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Preparation and Characterisation of the Siddha Polyherbal Formulation Koduveli Nei: Physicochemical, HPTLC, GC-MS and FTIR Characterisation

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ABSTRACT:

Background: Koduveli Nei (KN) is a polyherbal medicated castor-oil (Nei) preparation used in Siddha medicine, prepared as described in the classical text Vaatha Nithanam-800 for Vaatha-predominant musculoskeletal conditions, including lumbar spondylosis. Although Koduveli Nei is traditionally indicated for such spinal degenerative conditions, KN lacks validated pharmacopoeial standards, which limits quality assurance and regulatory acceptance; this study does not evaluate therapeutic efficacy but addresses this standardisation gap.

Objective: This study aimed to standardise a single prepared batch of KN following the Pharmacopoeial Laboratory for Indian Medicine (PLIM) protocol and WHO/Unani Pharmacopoeia guidelines.

Methods: Organoleptic characters, solubility profile, physicochemical parameters (rancidity, acid, saponification, iodine, and peroxide values, specific gravity, and refractive index), HPTLC fingerprinting at 254 nm, 366 nm, and 520 nm (derivatised), heavy metal content by ICP-OES (arsenic, cadmium, mercury, lead), microbial load and specific pathogen screening, aflatoxin content, and pesticide residue screening were assessed.

Results: All safety parameters tested were within permissible limits: heavy metals were below the instrument detection limit, microbial counts and pathogen screening met

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WHO/Unani Pharmacopoeia criteria, aflatoxin content was within commonly cited permissible limits, and pesticide residues were below the quantification limit. HPTLC fingerprinting showed 11, 10 and 14 peaks at 254 nm, 366 nm and 520 nm respectively, reproducible across all three replicate tracks. GC-MS profiling of Koduveli Nei in hexane, chloroform and methanol identified squalene, tocopherols and fatty acid methyl esters. FTIR of Koduveli Nei contained functional groups corresponding to the compounds identified by GC-MS. Conclusion: These findings suggest KN contains lipophilic antioxidant and anti-inflammatory constituents. The physicochemical, HPTLC, GC-MS and FTIR data together establish a preliminary analytical reference profile for this single batch of KN, supporting its safety as tested and providing a basis to guide further multi-batch standardisation of this classical Siddha formulation.

Key Findings

Koduveli Nei met WHO/PLIM safety limits for heavy metals, microbial load, aflatoxins, and pesticide residues, and yielded a reproducible three-wavelength HPTLC fingerprint (11/10/14 peaks) together with a GC-MS- and FTIR-supported lipophilic antioxidant and anti-inflammatory constituent profile in this single batch

1.INTRODUCTION

Siddha Medicine is one of the unique traditional medical systems in the world, originating in the southern part of India. This system of medicine is meant to treat diseases and is a life science of well-being. Treatment given to the patient with Siddha medicine is based on the stabilisation of *Uyir thathukal* (three humours) and *Udal thathukal* (seven physical constituents). According to the philosophical foundation of Siddha medicine, health (*Aarokkiyam*) is defined as a state of dynamic equilibrium among the three vital humours: *Vatham* (kinetic, governed by air and space), *Pitham* (metabolic, governed by fire and water), and *Kapham* (structural/lubricating, governed by water and earth) [1]. Siddha materia medica (*Gunapadam*) classifies therapeutic resources into three broad categories: *Thavara varkkam* (botanical sources), *Thathu varkkam* (minerals and inorganics), and *Jangama varkkam* (animal-derived materials). Each medicine is selected based on its taste, potency, post-digestive effect, and pharmacological action according to Siddha texts such as *Gunapadam Mooligai Vaguppu* and *Gunapadam Thathu Jeeva Vaguppu* [3,4]. Diagnosis and drug selection are made through the foundational framework of *Imboothams* (five elements) and the three humours (*Vatham, Pitham, Kapham*); therefore, a medicine or therapy is chosen not only for its chemical content but also for how its taste and potency are expected to correct humoral imbalance [4,5].

In the Siddha system of medicine, there are 32 types of internal and external medications, among which *Ennai* (*Thailam*) is one of the internally consumed forms. Despite the name stating *'Koduveli Nei'*, the preparation contains *Ammanaku ennai* (castor oil) as a base in which the other 15 herbs are processed [14]. Oil-based medicines have five modes of administration, including *Kudi Nei* (oral route) and four external applications: *Mudi Nei* (hair oil), *Pidi Nei* (topical application), *Thulai Nei* (nasal or ear drops), and *Silai Nei* (used for chronic ulcers) [4]. The fat-based nature of the medicine confers a major pharmacokinetic advantage: the lipophilic base of *Koduveli Nei* is believed to increase the bioavailability of its phytochemical constituents. Lipid binding helps the phytochemicals bypass first-pass metabolism and reach the small intestine, where the large surface area and rich blood supply favour absorption into the bloodstream [5,6]. Recent literature suggests that such oil- or ghee-based internal preparations, taken as the prepared medicine or as *Anubanam* (vehicle), enhance the bioactivity of herbal constituents (antioxidant, anti-inflammatory, hepatoprotective, etc.) through a fat matrix that favours tissue affinity and sustains gut tolerability [6].

As the name denotes, the medicine contains *Koduveli* (commonly referred to as *Ven Chitiramoolam* or *Ven Kodiveli*; *Plumbago zeylanica*) [13], processed along with 14 other herbs. *Koduveli Nei* is administered internally to manage *Vaatha* diseases of the spine, as described in the Siddha text *Vaatha Nithanam*-800 [7].

Koduveli Nei is one of the significant Varmam-based medications used in the management of pain in degenerative diseases [12], but it lacks validated pharmacopoeial standards. This poses challenges for quality assurance, global acceptance, and reproducibility across manufacturing batches [2].

Characterisation of *Koduveli Nei* is essential to ensure safety, efficacy, and consistency across batches. This is mandated under the regulatory framework administered by the Pharmacopoeial Laboratory for Indian Medicine (PLIM), Government of India, in coordination with World Health Organization (WHO) quality-control guidelines for medicinal plant materials. Standardisation protocols typically include organoleptic and physicochemical evaluation, chromatographic fingerprinting, and screening for contaminants such as heavy metals, microbial load, and mycotoxins (aflatoxins), mainly to guard against microbial proliferation and environmental contamination during cultivation, storage, and processing.

This study aimed to characterise *Koduveli Nei* based on the PLIM protocol and WHO/Unani Pharmacopoeia guidelines, with the objective of establishing physicochemical reference values, building a reproducible HPTLC chemical fingerprint, and confirming compliance with safety limits for heavy metals, microbial contamination, and aflatoxins.

2. MATERIALS AND METHODS

2.1 Sample

The test sample, *Koduveli Nei*, was selected from the Siddha text *Vaatha Nithanam*-800. It was prepared by incorporating 15 herbal drugs into castor oil following the classical Siddha method, and the prepared batch was procured and subjected to standardisation analysis. The ingredients of KN are presented in Table 1.

Table 1. Ingredients of the trial drug

S.No	Ingredient	Botanical name	Part used	Quantity
1	*Chithiramoolam*	*Plumbago zeylanica*	Root bark	4 *palam* (140 g)
2	*Mutsankan ver*	*Azima tetracantha*	Root	4 *palam* (140 g)
3	*Velulli*	*Allium sativum*	Bulb	4 *palam* (140 g)
4	*Omam*	*Trachyspermum ammi*	Fruit	4 *palam* (140 g)
5	*Chukku*	*Zingiber officinale*	Rhizome	1 *kazhachi* (5.1 g)
6	*Milagu*	*Piper nigrum*	Fruit	1 *kazhachi* (5.1 g)
7	*Thipili*	*Piper longum*	Fruit	1 *kazhachi* (5.1 g)
8	*Ellam*	*Elettaria cardamomum*	Seed	1 *kazhachi* (5.1 g)
9	*Kirambu*	*Syzygium aromaticum*	Flower bud	1 *kazhachi* (5.1 g)
10	*Nervalam*	*Croton tiglium*	Seed	1 *kazhachi* (5.1 g)
11	*Vasambu*	*Acorus calamus*	Rhizome	1 *kazhachi* (5.1 g)
12	*Venthayam*	*Trigonella foenum-graecum*	Seed	1 *kazhachi* (5.1 g)
13	*Jathipathiri*	*Myristica fragrans*	Aril	1 *kazhachi* (5.1 g)
14	*Malli*	*Coriandrum sativum*	Seed	1 *kazhachi* (5.1 g)
15	*Karujeeragam*	*Nigella sativa*	Seed	1 *kazhachi* (5.1 g)
16	*Aamanaku ennai*	*Ricinus communis*	Oil	1 *padi* (1.3 L)

2.2 Source, Collection and Authentication of Raw Drugs

The required raw drugs were purchased from a reputable raw-drug shop and authenticated by competent authorities, including a botanist and a Siddha pharmacologist (Medicinal Botany and *Gunapadam* Department). After authentication, all raw drugs were purified individually as described in Siddha literature. The trial drug was then prepared in the *Gunapadam* laboratory of the National Institute of Siddha.

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a) *Chithiramoolam (Plumbago zeylanica)*, **b)** *Mutsankan ver (Azima tetracantha)*, **c)** *Velulli (Allium sativum)*, **d)** *Omam (Trachyspermum ammi)*, **e)** *Chukku (Zingiber officinale)*, **f)** *Milagu (Piper nigrum)*, **g)** *Thipili (Piper longum)*, **h)** *Ellam (Elettaria cardamomum)*, **i)** *Kirambu (Syzygium aromaticum)*, **j)** *Nervalam (Croton tiglium)*, **k)** *Vasambu (Acorus calamus)*, **l)** *Venthayam (Trigonella foenum-graecum)*, **m)** *Jathipathiri (Myristica fragrans)*, **n)** *Malli (Coriandrum sativum)*, **o)** *Karujeeragam (Nigella sativa)*, **p)** *Aamanaku ennai (Ricinus communis)*

2.3 Purification of Raw Drugs

Kodiveli: The central part of the root bark is removed, and the outer part is powdered. This powder is placed in a cloth tied over a vessel containing milk. The milk is boiled until reduced to half its original volume, after which the powder is dried. [20]

Velulli: The garlic is dried in the sun, and its skin is peeled. [20]

Omam: It is soaked in lime water for three hours and then dried. [20]

Chukku: The outer skin is peeled from the dry ginger. [20]

Milagu: It is soaked in sour buttermilk for three hours (one *saamam*). [20]

Thipili: It is soaked in lemon juice for one day, then dried in sunlight.

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Elam: It is cleaned and dried in sunlight. [20]

Kirambu: It is cleaned and dried in sunlight. [20]

Nervalam: The seeds are boiled in cow-dung water and then soaked in lemon juice. The outer covering is subsequently removed, and the seeds are roasted in ghee.

Vasambu: The root is burnt, and the ash is collected. [20]

Venthayam: The seeds are soaked in rice water for 12 minutes (0.5 *nazhigai*) and then dried. [20]

Jathipathiri: The drug is collected and dried in sunlight. [20]

Karujeeragam: The seeds are cleaned and roasted. [20]

Aamanaku ennai: Before extracting the oil, the seeds are boiled in tender coconut water and dried. [20]

2.4 Standard Operating Procedure for Preparation of Koduveli Nei

Chitramoolam, Mutsankan ver, Velulli and *Omam* (140 g each) were added to 16 L of water and boiled until the volume was reduced to about 2 L, after which 1.3 L of *Aamanaku ennai* was added. To this, 5.1 g each of powdered *Chukku, Milagu, Thipili, Ellam, Kirambu, Nervalam, Vasambu, Venthayam, Jathipathiri, Malli* and *Karujeeragam* were ground with the decoction and added. The oil was boiled until it attained *mezhugu muthir patham* (the stage preceding wax consistency) and then filtered. The finished preparation was stored in an airtight glass container.

q & r) Boiling the decoction with ingredients 1–4, **s)** grinding ingredients 5–15 with the decoction, **t)** boiling the castor oil with the decoction and ground paste, **u)** residue of the oil after filtering, **v)** the prepared medicine.

2.5 Quality Evaluation and Standardization Procedures

2.5.1 Organoleptic Characters

Colour, odour, taste, size, shape, and special features such as touch and texture were recorded.

2.5.2 Solubility Profiles

A solubility study was performed based on the methods described by the Pharmacopoeial Laboratory for Indian Medicine.

2.5.3 Physicochemical Analysis

Physicochemical parameters — rancidity, acid value, saponification value, iodine value, peroxide value, specific gravity, and refractive index — were determined in duplicate as per the standard test procedures prescribed in the PLIM protocol (Lohar DR, *Protocol for Testing of Ayurvedic, Siddha and Unani Medicines*, Department of AYUSH, Government of India, 2008) [8,15].

2.5.4 HPTLC Fingerprinting Analysis

A sample weighing one gram was dissolved in 10 mL of ethanol, subjected to sonication for 15 minutes, and subsequently filtered; the resulting filtrate was used for chromatographic analysis. The extract was applied to a TLC plate using a Camag ATS4 applicator and developed with a mobile phase of toluene, ethyl acetate, methanol and formic acid (6.5:2.5:0.2:0.5, v/v/v/v) to a distance of 9 cm. The developed plate was photo-documented under UV light at 254 nm and 366 nm using a Camag TLC Visualizer, then derivatised by immersion in a 5% vanillin–sulphuric acid reagent and heated at 105°C until coloured bands appeared, followed by photo-documentation under white light. The plate was scanned densitometrically using a Camag Scanner 4 in absorption mode at 254 nm (D2 lamp), 366 nm (D2 lamp), and 520 nm (W lamp) in triplicate (three tracks) to produce HPTLC fingerprint profiles and peak tables at all three wavelengths. Visual UV band counts (photo-documentation, Table 9) and densitometric peak counts (Table 10) were obtained from separate acquisitions and are not directly comparable [16].

2.5.5 Heavy Metal Analysis (ICP-OES)

In a Teflon microwave digestion vessel, around 20 mg of sample was digested with 1 mL of ultrapure nitric acid for about 45 minutes using an Anton Paar microwave digestion unit. The resulting solution was diluted to 50 mL with ultrapure water. Calibration standards ranging from 2 to 10 µg/mL and blanks were prepared with ultrapure nitric acid. Analysis was performed using an Agilent ICP-OES 5100 VDV instrument, set at an RF power of 1.2 kW, with a plasma gas flow rate of 12 L/min and a nebulizer gas flow rate of 0.70 L/min, to estimate the levels of arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) [17].

2.5.6 Microbial Load and Pathogen Screening

Microbial quality testing followed the Unani Pharmacopoeia of India (2016) and WHO Standards (2007) guidelines for herbal-based medicines. The sample was homogenised using polysorbate, added at 1 mL to 9 mL peptone broth, and serially diluted. Using the pour-plate technique, aerobic bacterial and fungal populations were obtained in triplicate. Bacteria were inoculated on Casein Soyabean Digest Agar and incubated at 37°C for one to two days; fungi were inoculated on Sabouraud Dextrose Agar and incubated at 25°C for two to three days. Results were expressed as colony-forming units per gram (cfu/g), with an aerobic bacterial count above 10^5 cfu/g classified as failing the WHO standard. To screen for specific pathogens, the sample was cultured on selective media: EMB agar for *Escherichia coli*, MacConkey agar for *Salmonella* species, Cetrimide agar for *Pseudomonas aeruginosa*, and Mannitol Salt agar for *Staphylococcus aureus*. Any isolates were identified through colony appearance, Gram-stain reaction, and biochemical assays including oxidase, catalase, and gas-production tests.

2.5.7 Aflatoxin Estimation

Total aflatoxin content (B1, B2, G1, G2) was measured with a VICAM AflaTest fluorometer (Series 4EX). The sample was extracted using a 60:40 v/v methanol and 2% Tween-20/phosphate buffer mixture, then filtered and diluted before being passed through an AflaTest WB immunoaffinity column. After washing, the column was eluted with HPLC-grade methanol, mixed with AflaTest Developer reagent, and the fluorescence signal was recorded. Values were compared against WHO permissible thresholds (WHO/NHM/FOS/RAM/18.1, 2018).

2.5.8 Pesticide Residue Analysis

A 25 g sample was placed in a 250 mL bottle with 98 mL acetonitrile, 52 mL distilled water, and a scoop of sodium chloride, then agitated at 250 rpm for half an hour. It was filtered through Whatman paper into a graduated 250 mL cylinder. A 75 mL aliquot of this mixture was transferred into a separating funnel containing 150 mL distilled water, 60 mL petroleum ether, 20

mL saturated sodium chloride solution, and 50 mL hexane. After shaking and venting, the layers were left to separate. The upper (organic) layer was withdrawn, and a second extraction was performed with a further 40 mL of petroleum ether on the remaining mixture. Both extractions were combined, rinsed with 100 mL distilled water and sodium sulphate, and evaporated to dryness at 90°C. The residue was taken up in 15 mL diethyl ether and 85 mL petroleum ether, then cleaned up on a column packed sequentially with cotton, sodium sulphate, silica gel, aluminium oxide, florisil, and a final layer of sodium sulphate. The purified eluate was collected and evaporated to complete dryness at 90°C before analysis.

2.5.9 GC-MS Analysis

Sample preparation. *Koduveli Nei* was prepared for GC-MS analysis by simple dissolution in three solvents of increasing polarity — n-hexane, chloroform, and methanol; no transesterification, methylation, or other derivatisation step was performed before injection. The oil was dissolved directly at a ratio of 0.1 mL sample to 1 mL solvent.

Koduveli Nei was analysed in three extracts — hexane (non-polar), chloroform (mid-polar), and methanol (polar) — on a PerkinElmer Clarus 680 gas chromatograph coupled with a Clarus 600 mass spectrometer. The GC-MS platform was operated in electron-impact positive (EI+) scan mode over m/z 40–600. Chromatographic separation was achieved on a 30.0 m × 250 µm (i.d.) Elite-5MS fused-silica capillary column (5% biphenyl/95% dimethylpolysiloxane) with helium as carrier gas, a 10:1 split ratio, and an auto-injector temperature of 260°C. The oven programme began at 60°C, held for 2 min, ramped at 10°C/min to 300°C, and held for 6 min (total run time ≈ 32 min). The solvent delay was set to 2 min, with the transfer-line and ion-source temperatures maintained at 240°C. Mass spectra were acquired over an m/z range of 50–600. Total ion chromatograms (TIC) were acquired for each extract, and resolved peaks were selected by automated peak detection using a height/area threshold. Peaks were identified by comparing their mass spectra with the NIST (National Institute of Standards and Technology) 2014 mass spectral library.

2.5.10 FTIR

FTIR spectra were acquired in transmittance or ATR mode across 4000–400 cm^{-1} to obtain a whole-extract functional-group fingerprint that complements the compound-level resolution of GC-MS [21,22,26,45,46,49,53]. Diagnostic absorption regions commonly reported for extracts of this chemical composition include: O–H stretch (3200–3550 cm^{-1}, alcohols/phenols), aliphatic C–H stretch (2850–2960 cm^{-1}, consistent with long-chain fatty acid/ester methylene and methyl groups), ester/carboxylic C=O stretch (1710–1750 cm^{-1}), aromatic/chromanol C=C (1580–1620 cm^{-1}, consistent with the tocopherol chromanol ring), C–O stretch of esters and ethers (1030–1300 cm^{-1}), and a long-chain methylene rocking band near 720 cm^{-1} diagnostic of extended aliphatic chains such as those in fatty acid methyl esters and squalene [45,46,49,53].

3. RESULTS AND DISCUSSION

3.1 Organoleptic Characters

Koduveli Nei was a yellowish-brown, greasy, dense, viscous liquid with a strong characteristic odour (Table 2).

Table 2. Organoleptic characters of *Koduveli Nei*

S.No	Organoleptic character	Observation
1	Description	Brown dense viscous liquid
2	Odour	Strong odour
3	Touch	Greasy
4	Flow property	Not free-flowing; thick, highly viscous, and flows very slowly
5	Appearance and colour	Yellowish brown

3.2 Solubility Profiles

KN is an oil-based medicine. It is insoluble in water and soluble in conc. H_2SO_4, ethanol and methanol. Results are presented in Table 3.

Table 3. Solubility profile of *Koduveli Nei*

S.No	Solvent used	Solubility
1	Water	Insoluble
2	Conc. HCl	Sparingly soluble
3	Conc. H_2SO_4	Soluble
4	Ethanol	Soluble
5	Methanol	Soluble

3.3 Physicochemical Parameters

The physicochemical parameters of *Koduveli Nei* are presented in Table 4. The rancidity test was negative, and all other parameters were within the normal range expected for a ghee-based medicated preparation as per PLIM guidelines.

Table 4. Physicochemical analysis of *Koduveli Nei*

S.No	Parameter	Trial I	Trial II	Mean value
1	Rancidity test	Negative	Negative	Negative
2	Acid value	6.69	7.18	6.93
3	Saponification value	161.76	159.74	160.75
4	Iodine value	6.82	8.46	7.64
5	Peroxide value	11.05	11.05	11.05
6	Specific gravity	0.935	0.935	0.935
7	Refractive index	1.473	1.473	1.473

3.4 Heavy Metal Analysis (ICP-OES)

Arsenic, cadmium, mercury, and lead were all below the detection limit (BDL) in the *Koduveli Nei* sample (Table 5), suggesting no toxicologically significant heavy-metal contamination. Calibration across the 2–10 µg/mL range was linear for all four elements ($R^2 > 0.99$) (Table 6).

Table 5. Heavy metal content of *Koduveli Nei* by ICP-OES

Element	Result
Arsenic (As)	BDL
Cadmium (Cd)	BDL
Mercury (Hg)	BDL
Lead (Pb)	BDL

BDL: Below Detection Limit.

Table 6. ICP-OES calibration linearity for heavy metals

S.No	Element	Wavelength (nm)	R^2 value
1	Arsenic (As)	188.980	0.9972
2	Cadmium (Cd)	226.502	0.9964
3	Mercury (Hg)	184.887	0.9985
4	Lead (Pb)	220.353	0.9969

3.5 Microbial Load and Pathogen Screening

The total bacterial count of *Koduveli Nei* was 1×10^4 cfu/mL, within the WHO permissible limit, and the total fungal count was less than 3 cfu/mL (Table 7). Enterobacteriaceae and the specific pathogens tested — *Escherichia coli, Salmonella* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* — were all absent, confirming the microbiological safety of the formulation.

Table 7. Microbial load and pathogen screening of *Koduveli Nei*

S.No	Parameter	Result	Remarks
1	Total Bacterial Count (TBC)	1×10^4 cfu/mL	Within permissible limits
2	Total Fungal Count (TFC)	< 3 cfu/mL	—
3	Enterobacteriaceae	Absent	—
4	*Escherichia coli*	Absent	—
5	*Salmonella* spp.	Absent	—
6	*Staphylococcus aureus*	Absent	—
7	*Pseudomonas aeruginosa*	Absent	—

3.6 Aflatoxin Content

Total aflatoxin (B1+B2+G1+G2) in *Koduveli Nei* was estimated at 3 ppb (detection limit 1 ppb), well within the WHO permissible range of 0.5–15 µg/kg (ppb) for aflatoxins in foods and herbal materials (Table 8).

Table 8. Aflatoxin content of *Koduveli Nei*

S.No	Parameter	Method/Reference	Result
1	Total Aflatoxin B1+B2+G1+G2	VICAM AflaTest Fluorometer	3 ppb

Detection limit: 1 ppb. WHO reference limit: 0.5–15 µg/kg (WHO/NHM/FOS/RAM/18.1, February 2018, Department of Food Safety and Zoonoses).

3.7 HPTLC Fingerprinting

TLC of the ethanolic extract of *Koduveli Nei* resolved 8 bands under UV 254 nm, 9 bands under UV 366 nm, and 11 coloured bands under white light following derivatisation with vanillin–sulphuric acid reagent (Table 9, Figure 1).

Table 9. TLC band profile of *Koduveli Nei* (ethanolic extract)

UV 254 nm – Rf	UV 254 nm Colour	UV 366 nm – Rf	UV 366 nm Colour	White Light – Rf	White Light Colour
0.04	Dark	0.09	Dark blue	0.07	Pink
0.14	Dark	0.28	Salmon	0.13	Blue
0.25	Dark	0.34	Red	0.19	Light blue
0.37	Light	0.47	Cyan blue	0.24	Light pink
0.48	Dark	0.55	Brown	0.27	Pink
0.56	Dark	0.63	Green	0.42	Violet

0.62	Light	0.69	Light red	0.48	Yellow
0.79	Dark	0.75	Red	0.66	Purple
—	—	0.78	Blue	0.73	Purple
—	—	—	—	0.78	Light purple
—	—	—	—	0.85	Purple

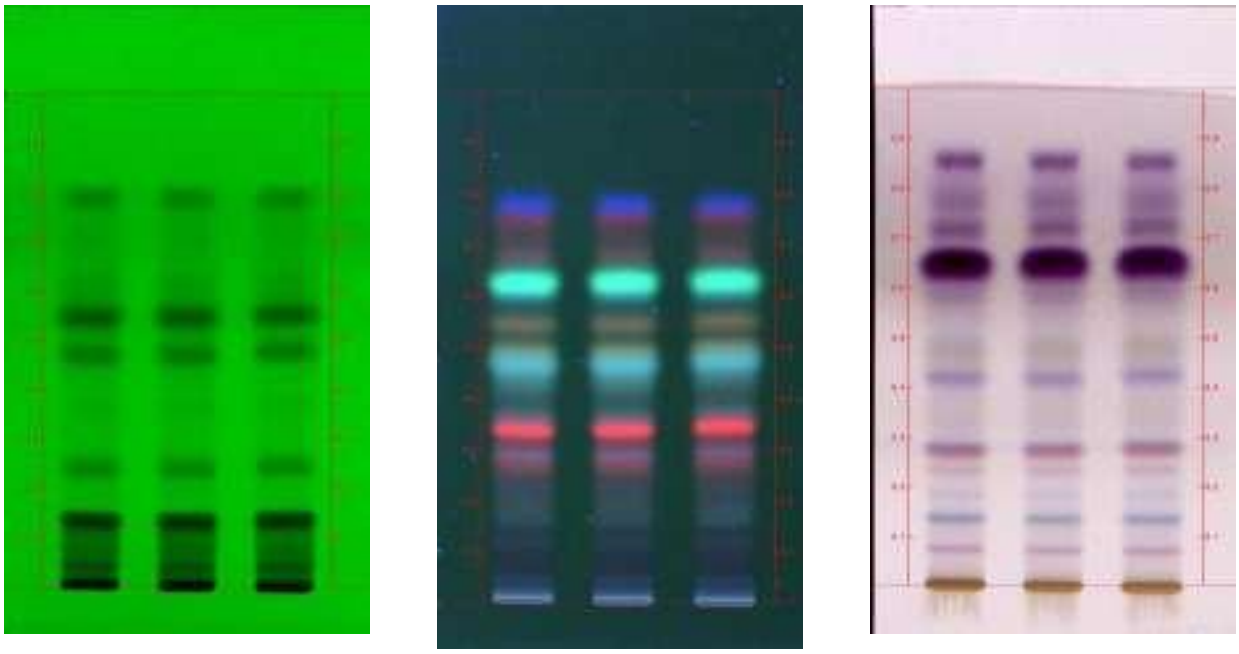


Figure 1. TLC plates of *Koduveli Nei* (ethanolic extract, three replicate tracks) under UV 254 nm (left), UV 366 nm (centre), and white light after derivatisation with 5% vanillin–sulphuric acid reagent (right).

HPTLC Fingerprint Profile

Densitometric scanning of the developed plate generated reproducible HPTLC fingerprint profiles across three tracks at each wavelength. At 254 nm, 11 peaks were resolved (Rf 0.01–0.89), with the major peak at Rf 0.56 (max height 415.4 AU, 23.20% of total peak area) and other prominent peaks at Rf 0.14 (20.59% max height; 18.30% area) and Rf 0.48 (13.87% max height; 16.38% area) (Figure 2). At 366 nm, 10 peaks were resolved (Rf 0.02–0.93), with the major peak at Rf 0.64 (max height 649.1 AU, 31.40% of total area) (Figure 3). At 520 nm, 14 peaks were resolved (Rf 0.01–0.87), with the major peak at Rf 0.67 (max height 509.5 AU, 33.38% of total area) (Figure 4). The consistent number, position, and relative intensity of peaks across the three replicate tracks at each wavelength confirm the reproducibility of the chromatographic method and establish a characteristic chemical fingerprint for *Koduveli Nei*.

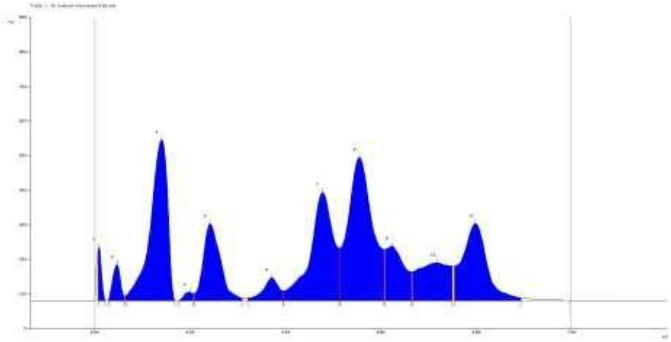


Figure 2. HPTLC densitogram of *Koduveli Nei* scanned at 254 nm, showing 11 resolved peaks.

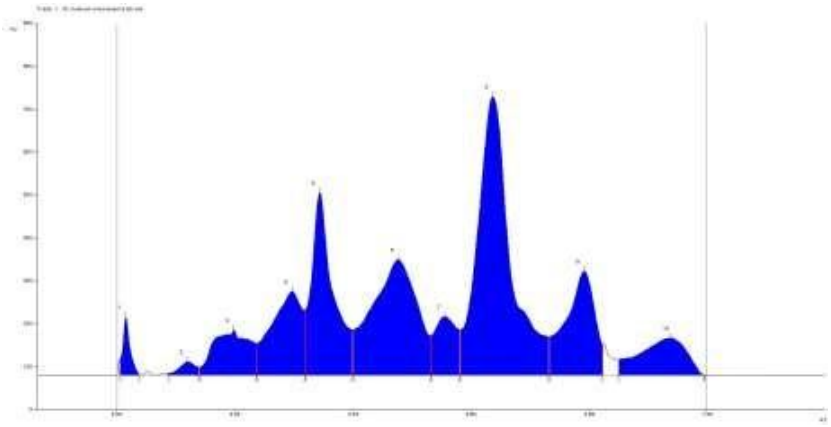


Figure 3. HPTLC densitogram of *Koduveli Nei* scanned at 366 nm, showing 10 resolved peaks.

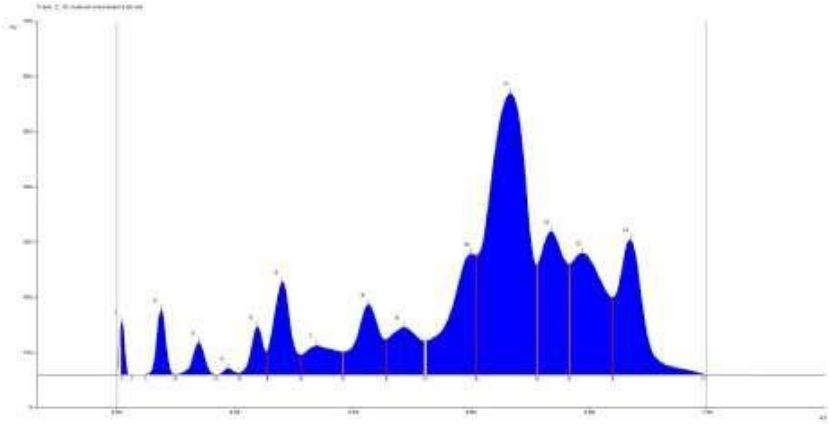


Figure 4. HPTLC densitogram of *Koduveli Nei* scanned at 520 nm, showing 14 resolved peaks.

Table 10. Major HPTLC peaks (≥5% area) resolved at 254, 366, and 520 nm

Wavelength	Peak	Max Rf	Max Ht %	Area %
254 nm	3	0.14	20.59	18.30
254 nm	5	0.24	9.87	8.74
254 nm	7	0.48	13.87	16.38
254 nm	8	0.56	18.36	23.20
254 nm	9	0.62	6.97	6.77
254 nm	10	0.72	4.85	8.00
254 nm	11	0.80	9.88	12.58
366 nm	3	0.20	4.61	5.59
366 nm	4	0.30	8.57	8.67
366 nm	5	0.34	18.68	13.55
366 nm	6	0.48	11.83	17.86
366 nm	8	0.64	28.54	31.40

366 nm	9	0.79	10.65	10.38
366 nm	10	0.93	3.80	5.97
520 nm	8	0.43	5.62	5.18
520 nm	10	0.60	9.69	9.88
520 nm	11	0.67	22.62	33.38
520 nm	12	0.73	11.51	11.04
520 nm	13	0.79	9.77	12.27
520 nm	14	0.87	10.85	10.71

3.8 Pesticide Residue Analysis

Table 11. Pesticide residue analysis of *Koduveli Nei*

Pesticide Residue	Sample KN	AYUSH Limit (mg/kg)
I. Organochlorine 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
II. Organophosphorus Pesticides		
Malathion	BQL	1 mg/kg
Chlorpyriphos	BQL	0.2 mg/kg
Dichlorovos	BQL	1 mg/kg
III. Organocarbamates		
Carbofuran	BQL	0.1 mg/kg
IV. Pyrethroids		
Cypermethrin	BQL	1 mg/kg
Bioallethrin	BQL	Non-permissible

BQL – Below Quantification Limit.

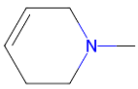
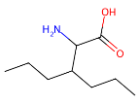
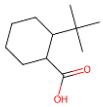
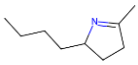
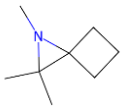
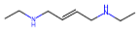
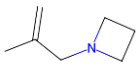
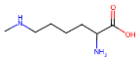
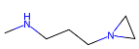
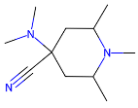
Result: No traces of organochlorine, organophosphorus, organocarbamate, or pyrethroid pesticide residues were detected in the sample analysed.

3.9 GC-MS Phytochemical Profiling

GC-MS resolved 18, 9, and 11 peaks in the hexane, chloroform, and methanol extracts respectively (38 peaks in total; one hexane peak unidentified), each dominated by a single major peak (35.9%, 54.9%, 65.8% of area, respectively). The hexane fraction was dominated by low-to-moderate-confidence (54–73% library match; below the ~80% threshold conventionally used

to call a hit "identified") nitrogenous heterocycles rather than a hydrocarbon/wax profile, and these identifications should be treated as tentative and unconfirmed pending an orthogonal technique. Eleven remaining minor peaks (each <2.5% area) were further short-chain amine/aziridine/lysine-type hits, and one peak (27.473 min, 1.89%) had no library match. The chloroform and methanol fractions both contained squalene [28,29] and γ-tocopherol [45,46], with the methanol fraction additionally containing a δ-tocopherol-type tocol and a 4α-methylsterol related to cholesta-8,24-dien-3-ol, consistent with a phytosterol-biosynthesis intermediate [45].

Table 12. GC-MS peak list, tentative NIST library identification, molecular weight, relative area, and library match quality for the n-hexane extract

Peak	RT (min)	Tentatively Identified Compound (NIST)	Structure	MW (g/mol)	Area (%)	Match (%)
1	17.029	1,2,3,6-Tetrahydro-1-methylpyridine		97	0.53	66.8
2	17.900	3-Propylnorleucine		173	0.53	54.3
3	18.120	Cyclohexanecarboxylic acid, 2-(1,1-dimethylethyl)-, cis-		184	12.36	53.7
4	19.925	5-Butyl-2-methyl-Δ1-pyrroline		139	35.87	69.1
5	21.686	1,2,2-Trimethyl-1-azaspiro[2.3]hexane		125	4.27	65.9
6	22.241	2-Butene-1,4-diamine, N,N'-diethyl-		142	10.70	70.9
7	22.782	1-(2-Methylallyl)azetidine		111	5.72	59.5
8	23.062	N(ε)-Methyl-L-lysine		160	2.20	61.6
9	23.367	N-[3-Methylaminopropyl]aziridine		114	4.44	72.8
10	23.992	4-Dimethylamino-4-cyano-1,2,5-trimethylpiperidine		195	4.56	60.7

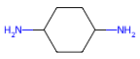
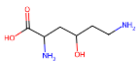
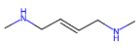
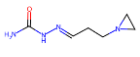
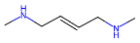
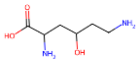
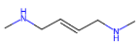
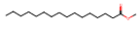
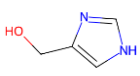
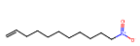
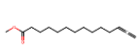
11	24.657	cis-1,4-Cyclohexanediamine		114	5.38	69.8
12	25.653	L-4-Hydroxylysine		162	2.13	72.9
13	26.333	2-Butene-1,4-diamine, N,N'-dimethyl-		114	3.65	61.6
14	26.653	3-[N-Aziridyl]propionaldehyde semicarbazone		156	3.64	60.6
15	27.248	2-Butene-1,4-diamine, N,N'-diethyl-		142	1.22	61.4
16	27.473	Not identified (no acceptable library match)		—	1.89	—
17	28.239	L-4-Hydroxylysine		162	0.51	60.6
18	28.674	2-Butene-1,4-diamine, N,N'-dimethyl-		114	0.40	64.6

Table 13. GC-MS peak list, tentative NIST library identification, molecular weight, relative area, and library match quality for the chloroform extract

Peak	RT (min)	Tentatively Identified Compound (NIST)	Structure	MW (g/mol)	Area (%)	Match (%)
1	15.739	Hexadecanoic acid, methyl ester (methyl palmitate)		270	2.09	76.5
2	16.949	1H-Imidazole-4-methanol		98	1.52	77.4
3	17.269	1-Undecene, 11-nitro-		199	2.31	69.3
4	18.035	Methyl 12,13-tetradecadienoate		238	13.84	67.5

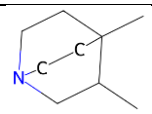
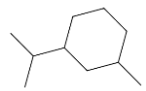
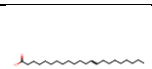
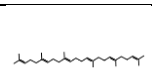
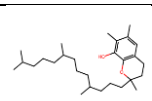
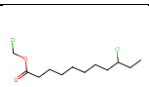
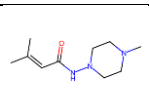
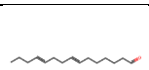
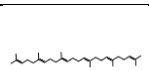
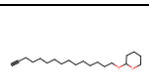
5	19.785	1-Azabicyclo[2.2.2]octane, 2,6-dimethyl-		139	54.85	71.4
6	20.366	Cyclohexane, 1-isopropyl-3-methyl-, trans-		140	12.88	76.3
7	22.542	(E)-13-Docosenoic acid (erucic acid isomer)		338	3.80	76.0
8	22.792	Squalene (2,6,10,15,19,23-hexamethyltetracosahexaene)		410	7.57	82.8
9	24.582	γ-Tocopherol		416	1.15	62.4

Table 14. GC-MS peak list, tentative NIST library identification, molecular weight, relative area, and library match quality for the methanol extract

Peak	RT (min)	Tentatively Identified Compound (NIST)	Structure	MW (g/mol)	Area (%)	Match (%)
1	16.969	Chloromethyl 9-chloroundecanoate		268	1.34	79.2
2	18.040	Methyl 13,14-octadecadienoate (18:2 methyl ester)		294	15.34	73.7
3	19.745	But-2-enoic acid, 4-(4-methylpiperazin-1-ylamino)-4-oxo-		213	65.75	69.2
4	21.681	7,11-Hexadecadienal		236	0.82	76.2
5	22.792	Squalene		410	7.67	82.8
6	23.192	2H-Pyran, 2-(3-heptadecynyloxy)tetrahydro-		336	1.70	81.6

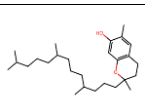
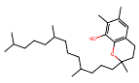
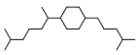
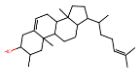
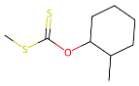
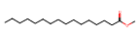
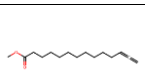
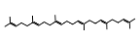
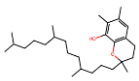
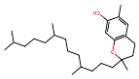
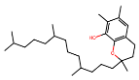
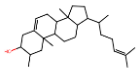
7	23.892	2H-1-Benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)- (δ-tocopherol-type tocol)		402	1.61	70.0
8	24.537	γ-Tocopherol		416	1.72	71.0
9	25.993	Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-		280	0.99	77.2
10	26.343	Cholesta-8,24-dien-3-ol, 4-methyl-, (3β,4α)- (4α-methylsterol)		398	1.71	78.5
11	26.478	Carbonic acid, dithio-, S-methyl O-(2-methylcyclohexyl) ester, trans-		204	1.35	75.2

Table 15. GC-MS compound, chemical structure, and reported biological activity

Peak	Compound (RT, min)	Structure	Reported Biological Activity
1	Hexadecanoic acid, methyl ester (methyl palmitate) (15.739)		Documented antimicrobial, antifungal, anti-inflammatory, antioxidant, and hepatoprotective activity reported in multiple recent studies [36-41].
2	Methyl 12,13-tetradecadienoate (18.035)		Unsaturated fatty acid methyl ester; polyunsaturated FAMEs of this chain length are broadly associated with membrane-modulating and anti-inflammatory activity [36,40,41].
3	(E)-13-Docosenoic acid (erucic acid isomer) (22.542)		Long-chain monounsaturated fatty acid; erucic-acid-type compounds have documented anti-inflammatory and lipid-modulating effects, with known dietary safety considerations at high intake [36,40,41].

4	Squalene (22.792)		Well-documented antioxidant, anti-inflammatory, hypolipidemic, antitumour, hepatoprotective, and antidiabetic triterpenoid [28-33].
5	γ-Tocopherol (24.582)		Potent chain-breaking antioxidant with anti-inflammatory and anticancer activity reported in recent comprehensive reviews [34,35].
6	Methyl 13,14-octadecadienoate (18:2 methyl ester) (18.040)		Linoleic-acid-type methyl ester; polyunsaturated C18 FAMEs are associated with anti-inflammatory and antimicrobial activity [36,40,41].
7	δ-Tocopherol-type tocol (23.892)		Structurally consistent with a δ-tocopherol-type tocochromanol; tocopherol homologues as a class exhibit antioxidant and anti-inflammatory activity [34,35].
8	γ-Tocopherol (24.537)		Potent chain-breaking antioxidant with anti-inflammatory and anticancer activity reported in recent comprehensive reviews [34,35].
9	Cholesta-8,24-dien-3-ol, 4-methyl- (4α-methylsterol) (26.343)		4α-Methylsterol structurally related to phytosterol biosynthetic intermediates; phytosterols as a class show cholesterol-lowering, anti-inflammatory, and anticancer activity [42-48].

3.10 Discussion

The physicochemical parameters of *Koduveli Nei* fell within the expected range for an oil-based Siddha *Nei* preparation, and a negative rancidity test indicates the sample was free of oxidative spoilage at the time of testing. All four heavy metals screened (As, Cd, Hg, Pb) were below the instrument detection limit, and calibration linearity ($R^2 > 0.99$ for all elements) supports confidence in this finding. This suggests there is no toxicologically significant heavy-metal contamination in this batch of the formulation.

Microbial load remained within WHO permissible limits, and the four specific pathogens screened (*E. coli, Salmonella* spp., *S. aureus, P. aeruginosa*) were all absent, indicating that microbial contamination was within acceptable limits for this preparation. The total aflatoxin content (3 ppb) was above the assay's detection limit of 1 ppb but remained well within the WHO permissible range of 0.5–15 µg/kg, and no organochlorine, organophosphorus, organocarbamate, or pyrethroid pesticide residues were

detected above the quantification limit. Together, these findings support the microbiological and chemical-contaminant safety of this single batch of *Koduveli Nei* as tested.

HPTLC fingerprinting provides a chemical fingerprint that complements the GC-MS and FTIR safety and identity data. The differing band/peak counts obtained under UV 254 nm, UV 366 nm, and vanillin–sulphuric acid-derivatised white light visualisation indicate the presence of structurally diverse constituents — including UV-active aromatic/conjugated compounds and vanillin–sulphuric acid-reactive terpenoid or steroidal constituents — consistent with the polyherbal, multi-ingredient composition of the formulation. The reproducibility of peak number, position, and relative area across three replicate tracks at each wavelength (Table 10) supports the suitability of this HPTLC method as a batch-identity fingerprint for future quality-control testing of *Koduveli Nei*. Although the well-documented naphthoquinone marker of the principal ingredient *Chithiramoolam* (*Plumbago zeylanica*) was not separately confirmed by an authentic reference standard in this study, one or more of the resolved HPTLC and GC-MS peaks may represent this or other principal marker constituents of the formulation; targeted marker validation is recommended as a follow-up study.

GC-MS analysis of the hexane extract returned a set of nitrogenous heterocyclic tentative hits at generally low-to-moderate library match confidence (54–73%), which fall below the ~80% threshold conventionally used to treat a spectral match as a confirmed identification; these hexane-extract identifications should therefore be regarded as tentative pending confirmation by an orthogonal method (e.g., authentic standard co-injection or high-resolution MS). By contrast, the chloroform and methanol extracts yielded higher-confidence identifications (match ≥70% for the majority of peaks) of squalene and tocopherol homologues, together with long-chain fatty-acid methyl esters. Squalene and γ-/δ-tocopherol are well-documented for antioxidant, anti-inflammatory, hypolipidemic, and hepatoprotective activity in the literature [28–35], and long-chain fatty-acid methyl esters are similarly associated with antimicrobial and anti-inflammatory effects [36,40,41]. The co-occurrence of these lipophilic constituents in *Koduveli Nei* is consistent with its castor-oil (*Ammanaku ennai*) base and supports the plausibility of the antioxidant and anti-inflammatory activity traditionally attributed to this formulation, although the present study did not include a bioactivity assay and this remains to be confirmed experimentally.

FTIR absorption bands consistent with O–H, aliphatic C–H, ester/carboxylic C=O, aromatic/chromanol C=C, and long-chain methylene functional groups corroborate the fatty-acid-ester and tocopherol-type constituents identified by GC-MS, providing an independent, whole-extract line of evidence for the chemical composition inferred from the chromatographic data.

Overall, this study establishes a preliminary physicochemical, chromatographic, and spectroscopic reference profile for a single prepared batch of *Koduveli Nei* and confirms that this batch met applicable WHO/PLIM safety limits for the heavy metals, microbial contaminants, aflatoxins, and pesticide residues tested. As a single-batch study without a therapeutic efficacy assessment, these findings should be regarded as a foundation for, rather than a substitute for, further multi-batch standardisation, marker-compound validation, and pharmacological evaluation of this classical Siddha formulation.

4. CONCLUSION

This study establishes a preliminary physicochemical, HPTLC, GC-MS, and FTIR-based analytical reference profile for a single prepared batch of the Siddha polyherbal formulation *Koduveli Nei*. The formulation met applicable WHO/PLIM safety limits for the heavy metals, microbial load, aflatoxins, and pesticide residues tested, and its GC-MS- and FTIR-supported lipophilic constituents — including squalene and tocopherol homologues — are consistent with the antioxidant and anti-inflammatory activity traditionally attributed to it. The reproducible three-wavelength HPTLC fingerprint developed here provides a candidate batch-identity tool for future quality control. Because this study examined a single batch and did not assess therapeutic efficacy, these findings should be extended through multi-batch standardisation, authentic marker-compound validation, and pharmacological studies before *Koduveli Nei* can be recommended for pharmacopoeial standardisation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

This study involved the physicochemical and chromatographic characterisation of a prepared formulation and did not involve human or animal subjects; ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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