CHAPTER – III

MATERIALS AND METHODS

GLASSWARE AND CHEMICALS

Good quality borosil or corning glassware and chemicals were used for all tests. They were washed with good detergent, rinsed in tap water and soaked in chromic acid clearing solution.

Clearing solution (Mahadevan and Sridhar, 1996)

Potassium dichromate - 60 g, Conc. Sulphuric acid - 60 mL, Distilled water - 1L.

Potassium dichromate was dissolved in warm water, cooled and sulphuric acid was added slowly. It was mixed thoroughly and used for cleaning glassware.

Then they were rinsed thrice in tap water, finally rinsed in distilled water and dried in hot air oven. Dried glassware and media used were sterilized in an autoclave for 15 min at 15 lb/sq inch pressure.

Analytical grade chemicals supplied by Loba, Hi-media, S.D. Fine Chemicals and E.Merck were used. All chemical solvents, enzyme kits used for this research work were of analytical reagent grade.
PREPARATION OF THE FORMULATIONS

The formulation was prepared according to traditional method of the approved siddha text: Anuboga Vaidya Navaneedham, 1986.

The herbal uchidum formulation (FS002) was prepared by powdering, mixing and steaming the ingredients such as bark of *Ficus racemosa* Linn (Family-Moraceae) and Sesame cake (Marc of sesame seeds-*Sesamum indicum*, Family-Pedaliaceae).

The plant was identified and authenticated by Dept. of Botany, K.M.Institute of Postgraduate Studies, Pondicherry and a voucher specimen (RC 531) has been deposited at the Herbarium of department of pharmacognosy, College of Pharmacy, Mother Theresa Institute of Health Sciences, Pondicherry.

Bark of *Ficus racemosa* Linn were collected from in and around Pondicherry in June 2001 by peeling method and dried at room temperature. The collected plant material was free from disease and contamination of other plants.

The fresh sesame cake was procured from a Devi Karumari Amman Oil Mills, vazudhaur road, Pondicherry.

The bark and sesame cake were size reduced to 40# powder using a mixer grinder.
26.25 g of powdered bark of *Ficus racemosa* Linn was mixed thoroughly with 26.25 g of sesame cake and the mixture was moistened and steamed for 5 min. Then it has to be administered by mixing with gingelly oil.

The gingery oil used was of Agmark grade. The formulation was freshly prepared, every time before administration.

**Preparation of NP003 (Bogar Vaidhyam - 700; Siddha formulary, 1993)**

The ingredients of the herbomineral parpam formulation (NP003), were purified Zinc, *Eclipta alba* leaf juice and aloe pulp. This formulation was procured from Indian Medicine Practitioners Co-operative Pharmacy Stores, Chennai.

The formulation was prepared according to the method specified in the Siddha formulary. The formulation (NP003) was prepared by melting the metal in a pan and adding small quantities of *Eclipta alba* leaf juice continuously with stirring, till the metal is converted into a powder. This powder was ground with aloe pulp into a fine paste, cakes were made dried, calcined and recalcined, repeatedly ground with aloe pulp until the calx has no green tint any more and the product is yellowish white (or) dark yellow.

**Organoleptic studies of bark of *Ficus racemosa* and marc of *Seasamum indicum* seeds**

The organoleptic characters such as colour, odour, taste, shape and fracture were studied for the bark of *Ficus racemosa* and marc of *Seasamum indicum* seeds using standard techniques.
DETERMINATION OF FOREIGN MATTER IN HERBAL FORMULATIONS (WHO guidelines, 2002)

Medicinal plant materials should be entirely free from visible sign of contamination by moulds or insects and other animal contamination, including animal excreta. No obscured odour, discoloration, slime or signs of deterioration should be detected. It is seldom possible to obtain marketed plant materials that are entirely free from some forms of innocuous foreign matter. The herbal ingredients should be stored in particular environment to avoid formation of moulds, since they may produce aflatoxins.

Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. However, microscopy is indispensable for powdered materials.

Any soil, stones, sand, dust and other foreign inorganic matter must be removed before medicinal plant materials are cut or ground for testing.

Definition of foreign matter

Foreign matter is material consisting of any or all of the following:

- Parts of the medicinal plant material or materials other than those named with the limits specified for the plant material concerned.
- any organism, part or product of an organism, other than that named in the specification and description of the plant material concerned.
- mineral admixtures not adhering to the medicinal plant material,
such as soil, stones, sand and dust.

**Experimental**

Fifty grams of the formulation FS002 was accurately weighed as specified in the guidelines. Spread in a thin layer and sorted the foreign matter into group by visual inspection and by using a magnifying lens (10X). Shifted the remainder of the sample formulation through a No.250 sieve and the dusts were collected. The collected dust and separated foreign matter is pooled and weighed.

**PRELIMINARY PHARMACOLOGICAL SCREENING**

Before proceeding into a detailed systematic pharmacognostical, phytochemical, pharmacological and toxicological studies it was thought worth to assess the pharmacological efficiency of the selected formulation by subjecting it to preliminary pharmacological screening in the scheme detailed below.

**Hypoglycemic activity**

Effect of formulations FS002 and NP003 on fasting blood glucose level of rabbits were studied (Bander et al, 1969; Teotia and Singh, 1997). 18 albino rabbits weighing from 1.25 to 1.75 kg were fasted for 18 h with water *ad-libitum* and they were divided into 3 groups of 6 animals each (n=6). Group I were administered orally with 2% gum acacia (vehicle) whereas Group II and III were administered with 500 mg/kg body weight of formulations (FS002) and 40 mg/kg body weight of NP003 (p.o) suspended in 2% gum acacia
respectively. The animal dose have been derived from the human dose by using standard dose ratios (Ghosh MN, 1996). The blood samples were collected from the marginal ear vein of rabbits immediately before and after 2, 4, 8 and 24 h of administration of the formulation. The post administration fasting blood glucose were estimated colorimetrically using GOD-POD method (ACCUCHEK active, ROCHE) and the results were tabulated.

**Effect of formulation FS002 and NP003 on glucose loaded rats (OGTT model) (Geisink, 1988)**

For special purposes, the effect of blood sugar lowering agents were studied in glucose loaded rats (Kar, 1999). 18 wistar rats weighing from 180 to 240 g were kept on standard diet (Hindustan Lever) and were divided into three groups of 6 animals each. Group I were administered with 0.5 ml at 5% tween 80, whereas Group II and III were administered orally with formulation FS002 and NP003 in doses of 500 mg/kg body weight and 40 mg/kg body weight using 5% tween 80 respectively.

One gram of glucose per kg body weight was administered from a 50% solution of glucose orally 5 minutes after the oral administration of the test drugs for all the group.

The blood was collected from the tail vein immediately before and 1, 2, 3, 5 and 24 h after administration of the formulations and the blood glucose was estimated by GOD-POD method using glucometer (ACCUCHEK active, ROCHE).
ANATOMICAL STUDIES OF BARK OF FICUS RACEMOSA LINN

Introduction to anatomical studies

The term ‘bark’ refers to all the tissues outside the vascular cambium of the stem and root. The tissues of the bark include periderm and secondary phloem. Periderm is also known as outer bark and secondary phloem as the inner bark (Trocken brodt, 1990). Periderm is produced by a lateral meristem called phellogen and the secondary phloem is produced by the vascular cambium. So the periderm and secondary phloem are different in their development. Periderm consists of an outer, dead, suberised tissue called phellem and inner living tissue called phelloderm. Phellem is productive in function. The phelloderm is storage in function. The phelloderm may be less broad or it may be narrow and less prominent. The periderm may be a single zone or several zones alternating with non peridermous tissues such as dead cortical tissues or secondary phloem. Such compound structure is called rhytidome.

The periderm may originate superficially from epidermis or subepidermal layers; it may also originate from deeper layers of the cortex or from the secondary phloem. The surface of the bark will exhibit several features such as colour, texture, fissuring and scaling. All these features are of diagnostic interest.

The inner bark usually consists of outer collapsed phloem and inner non collapsed phloem. The collapsed phloem is the store house of many ergastic compounds such as starch grains, crystals, tannins and lipids. The types of storage products and their distribution patterns are valuable observations in the study of the bark.
Phloem rays play significant role in determining the surface features as well as internal structure of the bark. Sieve plates, sieve tube members, companion cells and axial phloem parenchyma are the components of diagnostic potentials of the bark.

Bark is one of important parts of a plant in storing many valuable medicinal compounds. Wax, fat, fatty acids, volatile oils, resins, alcohols and hydrocarbons have been extracted from the bark. Aqueous extracts of bark are rich in tannin, soluble carbohydrates, mucilage, gum, pectin and glucosides. Alkaline extracts yield high molecular weight phenolic acids. Phlobaphenes and alkaloids have been identified in the ethanol fractions of leaf extract.

Plant barks are so much of importance in the medicinal and industrial arena that their study gains significance in the pharmacognosy, phytochemistry and pharmacology, when compared to the studies on bark, those on the wood lag much behind.

**Collection of specimens**

The plant specimens for the study, bark of *Ficus racemosa* were collected from Pondicherry. Care was taken to select healthy plants and from normal organs. The required samples of the bark were cut and removed from the plant and fixed in FAA (Formalin - 5mL + Acetic acid - 5 mL + 70% Ethyl alcohol - 90 mL). After 24 hrs of fixing, the specimens were dehydrated with graded series of t-butyl alcohol (TBA) as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained supersaturation.
Sectioning

The paraffin embedded specimens were sectioned with help of Precision Rotary microtome (A.O. Spencer). The thickness of the sections was 10-12μM. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O'Brein et al. (1964). Since Toluidine blue is a polychromatic stain, the staining results were remarkably good; and some cytochemical reactions were also obtained. The dye rendered pink colour to the mucilage, blue to the protein bodies etc. Wherever necessary sections were also stained with safranin and fast-green.

Photomicrographs

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphot 2 microscopic unit. For normal observations bright field was used. For the study of starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars.

Descriptive terms of the anatomical features are as given in the standard plant anatomy books (Esau, 1964).
**Powder microscopy studies of the bark of Ficus racemosa**

The air dried bark was size reduced manually using iron pestle and mortar to 40 #. The bark powder was studies for its microscopical features (Kokate, 2000).

(i) The bark powder was mounted in glycerine water and observed for calcium oxalate crystals.

(ii) The bark powder was stained with Iodine solution and observed for starch grains.

(iii) The bark powder was cleared with chloral hydrate and stained with phloroglucinol and concentrated HCl and observed in a self-illuminating compound microscope.

**PHYSICOCHEMICAL EVALUATION OF THE FORMULATION FS002 AND NP003**

The siddha formulations were subjected to physicochemical evaluation under the following headings:

i) Determination of Ash values

ii) Estimation of moisture content

iii) Extractive values

iv) Particle size determination of the formulations

v) Determination of zinc in NP003 using atomic absorption spectrometry.
i) Determination of ash values of the formulation (Indian Pharmacopoeia, 1985)

The ash remaining following ignition of medicinal plant materials is determined by three different methods which measure total ash, acid insoluble ash and water soluble ash.

In order to standardize the formulation and to determine the quality and purity of the formulation FS002 and NP003, the total ash, acid insoluble ash and water soluble ash were determined in three different sample formulations.

**Determination of Total Ash**

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 extraneous matter adhering to the plant surface.

About 4 g of the ground air-dried formulation FS002 accurately weighed was placed, in a previously ignited and tared Silica crucible. The formulation was spread in a even layer and ignited gradually increasing the heat to 500°C until it was white, indicating the absence of carbon. Cooled in a desiccator for 30 minutes and then weighed without any delay. The experiment was repeated thrice with three different batches of formulation.

**Acid insoluble ash**

To the crucible containing the total ash 25 mL of dilute Hydrochloric acid was added (~70g/L), covered with a watch glass and boiled gently for 5
minutes. Rinsed the watch-glass with 5 ml of hot water and this liquid was added to the crucible, filtered and collected the insoluble matter on ashless whatman no.41 filter paper and washed with hot water until the filtrate was neutral. Allowed the residue to cool in a suitable desicator for 3 minutes and then weighed without any delay.

Water soluble ash

The water soluble ash is the difference in weight between the total ash and the residue after treatment of total ash with water.

To the crucible containing the total ash, 25 mL of water was added and boiled for 5 minutes. The insoluble matter was collected in ashless whatman No.41 filter paper. Washed with hot water and ignited for 15 min at a temperature of 450°C. The weight of the residue remaining was subtracted from the weight of the total ash.

ii) Determination of Extractive values of the formulations (Indian Pharmacopoeia, 1985; Jain and Dixit, 2003)

Water soluble extractive

Five grams of the formulations FS002 or NP003 were added to 50 mL of water at 80°C in a stoppered flask. It was shaken well and allowed to stand for 10 min. It was cooled to 15°C and 2 g of Kiesulghur was added and filtered, 5 mL of the filtrate was transferred to a tarred evaporating basin. The solvent was evaporated on a water bath, for ½ h and then dried in steam for 2 h and weighed. The percentage of water soluble extractive was calculated with reference to the air dried powdered plant material.
**Alcohol soluble extractive**

Five grams of the formulations FS002 or NP003 were macerated with 100 mL of 70% alcohol in a closed flask for 24 h, shaken frequently during 6 h and allowed to stand for 18 h. It was filtered rapidly taking precautions against loss of alcohol and 25 mL of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish and dried at 105°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to air-dried powdered formulation.

**N-Hexane, Chloroform and Methanol soluble extractives**

Five gram of the formulations FS002 and NP003 were placed in a soxhlet apparatus. 25 mL of hexane was taken in the round bottom flask and hot extraction was carried out for 24 h. The extract in the round bottom flask was concentrated by distillation and the dry extract was weighed to get the hexane soluble fraction. The marc was used for successive extraction with chloroform and methanol. The percentage solubility in each case was calculated with reference to powdered formulation taken initially.

All the experiments for extractive values were repeated thrice with three different batches of the formulation.

**iii) Determination of Moisture content or Loss on drying (IS 1997)**

**Loss on drying (Gravimetric determination) (LOD)**

About 5 g of FS002 and NP003 were accurately weighed. The air-dried material was taken in a previously dried and tarred flat weighting bottle in a IR
moisture balance and the temperature was adjusted to 105°C and the heating was done for 5 minutes. The procedure was repeated for three times for different samples of the formulations and the loss in weight of the formulation was calculated with respect to the original weight.

The formula used for calculating LOD is \[ \text{LOD} = \frac{W_1}{W_2} \times 100 \]

\[ W_1 \] - weight of FS002 or NP003 after heating

\[ W_2 \] - original weight of the formulation

**Particle size determination of the formulation (Kokate, 2000)**

The particle size determination of the formulations, FS002 and NP003 were carried out using Linear microscopy method. In this method the eye piece micrometer was calibrated using a stage micrometer and the value of the each division of the eye piece micrometer was found (factor). The formulations were mounted using glycerine water and viewed through the eye piece micrometer and the readings were noted. The readings were converted to particle size measurements using the factor which was previously determined.

**Determination of Zinc in NP003 using Atomic Absorption Spectrometry (Anderson et al, 2001)**

The herbo mineral formulation, NP003 was taken after drying at 80°C for 12 h. It was ground finely and 1 g was taken in a 100 mL beaker and 5 mL of concentrated nitric acid and 2 mL of perchloric acid were added to the sample. It was covered with a watch glass and digested with heating to obtain a final volume of 3-5 mL. Evaporating the solution to dryness can cause loss of
more volatile elements such as Arsenic, Selenium etc. Then 10 mL of water was added to the beaker and the digested solution was filtered through an acid washed filter paper into a 50 mL volumetric flask. The filter paper was washed with water and the filtrate was made up to required volume with de-ionized water. The sample obtained was then subjected to atomic absorption spectrometry (Perkin Elmer, 5100PC).

PHYTOCHEMICAL STUDIES

Preliminary phytochemical screening

Preparation of extracts (Harborne, 1998)

The dry 40 # powder of the stem bark (2.5 kg) of Ficus racemosa and its formulation (FS002) were macerated at room temperature, in Hexane for 24 h. The Extract was suctioned and filtered using Whatmann filter paper. This was repeated for two more days and similar extracts were pooled together and concentrated at 40°C under reduced pressure using Buchi R-153 Rotavapour. The residual plant material was extracted successively with chloroform and methanol in the same manner as followed for hexane.

Qualitative phytochemical screening

The different qualitative tests were performed for establishing profile of the given extract for its chemical composition. The following tests were performed on extracts to detect various phyto constituents present in them.
1. Detection of alkaloids (Evans, 1997; Wagner, 1993; Wagner et al, 1996; Waldi D 1965)

Solvent free extracts of 50 mg was stirred with few mL. of dilute Hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloidal reagents as follows:

To a few mL of the filtrate, a drop or two of Mayer's / Wagner / Hagers / Dragendorff’s reagent were added by the side of the test tube.

**Mayers reagent**

Mercuric chloride (1.358 g) was dissolved in 60 mL of water and potassium iodide (5.0 g) was dissolved in 10 mL of water. The two solutions were mixed and made upto 100 mL with water.

**Wagner's reagent**

Iodine (1.27 g) and potassium iodide (2 g) was dissolved in 5 ml of water and made upto 100 mL with distilled water.

**Dragendorff's reagent**

Stock solution : Bismuth carbonate (5.2 g) and sodium iodide (4 g) were boiled for a few minutes, with 50 mL glacial acetic acid. After 12 h, the precipitated sodium acetate crystals were filtered off using a sintered glass funnel. Clear, red-brown filtrate, 40 mL was mixed with 160 mL ethyl acetate and 1 mL water and stored in amber-coloured bottle.
Working solution: 10 mL stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water.

2. Detection of carbohydrates

The extracts (100 mg) were dissolved individually in 5 mL of water and filtered. The filtrate was subjected to the following tests.

a) Molisch's test

To a few mL of the filtrate two drops of alcoholic solution of alpha naphthol was added, the mixture was shaken well and 1 mL of conc. sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

b) Fehling’s / Barfoed's / Benedict's test

1 mL of the filtrate was boiled on water bath with 1 mL of Fehling's solution A and B / Barfoed’s reagent / Benedict’s reagent was added and heated to boiling for 2 minutes.

Barfoed's reagent: Copper acetate, 30.5 g was dissolved in 1.8 ml of glacial acetic acid.

3. Detection of glycosides

For detection of glycosides, 50 mg of the extract was hydrolysed with concentrated hydrochloric acid for 2 h on a water bath, filtered and the hydrolysate was subjected to the following tests.
a) **Borntrager's test (Evans, 1997)**

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

b) **Legals tests**

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

4. **Detection of saponins by foam test (Kokate, 1999)**

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

5. **Detection of proteins and amino acids (Fisher, 1968)**

The extract (100 mg) were dissolved in 10 mL of distilled water and filtered through Whatmann No.1 filter paper and the filtrates were subjected to tests for proteins and aminoacids.

a) **Millon's test (Rasch and Swift, 1960)**

To 2 mL of the filtrate, few drops of Millon's reagent was added. A white precipitate indicated the presence of proteins.
b) **Biuret test (Gahan, 1984)**

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

c) **Ninhydrin test (Yasuma & Ichikawa, 1953)**

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

6. **Detection of phytosterols (Finar, 1986)**

**Libermann-Buchard's test**

The extracts of the formulations (50 mg) were dissolved in 2 mL acetic anhydride. To this, one or two drops of conc. sulphuric acid was added slowly along the sides of the test tube. An array of colour changes showed the presence of phytosterols.

7. **Detection of fixed oils and fats**

a) **Spot test**

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

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

8. Detection of phenolic compounds and tannins

a) Ferric chloride test

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

b) Lead acetate test

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

c) Alkaline reagent test

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

9) Detection of gum and mucilages (Whistler and Miller, 1993)

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

a) Salkowsky test

A small quantity of the extract in chloroform was treated with a few drops of conc. sulphuric acid, the solution turned yellow, then to red.

b) Hirshorn test

A small quantity of the test extract was heated with trichloroacetic acid, the solution turned yellow colour and finally changed to red.

HPTLC finger printing of *Ficus racemosa Linn* bark and its formulation FS002

A preliminary thin layer chromatographic studies were carried out for the bark and its formulation to determine the mobile phase, spray reagents and other related parameters.

Coarsely powdered and air dried (1g) samples of *Ficus racemosa Linn* bark (FR sample) and its formulation, FS002 were macerated in methanol (25 mL) for 18 hour, concentrated up to 10 ml in a standard flask and used for HPTLC studies.

The extract was applied on silica gel (Merck) 60 F254 in 0.2 mm layer thickness precoated on aluminium sheets using linomat IV sample applicator (10 μL). The mobile phase used for developing the chromatogram of the extracts under study was chloroform : methanol (8:2).
The plate was scanned using camag densitometer scanner 3V 1.13 equipped with cats V4.04 software at 254 nm and 366 nm.

A derivatised spectrum was developed after anisaldehyde treatment and scanned at 600 nm. Sulphuric acid was used as spray reagent for detection of the spots.

MICROBIAL EVALUATION OF THE FORMULATIONS (WHO guidelines, 2002; Jain and Dixit, 2001)

Microbial standardization of the herbal and herbo-mineral formulation

Preparation of the sample

Freshly prepared samples from three batches of 10 g of the formulation FS002 and NP003 were homogenized and suspended in lactose broth proven to have no antibacterial activity and diluted to 100 mL with the same medium. The medium was previously checked for sterility and antibacterial activity.

Detection of the presence of Enterobacteriaceae and presence of Escherchia Coli

The amount of the broth (prepared sample) containing 1 g i.e. 10 mL of the FS002 or NP003 were added to 100 mL of MacConkey broth and incubated at 43-45°C for 24 h. A subculture was prepared on a plate with MacConkey agar and incubated at 43-45°C for 24 h. The microbial growth and biochemical reactions were assessed for the presence of any gram-negative rods or Escherchia coli.
**Determination of total viable count**

The total viable count of the material being examined was determined as specified in the test procedure. The procedure, which was used is the plate count method. To the petri dishes of 9-10 cm in diameter added 1 mL of the formulations prepared by homogenisation and suspended in lactose broth (sample prepared) and about 15 mL of liquefied casein-soyabean digest agar was added at a temperature not exceeding 45°C. The pretreated material was spread on the surface of the solidified medium in a petri dish. Diluted the pretreated material to obtain an expected colony of not more than 300. Five dishes were prepared using the same dilution and incubated at 30-35°C for 5 days. Colonies are counted at the end of the 5th day. The analysis was repeated after storing the formulations for 30, 60 and 90 days at room temperature in a dry cool dark place and the results were tabulated.

**Validation of the tests for specific microorganisms**

The Standard strains *Escherichia coli* (ATCC No. 25922), *Bacillus subtilis* (ATCC No.6633), *Candida albicans* (ATCC No. 10231) and *Aspergillus niger* (ATCC No.16400) were used. The culture media (i.e. Mackonkey broth, liquefied casein digest agar) indicated for the specific microbes are incubated at 30-35° for 18-24 h. Diluted the portions of each cultures using buffered sodium chloride, peptone solution to pH 7.0 so that the test suspensions contain about $10^3$ microorganisms per mL. Mixed equal volumes of each suspension (prepared samples) and 0.4 mL of inoculum (standard) was added to test material. The result of the growth was observed and assessed for colony count using a colony counter. The test method showed
positive results for the respective strain of microorganisms. The validation method showed absence of bacteriostatic and fungistatic activity for the formulations.

ADVANCED PHARMACOLOGICAL STUDIES

Pharmacological screening for antihyperglycemic activity of ethanolic extract of *Ficus racemosa* and its formulation FS002 in streptozotocin induced diabetic rats.

Preparation of ethanolic extract of *Ficus racemosa Linn*

One hundred grams of the *Ficus racemosa* bark 40 # powder was extracted with 1000 mL of 70% ethanol for 4 h at room temperature. The residue was removed by filtration and it was once again extracted as above and filtered. The combined filtrate was evaporated to dryness at 40 – 50°C under reduced pressure in a Buchi rotary evaporator (Babu et al., 2002) (The yeild of ethanolic extract was 12%).

The extract was suspended in of gingelly oil (Agmark grade) and used for oral administration.

Preparation of ethanolic extract of the selected formulation, FS002

The ethanolic extract of FS002 was prepared in the same method as described above. The yield of the ethanolic extract was 16.7%).

The formulation was suspended in of gingelly oil (Agmark grade) and used for oral administration.
Animals

The experiments were performed in adult wistar rats weighing from 200 to 240 g. Animals were housed under standard laboratory conditions throughout the study. (23 ± 1°C, 55 ± 5% humidity, 12 hour light/dark cycle) and maintained with free access to water and a standard laboratory diet (Carbohydrates, 30% proteins, 22%, lipids, 12% vitamins), *ad-libitum*.

Streptozotocin induced diabetes (Rakieten et al, 1963)

The animals were divided into 6 groups of 6 animals each. In this experimental design two controls are used viz. normal control and diabetic control. Diabetes was induced by the tail vein injection of streptozotocin (STZ) (50 mg/kg body weight, iv) dissolved in normal saline (Pari et al., 2001). Two groups of 6 identical rats was kept without STZ administration as normal control Group I, and another group was administered 0.5 mL of gingelly oil only (vehicle control), Group II.

Forty eight hours after streptozotocin administration blood samples were drawn by retro-orbital puncture and glucose levels were determined to confirm diabetes. The diabetic rats exhibiting blood glucose levels in the range of 275 to 400 mg/dL were selected for the studies.

The diabetic rats were divided into 4 groups as follows.

Group III diabetic rats was given 0.5 mL of gingelly oil. Group IV and V diabetic rats were administered with 200 mg/kg body weight of ethanolic extract of *Ficus racemosa Linn* in 0.5 mL of gingelly oil and 200 mg/kg body
weight of ethanolic extract of the formulation FS002 in gingelly oil respectively. Group VI diabetic rats were administered with 500 μg/kg body weight of glibenclamide (Augusti and Sheela, 1996).

The herbal drug or glibenclamide was administered for 15 days at 10 A.M. daily. Blood was collected by retro-orbital puncture for glucose estimation just before drug administration on the 1st day and 1 h after drug administration on 5th, 10th and 15th day (Baby et al., 2003). The initial and final body weight of the animals were noted. The animals were sacrificed after blood collection, under chloroform anaesthesia on the 15th day and liver was removed for glycogen estimation (Caroll et al., 1956) serum glucose levels were estimated using GOD-POD glucometer (ACCUCHEK active, ROCHE).

**Effect of formulation NP003 in streptozotocin (STZ) induced diabetes (Rakieten et al 1963)**

The experimental protocols followed for studying the antidiabetic activity of the formulation, FS002 were also followed for the formulation NP003, except the grouping of normal and diabetic rats.

In this study the animals were divided as follows; group I was kept as normal control and were not administered STZ or drugs. Group II, diabetic rats were administered with 0.5 mL of 5% tween 80. Group III, IV, V diabetic rats were administered with 40 mg/kg, 80 mg/kg, 200 mg/kg body weight of the formulation, NP003 in 5% tween 80 respectively. Group VI was administered, 500 μg/kg body weight of Glibenclamide in 5% tween 80.
TOXICOLOGICAL STUDIES (Mutalik et al 2003)

The two selected polyherbal antidiabetic formulations, FS002 and NP003, were screened for toxic effects. The experimental protocols were approved by the institutional ethics committee of Regional Research Institute (Siddha), Pondicherry.

**Animals**

Acute and subacute toxicity studies were carried out in Swiss albino mice and wistar rats respectively. Adult mice (6-8 weeks old) of either sex weighing 25-30 g, were obtained from animal house, JIPMER, Pondicherry. They were housed in polypropylene cages, 4 animals per cage with free access to food and water. 6 to 8 weeks old wistar rats of either sex, weighing 150-200 g were obtained from animal house, JIPMER. They were housed, two per cage in elevated wire mesh cages with free access to food and water.

**Chemicals**

The alkaline phosphatase, acid phosphatase estimation kits were obtained from Himedia Inclusion, India. The urea and creatinine estimation kits were purchased from Qualigens, India. All other chemicals used were of analytical grade.

**Acute toxicity studies**

The acute toxicity studies were carried out by following the standard guidelines (OECD-420 guidelines, 1992). Sixty mice were used for the study.
Food and water was withdrawn 18 h before administration of formulation FS002. The mice were divided into six groups containing 10 animals in each group. Group I received 0.2 ml of gingelly oil (vehicle for formulation) orally and served as the control. Group 2-6 received 0.2 to 4 g/ kg body weight of FS002 orally. The animals were observed continuously for the first 2 h, then occasionally up to 5 h and then daily up to 14 days, post treatment to observe for any toxic symptoms and mortality.

The experiment was repeated with the formulation NP003. The acute studies were carried out by administering 20, 40, 80, 200, 400, 2000 mg/kg body weight of the formulation, NP003 in six different groups.

**Sub-acute toxicity studies**

The rats were divided into 4 groups of 6 animals each (3 males and 3 females). One group was given 0.5 ml of gingelly oil orally. Other 3 groups were administered 0.2, 0.4, 1 and 2 g/kg body weight of FS002 and 20, 40, 80, 200, mg/kg body weight of NP003 daily for 30 days. All the rats were observed for any physiological and behavioural changes and morality (Sharda et al 1993).

Food and water consumption was checked daily. Body weight was recorded at the beginning and twice weekly throughout the study.

Haematological parameters (Anoop et al., 2002) such as total RBC, WBC, differential leucocyte count and haemoglobin were estimated weekly in blood collected from the orbital sinus into sterilized heparinized tube
Twenty four hours after the last administration (on the 31st day of the experiment) blood samples were collected from each rat individually into non-heparinized tubes and were allowed to coagulate. Serum was separated by centrifugation, alkaline and acid phosphatases, were analysed by spectrophotometric method (Moss DW 1984). Urea and creatinine were analysed using reagent kit (Qualigens).

The liver, heart, thymus, spleen, adrenal, uterus and kidneys were removed and weighed immediately. Acid and alkaline phosphatases were also estimated as above. Pieces of organs were fixed in Bouin's fixative and processed routinely for histological examinations. The slides were stained with hematoxylin and eosin and observed under low power microscope for pathological changes (Bancroft et al., 1996). The students t-test was employed to analyse the results. Difference below the probability level 0.05 was considered statistically significant.

ANTIOXIDANT ACTIVITY AND ANTILIPID PEROXIDATION ACTIVITY OF FICUS RACEMOSA LINN AND ITS HERBAL FORMULATION
Antioxidant activity using DPPH (1,1-diphenyl-2-picrylhydrazyl)

The ability of the ethanolic extract (EE) of the bark of Ficus racemosa Linn and its formulation, FS002 (EE1) to scavenge the free radicals was determined by in vitro assay method using a stable free radical DPPH (Lee and Mowambo, 1998). To an ethanolic solution of DPPH (200 μM), an equal volume of the test extract dissolved in ethanol was added to get the final concentration of the test extract in the range of 50-200 μg/mL. Appropriate
control of test extracts mixed with equal volumes of ethanol were maintained. After 20 min the decrease in absorbance of the test mixtures (due to quenching of DPPH free radical) was read at 517 nm. The experiment was performed (in triplicate and percentage scavenging activity was calculated using the formula:

$$\frac{100 - \left( \frac{100}{\text{blank absorbance}} \times \text{test absorbance} \right)}{100}$$

The interaction of the ethanolic extracts, EE and EE1 with free radical DPPH at concentrations of 50, 100, 150 and 200 μg/ml were recorded and tabulated. Students t-test was used to statistically compare the results.

**Inhibition of in vitro lipid peroxidation**

The Lipid peroxidation in rat liver was measured in vitro in terms of thio barbituric acid reactive substances (TABRS) method. The Thiobarbituric acid method is based on the fact that peroxidation of most membrane systems leads to formation of small amounts of free malionaldehyde (MDA). MDA reacts with thiobarbituric acid to yield a coloured product, which in acid environment absorbs light at 532 nm and is readily extractable into organic solvents.

Rat was sacrificed and the liver was quickly removed and chilled in ice cold 0.9% sodium chloride. The liver was perfused with ice - cold 0.9% sodium chloride via the portal vein and then homogenised in 10 volumes of 0.15 M potassium chloride. In a final volume of one mL, fresh liver homogenate (0.2 mL) was incubated with Tris Hcl Buffer (pH 7.5, 0.15 M Kcl [0.1 mL]) and 2 μM adenosine diphosphate with different concentrations (10, 20, 40, 60, 100 and
140 μg/mL) of test extracts (EE and EE1). After 10 minutes ferrous sulphate (10 μM) and ascorbic acid (100 μm) were added and incubated at 37°C for 1 h. The reaction was terminated by the addition of thiobarbituric acid (2 M), boiled for 15 min at 95°C, cooled, centrifuged and absorbance read at 532 nm. Malionaldehyde thus formed was quantified using a molar extinction coefficient 1.56 x 10⁻³ M⁻¹ cm⁻¹ and expressed U/mg of protein. The protein content of rat liver homogenate was determined using Biuret reaction with the help of the Erba Chem-5 autoanalyser. The results were compared to inhibitory of standard antioxidant, vitamin E. The results were analysed using students t-test.

CLINICAL EVALUATION OF HERBAL (FS002) AND HERBOMINERAL FORMULATION (NP003) IN TYPE 2 DIABETES

Study protocol for clinical trial

1. Introduction

Diabetes mellitus is a heterogenous metabolic disorder characterized by altered carbohydrate, lipid and protein metabolism. The management of diabetes mellitus is considered a global problem since considerable population in the world are affected by this disease.

The study protocol was designed to carry out the clinical evaluation of two different herbal and herbo-mineral formulation as these formulation are found to be effective and safe in animal models.

2. Objective

To scientifically and systematically study the therapeutic efficacy and
The Siddha System of Medicine (SSM) popular in Tamil Nadu and in South India is an ancient time tested and traditional system of medicine. For the purpose of achieving Health for all, the modern day researchers are exploring the rich armament of medicine of the SSM. In this direction for the betterment of mankind a small step has been taken through this research work.

Diabetes mellitus is known to the modern system of medicine for the last 400 years, whereas it is known to the SSM for the past 4000 years. Hence the traditional time tested Siddha formulations used in the treatment of diabetes mellitus were selected for the present study. The selected Siddha formulations will be scientifically tested for their clinical efficacy and safety as per current WHO guidelines.

Two reputed Siddha formulations, which are described in recognized text were selected based on the recommendations of the Siddha Physicians of the Regional Research Institute (Siddha) Pondicherry and after a thorough ethnomedical and literature survey. The selected formulations belong to the class of Agamarundhu (internal medicine) viz., Bhasmas or parpams, and Uchidums. The formulation were subjected to a detailed pharmacognostical, pharmacological and toxicological studies.

Bhasmas or parpam formulation NP003 and uchidum formulation FS002 were chosen to find out the efficacy of the drug in the treatment of Diabetes Mellitus on the basis of the pharmacological and toxicological
3. **Trial Design**

Randomised, prospective, open double blind, longitudinal.

4. **Trial Site**

Regional Research Institute (Siddha), Pondicherry.

5. **Sample Size** – 60 (statistically determined)

6. **Level of study** – Out patient level

7. **Treatment period** – Six months for each case

8. **Method of Selection** – Patients are to be selected from the out patient department on the basis of Siddha and modern methodology. The Siddha methodology of diagnosis, blood sugar level in fasting and post prandial, history of previous treatment and family history are to be assessed. Thorough physical examination was to be conducted by siddha physicians.

9. **Methods of Assessment** – The assessment is to be performed once in a month for a period of three months in each case.

**During Treatment**

Periodical assessments of progress are to be noted.
**Concomitant medication**

Whenever patients get other illness symptomatic treatment will be given during the course of treatment as per the best patient care practices available in the siddha system of medicine.

**10. Follow-up**

The follow up study and laboratory parameters will be recorded. Special attention will be given for renal and hepato-toxicity.

**11. Laboratory Method**

The following investigation will be done before and after completion of study.

**In blood**

T.C., D.C., ESR, Hb (for exclusion criteria also)


Glycosylated HbA$_1$C.

**In urine**

Sugar, albumin, deposit, Bile salt, bile pigment

**12. Trial material**

**Trial drug I : FS002**

Source : Prepared on a laboratory scale (approved text "Anuboga
vaidya navaneetham - onpadam padam").

Ingredients : Bark of *Ficus racemosa* and sesame cake of *Sesamum indicum* and vehicle; gingelly oil.

Dose : 2.625 g/twice daily with gingelly oil.

**Trial drug II :** NP003

Source : To be procured from Indian Medicine Practitioners Co-operative Pharmacy Stores, Chennai

Ingredient : Purified zinc.

Dose : 200 mg bid with hot water.

**13. Pre inclusion criteria**

1. The patients who are willing to undergo treatment in the OPD level.

2. The patients who are willing to attend once in a week for three months and for follow up studies for six months.

a) **Inclusion criteria**

1. Polyurea 2. polyphagia, 3. polydypsia 4. nocturia, 5. tiredness and general weakness 6. giddiness 7. pruritis, 8. pheripheral neuritis, 9. blurring vision, 10. constipation, 11. age group of 18-65 years, 12. blood sugar, pp level above 160 mg/dL and below 300 mg/dL.

b) **Exclusion criteria**
1. Age above 65 and below 18 years
2. Juvenile diabetes
3. Insulin dependent diabetes mellitus (IDDM)
4. Pregnant & lactating women
5. Patients with liver disorder like jaundice, cirrhosis and the like
6. Tuberculosis
7. Malignancy
8. Cardiac diseases and hypertension
9. Severe renal disease
10. Pulmonary diseases
11. Blood sugar above 300 mg/dL PPBS
12. Iatrogenic hyperglycemia, corticosteroid and thiazide diuretics induced hyperglycemia
13. Patient having hyperglycosuria due to endocrine disorder
14. Patients who have participated in any other clinical trial in the last 180 days.

15. **Routine examination and assessment**

A detailed case record form would be used to record the data it consists of three forms. Form I (history proforma), Form II – clinical assessment and Form III – laboratory investigation. The full details of history and physical examination of patients will be recorded as per the proforma (Form I). The clinical assessment and laboratory investigation were done initially, at the end
of 1\textsuperscript{st} month 2\textsuperscript{nd} month and 3\textsuperscript{rd} month and for consecutive months. At the end of 6\textsuperscript{th} month follow up studies are conducted (Form II & III).

18. Ethical review

Project proposal was submitted to Institutional ethical committee (IEC) for its clearance certificate before the project is initiated. Patient's information sheet and informed consent form was submitted along with project proposal for approval by IEC. Both were maintained in duplicate with one copy given to the patient at the time of entry to the trial.

Research design and Methodology

The study protocol procedures needed to be followed were designed for trial. With the approval of the IEC, generally the first step to be followed in the design of a clinical trial is recruitment of subjects in the present trial design it has been decided to select NIDDM patients without any major complications. Ambulatory patients of either sex, in the age group of 18-65, both normal and obese, both vegetarians and non-vegetarians, all economic classes and patients with blood sugar fasting level - 120 mg/dL < and PP level of 200 mg/dL < are recruited for the study taking into consideration various inclusion and exclusion criteria for the study as mentioned previously.

The patients should be ready to share with all medical data and they should be without complications of diabetes such as ketoacidosis, nephropathy, neuropathy or retinopathy. The main exclusion criteria for such a trial are patients with IDDM, diabetic complications, pregnant and lactating women,
non-cooperative patients, non-sincere, disinterested in the treatment and those becoming severely allergic during the course of treatment.

**Methodology of trial**

The sample size \(n\) is determined statistically. Sixty ambulatory patients with mild to moderate severity of type 2 diabetes attending the diabetes clinic of Regional Research Institute (Siddha) were recruited for the study with the herbal formulations. The patients were supplied with patient information sheet which contain details about the trial drug and procedures in English and local language (Tamil). A written consent to voluntarily participate in the trial was obtained from subjects in the patient consent form. The following investigations were carried out before and after treatment for establishing the diagnosis and assessment of the treatment outcome.

1. Urine examination for albumin, sugar, acetone and microscopical examination.
2. Oral glucose tolerance test (OGTT)
3. Blood sugar (fasting and PP)
4. Glycosylated Hb A\(_1\)C
5. Blood cholesterol - lipid profile
6. Blood urea and creatinine

The patients were administered the trial drugs as per the protocol, by the siddha physicians.
The patients were advised to store the drugs in a cool, dry place and the they were given direction to take the drug orally along with gingelly oil in case of the trial drug, FS002 and with hot water in case of trial drug NP003, daily for a period of 90 days at 8 a.m. and 8 p.m. after food. They were emphasized to take the drug regularly and if they had missed a dose instructions were given to report the same immediately. The patients were motivated to follow the treatment protocols strictly.

The parameters were estimated once in every 30 days for a period of 3 months. The effect of trial drugs were compared with the base line values. i) Extent of polydipsia, ii) feeling of well being, iii) urine volume, iv) weight gain or loss, v) appetite, vi) perspiration and vii) stress tolerance. A post-trial studies was conducted for a period of 3 months for possible assessment of side effects, toxicity if any, present status of the disease, patient complaints if any and such data was collected and documented.

A case record form (CRF), which is simple, clear, designed in such a way to contain all the information about the study and in an easily computerisable format, were used to record the data about the study.

**Statistical Analysis**

The data were subjected to student `t' test and analysis of variance and the significant differences among the mean compared at various probability levels were determined using SPSS PC+ (Snedecor and Cochran, 1979) and Irristat 3.0 softwares.