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STANDARDIZATION OF THE DRUG AMUKKARA CHOORANAM – A SIDDHA POLYHERBAL FORMULATION

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ABSTRACT

Amukkara chooranam is a well-known polyherbal formulation in Siddha system of medicine which is selected by the author for standardisation. Aim and Objective of this study was to assess the quality of the drug Amukkara chooranam by conducting physicochemical analysis, preliminary phytochemical analysis and other analytical techniques. Physicochemical analysis and preliminary phytochemical analysis was done based on the standard text books. Other analytical technique like High Performance thin layer chromatography (HPTLC) finger printing was done using CAMAG. The physicochemical analysis of the drug Amukkara choornam showed 8.3±3.55% of loss on drying at 105°C, 2.64±0.25% of total ash, 11.8±2.99% of water soluble ash etc. which indicates that the drug has remarkable content of inorganic matter. Moreover the pH value of 5 indicates the drug is slightly acidic. The preliminary phytochemical analysis showed the presence of Starch, Sugar, Alkaloids, Tannic acid, flavonoids, steroids, triterpenoids, coumarin, saponins and phenol are present in the trial drug. The drug was free of microbial contamination; heavy metals and pesticide were below deductible limit. The results obtained indicate that the drug is of standard quality and can be used as reference standard in laying pharmacopoeia standard.

KEYWORDS: Amukkara chooranam, Physicochemical analysis, Standardization, Siddha medicine.

INTRODUCTION

Herbal based traditional remedies are highly recommended by World Health Organization (WHO) because of their safety, easy availability, low cost in the treatment of various diseases. In traditional system, these medicines have a richest bio-resource such as phenols, micro and macronutrients etc. They can act as a nutraceuticals, food supplements, pharmaceutical intermediates etc.[1] An herbal based formulation improves the quality of human life through its potent natural antioxidants[2] and bioactive compounds.[3] They provide remedy for various chronic diseases and metabolic disorders which are multifactorial and therapeutic intervention.[4]

World Health Organization (WHO) and National Centre for Complementary and Alternative Medicine (NCCAM) accentuates the need to ensure quality and safety of herbal medicine by modern techniques and applying suitable standards and has proposed guidelines for development of standard herbal medicine.[5] Quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine.[6] But we don't have a rigid quality control profiles for standardization of herbs and their formulations. It is mainly due to the lack of inadequate regulatory standards and implementation protocols.[7]

Development of standards for plant-based drugs being a challenging task, it needs innovative and creative approaches.[8] At each and every step of standardization viz; identification, organoleptic, pharmacognostic, physiochemical, phytochemical, presence of xenobiotics, microbial load and toxicity needs special attention because of complex nature of plant based medicines and the inherent variability of their constituents.[4]

Of these, the phytochemical profile is of special significance since it has a direct bearing on the activity of the herbal drugs. Multiple marker based standardization can be executed through HPTLC, a sophisticated analytical technique. It is a simple, fast, reproducible and economic method for standardization of herbal formulation which has an ability to determine the quality of drug thereby enhances the beneficial effects of herbal products. In addition to qualitative detection, HPTLC also provides quantitative information on the major active constituents of a drug, thus enabling an assessment of drug quality.

This is the first report on the standardization of Amukkara Chooranam, a Siddha polyherbal formulation comprises of 13 herbs such as *Withaniasomnifera, Smilax china, Hyosycymusniger, Piper cubeba, Picrorrhizakuroa, Terminalia chebula, Nigella sativa, Zingiberofficinale, Celastruspaniculatus, Alpiniaofficinarum, Piper longum, Accaciapennata, Saccharaumofficinarum.* It has been traditionally used for the management and treatment of chronic diseases such as cardio vascular diseases, diabetes mellitus etc. In the present study, we have elucidated the physio-chemical, phytochemical profile of TC using standard and modern techniques.

MATERIALS AND METHODS

The drug Amukkara chooranam[9] was prepared in the Gunapadam laboratory of National Institute of Siddha, Chennai, Tamilnadu, India, after proper purification.[10] The required quantity of the purified drugs was taken and grinded into fine powder and filtered by vasthrakayam procedure. The prepared medicine was also be authenticated by the Gunapadam Head of the Dept for its completeness. The analysis was conducted at Noble research solution Pvt. Ltd, Chennai, India. The ingredients of the drug Amukkarachoornam are given in the Table 1.

Table 1: Ingredients of Amukkara chooranam.

S.NO	Ingredients	Botonical Name
1	Amukkara	Withania somnifera
2	Parangipattai	Smilax china
3	Kurosaniomam	Hyoscyamus niger
4	Vaalmilagu	Piper cubeba
5	Kadugurohini	Picrrorhiza kuroa
6	Kadukkai	Terminalia chebula
7	Karunseeragam	Nigella sativa
8	Chukku	Zingiber officinale
9	Vaaluluvai	Celastrus paniculatus
10	Arathai	Alphinia officinarum
11	Thippili	Piper longum
12	Indu	Accacia pennata
13	Seenisarkarai	Saccharum officinarum

Identification of Raw drugs

Herbal drugs were purchased from reputedlocal raw drug store Chennai, India. Indu were collected from Ariyalur district, India. Herbal drugs were authenticated by Dr.D.Aravind M.D(s), M.Sc., Assistant Professor of Medicinal Botany, NIS, Chennai, India.

Preparation of the drug

The required quantity of the purified drugs was taken and grinded into fine powder and filtered by vasthrakayam procedure.[9]

Organoleptic characters

Colour, odour, taste and consistency of the drug were noted.

Physico-chemical parameters

All the physico-chemical parameters were carried out as per the methods mentioned in standard books. The parameters are as follows.

Particle Size

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 were 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.[11]

Percentage Loss on Drying

10gm of test drug was accurately weighed in evaporating dish. The sample was dried at 105oC for 5 hours and then weighed.[12,13]

Percentage loss in drying = Loss of weight of sample/ Wt of the sample X 100

Determination of Total Ash

3 g of test drug was accurately weighed in silica dish and incinerated at the furnace a temperature 400 ºC until it turns white in color which indicates absence of carbon. Percentage of total ash will be calculated with reference to the weight of air-dried drug.[12,13]

Total Ash = Weight of Ash/Wt of the Crude drug taken X 100

Determination of Acid Insoluble Ash

The ash obtained by total ash test will be boiled with 25 ml of dilute hydrochloric acid for 6mins. Then the insoluble matter is collected in crucible and will be washed with hot water and ignited to constant weight. Percentage of acid insoluble ash will be calculated with reference to the weight of air-dried ash.[12,13]

Acid insoluble Ash = Weight of Ash/Wt of the Crude drug taken X 100

Determination of Water Soluble Ash

The ash obtained by total ash test will be boiled with 25 ml of water for 5 mins. The insoluble matter is collected in crucible and will be washed with hot water, and ignite for 15mins at a temperature not exceeding 450ºC. Weight of the insoluble matter will be subtracted from the weight of the ash; the difference in weight represents the water soluble ash. Calculate the percentage of water-soluble ash with reference to the air-dried drug.[12,13]

Water Soluble Ash = Weight of Ash/Wt of the Crude drug taken X 100

Determination of Alcohol Soluble Extractive

About 5 g of test sample will be macerated with 100 ml of Alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowing to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105ºC, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.[12,13]

Alcohol sol extract = Weight of Extract/ Wt of the Sample taken X 100

Determination of Water Soluble Extractive

About 5 g of the test sample will be macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowing to stand and for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish, and dry at 105ºC, to constant weight and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug.[12,13]

Water soluble extract = Weight of Extract/ Wt of the Sample taken X 100

Determination of pH

About 5 g of test sample will be dissolved in 25ml of distilled water and filtered the resultant solution is allowed to stand for 30 mins and the subjected to pH evaluation.[12,13]

Preliminary Phytochemical Tests

Thepreliminary phytochemical testswere carried out as per the methods mentioned in standard books.

Test for Starch

2ml of extract was treated with weak dil. Iodine solution.[14]

Test for Reducing Sugar

5ml of Benedict's qualitative solution was taken in a test tube and allowed to boil for 2 minutes and added 8 to 10 drops of the extract and again boil it for 2 minutes. The colour changes are noted.[14]

Test for the Alkaloids

a) 2ml of the extract was treated with 2ml of dil. potassium Iodide solution.

b) 2ml of the extract was treated with 2ml of dil. picric acid.

c) 2ml of the extract was treated with 2ml of dil. phosphotungstic acid.[14]

Test for Tannic Acid

2ml of extract was treated with 2ml of dil. ferric chloride solution.[14]

Test for Amino Acid

2 drops of the extract was placed on a filter paper and dried well. 20ml of Burette reagent is added.[14]

Test for Coumarins

To the test sample, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of yellow colour.[14]

Test for Saponins

To the test sample, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of Saponins.[14]

Test for glycosides- Borntrager's Test

Test drug is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests. To 2 ml of filtered hydrolysate, 3 ml of chloroform is added and shaken, chloroform layer is separated and 10% ammonia solution is added to it. Pink colour indicates presence of glycosides.[14]

Test for flavonoids

To the test sample about 5 ml of dilute ammonia solution were been added followed by addition of few drops of conc. Sulphuric acid. Appearance of yellow color indicates the presence of Flavonoids.[14]

Test for phenols: Lead acetate test

To the test sample; 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.[14]

Test for steroids

To the test sample, 2ml of chloroform was added with few drops of conc. Sulphuric acid (3ml), and shaken well. The upper layer in the test tube was turns into red and sulphuric acid layer showed yellow with green fluorescence. It showed the presence of steroids.[14]

Triterpenoids

Liebermann–Burchard test: To the chloroform solution, few drops of acetic anhydride was added then mixed well. 1 ml concentrated sulphuric acid was added from the sides of the test tube, appearance of red ring indicates the presence of triterpenoids.[14]

Test for Cyanins

Anthocyanin

To the test sample, 1 ml of 2N sodium hydroxide was added and heated for 5 min at 100°C. Formation of bluish green colour indicates the presence of anthocyanin.[14]

High Performance Thin Layer Chromatography

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. In addition it is a reliable method for the quantitation of nano grams level of samples. 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 medicinal plant raw materials.[15]

a) 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.[15]

b) 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 extract and Rf values were tabulated.[15]

Toxic / Heavy Metal Analysis by Aas

Methodology

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 AC 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 sample AC.

Sample Digestion

Test sample AC 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.

Standard preparation

As & Hg- 100 ppm sample in 1mol/L HCl

Cd & Pb- 100 ppm sample in 1mol/L HNO3

Microbial Contamination Test by Pour Plate Method

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).

METHODOLOGY

Test sample was admixed with sterile distilled water and the mixture were been used for the sterility evaluation. About 1ml of the 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. Grown colonies of organism was then counted and calculated for CFU.

Test for Specific Pathogen

Methodology

One part of the test sample was dissolved in 9 mL of sterile distilled water and the 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.

Analysis of Pesticides Organochlorine, Organophosphorus and Pyrethroids

Extraction

About 10 g of test substance were extracted with 100 ml of 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 millilitres of toluene R and heat again until the acetone is completely removed. Resultant residue will be dissolved using toluene and filtered through membrane filter.[16,17]

Aflatoxin Assay by TLC (B1,B2,G1,G2)

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.

Test solution

Concentration 1 µg per ml.

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 from and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.[18]

RESULTS

Organoleptic Characters

The drug Amukkarachoornam was a moderately coarse powder pale brownish in colour with strongly aromatic in odour(Figure 1). The inferences are tabulated in Table 2.

Physico-Chemical Parameters

The loss on drying which indicates the moisture content of the drug was determined as 8.3 ± 3.55%. The total ash was found to be 2.64 ± 0.25% which indicates the inorganic content of the drug. The water soluble ash was calculated as 11.87 ± 2.99% and the value of acid insoluble ash was found to be 1.60 ± 0.03% which indicates that the drug contains negligible amount of siliceous matter. The water soluble extractive value and alcohol soluble extractive value were found to be 36.87 ± 1.72% and 34.9 ± 1.38%. The pH value is calculated as 5 which indicates that the drug is weekly acidic. The observed results were tabulated in Table 3.

Particle Size Determination by Microscopic Method

Microscopic observation of the particle size analysis reveals that the average particle size of the sample AC was found to be 107.4± 32.18 µm further the sample AC has particle with the size range of lowest 46.63 µm to highest 163.3 µm (Figure 2).

Preliminary Phytochemical Tests

The drug has high polar secondary metabolites like flavonoids, steroids, triterpenoids coumarin, phenol, betacyanin, saponins, sugar are shown in table 4.

High Performance Thin Layer Chromatography

HPTLC finger printing analysis of the sample AC reveals the presence of seven prominent peaks corresponds to presence of seven versatile phytocomponents present with in it. Rf value of the peaks ranges from 0.22 to 0.76. Further the peak 2 occupies the major percentage of area of 26.58% which denotes the abundant existence of such compound. Followed by this peak 4 and 7 occupies the percentage area of 25.80 and 14.06%.(Table 5) (Fig 3).

Heavy/Toxic Metal Analysis by Aas

Results of the present investigation have clearly showed that the sample AC has no traces of heavy metal lead. Further the results show the presence of Mercury, Arsenic and Cadmiumat 0.0008, 0.0004 and 0.001 ppm level. The reported heavy metals such as Mercury, Arsenic and Cadmium seem very low when compared to the allowed recommended limit. (Table 6).

Microbial Contamination by Pour Plate Method

No growth / colonies were observed in any of the plates inoculated with the test sample shown in (Table 7).

Test for Specific Pathogen

No growth / colonies were observed in any of the plates inoculated with the test sample shown in (Table 8).

Analysis of Pesticides Organochlorine, Organophosphorus and Pyrethroids

The results showed that there were no traces of pesticides residues such as Organo chlorine, Organo phosphorus and pyrethroides in the sample Ac. It further shows the above mentioned residues were not been detected in the sample AC provided for analysis. (Table 9).

Aflotoxin Assay by TLC (B1,B2,G1,G2)

The results shown that there was no spots were been identified in the test sample loaded TLC plated when compare to the indicates that the sample were free from Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2.(Table 10).

Table 2: Organoleptic characters.

State	**Solid**
Appearance	Pale Brownish
Nature	Moderately Coarse powder
Odor	Strongly Aromatic

Table 3: Physicochemical characters of Amukkarachooranam.

S.No	Parameter	Mean (n=3) SD
1.	***Loss on Drying at 105 °C (%)***	8.3 ± 3.55
2.	***Total Ash (%)***	2.64 ± 0.25
3.	***Acid insoluble Ash (%)***	1.60 ± 0.03
4.	***Water Soluble Ash (%)***	11.87 ± 2.99
5.	***Alcohol Soluble Extractive (%)***	36.9 ± 1.38
6.	***Water soluble Extractive (%)***	36.87 ± 1.72
7.	***PH***	5

Table 4: Preliminary Phytochemical Analysis of Parangipattai Chooranam.

Sl.no	Name of the test	Inference
1	Test for Alkaloids (Mayer's test)	-
2	Test for Flavonoids	+
3	Test for Glycosides (Borntrager's test)	-
4	Test for Steroids	+
5	Test for Triterpenoids (Liebermann–Burchard test)	+
6	Test for Coumarin	+
7	Test for Phenols (Lead acetate test)	+
8	Test for Tannin	-
9	Test for Proteins (Biuret test)	-
10	Test for Saponins	+
11	Test for Sugar (Benedict's test)	+
12	Test for Anthrocyanin	-
13	Test for Betacyanin	+

Table 5: HPTLC peak table.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.22	2.0	0.25	18.3	13.90	0.28	1.0	273.2	10.74
2	0.38	4.4	0.41	24.3	18.48	0.45	11.9	675.9	26.58
3	0.46	9.9	0.48	16.9	12.89	0.49	3.5	226.7	8.92
4	0.56	10.1	0.59	29.2	22.20	0.61	10.1	656.2	25.80
5	0.68	14.4	0.69	16.1	12.25	0.71	1.4	222.5	8.75
6	0.73	0.0	0.75	11.6	8.83	0.76	10.7	130.9	5.15
7	0.76	10.0	0.78	15.0	11.45	0.81	5.3	357.6	14.06

Table 6: Heavy metal analysis report.

Name of the Heavy Metal	Absorption Max Λ max	Result Analysis	Maximum Limit
Mercury	253.7 nm	0.0008 ppm	1 ppm
Lead	217.0 nm	BDL	10 ppm
Arsenic	193.7 nm	0.0004 ppm	3 ppm
Cadmium	228.8 nm	0.001 ppm	0.3 ppm

BDL – Below Detection Limit

Table 7: Sterility report.

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	

Table 8: Specific pathogen report.

Organism	Abbreviation
E-coli	*EC*
Salmonella	*SA*
Staphylococcus Aureus	*ST*
Pseudomonas Aeruginosa	*PS*

Table 9: Pesticide residue report.

Pesticide Residue **I.Organo Chlorine Pesticides**	Sample AC	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.Pyrethroid		
Cypermethrin	BQL	1mg/kg

BQL –Below quantification limit

Table 10: Aflatoxin report.

Aflatoxin	Sample AC	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

Fig. 1: Prepared medicine.

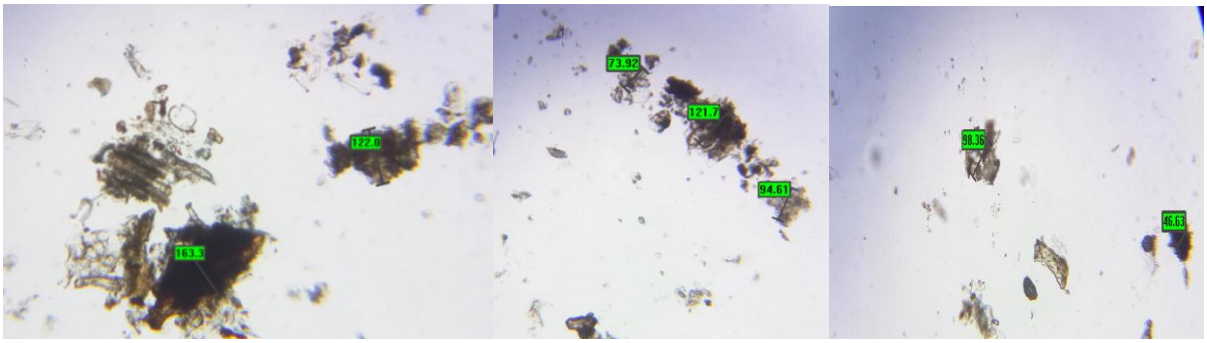

Fig. 2: Microscopic Observation of Particle Size for the sample AC.

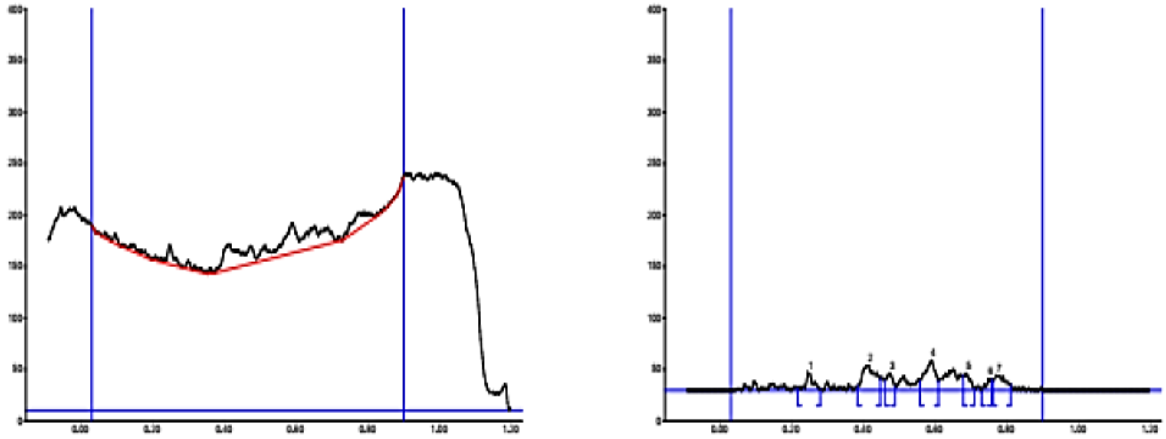

Fig. 3: HPTLC Finger printing.

CONCLUSION

From this current study of preclinical standardization of Amukkarachooranam which is mentioned in Siddha texts shows that the drug Amukkarachooranam was a moderately coarse powder pale brownish in colour with strongly aromatic in odour. The drug size has a particlesize with the range of lowest 46.63 µm to highest 163.3 µm. The loss on drying indicates the moisture content of the drug was determined as 8.3 ± 3.55%. The total ash was found to be 2.64 ± 0.25% which indicates the inorganic content of the drug. The water soluble ash was calculated as 11.87 ± 2.99% and the value of acid insoluble ash was found to be 1.60 ± 0.03% which indicates that the drug contains negligible amount of siliceous matter. The water soluble extractive value and alcohol soluble extractive value were found to be 36.87 ± 1.72% and 36.9 ± 1.38%. The pH value is measured as 5 which indicate that the drug is weekly acidic. The phytochemical analysis shows the drug has high polar secondary metabolites like flavonoids, steroids, triterpenoids, coumarin, phenol, betacyanin, saponins and sugar. HPTLC analysis shows that presence of four prominent peaks corresponds to presence of four versatile phytocomponents with in it and their Rf values were calculated.

This study also reveals that the chooranamis sterile and free of bacteria, fungi and specific pathogens like Salmonella, Staphylococcus aureus, E-coli, Pseudomonas aeruginosa and pesticide residues. In heavy metals analysis lead was not detected and mercury, arsenic, cadmium were present within the permissible limit there by ensures its safe usage. Aflatoxin like B1, B2, G1, G2 were not detected. As a result, Amukkarachooranam was proved its safety over the defined standardization method. The results obtained could be utilized as reference for developing standard formulation of great efficacy.

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