Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **43** Issue **1** Year **2022** Page **559-572***

Standardization And Analytical Evaluation Of Siddha Herbal Formulation Aavarai Kirutham In The Treatment Of Madhumega Avathai Vii: Diabetic Peripheral Neuropathy

M.K Sathesh kumar[1*], P.Sharmila[2], R.Gomathi[3], G.Thirunarayanan[4], T.Lakshmikantham[5]

[1]PG Alumni, National Institute of Siddha,Ministry of AYUSH, Tambaram Sanatorium, Chennai 600 047,Tamil Nadu. drmksathesh@gmail.com.*

[2]PG Alumni, National Institute of Siddha,Ministry of AYUSH, Tambaram Sanatorium, Chennai 600 047,Tamil Nadu. sharmi3794@gmail.com.

[3]PG Alumni, National Institute of Siddha,Ministry of AYUSH, Tambaram Sanatorium, Chennai 600 047,Tamil Nadu. kgmgomathi@gmail.com.

[4]Siddha Varmam Consultant, Varmam Research & Resource Centre, Vivekananda Kendra- NARDEP, Kanyakumari-629 702, Tamilnadu thirunarayanan.dr@gmail.com

[5]Head of the Department, Department of Maruthuvam, National Institute of Siddha,Ministry of AYUSH, Tambaram Sanatorium, Chennai 600 047,Tamil Nadu. drlakshmiramaswamy@gmail.com.

***Corresponding Author:** M .K. Sathesh kumar*

**PG Alumni, National Institute of Siddha,Ministry of AYUSH, Tambaram Sanatorium, Chennai 600 047,Tamil Nadu.,Email: drmksathesh@gmail.com Phone number: 9659327457*

ABSTRACT

Aavaarai kirutham is a Siddha herbal confection which literarily recommended for the treatment of diabetic peripheral neuropathy (Mathumega Avathaigal) in the authentic Siddha textbook " Sigicha rathina deepam". There are no standards for this antidiabetic herbal preparation. Standardization of the individual ingredients helps to prepare a quality test drug which responsible for the desired pharmacological effect. So, the present study has been performed to standardize unique ingredients and the herbal formulation 'Aavaarai kirutham' as a preliminary study by using three groups of test drug samples. The parameters used to standardize the preparation are organoleptic characters, Biochemical analysis, Physicochemical properties,HPTLC fingerprint analysis, Heavy metal analysis by ASS, Test for specific pathogens, Sterility test by pour plate method, Aflotoxin analysis, Pesticide residue analysis,and Thin layer chromatography. The organoleptic characteristics such as color, taste, odor, smell, and consistency of the ingredients and the test drug were evaluated. Physico-chemical parameters were determined as per WHO guidelines and reported as total ash, insoluble acid ash, water soluble extractive, ethanol soluble extractive, total sugar, reducing sugar content, fat content, and loss on drying. Thin layer chromatography developed for different solvent systems and detection of TLC fingerprint profiles were carried out under UV light at the wavelength of 366nm and also viewed after spraying with anisaldehyde sulfuric acid spray reagent. Colors and Rf Values of major spots were noted.

Keywords: Siddha, Aavarai kirutham, Standardization, Physiochemical, HPTLC

INTRODUCTION

The Siddha medical system is an ancient and traditional practice renowned for its distinctive characteristics, offering not only remedies for ailments but also profound insights into holistic well-being. Siddhars, revered as spiritual scientists, have meticulously categorized diseases into 4448 distinct entities, showcasing the system's comprehensive understanding of human health. [1] Meganeer is one among them. The ancient manuscript "Yugi Vaidhaya Chinthamani 800" intricately delineates the clinical manifestations of Madhumegam. Early indicators encompass polyuria, polyphagia, polydipsia, tastelessness, general debility, mood changes & irritability, and numbness These symptoms outlined in Yugi Vaidhya Chinthamani intricately parallel the presentation of Diabetes Mellitus.

Diabetes has emerged as a prominent global health challenge in the 21st century. Diabetes mellitus (DM) embodies a syndrome characterized by persistent high blood sugar levels due to insufficient insulin production, insulin resistance, or a combination of both factors. According to the International Diabetes Federation (IDF), an estimated 382 million individuals (8.3% of the global populace) were afflicted with diabetes in 2013, with projections foreseeing a rise to 592 million (10.1%) by 2035. Diabetic polyneuropathy (DPN), prevalent in about half of all diabetic patients over their lifetimes, stands as the most prevalent complication associated with diabetes. DPN significantly contributes to disability through complications such as foot ulcers, amputations, impaired gait, and injuries from falls. Approximately 20 to 30% of DPN patients endure neuropathic pain.

This study delves into the sensory characteristics, biochemical composition, physical properties, HPTLC fingerprinting, heavy metal content assessed through ASS, pathogen-specific testing, sterility evaluation via the pour plate method, aflatoxin examination, pesticide residue analysis, and thin-layer chromatography of the traditional Siddha formulation Aavarai Kirutham,documented in the Siddha text "Sigicha rathna deepam". This preparation holds potential for widespread application in the management of diabetic peripheral neuropathy (Mathumega Avathaigal).

MATERIALS AND METHODS

Preparation of trial drug - Aavarai Kirutham

Sources of raw drugs

The necessary herbal ingredients for the formulation of "Aavarai Kirutham" were procured from R.N.Rajan & Co., situated at No.1, Kumarappa Mastery Street, Chennai – 600 001, India. These raw materials underwent authentication by a botanist at the National Institute of Siddha, Tambaram Sanatorium, Chennai 600 047. Subsequently, the raw ingredients were subjected to purification procedures in accordance with established Siddha protocols. The formulation process took place at the Department of Gunapadam laboratory within the National Institute of Siddha.

Ingredients of Aavarai Kirutham

1. PurifiedAavarai samoolam (Cassia auriculata. Linn)
2. Purified Thenampoo (Cocos nusifera. Linn)
3. Purified Vaazhai kizhangu (Musa paradisiacal. Linn)
4. Purified Vaazhai poocharu (Musa paradisiaca. Linn)
5. Purified Jathikkai(Myristica fragrans. Houtt)
6. Purified Seeragam (Cuminum cyminum. Linn)
7. Purified Milagu (Piper longum. Linn)
8. Purified Kirambu (Syzygium aromaticum. Linn)
9. Purified Amukkara kizhangu (Withania somnifera. Linn)
10. Purified Lavangapattai (Cinnamomum verum. Linn)
11. Purified Sathisaaranai kizhangu (Trianthema decandra. Linn)
12. Purified Panai karkandu (Borassus flabellifer. Linn)
13. Purified Cow’s ghee. [4]

Method of purification of raw drugs [4] [5]

1. Aavarai samoolam (Cassia auriculata. Linn)

Wash it with water and then allow it to dry in the shade.

2. Thenam poo (Cocos nusifera. Linn)

Use a white cloth to clean it.

3. Vaazhai kizhangu (Musa paradisiacal. Linn)
Rinse with water followed by drying it in a shaded area.
4. Vaazhai poocharu(Musa paradisiaca. Linn)
Wash it with water and then let it air dry in the shade.
5. Jathikkai(Myristica fragrans. Houtt).
The outer peel should be peeled off, and the material should be sliced into small pieces before being dried in the shade.
6. Seeragam(Cuminum cyminum. Linn)
The fruit was sun-dried and subsequently fried to achieve a golden yellow hue.
7. Milagu (Piper nigrum. Linn)
Immerse in cow's buttermilk for a duration of 3 hours, then proceed to air-dry it in the shade.
8. Kirambu (Syzygium aromaticum. Linn)
Clean with a white cloth and dry under the shadow.
9. Amukkara kizhangu (Withania somnifera. Linn)
The tuber is boiled in milk, then dried and powdered.
10.Lavangapattai (Cinnamomum verum. Linn)
Thoroughly cleanse and allow to air-dry in a shaded area.
11.Sathisaaranai kizhangu (Trianthema decandra. Linn)
The tuber undergoes a process of boiling with milk, followed by drying and subsequent powdering.
12. Panai karkandu (Borassus flabellifer. Linn)
13. Cow's ghee. [4] [5]

Method of preparation

Step 1. Purified ingredients No 1 to No 4 were made with juices each 335 ml separately.
Step. 2. Cow's ghee 1340 ml was incorporated to the above mixture of juices and kept under sunlight for 24 hours.
Step. 3. The ghee and juice mixture was boiled till attained the vandal Mezhugu patham (pre-waxcame to settle stable and the boiled mixture was filtered.
Step. 4. Purified ingredients No 5 to 11 each 8.75 grams were finely powdered and then added to finely powdered ingredient 12 which weighed 175 grams.
Step. 5. The finely powdered mixture of ingredients No 5 to 12 was then added to the filtered (juices and ghee) mixture.
Step. 6. The filtered mixture was melted again till it attained ghee consistency and was stored airtight glass container. [4]

Organoleptic Parameters of Aavarai Kirutham

The organoleptic properties of the Aavarai Kirutham medication, including attributes such as color, taste, odor, and other sensory features, were meticulously observed and recorded.

Color

The Aavarai Kirutham was transferred to watch glasses and positioned against a white backdrop under white tube lighting. Its color was scrutinized through direct visual observation.

Odor

The Aavarai Kirutham was subjected to two separate olfactory assessments. A 2-minute interval was maintained between each smelling session to eliminate any lingering influence from the preceding aroma.

Taste

A minute quantity of Aavarai Kirutham was placed on the tip of the tongue.
Analyzed as per the standard procedure at the Department of Biochemistry National Institute of Siddha, Tambaram Sanatorium, Chennai- 600 047.

Preparation of extract

An exact measurement of 10 ml of Aavarai Kirutham was precisely dispensed into a clean 250 ml beaker, followed by the addition of 250 ml of distilled water. The mixture was vigorously boiled for a duration of 10 minutes. Subsequently, it was allowed to cool before being filtered into a 100 ml volumetric flask, where it was then adjusted to a total volume of 100 ml using distilled water. [6]

Table 1. Biochemical analysis

S. No	Experiment	Observation	Inference
1.	The appearance of the sample	Dark Yellow in Colour.	
2.	**Solubility** A small portion of the sample was thoroughly shaken and blended with distilled water.	Insoluble Sparingly soluble	Absence of Silicate
3.	**The action of heat** An aliquot weighing 500mg of the sample was placed into a dry test tube and subjected to gradual heating initially, followed by more intense heating.	White fumes evolved.	**Presence of Carbonate**
4.	**Flame test** A small quantity of the sample was transformed into a paste by mixing it with concentrated HCl in a watch glass, then introduced into the non-luminous section of the Bunsen flame.	No bluish-green flame appeared.	Absence of Copper
5.	**Ash test** A filter paper was immersed in a blend of the sample and cobalt nitrate solution, then introduced into the Bunsen flame and ignited.	No Yellow colored flame appeared	Absence of Sodium

Table 2. Test for acid radicles

S. No	Experiment	Observation	Inference
1.	Test for Sulphate Into a test tube, 2 ml of the previously prepared extract was dispensed, followed by the addition of 2 ml of a 4% solution of ammonium oxalate.	Cloudyappearance presents	Presence of **Sulphate**
2.	Test for Chloride 2 ml of the solution prepared earlier was combined with dilute HNO3 until the effervescence ceased. Subsequently, 2 ml of silver nitrate solution was introduced.	Cloudy appearance presents	Presence of **Chloride**
3.	Test for Phosphate A volume of 2 ml from the extract underwent treatment with 2 ml of Ammonium molybdate Solution and an additional 2 ml of concentrated HNO3.	The presence of a cloudy yellow appearance	Presence of **Phosphate**
4.	Test for Carbonate A volume of 2 ml from the extract underwent treatment with 2 ml of Magnesium sulfate Solution.	Cloudy appearance presents	Presence of **Carbonate**
5.	Test for Nitrate A single drop of the substance was heated alongside copper turnings and concentrated H2SO4, then observed by looking vertically down the test tube.	No characteristic changes formed	Absence of **Nitrate**
6.	Test for Sulphide 1 ml of substance was treated with 2 ml of Con.HCL.	Rotten egg-smelling gas evolved	Presence of **Sulphide**
7.	Test for Fluride and Oxalate A volume of 2 ml from the extract was combined with 2 ml of diluted acetic acid and 2 ml of calcium chloride solution, followed by heating.	Cloudy appearance presents	Presence of **Fluoride** and **Oxalate**
8.	Test for Nitrite Three drops of the extract were deposited onto the filter paper, followed by the placement of two drops each of acetic acid and benzidine solution.	No characteristic changes observed	Absence of **Nitrite**
9.	Test of Borate Two pinches of the substances were transformed into a paste using a mixture of sulfuric acid and 95% alcohol, then introduced into a blue flame.	Bluish bluish-yellow colored flame does not appear	Absence of **Borate**

Table 3. Test for basic radicles

1.	**Test for Lead** A volume of 2 ml from the extract was mixed with 2 ml of potassium iodide solution.	The yellow-colored precipitate was not obtained.	Absence of Lead.
2.	**Test for Copper** One pinch of substance was made into a paste with Con. HCL in a watch glass and introduced into the non-luminous part of the flame.	No blue-colored flame appeared	Absence of Copper
3.	**Test for Aluminum** Sodium hydroxide was gradually added in drops to the excess of 2 ml of the extract..	No characteristic changes observed	Absence of Aluminum
4.	**Test for Iron** a) Combine 2 ml of the extract with 2 ml of ammonium thiocyanate solution. b) Mix 2 ml of the extract with 2 ml of ammonium thiocyanate solution, then add 2 ml of concentrated HNO3.	The mild red colorappears	Presence of **Iron**
5.	**Test for Zinc** To 2ml of the extract, sodium hydroxide solution was added in drops to excess.	White precipitate did not appear.	Absence of Zinc
6.	**Test for Calcium** 2ml of the extract was added with 2ml of 4% ammonium oxalate solution.	cloudy appearance and a white precipitate is obtained	Presence of **Calcium**.
7.	**Test for Magnesium** 2ml of extract sodium hydroxide solution is added in drops to excess.	The white precipitate did not appear.	Absence of Magnesium
8.	**Test for Ammonium** To 2 ml of extract,a few ml of Nessler's reagent and excess sodium hydroxide solution are added.	Brown colordid not appear	Absence of Ammonium
9.	**Test for Potassium** 1ml of substance was treated with 2ml of sodium and then added with 2ml of cobalt nitrate in 30% glacial acetic acid.	No yellowish precipitate was obtained.	Absence of Potassium
10.	**Test for Sodium** 2 pinches of the substance were made into a paste by using HCL and introduced into the blue flame of the Bunsen burner.	No yellow color flame appeared.	Absence of Sodium
11.	**Test for Mercury** 2ml of the extract was treated with 2ml of sodium hydroxide solution.	No yellowish precipitate was obtained.	Absence of Mercury
12.	**Test for Arsenic** 2ml of the extract was treated with 2ml of sodium hydroxide solution.	No brownish-red precipitate was obtained.	Absence of Arsenic

Table 4. Misalliance biochemical analysis

1.	**Test for Starch** 2ml of the extract was treated with a weak iodine solution.	The bluecolor developed.	Presence of **Starch.**
2.	**Test for reducing sugar** 5ml of Benedict's qualitative solution was taken in a test tube and allowed to boil for two minutes Add 8 to 10 drops of the extract and again boil it for 2 minutes. The color is noted.	Nobrick red color developed.	Absence of Reducing sugar.
3.	**Test for alkaloids** 2ml of the extract was treated with 2ml of picric acid.	Yellow color developed.	Presence of **Alkaloid**
4.	**Test for Tannic acid** 2ml of the extract was treated with 2ml of ferric chloride solution.	Block color precipitate appears.	Presence of **Tannic acid.**
5.	**Test for Unsaturated Compounds** To the 2ml of extract 2ml of potassiumpermanganate solution wasadded.	Potassium permanganate is not decolored.	Absence of Unsaturated Compounds
6.	**Test for Amino acids** 2 drops of the extract were placed on filter paper and dried Well.	No violet color developed.	Absence of Amino acids
7.	**Test for the type of compound** 2ml of the extract is treated with 2ml of ferric chloride Solution.	No green color developed. No red color developed. No violet color developed. No blue color	Absence of Oxyquinole Epinephrine and pyrocatechol. Anti-pyrine, Aliphatic amino acid, and meconic acid are absent. Apomorphine, Salicylate, and Resorcinol are absent.

		developed.	Morphine, Phenol cresol, and Hydro quinine are absent.

Interpretation
Chemical analysis revealed the presence of Carbonate, Sulphate, Chloride, Phosphate, Carbonate, Sulphide, Iron, Calcium, Starch, Alkaloid, and Tannic Acid in Aavarai Kirutham, while heavy metals like Lead, Arsenic, and Mercury were notably absent.

Determination of specific gravity [7], [8]
Fill the dry specific gravity bottle with the prepared samples, ensuring that no air bubbles are trapped upon removing the cap from the sidearm. Insert the stopper securely and immerse the bottle in a water bath maintained at 50°C, allowing it to remain submerged for a duration of 30 minutes. Following this, meticulously wipe away any residue that may have emerged from the capillary opening. Remove the bottle from the bath, meticulously clean and completely dry it. Subsequently, remove the side cap promptly and conduct the weighing process. Calculate the disparity in weight between the sample and the reference standard.

Determination of Iodine value
Approximately 20 grams of the test sample were transferred into the iodine flask. Following this, 10 ml of chloroform was gently warmed and then allowed to cool for a duration of 10 minutes. Subsequently, approximately 25 ml of Wiji's solution was introduced into the same flask and thoroughly mixed. The flask was then left undisturbed for a period of 30 minutes and subsequently refrigerated for an hour. Next, about 10 ml of KI solution was added to the mixture, and it was titrated against 0.1 N sodium thiosulfate solution until a yellow color appeared. The addition of 1 ml of starch indicator followed, and titration against the sodium thiosulfate solution from the burette was performed once more. The endpoint was determined by the disappearance of the blue color. The above process was repeated without adding a sample, and the corresponding reading for blank titration was noted.

Determination of saponification value
Approximately 2 grams of the test sample were introduced into the round-bottomed flask. Subsequently, about 20 ml of 0.5 N alcoholic KOH solution was added to the flask. The same procedure was repeated without adding the sample to conduct a blank titration. Both the sample-containing and blank round-bottomed flasks were then subjected to reflux for a duration of 1 hour. Following reflux, both flasks were allowed to cool. The samples were then titrated using 0.5 N HCl solution with a phenolphthalein indicator. The endpoint was determined by the disappearance of the pink coloration.

Determination of Viscosity Value
Viscosity determination was conducted utilizing Ostwald viscometers. The process entails measuring the time necessary for a specified liquid volume to traverse through a capillary. The liquid is introduced into the viscometer, drawn into the upper reservoir via suction, and subsequently permitted to flow back into the lower reservoir under the influence of gravity. The duration required for the liquid to move between two marked points—one positioned above and the other below the upper reservoir—is precisely recorded.

Determination of Refractive Index
Determination of RL was carried out using a Refractometer.

Determination of Weight per ml
The determination of weight per milliliter was conducted employing the comparative weight calibration technique. This method involved calculating the weight of 1 milliliter of the formulation's base, followed by the calculation of the weight of 1 milliliter of the final formulation. The discrepancy in weight variations between the base and the final formulation was then computed as a measure of weight per milliliter.

Acid Value
Precisely 5 grams of the test sample were weighed and carefully transferred into a 250 mL conical flask. Subsequently, 50 mL of neutralized alcohol solution was added to the flask. The mixture was then heated for a duration of 10 minutes using a heating mantle. Following heating, the solution was removed from heat, and 1 or 2 drops of phenolphthalein indicator were introduced. This solution was then titrated against the KOH

solution dispensed from the burette. The appearance of a pink color signified the endpoint of the titration. The volume of KOH solution consumed was recorded, and the titration of the test sample was conducted in triplicate. The average of these successive readings was employed to calculate the acid value of the respective sample using the provided expression.

Acid value = Titter Value X 0.00561X 1000 / Wt. of the test sample (g)

Peroxide value

Accurately weigh 5 grams of the substance under examination and transfer it into a 250-milliliter conical flask equipped with a glass stopper. Add 30 milliliters of a mixture composed of 3 volumes of glacial acetic acid and 2 volumes of chloroform into the flask, and swirl until complete dissolution is achieved. Introduce 0.5-milliliter increments of saturated potassium iodide solution and allow the mixture to stand undisturbed for precisely 1 minute, intermittently shaking it. Following this, add 30 milliliters of water and begin titrating gradually with 0.01M sodium thiosulfate, ensuring continuous and vigorous agitation, until the yellow coloration nearly dissipates. Introduce 0.5 milliliters of starch solution and continue titrating, shaking vigorously until the blue color just vanishes. Repeat this procedure without including the substance under examination. The volume of 0.01M sodium thiosulfate utilized in the blank determination should not exceed 0.1 milliliters.

$$\text{Peroxide value} = 10\ (a - b)/w$$

HEAVY METAL ANALYSIS BY ASS-AAVARAI KIRUTHAM

Standard

Hg, As, Pb, and Cd – Sigma

Methodology

Atomic Absorption Spectrometry (AAS) stands as a widely utilized and dependable method for identifying metals and metalloids within environmental samples. The quantification of total heavy metal content in the sample was executed utilizing Atomic Absorption Spectrometry (AAS) Model AA 240 Series. This methodology was employed to ascertain the concentrations of heavy metals including mercury, arsenic, lead, and cadmium within the test specimen.

Sample Digestion

The test sample was digested with 1mol/L HCl for the determination of arsenic and mercury. Similarly, for the determination of lead and cadmium, the sample was digested with 1mol/L of HNO3.

Standard Preparation

1. As & Hg- 100 ppm sample in 1mol/L HCl.
2. Cd & Pb- 100 ppm sample in 1mol/L HNO3.

TEST FOR SPECIFIC PATHOGEN – AAVARAI KIRUTHAM

Methodology

The test sample underwent direct inoculation into specific pathogen media (EMB, DCC, Mannitol, Cetrimide) utilizing the pour plate technique. Subsequently, the plates were subjected to incubation at 37°C for a period ranging from 24 to 72 hours to facilitate observation. Identification of specific pathogens was based on discerning their characteristic coloration in conjunction with the pattern of colony formation observed across each differential medium.

Observation

Following the designated incubation period, no signs of growth were evident, indicating the absence of the specific pathogen.

STERILITY TEST BY POUR PLATE METHOD

Objective

The sterility of the product was assessed using the pour plate method. In the event of contamination or lack of sterility in the sample (formulation), its contact with the nutrient-rich medium would facilitate organism

growth. Following the prescribed incubation period, the organism's growth was identified by its distinctive colony formation pattern. These colonies are quantified as Colony Forming Units (CFUs).

Methodology
The test sample was mixed with sterile distilled water, and the resulting mixture was utilized for the assessment of sterility. Approximately 1 mL of the test sample was inoculated into a sterile petri dish, followed by the addition of around 15 mL of molten agar at 45°C. Thorough mixing of the agar and sample was achieved through gentle tilting and swirling of the dish. The agar was allowed to solidify undisturbed for approximately 10 minutes. Subsequently, the plates were inverted and placed in an incubator set at 37°C for a duration of 24-48 hours, with an additional extension up to 72 hours for observation of fungal growth. The colonies of the organism that developed were then enumerated and quantified as Colony Forming Units (CFUs).

Observation
No growth was observed after the incubation period. Reveals the absence of a specific pathogen.

AFLATOXIN ANALYSIS OF AAVARAI KIRUTHAM

Standard [9]
1. Aflatoxin B1, 2. Aflatoxin B2, 3. Aflatoxin G1, 4. Aflatoxin G2.

Solvent
Standard samples were solubilized in a combination of chloroform and acetonitrile (9.8 0.2) to yield a solution with concentrations of 0.5 micrograms per milliliter for both aflatoxin B1 and aflatoxin G1, and 0.1 micrograms per milliliter for both aflatoxin B2 and aflatoxin G2.

Test solution Concentration 1 µg per ml.
Procedure
The standard aflatoxin was applied onto the pre-coated TLC plate surface in volumes of 2.5 µL, 5 µL, 7.5 µL, and 10 µL, respectively. Similarly, the test sample was positioned on the plate, and both sets of spots were allowed to dry to initiate chromatogram development. This process occurred within an unsaturated chamber containing a solvent system comprised of chloroform, acetone, and isopropyl alcohol (in a ratio of 85 to 105). The chromatogram was allowed to develop until the solvent front moved a minimum distance of 15 cm from the origin. Subsequently, the plate was removed from the development chamber, the solvent front was marked, and the plate was left to air-dry. Spot location on the plate was determined through examination under UV light at a wavelength of 365 nm.

PESTICIDE RESIDUE AAVARAI KIRUTHAM ANALYSIS

Extraction [10] [11]
The test samples underwent extraction with 100 mL of acetone, followed by a brief homogenization process. Subsequent filtration was conducted, followed by the addition of additional acetone to the test mixture. Heating of the test sample occurred using a rotary evaporator, ensuring the temperature did not surpass 40°C, until the solvent had nearly evaporated entirely. A few milliliters of toluene were then added to the residue, and heating was resumed until all acetone was completely removed. The resulting residue was dissolved using toluene and subjected to filtration.

RESULTS AND DISCUSSION

Physiochemical Evaluation and Standardization of AAK
The organoleptic attributes of AAK affirm the purity and authenticity of both the raw drug and the finished formulation. These include its yellowish color, sticky texture, sour yet mildly sweet taste, strong characteristic odor, and turbid identity. AAK exhibits a specific gravity of 1.6171 and a viscosity of 64.46 Pas at 50°C. Its refractive index (RI) value of 1.34, along with a corresponding weight per milliliter of 1.1215 grams, adds to its distinctive identity. The degree of unsaturation is discerned through its iodine value, with AAK demonstrating a value of 75.56 mg I2/g. Saponification value, indicative of total free and combined acids, is measured at 177.7 mg of KOH per gram of fat for AAK. Additionally, the Acid Value is determined to be 1.01 mg KOH/g, while the Peroxide Value stands at 4.54 mEq/kg. Results from heavy metal analysis confirm the

absence of Mercury, Arsenic, and Cadmium in AAK. Notably, Lead is detected at a minimal level of 0.02 ppm, which falls below the prescribed limit. The results were tabulated in Table 05- 08.

Table 5. Organoleptic Parameters of Aavarai Kirutham

1.	Color	Yellow
2.	Odor	Characteristic
3.	Taste	Sour with mild sweet
4.	Touch	Sticky
5.	Appearance	Turbid

Fig. 1 Organoleptic Parameters of Aavarai Kirutham

Table 6. Biochemical analysis

ANALYTICAL TEST	**INFERENCE**
Sulphate	Presence of Sulphate
Chloride	Presence of Chloride
Phosphate	Presence of Phosphate
Carbonate	Presence of Carbonate
Nitrate	Absence of Nitrate
Sulphide	Presence of Sulphide
Fluoride and Oxalate	Presence of Fluoride and Oxalate
Nitrite	Absence of Nitrite
Borate	Absence of Borate
Test for basic radicals	
Lead	Absence of Lead
Copper	Absence of Copper
Aluminium	Absence of Aluminium
Iron	Presence of Iron
Zinc	Absence of Zinc
Calcium	Presence of Calcium
Magnesium	Absence of Magnesium
Ammonium	Absence of Ammonium
Potassium	Absence of Pottasium
Sodium	Absence of Sodium
Mercury	Absence of Mercury
Arsenic	Absence of Arsenic
Other constituents	
Starch	Presence of Starch
Reducing sugar	Absence of Reducing Sugar
Alkaloids	Presence of Alkaloids

Tannic acid	Presence of Tannic acid
Unsaturated compounds	Absence of Unsaturated compounds
Amino acid	Absence of Amino acid
Compound	Absence types of compound

Table 7. Analytical report of Aavarai Kirutham [7], [8]

S.No	Parameter	Aavarai Kirutham – AAK
1	Specific Gravity	0.6171
2	Viscosity at 50°C (Pa s)	64.46
3	Refractive index	1.34
5	Iodine value (mg I2/g)	75.56
6	Saponification Value (mg of KOH to saponify 1gm of fat)	177.7
7	Weight per ml	0.1215 g/ml
8	Acid Value mg KOH/g	1.01
9	Peroxidase Value mEq /kg	4.54

Table 8. Heavy Metal Analysis Report

Name of the Heavy Metal	Absorption Max Λ max	Result Analysis	Maximum Limit
Mercury	253.7 nm	BDL	1 ppm
Lead	217.0 nm	0.02	10 ppm
Arsenic	193.7 nm	BDL	3 ppm
Cadmium	228.8 nm	BDL	0.3 ppm

Qualitative Biochemical Analysis of AAK

Chemical analysis confirmed the existence of Carbonate, Sulphate, Chloride, Phosphate, Carbonate, Sulphide, Iron, Calcium, Starch, Alkaloid, and Tannic Acid in Aavarai Kirutham, while heavy metals like Lead, Arsenic, Cadmium, and Mercury were notably absent.[6]

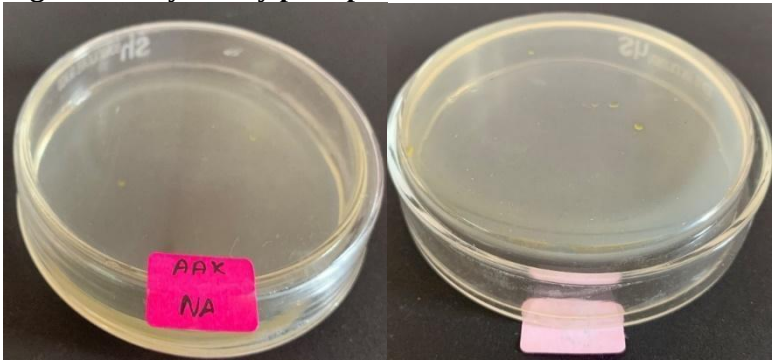
Sterility Test of AAK Formulation

The outcomes from the sterility assessment of AAK through the pour plate method demonstrate the formulation's high sterility. Following incubation, the culture exhibited the absence of pathogenic organisms including E. coli, Salmonella, Staphylococcus aureus, and Pseudomonas aeruginosa. The results were tabulated in Table 05 and illustrated in Figures 1-4.

Table 9. Colony Forming Units

Test	Result	Specification	As per AYUSH/WHO
Total Bacterial Count	Absent	NMT 10^5CFU/g	As per AYUSH specification.
Total Fungal Count	Absent	NMT 10^3CFU/g	

Fig. 2 Sterility test by pour plate method



Test for a specific pathogen

The assessment for specific pathogens indicates that the AAK formulation exhibits a high level of sterility. Subsequent to incubation, the culture demonstrates the absence of pathogenic microorganisms including E. coli, Salmonella, Staphylococcus aureus, and Pseudomonas aeruginosa.The results were tabulated in Table 05 and illustrated in Figures 1-4.

Table 10. Detail of Specific Medium and their abbreviation

Organism	Abbreviation	Medium
E-coli	EC	EMB Agar
Salmonella	SA	Deoxycholate agar
Staphylococcus Aureus	ST	Mannitol salt agar
Pseudomonas Aeruginosa	PS	Cetrimide Agar

Table 11. Microbial growth report

Organism	Specification	Result	Method
E-coli	Absent	Absent	As per AYUSH specification
Salmonella	Absent	Absent	
Staphylococcus Aureus	Absent	Absent	
Pseudomonas Aeruginosa	Absent	Absent	

Fig. 3 Culture plate with E-coli (EC) specific medium

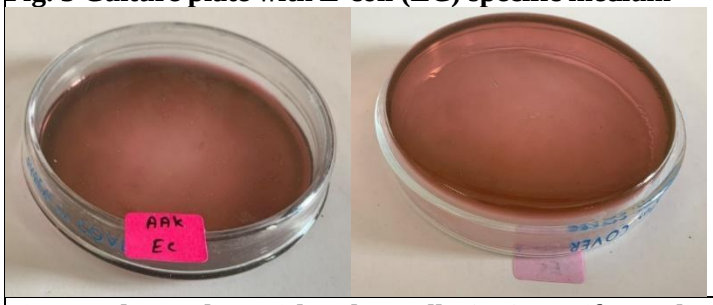


Fig. 4 Culture plate with Salmonella (SA) specific medium

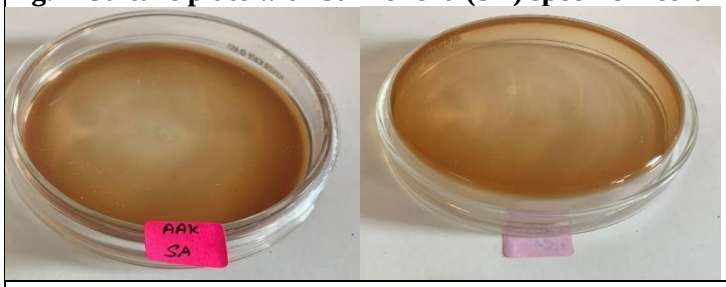


Fig .5 Culture plate with Staphylococcus Aureus (ST) specific medium

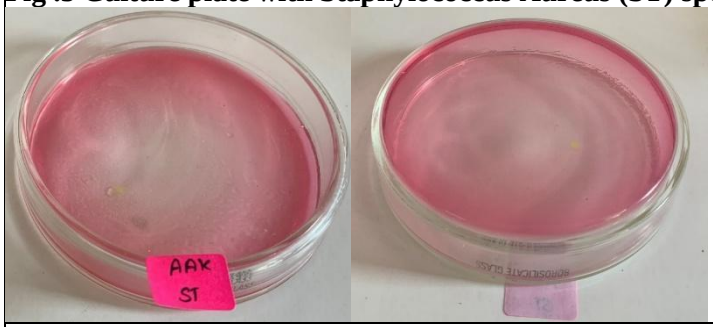
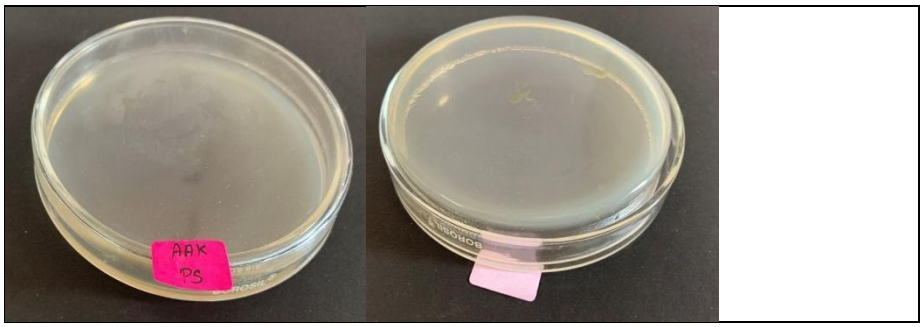


Fig.6 Culture plate with Pseudomonas Aeruginosa (PS) specific medium



Aflatoxin analysis of AAK

The results showed that there were no spots had been identified in the Aavarai Kirutham-loaded TLC plates when compared to the standard, which indicates that the sample was free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2. [9]

Table 12. Aflatoxin Specification

Aflatoxin	Sample AAK	AYUSH Specification Limit
B1	Not Detected - Absent	0.5 ppm
B2	Not Detected - Absent	0.1 ppm
G1	Not Detected - Absent	0.5 ppm
G2	Not Detected - Absent	0.1 ppm

Pesticide Residue of AAK Analysis

The results showed that there were no traces of pesticide residues such as Organochlorine, Organophosphorus, and pyrethroids in the Aavarai Kirutham for analysis. [10] [11]

Table 13. Pesticide Residue Result Analysis of the Sample AAK

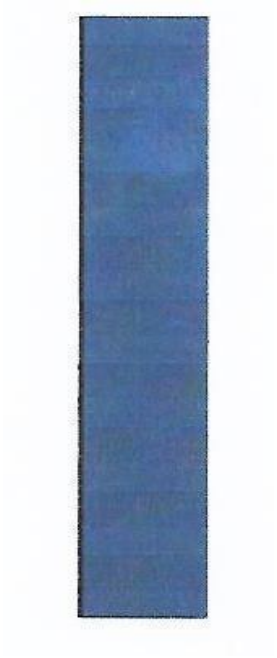
I. Organo Chlorine Pesticides	Sample AAK	AYUSH Limit (mg/kg)
1. Alpha BHC	BQL	0.1mg/kg
2. Beta BHC	BQL	0.1mg/kg
3. Gamma BHC	BQL	0.1mg/kg
4. Delta BHC	BQL	0.1mg/kg
5. DDT	BQL	1mg/kg
6. Endosulfan	BQL	3mg/kg
II. Organic Phosphorus Pesticides		
1. Malathion	BQL	1mg/kg
2. Chlorpyriphos	BQL	0.2 mg/kg
3. Dichlorvos	BQL	1mg/kg
III. Pyrethroid		
1. Cypermethrin	BQL	1mg/kg

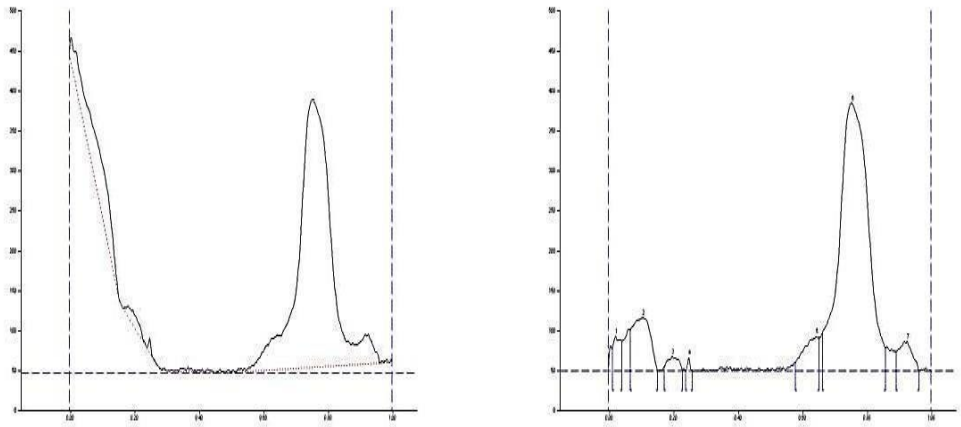
BQL- Below Quantification Limit

TLC and HPTLC analysis of AAK

Preliminary TLC analysis of the sample emits blue fluorescence indicating the presence of coumarins as illustrated in Figure 2. The results of HPTLC fingerprinting analysis of the sample AAK reveal the presence of seven prominent peaks corresponding to the presence of seven versatile phytocomponents within it. Rf value of the peaks ranges from 0.01 to 0.89. Further peak 6 occupies the major percentage of the area of 76.49 which denotes the abundant existence of such compound. The results were tabulated in Table 06 and illustrated in Figure 3.

TLC Visualization of AAK - TLC plate visualization at 366.





HPTLC fingerprinting of Sample AAK

Table. 13 Peak HPTLC fingerprinting

CONCLUSION

To standardize Aavarai Kirutham, a comprehensive assessment of its physical characteristics including color, odor, taste, and texture was conducted. Biochemical analysis revealed the presence of various constituents such as Carbonate, Sulphate, Chloride, Phosphate, Sulphide, Iron, Calcium, Starch, Alkaloid, and Tannic Acid. Microbial analysis confirmed the absence of harmful bacteria like E. coli, Salmonella, Staphylococcus aureus, and Pseudomonas aeruginosa, thereby enhancing the drug's efficacy. Safety was further ensured through the analysis of heavy metals, with Lead, Arsenic, and Mercury found to be absent in Aavarai Kirutham. Additionally, a novel, straightforward, precise, and robust HPTLC method revealed the presence of seven

distinct peaks corresponding to versatile phytoconstituents. These findings indicate that Aavarai Kirutham boasts maximum efficacy with minimal adverse effects, facilitating drug development. Standardization serves as a pivotal initial step for subsequent toxicological assessment and clinical research validation.

ACKNOWLEDGEMENT
The authors are thankful to the faculties Department of Maruthuvam, National Institute of Siddha, and Noble Research Solutions, Chennai for their technical support for this research work.

CONFLICT OF INTEREST
The authors have declared no conflict of interest.

REFERENCE

1. Dr. Shanmugavelu H.B.I.M, Noi Nadal Noi Mudal Naadal Part I, First Edition 1967 Dept. Of Indian Medicine and Homeopathy; Chennai, 106
2. International Diabetic Federation, https://www.diabetesatlas.org, 9thEdition 2019.
3. Singh R, Kishore L, Kaur Diabetic peripheral neuropathy: current perspective and future directions. Pharmacol Res. 2014 Feb; 80:21-35.
4. C. Kannusamipillai–Sigichaa Rathna Deepam Ennum Vaithiya nool, part-1, Rathnanayakar and Sons, Edition: 2007, page no: 209.
5. Purificatication of raw drugs"-Kannusami pillai -Sigichaa Rathna Deepam,part- 1,Edition: 2007,page no:28-33.
6. Prashant Tiwari, Bimlesh Kumar, Mandeep Kaur, Gurpreet Kaur, Harleen Kaur. Phytochemical screening and Extraction: A Review. International Pharmaceutical Science 2011; 1; 98-106.
7. India Pharmacopeia I Volume I, Government of India, Ministry of Health and Family Welfare, Indian Pharmacopeia Commission, 2014.
8. Indian standard methods of sampling and test for oils and fats Indian standard institution New Delhi 47-50. 1964.
9. Luciana de CASTRO. Determining Aflatoxins B1, B2, G1, and G2 In Maize Using Florisil Clean Up With Thin Layer Chromatography And Visual And Densitometry Quantification. Ciênc. Tecnol. Aliment. vol.21 no.1 Campinas. 2001.
10. WHO guidelines for assessing the quality of herbal medicines concerning contaminants and residues. WHO Geneva. 2007.
11. Lohar. D.R. Protocol for testing of ASU medicines. Pharmacopoeial Laboratory for Indian Medicines. Ministry of AYUSH. 2001
12. Pharmacopoeial Laboratory for Indian Medicine (PLIM) Guideline for standardization and evaluation of Indian medicine which includes drugs of Ayurveda, Unani, and Siddha systems, Department AYUSH, Ministry of Health & Family Welfare, Govt. of India.