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Phytochemical and Physicochemical Analysis of Nilavagaai Chooranam A Siddha Herbo-Mineral Preparation

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Abstract
Background: Standardization of Siddha medicine is necessary one in this scientific world. 'Nilavagaai chooranam' is a herbo- mineral preparation indicated for various diseases including skin diseases.
Aim and Objective: To do the phytochemical and physicochemical analysis for the drug 'Nilavagaai chooranam'
Material and Methods: The drug prepared as per the method mentioned in the classical Siddha literature. The drug is subjected to physico chemical analysis such as total ash, loss on drying, microbial load, heavy metal analysis, pesticide residues, aflatoxins, thin layer chromatography and high performance thin layer chromatography as per the pharmacopical laboratory standards of Indian medicine.
Results and Conclusion: The drug had no microbial contamination and shows positive for the presence of alkaloids, saponins, phenols, tannins, diterpenes, gum & mucilage, quinones etc. The ash value is 9.13%. The heavy metals such as lead and mercury are below detectable limit.
Keywords: Herbo-mineral, Siddha, Physicochemical analysis, High performance thin layer chromatography.

I. INTRODUCTION:

Siddha system of medicine is one of ancient system of medicine which is practiced among the Tamil speaking people. Sage Siddhars are the founder of this system. The medicine in this system is prepared from raw material of herbs, metals, minerals and animal products. 'Nilavagaai chooranam' is one of the Siddha drug chosen from the text Aathma Ratchamirdhamenum Vaidhya Saara Sangirakam[1]. It is indicated for various diseases like venereal itching, scabies, pitham diseases, ptyalism, some poisons and accumulation of gas in some part of the intestines. The use of scientific tools is essential to validate the traditional claim. Though Siddha drugs are considered to be safe and effective, it is the utmost duty of the physicians to standardizing the Siddha prepared medicine before trying out in human being. The drug is a herbo-mineral drug which contains only one mineral Indhuppu and the others are herbal ingredients.

II. AIM AND OBJECTIVE:

The aim of this study is to do phytochemical analysis, physicochemical analysis and HPTLC finger printing for the drug 'Nilavagaai chooranam'.

III. MATERIALS AND METHODS:

Ingredients of Nilavagaai chooranam[1] :

Nilavagaai samoolam(*Cassia senna*Linn.)	-10 palam (350 grams)
Milagu(*Piper nigrum*Linn.)	-each ¼ palam (8.75 grams)
Kadukkai(*Terminalia chebula*Retz.)	
Thandrikkai(*Terminalia bellerica*Roxb.)	
Seeragam(*Cuminum cyminum*Linn.)	
Vaaluvai(*Celastrus paniculatus*Willd.)	
Sirunaagapoo(*Mesua ferrea*Kosterm.)	
Elam(*Elettaria cardamomum*Linn.)	
Ilavangapattai(*Cinnamom verum*Presl.)	
Kadugurogini(*Picrorhiza kurroa*Pennell.)	
Sivadhai(*Operculina turpethum* Linn.)	
Thalisapathiri(*Taxus baccata*Mirb.)	

Jadhikkai(*Myristica fragrans*Houtt.) Kirambu(*Syzygium aromaticum*Linn.) Thippili(*Piper longum*Linn.) Chevviyam(*Root of Piper nigrum*Linn.) Indhuppu(*Sodium chloride*) Koogaineer(*Maranta arundinacea*Linn.) Chukku(*Zingiber officinale*Roscoe.)	-each ¼ palam (8.75 grams)
Senisarkarai(*Sugar)*	-Equal amount for Chooranam

Purification of raw drugs:
The raw drugs are purified as per the method mentioned in the Siddha literature.

Method of preparation[1]:
The purified raw drugs were dried and powdered separately, then mixed well together and then added with equal amount of white sugar and stored in clean dry air tight container.

Dosage : 1Gram (Thirikadi), twice a day
Adjuvant : Ghee
Duration : 48days

Analytical specification of chooranam:
1. Description: microscopic and macroscopic, 2.Loss on drying at 105^{o} C, 3. Total ash, 4.Acid-insoluble ash, 5.Water soluble ash, 6.Water soluble extractive, 7.Alcohol soluble extractive, 8. Detection of alkaloids, 9. Detection of carbohydrates, 10.Detection of glycosides, 11.Detection of saponins, 12.Detection of phenols, 13. Detection of tannins, 14.Detection of flavonoids, 15.Detection of proteins, 16.Detection of diterpenes, 17. Gum and Mucilage, 18.Test for quinones, 19.Test for aflatoxins: B1, B2, G1, G2, 20.TLC/HPTLC with marker, 21.Test for heavy / toxic metals: lead, cadmium, mercury, arsenic, 22.Pesticide residue: organochlorine pesticides, organophosphorus pesticide, pyrethroids, 23.Sterility test by Pour plate method, 24.Test for Specific pathogen.

Figure 1: ***Sample of Nilavagaai Chooranam (NVC)***

Table 1: Organoleptic characters of Nilavagaai Chooranam (NVC)

State	Solid
Appearance	Brownish green
Nature	Hard coarse powder
Odor	Strong Characteristic
Flow Property	Free flowing

Particle Size[2, 3]

Figure 2: ***Electron microscopic observation of particle size for the test sample- Nilavagaai Chooranam (NVC)***

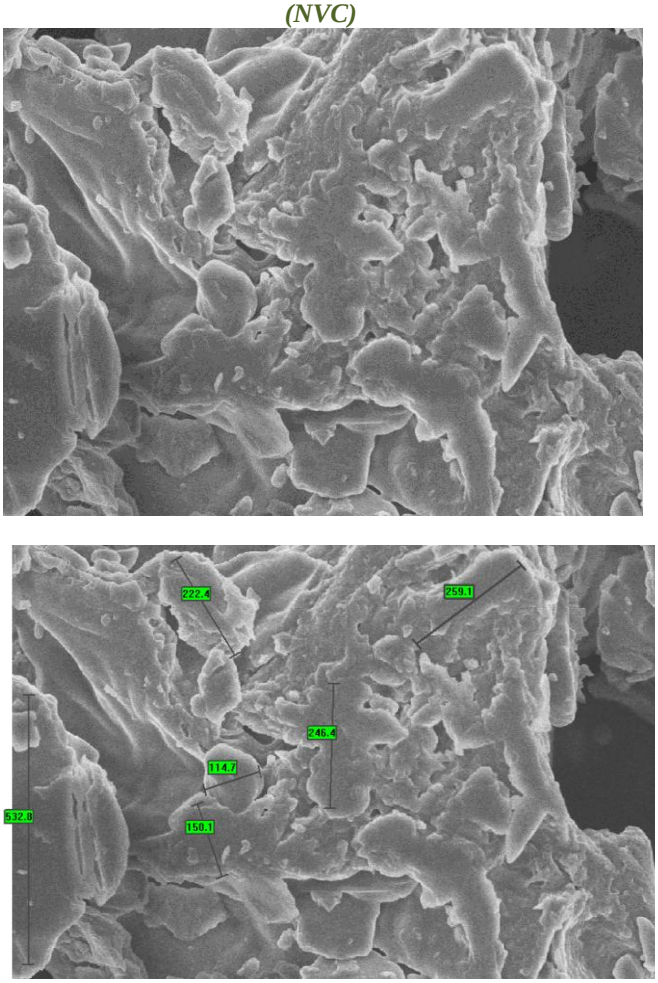

Table 2: Mean, Std. deviation and Std.error of Particle Size for the test sample- Nilavagaai Chooranam (NVC)

Mean	**253.8**
Std. Deviation	**147.5**
Std. Error	**60.23**

Report

Microscopic observation of the particle size analysis reveals that the average particle size of the sample was found to be 253.8 ± 147.5 µm

IV. RESULTS AND OBSERVATION:

PHYSICOCHEMICAL ANALYSIS OF NILAVAGAAI CHOORANAM

1. Loss on Drying:

An accurately weighed 2g of Nilavagaai chooranam formulation was taken in a tarred glass bottle. The crude drug was heated at 105^0C 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:

Weighed accurately 2g of Nilavagaai chooranam formulation was added in crucible at a temperature 6000C 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 obtained from the given sample, was boiled for 5min with 25ml of 1Nhydrochloric 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 2g 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 450^0C 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 powered Nilavagaai chooranam was macerated with 100ml of distilled water in a closed flask for twenty-four hours, shaking frequently. The Solution was filtered and 10 ml of filtrated solution was evaporated in a tarred flat bottom shallow dish, further dried at 100^0C 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 drugs, coarsely powdered Nilavagaai 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 filtrated solution was then evaporated in a tarred flat bottom shallow dish, dried at 100^0C and weighted. The percentage of alcohol soluble extractive was calculated with reference to air dried drug.

Table 3: Results of Physicochemical Parameters for the test sample- Nilavagaai Chooranam (NVC)

S.No	Parameters	Percentage
1	Loss on drying	4.56%
2	Total ash value	9.13%
3	Acid insoluble ash	1.11%
4	Water soluble ash	5.09 %
5	Water soluble extraction	18%
6	Alcohol soluble extraction	16%

The above stated physicochemical properties of the given sample certified to be present.

PRELIMINARY PHYTOCHEMICAL SCREENING OF NILAVAGAAI CHOORANAM

The Preliminary Phytochemical screening test was carried out for each extracts of **Nilavagaai chooranam** as per the standard procedure.

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 colored 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.

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3. Detection of glycosides:

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

Cardiac glycoside (Keller-Killiani test): Extract was shaken with distilledWater (5 mL). To this, glacial acetic acid (2 mL) containing a few drops of ferric chloride was added, followed byH2SO4 (1 mL) along the side of the test tube. The formation of brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring.

4. Detection of saponins

a) 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.

5. 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.

6. 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.
7. Detection of flavonoids
a) Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.
8. Detection of proteins
a) Xanthoprotein Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow color indicates the presence of proteins.
9. 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.
10. 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.
11. Test for quinones
Extract was treated with sodium hydroxide blue or red precipitate indicates the presence of Quinones.
The Preliminary phytochemical studies of aqueous extract of **Nilavagaai 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 **Nilavagaai chooranam**

Table 4: Results of Phytochemical analysis for the test sample- Nilavagaai Chooranam (NVC)

S.No.	Phytochemicals	Test Name	H2O Extract
1	Alkaloids	Mayer'sTest	+ve
2	Carbohydrates	Molisch's Test	-ve
3	Glycoside	Cardiac glycoside'sTest	-ve
4	Saponin	Foam Test	+ve
5	Phenols	Ferric Chloride Test	+ve
6	Tannins	Gelatin Test	+ve
7	flavonoids	Lead acetate test	-ve
8	Proteins	Xanthoprotein Test	-ve
9	Diterpenes	Copper Acetate Test	+ve
10	Gum & Mucilage	Extract + Alcohol	+ve
11	Quinones	NAOH + Extract	+ve

+ve/-ve present or absent if component tested
The above stated phytochemical properties for the given sample certified to be present.

Aflatoxin Assay[4]:
Standard
Aflatoxin B1
Aflatoxin B2
Aflatoxin G1
Aflatoxin G2
Solvent
Standard samples was 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 aflatoxin B1 and aflatoxin G1 and 0.1 µg per ml each of aflatoxin B2 and aflatoxin G2.

Test solution: Concentration 1 µg per ml
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 Allow the spots to dry and develop the chromatogram 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. Remove the plate from the developing chamber, mark the solvent from and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.

Table 5: Test results of Aflatoxin Assay for the test sample- Nilavagaai Chooranam (NVC)

Aflatoxin	Sample NVC	AYUSH Specification Limit
B1	Not Detected - Absent	0.5 ppm
B2	Not Detected - Absent	0.1 ppm
SG1	Not Detected - Absent	0.5 ppm
G2	Not Detected - Absent	0.1 ppm

Result: The results shown that there was no spots were been identified in the test sample loaded on TLC plates when compare to the standard, which indicates that he sample were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2.

TLC Analysis[5]

Instrument: CAMAG TLC SCANNER III
Sample Applicator:CamagLinomat - IV applicator with N2 gas flow.
Photo documentation System:Digi store - 2 documentation system with Win Cats & Video scan software.
Scanne :Camag HPTLC scanner - 3 (030618), Win Cats - IV.
Development Chamber :Camag HPTLC 10X10, 10 X 20 twin trough linear development chamber.
Quantity applied: 5, 10 µl for extracts and 5 µl for standards
Stationary phase:Aluminium Coated Silica Gel – Merck
Plate thickness: 0.2 mm.
Mobile Phase: Chloroform: n-Butanol: Methanol: Water: Acetic Acid (4:1:1:0.5:0.5)
Scanning wavelength: 254 nm
Laboratory condition: 26 ± 5^0C and 53 % relative humidity

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 different solvent system Chloroform: n-Butanol: Methanol: Water: Acetic Acid (4:1:1:0.5:0.5). After the run plates are dried and were observed using visible light Short-wave UV light 254nm and light long-wave UV light 365 nm

High Performance Thin Layer Chromatography Analysis[6]

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. In addition it is a reliable method for the quantitation of nano grams level of samples. 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 medicinal plant raw materials.

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 analysed. 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 extract and Rf values were tabulated.

Figure 3: ***TLC Visualization for the test sample Nilavagaai Chooranam (NVC) - TLC plate visualization at 366 nm***

Figure 4:*3D View of Chromatogram*

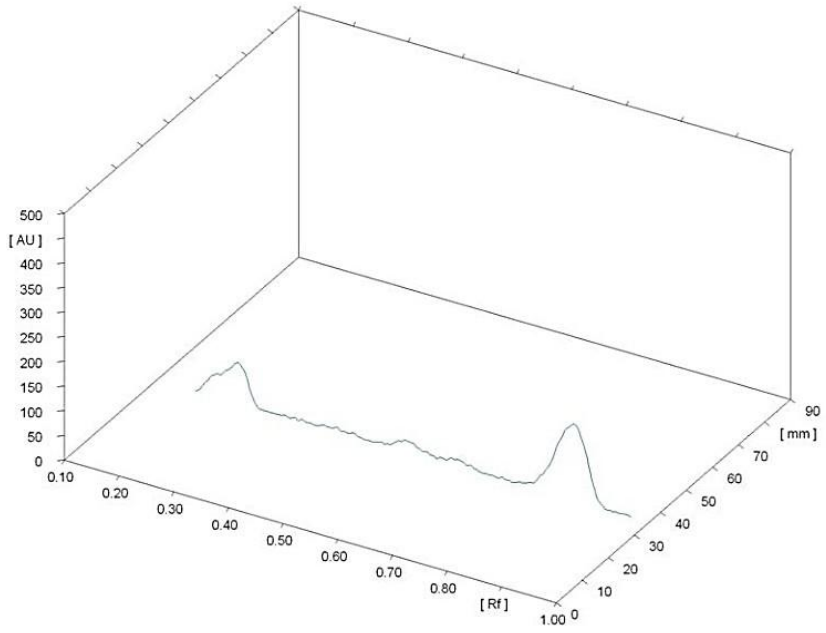


Figure 5: ***HPTLC finger printing for the test sample- Nilavagaai Chooranam (NVC)***

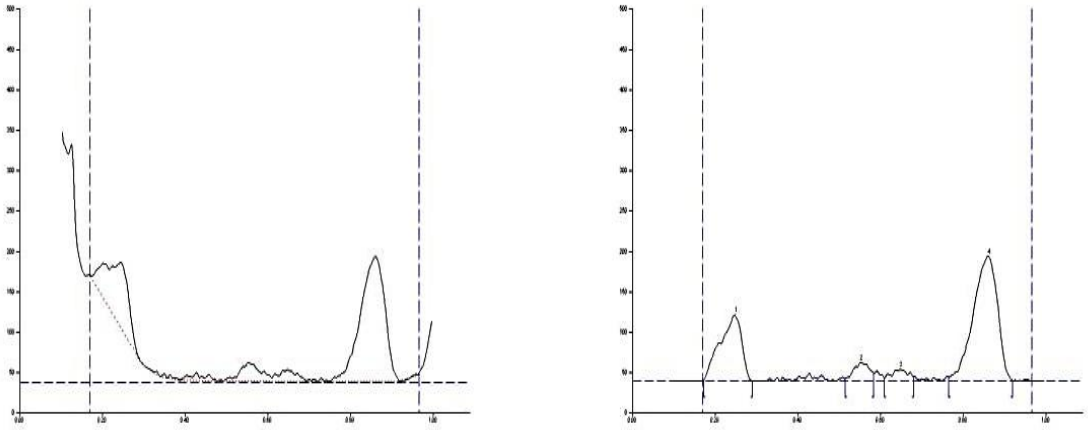


Table 6: Rf value of HPTLC finger print for the test sample- Nilavagaai Chooranam (NVC)

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.17	1.0	0.25	81.9	29.87	0.29	0.2	2745.5	28.69
2	0.52	3.3	0.55	22.8	8.33	0.59	10.2	577.2	6.03
3	0.61	4.6	0.65	14.8	5.40	0.68	5.8	406.6	4.25
4	0.77	5.1	0.86	154.6	56.40	0.92	0.2	5839.5	61.03

Report

HPTLC finger printing analysis of the sample reveals the presence of four prominent peaks corresponds to presence of four versatile phytocomponents present within it. Rf value of the peaks ranges from 0.16 to 0.64. Further the peak 4 and 1 occupies the major percentage of area of 56.40 and 29.87 % which denotes the abundant existence of such compounds.

HEAVY METAL ANALYSIS BY AAS

Standard: Hg, As, Pb and Cd – Sigma

Methodology

Atomic Absorption Spectrometry (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

Test sample was digested with 1mol/L HCl for determination of arsenic and mercury. Similarly for the determination of lead and cadmium the sample were digested with 1mol/L of HNO3. Standard reparation As& Hg- 100 ppm sample in 1mol/L HCl Cd&Pb- 100 ppm sample in 1mol/L HNO3

Standard preparation

As & Hg- 100 ppm sample in 1mol/L HCl

Cd &Pb- 100 ppm sample in 1mol/L HNO3

Table 7: Results of Heavy Metal analysis for the test sample- Nilavagaai Chooranam (NVC)

Name of the Heavy Metal	Absorption Max Λ max	Result Analysis	Maximum Limit
Mercury	253.7 nm	BDL	1 ppm
Lead	217.0 nm	0.36	10 ppm
Arsenic	193.7 nm	0.14	3 ppm
Cadmium	228.8 nm	BDL	0.3 ppm

BDL- Below Detection Limit

Report and Inference

Results of the present investigation have clearly shows that the sample has no traces of heavy metals such as Mercury and Cadmium. Further the results show the presence of arsenic and lead at 0.36 and 0.14 ppm whose level is below the prescribed limit

Pesticide Residue[7,8]

Extraction

Test sample were 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 40ºC until the solvent has almost completely evaporated. To the residue add a few milliliters of toluene R and heat again until the acetone is completely removed. Resultant residue will be dissolved using toluene and filtered through membrane filter.

Table 8: Test Result of Pesticide Residue for the test sample- Nilavagaai Chooranam (NVC)

Pesticide Residue	Sample NVC	AYUSH Limit (mg/kg)
I. Organo Chlorine Pesticides		
Alpha BHC	BQL	0.1mg/kg
Beta BHC	BQL	0.1mg/kg
Gamma BHC	BQL	0.1mg/kg
Delta BHC	BQL	0.1mg/kg
DDT	BQL	1mg/kg
Endosulphan	BQL	3mg/kg
II.Organo Phosphorus Pesticides		
Malathion	BQL	1mg/kg
Chlorpyriphos	BQL	0.2 mg/kg
Dichlorovos	BQL	1mg/kg
III.Pyrethroid		
Cypermethrin	BQL	1mg/kg

BQL- Below quantification Limit

Result: The results showed that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus and pyrethroids in the sample provided for analysis.

STERILITY TEST BY POUR PLATE METHOD

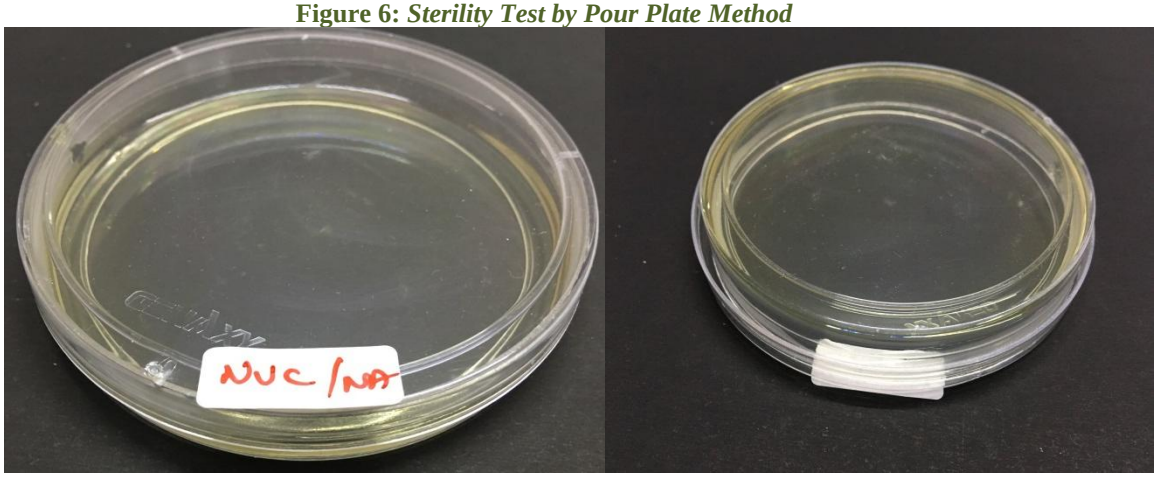
Objective

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

Test sample was admixed with sterile distilled water and the mixture were been used for the sterility evaluation. About 1ml of the test sample was inoculated in sterile petri dish to which about 15 mL of molten agar 45^{o}C were added. Agar and sample were mixed thoroughly by tilting and swirling the dish. Agar was allowed to completely gel without disturbing it. (About 10 minutes). Plates were then inverted and incubated at 37^{o} C for 24-48 hours. Grown colonies of organism was then counted and calculated for CFU.

Figure 6: ***Sterility Test by Pour Plate Method***



Observation
No growth was observed after incubation period. Reveals the absence of specific pathogen
Result
No growth / colonies were observed in any of the plates inoculates with the test sample.

Table 9: Results of Microbial Load for the test sample- Nilavagaai Chooranam (NVC)

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	

Test for Specific Pathogen
Methodology
One part of the test sample was dissolved in 9 mL of sterile distilled water and the test sample was directly inoculated in to the specific pathogen medium (EMB, DCC, Mannitol ,Cetrimide) by pour plate method. The plates were incubated at 37^{o}C for 24 - 72h for observation. Presence of specific pathogen identified by their characteristic color with respect to pattern of colony formation in each differential media.

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***

Observation
No growth was observed after incubation period. Reveals the absence of specific pathogen
Result
No growth / colonies were observed in any of the plates inoculated with the test sample.

Table 11: Test results for Specific Pathogen for the test sample- Nilavagaai Chooranam (NVC)

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

Figure 7: ***Culture plate with E-coli (EC) specific medium***

Figure 8: ***Culture plate with Salmonella (SA) specific medium***

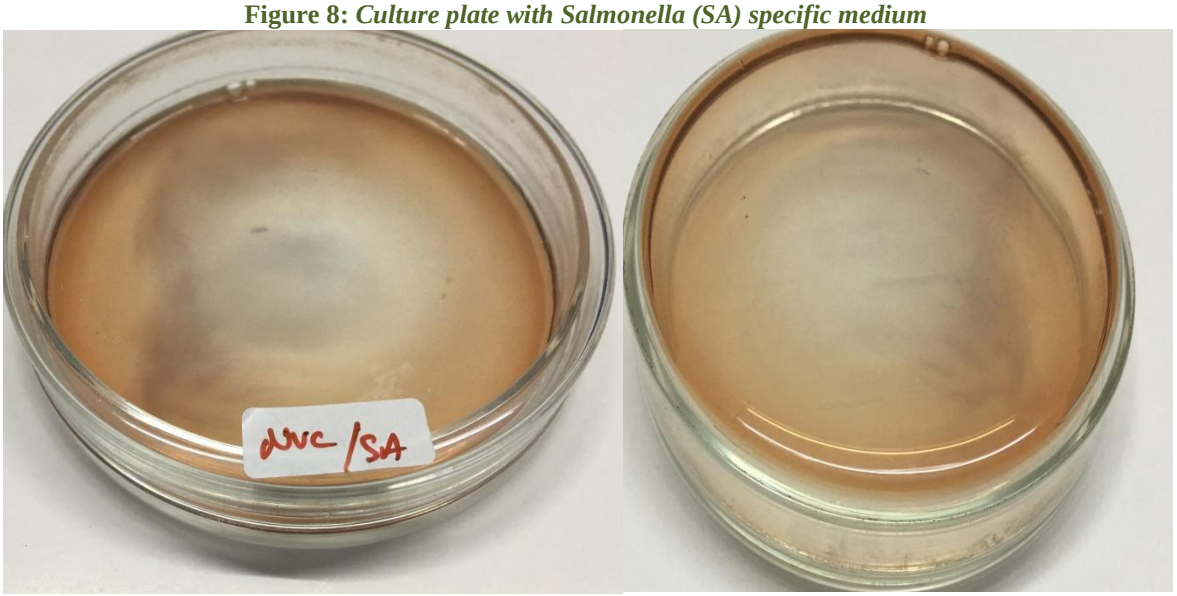


Figure 9: ***Culture plate with Staphylococcus Aureus (ST) specific medium***

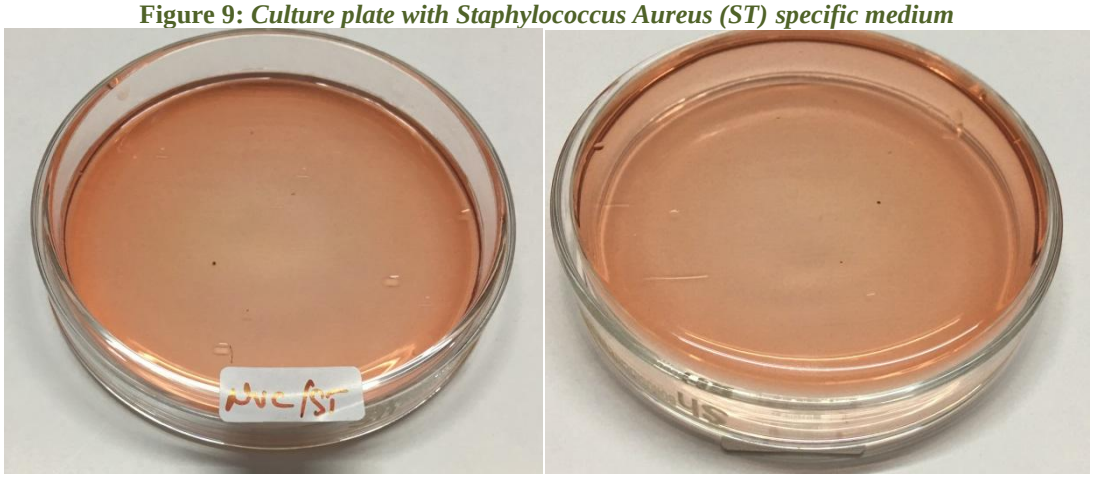
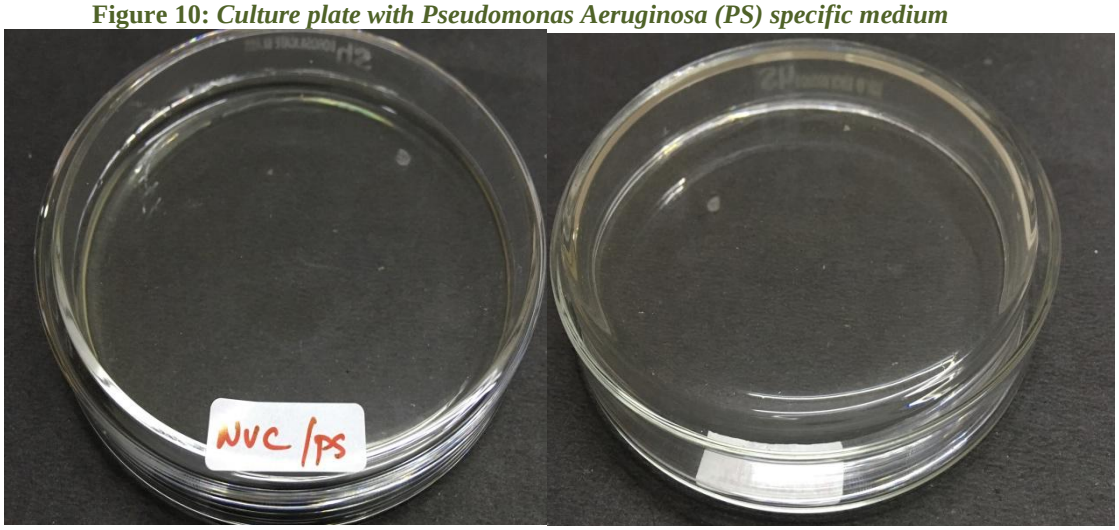


Figure 10: ***Culture plate with Pseudomonas Aeruginosa (PS) specific medium***



V. CONCLUSION:

Based on the above results, it can be assumed that the drug 'Nilavagaai chooranam' has validated the traditional claim.

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