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Marker based Chemo-profiling of a traditional formulation: *Pushyanuga Churna*

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ABSTRACT

Objectives: Standardization of traditional medicine is a multidimensional approach that ensures the quality and concentration of chemical constituents for their bio-potency. *Pushyanuga churna* is a traditional ayurvedic formulation prescribed for the treatment of various female reproductive disorders leucorrhoea, menorrhagia etc. As per AFI, the formulation consists of twenty five plants and one mineral. Owing to its therapeutic efficacy, it is prepared and marketed by various manufacturers and vaidyas. Hence, there is an increasing demand but uncertainty about the authenticity of ingredients.
Method: Chemo profiling-assisted characterization of the formulation using HPTLC can help in assuring the quality of the formulation which in turn decides its safety and efficacy. Quantitation of therapeutically reported biomarkers under set optimized chromatographic conditions can be used as a quality control tool.
Results: Fingerprints and quantitation of biomarkers have been used as tools for identification of *Pushyanuga Churna*. Hence in the present study, an HPTLC method was developed and validated following ICH guidelines for the simultaneous estimation of ursolic acid, β-sitosterol and lupeol from formulation prepared in-house along with its different manufacturers. Average content of markers was found to be 1.37±0.108, 0.73±0.025 and 1.24±0.136 mg/g in the in-house formulation and 1.57±0.027, 0.58±0.024 and 0.88±0.089 mg/g in marketed formulation respectively.

INTRODUCTION

Herbal industry lack rigid quality control profiles. This has become one of the major drawbacks for the globalization of available traditional knowledge. The medicines and practices also include valuable elements and plants sources that are no longer common. Herbal drugs, singularly and in combinations, contain a myriad variety of compounds in complex matrices in which no single active constituent is responsible for the overall efficacy. This creates a challenge in establishing quality control standards for raw materials and the standardization of finished formulation (Singh and Kumar, 2015). According to a report in the 44th World Health Assembly, emphasized the great importance of medicinal plants to the health of individuals and communities (Rastogi, 2012; Khare and Katiyar, 2012). Various Ayurvedic herbal and herbo-mineral preparations are used for the treatment of chronic and degenerative diseases with negligible side-effects (WHO, 2001). Standardization assures the quality, efficacy, performance and safety as these formulations are easily available in the market under the claims of being a cosmeceutical and nutraceutical product by various manufacturers (Kamboj, 2012). Chromatographic fingerprints play a vital role in the complex herbal medicines (Gong *et al*., 2005). Since they represent the chemical integrities of the herbal medicines and its products and therefore used for authentication and identification of herbal plants (Liang *et al*., 2004). Strict adherence to standardization procedures needs to be established for every plant medicine in the market because the scope for variation in different batches is enormous (jyotshna and Singh, 2013).

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Different ayurvedic formulations like *Pathadi Kwatha*, *Ashokaristha* etc are reported to treat various female reproductive disorders. *Pushyanuga churna* (AFI-I, 2003; API-II, 2008) is one such popular, non-hormonal Ayurvedic formulation described in Ayurvedic text *Charaka Samhita*. *Pushyanuga churna* consists of twenty five medicinal plants and one mineral drug i.e. *gairika* (red ochre). Ayurvedic Formulary of India prescribes *Pushyanuga churna* for disorders like menorrhagia, leucorrhoea, disorders of female genital tract etc. In India, due to the popularity of Ayurvedic medicine and marked therapeutic effect of *Pushyanuga Churna* in the female reproductive disorders, the formulation is being prepared and marketed by various manufacturers like Dabur, Baidyanath, Arkashala, Dhootpapeshwar and by local Vaidyas etc.

The word '*Pushya'* refers to *Pushya* nakshatra as according to *Acharya Charak*, collection of constituent drugs and the preparation of *Pushyanuga churna* should be done during *pushya nakshatra.* Sharan *et al*., suggests that medicinal plants get special properties during this *nakshatra* which can be utilized for healthy conception and treatment, and would lead to the production of a formulation with maximum potency (Sharan, 2016). Though the reports of experimental use of *Pushyanuga churna* and its ingredients in treating various gynaecological disorders are available (Devi, 2007; Khot *et al*., 2013), basic scientific standardization of the formulation is lacking. There is also a paucity of scientific data to support the assessment of quality, safety and pharmagnostic evaluation of *Pushyanuga churna.* Therefore, in the current research work, an attempt has been made to standardize *Pushyanuga churna*, in terms of its phytochemical, physicochemical parameters, chromatographic fingerprinting analysis and biomarker estimation. The current research data can be used in the standardization of the formulation. Similarly the chromatographic evaluation of the formulation can be used as a simple and rapid identification method for the prevention of adulteration and loss of quality. The data generated hence forms a report which can be used by the manufacturing units of *Pushyanuga Churna*.

MATERIALS AND METHOD

Chemicals and reagents

Chemicals of HPTLC grade were purchased from Merck Specialities Pvt. Ltd, Mumbai. The reference standards ursolic acid (98.5% purity), β-sitosterol (98.0 % purity) and lupeol (97.0% purity) were procured from Sigma Aldrich Chemical Company, Steinheim, Germany. The derivatizing agent i.e. 10% methanolic sulphuric acid was prepared according to the procedure described by Reich and Schibli (Reich and Schibli, 2006).

The following is the list of ingredients present in *Pushyanuga Churna* as per AFI

All the plant materials were collected manually from their respective habitats. Plant materials were authenticated from Agharkar Research Institute, Pune and by Dr. Sunita Shailajan, (HOD, Department of Botany) at, Ramnarain Ruia College, Mumbai. Samples were carefully segregated, cleaned, shade dried for a week and oven dried at 37 °C to constant weight. Further, the samples were powdered using an electric mixer grinder, sieved (BSS 85) and stored in airtight pearlpet bottles at room temperature.

Sr.	Name of plants	Parts used	Collection site
1	*Cissampelos pareira*	Roots	Tungareshwar, Maharashtra
2	*Syzygium cumini*	Endosperm	Boisar, Maharashtra
3	*Mangifera indica*	Endosperm	Boisar, Maharashtra
4	*Bergenia ligulata*	Rhizome	Vishnuprayag, Uttarakhand
5	*Berberis aristata*	Root/stem	Pipalkoti, Uttarakhand
6	*Hibiscus sabdariffa*	Root	Mosam, Maharashtra
7	*Salmalia malabarica*	Exudate	Khopoli, Maharashtra
8	*Mimosa pudica*	Whole plant	Yeoor, Maharashtra
9	*Nelumbo nucifera*	Androecium	Kale, Maharashtra
10	*Crocus sativus*	Style/stigma	Marketed, Badrinath, Uttarakhand
11	*Aconitum heterophylum*	Root	Marketed, Pydhonie, Maharashtra
12	*Cyperus rotundus*	Root tuber	Chena Creek, Maharashtra
13	*Aegle marmelos*	Stem bark	Junagarh, Gujarat
14	*Symplocos racemosa*	Stem bark	Mahabaleshwar, Maharashtra
15	*Red ochre*	-	Marketed, Pydhonie, Maharashtra
16	*Myrica esculenta*	Stem bark	Khirsu Village, Uttarakhand
17	*Piper nigrum*	Fruit	Marketed, Pydhonie, Maharashtra
18	*Zingiber officinale*	Rhizome	Marketed, Pydhonie, Maharashtra
19	*Vitis vinifera*	Dried fruit	Marketed, Pydhonie, Maharashtra
20	*Pterocarpus santalinus*	Heart wood	Marketed, Pydhonie, Maharashtra
21	*Ailanthus excelsa*	Stem bark	Pune, Maharashtra
22	*Holarrhena antidysenterica*	Stem bark	Yeoor Hills, Maharashtra
23	*Hemidesmus indicus*	Root	Yeoor Hills, Maharashtra
24	*Woodfordia fruticosa*	Flower	Ambi Valley, Maharashtra
25	*Glycyrrhiza glabra*	Root	Dang Forest, Gujarat
26	*Terminalia arjuna*	Stem bark	Yeoor Hills, Maharashtra

Preparation of *Pushyanuga churna*

In-house formulation of *Pushyanuga Churna* was prepared as per Ayurvedic Formulary of India. Before powdering the ingredient *Red ochre*, also called *gairika*, was passed through the process of *shodhana* to obtain *shodhita gairika*. *Shodhana* was carried out by giving *Bhavana* by milk. Equal quantities (w/v) of the ingredients were boiled and ground to a soft mass after

cooling. This process was repeated thrice and the mass was completely dried to obtain *shodhita gairaka*. Further, all the ingredients were mixed thoroughly in specified ratio (1 part each) to obtain a homogeneous blend of powders**.**

Marketed samples

Pushyanuga churna was purchased from different manufacturers like Dabur, Baidyanath, Arkashala, Dhootpapeshwar, Patanjali, and also from a local vaidya, for fingerprint analysis and compared with the in-house formulation

Quality Control evaluation

Physicochemical evaluation

The *churna* was subjected to analysis for the estimation of solvent extractive values along with proximate parameters like ash content, acid insoluble and water soluble ash content and loss on drying using standard pharmacopeial methods.

Preparation of Standard and Sample Solutions

Stock solutions of marker compounds (1000 µg/ mL) were prepared by dissolving 10 mg of accurately weighed standards in small amount of methanol by making up the volume to10 mL in a standard volumetric flask. The stock solutions were further diluted for the preparation of working solutions. For the preparation of sample, 10mL ethanol was added to accurately weighed formulation (1.0 g), vortexed and kept standing overnight. Next day, the extracts were filtered using Whatman filter paper no. 1 in dry stoppered test tubes and the filtrate (10 µL) was used for HPTLC analysis.

HPTLC - Instrumentation and operating conditions

Chromatographic separation was achieved on silica gel ^{60}F254 precoated HPTLC plates. Samples were spotted using the CAMAG Linomat 5 sample spotter (CAMAG Muttenz, Switzerland) equipped with syringe (Hamilton, 100µL). For the development of fingerprints, plates were developed in a glass twin trough chamber (CAMAG) pre-saturated for 20 mins with toluene: methanol: formic acid (8:1:0.3, v/v) mobile phase. The plates were derivatized in 10% methanolic sulphuric acid, 1% anisaldehyde and Liebermann Buchard reagent individually. For the simultaneous estimation of the biomarkers, ursolic acid, β-sitosterol and lupeol, a simple toluene: methanol mobile phase (8: 1, v/v) was developed and validated as per ICH guidelines. 10% Methanolic sulphuric acid reagent was used for derivatization. Densitometric scanning was performed using CAMAG TLC Scanner 4 at 366nm and CAMAG Reprostar 3 was used for photo-documentation. Fingerprint plate was also photo-documented at multiple wavelengths before derivatization and after derivatization inorder to visualize the maximum number of phytoconstituents.

Method Validation

The developed HPTLC method for the estimation of ursolic acid, β-sitosterol and lupeol was validated as per ICH guidelines.

Specificity and sensitivity

Specificity of the method was confirmed by comparing the marker bands present in the sample with that of the respective reference standards in terms of its R_f value and color. Sensitivity of the method was determined with respect to limit of detection (LOD, S/N ratio of 3:1) and limit of quantification (LOQ, S/N ratio of 10:1).

Preparation of calibration curve and quality control samples

For constructing the calibration curve, appropriate dilutions were prepared from the stock solutions. The working standards in the range of 5-150µg mL^{-1}, 15-35µg mL^{-1}, 45-105 µg mL^{-1} for ursolic acid, β-sitosterol and lupeol, respectively, were used to obtain a seven point linear calibration curve. Further, quality control samples (LQC, MQC and HQC) were prepared and analyzed for precision, accuracy and ruggedness studies.

Repeatability and precision

The repeatability of the method was affirmed by analyzing 5 µg/ mL samples of all the three markers on a HPTLC plate (n = 5) and precision were assessed by measurement of intra and inter-day variation in the area of the markers. The result was expressed as % RSD.

Accuracy and ruggedness

The accuracy of the method was assessed by spiking the QC samples in the formulation and calculating the percent recovery for each marker. Ruggedness was assessed by deliberately incorporating small variations in the optimized chromatographic conditions. Response and Rf were observed. Results were expressed in terms of percent mean deviation.

Assay

The content of all the three markers from the in-house formulation was determined by applying the samples (10 µL) along with pure standards.

Estimation of the markers

The quantity of the markers was calculated using the regression equation obtained from the regression analysis of the calibration curve.

Statistical Analysis

The statistical analysis of the results obtained was done using Microsoft Excel 2007.

RESULT AND DISCUSSION

Quality assurance is an integral part of medicine which in-turn ensures quality medication. Thus, there is a urgent need for the evaluation of such parameters which can be adopted by pharmaceutical industries (Shailajan *et al.*, 2011a). There are similar reports on the standardization of some popular Ayurvedic medicines (Shailajan *et al*., 2011b; Rashmibala and Kumar, 2011; Shailajan *et al*., 2010; Parameshwaran and Nandan; 2010)

Table 1: Proximate analysis the inhouse formulations.

Parameters	Results (N=3)	Limits –NMT%*
Total ash	12.57 ±0.25	NMT 15%
Acid insoluble	1.26 ±1.32	NMT 4%
Water soluble	9.42 ±1.68	NMT 13%
Loss on drying	5.75 ±0.92	NMT 11%

* NMT: Not More Than; Limits have been suggested.

Table 2: Detection of Preliminary Phytoconstituents.

Phytoconstituents	Aqueous extract	Ethanolic extract
Flavonoid	Present	Present
Tannin	Present	Present
Alkaloid	Present	Present
Glycosides	Absent	Absent
Oil	Absent	Absent
Resin	Present	Present

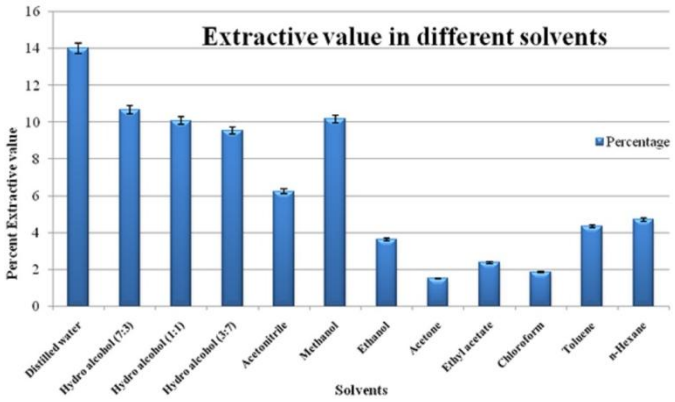

Fig. 1: Extractive values of Pushyanuga Churna in solvents of varying polarities.

Physicochemical analysis

The results for proximate parameters, preliminary phytochemical analysis and extractive values in various solvents have been summarized in the Tables 1, 2 and Figure1 respectively. The results obtained for total ash, acid insoluble and water soluble ash, along with loss on drying were found to be within the permissible limits for the formulation as prescribed by the Ayurvedic formulary of India. Among the phytoconstituents analysed, glycosides and oil was found to be absent whereas flavonoids, tannins, alkaloids and resins were found to be present (Table 2). Percent extractive value was found to be highest in distilled water, followed by hydro-alcohol (7:3) in the category of organic solvents. Hence it can be concluded that, *Pushyanuga churna* contains more polar components. But comparatively better values were also observed in non -polar solvents like n-hexane suggesting good quantities of non-polar components too (Fig. 1).

Method development

Ursolic acid, β-sitosterol, lupeol are phytomarkers which have been reported for numerous therapeutic activities including in the treatment of female reproductive disorders but the presence of these markers has not yet been established from *Pushyanuga churna.*

Hence, in the current research work, these markers have been quantitated using a simultaneous HPTLC method which can be used for the standardization of *Pushyanuga churna*. Optimized chromatographic conditions in order to obtain good separation amongst three triterpenoids viz. ursolic acid, β-sitosterol and lupeol are summarized in table 3.

Table 3: Optimized chromatographic conditions for quantitation of Ursolic acid, betasitosterol and Lupeol.

Parameters	Specifications
Stationary Phase	Merck silica gel 60 F254 HPTLC pre-coated plates
Sample Applicator	CamagLinomat 5
Development distance	85 mm
Derivatization	10% Methanolic sulphuric acid reagent
Densitometric scanner	Camag scanner 4
Software	winCATS planar chromatography manager software version 1.4.7
Lamp, wavelength	Mercury, 366 nm
Photo documentation	Camag Reprostar 3

Fig. 2: Plate Photo at 366 nm post derivatization (Post Fig) Track details Track 1: In-house formulation Track 2: Ursolic acid, β-sitosterol, Lupeol Track 3: Marketed formulation.

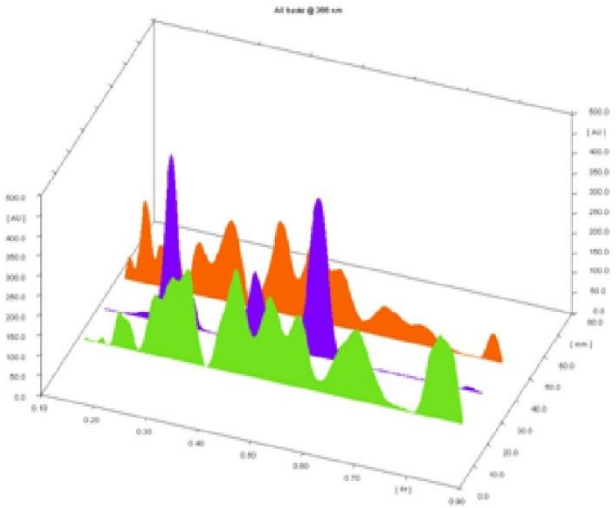

Fig. 3: 3D Overlay of plate at 366 nm (post Fig:) Track details: Track 1: In-house formulation Track 2: Ursolic acid, β-sitosterol, Lupeol Track 3: Marketed formulation.

Various solvent systems were tried, out of which toluene: methanol (8:1, v/v) showed good resolution. The method developed was validated as per ICH guidelines. Visualization of spot of these markers directly under UV or visible radiation is not possible as none of them have chromophoric groups.

Therefore, developed plate was derivatized in 10% methanolic sulphuric acid reagent. The plate was air dried and kept in oven for 5-7 min at 110 °C and densitometrically scanned at 366nm (Fig. 3).

Method validation

The described method has been extensively validated in terms of specificity, linearity, repeatability, precision, accuracy, recovery and ruggedness. The results of the method validation have been summarized in table 4. Ruggedness was assessed by deliberately incorporating small variations like change in analyst, plate batch, spotting volume and mobile phase in the optimized chromatographic conditions. The % CV obtained during precision and ruggedness studies were found to be < 2% which suggests that the method is rugged and reproducible. With recovery values found to be above 98% in all cases, the method can be regarded as selective, accurate, precise, and robust and it has a wide scope in the area of natural product separation, characterization, drug development, and their quality control/standardization.

Table 4: Results of method validation.

Parameters	Ursolic acid	β-sitosterol	Lupeol
R_f	0.31	0.49	0.60
LOD and LOQ (µg L^{-1})	1 and 5	5 and 15	15 and 45
Linear Range (µg mL^{-1})	5-150	15-35	45-105
System Suitability (% CV)	1.8243	1.6344	1.5781
Intraday Precision (% CV)	1.7021	0.50	0.89
Interday Precision (% CV)	1.99	0.93	1.15
Recovery	98.80%	99.32%	98.62%
Regression equation	Y= 19.75x+48.4	Y= 65.74x+91.0	Y= 21.75x+211.4

Detection and quantitation

The method was further applied in the simultaneous detection and quantitation of the biomarkers from the ethanolic extract of *Pushyanuga churna* prepared in-house and a marketed formulation. The content of ursolic acid, β- sitosterol and lupeol was found to be 1.37 ± 0.108, 0.73 ± 0.025 and 1.24 ± 0.136 mg/g from the in-house formulation respectively. In the marketed formulation, the content was found to be 1.57 ± 0.027, 0.58 ± 0.024 and 0.88 ± 0.089 mg/g respectively (Table 5).

Table 5: Content of Ursolic acid, β-sitosterol, Lupeol in the formulation.

	Ursolic acid	β-sitosterol	Lupeol
	Concentration (mg/g)Mean ± SD, n=3		
In-house formulation	1.37± 0.108	**0.73± 0.025**	**1.24± 0.136**
Marketed	**1.57± 0.027**	0.58± 0.024	0.88± 0.089

Fingerprint of Pushyanuga churna prepared in-house and compared with different manufacturers

A chromatographic fingerprint represents the phytochemical integrity of the sample thus providing it an identity. Using these fingerprints, the authentication and identification of Ayurvedic formulations can be accurately conducted even if the concentration varies (Kulkarni *et al*., 2014). Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug.

In the current research work a chromatographic fingerprint of an Ayurvedic formulation *pushyanuga churna* was established and compared to different manufacturers available in the market. Pre-derivatized plate showed less number of bands compared to post derivatization in different derivatizing reagents at different wavelength. Variations in fingerprint pattern may be due to the substitutes added to the formulation during preparation and also due to non-availability of the authentic ingredients. The variation can also be the result of variation in the quality of ingredients used and the varying collection conditions. Table 6 comprises the optimized chromatographic conditions for fingerprint.

Table 6: Optimized chromatographic conditions for fingerprint analysis of *Pushyanuga Churna*

Parameters	Specifications
Stationary Phase	Merck silica gel 60 F254 HPTLC pre-coated plates
Sample Applicator	Camag Linomat 5
Development distance	85 mm
Derivatization	10% Methanolic sulphuric acid reagent, 1% anisaldehyde, Liebermann buchard reagent
Densitometric scanner	Camag scanner 4
Software	winCATS planar chromatography manager software version 1.4.7
Lamp, wavelength	Mercury, 366 nm
Photo documentation	Camag Reprostar 3

Figure 4 (a, b, c, d) represent the photographic documentation of fingerprint patterns observed in various conditions. The presence of variation in bands can be traced back to the ingredients and used as a quality control tool by various manufacturing units of *Pushyanuga churna*. Track 1.i,e the formulation prepared in-house does show the presence of some bands which were found to be absent in many of the manufactured formulations. Among the marketed formulations, the formulation purchased from the local vaidya did show similar fingerprints. Among the manufacturer, formulation by Dhootpapeshwar followed by Baidyanath showed similarities to in-house formulation.

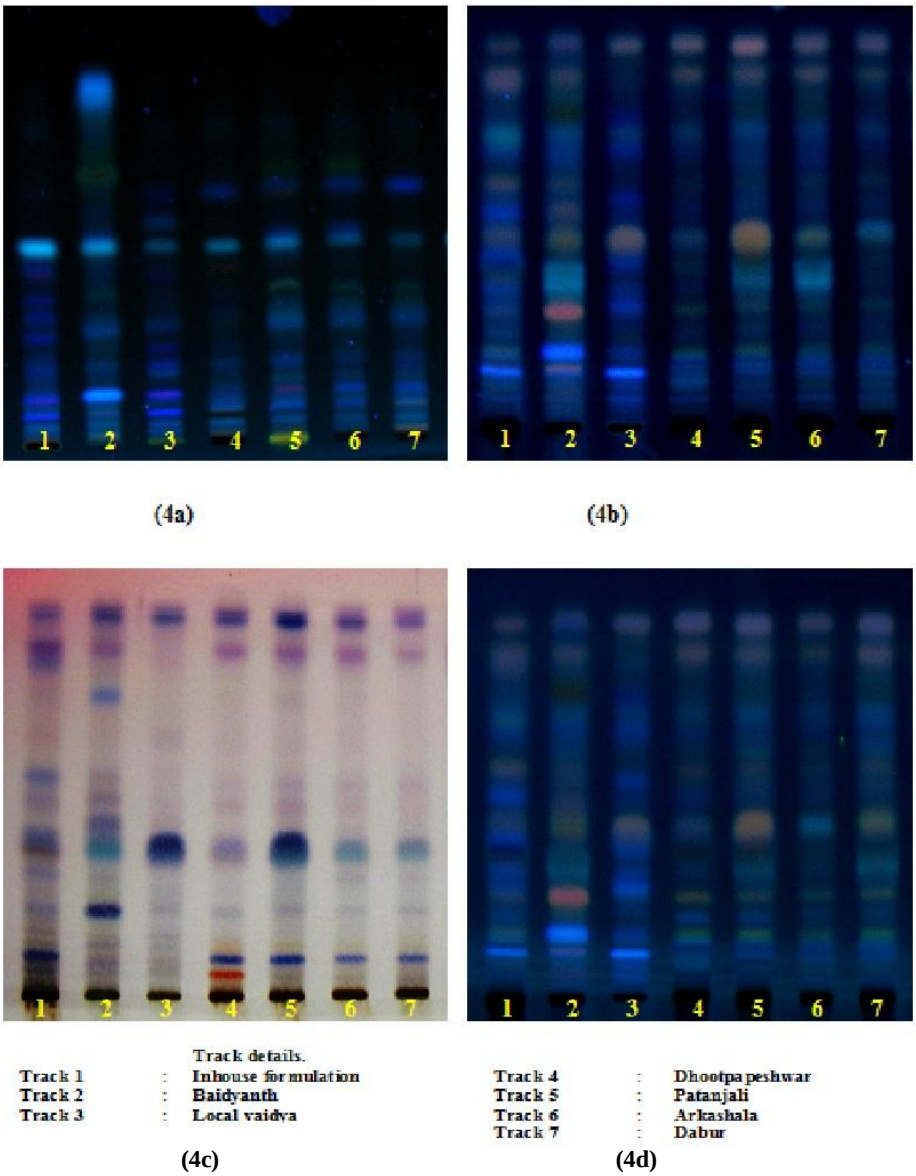

Fig. 4: Photo of plate after derivatization with various agents (a) Pre-derivatized plate 366 nm (b) 10% methanolic sulphuric acid at 366 nm (c) 1% Anisaldehyde acid at 550 nm (d) Liebermann buchard reagent at 366 nm.

CONCLUSION

Chemo profiling-assisted characterization of the polyherbal formulation *Pushyanuga churna* using HPTLC was generated for the first time to confirm the total number of chemical moieties which will help in identification of bioactive compounds. The markers ursolic acid, betasitosterol and lupeol quantitated in the current research are documented and reported to be associated with various therapeutic activities. The quantity of marker compounds can be used as a complementary approach for the quality control and stability assessment of *Pushyanuga churna.*

Any deviation from this fingerprint suggests that pharmacopoeial standards have been defaulted. Hence, the results from the current study will allow manufacturers to offer greater batch-to-batch consistency for reproducible clinical efficacy.

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