

Physicochemical and phytochemical standardization of polyherbal *Siddha* formulation ‘*Karisalai Chooranam*’

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Kayakarpam is one of the unique therapeutic formulation in *Siddha* system of medicine advocated for rejuvenation, longevity and elimination of disease causing factors. *Karisalai Chooranam* (KSC) is one of the *Kayakarpam* preparations described in *Siddha* medicine literature namely *Bogamunivar Vaidya Kaviyam-1000*. *Karisalai Chooranam* comprises equal proportion of six herbs such as *Eclipta alba* L. (Asteraceae), *Acalypha indica* L. (Euphorbiaceae), *Sphaeranthus indicus* L. (Compositae), *Indigofera tinctoria* L. (Fabaceae), *Centella asiatica* L. (Umbelliferae) and *Wedelia chinensis* (Asteraceae). Standardization and identification of marker compounds for *Karisalai Chooranam* has not been carried out. In the present study, the formulation has standardized by using standard physicochemical and phytochemical protocols as ash values, extractive values, chemical profiling; and marker quantification such as Wedelolactone, Quercetin, Asiaticoside and Rutin by using HPTLC finger printing. In addition, residue analyses (heavy metal content) by ICP-MS and pesticide analysis by LC-MS/MS were also carried out to strengthen the standardization process. Qualitative phytochemical screening revealed the presence of flavonoids, terpenes, steroids, saponins and cardiac glycosides. Microbial load, pesticide residues and heavy metals were found to be within permissible limits. The results are indicative of active ingredients responsible for therapeutic effect of KSC, thereby this study lends to the evidence for future pharmacological studies.

Keywords: Standardization, *Karisalai Chooranam*, HPTLC, Wedelolactone, Quercetin, Asiaticoside, Rutin

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Siddha system of medicine consists of large numbers of herbs with medicinal and pharmacological importance. Adaptogens/anti-stress herbs that are nontoxic produce a non-specific defensive response to stress, and have a normalizing influence on the body. Adaptogens help the body to adapt to stress, support its normal functions, and restore balance. They increase the body's resistance to physical, biological, emotional and environmental stressors¹. All adaptogens display effects that help to regulate the neuroendocrine and the immune systems, provide a defense against stress, and increase the ability of a person to maintain optimal homeostasis¹. A number of plants possess adaptogenic activity due to diverse classes of chemical compounds. Flavonoids and phenolic compounds are responsible for the antioxidant activity of the herbs. Antioxidants protect our body from free radical damage. Free radical damage within the cells has been linked to a range of

disorders including cancer, arthritis, atherosclerosis, stroke, Alzheimer's disease, diabetes, and emphysema in smokers, etc.² Ancient medical literature on *Siddha* gives a vivid and comprehensive description of this group of plant medicines which are rejuvenating herbs (*Kayakarpam*), tonics, *Rasayanas*, or restoratives. *Kayakarpam* is one of the unique therapeutic formulations in *Siddha* system advocated for rejuvenation, longevity and elimination of disease causing factors. The word *Kayakarpa* means (*Kayam*-body, *Karpa*-able, competent) to make our body competent and youthful. They can be divided into *Karpa* medicines, *Karpa* practices of lifestyle and *karpa* diet. In *Siddha* medicine, 108 herbs and herbomineral combinations are recommended for normal individuals to boost immunity, to promote general health, for prevention of diseases (*Pothu Karpam*) and also for restoration of health from specific type of diseases (*Sirappu Karpam*). Standardization of herbal formulations is an essential procedure in order to assess the quality of drugs,

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based on the concentration of their active principles, physicochemical and phytochemical properties. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicine. One of the major problems faced by the herbal industry is the unavailability of rigid quality control profiles for herbal materials and their formulations. The WHO in number of resolutions has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards. The phytochemical profile is of special significance since it has a direct bearing on the activity of the herbal drugs³⁻⁵. HPTLC is a sophisticated analytical method with multiple marker based standardization. It provides qualitative and quantitative information of a drug, thus enabling an assessment of drug quality. This is the first report on the standardisation of the *Karisalai Chooranam*. It comprises equal proportion of six herbs such as *Eclipta alba* L. (Asteraceae), *Acalypha indica* L. (Euphorbiaceae), *Sphaeranthus indicus* L. (Compositae), *Indigofera tinctoria* L. (Fabaceae), *Centella asiatica* L. (Umbelliferae) and *Wedelia chinensis* (Asteraceae). *Karisalai Chooranam* is a *Kayakalpa* medicine described by bogamunivar⁶. In the present study, we have elucidated the standardization parameters of KSC by physicochemical and phytochemical parameters.

Materials and methods

Physicochemical studies like total ash, water soluble ash, acid insoluble ash, fiber content, alcohol and hydro alcohol extract, pH value, loss on drying, microbial contamination, heavy metal analysis and pesticide residue were carried out as per the WHO⁷ and AYUSH guidelines⁸. Qualitative and quantitative phytochemical studies were also elucidated.

Plant material

Polyherbal formulation, *Karisalai Chooranam* consists of six ingredients. The plants were collected and purchased from local raw material traders, Chennai, Tamil Nadu, India. The six ingredients were authenticated by Dr D Aravind, Assistant Professor (Botany), National Institute of Siddha, Chennai. Plant collection number: NISMB1632015, Voucher is deposited in the Department of Medicinal Botany, National Institute of Siddha, Chennai.

Preparation of *Karisalai Chooranam*

The whole plants were thoroughly cleaned with water and dried in the shade. Equal quantities of the

dry plants (each 200 gm) were taken and made into fine powder. The powder was sieved and then mixed together in equal proportion in geometrical manner to get uniform mixer. The *chooranam* was stored in an air tight container.

Determination of loss on drying

The percentage of loss on drying (% LOD) was determined for *Karisalai Chooranam* because excess of water in medicinal plant materials will encourage microbial growth, fungi or insects, and cause deterioration following hydrolysis. The percentage of loss on drying was determined in which 5 gm of accurately weighed air-dried material was placed in a previously dried and tared flat weighing bottle. The sample was dried in an oven at 100–105 °C until 2 consecutive weightings did not differ by more than 5 mg⁹.

Determination of ash values

Total ash

About 4 gm of the powdered material was accurately weighed and placed in a previously ignited and tared silica crucible. The material was spread in an even layer and ignited by gradually increasing the heat to a temperature of 500–600 °C until it was white, indicating the absence of carbon. The material was cooled in a desiccator and weighed. The content of total ash was calculated in mg/gm of air-dried material.

Acid-insoluble ash

To the crucible containing the total ash, 25 ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5 min. The watch glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ash less filter paper and washed with hot water until the filtrate was neutral. The insoluble matter left on the filter paper was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in a suitable desiccator for 30 min, and then weighed without delay. The content of acid-insoluble ash was calculated in mg/gm of air-dried material.

Determination of water soluble ash

The ash was boiled for 5 min with 25 ml of water; the insoluble matter was collected on an ash less filter paper and washed with hot water. It was ignited for 15 min at a temperature not exceeding 450 °C. The

weight of the insoluble matter was subtracted from the weight of the ash; the difference in weight represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air dried drug⁹.

Determination of crude fibre

For determination of crude fibre, 2 gm of moisture and fat free material were treated with 200 ml of 1.25 % H₂SO₄, after filtration and washing, the residue was treated with 1.25 % NaOH, filtered, washed with hot distilled water and then 1 % HNO₃ and again washed with hot distilled water. The residue was ignited and the ash weighed. Loss in the weight gives the weight of crude fiber¹⁰.

Extractive values

The extractive values were recorded in alcohol and water with a view to study the distribution of various constituents of *Karisalai Chooranam*. Accurately weighed 4.0 gm of powdered air-dried material was placed in a glass-stoppered conical flask and macerated with 100 ml of the solvent for 6 hrs, shaking frequently, and then allowed to stand for 18 hrs. The mixture was filtered rapidly taking care not to lose any solvent. Twenty-five milliliter of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water bath. The residue was dried at 105 °C for 6 hrs, cooled in a desiccator for 30 min, and weighed without delay.

Pesticide residue

Herbal formulations are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administering of fumigants during storage. Specific pesticide residues like Organochlorine compounds, Organophosphorus compounds and Pyrethroids were analysed by the following procedure namely PrEN 15662:2007 (E)-QuEChERS method by LC-MS/MS¹¹. This analysis was carried out in SGS India Private Ltd, Chennai.

Heavy metal analysis

KSC was analysed for the presence of Lead (Pb), Cadmium (Cd), Mercury (Hg), Arsenic (As) by following the procedure namely SO-IN-MUL-TE-063 method by ICP-MS (Inductively coupled plasma mass spectrometry)¹². This analysis was carried out in SGS India Pvt. Ltd, Chennai.

Microbial contamination and Aflatoxins

Total bacterial count, identification of specified organisms such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp. count was carried out¹³. Aflatoxin - B1,B2,G1,G2 were evaluated according to the AOAC official method 999.07. This analysis was carried out in SGS India Pvt. Ltd, Chennai.

Phytochemical analysis

Qualitative analysis

KSC ethanol extract was subjected to phytochemical analysis such as flavonoids, terpenes, saponins, phenolic-compounds, tannins, steroids, cardiac-glycosides, proteins, mono-saccharides, reducing sugars, carbohydrates, and alkaloids by employing standard conventional protocols^{14,15}.

High Performance Thin Layer Chromatography (HPTLC)

HPTLC technique was used to identify and quantity of active principles such as Wedelolactone, Asiaticoside, Quercetin and Rutin in the Siddha medicine *Karisalai Chooranam*¹⁵.

Instrumentation

For HPTLC, CAMAG (Switzerland), Scanner (Model No.201433) Sample applicator (Model No. 210441) were used. Development Mode: Ascending mode.

Preparation of sample and biomarker compounds

Two gm of KSC was extracted with ethanol in a soxhlet extractor. Filtered, concentrated and made upto 10 ml in a standard flask. Marker compounds such as Wedelolactone, Asiaticoside, Quercetin and Rutin were weighed and dissolved in ethanol to get a concentration of 10 mg in 10ml each for analysis.

Chromatographic condition and calibration curves

Marker specific solvent system and their scanning wavelength were selected based on the literature. Standards such as Wedelolactone, Asiaticoside, Quercetin and Rutin were prepared (10mg in 10 ml Ethanol). Five concentration of each standard were prepared as mentioned in the Figs. 1-4, respectively. For Wedelolactone quantitation, solvent system, Toluene: Ethyl acetate: Formic acid (5: 1.5: 0.5), standard curve: 0.667, 1.000, 1.333, 1.667, 2.000 µg, sample solution: 2 gm in 10 ml, sample applied - 30 µl (triplicate), scanning wavelength: 254 nm were used. For Asiaticoside quantitation, solvent

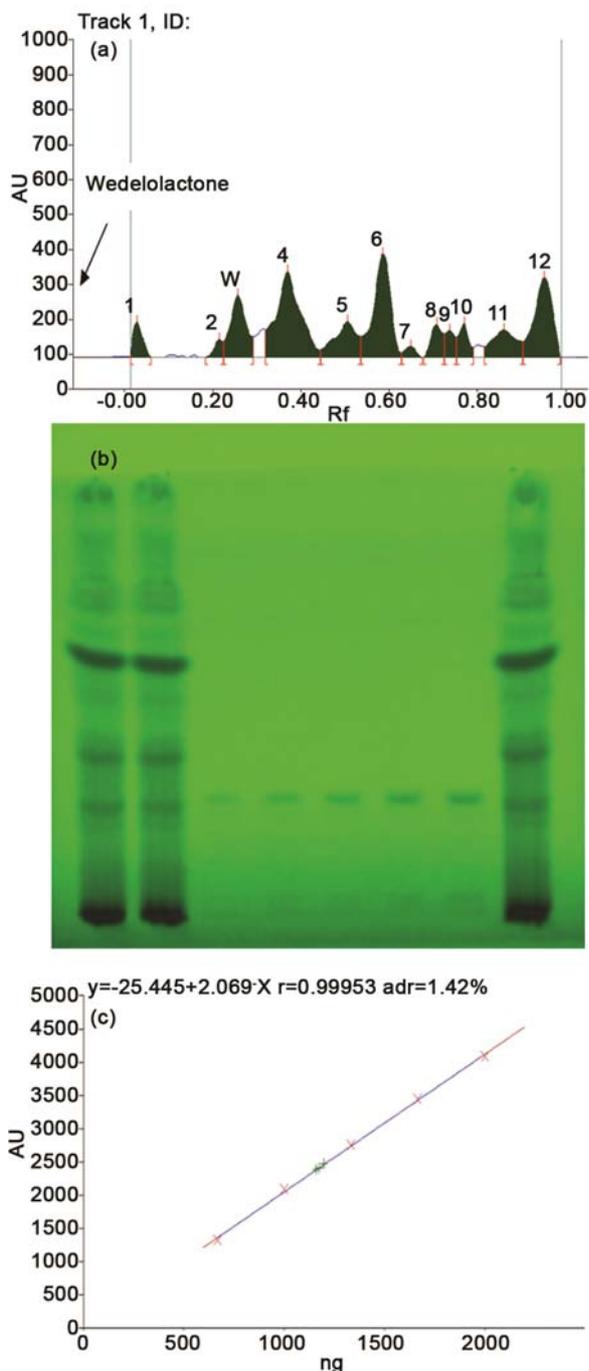


Fig.1—HPTLC – (A) Peak area of Wedelolactone in KSC, (B) HPTLC profile of KSC for Wedelolactone at 254 nm, (C) Calibration curve of standard Wedelolactone.

system, Chloroform: Glacial acetic acid: Methanol: Water (8:3.2: 1.2: 0.8), standard curve: 2, 4, 8, 1.5, 2.0 µg, sample solution: 2 gm in 10 ml, sample applied: 15 µl (triplicate), scanning wavelength: 600 nm were used. For Quercetin quantitation, solvent

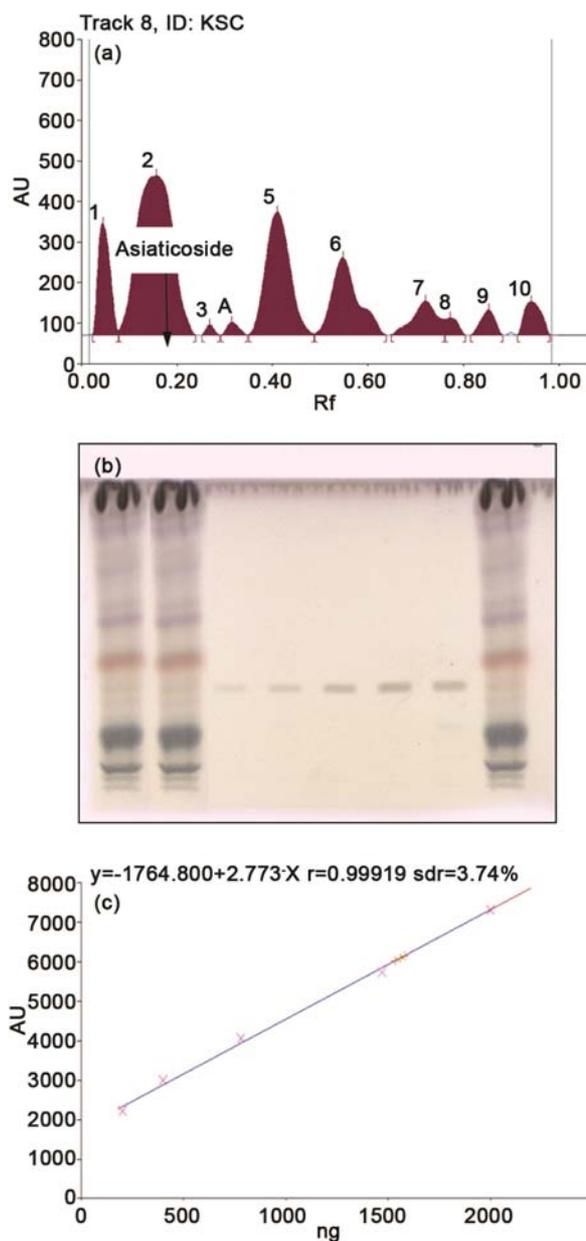


Fig. 2—HPTLC – (A) Peak area of Asiaticoside in KSC, (B) HPTLC profile of KSC for Asiaticoside at 600 nm, (C) Calibration curve of standard Asiaticoside.

system, Toluene: Ethyl acetate: Methanol (5: 3:2), standard curve: 2, 4, 6, 8, 10 µg, sample solution: 2 gm in 10 ml, sample applied: 15 µl (4 times), scanning wavelength: 254 nm were used. For Rutin quantitation, solvent system, Toluene: Ethyl acetate: Methanol (5: 3:2), standard curve: 2, 4, 6, 8, 10 µg, Sample solution: 2 gm in 10 ml, sample applied: 15 µl (4 times), scanning wavelength: 254 nm were used.

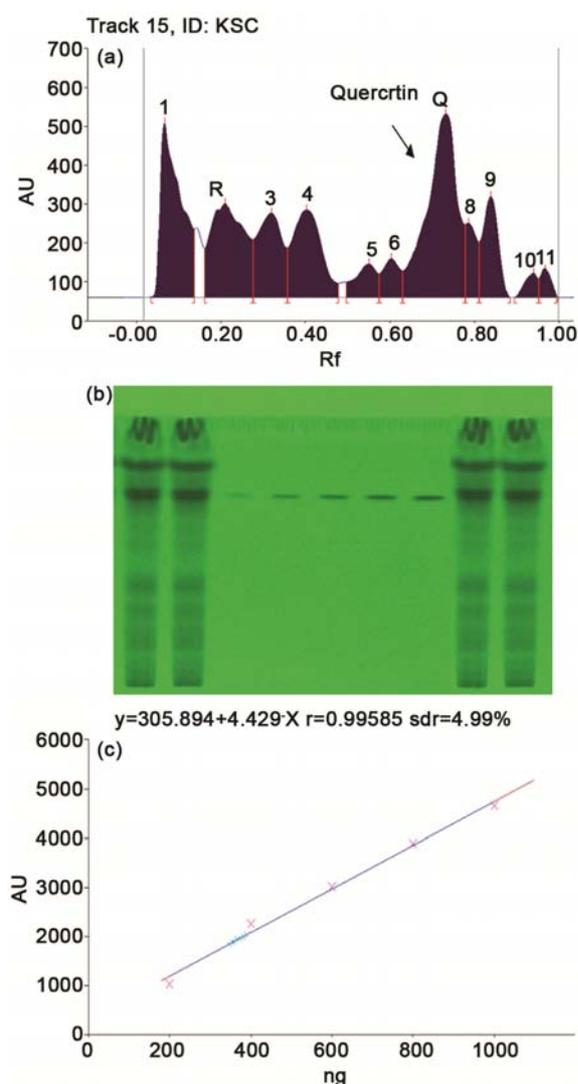


Fig. 3—HPTLC – (A) Peak area of Quercetin in KSC, (B) HPTLC profile of KSC for Quercetin at 254 nm, (C) Calibration curve of standard Quercetin.

Statistics

All experiments were carried out in triplicate and the results are expressed as Mean \pm Standard Error Mean (SEM).

Results

Physicochemical analysis

Physicochemical evaluation of KSC is carried out and it is found to have the following namely, loss on drying at 105 °C 36 %, total ash 9.8 %, water soluble ash 13.25 %, acid insoluble ash 5.3 %, alcohol extractive 6.82 %, hydro alcoholic extractive 5.95 %, crude fiber content 11.3 % w/w and pH value 6 (water).

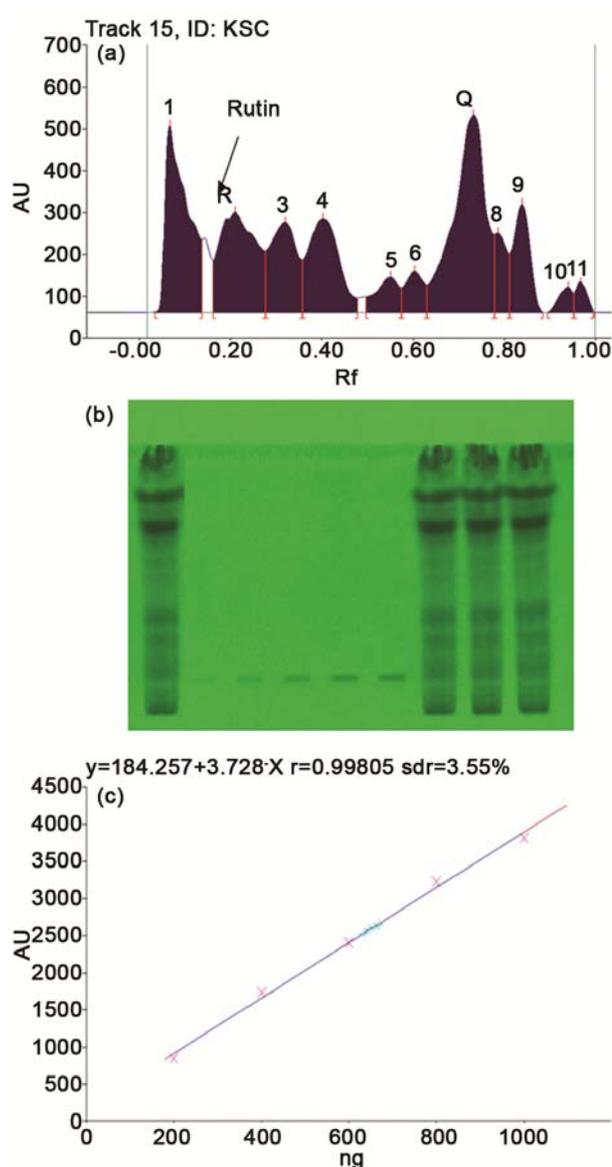


Fig. 4—HPTLC – (A) Peak area of Rutin in KSC, (B) HPTLC profile of KSC for Rutin at 254 nm, (C) Calibration curve of standard Rutin.

Pesticide residue analysis

Specific pesticide residues like organochlorine compounds, organophosphorus compounds and pyrethroids were estimated. The results are given in Table 1.

Heavy metal analysis

Quantitation of heavy metal contamination of KSC was carried out and it is found to have following namely Lead 1.8 ppm, Cadmium 0.08 ppm, Mercury 0.03 ppm, and Arsenic 0.58 ppm.

Table 1—Pesticide residues of *Karisalai Chooranam*

Pesticide residues	Unit (mg/Kg)
Organochlorine compounds	
Alpha HCH, Beta HCH, Gama HCH (Lindane), Delta HCH	BLQ *
Aldrin, Dieldrin, Trans chlordane Cischlordane, Endrin, Endrinaldehyde, Endrinketone	BLQ
Endosulphan-I, Endosulphan-II, Endosulphan Sulphate	BLQ
Heptachlor, Heptachlorepoxyde, Dicofol, Chlorthalonil	BLQ
Dicofol	0.01
o,p"DDT,p,p"DDT,o,p"DDD,p,p,DDD,o,p"DDE,p,p" DDE	BLQ
Organophosphorous compounds	
4-Bromo, 2-chlorophenol	0.08
Chlorfenvinphos	BLQ
Acephate, dimethoate, etrimfos, iprobenphos, methamidothos, monocrotophos, omethoate, parathion ethyl, Parathion methyl, phorate, phosalone, phosphamidon, profenophos, phorate sulphone, phorate sulphoxide	BLQ
Chlorpyrifos	0.98
Diazinon	BLQ
Dichlorvos, malathion	BLQ
Ethion	BLQ
Fenitrothion, Oxydemeton-methyl	BLQ
Quinalphos, Triazophos	BLQ *
Pyrethroids	
Permethrin (sum of isomers), Cypermethrin (including isomers)	BLQ
Cyfluthrin (including isomers), Ethofenprox, Lambda-cyhalothrin,	BLQ
Deltamethrin	BLQ
Fenvelerate & Esfenvelerate (RR&SS isomers)	BLQ
Fenvelerate & Esfenvelerate (RR&SR isomers)	BLQ

*BLQ – Below the limit of Quantitation

Microbial contamination

Total bacterial count in KSC sample was about 10^3 /gm. The specific pathogens like *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are absent in KSC. Fungal contamination and aflatoxins are not found in the sample.

Phytochemical analysis

Terpenes, flavonoids, saponins, steroids, cardiac glycosides, proteins, monosaccharides and Carbohydrates were found to be present in the KSC which is given in the Table 2. The HPTLC

Table 2—Qualitative phytochemical analysis of *Karisalai Chooranam*

Parameters	Methods	Results	Inference
Terpenes	Chloroform + conc. H ₂ SO ₄	Reddish brown ring	+++
Flavonoids	Add conc. HCL	Red colour	+++
Saponins	Frothing test	Frothing	++
Steroids	Liebermann–Burchard reaction Acetic anhydride + conc. H ₂ SO ₄	Violet to blue	+++
Cardiac glycosides	Glacial acetic acid + Ferric chloride solution	Brown ring	+
Proteins	Biuret test	Violet or pink colour	+
Monosaccharides	Barfoed's test	Red colour	+++
Reducing sugars	Fehling's test	No Yellow ppt.	-
Carbohydrates	Molisch test	Voilet ring	+
Tannin&Phenolic compounds	Acetic acid	Red coloured ring	-
Alkaloids	1% aqueous HCl+dragendorff's reagent	Turbidity or precipitation	-

+++High concentration, ++ moderate concentration, + low concentration, – absence.

Finger printing and quantitave analysis are shown in Figs. 1-4. HPTLC of KSC showed Comestan substance Wedelolactone 0.02 %, Titerpene glycoside substance Asiaticoside 0.04 % and Flavonoid substances such as Quercetin 0.02 % and Rutin 0.02 %.

Discussion

Karisalai Chooranam is a fine powder. It is brownish green in colour, possess herbal odour and slightly astringent and bitter in taste. The percentage of loss on drying and ash content of KSC are within the standard protocol limits. It provides information about the moisture level and inorganic content of KSC. The pesticide residues like organo chlorine compounds, organophosphorous compounds and pyrethroids are present within AYUSH limits. The concentration of the heavy metals present in KSC is within the limit of WHO and API. WHO prescribed the limit for presence of Lead is 10 ppm and for Cadmium 0.3 ppm and API prescribed limit for presence of Mercury is 1ppm and for Arsenic 3 ppm in herbal preparations. Bacterial contamination is within

AYUSH limits. AYUSH prescribed as the normal total bacterial count is 10^5 - 10^7 /gm and the total fungal count is 10^3 /gm. KSC has the bacterial count within the AYUSH limits.

Wedelolactone possesses neuroprotective potential by blocking oxidative stress-induced cell damage¹⁶ which is found to be present in the KSC. Asiaticoside has anti-stress¹⁷ and neuroprotective¹⁸ effect which is also found to be present in the KSC. Both Rutin and Quercetin are reported to have antioxidant and antistress (adaptogenic) activity in various acute and subacute stress models¹⁹.

The results of the present study indicate the physicochemical and phytochemical parameters of *Karisalai choornam* which have not been reported so far. These findings will certainly help in the standardization of *Karisalai chooranam*. Besides, this study identified the presence of biologically active compounds such as Wedelolactone, asiaticoside, quercetin and rutin in the *Karisalai Choornam* for the first time. Biological activities of these compounds may contribute to the antistress and antioxidant activities of the *Karisalai chooranam* which requires further pharmacological study.

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