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SYSTEMATIC STANDARDIZATION OF NOVEL SIDDHA FORMULATION *PASUNEER KADUKKAI CHOORANAM* BY MODERN ANALYTICAL TECHNIQUES

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ABSTRACT

The Siddha system of medicine is recognised as one of the most ancient Indian systems of medicine, with a long-standing history of human knowledge. Numerous indigenous siddha formulations, developed and postulated by ancient siddha practitioners, continue to be employed in the therapeutic treatment of several severe metabolic illnesses in humans, such as diabetes mellitus. The global market for siddha formulations appears to be noteworthy; nonetheless, it is imperative to prioritizes the assurance of preparation standards at now. The assurance of product quality and authenticity is essential throughout the whole production process, from the initial raw materials to the final completed product. The main aim of the present investigation is to standardize the novel formulation Pasuneer Kadukkai chooranam (PNKC) as per AYUSH guideline and to reveal the property of the formulation to the scientific community for better understanding about the standards of the formulation. The results obtained from the HPTLC analysis of the sample PNKC reveals the presence of four versatile phytocomponents present with in it. Rf value of the peaks ranges from 0.03 to 0.63. Result analysis on acid radical analysis of the formulation PNKC reveals the presence of carbonates, sulfates, phosphates and Nitrates. Further test on basic radicals reveals the presence of lead and arsenic. Results analysis on heavy metals analysis clearly shows that the sample shows the presence of Lead and Arsenic at 2.84 and 0.96 PPM level. Outcome of specific pathogen test of PNKC indicates the absence of pathogenic microbes such as E-coli, Salmonella, Staphylococcus Aureus and Pseudomonas Aeruginosa. Pesticide residue analysis signifies that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus, Organo carbamates and pyrethroids, further the formulation PNKC were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. It was concluded from the results of the present investigation that the formulation PNKC complies with the standard as prescribed by the guideline.

KEY WORDS: ***Siddha system, Pasuneer Kadukkai chooranam, Standardization, Physiochemical, AYUSH, HPTLC, Pesticide residue, Aflatoxin***

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1. Introduction

The significance of health has been paramount for humanity from ancient times. The global market for health-related items has seen significant activity, with these products being created in various regions worldwide and distributed on a global scale. The implementation of standardisation is necessary in order to ensure the consistent availability of a homogeneous product throughout all global regions. The process of standardisation ensures the production of a consistently superior product with assured components [1]. ASU systems employ plants, minerals, and animal products as primary therapeutic agents for the treatment of several medical conditions [2]. Herbal medicine, also known as botanical medicine or phytomedicine, pertains to the utilisation of various plant components such as seeds, berries, roots, leaves, bark, or flowers for therapeutic applications. The practise of herbalism has a rich historical lineage, existing as a distinct discipline separate from traditional medicine. The use of herbal medicine in the treatment and prevention of diseases is gaining popularity due to advancements in analysis, quality control, and clinical research, which have demonstrated its efficacy [3,4].

Natural goods are widely utilised on a global scale, constituting a significant proportion, around 70%, of the pharmaceuticals currently accessible on the worldwide market. Nevertheless, the biodiversity is facing a significant peril [5] as a result of excessive extraction of natural resources for the purpose of producing herbal medicines and healthcare items. Given the precarious status of several medicinal plants on the endangered list, it is advisable to consider embracing the ancient practise of mineral-based medicine that has been prevalent in India. The Siddha method, an old traditional Indian medicinal practise, incorporates the use of metals, metalloids, and minerals [6], which include hazardous and heavy elements like lead, mercury, and arsenic. The usage of these traditional remedies has elicited public concerns and raised anxieties among a significant portion of the community [7-9].

The exploration and creation of monographs for indigenous and unique preparations have been recognized as a valuable foundation for future researchers to identify their preferred medicine for their research endeavors. In order to address the need of medication standardization, the current study aims to establish standardized parameters for the traditional polyherbal siddha formulation known as Pasuneer Kadukkai chooranam (PNKC). This formulation has been traditionally utilized for the treatment of severe medical conditions. Currently, there is a lack of literature data about the standardization and phytochemical analysis of the formulation PNKC. This has motivated the researcher to do a systematic standardization of PNKC in accordance with the criteria provided by AYUSH.

2. Materials and Methods

2.1.Collection and Identification of raw materials

The required raw drugs are procured from a well reputed indigenous drug shop. The raw drugs were identified and its quality assessed by expert from the relevant department. The raw drugs were purified and the medicines are prepared in accordance with standardized protocol in order to formulate Pasuneer Kadukkai chooranam (PNKC).

2.2. Particle Size analysis [10]

Particle size determination was carried out by optical microscopic method. In which the sample were dissolved in the sterile distilled water (app 1/100th dilution). Diluted sample were mounted on the slide and fixed with stage of appropriate location. Light microscopic image was drawn with scale micrometer to arrive at the average particle size. Minimum 30 observations were made to ascertain the mean average particle size of the sample.

2.3. HPTLC analysis [11]

HPTLC method is a modern sophisticated and automated separation technique derived from TLC. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively. High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for

the evaluation of botanical materials efficiently and cost effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. Thus this method can be conveniently adopted for routine quality control analysis. It provides chromatographic fingerprint of phytochemicals which is suitable for confirming the identity and purity of phytotherapeutics.

2.3.1.HPTLC- Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analyzed. After elution, plates were taken out of the chamber and dried.

2.3.2. HPTLC- Scanning

Plates were scanned under UV at 366nm. The data obtained from scanning were brought into integration through CAMAG software. Chromatographic finger print was developed for the detection of phytoconstituents present in each sample and their respective Rf values were tabulated.

2.4. Test for acid and basic radicals [12,13]

In biochemical analysis the test for specific acid radical carried with respect to carbonates, chlorides, sulfates, sulfides, phosphates, fluoride, oxalate, borates and nitrates. Further test for basic radicals proceeded for identification of lead, mercury, arsenic, copper, ferric, ferrous, zinc, silver and magnesium.

2.5. Heavy metal analysis [14]

Atomic Absorption Spectrometry (AAS) is a very common and reliable technique for detecting metals and metalloids in environmental samples. The total heavy metal content of the sample was performed by Atomic Absorption Spectrometry (AAS) Model AA 240 Series. In order to determination the heavy metals such as mercury, arsenic, lead and cadmium concentrations in the test item. Test sample was digested with 1mol/L HCl for determination of arsenic and mercury. Similarly, for the determination of lead and cadmium the sample were digested with 1mol/L of HNO3 for standard such as As & Hg- 100 ppm sample in 1mol/L HCl, similarly for metals such as Cd & Pb- 100 ppm sample in 1mol/L HNO3.

2.6. Test for Specific pathogen

Test sample was inoculated in sterile petri dish to which about 15 mL of molten agar 45oC were added. Agar and sample were mixed thoroughly by tilting and swirling the dish. Agar was allowed to completely gel without disturbing it. (about 10 minutes). Plates were then inverted and incubated at 37o C for 24-48 hours and further extended for 72 hrs for fungal growth observation. Grown colonies of organism was then counted and calculated for CFU.

2.7.Test for Sterility

Test sample was directly inoculated in to the specific pathogen medium (EMB, DCC, Mannitol, Cetrimide) by pour plate method. The plates were incubated at 37oC for 24 - 72h for observation. Presence of specific pathogen identified by their characteristic colour with respect to pattern of colony formation in each differential media.

2.8. Pesticide residue Analysis [15,16]

Test sample were extracted with acetone and followed by homogenization for brief period. Further filtration was allowed and subsequent addition of acetone to the test mixture. Heating of test sample was performed using a rotary evaporator at a temperature not exceeding 40ºC until the solvent has almost completely evaporated. To the residue add a few milliliters of toluene and heat again until the acetone is completely removed. Resultant residue will be dissolved using toluene and filtered through membrane filter.

2.9. Aflatoxin Analysis [17]

Standard aflatoxin was applied on to the surface to pre coated TLC plate in the volume of 2.5 µL, 5 µL, 7.5 µL and 10 µL. Similarly, the test sample was placed and Allow the spots to dry and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone and isopropyl alcohol (85: 10: 5) until the solvent front has moved not less than 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent from

and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.

3. Results

3.1. Results of Particle Size analysis

Microscopic observation of the particle size analysis reveals that the average particle size of the sample was found to be 68.65 ± 25.21 µm. As shown in figure 1.

3.1. Results of HPTLC chromatogram

It was observed from the study that 38 physicians (76%) were giving pre-treatment procedure. As shown in Figure 1.

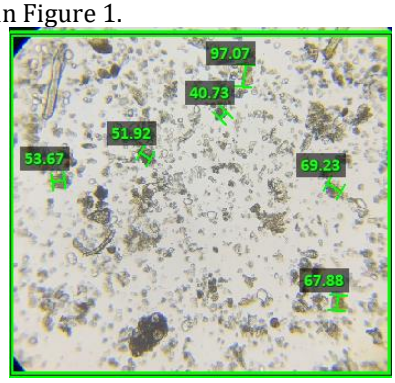


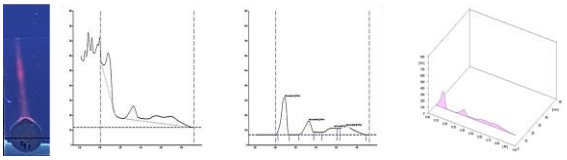
Figure 1: Microscopic Observation of Particle Size for the PNKC

3.2. Results of HPTLC chromatogram

HPTLC finger printing analysis of the sample reveals the presence of four prominent peaks corresponds to the presence of four versatile phytocomponents present with in it. Rf value of the peaks ranges from 0.03 to 0.63. As listed in table 1 and figure 2.

Table 1: Peak Table analysis of PNKC

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	25.9	0.09	251.8	56.26	0.14	0.0	5792.7	41.38
2	0.23	1.2	0.33	91.8	20.51	0.38	18.3	2665.1	19.04
3	0.46	17.8	0.58	47.6	10.63	0.61	43.0	2218.9	15.85
4	0.63	42.3	0.70	56.4	12.60	0.89	1.0	3322.0	23.73



TLC **HPTLC finger printing** **3D Chromatogram**

Figure 2: HPTLC finger printing of Sample PNKC

3.3. Results on acid and basic radicals

Result analysis on acid radical analysis of the formulation PNKC reveals the presence of carbonates, sulfates, phosphates and Nitrates. Further test on basic radicals reveals the presence of lead and arsenic. As shown in table 2.

Table 2: Results on acid and basic radical's analysis of PNKC

Test for Acid Radicals	
Specific Radical	**Test Report**
Test for carbonates	Positive- Indicates Presence
Test for chlorides	Negative - Indicates Absence
Test for sulfates	Positive- Indicates Presence
Test for sulphides	Negative - Indicates Absence
Test for phosphates	Positive- Indicates Presence
Test for Fluoride and Oxalate	Negative - Indicates Absence
Test for Borates	Negative - Indicates Absence
Test for Nitrates	Positive- Indicates Presence
Test For Basic Radicals	
Specific Radical	**Test Report**
Test for Lead	Positive- Indicates Presence
Test for Arsenic	Positive- Indicates Presence
Test for Mercury	Negative - Indicates Absence
Test for Copper	Negative - Indicates Absence
Test for Ferric	Negative - Indicates Absence
Test for Ferrous	Negative - Indicates Absence
Test for Zinc	Negative - Indicates Absence
Test for Silver	Negative - Indicates Absence
Test for Magnesium	Negative - Indicates Absence

3.4. Results of Heavy metal analysis

Result analysis on heavy metals analysis of PNKC clearly shows that the sample shows the presence of Lead and Arsenic at 2.84 and 0.96 PPM level as listed in the table 3.

Table 3: Results on Heavy metal analysis of PNKC

Name of the Heavy Metal	Absorption Max Λ max	Result Analysis	Maximum Limit
Lead	217.0 nm	2.84 PPM	10 ppm
Arsenic	193.7 nm	0.96 PPM	3 ppm
Cadmium	228.8 nm	BDL	0.3 ppm
Mercury	253.7 nm	BDL	1 ppm

3.5. Results on Specific pathogen

Result analysis on Specific pathogen test of PNKC indicates the absence of pathogenic microbes such as E-coli, Salmonella, Staphylococcus Aureus and Pseudomonas Aeruginosa. As listed in table 4 and figure 3.

Table 4: Results on Specific pathogen analysis of PNKC

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

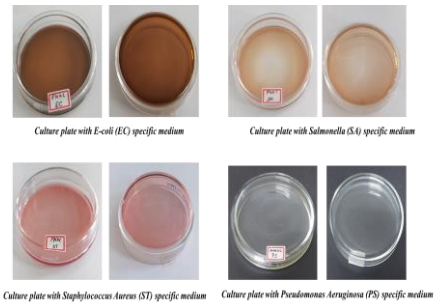


Figure 3: Specific pathogen analysis of PNKC

3.6. Results on sterility analysis

Result analysis on sterility test of PNKC reveals No growth / colonies was observed in any of the plates inoculates with the test sample. As listed in table 5 and figure 4.

Table 5: Results on sterility analysis of PNKC

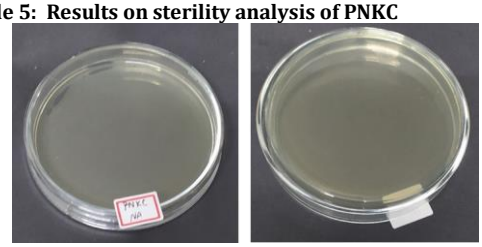


Figure 4: Sterility analysis of PNKC

Table 5: Results on sterility analysis of PNKC

Test	Result	Specification	As per AYUSH/WHO
Total Bacterial Count	Absent	NMT 10^5CFU/g	As per AYUSH specification
Total Fungal Count	Absent	NMT 10^3CFU/g	

3.7. Results on Pesticide residue Analysis

Table 6: Test Result Analysis of the Sample PNKC

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

Result analysis on pesticide residue analysis of PNKC reveals that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus, Organo carbamates and pyrethroids in the sample provided for analysis. As listed in table 6.

3.8. Results on Aflatoxin Analysis

Result analysis on pesticide residue analysis of PNKC shown that there were no spots were being identified in the test sample loaded on TLC plates when compare to the standard which indicates that the sample were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. As listed in table 7.

Table 6: Aflatoxin analysis of PNKC

Aflatoxin	**Sample PNKC**	**AYUSH Specification Limit**
B1	Not Detected - Absent	0.5 ppm
B2	Not Detected - Absent	0.1 ppm
G1	Not Detected - Absent	0.5 ppm
G2	Not Detected - Absent	0.1 ppm

4. Discussion

Standardization is a procedural method that ensures the preservation of uniformity in both the asserted effectiveness of a product and its ability to be reproduced consistently from one batch to another. One of the primary obstacles in achieving scientific standardization that aligns with industry norms is the presence of variations in the source, the absence of safety assessments, and the problems associated with quality control [18].

The process of standardization requires the verification of a natural plant product's authenticity at its source by employing approved agricultural methods [19], strategies for gathering from the wild, and suitable manufacturing practices for extraction methods and associated qualities [20-24]. The acknowledgment of a lead compound as a potential therapeutic candidate requires precise identification, authentication, and concentration of its active constituents [25,26]. This is particularly important when dealing with polyherbal formulations, where specific amounts of active components need to be determined [27,28].

In order to maintain a consistent chemical profile and biological activity of future drug candidates, regulatory approvals are required. These approvals involve quality assurance measures such as the identification of adulterants, pesticide residue, aflatoxin content, bacterial/fungal growth, and heavy metal contamination, among other factors. The results obtained from the HPTLC analysis of the sample PNKC reveals the presence of four versatile phytocomponents present with in it. Rf value of the peaks ranges from 0.03 to 0.63.

Result analysis on acid radical analysis of the formulation PNKC reveals the presence of carbonates, sulfates, phosphates and Nitrates. Further test on basic radicals reveals the presence of lead and arsenic. Results analysis on heavy metals analysis clearly shows that the sample shows the presence of Lead and Arsenic at 2.84 and 0.96 PPM level. Outcome of specific pathogen test of PNKC indicates the absence of pathogenic microbes such as E-coli, Salmonella, Staphylococcus Aureus and Pseudomonas Aeruginosa. Pesticide residue analysis signifies that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus, Organo carbamates and pyrethroids, further the formulation PNKC were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. It was concluded that the formulation PNKC complies with the standard as prescribed by the guideline.

5. Conclusion

There has been a global endeavour to oversee the quality and govern the expanding industry of herbal medications and traditional medicine. Health agencies and governments from several nations have demonstrated a proactive commitment to the provision of standardized botanical drugs. The present investigation's results indicate that the formulation PNKC adheres to the standard outlined in the guideline.

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