

Standardization of Anda Neer prepared as per Siddha Literature

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Abstract

Siddha drugs are natural products obtained from herbal, mineral and animal kingdom. Most of the Siddha medicines are effective but they have not been standardized yet. So, there be in a need to progress a standardization technique. Anda Neer is a classical Siddha preparation for curing redness of eye mentioned in the textbook of Siddhar Aruvai Maruthuvam written by Dr. K. S. Uthamarayan.. The aim of this study to estimate quality of Anda Neer by conducting physico-chemical analysis, and other analytical techniques. This study was done based on Pharmacopoeial laboratory for Indian Medicine guidelines. Other analytical technique like High performance thin layer chromatography was done through CAMAG software. The physical parameters like state, odour, touch, flow Property and appearance/colour revealed that it was liquid, characteristic odour, non-greasy to touch, free flowing, colourless and transparent. So the drug AN is good in nature and safe to use. The pH value of AN was 7 which indicates that the drug is neutral in nature. A more acidic or alkaline pH value induces lacrimation, ocular pain, and discomfort. In the clarity test observation it was found as clear solution with no major visible particles. HPTLC finger print analysis of the extracts showed the presence of possible number of components. The test drug is devoid of microbial contamination, pesticide residues and aflatoxin assay were below quantification limit. From the result we infer that the drug is of standard quality and shall be used as reference in pharmacopoeia standards.

Keywords: Standardization; Anda Neer; Zoological formulation of kalikkam; Siddha

1. Introduction

In Siddha system there are 32 types of external therapies/ medicines explained by Saint Theraiyar in his text Theraiyar Tharu.Kalikam (Liquid Ophthalmic Application/eye drop) is one of the 32 forms of Pura Marunthu (external applications). Pura Marunthu plays a major role in Siddha system of Medicine.^[1] Kalikam is the one of the way for the systemic absorption through ocular route. Apart from treating eye diseases, Kalikam is also having proven records in treating conditions such as Long Sight, Short Sight, Headache, Stomach Pain, Skin Disease, Leucoderma, psoriasis etc. Kalikam – cures the abnormalities of the disease causing three dosas Vatha, Pitha, Kabam and their imbalance.

Thus, the present study deals with standardization of Siddha zoological formulation, Anda Neer a Siddha drug mentioned in the text Siddhar Aruvai Maruthuvam which is used to treat eye inflammation of conjunctiva.^[2] Till now there is no clear documentation available on standardization .This is proved through the systematic standardization of the test drug by physico-chemical and HPTLC finger printing aspects according to PLIM guidelines.

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2. Material and methods

2.1. Selection of the trial drug

For this present study, the zoological formulation “*Anda Neer*” a compound drug preparation for *Kan Sivappu* (Eye inflammation of conjunctiva) has been chosen from classical *Siddha* literature – “*Siddhar Aruvai Maruthuvam* written by Dr. K. S. Uthamarayan.”^[2] Published by *Department of Indian Medicine & Homoeopathy, Chennai, Govt. of Tamil Nadu*, page: No: 262.

2.2. Collection Of the Raw Materials

The fresh eggs were to be purchased from super market, Palayamkottai. Sugar candy will be collected from the raw drug shop.

2.3. Identification and authentication of the drugs:^[1]

Fresh eggs were identified by egg float test as per *Siddha* literature. The raw materials Identification and authentication will be obtained from faculties Of Gunapadam department, Government *Siddha* medical college, Palayamkottai.

2.4. Preparation of the ANDA NEER:^[2]

Boil hens egg well, remove a small piece of eggshell and kundri size of white yolk on the top side and through it yellow yolk completely scratched out with a needle, wash it with water and filled with powdered sugar candy, cover the hole with eggshell and seal the eggshell with honey wax, cover it with a Cloth and bury it in the soil for 12 hours at night time. After 12 hours *Anda Neer* has been Collected from egg.

Indications: It cures eye inflammation of conjunctiva

Preparation process of AN is illustrated in fig1 to 12 as below

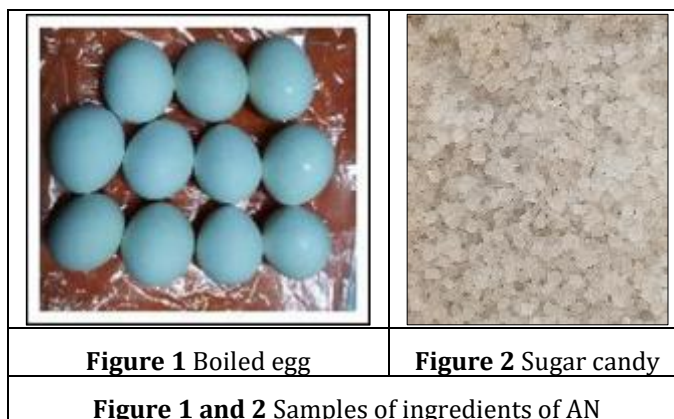


Figure 3 kundri size hole made top of it



Figure 4 Powdered sugar candy



Figure 5 after removing yellow yolk



Figure 6 hole filling with powdered sugar candy



Figure 7 hole is covered with eggshell & Eggshell sealed with honey wax



Figure 8 Egg covered with cloth&bury in soil for 12hours



Figure 9 The process of burying the eggs in the soil



Figure 10 After 12hours remove From soil & cloth



Figure 11 AN was seen After removal of covered eggshell



Figure 12 Sample of prepared AN

These following studies were done at Noble Research Solutions, Perambur at Chennai.

2.5. Physico-chemical Evaluation ^[3-5]

The samples were analysed for the Physico-chemical parameters like Description-Color, Odour, pH, Clarity test

2.5.1. Determination of pH

About 5g of test sample AN was dissolved in 25ml of distilled water and filtered. The resultant solution was allowed to stand for 30mins and then subjected to pH evaluation

2.5.2. Determination of Clarity test

Clarity testing was carried out to check the particulate matter in the sample AN. In this test transparent particles or white particles observed against the black background and the black or dark particles observed against the white background.

2.6. Thin Layer Chromatography (TLC) Analysis ^[6]

Test sample was subjected to thin Layer chromatography (TLC) as per Conventional one dimensional ascending Method using silica gel 60F254, 7X6 cm (Merck) were cut with ordinary household Scissors. Plate markings were made with Soft pencil. Micro pipette was used to spot The sample for TLC applied sample volume 10-micro litter by using pipette at distance Of 1 cm at 5 tracks. In the twin trough Chamber with the specified solvent system After the run plates are dried and were Observed using visible light Short-wave UV Light 254nm and light long-wave UV light 365 nm.

2.7. High Performance Thin Layer Chromatography Analysis (HPTLC) ^[6]

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.

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 analysed. After elution, plates were taken out of the chamber and dried.

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.

2.8. Sterility Test by Pour Plate Method^[7]

Objective

The pour plate techniques were adopted to determine the sterility of the product. Contaminated / un sterile sample (formulation) when come in contact with the nutrition rich medium it promotes the growth of the organism and after stipulated period of incubation the growth of the organism was identified by characteristic pattern of colonies. The colonies are referred to as Colony Forming Units.(CFUs).

2.8.1. Methodology

Test sample was inoculated in sterile petri dish to which about 15 mL of molten agar 45°C 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 37° 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.

2.9. Test for Specific Pathogen^[8]

2.9.1. Methodology

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

Table 1 Detail of Specific Medium and their abbreviation

Organism	Abbreviation	Medium
<i>E-coli</i>	EC	EMB Agar
<i>Salmonella</i>	SA	Deoxycholate agar
<i>Staphylococcus aureus</i>	ST	Mannitol salt agar
<i>Pseudomonas aeruginosa</i>	PS	Cetrimide Agar

2.10. Analysis of Pesticide Residue Organochlorine, Organophosphorus,

2.10.1. Organocarbamates, Pyrethroids^[9-10]

Extraction

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 40°C until the solvent has almost completely evaporated. To the residue add a few millilitres of toluene and heat again until the acetone is completely removed. Resultant residue was dissolved using toluene and filtered through membrane filter.

2.11. Aflatoxin assay by Thin Layer Chromatography (TLC) (B1, B2, G1, G2)^[11]

2.11.1. Solvent

Standard samples was dissolved in a mixture of chloroform and acetonitrile (9.8 : 0.2) to obtain a solution having concentrations of 0.5 µg per ml each of aflatoxin B1 and aflatoxin G1 and 0.1 µg per ml each of aflatoxin B2 and aflatoxin G2.

2.11.2. Procedure

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 front 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. Physico-chemical parameters

Results tabulated in table 2 and illustrated in Figure 13.



Figure 13 Physico-chemical parameters

Table 2 Physico-chemical parameters

1	State	Liquid
2	Odour	Characteristic
3	Touch	greasy
4	Flow Property	Free Flowing
5	Appearance/ Colour	Colourless / Transparent
6	PH	7

3.2. Clarity test Report

Results illustrated in Figure 14 and 15



Figure 14 Clarity at Dark Background



Figure 15 Clarity at White Background

High Performance Thin Layer Chromatography Analysis (HPTLC) of AN

Results illustrated in Figure 16 and 17 and tabulated in Table 3.

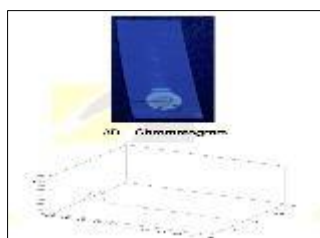


Figure 16 TLC Visualization of AN at 366 nm 3D – Chromatogram

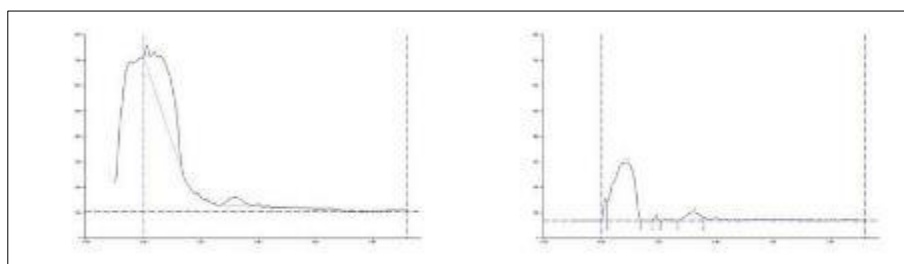


Figure 17 HPTLC finger printing of Sample AN

Table 3 Peak value

Peak	Start Rf	Start Height	Max Rf	Max Height	Max%	End Rf	End Height	Area	Area%
1	0.02	75.1	0.09	229.8	83.29	0.14	1.7	8158.2	90.09
2	0.18	0.9	0.19	11.5	4.18	0.20	0.5	76.6	0.85
3	0.27	0.4	0.32	34.6	12.53	0.36	12.4	820.8	9.06

3.3. Analysis of Pesticide residue Organochlorine, Organophosphorus, Organo carbamates, Pyrethroids

The results showed that there were no pesticide traces such as Organo chlorine, Organo phosphorus, Organo carbamates and Pyrethroids in the sample AN provided for analysis. Results tabulated in Table 4.

Table 4 Pesticide residue report

Pesticide Residue	Sample AN	AYUSH Limit (mg/kg)
I. Organo Chlorine Pesticides		
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	10µg/kg	1mg/kg
Chlorpyriphos	BQL	0.2 mg/kg
Dichlorovos	BQL	1mg/kg
III. Organo carbamates		
Carbofuran	BQL	0.1mg/kg
IV. Pyrethroid		
Cypermethrin	BQL	1mg/kg

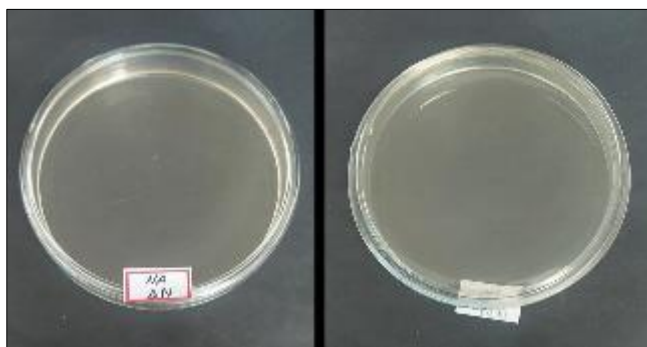
BQL- Below Quantification Limit

3.4. Microbial contamination test by pour Plate method

Results illustrated in Figure 18 and tabulated In Table 5.

Table 5 Sterility Report

Test	Result	Specification	As per AYUSH/WHO
Total Bacterial Count	Absent	NMT 105CFU/g	As per AYUSH specification
Total Fungal Count	Absent	NMT 103CFU/g	

**Figure 18** Microbial contamination test by pour plate method**3.5. Test for Specific Pathogen**

Shown in Table 6 and illustrated in Figure No growth was observed in any of 19,20,21 and 22. the plates inoculated with the test sample

Table 6 Specific Pathogen Report

Organism	Specification	Result	Method
<i>E-coli</i>	Absent	Absent	As per AYUSH specification
<i>Salmonella</i>	Absent	Absent	
<i>Staphylococcus aureus</i>	Absent	Absent	
<i>Pseudomonas aeruginosa</i>	Absent	Absent	

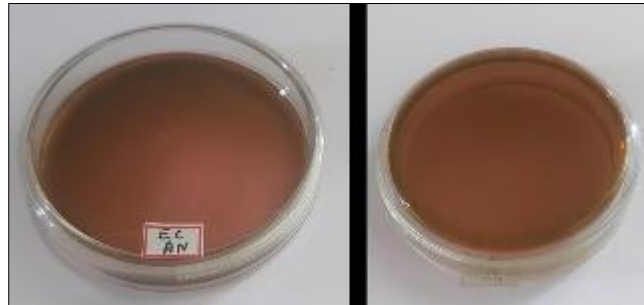


Figure 19 Culture plate with E-coli (EC) specific medium

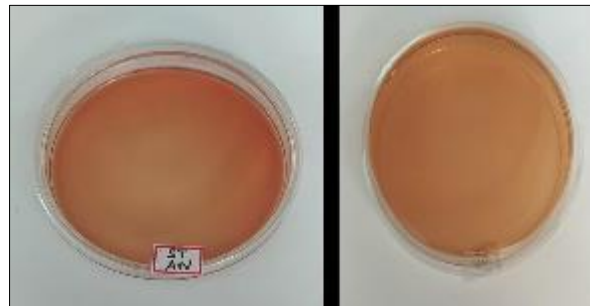


Figure 20 Culture plate with Salmonella (SA) specific medium

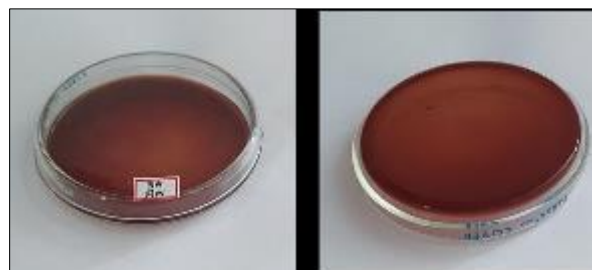


Figure 21 Culture plate with Staphylococcus Aureus (ST) specific medium

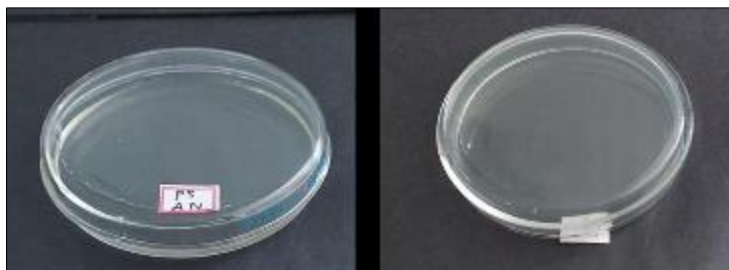


Figure 22 Culture plate with *Pseudomonas Aeruginosa* (PS) specific medium

3.6. Aflatoxin Assay by Thin Layer Chromatography (TLC) (B1, B2, G1, G2)

Results tabulated in Table 7.

Table 7 Aflatoxin report

Aflatoxin	Sample AN	AYUSH Specification Limit
AB1	Not Detected - Absent	0.5ppm
B2	Not Detected - Absent	0.1ppm
G1	Not Detected - Absent	0.5ppm
G2	Not Detected - Absent	0.1ppm

4. Discussion

Standardization of the drugs is more essential to derive the efficacy, potency of the drug. The standardization of Anda Neer (AN) was achieved through various procedures like analysing the physico-chemical characters. The physical parameters like state, odour, touch, flow Property and appearance/colour revealed that it was liquid, characteristic odour, non-greasy to touch, free flowing, colourless and transparent. So the drug AN is good in nature and safe to use.

The pH value of AN was 7 which indicates that the drug is neutral in nature. A more acidic or alkaline pH value induces lacrimation, ocular pain, and discomfort.^[12]

In the clarity test observation it was found that there are no particulate matters found in the sample AN, were in the formulation appears slightly turbid in nature and hence it here by described as clear solution with no major visible particles.

The results of HPTLC finger printing analysis of the sample AN reveals the presence of 3 prominent peaks corresponds to presence of three components present with in it. Rf value of the peaks ranges from 0.02 to 0.27 with percentage area of 0.85% to 90.09%.

The result of the Sterility test shows no growth was seen in any of the plates inoculates with the test sample AN. The revealed that the drug AN is free from the viable microorganisms and the absence of total bacterial and fungal colonies which indicates that the drug AN have good quality and safer drug.

The result of the Specific pathogen was observed that there was no growth in any of the plates inoculated with the test sample AN which confirms that there are no viable aerobic microorganisms present in the sample.

The results showed that there were no pesticide traces such as Organo chlorine, Organo phosphorus, Organo carbamates and Pyrethroids in the AN for analysis. This result suggests that AN have good quality.

The results shown no spots 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. So the drug AN Is non-toxic and there is no contamination and does not possess carcinogenic property. As a result, ANDA NEER was proved for its safety over the defined standardization method.

5. Conclusion

Standardization of ANDA NEER was done as per PLIM guidelines and standardized procedure. The obtained results of standardization of Siddha zoological formulation AN by different parameters such as physico-chemical parameters, TLC visualization of drug at 366nm, HPTLC finger printing analysis will be useful as tool for authentication and analysis their safety and quality of drug. These standardization parameters could be considered as a reference standard of this drug for quality control assessment in future.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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