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Author: Sasvatha Sivapathis, Anpuchelvi. S

Siddha Ayurvedic Base Hospital Batticaloa, University of Jaffna, Sri Lanka

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PHYTO – CHEMICAL, PARTICLE SIZE AND HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHY ANALYSIS OF SIDDHA FORMULATION ATTATHI CHOORANAM (ATC)

[1]Sasvatha Sivapathis, [2]Anpuchelvi. S

[1]Siddha Ayurvedic Base Hospital Batticaloa, [2]Unit of Siddha Medicine,

University of Jaffna, Sri Lanka

ABSTRACT

Siddha medicine is a valuable medicine that has been in practice since the ancient era. Preliminary standardization steps are essential for the identification of active compounds of genuine drugs and for setting analytical standards. The therapeutic potentials of herbal drugs are attributed to the presence of Phyto-chemicals. The study was done for documentation of qualitative & quantitative parameters of Phyto-chemicals, particle size, and High-Performance Thin Layer Chromatography analysis (HPTLC) properties of Siddha formulation ATTATHI CHOORANAM (ATC). Preliminary Phyto-chemical screening of ATC established the presence of chemical constituents like Alkaloids, Flavonoids, Glycosides, and Proteins. Quantitative analysis for Phyto-chemicals in aqueous and ethanol extracts showed, that Glycosides (3.7 ± 0.2 µg / ml), Flavonoids (7 ± 0.3 µg / ml) and Protein (52.5 ± 0.2 µg / ml) were maximum quantifiable in ethanol extraction whereas Alkaloids (2.6 ± 0.1 µg / ml) in aqueous extraction. The average particle size of sample ATC 106.711 µm in diameter and the overall distribution of particle is from 4 µm to 600 µm.

Keywords: Attathi Chooranam, Phyto-chemical particle size, HPTLC analysis.

INTRODUCTION

Plants provide essential nutrients and also other bioactive and non-nutritive phytochemicals that are produced by plants via several chemical pathways. Phytochemicals of medicinal plants that contribute to health promotion and disease prevention 3,11. Researchers have previously conducted studies about traditional herbal medicines, which play a major role in maintaining health. Pharmaceutical and natural compounds and newer drugs analysis is commonly used in all the stages of drug discovery and developmental process. Siddha medicine is one of the ancient medicines has been practicing since the civilizations of South India & Sri Lanka. Attathi chooranam (ATC) is a classical Siddha drug mentioned in the text Anupoka vaithiya navaneetham (part-8). Ingredients of ATC contain eight plants and one mineral in it. This Herbo-mineral drug is very effective in curing vatham and pitham related diseases1. According to WHO guidelines, an herbal product needs to be standardized before releasing it into the market11. More-over in industrialized societies, medicinal plants are abundantly depended for the development of newer drugs and nutraceutical products. Therefore, preliminary standardization3 steps are essential for the identification of genuine drug and setting analytical standards. This study was performed on the phytochemical properties4,5 (both qualitatively & quantitatively) particle

size and high-performance thin layer chromatography analysis6 (HPTLC) of the Herbo mineral compound drug of powder of ATC with different tests8,10.

Aim and Objectives

This study aims to determine the phytochemical, particle size and high-performance thin layer chromatography analysis of properties in ATC

Materials and Methods

Materials

Collection and identification of plant materials

The ingredients were authenticated by the Professors of department of Gunapadam and Medicinal Botany at Government Siddha Medical College and Hospital, Palayamkottai.

Purification of raw drugs:

The raw drugs were purified as per the methods mentioned in the Siddha literature.

Preparation of the drug Attathi chooranam (ATC)

All the ingredients for drugs were dried well in shadow and made into micronized powder with the proportion mentioned in the literature. (Table No.1 & Figure I).

Methods

Test procedure

The qualitative estimation of Phyto-chemicals and High-Performance Thin Layer Chromatography analyses were conducted at Siddha Regional Research Institute, Poojappura, Trivandrum, Kerala. The Quantitative estimation and particle size analysis were conducted Inbiotics, at William hospital campus, MS road, Nagercoil, KK district, Tamil Nadu.

Table 1: Ingredients of ATC

	INGREDIENTS	BOTANICAL NAME	FAMILY NAME	PART USED	QUANTITY
1	*Thippili*	*Piper longum* Linn.	Piperaceae	Dry fruit	8 parts
2	*Sukku*	*Zingiber officinale* Rosc.	Zingiberaceae	Dry rhizome	7parts
3	*Karujcheeragam*	*Nigella sativa* Linn.	Ranunculaceae	Seeds	6parts
4	*Natseeragam*	*Carum cyminum* Linn.	Apiaceae	Seeds	5parts
5	*Milaku*	*Piper nigrum* Linn.	Piperaceae	Dry fruit seeds	4parts
6	*Inthuppu*	Sodium chloride impure (Chemical name)			3parts
7	*Perumkayam*	*Ferula asafoetida* Linn.	Umbelliferae	Gum resin	2parts
8	*Omam*	*Carum copticum* Benth &Hook. F.	Apiaceae	Seeds	1part
9	*Sarkarai*	*Saccharum officinarum*	Poaceae	Jaggery	16 parts

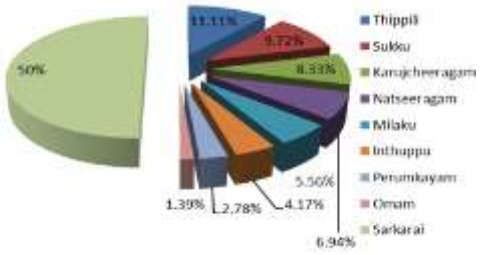


Figure I: Diagrammatic ingredients ratio presentation of ATC

Qualitative estimation of Phyto-chemicals

5 g of ATC was taken in a 250 ml clean beaker and 50 ml of distilled water was added, boiled well and allowed it to cool. It was filtered in a 100 ml volumetric flask and made up to 100 ml with distilled water. The ATC was subjected to following screenings.

Alkaloids

To the test substance, 1 ml of Dragendroff's reagent and 1 % H_2SO_4 was added.

Flavones

Shinado's test: To the substance in alcohol, a few magnesium turnings and a few drops of con HCl was added and boiled in a water bath for two minutes.

Glycosides

Mix the substance with a little enthrone on a watch glass, then one drop of con H_2SO_4 was added and made into a paste and warm gently in a water bath.

Terpenoids

Noller's test: Warm the substance with 2 or 3 Tin metal bits and 2 ml of thionyl chloride.

Saponins

Substance was diluted separately with 20ml of distilled water and it was agitated on a graduated cylinder for 15 min.

Amino acids

Treat the substance in alcohol or water with ninhydrin in alcohol

Tannins

Substance was treated with alcoholic and lead acetate solution.

Quantitative estimation of phytochemicals of ATC

Alkaloids:

5 ml pH 4.7 phosphate Buffer was added to 1 ml of Methanolic extract, and 5 ml BCG solution and shake the mixture with 4 ml of chloroform. The extracts were collected in a 10 ml volumetric flask and then diluted to adjust volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm against blank prepared as above but without extract. Atropine is used as a standard material and compared the assay with Atropine equivalents2.

Glycosides:

10 ml of the extract and 10 ml of Baljet's reagent are taken and allowed to stand for one hour. Then dilute the solution with 20 ml distilled water and mix. Read the intensity of the colour obtained against blank at 495 nm using a spectrophotometer. The difference between test and control is taken for calculation. Standard graph can be prepared using standard digitoxin8.

Flavanoids:

Total flavonoid content was determined by Aluminium chloride method using catechin as a standard. 1 ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). After 5 minutes, 0.3 ml of 5 % Sodium nitrite and 0.3 ml of 10 % Aluminium chloride was added. After 6 minutes incubation at room temperature, 2 ml of 1.0 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically. Results were expressed as catechin equivalents (mg catechin/g dried extract)2.

Estimation of protein by Lowry's method

Principle:

The blue colour developed by the reduction of the phosphomolybdicphosphotungstic components in the Folin–ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method5.

Reagents:

• Folin–ciocalteau reagent (reagent D)- reflux gently for 10 hours a mixture consisting of 100 g Sodium tungstate (Na2WoO4.2H2O), 25 g Sodium molybdate (Na2WoO4.2H2O), 700 ml water, 50 ml of 80 % phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5 L flask. Add 150 g lithium sulfate, 50ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 L and filter. The reagent should have no greenish 20 % Sodium carbonate in 0.1 M sodium hydroxide (Reagent A).

• 0.5 % Copper Sulphate (CuSO4.5H2O) in 1 % potassium sodium tartrate (Reagent B).

• Alkaline copper solution.: Mix 50 ml of A and 1ml of B prior to use (Reagent C)

• Protein Solution (Stock Standard): Weigh accurately 50 mg of bovine serum albumin (fraction V) and dissolve in distilled water and make up to 50 ml in a standard flask.

• Working Standard Solution: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. 1.0 ml of this solution contains 200 µg protein.

Procedure

Extraction of protein from Sample:

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grind well with a pestle and mortar in 5-10 mL of the buffer. Centrifuge and use the supernatant for protein estimation.

Estimation of Protein:

Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard into a series of test tubes. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Make up the volume to 1.0 ml in all the test tubes. A tube with 1.0 ml of water serves as the blank. Add 5.0 ml of reagent C to each tube including the blank. Mix well and allow it to standing for 10 mins. Then add 0.5 ml of reagent D, mix well and incubate at room temperature in dark for 30 min, blue colour is developed. Take the reading at 660 nm. Draw a standard graph and calculate the amount of protein in the sample.

Particle size analysis

The analytical sample was prepared and injected into the sample holder for the analysis of particle size and distribution by Particle size analyzer [Shimadzu SALD 2300 (WingSALD II: Version 3.1.1). The analytical measurements are given in the table 2.

RESULTS AND DISCUSSION

Phytochemical analysis of ATC

Qualitative estimation of phytochemicals revealed the presence of Alkaloids, Flavonoids, Glycosides, and Proteins (Table 3)

Table 2: Analytical measurements

Light source	Red Semiconductor laser (Wavelength: 680 nm)
Light detector	Detector elements for UV semiconductor laser Total 84 elements (78 forward, 1 side, 5 back)
System Compliance	Class 1 Laser Product, CE compliant
Required power supply	115 or 230 VAC as ordered 100 VA
Dimensions & weight	W600mm×D280mm×H430mm, 31kg
Operating Environment	Temperature: 10 to 30°C, Humidity: 20 to 80 % (no condensation)

Table 3 - Qualitative estimation of phytochemical constituents

No	Phytochemicals	Results
1.	Alkaloids	+
2.	Flavonoids	+
3.	Glycosides	+
4.	Terpenoids	-
5.	Phenols	-
6.	Acids	-
7.	Saponins	-
8.	Amino acids	-
9.	Proteins	+
10.	Tannins	-

The quantitative estimation results listed in tables 4 & 5 showed that Glycosides, Flavonoids and Protein were maximum quantifiable in ethanol extraction whereas Alkaloids in aqueous extraction.

Table 4 - Quantitative estimation of phytochemical constituents

SAMPLE: ATC (Aq)	
Test	Aqueous
Alkaloids µg / ml	2.6 ± 0.1
Glycosides µg / ml	3.5 ± 0.2
Flavonoids µg / ml	4 ± 0.3
Protein µg / ml	40.2 ± 0.2

Table 5- Quantitative estimation of phytochemical constituents

SAMPLE: ATC(Eth)	
Test	Ethanol
Alkaloids µg / ml	1.6 ± 0.1
Glycosides µg / ml	3.7 ± 0.2
Flavonoids µg / ml	7 ± 0.3
Protein µg / ml	± 0.2

Particle size analysis

The average particle size of sample ATC 106.711 µm in diameter and the overall distribution of particle is from 4 µm to 600 µm. the cumulative % of sample AC was fall in 10, 50, 97 % where 10% of total particle fallen in 14.304 µm, 50% of total particle was fallen in 106.711 µm and 97% of total particle was fallen in 441.161 µm.

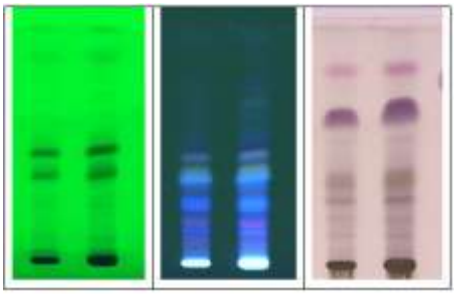
High performance Thin Layer chromatography of of Attathi chooranam.

High-performance thin layer chromatography is one of the sophisticated instrumental techniques based on the full capabilities of thin layer chromatography. It presents results as images and offers simplicity, cost-effectiveness, parallel analysis of samples, high sample capacity, rapid results, and the option for multiple detection methods. HPTLC is confirmed and established its identity. It is also an ideal screening tool for adulterations and is highly suitable for evaluation and monitoring of cultivation, harvesting, and extraction processes and testing of stability.

Alcohol extract

Solvent system: Toluene: Ethyl acetate: Formic acid (5: 1: 0.1)

Volume applied; Track 5- 6 µl: Track 6 – 12 µl



Under UV short	Under UV long	Under white light after derivatisation

Figure II: HPTLC of ATC

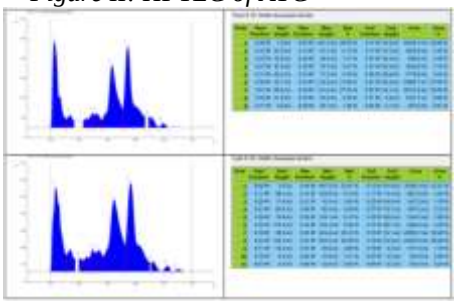


Figure III: 254 nm

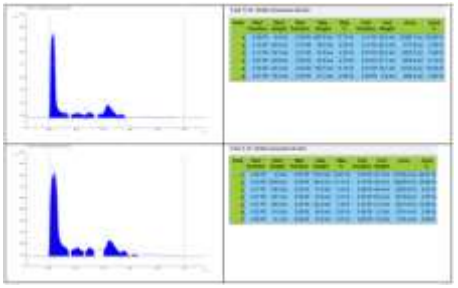


Figure IV: 366 nm
575 nm

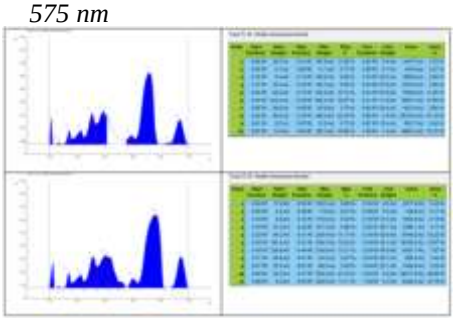
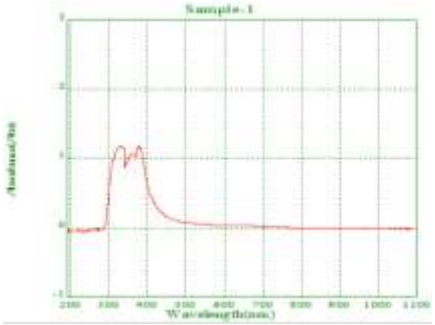


Figure V: 575 nm



CONCLUSION

Analytical tests are essential tool for authentication, standardization and quality control assessment of raw drugs. Selection of such genuine drugs in the manufacturing of traditional medicines are need of the present hour. The results revealed that ATC has significant presence of Glycosides (3.7 ± 0.2 µg / ml), Flavonoids (7 ± 0.3 µg / ml) and Protein (52.5 ± 0.2 µg / ml) in ethanol extraction whereas Alkaloids (2.6 ± 0.1 µg / ml) in aqueous extraction. The above phytochemicals will be very effective to cure the diseases and its complications. The average particle size of sample ATC 106.711 µm in diameter and the overall distribution of particle is from 4 µm to 600 µm. HPTLC is confirmed and established its identity of ATC. According to this study that can conclude; observed phytochemicals have medicinal values for curable diseases induced by Vatha and Piththam.

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