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Physicochemical and Phytochemical Evaluation of Siddha Formulation Vani Vallari Nei for Safety and Clinical management of cerebral palsy

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ABSTRACT

Background: The Siddha system of medicine, one of the oldest traditional healing practices in South India, utilizes ghee-based (Nei) formulations for enhancing drug delivery and therapeutic efficacy, particularly in neurological conditions like Cerebral palsy (CP). Vani Vallarai Nei (VVN), a classical Siddha preparation containing Centella asiatica, Picrorhiza kurroa, Clitoria ternatea, and Acorus calamus processed in clarified ghee, is traditionally indicated for improving memory and managing neurological disorders including cerebral palsy. Despite its therapeutic relevance, scientific validation of its quality and safety has been limited.

Materials and Methods: The formulation, VVN was subjected to comprehensive standardization in line with WHO and AYUSH guidelines. The raw drugs were authenticated, purified, and formulated using classical Siddha procedures. Physicochemical characterization included parameters such as specific gravity, pH, moisture content, iodine value, saponification value, unsaponifiable matter, acid and peroxide values, and total fatty matter.

Results: Phytochemical screening of different extracts was carried out, followed by HPTLC fingerprinting to establish identity. Safety profiling involved heavy metal quantification, microbial load assessment, aflatoxin screening, pesticide residue evaluation, and stability testing over 12 months. The formulation exhibited acceptable physicochemical parameters with high fatty matter content and absence of rancidity. Phytochemical analysis revealed the presence of phenolics, tannins, terpenoids, cholesterol, quinones, and fixed oils. HPTLC provided distinct chromatographic fingerprints, supporting authentication. Heavy metals, microbial contaminants, and aflatoxins were either absent or within permissible limits, while pesticide analysis indicated compliance with AYUSH standards. Stability evaluation confirmed microbial safety and toxin-free status up to one year.

Conclusion: In conclusion, the results affirm that Vani Vallarai Nei is a stable, safe, and standardized Siddha formulation. These findings provide a strong scientific basis for its traditional use in managing neurological conditions, highlighting its potential as a supportive therapy for cerebral palsy.

Keywords: *Siddha medicine, Vani Vallarai Nei, Standardization, Neurological disorders, Cerebral palsy, Ghee-based formulations.*

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1. INTRODUCTION

The Siddha system of medicine, one of the oldest traditional medical systems practiced in South India, particularly in Tamil Nadu, emphasizes a holistic approach to health and disease management. Rooted in centuries of practice and documented in classical texts, Siddha therapy combines herbal formulations, mineral preparations, and dietary principles to restore balance in the body. Among its many dosage forms, ghee-based (nei) preparations hold a unique place because of their

capacity to extract fat-soluble bioactive constituents, improve palatability, and enhance drug delivery to the central nervous system. Such formulations are particularly valuable in the management of neurological disorders where lipid solubility and brain bioavailability are critical [1,2]. With the increasing global recognition of traditional systems, the demand for standardization and scientific validation has grown considerably. Herbal and polyherbal formulations, though widely used, often face challenges of inconsistency, contamination, and variability in preparation [3]. The Pharmacopoeial Laboratory for Indian Medicine (PLIM), under the Ministry of AYUSH, was established to provide guidelines and ensure quality control of Ayurveda, Siddha, and Unani (ASU) medicines. These regulatory measures focus on critical aspects such as raw drug authentication, purification techniques, assessment of physicochemical and phytochemical parameters, microbial contamination, heavy metals, pesticide residues, and aflatoxin limits [4]. Implementation of these quality standards is vital for both domestic and international acceptance of Siddha therapies, especially in the context of pediatric applications where safety profiles must be rigorously maintained.

Neurological disorders represent one of the most pressing global health concerns, with conditions such as epilepsy, cognitive impairment, and developmental disabilities significantly affecting quality of life. In Siddha medicine, several herbal formulations, including Vani Vallarai Nei, are traditionally prescribed for their neuroprotective, cognition-enhancing, and anticonvulsant properties. Key ingredients such as *Centella asiatica*, *Clitoria ternatea*, and *Acorus calamus* are well documented for their effects on memory enhancement, reduction of oxidative stress, and modulation of neurotransmission [5,6]. The Nei base (clarified ghee) not only stabilizes these phytoconstituents but also facilitates their transport across the blood–brain barrier, thereby improving therapeutic efficacy in neurological conditions[7]. This unique pharmacological advantage distinguishes ghee-based Siddha preparations from other formulations.

Among the neurological conditions of public health concern, cerebral palsy (CP) is a non-progressive neurodevelopmental disorder caused by injury to the developing fetal or infant brain. It is characterized by motor dysfunction and often accompanied by impairments in cognition, communication, and behavior. According to global health estimates, the prevalence of cerebral palsy ranges from 2 to 3 per 1,000 live births, with higher rates in low- and middle-income countries due to perinatal complications and limited neonatal care [8]. In India, the burden is particularly significant given the large pediatric population, with CP representing one of the leading causes of long-term disability in children [9,10]. Management of cerebral palsy remains a major challenge, as conventional therapies often provide limited relief and are associated with adverse effects such as sedation, dizziness, or gastrointestinal intolerance. In this context, Siddha medicines such as Vani Vallarai Nei offer a promising complementary approach. Their polyherbal composition provides antioxidant, anti-inflammatory, and neuroprotective effects that may aid in improving motor and cognitive functions [11,12]. Furthermore, Nei-based formulations are palatable, easy to administer to children, and possess the added advantage of nourishing properties that align with the Siddha philosophy of supporting growth and development.

Rigorous evaluation of such formulations, including quality control, microbial and toxin safety, and stability testing, is therefore essential to validate their clinical utility and promote wider adoption. Taken together, the integration of traditional Siddha wisdom with modern quality control frameworks like those established by PLIM represents a vital step toward developing safe, effective, and scientifically validated therapies. Given the growing population of children affected by cerebral palsy and the limitations of existing pharmacological interventions, the systematic study and standardization of Siddha formulations such as Vani Vallarai Nei is both timely and necessary.

2. MATERIALS AND METHODS

2.1 Ingredients and Authentication

The classical Siddha formulation Vani Vallarai Nei (VVN) is composed of carefully selected herbal ingredients processed in ghee, which serves both as a medium for extraction of bioactive compounds and as a vehicle for drug delivery. The major botanical components include *Centella asiatica* (commonly known as Vallarai), where the entire aerial and subterranean parts of the plant are utilized (5200 mL of juice/extract). This herb is well known in traditional medicine for its neuroprotective and memory-enhancing properties. The root of *Picrorhiza kurroa* (Kadugurogini, 350 g) is incorporated for its reputed hepatoprotective and anti-inflammatory activities, supporting the overall therapeutic potential of the formulation. Flowers of *Clitoria ternatea* (Sangu Poo, 350 g), recognized for their antioxidant and cognition-enhancing effects, are added after careful separation of stamens and sepals to ensure purity of the drug material. The rhizome of *Acorus calamus* (Vasambu, 350 g) is included due to its traditional use in treating neurological ailments and its role in improving sensory functions [13]. Finally, 1300 mL of clarified ghee forms the lipid base, facilitating the absorption and stabilization of the herbal constituents. All raw drug materials were procured from a reputed country drug store in Chennai, Tamil Nadu, which is known for supplying indigenous medicinal plants. To ensure reliability, the collected ingredients underwent macroscopic and organoleptic verification, followed by authentication by a qualified botanist from the National Institute of Siddha, Chennai (PIN 600047). This systematic authentication step helped in confirming the botanical identity of each component, thereby ensuring the accuracy and quality of the formulation used for subsequent analyses.

2.2 Purification of Raw Drugs

Each raw ingredient employed in the preparation of Vani Vallarai Nei (VVN) was subjected to a systematic purification procedure in line with Siddha pharmacopoeial practices, ensuring removal of extraneous matter and enhancement of medicinal efficacy [14].

- Vallarai (*Centella asiatica*): Fresh plant material was thoroughly washed under running water to eliminate adhering soil particles, dust, and other impurities. This step ensured that only clean, uncontaminated plant parts were used for subsequent processing.
- Kadugurogini (*Picrorhiza kurroa*): The roots were immersed in an extract of *Vitex negundo* for a duration of three hours. This traditional purification step is intended to reduce unwanted phytoconstituents and enhance therapeutic quality. Following immersion, the roots were carefully dried under direct sunlight until complete removal of moisture.
- Sangu Poo (*Clitoria ternatea*): From the harvested flowers, non-essential parts such as sepals, stamens, and stalks were meticulously removed. Only the petals, considered to hold the active medicinal principles, were retained for the preparation of the formulation.
- Vasambu (*Acorus calamus*): The rhizomes were subjected to controlled burning until they transformed into charcoal. The charred material was then collected for further use. This detoxification method is a classical Siddha practice to reduce potential toxicity while retaining its therapeutic essence.

These purification steps were carried out to ensure the quality, safety, and pharmacological consistency of the ingredients, thereby minimizing the risk of contamination or variability in the final formulation.

2.3 Method of Preparation of Vani Vallarai Nei (VVN)

The formulation of Vani Vallarai Nei (VVN) was carried out following traditional Siddha pharmaceutical procedures. Initially, the purified raw drugs were combined with 7800 mL of water and subjected to boiling until the volume was reduced to one-eighth of its original quantity, thereby forming a concentrated decoction. This reduction process ensured maximum extraction of the active phytoconstituents from the raw ingredients [14]. The resulting decoction was then carefully filtered to remove coarse particles and impurities.Subsequently, freshly obtained juice of *Centella asiatica* (Vallarai) along with clarified ghee was added to the filtered decoction. The mixture was continuously heated over a mild flame with constant monitoring to avoid overheating, allowing the formulation to gradually reach the required semisolid consistency, traditionally described as “Kadugu Thiral Pakkuvam” (mustard-like stage of thickness). At this stage, the ghee absorbed the medicinal essence of the herbal ingredients while attaining stability.The medicated ghee was then filtered again to remove any residual particulate matter, ensuring clarity and uniformity. Finally, the filtered preparation was placed beneath a heap of grains for a period of ten days. This storage method, rooted in classical Siddha practice, is believed to enhance the preservation, potency, and therapeutic qualities of the formulation before it is made ready for clinical use.

2.4 Physicochemical Evaluation

A comprehensive physicochemical evaluation of Vani Vallarai Nei (VVN) was performed at the Regional Research Institute of Unani Medicine (RRIUM), Chennai, in accordance with standard quality control protocols for Siddha. The analysis encompassed a wide spectrum of parameters aimed at assessing the safety, stability, and consistency of the formulation [15-18]. The preliminary examination involved organoleptic characterization, where the preparation was assessed for its physical state, color, odor, and texture. Additionally, rancidity tests were performed to determine the freshness and stability of the ghee base. Further physicochemical parameters included measurement of specific gravity, pH, moisture content (loss on drying at 105 °C), congealing point, refractive index, viscosity, iodine value, saponification value, unsaponifiable matter, acid value, peroxide value, free fatty acids, and total fatty matter content. These evaluations provided critical information regarding the physical integrity, chemical stability, and lipid profile of the medicated ghee.

Advanced quality control measures included mineral oil detection, heavy/toxic metal estimation, pesticide residue analysis, microbial contamination assessment, and screening for specific pathogens and aflatoxins, ensuring compliance with WHO and AYUSH safety standards. Additionally, High-Performance Thin Layer Chromatography (HPTLC) was employed for fingerprint profiling, which serves as an important tool for authentication and standardization by detecting unique phytoconstituents. A shelf-life study was also conducted at periodic intervals to monitor microbial load, aflatoxin levels, and overall stability during storage.

2.5 Preliminary Phytochemical Screening

Preliminary phytochemical screening of Vani Vallarai Nei (VVN) was carried out to identify the major classes of secondary metabolites present in the formulation. Successive extracts were prepared using petroleum ether, alcohol, and water as solvents to ensure extraction of both polar and non-polar phytoconstituents. Standard qualitative tests were performed included color reactions and precipitation techniques specific to different phytochemical groups [19,20]. The extracts were tested for the presence of alkaloids, carbohydrates, glycosides, proteins and amino acids, flavonoids, phenolic compounds, tannins, phytosterols, cholesterol, terpenoids, quinones, gums and mucilage, and fixed oils and fats.

2.6 HPTLC analysis

High-Performance Thin Layer Chromatography (HPTLC) was carried out to establish a fingerprint profile of Vani Vallarai Nei (VVN). Alcoholic (8 µL) and chloroform (2 µL) extracts of the formulation were applied to aluminium-backed silica gel 60 F254 TLC plates (5 × 10 cm, E. Merck) using a Camag ATS4 sample applicator [21,22]. The plates were developed in a twin-trough chamber (10 × 10 cm) pre-saturated with the mobile phase consisting of hexane and ethyl acetate in the ratio of 7.5:2.5 (v/v), to a distance of 8 cm. After development, the plates were air-dried at room temperature and visualized under UV light at 254 nm and 366 nm, followed by post-derivatization with vanillin-sulphuric acid reagent and heating at 110 °C for 5 minutes to observe spots under visible light. Densitometric scanning of the developed plates was performed to record chromatographic fingerprints at both 254 nm and 366 nm in absorbance and fluorescence modes.

2.7 Heavy Metal Analysis

Heavy metal determination in Vani Vallarai Nei (VVN) was conducted to assess the safety of the formulation with respect to toxic element contamination [23,24]. The analysis was performed using atomic absorption spectrophotometry (AAS), following the standard procedures recommended by the World Health Organization (WHO, 2007) and the Ayurvedic Pharmacopoeia of India. Test samples were digested with nitric acid and perchloric acid under controlled heating, and the clear digest was subjected to instrumental analysis. The concentrations of lead, cadmium, arsenic, and mercury were quantified and compared with the permissible limits specified by WHO and AYUSH regulatory guidelines.

2.8 Microbial Load Determination

Microbial quality assessment of the sample VVN were carried out by, ten milliliters of VVN were emulsified with polysorbate (20 or 80) and made up to 100 mL with distilled water. The emulsion was prepared by gentle warming below 45 °C for less than 30 minutes to avoid degradation [25]. From this, 1 mL aliquots were inoculated into 9 mL of sterile peptone broth and subjected to serial dilution. Microbial enumeration was carried out using the pour plate method, employing nutrient agar for bacterial growth and Sabouraud dextrose agar for fungal isolation. Plates were incubated at 37 °C for 24–48 hours for bacteria and at 25 °C for 48–72 hours for fungi. Colony counts were expressed as colony-forming units per milliliter (CFU/mL) and compared with WHO permissible limits. Samples with bacterial counts above 1 × 10^5 CFU/g were considered unsatisfactory. For pathogenic screening, aliquots were streaked onto selective media such as MacConkey agar (Escherichia coli), Mannitol salt agar (Staphylococcus aureus), cetrimide agar (Pseudomonas aeruginosa), and EMB agar (Enterobacteriaceae). Preliminary identification was confirmed by colony morphology, Gram staining, and biochemical reactions.

2.9 Aflatoxin estimation

Aflatoxin estimation was carried out using the VICAM AflaTest fluorometric method [23]. One gram of the formulation was mixed with 0.4 g of sodium chloride and extracted with a mixture of methanol and 2% Tween 20 or phosphate buffer (60:40 v/v). The mixture was vortexed at high speed for three minutes and filtered through fluted filter paper. To the filtrate, 20 mL of purified water was added and mixed again for one minute, followed by passage through a 1.5 µm glass microfiber filter. Ten milliliters of this diluted extract were passed through AflaTest WB immunoaffinity columns at a flow rate of 1–2 drops per second. Columns were washed sequentially with Tween solution and purified water before elution with 1 mL of HPLC-grade methanol. The eluate was collected in VICAM cuvettes, mixed with developer solution, and immediately analyzed using a VICAM Series 4EX fluorometer. Aflatoxins B1, B2, G1, and G2 were quantified, with a detection limit of 1 ppb, and the results were compared with WHO permissible limits of 0.5–15 µg/kg.

2.10. Stability testing of Vani Vallarai Nei (VVN)

Stability testing of Vani Vallarai Nei (VVN) was conducted to assess its microbial and toxin-free status during storage over a defined period. Samples were stored under recommended laboratory conditions and analyzed at predetermined intervals: immediately after preparation (0 month), at 6 months, and at 12 months [25]. The study was carried out in accordance with WHO guidelines for stability evaluation of herbal formulations (WHO, 2007) and the procedures adopted by the Drug Standardisation Research Unit, Regional Research Institute of Unani Medicine (RRIUM), Chennai. For microbial load assessment, 10 mL of the sample was emulsified with 1% polysorbate 20 and made up to 100 mL. From this, serial dilutions were prepared and plated using the pour plate technique. Nutrient agar was used for total bacterial count (TBC) and Sabouraud dextrose agar for total fungal count (TFC). Plates were incubated at 37 °C for 24–48 hours for bacteria and at 25 °C for 48–72 hours for fungi. Pathogen-specific screening was conducted for *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* using selective media.

3. RESULTS

3.1 Physicochemical Analysis of Vani Vallarai Nei

The physicochemical evaluation of Vani Vallarai Nei (VVN) demonstrated parameters well within acceptable limits, confirming its stability and quality. The formulation was free from rancidity, indicating the absence of lipid degradation and ensuring product freshness. The specific gravity was measured at 0.9189 g/mL, reflecting the consistency of the ghee-

based preparation. The pH value of 6.12 suggests a slightly acidic nature, which is compatible with oral administration and stability of herbal constituents. The moisture content, expressed as loss on drying, was only 0.4 percent, highlighting the low water activity that contributes to extended shelf life. The iodine value was determined as 8.81, indicating a moderate degree of unsaturation in the fatty components. The saponification value of 110.94 confirmed the presence of medium- to long-chain fatty acids, a typical characteristic of ghee-based formulations. The unsaponifiable matter was measured at 1.06, denoting the presence of minor bioactive compounds such as sterols and hydrocarbons. The acid value (0.7032 mg KOH/g) and peroxide value (1.79 meq/kg) were both within permissible ranges, reflecting minimal free fatty acid content and negligible oxidative rancidity. Importantly, the formulation contained a high total fatty matter of 97.98 percent, consistent with the ghee base, which enhances solubility and bioavailability of herbal actives.

Table 1: Physicochemical characteristics of Vani Vallarai Nei (VVN)

Parameter	Result	Unit/Remarks
Rancidity	Absent	Stable, no lipid degradation
Specific gravity	0.9189	g/mL
pH	6.12	Slightly acidic
Loss on drying	0.4	% (moisture content)
Refractive index	--	Not determined
Iodine value	8.81	Indicates unsaturation level
Saponification value	110.94	mg KOH/g (fatty acids)
Unsaponifiable matter	1.06	%
Acid value	0.7032	mg KOH/g
Peroxide value	1.79	meq/kg
Total fatty matter	97.98	%

3.2 Preliminary Phytochemical Analysis

The phytochemical screening revealed the presence of several bioactive compounds in Vani Vallarai Nei. Alcoholic extracts tested positive for phenolic compounds, tannins, cholesterol, terpenoids, and quinones, while petroleum ether extracts confirmed the presence of tannins, cholesterol, quinones, and fixed oils and fats. The aqueous extract showed negative results for all tested groups, suggesting limited solubility of the phytoconstituents in water. Notably, alkaloids, carbohydrates, glycosides, proteins, amino acids, flavonoids, phytosterols, and gums were absent in all three extracts. The predominance of tannins, phenolic compounds, terpenoids, cholesterol, and fixed oils may contribute to the formulation's pharmacological effects, particularly its antioxidant and neuroprotective activities.

Table 2. Preliminary phytochemical constituents of Vani Vallarai Nei (VVN)

Phytochemical group	Petroleum ether extract	Alcohol extract	Water extract
Alkaloids	–	–	–
Carbohydrates	–	–	–
Glycosides	–	–	–
Proteins & amino acids	–	–	–
Flavonoids	–	–	–
Phenolic compounds	–	+	–
Tannins	+	+	–
Phytosterols	–	–	–
Cholesterol	+	+	–
Terpenoids	–	+	–
Quinones	+	+	–
Gums & mucilage	–	–	–
Fixed oil & fat	+	+	–

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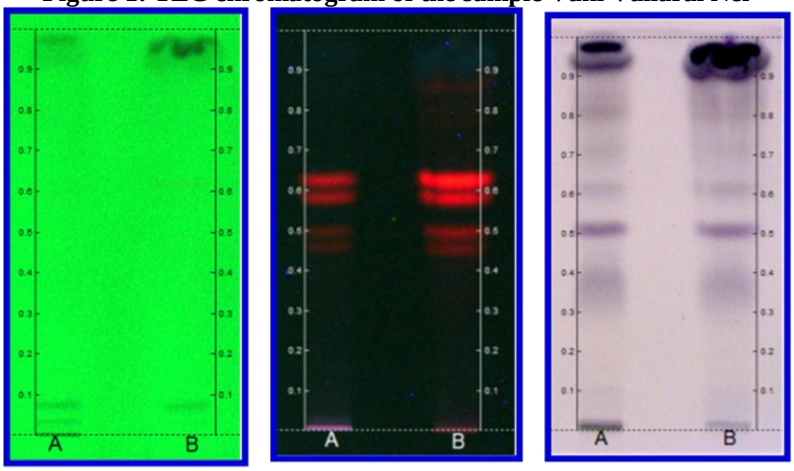
3.3. The HPTLC analysis of Vani Vallarai Nei

The HPTLC analysis of Vani Vallarai Nei produced distinct chromatographic fingerprints for both alcoholic and

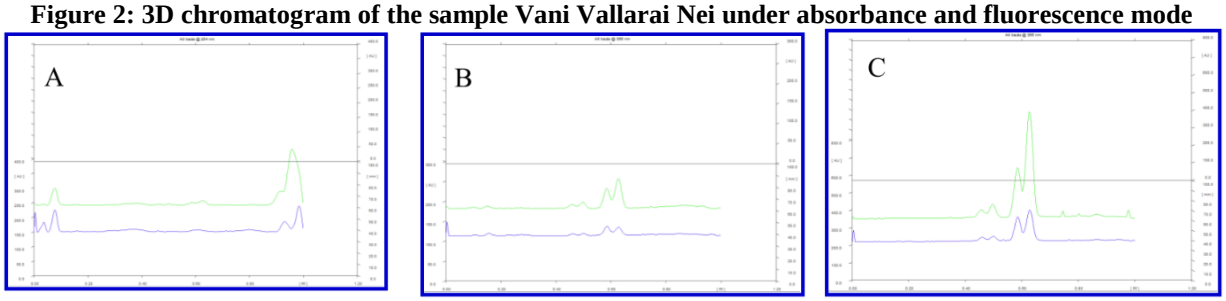
chloroform extracts. Multiple bands were observed at different Rf values under UV 254 nm, UV 366 nm (absorbance and fluorescence modes), and after derivatization with vanillin-sulphuric acid. The presence of several resolved spots indicates the complexity of phytoconstituents in the formulation and provides a characteristic fingerprint for its authentication and standardization.

Figure 1: TLC chromatogram of the sample Vani Vallarai Nei



UV – 254 nm UV – 366 nm V - S Reagent
Solvent System: Hexane : Ethyl acetate (7.5: 2.5)
Track A: Alcohol extract (8 µl); Track B: Petroleum ether extract (2 µl)

Figure 2: 3D chromatogram of the sample Vani Vallarai Nei under absorbance and fluorescence mode

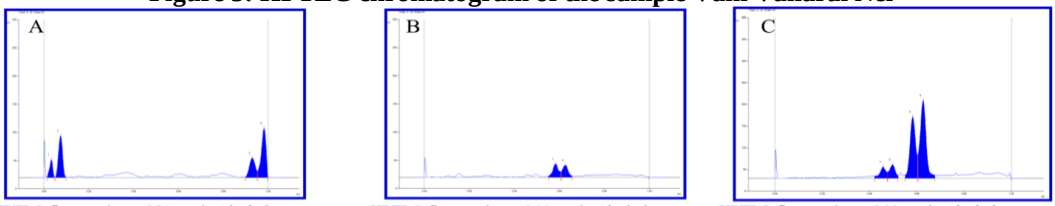


Densitometric chromatogram at 254 nm in Alcohol and Chloroform extract (Absorbance mode)

Densitometric chromatogram at 366 nm in Alcohol and Chloroform extract (Absorbance mode)

Densitometric chromatogram at 366 nm in Alcohol & Chloroform extract (Fluorescence mode)

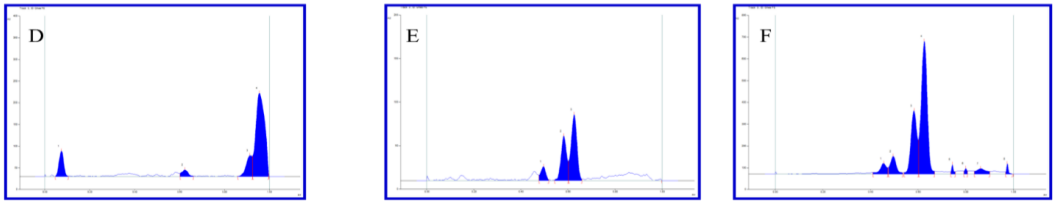
Figure 3: HPTLC chromatogram of the sample Vani Vallarai Nei



HPTLC finger print at 254 nm in Alcohol extract (Absorbance mode)

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

HPTLC finger print at 366 nm in Alcohol extract (Fluorescence mode)



HPTLC finger print at 254 nm in Chloroform extract (Absorbance mode)

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

HPTLC finger print at 366 nm in Chloroform extract (Fluorescence mode)

3.4 Heavy Metal Analysis

The heavy metal profiling of Vani Vallarai Nei indicated that the levels of toxic metals were within the permissible limits set by WHO/AYUSH standards. Lead and cadmium were found to be absent in the sample, confirming the absence of contamination from these hazardous elements. Arsenic was detected at 0.269 mg/L, well below the permissible limit of 3 ppm. Mercury was present at 0.385 mg/L, also within the acceptable range of 1 ppm. These findings suggest that the formulation is free from heavy metal toxicity concerns and is safe for internal administration.

Table 3. Heavy Metal Analysis of Vani Vallarai Nei (VVN)

Name of the Element	Result mg/L	Permissible limit (ppm)	Inference
Lead	Nil	10	Within the permissible limit
Cadmium	Nil	0.3	
Arsenic	0.269	3	
Mercury	0.385	1	

3.5 Microbial load analysis

The microbial evaluation of Vani Vallarai Nei (VVN) showed that the total bacterial count was 1 × 10^5 CFU/mL, which is within the permissible limit set by WHO for herbal formulations. Fungal contamination was minimal, with fewer than three colonies observed per plate. Screening for specific pathogenic bacteria including Enterobacteriaceae, Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pseudomonas aeruginosa revealed negative results, indicating the microbiological safety of the preparation.

Table 4. Microbial load analysis of Vani Vallarai Nei (VVN)

Parameter	Result	WHO reference limit	Inference
Total bacterial count	1 × 10^5 CFU/mL	≤ 1 × 10^5 CFU/g	Within permissible limit
Total fungal count	< 3 CFU/mL	≤ 1 × 10^3 CFU/g	Within permissible limit
Enterobacteriaceae	Absent	Absent	Complies
Escherichia coli	Absent	Absent	Complies
Salmonella spp.	Absent	Absent	Complies
Staphylococcus aureus	Absent	Absent	Complies
Pseudomonas aeruginosa	Absent	Absent	Complies

3.6 Aflatoxin analysis

The aflatoxin screening of Vani Vallarai Nei using the VICAM AflaTest method showed levels of B1, B2, G1, and G2 below the detection limit of 1 ppb. Compared with the WHO permissible range of 0.5–15 µg/kg, the formulation was considered safe and free from aflatoxin contamination.

Table 5. Aflatoxin content in Vani Vallarai Nei (VVN)

Parameter	Method	Result	WHO reference limit	Inference
Total aflatoxin (B1+B2+G1+G2)	VICAM AflaTest method	Below detection limit (< 1 ppb)	0.5–15 µg/kg	Safe, no contamination

3.6 Stability study results of Vani Vallarai Nei (VVN)

The stability evaluation of Vani Vallarai Nei at 0, 6, and 12 months demonstrated that the formulation remained microbiologically safe and free from aflatoxin contamination throughout the storage period. The total bacterial count (TBC) was consistently below the WHO permissible limit of 1 × 10^5 CFU/g, with values of 1.2 × 10^4 CFU/mL at 0 month, 1 × 10^4 CFU/mL at 6 months, and 2 × 10^4 CFU/mL at 12 months. Total fungal counts (TFC) remained negligible, ranging from less than 1 to less than 5 CFU/mL. Screening for *Enterobacteriaceae*, *E. coli*, *Salmonella spp.*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* showed no detectable growth at any time point. Aflatoxin analysis indicated that levels of B1, B2, G1, and G2 were consistently below the detection limit of 1 ppb across all intervals, confirming the absence of toxin contamination. These results confirm that Vani Vallarai Nei retains its microbial safety and toxin-free status for at least 12 months under standard storage conditions.

Table 6. Stability study results of Vani Vallarai Nei (VVN) over 12 months

Parameter	0 month (Feb 2024)	6th month (Aug 2024)	12th month (Feb 2025)	WHO reference limit	Inference
Total bacterial count	1.2 × 10^4 CFU/mL	1 × 10^4 CFU/mL	2 × 10^4 CFU/mL	≤ 1 × 10^5 CFU/g	Within permissible limit
Total fungal count	< 3 CFU/mL	< 5 CFU/mL	< 1 CFU/mL	≤ 1 × 10^3 CFU/g	Within permissible limit
Enterobacteriaceae	Absent	Absent	Absent	Absent	Complies
Escherichia coli	Absent	Absent	Absent	Absent	Complies
Salmonella spp.	Absent	Absent	Absent	Absent	Complies
Staphylococcus aureus	Absent	Absent	Absent	Absent	Complies
Pseudomonas aeruginosa	Absent	Absent	Absent	Absent	Complies
Total aflatoxin (B1+B2+G1+G2)	< 1 ppb	< 1 ppb	< 1 ppb	0.5–15 µg/kg	Safe, no contamination

4. DISCUSSION

Standardization of Siddha preparations is essential to ensure consistency, safety, and efficacy of formulations that are otherwise prone to variability in raw drug quality and processing. It builds confidence among practitioners, patients, and regulators by providing reproducible benchmarks. Standardization also minimizes risks of contamination and adulteration, particularly in pediatric and long-term therapies [26]. Ultimately, it enables Siddha medicines to align with global quality standards, facilitating wider clinical acceptance and integration into mainstream healthcare. The present investigation provides a comprehensive quality and safety profile of the classical Siddha formulation *Vani Vallarai Nei* (VVN), validating its traditional claims with modern analytical parameters. The findings affirm that nei-based Siddha formulations can be standardized through rigorous physicochemical, phytochemical, and toxicological evaluation, thereby meeting current global expectations for herbal product regulation and clinical use [27]. The physicochemical profile of VVN reflects a formulation of high stability and integrity. Parameters such as specific gravity, acid and peroxide values, and saponification and iodine indices fall well within pharmacopeial limits. These values not only demonstrate the absence of oxidative rancidity but also suggest the presence of a favorable lipid profile that can act as a vehicle for lipophilic bioactive molecules. Similar physicochemical stability has been observed in other medicated ghee preparations, which have shown resistance to oxidative degradation during prolonged storage and favorable physicochemical compatibility with phytoconstituents [28].

Phytochemical analysis revealed the presence of phenolic compounds, tannins, terpenoids, and quinones in the formulation, while alkaloids and flavonoids were absent. The dominance of phenolic and tannin fractions aligns with their well-recognized antioxidant potential, which is critical in protecting neuronal tissues against oxidative stress. Studies on polyphenolic compounds have shown significant neuroprotective effects through free-radical scavenging and modulation of inflammatory pathways [29,30]. Terpenoids and quinones, on the other hand, have been associated with neurotrophic modulation and anti-inflammatory signaling in experimental systems, supporting the potential of VVN as a neuroprotective agent [31,32].

HPTLC fingerprinting provided a unique chromatographic “barcode” for VVN, confirming its complexity and consistency across batches. The use of densitometric chromatographic profiling has been widely recommended for quality assurance in traditional medicines, as it allows authentication, detection of adulterants, and reproducibility of bioactive markers [33]. The observed multiple spots under UV and fluorescence conditions in both alcohol and chloroform extracts highlight the chemical diversity of the formulation and establish a robust identity profile for routine quality control. The safety assessment of VVN indicated compliance with international limits for heavy metals, microbial contamination, aflatoxins, and pesticide residues. The absence of lead and cadmium, and sub-permissible levels of arsenic and mercury, ensure that the formulation can be considered safe for oral consumption, especially in pediatric populations. Concerns over heavy metal contamination have long been raised as barriers for the acceptance of traditional formulations; however, well-monitored ghee-based preparations such as VVN can overcome these limitations through proper sourcing and processing of raw drugs [34].

Microbial safety was confirmed by the absence of pathogenic bacteria such as *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. The total bacterial and fungal counts remained within WHO guidelines, further confirmed by stability studies conducted over 12 months. These findings demonstrate that the formulation retains its microbiological integrity throughout storage, an essential factor for pediatric use. The significance

of long-term stability studies in herbal medicines has been emphasized by international regulatory bodies to ensure that efficacy and safety are not compromised over time [35,36].Importantly, the aflatoxin screening demonstrated that VVN is free from contamination with Aspergillus-derived toxins, which are a major health hazard in herbal formulations. Aflatoxin levels were below the detection limit across all intervals, underscoring the robustness of the preparation process and packaging. This aligns with earlier reports on the importance of aflatoxin monitoring in herbal preparations, particularly for pediatric applications where even trace contamination can have serious health consequences [37,38].

The integration of these findings suggests that VVN holds translational potential in the supportive management of neurological conditions such as cerebral palsy. While the current study establishes its quality, identity, and safety, further work is needed to delineate its pharmacological activity and clinical benefits. In particular, mechanistic studies on its antioxidant and anti-inflammatory pathways, alongside pilot clinical evaluations in children with CP, would provide evidence-based support for its use. Future studies could also benefit from incorporating metabolomic and proteomic profiling to better understand the bioactive synergy of its phytoconstituents [39,40].In conclusion, this work provides a scientific foundation for the Siddha formulation Vani Vallarai Nei, demonstrating its physicochemical stability, phytochemical richness, microbial safety, absence of contaminants, and shelf-life consistency. Such a multi-pronged validation approach is critical for bridging traditional knowledge with contemporary regulatory and clinical frameworks, ensuring that classical preparations can be safely and effectively integrated into modern healthcare systems.

5. CONCLUSION

In conclusion, the present study provides the first comprehensive standardization and safety evaluation of the Siddha formulation *Vani Vallarai Nei* (VVN), integrating classical preparation methods with modern analytical validation. The formulation demonstrated favourable physicochemical characteristics, reproducible phytochemical and HPTLC profiles, and compliance with international standards for heavy metals, microbial load, aflatoxins, and pesticide residues. Stability testing further confirmed its safety and quality over 12 months, supporting its long-term usability. These findings not only substantiate the traditional claims of VVN in managing neurological disorders but also highlight the importance of rigorous quality control in ensuring the safety, efficacy, and global acceptance of Siddha medicines. Future pharmacological and clinical investigations, particularly in children with cerebral palsy, are warranted to establish therapeutic benefits and promote wider integration of this classical preparation into evidence-based healthcare.

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