

STANDARDIZATION OF SIDDHA POLYHERBAL FORMULATION KALLADAIPPU CHOORANAM – BY MODERN TECHNIQUES

¹Dr.K.Kanimozhi, ²Dr.V. Manimekalai, ³Dr.M.D. Saravana Devi MD(s)

^{1,2}PG scholar, ³Professor, Head of the Department
Department of PG Gunapadam, Govt Siddha medical college
Chennai, Tamilnadu, India.

Abstract- *Kalladaippu chooranam(KAC)* is a Siddha polyherbal formulation which is described in the siddha literature useful for the treatment of Renal calculi and its related symptoms. *Kalladaippu chooranam* was made as per the preparation given in the siddha literature *Theraiyar vagadam* (Page no. 175). The objective of the study is to evaluate the physicochemical, biochemical and phytochemical analysis and HPTLC of the prepared drug as per the PLIM guidelines. The organoleptic character of the drug provides the purity and quality of the formulation. The results obtained from the physicochemical evaluation shows that the Loss on drying of *Kalladaippu chooranam* at 105°C was 9.85% , Total ash value of *Kalladaippu chooranam* was 5.68%, Acid insoluble ash value was 1.61% ,Water soluble ash value was 1.61%, Water soluble extract value was 11.71%, Alcohol soluble extract value was 7.61%, pH was 7.8 , indicates the alkaline nature of drug. Biochemical analysis of *Kalladaippu chooranam* showed the presence of Carbonates and sulfates. High Performance Thin Layer Chromatography analysis of *Kalladaippu chooranam* reveals the presence of five prominent peaks corresponds to the presence of five versatile phytochemicals present with in it. Rf value of the peaks ranges from 0.01 to 0.88. Phytochemical analysis of *Kalladaippu chooranam* divulges the appearance of Alkaloids, Carbohydrates, Saponins, Phenols, Tannins, Flavonoids, Diterpenes. From the results , the drug has no microbial contamination. Heavy metals and pesticide residue were not exceeds the maximum permissible limit. The above analysis concluded that the results obtained were within the normal limits in all the parameters, and the drug has effective biologically active components used to treat Renal calculi and its related ailments.

Key words: Renal calculi, *Kalladaippu chooranam*, Physicochemical, polyherbal, siddha medicine.

INTRODUCTION:

Siddhars who given the siddha literature were contributed not only a system of medicine but also to the knowledge of eternity, alchemy, and yogic living^[1]. The Siddha system such an antiquated form of medicine consists of comprehensive treatment modalities for various diseases. Siddha system is wide in therapeutics. In this system, medicine is classified into two types namely Internal medicine and External medicine^[2]. *Chooranam* is one of the form of internal medicines in which, purified raw materials are powdered separately, sieved and mixed according to a given ratio; for certain preparations the purified raw materials are powdered and sieved . shelf life of three months. *Chooranam* is a fine powdered particles which are taken along with one of the adjuvants such as honey, milk, ghee, hot water, some other juices or decoction^[3].

Kalladaippu chooranam(KAC) ,a polyherbal formulation,mentioned in the ancient siddha literature *Theraiyar vagadam* (page no. 175) which has shown highest potential in the treatment of Renal calculi and its related ailments. But scientific evidences for *Kalladaippu chooranam* have not been reported^[4]. Standardization of drug means confirmation of its identity and resoluteness of its standard and purity. Lack of quality control can affect the potency and well being of drugs may lead to health problems in the consumers^[5]. So there is a need to develop a standardization technique by using preliminary guidelines. Therefore, the current investigation was done to detect physicochemical screening – organoleptic nature, loss on drying, Total ash, Acid insoluble Ash, Alcohol soluble extractive, water soluble extractive, High performance Thin Layer Chromatography (HPTLC), Heavy metal analysis, Sterility testing, Specific pathogen, Pesticide residue, Aflatoxin, Biochemical and phytochemical analysis of siddha formulation *KC* according to PLIM guidelines.

MATERIALS AND METHODS:

Drug selection:

Kalladaippu chooranam was acquired as a test medicine for its activity against renal calculi as mentioned in the *Theraiyar vagadam* (Pg no. 175)^[6]

Ingredients of *Kalladaippu chooranam* with Botanical Name

Table no.1. Composition of KAC

S.NO	NAME OF THE DRUG	BOTANICAL NAME	QUANTITY
1.	<i>Elarisi</i>	<i>Elettaria cardomomum</i>	1 palam (35 gm)
2.	<i>Sandhanam</i>	<i>Santalum album</i> Wood	1 palam (35 gm)

3.	<i>Kodiveli</i>	<i>Plumbago indica</i> Root bark	1 palam (35 gm)
4.	<i>Velipparuthi</i>	<i>Pergularia daemia</i> Root bark	1 palam (35 gm)
5.	<i>Ponnanganni</i>	<i>Alternanthera sessilis</i> Root bark	1 palam (35 gm)
6.	<i>Aavaarai pattai</i>	<i>Cassia auriculata</i>	1 palam (35 gm)
7.	<i>Alli kizhangu</i>	<i>Nymphaea nouchali</i>	1 palam (35 gm)
8.	<i>Aalam vizhuthu</i>	<i>Ficus benghalensis</i>	1 palam (35 gm)
9.	<i>Athimadhuram</i>	<i>Glycyrrhiza glabra</i>	1 palam (35 gm)

Collection of the drugs:

The raw drugs *Elarisi*(*Elettaria cardomomum*), *Sandhanam* (*Santalum album* Wood) , *Kodiveli ver pattai* (*Plumbago indica*), *Aavaarai pattai* (*Cassia auriculata*), *Alli kizhangu* (*Nymphaea nouchali*) , *Athimadhuram* (*Glycyrrhiza glabra*) were bought from Country Raw drug store in Paris corner ,Chennai, *Velipparuthi* (*Pergularia daemia*), *Ponnanganni* (*Alternanthera sessilis*) were freshly collected from the area Maangaadu, Chennai, *Aalam vizhuthu* (*Ficus benghalensis*) was freshly collected from the area Arumbakkam, Chennai.

Recognition and verification of drugs:

The raw drugs were conceded and verified by Gunapadam experts and Botanist of the department. Each raw drug samples have been labeled as 1087 -1095 / PGG/ 320220100505/ GSMC – CH/ 2020- 2023 . For future reference ,the labelled specimens were stored in the PG Gunapadam Department laboratory, Government Siddha Medical College , Chennai-106.

Purification of raw drugs:

Purification process were done as per classical Siddha literature *Sarakkugalin suthi sei murai*.

Preparation of *Kalladaippu chooranam*(KAC):

Equal ratio of the above given drugs were taken purified, dried and pounded into fine powder using stone mortar. The obtained powder was purified by Pittaviyal method as per Siddha classical literature. A mud pot was taken and it was half filled by mixture of milk with equal quantity of water. The mouth of the pot was sealed with a cloth. This chooranam was placed over the cloth and tied firmly around the mouth of mud pot by another pot. The gap between mud pots was tied with a wet cloth to avoid evaporation. The mud pot was kept on fire and boiled until the cow's milk 3 /4 part reduced in the lower pot. The same drug was later dried and powdered then sieved again. Then it was kept in a clean air tight glass container.

Dosage:

1 to 2 grams twice a day

Adjuvant: Butter or Milk

Indications: Renal calculi^[6]

Qualitative Analysis Investigation

The analysis of the trial drug KAC was evaluated as per the PLIM (Pharmacopoeial Laboratory for Indian Medicines) guidelines. Physico-chemical, Phyto-chemical analysis, Biochemical analysis, Heavy metal analysis, TLC & HPTLC analysis and Sterility method, specific pathogen test, Pesticide residue analysis, and Aflatoxins assay were done at Noble Research Institute, Perambur, Chennai.

Organoleptic characters:

Taste, nature, odour, touch, flow property, appearance of the drug was noted.

These following studies were done at Noble Research Solutions, Perambur at Chennai.

PHYSICOCHEMICAL ANALYSIS^[7,8]

Physicochemical studies of the trial drug have been done according to WHO Guidelines. Physicochemical studies like total ash, water soluble ash, acid soluble ash, acid insoluble ash, water and alcohol soluble extract, loss on drying at 105% were done at The Tamilnadu DR. M.G.R.Medical University, Chennai.

1. Loss on drying:

An accurately weighed 1g of *Kalladaippu chooranam* formulation was taken in a tarred glass bottle. The crude drug was heated at 105°C for 6 hours in an oven till a constant weight. The Percentage moisture content of the sample was calculated with reference to the shade dried material.

2. Determination of total ash:

Accurately weighed 2g of *Kalladaippu chooranam* formulation was added in crucible at a temperature 6000° C in a muffle furnace till carbon free ash was obtained. It was calculated with reference to the air dried drug.

3. Determination of acid insoluble ash:

Ash above obtained, was boiled for 5min with 25ml of 1M Hydrochloric acid and filtered using an ash less filter paper. Insoluble matter retained on filter paper was washed with hot water and filter paper was burnt to a constant weight in a muffle furnace. The percentage of acid insoluble as was calculated with reference to the air dried drug.

4. Determination of water soluble ash:

Total ash 1g was boiled for 5min with 25ml water and insoluble matter collected on an ash less filter paper was washed with hot water and ignited for 15 min at a temperature not exceeding 4500C in a muffle furnace. The amount of soluble ash is determined by drying the filtrate.

5. Determination of water soluble extractive:

5gm of air dried drug, coarsely powdered *Kalladaippu chooranam* was macerated with 100ml of distilled water in a closed flask for twenty-four hours, shaking frequently. The Solution was filtered and 25 ml of filtrated was evaporated in a tarred flat bottom shallow dish, further dried at 1000C and weighted. The percentage of water soluble extractive was calculated with reference to the air dried drugs.

6. Determination of alcohol soluble extractive:

1 gm of air dried drug coarsely powdered *Kalladaippu chooranam* was macerated with 20 ml alcohol in closed flask for 24 hrs. With frequent shaking, it was filtered rapidly taking precaution against loss of alcohol 10ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 1000C and weighted. The percentage of alcohol soluble extractive was calculated with reference to air dried drug.

Determination of pH

About 5gm of test sample was dissolved in 25 ml of distilled water and filtered the resultant solution was allowed to stand for 30 minutes and then subjected to pH evaluation.

Particle size determination^[9]**Methodology**

Particle size determination was carried out by optical microscopic method. In which the sample were dissolved in the sterile distilled water (app 1/100th dilution). Diluted sample were mounted on the slide and fixed with stage of appropriate location. Light microscopic image were drawn with scale micrometer to arrive at the average particle size. Minimum 30 observations were made to ascertain the mean average particle size of the sample.

Solubility Test:

A test sample *KAC* was taken in a dry test tube and to it 2 ml of the solvent was added and shaken well for about a minute and the results are observed. The test was done for solvents like water, Ethanol, Chloroform, Hexane, DMSO and Ethyl acetate and the results are observed individually.

PHYTOCHEMICAL ANALYSIS^[10]

The preliminary phytochemical screening test was carried out for each extracts of *Kalladaippu chooranam* as per the standard procedure mentioned hereunder.

1. Detection of alkaloids:

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

a) Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow colour precipitate indicates the presence of alkaloids.

b) Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (Potassium Bismuth Iodide). Formation of a red precipitate indicates the presence of alkaloids.

c) Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

2. Detection of carbohydrates:

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch's Test: To 2 ml of plant sample extract, two drops of alcoholic solution of α -naphthol are added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

b) Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

3. Detection of saponins

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

4. Detection of phenols -Ferric Chloride Test:

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

5. Detection of tannins Gelatin Test:

The extract is dissolved in 5 ml of distilled water and 2 ml of 1% solution of Gelatin containing 10% NaCl is added to it. White precipitate indicates the presence of phenolic compounds.

6. Detection of Flavonoids

a) Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

b) Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.

7. Detection of diterpenes Copper Acetate Test:

Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green color indicates the presence of diterpenes.

8. Test for Quinones:

Extract was treated with sodium hydroxide blue or red precipitate indicates the presence of Quinones.

9. Gum and Mucilage:

To 1ml of extract add 2.5ml of absolute alcohol and stirring constantly. Then the precipitate was dried in air and examine for its swelling properties. Swelling was observed that will indicate presence of gum and mucilage.

The Preliminary phytochemical studies of aqueous extract of *Kalladaippu chooranam* were done using standard procedures. The results were presented in tables. The present study reveals that the bioactive compounds were present in all the extracts of *Kalladaippu chooranam*.

Thin Layer Chromatography(TLC) Analysis^[11]

Test sample was subjected to thin layer chromatography (TLC) as per conventional one dimensional ascending method using silica gel 60F254, 7X6 cm (Merck) were cut with ordinary household scissors. Plate markings were made with soft pencil. Micro pipette were used to spot the sample for TLC applied sample volume 10-micro liter by using pipette at distance of 1 cm at 5 tracks. In the twin trough chamber with the specified solvent system After the run plates are dried and was observed using visible light Short-wave UV light 254nm and light long-wave UV light 365 nm.

High Performance Thin Layer Chromatography(HPTLC) Analysis^[12]

HPTLC method is a modern sophisticated and automated separation technique derived from TLC. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively. High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials efficiently and cost effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. Thus this method can be conveniently adopted for routine quality control analysis. It provides chromatographic fingerprint of phytochemicals which is suitable for confirming the identity and purity of phytotherapeutics.

Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analyzed. After elution, plates were taken out of the chamber and dried.

Scanning

Plates were scanned under UV at 366nm. The data obtained from scanning were brought into integration through CAMAG software. Chromatographic finger print was developed for the detection of phytoconstituents present in each sample and their respective R_f values were tabulated.

Biochemical analysis of Acid and Basic radicals^[13]**Test for Acid Radicals****Test for Carbonates**

To 1 ml of the test solution about 1 ml of concentration (conc.) HCL was added. Formation of brisk effervescence indicates the presence of carbonates

Test for chlorides

To 2 ml of test solution, about 1 ml of silver nitrate solution was added. Appearance of White precipitate indicates the presence of chlorides.

Test for sulfates

To 1 ml of the test sample add diluted H₂SO₄ till effervescence ceases followed by this about 1 ml of barium chloride solution was added. Appearance of white precipitate indicates the presence of sulfates.

Test for sulfides

To 1 ml of the test sample about 2 ml of HCL was added with slight warming the mixture. Formation of colorless gas with the smell of rotten egg indicates the presence of sulfides.

Test for phosphates

To 2 ml of test solution treated with 2 ml of ammonium molybdate solution followed by addition of 2ml of concentrated nitric acid. Formation of yellow precipitate Indicates the presence of phosphates

Test for Fluoride and Oxalate

To 2 ml of the test solution about 2 ml of dil acetic acid and 2ml of calcium chloride solution was added. Formation of white precipitate Indicates the presence of Fluoride/ Oxalate

Test for Borates

2ml of the test solution was added with sulphuric acid and 95% alcohol followed by exposure to flame. Appearance of green flame Indicates the presence of Borates

Test for Nitrates

0.5 ml of test solution heated with copper turning followed by addition of sulphuric acid. Appearance of reddish brown gas Indicates the presence of Nitrates

Test for Specific Basic Radical**Test for Lead**

1 ml of the test solution added with 2 ml of potassium chromate solution. Formation of yellow precipitate indicates the presence of lead.

Test for Arsenic

1 ml of the test solution added with 2 ml of 10% (2N) sodium hydroxide (NaOH) solution. Formation of brownish red precipitate indicates the presence of Arsenic

Test for Mercury

1 ml of the test solution added with 2 ml of 10% (2N) sodium hydroxide (NaOH) solution. Formation of yellow precipitate indicates the presence of mercury.

Test for Copper

1 ml of the test solution added with 1 ml of Ammonium hydroxide (NH₄OH) solution. Formation of blue precipitate indicates the presence of copper.

Test for Ferric

To 1 ml of test solution, about 2 ml of potassium ferrocyanide was added. Formation of blue precipitate indicates the presence of ferric.

Test for Ferrous

To 1 ml of test solution, about 1 ml of potassium ferric cyanide solution was added. Formation of blue precipitate indicates the presence of ferrous.

Test for Zinc

1 ml of the test solution added with 2 ml of sodium hydroxide (NaOH) drop wise until indication appears. Formation of white precipitate indicates the presence of Zinc.

Test for Silver

1 ml of the test solution was added with 1 ml of conc. HCL followed by appearance of curdy white precipitate. Boil the precipitate with water. It does not dissolve. Add NH₄OH solution in it and add 1 ml dilute HNO₃. Formation of curdy white precipitate indicates the presence of silver.

Test for Magnesium

1 ml of the test solution added with 2 ml of sodium hydroxide (NaOH) drop wise until indication appears. Formation of white precipitate indicates the presence of Magnesium.

Heavy Metal Analysis by Atomic Absorption Spectrometry(AAS)^[14]

Standard: Hg, As, Pb and Cd – Sigma

Methodology:

Atomic Absorption Spectroscopy [AAS] is a very common and reliable technique for detecting metals and metalloids in environmental samples. The total heavy metal content of the sample was performed by Atomic Absorption Spectrometry [AAS] Model AA 240 series. In order to determination the heavy metals such as mercury, arsenic, lead and cadmium concentrations in the test item.

Sample Digestion:

KAC sample was digested with 1 mol/L Hcl for determination of arsenic and mercury. Similarly, for the determination of lead and cadmium the KAC sample were digested with 1 mol/L of HNO₃.

Standard Preparation: As & Hg - 100 ppm sample in 1 mol/L Hcl Cd and Pb - 100 ppm sample in 1 mol/L HNO₃

Sterility test by Pour plate method^[15]**Objective:**

The pour plate techniques were adopted to determine the sterility of the product. Contaminated / un sterile sample [formulation] when come in contact with the nutrition rich medium it promotes the growth of the organism and after stipulated period of incubation the growth of the organism was identified by characteristic pattern of colonies. The colonies are referred to as Colony Forming Units [CFUs].

Methodology:

KAC was admixed with sterile distilled water and the mixture was used for the sterility evaluation. About 1ml of the KAC extract was inoculated in sterile petri dish to which about 15 ml of molten agar 45 C was added. gar and KAC were mixed thoroughly by tilting and swirling the dish. gar was allowed to completely gel without disturbing it about 10 minutes . Plates were then inverted and incubated at 37 C for 24 - 48 hours and further extended for 72 hours for fungal growth observation. Grown colonies of organism was then counted and calculated for CFU.

Test for specific pathogens^[16]

The tests for specific pathogens are designed for the estimation of the number of viable aerobic micro-organisms present and for detecting the presence of designated microbial species in pharmaceutical substances. The term „growth“ is used to designate the presence and presumed proliferation of viable microorganisms.

Methodology:

One part of the KC sample was dissolved in 9ml of sterile distilled water and the test sample was directly inoculated into the specific pathogen medium [EMB, DCC, Mannitol, Cetrimide] by pour plate method. The plates were incubated at 37°C for 24 - 72 hour for observation. Presence of specific pathogen was identified by their characteristic colour with respect to pattern of colony formation in each differential media.

Table no: 2. Details of Specific Medium and their abbreviation

S.NO	ORGANISM	ABBREVIATION	MEDIUM
1.	Escherichia coli	EC	EMB Agar [Eosin Methylene Agar]

2.	Salmonella species	SA	Deoxycholate Agar
3.	Staphylococcus aureus	ST	Mannitol salt Agar
4.	Pseudomonas aeruginosa	PS	Cetrimide Agar

Analysis of Pesticide Residue Organochlorine, Organophosphorous, Organocarbonates, Pyrethroids^[17-18]

Extraction:

Kalladaippu chooranam test sample was extracted with 100 ml of acetone and followed by homogenization for brief period. Further filtration was allowed and subsequent addition of acetone to the test mixture. Heating of test sample was performed using a rotary evaporator at a temperature not exceeding 40 C until the solvent has almost completely evaporated. To the residue a few millimetres of toluene was added and it was heated again until the acetone was completely removed. Resultant residue was dissolved using toluene and filtered through membrane filter.

Aflatoxin assay by Thin Layer Chromatography (TLC)(B₁, B₂, G₁,G₂)^[19]

Solvent:

Standard samples were dissolved in a mixture of chloroform and acetonitrile [9.8:0.2] to obtain a solution having concentrations of 0.5 µg per ml each of aflatoxins B₁ and aflatoxins G₁ and 0.1 µg per ml of each of aflatoxins B₂ and aflatoxins B₂ and aflatoxins G₂.

Procedure:

Standard aflatoxin was applied on to the surface to pre-coated TLC plate in the volume of 2.5 µL, 5 µL, 7.5 µL and 10 µL. Similarly, the test sample was placed and the spots was allowed to dry and the chromatogram was developed in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone and isopropyl alcohol [85: 10: 5] until the solvent front has moved not less than 15 cm from the origin. The plate was removed from the developing chamber, the solvent was marked in the front and the plate was allowed to air-dry. The spots were located on the plate by examination under UV light at 365nm.

RESULTS & DISCUSSIONS

Organoleptic characters

The inferences are illustrated in Figure 1 and tabulated in Table no.3 .



Figure no. 1

Table No. 3 Organoleptic characters

S.no	Parameter	Result
1	State	Solid
2	Nature	Fine
3	Odour	Aromatic
4	Touch	Soft
5	Flow property	Non free flowing
6	Appearance	Pale Brownish
7	Taste	Sweet with slight bitter

Physicochemical parameters

The physicochemical parameters of *KAC* were analysed and the results given in table no.4 and table no.5 .

Table no.4 Results of Physicochemical evaluation of *Kalladaippu chooranam*

S.NO	PARAMETERS	PERCENTAGE
1.	Loss on drying	9.85%
2.	Total ash value	5.68%
3.	Acid insoluble ash,	1.24%
4.	Water soluble ash	1.61%
5.	Water soluble extraction	11.71%
6.	Alcohol soluble extraction	7.61%

Particle Size Determination Results

Microscopic observation of the particle size analysis reveals that the average particle size of the sample was found to be $79.6 \pm 21.02 \mu\text{m}$ given in Figure no. 2

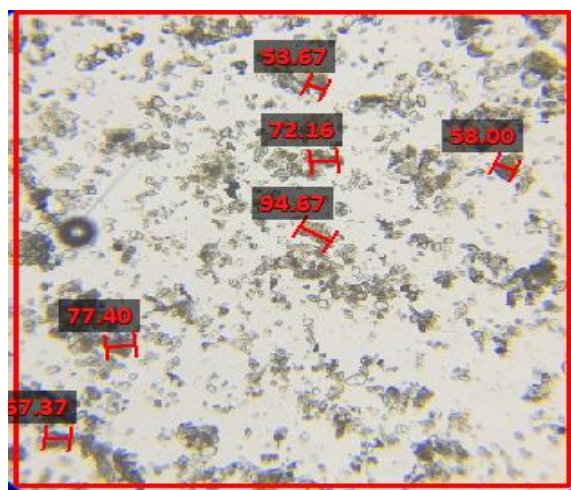


Figure no. 2 Microscopic Observation of Particle size

Table no: 5 Solubility Profile Results

S.No	Solvent Used	Solubility / Dispersibility
1	Chloroform	In Soluble
2	Ethanol	Soluble
3	Water	Soluble
4	Ethyl acetate	In Soluble
5	Hexane	In Soluble
6	DMSO	Soluble

pH determination

Required quantity of test sample was admixed with distilled water and the subjected to screening using pH meter.

<i>KAC</i>	Ph	7.8
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Qualitative Phytochemical screening of *KAC*

Results tabulated in Table no.6 and illustrated in Figure no. 3

Table no.6 Results of qualitative Phytochemical Screening

S.No	Phytochemicals	Test name	H ₂ O Extract
1	Alkaloids	Wagner Test	+ve
2	Carbohydrates	Molisch's Test Benedict Test	+ve +ve
3	Saponins	Foam Test	+ve
4	Phenols	Ferric Chloride Test	+ve
5	Tannins	Gelatin Test	+ve

6	Flavanoids	Lead acetate	+ve
7	Diterpenes	Copper Acetate Test	+ve
8	Quinones	Test for Quinones	-ve
9	Gum & Mucilage	Test for Gum & Mucilage	+ve

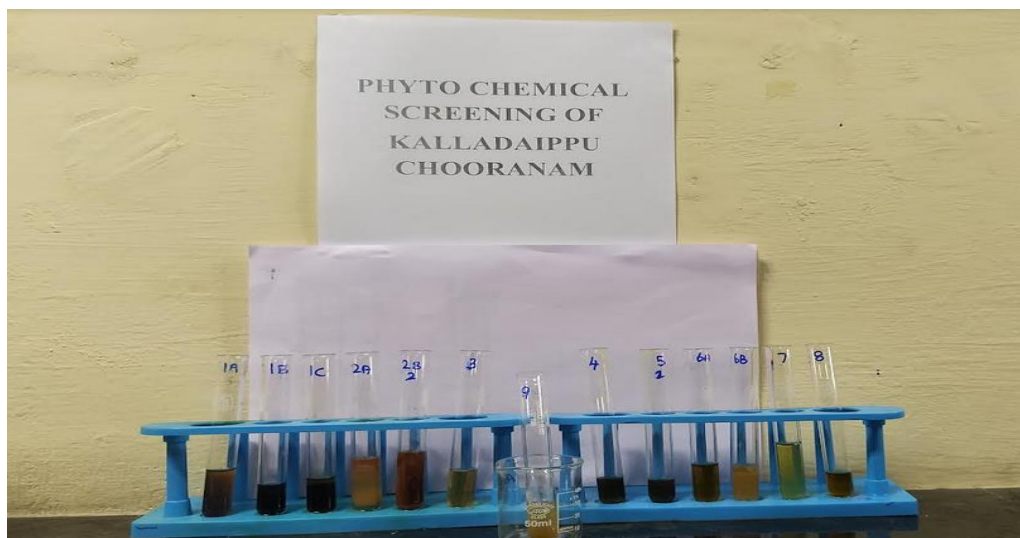


Figure no. 3 Qualitative Phytochemical analysis of KAC

High Performance Thin Layer Chromatography Analysis of KAC

Results illustrated in Figure no.4 and tabulated in Table no. 7



Figure no.4 TLC Chromogram of KAC

TLC Visualization of KAC at 366 nm

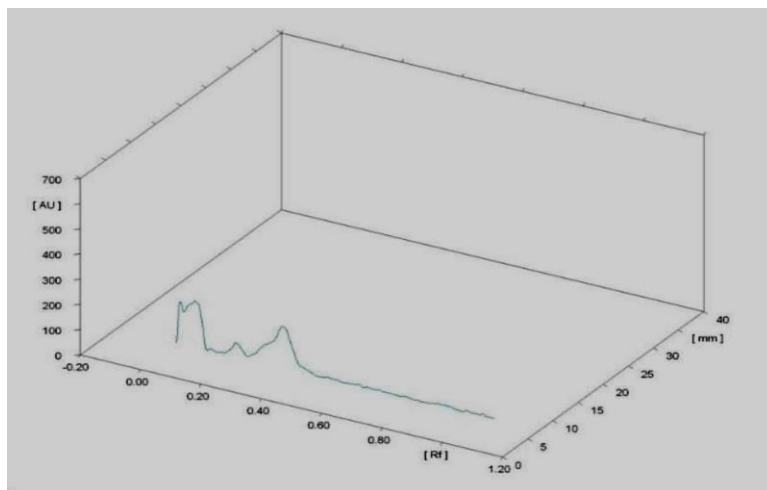


Figure no.5 HPTLC Chromatogram 3D- Chromatogram

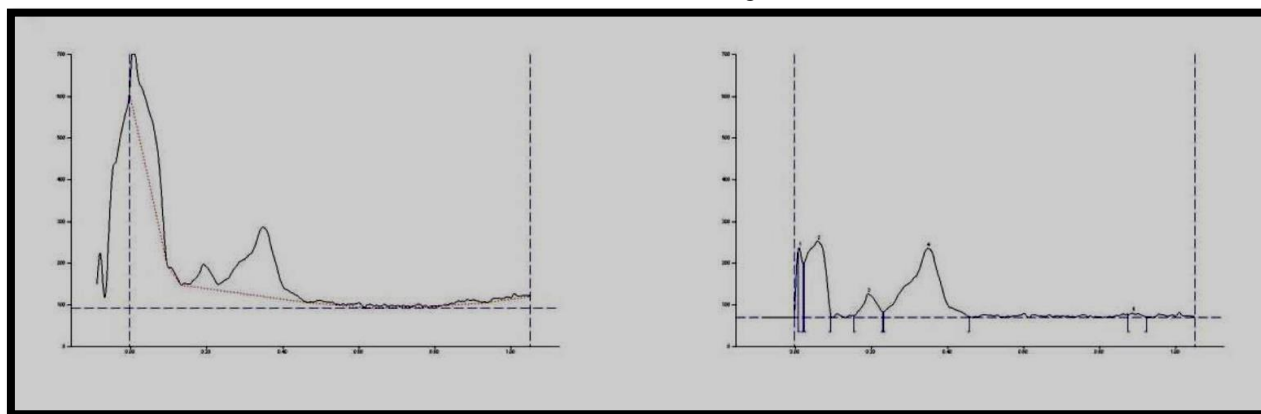


Figure no.6 HPTLC Fingerprinting

Table.no.7 Peak Value

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.01	163.1	0.01	167.2	28.55	0.02	129.3	1049.2	7.29
2	0.02	131.3	0.06	184.0	31.41	0.09	5.8	4782.8	33.25
3	0.15	4.8	0.19	57.1	9.75	0.23	13.7	1142.9	7.94
4	0.23	13.9	0.35	166.2	28.38	0.46	2.6	7234.4	50.29
5	0.88	8.2	0.89	11.2	1.91	0.93	1.4	175.4	1.22

Biochemical analysis of KAC

Results tabulated in Table no.8 and Table no .9

Table: 8 Results of Acid radicals of KAC

S.No	Specific radical	Test report
1	Test for carbonates	Positive
2	Test for Sulfates	Positive

Table : 9 Results for Basic Radicals

S.No	Specific radical	Test report
1	Lead	Positive

Heavy metal analysis by Atomic Absorption Spectrometry (AAS)

Results listed in the Table 10

Name of the Heavy Metal	Absorption Max λ max	Result Analysis	Maximum Limit
Lead	217.0 nm	1.81 PPM	10 ppm
Arsenic	193.7 nm	BDL	3 ppm
Cadmium	228.8 nm	BDL	0.3 ppm
Mercury	253.7 nm	BDL	1 ppm

BDL- Below Detection Limit

Microbial Contamination Test by Pour Plate Method

Results illustrated in Figure 7,8 and Tabulated in Table 11

Sterility test by pour plate method

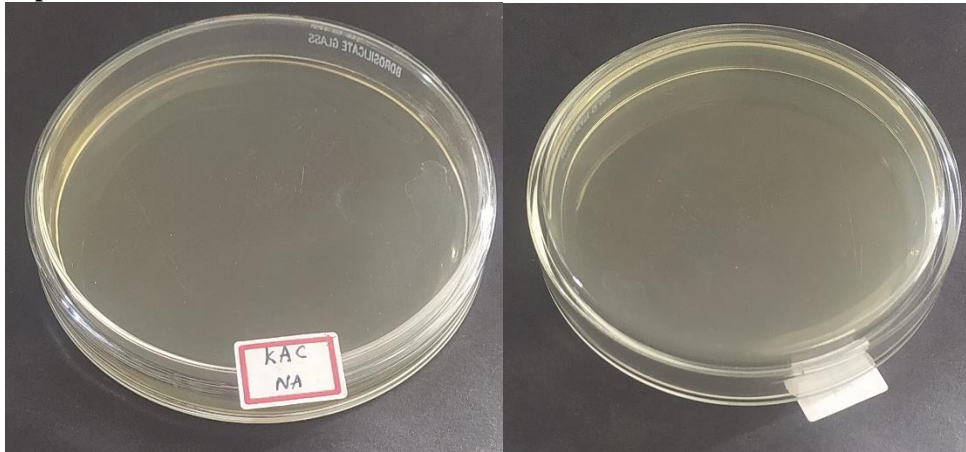


Figure : 7

Figure : 8

Table 11 : Result of sterility test of KAC

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

Test for Specific Pathogen

No growth was observed in any of the plates inoculated with test sample shown in Table 12 and illustrated in Figure 9, 10

Table 12 : Result for specific pathogen

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



Figure 9, 10: Culture plate with E-coli (EC) specific medium

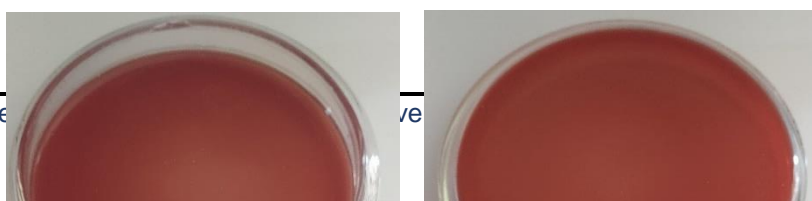


Figure 11, 12 : Culture plate with Salmonella (SA) specific medium

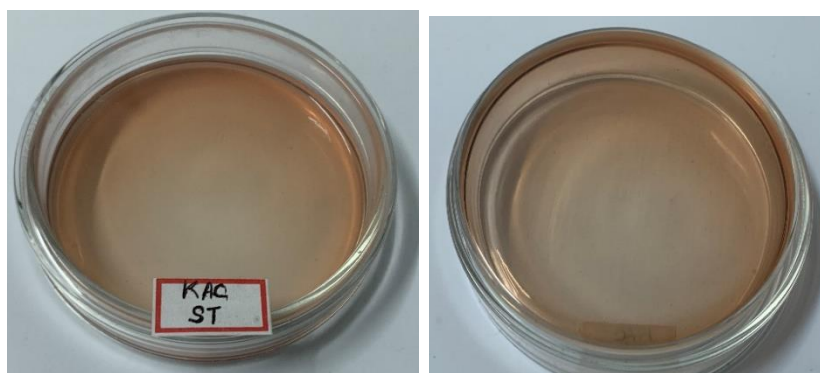


Figure 13, 14 : Culture plate with Staphylococcus Aureus (ST) specific medium

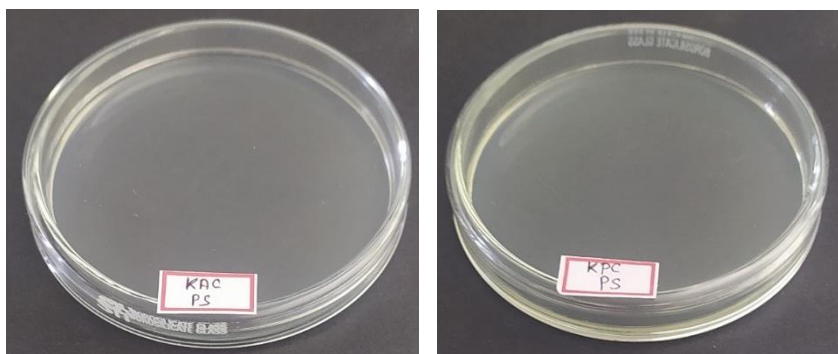


Figure 15, 16 : Culture plate with Pseudomonas Aeruginosa (PS) specific medium

Analysis of Pesticide Residue Organochlorine, Organophosphorous, Organocarbamates, Pyrethroids

The results showed that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus, Organo carbamates and pyrethroids in the sample provided for analysis. Results tabulated in Table 13.

Table 13 : Results of pesticide residue of KAC

Pesticide Residues	Sample KAC	AYUSH Limit (mg/kg)
1.Organo Chlorine Pesticides		
Alpha BHC	BQL	0.1 mg/kg
Beta BHC	BQL	0.1 mg/kg
Gamma BHC	BQL	0.1 mg/kg
Delta BHC	BQL	0.1 mg/kg
DDT	BQL	1 mg/kg
Endosulphan	BQL	3 mg/kg
1. Organo Phosphorous Pesticides	Or	

Malathion		50µg/k g	1 mg/kg
Chlorpyriphoos		BQL	0.2 mg/kg
Dichlorovos		BQL	1 mg/kg
2. gano Carbonates	Or		
Carbofuran		BQL	0.1 mg/kg
3. ethroid	Pyr		
Cypermenthin		BQL	1 mg/kg

BQL-Below Qualification Limit

Result for Aflatoxin by TLC:

The results shown that there were no spots were being identified in the test sample loaded on TLC plates when compare to the standard which indicates that the sample were free from Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. Whereas results indicate the presence of Aflatoxin B2 at 0.015 mg/kg.

Aflatoxin study of KAC

Results tabulated in Table 14

Table 14 : Aflatoxin report

Aflatoxin	Sample KAC	AYUSH Specification Limit
B1	0.015 mg/kg	0.5 ppm (0.5mg/kg)
B2	Not Detected - Absent	0.1 ppm (0.1mg/kg)
G1	Not Detected - Absent	0.5 ppm (0.5mg/kg)
G2	Not Detected - Absent	0.1 ppm (0.1mg/kg)

DISCUSSION:

Standardization of the drugs is much necessary to derive the efficacy , potency of the drug. The standardization of *Kalladaippu chooranam* was carried out through various procedures like physicochemical analysis, phytochemical analysis, biochemical analysis, and analysing the organoleptic characters, pesticide residues , aflatoxins and HPTLC. The organoleptic characters like state , nature, odour, touch ,flow property and appearance revealed that it was solid , fine in nature with aromatic odour, sweet with pungent taste, soft to touch , non free flowing and pale brownish in colour .The fine quality of the chooranam provides easy absorption and good bio availability. So the drug *KAC* is good in nature and safe to consume.

The results obtained from the physicochemical evaluation revealed that Loss on drying value of *KAC* was 9.85% which represent low moisture content could increase the stability and shelf life of drug which is suitable for medicine preparation. The total ash value of *KAC* was 5.68% which represents the purity of drug. Water soluble ash value of *KAC* was 1.61% which represents Acid insoluble ash value of *KAC* was 1.24% which guaranteed that the trial drug is not contaminated with siliceous material like sand , dust. The water soluble extractive value of *KAC* was 11.71% which indicates easy facilitation of diffusion and osmosis mechanism. The Alcohol soluble extractive value of *KAC* was 7.61% which indicates that the test drug has good quality , purity and no adulteration. The pH value of *KAC* was 7.8 which indicates that the drug is alkaline in nature . In oral administration , the alkaline nature of drug enhances fast absorption in stomach . Particle size analysis ensures the average particle size of the sample was $79.6 \pm 21.02 \mu\text{m}$. So the drug *KAC* is easily available and convenient for oral administration . *KAC* is well soluble in major solvents like water, ethanol, DMSO. So that it ensures its efficiency of solubility and increases the bio- availability in the stomach .

The result of the qualitative phytochemical analysis indicates that the formulation *KAC* reveals the presence of alkaloids, flavonoids, saponins, diterpenoids, phenols, tannins and sugar. Alkaloids are used for bleeding disorders and eye diseases, and antiseptics, sedatives, stomatics, anti-inflammatory and analgesics^[20]. The results of recent studies have shown that the plant flavonoids could effectively inhibit the formation of CaOx stones in vitro and in vivo, correlating with their diuretic, antioxidant, anti-inflammatory, antibacterial properties and other protective effects^[21]. Saponin rich fractions has also been found to be a great inhibitor of calcium stone formation not only in vitro but in vivo too^[22]. Naturally occurring diterpenes exert several biological activities such as anti-inflammatory action, anti-microbial and anti-spasmodic activities^[23].Tannic acid was able to improve renal recovery after warm ischemia with an antioxidant effect putatively, extended by the promoted cell regeneration during hypoxia^[24] The results of HPTLC finger printing analysis of the sample *KAC* divulges the presence of 5 prominent peaks corresponds to presence of five versatile phyto-components present with in it. Rf value of the peaks ranges from 0.01 to 0.88 % . This method was used to develop a chemical fingerprint for authentication and good confirmation of the presence of bioactive compounds in the sample *KAC*. Hence, the result supports the ethno medical uses of the drug *KAC* to treat Renal calculi.

The result of the Biochemical analysis of the test for Acid radicals reveals the presence of Carbonates, Sulphates in the test drug *KAC*. Results of the Heavy metal analysis have clearly shown that the sample *KAC* has no heavy metals traces such as Lead, Arsenic, Mercury and Cadmium. These results indicate that the trial drug is extremely safe as it contains heavy metals below detection limits. This reveals the safety of the drug. The result of the Sterility test shows no growth was seen in any of the plates inoculates with the test sample *KAC*. The revealed that the drug *KAC* is free from the viable microorganisms and the absence of total bacterial and fungal colonies which indicates that the drug *KAC* have good quality and safer drug.

The result of the Specific pathogen was observed that there was no growth in any of the plates inoculated with the test sample *KAC* which confirms that there are no viable aerobic microorganisms present in the sample. The results showed that there were no pesticide traces such as Organo chlorine, Organo phosphorus, Organo carbamates and Pyrethroids in the *KAC* for analysis. This result suggests that *KAC* have good quality.

The results shown no spots in the test sample loaded on TLC plates when compare to the standard which indicates that the sample were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. So the drug *KAC* is non-toxic and there is no contamination and does not possess carcinogenic property. As a result, *Kalladaippu Chooranam* was proved for its safety over the defined standardization method.

Conclusion:

From the results obtained from the above discussion, the final conclusion is that the Siddha formulation *KAC* have potent biologically active components which may potential in treatment of various disorders. Analysis of those specifications with the help of modern technological tools helps in standardization of *KAC*. Hence this present investigation gives rise to an evidence-based data with respect to standards, purity, physico- chemical, phytochemical and biochemical nature of the formulation *KAC*.

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