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Standardization of *Amukkirai Chooranam*, a Traditional Herbal Preparation in Siddha System of Medicine

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: *Amukkirai chooranam* (*A. chooranam*) is an important traditional herbal preparation and its main component is the root powder of *Withania somnifera* Dunal (*Solanaceae*). It is the best remedy for rheumatism, weakness, stress, sleeping disorders, gastric ulcer, anaemia, etc.
Aims: The goal of this study was to standardize this traditional herbal preparation on the basis of qualitative and quantitative methods.
Study Design: Experimental laboratory study on Phytochemical and Pharmacognostic investigations.
Place and Duration of Study: The Phytochemical and pharmacognostic investigations were carried out at the Herbal Technology Section, Industrial Technology Institute (ITI), Colombo, Sri Lanka, between Jan 2012 to August 2012.
Methodology: The *A. chooranam* was prepared according to the procedure given in Traditional Text. The preparation has been standardized on the basis of macroscopic and organoleptic

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characters, physicochemical properties, phytochemical screening, fluorescence analysis, and elemental analysis, aqueous and ethanol extractive values, TLC and HPLC fingerprint analysis. Data were analysed by statistical software - Statistical Package for Social Sciences (SPSS) version 17.

Results: The present study reveals that the *A. chooranam* was light whitish brown in colour having characteristic odour with bitter taste and smooth texture. The moisture content at 105ºC in *A. chooranam* was found to be 9.12±0.10%. Analytical results showed total ash, water soluble, acid insoluble and sulfated ash values of 5.76±0.09%, 2.93±0.15%, 0.63±0.04% and 1.92±0.04% respectively. The percentage yields of cold and hot aqueous extractive values (15.02±0.46 & 30.82±1.09) were greater than the percentage yield of cold and hot ethanol extractive values (03.07±0.31 & 10.00±0.22) of *A. chooranam*. Heavy metal analysis revealed that their concentrations in *A. chooranam* were below the WHO/FDA permissible limits.

Conclusion: *A. chooranam* exhibits a set of diagnostic characters, which will help to identify the standard preparation and build a monograph of the Siddha pharmacopeia of Sri Lanka.

Keywords: Amukkirai chooranam; chromatography; herbal preparation; quality control; Solanaceae; standardization; siddha medicine; Withania somnifera.

1. INTRODUCTION

The Majority of the world population depends on traditional medicine for primary health care. Plants have been extensively used as a rich source of medicine as they contain organic compounds with therapeutic value [1]. Standardization of herbal formulations is an essential factor in order to assess the quality, purity, safety and efficacy of drugs based on the concentration of their active principles. It is very important to establish a system of standardization for every plant medicine in the market, since the scope for variation in different batches of medicine is enormous [2]. The quality assurance is necessary if plant products are to fill the need for cheap and reliable medicines or if natural products are to be used as templates for new drug molecules [3].

Amukkirai chooranam is an important traditional herbal preparation and its main component is the root powder of *Withania somnifera* Dunal. It is the best remedy for rheumatism, weakness, stress, sleeping disorders, gastric ulcer, anaemia, etc [4]. The term *chooranam* (powder preparation for internal use) is applied to the powder prepared by a single or a combination of two or more herbal ingredients [5,6].

Withania somnifera (*Ashwagandha* in Sanskrit, Indian ginseng/ Winter cherry in English, and *Amukkirai* in Tamil) belongs to the family *Solanaceae*/ nightshade. Laboratory analysis has revealed over 35 chemical constituents contained in the roots of *W. somnifera*. The biologically active chemical constituents are alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanoloides with a glucose at carbon 27 (sitonidoside XI and X). The other alkaloids are somniferine, somnine, somniferinine, withananine, pseudo-withanine, tropine, pseudo-tropine, cuscohygrine, anferine and anhydrine. *Withania somnifera* is also rich in iron. Much of Ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D [7].

Numerous studies indicated that ashwagandha possesses antioxidant, antitumor, anti-stress, anti-inflammatory, anti-ageing, hematopoetic, anxiolytic, immune-modulatory, anti-depressive, rejuvenating properties and also influences various neurotransmitter receptors in the central nervous system [8].

Roots are straight, and unbranched, thickness varying with age. Roots bear fiber like secondary roots; outer surface buff to grey-yellow with longitudinal wrinkles. Crown consists of 2-6 remains of stem base. The stem bases are variously thickened, nodes prominent only on the side from where petiole arises, cylindrical, green with longitudinal wrinkles, fracture, short and uneven, odour characteristic, and taste bitter and acrid. The transverse section of stem base shows pith, pericyclic fibres, and xylem with tracheids, fibres and starch grains [7,9].

Macroscopic analysis showed the powder of *W. somnifera* root showed brownish yellow in colour, woody smell and no taste. Microscopic analysis of the powder of *W. somnifera* roots has revealed walled parenchyma cells, fragments of thick walls

of tracheida/ vessels and parts of tracheids/ vessel, with irregular shape parenchyma cells and their parts, number of acentric starch granules and large numbers of droplet like granules that have come out from the cells [10].

The process of standardization can be achieved by stepwise pharmacognostic studies [11]. The pharmacognostical parameters are major and reliable criteria for confirmation of the identity and determination of quality and purity of the crude drugs [12]. Due to a lack of modern pharmacopoeial standards, *A. chooranam* prepared using traditional methods may not have the desired quality and batch-to-batch consistency. Hence, there is a need for standardization of *A. chooranam,* following scientific parameters including macroscopic and organoleptic characters, physico-phytochemical analysis, fluorescence analysis, elemental analysis, and chromatographic pattern.

2. MATERIALS AND METHODS

2.1 Plant Material

A. chooranam consists of root powder of *W. somnifera* (*Solanaceae*). Six root powder samples were used for the standardization study. For the preparation of *A. chooranam*, root of *W. somnifera* were purchased from a reputed vendor of herbal material (MS Marunthakam) during the month of January 2012 in Jaffna District. The botanical identity of the plant (Voucher specimen no. 2453) was confirmed by the Botanist and the voucher specimen was deposited at Bandaranayke, Memorial Ayurvedic Research Institute (BMARI), Navinna, Maharagma, Sri Lanka.

2.2 Preparation of *Amukkirai chooranam*

The *A. chooranam* was prepared according to the procedure given in a traditional text [13]. The purchased *W. somnifera* roots were cut into small pieces and boiled with cow's milk (1:1 w/ v). Then the roots were air-dried thoroughly under shade (at room temperature) for 2-3 weeks to avoid direct loss of phytoconstituents from sunlight. The shade dried materials were powdered using the pulverizer and sieved up to 80 meshes. It was then homogenized to fine powder and stored in air tight container for further analysis.

All the reagents and chemicals used were procured from Institute of Industrial Technology {ITI}, Colombo-07 {these were purchased from Sigma-Aldrich (USA)}. All other chemicals used for the preparation of solvents were in analytical grade.

2.3 Determination of Foreign Matter

Accurately weighed 100 g of the powdered preparation was taken and spread out in a thin layer to sort out the foreign matter using a magnifying lens (6 X). Plant material collected should be free from foreign matter like soil, stones, sand, dust and insect parts or animal excreta. They were separated and weighed and the percentage was calculated.

2.4 Standardization Studies

2.4.1 Macroscopic and organoleptic evaluation

Macroscopic and organoleptic evaluation of *A. chooranam* was used for identification of sensory characteristics like colour, odour, taste, texture, etc [14].

2.4.2 Physicochemical evaluation

Physicochemical parameters such as pH, ash values, moisture content, and extractive values of *A. chooranam* were determined as per guidelines of World Health Organization [15].

2.4.2.1 Determination of pH range

The powder samples of preparation (about 1g and 10g) were weighed separately and immersed in 100ml of water in separate beakers (1% w/v and 10% w/v). The beakers were closed with aluminum foil and left for 24 hours at room temperature. Later the supernatant solutions were decanted into another beaker and the pH of the formulations was determined using standard simple glass electrode pH meter (Consort) at room temperature [16].

2.4.2.2 Loss on drying (Gravimetric determination)

One gram of powder sample of preparation was placed in an accurately weighed moisture disc (Electronic measurement scale – Mettler Toledo) and weighed. For estimation of loss on drying, it was dried at 105℃ for 5 hours in an oven (Memmert), cooled in a desiccator for 30 minutes, and weighed without delay. The loss of weight was calculated in mg per g of air-dried material.

2.4.2.3 Extractive values

Extractive values of *A. chooranam* were performed using water, methanol, ethanol, hexane, toluene, ethyl acetate, and n-butanol as described by guidelines of W.H.O.

2.4.2.4 Ash values

Total ash, water soluble ash, acid insoluble ash and sulfated ash values of *A. chooranam* was carried out according to methods described by W.H.O [17].

2.4.3 Fluorescence analysis

The *A. chooranam* was exposed to ultraviolet light at wavelengths of 254 and 366 nm separately. Fluorescence analysis was carried out in accordance with the procedure as described by Sriwastava et al. [18]. One milligram of powdered drug was placed on a grease free clean microscopic slide and 1-2 drops of the freshly prepared reagent solution were added, mixed by gentle tilting and the slide was left for 1-2 minutes. The slide was then placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365nm) ultraviolet radiations. The fluorescent characteristics of powder and colours observed by application of different reagents in different radiations were recorded. In the present study, one milligram of powdered drug was treated with 1ml 1N HCl, 1N NaOH, 1N NaOH in 1 ml methanol (MeOH), 50% KOH, 50% sulfuric acid, concentrated sulfuric acid, 50% HNO_3, concentrated HNO_3, acetic acid, and iodine separately and observed under UV 366, UV 254 in day light while still wet [18].

2.4.4 Preliminary phytochemical screening

The *A. chooranam* (10g) was subjected to hot and cold extraction technique with ethanol, methanol and water (50ml), filtered and evaporated to dryness under reduced pressure in a Rota evaporator, stored at 20^0C until required. The different extracts were subjected to qualitative tests for the identification of various phytochemical constituents as per the standard procedures [19-23].

2.4.5 Quantitative estimations

2.4.5.1 Estimation of total alkaloid

Quantitative analysis of alkaloid was determined using the procedure reported as described by Edeoga et al. [24].

2.4.5.2 Estimation of total flavonoids

Flavonoids were determined using the procedure reported as described by Edeoga et al. [24].

2.4.5.3 Estimation of total saponin (Determination of foaming index)

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of powder sample and their extracts is measured in terms of a foaming index. Saponins were determined according to the method described by World Health Organization [15].

2.4.6 Determination of heavy metal and other elements

A. chooranam was subjected to heavy metal analysis by Atomic Absorption Spectrophotometer (AAS). A 0.3 g powder sample preparation was placed in digestion tubes stirred with 5ml nitric acid and left overnight for complete digestion. Next day, added 5ml HNO_3, to each tube vessel closed with lid, and heated in 150°C oven for 2hrs till the solution became transparent. Cooled samples were transferred into 10 ml volumetric flask. Added 4ml H_2O to vessel, covered with lid. Diluted to volume with water and used for the estimation of the heavy metal concentration.

The digested samples were then analyzed for Cd, Pb and other elements (Na, Ca, Mg & Fe), using a graphite furnace atomic absorption spectrophotometer (AAS; GBC 932 plus, Australia). A single-beam hollow cathode lamp (GBC) was used for estimation of Cd and Pb. Concentration of Hg was determined through AAS using Cold vapour technique whereas air-acetylene flame was used for determination of 'As' concentration.

All necessary precautions were taken to avoid any possible contamination of the sample as per the AOAC guidelines. The metal quantification was based on calibration curves, which were determined through a series of concentrations prepared using the chemical standard with 1 mg/ ml concentration. The concentrations of the respective metals in samples were expressed as mg of metal per kg (ppm) [25].

2.4.7 Analysis of Thin Layer Chromatography (TLC) profiles

Five µL of each extract was spotted on to TLC plate coated with silica gel (pre-coated, GF_{254}) and separated using a variety of solvent system. Different solvent systems were used for separation. The best separation of the methanol and ethanol (cold and hot) extracts occurred in the solvent system comprised of Methanol: Dichloromethane: Cyclohexane (0.2: 5.8: 4 v/ v) as the mobile phase. After development, numbers of spots were observed under UV light (short and long). Visualization was attempted by spraying Anisaldehyde sulfuric acid reagent and heating the plate for 5-10 min. (100-105℃). The colour and R_f values of the spots were recorded carefully and the chromatogram was documented by graphical copying [15].

2.4.8 Analysis of High-performance Liquid Chromatography (HPLC) profiles

For determination of the HPLC profiles, the aqueous extract *A. chooranam* was re-dissolved in distilled water (20mg/ ml concentration). After filtration through 0.45µm, 13-mm Millipore filters, 20 µL of each sample were injected in to an Hypersil® ODS (C_{18}), (250x4.0mm, 5µm) reversed phase column of a High-Performance Liquid Chromatography System (Schimadzu, Kyoto, Japan) connected to a UV-Vis detector (Model SPD-10AVP). The HPLC analysis was performed using a linear gradient of methanol and water (solvent system) in a ratio of 50: 50 for sample for 20 min, with a flow rate 1 ml/ min, detection at 254nm. The peak areas and peak heights were analyzed by the software package (CLASS-VP) provided with the HPLC system [26].

2.5 Statistical Analysis

For statistical analysis, six replicates were conducted for each activity and the experiments were repeated twice. Data were analysed by statistical software-Statistical Package for Social Sciences (SPSS) version.

3. RESULTS AND DISCUSSION

Foreign matter was found to be 0.5%. The permissible limit as per standards is not more than 2%.

The macroscopic and organoleptic characters of the *A. chooranam* and its aqueous and alcoholic extracts are shown in Table 1. As apparent from Table 1 the *A. chooranam* was light whitish brown in colour (Fig. 1) having characteristic odour with bitter taste and smooth texture. Its ethanol, methanol and aqueous extracts had similar organoleptic characters except for the colour.

Determination of extractive values, ash residues and active components (saponins, alkaloids and essential oil content) plays a significant role for standardization of the indigenous crude drugs [27]. Physicochemical parameters of the *A. chooranam* are shown in Table 2(2.1 & 2.2). The loss on drying value obtained is an indicative of amount of moisture content present in the drug. The loss on drying at 105℃ in *A. chooranam* was found to be 9.12±0.10%. The total ash value is an indicative of total amount of inorganic material after complete incineration and the acid insoluble ash value is an indicative of silicate impurities, which might have arisen due to improper washing of drug [28]. Extractive values help us in determining the amount of active constituents and is done on plant material for which as yet no suitable chemical or biological assay exists. Analytical results showed that total ash, water soluble, acid insoluble and sulfated ash values were found to be 5.76%, 2.93%, 0.63% and 1.92% respectively. The percentage yields of cold and hot aqueous extractive values (15.02±0.46 & 30.82±1.09) were greater than the percentage yield of cold and hot ethanol extractive values (03.07±0.31 & 10.00±0.22) of *A. chooranam*. In the present study, the yields of cold n-Butanol, ethyl acetate, toluene, and hexane extractive values were found to be very low. Nasreen and Radha, 2011 mentioned the root of *W. somnifera* yielded 5.1% of total ash, 0.24% acid insoluble ash, 2.31% of loss on drying, 16.8% of alcoholic soluble matter and 15.53% water extractable matter [9]. Kaur et al., 2010 stated the values of physicochemical parameters from *Ashwagandha Churna* were 5.85% total ash, 0.71% acid insoluble ash, 9.60% loss on drying, and 6.98% alcoholic soluble matter and 17.82% water extractable matter [29]. These values were slightly differing from certain physicochemical parameters such as total ash and aqueous extractive values which were obtained in present study. These differences may be due to the preparation method of *chooranam*, or seasonal variation in the environment or geographical variations.

Table 1. Macroscopic and organoleptic characters of the *Amukkirai chooranam* and its extracts

***Amukkirai chooranam* & its extracts**	**Appearance**	**Colour**	**Taste**	**Touch**	**Odour**
Amukkirai chooranam	Powder	Whitish brown	Bitter	Coarse	Characteristic
Hot aqueous extract	Liquid	Brown	Bitter	Smooth	Characteristic
Cold aqueous extract	Liquid	Light brown	Bitter	Smooth	Characteristic
Hot ethanol extract	Liquid	Orange	Bitter	Smooth	Characteristic
Cold ethanol extract	Liquid	Dark yellow	Bitter	Smooth	Characteristic
Hot methanol extract	Liquid	Orange	Bitter	Smooth	Characteristic
Cold methanol extract	Liquid	Dark yellow	Bitter	Smooth	Characteristic

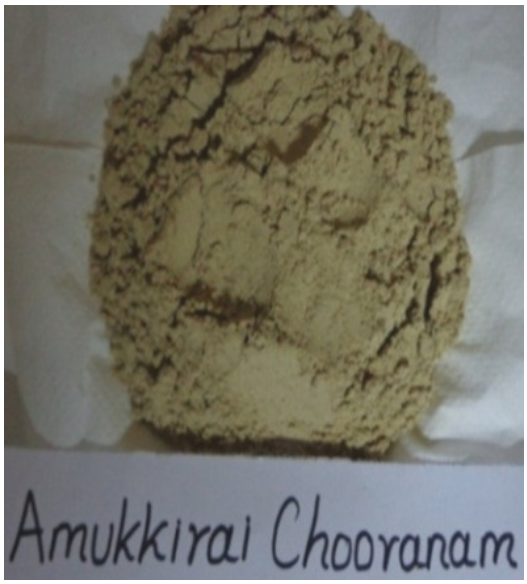

Fig. 1. Macroscopic character of *Amukkirai Chooranam*

Table 2.1. Physicochemical parameters of the *Amukkirai chooranam*

Physicochemical parameters	**Values (% w/w)**	**Standard value for *W. somnifera* root (% w/w)**
Loss on drying at 105ºC	09.12±0.10	NMT 08 %
Total ash value	05.76±0.09	NMT 07 %
Water soluble ash	02.93±0.15	NMT 15 %
Acid insoluble ash	00.63±0.04	NMT 01 %
Sulfated ash value	01.92±0.04	-
pH of 1% w/v formulation solution	05.73±0.02	-
pH of 10% w/v formulation solution	05.64±0.03	-

Values are expressed as mean% ± S.D., n=6; NMT- not more than are standard values

Table 2.2. Extractive values of the *Amukkirai chooranam*

Name of the solvent	**Extractive values (% w/w)**	**Standard value for *W. somnifera* root (% w/w)**
Hot aqueous extract	30.82±1.09	NLT 15 %
Hot ethanol extract	10.00±0.22	NLT 15 %
Cold aqueous extract	15.02±0.46	NLT 15 %
Cold ethanol extract	03.07±0.31	NLT 15 %
Cold n-Butanol extract	01.80±0.08	
Cold ethyl acetate extract	01.53±0.14	
Cold toluene extract	01.57±0.03	
Cold hexane extract	01.60±0.17	

Values are expressed as mean% ± S.D., n=6; NLT- not less than are standard values

The fluorescence analysis is adequately sensitive and enables the precise and accurate determination over a satisfactory concentration range without several time-consuming dilution steps prior to analysis of pharmaceutical samples [30]. The fluorescence colour is specific for each compound. A non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent [31]. The colour of the extracts from organic and inorganic solvents were observed both under ordinary and UV light. There is little difference between extracts and the light sources. The fluorescence analysis of *A. chooranam* treated with different chemical reagents is showed in Table 3.

The preliminary phytochemical screening of cold and hot methanol, ethanol and aqueous extracts of *A. chooranam* are given in Table 4. Table 5, summarizes the height and weight of the foam for the determination of saponin content in *A. chooranam*. Plants are a rich source of secondary metabolites with interesting biological activities. In general these secondary metabolites

are an important source with a variety of structural arrangements and properties [32]. The results of the preliminary phytochemical screening of *A. chooranam* (Table 4) revealed the presence of alkaloids, flavonoids, steroids, tannins, phenols coumarins, saponins, etc and micro nutrition reducing sugar, proteins in cold and hot ethanol, methanol and aqueous extracts. Although these phytochemicals were presence in both extracts, ethanol extract has higher constituents than aqueous extract. Nasreen and Radha, 2011 reported the presence of terpenoids, alkaloids, saponins, carbohydrates, glycosides, flavonoids, tannins and steroids in hydromethanolic root extract of the same plant [9]. Sigh et al. [33] identified the presence of alkaloids, flavonoids, steroids, tannins, terpenoids, saponins and sugars in alcoholic root extract of *W. somnifera*. Another three studies mentioned the presence of alkaloids, flavonoids, saponins, steroids, and tannins in crude extract [34,35] and ethanolic extract [36] of *W. somnifer*a.

The quantified values of the phytoconstituents can be used as a major tool for obtaining a quality control profile of drug. The total alkaloid and flavonoid contents were found to be 0.81±0.01% and 14.43±0.40% (w/ w) in the *A. chooranam* (powder preparation). The saponin content was relatively low in this preparation. Dasgupta and Amartya, 2012 found that two marketed *Ashwagandha churna* formulation contained 28.3% and 0% total alkaloid content respectively [37]. Nasreen and Radha, 2011 reported that total alkaloid content of root powder of *W. somnifera* was found 0.9818mg/100g [9].

Table 3. Powder fluorescence test of *Amukkirai chooranam*

***Amukkirai chooran*am**	**UV 254 nm**	**UV 366 nm**	**In day light**
Dry powder Powder as such	Light brown	Light brown	Whitish brown
NaOH (1N) in water	Green	Light brown	Light yellow
P + HCl (1N)	Brownish yellow	Dark brown	Yellow
P + NaOH (1N) in MeOH	Yellow	Brownish yellow	Yellow
P + 50% KOH	Light brown	Light brown	Yellow
P + H_2SO_4	Light brown	Dark green	Light green
P + 50% HNO_3	Dark brown	Dark brown	Yellow
P + concentrated HNO_3	Brownish yellow	Dark brown	Yellow
P + CH_3COOH	Dark brown	Light brown	Yellow
P + concentrated H_2SO_4	Dark brown	Light brown	Yellow
P + iodine in water	Dark brown	Dark brown	Black

Table 4. Phytochemical screening of cold and hot aqueous and alcoholic extracts of *Amukkirai chooranam*

Components	**CE**	**HE**	**CM**	**HM**	**CA**	**HA**
Phenolic compounds	+++	+++	+++	+++	+++	+++
Tannins- *Ferric chloride test*	+++	+++	+++	+++	+++	+++
Flavonoids- *Shinoda test*	+++	+++	+++	+++	+	+
Coumarins	+++	+++	+++	+++	+++	+++
Steroid-glycosides- *LibermannBurchard's test*	+++	+++	+++	+++	++	++
Alkaloids- *Mayer's Test*	+++	+++	+++	+++	+++	++
Dragendroff's Test	+++	+++	+++	+++	+++	++
Protein- *Xanthoproteic Test*	++	++	++	++	++	++
Quinones	0	0	0	0	0	0
Anthraquinones	0	0	0	0	0	0
Saponins- *Foam test*	++	++	++	++	+	+
Reducing sugars- *Fehling's test*	+++	+++	+++	+++	+++	+++
Fixed oil and Fats	0	0	0	0	++	++

+++ = appreciable amount, ++ = average amount, + = trace amount, 0 = absent
CE- Cold ethanol, HE- Hot ethanol, CM-Cold methanol, HM- Hot methanol, CA-Cold aqueous, HA- Hot aqueous

Table 5. Estimation of saponin-determination of foaming index

No of test tube	Quantity of extract with water	Height of the foam (cm)	Volume of the foam (cm^3)
T1	1 ml extract + 9 ml H_2O	0.1	0.18
T2	2 ml extract + 8 ml H_2O	0.15	0.26
T3	3 ml extract + 7 ml H_2O	0.15	0.26
T4	4 ml extract + 6 ml H_2O	0.2	0.35
T5	5 ml extract + 5 ml H_2O	0.25	0.44
T6	6 ml extract + 4 ml H_2O	0.25	0.44
T7	7 ml extract + 3 ml H_2O	0.3	0.53
T8	8 ml extract + 2 ml H_2O	0.3	0.53
T9	9 ml extract + 1 ml H_2O	0.35	0.62
T10	10 ml extract	0.4	0.71
	Foaming Index	**< 100**	

The heavy metals and other elemental analysis of *A. chooranam* are shown in Table 6. The therapeutic value of any drug depends not only on its clinical efficacy, but also in its lack of toxic side effects [25]. In this study, analysis of heavy metals (Pb, As, Cd and Hg) showed the absence of harmful levels of these metals in *A. chooranam*. That is *A. chooranam* being within the permissible limits as per WHO/FDA. Results in the present study are similar to those of other investigators. Thus, earlier study by Khan et al. [38] stated that, the concentration of heavy metals like Mn, Zn, Fe, Ni, Cu, Cr, Pb and Cd in root of *W. somnifera* were well below the critical limit. Nasreen and Radha, 2011 found that, the heavy metals (As, Hg, Cd and Pb) analysis of crude powder of *W. somnifera* root was not exceeding the permissible limit given by the WHO guidelines [9]. Dasgupta and Amartya, 2012 reported that, the presence of heavy metals below the WHO/ FDA permissible limits in two marketed *Ashwagandha Churna* formulation [37].

Other elements such as Ca, Na and Mg showed the detectable levels in *A. chooranam*. Iron content (< 0.1 mg/ Kg) was not detected in this preparation. Calcium (12.5 mg/ Kg) and Sodium (19.8 mg/ Kg) levels of *A. chooranam* were found to be high. Magnesium level was (2.6 mg/ Kg) found to be low.

The TLC profile of *A. chooranam* (cold and hot methanol and ethanol extracts) were developed in Methanol: Dichloromethane: Cyclohexane (0.2: 5.8: 4 v/ v) solvent system. At the same time, cold and hot aqueous extracts of *A. chooranam* did not get mobile phase or colour of spots in the same solvent system, because the aqueous extract has higher polar content than other solvents. Table 7 summarizes the R_f values and colour of spots visible in the TLC profiles of the cold and hot methanol and ethanol extracts of *A. chooranam* in same solvent system. The analytical data along with the TLC patterns can be used for fixing standards to this *A. chooranam*.

Table 6. Heavy metal contents of *Amukkirai chooranam*

Heavy metals	*Amukkirai chooranam* mg/ Kg	Permissible limits(ADI / FDA values) {mg of metal per Kg (ppm)}
Lead (Pb)	0.76	10 mg/ Kg
Arsenic (As)	0.35	0.5 ppm
Cadmium (Cd)	< 0.1/ ND	0.3 mg/ Kg
Mercury (Hg)	< 0.05 / ND	1.0 ppm

Values are expressed mg /Kg, N.D. – Not detected

Table 7. Thin layer chromatography analysis (R_f values and colour of the spots) of different extracts of *Amukkirai chooranam*

Solvent front 8.5cm		Solvent front 7.0cm	
Cold methanol extract	**Hot methanol extract**	**Cold ethanol extract**	**Hot ethanol extract**
0.03# Bn	0.02# Bn	0.07* DP	0.04* DP
0.09* DP	0.08* DP	0.13$ P	0.13$ P
0.13$ P	0.12$ P	0.21$ DP	0.21$ DP
0.18$ P	0.17$ P	0.29$ Be	0.30$ Be
0.22# P	0.21# P	0.41# Pi	0.41# P
0.29* DP	0.31* DP	0.64* Be	0.63* Be
0.35* DP	0.35* DP	0.79* DP	0.77* DP
0.41# P	0.42# P	0.86# P	0.84# P
0.53# P	0.53# P	0.90# P	0.91# P
0.73# P	0.73# P	0.96* DP	0.97* D
0.78$ DP	0.79$ DP	-	-

** - intense, $ - Moderately intense, # - Faint; Bn- Brown, DP- Dark pink, P- Pink, Be- Blue*

Analysis of high performance liquid chromatography (HPLC) of aqueous extracts (Retention time of main peaks) of *A. chooranam* are shown in Table 8. HPLC profile of *A. chooranam* (aqueous extract) was developed in methanol and water (50: 50) solvent system. HPLC profile showed the presence of polar and non-polar compounds in the aqueous extract of *A. chooranam*. Aqueous extract of *A. chooranam* was subjected to reverse phase chromatography (HPLC). As apparent in Fig. 2; most of the major peaks (8 areas) appeared within 20 minutes.

Table 8. Retention time of main peaks in HPLC profile of *Amukkirai chooranam*

Peak no	Retention time (min)
1	3.229
2	4.266
3	6.06
4	8.41
5	9.168
6	11.26
7	13.953
8	15.608

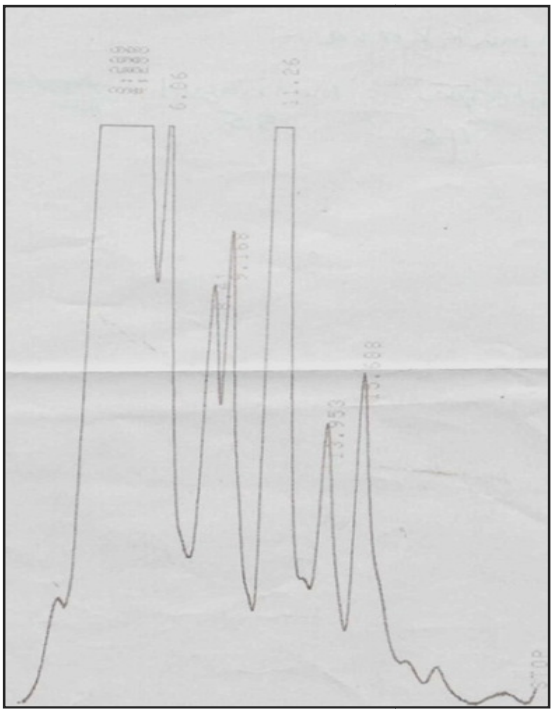

Fig. 2. HPLC profiles of aqueous extracts of *Amukkirai chooranam*

4. CONCLUSION

Herbal drug which are used in various traditional medicine needs detailed investigation with ethno pharmacological approach. Standardization of *A. chooranam* (single herbal formulation) has been carried out according to WHO guidelines and standard procedures. These analytical findings and data analysis reveals that the *A. chooranam* exhibits a set of diagnostic characters, which will help to identify the preparation and build a reference of the monograph of the Siddha pharmacopeia of Sri Lanka. Further studies may be carried out based on identification and separation of active ingredients with the help of bio markers.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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