Research Article

Chromatographic assessment of a marketed ayurvedic formulation *Pushyanuga churna*: A modern approach

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ABSTRACT

Aim: *Pushyanuga churna* (PC) is an Ayurvedic formulation composed of twenty-five plant ingredients and one mineral described in AFI for its use in various female reproductive disorders. Owing to its therapeutic efficacy, it is prepared and marketed by different manufacturers. But, as there is paucity of scientific data on its standardization and quality control parameters which may lead to undesired quality and variation in its consistency, standardization of this formulation using modern bioanalytical techniques is required. **Material and Methods:** *Pushyanuga churna* (PC) was purchased from the market, manufactured by different companies subjected to quality control parameters. HPTLC-fingerprint for different marketed of PC was developed. Further, a simple, rapid, accurate and sensitive HPTLC method was developed for the estimation of two therapeutically potent biomarkers viz. gallic acid and bergenin simultaneously using a toluene: ethyl acetate: methanol: formic acid as mobile phase. The developed method was validated as per ICH guidelines. **Results and Discussion:** Preliminary phytochemical, physicochemical analysis and chromatographic fingerprint for different manufacturers of *Pushyanuga churna* were established. It was observed that all the marketed samples did not show uniformity in results. Quantitation of two bioactive markers were evaluated and the maximum content of gallic acid and bergenin were found in marketed *Pushyanuga churna* 2 (2.346 ± 0.026 mg/g) and marketed *Pushyanuga churna* 4 (2.283 ± 0.175 mg/g) respectively. **Conclusion:** The data obtained from scientific evaluation of PC can be adapted to lay down new pharmacopoeial standards for batch-to-batch consistency.

KEY WORDS: Bergenin, Gallic acid, High-performance thin-layer chromatography, *Pushyanuga churna*

INTRODUCTION

Ayurvedic medicines have gained a lot of popularity worldwide due to its curative properties and minimal side effects.[1] This is due to active constituents present in the formulation and is used as phytopharmaceutical agents.[2] Ayurvedic formulations are combinations of more than one herb that work synergistically to achieve greater therapeutic efficacy.[3] Hence, standardization of herbal formulation is essential to assure its safety, efficacy, and concentration of chemical constituents for their biopotency.[4]

Different ayurvedic formulations have been reported to treat various female reproductive disorders such as *Pathadi Kwatha* and *Ashokaristha*. *Pushyanuga churna* (PC) is one such ayurvedic polyherbal formulation composed of 25 plant ingredients and one mineral described in Ayurvedic Formulary of India.[5] Ayurvedic texts prescribe it for various female reproductive disorders such as Ashwagandha (menorrhagia), Shweta pradara (leukorrhea), Rajodosa (menstrual disorders), Arsa (piles), and Yonidosa (disorders of female genital tract).[5] Due to its clinical efficiency, PC is being prepared and marketed by different manufacturers such as Dabur, Baidyanath, Arkashala, Dhootpapeshwar, Patanjali, and Kottakkal.[6]

As the formulation contains 25 plant ingredients, collection of authentic plant parts becomes difficult, also pre- and post-harvesting conditions, manufacturing process may affect its quality and this, in turn, may curtail its therapeutic potency.

Therefore, in the current research work, marketed formulations of *Pushyanuga churna* were subjected to quality evaluation in terms of physicochemical parameters, phytochemical evaluation, and chromatographic characterization in terms of its marker content. Polyherbal formulation PC may

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contain many secondary active metabolites such as ursolic acid, β-sitosterol, and lupeol[6] which are responsible for its therapeutic efficiency. Bergenin and gallic acid which are reported to possess various pharmacological activities have been quantitated from PC. The research data may provide substantial information to the manufacturers and help in scientific evaluation of the traditional formulation PC.

MATERIALS AND METHODS

Marketed Samples

Marketed samples of PC were purchased from local medical shops of six brands coded as Marketed PC-1, Marketed PC-2, Marketed PC-3, Marketed PC-4, Marketed PC-5, and Marketed PC-6.

Chemicals and Reagents

Chemicals of HPLC grade were purchased from Merck Specialties Pvt., Ltd., Mumbai. Reference standards, gallic acid (≥98% purity) was procured from Sigma-Aldrich, Steinheim, Germany, and bergenin (≥97.0% purity) was procured from Chengdu Biopurify Phytochemicals Ltd., China. 10% methanolic sulfuric acid as derivatizing reagent was prepared according to Reich and Schibli.[7]

Evaluation of Quality Control Parameters

Organoleptic evaluation

The organoleptic characters of the marketed formulations were carried out based on the method described by Wallis.[8] Organoleptic evaluation refers to the evaluation of the formulation by color, odor, taste, texture, etc. For determining the odor of an innocuous material, small portion of the sample was placed in the beaker of suitable size, examined by slow and repeated inhalation of the air over the material. If no distinct odor was perceptible, the sample was crushed between the thumb and index finger, using gentle pressure.[9]

Physicochemical evaluation

Physicochemical evaluation of marketed formulations were carried out using parameters such as pH, loss on drying, total ash, acid-insoluble and water-soluble ash content, and alcohol- and water-soluble extractive content using standard pharmacopeial method.[10]

Preliminary phytochemical screening

Ethanolic extract of marketed formulations of PC was subjected to preliminary phytochemical screening for evaluation of some major phytoconstituents using reported method.[11]

Determination of physical characteristics

Samples were subjected to physical characteristic parameters such as bulk density, tap density, Hausner ratio, and Carr's index as per the reported methods[12,13] and acceptance limits [Table 1] were taken as per USP.[14]

Bulk density

It is the ratio of given mass of powder and its bulk volume. Marketed formulations of PC were added to a cylinder with the aid of a funnel for any loss. The initial volume was noted.

Bulk density = W/V_0 g/mL

Where, W = Mass of the powder and V_0 = Untapped volume

Tapped density

The initial volume gave the value of bulk density, and the sample was then tapped until no further reduction in volume was noted giving the value of tapped density, respectively.[9,15]

Tapped volume = W/V_f g/mL,

Where, W = Mass of the powder and V_f = Tapped volume.

Hausner's ratio

It indicates the flow properties of the powder. The ratio of tap density to the bulk density of the powder is called Hausner ratio.

Hausner's ratio = Tapped density/bulk density

Carr's index (percentage compressibility)

It is the propensity of the powder to be compressed. The percentage compressibility of a powder is a direct measure of the potential powder arch or bridge strength and stability, and is calculated according to the following equation;

Table 1: Physical characteristics USP limits[14]

Flow property	Compressibility index (%)	Angle of response (degree)	Hausner's ratio
Excellent	≤10	25–30	1.00–1.11
Good	11–15	31–35	1.12–1.18
Fair aid not added	16–20	36–40	1.19–1.25
Passable may hang up	21–25	41–45	1.26–1.34
Poor must agitate, vibrate	26–31	46–55	1.35–1.45
Very poor	32–37	56–65	1.46–1.59
Very, very poor	>38	>66	>1.60

% compressibility = [(Tapped density – bulk density)]/ tapped density] ×100

Angle of response

Angle of response is a characteristic related to interparticulate friction or resistance to movement between particles. The fixed funnel method employs a funnel that is secured with its tip at a given height, which was taken 2.0 cm (H), above the graph paper that is placed on flat horizontal surface. Powder was carefully poured through the funnel until the apex of the conical pile just touched the tip of the funnel and is calculated according to the following equation;

$\tan(\alpha) = \text{height}/0.5 \text{ base}$.

High-performance Thin-layer Chromatography (HPTLC) - Optimized Conditions

Optimization of extraction technique from different marketed formulations of PC

Extraction of phytoconstituents from different marketed formulations of PC was optimized to achieve good fingerprint and also to resolve the marker compounds efficiently. To the accurately weighed 1 g of each marketed formulation, hydroalcohol was added in the ratio of 2:8 v/v, vortexed for 5 min, and kept standing overnight. Next day, it was filtered through Whatman filter paper No. 1 and the filtrate (10 µL) was then used for HPTLC analysis.

Table 2: Optimized chromatographic conditions for fingerprint analysis of PC

Parameters	Specifications
Stationary phase	Merck silica gel $60F_{254}$ HPTLC precoated plates
Sample applicator	Camag Linomat 5
Development distance	85 mm
Derivatization	10% methanolic sulfuric acid reagent
Densitometric scanner	Camag scanner 4 software winCATS planar chromatography manager software version 1.4.7 Lamp
Wavelength	366 nm, 254 nm
Photodocumentation	Camag Reprostar 3

HPTLC: High-performance thin-layer chromatography, PC: *Pushyanuga churna*

HPTLC fingerprint

Chromatographic separation was achieved on silica gel $60F_{254}$ precoated HPTLC plates. Samples were spotted using the CAMAG Linomat 5 sample spotter (CAMAG, Muttenz, Switzerland) equipped with syringe (Hamilton, 100 µL). For the development of fingerprints, plate was developed in a glass twin trough chamber (CAMAG) pre-saturated for 20 min with toluene:ethyl acetate:formic acid (8:2:1 v/v) as mobile phase. The plates were derivatized in 10% methanolic sulfuric acid. Densitometric scanning was performed using CAMAG TLC Scanner 4 at 254 nm and CAMAG Reprostar 3 was used for photodocumentation. Fingerprint plate was photodocumented before and after derivatization at 254 nm and 366 nm to visualize maximum number of phytoconstituents [Table 2].

Chromatographic evaluation of phytochemical markers

For simultaneous estimation of the biomarkers gallic acid and bergenin from marketed formulations of PC, a validated method as per ICH guidelines was used.[16] Toluene:ethyl acetate:methanol:formic acid (6:6:2:1 v/v) was used as mobile phase to resolve and quantitate the marker compounds from hydroalcoholic extract of different marketed formulations of PC [Table 3].

Statistical Analysis

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

RESULTS

Quality evaluation of herbal formulations is imperative to justify their acceptability in modern system of medicine.[17]

Organoleptic evaluation of the marketed formulations of PC showed slight variation in its color and texture. Results have been summarized in Table 4.

All marketed formulations of PC were subjected to physicochemical evaluation and preliminary phytochemicals screening, and results were notified in Tables 5 and 6, respectively.

The Carr's index of all the marketed formulations of PC were found to be in the range of 18–35, suggesting

Table 3: Optimized chromatographic conditions for quantitation of biomarkers from marketed formulations of PC

Parameters	Specifications
Stationary Phase	Merck silica gel $60F_{254}$HPTLC precoated plates
Sample Applicator	Camag Linomat 5
Development distance	85 mm
Densitometric scanner	Camag scanner 4 software win CATS planar chromatography manager software version 1.4.7 Lamp
Wavelength	254 nm
Photodocumentation	Camag Reprostar 3

HPTLC: High-performance thin-layer chromatography, PC: *Pushyanuga churna*

Table 4: Organoleptic evaluation of different marketed formulations of PC

Parameters	Marketed PC1	Marketed PC2	Marketed PC3	Marketed PC4	Marketed PC5	Marketed PC6
Appearance	Powder	Powder	Powder	Powder	Powder	Powder
Color	Light brown	brown	Dark brown	Dark brown	Reddish-brown	Light brown
Odor	Musty	Musty	Musty	Characteristic	Musty	Aromatic
Taste	Bitter	Bitter	Bitter	Slightly bitter	Bitter	Bitter
Texture	Fine powder	Moderately fine	Moderately fine	Fine powder	Fine powder	Fine powder

PC: *Pushyanuga churna*

Table 5: Physicochemical evaluation of different marketed formulations of PC

Parameters	Marketed PC1	Marketed PC2	Marketed PC3	Marketed PC4	Marketed PC5	Marketed PC6
pH (5–6)	5.3±0.21	4.9±0.22	4.7±0.25	5.1±0.23	4.8±0.21	4.8±0.27
LOD (NMT 11%)	6.68±0.38	6.75±0.54	7.62±0.385	6.20±0.24	8.51±0.16	3.04±0.21
Total Ash (NMT 15%)	13.70±0.16	12.33±0.74	11.65±0.14	16.26±0.34	12.63±0.36	16.52±0.22
Acid-insoluble ash (NMT 4%)	1.32±0.12	2.59±0.42	1.44±0.21	3.64±0.19	2.54±0.35	6.27±0.28
Water-soluble Ash	4.39±0.18	2.94±0.38	8.07±0.48	6.59±0.59	8.93±0.74	4.63±0.25
Alcohol-soluble extractive (NLT 12%)	13.72±0.61	13.47±0.58	11.45±0.84	9.13±0.54	12.20±0.15	9.70±0.58
Water-soluble extractive (NLT 13%)	65.76±0.52	67.98±0.23	66.57±0.52	59.79±0.58	65.23±0.25	48.19±0.25

PC: *Pushyanuga churna*

Table 6: Preliminary phytochemical screening of different marketed formulations of PC

Phytochemical constituents	Test performed with	Inference					
		Marketed PC1	Marketed PC2	Marketed PC3	Marketed PC4	Marketed PC5	Marketed PC6
Flavonoids	$(CH3COO)_2$ Pb solution	Present	Present	Present	Absent	Present	Present
	Increasing amount of NaOH solution	Absent	Absent	Absent	Present	Absent	Absent
Phenolic compounds	$K2Cr_2O_7$ solution	Present	Present	Present	Present	Present	Present
	$KMnO_4$ solution	Present	Present	Present	Present	Present	Present
Alkaloids	Wagner's reagent	Present	Present	Present	Absent	Present	Present
	Mayer's reagent	Present	Present	Present	Absent	Present	Present
Saponins	Water with vigorous shaking	Present	Present	Present	Present	Present	Present
Terpenoids	Chloroform and carefully addition of concentrated H_2SO_4	Present	Present	Present	Present	Present	Present
Glycosides	Water and NaOH	Present	Present	Present	Present	Present	Present

PC: *Pushyanuga churna*

Table 7: Physical characteristics of different marketed formulations of PC

Parameters	Marketed PC1	Marketed PC2	Marketed PC3	Marketed PC4	Marketed PC5	Marketed PC6
Bulk density (g/ml)	0.357±0.003	0.455±0.012	0.400±0.009	0.455±0.010	0.408±0.015	3.336±0.200
Tap density (g/ml)	0.556±0.029	0.556±0.066	0.553±0.033	0.553±0.018	0.537±0.075	5.004±0.003
Hausner ratio	1.556±0.018	1.222±0.017	1.389±0.011	1.222±0.024	1.316±0.025	1.500±0.002
Carr's index	35.714±0.049	18.182±0.016	28.000±0.027	18.182±0.012	24.00±0.028	33.333±0.185
Angle of response (degrees)	41.47±0.021	34.95±0.024	32.37±0.034	41.60±0.042	41.12±0.035	41.12±0.021

PC: *Pushyanuga churna*

the poor flowability which was further confirmed by high values of Hausner ratio summarized in Table 7.

The chromatographic fingerprint was established for different marketed formulations of PC [Figure 1]. Further, a simple, rapid, accurate, and sensitive HPTLC method was developed for the estimation of two therapeutically potent biomarkers, namely gallic acid and bergenin, simultaneously using a toluene:ethyl acetate:methanol:formic acid as mobile phase [Figures 2 and 3].

The maximum content of gallic acid and bergenin was found in marketed PC2 (2.346 ± 0.026 mg/g) and marketed PC4 (2.283 ± 0.175 mg/g), respectively [Table 8].

DISCUSSION

Physicochemical Evaluation

Physicochemical parameters of all marketed formulations of PC showed loss on drying within the acceptance limits [Table 5].

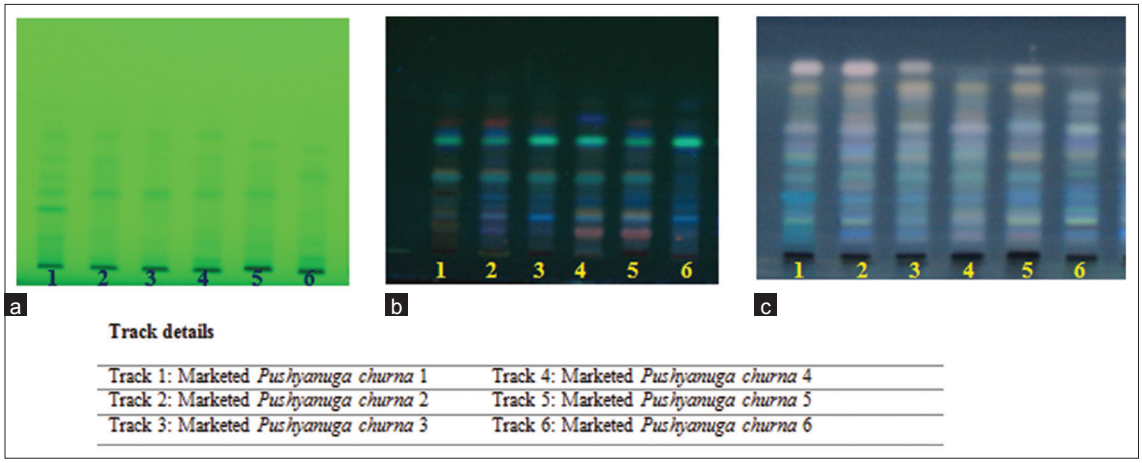

Figure 1: High-performance thin-layer chromatography - fingerprint plate photo. (a) Plate before derivatization at 254 nm (b) Plate before derivatization at 366 nm (c) Plate after derivatization at 366 nm

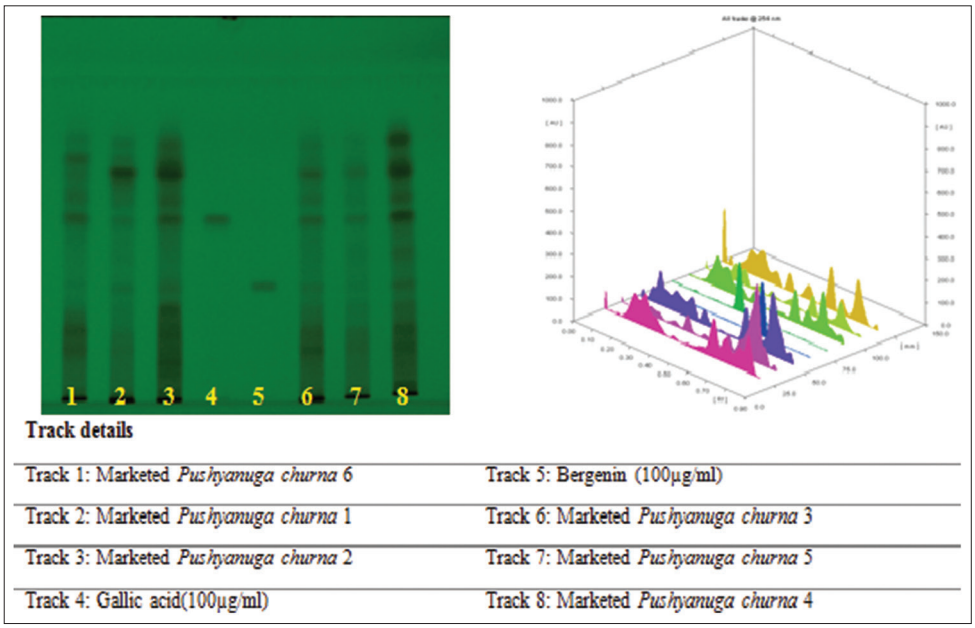

Figure 2: High-performance thin-layer chromatography (HPTLC) quantitation at 254 nm of different marketed formulations of *Pushyanuga churna*. (a) HPTLC plate photo at 254 nm (b) overlay of the chromatograms

Higher values of moisture content in marketed PC 3 and marketed PC 5 samples showed susceptibility for bacterial, fungal, or yeast growth as compared to marketed PC1, marketed PC2, marketed PC4, and marketed PC6 sample.[15]

Total ash content of marketed formulations were found to be within acceptance limit except marketed PC4 and marketed PC6 formulations. Higher content of total ash in marketed PC4 and marketed PC6 indicates the presence of more amount of foreign inorganic matter. These inorganic contents can be considered as "impurities" which may be due to careless collection, contamination, substitution, or adulteration.[9]

Higher water extractive value of all marketed formulation reveals the presence of more amount of polar compounds.[6] Less alcohol extractive values of marketed PC3, marketed PC4, and marketed PC6 indicate improper post harvesting conditions and addition of exhausted material.

Preliminary Phytochemicals Screening

Preliminary phytochemicals screening showed the presence of flavonoids, phenolic compounds alkaloids, saponins, terpenoids, and glycosides in marketed formulations of PC which are considered to be responsible for its therapeutic activity.

Physical Characteristics

The results of physical properties [Table 7] of marketed formulations showed broad range of variation which may be due to different and improper preparation, grinding method, storage, and packing of finished product.[14,18] The variation in particle properties of marketed formulation revealed that

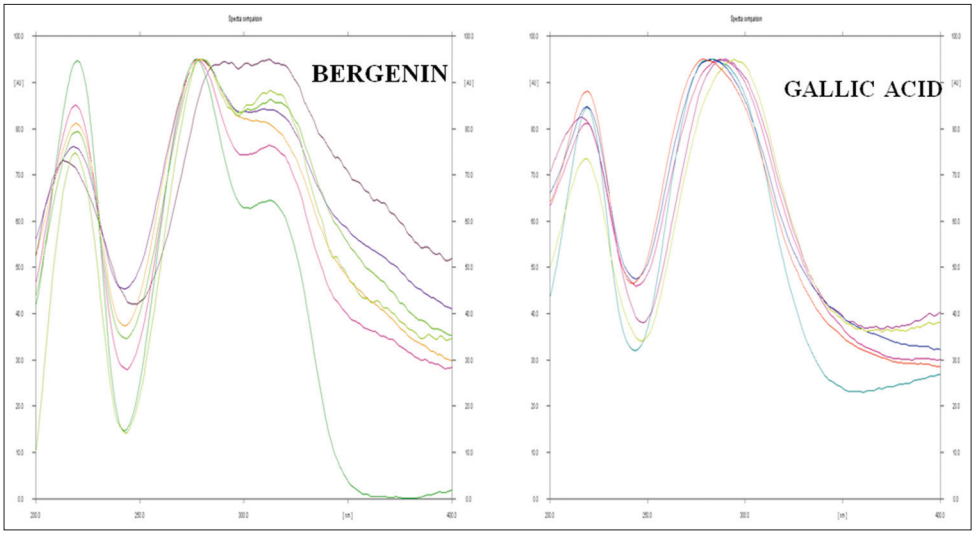

Figure 3: Spectral analysis of marketed formulations of *Pushyanuga churna* for bergenin and gallic acid at 254 nm

Table 8: Content of bergenin and gallic acid in the marketed formulations of PC

Sample	Gallic acid	Bergenin
	Concentration (mg/g) mean±SD, n=3	
Marketed PC1	0.537±0.108	0.801±0.109
Marketed PC2	2.346±0.026	1.950±0.150
Marketed PC3	0.945±0.017	0.922±0.028
Marketed PC4	2.078±0.028	2.283±0.175
Marketed PC5	0.417±0.019	0.016±0.027
Marketed PC6	1.470±0.025	0.114±0.179

PC: *Pushyanuga churna*

marketed PC 2, marketed PC 3, marketed PC 4 and marketed PC 5 have better flow properties and high solubility. Therefore, ease for administration and absorption of churna through oral route will be better in these marketed formulations.

Chromatographic Fingerprint

Chromatographic fingerprint of hydroalcoholic extracts of the marketed formulations showed variation in fingerprint profile [Figure 1]. The observed variation might be due to the variation in the quality of ingredients or unavailability of the authentic ingredients. This variation can also be attributed to differences in their geographical distribution, different vernacular names of plant ingredients, lack of knowledge about authentic source and authentic plant, and improper post-processing methods utilized during the preparation of the formulation. These fingerprints can be used to prevent ingredient-based adulterations which might play a key role in the therapeutics of the formulation.

Gallic Acid and Bergenin

For simultaneous estimation of gallic acid and bergenin from marketed formulations of PC, a validated method reported by our group was employed.[16] Chromatographic evaluation using mobile phase toluene:ethyl acetate:methanol:formic acid gave the best resolution of bergenin and gallic acid from the other components of the hydroalcoholic extract of marketed formulations of PC.

Therapeutically important biomarkers gallic acid and bergenin have been reported to possess various pharmacological activities. Gallic acid possesses cytotoxicity against cancer cells, antioxidant, anti-inflammatory, hepatoprotective, neuroprotective, analgesic activity,[6,19] etc.

Bergenin is reported to possess biological activities such as antiulcerogenic, anti-HIV, antifungal, hepatoprotective, antiarrhythmic, neuroprotective, anti-inflammatory, immunomodulatory, and burn wound healing properties.[20] Furthermore, ursolic acid, β-sitosterol, and lupeol have been reported from PC.[6] Based on the concentration of bioactive markers, formulations can be selected having maximum content supporting its efficacy.

The variation in the marker content among the marketed formulations may be due to variation in climatic conditions of different geographical regions in India. It can be due to collection of plant material in different seasons, for example, collection of *Myrica nagi* bark in summer and winter. Furthermore, it may be due to the substitution of some plant material with related species or totally different plant with the same therapeutic value.

For example, in marketed PC 1, *Bergenia ligulata* has been substituted with *Bergenia ciliata*, similarly in marketed formulation 6, *Cissampelos pareira* has been substituted with *Cyclea peltata. Mimosa pudica,* one of the most important ingredients of PC,

is reported to be effective in the treatment of heavy menstrual blood loss (menorrhagia), leukorrhea, and dysfunctional uterine bleeding[21] which is absent in marketed PC 3 and 4 attributing toward the marker variation in formulations.

As there are 25 plant ingredients in PC, each plant has its own bioactive markers which are acting synergistically and giving therapeutic value to PC. All these bioactive markers should be evaluated for their activity against female reproductive disorders.

Therefore, we can conclude that the formulation should be evaluated and authenticated in terms of marker compounds present in the formulation, as these marker compounds are responsible for therapeutic efficacy of the formulation, and high therapeutic efficacy is the ultimate goal of consuming a formulation.

CONCLUSION

This method can be designed as a standard protocol to assure the quality of marketed ayurvedic formulation of PC. Industries manufacturing PC can use marker-based standardization to assure quality and therapeutic value of the formulation with reference to the content of phytochemical marker. These standardized parameters can be followed during different stages of preparation and processing of PC which would increase global acceptance of ayurvedic formulations.

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