceeded with the chromatography.
2. The column is not considered suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 mL.

3. If necessary calibrated the column, using a solution containing, in toluene, at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determined which fraction of the eluate contained both pesticides.

**Purification of the test solution (solution B):**

1. Injected a suitable volume of solution A (100 µl to 500 µl) and proceeded with the chromatography.

2. Collected the fraction as determined above (solution B).

**Organochlorine and pyrethroid insecticides:**

1. In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduced a piece of defatted cotton and 0.5 g of silica gel treated as follows:

2. Heated silica gel for chromatography in an oven at 150°C for 4 hours.

3. Allowed to cool and added dropwise a quantity of water corresponding to 1.5% of the weight of silica gel used; shaked vigorously until agglomerates have disappeared and continued shaking for 2 hours using a mechanical shaker.

4. Conditioned the column using 1.5 mL of hexane.

*Note: Prepacked columns containing about 500 mg of a suitable silica gel was also used and they were previously validated.*
Solution C:

1. Concentrated solution B in a current of helium or oxygen-free nitrogen almost to dryness and diluted to a suitable volume with toluene (200 µl to 1 mL according to the volume injected in the preparation of solution B).

2. Transferred quantitatively onto the column and proceeded to the chromatography using 1.8 mL of toluene as the mobile phase. Collected the eluate (solution C).

Quantitative analysis:

Organophosphorus insecticides (By GC):

Instrument:

Gas chromatograph with accessories as mentioned under chromatographic system.

Reagents:

Toluene

Chromatographic system:

Column : A fused-silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 µm thick of poly(dimethyl)siloxane (DB-1).

Carrier gas : Helium at flow rate of 0.9 mL / min.
**Temperature:**

Column : 80°C for 1 minute, then raising it at a rate of 30°C / minute to 150°C, maintaining at 150°C for 3 minutes, then raising the temperature at a rate of 4°C per minute to 280°C and maintaining at this temperature for 2 minute.

Injection port : 250°C

Detector : 275°C

Detection : A phosphorus-nitrogen flame-ionisation detector or a atomic emission spectrometry detector.

**Test solution:**

Concentrated solution B in a current of helium almost to dryness and diluted to 50 to 250 μl with toluene.

**Standard stock solution A:**

Weighed accurately about 5.0 mg of each of the standards as listed in the table-1 in separate 10 mL volumetric flasks, dissolved in toluene and made up the volume with the same solvent, and mixed.
Standard stock solution B:

Pipetted a volume of standard stock solution A (for each standard) and diluted to volume with toluene, as indicated in the table-3.

Table-4 Standard stock solution A and B for Organophosphorus insecticides

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Substance</th>
<th>Volume of standard stock solution A taken (mL)</th>
<th>Volume of standard stock solution B to be prepared (mL)</th>
<th>Relative retention time with respect to carbophenothion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Isoproturon</td>
<td>0.050</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>Metoxuron</td>
<td>0.050</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>Atrazine</td>
<td>0.100</td>
<td>0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>Fonofos</td>
<td>0.050</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Diazion</td>
<td>0.500</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>6</td>
<td>Metribuzin</td>
<td>5.000</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>7</td>
<td>Methyl-Parathion</td>
<td>0.200</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>8</td>
<td>Chlorpyrifos-Methyl</td>
<td>0.100</td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>9</td>
<td>Primiphos</td>
<td>4.000</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>10</td>
<td>Fenitrothion</td>
<td>0.500</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td>11</td>
<td>Malathion</td>
<td>1.000</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>12</td>
<td>Parathion</td>
<td>0.500</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>13</td>
<td>Chlorpyrifos</td>
<td>0.200</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>14</td>
<td>Quinalphos</td>
<td>0.050</td>
<td>0.82</td>
<td>0.82</td>
</tr>
<tr>
<td>15</td>
<td>Methidathion</td>
<td>0.200</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>16</td>
<td>Carbophenothion</td>
<td>0.300</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>17</td>
<td>Anilophos</td>
<td>0.100</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>18</td>
<td>Azinophos methyl</td>
<td>1.000</td>
<td>1.13</td>
<td>1.13</td>
</tr>
<tr>
<td>19</td>
<td>Phosalone</td>
<td>0.1000</td>
<td>1.14</td>
<td>1.14</td>
</tr>
<tr>
<td>S. No.</td>
<td>Substance</td>
<td>Volume of standard stock solution A taken (mL)</td>
<td>Volume of standard stock solution B to be prepared (mL)</td>
<td>Relative retention time with respect to carbophenothion</td>
</tr>
<tr>
<td>--------</td>
<td>---------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>20</td>
<td>Ethion</td>
<td>2.000</td>
<td></td>
<td>0.97</td>
</tr>
<tr>
<td>21</td>
<td>Chlorfenvinphos</td>
<td>1.500</td>
<td></td>
<td>1.10</td>
</tr>
</tbody>
</table>

Reference solution-I:

Transfer 0.5 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

Reference solution-II:

Transfer 1 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

Reference solution-III:

Transfer 1.5 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

Procedure:

1. Injected the chosen volume of each of reference solution-I, reference solution-II and reference solution-III, into chromatograph, recorded the chromatograms and plotted the calibration curve.

2. When the chromatograms were recorded in the prescribed conditions, the relative retention times were approximately those listed in Table-1.
3. Injected the test solution, into chromatograph and recorded the chromatograms.

4. Calculated the content of each of Organophosphorus insecticides from the peak areas and the concentrations of the solutions.

**Organochlorine and pyrethroid insecticides; dithiocarbamates (by GC):**

**Instrument:**

Gas chromatograph with a device allowing direct cold on-column injection and other accessories as mentioned under chromatographic system.

**Reagents:**

Toluene

**Chromatographic system:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>A fused-silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 µm thick of poly (dimethyl) (diphenyl)siloxane.</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium at flow rate of 1.4 mL / min.</td>
</tr>
<tr>
<td><strong>Temperature:</strong></td>
<td></td>
</tr>
<tr>
<td>Column</td>
<td>80°C for 1 minute, then raising it at a rate of 30°C / minute to 150°C, maintaining at 150°C for 3 minutes, then raising the temperature at a rate of 4°C per minute to 280°C and maintaining at this temperature for 2 minute.</td>
</tr>
</tbody>
</table>
Injection port : 250°C
Detector : 275°C
Detection : An electron-capture detector.

Test solution:
Concentrated solution C in a current of helium or oxygen-free nitrogen almost to dryness and diluted to 50 to 250 µl with toluene.

For Organochlorine and pyrethroid insecticides:

Standard stock solution A:
1. Weighed accurately about 5.0 mg of each of the standards as listed in the table-2 in separate 10 mL volumetric flasks, dissolved in toluene and made up the volume with the same solvent, and mixed.

Standard stock solution B:
1. Pipetted a volume of standard stock solution A (for each standard) and diluted to volume with toluene, as indicated in the table-4.

Table 5. Standard stock solution A and B for Organochlorine and pyrethroid insecticides

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Substance</th>
<th>Volume of standard stock solution A taken (mL)</th>
<th>Volume of standard stock solution B to be prepared (mL)</th>
<th>Relative retention time with respect to carbophenothion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dichlorvos</td>
<td>1.000</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>alpha-HCH</td>
<td>0.300</td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>Hexachlorobenzene</td>
<td>0.100</td>
<td></td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>Dichlorophenoxy acetic acid</td>
<td>0.050</td>
<td>10</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>Beta-HCH</td>
<td>0.300</td>
<td></td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Name</td>
<td>Amount</td>
<td>Retention Time</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-----------------------</td>
<td>--------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Lindane</td>
<td>0.600</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Quintozene</td>
<td>1.000</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Pentachloroaniline</td>
<td>1.000</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Heptachlor</td>
<td>0.050</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Alachlor</td>
<td>0.200</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Aldrin</td>
<td>0.050</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Heptachlor epoxide</td>
<td>0.050</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2,4-DDE</td>
<td>1.000</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Alpha-Endosulfan</td>
<td>3.000</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Chlordane</td>
<td>0.050</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Dialdrin</td>
<td>0.050</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>4,4-DDE</td>
<td>1.000</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2,4-DDD</td>
<td>1.000</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Endrin</td>
<td>0.050</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Beta-Endosulfan</td>
<td>3.000</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>2,4-DDT</td>
<td>1.000</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Pyrethrins</td>
<td>3.000</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Carbophenothion</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>4,4-DDT</td>
<td>1.000</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Bromopropylate</td>
<td>3.000</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Piperonyl Butoxide</td>
<td>3.000</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Permethrin</td>
<td>1.000</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Cypermethrin</td>
<td>1.000</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Fenvalerate</td>
<td>1.500</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Deltamethrin</td>
<td>0.500</td>
<td>1.50</td>
<td></td>
</tr>
</tbody>
</table>

**Reference solution-I:**
Transferred 0.5 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

**Reference solution-II:**

Transfer 1 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

**Reference solution-III:**

Transfer 1.5 mL each of standard stock solution B into a 50 mL volumetric flask, and 1 mL of internal standard solution (Containing 50 µg/mL carbophenothion) and made up the volume with toluene, and mixed.

**Procedure:**

1. Injected the chosen volume of each of reference solution-I, reference solution-II and reference solution-III, into chromatograph, recorded the chromatograms and plotted the calibration curve.
2. When the chromatograms were recorded in the prescribed conditions, the relative retention times were approximately those listed in Table-2.
3. Injected the test solution, into chromatograph and recorded the chromatograms.
4. Calculated the content of Organochlorine and pyrethroid insecticides, from the peak areas and the concentrations of the solutions.

**For dithiocarbamates insecticides:**

**Standard stock solution A:**
1. Weighed accurately about 5.0 mg of each of the standards as listed in the table-3 in separate 10 mL volumetric flasks, dissolved in toluene and made up the volume with the same solvent, and mixed.

**Standard stock solution B:**

1. Pipetted a volume of standard stock solution A (for each standard) and diluted to volume with toluene, as indicated in the table-5

**Table-6 Standard stock solution A and B for carbophenothion**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Substance</th>
<th>Volume of standard stock solution A taken (mL)</th>
<th>Volume of standard stock solution B to be prepared (mL)</th>
<th>Relative retention time with respect to carbophenothion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Dithiocarbamates</td>
<td>2.000</td>
<td>10</td>
<td>1.10</td>
</tr>
</tbody>
</table>

**Reference solution-I:**

Transfered 0.5 mL each of standard stock solution B into a 50 mL volumetric flask and 1 mL of internal standard solution (Containing 50 µg/ml carbophenothion) and made up the volume with toluene and mixed.

**Reference solution-II:**

Transfered 1 mL each of standard stock solution B into a 50 mL volumetric flask and 1 mL of internal standard solution (Containing 50 µg/ml carbophenothion) and made up the volume with toluene and mixed.
**Reference solution-III:**

Transfered 1.5 mL each of standard stock solution B into a 50 mL volumetric flask and 1 mL of internal standard solution (Containing 50 µg/ml carbophenothion) and made up the volume with toluene and mixed.

**Procedure:**

1. Injected the chosen volume of each of reference solution-I, reference solution-II and reference solution-III, into chromatograph, record the chromatograms and plotted the calibration curve.

2. When the chromatograms were recorded in the prescribed conditions, the relative retention times were approximately those listed in Table-3.

3. Injected the test solution, into chromatograph and recorded the chromatograms.

4. Calculated the content of dithiocarbamates insecticides, from the peak areas and the concentrations of the solutions.

**Determination of heavy metal contamination**

The medicinal plant materials can be contaminated with arsenic and heavy metals which can be attributed to many causes including environmental pollution and places of pesticides. As these components even in trace amounts are dangerous, they have to be removed from the herbal drugs limit is for these materials have been prescribed in almost all the Pharmacopoeia throughout the world. As prescribed by WHO the following procedures have been recommended for their respective limit tests.

**Lead:**
Instrument: AAS with Graphite Furnace and Microwave Reaction System

Reagents and Standard:

- Nitric acid ultrapure grade (Make Merck)
- Magnesium nitrate Hexahydrate AR grade
- Ammonium dihydrogen phosphate AR grade
- Hydrogen Peroxide Sol. (30%)
- Milli Q water
- Lead standard 1000 ppm

Preparation of reagents:

Diluent (0.5% v/v nitric acid):

Diluted 5 ml of nitric acid to 1000 ml with water.

Matrix modifier:

Weighed and transfered accurately about 0.20 g of ammonium dihydrogen phosphate and 0.01 g of magnesium nitrate hexahydrate to a 100 mL volumetric flask, dissolved in and made up the volume with diluent and mixed.

Standard Preparation (30 ppb Pb):

1. Transfered accurately 0.25 mL of Lead standard solution (1000 ppm Pb) to a 250 mL volumetric flask and mixed, made up the volume with diluent.
2. Transfered 0.75 mL of the resulting solution to a teflon tube, added 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) and digested in microwave reaction system as per microwave parameters given under 8.1.3.6.1.
3. Transferred the resulting digested solution to a 25 mL volumetric flask, rinsed the tube with 10 ml of diluent and transferred it to the same volumetric flask, and made up to volume with diluent and mixed.

**Test preparation:**

1. Weighed and transferred accurately about 0.05 g of the test sample to a teflon tube. Added 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) and digested in microwave reaction system as per microwave parameters given under 8.1.3.6.1.

2. Transferred the resulting digested solution to a 25 mL volumetric flask, rinsed the tube with 10 ml of diluent and transferred it to the same volumetric flask and made up to volume with diluent and mixed.

**Reagent blank:**

1. Transferred 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) to a teflon tube and digested in microwave reaction system as per microwave parameters given under 8.1.3.6.1.

2. Transferred the resulting digested solution to a 25 mL volumetric flask, rinsed the tube with 10 ml of diluent and transferred it to the same volumetric flask and made up to volume with diluent and mixed.

**Instrumental parameters:**

**Table 7. Microwave parameters heavy metal contamination**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Power</th>
<th>Ramp</th>
<th>Hold time (min.)</th>
<th>Fan</th>
</tr>
</thead>
</table>

---
AAS (with Graphite furnace) Parameters:

Spectrometer:-

(i) Lam\(\text{p}\) setting

Element : Lead

Wavelength : 283.31 nm

Slit width : 2.7/1.05nm

Signal type : AA-BG

Measurement : Peak Area

(ii) Read parameter setting:-

Read time : 5 Seconds

Delay time : 0 Seconds

BOC time : 2 Seconds

Replicate : 3 (Same for all samples)

Calibration equation : Linear through Zero

Units:-

Calibration : µg/L

Sample : µg/L

a) Graphite Furnace:-

(i) Temperature programming:
### Read step 4

**Auto sampler parameter:-**

- Sample Volume: 25µl
- Volume of matrix modifier: 20µl

<table>
<thead>
<tr>
<th>Standard Preparation (30 ppb Pb)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of diluent (in µL)</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Std conc. obtained (in ppb)</td>
<td>6</td>
<td>12</td>
<td>18</td>
<td>24</td>
<td>30</td>
</tr>
</tbody>
</table>

**Procedure:**

1. Introduced diluent (as blank) into the atomic generator for auto zero.

2. Introduced each of the reference solutions in triplicate and recorded the steady reading.
   
   Drew linearity curve (linear through zero).

3. Introduced the reagent blank into the atomic generator for auto zero.
4. Introduced the test solution in triplicate into atomic generator and recorded the steady reading.

5. Calculated the linear equation of the graph using a least-squares fit and derived from it the conc. (C) of lead in the test solution in µg/L.

*Note: Coefficient of determination should not be less than 0.95. The LOQ of the method is 2.6 ppb in test solution.*

Calculation:

\[
\frac{Tc}{1000} \times \frac{25}{W_T}
\]

Where,

\(Tc\) = Average conc. (ppb) found in test.

\(W_T\) = Weight of test sample taken (in g).

**Cadmium:**

**Instrument:** AAS with Graphite Furnace and Microwave Reaction System

**Reagents and Standard**

- Nitric acid ultrapure grade (Make Merck)
- Magnesium nitrate Hexahydrate AR grade
Ammonium dihydrogen phosphate AR grade

Hydrogen Peroxide Sol. (30%)

Milli Q water

Cadmium Standard above 99% purity

**Preparation of reagents:**

**Diluent (0.5% v/v nitric acid):**

Diluted 5 ml of nitric acid to 1000 ml with water.

**Matrix modifier:**

Weigh and transfer accurately about 0.20 g of ammonium dihydrogen phosphate and 0.05 g of magnesium nitrate hexahydrate to a 100 ml volumetric flask dissolved in and made up the volume with diluent and mixed.

**Standard preparation (1.125 ppb Cd):**

1. Weighed and transferred accurately 0.100 g of Cadmium Standard to a 100 mL volumetric flask, dissolved in 3 mL nitric acid and 3 mL water and made up the volume with diluent.
2. Transferred 0.1 mL of the resulting solution to 100 mL with diluent and mixed.
3. Further, diluted 5 mL of the above resulting solution to 50 mL with diluent and mixed.
4. Transferred 0.225 mL of the above solution to a teflon tube, added 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) and digested in microwave reaction system as per microwave parameters given under 8.2.7.1.
5. Transferred the resulting digested solution to a 20 mL volumetric flask, rinsed the tube
with 10 ml of diluent and transferred it to the same volumetric flask and made up to
volume with diluent and mixed.

Test preparation:

1. Weighed and transferred accurately about 0.05 g of the test sample to a teflon tube.
2. Added 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) and digested in
microwave reaction system as per microwave parameters given under 8.2.7.1.
3. Transferred the resulting digested solution to a 20 mL volumetric flask, rinsed the tube
with 10 ml of diluent and transferred it to the same volumetric flask and made up to
volume with diluent and mixed.

Reagent blank:

1. Transferred 4 ml of nitric acid and 1 ml hydrogen peroxide sol. (30%) to a teflon tube and
digested in microwave reaction system as per microwave parameters given under 8.2.7.1.
2. Transferred the resulting digested solution to a 20 mL volumetric flask, rinsed the tube
with 10 ml of diluent and transferred it to the same volumetric flask and made up to
volume with diluent and mixed.

Instrumental parameters:

Microwave parameter:-

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Power</th>
<th>Ramp</th>
<th>Hold time (min.)</th>
<th>Fan</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>10</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

AAS (with Graphite furnace) parameters:

a) Spectrometer:-
(i) **Lamp setting**

Element: *Cadmium*

Wavelength: 228.80 nm

Slit width: 1.8/0.06nm

Signal type: AA-BG

Measurement: Peak Area

(ii) **Read parameter setting**:

Read time: 5 Seconds

Delay time: 0 Seconds

BOC time: 2 Seconds

Replicate: 3 (Same for all samples)

Calibration equation: Linear through Zero

(iii) **Units**:

Calibration: µg/L

Sample: µg/L

b) **Graphite Furnace**:

(i) **Temperature programming**:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp °C</th>
<th>Ramp time (Sec.)</th>
<th>Hold time (Sec.)</th>
<th>Gas flow (mL/min.)</th>
<th>Gas type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>5</td>
<td>20</td>
<td>250</td>
<td>Normal (Argon)</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>15</td>
<td>15</td>
<td>250</td>
<td>Normal (Argon)</td>
</tr>
<tr>
<td>3</td>
<td>650</td>
<td>10</td>
<td>20</td>
<td>250</td>
<td>Normal (Argon)</td>
</tr>
<tr>
<td>4</td>
<td>1350</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>Normal (Argon)</td>
</tr>
<tr>
<td>5</td>
<td>2600</td>
<td>1</td>
<td>3</td>
<td>250</td>
<td>Normal (Argon)</td>
</tr>
</tbody>
</table>
Read step 4

Auto sampler parameter:

Sample volume : 25 µl
Volume of matrix modifier : 7 µl

<table>
<thead>
<tr>
<th>Standard preparation (1.125 ppb Cd) volume (in µl)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of diluent (in µL)</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Std conc. obtained (in ppb)</td>
<td>0.225</td>
<td>0.450</td>
<td>0.675</td>
<td>0.90</td>
<td>1.125</td>
</tr>
</tbody>
</table>

Procedure:

1. Introduced diluent (as blank) into the atomic generator for auto zero.

2. Introduced each of the reference solutions in triplicate and recorded the steady reading.

   Draw linearity curve (linear through zero).

3. Introduced the reagent blank into the atomic generator for auto zero.

4. Introduced the test solution in triplicate into atomic generator and recorded the steady reading.

5. Calculated the linear equation of the graph using a least-square fit and derived from it the conc. (C) of Cadmium in the test solution in µg/L.

*Note: Coefficient of determination should not be less than 0.95. The LOQ of the method is 0.06 ppb in test solution.*

Calculation:
Tc = 20

Cadmium (in ppm)  =  \frac{T_c}{1000 W_T}

Where,

Tc  =  Average conc. (ppb) found in test.

W_T  =  Weight of test sample taken (in g).

Mercury:

**Instrument: AAS with FIAS flame and Microwave Reaction System**

**Reagents and Standard**

- Hydrochloric acid ultrapure grade (Make Merck)
- Potassium permanganate AR grade
- Ascorbic acid AR grade
- Nitric acid ultrapure grade (Make Merck)
- Hydrogen Peroxide Sol. (30%) AR grade
- Sodium borohydride AR grade
- Sodium Hydroxide AR grade
- Milli Q water
- Mercury Standard Solution 10 ppm
Preparation of reagents:

Diluent: Water

Matrix modifier (5% KMnO₄):

Weighed and transferred accurately about 0.5 g of potassium permanganate to a 10 mL volumetric flask dissolved in and made up the volume with diluent and mixed.

0.01 % Sodium hydroxide:

Weighed and transferred accurately about 0.1 g of sodium hydroxide in a 1000 mL volumetric flask dissolved in and made up the volume with water and mixed.

0.2% Sodium borohydride in 0.01 % Sodium Hydroxide (Reductant solution):

Weighed and transferred accurately about 2.0 g of sodium borohydride and 1.0 g of ascorbic acid to a 1000 mL volumetric flask dissolved in and made up the volume with 0.01% sodium hydroxide and mixed.

3% Hydrochloric acid (Carrier solution):

To 500 mL water, added 30 mL of Hydrochloric acid and made up the volume with water to 1000 mL.

Stock standard preparation (100 ppb Hg)

Transferred accurately 1 mL of Mercury Standard Solution (10 ppm Hg) solution to a 100 mL volumetric flask, added 50 mL diluent and 1 mL of nitric acid mixed in and made up the volume with diluent.

Linearity standard preparations:
1. Transferred accurately 0.5, 1.0, 1.75, 2.50 and 3.25 mL of the above stock standard preparation (100 ppb Hg) to separate teflon tubes. To each of the teflon tube added 4 ml nitric acid and 4 ml hydrogen peroxide solution 30% and digested in microwave reaction system as per microwave parameters given under 8.3.8.1.

2. Transferred each of the digested standard preparations to separate 25 ml volumetric flasks, rinsed the teflon tubes with 5 ml diluent and transferred to the respective volumetric flasks. To each of volumetric flasks, added 0.4 ml of hydrochloric acid, and 20 μL of matrix modifier and made up to volume with diluent to obtain linearity standard solutions having conc. of 2, 4, 7, 10 and 13 ppb of Hg respectively.

**Test preparation:**

1. Weighed and transferred accurately about 0.20 g of the test sample to a teflon tube. To the teflon tube added 4 ml nitric acid and 4 ml hydrogen peroxide solution 30% and digested in microwave reaction system as per microwave parameters given under 8.3.8.1.

2. Transferred the digested solution to a 25 ml volumetric flask. Rinsed the teflon tube with 5 ml diluent and transferred to the volumetric solution. To it, added 0.4 ml of hydrochloric acid, and 20 μL of matrix modifier and made up to volume with diluent.

**Reagent Blank:**

1. Transferred 4 ml of nitric acid and 4 ml of hydrogen peroxide to a teflon tube and digested in microwave reaction system as per microwave parameters given under 8.3.8.1.
2. Transfered the digested solution to a 25 ml volumetric flask. Rinsed the teflon tube with 5 ml diluent and transfer to the volumetric solution. To it, added 0.4 ml of hydrochloric acid, and 20 µL of matrix modifier and made up to volume with diluent.

**Instrumental parameters:**

**Microwave parameters:**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Power</th>
<th>Ramp</th>
<th>Hold time (min.)</th>
<th>Fan</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1000</td>
<td>10</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>

**AAS (with FIAS Flame) parameters:**

a. **Spectrometer:**

(i) **Lamp setting**

Element : Mercury  
Wavelength : 253.65 nm  
Slit width : 2.7/1.05 nm  
Signal type : AA  
Measurement : Peak Height

(ii) **Read parameter setting:**

Read time : 25 Seconds  
Delay time : 0 Seconds  
BOC time : 2 Seconds  
Replicate : 3 (Same for all samples)  
Calibration equation : Linear through Zero
(iii) **Units:-**

- **Calibration**: µg/L
- **Sample**: µg/L

b. **FIAS programming:-**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time Sec.</th>
<th>Pump 1 speed</th>
<th>Pump 2 speed</th>
<th>Valve position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-fill</td>
<td>15</td>
<td>120</td>
<td>0</td>
<td>Fill</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>120</td>
<td>0</td>
<td>Fill</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>120</td>
<td>0</td>
<td>inject</td>
</tr>
</tbody>
</table>

**Read step 2**

- **Sample volume**: 500µl
- **Gas flow (Argon)**: 150 mL/min.

*Flame programme:* Flame Off

**Procedure:**

1. Diped the line of reductant in reductant solution (0.2% sodium borohydride in 0.01% sodium hydroxide) and line of carrier in carrier solution (3% Hydrochloric acid) and ran programme as given in FIAS programming.

2. Introduced reagent blank (as blank) into the atomic generator for auto zero.
3. Introduced each of the reference solutions in triplicate and recorded the steady reading.

   Draw linearity curve (linear through zero).

4. Introduced the reagent blank into the atomic generator for auto zero.

5. Introduced the test solution in triplicate into atomic generator and recorded the steady reading.

6. Calculated the linear equation of the graph using a least-squares fit and derived from it the conc. (C) of Mercury in the test solution in µg/L.

   *Note: Coefficient of determination should not be less than 0.95.*

The LOQ of the method is 1.9 ppb in test solution

**Calculation:**

\[
\text{Mercury (in ppm)} = \frac{T_c \times 25}{1000 \times W_T}
\]

Where,

\[
T_c = \text{Average conc. (ppb) found in test.}
\]

\[
W_T = \text{Weight of test sample taken (in g).}
\]

**Arsenic**

Arsenic is abundant in nature and its presence in herbal medicines should be no different to its wide occurrence in foods. A popular test method relies on the digestion of the plant matrix followed by subjection of the digestate to a comparative colorimetric test in a special apparatus.
The test method described below uses colorimetry and does not use toxic mercuric bromide paper. The method uses $N$-$N$-diethylmethyldithiocarbamate in pyridine and it reacts with hydrogen arsenide to afford a red–purple complex. The limit is expressed in terms of arsenic (III) trioxide ($\text{As}_2\text{O}_3$).

**Procedure**

Placed glass wool in the exit tube B up to about 30 mm in height, moistened the glass wool uniformly with a mixture of an equal volume of lead (II) acetate and water, and applied gentle suction to the lower end to remove the excess mixture. Inserted the tube vertically into the centre of the rubber stopper, and attached the tube to the generator bottle so that the small perforation in the lower end extends slightly below the stopper. At the upper end attached the rubber stopper to hold the tube vertically. Made the lower end of the exit tube level with that of the rubber stopper.

**Preparation of the test solution**

**Method**

Weighed the specified amount of the sample and placed it in a crucible of porcelain. Added 10 ml of a solution of magnesium nitrate hexahydrate in 95% ethanol, burned the ethanol, heated gradually, and ignited to incinerate. After cooling, added 3 ml of hydrochloric acid, heated in a water bath to dissolve the residue, and designated it as the test solution.

**Standard solutions**

- *Absorbing solution for hydrogen arsenide.* Dissolved 0.50 g of silver $N$-$N$-diethyl-dithiocarbamate in pyridine to made 100 ml. Preserved this solution in a glass-
stoppered bottle protected from light, in a cold place.

- **Standard arsenic stock solution.** Weigh accurately 0.100 g of finely powdered arsenic (III) trioxide standard reagent dried at 105 °C for 4 hours, and added 5 ml of sodium hydroxide solution (1 in 5) to dissolve. Added dilute sulfuric acid to neutralize, added a further 10 ml of dilute sulfuric acid and added freshly boiled and cooled water to make exactly 1000 ml.

- **Standard arsenic solution.** Pipetted 10 ml of standard arsenic stock solution, added 10 ml of dilute sulfuric acid and added freshly boiled and cooled water to make exactly 1000 ml. Each ml of the solution contains 1 μg of arsenic (III) trioxide (As₂O₃). Prepared standard arsenic solution just before use and preserved in a glass-stoppered bottle.

**Procedure**

Placed the test solution in the generator bottle and wash down the solution in the bottle with a small quantity of water. Added 1 drop of *methyl orange*, and after neutralizing with *ammonia*, added 5 ml of diluted hydrochloric acid (1 in 2) added 5 ml of *potassium iodide*, and allowed to stand for 2 to 3 minutes. Added 5 ml of *acidic tin (II) chloride* and allowed to stand for 10 minutes. Then added water to make 40 ml, added 2 g of zinc for arsenic analysis and immediately connected the rubber stopper fitted.

**Preparation of standard colour.** Measured accurately 2 ml of Standard Arsenic Solution into the generator bottle. Added 5 ml of diluted hydrochloric acid (1 in 2) and 5 ml of *potassium iodide* and allowed to stand for 2 to 3 minutes. Added 5 ml of *acidic tin (II) chloride*, allowed to stand at room temperature for 10 minutes and then proceeded as directed above. The colour produced corresponds to 2 μg of arsenic (III) trioxide (As₂O₃) and is used as the standard.

**Determination of Microbial contamination**:71:
Since it became known in 1960’s that patience could contract serious infections from oral and ophthalmic drugs contaminated with *Salmonella* and *Pseudomonas*, great efforts have been made both by scientist and by the authorities to put limitations on the degree of microbial contamination, even in medicinal preparations which do not obligatorily need to be sterile. The principal demand is for the demonstrated absence of pathogenic bacteria and so called indicator bacteria, example *Escherichia Coli*.

The permitted number of apathogenic bacteria in oral medicaments is very much lower than in foodstuffs and should perhaps relate to the finished product. The limit in natural starting materials should be 10⁴ bacteria /g and 100 mould or yeast cells /g, especially when a reduction of the number of organisms can be expected from further processing.

**Sample Preparation:**

Dissolved 10 g of sample in 90 ml of Sodium chloride peptone solution to prepare 1:10 dilution or to make further suitable dilutions.

**Tests for Total Viable Aerobic Count**

1. Transfered 1 ml of prepared sample in duplicate to the two sets of plates.
2. Aseptically pored around 20 ml of Soyabean Casein Digest Agar in two plates and Sabouraud Dextrose Agar in another two plates.
3. Mixed well to distribute the sample properly and allow solidifying the medium.
4. Inverted the plate and incubated the plates of at Soyabean Casein Digest Agar at 30 - 35°C for 3 days and Sabouraud Dextrose Agar at 20 -25°C for 5 days.
5. After completion of incubation period counted the number of colonies observed on plates.
6. Enumerate the counts as cfu/g by using the formula given below
7. Taken the mean of two plates for both SCDA and SDA and reported the counts on SCDA as Total Viable Aerobic Counts and the counts on SDA as total yeast and mold counts.

Test for *Escherichia coli*

1. Prepared a sample using a 10 fold dilution of not less than 1 g of the product to be examined in sodium chloride peptone solution.
2. Prepared 3 tubes of suitable quantity of soyabean casein digest broth.
3. Transfered quantities corresponding to 0.1 g, 0.01 g and 0.001 g to the tubes of soyabean casein digest broth.
5. Shook all the tubes and transfered 1 ml from each tube to 100 ml of MacConkey broth and incubated at 42-44 °C for 18-24 hrs.
6. Subcultured from MacConkey broth to plates of MacConkey agar and incubated at 30 -35°C for 18-72 hrs.
7. Growth of colonies indicates the possible presence of *E. coli*. This was confirmed by identification test.
8. Noted the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result.
9. Determine the probable number of bacteria from the following table.
5.2 PHYTO-CHEMICAL STANDARDIZATION

PRELIMINARY PHYTOCHEMICAL SCREENING 60-61,72,73

The different qualitative chemical tests can be performed for establishing profile of given extract for its chemical composition. The following tests may be performed on extracts to detect various phytocomponents present in them.

❖ Detection of Alkaloids

Solvent free extract, 50 mg is stirred with few ml of dilute hydrochloric acid and filtered. The filtrate is tested carefully with various alkaloidal reagents as follows

➢ Mayer’s test

To a few ml of filtrate, 1-2 drop of Mayer’s reagent (Potassium mercuric iodide solution) was added by the sides of the test tube. A white or creamy precipitate indicated the test as positive.

➢ Wagner’s test

To a few ml of filtrate, few drops of Wagner’s reagent (Iodine potassium iodide solution) was added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

➢ Hager’s test

To a few ml of filtrate, 1 or 2 ml of Hager’s reagent (saturated solutions of picric acid) was added. A prominent yellow precipitate indicated the test as positive.

➢ Dragendorff’s test
To a few ml of filtrate, 1 or 2 ml of Dragendorff’s reagent (Potassium bismuth iodide solution) was added. A reddish brown indicated the test as positive.

**Detection of Carbohydrates and Glycosides**

The extract (100 mg) was dissolved in 5 ml of distilled water and filtered. The filtrate was subjected to the following tests.

- **Molish’s test**
  
  To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol was added, the mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

- **Fehling’s test**
  
  One ml of filtrate was boiled on water bath with 1 ml each of Fehling solutions A and B. A red precipitate indicated the presence of sugar.

- **Barfoed’s test**
  
  To 1 ml of filtrate, 1 ml of Barfoed’s reagent was added and heated on a boiling water bath for 2 minute. Red precipitate indicated the presence of sugar.

- **Benedict’s test**
  
  To 0.5 ml of filtrate, 0.5 ml of Benedict’s reagent was added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicated the presence of sugar.

**For detection of glycosides**

50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hour on a water bath, filtered and the hydrolysate was subjected to the following tests.
➢ **Borntrager’s test**

To 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides.

➢ **Legal’s test**

Fifty mg of extract was dissolved in pyridine, sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycoside was indicated by pink colour.

❖ **Detection of Saponins**

The extract (50 mg) was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicated the presence of saponins.

❖ **Detection of Proteins and Amino acids**

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

➢ **Millon’s test**

To 2 ml of filtrate, few drops of Millon’s reagent was added. A white precipitate indicated the presence of proteins.

➢ **Biuret test**
An aliquot of 2 ml of filtrate was treated with one drop of 2% Copper sulphate solution. To this, 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicated the presence of proteins.

- **Ninhydrin test**

  Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) was added to 2 ml of aqueous filtrate. A characteristic purple colour indicated the presence of amino acids.

- **Detection of Phytosterols**

  - **Libermann-Burchard’s test**

    The extract (50 mg) was dissolved in 2 ml acetic anhydride. To this, 1-2 drops of concentrated sulphuric acid was added slowly along the sides of the test tube. An array of colour changes shows the presence of phytosterols.

- **Detection of Fixed Oils and Fats**

  - **Spot test**

    A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil.

- **Saponification test**

  A few drops of 0.5 N alcoholic potassium hydroxide solution was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 hour. Formation of soap or partial neutralisation of alkali indicated the presence of fixed oils and fats.
Detection of Phenolic compounds and Tannins

Ferric chloride test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

Gelatine test

The extract (50 mg) was dissolved in 5 ml of distilled water and 2 ml of 1% solution of gelatine containing 10% sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

Lead acetate test

The extract (50 mg) was dissolved in 5 ml of distilled water and to this, 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

Magnesium and hydrochloric acid reduction

The extract (50 mg) was dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) was added. If any pink to crimson colour developed, presence of flavanol glycosides was inferred.

Detection of Gum and Mucilages
The extract (100 mg) was dissolved in 10 ml of distilled water and to this, 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitate indicated the presence of gums and mucilages.

Detection of Volatile Oil

In a volatile oil estimation apparatus, 50 gram of powdered material (crude drug) was taken and subjected to hydro-distillation. The distillate was collected in graduated tube of the assembly, wherein the aqueous portion automatically separated out from the volatile oil.

FLUORESCENCE ANALYSIS

A molecule can be excited from its ground electronic state to an excited electronic state by absorbing energy in the form of visible or ultraviolet light. Many molecules are capable of emitting this energy as radiation, thus returning to the ground state. The emitted radiation is called fluorescence. For fluorescence analysis the whole plant powder was treated with different solvents (1N aqueous sodium hydroxide and 1N alcoholic sodium hydroxide, acids like 1N hydrochloric acid, 50% sulphuric acid and nitric acid) separately and then these extracts were subjected to fluorescence analysis in visible/day light and UV light (254nm & 365nm).

THIN LAYER CHROMATOGRAPHY (TLC) ANALYSIS

The term chromatography (Greek Kromatos-colour and Graphos-written) meaning colour writing. Chromatography represents a group of methods for separating molecular mixtures that depend on the differential affinities of the solute between two immiscible phases. IUPAC has defined chromatography as “A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid or a liquid supported on a solid or a
gel and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid”.

In TLC, the separation is carried on a glass or plastic plate, which is coated with a thin uniform layer of finely divided inert adsorbent such as silica gel or alumina. The special advantages of TLC compared to PC (Paper chromatography) include versatility (a number of different adsorbents besides cellulose may be used), greater speed (due to more compact nature of the adsorbent when spread on a plate) and sensitivity (separations on less than µg amounts of materials can be achieved). One more advantage over PC is that glass plates may be spread with conc. H₂SO₄, a useful detection reagent for steroids and lipids. One of the disadvantages of TLC was the labour of spreading glass plates with adsorbent, a labour somewhat eased by the automatic spreading devices.

**Procedure**

The glass plates have to be carefully cleaned with acetone to remove grease. Then the slurry of adsorbent in water was vigorously shaken for a set time interval before spreading. Finally, plates after spreading were air dried and then activated by heating in an oven at 100-110°C for 30 minutes. The solution of the sample in a volatile solvent was applied by using a capillary tube or a micropipette to a spot keeping 1.2 cm from the bottom of the TLC plate. The position of the sample spot was indicated by making a ‘origin line’ on the plate with the lead pencil. When the spot has dried, the plate is placed vertically in a suitable tank (which is paper-lined so that the atmosphere inside is saturated with the solvent phase) with its lower edge
immersed in selected mobile phases. The solvent rises by capillary action, resolving the sample mixture into discrete spots.

**Adsorbent:** Silica gel 60 F$_{254}$

**Solvent system:**

Toluene: Ethyl acetate: Diethylamine (70:20:10) for Alkaloids

Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26) for Flavonoids

Chloroform: Glacial acetic acid: Methanol: Water (64:32:12:8) for Saponins

Diethyl ether: Toluene (1:1; Saturated with 10 % acetic acid) for Coumarins

Toluene: Ethyl acetate (93:7) for Essential oils

Ethyl acetate: Methanol: Water (100:13.5:10) for Glycosides

**Detection:**

The developed chromatogram was first inspected under UV light. After preliminary inspection in UV light, each chromatogram was analyzed for the presence of drug constituents by spraying with an appropriate group reagent.

**$R_f$ value**

The $R_f$ value is the distance of a compound moves in chromatography relative to the solvent front. It is calculated by using the formula

$$R_f = \frac{\text{Distance travelled by Component from base line}}{\text{Distance travelled by Solvent front from base line}}$$
HPTLC is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed our work and allows us to do many things at a time usually not possible with other analytical techniques.

For scanning use of UV / Visible / Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer.

**Steps involved in HPTLC**

1. Selection of chromatographic layer
2. Sample and standard preparation
3. Layer pre-washing
4. Layer pre-conditioning
5. Application of sample and standard
6. Chromatographic development
7. Detection of spots
8. Scanning
9. Documentation of chromatic plate

**Development of HPTLC finger print**
The samples were applied at a concentration of 2-10 μl and standard at about 5μl were applied using CAMAG Linomate V Sample applied on aluminium sheets precoated with silicagel 60F254 (Merck) of 0.2mm layer thickness and 5x20 cm will be used as a stationary phase in different track.

The plate was developed in the mobile phase to a distance of 120mm for developing the chromatogram. The developments were carried out in CAMAG twin trough glass chamber.

Maintain the band width as 6mm and applied the band on the plate at a distance of 6mm. The different tracks were scanned using CAMAG densitometer scanner 3VI.13 equipped with CATS V 4.04 software at a wavelength of 254 and 366 nm using deuterium lamp and recorded the finger print profile.

**Reagents:**

- Toluene AR grade
- Ethyl acetate AR grade
- Formic acid AR grade
- Methanol AR grade
- Diphenylboryloxyethylamine AR grade
- Ethanol (96%) AR grade
- Polyethylene glycol 4000 AR grade

**Mobile phase:**
Prepared a mixture of ethyl acetate, formic acid and water (8 :1 :1).

**Dipping reagent I:**

1. Dissolved 1.0 g of diphenylboric acid-2-aminoethyl ester in methanol to produce 100 mL.
2. Added 100mL of dichloromethane AR to the solution and mixed well.

**Dipping reagent II:**

1. Dissolved 5.0 g of polyethylene glycol 4000 in sufficient ethanol to produce 100 mL.
2. Added 100 mL of dichloromethane AR to the solution and mixed well.

**Reference solution:**

1. Weighed and transferred accurately about 25.0 mg standard of curcumin, 25.0 mg standard of phyllanthin, 25.0 mg standard of Anthocyanin, 25.0 mg standard of chebulinic acid, 25.0 mg standard of cyperene and 25.0 mg standard of β-carophyllene in a 10 mL volumetric flask.
2. Added about 8 mL methanol and sonicated for 15 minutes and made up the volume with the same solvent and mixed.
3. Kept the volumetric flask aside for at least 30 minutes at room temperature to allow undissolved particles if any, to settle down. Filtered the solution through 0.45µm membrane filter (Millipore HVLP type).

**Test solution:**

1. Weighed accurately about 2.5 g of dried and powdered test sample.
2. Added 50 mL of methanol and sonicated for 15 minutes with slight warming.

3. Cooled the solution and filtered through a Whatman filter paper No. 1.

**Procedure:**

**Development chamber:**

Took clean, dried twin trough HPTLC development chamber. Put a piece of filter paper (Whatman No.1) into one trough of the development chamber.

**Application of spots:**

1. Taken, pre-coated Aluminium HPTLC plate [coated with 0.25 mm layer of chromatographic silica gel 60 F$_{254}$] 10 cm of height and applied 10 µl each of the filtered test solution and the reference solution separately on the plate at a height of about 1-2 cm distance from the base of the plate with the help of applicator.

2. Allowed the spots to dry in air.

**Saturation of development chamber and HPTLC plate:**

1. Transfered mobile phase through the filter paper trough of development chamber.

2. Placed plate into other trough (without mobile phase) of development chamber.

3. Closed the chamber with chamber lid and saturated the chamber as well as plate with mobile phase for 30 minutes.

4. After 30 minutes, opened the chamber lid took out the saturated plate and tilted the development chamber such that there is equal distribution of mobile phase in both troughs of development chamber.

**Development:**
Placed the saturated plate into same trough (now filled with mobile phase) and developed using the mobile phase until the solvent front had moved up to 8 cm to 9 cm from application position.

**Scanning and Derivatization:**

1. Took out the plate from development chamber.

2. Marked the solvent front at one corner with a pencil, dried the plate in air and scanned the plate using TLC Scanner at 254 nm.

3. Filled the TLC dipping chamber reagent-I and dipped the developed plate in the dipping chamber (containing reagent –I) up to at least 5 mm above the solvent front, and dried it.

4. Now changed the reagent of the dipping chamber to dipping reagent II and again dipped the plate in the dipping reagent II.

5. Dried the plate in air and exposed the plate to ammonia vapours for 1 minute.

**Photograph:**

Taken the photograph of the derivatized plate with the help of digital camera and tested under UV light at 366 nm.

**Acceptance criteria:**

1. The \( R_f \) of main peaks obtained with the test solution from \( R_f \) 0.1 onwards correspond to those obtained with the reference solution after scanning at 254 nm.

2. At 366 nm the positions as well as the colours of fluorescent spots obtained with the test solution from \( R_f \) 0.1 onwards correspond to those obtained with the reference solution after derivatization by dipping in reagent I and reagent II.
5.4 TOXICOLOGICAL STUDIES

1. Acute toxicity studies of Neerzhivu choornam formulation

Experimental animals\textsuperscript{90, 91}

Albino Wistar rats (180-220g) of either sex bred in the animal house were used in this study. The animals were fed on a standard pellet diet (Hindustan Unilever Ltd, Mumbai-400 099) and had free access to ozonised filter water \textit{ad libitum}. The animals were maintained in their respective groups under controlled conditions of temperature and humidity\textsuperscript{11}. All the studies were conducted in accordance with CPCSEA guidelines and the experiments were carried out as per the approval of institutional ethics committee (IAEC-XII/SRU/78/2008).

Dose and drug solution

Traditionally 1 to 2g of the Neerizhivu choornam is used in diabetes. Further for this study, in-house prepared Neerizhivu choornam was suspended in 1% gum acacia solution to have a desired dose of 125, 250 and 500 mg/kg BW in 1ml solution. Glibenclamide was obtained as a gift sample from USV Ltd, Mumbai, India. All other reagents and chemicals used were of analytical grade and procured locally.

Acute toxicity studies\textsuperscript{92}

The study was carried out according to the OECD guidelines 423. Female Wistar rats of weight (180-220g) were taken for the study and kept for overnight fasting. Next day, body weight was taken and Neerizhivu choornam was administered orally at a dose of 2000mg/kg in distilled water. Then the animals were observed for mortality and morbidity at 0, \(\frac{1}{2}\), 1, 2, 4, 6, 8, 12 and 24 hr. Feed was given to the animals after 4 hr of the dosing and the body weight was checked prior and at 6 hr after dosing. The animals were observed twice daily for 14 days and
body weight was taken. The same experiment was repeated again on 3 rats as there was no observable clinical toxicity for the animals on the acute toxicity study.

5.5 PHARMACOLOGICAL SCREENING

Diabetes mellitus is a metabolic disorder characterized by increased blood glucose levels associated with discharge of glucose in urine. There are two major types of Diabetes mellitus i.e,

1. Insulin dependent diabetes mellitus (IDDM)

2. Non Insulin dependent diabetes mellitus (NIDDM)

Insulin dependent diabetes mellitus also called type 1 diabetes, occurs due to complete loss of pancreatic β islet cells and hence there is insulin deficiency. Non insulin dependent diabetes mellitus also called as type 2 diabetes, is due to insulin resistance. Insulin resistance is developed due to defects at the receptor level or insulin signaling at the post receptor level. This defect may be in the effectors cells such as the skeleton muscle, the adipose tissue or in the β islet cells. A large number of drugs including herbs and herbal formulation with suspected anti-diabetic activity have been successfully tested in the laboratory.

Streptozotocin induced diabetes

Streptozotocin is a broad spectrum anti-biotic which causes β islet cells damage by free radical generation. Streptozotocin induces diabetes in almost all species of animals excluding rabbits and guinea pigs. The diabetogenic dose of Streptozotocin varies with species. In mice, the dose level is 200mg/kg through i.p and in beagle dogs 15mg/kg through i.p. for three days.

3. Anti-hyperglycemic studies of Neerzhivu choornam formulation.

Hypoglycemic activity of Neerizhivu choornam in normal rats
Normal fasted rats: Normal albino rats (180-220 mg) were first used for the screening of the herbal formulation Neerizhivu choornam for hypoglycemic activity. Overnight fasted normal rats were randomly divided into 5 groups, of 6 rats each. The group I served as control, which received vehicle i.e. 1% W/V Gum acacia solution (1ml/kg, orally). Group II, III and IV were treated orally with test Neerizhivu choornam 125, 250 and 500 mg/kg, respectively. Group V received standard drug Glibenclamide 5 mg/kg orally.

Table 8. Experimental protocol for hypoglycemic activity of Neerizhivu choornam in normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Treated with 1% Gum acacia solution, 1ml/kg</td>
</tr>
<tr>
<td>Group II</td>
<td>Treated orally with Neerizhivu choornam, 125mg/kg</td>
</tr>
<tr>
<td>Group III</td>
<td>Treated orally with Neerizhivu choornam, 250mg/kg</td>
</tr>
<tr>
<td>Group IV</td>
<td>Treated orally with Neerizhivu choornam, 500mg/kg</td>
</tr>
<tr>
<td>Group V</td>
<td>Treated orally with Glibenclamide, 5mg/kg</td>
</tr>
</tbody>
</table>

Blood samples were collected from tail vein prior and 1, 2, 4 and 6 hour after treatment using CONTOUR™TS blood glucose meter with same test strips. Fasting blood glucose was estimated by glucose oxidase and peroxidise (GOD/POD kit) method. Intensity of the red quinoneimine was measured at 540 nm in auto analyzer. The percentage (%) fall in blood glucose level was also calculated at peak hour of effect96-97.

Antidiabetic activity of Neerizhivu choornam in Streptozotocin (STZ) induced diabetic rats98-100

Induction of experimental diabetes
Adult albino Wistar rats (180-220g) of either sex were made diabetic with an intraperitoneal injection of 65mg/kg body weight of Streptozotocin (Sigma Aldrich chemical company, Mumbai) dissolved in 0.1 M cold citrate buffer, pH4.5, immediately before use. Streptozotocin injected animals exhibited massive glucosuria and hyperglycemia within few days. Diabetes was confirmed in STZ rats by measuring the fasting blood glucose concentration, on 4th day after the injection with STZ. Adult albino Wistar rats with blood glucose levels more than 200mg/dl were considered to be diabetic and were used in this experiment. The Neerizhivu choornam at the dose of 125, 250 and 500mg/kg body weight were administered orally after suspending in 1% gum acacia solution. The blood samples were collected from tail vein and the blood glucose levels were determined using CONTOUR™TS blood glucose meter with same test strips.

**Experiment no1.**

**Evaluation of Neerizhivu choornam for anti-hyperglycemic properties in STZ induced diabetic rats (single dose, short term study)**

After induction of diabetes, the rats were divided into 6 groups of six animal each and screened for anti-hyperglycemic activity of the various concentration of Neerizhivu choornam in overnight fasted diabetic rats. The blood samples were collected from tail vein and the blood glucose levels were determined using CONTOUR™TS blood glucose meter with same test strips.
Table 9. Experimental protocol for anti-hyperglycemic properties in STZ induced diabetic rats (single dose, short term study)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I</td>
<td>Healthy rats, treated with 1%w/v gum acacia/P.O</td>
</tr>
<tr>
<td>Group-II</td>
<td>Diabetic rats, 1%w/v gum acacia/P.O</td>
</tr>
<tr>
<td>Group-III</td>
<td>Treated with Neerizhivu choornam,125mg/kg/P.O</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Treated with Neerizhivu choornam,250mg/kg/P.O</td>
</tr>
<tr>
<td>Group-V</td>
<td>Treated with Neerizhivu choornam,500mg/kg/P.O</td>
</tr>
<tr>
<td>Group-VI</td>
<td>Treated with Glibenclamide,5mg/kg/P.O</td>
</tr>
</tbody>
</table>

Experiment no2.

Evaluation of Neerizhivu choornam for anti-hyperglycemic properties in STZ induced diabetic rats in presence of glucose load (Oral Glucose Tolerance Test).

Overnight fasted rats were divided into 6 groups of six animal each as mentioned as above and received the respective treatments. After 30 minutes of drug administration the rats of all the groups were orally administered with 2g/kg of glucose. Blood samples were collected from tail vein just prior to drug administration and at 30, 60, 120 and 240 minutes after glucose loading. Blood glucose levels were measured immediately using CONTOUR™TS blood glucose meter with same test strips.

Experiment no3.

Evaluation of Neerizhivu choornam for anti-hyperglycemic properties in STZ induced diabetic rats (multiple dose, long term study)
In multiple dose studies the Neerizhivu choornam at the dose of 125, 250 and 500mg/kg bodyweight once daily was given for 28 days and blood glucose levels were monitored only at seven days intervals. Blood sample were collected from tail veins of the animals. Blood glucose levels were determined using CONTOUR™TS blood glucose meter with same test strip at intervals of seven days. After 4 weeks of drug treatment, parameters such as fasting blood glucose, a portion of pancreatic tissue was homogenized and the extract was used for the estimation of activity of enzymes namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), lipid peroxidase (LPO), serum glutamate oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT), alkaline phosphophatase (ALP) by colorimetric method. The body weights of all the animals of all the groups were recorded before starting the treatment and at end of the treatment period103-105.

Table 10. Experimental protocol for anti-hyperglycemic properties in STZ induced diabetic rats (multiple dose, long term study)

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Normal control and received vehicle i.e. 1% Gum acacia Solution,</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>Diabetic control and received (STZ) + 1% Gum acacia solution,</td>
</tr>
<tr>
<td>Group 3</td>
<td>Treated orally with Neerizhivu choornam,125mg/kg/BW</td>
</tr>
<tr>
<td>Group 4</td>
<td>Treated orally with Neerizhivu choornam,250mg/kg/BW</td>
</tr>
<tr>
<td>Group 5</td>
<td>Treated orally with Neerizhivu choornam,500mg/kg/BW</td>
</tr>
<tr>
<td>Group 6</td>
<td>Received Glibenclamide,5mg/kg/BW</td>
</tr>
</tbody>
</table>
Estimation of blood parameters

Blood samples were collected from the retro-orbital plexus of the rats and the blood glucose level was estimated by GOD-POD method, total cholesterol were estimated by CHOD-PAP method and triglycerides level was estimated by GPO-ESPAS method using Ranbaxy diagnostic kits, New Delhi following the kit’s procedure. Serum insulin levels were estimated by Radio-immuno assay method by using R.I.A kit. (Baba atomic research centre, Mumbai, India). the results are expressed as µU of insulin ml.

Haemoglobin and glycosylated Hb (HbA₁C)

Haemoglobin was estimated by the method of Drabkin’s method. Intensity of the color formed by oxidized haemoglobin with potassium ferricyanide was measured at 530 nm in UV-Visible spectrophotometer (Shimadzu, Japan). Glycosylated Hb (HbA₁C) was estimated by
following the method of Sudhakar Nayak and Pattabiraman, 1982. Briefly, saline washed red cells were treated with water/CCl₄ for lysis and incubated at 37°C for 15 minutes and oxalate or HCl solution was then added and mixed. The filtrate was heated in a boiling water bath for 4 hrs, cooled with ice-cold water, treated with 40% TCA and again centrifuged at 1000g for 10 minutes. The supernatant obtained was then heated with 80% phenol and sulphuric acid and the colour developed using thiobarbituric acid was read at 480nm after 30 minutes.

**Histopathological study of pancreas**

Pancreas were isolated and preserved in 10% formalin. All paraformaldehyde fixed tissues were embedded in paraffin, sections 6µm thick cut with a cryostat microtome and then stained with haematoxylin and eosin. Histopathological observation of the tissues was carried out under a light microscope. Photomicrograph were taken to substantiate the findings. The alteration and changes in the histology of pancreas were shown in vide plate and the results with photomicrograph were given in the result section.

**Histopathology of other vital organ**

The histopathological changes occurred during the experimental studies on other vital organ such as lung, liver, heart, kidney and spleen were isolated and subjected for routine histopathological examination. Histopathological observations of the tissues were carried out under a light microscope. Photomicrographs were taken to substantiate the findings. The alteration and changes in the histology of other vital organ were shown in vide plate and the results with photomicrograph were given in the result section.

**Statistical analysis**

The data obtained was analyzed using prism software and the results were expressed as mean ± SEM, n=6. Statistical significance was determined by using one way analysis of variance.
(ANOVA) followed by dunnett’s test. The Neerizhivu choornam and Glibenclamide treated groups were compared with the corresponding normal or diabetic control. P<0.01 and p<0.05 were considered to be significant.

5.3 PHYTO-FORMULATION STUDIES

Need for phytoformulation studies

- To increase the stability
- To improve the patient compliance
- To improve the accuracy of dosage forms
- To mask the unpleasant odour and taste
- To enhance the Bio availability
- To make convenient, modern, elegant, patient attracting medicament

Tablet formulation\textsuperscript{82-84}

Tablets are the solid dosage form of powdered herbs, herbal extracts or their constituents prepared by moulding or compression. Certain additives are also added to the medicaments in the formulation of tablets to improve the disintegration and dissolution status. Tablets are usually in circular in shape or bi-convex.

Tablet is a convenient modern dosage form which ensures correct dosage administration. It also prevents the deterioration of medicaments as compared with other solid dosage forms. The shape, color, odour and taste can be conveniently made according to the medicament as well as patience compliance.

Coating material\textsuperscript{85}

Coating of a tablet is required
To mask a unpleasant taste and odour,
To improve the appearance of the tablet,
To protect the medicament from atmospheric effects,
To control the site of action of drugs,
To produce sustain release of the product

The coating is generally carried out either by using pan coating or press coating. After coating, polishing is done in a polishing pan. The pan coating technique is used for sugar coating, film coating and enteric coating.

1. **Preparation of three different batches of tablets of “Neerzhivu choornam”**

**Tablet Specification:**

- **Composition**: Each Film coated tablet contains Neerizhivu choornam 1000 mg
- **Excipients**: q.s.
- **Colour**: Yellow oxide of iron
- **Average Weight**: 1330 mg
- **Thickness**: 7.6 mm
- **Hardness**: 2.5Kg/cm³
- **D.T.**: 45 second (Uncoated)
- **D.T.**: 2.0 minute (Coated)
- **Assay**: 95 to 105% of label claim 30 mg of phenolic.

**Experiment**: Three batches were manufactured using different polymers or change in polymer quantities to establish the formulation.

**Procedure:**
1. Sifted Neerizhivu choornam through 20#

2. Sifted maize starch, lactose IP & PVP K90 through 40# & mixed each other up to 5 minutes.

3. Mixed stage 1 & stage 2 for 5 minutes.

4. Binded with above material through IPA (250 ml). Kept for air drying, dried material passed through 20#

5. Sifted Croscarmellose sodium & MCC pH 102 through 40# & mixed with stage 4.

6. Sifted magnesium stearate & Aerosol through 40# and mixed with stage 5, for 5 minutes. Then blend ready for compression.

**Compression Parameter:**

**Average weight** - 21.5 X 10 mm (1330 mg)

**Thickness** - 7.6 mm

**Hardness** – 2.5 kg/cm³

**D.T.** – 45 seconds
Finalization of Process

*Product Formulation First batch:-*

Product Name :- Neerizhivu choornam 1000 mg Tablet

B.NO. :- FD/189/12

Batch Size:- 665 gm. (500 Tablet)

<table>
<thead>
<tr>
<th>Sr. NO.</th>
<th>Ingredients</th>
<th>Qty./Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neerizhivu choornam</td>
<td>500 gm.</td>
</tr>
<tr>
<td>2</td>
<td>Lactose IP</td>
<td>25 gm.</td>
</tr>
<tr>
<td>3</td>
<td>Starch IP</td>
<td>40 gm.</td>
</tr>
<tr>
<td>4</td>
<td>PVP K-90</td>
<td>25 gm.</td>
</tr>
<tr>
<td>5</td>
<td>Microcrystalline Cellulose pH 102</td>
<td>30 gm.</td>
</tr>
<tr>
<td>6</td>
<td>Croscarmellose Sodium</td>
<td>25 gm.</td>
</tr>
<tr>
<td>7</td>
<td>Colloidal Silicone Dioxide</td>
<td>10 gm.</td>
</tr>
<tr>
<td>8</td>
<td>Magnesium Stearate</td>
<td>10 gm.</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>665 gm.</strong></td>
</tr>
</tbody>
</table>


Product Formulation Change in Process:

This formulation was manufactured by changing in polymer PVPK 30.

Product Name: Neerizhivu choornam 1000 mg Tablet

B.NO.: FD/190/12

Batch Size: 665 gm. (500 Tablet)

<table>
<thead>
<tr>
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</tr>
<tr>
<td>Total</td>
<td></td>
<td>665 gm.</td>
</tr>
</tbody>
</table>
**Product Formulation Third Process:-**

This formulation was manufactured by changing in quantity of PVPK 90 and starch.

**Product Name :- Neerizhivu choornam 1000 mg Tablet**

**B.NO. :- FD/191/12**

**Batch Size:- 665 gm. (500 Tablet)**

<table>
<thead>
<tr>
<th>Sr. NO.</th>
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<td><strong>Total</strong></td>
<td></td>
<td><strong>665 gm.</strong></td>
</tr>
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</table>
Capsule formulation

Powdered herbs are most easily taken as capsules but can be sprinkled on food or taken with water. Externally they can be applied as dusting powder to the skin or mixed with tinctures as a poultice. Gelatin or vegetarian capsule shells are mostly used as a material for capsule filling. It is easiest and convenient dosage form of administration of bitter herbal drugs. The coating and polishing technique can be avoided during capsule formulation.

2. Preparation of three different batches of capsule from Neerzhivu choornam

Capsule Specification:

**Composition**- Each capsule contain Neerizhivu choornam 1000 mg

**Excipients**- q.s.

**Colour**- Yellow oxide of iron

**Average Fill weight** - 1325 mg

**Diameter**: CAP-7.65 mm

**Body**-7.35 mm

**D.T.**– 7.0 minutes

**Assay** –Each capsule contain 95 to 105 % of label claim 30 mg of phenolic.

**Experiment**:

Three batches were manufactured using different polymers or change in polymer quantities to establish the formulation.
Procedure:-

1. Sifted Neerizhivu choornam through 20#

2. Sifted maize starch, lactose IP & PVP K90 through 40# & mixed each other up to 5 minutes.

3. Mixed stage 1 & stage 2 for 5 minutes.

4. Binded with above material through IPA (250 ml). Kept for air drying, dried material passed through 20 #

5. Shifted Croscarmellose sodium & MCC pH 102 through 40 # & mixed with stage 4.

6. Sifted magnesium stearate & Aerosol through 40 # and mixed with stage 5, for 5 minutes. Then blend ready for Filling of capsule.

7. Used ‘0’ size of empty Hard Gelatin capsule for filling.

Filling Parameter:

Average Net fill weight - 1325 mg

Average weight of empty shells - 96 mg

Diameter: CAP-7.65 mm

Body-7.35 mm

D.T.- 7.0 minutes
Finalization of Process

*Product Formulation First batch:-*

Product Name :- Neerizhivu choornam 1000 mg Capsule

B.NO. :- FD/201/12

Batch Size:- 665 gm. (500 Capsules)

<table>
<thead>
<tr>
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<tr>
<td>Total</td>
<td></td>
<td>665 gm.</td>
</tr>
</tbody>
</table>
**Product Formulation Change in Process:-**

This formulation was manufactured by changing the polymer PVPK 30.

**Product Name :- Neerizhivu choornam 1000 mg Capsule**

**B.NO. :- FD/202/12**

**Batch Size:-  665 gm. (500 Capsules)**

<table>
<thead>
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<td>665 gm.</td>
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</tbody>
</table>
**Product Formulation Third Process:**

This formulation was manufactured by changing the quantity of PVPK 90 and starch.

**Product Name:** Neerizhivu choornam 1000 mg Capsule

**B.NO.:** FD/203/12

**Batch Size:** 665 gm. (500 Capsules)

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<td></td>
<td><strong>665 gm.</strong></td>
</tr>
</tbody>
</table>

3. Stability Studies of Tablets of different Batches of Neerizhivu chooranam.

Stability Study on different storage conditions of 40/75
Product Name :- Neerizhivu choornam 1000 mg Tablet

B.NO. :: FD/189/12

Batch Size:- 665 gm. (500 Tablet)

Storage condition:40°C±2°C/75%RH±5%RH

4. Stability Studies of Capsules of different Batches of Neerizhivu chooranum.

Stability Study of Capsule on different

Batches on storage conditions of 40/75

Product Name :- Neerizhivu choornam 1000 mg Capsule

B.NO. :: FD/201/12

Batch Size:- 665 gm. (500 Capsules)

Storage condition:40°C±2°C/75%RH±5%RH