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Standardization Of Asthiposhak Vati –A Ayurvedic Herbomineral Formulation

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Abstract
Calcium is a vital micronutrient that is generally considered to affect bone health and human metabolism. Several people across the globe suffer from calcium deficiency due to poor calcium consumption and vitamin D inadequacy. Calcium insufficiency causes a disease such as osteoporosis, rickets, epilepsy, and anemia. There are several Ayurvedic and allopathic supplements that can be used to address this calcium insufficiency.
One of the herbal-mineral medications used frequently by Ayurvedic doctors to treat calcium deficiency is Asthiposhak Vati. Where "Asthi" refers to the bone and "Poshak" to nutrition It refers to a substance that provides the bones nutrition. As this is Herbo mineral formulation which contains many herbals as well as minerals. The chief ingredient of this formulation is Kukkutanda Bhasma (Egg Hen Shell) is one of the calcium-rich mineral medicinal formulations mentioned in Ayurveda.It is included under Sudha Varga as it contains Calcium compound.
Lack of methods for standardization and quality control of raw materials and formulations, as well as safety and toxicity problems caused by the presence of heavy metals and minerals, are key barriers to the success of Ayurvedic medicines. Due to a lack of reference standards, standardization of Ayurvedic formulations is lagging behind. To achieve global harmonization, the WHO has established standards for evaluating the efficacy and safety of herbal medicines.
The objective of the current research was to establish a methodology for standardization of Asthiposhak Vati and its raw materials. As per WHO guidelines, standardization was done using systematic Pharmacognostical and physicochemical characteristics. The set standards were considered to be adequate for evaluating the Asthiposhak Vati and can be utilized as reference standards for forward for quality control and quality assurance in future.
Keywords: *Asthiposhak Vati, Calcium Deficiency, Kukkutanda Bhasma, Herbo mineral, Standardization, HPTLC.*

INTRODUCTION:

India has frequently been described as the Medicinal Garden of the universe because nature has gifted our nation with an amazing diversity of medicinal plants. Ayurveda, Siddha, Unani, Homeopathy, Yoga, and Naturopathy are only a few of the established Indigenous systems of medicine used in India, which holds a unique place in the world.(1)
More than 3,000 years ago, India gave birth to the natural medical system known as Ayurveda. The Sanskrit terms Ayur (life) and Veda (science or knowledge) are used to form the word ayurveda. Ayurveda, thus, means "knowledge of life." (2)The term "Rasa dravyas" refers to substances that are dealt by a specialized branch of Ayurveda known as "Rasa Shastra," which literally translates as "Science of Mercury." Ayurveda now includes Rasa-Shastra as a core part. Rasa refers primarily to Parada (Mercury). Rasa-Aushadhis (Herbo-mineral metallic preparations) are compounds formed by mercury and incinerated metals and minerals. In this dynamic age. Rasa Aushadhis have given Ayurveda a completely modern healthcare look. Rasa Aushadhis' innate advantages, like their immediate effect, low dosage, longer shelf life, and improved palatability, have made it easier for them to attract both patients and pharmaceutical companies. (3,4)Purification and detoxification procedures used during drug manufacturing are said to reduce or eliminate the toxicity of Herbo mineral. Purification, detoxification, and incineration processes are all recommended to provoke attributes that are necessary for ensuring the material's safe and easy integration into the live body (5)
The acharyas divided a wide range of minerals into varga, including Maharasa, Uprasa, Sadharana Rasa, Dhatu-updhatuvarga, Sudha varga, and Vishopvishavarga, and others. The minerals that are calcium-rich, such as Sudha, Shankha (conch shell), Shukti (pearl oyster), and Khatika (cowrie shell), fall under the purview

of Sudha Varga. Kukkutanda twaka, or eggshell, is a constituent of Sudha varga and is primarily composed of calcium compounds.(6)

Sustaining quality standards is essential for wider acceptance in an era of growing demand for indigenous medicines. Lack of methods for regulating the quality and standardization of raw materials and formulations, as well as safety and toxicity problems brought on by the presence of heavy metals and minerals, are key obstacles to the success of Ayurvedic medicines. Standardization is a process that guarantees each dose of ingredients has a specific quantity, quality, and therapeutic efficacy. In order to achieve global harmonization, the WHO has set guidelines for evaluating the efficacy and safety of herbal medicines. (7)

800 million individuals worldwide are malnourished. More than 3.5 billion people worldwide are at danger of calcium deficiency, with 90% of those at risk living in Africa and Asia. Nutritional bone disease affected 52% of the population in one of the longest (1963–2005) and largest studies from India. The three most prevalent diseases are Osteomalacia (35.3%), rickets (7.6%), and osteoporosis, which are all brought on by vitamin D insufficiency, insufficient sun exposure, and a calcium-deficient diet. (8)

In the earth's crust, calcium is the fifth most prevalent element.(9) The most abundant stored nutrient in the human body is calcium. The bones and teeth store a majority of the energy (1.2–1.4 kg). Extracellular serum calcium makes up less than 1% of the total. Calcium is involved in the contraction and dilatation of blood vessels, muscular contraction and nerve transmission, blood coagulation, and the release of hormones, according to research. (10)

Tablets play a vital part in Ayurvedic pharmaceutics due to various advantages such as ease of administration, palatability, and a suitable form for dispensing and transportation. The terms Vati and Gutika refer to ayurvedic medications made in the form of tablets or pills. These contain one or more medications with a botanical, animal, or mineral origin (11,12)

"Asthi" signifies bone, while "Poshak" denotes nutrition. It means a formulation, which provide nutrition to the bone is known Asthiposhak Vati. A *Asthiposhak Vati* is Herbo-mineral preparation of Ayurveda mentioned to enhanced calcium level in calcium deficient patient. The preparation of *Asthiposhak Vati* mainly contains Kukkutanda Twak Bhasma (Hen eggshell Bhasma), Shigru (*Moringa olifera*), Arjuna (*Terminalia arjuna*), Ashwagandha (*Withania somnifera*), Haritaki (*Terminalia chebula*), Laksha (*Laccifer lacca*), Dinka (*Cestrum diurnum*), Yawa (*Amorphophallus paeoniifolius*), Guduchi (*Tinospora cordifolia*), Babul Twak (*Vachellia nilotica*)

Figure 1, Container of Asthiposhak Vati

Figure 2, Asthiposhak Vati

Asthiposhak Vati is made by mixing these main ingredients in the same ratio after being purified with a special herbal juice using the Bhavana method. The formulation has been suggested for the treatment of calcium deficiency. Recent studies on the presence of heavy metals in Ayurvedic or herbal medicines have sparked controversy and raised questions, necessitating the urgent need to create quality standards for herbal-mineral formulations and guidelines for Rasa Aushadhis (Asthiposhak Vati) so that they can be used without concern for toxicity and contamination.

Hence in the present study effort has been made to highlight the significance of these pharmaceutical procedures and to standardize the method of preparation of Asthiposhak Vati through Pharmacognostical and pharmaceutical evaluation.

MATERIAL AND METHODOLOGY:

1] Macroscopic study: Macroscopic study refers to organoleptic evaluation like colour, odour, taste, and texture were used to confirm uniformity in visual identity of raw materials and finished product. The results are as tabulated in Table (13)

2] Physiochemical Evaluation: The physicochemical parameters include tests like pH, Loss on drying, Extractive vale (Water & alcohol soluble extractive) and determination of total ash and Acid insoluble ash value of the relevant raw materials and finished product values were determined. The results are as tabulated in Table. (14)

3] Phytoconstituent Study: A phytochemical screening was conducted on the extract using standard qualitative methods to confirm the presence of different phytoconstituent such as carbohydrate, proteins, amino acid, anthraquinone glycosides, cardiac glycosides, alkaloids, tannins, flavonoid, saponins. (14)

4] Modern Pharmaceutical Parameter

a) Physical Dimensions (Diameter and Thickness):

The diameter and thickness of Asthiposhak Vati was measured with Vernier calliper. (15)

b) Hardness: The hardness test was performed by using Monsanto hardness tester. The instrument measures the force required to break the tablet when the force (Kilogram-force) generated by anvils to the tablet. The tablet was placed between two anvils; the force applied to the anvils, and the crushing strength that causes the tablet to break was recorded, and the crushing strength test was performed on 5 tablets. (15)

c)Friability: The friability test was performed by using tablet friability tester. Take the tablet's weight equal to or less than 6.5 g if the unit weight is equal to or less than 650 mg. Each tablet was weighed and tested at a speed of 25 rpm for 4 min (100 rotations). After removing of dust, tablets were re-weighed, and friability percentage was calculated by the given equation and the average value of all formulation is given in table.(15)

$$\% \text{ Friability} = \frac{\text{Tablet wt before friability} - \text{Tablet wt after friability}}{\text{Tablet wt after friability}} \times 100$$

d) Disintegration: Disintegration time was determined in 500 ml of 0.1 N HCl at 37°C. Introduce one tablet in each of the six tubes of the basket. Suspend the assembly in a beaker containing a specified amount of 0.1N HCl and operate the apparatus for a specified time. Observed all the tablets. (16)

e) Dissolution: The individual tablets were placed into vessels containing 900 ml of 0.1 N HC1 at 37°C and constantly stirred by rotating paddles at 75 rpm for 90 minutes. Aliquots (5 ml) are withdrawn from each vessel at 0, 30, 60, and 90 minutes. All samples were filtered through 0.45µm cellulose acetate membranes and refrigerated at 4°C until analysed by atomic absorption spectrophotometry. (17)

f) Particle Size Analysis: Particle size strongly relates to flowability, content, bioavailability, dissolution, absorption behaviour, which are crucial parameter in pharmaceutical industry.

Particle size was measure by using Horiba particle size analyser. 1 mg of Asthiposhak Vati mixed in 10 ml of distilled water, sonicated for 15 minutes. Then it was poured into the sample chamber, where it passes through the laser beam as homogeneous stream of particles. based on this scattering pattern of sample, particle size distributions are calculated.(18)

g) Zeta Potential:

Zeta Potential was measure by using Horiba particle size analyser .1% concentration of Asthiposhak Vati was prepared in distilled water. The particles were well dispersed before analysis and the sample was taken in a 1ml syringe and injected slowly into the capillary cell (cuvette) through the sample port. Care was taken to see that air bubbles are not formed during this process. As the sample comes out from the second port of the capillary cell, the injection process is stopped. The sample ports are then covered with lids and the capillary cell was then placed into the sample holder of the zeta sizer instrument for analysis.(18)

5] Fourier Transform Infra-Red Spectroscopy (FTIR): FTIR was performed to detect the presence of functional groups or organic legends present in Asthiposhak Vati. FTIR analysis of the Vati was carried out using potassium bromide (KBr) pellet in a Fourier transform infrared (FT-IR) spectrophotometer, model Shimadzu (8400S) Japan. The Sample was placed in the Potassium bromide pellet of FTIR spectrometer, and the interference pattern was detected by the infrared detector as variations in the infrared energy level, and the obtained spectral information was calculated. (19)

6] Inductively Couple Plasma Mass Spectrometry (ICPMS):

ICPMS study of Asthiposhak Vati was done for quantification of Heavy metals and minerals. The test was carried at Arbro Pharmaceuticals. pvt. Ltd (Analytical division), Industrial Area, Kirti Nagar Delhi to evaluate Heavy metal and mineral content.

Make: *PerkinElmer's* *Model: Nexion 2000*

7] Scanning Electron Microscopy (SEM): Scanning electron microscope (SEM) is one of the most widely used instrumental methods which is used to characterize the surface features and evaluate the morphological changes.

SEM study of Asthiposhak Vati and its powder was done for determination of Surface morphology. The test was carried at Savitribai Phule Pune University (SPPU).(19)

8] X-Ray Diffraction [XRD]: X-ray diffraction analysis (XRD) is a technique used in materials science to determine the crystallographic structure of a material.

XRD study of Asthiposhak Vati was done for determination of crystalline structure. The test was carried at CSIR-NCL Pune.(19)

9] High Performance Thin Layer Chromatography.

Extraction of Quercetin and Berberine from Asthiposhak Vati formulations:

Preparation of sample (Asthiposhak Vati): Weigh accurately ten tablets and triturated to make powder. Take 500 mg powder of Asthiposhak Vati preparation into 10 ml of volumetric flask and dissolve in methanol to make solution of 50,000 ng/µl concentration by sonicating for 15 min which help to extract Quercetin and Berberine content into methanol. Filter the solution with the syringe filter and centrifuge for 10 mins at speed of 80 RPM.

Preparation of sample extract:

1000 mg of powder extract *(Moringa Olifera)* dissolved in 10ml methanol (sonicate for 15min) to make stock solution of 100000ng/ µl concentration, and centrifuge for 10 min at speed of 80 RPM.

10 mg of powder extract (*Tinospora Cordifolia*) dissolved in 10ml methanol (sonicate for 15min) to make stock solution of 1000ng/ µl concentration, and centrifuge for 10min at speed of 80 RPM.

Preparation of standard (Quercetin): Weigh 2.5 mg standard Quercetin and transfer into 25 ml of volumetric flask add methanol to make solution of 100ng/µl concentration, dissolve it by sonicating for 15 mins.

Preparation of standard (Berberine): Weigh 10 mg Berberine dissolve in 10 ml of methanol to make stock solution of 1000ng/ µl concentration, dissolve it by sonicating for 15 mins.

Chromatographic conditions:

Chromatographic separation was achieved on HPTLC plates (10×10 cm) pre-coated with precoated silica gel 60F256 of 0.2 mm thickness with aluminum sheet support was used for application. Silica plates prewashed with analytical grade methanol and activated at 60°c for 10 mins in the oven. The sample application done by using CAAMAG LINOMAT SYRINGE 695.0014 with maintaining space between two bands was 6 mm and the slit dimension was kept 5mm × 0.45mm. The After development, the plates were dried, and scanning was done at speed of 20mm/s by CAMAG TLC SCANNER 3 using the deuterium lamp with WIN cats' version 2.0 software.

Optimization of Mobile phase: The standard stock solution containing 100µg/ml of Quercetin and 100ng/µl Berberine was spotted on to TLC plate and developed in different solvent systems. Many preliminary trials were carried out for selection of mobile phase. Mobile phase composition was optimized to provide accurate, precise, and reproducible results for the determination of Quercetin and Berberine.

Method validation:

The proposed HPTLC method for simultaneous estimation of QT and Berberine from Herbo mineral preparation Asthiposhak Vati was validated as per ICH guidelines Q2 (R1) by performing Linearity, Limit of detection, Limit of quantitation, Interday & Intraday precision, Accuracy, Robustness and Specificity.

Linearity:

The Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. It was determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (r2) and equation of the line.

Standard solution of QT and Berberine were prepared in methanol at concentrations of 100 ng/µl and 1000 ng/µl, respectively. A Camag Linomat V sample applicator was used to spot various quantities of standard solutions on the TLC plates in triplicate to achieve bands with concentrations between 100 and 500 ng/band for QT and 100 and 600 ng/spot for BE. The plates were then developed, and to get the regression equations, the data of peak areas vs drug concentrations were subjected to linear least squares regression analysis.

Limit of Detection and Limit of Quantitation: The limit of detection and limit of quantification were obtained with signal – to- noise ratios of 3 and 10 respectively. The LOD and LOQ were found to be 12.56ng/spot and38.06 ng/spot respectively for Quercetin, 8.7ng / spot and 26.44 ng / spot respectively for Berberine.

Precision:

The precision of the system was determined by measuring repeatability of sample application and measurement of peak areas for three replicates at each concentration level. The Inter-day and Intraday Precision evaluated by repeating of application the bands of standard solution per plate. To evaluate intraday precision, three mixed standards were prepared. Suitable volumes (0.2µL) for berberine and (2 µL) for quercetin were applied to HPTLC plates to obtain standard bands corresponding to three concentrations (200, 400, and 600 ng) for berberine and (200, 300, and 400 ng) for QT in triplicate on the same day at 3 different time of day like morning, afternoon, and evening. For the intraday precision (intermediate precision), the assays were performed on three consecutive days and the peak areas were recorded. The precision of the system and method was expressed as relative standard deviation (RSD) % of peak area.

Accuracy:

The accuracy of the method was established by calculating QT and Berberine recoveries using the standard addition method. To a prequantified sample solution, known concentrations of standard solutions of QT and Berberine were added at 80%, 100%, and 120% level (extracts). The concentrations of QT and Berberine were estimated by adding the obtained data into the relevant regression line equations.

Robustness- Robustness of developed method examined by making smallest change into the mobile phase composition, volume of mobile phase and Duration of chamber saturation. The change in the method examined by calculating Standard deviation and % RSD. The result shows that a little change into method shows significant variation into peak areas and Rf values of Quercetin and Berberine in the preparation. Robustness of method was done by applying concentration level of 400 ng / spot for Quercetin and 400 ng / spot for Berberine and calculate % RSD by peak areas.

RESULTS AND DISCUSSION:

1] Macroscopic Study: Organoleptic Evaluation

Table 1,

Sr. No	Parameter	Results
1	Description	Brown Colour Round Shape Biconvex Uncoated Tablets
2	Colour	Greyish Brown
3	Odour	Characteristic
4	Taste	Bitter
5	Touch	Fine

Observed Organoleptic properties

2] Physiochemical Evaluation: Physicochemical parameters of Asthiposhak Vati are tabulated in Table 2, 3 and 4. The
physiochemical parameters of Asthiposhak Vati were determined as per the Standard protocol.

Sr. No	Parameters	Results
1	pH	8.2 pH (Alkaline in Nature)
2	Total Ash Value	34.15%
3	Acid insoluble ash value	1.55%
4	Loss on Drying (LOD)	2.2%

Table 2, Physiochemical evaluation

Extractive Value: Water & Alcohol Soluble extractive value:

Sr. No	Drug name	% Total water-soluble extractive value	% Total alcohol soluble extractive value:
1	Shigru	28.275	14.4
2	Arjuna	10.625	11.425
3	Ashwagandha	11.4	27.85
4	Haritaki	34.2	37.81
5	Guduchi	11.2	5.925
6	Babul	7.85	19.225
7	Laksha	2.45	25.175
8	Dinka	47.375	5.75
9	Asthiposhak Vati	12.05	1.75

Table 3, Water Soluble extractive value

3] Phytoconstituent Study: Phytochemical screening of the extract shows presence of Carbohydrates, Alkaloids, Tannins/Phenolic and Flavonoids given in Table 5.

Sr. No	Phytochemical constituents	Tests/ Reagents	Inference
1	Carbohydrates	Molish test	+
2	Alkaloids	Hager's test	+
3	Tannins/ Phenolic	Ferric chloride test	+
4	Flavonoids	Shinoda test	+

Table 5, Phytochemical Screening

4] Modern Pharmaceutical Parameter:

- **Diameter, Thickness, Hardness and Friability:** The results for Diameter, Thickness, Hardness and Friability given in Table 6.

Sr. No	Parameter	Average	Limit	References
1	Diameter	10.31 mm	------	IP
2	Thickness	5.613 mm	------	IP
3	Hardness	5.22 kg/cm2	4-10 kg/ cm2	IP
4	Friability	0.14%	Less than 1%	IP

Table 6, Diameter, Thickness, Hardness and Friability

- **Disintegration:** Disintegration time of Asthiposhak Vati was found to be 11 Min.35 sec. The limit of disintegration time for Uncoated tablets was 15 min.
- **Dissolution:** The drug dissolution studies showed a linear graph and maximum drug release i.e., 51% was demonstrated at a 90 min interval which was given in figure 3.

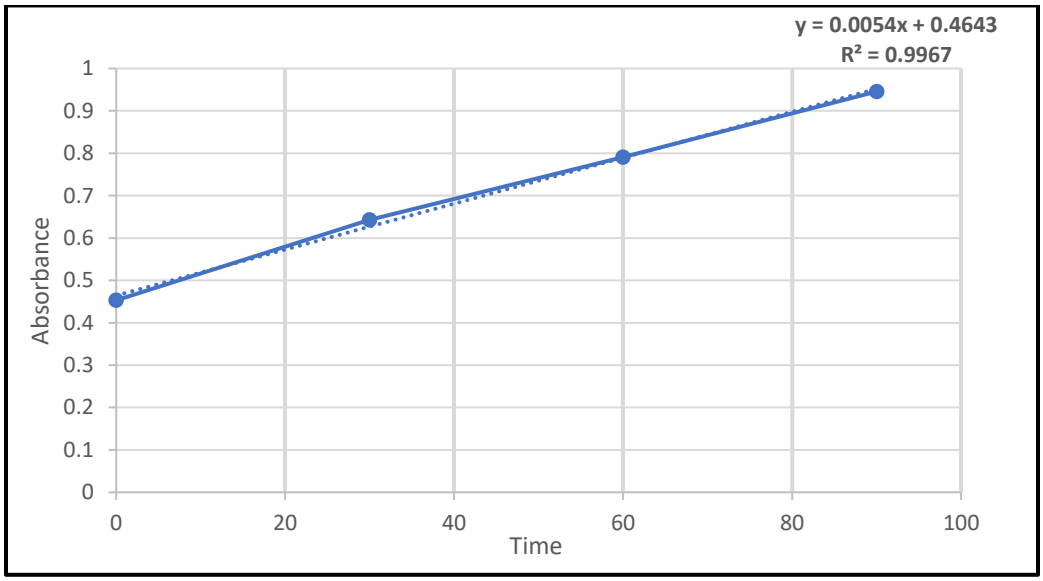

Figure 3, Graph of Dissolution

- **Particle Size Analysis:**

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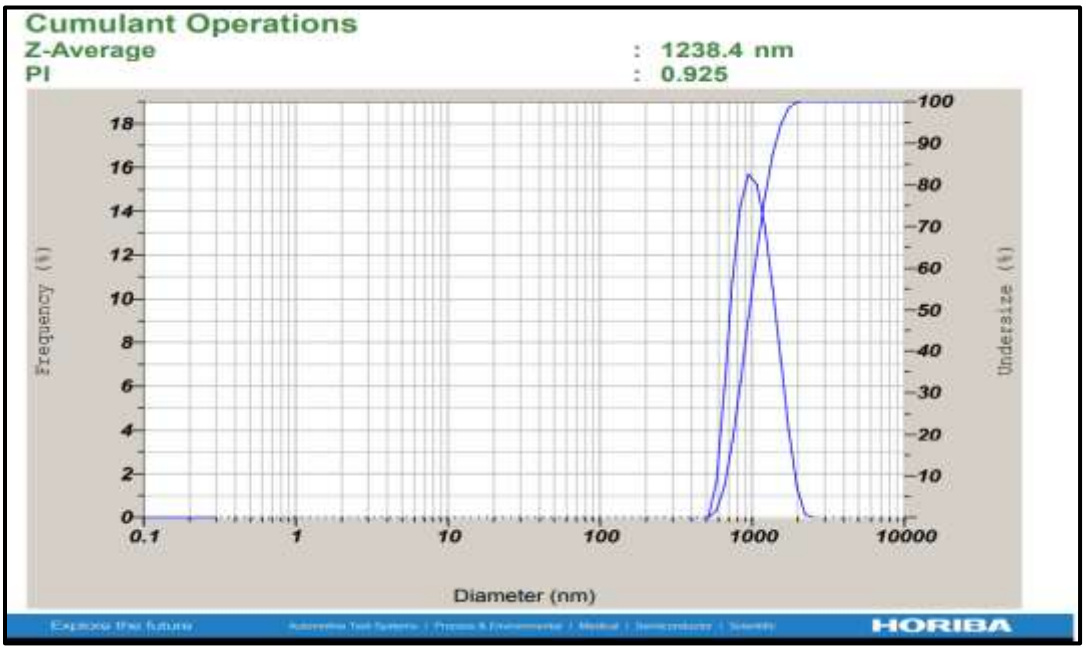

Figure 4, Graph of Particle Size

The size of the particles in the drug play's major role in its therapeutic action and efficacy. Particle size and surface area of solid drug are inversely related to each other The mean of Particle Size of Asthiposhak Vati was found to be 1238.4 nm shown in figure 4.

- **Zeta potential:**

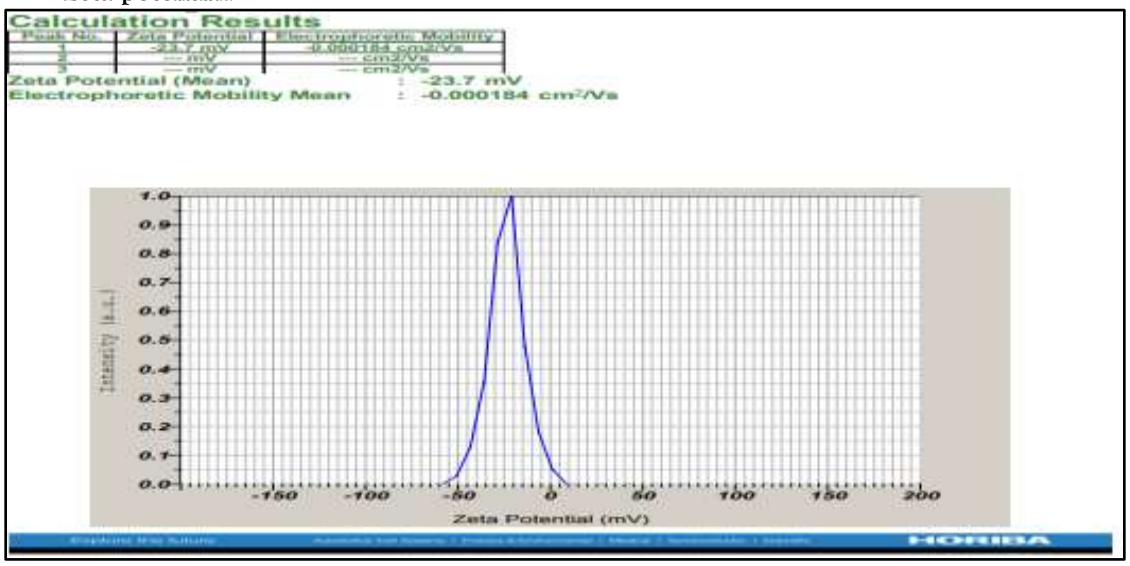

Figure 5, Graph of Zeta Potential

Asthiposhak Vati sample showed a Zeta potential value of – 23.7 mV in figure 5, which indicates moderate colloidal stability. High zeta potential indicates easy dispersion, whereas less zeta potential indicates strong aggregation of particles in suspension**.**

5] Fourier transform infrared spectroscopy (FTIR):

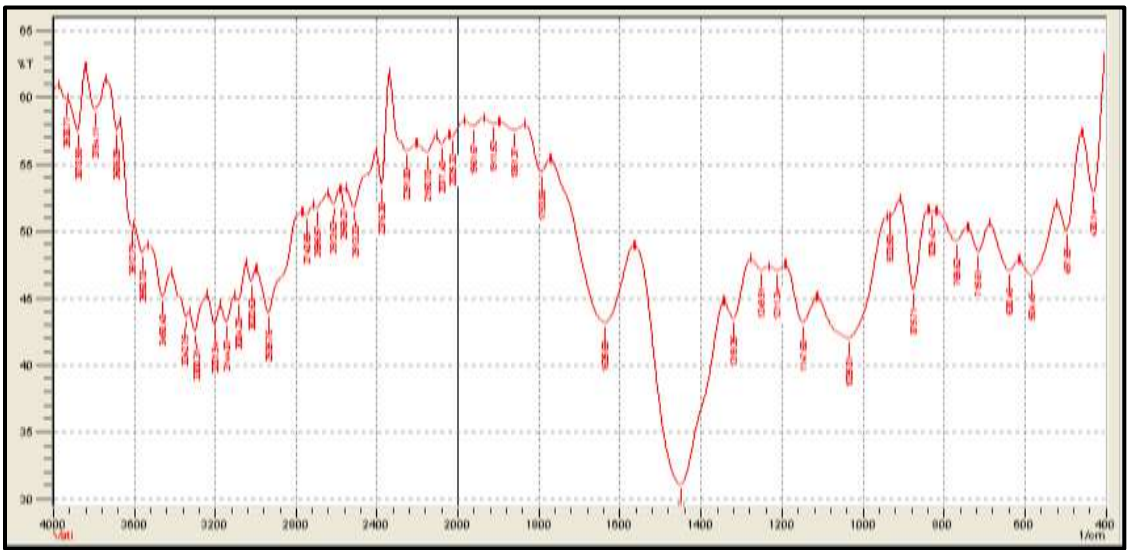

Figure 6, FTIR Spectra

Sr. No	Peak Position	Assigned Functional group	Reported in Literature
1	715	CO_3^{2-} In plane bend	712
2	875	CO_3^{2-} Out of plane bend	875
3	1450	CO_3^{2-} Asymmetric stretch	1427
4	638.46	Sulphate ion	612-674
5	430	Poly sulfite ion	416
6	1635	Ca region	1632.13
7	2921	Cyanide related ion	2935
8	3458	-OH, stretching vibration	3440
9	3686	Hydroxy group present in Ca (OH)2	3642

Table 7, **FTIR Frequency Value**

FTIR spectra of both samples were taken in the region of 400–4000 cm^{-1}. General overview indicates the presence of large number of functional groups. In Asthiposhak Vati sharp peaks were obtained at and around 715, 875, 1450, 638.46, 430, 1635, 2921, 3458, 3686 shown in figure 6 and values given in Table7.These peaks indicate the presence of organic compounds such as OH, methyl, cyanide, Ca, sulfate, carbonate, and polysulfide ion.

6] Inductively Couple Plasma Mass Spectrometry (ICPMS) Results: The samples were analyzed for the presence of various heavy metals using Inductively couple plasma mass spectrometry (ICPMS). The results value is given in table 8 which shows that the essential macro minerals – Calcium, Sodium, potassium, and

magnesium concentrations were high as compared to the other mineral elements determine. Hence from these results it is confirmed that Asthiposhak Vati contains high concentration of Calcium.

Sr. No	Parameter	Results	LLOQ
1	Lead (as Pb)	0.397 mg/kg	0.080 mg/kg
2	Cadmium (as Cd)	Below Quantification limit	0.080 mg/kg
3	Mercury (as Hg)	15.630 mg/kg	0.080 mg/kg
4	Arsenic (as As)	9.599 mg/kg	0.080 mg/kg
5	Iron (as Fe)	483.217 mg/kg	0.080 mg/kg
6	Magnesium (as Mg)	2200.259 mg/kg	0.080 mg/kg
7	Copper (as Cu)	1.227 mg/kg	0.080 mg/kg
8	Boron (as B)	3.445 mg/kg	0.080 mg/kg
9	Zinc (as Zn)	5.530 mg/kg	0.080 mg/kg
10	Lithium (as Li)	0.331 mg/kg	0.080 mg/kg
11	Calcium (as Ca)	179267.81 mg/kg	0.080 mg/kg
12	Sodium (as Na)	1124.15 mg/kg	0.080 mg/kg
13	Potassium (as K)	2924.79 mg/kg	0.080 mg/kg
14	Phosphates	3745.06 mg/100 g	
15	Phosphorus	1635.40 mg/100 g	

Table 8, Inductively Couple Plasma Mass Spectrometry (ICPMS) Values

7] Scanning Electron Microscopy (SEM)

The Morphology of the Asthiposhak Vati was shown in Figure 7 to 9 showed clusters of rough, irregularly arranged, and spongy particles, aggregated together with microspores scattered on the surface of the sample. The small size of the grains and aggregates could provide higher specific surface areas since the size of the particle should directly respond to the surface area.

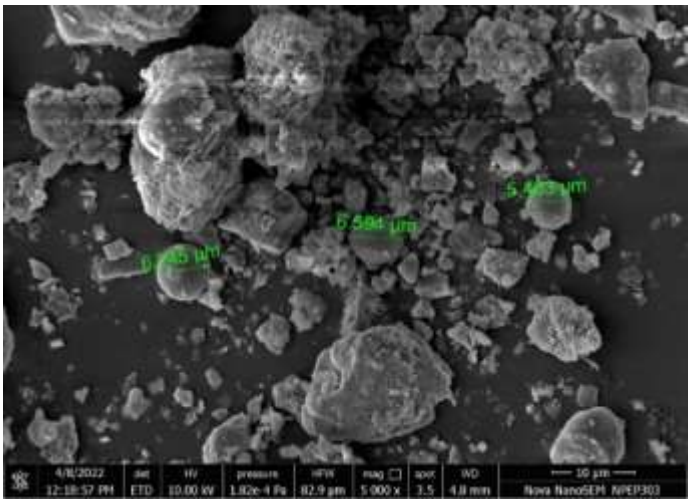

Figure 7, SEM of Asthiposhak Vati Powder at 10 µm

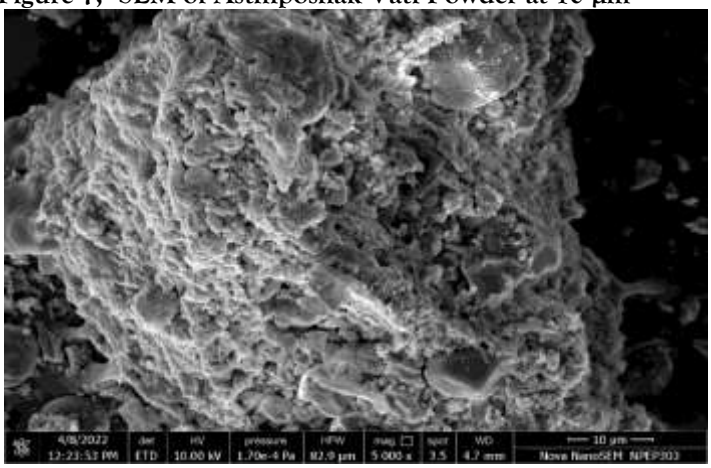

Figure 8, SEM of Asthiposhak Vati Powder at 10 µm

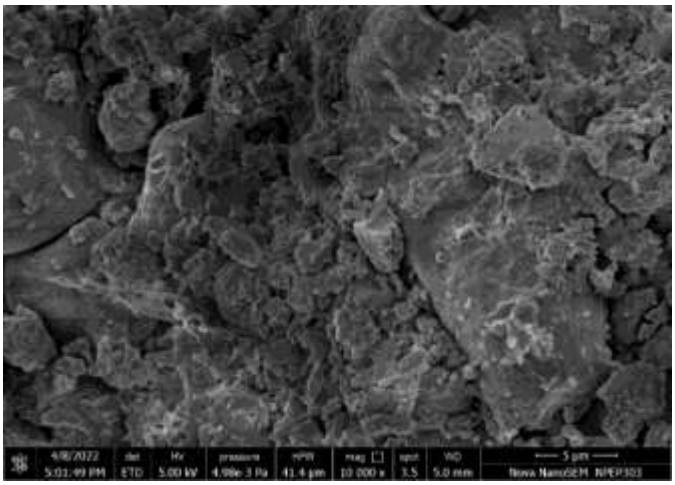

Figure 9, SEM of Asthiposhak Vati at 5 µm

8] X-Ray Diffraction:

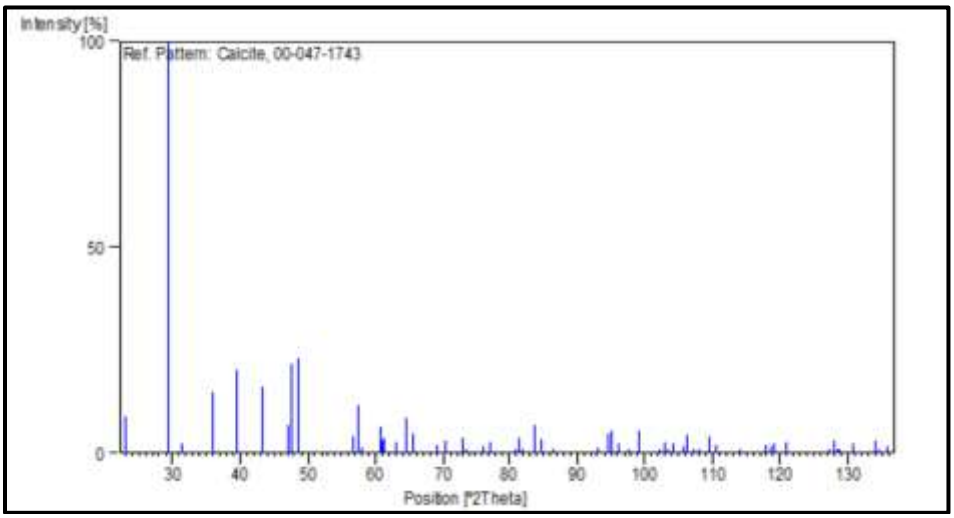

Figure 10, Graph of X-Ray Diffraction

Sr. No	Position [2 TH]	d-spacing [A]	I [%]	H	K	L
1	23.054	3.854	8.9	0	1	2
2	29.400	3.035	100	1	0	4
3	31.425	2.843	2.2	0	0	6
4	35.969	2.494	14.7	1	1	0
5	39.409	2.284	20.2	1	1	-3
6	43.158	2.094	16.1	2	0	2
7	47.114	1.927	7.0	0	2	4
8	47.506	1.912	21.7	0	1	8
9	21.7	1.875	23.2	1	1	-6
10	56.563	1.625	4.2	1	2	-1
11	57.398	1.604	11.8	1	2	2

Table 9, XRD Peak Value

Sr. No	Compound name	Chemical Formula	a/b/c(angstrom)	Crystal System
1	**Calcium Carbonate**	**CaCO3**	**4.989/4.989/17.061**	**Rhombohedral**

Table 10, XRD Crystal Structure

As per the graph and value given in figure, 10 and Table 9, it was confirmed that Asthiposhak Vati sample contains calcium carbonate. Asthiposhak Vati was found to be in calcite (calcium carbonate [CaCO3]), and the crystal structure was rhombohedral in nature which is given in table 10

9] High Performance Thin Liquid Chromatography (HPTLC):

For quantitative analysis through HPTLC techniques, optimization of solvent system was done using combination of solvent system of varying polarity and the most suitable solvent system were taken for the quantitative analysis of Quercetin and Berberine was Toluene: Ethyl acetate: Formic acid (5:4:1) and found to be suitable solvent system for quantitative analysis through HPTLC.

The content of quercetin and berberine in M. Oleifera and T. Cordifolia was found to be 0.15 %w/w and 2.6%w/w respectively which is given in table 20. The HPTLC fingerprinting of standard Quercetin under 254 nm and Berberine under 366 nm with Asthiposhak Vati were presented in the figure 11, figure 12. The respective HPTLC densitogram of Standard Quercetin (Retention factor- 0.74) and Berberine (Retention factor- 0.81) were presented in figure 13 and figure 15 respectively. The HPTLC densitogram of Asthiposhak Vati formulation containing Quercetin (Retention factor- 0.74) and berberine (Retention factor- 0.81) were presented in figure 14 and figure 16 respectively. The interpretations of result were done using standard calibration curve of quercetin and berberine (Figure 17 and Figure 19). Method validation parameter of Quercetin and Berberine were presented in Table 13. The results suggest that the sample contained considerable amount of Quercetin and Berberine.

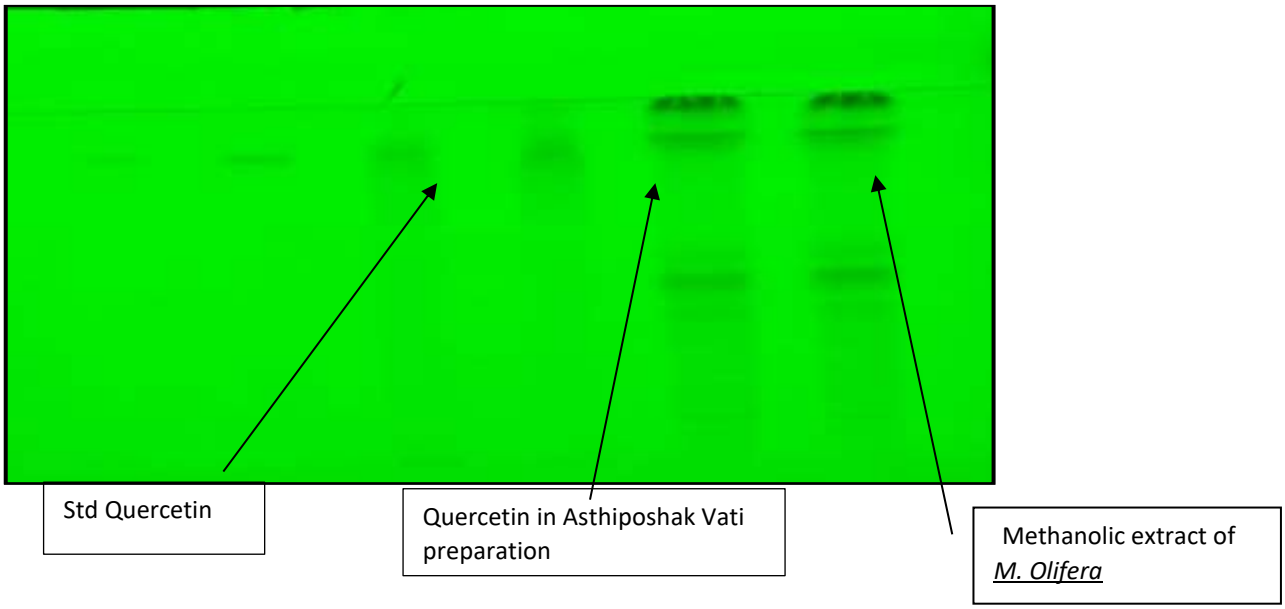

Figure 11, HPTLC fingerprinting of standard Quercetin with Asthiposhak Vati under 254 nm

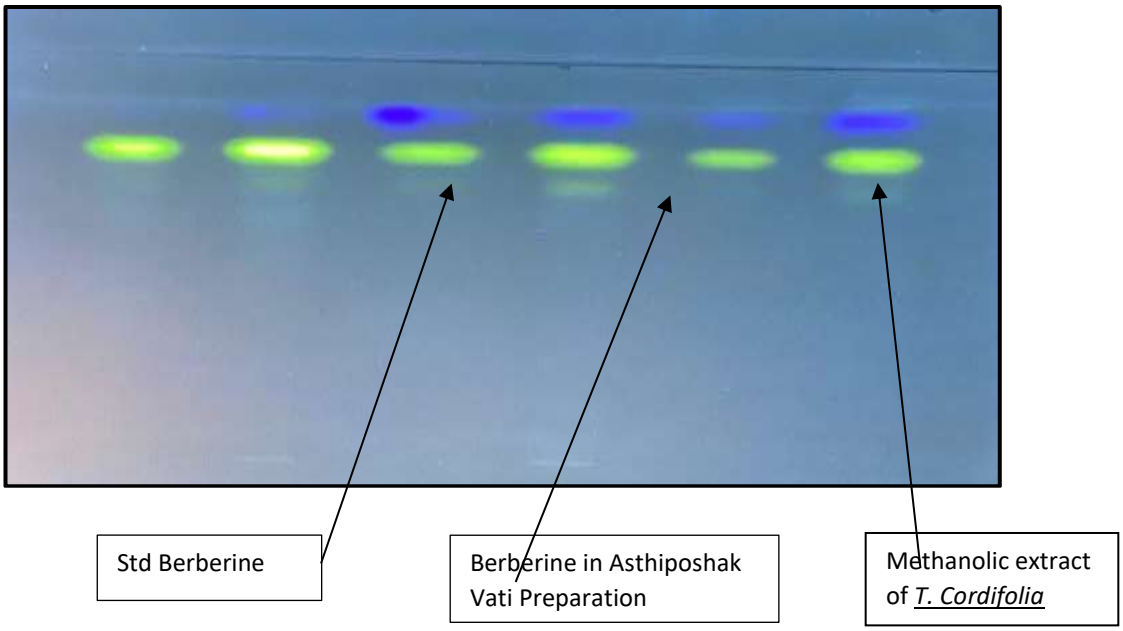

Figure 12, HPTLC fingerprinting of standard Berberine with Asthiposhak Vati under 366 nm

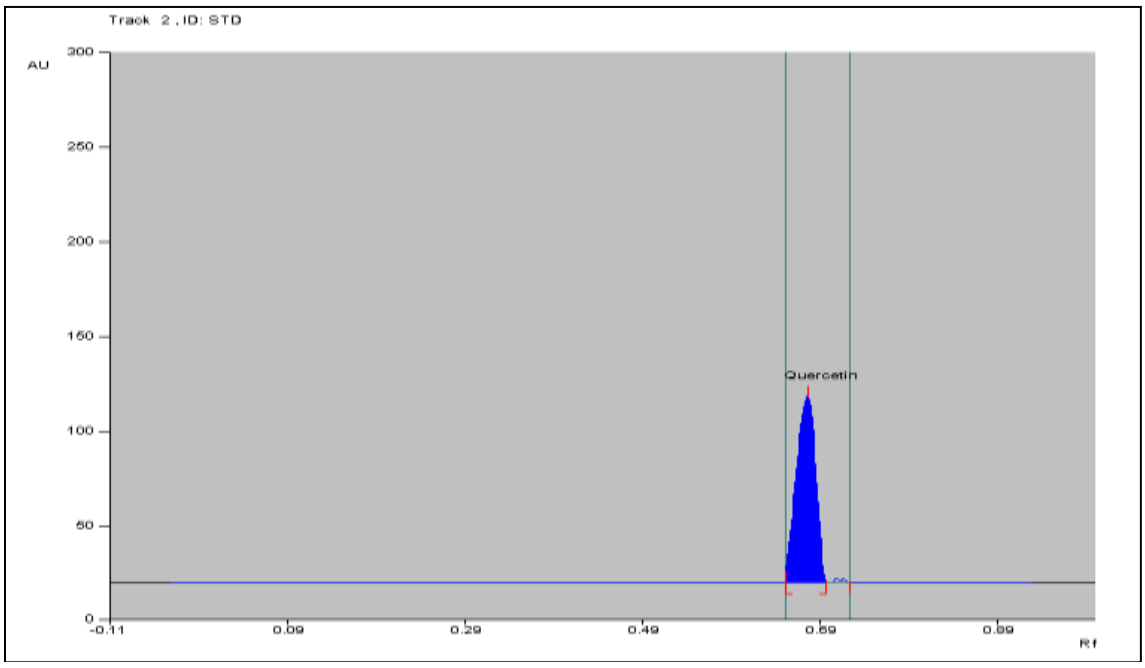

Figure 13, Typical densitogram of standard Quercetin (Retention factor:0.74)

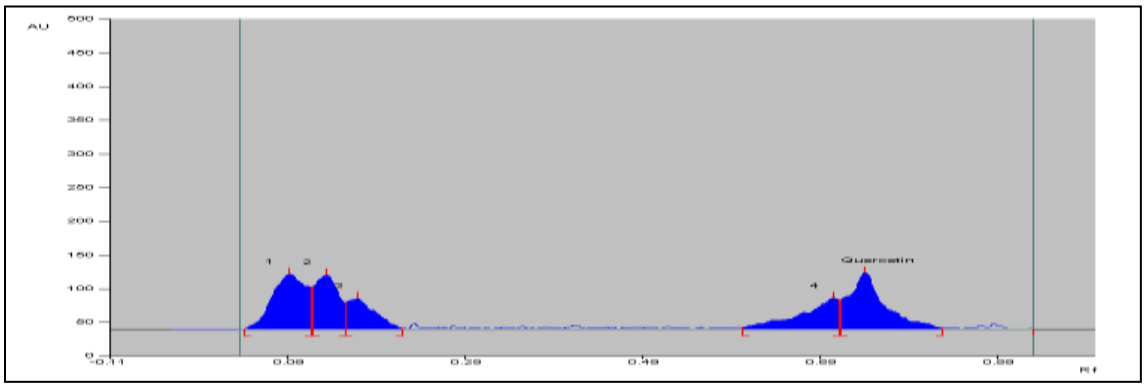

Figure 14, Typical densitogram of Asthiposhak Vati formulation containing Quercetin (Retention factor: 0.74)

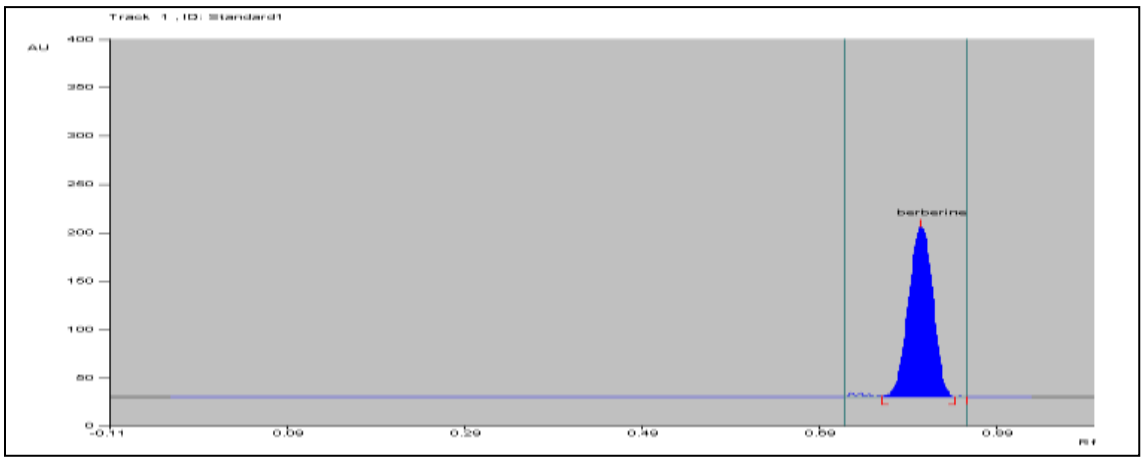

Figure 15, Typical densitogram of Standard Berberine (Retention factor:0.81)

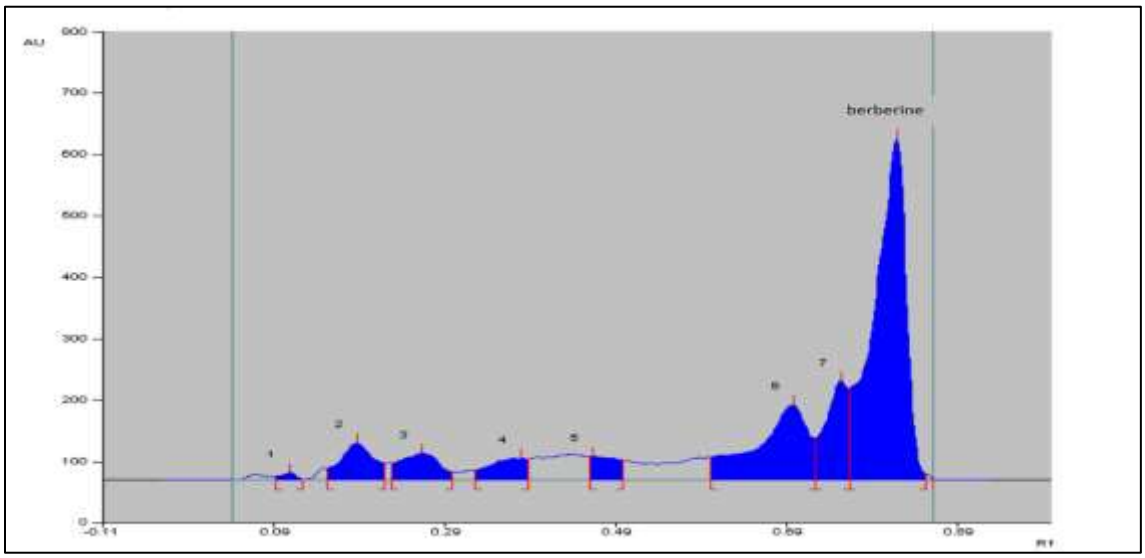

Figure 16, Typical densitogram of Asthiposhak Vati formulation containing Berberine (Retention factor: 0.81)

Linearity: The linear regression data obtained for the calibration curves (n=5) showed an excellent linear relationship over a wide concentration range of 100-500 ng/band for QT and Berberine100-600 ng/band

(Table 11 &12). 2D Spectra of linearity of standard Quercetin and berberine were presented in (Figure 18, 20)

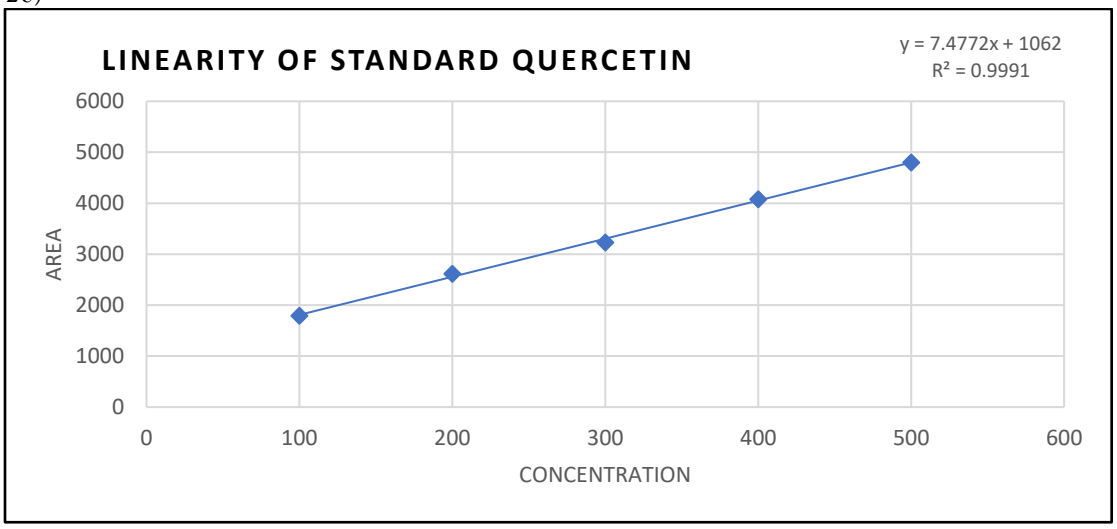

Figure 17, Linearity curve of standard Quercetin in the range of 100-500 ng

Sr. No.7	Conc. (ng/ band)	Area	Rf
1	100	1796.10	0.67
2	200	2613.97	0.65
3	300	3232.27	0.65
4	400	4079.67	0.65
5	500	4801.86	0.65

***Table 11, Standard* Calibration data for Quercetin**

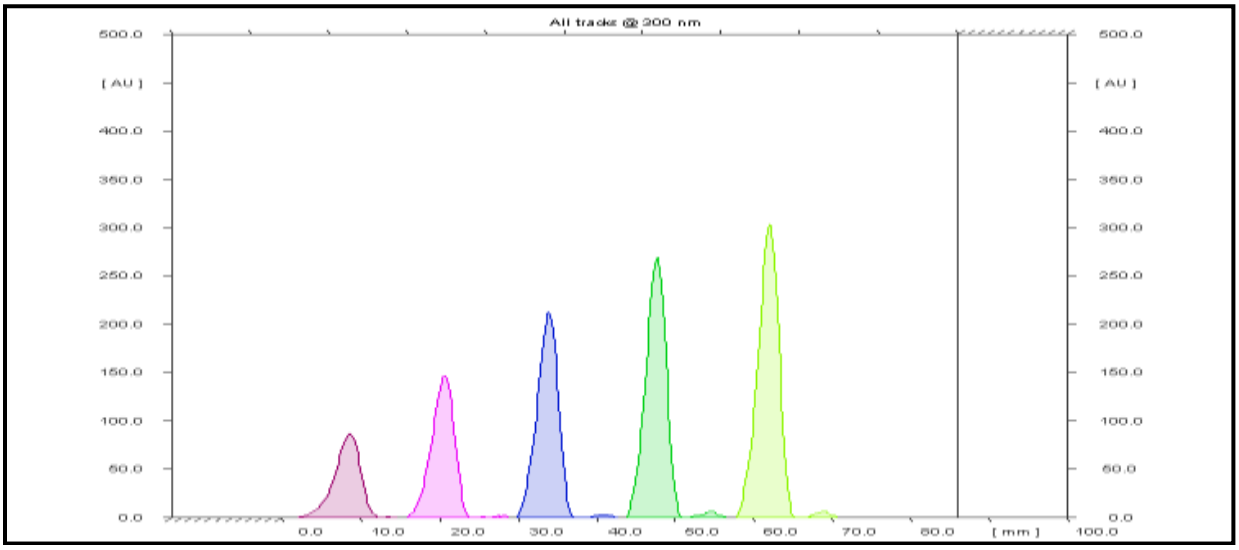

Figure 18, 2D Spectra of linearity of standard Quercetin

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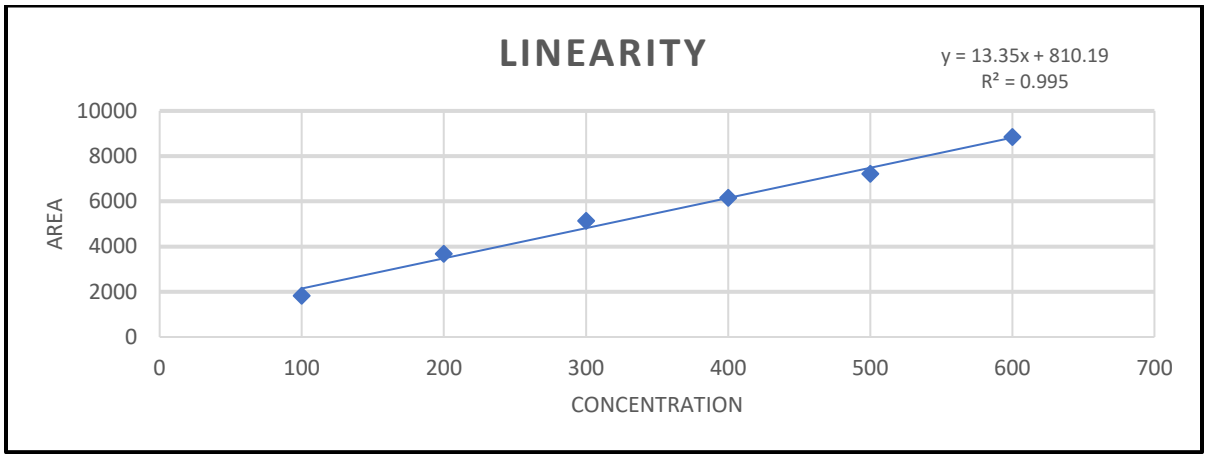

Figure 19, linearity curve of Standard Berberine in the range 100 to 600 ng

Sr. No.	Conc. (ng/ band)	Area	Rf
1	100	1834.54	0.75
2	200	3679.57	0.76
3	300	5138.57	0.76
4	400	6166.09	0.76
5	500	7223.15	0.76
6	600	8844.71	0.76

Table 12, Standard calibration data for Berberine

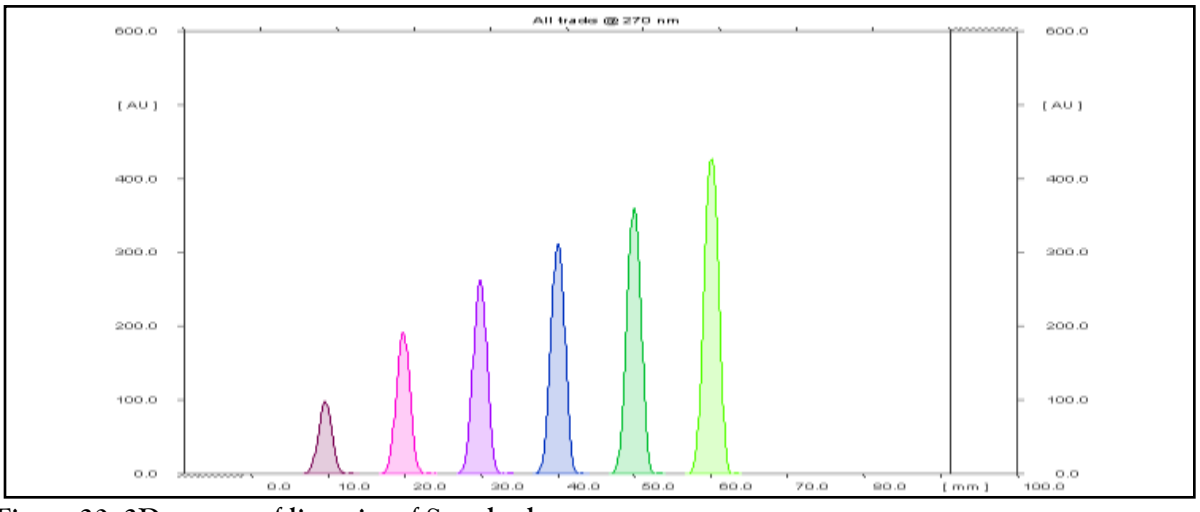

Figure 20, 2D spectra of linearity of Standard

Limit of Detection and Limit of Quantitation: Limit of Detection for Quercetin and Berberine was found to be 12.56ng/spot and 8.7ng / spot respectively. Limit of Quantitation for Quercetin and Berberine was found to be 38.06 ng/spot and 26.44 ng / spot respectively

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Sr. no	Parameters	Observed values	
		Quercetin	Berberine

1.	Calibration range (ng/ band)	100 to 500	100 to 600
2.	Volume applied (µl)	1 to 5	0.1 to 0.6
3.	Mobile phase	Toluene: Ethyl acetate: Formic acid (5:4:1)	Toluene: Ethyl acetate: Formic acid (5:4:1)
4.	Scanning wavelength	300nm	270nm
5.	Rf value	0.68	0.76
6.	Regression equation	Y=1062+7.477x.	Y = 810.2+ 13.35x
7.	Correlation coefficient	0.999	0.995
8.	intercept	1062	810.2
9.	slope	7.477	13.36
10.	Limit of Detection (LOD)	12.56ng/spot	8.7ng / spot
11.	Limit of Quantitation (LOQ)	38.06 ng/spot	26.44 ng / spot

Table 13, Method validation parameters for Quercetin and Berberine.

Precision: The measurement of peak area in the Interday and intraday precision studies showed low % RSD (Table)

Inter-day precision					**Intraday precision**				
Time	**Conc. (ng / spot)**	**Mean area**	**S.D.**	**%RSD**	**Days**	**Conc. (ng / spot)**	**Mean area**	**S.D.**	**%RSD**
Morning	200	1918.8	7.30	0.38%	**1st**	200	1907.08	15.00	0.79%
	300	3231.56	32.08	0.99%		300	3205.0	13.22	0.41%
	400	4452.8	24.20	0.54%		400	4475.0	56.34	1.26%
Afternoon	200	1853.73	35.29	1.90%	**2nd**	200	1907.66	13.01	0.68%
	300	3146.6	24.24	0.77%		300	3204.01	16.22	0.51%
	400	4354.5	29.46	0.68%		400	4471.33	47.96	1.07%
Evening	200	1784.06	12.14	0.68%	**3rd**	200	1923.0	29.61	1.54%
	300	3165.5	2.9	0.09%		300	3195.66	8.73	0.27%
	400	4137.95	11.05	0.27		400	4476.33	59.19	1.32%

Table 14, Inter day and intraday precision analysis of Quercetin

Inter-day precision					**Intraday precision**				
Time	**Conc. (ng / spot)**	**Mean area**	**S.D.**	**%RSD**	**Days**	**Conc. (ng / spot)**	**Mean area**	**S.D.**	**%RSD**

Morning	200	3569.33	25.80	0.73%	**1st**	200	3357.46	28.18	0.84%
	400	3842.33	15.50	0.40%		400	5663.3	32.95	0.58%
	600	5163.33	26.63	0.52%		600	7323.7	18.78	0.26%
Afternoon	200	3258.34	26.76	0.82%	**2nd**	200	3268.06	32.79	1.00%
	400	3853.33	21.57	0.56%		400	5550.26	29.65	0.53%
	600	4866.86	23.25	0.48%		600	7196.63	67.30	0.94%
Evening	200	3248.13	34.91	1.07%	**3rd**	200	3140.39	43.62	1.39%
	400	3943.83	17.06	0.43%		400	5470.8	39.84	0.73%
	600	5163.23	36.97	0.72%		600	7358.68	29.83	0.41%

Table 15, Inter day and intraday precision analysis of Berberine

Accuracy: The accuracy of the proposed HPTLC method demonstrated through recovery studies performed by spiking sample with pure drugs at 80%, 100%, and 120% indicated good recovery of the QT and Berberine with % recovery in the range of 98.1-99.4% of 98% to 102% of Quercetin and 99% to102 %of Berberine. (Table 16 and 17) respectively.

Level of recovery	**Amount of std applied in µl**	**Amount of std added in ng**	**Amount of sample applied in µl**	**Amount of sample added in ng**	**Peak area**	**% Recovery**
80	2.4	2899	4	314	3220	99.8%
	2.4	2899	4	314	3205	99.16%
	2.4	2899	4	314	3196	98.88%
100	3	3232	4	314	3209	99.28%
	3	3232	4	314	3231	99.6%
	3	3232	4	314	3215	99.47%
120	3.6	4192	4	314	3253	100.64%
	3.6	4192	4	314	3330	103.03%
	3.6	4192	4	314	3301	102.13%

Level of Recovery	**%Recovery**	**S.D.**	**R.S.D.**
80 %	99.28	12.12	0.38%

100 %	99.45	11.37	0.35%
120 %	102.48%	38.88	1.18%

Table 16, Recovery data of Quercetin in the Asthiposhak Vati preparation.

Level of recovery	**Amount of std applied in µl**	**Amount of std added in ng**	**Amount of sample applied in µl**	**Amount of sample added in ng**	**Peak area**	**% Recovery**
80	2.4	2899	4	314	3220	99.8%
	2.4	2899	4	314	3205	99.16%
	2.4	2899	4	314	3196	98.88%
100	3	3232	4	314	3209	99.28%
	3	3232	4	314	3231	99.6%
	3	3232	4	314	3215	99.47%
120	3.6	4192	4	314	3253	100.64%
	3.6	4192	4	314	3330	103.03%
	3.6	4192	4	314	3301	102.13%

Level of Recovery	**%Recovery**	**S.D.**	**R.S.D.**
80 %	98.03%	105.39	1.73%
100 %	99.52%	95.84	1.56%
120 %	102.48%	53.07	0.85%

Table 17, Recovery data of Berberine in the Asthiposhak Vati preparatio

Robustness: The result was presented in table 18 and 19 which shows that a little change into method shows significant variation into peak areas and Rf values of Quercetin and Berberine respectively in the preparation. %RSD value was found to be less than 2% for Quercetin and Berberine.

Sr.no	**Factors**	**Result**

1	**Mobile phase composition (±) 0.1**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**
	4.9	3.9	0.9	-0.1	3076	0.67	20.55	0.67 %
	5	4	1	0	3239	0.65	15.84	0.49%
	5.1	4.1	1.1	+0.1	3421	0.63	32.33	0.95 %
2.	**Duration of chamber saturation (Minutes)**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**
	10 min			-5	3217	0.60	12.38	0.38%
	15 min			0	3242	0.65	13.08	0.40%
	20 min			+5	3275	0.70	12.02	0.37 %
3.	**Volume of mobile phase (±) 1 ml**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**
	4.5	3.5	1	-1	3195	0.61	12.59	0.39%
	5	4	1	0	3254	0.66	32.64	1.00%
	5.5	4.5	1	+1	3275	0.72	11.14	0.34 %

Table 18, Robustness data of developed HPTLC method of Quercetin (n=6

Sr.no	**Factors**				**Result**			
1	**Mobile phase composition (±) 0.1**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**
	4.9	3.9	0.9	-0.1	5813.66	0.71	25.65	0.44%
	5	4	1	0	6281.0	0.75	1.2	0.24%
	5.1	4.1	1.1	+0.1	7060.83	0.69	26.61	0.38%
2.	**Duration of chamber saturation (Minutes)**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**

	10 min			-5	6837.09	0.69	11.72	0.17%
	15 min			0	6221.83	0.76	36.84	0.59%
	20 min			+5	5941.09	0.83	42.63	0.73%
3.	**Volume of mobile phase (±) 1 ml**			**Level**	**Peak Area**	**Rf**	**S.D.**	**%RSD**
	4.5	3.5	1	-1	2094.16	0.59	36.32	1.73%
	5	4	1	0	6316.33	0.63	10.94	0.17%
	5.5	4.5	1	+1	7332.83	0.72	11.78	0.16%

Table 19, Robustness data of developed HPTLC method of Berberine (n=6)

% Estimation:

The % estimation of Quercetin and Berberine in the Asthiposhak Vati preparation was calculated and mentioned in the Table No.20 the % estimated by using formulas.

$$\textbf{Amount of drug estimated in sample (mg)} = \frac{\text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times \text{Conc of Standard (mg/ml)}$$

$$\textbf{\% Estimation} = \frac{\text{Amount of drug estimated (mg)}}{\text{Amount of pure drug taken (mg)}} \times 100$$

Sr. No.	**Compound**	**% Estimation**
1.	Quercetin	0.15
2.	Berberine	2.6

Table 20, Content of Quercetin and Berberine in the formulation.

DISCUSSION:

- The standardization of herbal-mineral preparation, which was done using the basic organoleptic test, phytochemical and physicochemical tests, heavy metals test, HPTLC, and preclinical study.
- Physicochemical, and phytochemical analysis was performed to determine whether a crude drug is authentic; as a result, it is crucial in minimizing potential adulteration processes.
- The particle size of the Asthiposhak Vati was found to be 1238.4nm. The drug's therapeutic activity and effectiveness are significantly influenced by the size of the drug's particle. In a solid medication, the relationship between particle size and surface area is inversely to each other.
- The Zeta potential of the Asthiposhak Vati sample was - 23.7 mV, which denotes moderate colloidal stability.

- The Asthiposhak Vati formulation's FTIR spectra revealed the presence of calcium carbonate, confirming that Kukkutanda Bhasma serves as the principal ingredient and is primarily used for calcium supplementation.
- Calcite (Calcium Carbonate), CaCO3, had a rhombohedral crystal structure according to the results of the X-ray diffractogram (XRD) analysis.
- The Asthiposhak Vati SEM research demonstrates the morphology as aggregates of rough, irregularly organized, and spongy particles, with microspores dispersed across the surface of the sample. Given that the size of the particle should directly relate to the surface area, the small size of the grains and aggregates could provide larger specific surface areas.
- ICPMS was used to analyse the sample for the presence of different heavy metals and minerals Inductively coupled plasma mass spectrometry is widely used because of its advantages over other methods, such as atomic absorption, flame photometry, ICPOES, and ICPAES. It is an advanced technique which can analyze multiple elements simultaneously with high selectivity, sensitivity, and much lower detection limit. It also offers numerous features which make it particularly attractive for the clinical laboratory this include wide linear dynamic range, wide elemental coverage, multi-element capability, high sample throughput and simple sample preparation. From this ICPMS study, it was discovered that the concentrations of the important macrominerals calcium, sodium, potassium, and magnesium were higher than those of the toxic heavy metals' mercury, lead, and arsenic. Therefore, it is evident from these findings that Asthiposhak Vati has a high calcium concentration.
- Chromatography by HPTLC is one approach for standardizing herbs and herbal formulations utilizing current scientific instruments. The HPTLC method was developed for the simultaneous quantification of quercetin and berberine from *Moringa olifera* and *Tinospora cordifolia*, respectively, in Asthiposhak Vati. The method was developed and validated using ICH Q2 (R1) guidelines by examining linearity, intraday and Interday precision, accuracy, and robustness parameters.
- As per the results, the calibration curve for quercetin was linear in the range 1-5g/l and 0.1 to 0.6g/l for berberine. The correlation coefficient for Quercetin was determined to be 0.999 and 0.995 for Berberine, indicating good linearity. Precision, robustness, and accuracy were measured using SD, RSD, and %RSD. Asthiposhak Vati recovery tests were conducted at 80%, 100%, and 120% for both Quercetin and Berberine, and recovery revealed 99.28%, 99.45%, and 102.48% for quercetin and 98.03%, 99.52%, and 102.48% for berberine. The values of% RSD are less than 2% for all the validation criteria, including precision, accuracy, LOD, LOQ, and robustness.
- The HPTLC densitometric approach shown here is very simple, precise, specific, sensitive, and accurate. It may be used to determine the concentrations of significant quercetin and Berberine in raw materials and Ayurvedic Herbo mineral products containing *Moringa olifera* and *Tinospora cordifolia*, respectively.

CONCLUSION: The objective of present study's was to provide scientific proof of the standardization of the herbal-mineral ayurvedic formulation Asthiposhak Vati. Keeping this in mind, the current research aimed to evaluate the Asthiposhak Vati Standardization Parameter. Phytochemical, modern scientific Physico-chemical, Modern Pharmaceutical, and Ayurvedic parameters have all been used to validate the pharmaceutical and analytical procedure of the Ayurvedic preparation Asthiposhak Vati (Tablet). As a result, the HPTLC densitometric approach shown here is very simple, precise, specific, sensitive, and accurate. It may be used to determine the concentrations of significant quercetin and berberine in raw materials and Ayurvedic Herbo mineral products containing *Moringa olifera* and *Tinospora cordifolia*, respectively. The validated method may be used to establish guidelines for the preparation, qualitative evaluation, and quantitative evaluation of Asthiposhak Vati (Tablet), enabling us to achieve the optimal levels of efficacy, quality, and quantity of the finished product. Based on the findings of this investigation, it may be concluded that the herbal-mineral ayurvedic formulation "Asthiposhak Vati" satisfies the quality parameter. The existing observations in this study could be used as a reference for future research considering that there is no standard data published anywhere for this formulation.

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