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Jitendra Modi
Department of Pharmacognosy; S.K Patel College of Pharmaceutical Education and Research, Ganpat University, Kherva – 382711, Mehsana, Gujarat, India

Hardik Soni
Vasu Research Centre (A Division of Vasu Healthcare Pvt Ltd.), 896/A, GIDC, Makarpura, Vadodara – 390010, Gujarat, India

Kruti Pandya
Vasu Research Centre (A Division of Vasu Healthcare Pvt Ltd.), 896/A, GIDC, Makarpura, Vadodara – 390010, Gujarat, India

Ghanshyam Patel
Vasu Research Centre (A Division of Vasu Healthcare Pvt Ltd.), 896/A, GIDC, Makarpura, Vadodara – 390010, Gujarat, India

Nikunjana Patel
Department of Pharmacognosy; S.K Patel College of Pharmaceutical Education and Research, Ganpat University, Kherva – 382711, Mehsana, Gujarat, India.

Correspondence
Kruti Pandya
Vasu Research Centre (A Division of Vasu Healthcare Pvt Ltd.), 896/A, GIDC, Makarpura, Vadodara – 390010, Gujarat, India
Email:krutipandya@vasuresearch.com, krutishah85@gmail.com

A detail phyto-chemical evaluation of herbo-mineral formulation used in respiratory diseases

Jitendra Modi, Hardik Soni, Kruti Pandya, Ghanshyam Patel, Nikunjana Patel

ABSTRACT

Herbal formulations have reached extensive acceptability as therapeutic agents for several diseases. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analysis of the active compound and other major constituents, is a major challenge to scientists. Standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assured for the manufacturing of herbal drugs. The WHO specifies guidelines for the assessment of the safety, efficacy and quality of herbal medicines as a prerequisite for global harmonization. Present study was focused on an exhaustive standardization of Herbo-mineral syrup preparation Zeal Cough Syrup which was carried out employing the basic organoleptic test, physico-chemical tests, and bio-assays by sophisticated instruments like HPLC, HPTLC. HPTLC fingerprinting, assays of marker compounds etc. were carried out to confirm their quality and potency. The presence of the raw materials in the finished product was carried out with the aid of sophisticated instruments. The study results revealed that the syrup formulation was well standardized at various levels such as Physical consistency, Chemical profile, Microbial and Heavy metal limits.

Keywords: Standardization, Herbo-mineral Syrup Formulation, HPLC, HPTLC, Zeal Cough Syrup

1. Introduction

Standardization is a system that ensures a predefined amount of quantity, quality and therapeutic effect of ingredients in each dose [1]. Herbal product cannot be considered scientifically valid if the drug tested has not been authenticated and characterized in order to ensure reproducibility in the manufacturing of the product. Therapeutic activity of an herbal formulation depends on its phytochemical constituents. The development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/ bioactive compounds and other major constituents, is a major challenge to scientists. In view of the above, standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for the manufacturing of an herbal drug [2]. The authentication of herbal drugs and identification of adulterants from genuine medicinal herbs are essential for both pharmaceutical companies as well as public health [3-5].

In the present study, Herbo-mineral syrup preparation (Zeal Cough Syrup) has been selected to establish its standardization status. The key ingredients used in the formulation are extract of *Ocimum sanctum* (Tulasi) Aerial [6], *Adhatoda vasica* (Vasa) Leaves [6], *Glycyrrhiza glabra* (Yashtimadhu) Root [6], *Solanum xanthocarpum* (Kantakari) Panchang [6], *Zingiber officinale* (Shunthi) Rhizome [6], Trikatu Churna [6], Navasar [6] (Ammonium chloride), *Mentha sylvestris* (Pudina) [6] satva, *Eucalyptus globulus* (Nilgiri) [7] oil.

2. Material and Methods

2.1 Organoleptic parameters: Organoleptic parameter like appearance, colour and odour were used to confirm uniformity in visual identity of raw materials and finished product. The results are as tabulated in Table 1.

2.2 Physicochemical parameters for extracts:
The physiochemical parameters includes tests like Moisture content / Loss on drying [8], pH [8], Alcohol soluble extractive [8], Water soluble extractive [8], Determination of ash [8], Acid insoluble ash [8], and Water soluble ash [8] of the relevant raw materials. The results are as tabulated in Table 2 & 3.

2.3 Estimation of Actives: Assay analysis includes estimation of Tannin [9], Alkaloid [9], Glycyrrhizin [9], Solasodine, Piperine [9], Ammonium chloride [10] in respective ingredients. The results are as tabulated in Table 4.

2.4 Evaluation of Standardization Parameters selected for Finished Product: The finished product was analyzed by parameters like Appearance, pH [8], Specific gravity [8], Viscosity [8], Assay of Ammonium chloride (Navsar) [10]. The results are as tabulated in Table 5.

2.5 Microbial Analysis [11]: Bio-burden analysis consists of parameters like Total Bacterial Count, Total Fungal Count, Presence of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella enterica*. The results are as given in Table 6.

2.6 Heavy Metal Analysis [12]: Sample preparation for heavy metal analysis was done by MARS Express microwave digestive system. The standard solutions of Pb, Cd, As and Hg were prepared. Then samples were analyzed for the presence of Pb, Cd, As, Hg using Atomic absorbance spectrophotometer AA 6300, SHIMADZU and HVG-1 by using a calibration curve of standard. The results are as given in Table 6.

3. HPLC analysis for estimation of active components:
3.1 Apparatus, equipment and reagents: HPLC system's pump was from Shimadzu LC 20ATVP, Shimadzu, Japan with 20 mL Rheodyne injector, Phenomenex (Torrance, CA) Luna C18 (250 cm x 4.6 mm id) column and SPD-20 AT UV-Visible and spinchrom/LC solution software were used. All the reagents were of the HPLC grade.

3.2 Estimation of Glycyrrhizin in *Glycyrrhiza glabra* (Yashtimadhu) extract:
Chromatographic Condition
Stationary phase: Phenomenex C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was used at ambient temperature
Mobile Phase: Buffer: acetonitrile (60:40, v/v)
Flow rate: 1 ml/min.
Injection volume: 20 µL
Detection: At 254 nm with UV detector
Preparation of solutions -
Preparation of standard solution: The standard solution of Glycyrrhizin was prepared in Water.
Preparation of sample solutions: The sample solution of *Glycyrrhiza glabra* extract was also prepared in Water.

3.3 Estimation of Ursolic acid in *Ocimum sanctum* (Tulasi) Extract
Chromatographic Condition
Stationary phase: Phenomenex C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was used at ambient temperature
Mobile Phase: Methanol: Acetonitrile (30:70) v/v)
Flow rate: 0.6 ml/min.
Injection volume: 20 µL
Detection: At 210 nm with UV detector
Preparation of solutions -
Preparation of standard solution: Standard solution of Ursolic acid was prepared in methanol.
Preparation of calibration curve: The working standard solution of 1 µg/ml to 5 µg/ml was prepared and calibration curve was plotted for concentration v/s area.
Preparation of sample solutions: Sample solution of Tulasi extract was prepared in methanol.

4. HPTLC analysis for Zeal Cough Syrup and its raw materials:
HPTLC is one of the most advanced separation technique available today which gives better precision and accuracy with extreme flexibility for various steps (stationary phase, mobile phase, development technique and detection). HPTLC analysis was carried out using a Hamilton 100 µl HPTLC syringe, Camag Linomat V automatic spotting device, Camag twin trough chamber, Camag TLC Scanner-4, WINCAT integration software, aluminium sheet precoated with Silica Gel F254 (Merck) 0.2 mm thickness.

4.1 Steps involved in HPTLC analysis:
4.1.1 Selection of plate and adsorbent: Precoated aluminum plates with Silica Gel F_{254} of 20 x 20 cm and 0.2 mm thickness, was used for the detection. The plates were pre washed by methanol and activated at 60 °C for 5 min prior to chromatography.

Application Mode	CAMAG Linomat 5 – Applicator
Filtering System	Whatman filter paper No.41
Stationary Phase	MERCK - TLC Silica gel 60 F_{254} on Aluminum sheets
Application (Y axis) Start Position	10mm
Development (Y axis) End Position	90 mm from plate base
Band length	8 mm
Development Mode	CAMAG TLC Twin Trough Chamber
Chamber Saturation Time	30 minutes
Visualization	@254 nm, @366 nm, @ Visible (after spray of Anisaldehyde Sulphuric acid reagent)
Derivatization mode	CAMAG – Dip tank for about 1 minute
Drying Mode, Temp. & time	TLC Plate Heater Preheated at 100±5 ^{0}C for 3 minutes

4.1.2 Sample solution
Extract: Extract 1.0 g of the sample raw material (Reference Standard / Test Drug) with 10mL of Methanol with constant shaking for 05 minutes. Heat on water bath at 90-100 ^{0}C for 5 minutes, Filter it through Whatman filter paper No.41. Use the filtrate for HPTLC Profiling.
4.1.3 Preparation of Spray reagent (Anisaldehyde sulphuric acid reagent): 0.5 mL of Anisaldehyde EP is mixed with 10 mL of Glacial acetic acid AR, followed by 85 mL Methanol AR and 5 mL Sulphuric acid 98% GR.

Track 1: 8 µl/mL methanol extract of the reference standard of the raw material
Track 2: 8 µl/mL methanol extract of test drug under observation
Track 3: 8 µl/mL methanol extract of Herbo-mineral formulation.

4.1.4 Preparation of solution for Finished Product: To 10 mL of the syrup, 10 mL of Ethyl acetate was added. The Ethyl acetate layer was separated and kept aside and 10 mL of Ethyl acetate was again added and the cycle was repeated thrice (till a colourless solution is obtained). The Ethyl acetate layers were then collected and evaporated to dryness. The residue was dissolved in 5 mL methanol and used as the reference solution for HPTLC Profiling.

5. Result

Table 1: Organoleptic parameters and ingredient's part used

Ingredient	Parts used	Organoleptic characters		
		Colour	Odour	Taste
TE	Aerial	Greenish Brown	Characteristic	Astringent
VE	Leaves	Brown	Characteristic	Bitter
YE	Root	Brown	Peculiar	Sweet
KE	Panchang	Brown	Characteristic	Bitter
SE	Rhizome	Creamish Brown	Characteristic	Characteristic
TC	Formulation	Light Greenish	Characteristic aromatic	Pungent
NAV	Mineral	White Crystalline	Characteristic	Salty
PU	Satva	Colourless	Mentholated aroma	Strong pungent
NO	Oil	Pale Yellow	Aromatic like turpentine	NA

TE: Tulasi Ext, **VE**: Vasa Ext, **YE**: Yashtimadhu Ext, **KE**: Kantakari Ext, **SE**: Shunthi Ext, **TC**: Trikatu Churna, **NAV**: Navasar, **PU**: Pudina, **NO**: Nilgiri oil; **NA**: Not Applicable

Table 2: Physico-chemical parameters

Sr No.	Ingredients	Physicochemical parameter		
		M/S (by KF) %	pH	M/S (by LOD) %
1	TE	NA	5.11±0.02	3.63±0.02
2	VE	NA	6.62±0.05	4.93±0.12
3	YE	NA	5.48±0.12	3.52±0.56
4	KE	NA	4.92±0.04	3.15±0.45
5	SE	NA	5.42 ± 0.03	4.95±0.12
6	TC	NA	5.32±0.03	7.70±0.08
7	NAV	NA	5.63±0.06	0.09±0.02
8	PU	NA	NA	NA
9	NO	0.24±0.02	NA	NA

TE: Tulasi Ext, **VE**: Vasa Ext, **YE**: Yashtimadhu Ext, **KE**: Kantakari Ext, **SE**: Shunthi Ext, **TC**: Trikatu Churna, **NAV**: Navasar, **PU**: Pudina, **NO**: Nilgiri oil; **NA**: Not Applicable; **M/S:** Moisture**; KF:** Karl Fischer; **LOD**: Loss on Drying

Table 3: Extractive values and Ash value of Ingredients of Zeal Cough Syrup

S. No	Ingredients	WSE (%)	ASE (%)	TA (%)
1	TE	95.68±0.02	70.0 ± 1.02	12.11±0.01
2	VE	90.96±0.21	69.8 ± 0.21	14.82±0.21
3	YE	97.28±0.05	60.2 ± 0.93	3.74±0.03
4	KE	90.72±0.12	73.6 ± 0.43	9.85±0.01
5	SE	78.32±0.23	61.4 ± 0.94	9.91±0.23
6	TC	78.42±0.21	76.6 ± 0.54	5.32 ± 0.04

TE: Tulasi Ext, **VE**: Vasa Ext, **YE**: Yashtimadhu Ext, **KE**: Kantakari Ext, **SE**: Shunthi Ext, **TC**: Trikatu Churna; **NA**: Not Applicable; **WSE**: Water Soluble Extractive; **ASE**: Alcohol Soluble Extractive; **TA**: Total Ash

Table 4: Assay estimation in extract raw material of Zeal Cough Syrup

Sr No.	Ingredients	Tannin	Alkaloid	Glycyrrhizin	Solasodine	Piperine	NH_4Cl
1	TE	7.89 ± 0.02	NA	NA	NA	NA	NA
2	VE	NA	0.64 ± 0.01	NA	NA	NA	NA
3	YE	NA	NA	34.15 ± 0.08	NA	NA	NA
4	KE	NA	NA	NA	3.46±0.15	NA	NA
5	TC	NA	NA	NA	NA	2.08 ± 0.21	NA
6	NAV	NA	NA	NA	NA	NA	100.12±0.21

TO: Tulasi Ext, **VE**: Vasa Ext, **YE**: Yashtimadhu Ext, **KE**: Kantakari Ext, **SE**: Shunthi Ext, **TC**: Trikatu Churna, **NAV**: Navasar; **NA**: Not Applicable

Table 5: Standardization parameters for the finished product Zeal Cough Syrup

Parameter	Limits	Results		
		Batch 1	**Batch 2**	**Batch 3**
Description	Dark green to green coloured syrup with Characteristic mentholated odour and Characteristic pungent sweet taste	Green coloured syrup with characteristic mentholated odour & characteristic pungent taste.	Green coloured syrup with characteristic mentholated odour & characteristic pungent taste.	Green coloured syrup with characteristic mentholated odour & characteristic pungent taste.
pH	4.5 to 6.5	5.41	5.38	5.46
Specific gravity	1.10 to 1.20 g/mL	1.142 g/mL	1.148 g/mL	1.146 g/mL
Viscosity	13 to 28 Centipoise	21.54 Centipoise	21.68 Centipoise	20.58 Centipoise
Assay of Ammonium chloride	97.0 to 103.0%	101.42 %	101.56 %	101.48 %

Table 6: Results of Heavy metal content and Bio burden in raw material of Zeal Cough Syrup

Ingre-dients	Trace elements				Bio-burden					
	Pb 10ppm	**Cd** 0.3ppm	**As** 3.0ppm	**Hg** 1.0ppm	**TBC** NMT 10^7 cfu/g	**TFC** NMT 10^5 cfu/g	***E. coli*** Ab	***P.a*** Ab	***S.e*** Ab	***S.a*** Ab
TE	0.435	0.067	0.067	Absent	7×10^2	Absent	Absent	Absent	Absent	Absent
VE	1.523	0.075	0.076	Absent	8×10^2	Absent	Absent	Absent	Absent	Absent
YE	1.788	0.136	0.070	Absent	5×10^2	Absent	Absent	Absent	Absent	Absent
KE	0.652	0.023	0.050	Absent	4×10^2	Absent	Absent	Absent	Absent	Absent
SE	0.989	0.035	0.156	Absent	12×10^2	Absent	Absent	Absent	Absent	Absent
TC	1.646	0.055	0.127	Absent	4×10^2	Absent	Absent	Absent	Absent	Absent
NAV	0.564	0.012	0.054	Absent	NA	NA	NA	NA	NA	NA
PU	0.521	0.212	0.252	Absent	NA	NA	NA	NA	NA	NA
NO	0.251	0.001	1.251	Absent	NA	NA	NA	NA	NA	NA
ZCS	1.252	0.521	1.256	Absent	13×10^2	Absent	Absent	Absent	Absent	Absent

TE: Tulasi Ext, **VE**: Vasa Ext, **YE**: Yashtimadhu Ext, **KE**: Kantakari Ext, **SE**: Shunthi Ext, **TC**: Trikatu Churna, **NAV**: Navasar, **PU**: Pudina, **NO**: Nilgiri oil; **ZCS**: Zeal Cough Syrup, **NA**: Not Applicable; **ppm**: parts per million, **cfu/g**- colony forming unit per gram, **Pb**: Lead, **Cd**: Cadmium, **As**: Arsenic, **Hg**: Mercury, **TBC**: Total bacterial count, **TFC**: Total fungal count, ***E. coil***: *Escherichia coli,* ***P.a***.: *Pseudomonas aeruginosa,* ***S.e***: *Salmonella enterica.*, ***S.a***: *Staphylococcus aureus;* **Ab**: Absent

6. HPLC Analysis

6.1 HPLC study of *Glycyrrhiza glabra* Root Ext.

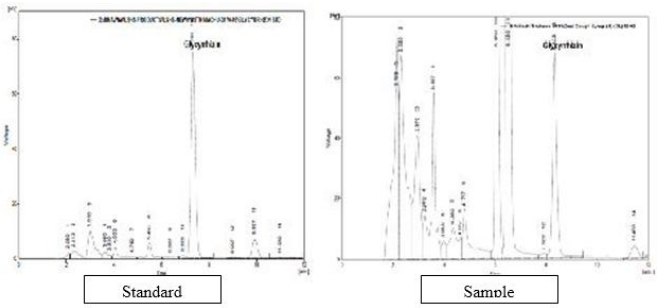

Fig 1: HPLC chromatogram of standard glycyrrhizin & *Glycyrrhiza glabra* extract. The result indicated 27.32% of glycyrrhizin in *Glycyrrhiza glabra* Root extract

6.2 HPLC study of Piperine

HPLC chromatogram of standard piperine and Zeal Cough Syrup

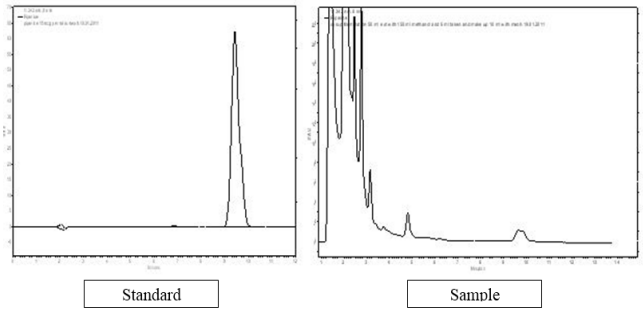

Fig 2: HPLC chromatogram of standard piperine and piperine in Zeal Cough Syrup. The results indicate that Zeal Cough Syrup contains 0.0973% of Piperine.

7. HPTLC

In HPTLC analysis, the sample shows comparison of individual extract & Powder with finished product. The visualization of TLC plates was carried out in all 3 different wavelengths i.e., 254 nm, 366 nm and 540 nm. From this only the best visualization result was selected and included in our study along with its 3D image. The R_f value thus found during this study indicates the prominent presences of raw material in the finished product which is used to establish its quantitative presence.

Tracks of HPTLC fingerprinting plates were spotted in following way:

Track 1: 8 µl/mL methanol extract of the reference standard of the Extract

Track 2: 8 µl/mL methanol extract of test drug under observation

Track 3: 8 µl/mL methanol extract of Herbo-mineral formulation.

7.1 *Ocimum sanctum* (Tulasi) Extract (TE)

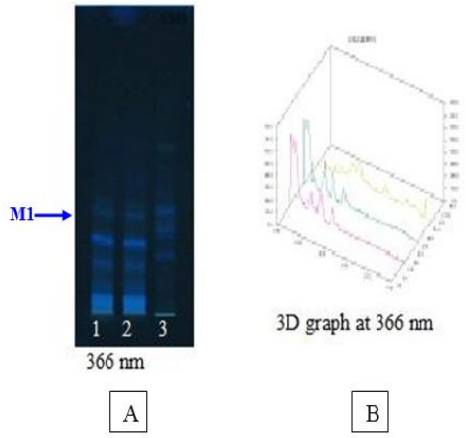

Fig 3: It shows the HPTLC Chromatogram of TE.

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.

A: HPTLC Plate of TE at 366 nm under UV.

B: 3D image of the Fingerprinting of TE and finished product (366 nm). The results indicate that HPTLC Chromatogram of TE and finished product has shown the similar R_f value of 0.32 at 366 nm.

7.2 *Adhatoda vasica* (Vasa) Extract (VE)

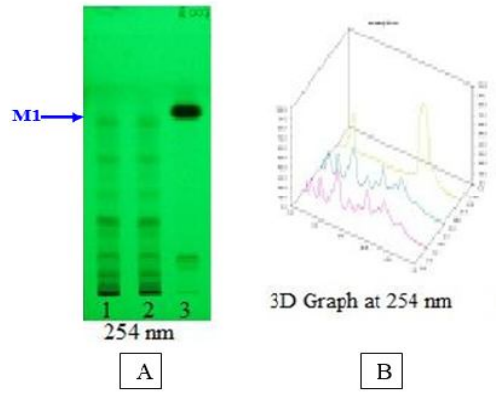

Fig 4: It shows the HPTLC Chromatogram of VE.

Track 1: reference standard Track 2: test drug Track 3: Herbo-mineral formulation.

A: HPTLC Plate of VE at 254nm under UV.

B: 3D image of the Fingerprinting of VE and finished product (254 nm). The results indicate that HPTLC Chromatogram of VE and finished product has shown the similar R_f value of 0.61 at 254 nm.

7.3 *Glycyrrhiza glabra* (Yashtimadhu) Extract (YE)

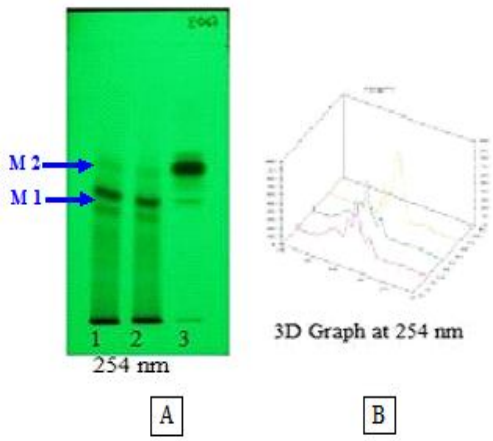

Fig 5: It shows the HPTLC Chromatogram of YE.

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.

A: HPTLC Plate of YE at 254 nm under UV.

B: 3D image of the Fingerprinting of YE and finished product (254 nm). The results indicate that HPTLC Chromatogram of YE and finished product has shown the similar R_f value of 0.39 and 0.50 at 254 nm.

7.4 *Solanum xanthocarpum* (Kantakari) Extract (KE)

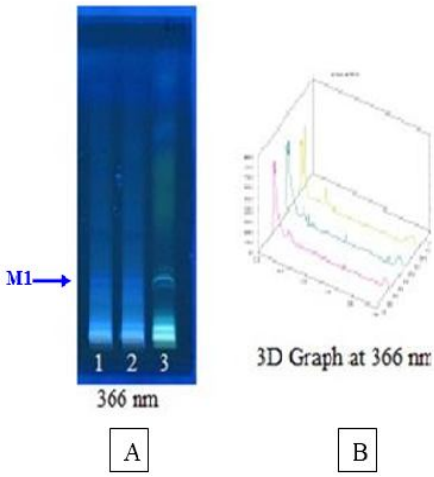

Fig 6: It shows the HPTLC Chromatogram of KE.

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.

A: HPTLC Plate of KE at 366 nm under UV.

B: 3D image of the Fingerprinting of KE and finished product (366 nm). The results indicate that HPTLC Chromatogram of KE and finished product has shown the similar R_f value of 0.20 at 366 nm.

7.5 *Zingiber officinale* (Shunthi) Extract (SE)

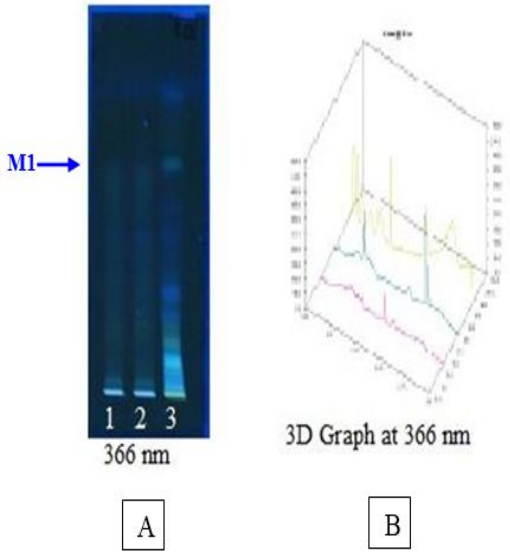

Fig 7: It shows the HPTLC Chromatogram of SE.

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.
A: HPTLC Plate of SE at 366 nm under UV.
B: 3D image of the Fingerprinting of SE and finished product (366 nm). The results indicate that HPTLC Chromatogram of SE and finished product has shown the similar R_f value of 0.57 at 366 nm.

7.6 Trikatu Churna (TC)

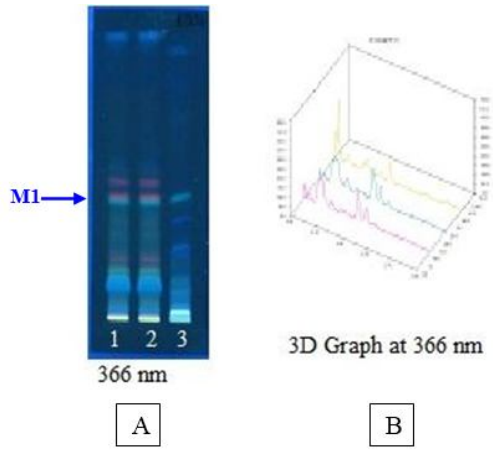

Fig 8: It shows the HPTLC Chromatogram of TC.

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.
A: HPTLC Plate of TC at 366 nm under UV.
B: 3D image of the Fingerprinting of TC and finished product (366 nm). The results indicate that HPTLC Chromatogram of TC and finished product has shown the similar R_f value of 0.44 at 366 nm.

7.7 *Mentha sylvestris* (Pudina) Satva (PU)

Track 1: reference standard **Track 2:** test drug **Track 3:** Herbo-mineral formulation.
A: HPTLC Plate of PU at 540 nm under UV.
B: 3D image of the Fingerprinting of PU and finished product (540 nm). The results indicate that HPTLC Chromatogram of PU and finished product has shown the similar R_f value of 0.45 at 540 nm.

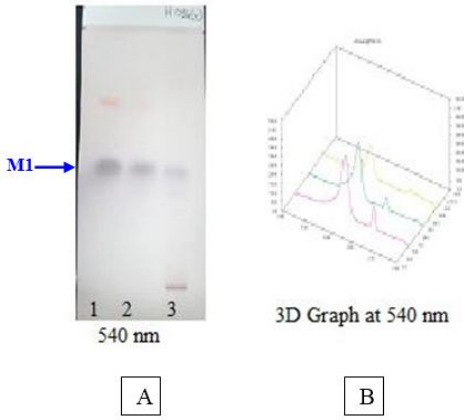

Fig 9: It shows the HPTLC Chromatogram of PU.

8. Discussion & Conclusion

Standardization of bio-active is the process of delivering a product with a specified level of one or more phyto-constituents where one can make sure about related product quality and efficacy; broadly it covers the qualitative and quantitative part of analysis. Qualitative analysis mainly covers the identification of the constituent(s) present in a particular product, whereas the quantitative analysis is accomplished by measuring the level of a chemical in a crude herbal extract which are, present in that particular product and establishing a standard amount of that chemical for future production. The concept of standardized extracts definitely provides a solid platform for scientific validation of herbals.

The testing of a finished product in compliance with predetermined standard prior to release of the product for packaging and subsequent distribution is a critical factor for quality assurance.

Zeal Cough Syrup is a Herbo-mineral Ayurvedic propriety product manufactured and marketed by Vasu Healthcare Pvt. Ltd. As a part of standardization procedure, the finished product and the raw materials of three different batches were analyzed for physical and chemical parameters.

The testing method for each parameter has been standardized and validated. The protocols for the same have been adopted from standard reference books.

Organoleptic characters like physical appearance, colour odour and taste of the raw materials and Finish product were first evaluated for identification and purity before any further tests are undertaken.

pH and moisture play important role in reflecting quality of product. Variation in them can encourage microbial growth and can cause deterioration followed by hydrolysis.

Extractive Value determines the amount of active constituents in a given amount of medicinal plant material when extracted with a solvent media such as Water and Alcohol. These values provide an indication of the extent of polar, medium polar and non-polar compounds present in the plant material. Thus from the above study we can conclude that all our extracts have good solubility in water which is a polar solvent.

The total ash usually consists of carbonates, phosphates silicates and silica that include the physiological ash which is derived from the plant tissue itself and non-physiological ash which is the residue of the adhering material to the plant

material e.g. sand and soil. Total ash was performed to measure the total amount of material remaining after ignition. This test is important to control adulteration. Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the washed insoluble matter.

Thus our results show that the ash values were much within the prescribed limits.

WHO has specified the limits for the presence of contaminants like four pathogenic micro-organisms viz *E. coli*, *Staphylococcus aureus*, *P. aeruginosa* & *Salmonella enterica* along with yeast-molds and four heavy metals viz; Lead, Cadmium, Arsenic & Mercury as the consumption of which can lead to complications in one's routine life. Zeal Cough Syrup was found in full compliance of the permissible microbial and heavy metal limits.

HPTLC study confirmed the qualitative as well as quantitative presence of the raw material in the finished product.

Present standardization study reveals compliance with all the above discussed parameters and hence it can be concluded that Zeal Cough Syrup is a well standardized product at essential physico-chemical parameters.

9. Acknowledgement

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