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ORIGINAL ARTICLE **OPEN ACCESS**

Standardization and Quantitative Estimation of Active Biomarker from Polyherbal Formulation by RP-HPLC Method

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ABSTRACT
Sukshma vara vati (SV) is an Ayurvedic polyherbal formulation contains three major ingredients such as Terminalia chebula (Haritaki), Terminalia belerica (Bibhitaki), Emblica officinalis (Amalaki) that is employed for cough and colds, allergic disorders, acts as immunomodulator to strengthen the body's immunity and protect against common bacterial infections. The present research study deals with standardization of sukshma vara vati and the biomarker method for quality control of well-known ayurvedic formulation was developed by reversed phase high performance liquid chromatography determination using Gallic acid as a standard, which are important and major content in formulation. The marketed formulation was procured from Chaitanya Pharmaceutical Pvt. Ltd., Satpur. The standardization of this formulation, the organoleptic characteristics, physico-chemical properties, quality control testing and phytochemical screening was carried out to ascertain the quality, purity and safety of this herbal formulation. Phytochemical screening shows that presence of tannins, alkaloids, flavanoids, saponins, glycosides. Total phenolic content were also performed. The RP-HPLC method was developed for the estimation of isolated biomarker (Gallic acid) in marketed formulation. The chromatogram of gallic acid under experimental condition showed a single peak of the drug at 4.570min. The percent yield of isolated biomarker and polyherbal formulation was found to be 33.75 % and 1.17 % respectively. The result of statistical analysis shows that the current RPHPLC method for determining of Gallic acid is simple, accurate, and suitable for routine analysis of Gallic acid in Sukshma vara vati formulation.
***Keywords**: Standardization, Polyherbal formulation, Biomarker, RP-HPLC, Gallic acid.*

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INTRODUCTION

"Standardization" of herbal medicine is not easy as there are many factors influencing biological efficacy and reproducible treatment outcomes [1-3]. In order to obtain quality-oriented herbal products, attention should be paid to the correct identification of plants, sites and areas of their harvest, the processes of their extraction, purification and the rationalization of combinations of polyherbal drugs. Standardization concerns the addition of excipients or the mixing of herbs or herbal preparations to adapt the herbal formulations to the prescribed content of ingredients or groups of substances with known therapeutic effects. Standardization of herbal medicines is the process of defining a set of internal standards or characteristics, fixed parameters, qualitative and quantitative values that are guaranteed to be quality, effective, safe and repeatable. It is the process of developing technical standards and reaching agreement. Concrete standards are established through experimentation and observation that will lead to the process of prescribing the set of characteristics that a given drug exhibits[4]. The characteristic compound is used as a marker to evaluate the presence of other therapeutic biochemical compounds[5].

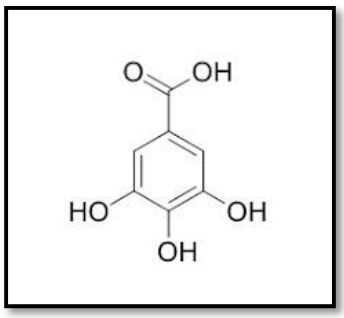


Fig 1: Structure of Gallic acid

The herbal formulation used extensively in the Ayurveda system of medicine contains an enormous number of active biomarkers, e.g., tannins such as Ellagic acid and Gallic acid in Sukshma vara vati. Gallic acid is a common component of plants; therefore, it is an active ingredient in herbal preparations used as health products and or also used as a biomarker. Gallic acid (3,4,5-trihydroxybenzoic acid) is a naturally occurring plant phenol, that is found in Amla, Haritaki, Behera, tea, grapes etc. in the free state and as part of the tannin molecules [5-10]. Gallic acid has a cytotoxic, anti-inflammatory, antimutagenic, hepatoprotective and neuroprotective effect on cancer cells, an antitumor potential and an analgesic effect.

MATERIAL AND METHODS

Procurement of Marketed Formulation: The marketed formulation of Sukshma vara vati was procured from Chaitanya Pharmaceuticals Pvt. Ltd., Satpur, Nashik.

Organoleptic Evaluation: Organoleptic evaluation parameters include colour, smell, taste, size, shape and particularities such as touch, texture etc. The first view of a plant or extract is so specific that it tends to identify itself. If this is not enough, the plant or extract may have a characteristic smell or taste.

PHYSICOCHEMICAL CHARACTERISTICS [11]:

1. **Determination of Ash:** The value of ash is an important parameter for confirming the acceptability and purity of improper collection and storage of medicines. High ash content indicates that the API is contaminated, substituted or adulterated.
   a) **Total Ash:** The ash content of the drug is the residue remaining after incineration. The total ash content is calculated in mg/gm air-dried material.
   b) **Acid Insoluble Ash**: It determines the amount of silica, especially sand and siliceous earth.
   c) **Water Soluble Ash:** Water soluble ash value is used for the determination of inorganic contents.
   d) **Sulphated Ash:** The sulphated ash test is used to measure the amount of non-volatile residue in a sample when the sample is ignited in the presence of sulphuric acid. This test is generally used to determine the content of inorganic impurities in organic substances.
2. **Loss on Drying:** It determines the wet content of the drugs i.e. Moisture. It also estimates the water and volatile content of drugs. Loss on drying prevents the declining or decaying of drugs. High moisture has lesser stability because of greater degradation of the product.
3. **Extractive Values:**
   a) Alcohol Soluble Extractive Value: To estimate the amount and detect adulteration from incorrect processing drugs or exhausted drugs.
   b) Water Soluble Extractive Value: Water soluble extractive value is the detection of adulteration from incorrect processing drugs or exhausted drug during drying or storage.

QUALITY CONTROL TESTS [12-20]

Quality control tests of tablets were done with the following parameters

1. **Weight Variation**: Tablet weight variation was performed to ensure that each tablet contained the correct amount of the drug.
2. **Hardness:** The conformation of the tablets to capping, abrasion, or breaking under the conditions of storage, transport and handling prior to use depends on the hardness of the tablet (kg/ cm2).
3. **Thickness:** The thickness of the tablet is determined by the diameter of the tablet. A micrometre was used to check the thickness of the tablet. The thickness must be controlled to be consumer acceptance and to facilitate packaging.
4. **Friability:** This test is designed to determine the physical strength of the tablet. The friability test was performed using a tablet friability tester (Roche fibrillator).
5. **Disintegration Test:** One tablet is placed in each tube and the basket is placed in a 1L beaker of water, simulated gastric fluid or simulated intestinal fluid at 37˚C ± 2˚ C,

PHYTOCHEMICAL SCREENING [21]

Phytochemical analysis is very important for assessing the potential medicinal value of plants and determining the active ingredients responsible for the known biological activity of the plants. In addition, it also provides the base for isolating specific compounds and investigating more thoroughly. The extraction of phytochemicals from plant materials depends mainly on the type of solvent used. Likewise, tests used for phytochemical analysis can determine the presence of phytochemicals in a sample.

EXTRACTION, ISOLATION AND PURIFICATION OF GALLIC ACID [22]

Extraction: Sukshma vara tablets were crushed to a fine powder (100gm) and extracted in a Soxhlet extractor with methanol for 15 hours. The extract was dried under reduced pressure using a rotary evaporator to obtain a viscous mass, and it was suspended in 100 ml of distilled water. Then it was transferred to another separatory funnel, and extracted with hexane (300ml) and then with ethyl acetate (300ml). The solvent was evaporated under reduced pressure using a rotary evaporator to obtain an extract of hexane and ethyl acetate. The ethyl acetate extract was chromatographed on a silica gel G column, eluting with methanol using column chromatography.

Isolation of the fractions by column chromatography: A methanol solvent was used to build the silica in the glass column. The activated silica was loaded into the column. 2 grams of ethyl acetate extract was applied to the top of the column. The column was eluted using with mixture of toluene: ethyl acetate: methanol: formic acid (6: 6: 0.4: 1.6). Fractions were collected for the selected mobile phase. TLC was performed for each fraction to determine homogeneity and detection with various reagents for separating the active ingredients. The chromatographic pattern of each fraction was carefully examined and the fractions belonged to the same elution solvent, which gave identical patterns in terms of Rf value, and the colour reaction was mixed. Altogether ten major fractions were collected after monitoring by TLC for the presence of Gallic acid (RF 0.45) in solvent systems, Toluene: ethyl acetate: methanol: formic acid (6:6:0.4:1.6).

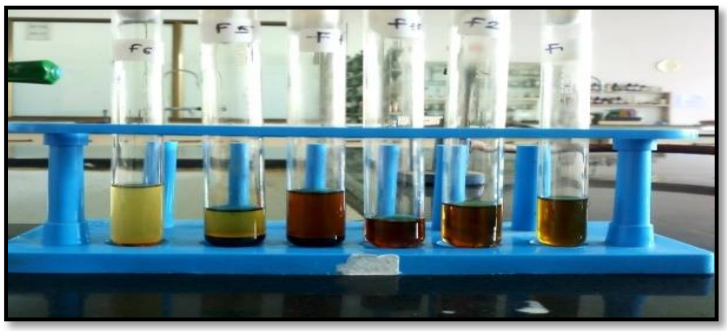


Fig 2: Isolated fractions by column chromatography

Identification of Bioactive Fraction by Thin Layer Chromatography:

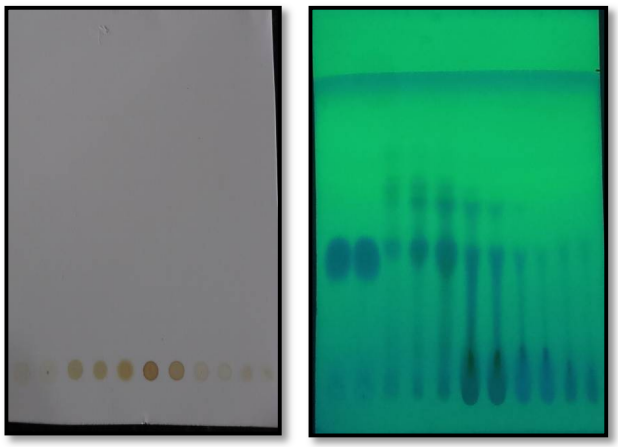


Fig 3: Thin layer chromatography of standard Gallic acid and isolated fraction i) In normal light ii) In UV-Visible light

Purification: The fraction F- 2, 3, 4, 5 containing Gallic acid was further purified by column chromatography. Again 10 fractions were collected and performed preparative thin layer chromatography in the solvent system toluene-ethyl acetate-formic acid (6:6:1).

Fig 4: Isolated fraction again purified by column chromatography

An amount of 20 mg of sample was loaded on the preparative thin layer chromatographic plate (Silica gel GF254, 20 x 20 cm, 1 mm thickness). Fraction 6, 7, 8, 9 does not contain any type of impurity and was selected for further analysis. The band corresponds to Gallic acid. Then the solvent was evaporated and the crystals of Gallic acid (3 mg) were isolated. It was finally purified by re-crystallization with hot water [23].

Fig 5: Isolated biomarker

Fourier Transform Infrared Spectroscopy: FTIR is a very useful technique that can be used to confirm the identity of pure compounds, but is of limited value if used in mixtures of compounds.

Total Phenolic Content: Phenolic compounds are important components of redox plants and are responsible for antioxidant activity. The hydroxyl groups in plant extracts are responsible for promoting free radical scavenging activity. The Folin-Ciocalteu reagent was used as a basis for measuring the phenol content of each extract.

QUANTITATIVE ESTIMATION OF GALLIC ACID [24-30]

Quantitative measurement by chromatographic analysis is based on the measurement of the peak height or peak area of a sample of unknown concentration.

Preparation of Standard Stock Solution

An accurately weighed quantity of pure Gallic acid (10mg) was transferred to a 10mL volumetric flask, dissolved and diluted to the mark with Water: ACN (80:20) pH 3.0 to obtain a standard stock solution of 1000µg/ml.

Sample preparation for Pure Gallic Acid

An Aliquot of 300 µl sample is withdrawn from the above stock solution by a micropipette and transferred to a 10 ml volumetric flask and the final volume is made up of the same mobile phase. A prepared sample solution of 30 µg/ml was analysed.

Sample preparation for Marketed formulation

20 tablets were taken and crushed in mortar pastel. From that, accurately weighed 16.6mg tablet powder was transferred to a 10ml standard flask. Volume is made up to the mark with Water: ACN (80:20) pH 3.0, sonicated for 10 min. It is filtered with 0.22µ filter to obtain a sample stock solution. An Aliquot of 300 µl sample is withdrawn by a micropipette and transferred to a 10 ml volumetric flask and the final volume is made up with the mobile phase. A prepared sample solution of 30 µg/ml was analysed.

Sample Preparation for Extracted Biomarker

An accurately weighed quantity of extracted Gallic acid (10mg) was transferred to a 10mL volumetric flask, dissolved and diluted to the mark with Water: ACN (80:20) pH 3.0, sonicated for 10 min. It is filtered with 0.22µ filter to obtain a standard stock solution of 1000µg/ml. An Aliquot of 300 µl sample is withdrawn by a micropipette and transferred to a 10 ml volumetric flask and the final volume is made up with the mobile phase. A prepared sample solution of 30 µg/ml was analysed.

Table 1: Chromatographic Condition for Gallic Acid by RP-HPLC

Mobile phase	Water: ACN (80:20) pH3
Selection of column	Phenomenex C18 (4.6mm x 290mm, Particle size: 5µm)
Injection volume	20 µL
Flow rate	1.0 ml/min
Column temperature	Room Temperature
Detection wavelength	272 nm
Retention time	4.5 min

RESULTS AND DISCUSSION

Organoleptic Properties

The observations for the organoleptic evaluation of Sukshma vara vati formulation were reported in Table 2.

Table 2: Results for organoleptic evaluation of Sukshma vara vati formulation

S.N.	ORGANOLEPTIC PROPERTY	FORMULATION
1	Appearance	Tablet
2	Colour	Light brown
3	Odour	Characteristics
4	Taste	Salty and Sour
5	Shape	Round
6	Texture	Smooth surface
7	Touch	Hard

PHYSICO-CHEMICAL CHARACTERISTICS

The observations for the physico-chemical evaluation of Sukshma vara vati formulation were reported in Table 3.

Table 3: Results for physicochemical evaluation of Sukshma vara vati formulation.

S.N.	PROPERTIES	RESULTS	STANDARD (IP)
1	Total Ash	6.66 %	NMT 7%
2	Acid Insoluble Ash	4.33%	NMT 5%
3	Water soluble Ash	13.33%	-
4	Sulphated Ash	10.5%	NMT 15%
5	Loss On Drying	2.0%	NMT 2%
6	Alcohol Soluble Extractive Value	26.20%	NLT 25%
7	Water Soluble Extractive Value	38.60%	NLT 35%
8	PH	3.5	-
9	Solubility		
	I) Distilled water	Soluble	-
	II) Methanol	Freely soluble	-

QUALITY CONTROL TESTS

The observations for the quality control evaluation of Sukshma vara vati formulation were reported in Table 4.

Table 4: Results for quality control testing of Sukshma vara vati formulation

S.N.	PARAMETERS	RESULTS		STANDARD
1	Weight uniformity test Avg. weight Min. weight Max. weight	304.50mg 289.28mg 319.72mg	± 5%	± 5%
2	Hardness	4.0 kg/cm^2		4-6 kg/cm^2
3	Thickness	0.29cm		-
4	Diameter	0.79cm		-
5	Friability	0.165%		NMT 1%
6	Disintegration test	56minutes 14seconds		NMT 1hour

PHYTOCHEMICAL SCREENING

The observations for the phytochemical screening analysis of Sukshma vara vati formulation were reported in Table 5.

Table 5: Results for Phytochemical screening of Sukshma vara vati formulation

S. N.	Phytoconstituents	Results
1	Carbohydrates	+
2	Reducing sugars	+
3	Saponins	+
4	Proteins	-
5	Amino acids	+
6	Steroids	+
7	Alkaloids	+
8	Tannins	+
9	Flavonoids	+
10	Cardiac glycosides	-

IDENTIFCATION OF ISOLATED BIOMARKER (Gallic acid)

Thin Layer Chromatography:

1. **Preliminary TLC study**

Figure 6: Thin layer chromatography of standard Gallic acid

The results of TLC analysis of the standard gallic acid reveal the presence of a single dark violet coloured spot corresponds with Rf value ranging from 0.40 to 0.45 using in optimized mobile phase such as Toluene: Ethyl acetate: Methanol: Formic acid (6: 6: 0.4: 1.6). This mobile phase composition used for further isolated fractions of biomarker characterization.

2. **Identification of isolated fractions of biomarker compound by TLC:**

Figure 7: TLC Chromatogram of std. Gallic acid and an isolated fraction of biomarker compound

The result revealed the presence of a single dark violet-coloured spot, showing the presence of Gallic acid in the marker compound. The results are illustrated in Figure 7.

Melting Point Determination: The melting point of the biomarker was determined by using a melting point apparatus.

Table 6: Results for Melting Point Determination

S.N.	MELTING POINT OF BIOMARKER	STANDARD
1	256^{0}C - 258^{0}C	258^{0}C - 260^{0}C
2	254^{0}C - 256^{0}C	
3	256^{0}C - 258^{0}C	

The melting point of the isolated biomarker from formulation was found to be 256^{0}C-258^{0}C and the melting point of the standard Gallic acid sample was found to be 258^{0}C-260^{0}C. That means it indicates that the isolated biomarker shows the presence of Gallic acid.

FTIR Analysis

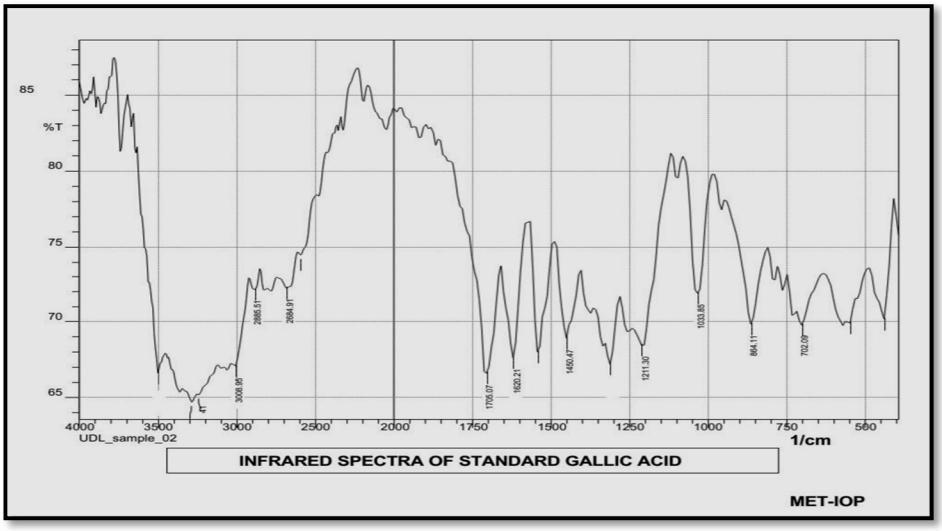


Figure 8: Infrared spectrum of standard Gallic acid

Table 7: Interpretation of IR spectrum of standard Gallic acid

Sr. No.	Peak (cm-1)	Type of vibrations	Intensity	Frequency (cm-1)
1	702.09	Aromatic C-H (Out-of- plane bend)	S	900-690
2	1033.85	C-O stretch	S	1300-1000
3	1211.3	C-O stretch	S	1300-1000
4	1450.47	-CH3 bend	M	1450 & 1375
5	1620.21	C =C stretch(alkene)	S	1680-1600
6	1705.07	C =O stretch(ketone)	S	1725-1705
7	2684.91	O-H stretch(COOH)	M	3400-2400
8	2885.51	O-H stretch(COOH)	M	3400-2400
9	3008.95	C-H stretch(alkenes)	M	3100-3000
10	3240.41	O-H stretch(H-bonded)	S	3400-3200
11	3286.7	O-H stretch(H-bonded)	S	3400-3200

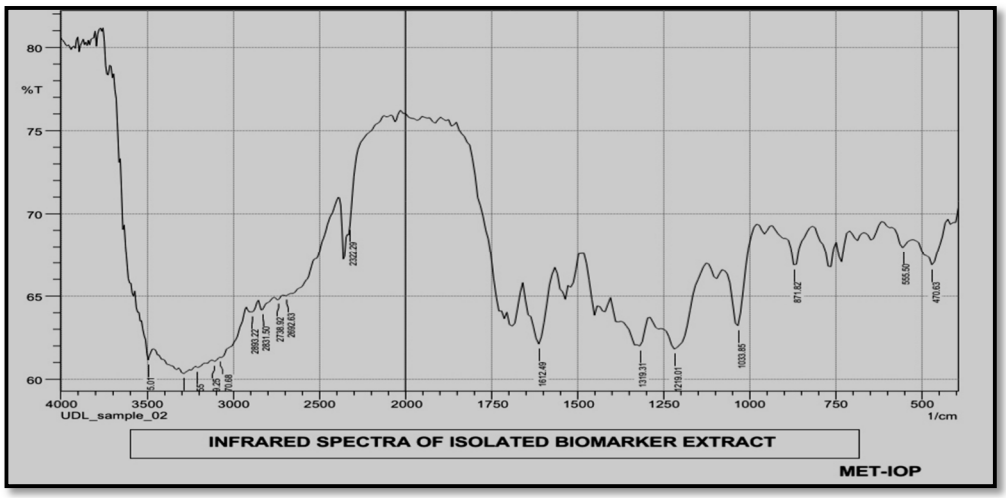


Figure 9: Infrared spectrum of isolated biomarker compound

Table 8: Interpretation of IR spectrum of isolated biomarker compound

Sr. No.	Peak (cm-1)	Type of vibrations	Intensity	Frequency (cm-1)
1	871.82	Aromatic C-H (Out-of- plane bend)	S	900-690
2	1033.85	C-O stretch	S	1300-1000
3	1219.01	C-O stretch	S	1300-1000
4	1612.49	C =C stretch (alkene)	S	1680-1600
5	1710.29	C =O stretch (ketone)	S	1725-1705
6	2831.5	O-H stretch (COOH)	M	3400-2400
7	2893.22	O-H stretch (COOH)	M	3400-2400
8	3070.68	C-H stretch (alkenes)	M	3100-3000
9	3209.55	O-H stretch (H-bonded)	S	3400-3200
10	3286.7	O-H stretch (H-bonded)	S	3400-3200

Determination of Total Phenolic Content:

The total phenolic content for methanolic extracts was estimated by Folin Ciocalteu's method using Gallic acid as standard in terms of the Gallic acid equivalent (GAE) in mg/g of the extract. The blue colouration produced has maximum absorption in the region of 725 nm and is proportional to the total quantity of phenolic compounds originally present. The Gallic acid solution of concentration (20-100 ppm) confirmed Beer's Law at 725 nm with a regression coefficient (R2) = 0.993. The total phenolic content was calculated with the help of the calibration curve shown in Figure 18. The plot has a slope (m) = 0.004 and intercept = 0.0542. The equation of the standard curve is y = 0.004x + 0.082. The total phenolic contents (Gallic acid equivalents, mg/g) in the methanolic extracts were calculated to be 67.50 ± 3.110 mg/g.

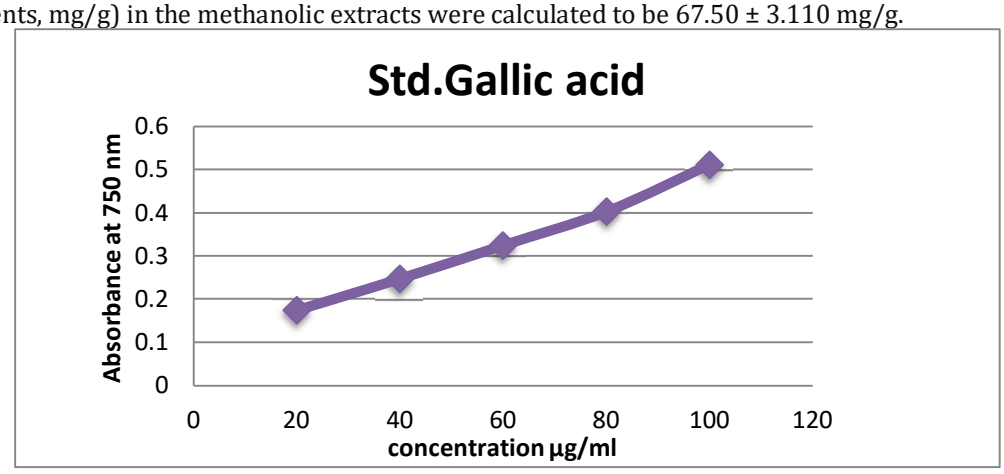


Figure 10: Calibration curve of std. Gallic acid

Table 9: Calibration curve of std. Gallic acid

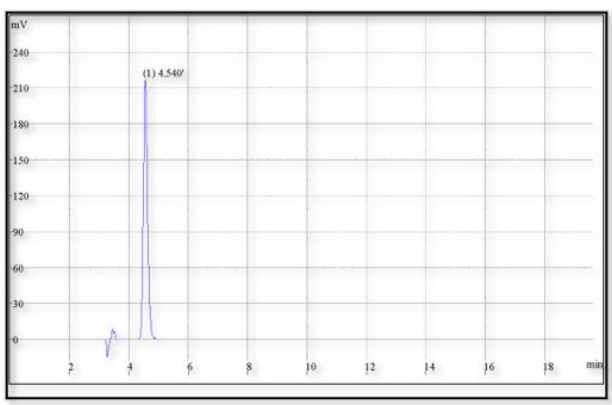
Sr. No.	Concentration of extracts	Total Phenolic content
Trial-1	1mg/ml(methanol)	68.5
Trial-2	1mg/ml(methanol)	66.3
Trial-3	1mg/ml(methanol)	60
Mean± S.E.M	-	67.50 ± 3.110

QUANTITATIVE ESTIMATION OF BIOMARKER BY HPLC:

The quantitative estimation of biomarker by rp-hplc method was developed by using Mobile phase Water: ACN (80:20). The chromatogram of Gallic acid under experimental condition showed a single peak of the drug at 4.540 min.

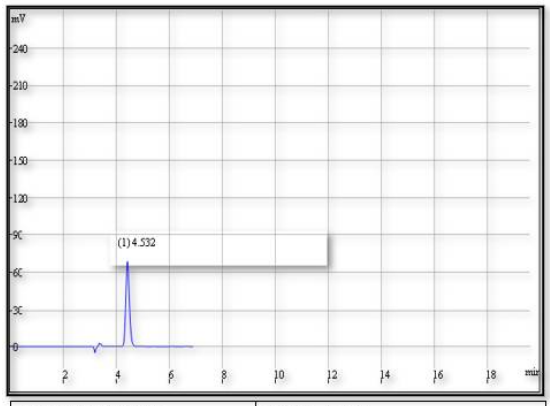
Table 10: Chromatographic Condition for Gallic acid by RP-HPLC

Mobile phase	Water : ACN (80:20) pH3
Selection of column	Phenomenex C18 (4.6mm x 290mm, Particle size: 5µm)
Injection volume	20 µl
Flow rate	1.0 ml/min
Column temperature	Room Temperature
Detection wavelength	272 nm
Retention time	4.5 min



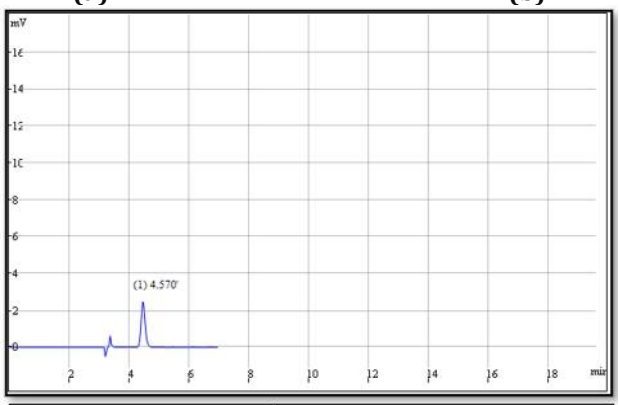
Retention time	Peak Area
4.540 min	1910398

(a)



Retention time	Peak Area
4.532 min	644831

(b)



Retention time	Peak Area
4.570 min	22481

(c)

Fig 11: HPLC Spectra of **(a)** Gallic Acid 30ppm Standard biomarker **(b)** Gallic Acid 30ppm Extracted sample **(c)** Gallic Acid 30ppm Marketed formulation (Sukshma Vara)

Yield of biomarker compound

The quantitative estimation of isolated biomarker compounds from the Sukshma vara vati formulation was performed. The per cent yield of the isolated biomarker compound was found to be 33.75% and the % yield of Sukshma vara vati formulation was found to be 1.17%.

Table 22: Quantitative estimation of biomarker and marketed formulation by HPLC

Sr. No.	Sample Name	Area of Sample	Area of Standard	% Yield
1.	Extracted Sample	644831	1910398	33.75%
2.	Marketed formulation (Sukshma Vara)	22481	1910398	1.17%

The HPLC method developed for the quantitative estimation of Gallic acid is simple, rapid and precise for the routine estimation of Sukshma vara vati. As Sukshma vara vati is a good source of Gallic acid, these findings can be used as a routine chromatographic fingerprinting method for the standardization of the finished formulation Sukshma vara vati.

CONCLUSION

After analysis of Ayurvedic formulation of Sukshma vara vati, it can be concluded that the herbal formulation can standardized by modern scientific quality control protocol. This standardization protocol is prominent in maintaining or fixing quality standards of herbal formulation. The result of quality control parameters for this herbal formulation was complying with the standards of Indian pharmacopoeia for the tablet dosage form. The present study reported that formulation shows significant Antioxidant activity, which stabilizes free radicals before they attack a target in biological cells. TLC chromatogram confirmed the presence of an active ingredient in the herbal formulation. The chemical structure of the formulation was observed by IR spectrum and was confirmed with respect to standard drugs. Hence those chemical constituents present in the formulation, are responsible for therapeutic activity. Thus, the present standardization study reveals compliance with all the above-selected parameters. The HPLC method developed for the quantitative estimation of Gallic acid is simple, rapid and precise for the routine estimation of Sukshma vara vati. As Sukshma vara vati is a good source of gallic acid, these findings can be used as a routine chromatographic fingerprinting method for the standardization of the finished formulation Sukshma vara vati.

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