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# STANDARDIZATION OF SIDDHA POLYHERBAL FORMULATION "INJI RASAYANAM"

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**ABSTRACT**

Inji Rasayanam is a classical Siddha polyherbal formulation indicated for the management of Soothagavaayu, which is referred to as Polycystic Ovarian Disease (PCOD). Although its traditional efficacy is well documented, the lack of standardized quality control parameters possesses a challenge to its global acceptance. The present study aimed to establish the standardization profile of Inji Rasayanam through physicochemical evaluation, phytochemical analysis, HPTLC fingerprinting, microbial load assessment, and aflatoxin analysis. The physicochemical parameters, such as total ash value, acid-insoluble ash, loss on drying, alcohol extractive value, and water extractive value, were analyzed according to standard protocols. Phytochemical screening revealed the presence of carbohydrates. The HPTLC fingerprinting profile showed 10 peaks at 254 nm and 6 peaks at 366 nm, indicating the presence of multiple phytoconstituents. Microbial load and aflatoxin levels were found to be within WHO permissible limits.These observations contribute to the scientific framework and confirm the quality and safety of Inji Rasayanam.

***Keywords:*** *Inji Rasayanam, Siddha medicine, PCOD, physicochemical, phytochemical, HPTLC.*



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## INTRODUCTION

The siddha system of medicine is one of the oldest, traditional Dravidian- rooted medical system originated from ancient Tamil civilization and believed to have been founded by sage Agasthiar.This system of medicine is based on a holistic concept that "the human body is a replica of the universe". According to Siddha philosophy, the human body is composed of 96 Thathuvams, which represent the fundamental principles governing physiological and pathological processes. Health is maintained through the equilibrium of three vital humors namelyVaatham, Pitham and Kabam collectively known as mukkutram.Any imbalance of these humors leads to the manifestation of disease [1]. There are many polyherbo-mineral formulations are available in ancient Siddha literature for Soothaga Vaayu (PCOD). One of the effective formulations for PCOD is Inji Rasayanam as mentioned in siddha literature "Anuboga Vaithya Navanitham Part-8". Although this formulation has been traditionally used in clinical practice, scientific validation and quality evaluation are essential to ensure its safety, efficacy, and reproducibility. Standardization acts as a vital bridge between traditional knowledge and modern evidence-based medicine. It involves establishing defined parameters such as organoleptic evaluation, physicochemical characteristics, phytochemical profiling, chromatographic fingerprinting, and safety assessment including microbial load and aflatoxin analysis. Among the analytical techniques used for herbal drug standardization, High Performance Thin Layer Chromatography plays an important role in identifying bioactive markers and establishing a characteristic fingerprint profile of the formulation [2]. Therefore, the present study aims to establish a comprehensive standardization profile for Inji Rasayanam in accordance with Ministry of AYUSH protocols and WHO guidelines. This study helps to validate the

formulation scientifically and supports its safe use in the management of Soothaga Vaayu (PCOD) [3].

## MATERIALS AND METHODS

### Authentication, Collection and Purification of Drugs

The trial drug Inji Rasayanam consists of fifteen ingredients. All the drugs were procured from a well-reputed raw drug store. The botanical authentication of the ingredients was validated by Department of Siddha Pharmacology at Government Siddha Medical College, Chennai.

Purification of the raw drugs was performed according to the procedures mentioned in Siddha classical text "Sikitcha Ratna Deepam Enum Vaithiya Nool" and "Marunthu sei iyalum kalayum".

Fresh ginger was purified by removing the outer peel. The purified ginger was chopped into small pieces, and roasted with ghee and finely powdered. All other purified ingredients were also powdered to obtain a fine chooranam. The prepared chooranam was further processed using the pittaviyal method. Then the purified chooranam was taken along with sugar, honey were retransferred to a stone mortar and pounded it until itreached llagapatham consistency [4].

Table 01: Ingredients of Inji Rasayanam

| S.NO | INGREDIENTS | BOTANICAL NAME | Quantity | Part used |
|---|---|---|---|---|
| 1. | INJI | *Zingiber officinale* | 60g | Rhizome |
| 2. | SUKKU | *Zingiber officinale* | 2g | Rhizome |
| 3. | ARISI THIPPILI | *Piper longum* | 2g | Dried unripe fruit |
| 4. | SEERAGAM | *Cuminum cyminum* | 2g | Seed |
| 5. | ELAM | *Elettaria cardamomum* | 2g | Seed |
| 6. | SIRUNAGAPOO | *Mesua ferrea* | 2g | Dried flower |
| 7. | ATHIMATHURAM | *Glycyrrhiza glabra* | 2g | Root |
| 8. | LAVANGAPATHIRI | *Cinnamomum tamala* | 2g | Dried leaves |
| 9. | THALISAPATHIRI | *Abies spectabilis* | 2g | Dried leaves |
| 10. | MOONGILUPPU | Bamboo salt | 2g | |
| 11. | VITHAI NEEKINA THIRATCHAI PALAM | *Vitis vinifera* | 2g | Dried fruit |
| 12. | MILAGU | *Piper nigrum* | 4g | Seed |
| 13. | SARKARAI | *Saccharum officinarum* | 60g | |
| 14. | THAEN | Honey | 20g | |
| 15. | NEI | Ghee | 20g | |

### Physicochemical Analysis

Physicochemical parameters such as Total ash value,acid- insoluble ash, pH, loss on drying,water extractive value, alcohol extractive value, total solid, total fat content, total sugar, non-reducing sugar and non-reducing sugar were evaluated according to AYUSH protocol. These parameters were determined to assess the purity, quality and stability of the formulation.

### Phytochemical Analysis

The preliminary phytochemical screening of Inji Rasayanam showed the presence of carbohydrates.

### HPTLC fingerprinting

TLC methodology

HPTLC was performed as per guidelines provided by UPI part 1, volume 1 -VI. A total of 2 g of Inji Rasayanam was weighed and extracted with 20mL of hydroalcohol. The mixture was heated in a water bath for 10 minutes and kept for 24 hours for complete extraction. The extract was then filtered and concentrated to a final volume of 10 mL. An aliquot of 5 µL of the sample solution was applied onto a silica gel 60 F254 pre-coated aluminum plate using a CAMAG automated sample applicator.

TLC plate was developed using Toluene: Ethyl acetate: Formic acid (7.3: 2.7: 0.1) as mobile phase. After development, the plate was dried and observed under UV light at 254 nm and 366 nm. The plate was then derivatized using vanillin–sulphuric acid reagent and heated at 105 °C for 5 minutes to visualize colored bands. The densitometric chromatogram was recorded using an HPTLC scanner.

### Microbial Load Determination

The microbial quality, including the isolation and identification of pathogenic bacteria from commercial and homemade herbal medicines, was tested according to the regulations of the WHO standards (2007). The tests were used to quantify the number of bacteria and fungi isolated that are able to grow aerobically in 1 g of sample. The samples were homogenized by mixing vigorously with water. 1-gram of sample was transferred to 9 mL of peptone broth. Then, serial dilutions were made to achieve an appropriate concentration. All microbial analyses were carried out in triplicate. Briefly, serial dilutions were made, and viability was assessed using the pour plate method on Casein soyabean digest agar and Sabouraud dextrose agar for bacterial counts and fungal identification, respectively. All dehydrated media were prepared according to the manufacturer's instructions and seeded and incubated at 37 °C for 24 to 48 hours for bacterial screening and at 25 °C for 48 to 72 hours for fungal screening. At the end of the incubation period, the number of colony-forming units per gram (CFU/g) was calculated by multiplying the average number of


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colonies by the dilution factor. The obtained CFU/g of sample was compared with WHO standards. Samples that presented bacterial growth greater than $10^5$ CFU in 1 g of herbal medicine were considered unsatisfactory or inadequate according to WHO guidelines for aerobic bacteria.

**Identification of Bacteria**

For bacterial isolation and identification, the samples were diluted in water and homogenized by vigorously mixing. The 1-mL aliquots were transferred to 9 mL of peptone broth and cultured at the recommended time and temperature. All microbial analyses were carried out in triplicate. For investigating *Escherichia coli*, *Salmonella spp*., *Pseudomonas aeruginosa* and *Staphylococcus aureus*were identified using EMB agar, MacConkey agar, Deoxycholate citrate agar, Cetrimide agar and Mannitol salt agar. Identification was further confirmed by colony morphology, Gram staining, and biochemical tests (oxidase, gas and catalase production).

**Aflatoxin test using afla-test fluorometer**

Aflatoxins are a group of naturally occurring toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*, two common mold species. AflaTest is a quantitative method for the detection of aflatoxin in B1, B2, G1, G2, M1, and M2. Five grams of Siddha formulation (Inji Rasayanam) and 0.4 g of sodium chloride was mixed with methanol: 2% Tween 20 or phosphate buffer (60:40 v/v). Vortex the extract mixture at high speed 3 minutes. Filter the extract through fluted filter paper. Add 10 ml of filtered extract in measuring cylinder, in that 20 mL purified water was added and vortex on high for 1 minute. Then filter the diluted extract through a pre-wet glass microfiber filter (1.5µm). Pass 10 mL of diluted extract through AflaTest WB column. Apply pressure to get 1-2 drops per second. Wash the column with 10 mL 2% Tween 20. Wash column with 10 mL purified water twice. Elute AflaTest WB columns by passing 1 mL HPLC-grade methanol (100%) through column, apply pressure to get 1 drop per second. Collect eluate in sterile VICAM cuvette. In that add 1.0 mL of AflaTest Developer and mix well, then immediately place in fluorometer (VICAM fluorometer-series 4EX). Fluorometer will read concentration after 60 seconds [5].

## RESULTS

**Physicochemical Parameters**

Table 02: Physicochemical analysis of Inji Rasayanam

| S.No. | Test Parameter(s) | Result(s) |
|---|---|---|
| 1 | **Ash** (%w/w) Total ash | 4.92 % |
| 2 | **Acid-insoluble ash** | 2.73 % |
| 3 | **pH** | 5.51 |
| 4 | **Loss on Drying** | 8.91 % |
| 5 | **Water Extractive Value** | 53.64 % |
| 6 | **Alcohol Extractive Value** | 18.86 % |
| 7 | **Total Solid** | 12.71 % |
| 8 | **Total Fat content** | 17.87 % |
| 9 | **Reducing Sugar** | 17.16 % |
| 10 | **Total Sugar** | 27.32 % |
| 11 | **Non-Reducing Sugar** | 10.16% |

**Preliminary Phytochemical analysis**

Table 03: Phytochemical analysis of Inji Rasayanam

| S.NO | Preliminary Test | Methanol extract | Hydro alcohol extract |
|---|---|---|---|
| 1 | **Alkaloids**<br>Wagner's test<br>Picric acid test | <br>-<br>- | <br>-<br>- |
| 2 | **Carbohydrates**<br>Fehling's test | <br>+ | <br>+ |
| 3 | **Glycosides**<br>Borntrager's test<br>Aqueous NaOH test | <br>-<br>- | <br>-<br>- |
| 4 | **Proteins & amino acids**<br>Biuret test<br>Ninhydrin test | <br>-<br>- | <br>-<br>- |
| 5 | **Flavonoids**<br>Lead acetate test | <br>- | <br>- |
| 6 | **Phenolic compounds**<br>Lead acetate test | <br>- | <br>- |
| 7 | **Tannins**<br>Braymer's test<br>10% NaOH test | <br>-<br>- | <br>-<br>- |
| 8 | **Phytosterols**<br>Salkowski's test | <br>- | <br>- |
| 9 | **Cholesterol** | - | - |
| 10 | **Terpenoids** | - | - |
| 11 | **Quinones** | - | - |
| 12 | **Anthocyanin** | - | - |
| 13 | **Carboxylic acid** | - | - |
| 14 | **Gums & mucilage** | - | - |
| 15 | **Fixed oil & fat** | - | . |

Thin Layer Chromatography



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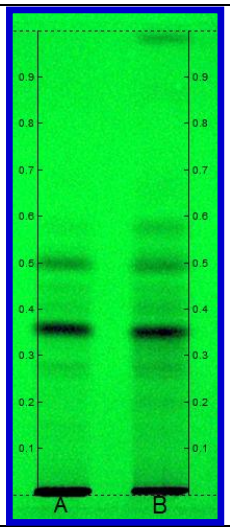
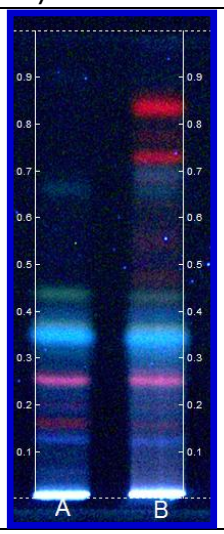
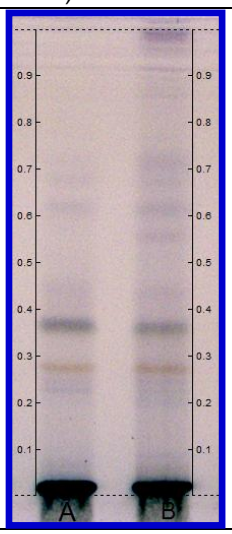
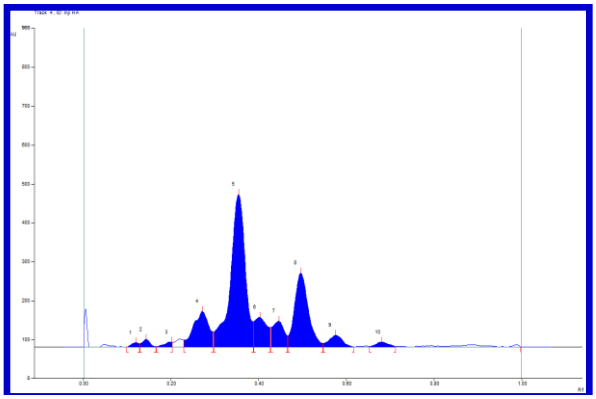
Table 04: Toluene: Ethyl acetate: Formic acid (7.3: 2.7: 0.1)

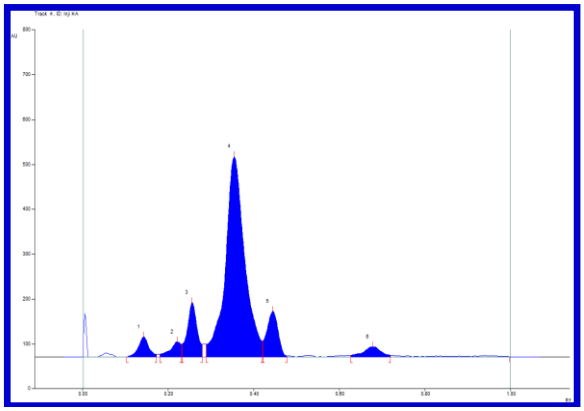
| | | |
|---|---|---|
|  |  |  |
| UV – 254 nm | UV – 366 nm | V - S Reagent |
| Track A: Hydroalcohol extract (5 µl) | | |



HPTLC finger print at 254 nm in Hydro alcohol extract (Absorbance mode.

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.10 Rf | 0.2 AU | 0.12 Rf | 11.0 AU | 1.22 % | 0.13 Rf | 8.3 AU | 153.7 AU | 0.65 % |
| 2 | 0.13 Rf | 8.5 AU | 0.14 Rf | 19.4 AU | 2.15 % | 0.17 Rf | 0.8 AU | 289.8 AU | 1.22 % |
| 3 | 0.17 Rf | 0.9 AU | 0.20 Rf | 12.6 AU | 1.40 % | 0.20 Rf | 12.4 AU | 196.4 AU | 0.82 % |
| 4 | 0.23 Rf | 18.3 AU | 0.27 Rf | 91.4 AU | 10.11 % | 0.30 Rf | 39.1 AU | 2687.3 AU | 11.28 % |
| 5 | 0.30 Rf | 39.1 AU | 0.36 Rf | 392.3 AU | 43.43 % | 0.39 Rf | 65.1 AU | 11075.7 AU | 46.49 % |
| 6 | 0.39 Rf | 65.6 AU | 0.40 Rf | 76.3 AU | 8.45 % | 0.43 Rf | 50.1 AU | 1825.8 AU | 7.66 % |
| 7 | 0.43 Rf | 50.4 AU | 0.45 Rf | 66.8 AU | 7.39 % | 0.47 Rf | 28.7 AU | 1480.0 AU | 6.21 % |
| 8 | 0.47 Rf | 28.8 AU | 0.50 Rf | 190.4 AU | 21.08 % | 0.55 Rf | 9.4 AU | 5020.9 AU | 21.08 % |
| 9 | 0.55 Rf | 9.4 AU | 0.58 Rf | 30.2 AU | 3.34 % | 0.62 Rf | 0.1 AU | 787.3 AU | 3.30 % |
| 10 | 0.65 Rf | 1.2 AU | 0.68 Rf | 13.0 AU | 1.43 % | 0.71 Rf | 1.6 AU | 305.6 AU | 1.28 % |

$R_f$ values at 254 nm in Hydro alcohol extract (Absorbance mode)



HPTLC finger print at 366 nm in Hydro alcohol extract (Absorbance mode)

| Peak | Start Position | Start Height | Max Position | Max Height | Max % | End Position | End Height | Area | Area % |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.10 Rf | 0.1 AU | 0.14 Rf | 44.9 AU | 5.82 % | 0.17 Rf | 5.7 AU | 916.6 AU | 3.86 % |
| 2 | 0.18 Rf | 6.4 AU | 0.22 Rf | 33.4 AU | 4.34 % | 0.23 Rf | 28.7 AU | 709.1 AU | 2.98 % |
| 3 | 0.23 Rf | 28.8 AU | 0.26 Rf | 121.0 AU | 15.70 % | 0.28 Rf | 28.0 AU | 2321.5 AU | 9.77 % |
| 4 | 0.29 Rf | 27.9 AU | 0.36 Rf | 446.5 AU | 57.95 % | 0.42 Rf | 35.2 AU | 16778.2 AU | 70.61 % |
| 5 | 0.42 Rf | 35.6 AU | 0.45 Rf | 101.8 AU | 13.21 % | 0.48 Rf | 1.9 AU | 2235.1 AU | 9.41 % |
| 6 | 0.63 Rf | 3.7 AU | 0.68 Rf | 22.9 AU | 2.97 % | 0.72 Rf | 3.7 AU | 800.1 AU | 3.37 % |

$R_f$ values at 366 nm in Hydro alcohol extract (Absorbance mode)

**Microbial load**

Tab 05: Microbial load determination

| S. No. | Parameters | Results |
|---|---|---|
| 1 | Total Bacterial Count (TBC) | $2x10^4$ cfu/g |
| 2 | Total Fungal Count (TFC) | $1x10^2$ cfu/g |
| 3 | Enterobacteriaceae | Absent |
| 4 | *Escherichia coli* | Absent |
| 5 | Salmonella Spp | Absent |
| 6 | *Staphylococcus aureus* | Absent |
| 7 | *Pseudomonas aeruginosa* | Absent |

## DISCUSSION

The colour of the Inji Rasayanam was found to be brownish black, Semi-solid in consistency and sweet with pungent in taste. The main ingredient in Inji Rasayanam was Ginger. Ginger contains bioactive phytochemicals like 6-gingerol, 6-shogaol contains anti-oxidant, anti- inflammatory properties which are beneficial for managing soothagavaayu (PCOD) symptoms. Standardization of the drug was performed as per Ayush and WHO guidelines. Inji Rasayanam is a poly herbal formulation comprised of 15 ingredients was analysed using pharmacological standard procedure. The total ash value (4.92%) was within permissible range which indicates the total inorganic, non- combustible material and to identify the adulteration in the drug to meet the quality standards. The acid- insoluble ash content (2.73%) suggesting less silicious matter in the drug. The pH value was (5.51) suggesting a mildly acidic nature. The test for loss on drying (8.91%) which indicates the moisture content present in the drug and plays an important role in determining its stability and shelf life. The water extractive value was 53.64%, whereas the alcohol extractive value was 18.86%, suggesting that a greater proportion of the phytoconstituents are soluble in water. The total solid content, total fat content, reducing sugar, total sugar, and non-reducing sugar were found to be 12.71%, 17.87%, 17.16%, 27.32%, and 10.16% respectively [2, 3, 6]. Phytochemical parameter screening revealed the presence of carbohydrates. The presence of carbohydrates may contribute to the nutritive and therapeutic value of the formulation. TLC photo documentation profiles of hydro alcoholic extract of Inji Rasayanam at UV 254 nm showed 10 peaks and 6 peaks at UV 366 nm. The total bacterial count($2x10^4$ cfu/g) and total fungal count ($1x10^2$ cfu/g)were within the permissible limits. E. Coli, salmonella spp, Staphy aures, Pseudomonas aeruginosa were absent. Total aflatoxin (1ppb) was within WHO limits.

## CONCLUSION

From the above observations, the characteristic physicochemical constants and HPTLC fingerprint profile obtained in this study can serve as reference standards for quality control and authentication of Inji Rasayanam. Thus, the findings of this study scientifically validate the traditional formulation and support its safe therapeutic use in the management of Polycystic Ovary Syndrome, referred to in Siddha literature as Soothaga Vaayu.

## CONFLICT OF INTEREST

None

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Self-Funded

## ETHICAL APPROVAL

Approved

## AUTHOR CONTRIBUTION

Dr. R. Menaka designed and supervised the project. Dr. S. Kowsalya Collected and analysed the data and prepared the manuscript.

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