Standardization of *Keezhanelli chooranam* (KNC): A Single-Herbal Siddha Formulation

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Abstract

Standardization of herbal formulations is essential to ensure the quality, safety, and efficacy of traditional medicines. This study aimed to establish standardization parameters for *Keezhanelli Chooranam* (KNC), a Siddha formulation prepared from *Phyllanthus amarus*, traditionally used in the management of *Madhumegam* (diabetes mellitus). KNC was prepared according to classical Siddha methods and analyzed using modern techniques in accordance with AYUSH guidelines. Physico-chemical evaluation revealed a brownish, moderately fine powder with characteristic odour, particle size of 94.67 ± 25.5 µm, pH 6.6, and good solubility in water, ethanol, and DMSO. Loss on drying (5.467 ± 0.30%), total ash (0.31 ± 0.026%), extractive values, and absence of acid-insoluble ash were within acceptable limits. ICP-OES analysis confirmed the absence of toxic heavy metals. HPTLC profiling showed multiple phytochemical peaks with Rf values ranging from 0.01 to 0.66. Microbial studies confirmed sterility of the formulation. The results indicate that KNC meets standard quality and safety requirements, supporting its suitability for further pharmacological and clinical evaluation.

Keywords: *Keezhanelli Chooranam*, *Keezhanelli*, *Phyllanthus amarus,* Standardization, Siddha Medicine

Introduction

Sri Lanka has a rich heritage of traditional systems of medicine, including Ayurveda, Siddha, Unani and Desiya Chikitsa[1]. These systems have made significant contributions to the healthcare of humankind. However, a major limitation of these systems is the lack of standardized quality control parameters[2]. As the global demand for herbal medicinal products continues to grow rapidly, there is an urgent need to establish reliable quality control measures for herbal medicines originating from these traditional systems[3].

Standardization is essential to ensure the quality, purity, safety, and efficacy of herbal formulations[4,5]. *Keezhanelli chooranam* (KNC) is a traditional Siddha formulation prepared from *Keezhanelli* (*Phyllanthus amarus*) and this formulation is documented in the Siddha Pharmacopoeia of India[6].

Siddha literature highlights its potential for treating *Madhumegam* (Diabetes mellitus), *Kannoikal* (eye diseases), *Mega noi* (sexually transmitted diseases) and *Mega Pun* (wounds associated with sexually transmitted diseases)[7]. Despite its potential therapeutic uses, there is no documented evidence regarding the standardization of *Keezhanelli Chooranam*. Therefore, this study was undertaken to establish a contemporary approach to the standardization of this novel Siddha formulation, as per AYUSH guidelines, to ensure consistent quality and control over the manufacturing process. Therefore, this study was carried out to prepare *Keezhanelli chooranam* as per the standard operating procedure and also, to evaluate the standardization parameters according to the AYUSH guidelines.

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Study methodology

Preparation of the drug

Raw material collection

The *Keezhanelli* (*Phyllanthus amarus*) plant was collected from vegetable gardens in the Palali area of the Jaffna District. The whole plant of *Phyllanthus amarus* was collected during the months of January - February 2024, corresponding to its optimal vegetative growth phase

Identification and Authentication

The identification and authentication of the plant material were carried out by the National Herbarium, Department of National Botanical Gardens, Peradeniya (Ref. No: 2024/388).

Preparation Method of Keezhanelli chooranam

The whole plant of *Phyllanthus amarus* was collected, cleaned (purified and washed), and then dried under sunlight for several weeks. The dried raw material was weighed, finely powdered, and sieved through a fine mesh (80 - 100 mesh). The formulation was prepared at the Siddha Drug Production Unit, Kaithady Siddha Teaching Hospital.

Organoleptic evaluation

Description

The KNC was placed on a watch glass for macroscopic examination of its morphology and texture[4,6].

Color

The KNC was placed on a watch glass against a white background under tube light for its color observation[4,6].

Odor

The KNC was smelled twice with a two-minute interval between assessments to minimize olfactory adaptation[4,6].

Particle size

Particle size determination was carried out using the optical microscopic method. Samples were dissolved in sterile distilled water (approximately 1/100 dilution). Diluted samples were mounted on a slide and fixed on the stage at the appropriate location. Light microscopic images were drawn with a scale micrometer to determine the average particle size[8].

Physicochemical analysis

Determination of moisture content (Loss on drying)

Accurately weigh 4 g of the drug. Place it in a pre-weighed beaker and dry at 105º C constantly for 5 hours. Now weigh the drug. Continue drying and weighing every hour. This process has to be continued until the two corresponding weights don't exceed 0.25 percent. Constant weight is said to be obtained when drying and cooling for 30 minutes in a desiccator didn't show a difference more than 0.01 g[4,6].

Calculation:

$$\text{Percentage of loss on drying at } 105\,^{\circ}\text{C} = \frac{\text{Loss in weight of the sample}}{\text{Weight of the sample taken}} \times 100$$

Determination of total ash

Incinerate about 3 g accurately weighed of the ground drug in a pre-weighed silica dish. Calculate the percentage of ash with reference to the air dried drug[4,6].

Calculation:

$$\text{Percentage of total ash} = \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100$$

Determination of water soluble ash

Boil the ash obtained from above test for few minutes with 25 ml of distilled water; repeat the process for one more time. Filter the insoluble matter on an ash less filter paper and ignite in a silica crucible to constant weight. Calculate the percentage of water soluble ash with reference to the air dried drug[4,6].

Calculation:

$$\text{Percentage of water soluble ash} = \frac{\text{Weight of the total ash - weight of water insoluble residue}}{\text{Weight of the sample}} \times 100$$

Determination of acid insoluble ash

Boil the ash obtained from the above test for 5 min with 25 ml of dilute hydrochloric acid. Filter the insoluble matter on an ash less filter paper, wash with hot water and ignite in a silica crucible to constant weight. Calculate the percentage of acid insoluble ash[4,6].

Calculation

Percentage of Acid insoluble ash =

$$\frac{\text{Weight of the acid insoluble residue}}{\text{Weight of the sample}} \times 100$$

Determination of alcohol soluble extractive

About 5 g of the sample was macerated with 100 ml of alcohol in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing it to stand for the remaining 18 hours. Filter rapidly, avoiding solvent loss. Evaporate 25 ml of the filtrate to dryness in a tared flat-bottomed dish, dry at 105°C to constant weight, and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug[4,6].

Determination of water-soluble extractive

About 5 g of the sample was macerated with 100 ml of chloroform-water in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing it to stand for the remaining 18 hours. Filter rapidly, avoiding solvent loss. Evaporate 25 ml of the filtrate to dryness in a tared flat-bottomed dish, dry at 105°C to constant weight, and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug[4,6].

Determination of pH

10 grams of sample was weighed and mixed with 90 mL of distilled water. The mixture was stirred for three hours of time using rotary shaker. Then the pH was measured using pH meter[4,6].

HPTLC analysis

High-Performance Thin Layer Chromatography (HPTLC) was carried out using a CAMAG HPTLC Scanner III. The stationary phase consisted of Merck Silica Gel 60 F_{254} plates, while the mobile phase comprised chloroform: *n*-butanol : methanol : water : acetic acid (4:1:1:0.5:0.5). The sample was dissolved in methanol**,** and 10 µL of the solution was applied to the plate. The developed chromatogram was scanned at 254 nm and 365 nm wavelengths to detect and compare the phytochemical constituents present in the formulation[9].

Heavy metal analysis

The heavy metal content of *Keezhanelli chooranam* (KNC) was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) with a Perkin-Elmer 5300 DV instrument. This method was employed to quantitatively determine the presence of heavy metallic elements and to ensure the formulation complied with safety standards[10].

Microbiological tests

Sterility test (Pour Plate Method)

The sterility of KNC was evaluated using the pour plate method. The sample was inoculated into sterile Petri dishes, and 15 mL of molten agar (maintained at 45°C) was poured and gently swirled to ensure even mixing. After solidification for about 10 minutes, the plates were inverted and incubated at 37°C for 24 - 48 hours. For fungal observation, the incubation period was extended to 72 hours. The number of microbial colonies formed was counted and expressed as colony-forming units (CFU) to assess microbial contamination[4,6].

Test for specific pathogens

Specific pathogenic contamination was assessed by inoculating the sample into selective media using the pour plate method. The following media were used: Eosin Methylene Blue (EMB) agar for *Escherichia coli***,** Deoxycholate Citrate (DCC) agar for *Salmonella* species, Mannitol Salt Agar for *Staphylococcus aureus***,** and Cetrimide agar for *Pseudomonas aeruginosa***.** The inoculated plates were incubated at 37°C for 24 - 72 hours, and the pathogens were identified based on their characteristic colony color and morphological features specific to each differential medium[4,6].

Development steps of KNC

Figure 1 and 2 shows the Fresh raw materials and dried and purified raw materials of *Phyllanthus amarus* which was used to prepare *Keezhanelli chooranam.*

Fig.1: Fresh raw materials of *Phyllanthus amarus*

Fig.2: Dried and purified raw materials of *Phyllanthus amarus*

Organoleptic evaluation

Table 1 summarizes the organoleptic characteristics of the *Keezhanelli Chooranam* was a brownish, moderately fine powder with a characteristic odour and soft consistency.

Table 1: Organoleptic character of the KNC

Parameters	Interpretation
State	Solid
Nature	Moderately fine
Odor	Characteristic
Touch / Consistency	Soft
Flow Property	Non Free flowing
Appearance	Brownish

Particle size

Microscopic observation of the particle size analysis reveals that the average particle size of the KNC was found to be 94.67 ± 25.5 µm further the sample has particle with the size range of lowest 56.20 µm to highest 100.8 µm (Figure 3).

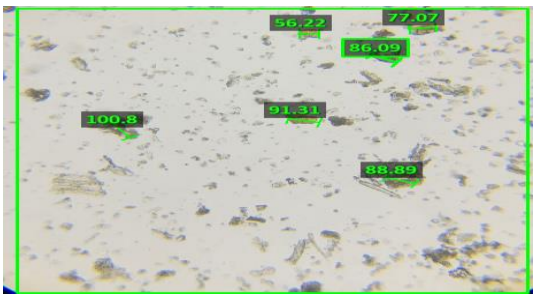


Fig.3: Microscopic observation of particle size for the sample KNC

Solubility of the KNC

The *Keezhanelli chooranam* (KNC) exhibited solubility in ethanol, water, and dimethyl sulfoxide (DMSO), indicating the presence of both polar and semi-polar phytoconstituents. The solubility in ethanol and DMSO suggests that KNC contains compounds such as alkaloids, flavonoids, and phenolic constituents, while its partial solubility in water indicates the presence of hydrophilic components like glycosides and carbohydrates. This broad solubility profile implies that KNC possesses a diverse range of bioactive constituents, contributing to its potential pharmacological activities.

Physicochemical analysis of the KNC

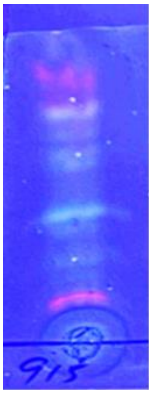
According to Table 3, the physicochemical analysis of *Keezhanelli chooranam* (KNC) showed acceptable quality, with low moisture content, minimal ash values, high water-soluble extractives, moderate alcohol-soluble extractives, and a near-neutral pH indicating good formulation stability and biological compatibility.

HPTLC analysis

As shown in Table 4 and figure 4 and 5, presence of ten prominent peaks corresponds to the presence of versatile phytocomponents present with in it. The major Rf value of the peaks ranges from 0.01 to 0.66.

Table 3: Physicochemical analysis of the KNC

Parameter	Mean (n=3) SD	Interpretation
Loss on Drying at 105 °C (%)	5.467 ± 0.30	Acceptable; ideally <10% to prevent microbial growth
Total Ash (%)	0.31 ± 0.026	Low, indicates minimal inorganic or adulterant content.
Acid insoluble Ash (%)	0 ± 0	Ideal; confirms absence of siliceous (gritty) impurities.
Water soluble Extractive (%)	9.467 ± 0.40	High; reflects rich water-soluble phytochemicals
Alcohol Soluble Extractive (%)	6.1 ± 0.2	Moderate; suggests presence of non-polar actives like resins.
pH	6.6	Near-neutral; suitable for biological compatibility and formulation stability.



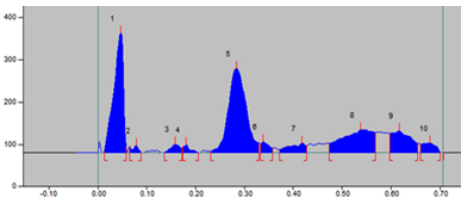


Fig.5: HPTLC fingerprinting of KNC

Fig.4: TLC plate under visible light and 366 nm

Table 4: HPTLC peak table of KNC

Peak No	Start R_f value	Max R_f value	Max %	Area	Area %
1	0.01	0.05	39.64	4247.6	26.72
2	0.06	0.08	2.37	166.3	1.05
3	0.14	0.16	2.75	288.8	1.82
4	0.17	0.18	2.54	188.7	1.19
5	**0.23**	**0.26**	**27.84**	**5466.2**	**34.38**
6	0.33	0.34	3.65	345.8	2.17
7	0.37	0,42	3.15	564.7	3.55
8	0.47	0.54	7.67	2612.0	16.43
9	0.60	0.62	7.21	1537.1	9.67
10	0.66	0.68	3.18	482.1	3.03

Heavy metals

Heavy metals analysis of KNC through the ICP OES is shown in Table 5.

Table 5: Heavy metals analysis of KNC through the ICP OES

Elements	Requirement	KNC
Arsenic	3ppm	BDL
Mercury	1ppm	BDL
Cadmium	0.3ppm	BDL
Lead	10ppm	BDL

The heavy metals analysis showed (Table 5) the presence of mercury, lead, arsenic and cadmium below the limit of quantification.

Elements analysis of KNC through the ICP OES is shown in Table 6.

Table 6: Elements analysis of KNC through the ICP OES

Element	Concentration (mg/L)
Carbon	175.150
Calcium	1.163
Copper	BDL
Iron	BDL
Potassium	21.110
Magnesium	1.321
Sodium	71.390
Phosphorus	121.300
Sulphur	1.101
Zinc	1.280

The elemental analysis of *Keezhanelli chooranam* (KNC) revealed that toxic heavy metals such as arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), and lead (Pb) were not detected (below detectable limits), indicating that the formulation is free from heavy metal contamination and safe for therapeutic use. The sample showed a high carbon content, suggesting a strong organic composition and the presence of various carbon-based bioactive compounds. Calcium (Ca) and magnesium (Mg) were present in small quantities, both of which are essential minerals that play vital roles in physiological functions. Potassium (K) content was moderately high, contributing to cellular activity and maintaining electrolyte balance. Sodium (Na) levels were found to be elevated, which may influence osmotic balance and could be relevant for the formulation's stability. Phosphorus (P) was present in high concentration, possibly indicating the existence of phospholipids or other energy-related compounds, while sulfur (S) was detected in trace amounts, suggesting the presence of sulfur-containing amino acids or secondary metabolites.

Microbiological Tests

Sterility test results of KNC is shown in Figure 6 and 7 and Table 7.

Sterility test by pour plate method

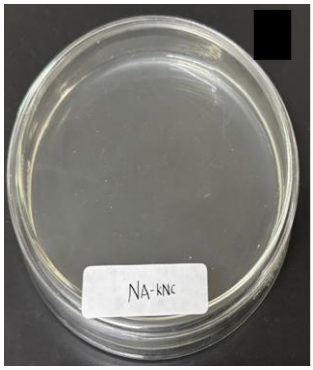


Fig.6: Control

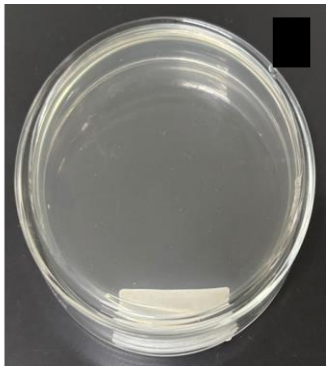


Fig.7: KNC

Table 7: Sterility test results of KNC

Test	Result	Specification
Total Bacterial Count	Absent	NMT 10^5CFU/g
Total Fungal Count	Absent	NMT 10^3CFU/g

Test for specific pathogens

Results of the Specific pathogens results of KNC is shown in Table 8.

Table 8: Specific pathogens result of KNC

Organism	Specification	Result
E-coli	Absent	Absent
Salmonella	Absent	Absent
Staphylococcus aureus	Absent	Absent
Pseudomonas aeruginosa	Absent	Absent

Culture plates showing the presence or absence of specific pathogens: *Escherichia coli* (EC) (Figure 8), *Staphylococcus aureus* (ST) (Figure 9), *Pseudomonas aeruginosa* (PS) (Figure 10), and *Salmonella* (SA) (Figure 11). Plates without and with the respective selective media.

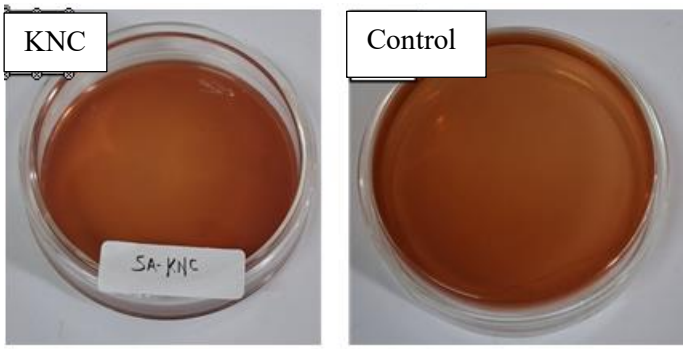


Fig.8: *Escherichia coli*

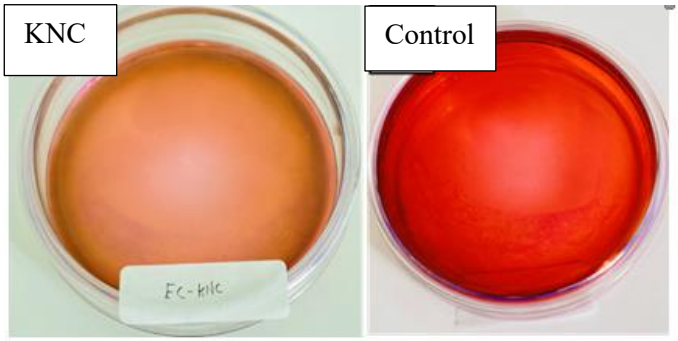


Fig.9: *Escherichia coli*

Fig.10: *Staphylococcus aureus*

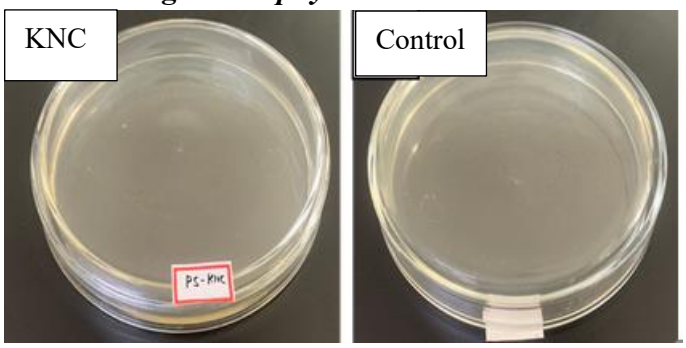


Fig.11: *Salmonella*

Discussion

The present study focused on the standardization and quality evaluation of *Keezhanelli chooranam* (KNC), a traditional Siddha formulation prepared from *Phyllanthus amarus*. The comprehensive analysis included organoleptic, physicochemical,

phytochemical, elemental, and microbiological parameters, all of which play a vital role in establishing the quality, purity, and safety of Siddha formulations as per AYUSH guidelines.

The organoleptic evaluation of KNC revealed a brownish, moderately fine powder with a characteristic odour and soft consistency, aligning with the expected physical features of a finely prepared *Chooranam*. The observed organoleptic characteristics, including colour, texture, taste, and fine powder nature, indicate the genuineness, purity, and overall quality of the test drug KNC[11]. The particle size of 94.67 ± 25.5 µm confirmed the moderate fineness of the formulation. Particle size influences solubility, processing properties, bioavailability, product uniformity, stability, and therapeutic efficiency[12]. The solubility profile in ethanol, water, and DMSO indicated the presence of both polar and semi-polar phytoconstituents, suggesting a diverse range of bioactive compounds such as alkaloids, flavonoids, glycosides, and phenolics[13,14]. Drugs that are poorly soluble in water often require higher doses to attain therapeutic plasma concentrations after intake[15].

Physicochemical parameters serve as essential quality indicators for herbal formulations[4]. The loss on drying value (5.467 ± 0.30%) was within the acceptable limit (<10%), confirming the minimal moisture content and reduced risk of microbial growth[4]. The total ash (0.31 ± 0.026%) and absence of acid-insoluble ash indicated low inorganic contamination and absence of earthy or siliceous materials, reflecting purity of the sample[4]. The extractive values further highlighted the presence of both water-soluble and alcohol-soluble constituents, which are indicative of the phytochemical richness of *Phyllanthus amarus*. The near-neutral pH (6.6) supports formulation stability and biological compatibility, making it suitable for internal use[16,17].

The HPTLC profile revealed ten prominent peaks with Rf values ranging from 0.01 to 0.66, confirming the presence of multiple phytoconstituents[18,19,20]. This chromatographic fingerprint can serve as a reference standard for future identification and quality control of KNC batches. Similar multi-component patterns have been reported in other standardized Siddha formulations, supporting the reliability of HPTLC as a powerful tool for phytochemical profiling.

Heavy metal analysis through ICP-OES demonstrated that toxic metals such as mercury, lead, cadmium, and arsenic were below detectable limits, ensuring the safety of the formulation. The presence of essential elements like calcium, magnesium, potassium, and phosphorus reflects the mineral richness of the formulation, which may contribute to its therapeutic efficacy. These findings are consistent with similar studies on Siddha formulations that emphasize the importance of ensuring heavy metal-free preparations for patient safety[4,21,22].

Microbiological evaluation confirmed the absence of total bacterial and fungal counts, as well as specific pathogens such as *E. coli*, *Salmonella*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. This indicates the formulation's sterility and compliance with AYUSH standards for microbial safety in herbal products[4].

Overall, the study successfully established a comprehensive standardization profile for *Keezhanelli chooranam*. The results confirm that the formulation meets the acceptable quality limits and is safe for therapeutic application. The combined application of traditional Siddha preparation techniques and modern analytical methods ensures reproducibility, authenticity, and quality assurance. Further studies, including pharmacological and clinical evaluations, are recommended to validate its therapeutic efficacy and to support its inclusion in evidence-based Siddha pharmacopeial standards.

Conclusion

The present study successfully established comprehensive standardization parameters for *Keezhanelli chooranam* (KNC), a classical single-herb Siddha formulation. All evaluated physicochemical, phytochemical, elemental, and microbiological parameters were found to be within prescribed AYUSH limits, confirming the formulation's quality, purity, and safety. The generated HPTLC fingerprint can serve as a reference

standard for future batch-to-batch quality control. These findings support the scientific validation of KNC and provide a foundation for further pharmacological and clinical investigations to substantiate its therapeutic efficacy.

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