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Simultaneous marker based standardization of in-house gel by HPTLC with gallic acid and Berberine

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Abstract
The present paper deals with marker based standardization of in-house gel containing ethanol extract of dried peels of *punica granatum* and dried root bark of *berberis aristata*. The bioactive markers were gallic acid and Berberine used to develop and validate the High Performance Thin Layer Chromatographic method. The method was performed on TLC aluminum plates Precoated with silica gel 60F254. Good separation was achieved in the mobile phase composed of toluene: ethyl acetate: methanol: formic acid (3.5: 5: 1:0.5) and detected at wavelength 254nm in reflectance/absorbance mode. The developed method was well validated by different parameters mentioned in ICH guidelines such as linearity, accuracy, precision, robustness.

Keywords: Gallic acid, Berberine, HPTLC, validation, ICH guidelines, markers

Introduction
Standardization of polyherbal formulation is of supreme importance in order to defend their acceptability in modern system of medicine and the quality assessment. Standardization is a technique which ensures that every envelope of medicine that is being sold has the suitable substances in the correct amount and will encourage its therapeutic effect.
From past few decades, herbal drugs have been gaining importance because of the vast chemical diversity that they offer. But standardization of herbal medicine is not routine practice carried out by manufacturer because of their complex nature [1-3].
Standardized herbal products of consistent quality and containing well-defined constituents are required to provide consistent beneficial therapeutic effects. So, standardization is paramount importance in acceptance of herbal products at global level. Development of authentic analytical methods which can consistently profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a big challenge to scientists [4].
Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques. HPTLC has emerged as an important tool for the qualitative, semi-quantitative and quantitative phytochemical analysis of herbal drugs and formulations. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers [5, 6].
In the present investigation, a simple, optimized and validated HPTLC method for standardization of in house gel was developed. Two marker compounds (gallic acid and Berberine) were selected from the raw materials used for preparation of the gel. The method was validated according to the International Conference on Harmonization (ICH) guidelines.

Material and Methods
Solvents and chemicals
Standard gallic acid and Berberine were procured from Yucca enterprises. All chemicals and reagents used were of analytical grade and purchased from Rankem and S. D. Fine Chemicals, India. Silica gel 60F HPTLC pre-coated plates were purchased from Merck.

HPTLC Instrumentation and Method development [7-9].
The stationary phase used was Precoated silica gel aluminium plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat V (Switzerland)on to which the test solutions were spotted in the form of bands of width 6 mm with a Camag Microlitre syringe.
The plates were pre-washed by methanol and activated at 60 °C for 5 min prior to chromatography. The slit dimension was kept at 5mm × 0.45 mm, bandwidth was set at 20 nm,

each track and 10 mm/s scanning speed was employed. The composition of the mobile phase was toluene: ethyl acetate: methanol: formic acid (3:4:1.0:0.5) for gallic acid and Berberine. The linear ascending development was carried out in a twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 minutes at room temperature (25 ± 2 °C). The length of the chromatogram run was 80 mm. Subsequently, the plate was allowed to dry at room temperature. The separated bands on the HPTLC plates were scanned over the wavelength of 200 – 400 nm. The source of radiation utilized was the tungsten lamp (or deuterium Illumination). The maximum absorbance was found at 278 nm. The images were captured on Camag reprostar 3 with win-CATS software 4.05.

Preparation of standard solution

A stock solution of gallic acid and Berberine (1 mg/ml) were prepared by dissolving 10 mg of accurately weighed gallic acid and Berberine in methanol separately and making up the volume to 10 ml with methanol. The stock solution was further diluted with methanol to give a standard solution of gallic acid and Berberine (100 μg/ml). This concentration was used as the working standard for the HPTLC method.

Sample preparation

Accurately weigh 100 mg of topical gel containing ethanolic extract of dried fruit peel powder of *Punica granatum and* dried root bark *Berberis aristata* in a 50 ml volumetric flask. Dissolve the gel in 25 ml of ethanol and sonicated for 15 mins and make up the volume with methanol. Further, the dilution was made to obtain 50 μg/ml concentration. All samples were filtered through a 0.22 μ membrane filter from Millipore.

Method validation [10-14].

The validation of the developed HPTLC method was carried out according to ICH guidelines. All the parameters were evaluated as mentioned in Sharma *et al*. 2016 [10].

Linearity

The linearity was analyzed for different concentration ranging from 1- 1000 ng/spot were spotted. The data of the peak areas plotted against the corresponding concentrations were treated by least-square regression analysis.

LOD and LOQ were determined by using standard deviation method. Detection limit =3.3σ /S and quantitation limit=10 σ /S where σ is the residual standard deviation of a regression line and S is the slope of the calibration curve.

Precision studies

Precision of the method was evaluated by repeatability (intra-day) and reproducibility (inter-day). The triplicates of three different concentrations of each gallic acid and Berberine were spotted and analyzed on same day for intra-day study and two different days for inter-day study.

Accuracy studies

Recovery study method was employed to evaluate accuracy of the method. The samples were spiked with 80, 100 and 120 % of median concentrations (250 ng of gallic acid and Berberine) of standard. Accuracy was calculated from the following equation:

[(spiked concentration − mean concentration)/spiked concentration] × 100.

Robustness

For the determination of the robustness of method, chromatographic parameters, such as mobile phase composition and detection wavelength, were intentionally varied to determine their influence on the retention factor and quantitative analysis.

The mobile phase composition was altered by ± 5 % changes in the composition of methanol. The chamber saturation time was altered from 15 min to 30 min.

Results and Discussion

Method optimization

The proposed method gave very good separation and resolution of the standard Gallic and bebeerine (R_F value = 0.5 and 0.3) as indicated in (Fig. 1).

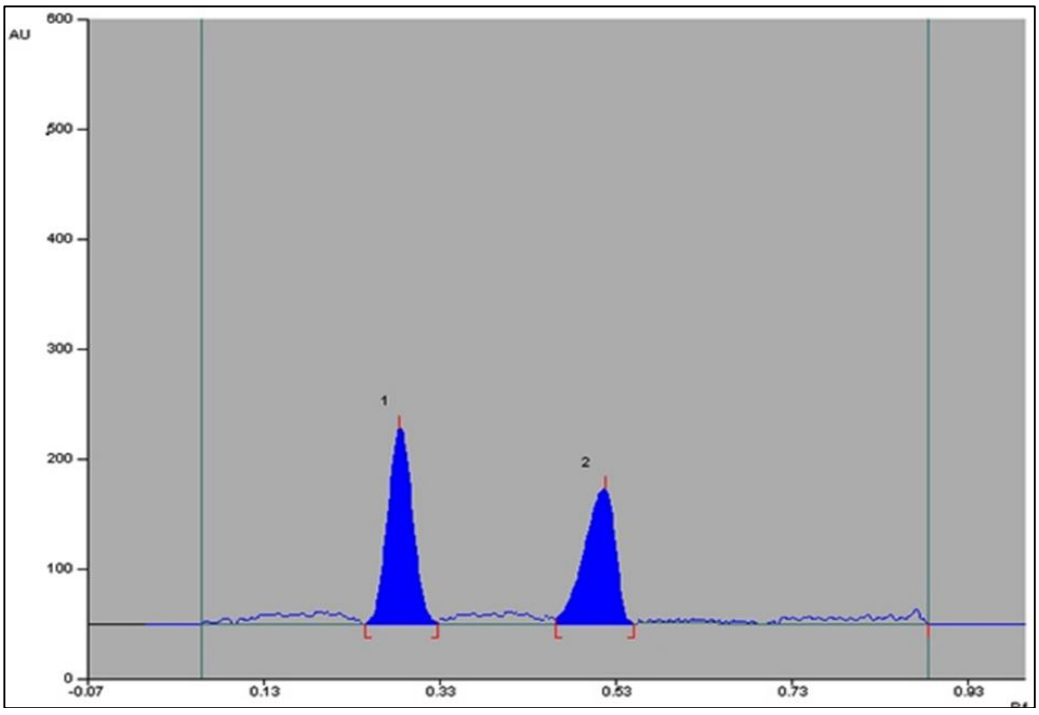

Fig 1: Haptic Profile of Standard Gallic Acid and Berberine

Method validation [10-14].

Linearity, limit of detection and quantitation

Under the above described experimental conditions, linear correlation between the peak area and applied concentration was found to occur in the concentration range 200-1000 ng/spot for gallic acid and 150-950ng /spot for Berberine. (Fig 1 and Fig 2)

The experimentally derived LOD and LOQ for both the markers were determined as mentioned in Table 1.

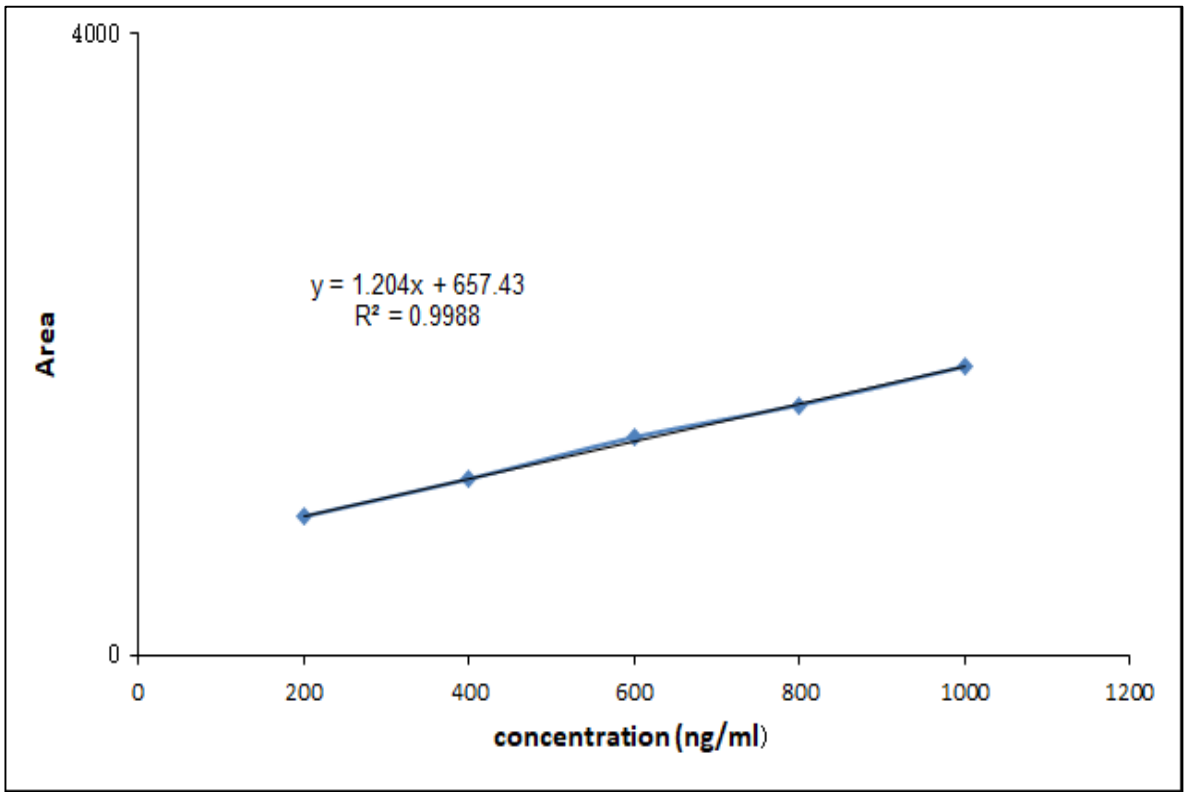

Fig 2: Linearity graph of Standard Gallic acid

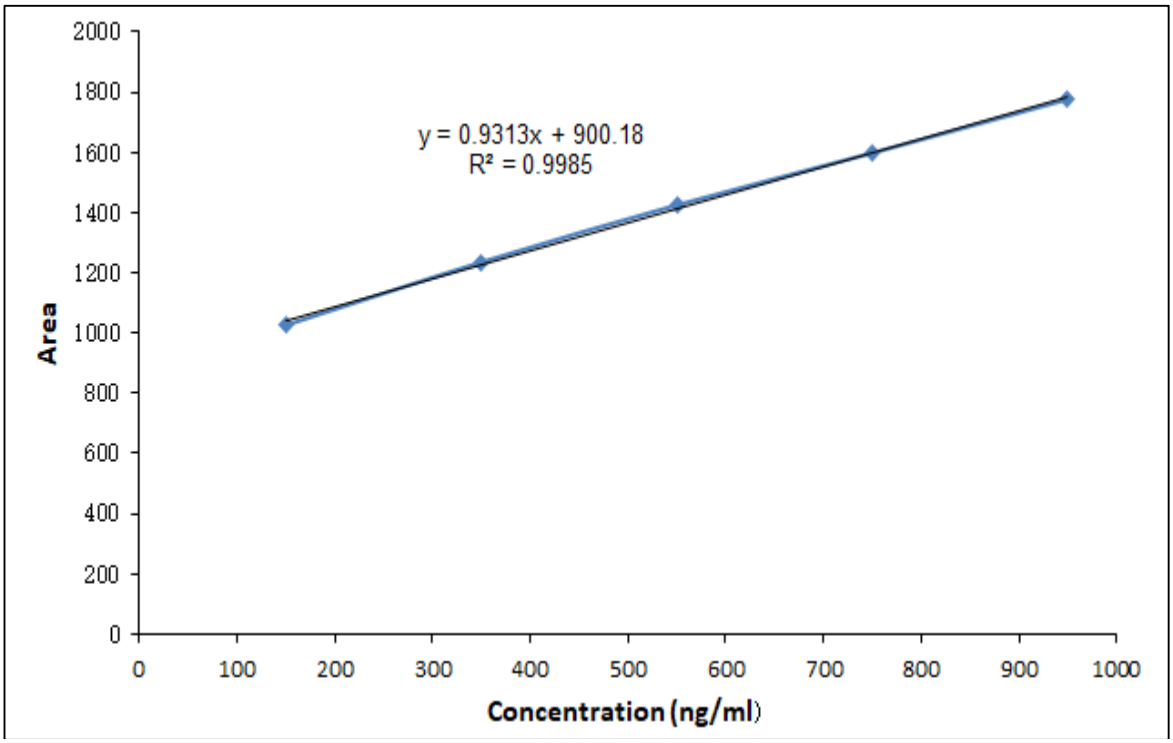

Fig 3: Linearity graph of Standard Berberine

Table 1: LOD and LOQ of both markers; gallic acid and Berberine

Markers	LOD (ng/spot)	LOQ (ng/spot)
Gallic acid	51.2	155.2
Berberine	43.06	130.50

Precision

Precision data on repeatability (intra-day) and instrumental variation for three different concentration levels are summarized in Table 2 and Table 3. Precision studies showed R.S.D. less than 1%, indicating a good precision.

Table 2: Results of Precision Studies of gallic acid

Type of Precision	Intra-day			Inter-day		
	AUC for concentration of Gallic acid			AUC for concentration of Gallic acid		
Sr. No	200	400	600	200	400	600
Mean	885.98	1142	1403.11	896.99	1136	1401.44
% RSD	0.31	0.28	0.69	0.15	0.47	0.37

AUC concentration of Gallic acid: ng/spot

Table 3: Results of Precision Studies of Berberine

Type of Precision	Intra-day			Inter-day		
	AUC for concentration of Berberine			AUC for concentration of Berberine		
Sr. No	350	550	750	350	550	750
Mean	1236.58	1428.26	1599.47	1233.25	1424.27	1598.22
% RSD	0.55	0.47	0.29	0.14	0.33	0.47

AUC concentration of Berberine: ng/spot

Accuracy

The sample containing 250 ng of gallic acid and Berberine were spiked with the known amount of standard, and the percent ratios between the recovered and expected concentrations were calculated. Recoveries were obtained in the range of 81.80-118.97%, depicting the HPTLC proposed method for simultaneous estimation is accurate for the quantification of all three marker compounds.

Table 4: Recovery studies of Gallic acid and Berberine

Recovery studies of Gallic acid		Recovery studies of Berberine	
In 600 ng Gallic acid	**Recovery ± S.D. (%)**	**In 550 ng Berberine**	**Recovery ± S.D. (%)**
480 (80%)	107.22 ± 0.68	440 (80%)	107.7 ± 0.88
600 (100%)	98.59 ± 0.59	550 (100%)	9.84 ± 0.96
720 (120%)	96.85 ± 0.77	660 (120%)	97.16 ± 0.81

Robustness

No changes were observed in retention time and peak shape of both the standards with the changes made with mobile phase and chamber saturation time. The resolution and the separation of markers were also unaltered.

Analysis of in-housed formulations containing ethanol extract of dried peel of punica and bark of berberis

Standardization of in-house gel was carried out by the developed and validated HPTLC method. The developed method was able to be provide a well resolved chromatogram with no alterations in peaks of gallic acid and Berberine. (Fig.4). the percent content of gallic acid and Berberine in the gel was found to be 0.054%±0.09 and 0.094%±0.12.

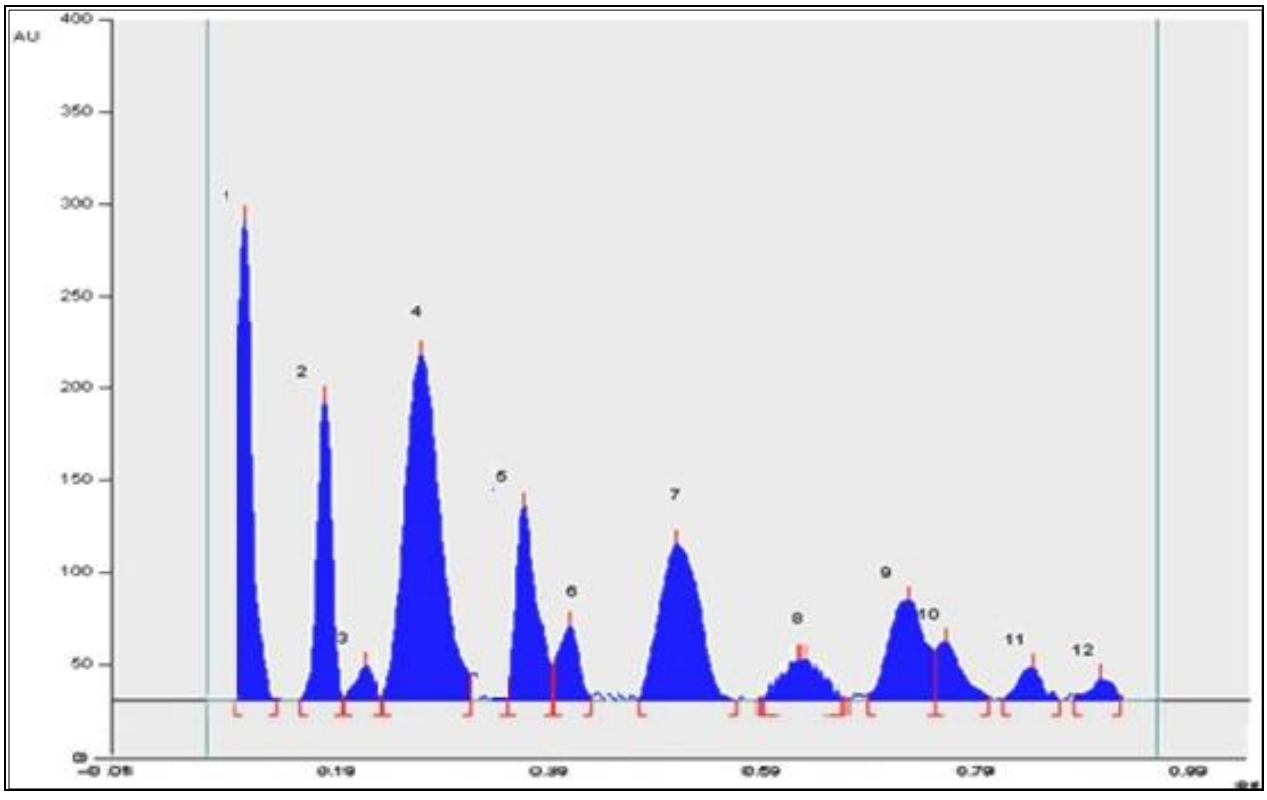

Fig 4: HPTLC profile of in-house gel containing well resolved peaks of Gallic acid and Berberine

Conclusion

The standardization of in-house gel containing ethanol extract of dried fruit peel powder of *Punica granatum* and dried roots of *Berberis aristata* was successfully carried out by HPTLC method. The method was developed and validated as per ICH guidelines for standards gallic acid and Berberine. These two standards are primarily the Bioactives of *Punica granatum* and dried roots of *Berberis aristata* extracts respectively. This method as certainly employed for the routine quality control testing of the formulations containing these marker compounds.

It is possible to formulate the herbal medicines with the labeled content of active ingredients. Thus, the standardization of traditional medicines brings scientific approach and thus enables to prove the authenticity and effectiveness of the ancient method of treatment.

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