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Original Article

Chromatography Assisted Validation of Alternate Plant Materials in a Polyherbal Formulation: A Prospective Positive Substitution

Sunita Shailajan[1,*], Swati Singh[1], Sasikumar Menon[2]
[1]Herbal Research Lab (Industrial Co-ordination Centre), Ramnarain Ruia Autonomous College, Matunga, Mumbai, Maharashtra, INDIA.
[2]Therapeutic Drug Monitoring Laboratory, Sion (East), Mumbai, Maharashtra, INDIA.

ABSTRACT

Introduction: *Bergenia ligulata* Wall. is a popular medicinal herb from family Saxifragaceae, belonging to category of medicinal plants, the authentic species of which are not available easily. Though Ayurvedic Formulary of India prescribes the use of *Bergenia ligulata*, manufacturers have been using different species of *Bergenia* in many traditional Ayurvedic formulations including *Pushyanuga churna* (*PC*). Commercial exploitation of *Bergenia ligulata* has resulted in imbalance between supplies of this plant drug as compared to its demand. Commercially therefore, *Bergenia ciliata* which is more abundantly available, is substituted for *Bergenia ligulata*. **Methods:** The present study was undertaken to validate the use of *Bergenia ciliata* in a polyherbal formulation (PC), as a positive substitute for *Bergenia ligulata*. One in-house PC containing *B. ligulata*, along with two commercially available formulations of PC containing different species of *Bergenia* were compared for their bergenin content using validated HPLC-method to evaluate the phytochemical equivalence. **Results:** Bergenin content in the in-house formulation containing *B. ligulata* was found to be 0.27mg/gm whereas the commercial samples containing *B. ciliata* contained 0.24mg/gm of bergenin while another marketed formulation without label claim contained 0.162mg/gm of bergenin. The results clearly support the use of *B. ciliata* as a positive substitute for *B. ligulata* in PC. **Conclusion:** Positive substitution can be adopted by manufacturing units for the medicinal plant ingredients, which are scarcely available, to prevent their over-exploitation from the wild. Such substitutions can be justified if phytochemical equivalence could be demonstrated by validated chromatographic techniques. Nevertheless, evidence based efficacy studies need to be conducted additionally to corroborate such usage.

Key words: *Bergenia ligulata*, *Pushyanuga churna*, Bergenin, Disputed drugs, Substitution, HPLC.

Correspondence:
Prof. (Dr.) Sunita Shailajan
Herbal Research Lab (Industrial Co-ordination Centre), Ramnarain Ruia Autonomous College, Matunga, Mumbai-400 019, Maharashtra, INDIA.
Phone no: +91 9821863676
E-mail: sunitashailajan@gmail.com
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INTRODUCTION

An Ayurvedic polyherbal formulation contains myriad of diverse phytochemicals. In the complex matrices of phytomedicines, no single phytoconstituent individually is responsible for the overall efficacy.[1] As stated by the ancient seer Acharaya Charaka, in plant derived drugs there may be one phytoconstituent which may act as "main" and then there are other 'additional' phytoconstituents.[2] Together, the additive or synergistic effect of these phytoconstituents elicits the multidimensional therapeutic action of polyherbal formulations in healing the disease state.

Variability in biochemical composition of phytoconstituents, coupled with the variation in collection procedures, variations in plant parts collected and difference in processing methods adopted by manufacturers results in inconsistencies in quality and efficacy of phytomedicines. In addition to inconsistencies in the quality of polyherbal formulations, presence of adulterants, herb-drug interactions and various toxic side effects have also been reported.[3] These factors affect the efficacy of polyherbal formulations.

The major source of adulterations or substitutions in polyherbal formulation is the presence of 'disputed drugs' and the non-availability of genuine herb. Disputed drugs refers to different plant species having the same Sanskrit name but unresolved botanical identities.[4] These disputed plant drugs of different plant species may have different therapeutic potency as compared to the original plant drug.

Bergenia ligulata Wall. a plant from the family Saxifragaceae, belongs to the category of disputed drugs.[5] *Bergenia* species has been ascribed divine status in various Ayurvedic lexicons which include *Charak-Samita, Ashtang-Hridaya and Sushruta Samhita*.[6] The plant, *Bergenia ligulata* is popularly known as '*Pashanbheda*' and usually grows in rocky areas on cliffs. The common name of *Bergenia* ascribes its ability to dissolve (*bheda*) the stone (*Pashan*). It is also popularly known as stone flower or stone breaker.[7] Owing to its broad spectrum of pharmacological activity, the plant is also referred as 'Golden herb of Himalaya'.[8] As per the new IUCN criteria, the plant is featured in 'endangered and vulnerable medicinal plant list'.[9] Mass production of traditional medicines has resulted in indiscriminate exploitation of this herb for health care formulations. Consequently, an imbalance has been arisen in supply of *B. ligulata* as compared to its demand. The increased demand has resulted in the over exploitation and substitution of *Bergenia ligulata* with other species of *Bergenia*. The validation of substitute medicinal plant is, however, a prerequisite to ensure acceptability of polyherbal formulations made from such substituted plant ingredients and also to ensure adequate therapeutic efficacy. As per the comprehensive report by Gurav and Gurav,[6] there are about nine different species of *Bergenia*, which are known as '*Pashanbheda*' in different regions and sold under the same name in different parts of India. The plant, *B. ligulata* is used in many traditional formulations of Ayurveda including *Pushyanuga churna* (PC), *Vastyamayantaka ghrita, Traikantaka ghrita* etc.[10]

Pushyanuga churna (PC) is an important traditional phytomedicine prescribed for the management of female reproductive disorders such as menorrhagia, leucorrhoea, menstrual disorders etc.[10] *Bergenia ligulata*, which is reported for its clinical application in excessive uterine haemorrhage, menorrhagia and diabetes,[11] is an active ingredient of PC.[12] The Ayurvedic Formulary of India, prescribes the use of rhizome of *Bergenia ligulata* to prepare PC.[10] It is observed that, in different marketed formulations of PC, various other species and different plant parts of *Bergenia* are used which can significantly affect the quality and therapeutic efficacy of PC. As per the label claim of commercially available formulations of PC, it is seen that, the major substitution of *Bergenia ligulata* is by *Bergenia ciliata* (Haw.) Sternb.

Therapeutic properties of *Bergenia* species is attributed to their secondary metabolites like bergenin, paashaanolactone, arbutin, afzelechin, gallic acid, catechin, stigmasterol, β-sitosterol, etc.[13] Bergenin a C-glucoside of 4-O-methyl gallic acid, is the major bioactive phytochemical reported in *Bergenia* species. It is reported to be protective against oxidative stress in various clinical conditions.[14]

In order to scientifically evaluate the veracity of using different *Bergenia* species as substitutes in PC, one in-house PC containing *B. ligulata* along with two marketed PC formulations containing different species of *Bergenia* were compared for their bergenin content. A literature survey revealed that various chromatographic methods have been reported for quantitative estimation of bergenin from various *Bergenia* species.[15,16] All three formulations were subjected to a validated isocratic HPLC analysis to quantify their bergenin contents. The present investigation thus verifies the influence of using different *Bergenia* species on the bergenin content in a polyherbal formulation. The results of the study can help to optimize the judicious use of *Bergenia* species without affecting the efficacy of formulation. The bioanalytical strategy of quantifying bergenin will provide an alternative approach to scientifically validate the use of different species of *Bergenia* if *B. ligulata* is in short supply. This may also help the replenishment of natural populations of *B. ligulata* and protect it from overexploitation.

MATERIAL AND METHODS

Reference standard and chemicals: Bergenin (≥ 97% purity) was procured from Sigma-Aldrich Chemicals (St. Louis, USA). HPLC grade solvents were procured from Merck Specialties Pvt. Ltd., Mumbai, India. All other chemicals used were of analytical grade procured from Qualigens Fine Chemicals Pvt. Ltd., Mumbai, India.

In-house formulation and commercially available formulations: PC consists of twenty-five medicinal plants and one mineral. In-house formulation of PC was prepared as per Ayurvedic Formulary of India.[10] All the plant ingredients were collected manually from their respective natural habitats. Plant materials were authenticated from Agharkar Research Institute, Pune and by Dr. Sunita Shailajan, (Head, Department of Botany) at, Ramnarain Ruia Autonomous College, Mumbai. [17] Voucher specimen for the same were deposited in College. The plant materials were carefully segregated, cleaned, shade dried for a week and oven dried at 37°C to constant weight. The dried plant materials were powdered using an electric grinder and passed through the mess (BSS 85) to obtain standardized particle size. All the sieved ingredients were mixed thoroughly in specified ratio (1 part each) to obtain a homogeneous blend of powders and stored in airtight pearlpet bottles at room temperature, protected from light and moisture.[10]

The two commercially available formulations of PC were purchased from a registered Ayurvedic pharmacy at Matunga, Mumbai (Batch number: AUS-510/DB041809 and APNCO20).

Sample- *Pushyanuga churna*	Species of *Bergenia* used
In-house	*Bergenia ligulata*
Commercial formulation-I	*Bergenia ciliata*
Commercial formulation-II	No label claim

Preparation of calibration standard and QC samples for chromatographic analysis: A stock solution of bergenin (1mg/mL) was prepared in methanol. For the preparation of standard curve, seven calibrant dilutions were made using methanol in the range of 0.5-20 µg/mL. Quality control (QC) samples were prepared at low, intermediate and high concentration levels of 0.7, 3.0, 15.0µg/mL respectively in methanol. All the stock and working solutions were stored at 5 ± 3°C and brought to room temperature prior to use.

Chromatographic conditions: A Jasco's HPLC system equipped with two PU-1580 pumps (HG-1580-31) and a photo diode array detector (MD-1510) was used for HPLC-analysis. The detector was connected to Jasco-Borwin chromatography software version 1.50. The chromatographic separation was performed on a Phenomenex C_{18} column (250 mm X 4.6 mm i.d., 5µm particle size) using a mobile phase, methanol:water (30:70 v/v) containing 0.1% ortho phosphoric acid (OPA). The mobile phase was degassed by sonication for 20 min. A flow rate of 1 ml/ min was maintained throughout the run. The injection volume used was 20µL. After 10 min of equilibration, the samples were injected into HPLC system and the column eluent was monitored at a wavelength of 275 nm.

***Method validation*:** Validation of the method was performed as per ICH guidelines[18] to demonstrate selectivity, specificity, system suitability, linearity, precision, accuracy and robustness of the method.

Selectivity and specificity: During the HPLC analysis, UV scan was performed using the wavelength range of 200-400 nm at the retention time window of the bergenin using a PDA detector (Jasco, MD-1510). Specificity of the intended method was established by comparing the retention time and absorption spectra of peaks obtained for reference standard with that of the target peaks from the analyzed samples.

System suitability: Under the chromatographic conditions specified above, system suitability experiments were performed by injecting five consecutive injections of the bergenin standard at 2 µg/mL. The acceptance criteria was set at % CV of ≤ 2 for area values and retention time obtained for the five consecutive injections (*n*=5).

Linearity and sensitivity: Linearity of the HPLC response was established by triplicate analysis of seven different working concentrations of bergenin ranging from 0.5µg/mL to 20 µg/mL. The mean area response obtained for each concentration was plotted against the respective concentrations to obtain the calibration curve. The linear regression equation was obtained using least square regression analysis. Back calculated concentrations within the range of 85% - 115% with percentage CV of ≤ 2% were accepted. The sensitivity of the method was evaluated by determining the Limit of Detection (LOD) and Limit of Quantitation (LOQ).

Intra-day and inter-day precision: Intra-day precision was assessed by replicate analysis of three quality control samples of bergenin (*n*=5) on the same day, while inter-day precision was assessed by analyzing the variability within replicate injections (*n*=5) of three quality control samples on three consecutive days. Back calculated concentrations within the range of 85% - 115% and % CV of ≤ 2% were accepted.

Stability: Five quality control samples of bergenin (*n*=5) were stored at different storage conditions; at room temperature for 6 h (for bench top stability), at 4°C for 12 h (for short term stability) and at 4°C for 7 and 15 days (for long term stability). The area responses obtained after analysis of these samples were compared with those obtained for freshly prepared samples. These results were used for evaluating the stability of bergenin. The Relative standard deviation (RSD) were calculated and values within a range of ± 5% were accepted.

Recovery: Three quality control levels namely; low, intermediate and high (0.7, 3.0, 15.0 µg/mL respectively) of bergenin were spiked into replicates of formulation samples and extracted as per the optimized extraction procedure. These spiked samples were analyzed (*n*=5) using the HPLC technique. The % recovery was estimated and values within the range of 90-110% were accepted.

Robustness: Robustness of the method was evaluated for the use of column from two different batches, analysis by different analysts, changes in the flow rate (1.0 ± 0.1 ml/min) and changes in mobile phase composition (70 ± 1 : 30 ±1). Replicates of the

three quality control samples (n=5) of bergenin were analyzed to assess the effect of these deliberate changes. Values within a % difference range of ± 5% were accepted.

Assay: One gm of each PC-formulation was extracted in methanol (10.0 mL), vortexed for 30 s, sonicated for 10 min and then vortexed again for 5 min. The solution was then allowed to stand overnight. The next day, samples were filtered through a 0.45 µm Millipore nylon membrane filter and used for HPLC-analysis.

Statistical analysis: Microsoft Excel was used for statistical evaluation of data to determine arithmetic mean, standard deviation (SD), coefficient of variation (% CV), accuracy (% nominal) and for regression analysis.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions: The main step in the chromatographic analysis was to obtain distinctive response for bergenin from the complex matrix of PC. The mobile phase composition, the type of the column, flow rate and the wavelength for detection were varied to achieve the optimal chromatographic conditions that would give the best separation. Several analytical columns with different lengths and internal diameters, viz. Cosmosil C_8 and C_{18} (150 X 4.6 mm ID, 5µm), Phenomenex C_{18} column (i.d.250x4.6mm, 5µm), Dionex Acclaim® 120 Reverse phase C_8 column (250 X 4.6 mm ID, 5 µm) were evaluated**.** It was observed that good separation, better resolution, higher sensitivity and selectivity were obtained on a Phenomenex C_{18} column (250mm X 4.6 mm ID, 5µm) owing to better binding of analyte to the stationary phase. A column of 250 mm length gave the optimal retention time and good peak shape for bergenin. After trails with various combinations of acetonitrile, methanol and water, it was found that methanol and water at a ratio of 30:70, v/v. Further, it was observed that peak response and resolution increased considerably when the pH was set at 3 with 0.1 % OPA. Therefore, a mixture of methanol and water (30:70, v/v) containing 0.1% OPA was adopted as optimum composition for the mobile phase. The mobile phase was used in isocratic elution mode with a flow rate of 1 mL/min. All the PC samples analyzed showed a peak at the retention time of 8 min and UV maximum absorption at 275 nm which corresponded with the standard bergenin. The representative chromatogram is illustrated in Figure 1.

Method validation

Linearity and sensitivity: The calibration curve of bergenin exhibited good linear response within the linearity range, with the coefficient of correlation (r^2) values of more than 0.99 indicating good positive linear relationship (Table 1). LOD and LOQ values were determined at signal to noise ratios (S: N) of 3: 1 and 10: 1, respectively. The LOD and LOQ of bergenin were found to be 0.3 µg/mL and 0.5 µg/mL, respectively.

Precision and accuracy: Repeated analysis of replicates of quality control samples of bergenin were conducted to evaluate the intra-day (within a day) and inter-day (between days) variability. The results showed % CV values below 2, and % nominal values within the acceptance limits of 85-115% indicating the method to be precise, reliable and reproducible (Table 1).

Stability: Stability of bergenin in methanolic solution was studied by analyzing the replicates of three QC levels which were stored at different temperatures; 6 hr at room temperature (25 ± 2°C) for bench top stability, 12 hr at 4°C for short term stability and at 4°C for 7 and 15 days for long term stability. Bergenin was found to be stable at the different storage conditions (Table 2).

Robustness: A change of analyst or use of different batches of the HPLC column did not affect the area response and the retention time of the standard bergenin. Changes in flow rate and changes in mobile phase composition, however, affected the retention time of bergenin. The changes in area response and retention time were within the acceptable limits of the % CV (< 2%) and the difference in mean values (± 5%), as summarized in Table 3. This indicates that the method is adequately robust.

Recovery: The average recovery of bergenin from replicates analysis of QC levels was found to be 100.9%, 98.6% and 97.4% for LQC, MQC and HQC respectively, indicating that the method had high sensitivity and reliability (Table 1).

Assay of bergenin from methanolic extract of PC

The validated analytical method for bergenin was applied to estimate bergenin content from different PC samples. The bergenin content in the in-house formulation containing *Bergenia ligulata* was found to be 0.27 mg/gm, whereas the two commercial samples were found to contain 0.24 mg/gm bergenin (sample in which *Bergenia ciliata* is substituted)

Table 1: Method validation data.

Method property	Value
Mean retention time (Rt)	4.012min
Calibration range	0.5µg/mL to 20 µg/mL
Equation	y = 90749.02 x – 1179.19
Regression coefficient(r^2)	0.999
Limit of Detection(LOD)	0.3µg/mL
Limit of Quantification (LOQ)	0.5µg/mL
LQC	0.7µg/mL
MQC	3µg/mL
HQC	15µg/mL
Intraday precision (% CV., n=3)	0.580-1.611
Intermediate precision (% CV., n=3)	0.422-0.733
Recovery (%)	97.35% to100.89 %

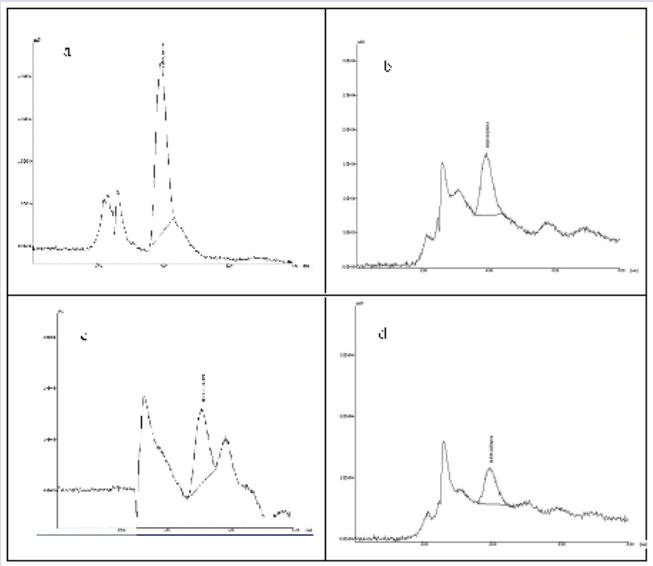

Figure 1: Representative HPLC chromatograms of bergenin from PC-formulations (a= Bergenin- 10µg/mL, b= PC In-house, c= PC Commercial Sample-1, d= PC Commercial Sample-2).

Table 2: Stability of the bergenin at different storage conditions.

Stability	LQC		MQC		HQC	
	Area (% CV)	% difference	Area (% CV)	% difference	Area (% CV)	% difference
Freshly prepared solution	0.84	--	1.37	--	0.81	--
Bench top stability- Room temperature (25 ± 2°C) for 6 h	0.81	0.19	1.64	-1.48	1.20	-0.76
Short-term stability (4°C for 12 h)	0.62	0.27	1.57	-0.94	0.95	0.12
Long-term stability (4°C for 7 days)	1.37	0.31	1.37	0.07	0.85	-0.06
Long-term stability (4°C for 15 days)	1.09	0.56	1.63	-1.48	1.22	-1.29

*Mean ± SD, µg/ml; *n*=3

Table 3: Robustness.

Parameters	Area (% CV)			% difference
Change in analyst				
	Analyst 01		Analyst 02	
LQC	1.324		1.398	0.72
MQC	0.992		0.997	-0.73
HQC	1.452		0.239	1.78
Change in column				
	Column 01		Column 02	
LQC	0.209		0.191	-0.07
MQC	0.993		0.960	-0.59
HQC	1.350		1.006	2.03
Change in flow rate				
	0.9 mL/min	1.0 mL/min	1.1 mL/min	
LQC	1.34	1.77	1.55	-0.59 to-0.39
MQC	1.20	1.16	1.43	-0.08 to 0.24
HQC	0.23	1.47	0.80	-0.09 to 0.64
Change in mobile phase composition*				
	I	II	III	
LQC	1.319	1.557	1.377	-0.34 to 0.27
MQC	1.493	2.774	1.407	-0.07 to 2.32
HQC	0.833	1.198	0.834	0.70 to 0.74

*I-III in Table represents different mobile phase compositions.
I - (Methanol: distilled water,0.1% OPA; 29:71, *v/v*); II (Methanol: distilled water,0.1% OPA; 30:70, *v/v*) and III (Methanol: distilled water,0.1% OPA; 31:69, *v/v*)
*Mean ± SD, µg/ml; *n*=5

and 0.162 mg/gm (the sample with no label claim), as shown in Table 4. It is evident from the results that the bergenin content was more in the formulation containing *B. ligulata* suggesting that *B. ligulata* is a better source for bergenin. The bergenin content of in-house formulation containing *B. ligulata* and the commercial sample-I containing *Bergenia ciliata* were comparable. The results correlate well with those reported by Rawat *et al.*[15]

PC is a polyherbal formulation, formulated as a heterogeneous mixture of twenty-five plant ingredients and one mineral. The unique blend of herbs with their varying biopotency gives synergistic therapeutic effects to PC

Table 4: Content of bergenin in PC-samples.

PC-Samples	Content (mg/g)	% CV (n=3)
PC-In-house	0.277	1.51
PC commercial sample-I	0.240	1.32
PC commercial sample-II	0.162	1.06

through the phenomenon of 'positive herb-herb interaction'.[19] Earlier research has shown that different plant parts of *Bergenia* exhibits wide spectrum of pharmacological activity ranging from anti-inflammatory, antibacterial, antidiabetic, free radical scavenging, hepatoprotective etc. The plant is also useful in managing haemorrhage and menorrhagia.[11,20]

In the current investigation, a bioactive marker bergenin content is estimated in different PC formulations containing different species of *Bergenia*. The comparison of bergenin content is used to verify the validity of substitution of different *Bergenia* species in PC formulations. In addition to the effect of using different plant species, variations in phytochemical content in a medicinal plant based formulation can occur due to use of different morphological parts of the plant, use of plants in different phenological stages, use of plants from different geographical location and use of plants harvested in different seasons.[21] From the results of the current study, it is possible to deduce that the low content of bergenin observed in commercial sample-II may be due to the use of *Bergenia* species with low content of Bergenin or it may be because of use of different parts of the plant.

Since *Bergenia ligulata* is an endangered and vulnerable medicinal plant, substitution of *B. ligulata* with *B. ciliata* significantly reduces the overexploitation of *B. ligulata* by manufacturers. In the case of PC, the use of right species of *Bergenia* plant can be ensured by evaluating the bergenin content since the therapeutic use of PC in management of gynecological disorders can be mainly attributed to bergenin content. The bioanalytical strategy reported in the current work can be applied to other *Bergenia* species to validate their potential use as positive substitutes of *B. ligulata* in herbal formulations.

CONCLUSION

The scenario in the herbal drug industry has changed tremendously during the past few decades with emerging market for phytomedicine in primary healthcare systems. The major step in global harmonization is to develop a strong commitment by manufactures to safeguard the consumers' health by providing efficacious formulations of good quality. Accurate identification of plant raw materials and validation of its substitutes in terms of their comparative therapeutic effect is necessary to cross the barrier of impediments associated with nonavailability of some plant raw materials.

In a polyherbal formulation, some drug other than the principle medicinal plant may be substituted if the genuine raw material written in classic text is not available.[2,22] On the basis of results of this study, we can conclude that substitution of plant raw material can be made without significantly affecting the phytochemical content. Resurgence of phytomedicines has increased the demand for plant raw materials. To satisfy the demand for plant raw materials, especially those in short supply, substitution with appropriate alternate species can be made if the complete profile of the alternative plant ingredient is available with its phytochemistry and pharmacological effect. There is an urgent need to shift approach from 'negative substitution to positive substitution' for the medicinal plant ingredients, which are not available in plenty. Positive substitution will also help in preventing overexploitation of the original plant species. At the same time, pharmacological evidence based efficacy studies should support and justify such substitutions.

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ABBREVIATIONS

PC: *Pushyanuga churna;* **IUCN:** International Union for Conservation of Nature; **HPLC:** High Performance Liquid Chromatography; **QC:** Quality Control; **ICH:** International Conference on Harmonization; **Rt:** Retention Time; **% CV:** Percent coefficient of variation; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **RSD:** Relative standard deviation; **PDA:** Photo Diode Array; **min:** Minute(s); **Ltd.:** Limited.

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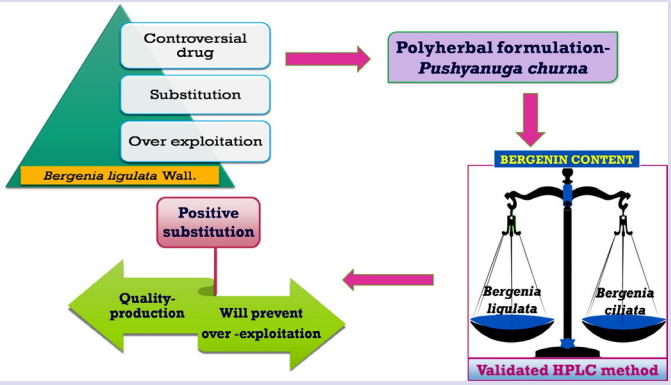
PICTORIAL ABSTRACT



SUMMARY

The present project work was undertaken to give prospective of positive substitution of medicinal plants by using their allied species in polyherbal formulation.

ABOUT AUTHORS

Prof. (Dr.) Sunita Shailajan is former Head Department of Botany and Dean of Research, Development and Innovation at Ramnarain Ruia Autonomous College. Currently working as a principal Investigator in DST-SERB project, (Govt. of India). She has 38 years of teaching experience along with strong support of research. She has completed 14 Government funded projects, sponsored by agencies like DST-SERB, DBT, UGC, NMPB, AYUSH, BARC- BRNS, ICMR and 01 project is still in progress. She is a nominated member of the prestigious HPTLC Association (International Association for the Advancement of High Performance Thin Layer Chromatography), Switzerland. She has contributed 26 SOP's of Ayurvedic formulations in Ayurvedic Pharmacopoeia of India under AYUSH project from Govt. of India and 08 monographs of medicinal plants in different volumes of ICMR. She has 132 publications in reputed journals.

Ms. Swati Singh is a Ph.D. student in Bioanalytical Sciences at Ramnarain Ruia Autonomous College, affiliated to University of Mumbai, Maharashtra, India wherein she is working on the prevalent problem of polycystic ovarian syndrome. She has completed M.Sc. in the subject of Bioanalytical Sciences at Ramnarain Ruia Autonomous College, University of Mumbai. She has one publication and presented her work in this field at various National and International Conferences.

Dr. Sasikumar Menon is currently the Director of Institute of Advanced Research in Interdisciplinary Sciences (TDM Lab.) at Sion and Associate Professor in Pharma Analytical Sciences, KAUSHAL Kendra Ramnarain Ruia Autonomous College, Mumbai. He has been teaching in Dept. of Zoology of Ramnarain Ruia College since 1983. Dr. Menon is a Recognized Research Guide for University of Mumbai and has guided 23 students for PhD and 02 M.Sc by research. His research interests include drug toxicology, herbal drugs, drug action, reproductive physiology, male contraceptives, ecology and biodiversity conservation. Dr. Menon has been the Principal Investigator / Study Director for more than 350 drug trials in human and more than 60 trials on cosmeceuticals and nutraceuticals in human. also, more than 150 drug toxicological studies in animals. He has also been a Co-Investigator of Projects funded by DBT, DST and AYUSH, Ministry of Health. GOI, UGC etc. Dr. Menon has over 95 Research Publications in national and international Journals and also has 2 Indian Patents to his credit. Furthermore, he is a Reviewer for many National and International journals.