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PHARMACOGNOSTIC EVALUATION OF *TRIPHALA* HERBS AND ESTABLISHMENT OF CHEMICAL STABILITY OF *TRIPHALA* CAPLETS

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ABSTRACT: The use of herbal medicines for the betterment of mankind is well practiced from centuries. *Triphala* is a well known *Rasayana* drug used in Indian systems of medicine. Due to its wide spread usage in herbal medicine it drives the attention of regulatory bodies for its safety and efficacy and hence quality become major concern. In the present study we have standardized herbs by Pharmacognostical evaluation and selected chemical markers as part of quality control tool. Gallic acid and total tannins are selected as stability indicating assay for triphala caplet. The drug was subjected to short time accelerated stability study (40^0C/75%RH) as per ICH guidelines. Presence of stone cells, starch grains and calcium oxalate crystals are noted for differentiate along with the xylem elements (fibers and vessels) based on their structure. The content of these active constituents were monitored throughout the study and were found to be stable (Gallic acid: 3.72 to 5.24 %w/w, Total tannins: (17.16%w/w to 23.49%w/w). The HPTLC chromatographic fingerprint was also found to be complying with ICH guidelines. The drug product was found to be safe and stable for the prescribed shelf life. The results suggest that the methods can be appropriate emphasis as quality part to ensure the potency of any herbal formulation containing Triphala.

INTRODUCTION: *Triphala* is well known polyherbal formulation in the Indian systems of medicine. The word *triphala* is named by its formulation of three fruits namely *Haritaki* [*Terminalia chebula* Retz.], *Bibhitaki* [*Terminalia bellirica* (Gaertn.) Roxb.] and *Amalaki* [*Phyllanthus emblica* L.] which are classified under *Rasayana* drugs. *Rasayana* drugs have the property to promote health, longevity and immunity due to this property it is widely used in 219 formulations. [1, 2]

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Terminalia bellirica and *Terminalia chebula* belongs to family Combretaceae and *Phyllanthus emblica* belongs to family Phyllanthaceae. Anatomical studies reveals that the fruits can be differentiated based on their histological characters. Earlier studies were reported on Comparative Evaluation of different combinations formulation of *triphala* which highlighted few different characters [3].

FIG.1: EXTERNAL MORPHOLOGY OF TRIPHALA FRUITS; a. *PHYLLANTHUS EMBLICA;* b. *TERMINALIA CHEBULA;* c. *TERMINALIA BELLIRICA.*

Triphala contains major four phenolics chemical constituents such as gallic acid, tannic acid, syringic acid and epicatechin along with ascorbic acid. *P. emblica* contained ascorbic acid, gallic acid, *T. bellirica* contained gallic acid, tannic acid and ascorbic acid, while *T. chebula* contained gallic acid, tannic acid, syringic acid and epicatechin and ascorbic acid.

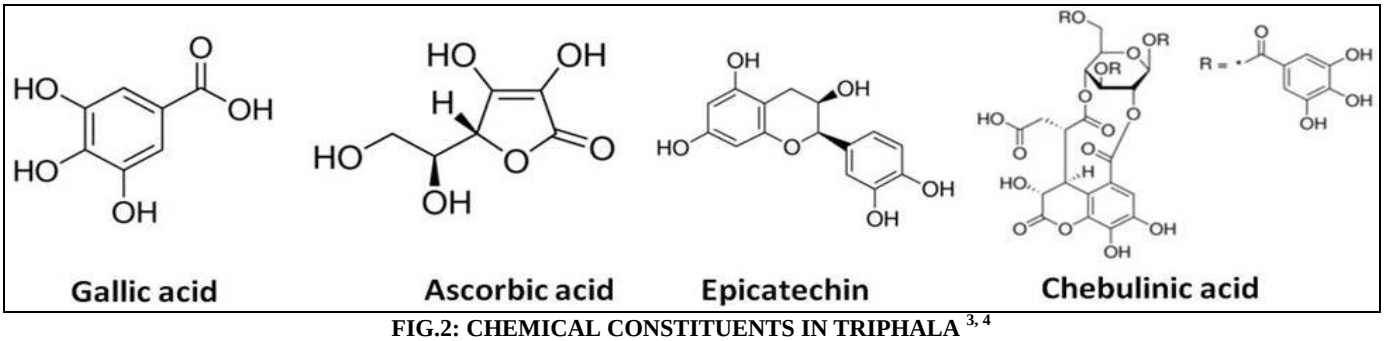


FIG.2: CHEMICAL CONSTITUENTS IN TRIPHALA [3, 4]

Triphala formulation is a rich source of antioxidants, frequently used to treat many diseases such as anaemia, jaundice, constipation, asthma, fever and chronic ulcers. Methanolic extract of triphala (70%) has shown high antioxidant activity in the in-vitro studies. Some reports have shown the radio- protective activity of triphala in mice exposed to gamma radiation [5, 6] The chloroform, water and acetone extracts of triphala have shown distinct anti-mutagenic activity against Salmonella typhimurium [7] and is found to act as purgative [8]. *Terminalia bellirica* is reported to provide protection from myocardial necrosis [9] and also found to be useful in many ocular diseases. Fully ripe or dried fruit, mixed with honey is used as an external application in ocular diseases [6]. *Phylanthus emblica* is found to possess significant anti -inflammatory [10], cytoprotective [11], antimutagenic [7], hypolipidaemic [12] and gastroprotective [13] activity. Aqueous extract of *P.emblica* was found to be a potent inhibitor of lipid peroxide formation and as a scavenger of hydroxyl and superoxide radicals *in vitro* [10]. Similarly, *Terminalia chebula* is potent antibacterial [14], anticaries [15], anticancer [16], antimutagenic [17] agent and also inhibits occurrence of local anaphylaxis [18].

In the present study, the raw herbs used in *triphala* are standardized (powder microscopy, and chemical) to evaluate the quality of the materials as per API. Heavy metal content was also evaluated to establish the safety of the product. The evaluation of drug stability for commercially available product (Triphala caplets) was done for 6 months accelerated stability study (Short term) as per ICH guidelines. Quantification of Gallic acid, which is one of the major active constituent of *Triphala* caplet, was performed by HPLC and chromatographic fingerprint was performed by HPTLC. The product was found to be stable and no degradation of compounds was observed.

MATERIALS AND METHODS:

Collection and preparation of materials:

The fruits of the three plants were purchased from local vendors. Fruits were authenticated by Botanist based on Organoleptical, macroscopical and microscopical characterization with reference to Botanical and Ayurvedic literature. Authentic fruits were processed and powder was prepared with mesh size of 18 for microscopic analysis. [19]

Reagents and Instrumentation:

Toluidine blue O, Safranin, Phloroglucinol-Hcl, and Iodine were procured from Hi-Media. and observed under Leica DM1000 microscope and captured images using the camera Leica MC120HD. Starch grains and calcium oxalate crystals were observed under polarizer and analyzer. Standard Gallic acid (Total purity ≥95%) was procured from Sigma Aldrich. Hexane-1-sulfonic acid sodium salt, Glacial acetic acid (Analytical grade) and Triethyl amine (Analytical grade) were procured from Merck. Mili-Q water generated by the Waters System Model ZMQX5V0001 was used for analysis. The HPLC system, Supplied by Shimadzu prominence quaternary pumps, PDA auto injector and,

Phenomenex C_{18} Luna (250 x 4.6 mm) particle size: 5 µ column was used. Evaluation of analysis was done by peak areas with linear regression. All solutions used for HPLC analysis were filtered through 0.45 µm membrane filter using Millipore filtration unit.

The HPTLC system, supplied Camag Swtizerland equipped with linomat 5 sample applicator for spotting of sample and TLC visualizer for capturing the TLC images.

The UV-Vis spectrophotometer system, supplied by Shimadzu model UV-1700 pharma spec consists of UV and white light having absorbance capacity from 190nm to 800nm. UV probe software was used for data evaluation.

Evaluation of Product Stability: [20]

The evaluation of drug stability was performed as per ICH guidelines. Triphala caplets were stored at 30°C/65%RH ±2°C/5% RH and at accelerated condition 40°C/75% RH ±2°C/5% RH up to 6 months.

Standardization of raw herbs used for *triphala*: [21, 22]

The powder microscopic studies were performed *Marimuthu et al* [19]. Physico-chemical drug standardization was done as per Indian Pharmacopoeia for the parameters such as Loss on Drying, Total Ash, Acid Insoluble Ash, Water soluble extractive and Alcohol soluble extractives. Chromatographic (HPTLC) standardization was done for evaluating fingerprint of the raw drugs. Toluene: Ethyl acetate: Glacial acetic acid (5:4:1) was used as mobile phase and derivatised using 1% ferric chloride solution for further evaluation.

Analytical method of analysis:

HPLC:

Preparation of mobile phase: 1.8822 g Hexane-1-sulfonic acid sodium salt was transferred in to 1000 ml volumetric flask and 500 ml of purified water was added and dissolved by sonication for 5 minutes. 10 ml of glacial acetic acid and 1.3 ml of tri-ethylamine was added, mixed well and the volume was made up to 1000 ml with purified water and use for analysis.

Standard Preparation:

Gallic acid Standard (0.25mg/ml): 100mg of standard Gallic acid was taken in a 100 ml volumetric flask; about 70 ml of purified water was added and dissolved by sonication. The volume was made up to the mark with purified water. 2.5 ml of this solution was taken into 10 ml volumetric flask and made up the volume with purified water.

Sample Preparation (5.0 mg/ml):

500mg of triphala caplet was taken in a 100 ml volumetric flask; about 70 ml of purified water was added and dissolved by sonication.

Quantification of Gallic acid by HPLC:

20µ aliquots of sample were injected in to HPLC. The HPLC analysis was continued for 20 min with the following specification.

Column: Phenomenex C18 Luna (250 x 4.6) mm, particle size: 5 µ

Wave length: 254nm

Mobile phase: 10 mM Hexane-1-sulfonic acid sodium salt, 1% Acetic acid and 0.13% Triethylamine in Purified water

Flow rate: 1.0 ml/min

Column temperature: 25^0C

Run time: 20 min

UV-VIS Spectrophotometer

Sample Preparation (0.3mg/ml):

About 150mg of finely powdered sample was taken in to 250 ml flat bottomed flask. 100 ml of purified water was added and refluxed at 98°C±2°C using water bath for 1 hour, cooled and decanted the dissolved extract into 250 ml volumetric flask. Same process was repeated with each 100 ml of purified water for 2 more times and the volume was made up to the mark with water. The test was performed using 0.2 ml of sample solution. Quantification of tannins was performed as per A M Diaz *et al.* [23]

HPTLC:

Preparation of Standard and Sample: About 1 g of this powdered standard and sample were weighed into a 250 ml flat bottomed flask and 25 ml of methanol was added and sonicated for 10 - 15

minutes. The obtained extract was filtered through whatman No.1 filter paper and used for analysis.

Standard and sample application:

10 µl of sample and standard were spotted on CAMAG Linomat V as 12 mm band width on a pre-coated Silica gel 60 F 254 plate of thickness (0.2 mm). The plate was developed in the solvent system Toluene: Ethyl acetate: Glacial Acetic acid: Formic acid (20: 45: 20: 05) and the dried plate was visualized under UV 254 nm and 366 nm using UV cabinet. The developed plate was dipped in vanillin sulphuric acid reagent and heated at 110°C for 5 to 10 minutes and observed under white light. The fingerprint obtained with the sample was compared with the standard solution.

RESULTS AND DISCUSSIONS: The use of herbal medicine as an effective mode for healthcare sector has been increasing exponentially. The lack of the quality control profiles for the herbal formulations drives researchers to explore more about the plant species. Due to the complexity and inherent variability of the phyto-constituents, it will be difficult to establish quality control parameters without the chromatographic techniques. The quality control parameters of finished products have major implications on safety and efficacy of the drug. Based on powder microscopical study shows the presence of calcium oxalate crystals, starch grains, fibers, vessels, sclereid and stone cells are noted characters, whereas both the species of *Terminalia* shows the presence of starch grains and whereas lack in *Phyllanthus emblica* (**Fig 3-5**). Calcium oxalate crystals are abundant in all three species and other xylem elements, stone cells are listed in **Table 1.**

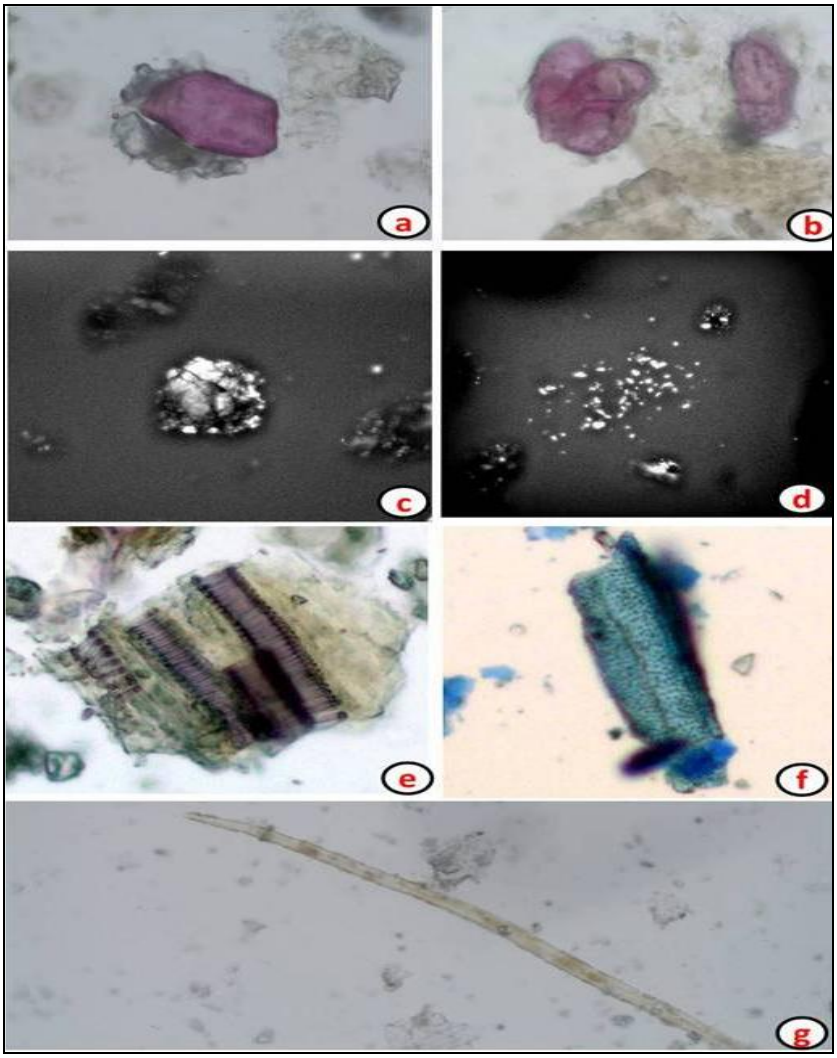


FIG.3: POWDERED MATERIALS OF *PHYLLANTHUS EMBLICA* FRUITS SHOWS THE PRESENCE OF

a. Isolated stone cell; b. grouped stone cell; c-d. calcium oxalate crystals; Druse and crystal sands respectively; e. spiral vessel; f. pitted

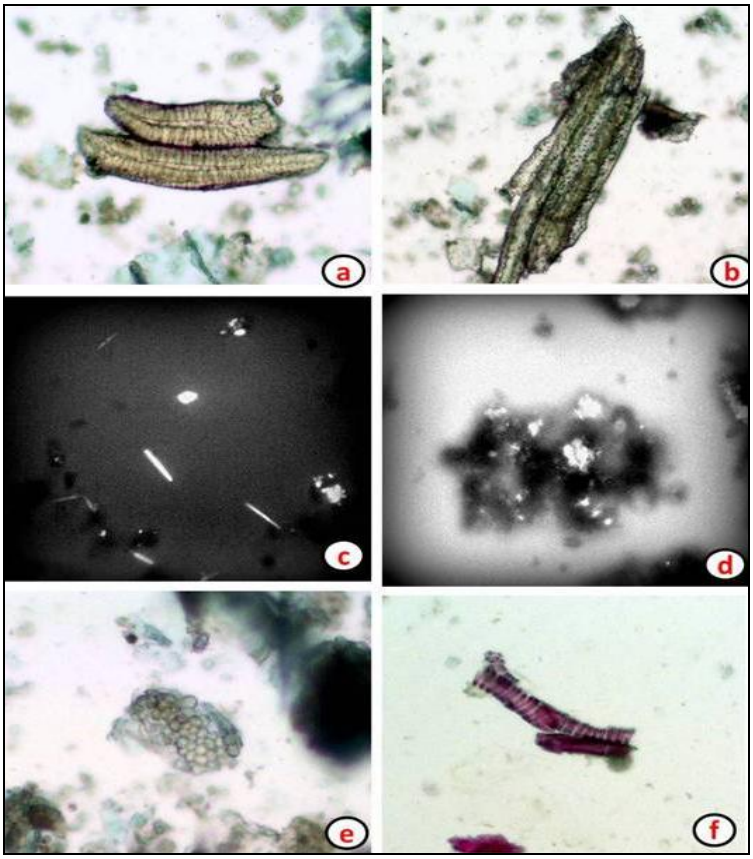


FIG.4: POWDERED MATERIALS OF *TERMINALIA CHEBULA* FRUITS SHOWS THE PRESENCE OF a. stone cell; b. Sclereid patch from the fruits rind; c-d. calcium oxalate crystals; acicular and druse respectively; e. simple starch grains in clusters; f.spiral vessel element

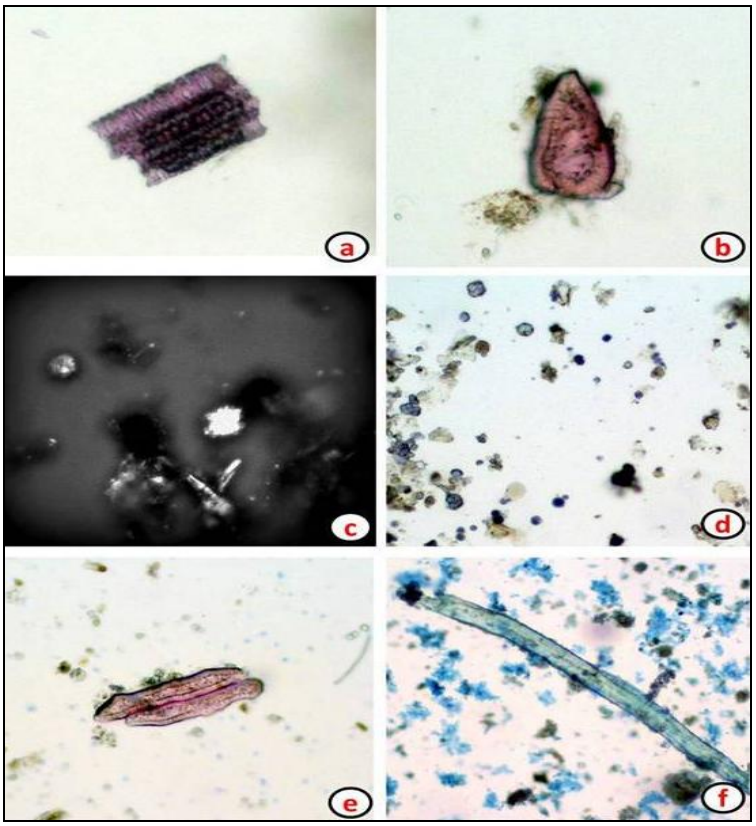


FIG.5: POWDERED MATERIALS OF *TERMINALIA BELLIRICA* FRUIT SHOWS THE PRESENCE OF a. pitted spiral vessel; b. stone cell; c. druse calcium oxalate crystals; d. simple and compound starch grains in clusters; e. sclereid cells of fruits rind; f. xylem fiber with lumen

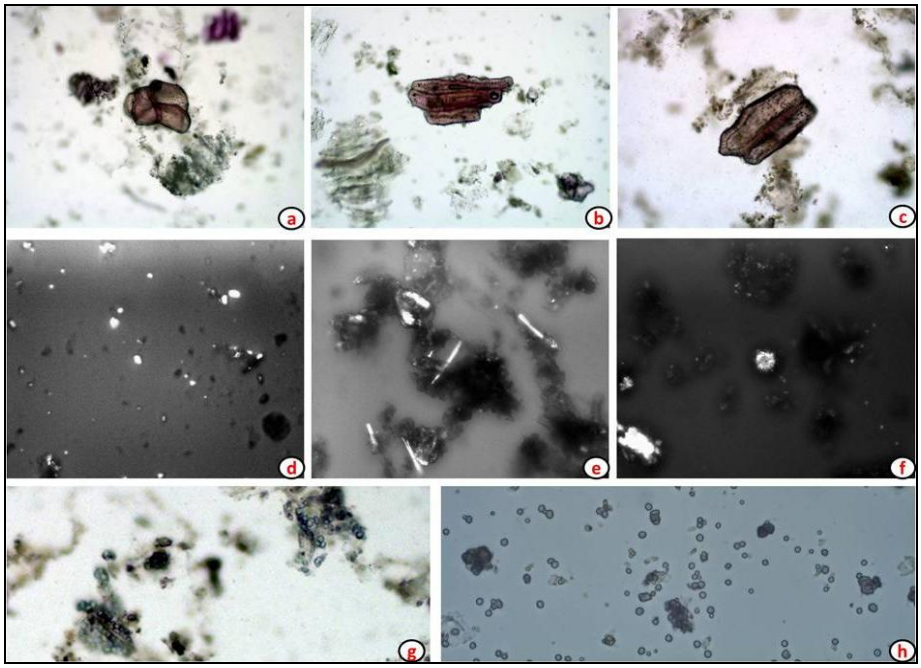


FIG.6: TRIPHALA POWDER MICROSCOPICAL ANALYSIS SHOWS

The stone cells a. *Phyllanthus emblica*; b. *Terminalia chebula*; c. *Terminalia bellirica*; Calcium oxalate crystals viewed under polarizer and analysizer shows, d. sand crystals of *Phyllanthus emblica* e. acicular crystals of *Terminalia chebula*; f. druse crystal of *Terminalia bellirica*; g and h. starch grains of *Terminalia* species.

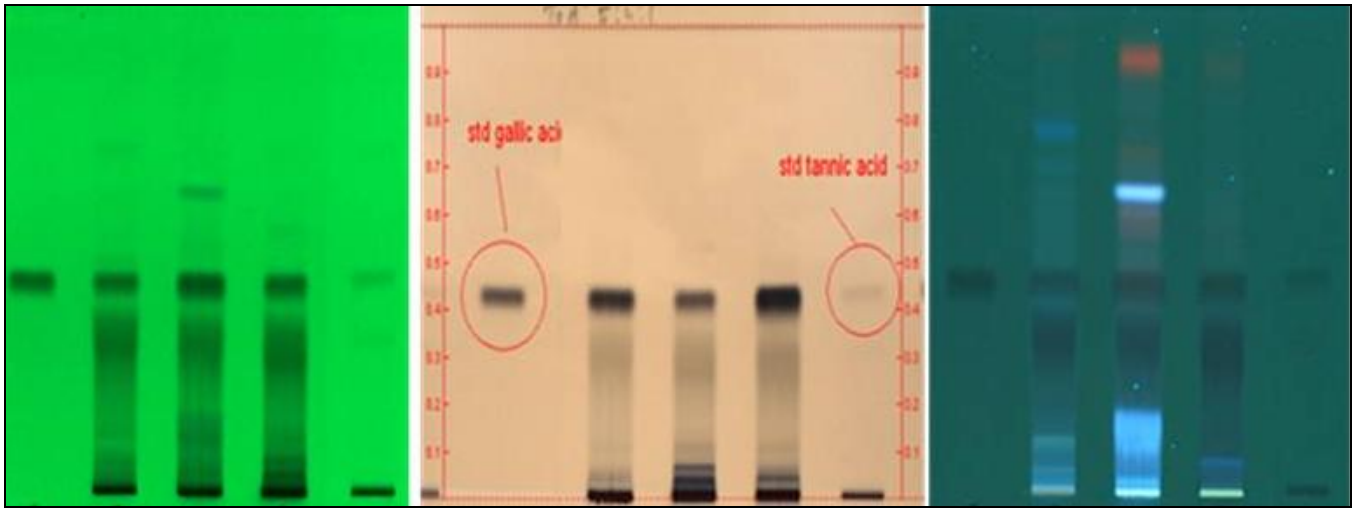
Although few powder characters show similar in all three species it will be difficult to identify in *triphala*. By morphological characterization the crude drugs can be identified before powdering and blending for the usage in formulation. Triphala is one the widely used formulation in ISM. It is equally important to establish the quality control parameters for the raw materials used for the formulation also. The three key ingredients of *triphala* namely *Haritaki*, *Bibhitaki* and *Amalaki* are physic-chemically standardized as per Ayurvedic pharmacopoeia of India and Phytochemical techniques by Raman. The water soluble extractives, alcohol soluble extractives, total ash, acid insoluble (**Table 2**) are analyzed. *Haritaki*, *Bibhitaki* and *Amalaki* physico-chemical tests analyzed conforms the limits of API and are safe enough for further processing of the drug. The HPTLC chromatographic fingerprints of these herbs reveal the authenticity of the herbs and also found to contain key chemical constituents such as tannin acid and Gallic acid (**Fig. 7**).

TABLE 1: KEY TO IDENTIFY INDIVIDUAL INGREDIENTS IN *TRIPHALA* BY POWDER MICROSCOPY

Sl. No	Characters	*Amalaki*	*Haritaki*	*Bibhitaki*
1	Calcium Oxalate	Druse and Crystal sand	Acicular and Druse Crystals	Druse Crystals
2	Starch grains	Absent	Simple	Simple and Compound
3	Stone cells	Isolated or grouped	Horizontally elongated	Spherical to elongated
4	Fiber	Present	Present	Present
5	Vessels	Pitted Vessels	Spiral Vessels	Pitted Vessels

TABLE: 2 STANDARDIZATION OF RAW HERBS USED FOR *TRIPHALA* CAPLETS

Parameters	*Phyllanthus emblica* Fruit %w/w	*Terminalia bellirica* Fruit %w/w	*Terminalia chebula* Fruit %w/w
Alcohol Extractive value	41.0 to 44.0	41.0 to 44.0	41.0 to 54.0
Water Extractive value	50.0 to 60.66	50.0 to 60.66	62.0 to 66
Loss on Drying	5.80 to 7.90	5.80 to 7.90	5.00 to 9.20
Ash value	2.65 to 3.45	2.65% to 3.45	1.50 to 3.00
Acid insoluble ash	0.20 to 1.55	0.20% to 1.55	0.050 to 0.30



TRACK 1: STD GALLIC ACID, TRACK 2: *PHYLANTHUS EMBLICA*, TRACK3: *TERMINALIA BELLIRICA*, TRACK 4: *TERMINALIA CHEBULA*, TRACK 6: TANNIC ACID

FIG.7: HPTLC FINGERPRINTS OF RAW HERBS USED IN *TRIPHALA* FORMULATION

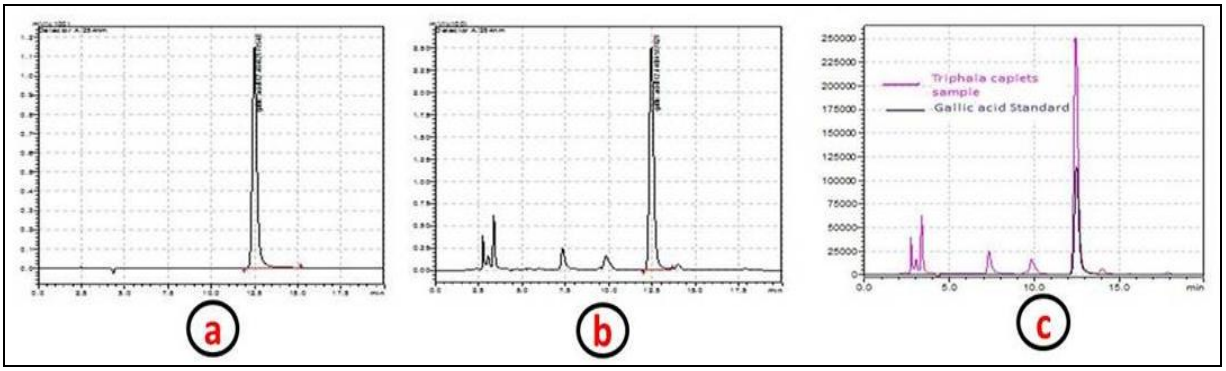
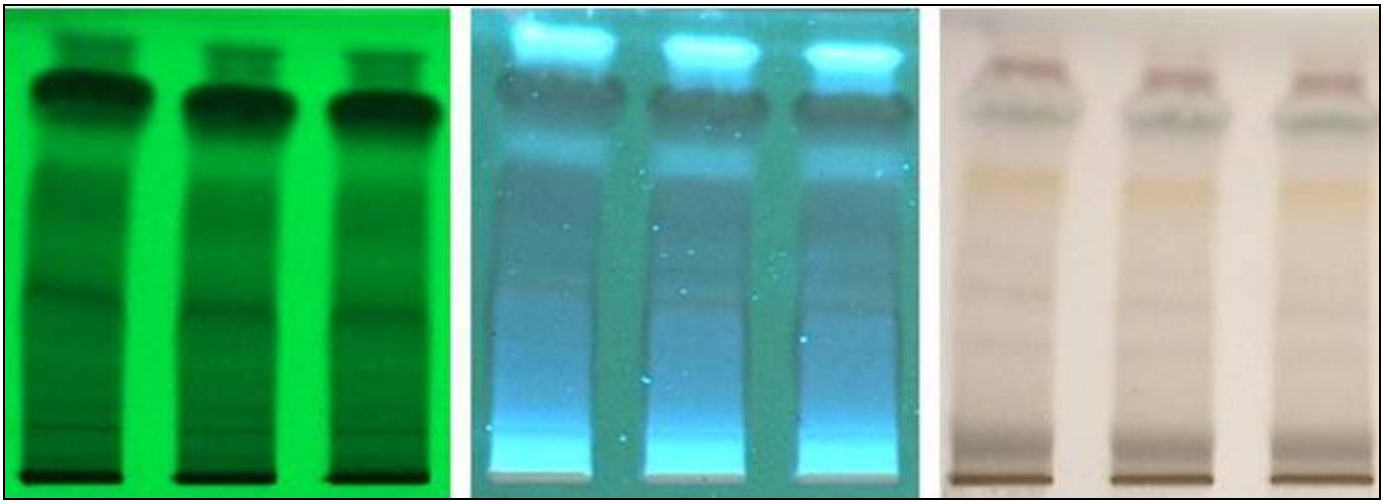


FIG.8: HPLC CHROMATOGRAM (a) STANDARD GALLIC ACID, (b) *TRIPHALA* CAPLETS AND (c) OVERLAY OF STANDARD GALLIC ACID AND *TRIPHALA* CAPLET



TRACK 1: *TRIPHALA* CAPLET REFERENCE STANDARD, TRACK 2: *TRIPHALA* CAPLET (30 ºC/65%RH), TRACK 3: *TRIPHALA* CAPLET (40 ºC/ 75%RH

FIG.9: HPLC FINGERPRINTS OF *TRIPHALA* CAPLETS DURING STABILITY STUDIES

Establishment of the drug stability plays a vital role in the safety and efficacy of the drug. Gallic acid and tannins are the stability indicating assay for *triphala* caplets. The Gallic acid and tannins are reported for their therapeutic activity of the *triphala*. The drug was subjected to short time accelerated stability study (40^0C/75%RH) as per ICH guidelines. The content of these active

constituents were monitored throughout the study and were found to be stable (Gallic acid: 3.72 to 5.24 %w/w (**Fig. 8**), Total tannins: (17.16%w/w to 23.49%w/w).

Along with the key actives the physical parameters such as description, loss on drying were also observed and found to comply with ICH guidelines. There was no degradation observed during the study hence proving the product more safe and efficacious. There was no significant change in the HPTLC chromatographic fingerprint of the *triphala* caplets throughout the study (**Fig. 9**).

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