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Analytical Method Development Of High-Performance Thin Layer Chromatography Method For Simultaneous Quantitative Estimation Of Gallic Acid and Diosgenin Present in Polyherbal Formulation

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ABSTRACT-High-Performance Thin-Layer Chromatography (HPTLC) is an advanced chromatographic technique used for separating and analyzing complex mixtures of chemical compounds. It is an extension of traditional thin-layer chromatography (TLC), which is a simple and effective separation technique commonly used in laboratories. HPTLC offers higher resolution, sensitivity, and automation, making it more suitable for various analytical and qualitative applications.A sensitive, fast, and reproducible high performance thin-layer chromatographic method has been developed for simultaneous analysis of Gallic Acid and Diosgenin from Polyherbal Formulation, using TLC aluminium plates precoated with Silica gel $G_{60}F_{254}$. Among the different combinations of mobile phases used, best separation was achieved in Toluene-Ethyl acetate-Formic acid (7 : 3 : 1,v/v/v).Densitometric scanning of the plates directly at 278 nm was used for analysis of Gallic Acid.for analysis of Diosgenin, plates were scanned at 450 nm after spraying with Anisaldehyde-Sulphuric acid reagent. The retardation factor value of Gallic acid and Diosgenin was found to be 0.38 and 0.47 respectively. The present method is being reported for the first time and can be used for routine quality control and quantification of these marker compounds in Polyherbal Formulation of Fruits Extracts of Terminalia bellerica and Tribulus terrestris.

Keywords-Polyherbal Formulation, TLC, HPTLC, Gallic Acid, Diosgenin.

I. INTRODUCTION-

[1].Preparation of highly standardized herbal products with respect to chemical composition and biological activity is considered to be a valuable approach in the field of Standardization is an essential factor for polyherbal formulation in order to assess the quality of the drugs based on the concentration of their active principle.[2,3]. High performance thin layer chromatography (HPTLC) is an invaluable quality assessment tool for the standardization of herbal products. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. Additionally, numerous samples can be run in a single analysis thereby dramatically reducing analytical time. [4].

HPTLC is the most simple separation technique available today which gives better precision and accuracy with extreme flexibility for various steps (stationary phase, mobile phase, development technique and detection). HPTLC finger printing technique is useful to identify and to check the purity of raw herbal extracts as well as finished product. Hence Hence forth it is very useful tool in standardizing process of raw herbal extracts and finished products.[5].

HPTLC finds applications in various fields, including pharmaceuticals, herbal medicine analysis, food and beverage analysis, environmental monitoring, forensic science, and more. It is particularly valuable in qualitative analysis, fingerprinting of complex mixtures, and the quantification of individual components in a mixture.Overall, HPTLC is a powerful and versatile analytical technique that has proven to be a valuable tool in many scientific and industrial applications for separating and identifying complex mixtures of compounds.[6]

High-Performance Thin-Layer Chromatography (HPTLC) is a suitable analytical technique for quantifying marker compounds in polyherbal

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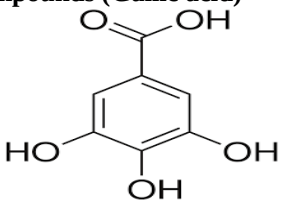
formulations. The process involves separating the components of the formulation on a thin-layer chromatography plate and then quantifying the marker compound(s) based on their spot intensities or areas. It is important to note that proper calibration curves using standard solutions are essential for accurate quantification. Additionally, the method's validation ensures the reliability and reproducibility of the results, making the HPTLC analysis a robust technique for quantifying marker compounds in polyherbal formulations.[7].

Gokharu, also known as Gokshura or Tribulus terrestris, is a medicinal plant widely used in traditional medicine systems, including Ayurveda, Chinese medicine, and traditional European medicine. It is a herbaceous plant belonging to the Zygophyllaceae family and is native to regions in Asia, Europe, and Africa. The plant has spiny fruits and leaves and is known for its medicinal properties, particularly in supporting urinary and reproductive health. [8].

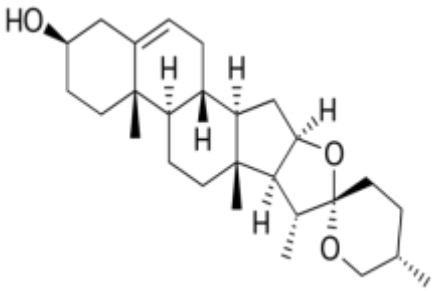
Beheda, also known as Bibhitaki or Terminalia bellerica, is a medicinal plant commonly used in traditional medicine systems, including Ayurveda, for its therapeutic properties. It is one of the three fruits in the popular Ayurvedic formulation called "Triphala," along with Amla (Emblica officinalis) and Haritaki (Terminalia chebula). Beheda is a large deciduous tree belonging to the Combretaceae family and is native to South Asia. [9].

Marker Compounds (Gallic acid)-



Gallic acid is a trihydroxybenzoic acid. It is calssified as a phenolic acid.. It is found in Gallnuts, Sumac, Witch hazel, Tea leaves, Oak bark and other plants. It is a white solid, although samples are typically brown owing to partial oxidation. Salts and esters of Gallic acid are termed "Gallates". [10].

Diosgenin-



Diosgenin, a phytosteroid sapogenin, is the product of hydrolysis by acids, strong bases, or enzymes of saponins. The sugar-free (aglycone) product of such hydrolysis, Diosgenin is used for the commercial synthesis of Cortisone, Pregnenolone, Progesterone, and other Steroid products. [11].

II. MATERIAL AND METHODS-

2.1 Collection, Identification and Authentification of Plant material-

Dried Fruits of Terminalia bellerica and Tribulus terrestris was collected from Dagadu Teli Chandwadkar, Raviwar Karanja, Nashik and the plant material were identified and authenticated by Dr. D. S. Khandbahale, Assistant Professor of Botany Department, KTHM College, Nashik.

2.2 Extraction-

All crude drug powders of formulations, was extracted with Hydro-alcoholic solvent. The extracts were concentrated and dried.

Table No. 1 : Extracts of the selected Plant parts of Polyherbal Formulation

Sr.no	Plant Name	Extract	% Yield (w/w)
1	Terminalia bellerica (Fruit)	Hydro-alcoholic	21.05%
2	Tribulus terrestris (Fruit)	Hydro-alcoholic	10.15%

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.2.3Procurement of markers-

Marker compound means chemical constituents within a medicine that can be used to verify its potency or identity.chemical markers Gallic acid and Diosgeninwere procured from Yucca Enterprises, Mumbai.

2.4 Devlopment of Solvent system by TLC-

The TLC technique is used for qualitative determination of possible number of phytoconstituents from the formulation. Solvent systems were optimized in order to get maximum separation of various phytochemicals. Solvent system optimized in TLC study was chosen for HPTLC parameters were set.

2.5 Development of HPTLC methods for each chemical markers-

HPTLC method was developed for polyherbal formulations taking procured marker compounds as working standards.

1.Steps involved in performing HPTLC of Polyherbal formulation-

Precoated aluminium sheets TLC plates were used.

1.1 Activation of Plate-

Plates were activated for 30 minutes at 105℃ in oven.

1.2 Sample Preparation-

In 10 ml of Methanol, 400 mg of Herbal tablet Formulation was dissolved. And after 15 min. sonicated for complete dissolution. Filtrate is used for analysis.

Procedure-

Using capillary tube sample was applied on baseline of HPTLC plates above 1cm from bottom and dry the spot. The chamber is saturated with solvent system for 30 minutes. Plate is kept in chamber after the saturation of chamber. The plat is allowed to run in solvent system up to 7-9 cm distance. Plates are removed and visualized under UV and using specific reagent.

Procedure for HPTLC method development is outlined as follow:

- 10×10 cm of precoated Silica gel plates backed with aluminium were taken.
- Application of the samples and test solutions were spotted at application position on Y axis was 8 mm and 14 mm on X-axis, using LINOMAT 100 µl syringe.Wavelength was set at 280 nm.Band length was set as 8 mm.The distance between two spots should not be less than 11 mm, so it was set as 12 mm.
- Firstly, the syringe was rinsed with the solvent, after that the sample was filled in it for spotting.Once the sample application is done, the plates were then dried.
- In the Twin trough chamber, the mobile phase (Tolune: Ethyl acetate: Formic acid) is filled and the mobile phase is equally distributed in both the troughs, filter paper is kept in it for chamber saturation.
- The plate was kept in the chamber at an angle of 45º and allowed the mobile phase to run to the solvent front assigned. Once the development of the plate was completed, the plate is carefully removed and dried.
- Separation of the spots was observed in the UV chamber and recorded. The plate was further proceeded for the scanning in the scanner and scanned on the wavelength which was already assigned. Documentation of the detection is done and peaks are reported with the Rf values and peak area.

2. Instrument-

- A Hamilton 100 µl HPTLC syringe
- A Camag Linomat 5 (semi-automatic spotting device)
- A Camag twin-trough chamber
- A Camag TLC Scanner 3 A Camag data evaluation 32 bit software (latest version)

2.1 Spotting Parameters-

Start position : 15 mm from bottom edge
Band width : 4 mm
Space between two bands : 5 mm

2.2 Experimental Conditions for HPTLC-

Stationary phase : pre-coated TLC plates of silica gel 60 F_{254} (E. Merck)
Separation technique : Ascending
Development chamber : Twin-trough chamber
Mobile phase : Tolune:Ethyl acetate:Formic acid(7:3:1)
Derivatization : Anisaldehyde- Sulphuric acid reagent
Chamber saturation time : 30 min.
Temperature : 29°C
Migration distance : 80 mm

2.3 Densitometric Scanning (CAMAG scanner 3)-

Wavelength : Gallic acid 278 nm, Diosgenin 450 nm

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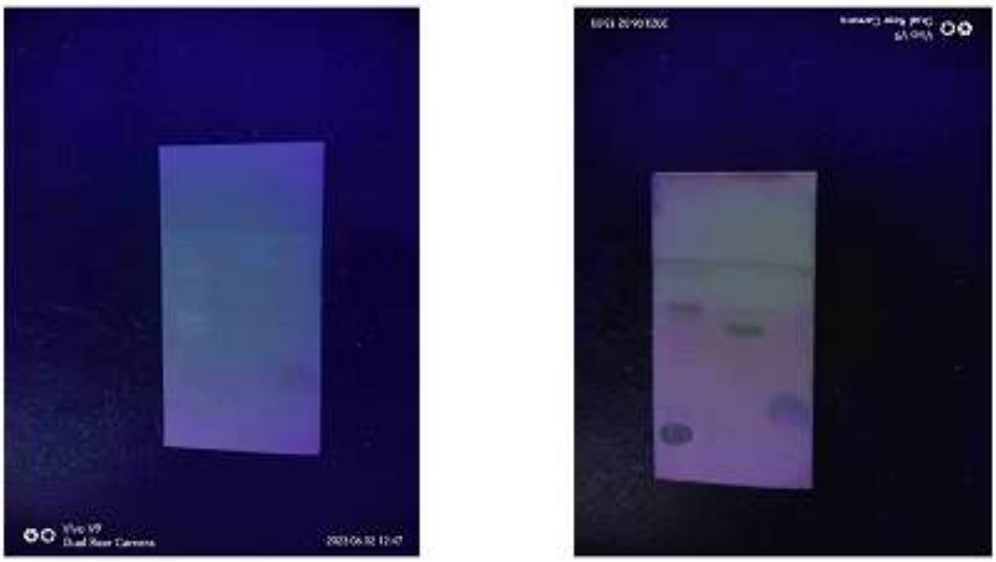
Lamp used : Mercury
Slit dimension : 3 X 0.45mm

2.4 Preparation of Solution-
(i) Preparation of Standard Solution of Chemical Marker Compounds-
Accurately weighed 10mg of Gallic acid and Diosgenin were dissolved in 10 ml of methanol(1.0mg/ml) in a volumetric flask seperately.
(ii) Preparation of Sample Solutions-
Sample solutions were generated by dissolving 400 mg of prepared polyherbal formulations in 10 ml of methanol (4.0 mg/ml) in a volumetric flask seperately.

2.5 Estimation of Chemical Markers in polyherbal formulations-
(i) Estimation of Gallic acid-
5µl of test sample solution (0.005 mg/ml) was used for spotting. The plate was developed in the mobile phase. After development the plate was dried and immediately scanned at 278 nm using the Camag scanner 3. Peak areas were noted and concentration of gallic acid in the formulation was calculated.
(ii) Estimation of Diosgenin-
5 µl of test sample solution (0.005 mg/ml) was used for spotting. The plate was developed in the mobile phase. After development the plate was dried and immediately scanned at 450 nm using the Camag scanner 3. Peak areas were noted and concentration of Diosgenin in the formulation was calculated.

III. RESULTS AND DISCUSSIONS-

3.1 Preliminary TLC study for Solvent System Development-Solvent systems were optimized to achieve best resolution of the marker compounds from other components of the formulation through TLC study. The specific solvent system, Tolune:Ethyl acetate:Formic acid (7:3:1) gave better resolutions of the marker compounds in polyherbal formulation.



TLC Method development of Polyherbal Formulation
Fig no. 1

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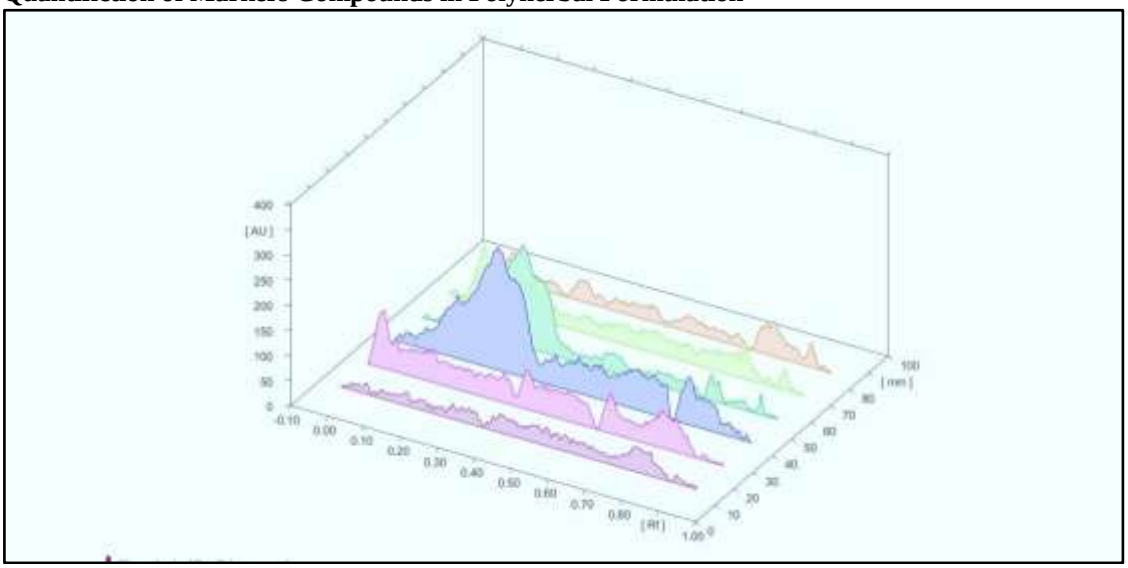
3.2 Quantifiction of Markers Compounds in Polyherbal Formulation-



Track 1 and 2-Diosgenin
Track 3 and 4-Gallic acid
Track 5 and 6-Polyherbal Formulation
Fig no.2 tracks at wavelength (200 to 700 nm)

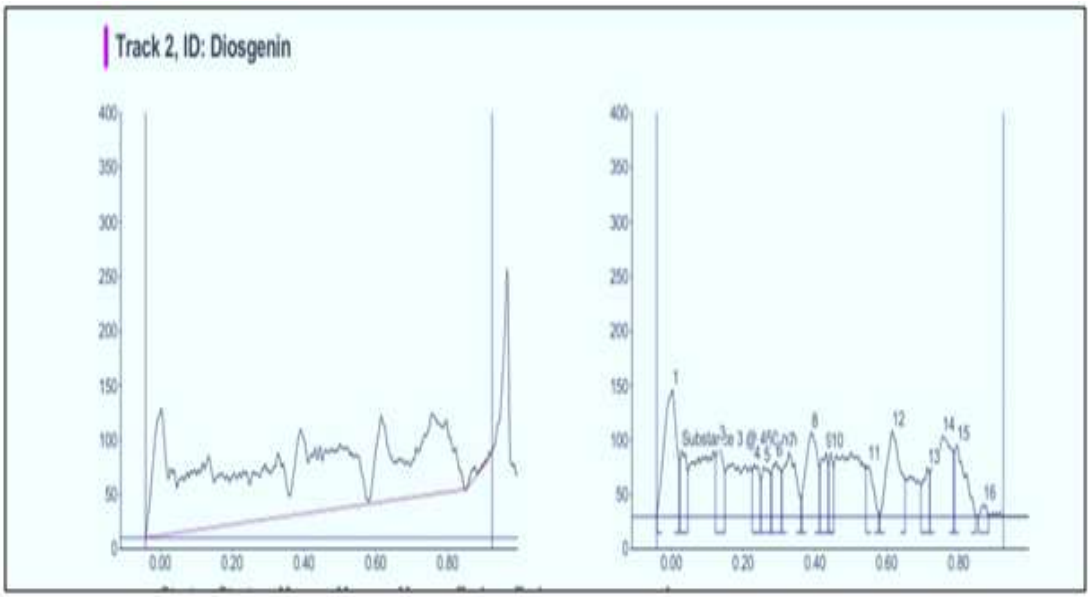


Fig no. 3 Chromatograms of Diosgenin at wavelength (450 nm)

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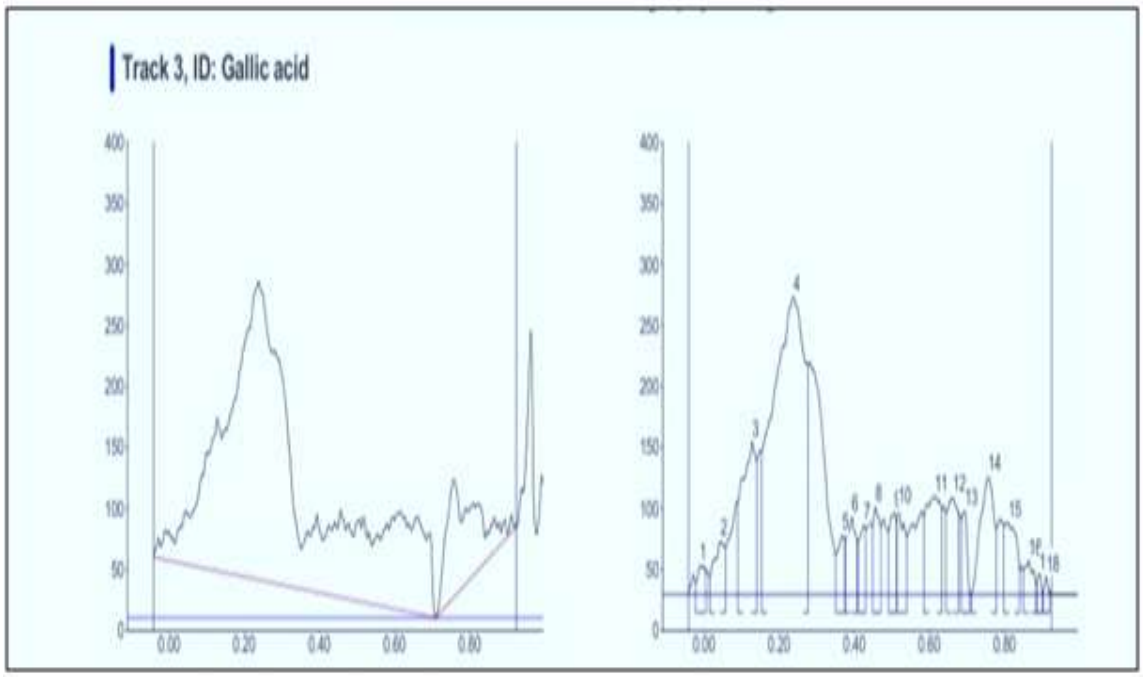

Fig no.4 Chromatograms of Gallic acid at wavelength (278 nm)

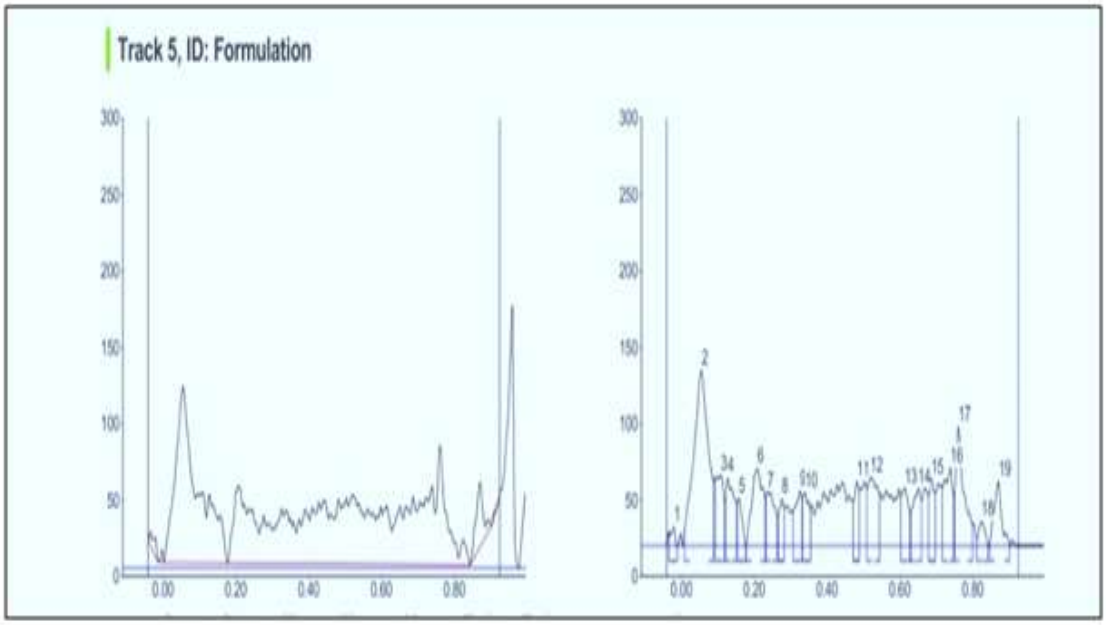

Fig no.5 Chromatograms of Polyherbal formulation at wavelength (450 nm)

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Sr.no	No. of peaks	Max. Rf value	Height of peak	Area of peak
1	1	-0.02	0.4	125.2
2	2	0.06	42.3	3831.9
3	3	0.11	29.7	842.4
4	4	0.13	27.8	857.7
5	5	0.16	0.0	333.7
6	6	0.21	32.2	1290.2
7	7	0.24	19.5	711.3
8	8	0.28	24.5	363.5
9	9	0.33	31.1	554.6
10	10	0.34 (Gallic acid)	24.6	516.2
11	11	0.48 (Diosgenin)	36.3	473.0
12	12	0.52	34.0	1051.0
13	13	0.61	21.8	645.3
14	14	0.65	29.9	709.7
15	15	0.69	34.8	530.7
16	16	0.74	33.7	1022.3
17	17	0.76	14.3	1462.7
18	18	0.83	0.4	223.8
19	19	0.87	0.2	710.3

Table no.1 No. of peaks detected in Sample, their Rf values, peak height and peak area

Quantitative estimation of marker compounds in polyherbal formulation by following formula-
% Assay-[AUC of test/AUC of standard]×[dil.of standard/dil.of test]×100

Sr. no	Name of compound	Volume (w/v)	Area	% w/w
1	Diosgenin	5 µl	516.2	1.456%
2	Gallic acid	5 µl	473.0	1.027%

Table no.2 Qauntification of Biomarker in Polyherbal formulation

IV. CONCLUSION-

This is the first report for the standardization of polyherbal formulations, Incorporation of this will authenticate quality thereby reducing further problems. Quality is inspected at right starting point then it will eliminate all bottlenecks in quality control of the polyherbal formulations to obtain better formulations. These methods also can be applied by the herbal manufacturers to estimate all the markers like Gallic acid and Diosgenin in their products as routine quality control and to keep a check on to the batch to batch variation.

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