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RESEARCH ARTICLE

Standardization of Vellarugu Chooranam – A Poly Herbal Formulation

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ABSTRACT

Siddha medicine is a prehistoric system of medicine predominant in South India. Siddha medicine have 32 types of Internal medicine and 32 types of External medicine. Vellarugu Chooranam is one of the Siddha formulations from Agathiyar Irandayiram Part- 3 which has indications cure Kadi vishangal(Poisonous bites), Karappan(Eczema), Soolai(Acute pain), Kiranthi(Veneral diseases).(1) Standardisation means validation of drugs identity and determination of its quality and purity. Standardisation includes Physico- chemical analysis, Biochemical analysis, TLC analysis, HPTLC analysis, Pesticide residual, Sterility test, and Aflatoxin assay. This study shown the presence of sulphate, phosphate, aluminium, iron, calcium, and alkaloids. This study revealed the data regarding the physicochemical characteristics, quality and safety of VC which indicates the standard quality of the drug.

Keywords: Standardization, Physico-chemical analysis, Siddha Medicine,Vellarugu Chooranam.

INTRODUCTION

The Siddha system of medicine is the earliest system of medicines and being practiced by a huge population in South India.Drugs used by the Siddhars can be classified into three groups: Thaavaram (herbal product), Thaathu (inorganic substances), and Jangamam (animal products)(2).Siddha medicine have 32 types of Internal medicines and 32 types of External medicines. Chooranam is one of the Internal medicines and it has a shelf life at Three months.Chooranam is a thin dry powder of drugs. Chooranam is a powder of a single drug or a mixture of two or more drugs which are powdered separately and then mixed together for homogeneity. All ingredients should be purified before preparation of chooranam. The Chooranam should be very fine, amorphous and should be perfectly dry.(3)Vellarugu Chooranam is one of the Siddha formulations from Agathiyar Irandayiram Part-3 which has specific indication to cure Kadivishangal(Poisonous bites), Karappan(Eczema), Soolai(Acute pain), Kiranthi(Veneral diseases). (1)It is prepared from 10 medicinal plants. According to WHO guidelines, a drugshould be standardized before releasing into the market with respect to safety. In this paper an attempt was made to estimate a Siddha formulation, Vellarugu chooranam by analytical methods and chromatographic studies. Hence in this paper an attempt was made to evaluate and standardize Vellarugu chooranam by identifying the ingredients microscopically and using Physico- chemical analysis, Biochemical analysis, TLC analysis, HPTLC analysis, Pesticide residual, Sterility test, and Aflatoxin assay.

MATERIALS AND METHODS

Identification And Authentication
All ingredients of *Vellarugu chooranam* were identified and authenticated by the Dept. of Medicinal Botany, National Institute of Siddha, Tambaram Sanatorium, Chennai.
Preparation of Vellarugu Chooranam:
Ingredients of "Vellarugu Chooranam":

S.No	Ingredients	Botanical Name	Used Parts
1	Vellarugu	*Enicostemmaaxillare*	Whole plant
2	Sirukurinjanver	*Gymnemasylvestae*	Root
3	Vakkanapattai	*Diospyros montana*	Stem Bark
4	Milagaranaiver	*Toddaliaasiatica*	Root
5	Kodiveliver	*Plumbago zeylanica*	Root
6	Veppampattai	*Azadirachtaindica*	Stem Bark
7	Sengaththaaripattai	*Capparis sepiaria*	Stem Bark
8	Agasakarudankilangu	*Corallocarpusepigaeus*	Root Tuber
9	Milagu	*Piper nigrum*	Dried Fruit
10	Sangam ver	*Azimatetracantha*	Root

Procurement of Raw Drugs:
All the raw drugs were purchased from K.Ramasamychetty country drug shop, Park town, Chennai.

Purification of Raw Drugs:
The raw drugs are purified as per the procedures stated in the Siddha literatures.

Method of Preparation
Take the above drugs in equal quantity after purification is grinded well to obtain fine powder. After this process, the powder was sieved through white cloth and Prepared Chooranam was stored in air tight container. [4]
Dosage : 3 Viral alavu (800-1gram), twice a daily
Adjuvant: Honey

Indications
Kadivishangal (Poisonous bites)
Karappan (Eczema)
Soolai (Acute pain)
Kiranthi (Veneral diseases) (2)

Standardization of Vellarugu Chooranam
The standardization of test drug is essential to exhibit the purity and quality of drug. This is basically done by physicochemical, Phytochemical and biochemical analysis. The physicochemical analysis, Phytochemical analysis, and instrumental analysis has been done at Noble Research Solution, an ISO 9001-2015 accredited business located in Chennai. Biochemical analysis was done at National Institute of Siddha.

Organoleptic Characters
The organoleptic characters such as colour, taste and odour were done as per PLIM guidelines noted in table -1 Organoleptic characters.(5,6)

Physico-Chemical Parameters
The physico-chemical examinations consist of determination of total ash, acid insoluble ash, extractable matter in water and alcohol, loss on drying at 105°C. All the physico-chemical parameters were determined by standard methods.

Percentage Loss On Drying
4g of test drug was weighed in a formerly weighed 100ml beaker and heated inan oven at 105ºC for 5hours. Cooled in a desiccator and weighed. Repeated this procedure till constant weight was attained. The percentage loss in weight of test drug was calculated.

Determination of Total ASH
4g of test drug was weighed accurately in a previously ignited and tarred silica dish. The material was evenly spread and ignited in a muffle furnace at400⁰Cuntilitbecame white indicating the absence of carbon. The dish is cooled in desiccator and weighed. As carbon free ash cannot be obtained in this manner, the dish was cooled and the residue moisturized with sufficient amount of water. Dried on a water bath and then it is ignited in the electric furnace to get the constant weight. Cooled the dish inadesicctor and then weighed.

Determination of Acid Insoluble ASH
The total ash of test drug was found as described above. To the dish containing the total ash was added 25 ml of dilute hydrochloric acid.Boiledgentlyfor6minutesandfiltered.Collectedtheinsolublematteronanashless filter paper and washed with distilled water until the residue was free from acid. Transferred the filter paper containing the insoluble matter to the original dish. Dried and ignited to the constant weight. Cool the dish in a desiccator, and then weighed.

Determination of Water-Soluble Extractive
4 g of test drug was weighed exactly in a glass stoppered flask. Add100ml of distilled water and shakened occasionally for 6 hours and then allowed to stand for 18hours.Filteredrapidly taking care not to lose any solvent and pipetted out 25 ml of the filtrate in a reweighed 100 ml beaker and evaporated to dryness on a water bath. Kept it in an air oven at 105ºC for 6 hours. Cooled in adesiccator and weighed. Repeated this experiment twice, and taken the average value.

Determination of Alcohol Soluble Extractive
4g of sample was weighed exactly inaglasss to ppered flask. Added 100ml of distilled alcohol (approximately95%)and shaken occasionally for 6 hours and then allowed to stand for 18 hours. Filtered quickly

taking care not to lose any solvent and pipetted out 25 ml of the filtrate in a pre-weighed 100 ml beaker and evaporated to dryness on a water bath. Kept it in an air oven at 105ºC for 6 hoursand cooled in desiccator and weighed. Repeated this experiment twice, and taken the average value.

Biochemical Analysis

The presence of biochemical substances in VC such as Sulphate, phosphate, iron, calcium and alkaloids were carried out by standard methods.

Phyto - Chemical Analysis

The presence of phytochemicals in VC such as alkaloids, carbohydrates, glycosides, saponins, tannins, gum and mucilage were carried out as per PLIM guidelines[7]

Detection of Alkaloids

Extracts were dissolved in dilute Hydrochloric acid individually and filtered.

Mayer's Test

Filtrates were treated with Mayer's reagent. Formation of yellow colored precipitate denotes the presence of alkaloids.

Hager's Test

Filtrates were treated with Hager's reagent. Presence of alkaloid was confirmed by the formation of yellow colored precipitate.

Detection of carbohydrates - Molisch's Test

To 2 ml of sample extract, 2 drops of alcoholic solution of α- naphthol was mixed. The mixture was mixed well by shaking and few drops of concentrated sulphuric acid was added along the sides of test tube slowly. Formation of violet ring indicates the presence of carbohydrates.

Detection of Glycosides - Modified Borntrager's Test

The extract was treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and then extracted with equal volume of benzene. The benzene layer is separated and processed by an ammonia solution. Rose-pink color appearance in the ammonical layer indicates the presence of anthranol glycosides.

Cardiac Glycoside - Keller- killiani test

Extract was shaken with distilled water (5 ml). To this, add glacial acetic acid (2 ml) containing a few drops of ferric chloride followed by H2SO4 (1 ml) along the side of the test tube. The formation of a brown ring at the interface is a positive sign indicating the presence of cardiac glycosides, and a purple ring may appear beneath the brown ring.

Detection of Saponins - Froth Test

The extract was diluted to 20 ml with distilled water and stirred in a graduated cylinder for 15 min. The formation of a 1 cm high foam indicates the presence of saponins.

Detection of phenols - Ferric Chloride Test

The extract was treated with 3-4 drops of ferric chloride solution. The formation of a blue-black color indicates the presence of phenol.

Detection of tannins - Gelatin Test

The extract is dissolved in 5 ml of distilled water and 2 ml of 1% solution of Gelatin containing 10% NaCl is added to it. Presence of white precipitate denotes the phenolic compounds.

Detection of flavonoids - Alkaline Reagent Test
Extract was treated with few drops of sodium hydroxide solution. Presence of flavonoid is confirmed by formation of intense yellow color, which becomes colorless on addition of dilute acid.

Detection of proteins and amino acids - Xanthoproteic Test
The extract was treated with a few drops of concentrated nitric acid. The formation of yellow color indicates the presence of protein.

Gum and Mucilage
To 1ml of extract, add 2.5ml of absolute alcohol, mix it constantly. Then the precipitate was dried in air and examine for swelling. Swelling indicates presence of gum and mucilage.

Quantitative Analysis
Microbial Contamination
Test sample was admixed with sterile distilled water and the mixture had been used for the sterility evaluation. 1 ml of test sample was inoculated into a sterile Petri dish, to which approximately 15 ml of molten agar was added at 45°C. Agar and sample were mixed completely by tilting and swirling. Agar was allowed to become completely gel form without disturbing it for about 10 minutes. Plates were then inverted and incubated at 37oC for 24-48 hours and further extended upto72 hours for fungal growth observation. Colonies of organism grown was counted and calculated for CFU.(8)

Test For Heavy / Toxic Metals
Standard: Hg, As, Pb and Cd – Sigma

Methodology
Atomic absorption spectrometry is a common and reliable method for detecting metals and metalloids in samples. The total heavy metal content of the samples was determined using an AA 240 series atomic absorption spectrometry (AAS). It measures the concentration of heavy metals like mercury, arsenic, lead, cadmium etc. in the tested products. Sample Digestion: To determine arsenic and mercury, the test samples were digested with 1 mol/L HCl. Similarly, for the determination of lead and cadmium, the sample were digested with 1mol/L of HNO_3. (8)

Standard Reparation
- As & Hg- 100 ppm sample in 1mol/L HCl
- Cd &Pb- 100 ppm sample in 1mol/L HNO_3.

Determination of Pesticide Residue
VC was extracted with 100 ml of acetone followed by homogenization for brief period. Further filtration was allowed, consecutive addition of acetone to the test mixture. The sample studied was heated using a rotary evaporator at a temperature not exceeding 40ºC until almost complete evaporation of the solvent. A few milliliters of toluene were added to the residue and heated again until the acetone was completely eliminated. Resultant residue was dissolved using toluene and filtered through membrane filter.(9,10)

Aflatoxins
Standard: Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. Solvent: Standard samples were dissolved in a mixture of chloroform and acetonitrile (9.8:0.2) to obtain solutions with concentrations of 0.5 µg per ml for each aflatoxin B1 and aflatoxin G1, and 0.1 µg per ml for each aflatoxin B2 and alphatoxin G2. Test Solution: Concentration 1 µg per ml.

Procedure

Standard aflatoxin was applied on the surface of pre coated TLC plate at the volume of 2.5 µL, 5 µL, 7.5 µL and 10 µL. Similarly, the test samples 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 in the ratio of 85: 10: 5 respectively 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. Examine and locate the spots on the plate under UV light at 365 nm.(11)

TLC Analysis

Test sample was exposed to thin layer chromatography (TLC) as per conventional one-dimensional ascending technique using silica gel 60F254, 7X6 cm (Merck) were cut with normal household scissors. Plate markings were made with soft pencil. Micro pipette is used to spot the sample for TLC applied sample volume 10- micro liter by using pipette at a distance of 1 cm at 5 tracks. In the twin trough chamber with specified solvent system after the run plates were dried and observed by visible light Short-wave UV light 254nm and light long-wave UV light 365 nm.[12]

High Performance Thin Layer Chromatography Analysis

HPTLC method is a modern sophisticated and automated separation procedure derived from TLC. Pre-coated HPTLC graded plates and auto sampler are used to complete precision, sensitive, significant separation both qualitatively and quantitatively. High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the estimation of botanical materials. HPTLC method offers high degree of selectivity, sensitivity and rapidity collectively within single-step sample preparation. Thus, this method can be conveniently implemented for routine quality control analysis. It gives chromatographic fingerprint of phytochemicals which is apt for confirming the identity and purity of phyto therapeutics.

Chromatogram Development

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

Chromatographic Scanning

Plates were scanned under UV at 366nm. The data gotten from scanning was brought into integration through CAMAG software. Chromatographic finger print was developed for the detection of phyto constituents present in each sample and their respective Rf values were tabulated [13].

RESULTS

Standardization of the drug is essential to derive the quality and safety of the drug which was analyzed by many methods. The results of organoleptic characters (Table No: 1), physicochemical analysis (Table No: 2), Biochemical analysis (Table No: 4), Microbial contamination (Table No: 5), Heavy metal analysis (Table No: 6), Pesticide residue (Table No: 7), Aflatoxin (Table No: 8), and HPTLC analysis (Table No: 9) of VC is tabulated below.

DISCUSSION

Moisture content of the drug indicates the stability and its shelf life. High moisture content can adversely affect the active ingredients of the drug.[14] The percentage of loss of drying of Vellarugu Chooranam is 6.7% (Normal range 1-20%). Therefore, low moisture of VC might get maximum stability and shelf life. Ash values are helpful in determining the quality and purity of crude drugs, especially in powder form. [15]The total Ash content and Acid Insoluble Ash values of Vellarugu Chooranam are 2.46% (Normal range: 1-25%) and 0.36% (Normal range: 0.1-10%). This denotes the purity of the drug. The water soluble and alcohol soluble extract values are responsible for

indication of the extent of polar and non-polar compounds which are existing in VC. The extract values of alcohol in VC is 17.5% and water is 20.97%. Decreased water soluble ash value (2.8%) shows easy facilitation of diffusion and osmosis mechanisms. Qualitative analysis of Vellarugu Chooranam for Acid radicals, Basic radicals, and other components reveals the presence of Sulphate, Phosphate, Aluminium, Iron, Calcium and Alkaloids. Phytochemical analysis revealed the presence of Alkaloids, Carbohydrates, Glycoside, Saponin, Tannins, Gum and mucilage in Vellarugu Chooranam. The results of Heavy metal analysis done by AAS method, results indicated that Heavy metals such as Mercury, Lead, Arsenic and Cadmium were found below the detectable limit. So, Vellarugu Chooranam is safe for oral consumption. Test for specific pathogens for VC concludes that there is absence of E-coli, Salmonella, Staphylococcus aureus, and Pseudomonas aeruginosa. Test for Aflatoxin revealed that the drug VC is free of Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2. Pesticide residue analysis of VC indicated that it has below detectable limit of Organophosphorus, Organochlorine, and Pyrethroid contents. The quantitative analysis of Vellarugu Chooranam through TLC and HPTLC results showed that the HPTLC finger printing analysis of the sample reveals the presence of seven prominent peaks corresponds to presence of seven versatile phytocomponents present within it. Rf value of the peaks ranges from 0.04 to 0.90.

CONCLUSION

From the above observations, it is resolved that the results of physico-chemical analysis, preliminary phytochemical studies, TLC photo documentation & HPTLC finger print studies can be used as a diagnostic tool to determine the quality & purity of the drug and to lay down pharmacopoeal standards for Vellarugu Chooranam .Further present investigation had generated an evidence-based data with respect to purity, standards and phytochemical nature of the formulation VC. Therefore, the necessity of standardization of various herbal formulations will pave way to explore the therapeutic effects as claimed in Siddha literature as well as to confirm the quality and safety of drug and thereby improving the scientific integrity of Siddha medicine.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Table.1: Organoleptic Character of Vellarugu Chooranam

State	Solid
Nature	Moderately fine
Odor	Characteristic
Touch	Soft
Flow Property	Moderately free flowing
Appearance	Pale brownish

Table.2: Physico- Chemical properties of *Vellarugu Chooranam.*

S. No	Parameter	Mean (n=3) SD
1.	*Loss on Drying at 105 °C (%)*	6.7± 0.65
	Total Ash (%)	2.46± 0.35
3.	*Acid insoluble Ash (%)*	0.36 ± 0.15
4.	*Water soluble Extractive (%)*	20.97± 1.16
5.	*Alcohol Soluble Extractive (%)*	17.5 ± 2.28
6.	*Particle Size*	Completely passes through sleeve size of 1 mm(90% passes through 400 micro meter sleeve and 10% Passes through 1 mm sleeve)

Table.3: Results of Bio - Chemical Analysis of Vellarugu Chooranam

PARAMETER	RESULTS
Test for Acid Radicles	
Test for Sulphate	**Present**
Test for Phosphate	**Present**
Test for Carbonate	Absent
Test for Sulphide	Absent
Test for Basic Radicles	
Test for Lead	Absent
Test for Aluminium	Present
Test for Iron	**Present**
Test for Zinc	Absent

Test for Calcium	**Present**
Test for Magnesium	Absent
Test for Ammonium	Absent
Test for Mercury	Absent
Test for Arsenic	Absent
Miscellaneous	
Test For Starch	Absent
Test for Reducing Sugar	Absent
Test for Alkaloids	**Present**
Test for Tannic Acid	Absent

Table.4: Results of Phyto - Chemical Analysis of Vellarugu Chooranam.

S. No	Phyto-Chemicals	Test Name	H_2O Ext.
1.	Alkaloids	Mayer's Test	**Present**
		Hager's Test	Absent
2.	Carbohydrates	Molisch's Test	**Present**
3.	Glycoside	Modified Borntrager's Test	**Present**
		Cardiac Glycoside- Keller-Killiani test)	Absent
2.	Saponins	Froth Test	Absent
		Foam Test	**Present**
3.	Phenols	Ferric Chloride Test	Absent
4.	Tannins	Gelatin Test	**Positive**
5.	Flavonoids	Alkaline Reagent Test	Absent
6.	Proteins and amino acids	Xanthoproteic Test	Absent
7.	Gum and Mucilage	Extract + alcohol	**Positive**

Table.5: Microbial Contamination Test of *Vellarugu Chooranam*

S. No	Parameters	Result
1.	Total Bacterial Content	Absent
2.	Total Fungal Content	Absent
3.	E. Coli	Absent
4.	Salmonella Spp.	Absent
5.	Staphylococcus Aureus	Absent
6.	Pseudomonas aeruginosa	Absent

Table.6: Heavy Metal Analysis of *Vellarugu Chooranam.*

Name of the Heavy Metal	Absorption Max Λmax	Result Analysis	Maximum Limit
Mercury	253.7 nm	BDL	1 ppm
Lead	217.0 nm	8.52	10 ppm
Arsenic	193.7 nm	BDL	3 ppm
Cadmium	228.8 nm	BDL	0.3 ppm

BDL- Below Detection Limit

Table.7: Pesticide Residue Analysis of *Vellarugu Chooranam.*

PesticideResidue		
I.OrganoChlorinePesticides	**SampleVC**	**AYUSHLimit(mg/kg)**
AlphaBHC	BQL	0.1mg/kg

BetaBHC	BQL	0.1mg/kg
GammaBHC	BQL	0.1mg/kg
DeltaBHC	BQL	0.1mg/kg
DDT	BQL	1mg/kg
Endosulphan	BQL	3mg/kg
II. Organo Phosphorus Pesticides		
Malathion	BQL	1mg/kg
Chlorpyriphos	BQL	0.2mg/kg
Dichlorovos	BQL	1mg/kg
III. Organocarbamates		
Carbofuran	BQL	0.1mg/kg
III. Pyrethroid		
Cypermethrin	BQL	1mg/kg

BQL-Below Quantification Limit

Table.8: Aflatoxin Analysis of *Vellarugu Chooranam.*

Aflatoxin	Sample VC	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

Figure.1: Organoleptic Character of Vellarugu Chooranam

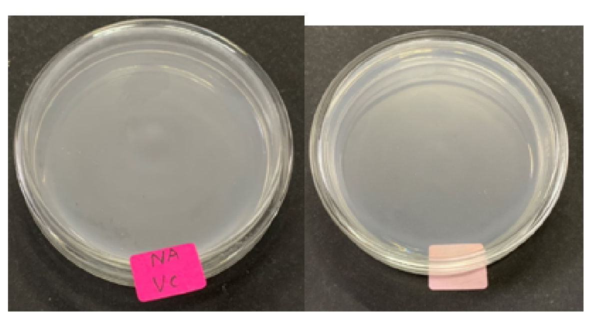

Figure.2: Microbial Contamination Test of *Vellarugu Chooranam.*
No growth / colonies was observed in any of the plates inoculates with the test sample.

TLCVisualizationofVC-TLCplatevisualizationat366nm

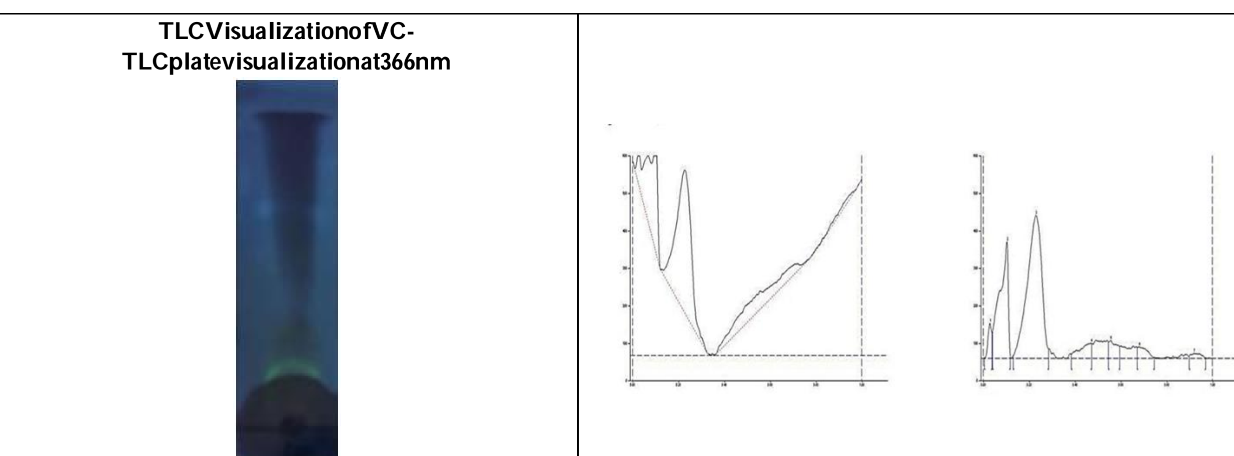

Figure.3: HPTLC finger printing of Sample VC

Figure.4:High Performance Thin Layer Chromatography (HPTLC) Analysis of Vellarugu Chooranam. High Performance Thin Layer Chromatography (HPTLC) Analysis of Vellarugu Chooranam:

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.00	2.4	0.03	93.7	10.16	0.04	67.5	789.3	3.55
2	0.04	72.1	0.10	311.6	33.80	0.12	7.7	6006.6	26.98
3	0.13	8.0	0.23	381.8	41.42	0.29	25.5	12441.3	55.89
4	0.38	11.9	0.47	41.0	4.44	0.47	40.4	1091.3	4.90
5	0.55	45.0	0.56	48.4	5.25	0.60	33.1	915.6	4.11
6	0.67	30.0	0.68	31.4	3.41	0.75	3.0	692.0	3.11
7	0.90	7.5	0.92	13.9	1.51	0.97	1.2	325.0	1.46

Figure.5: HPTLC finger printing analysis of the sample reveals the presence of seven prominent peaks corresponds to presence of seven versatile phytocomponents present within it. Rf value of the peaks ranges from 0.04 to 0.90.