DETAILED RESEARCH PROPOSAL

Methodology
The efficacy and safety profile of the individual formulation would be carried out in the Sri Sairam Siddha Medical College & Research Centre. The expertise available in this direction in the Institute from the department of Biochemistry, Pharmacology and Geomedical will be taken on mutual understanding since the test formulations is a proprietary Siddha polyherbal formulation. A qualified Siddha expert has been appointed in our Institute as Professor in allied health sciences to guide the Investigators. Moreover the collaborating industrial partner has an array of Siddha practitioners in their concern to promote traditional System of Medicine. Sufficient input will also be obtained from them.

Standardization of Test formulation
To scientifically standardize the formulation, the various ingredients in the formulation are checked for genuinity using modern analytical techniques and standardized. Further, shelf life studies including accelerated stability studies, real time stability studies and other standard procedures such as contamination with aflatoxin, microorganism etc., as per GLP and GMP standards following WHO and FDA guidelines would be carried out.

Selected Siddha formulation

- *Caesalpinia bonducella* –Kazharchikkai- Seed- Siddha Materia Medica
- *Vitex negundo* Nochi -Whole Plant- Siddha Materia Medica
- *Pandanus odoratissimus* –Thaazhai- Male Flower- Siddha Materia Medica
- *Acorus calamus* -Vasambu -Root Siddha- Materia Medica
- *Clitoria ternatea* -Kakkattan Root -Siddha Materia Medica

Screening of individual herb

Collection of crude materials
Identification of crude materials

Botanical parameters

- Foreign matter: Includes foreign plants, foreign insects, moulds etc, foreign minerals etc.
- Pharmacognostical studies by both macroscopic and microscopic methods

Identification of Bench mark compound
Authentications by the Botanist
Standardization Process

The following parameters are analyzed for conforming the quality and standards of herbs.

Collection of authenticated crude materials and dry it under shade.

1. Physico chemical parameters:
   a. Ash values: Total, acid insoluble, water soluble.
   b. Extractive values: Water soluble extractive and alcohol extractive values
   c. Moisture content: Loss on drying
   d. Volatile oils: By steam distillation.
   e. Qualitative analysis of phyto chemicals preliminary test.
   f. HPLC / HPTLC finger print.
   g. Determination of minerals by geo-chemical methods.

2. Pharmacognostical parameters:
   a. Bitterness value: Unit equivalent bitterness of standard solution of Quinine hydro chloride.
   c. Astringent property: Tannins that bind to standard Freiberg hide powder.
   d. Swelling Index: In water.
   e. Foaming Index: Foam height produced by 1 gm material under specified conditions.

3. Toxicological parameters:
   a. Arsenic: Stain produced on HgBr$_2$ paper in comparison to standard stain
   b. Pesticide residues: Includes total organic chloride & total organic phosphorous.
   c. Heavy metals: Like Lead, Cadmium and Mercury etc.
   d. Microbial contamination: Total viable aerobic count of pathogens like Entero bacteriaceae, E.coli, Salmonella, P.aeruginosa, S.aureous.
   e. Aflatoxins: By TLC using standard aflatoxins (B$_1$)
   f. Radioactive contamination.
   g. Micronutrients: Iron, Manganese, Copper, Cobalt, Chromium, Sodium, Potassium, Lithium, Vanadium, Molybdenum and Selenium by ICP, AAS and Titration.

Evaluation of extract, through following techniques

1. Color
2. Odor
3. Freedom from microbial contaminants and Pesticides
4. pH
5. Phyto chemical analysis – Preliminary test
6. HPTLC/HPLC
After analyzing the above parameters, extract will be isolated from the herbs by adapting following steps.

- Choosing quality plant
- Taking active parts / organs to be used
- Died and checked for pesticides.

Preparation of the extracts by solvents used

1. Aqueous
2. Ethanol
3. Methanol
4. Propylene glycol
5. Butylene glycol
6. Benzene
7. Chloroform
8. Vegetable oil

Normally 200 gm of dried herbal parts / kg solvent used

**Evaluation of Herbal Extract**

A herbal extract is extracted to be clear, free of foreign particles, with no sludge or precipitate and should stay like this, even if it is stored for a long time.

**Color of the extract**

The color of the extract depends upon the nature of plant. For example, carotenoids from carrot give a red extract, and copper derivatives of spirulina give a blue color extract. However, if the color of all the extracts is the same brownish color, it indicates unsatisfactory extraction techniques like oxidation, beginning of degradation or beginning of the polymerization of sugar derivatives. So, the dark color of extracts, especially, if it found in all the extracts of the range, is not always a guarantee of quality, as it is widely thought.

**Smell of the extract**

Similarly, the smell of the extract is related to the nature of the plant and if the extract does not smell like the plant, it indicates that the extract has been ill-treated. For e.g. chamomile extract should smell like chamomile flowers.

However, glycolic extracts and even most hydro glycolic extracts are never fragranced to the point that they replace perfumes in the final formulation.

From the above discussion, eyes and nose can be considered as the first laboratory equipment to evaluate extracts.
Freedom from microbial contaminants

The extracts should be free from microbial contaminants. Thought, we cannot speak of absolute sterility, yet an extract should not have more than 100 non-pathogenic germs per gram.

The extracts can be kept safe by using the right preservatives at the light dosage level. This can be done either by the direct injection into a specific gelose media or a filtration on a 0.2µ membrane and then, inoculation to the media.

Dry residue

Another indicator of the quality of an extract is dry residue. If a large amount of drug is used to make the extract, there should be a high percentage of dry residues. This dry residue comes from the extractible soluble part of the plant in that solvent. Dry residue varies from plant to plant depending upon the chemical composition.

The maximum theoretically extractible part of a dried plant is in general 10 to 20%, which means that herbal extracts made with 20% of the dried plant cannot show a dry residue above 4%.

All other figures (lower or higher) have to be checked often by a complimentary analytical method. The dry residue percentage has been correlated by an UV spectrum to check whether there is indeed some active constituents in the extracts or whether this dry residue is only obtained by adding preservatives, salts or sugars.

The amount of dry residue can be estimated by placing 1 gram sample in an oven at 118°C for 2 hours. It is then, checked that a constant mass is obtained even after two hours in the oven.

Refractometry

Refractometry is also employed in the evaluation of an herbal extract. Refractometry is linked to the results obtained on the dry residue test. The more residues in solution, the higher are the refractive index. However, the final results/figure also depends on the nature of the solvent i.e. the refractive index of propylene glycol is higher than that of water.

PH of the extract

PH of the extract is also an indicator of the quality of an extract. Generally the pH of cosmetic extracts range from 5 to 7. Excessive pH values whether acidic or alkaline may lead to bad conservation.

UV and visible spectrophotometry

UV and visible spectrophotometry offer very fast and reproductive ways to assay the presence of preservatives and also the amount of preservative, if present. Further, it also helps to characterize the extract and evaluate its strength. This is made possible as many active matters of plant absorb UV rays at a set wavelength which is invariable and characteristic of the plant. The more active the matter is the stronger is the peak.
**Colorimetry assays**

Colorimetry assays can also be used for evaluation of drug extracts. The general principle of colorimetry assay is to allow contact between the components or the family of components to be evaluated with a specific reagent to obtain a coloured complex, which should follow Beer-Lambert’s law.

The intensity of the colouration is measured by spectrophotometry and is directly proportional to the analyzed substance. One may calculate this concentration in relation with the standard range of the pure product.

This technique is simple although not very precise or accurate. However, it may be performed to make a comparative analysis between two competitive extracts of the same plant. A more accurate computation may be effected by densitometry or by PHLC.

**Thin layer chromatography**

Thin layer chromatography offers a good method for evaluation of drug extract. Thin layer chromatography is a first step towards the identification of the chemicals present in the herbal extracts. It is a physio-chemical separation method. The thin layer which is also called the stationary phase, is made of a substance finely dispersed on a rigid plate (glass, aluminium, plastic). It is very often made of silica. The solution of the unknown mixture is spotted on the plate as a drop or as a lime-sized ball.

India can emerge as the major country and play the lead role in production of standardized, therapeutically effective herbal formulation. India needs to explore the medicinally important plants. This can be achieved only if the herbal products are evaluated and analyzed using sophisticated modern techniques of standardization such as UV-visible, TLC, HPLC, HPTLC, GC-MS, spectro-fluorimetric and other methods. After evaluating the different extract, the potential extract is chosen and subjected to safety and efficacy study.

The best proven safety / efficacious four different herbal extract are chosen and combined as a Poly Herbal Formulation (PHF). Then PHF is subjected for safety and efficacy profile on animal.

**Poly Herbal Formulation (PHF)**

Among the six extracts, the best three safer and efficacious extracts will be chosen and combined as a Poly Herbal Formulation (PHF). This PHF will be screened for HPLC/ HPTLC finger print and Stability test such as Physical, Chemical & Biological methods.

**SCREENING OF PHF:**

HPLC / HPTLC finger print.
Stability test: Physical, Chemical & Biological method.
Antimicrobial studies.
To standardize, as well as quality control the medicinal plant, the first step is the identification of primary and secondary metabolites such as alkaloids, glycosides, polyphenol, tannins, flavanoids and saponin. Then, it will be isolated by using Modern chromatographic techniques such as HPLC and HPTLC. The identification and characterization of molecules will be done by using UV, NMR, FT-IR, GC-MS and LC-MS.

**HERBO-MINERAL FORMULATION (HMF) - (Thurusu mezhugu)**

Most of the medicines are mixture of compounds and because of its synergistic action; toxicity is being diminished thereby, increasing bioavailability through the cells of the body. Various commercially available medicines such as Thurusu mezhugu, Linga Chenduram, Poorna Chandrodaya Chenduram, Kshaya Kulanthaga Chenduram, Velli Parpam, Naga Chenduram Naga Parpam, etc., These medicines are particularly used for treating infectious diseases. So far, nothing is known about the chemical composition of these medicines and the scientific basis of its application in treating infectious diseases. Proper standardization techniques for checking the quality are inadequate on these medicines to meet the criteria to support its use worldwide.

**The aim of our present study is to:**

- Analyze the HMF using several modern analytical techniques to help in quality control and standardization of the drugs
- Collating the data from multiple approaches to suggest the likely speciation of metals present in these complex powders and their bio-absorption potential
- To assess the content and likely potency of very expensive preparations based on noble and other metals

**STANDARD METHOD OF PREPERATION**

**Purification and detoxification of raw materials:**

**Ingredients**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified Thurusu (Blue vitriol - Copper sulphate)</td>
<td>14 g</td>
</tr>
<tr>
<td>Saadhikkai (Nut Meg - <em>Myristica fragrans</em>)</td>
<td>28 g</td>
</tr>
<tr>
<td>Saadhipathri (Arillus of the nut - <em>Myristica fragrans</em>)</td>
<td>28 g</td>
</tr>
<tr>
<td>Lavangam (Clove – <em>Syzygium aromaticum</em>)</td>
<td>28 g</td>
</tr>
<tr>
<td>Elarishi (Cardamom seeds – <em>Elettaria cardamomum</em>)</td>
<td>28g</td>
</tr>
<tr>
<td>Kadukkai (Chebulic Myrobalan – <em>Terminalia chebula</em>)</td>
<td>28g</td>
</tr>
<tr>
<td>Kottai pakku (Betel – nut palm - <em>Areca catechu</em>)</td>
<td>28g</td>
</tr>
<tr>
<td>Kasukkatti (Extract of Black catechu - <em>Acacia catechu</em>)</td>
<td>28g</td>
</tr>
</tbody>
</table>
**Purification of Thurusu**

Crystals of raw Thurusu – 35g will be soaked in the amalgam of lead and keep alone for cooling. Then, the crystals are taken out and again soaked in the amalgam of lead. Repeat the same procedure for 11 times. After this process, the Thurusu will be fried in a mud plate till it becomes white and grind it to become fine powder to get purified form of white Thurusu powder.

**Preparation of Thurusu mezhugu**

All the above herbal ingredients are powdered separately and mixed each in above said proportion. To this herbal powder, the purified Thurusu powder is added and mixed well. Then, the juice of lemon fruit (125 Nos) is added little by little to the powder and triturated well to get paste form of Thurusu mezhugu. This medicine is kept in air tight glass container.

**SCREENING OF HERBO-MINERAL FORMULATION**

Collection of Plant crude materials / Ores / Salts / Secondary minerals  
Identification of Plant crude materials / Ores / Salts / Secondary minerals  
Authentication of the materials by the Botanist / Metallurgist / Chemist  
Standardize the procedure of preparation including purification process and elemental analysis at each stage of processes  
\[ \text{ICP-MS} \]  
\[ \text{XRD} \]  
Electron microscopes – (SEM) Refracted & Reflected microscopic properties analyzed.  
\[ \text{TGA / DSC Spectrometer} \]

Antimicrobial studies

PHF and HMF also will be tested with the following studies

*In vitro* toxicological profile: Photo toxicity test, Cytotoxicity/Hepatotoxicity, Ames test, HPRT test, Mouse Lymphoma assay, Commet assay, Micronucleus test, Chromosome aberration test. These tests will be performed under OECD guidelines.

**IN VITRO TOXICOLOGICAL PROFILE (SAFETY PROFILE) for PHF and HMF**

1. **Phototoxicity Test**

The standard *in vitro* 3T3 NRU Phototoxicity Test is performed according to OECD guideline 432. The phototoxic potential of the PHF and HMF are investigated by comparison of the cytotoxicity (EC\(_{50}\) values) under illumination and in the dark.

**Study Design**

Range finding pre-test  
Balb/c 3T3 cells  
8 dose levels
Treatment: 1 h in the dark, followed by 50 min at 1.7 mW/cm²
Control
Measurement of Neutral Red uptake

2. Cytotoxicity / Hepatotoxicity Test

Test Systems
Low metabolic competence:
V79 cells (parental)
High metabolic competence:
Primary hepatocytes
Specific metabolic competence:
V79 Cell Battery

**Three different Endpoints:**
For cytotoxicity can be measured:
Mitochondrial activity:
MTT-Assay
Membrane integrity:
Neutral Red Uptake
Total protein content:
Sulforhodamine B-Assay

The relative cell viability upon incubation with test item compared to the solvent control is determined (dose-effect curve and calculation of the EC₅₀).

**Study Design**
V79 cells, primary hepatocytes, etc.
Ten concentrations of test item
Eight replicates
Incubation time: 4 to 72 h
Positive control
Negative control

3. AMES Test

The standard Ames test (bacterial reverse mutation test) is performed according to OECD guideline 471. The mutagenic potential of the test item in a bacterial test system is investigated (dose-effect curve, 5 *S. typhimurium* strains).

**Study design**
5 *S. typhimurium* strains: TA97a, TA98, TA100, TA102, TA1535,
and/or TA1537, TA1538, E. coli WP2 uvrA
cytotoxicity and solubility pre tests
W and w/o S9 (rat), preincubation assay
5 dose levels
3 plates per dose level
4 plates as viability control
3 plates per negative control
3 plates per positive control

4. HPRT Test

The HPRT (hypoxanthine guanine phosphoribosyl transferase test; mutagenicity assay in vitro: mammalian cell gene mutation test) detects mutagenic effects of the test item in mammalian cells. The test is performed according to OECD guideline 476.

Study Design
Cytotoxicity and range finding pre-test
V79 cells
Four dose levels
Two replicate cultures per dose level
Three subcultures per replicate
Treatment: 4 h or 24 h w/o metabolic activation, 4 h with
Metabolic activation
Plating efficiency control
Control

5. Mouse Lymphoma Assay

The mouse lymphoma assay (mutagenicity assay in vitro: mammalian cell gene mutation test) detects mutagenic and clastogenic effects of the test item in mammalian cells. The assay is performed according to the OECD guideline 476.

Study Design
Cytotoxicity and range finding pre-test
L5178Y/TK- mouse lymphoma cells, heterozygous at the
Thymidine kinase (TK) locus
Two replicate cultures per dose level
Three subcultures per replicate
Treatment: 3 h w and w/o metabolic activation, 24 h w/o
Metabolic activation
4 dose levels
Negative (vehicle) control
Positive control
Plating efficiency control
Selection period: 10 – 14 days

6. Comet Assay

The comet assay (single-cell gel electrophoresis assay, SCGE) measures DNA strand breaks at the level of single cells from any tissue or cell culture. The assay is
performed according to “Guidelines for in vitro and in vivo Genetic Toxicology Testing: Single Cell Gel/Comet Assay“ by R. Tice et al. (2000).

**Study Design**
Cytotoxicity and range finding pre-test
V79 cells or others
Duplicates
5 dose levels
Treatment: 3 – 6 h w and w/o metabolic activation, 24 h w/o
Metabolic activation
Negative (vehicle) control
Positive control
Microscopic analysis: 100 cells per duplicate

7. Micronucleus Test
The micronucleus test in vitro detects clastogenic effects of the test item in mammalian cells. The tests are performed according to the OECD draft guideline by J. M. Parry.

**Study Design**
Cytotoxicity and range finding pre-test
V79 cells
Duplicates
3 dose levels
With and without rat S9
Incubation time: 3 h w and w/o S9, 24 h w/o S9
Control
Microscopic analysis: 1000 cells per duplicate
Detailed study report

8. Chromosome Aberration Test
The chromosome aberration test (mutagenicity: in vitro mammalian cytogenetic test) is performed according to OECD guideline 473.

**Study Design**
Cytotoxicity and range finding pre-test
V79 cells
3 dose levels
Minimum of two replicates per dose level
With and without rat S9
Incubation time: 3 h w and w/o S9, 24 h w/o S9
Control
Evaluation of 200 metaphases per concentration
**IN VITRO ANTI FILARIAL PROFILE (EFFICACY):**

Protocols of selected Siddha formulation (PHF and HMF)

**W. bancrofti infection:** W. bancrofti Infective (third stage) larvae are used throughout this study. The worms were allowed to develop in Culex fatigans mosquitoes, the natural vector, which were previously reared in the laboratory and fed on donors with known levels of microfilaraemia. The engorged mosquitoes were then, placed in an air-conditioned environment and fed during the next 15-17 days, the time required for development of the ingested microfilariae to the third stage. The motile larvae are dissected free of the mosquitoes, washed thoroughly in physiological saline, and kept overnight suspended in phosphate saline in the cold. Normally, all larvae are used in the following day for the study. On warming to room temperature, the larvae regained active movement within a few minutes. All larvae measured approximately 15-20 mm in length.

**Brugia malayi infection:** Brugia malayi is established and maintained in jirds and mastomys. B. malayi–infected mastomys will obtained from CDRI, Lucknow, are used for infecting other animals. For maintaining the cycle of infection, 4-days old mosquitoes are used to feed on infected mastomys having 80–100 microfilariae (mf) in their circulation. Colonies of the liver pool black eye strain mosquitoes, Aedes aegypti (SS strain), will be obtained from Hindustan Ciba Geigy Research Center, Mumbai, India, were maintained at the MGIMS animal house facility. After 12–14 days, the mosquitoes are dissected and checked for L3 stage larvae. For mass dissection, the mosquitoes are collected using a mosquito suction gun (Hausherr’s Machine Works, Toms River, NJ), stunned by shaking, placed in a petri dish with 2–3 mL of insect saline (0.6% NaCl), and are gently crushed to release the L3 larvae. The contents are transferred to a Bearmann apparatus and kept at 35–40°C for 45–60 min. The third stage larvae collected at the bottom are removed, counted with the help of a dissecting microscope and used for infecting fresh animals. Male Mastomys (normal), 6–8 weeks old, are infected by subcutaneous injection, and male jirds (normal) of the same age are infected by intraperitoneal injection of 100 L3, respectively, so that the microfilariae are available after 3 months or more after infection, which are collected by peritoneal lavage (in jirds).

**In vitro screening for antifilarial activity:** Test drugs are diluted in suitable solvents like methanol / double distilled water to obtain the desired final concentration range as previously optimized in our lab so as to obtain dose dependant effects against microfilariae in sterile 24 well culture plates (Nunc, Denmark) containing 900 ml of RPMI medium. Wells without any extract but with similar solvents in 900 ml of the medium were kept as corresponding controls. Approximately 100 microfilariae in 100 ml of RPMI medium were introduced into each well for every test samples and also for corresponding control samples (each individual samples in triplicates). The plates were
incubated at 37°C for 48 h in CO2 (5%) incubator. Mf motility is assessed by microscopy after 48 h of exposure (incubation for this time period was optimized during screening); the observations are recorded as the number of non motile mf out of all the 100 mf taken in each well for the study and represented as percentage (%) reduction in mf motility. All these conditions of assay procedure have been standardized in our laboratory to obtain reproducible results. Each experiment (in triplicate) was repeated thrice and results were represented as Mean + SEM of per cent reduction in motility of three such observations.

Assessment of parasite viability:

(i) Visual inspection. Parasite viability and killing are assessed visually by using a Leitz inverted microscope, and the observations are scored as -, inactive or dead; +, less active; + +, moderately active; and + + +, highly active.

(ii) MTT reduction assay. Parasite viability is also assessed quantitatively by the MTT reduction assay. The procedure used is essentially as described by Comley et al, with a slight modification. Briefly, after microscopic assessment of parasite viability, adult worms are transferred to 0.5 ml of phosphate-buffered saline (PBS) containing 0.5 mg of MTT (Sigma) per ml. The mf or L3 are suspended in 0.1 ml of PBS containing 0.5 mg of MTT ml⁻¹. Adult worms, mf, and L3 are incubated for 2 h at 37°C in the dark. The mf are pelleted and washed once with PBS by centrifugation. To the mf pellet, 100 µl of dimethyl sulfoxide (DMSO) is added to dissolve the dark blue crystals of formazan, and the mixture is then transferred to a 96-well microtiter plate (Costar). Adult worms incubated in MTT are washed once with PBS and blotted, and each adult worm is transferred into each well of a microtiter plate containing 200 µl of DMSO. The parasite-DMSO mixture is incubated for 1 h at room temperature for complete solubilization of formazan from the parasites into the solvent. Formazan quantification is performed with an automatic microtiter plate reader (Flow Laboratories, McLean, Va.) at a 595-nm wavelength by using DMSO as a blank and frozen worms as negative controls. Greater than 50% inhibition in MTT reduction is considered significant. These absorbance values relative to those for the controls are compared for significance (Student's t statistic) by using Statworks software of the Macintosh Plus computer. The filaricidal activity of TGase inhibitors against L3 is determined by microscopic examination.

In vitro antibody–dependent cellular cytotoxicity assay:

L3 and mf. Peritoneal exudates cells (PECs) are collected from normal jirds, and samples with > 95% viability are used. The cytotoxicity assay is carried out as described by previous methods. Briefly 100 mf or 10 L3 larvae of B. malayi in 50 µL of RPMI 1640 are incubated with 50 µL of normal jird PECs (5 × 10⁵ cells/100 mf/10 L3) and 50 µL of normal jird serum or sera from each of the immunized group of jirds in a 96-well culture plate. The 96-well plate is incubated for 48 hours at 37°C in a 5% CO₂ atmosphere. The parasites are examined under a microscope after 48 hours to check for viability. The parasites are considered dead if not motile and adhered by PECs. The percentage
cytotoxicity is expressed as the ratio of the number of dead parasites to that of the total number recovered within the experimental period multiplied by 100.