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Development of quality standards of Triphala Kwatha churna with its ingredients through HPTLC and mass spectroscopy

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In recent years there has been rapid growth in the field of herbal medicine. Drugs belonging to Asian traditional systems of medicine are accepted universally only after standardisation. It is therefore, very important to develop essential techniques for standardisation of herbal drugs. The present study has focused on development of quality standards of Triphala Kwatha churna under WHO/API guidelines along with HPTLC and mass spectroscopy. This polyherbal Kwatha churna is used to treat constipation and other gastric disorders. In this study, an in-house prepared Triphala Kwatha churna was botanically and chemically standardised by HPTLC and mass spectroscopic studies with the reference standard gallic acid. The botanical standardisation of the above formulation was done by evaluation of macroscopic and microscopic studies of the powder formulation along with its ingredients. Physicochemical parameters like LOD, ash value, acid insoluble ash, water and alcohol extractives were determined. Safety parameters, viz., heavy metals, microbial content, specific pathogens, pesticide residue and aflatoxins detection have been also estimated.

The results obtained in the present study defining quality control parameters help to develop the quality standards of Triphala Kwatha churna formulation. The HPTLC fingerprint profiling of the formulation along with its ingredients complies with the reference standards gallic acid. This observation is also found in Mass Spectroscopic study of the methanolic extract of the formulation with this reference standard. Preliminary phytochemical screening test revealed the presence of bioactive constituents including phenols, flavonoids, tannins and carbohydrates.

Keywords: HPTLC finger-print, Mass spectroscopy, Physicochemical parameters, Phytochemical screening, Quality standards, Triphala Kwatha churna

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Ayurveda is an ancient Indian system of herbal medicines acclaimed throughout the world in different formulations. The use of Ayurvedic drugs has gradually spread throughout the world, to countries at different levels of development - developed and developing[1]. Standardisation of herbal formulations is essential for assessing and ensuring quality of the drugs and hence for their acceptability and safety. The standardisation is based on physico-chemical properties, phytochemical screening, use of modern techniques like HPTLC and spectral analysis to detect chemical constituents[2]. Triphala (Sanskrit; tri = three and phala = fruits) is a well-established and respected polyherbal drug used in traditional Indian medicine for over a thousand years[3]. It consists of the pericarps of dried fruits of the following three Indian plant species in equal proportions (1:1:1), viz., Amalaki or the Indian Gooseberry – *Emblica officinalis* Gaertn. (Euphorbiaceae family), Bibhitaki - *Terminalia bellirica* (Gaertn.) Roxb. (Combretaceae family) and Haritaki – *Terminalia chebula* Retz. (Combretaceae family). In Ayurvedic medicine, it is classified as a *tridoshic rasayana* promoting longevity and rejuvenation in patients of all ages and constitutions. Its various applications in the medicine include use as laxative, anti-inflammatory, eye rejuvenator and antiviral. It is reputed to be effective in treatment of headache, dyspepsia, and leucorrhea. It is used asa blood purifier and possesses analgesic, ascites, hypoglycemic, anti-arthritic and anti-aging properties[3-7].

Over the years, Triphala and its three ingredients have been the subject of several investigations to assess their

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biological properties and determine their chemical constituents[8-13].

Materials and Methods

Collection and authentication of plant material

The three ingredients of pharmacopoeial quality were purchased from well-reputed suppliers in Kolkata. The samples were identified by the Pharmacognosy Department of the Institute. The following vouchers specimens have been deposite in the Institute: *Emblica officinalis* Gaertn. - (CARIDD/EO/MD13/2019); *Terminalia chebula* Roxb. - (CARIDD/TC/MD14/ 2019), *Terminalia bellirica* (Gaertn.) Roxb. - (CARIDD/TB/MD15/2019)

Plant sample processing

Triphala Kwatha churna is a coarse powder preparation made with Amlaki (*Emblica officinalis* Gaertn.) pericarp, Haritaki (*Terminalia chebula* Roxb.) pericarp and Bibhitaka (*Terminalia bellirica* (Gaertn.) Roxb.) pericarp in equal proportions (1:1:1). All the ingredients were of pharmacopoeial quality. All the ingredients were cleaned by separately washing under running tap water followed by washing three times by sterile distilled water. Samples were air-dried in the shade at ambient temperature for 7 days. To avoid any contamination, these were stored in air-tight containers. These were powdered individually in a pulveriser to a coarse powder. The total coarsely powdered samples of the three ingredients were taken for assessment of the physicochemical parameters.

For preparation of Triphala Kwatha churna these coarsely powdered ingredients were passed through sieve number 22, the amount passing through the sieve being taken for the preparation of the formulation. Each of the sieved ingredients was weighed separately and mixed together thoroughly in equal proportions in a mechanical shaker. Kwatha churna particles passed through a sieve - 710 µm mesh aperture, and not more than 42% by weight through a 250 µm mesh aperture sieve. The Kwatha churna was stored in a container and closed tightly to protect from air, light and moisture.

Physicochemical parameters

Standard protocols as given in Ayurvedic Pharmacopoeia of India/ WHO protocols[6,7] were followed to determine physicochemical parameters Table 1.

Preliminary phytochemical analysis were performed using standard chemical tests[2,6,14,15].

Solvents and chemicals

GR grade (E. Merck Ltd., Mumbai, India) chemicals and solvents were used.

Macroscopy of plant material - organoleptic parameters

Naked eye observation with a simple microscope Olympus OIC DM was done to note texture, shape, size, colour of the plant material.

Cytomorphology of plant material

For powder microscopy, finely powdered samples (~2 g) were separately treated with the following reagents as appropriate - aqueous saturated chloral hydrate (for maceration), 50% glycerin, phloroglucinol in concentrated HCl (for staining lignified tissues) and 0.02 N iodine reagent (for starch grains); these were mounted on slides with glycerin following standard protocol. Then these slides were observed under the binocular compound microscope (Olympus OIC-07964) at 10X and 40X magnifications. Magcam DC14 camera attached with Olympus CX21i trinocular compound microscope[16] was used to take photomicrographs of the different cellular structure and inclusions.

HPTLC Study

Equipment

This study was performed with a CAMAG HPTLC system (Switzerland), which comprised of a CAMAG ATS-4 applicator, CAMAG TLC scanner 3, CAMAG Wincats Software, Version 1.44, CAMAG Reprostar 3, a CAMAG TLC plate heater, and a CAMAG UV cabinet.

Equipment used for Mass Spectroscopic studies

The batch of Triphala Kwatha churna was analysed by Mass Spectrometry on Waters Xevo-G2-Xs-QT

Table 1 — Results of quality standard parameters of Triphala Kwatha churna and its crude ingredients. Physicochemical parameters

Testing parameters	Amlaki (p) (w/w %)	Haritaki (p) (w/w %)	Bibhitaka (p) (w/w %)	Triphala Kwatha churna (w/w %)
Loss on drying	12.8 ± 0.2	14.7 ± 0.173	16.3 ± 0.265	6.9 ± 0.06
Ash value	4.8 ± 0.2	2.20 ± 0.087	5.45 ± 0.13	3.1 ± 0.017
Acid insoluble ash	1.4 ± 0.046	1.15 ± 0.01	1.75 ± 0.087	0.25
Water extractive	52.1 ± 0.625	62.3 ± 0.871	42.6 ± 0.603	50.1 ± 0.793
Ethanol extractive (90%)	40.7 ± 0.436	48.3 ± 0.723	18.3 ± 0.625	46.3 ± 0.436
pH (10% aqueous suspension)	2.8 ± 0.08	3.84 ± 0.056	4.1 ± 0.07	3.07 ± 0.03

instrument. The dried sample of the methanolic extract of TKC was taken in 50% aqueous methanol and was directly introduced into the ionization chamber using the automated electrospray technique. The resultant ions were analysed by a time-of-flight Quadrople analyser. The ESI-MS of the marker compound gallic acid was also recorded in the same way. The respective operational parameters are given below.

Method used

ESI-MS in positive ion mode was performed. Sample flow-direct infusion was at 5 microlitre per min. acquisition time being 1 min.; mass range acquired was 50 to 1000 m/z. Other parameters were: capillary voltage 3 kv; sampling cone 40; source offset 80; source temperature 100^0C; desolvation temperature 250^0C; cone gas 49 lit/h.; desolvation gas 300 lit/h.

Results and Discussion

Organoleptic Evaluation of Amlaki (P), Haritaki (P), Bibhitaka (P), Triphala Kwatha churna

These were carried out regarding colour, texture, odour and taste. These are mentioned sequentially. (P) refers to pericarps.

Amlaki (P): Dark yellow; Fine, smooth; characteristic odour; sour and astringent taste.

Haritaki (P): Yellowish brown; Fine, smooth; characteristic odour; astringent taste.

Bibhitaka (P): Dark yellow; Fine, smooth; characteristic odour; astringent taste.

Triphala Kwatha churna: Yellowish brown; coarse powder; characteristic odour; astringent taste.

Macroscopic studies of the ingredients

The characteristics are listed below. The ingredients and the formulation are shown in (Fig. 1).

Amlaki pericarp – Drug consists of curled pieces of pericarp of dried fruit; 1.0-2.0 cm long; gray to black in colour; the pieces showed broad, highly shriveled and wrinkled external convex surfaces to somewhat concave, transversely wrinkled lateral surfaces; texture rough, cartilaginous, tough. Taste sour and astringent.

Haritaki pericarp - Fruit pericarp yellowish brown in colour, ovoid with both ends pointed; wrinkled and ribbed longitudinally (more or less five-ribbed); pericarp fibrous, non-adherent to the seed, odour characteristic; taste astringent.

Bibhitaka pericarp - Pericarp grey or grayish brown with slightly wrinkled appearance; spherical to ovoid; 1.0-2.0 cm in diameter, taste astringent.

Microscopic studies of Triphala Kwatha churna

Microscopic studies of the formulation showed lignified stone cells, various sizes of elongated tracheids, epicarp containing crystals, isodiametric parenchyma cells with irregularly thickened walls, groups of hair with basal cell, pitted, spiral vessel, thick-walled fiber with large lumen, prismatic calcium oxalate crystals and starch grains (Fig. 2).

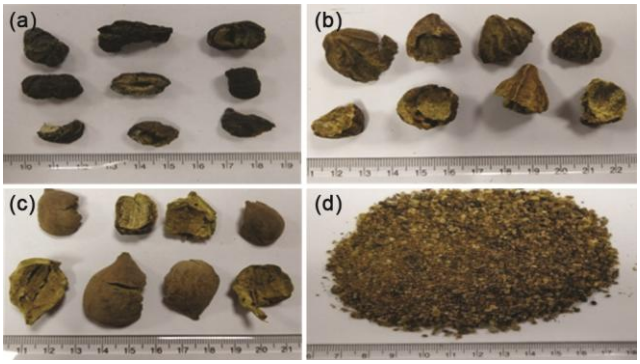

Fig. 1 — Macroscopic examination of (a) Amlaki pericarp; (b) Haritaki pericarp; (c) Bibhitaka pericarp; (d) Triphala Kwatha churna formulation

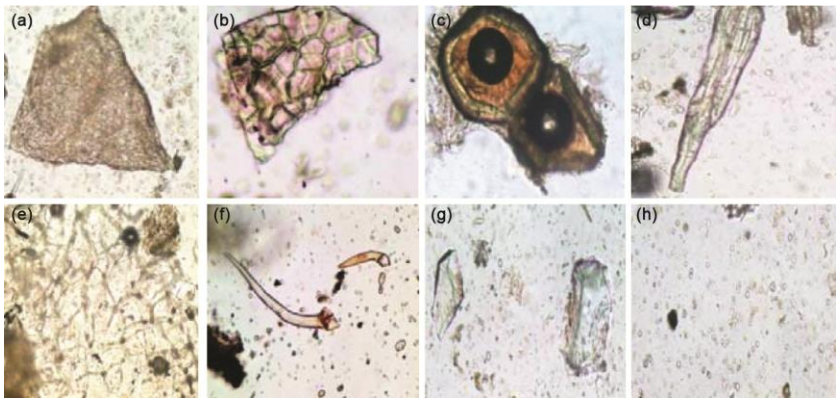

Fig. 2 — Photomicrographs of Triphala Kwatha churna formulation A., B. Epicarp containing crystals; C. Lignified stone cells; D., Elongated tracheids; E. Isodiametric parenchyma cells with irregularly thickened wall; F. Groups of hair with basal cell; G. Prismatic calcium oxalate crystals; L. Starch grains.

Safety parameters of Triphala Kwatha churna

Heavy metals testing have been carried out according to API protocols. The results indicate that none of these heavy metals have been detected within limit of detection 0.05 ppm. Acceptance limits mentioned in API are as follows: Lead (Pb) NMT 10 ppm; Arsenic (As) NMT 3.0 ppm; Cadmium (Cd) NMT 0.3 ppm; Mercury (Hg) NMT 1.0 ppm.

Table 2 — Pesticide residues testing

Tests	Acceptance Limit as in API NMT (mg/kg)
Pesticides Residue [$]	-
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of *cis*-, *trans*- and Oxythlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrifos-methyl	0.1
Cypermenthrin (and Isomers)	1.0
DDT (sum of p,p-DDT, o,p-DDT, p,p-DDE and p,p-TDE)	1.0
Deltamenthrin	0.5
Dichlorvos	1.0
Diazinon	0.5
Dithiocarbamates (as CS2)	2.0
Endosulfan (sum of isomers and endosulfan sulphate)	3.0
Ethion	2.0
Endrin	0.05
Fenitrothion	0.5
Fenvalerate	1.5
Fonofos	0.05
Heptachlor (sum of Heptachlor and Heptachlorepoxide)	0.05
Hexachlorbenzene	0.1
Hexachlorocyclohexane isomers (other than ɤ)	0.3
Lindane (?- Hexachlorocyclohexane)	0.6
Methidathion	0.2
Malathion	1.0
Parathion	0.5
Parathion-methyl	0.2
Pyrethrins (sum of)	3.0
Quintozene (sum of pentachloroaniline and methyl pentachlorophnyl sulphide)	1.0
Permethin	1.0
Phosalone	0.1
Piperonyl Butoxide	3.0
Pirimiphos-Methyl	0.2

[$] Not detected within
Limit of Detection = 10 ppb (GC-MS MS).
NMT - Not More Than

Pesticide testing

Pesticide testing used GC-MS-MS protocols, as per API, showed that all the pesticides listed in Table 2, were not detected at the Limit of Detection of 10 ppb (GC-MS MS). The acceptance limits as in API are also listed in mg/kg in the Table.

Pathogens testing

Microbiological examination results are given in Table 3 - total aerobic bacterial count as well as yeast and mould count were as per API protocol. Pathogen testing as per API protocol showed that *Escherichia coli*, *Salmonella spp.*, *Staphylococcus aureus* and *P. aeruginosa* were absent. Acceptance limits in API specify that these should be absent.

Aflatoxins testing

Aflatoxins testing were carried out as per API protocols. The aflatoxins B1, B2, G1, G2 were not detected, with the limit of detection of 1 ppb. API permissible limits are B1 < 2 ppm; all four Aflatoxins, B_1, B_2, G_1 and G_2 altogether < 5 ppb.

Phytochemical Evaluation

Sample preparation

A suspension of 4 g of Triphala Kwatha churna was refluxed with 80 mL 90% ethanol (90 ethanol: 10 distilled water; v/v) for 1 h. Filtration through fluted Whatman No. 40 filter paper gave extract (TKcE). For tests with this extract, which had to be carried out in aqueous medium, 20 mL of the alcoholic extract was evaporated on the water bath, and taken up in 20 mL distilled water. The aqueous extract was prepared by shaking a suspension of 4 g. of Triphala Kwatha churna in 80 mL distilled water for 6 h. at ambient temperature, keeping overnight for 18 h, then filtering through fluted filter paper (Whatman No. 40) – extract TKcW. For tests in ethanolic medium, 10 mL of this extract was evaporated to dryness on the water bath and taken up in ethanol. About 2 mL of the extracts was used for each test.

Results are given in Table 4. These tests were performed using standard procedures.

Table 3 — Microbiological Examination

Tests	**Protocol**	**Results (in cfu/g)**	**Acceptance Limit (in cfu/g)**
Total aerobic bacterial count	As per API	11,682	NMT 10^5
Yeast and mould count	As per API	264	NMT 10^3

Table 4 — Phytochemical Evaluation of 90% alcoholic and aqueous extracts of Triphala Kwatha churna. These tests were performed using standard reagents and test protocols[2,6,14,15].

Sl. No.	Test/Reagent used	90% alcoholic extract [TKcE]	Aqueous extract [TKcW]
1a.	Alkaloids - Mayer's test (in dil. HCl)	-ve	-ve
2a.	Molisch's test for Carbohydrates	+ve	+ve
2b.	Fehling's test for Reducing sugars	+ve	+ve
3.	Shinoda test for Flavonoids	-ve	-ve
4.	Salkowski's test for Steroids	-ve	-ve
5.	$FeCl_3$ test - Phenolic compounds/ tannins	+ve	+ve
6.	Ninhydrin test for Amino Acids	-ve	-ve
7.	Biuret test (Pietrowski's test) for Proteins	-ve	-ve
8.	Lead acetate test	+ve	+ve
10.	Saponins - Frothing test	+ve	+ve

Mass Spectrometric analysis

The ESC-MS of gallic acid showed a M+1 peak at m/z 171.033 corresponding to the formula $C_7H_7O_5$ $[M+H]^+$. Prominent peaks were obtained at m/z 153.084 $[171\text{-}CO_2]^+$; 127.0388 $[153\text{-}H_2O]^+$; 109 $[127\text{-}H_20]^+$ and 185 $[M+H+H_2O]^+$. In the batch TKC1, all the peaks corresponding to gallic acid, mentioned above, are present. This confirms that gallic acid is one of the constituents.

Additional peaks appear at 193.049, 225.1517, 281.0570, 297.9842, 338.3473, 360.0254, 360.5264, 445.0346, 549.0246, 563.9912, 564.9974. These are due to the other chemical constituents present. All the results are shown in Figure 3a-c.

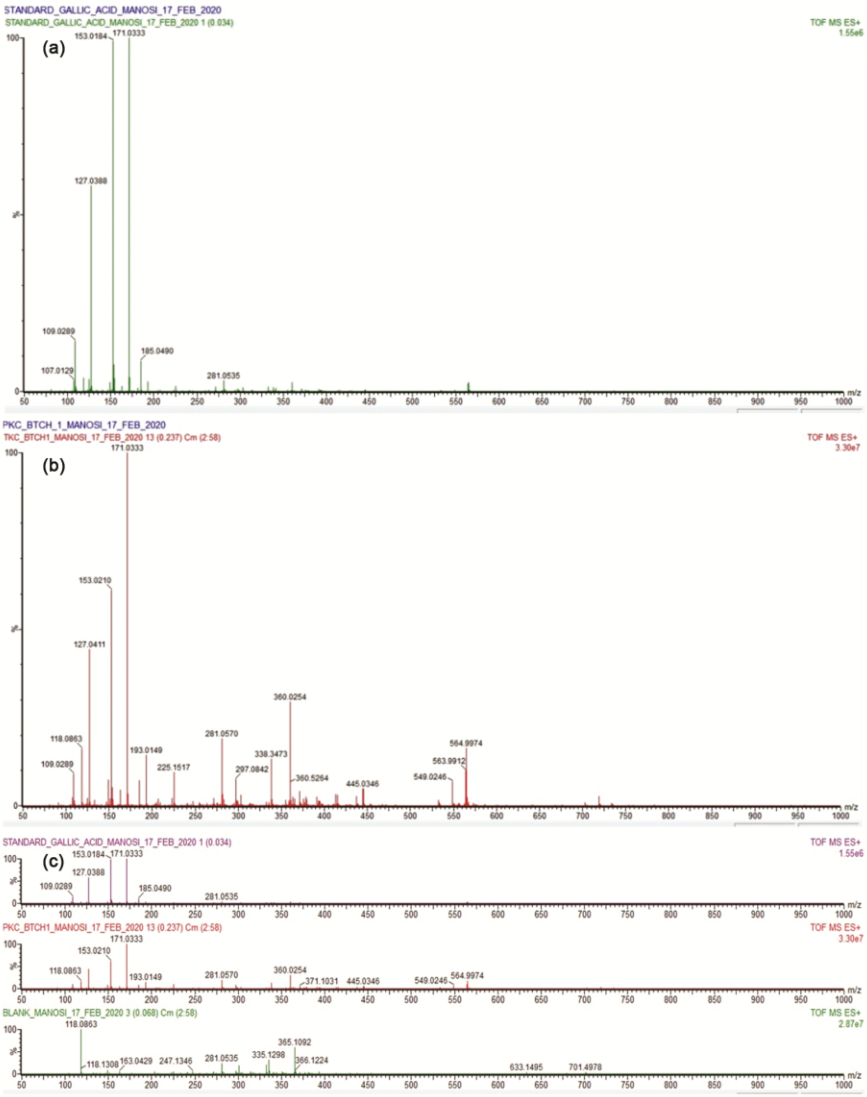

Fig. 3a — Mass spectrum of Gallic acid.(b) Mass spectrum of Triphala Kwatha Churna – peaks (c) Mass spectra of (a) Gallic acid; (b) Triphala Kwatha Churna; (c) the lowest trace showing the background peaks - these also appear the MS of the marker gallic acid (upper trace), and TKC sample (middle trace), and are to be discounted.

HPTLC Profile of Triphala Kwatha churna and its ingredients

Sample preparation

The suspensions of 1 g of each of the samples of Triphala Kwatha churna and its three ingredients were separately subjected to reflux with 20 mL methanol, for 1 h. 10 mL of each the filtered extracts was used for HPTLC analyses.

Standard preparation

1mg of gallic acid (Marker) was dissolved in 10 mL of methanol in a volumetric flask. This solution was also applied on the HPTLC plate

Stationary phase

Aluminum-supported precoated silica gel 60 F254 plates (Merck, India, Batch No. 1.05554.0007) were used. Plate preconditioning – temperature 27°C; relative average humidity was 48%. Mobile Phase - Hexane: Ethyl acetate: Methanol: Formic acid = 4 : 5 : 0.5: 0.5 (v/v) [G R grade solvent used, MERCK, India].

Sample application

Applied volume – 5 µL as 8 mm bands were applied at 12 mm from the base of the plate with a CAMAG ATS4. Plate size was 10 x 10 cm.

Development

The plates were developed upto 80 mm in CAMAG Twin trough chamber.

Observation

CAMAG TLC visualiser and CAMAG TLC Scanner 4 were used for visualisation and scanning respectively. The HPTLC chromatograms are given in Fig. 4a (visualised at 254 nm) and Fig. 4b (visualised at 366 nm). Fig. 4c shows white light visualisation after derivatisation with 20% aqueous sulphuric acid followed by warming.

Photography of developed HPTLC plate

Photography of developed HPTLC plate are presented in Fig. 4; Fig. 4a - visualization at 254 nm, Fig. 4b - 366 nm, Fig. 4c - visualised with white light after derivatisation with 20% aqueous sulphuric acid followed by warming.

General comments

In each case, a large number of bands were observed - Table 5 lists the intense bands with R_f values. All the

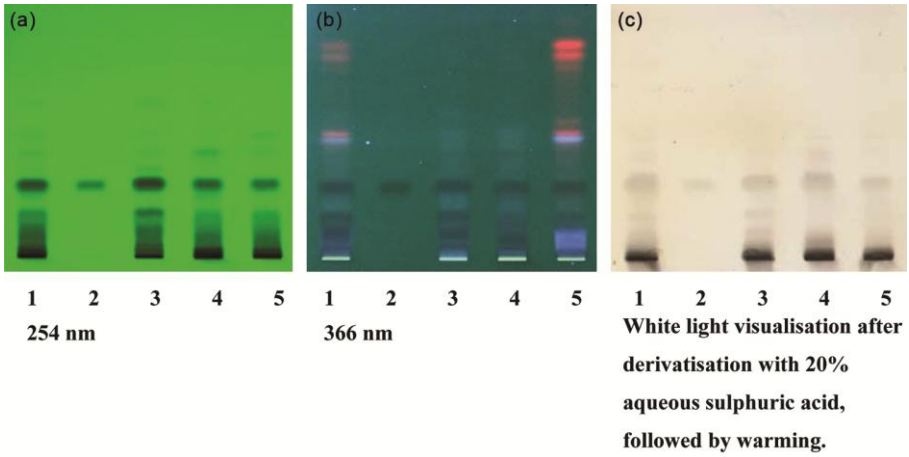

Fig. 4 — Visualisation of HPTLC traces under different conditions. In all the Figures the sequence of tracks are of methanolic extracts: Track -1 Triphala Kwatha Churna formulation, Track -2- Gallic acid marker standard, Track -3- Amlaki pericarps, Track - 4- Haritaki pericarps, Track -5- Bibhitaka pericarps.

Table 5 — HPTLC results

Sample name	R_f values (Observed at 254 nm)	R_f values (Observed at 366 nm)	R_f values (Derivatised with 20% aq. sulphuric acid and warmed)
Triphala Kwatha churna	0.03, 0.07, 0.10, 0.14, 0.22, 0.35, 0.48, 0.56	0.04, 0.11, 0.14, 0.23, 0.35, 0.50, 0.65, 0.87, 0.93	0.03, 0.08, 0.11, 0.18, 0.35
Gallic acid (Marker)	0.35	0.35	0.35
Amlaki (p)	0.03, 0.07, 0.14, 0.22, 0.35, 0.48, 0.68	0.04, 0.07, 0.14, 0.23, 0.35	0.04, 0.12, 0.23, 0.35
Haritaki (p)	0.04, 0.11, 0.18, 0.35, 0.48, 0.68	0.04, 0.07, 0.14, 0.23, 0.35	0.03, 0.08, 0.12, 0.35
Bibhitaka (p)	0.03, 0.07, 0.11, 0.18, 0.23, 0.35, 0.51, 0.56	0.04, 0.08, 0.13, 0.18, 0.23, 0.35, 0.50, 0.58, 0.87, 0.93	0.03, 0.08, 0.17, 0.35

samples subjected to HPTLC analysis showed the presence of the marker compound gallic acid.

Conclusions

The results obtained in the present study define quality control parameters, which will develop the quality standards of Triphala Kwatha churna formulation. HPTLC fingerprint profiling of the formulation along with its ingredients complies with the reference standard gallic acid. The same observation is also found in Mass Spectroscopic analysis of the methanolic extract of the formulation with its reference standard. Preliminary phytochemical screening revealed presence of flavonoids, phenols, carbohydrates and tannins.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

MD is the communicating author. She carried out the physicochemical, chemical, analytical and HPTLC experimental works. RS provided the concept of the work. RB carried out the phytopharmacognostical experimental works. AB was involved in designing some the experimental work and interpretation of the results. SR carried out the mass-spectroscopical work at the CIF, Bose Institute. JH, Director of the Institute, overviewed the whole of the research work.

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