7. MATERIALS AND METHODS

Aavarai kudineer formulation is a Siddha anti-diabetic liquid 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 Aavarai kudineer formulation 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.

Table 7.1 Preparation of Aavarai Kudineer Formulation (AKF)

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the drug</th>
<th>Biological source &amp; Family</th>
<th>Quantity/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aavarai ilai</td>
<td>Leaves of <em>Cassia auriculata</em> (Leguminosae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>2</td>
<td>Kondrai ilai</td>
<td>Leaves of <em>Cassia fistula</em> (Caesalpinaceae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>3</td>
<td>Naval kottai</td>
<td>Seeds of <em>Syzygium cumini</em> (Myrtaceae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>4</td>
<td>Kadalazhingil</td>
<td>Roots of <em>Salacia chinensis</em> (Celastraceae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>5</td>
<td>Korai kizhangu</td>
<td>Rhizomes of <em>Cyperus rotandus</em> (Cyperaceae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>6</td>
<td>Kosthum</td>
<td>Rhizomes of <em>Costus specious</em> (Coataceae)</td>
<td>14.29g</td>
</tr>
<tr>
<td>7</td>
<td>Marutham pattai</td>
<td>Bark of <em>Terminalia arjuna</em> (Combretaceae)</td>
<td>14.29g</td>
</tr>
</tbody>
</table>
7.1 COLLECTION AND AUTHENTICATION OF THE INGREDIENTS OF AKF

Leaves of *Cassia auriculata* and *Cassia fistula*, seeds of *Syzygium cumini*, roots of *Salacia chinensis*, rhizomes of *Costus speciosus* and *Cyprus rotundus*, bark of *Terminalia arjuna* were collected from various parts of Puducherry state, India in the month of December–2012. All these plants were authentified by Dr. B. R. Ramesh, Director of Research, Institut Franceais de Pondichery, French institute of Pondicherry, Louis st., Puducherry. Herbarium for each drug was prepared by conventional method, kept in French Institute and our department library for further references (Accession number: HIEP 26662-26667 and 26659). Photograph of authentication certificate and herbarium were presented in result section.

7.2 Methods of preparation:

Aavarai ilai, Kondrai ilai, Naval kottai, Kadalazhingil, Korai kizhangu, Kosthum and Marutham pattai (each equal grams) 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:** 30-60ml two times a day orally after boiling with water as per Siddha literature.

7.3 Standardization of Aavarai kudineer formulation

The prepared in-house Aavarai kudineer formulation 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.
7.3.1 PHARMACOGNOSTICAL STANDARDIZATION OF AAVARAI KUDINEER FORMULATION (AS PER WHO GUIDELINES)

7.3.1.1 EXO-MORPHOLOGICAL STUDIES

The exo-morphological examination of leaves of *Cassia auriculata* & *Cassia fistula*, seeds of *Syzygium cumini*, roots of *Salacia chinensis*, rhizomes of *Costus speciosus* & *Cyprus rotundus*, bark of *Terminalia arjuna* was done by observing the sample collected in naked as well as luminescent light for their colour, size, and shape. The odour and taste of the material were also studied. All these observations were noted and given in the result section.

7.3.1.2 HISTO-MORPHOLOGICAL STUDIES

Materials and methods for anatomical studies

The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin -5ml + Acetic acid 5ml + 70% Ethyl alcohol-90ml). After 24 hours of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol as per the schedule given by Sass, 1940. Infiltrations of specimens were carried out by gradual addition of paraffin wax (melting point 58-60ºC) until tertiary butyl alcohol solution attained super saturation. The specimens were cast into paraffin blocks.

**Sectioning**

The paraffin embedded specimens were sectioned with the help of a Razor blade (Hand sectioning). The thickness of the section was maintained uniform. De-waxing of the section was done by customary procedure. The sections were stained with toluidine blue, a polychromatic stain. The staining results were remarkably good and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to
mucilage, blue to protein bodies etc. Where ever necessary, sections were also stained with safranin and fast-green and IKI (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, Paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with the 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffery’s maceration fluid were prepared. Glycerine mounted temporary preparations were made for macerated /cleared materials. Powdered materials of different parts were cleared with sodium hydroxide and mounted in glycerine medium after staining. Different cell component were studied and measured.

**Photomicrographs**

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken in Nikon labphoto-2 microscope unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under the polarized light they appear bright against dark background. Descriptive terms of the anatomical feature were given in the standard anatomy books.

7.3.1.3 **POWDER MICROSCOPICAL STUDIES**

The leaves of *Cassia auriculata & Cassia fistula*, seeds of *Syzygium cumini*, roots of *Salacia chinensis*, rhizomes of *Costus speciosus & Cypris rotundus*, bark of *Terminalia arjuna* 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.

7.3.1.4 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 in 254nm and in 365nm.

7.3.1.5 QUANTITATIVE PHYSICO-CHEMICAL ANALYSIS

DETERMINATION OF TOTAL ASH, ACID INSOLUBLE AND WATER SOLUBLE 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:**

**Determination of Total ash**

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

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

**Determination of Acid insoluble ash**

To the crucible containing the total ash, add 25 ml of hydrochloric acid (70g/l) TS, cover with a watch-glass and boil gently for 5 minutes. Rinse the watch-glass with 5ml of hot water and add this liquid to the crucible. Collect the insoluble matter on an ash less filter-paper and wash with hot water until the filtrate is neutral. Transfer the filter-paper containing the insoluble matter to the original crucible, dry on a hot-plate and ignite to constant weight. Allow the residue to cool in a suitable desiccator for 30 minutes and then weigh without delay. Calculate the content of acid-insoluble ash in mg per gram of air-dried material.

**Determination of Water-soluble ash**

To the crucible containing the total ash, add 25 ml of water and boil for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter-paper. Wash with hot water and ignite in a crucible for 15 minutes at a temperature not exceeding 450°C. Substract the weight of this residue in mg from the weight of total ash. Calculate 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.

**Determination of Water soluble extractive**

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

**Determination of 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 weigh to obtain the total weight including the flask. Shake well and allow to stand for 1 hour. Attach a reflux condenser to the flask and boil gently for 1 hour, cool and weigh. Readjust to the original total weight with the solvent specified in the test procedure for the plant material concerned. Shake well and filter rapidly through a dry filter. Transfer 25 ml of this filtrate to a tared flat-bottomed dish and evaporate to dryness on a water bath. Dry at 105°C for 6 hours, cool in a desiccator for 30 minutes, then weigh without delay. Calculate the content of extractable matter in mg per gram of air dried material.

Cold Maceration

Place about 4.0 gram of coarsely powdered air-dried material, accurately weighed, in a glass-stoppered conical flask. Macerate with 100 ml of the solvent specified for the plant material concerned for 6 hours, shaking frequently and then allow to stand for 18 hour. Filter rapidly taking care not to lose any solvent, transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on water-bath. Dry at 105°C for 6 hours; cool in a desiccator for 30 minutes and weigh without delay. Calculate the content of extractable matter in mg per gram of air-dried material.

For ethanol-soluble extractable matter, use the concentration of solvent (usually 90 % ethanol) specified in the test procedure for the plant material concerned; for water-soluble extractable matter, use water as the solvent.
DETERMINATION OF LOSS ON DRYING\(^{173}\)

Place about 2-5 gram of the prepared air-dried material or the quantity specified in the test procedure for the plant material concerned, accurately weighed, in a previously dried and tared flat weighing bottle. Dry the sample by one of the following techniques:
- In an oven at 100-105\(^{0}\) C.
- In a desiccators over phosphorus pentoxide at room temperature.

Dry until two consecutive weighing do not differ by more than 5 mg, unless otherwise specified in the test procedure. Calculate the loss of weight in mg per gram of air-dried material.

7.3.1.6 SAFETY PROFILE/TOXICOLOGICAL EVALUATION

DETERMINATION OF MICROBIAL CONTAMINATION\(^{174}\)

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 \(10^3\) 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:

10 g of sample is dissolved 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 (Total bacterial load, Total fungal count)

1. 1 ml of prepared sample is transferred 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

   \[ \text{No. of colony forming units on plate} \times \text{Dilution factor} \]

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. Transferred 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 transferred 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.

**Test for salmonella spp.**

1. Enrichment was done by transferring 1gm of the sample into 10ml of selenite cysteine broth.

2. Incubated for 24 hr at 37°C.

3. The enriched culture after incubation was then streaked on duplicate plates of freshly prepared Bismuth Sulphite Agar (BSA), (one lightly and one heavily), and incubated alone with the control plate of Bismuth Sulphite Agar (BSA) at 37°C for 24 hr.

4. After incubation, typical black and green colonies were regarded as positive for *Salmonella*.

5. The colonies were then streaked on the nutrient agar slants for further biochemical identifications using Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) tests.
DETERMINATION OF HEAVY METAL CONTAMINATION

Determination of Heavy metal analysis (Lead, Cadmium, Mercury and Arsenic)

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.

Determination of Lead:

Instrument: AAS with Graphite Furnace and Microwave Reaction System

Reagents and Standard:

- Nitric acid ultrapure grade (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:

Diluents (0.5% v/v nitric acid):

5 ml of nitric acid is diluted to 1000 ml with water.

Matrix modifier:

About 0.20 g of ammonium dihydrogen phosphate and 0.01 g of magnesium nitrate hexahydrate is weighed and transferred accurately to a 100
mL volumetric flask, dissolved in and made up the volume with diluents and mixed.

**Standard Preparation (30 ppb Pb):**

1. 0.25 mL of Lead standard solution (1000 ppm Pb) is transferred accurately to a 250 mL volumetric flask and mixed, made up the volume with diluents.

2. 0.75 mL of the resulting solution is transferred 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 diluents 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 7.2 Microwave parameters for determination of Lead**

<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:**

**Spectrometer:**

**Lamp setting**

Element: Lead, Wavelength : 283.31 nm, Slit width : 2.7/1.05nm, Signal type : AA-
BG, Measurement : Peak Area

**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:**

**Table 7.3 Temperature programming for determination of Lead**

<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>700</td>
<td>10</td>
<td>20</td>
<td>250</td>
<td>Normal (Argon)</td>
</tr>
<tr>
<td>4</td>
<td>2300</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: 25µl

Volume of matrix modifier: 20µl

| Standard Preparation (30 ppb Pb) volume (in µL) | 5  | 10  | 15  | 20  | 25  |
| Volume of diluent (in µL)                      | 20 | 15  | 10  | 5   | 0   |
| Std conc. obtained (in ppb)                    | 6  | 12  | 18  | 24  | 30  |

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:

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

Where,

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

\(W_T\) = Weight of test sample taken (in g).
Determination of 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, dissolveed 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. Transfered 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. Transfered the resulting digested solution to a 20 mL volumetric flask, rinsed the tube with 10 ml of diluent and transfered it to the same volumetric flask and made up to volume with diluent and mixed.

**Test preparation:**

1. Weighed and transfered 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. Transfered the resulting digested solution to a 20 mL volumetric flask, rinsed the tube with 10 ml of diluent and transfered it to the same volumetric flask and made up to volume with diluent and mixed.

**Reagent blank:**

1. Transfered 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. Transfered the resulting digested solution to a 20 mL volumetric flask, rinsed the tube with 10 ml of diluent and transfered it to the same volumetric flask and made up to volume with diluent and mixed.
Instrumental parameters:

7.4 Microwave parameter for determination of Cadmium

<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:-

Table 7.5 Temperature programming for determination of Cadmium

<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:

\[
\text{Cadmium (in ppm)} = \frac{T_c \times 20}{1000 \times W_T}
\]

Where,

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

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

Instrumental parameters:

Table 7.6 Microwave parameters for determination of Mercury

<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

Table 7.7 FIAS programming for determination of Mercury

<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. Dipped 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:

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

Where,

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

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

Determination of 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 \(N-N\)-diethyl methyl di thiocarbamate 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 make 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 (As$_2$O$_3$). 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 (As$_2$O$_3$) and is used as the standard.
DETERMINATION OF PESTICIDE RESIDUES$^{176-177}$

(Chlorpyriphos, DDT, Endosulfan, Malathion and Parathion)

**Determination of Pesticide residues:**

Pesticides are simple substances or mixtures used to eliminate undesirable vegetable and animal life in agricultural and urban ecosystems. Owing to the great variability 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 more of action in organisms. Chemically the compounds can be derived into three groups; Biological, Inorganic and Organic Pesticide. Mainly the organochlorine, organophosphorues, 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 7.8 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>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><strong>Endosulfan</strong> (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><strong>Malathion</strong></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>33.</td>
<td><strong>Parathion</strong></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><strong>Quintoze (sum of qintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)</strong></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 0°C, protected from light.

DETERMINATION OF ORGANOCHLORINE, ORGANOPHOSPHORUS AND PYRETHROID INSECTICIDES

(by size exclusion chromatography):

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:**

**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 preceded 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. Transfered 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.

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.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Substance</th>
<th>Volume of standard stock solution A to be prepared (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>10</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>Metoxuron</td>
<td>0.050</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>Atrazine</td>
<td>0.100</td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>Fonofos</td>
<td>0.050</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Diazion</td>
<td>0.500</td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td>6</td>
<td>Metribuzin</td>
<td>5.000</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>7</td>
<td>Methyl-Parathion</td>
<td>0.200</td>
<td></td>
<td>0.68</td>
</tr>
<tr>
<td>8</td>
<td>Chlorpyrifos-Methyl</td>
<td>0.100</td>
<td></td>
<td>0.69</td>
</tr>
<tr>
<td>9</td>
<td>Primiphos</td>
<td>4.000</td>
<td>10</td>
<td>0.73</td>
</tr>
<tr>
<td>10</td>
<td>Fenitrothion</td>
<td>0.500</td>
<td>10</td>
<td>0.73</td>
</tr>
<tr>
<td>11</td>
<td>Malathion</td>
<td>1.000</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>12</td>
<td>Parathion</td>
<td>0.500</td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>13</td>
<td>Chlorpyrifos</td>
<td>0.200</td>
<td></td>
<td>0.76</td>
</tr>
<tr>
<td>14</td>
<td>Quinalphos</td>
<td>0.050</td>
<td></td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Concentration</td>
<td>Relative Retention Time</td>
<td>RSD</td>
</tr>
<tr>
<td>---</td>
<td>-------------------</td>
<td>---------------</td>
<td>-------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>15</td>
<td>Methidathion</td>
<td>0.200</td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td>16</td>
<td>Carbophenothion</td>
<td>0.300</td>
<td>10</td>
<td>1.00</td>
</tr>
<tr>
<td>17</td>
<td>Anilophos</td>
<td>0.100</td>
<td></td>
<td>1.11</td>
</tr>
<tr>
<td>18</td>
<td>Azinophos methyl</td>
<td>1.000</td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>19</td>
<td>Phosalone</td>
<td>0.1000</td>
<td></td>
<td>1.14</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 7.9.
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:**
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) (diphenyl)siloxane.

Carrier gas: Helium at flow rate of 1.4 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: 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 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.

**Table 7.10 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>10</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>alpha-HCH</td>
<td>0.300</td>
<td>10</td>
<td>0.48</td>
</tr>
<tr>
<td>3</td>
<td>Hexachlorobenzene</td>
<td>0.100</td>
<td>10</td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>Dichlorophenoxyacetic 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>10</td>
<td>0.53</td>
</tr>
<tr>
<td>6</td>
<td>Lindane</td>
<td>0.600</td>
<td>10</td>
<td>0.65</td>
</tr>
<tr>
<td>7</td>
<td>Quintozene</td>
<td>1.000</td>
<td>10</td>
<td>0.54</td>
</tr>
<tr>
<td>8</td>
<td>Pentachloroaniline</td>
<td>1.000</td>
<td>10</td>
<td>0.61</td>
</tr>
<tr>
<td>9</td>
<td>Heptachlor</td>
<td>0.050</td>
<td>10</td>
<td>0.66</td>
</tr>
<tr>
<td>10</td>
<td>Alachlor</td>
<td>0.200</td>
<td>10</td>
<td>0.71</td>
</tr>
<tr>
<td>11</td>
<td>Aldrin</td>
<td>0.050</td>
<td>10</td>
<td>0.78</td>
</tr>
<tr>
<td>12</td>
<td>Heptachlor epoxide</td>
<td>0.050</td>
<td>10</td>
<td>0.83</td>
</tr>
<tr>
<td>13</td>
<td>2,4-DDE</td>
<td>1.000</td>
<td>10</td>
<td>0.84</td>
</tr>
<tr>
<td>14</td>
<td>Alpha-Endosulfan</td>
<td>3.000</td>
<td>10</td>
<td>0.82</td>
</tr>
<tr>
<td>15</td>
<td>Chlordane</td>
<td>0.050</td>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>16</td>
<td>Dialdrin</td>
<td>0.050</td>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>17</td>
<td>4,4-DDE</td>
<td>1.000</td>
<td>10</td>
<td>0.88</td>
</tr>
<tr>
<td>18</td>
<td>2,4-DDD</td>
<td>1.000</td>
<td>10</td>
<td>0.9</td>
</tr>
<tr>
<td>19</td>
<td>Endrin</td>
<td>0.050</td>
<td>10</td>
<td>0.92</td>
</tr>
<tr>
<td>20</td>
<td>Beta-Endosulfan</td>
<td>3.000</td>
<td>10</td>
<td>0.93</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:

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-7.10.

<table>
<thead>
<tr>
<th></th>
<th>Substance</th>
<th>Volume</th>
<th>Dilution</th>
<th>RRT</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>2,4-DDT</td>
<td>1.000</td>
<td>10</td>
<td>0.96</td>
</tr>
<tr>
<td>22</td>
<td>Pyrethrins</td>
<td>3.000</td>
<td>10</td>
<td>0.91</td>
</tr>
<tr>
<td>23</td>
<td>Carbophenothion</td>
<td>1.000</td>
<td>10</td>
<td>1.02</td>
</tr>
<tr>
<td>24</td>
<td>4,4-DDT</td>
<td>3.000</td>
<td>10</td>
<td>1.20</td>
</tr>
<tr>
<td>25</td>
<td>Bromopropylate</td>
<td>3.000</td>
<td>1.02</td>
<td>1.21</td>
</tr>
<tr>
<td>26</td>
<td>Piperonyl Butoxide</td>
<td>3.000</td>
<td>1.02</td>
<td>1.27</td>
</tr>
<tr>
<td>27</td>
<td>Permethrin</td>
<td>1.000</td>
<td>1.02</td>
<td>1.28</td>
</tr>
<tr>
<td>28</td>
<td>Cypermethrin</td>
<td>1.500</td>
<td>1.02</td>
<td>1.44</td>
</tr>
<tr>
<td>29</td>
<td>Fenvalerate</td>
<td>0.500</td>
<td>1.02</td>
<td>1.5</td>
</tr>
</tbody>
</table>
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 7.11 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 ploted the calibration curve.

2. When the chromatograms were recorded in the prescribed conditions, the relative retention times were approximately those listed in Table 7.11.

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.
7.3.2 PHYTOCHEMICAL STANDARDIZATION

As per Indian Herbal Pharmacopoeia the following parameters have been tested for AKF

- Qualitative phytochemical screening
- Phytochemical estimation
- TLC identification
- HPTLC fingerprinting with bio-marker
- GC-MS analysis

7.3.2.1 QUALITATIVE PHYTOCHEMICAL SCREENING\textsuperscript{178-179}

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

\begin{itemize}
\item **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

\begin{itemize}
\item **Mayer’s test**

To a few ml of filtrate, 1-2 drop of Mayer’s reagent (Potassium mercuric iodide solution) are added by the sides of the test tube. A white or creamy precipitate indicates the test as positive.
\end{itemize}
\end{itemize}
Wagner’s test

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

Detection of Carbohydrates and Glycosides

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

Molish’s test

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

Fehling’s test

One ml of filtrate is boiled on water bath with 1 ml each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.
Barfoed’s test

To 1 ml of filtrate, 1 ml of Barfoed’s reagent is added and heated on a boiling water bath for 2 minutes. Red precipitate indicates presence of sugar.

Benedict’s test

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

For detection of glycosides

50 mg of extract is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests.

Borntrager’s test

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

Legal’s test

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

Detection of Saponins

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

- **Detection of Proteins and Amino acids**

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

  - **Millon’s test**

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

  - **Biuret test**

    An aliquot of 2 ml of filtrate is 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 indicates the presence of proteins.

  - **Ninhydrin test**

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

- **Detection of Phytosterols**

  - **Libermann-Burchard’s test**

    The extract (50 mg) is dissolved in 2 ml acetic anhydride. To this, 1-2 drops of concentrated sulphuric acid are 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 is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil.

Saponification test

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

Detection of Phenolic compounds and Tannins

Ferric chloride test

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

Gelatine test

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

Alkaline reagent test

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.
➢ **Lead acetate test**

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

➢ **Magnesium and hydrochloric acid reduction**

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

❖ **Detection of Gum and Mucilages**

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

❖ **Detection of Volatile Oil**

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

Determination of Total Phenolic Content\textsuperscript{180-181}:

**Preparation of 16% w/v sodium carbonate solution:**

Dissolve 16.0 g of anhydrous sodium carbonate in sufficient purified water to produce 100 ml.

**Blank solution:**

Use purified water.

**Standard solution:**

Weigh and transfer accurately about 40.0 mg of Gallic acid WS in 100 mL volumetric flask and make up the volume with purified water.

Dilute 10 ml of the above solution to 100 ml with purified water.

**Test solution:**

Weigh and transfer accurately about 2.0 g of test sample in a 500 mL round-bottomed flask, add 200 mL of purified water and reflux it for 45 minutes in boiling water bath.

After 45 minutes, allow the flask to cool, transfer the solution into a 250 mL volumetric flask and make up the volume up with purified water.

Centrifuge a part at 5000 rpm for 15 minutes. Transfer 2 mL of the supernatant into a 25 mL volumetric flask and make up the volume with purified water.

**Procedure:**

Pipette 2 mL each of standard solution, test solution and blank solution, respectively in three distinct 25 mL volumetric flasks.

Add 1 mL of Follin’s reagent in each of the volumetric flasks and make up the volume with 16% w/v sodium carbonate solution in each case.
Keep the volumetric flasks for 30 minutes in dark to allow the formation of blue colored complex.

**System suitability:**

Measure the absorbance of standard solution (five replicates) at 760 nm against the blank.

Test is not valid when relative standard deviation of the absorbance values of five replicates is more than 2.0%.

Measure the absorbance of test solution at 760 nm against the blank.

Calculate the quantity of total phenolics as gallic acid, on dried basis, in % w/w, present in the test sample using the formula given below:

\[
\text{Total phenolics} = \frac{A_{BT} \times W_S \times 10 \times 2 \times 250 \times 25 \times 25 \times P \times 100}{A_{BS} \times 100 \times 100 \times 25 \times W_T \times 2 \times 2 \times 100 \times (100 - \text{LOD})}
\]

Where,

- \( A_{BT} \) = Absorbance of the test solution.
- \( A_{BS} \) = Mean of absorbance of the standard solution.
- \( W_S \) = Weight of gallic acid working standard taken (in mg).
- \( W_T \) = Weight of the test sample taken (in mg).
- \( P \) = Potency of gallic acid working standard (in % w/w).
- \( \text{LOD} \) = Loss on drying of the test sample (in % w/w).
7.3.3 PHYTO-ANALYTICAL STANDARDIZATION

7.3.3.1 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 original 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 silica gel (or other adsorbent) in water has to be vigorously shaken for a set time interval before spreading. Finally, plates after spreading have to be 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 F254

**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

$$R_f = \frac{\text{Distance travelled by Component from base line}}{\text{Distance travelled by Solvent front from base line}}$$

7.3.3.2 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) ANALYSIS

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{\textsubscript{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 vapors 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\text{f} of main peaks obtained with the test solution from R\text{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\text{f} 0.1 onwards correspond to those obtained with the reference solution after derivatization by dipping in reagent I and reagent II.

7.3.3.3 GC-MS ANALYSIS OF AKF

The in-house prepared formulation of AKF was extracted with methanol and analyzed through GC-MS for identification of different compounds.

GC-MS analysis was carried out using Perkin Elmer GC Clarus 500. It consists of an automatic operation controller (AOC-20i auto injector) and Gas Chromatograph interfaced to a Mass Spectrometer. Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) capillary column (30x0.25mmx0.25\mu m df) employing for separation of components and operating in mass detector turbo mass gold mode at 70eV. Helium was used as carrier gas at a constant flow of 1ml/min and an injection volume of 2\mu l was employed. The temperature of Injector and ion source were maintained at 270°C and 200°C respectively. The oven temperature was programmed from 50°C for 2 min, inversed to 150° C at 8° C/min and to 240° C at 8° C/min, and held at 270° C for 20min. Mass spectra were obtained at 70eV. A scan interval is 0.5 seconds and fragments from 40 to 450 Da. The amount of each component was calculated on relative percentage basis comparing its average peak area to the total areas. The Total Ion
Chromatogram (TIC) was created by summing up intensities of all mass spectral peaks. The TIC was compared with GC chromatogram. Turbo Mass Version 5.2.0 Software was adopted to handle mass spectra and chromatograms\textsuperscript{191-192}.

**Identification of compounds**

The compounds were identified by interpretation of the spectrum of the unknown compounds with the spectrum of the known compounds mentioned in The National Institute of Standard and Technology (NIST) library database.
7.3.4 TOXICOLOGICAL STUDIES

Experimental animals

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 ad libitum. The animals were maintained in their respective groups under controlled conditions of temperature and humidity. 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/XXXIV/SRU/78/2013 and IAEC-MTPG&RIHS/6040/16).

Dose and drug solution

Traditionally 1 to 2g of the AKF is used in diabetes. Further for this study, in-house prepared AKF was extracted with distilled water, concentrated under vacuum, dried and dissolved in 1% CMC 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.
7.3.4.1 ACUTE TOXICITY STUDIES OF AAVARAI KUDINEER FORMULATION (AKF)

PROCEDURE FOR ACUTE TOXICITY STUDIES\textsuperscript{195}:

1. The study was carried out according to the OECD guidelines 423.

2. Female Wistar rats of weight (180-220g) were taken for the study and kept for overnight fasting.

3. Next day, body weight was taken and AKF was administered orally at a dose of 2000mg/kg in distilled water.

4. Then the animals were observed for mortality and morbidity at 0, \(\frac{1}{2}\), 1, 2, 4, 6, 8, 12 and 24 hr.

5. 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.

6. The animals were observed twice daily for 14 days and body weight was taken.

7. The same experiment was repeated once again on 3 rats (female) if there is no observable clinical toxicity for the animals on the acute toxicity study

7.3.5 PHARMACOLOGICAL SCREENING

ANTIDIABETIC SCREENING OF AAVARAI KUDINEER FORMULATION (AKF) IN NORMAL AND STREPTOZOTOCIN INDUCED DIABETIC RATS.

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\textsuperscript{196}.

**Streptozotocin induced diabetes\textsuperscript{197}**

*Streptozotocin* or *streptozocin* (STZ) is a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals. It is used in medicine for treating certain cancers of the Islets of Langerhans and used in medical research to produce an animal model for hyperglycemia in a large dose as well as Type 1 diabetes with multiple low doses. 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, in rat 65mg/kg and in beagle dogs 15mg/kg through i.p. for three days.
7.3.5.1 HYPOGLYCEMIC ACTIVITY OF AKF IN NORMAL RATS

Anti-diabetic activity of Avarai Kudineer Formulation (AKF) in normal fasted rats

Normal fasted rats: Normal albino rats (180-220 mg) were first used for the screening of the herbal formulation AKF 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% CMC (1ml/kg, orally). Group II received standard drug Glibenclamide 5 mg/kg orally. Group III, IV and V were treated orally with test AKF 125, 250 and 500 mg/kg, respectively (Table 7.12).

Table 7.12 Treatment protocol for hypoglycemic activity of AKF in normal rats

<table>
<thead>
<tr>
<th>Group I</th>
<th>Treated with 1%CMC, 1ml/kg/p.o</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group II</td>
<td>Treated orally with Glibenclamide,5 mg/kg</td>
</tr>
<tr>
<td>Group III</td>
<td>Treated orally with AKF,125 mg/kg</td>
</tr>
<tr>
<td>Group IV</td>
<td>Treated orally with AKF,250 mg/kg</td>
</tr>
<tr>
<td>Group V</td>
<td>Treated orally with AKF,500 mg/kg</td>
</tr>
</tbody>
</table>

Blood samples were collected from tail vein prior and 1, 2 and 4 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

\[^{199-200}\]
Evaluation of AKF for anti-hyperglycemic properties in presence of glucose load (Oral Glucose Tolerance Test).

Overnight fasted rats were divided into 5 groups of six animals 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.

7.3.5.2 Anti-diabetic activity of AKF in Streptozotocin (STZ) induced diabetic rats

Induction of experimental diabetes

Adult albino Wistar rats (180-220g) of either sex were made diabetic with an intra-peritoneal injection of 65mg/kg body weight of Streptozotocin (Sigma Aldrich chemicals, USA) dissolved in 0.1 M cold citrate buffer, pH4.5, immediately before use. Streptozotocin injected animals exhibited massive glucosuria and hyperglycemia within 2-4 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 AKF at the dose of 125, 250 and 500mg/kg body weight were administered orally after suspending in 1% CMC 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.
Evaluation of AKF 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 animals each and screened for anti-hyperglycemic activity of the various concentration of AKF in overnight fasted diabetic rats as mentioned in the table 7.13. 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 7.13 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% CMC/P.O</td>
</tr>
<tr>
<td>Group-II</td>
<td>Diabetic rats, treated with 1% CMC/P.O</td>
</tr>
<tr>
<td>Group-III</td>
<td>Treated with Glibenclamide, 5 mg/kg/P.O</td>
</tr>
<tr>
<td>Group-IV</td>
<td>Treated with AKF, 125 mg/kg/P.O</td>
</tr>
<tr>
<td>Group-V</td>
<td>Treated with AKF, 250 mg/kg/P.O</td>
</tr>
<tr>
<td>Group-VI</td>
<td>Treated with AKF, 500 mg/kg/P.O</td>
</tr>
</tbody>
</table>

Evaluation of AKF for anti-hyperglycemic properties in STZ induced diabetic rats
(multiple dose, long term study)

In multiple dose studies the AKF 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 phosphatase (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 period²⁰⁶⁻²⁰⁸.

**Table 7.14 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%CMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>Diabetic control and received (STZ) + 1%CMC</td>
</tr>
<tr>
<td>Group 3</td>
<td>Treated orally with Glibenclamide, 5 mg/kg/BW</td>
</tr>
<tr>
<td>Group 4</td>
<td>Treated orally with AKF, 125 mg/kg/BW</td>
</tr>
<tr>
<td>Group 5</td>
<td>Treated orally with AKF, 250 mg/kg/BW</td>
</tr>
<tr>
<td>Group 6</td>
<td>Treated orally with AKF, 500 mg/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 (HbA$_1$C)

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 (HbA$_1$C) was estimated by following the method of Sudhakar Nayak and Pattabiraman, 1982$^{209}$. 

---

28 Days Repeated efficacy Study In STZ Induced Diabetic Wistar Rat
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 minutes²¹⁰-²¹².

**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 was 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 section²¹³.

**Histopathology of other vital organ**

Since the test formulation AKF was not subjected for sub-acute or chronic toxicity study profile 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 section²¹⁴.
Statistical analysis \(^{215-216}\)

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 multiple comparison tests. The AKF 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.

7.3.5.3 INVITRO ANTI-DIABETIC ACTIVITY OF AKF

Invitro anti-diabetic screening of AKF by \(\alpha\)-glucosidase & \(\alpha\)-amylase inhibitory methods

Inhibition of \(\alpha\)-glucosidase and \(\alpha\)-amylase enzymes involved in the digestion of carbohydrates can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in the management of post prandial blood glucose level in type 2 diabetic patients and border line patients. Currently there is renewed interest in functional foods and plant based medicines modulating physiological effects in the prevention and cure of diabetes and obesity. Hence the attractive targets like \textit{in-vitro} inhibition of \(\alpha\)-glucosidase and \(\alpha\)-amylase enzymes are currently in vogue. Therefore, natural \(\alpha\)-glucosidase and \(\alpha\)-amylase inhibitors from plant sources offer an attractive strategy for the control of post prandial hyperglycemia.
a) *IN-VITRO α-GLUCOSIDASE INHIBITORY ACTIVITY* 217-218

**Requirements**

- α-glucosidase
- 100mM phosphate buffer pH 7
- Bovine serum albumin
- Sodium azide
- Para nitro phenyl- α-D-glucopyranoside
- Acarbose
- Dimethylsulfoxide (DMSO)
- ELISA microplate

**Preparation of the extracts and standards**

Stock Solutions of extract/acarbose (1 mg/ml) were prepared by first dissolving in minimal volumes of DMSO and diluting with distilled water and then serial dilutions were carried out.

**Procedure**

Yeast α-glucosidase was dissolved at a concentration of 0.1 U/ml in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 200 mg/ml, and sodium azide 200 mg/ml which was used as enzyme source; para-nitrophenyl-α-D-glucopyranoside were used as substrate. Weighed AKF extract was made into a concentration of 1 mg/ml and serial dilutions of 500, 250, 125, 62.5, 31.25μg/ml were made with equal volumes of dimethyl sulfoxide and distilled water. 10μl of AKF extract dilutions were incubated for 5 min with 50μl enzyme source. After the incubation, 50μl of substrate should be added
and further incubated for 5min at room temperature. The pre-substrate and post-substrate addition absorbance was measured at 405 nm with ELISA microplate reader.

Percent α-glucosidase inhibition was calculated as follows:

\[
\% \text{ Inhibition} = \left( \frac{\text{Absorbance of Control} - \text{Absorbance of Test}}{\text{Absorbance of Control}} \right) \times 100
\]

The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (IC\text{50}) was calculated by graphical method. Experiments were performed in triplicate. Acarbose were dissolved in distilled water and serial dilutions were done and it was used as positive control.

b) \textit{IN-VITRO α-AMYLASE INHIBITORY ACTIVITY}\textsuperscript{219-220}

\textbf{Requirements}

- α-amylase
- 20mM phosphate buffer pH 6.9
- 0.5% starch solution (in 20mM phosphate buffer pH 6.9)
- 96mM 3,5-dinitro salicylic acid
- Acarbose
- Dimethyl sulfoxide (DMSO)
- ELISA microplate

\textbf{Preparation of the extracts and standards}

Stock Solutions of extract/acarbose (1mg/ml) were prepared by first dissolving in minimal volumes of DMSO and diluting with distilled water and then serial dilutions should be carried out.
Procedure

A total of 500 μl of test samples and standard drug (31.25-500μg/ml) were added to 500 μl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 μl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle.

\[
\% \text{ Reaction} = \left( \frac{\text{Absorbance of Test}}{\text{Absorbance of Control}} \right) \times 100
\]

\[
\% \text{ Inhibition} = 100 - \% \text{ Reaction.}
\]

For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The concentration of the extract required to inhibit the activity of the enzyme by 50% (IC\textsubscript{50}) was calculated by graphical method. Experiments were performed in triplicate.
7.3.5.4 *IN-VITRO ANTI-OXIDANT ACTIVITY OF AKF*

**a) DPPH radical scavenging activity of AKF**

In the DPPH test, the anti-oxidants reduce the purple coloured DPPH radical (2, 2- diphenyl-1-picrylhydrazyl hydrate) to a yellow-coloured 2, 2- diphenyl-1-picrylhydrazine, and the extent of the reaction was depend on the hydrogen donating ability of the antioxidants. The changes in colour (from deep-violet to light-yellow) was measured at 517 nm wavelength. As DPPH is sensitive to light, it is exposed to minimum possible light.

**Materials**

- DPPH (2, 2-Diphenyl-1-picrylhydrazyl; Mol.wt 395.326)
- Ascorbic acid as standard
- UV visible spectrophotometer
- Dimethyl sulfoxide (DMSO)
Preparation of the extracts and standards

Stock solutions of extract/ascorbic acid (1 mg/ml) were prepared in DMSO and then serial dilutions were carried out by distilled water.

Procedure

Radical scavenging activity of AKF extract were measured according to the method of James (2010). Briefly, 1 ml each of different concentrations (500, 250, 125, 62.5, 31.25 µg/ml in DMSO and distilled water) of extracts or standard (Ascorbic acid) were added to 2.5 ml of a 0.3 mM DPPH-methanol solution. The mixture should be shaken vigorously and allowed to stand at room temperature in the dark for 30 minute. The absorbance was measured at 517 nm.

The % DPPH radical scavenging activity was calculated using the following formula:

\[
\text{DPPH radical Scavenging activity} = \left( \frac{A_{517}^{\text{control}} - A_{517}^{\text{test}}}{A_{517}^{\text{control}}} \right) \times 100
\]

Where, \( A=\text{Absorbance} \).

Lower absorbance of the reaction mixture indicates higher radical scavenging activity. All the tests were performed in triplicate and the graph were plotted with the mean values. The concentration of sample required to scavenge 50% of DPPH (IC\(_{50}\)) was determined. The IC\(_{50}\) values were calculated by graphical method.
b) Nitric oxide scavenging activity of AKF

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of the Griess illosvoy reaction. In the present investigation, Griess illosvoy reagent is modified by using naphthyl ethylenediamine-di-hydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduce the production of nitric oxide.

\[
\begin{align*}
H_2NO_2S & \quad \text{Sulphanilamide} \\
& \quad \text{NED} \\
N= & \quad \text{Azo compound}
\end{align*}
\]

**Materials**

- Sodium nitroprusside solution (mol.wt 297.96)
- Phosphate buffer saline, pH 7.4
- Sulphanilic acid reagent
- Naphthyl ethylenediamine-di-hydrochloride (NED)
- Ascorbic acid
Preparation of the extracts and standards

Stock Solutions of extract/standard (1mg/ml) were prepared in DMSO and then serial dilutions by distilled water were carried out.

Procedure

The reaction mixture (3ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and extract or standard solution (0.5 ml) were incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite were pipetted and mixed with 1ml of sulphanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5min for completing diazotization. Then, 1ml of naphthyl ethylenediamine-di-hydrochloride (0.1%) was added, mixed and allowed stand for 30 min. A pink coloured chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions.

The amount of nitric oxide radical inhibited by the extract was calculated using the following equation:

\[
\% \text{ of Scavenging activity} = \left( \frac{A_{540}^{\text{control}} - A_{540}^{\text{test}}}{A_{540}^{\text{control}}} \right) \times 100
\]

Where A=Absorbance

Determination of IC_{50}

The IC_{50} value is the concentration of sample required to inhibit 50% of nitric oxide radical. It was calculated by plotting graph, log concentration (on X-axis) versus % inhibition (on Y-axis).
7.4 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**

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 dissolutions status. Tablets are usually in circular in shape or bi-convex.

Tablet is a convenient modern dosage forms 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**

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.

**Wet granulation method for AKF tablet preparation**

Initially, mixed herbs, avicel PH 102, povidone K30, sodium starch glycolate, and colloidal silicon dioxide were mixed together by the geometric dilution method. Water was then added into the mixture powders until a damp mass occurred, sieved through an 18-mesh sieve to produce granules. The granules were dried in hot air oven at 60°C for 4 hrs. The dried granules were sieved all over again through a 20-mesh sieve and magnesium stearate was added and mixed together for three minutes. The mixture powders were tested and evaluated by preformulation studies: angle of repose, bulk density, tapped density, compressibility index, and Hausner ratio before tablets compression. Then, the mixture powders were compressed into tablets using a single punch tableting machine (Charatchai Machinery Model: CMT 12, Thailand) with a die diameter of 10.3 mm. The ingredients ratio is shown in Table 1 which designed as W1 and W2 formulas, respectively. After that, the tablets were tested the physical properties: weight variation, friability, tablets thickness, tablets hardness, and disintegration time.
7.4.1 Pre-formulation studies of AKF\textsuperscript{230}

**Angle of repose**

The angle of repose was tested by the fixed funnel method. The 5 g of powder mixture was poured into glass funnel. The lower tip of glass funnel was 5 cm height from the ground. The height (h) and radius (r) of pile were measured, and then calculated follow Equation 1.

\[
\tan \theta = \frac{h}{r} \quad \text{(Eq. no: 1)}
\]

\(\theta\) = angle of repose

h = height (cm)

r = radius (cm)

The study was carried out in triplicate

**Bulk density**

The 20 g of powder mixture was weighted accurately, gently poured into 100 ml glass cylinder without compacting. The volume of powder mixture was recorded, and then calculated follow Equation 2. The study was carried out in triplicate.

\[
\text{Bulk density} = \frac{m}{V_0} \quad \text{(Eq.2)}
\]

m = mass (g)

\(V_0\) = unsettled apparent volume (cm\(^3\))

**Tapped density**

The glass cylinder with powder mixture from bulk density testing was used to test tapped density. It was tapped using tapped density tester (Erweka D-63150, Germany) for 1,250 strokes. The volume of tapped powder mixture was recorded, and then calculated follow
Equation 3. The study was carried out in triplicate.

\[ \text{Tapped density} = \frac{m}{V_f} \]  
(Eq.3)

\[ m = \text{mass (g)} \]

\[ V_f = \text{final tapped volume (cm}^3\text{)} \]

**Compressibility index**

Data from bulk density and tapped density testing were used for calculate compressibility index by following Equation 4.

\[ \text{Compressibility index} = \left( \frac{V_0 - V_f}{V_0} \right) \times 100 \]  
(Eq.4)

\[ V_0 = \text{unsettled apparent volume (cm}^3\text{)} \]

\[ V_f = \text{final tapped volume (cm}^3\text{)} \]

**Hausner ratio**

Hausner ratio was calculated follow Equation 5.

\[ \text{Hausner ratio} = \frac{V_0}{V_f} \]  
(Eq.5)

\[ V_0 = \text{unsettled apparent volume (cm}^3\text{)} \]

\[ V_f = \text{final tapped volume (cm}^3\text{)} \]
**Physical property evaluations**

**Weight variation**

Twenty tablets were individually accurately weighed. Each tablet weight was recorded. Results were reported as mean ± standard deviation (SD) in milligrams (mg) units.

**Friability**

The tablets had any dust removed before testing. Ten tablets were accurately weighed together, and friability was tested using a friability tester (K.S.L. Engineering, Thailand). After 4 minutes of rotation at 25 rpm, any loose dust from the tablets was removed before accurately weighing again. If friability was not more than 1.0%, it was considered acceptable. The friability was calculated follow Equation 6.

\[
Friability = \frac{(W_{\text{before}} - W_{\text{after}})}{W_{\text{before}}} \times 100
\]

(Eq.no:6)

\( W_{\text{before}} \) = weight of tablets before test (g)

\( W_{\text{after}} \) = weight of tablets after test (g)

**Thickness**

Ten tablets were individually measured using the thickness tester (Mitutoyo Corp. Model: ID-C112TB Absolute, Japan). Results were reported as mean ± SD in millimeter (mm) units.

**Hardness**

Ten tablets were measured using a hardness tester (Erweka D- 63150 Model: TBH220TD, Germany). Results were reported as mean ± SD in kilopond (kP) units.

**Disintegration time (DT)**

Six tablets were tested by a disintegration tester (K.S.L. Engineering, Thailand) following the United State Pharmacopeial method, and water was used as the disintegration
medium at 37 °C. DT of each tablet was recorded in minutes.

7.4.2 Preparation of tablets of AKF

Preparation of three different batches of tablets of “Aavarai Kudineer Formulation”

Tablet Specification:

**Composition** - Each Film coated tablet contains Aavarai Kudineer Formulation 500 mg

**Excipients** - Quantity sufficient

**Colour** - Yellow oxide of iron

**Average Weight** - 630 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 of 30 mg of phenolic content.

**Experiment:**

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

**Procedure:**

1. Sifted Aavarai Kudineer Formulation through 20#

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

3. Mixed stage 1 and stage 2 for 5 minutes.
4. Bound 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 and 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 (630 mg)

**Thickness** - 7.6 mm

**Hardness** – 2.5 kg/cm³

**Disintegration time (D.T.)** – 45 seconds
Finalization of Process

Product Name: Aavarai Kudineer Formulation 500 mg Tablet

B.NO. :- FD/AKF/200/15

Batch Size: - 665 gm. (500 Tablet)

Table 7.15 Product Formulation First batch (Batch No. FD/200/15)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ingredients</th>
<th>Qty./Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aavarai Kudineer Formulation</td>
<td>500 gm.</td>
</tr>
<tr>
<td>2</td>
<td>Starch IP</td>
<td>40 gm.</td>
</tr>
<tr>
<td>3</td>
<td>Lactose IP</td>
<td>25 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></td>
<td>Total</td>
<td>665 gm.</td>
</tr>
</tbody>
</table>
Product Formulation second batch (Change in Process)

This formulation was manufactured by changing in polymer PVPK 30.

Product Name : Aavarai Kudineer Formulation 500 mg Tablet

B.NO. :- FD/AKF/201/15

Batch Size:– 665 gm. (500 Tablet)

Table 7.16 Product Formulation Second batch (Batch No. FD/201/15)

<table>
<thead>
<tr>
<th>Sr. NO.</th>
<th>Ingredients</th>
<th>Qty./Batch</th>
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<td>10 gm.</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>665 gm.</td>
</tr>
</tbody>
</table>
Product Formulation third batch (Change in Process)

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

Product Name: Aavarai Kudineer Formulation 500 mg Tablet

B.NO. : FD/AKF/202/15

Batch Size: 665 gm. (500 Tablet)

Table 7.17 Product Formulation Third batch (Batch No.FD/202/15)

<table>
<thead>
<tr>
<th>Sr. NO.</th>
<th>Ingredients</th>
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<td>Total</td>
<td></td>
<td>665 gm.</td>
</tr>
</tbody>
</table>
7.4.3 Preparation of capsules of AKF

Capsule formulation\textsuperscript{232-233}

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.

**Preparation of three different batches of capsule from Aavarai Kudineer**

**Formulation**

**Capsule Specification:**

**Composition**-Each capsule contain Aavarai Kudineer Formulation 500 mg

**Excipients** – Quantity sufficient

**Colour**- Yellow oxide of iron

**Average Fill weight** - 625 mg

**Diameter:** Cap-7.65 mm

Body-7.35 mm

**Disintegration time (D.T.)** – 7.0 minutes

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

**Experiment:**

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

1. Sifted Aavarai Kudineer Formulation 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** - 625 mg

**Average weight of empty shells** - 96 mg

**Diameter:** Cap-7.65 mm

**Body**-7.35 mm

**Disintegration time (D.T.)**– 7.0 minutes
Finalization of Process

**Product Formulation First batch:**

Product Name: Aavarai Kudineer Formulation 500 mg Capsule

B.NO: FD/AKF/203/15

Batch Size: 665 gm. (500 Capsules)

**Table 7.18 Product Formulation First batch (Batch No.FD/203/15)**

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


Product Formulation second batch (Change in Process)

This formulation was manufactured by changing the polymer PVPK 30.

Product Name: Aavarai Kudineer Formulation 500 mg Capsule

B.NO : FD/AKF/204/15

Batch Size:- 665 gm. (500 Capsules)

Table 7.19 Product Formulation Second batch (Batch No.FD/204/15)

<table>
<thead>
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<td><strong>Total</strong></td>
<td></td>
<td><strong>665 gm.</strong></td>
</tr>
</tbody>
</table>
Product Formulation third batch (change in process)

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

Product Name: Aavarai Kudineer Formulation 500 mg Capsule

B.NO: FD/AKF/205/15

Batch Size: 665 gm. (500 Capsules)

Table 7.20 Product Formulation Third batch (Batch No.FD/205/15)

<table>
<thead>
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</tr>
<tr>
<td>Total</td>
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<td>665 gm.</td>
</tr>
</tbody>
</table>
7.4.4  Stability studies of AKF

Stability Studies of Tablets of different Batches of Aavarai Kudineer Formulation.

Stability Study on different storage conditions of 40°C/75%RH

Product Name: Aavarai Kudineer Formulation 500 mg Tablet

B.NO: FD/AKF/200/15 to FD/AKF/202/15

Batch Size: 665 gm. (500 Tablets)

Storage condition: 40°C±2°C/75%RH±5%RH

Stability Studies of Capsules of different Batches of Aavarai Kudineer Formulation.

Stability Study of Capsule on different storage conditions of 40°C/75%RH

Product Name: Aavarai Kudineer Formulation 500 mg Capsule

B.NO: FD/AKF/203/15 to FD/AKF205/15

Batch Size: 665 gm. (500 Capsules)

Storage condition: 40°C±2°C/75%RH±5%RH