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ORIGINAL

Identification, Validation and Standardization of Bioactive Molecules Using UPLC/MS-QToF, UHPLC and HPTLC in Divya-Denguenil-Vati: A Penta-Herbal Formulation for Dengue Fever

Acharya Balkrishna[1,2] · Monali Joshi[1] · Meenu Tomer[1] · Sudeep Verma[1] · Seema Gujral[1] · Vallabh Prakash Mulay[1] · Jyotish Srivastava[1] · Anurag Varshney[1,2,3]

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Abstract

For the last fifty years, Dengue has been one of the most common mosquito-borne arboviral infections which has spread over the tropical and subtropical world. Divya-Denguenil-Vati (DNV) has been formulated by blending five specific herbs for effective resolution of Dengue fever. In the present study, we aimed to identify, develop, validate, and standardize methods for Divya-Denguenil-Vati (DNV), on UHPLC and HPTLC analytical platforms, with rapid, sensitive, accurate and rugged attributes. At first, 97 phyto-constituents were identified by UPLC/MS-QToF in Divya-Denguenil-Vati. UHPLC method was then developed and validated for simultaneous determination of gallic acid, 5-HMF, protocatechuic acid, magnoflorine, methyl gallate, berberine, rutin, ellagic acid, β-ecdysone and rosmarinic acid in DNV. Four selected markers, gallic acid, rosmarinic acid, magnoflorine and rutin were further developed and validated on HPTLC. Analytical processes were validated as per ICH Q2 (R1) guidelines; and were found linear ($r^2 > 0.99$), sensitive, precise (%RSD < 5%), and accurate, as indicated by high recovery values (88–105%). The limit of detection and quantification were also established for these phyto-metabolites, with their respective RSDs within 5% limits. Finally, these validated methods were employed to test twenty six different commercial batches of DNV. The quality, reproducibility and consistency of DNV have been well established using these developed and reliable analytical tools. These analytical strategies successfully set a path forward for robust quality evaluation and standardization of Divya-Denguenil-Vati, and other related herbal formulations.

Keywords UPLC/MS-QToF · UHPLC · HPTLC · Validation · Ayurveda · Denguenil

Introduction

Dengue is a mosquito-borne viral disease where the virus is transmitted by female mosquitoes, mainly of the species *Aedes aegypti* and, to a lesser extent, *Aedes albopictus*. It has spread in over a hundred tropical and subtropical countries in the last few decades [1]. In 2020, Dengue affected countries such as Brazil, Bangladesh, India, Indonesia, Maldives, Nepal, Singapore, Sri Lanka and many more, where a large number of cases were reported [2]. Dengue virus belongs to the Flaviviridae family which has four distinct but very closely related serotypes namely DENV-1, DENV-2, DENV-3 and DENV-4 [3]. Dengue infection could be completely subclinical and could also manifest with severe flu-like symptoms. Usual febrile symptoms of dengue, prolonged high fever, backaches, muscle and joint pains that overlap with other concurrent mosquito-borne infections such as chikungunya and malaria [4]. At times, patient could enter into critical phase after 3–7 days of illness where Dengue Haemorrhagic Fever (DHF), characterized by plasma leakage into the tissues, an acute drop in the platelet counts, and internal bleeding that could be fatal, if not hospitalized in time [5].

At present, there is no effective treatment for dengue fever, analgesics along with anti-pyretics are the usual

✉ Anurag Varshney
anurag@prft.co.in

1 Drug Discovery and Development Division, Patanjali Research Institute, NH-58, Haridwar, Uttarakhand 249 405, India

2 Department of Allied and Applied Sciences, University of Patanjali, Patanjali Yogpeeth, Roorkee-Haridwar Road, Haridwar, Uttarakhand 249 405, India

3 Special Centre for Systems Medicine, Jawaharlal Nehru University, New Delhi 110 067, India

therapy to provide symptomatic relief from the Dengue infection. As there are several potential herbal medicines against viral fevers, countries like India use its vast herbal bioresources for a sustainable solution to the menace of Dengue [6]. Divya-Denguenil-Vati (DNV) is one such formulation, which is a unique blend of five herbs, namely *Tinospora cordiofolia* (Heart-leaved moonseed, Giloy, Saptaśirikā aromapatrā), *Aloe barbadensis* (Aloe vera, Majjapatrakam aṅgārasumam), *Carica papaya* (Papaya, Kumbhakarkaṭikā kṛṣṇerūḥ), *Punica granatum* (Pomegranate, Dāḍimakam dantabījam) and *Ocimum sanctum* (holy basil, Tulsi, Sumañjarikā rāmā). These herbs have been described for their efficient therapeutic uses in the ancient medicinal texts. One of our previous studies has validated and established anti-dengue properties of poly-herbal medicine, Divya-Denguenil-Vati in the zebrafish model of disease [7].

Standardization and establishment of quality parameters are a significant step to ensure the safety, purity, potency, and efficacy of the medicine. Inherent variation and inconsistency, in terms of bioactive molecules, present in raw materials used to formulate herbal medicines pose a challenge for their quality evaluation and standardization. Therefore, these herbal medicines have lesser acceptability as compared to their synthetic counterparts.

Few attempts have been made to develop methods for simultaneous determination of phyto-metabolites in poly-herbal medicines by RP-HPLC and HPTLC such as Coronil [8], Divya Swasari Vati [9, 10], Ukgansan [11] and SJT-DI-02 [12]. The herb extracts utilized in the production of these formulations are not the same as those used in DNV. For the measurement of bioactive compounds, there are various official US and European pharmacopeial procedures available, but they were developed for single herbs. Therefore, there is a pressing need to establish a simple, dependable, and repeatable approach for standardizing poly-herbal dosage forms. The present study attempts to identify signature markers and develop, optimize and validate a method for the first time in DNV. An integrated approach of identification by UPLC/MS-QToF, development, validation and standardization by UHPLC and HPTLC was adopted to achieve a sensitive, precise, accurate and rugged analytical method for DNV. In addition, several commercial batches of DNV were used to gauge the method's usefulness and reproducibility by both the techniques.

Materials and Methods

Chemicals and Reagents

The HPLC and LCMS grade solvents acetonitrile and methanol were obtained from J. T. Baker (USA) and Honeywell (Germany). Analytical grade solvents, glacial acetic acid, and formic acid, toluene and ethyl acetate were purchased from Rankem, India. Deionized water, purified by a Milli-Q system (Millipore, USA), was used throughout the study. Reference standards with defined purity in parenthesis, for gallic acid (97.3%), magnoflorine (99.0%), berberine (88.4%), ellagic acid (99.6%), rosmarinic acid (98.0%) were procured from Sigma Aldrich (USA). 5-HMF (97.3%), methyl gallate (99.5%) from Tokyo Chemical Industries (Japan) and protocatechuic acid (99.5%), rutin (98.7%) from Natural Remedies (India) and β-ecdysone (99.9%) were purchased from PHY-proof (Germany), respectively. Divya-Denguenil-Vati (DNV) batches were sourced from Divya Pharmacy, Haridwar (India), and were stored in airtight bottles. DNV (batch # 015) was used for UPLC/MS-QToF identification, as well as UHPLC and HPTLC validation studies. Twenty-six commercial batches of DNV, # 014, # 015, # 029 to # 034, and # 036 to # 053 were analyzed to evaluate quality consistency across different DNV batches.

Sample Preparation

Ten DNV tablets from a given batch were powdered using mortar and pestle to get a homogenized sample for analysis. 500 mg of powdered DNV was added to 10.0 mL, 50:50 methanol:water for UPLC/MS-QToF and in ratio of 80:20 for HPLC and HPTLC. The solution was then vortexed to extract desired phyto-constituents into the solution. The resulting solution was sonicated for 30 min, centrifuged for 5 min at 10,000 rpm and then filtered by 0.45 µm nylon filter before injecting.

UPLC/MS-QToF Method

Ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/MS-QToF) has been used to identify phyto-constituents present in DNV. Experiments were performed using a Xevo G2-XS QToF mass spectrometer (Waters Corporations, USA) connected to the ACQUITY UPLC I-Class System via electrospray ionization (ESI) interface in the positive and negative mode of ionization. A wide mass range (*m/z* 50–1200) was selected for the acquisition of accurate mass precursor and fragment ion data. The capillary voltage, cone voltage, source temperature and desolvation temperature were maintained at 1.0 kV (positive mode) and 2 kV (negative mode), 40 V, 120 °C and 500 °C, respectively. High-purity nitrogen gas was used for desolvation and cone, with gas flow rates 900 and 50 L h^{-1}. Argon was used as collision gas. The low collision energy (low CE) of 5 eV and high collision energy (High CE) of 15–60 eV were applied in the collision cell. Analyses were performed in full scan mode and the scan time was set to 0.5 s. To ensure mass accuracy of the optimized MS

conditions, leucine-enkephalin (Waters Corporations, USA) (*m/z* 556.2766 in positive mode and *m/z* 554.2620 in negative mode) was used as a reference (lock mass) at a concentration of 200 pg mL^{-1} and a flow rate of 10 µL min^{-1}. The Lock–Spray scan time was set at 0.25 s with an interval of 30 s. The instrument was calibrated by sodium formate solution as the calibration standard to achieve mass accuracies of < 0.2 mDa in the range of 50–1200 *m/z*. Chromatographic separations were achieved using an ACQUITY UPLC HSS T3 (Waters Corporation, USA) (100 × 2.1 mm, 1.7 µm) column. The column temperature was maintained at 40 °C throughout the analysis, whereas samples were kept at 20 °C for analysis. The elutions were carried out at a flow rate of 0.3 mL min^{-1} using gradient elution of mobile phase 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Methanol: isopropyl alcohol:water in 50:25:25 ratios were used as wash solvent for rinsing the injection needle. The elution gradient program of mobile phase B was, 2% for 0–5 min, 2–10% for 5–10 min, 10-25% for 10–35 min, 25–60% for 35–45 min, 60–80% for 45–55 min, 80% for 55–60 min. 1 µL of the sample solution was injected for scanning, and the chromatograms were recorded for 60 min.

UHPLC Method

Ultra high-performance liquid chromatography (UHPLC) was used to develop and validate a method for quantification of the marker components in DNV. Method development and validation was performed by Prominence-XR UHPLC system (Shimadzu, Japan) equipped with Quaternary pump (NexeraXR LC-20AD XR), DAD detector (SPD-M20 A), Auto-sampler (Nexera XR SIL-20 AC XR), Degassing unit (DGU-20A 5R) and Column oven (CTO-10 AS VP). Shodex C18-4E (5 µm, 4.6 × 250 mm) column was used for chromatographic separation. Chromatograms of DNV samples were recorded at 270 nm for gallic acid, 5-HMF, protocatechuic acid, magnoflorine, methyl gallate, and berberine, at 255 nm for rutin, ellagic acid, β-ecdysone and at 325 nm for rosmarinic acid.

HPTLC Method

The HPTLC system (CAMAG, Switzerland) equipped with an automatic TLC sampler (ATS_4), TLC scanner 4, TLC Visualizer was used for the analysis. HPTLC was performed on a pre-coated silica gel 60 F_{254} (cat # 1.05554.0007) aluminium-backed TLC plate. For HPTLC fingerprinting, 10 µL of each standard and sample, in duplicates were applied as 8 mm band using the spray-on technique on TLC plate. The plate was then developed using a twin trough chamber (CAMAG, Switzerland) pre-saturated with the mobile phase. The migration distance of the mobile phase was up to 70 mm. The developed TLC plate was air dried, and imaged at 254 nm by TLC visualizer (CAMAG, Switzerland). TLC plates were scanned and chromatograms were recorded at 265, 280, and 330 nm.

Standard Preparation for UHPLC and HPTLC

1000 µg mL^{-1} stock solution of each marker compound was prepared by dissolving accurately weighed reference standards in methanol. 0.05 mL of 1000 µg mL^{-1} from each standard stock solution was taken and diluted to 1 mL with methanol:water in ratio 80:20 to prepare 50.0 µg mL^{-1} of mix working standard solution.

Validation of UHPLC and HPTLC

Validation of the UHPLC and HPTLC methods was carried out following the International Council on Harmonisation (ICH) [13] and pharmacopeial guidelines [14].

System Suitability and Specificity

System performance was ascertained before starting analysis on the instrument. Area % RSDs [Not More Than: NMT 2] of six replicates ($n = 6$), tailing factor (NMT 2) and theoretical plates (Not Less Than: NLT 5000) were calculated to prove that system suitability were well within acceptability criteria. The specificity of an analytical method can be defined as the ability to distinguish target and non-target analytes in the presence of matrix components. Specificity was observed by comparing chromatograms of sample, standard and diluent blank. The peak purity of each compound was estimated using LabSolution software. The 3-point peak purity was evaluated by comparing the similarity index, threshold value and the purity index. If the similarity index (SI) is greater than threshold value (t) and purity index (PI) is greater than and equal to zero, then the peaks are considered to be pure.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ are important parameters of an analytical method. LOD is the amount of analyte which can be detected but not necessarily be quantified, LOQ is the lowest amount of analyte which can be quantified accurately and repeatedly with suitable precision. In UHPLC studies, LOD and LOQ were computed by the signal-to-noise (S/N) ratio method. The concentration with an S/N ratio of 3:1 is referred to as LOD, and the concentration with an S/N ratio of 10:1 is referred to as LOQ. The LOD and LOQ values were verified by injecting six replicates ($n = 6$) of minimum detectable and minimum quantifiable concentrations of analyte, respectively. The limit for %RSDs of peak area was set at NMT

33% for LOD and NMT 10% for LOQ, as recommended by ICH guidelines [13].

Linearity and Range of Method

Linearity is plotted and the correlation coefficient (r^2) is calculated to check the relationship between concentration and peak area of the analytes in DNV. The limit of the correlation coefficient was set to be NLT 0.99 for both UHPLC and HPTLC. The range of testing for the method was also judged based on the linear curve obtained between analyte concentrations and responses of the individual markers.

Precision, Accuracy and Robustness

Precision and accuracy of the optimized procedure were determined by calculating RSDs of intraday, interday and recovery studies. Interday and intraday precision were calculated by injecting different replicates $n = 6$ for UHPLC, and $n = 15$ and $n = 30$ for HPTLC. Recovery was calculated by spiking reference standards at three different levels, 80%, 100% and 120% of the assay values to check the accuracy of the developed and optimized method. The concentrations of spiked samples were estimated by comparing test samples to a standard curve prepared using known analyte concentrations. Robustness was demonstrated by making deliberate changes to the method, to check its reliability [13].

Data Analysis

Characterization of the marker analytes on UPLC/MS-QToF was performed by using UNIFI software, version 1.9.4.053 (Waters Corporation, USA). UHPLC-PDA analysis was performed on LabSolutions (Shimadzu, Japan). HPTLC studies were performed and analyzed using winCATS software (CAMAG, Switzerland). Statistical analyses and displays were generated using GraphPad Prism 8.0 (GraphPad Software, USA) software suite.

Results

Characterization of Phytochemical Markers in DNV by UPLC/MS-QToF

UPLC/MS-QToF has emerged as the most rapid and sensitive technique for the characterization of phytochemicals in poly-herbal formulations [8, 9]. In case of DNV, total 97 phyto-compounds were identified, as confirmed through the in-house library and available literature (Table 1). High-energy fragmentation patterns, *m/z* ratio, and mass error were considered primary criteria for confirming the compounds. Chromatograms recoded in the positive (Fig. 1a), and negative modes (Fig. 1b) were identified with peak numbers, as per their time of elution or retention times (t_R). DNV predominantly contains, 21 polyphenols, 16 alkaloids, 14 flavonoids, 13 terpenoids, and 10 tannins. In addition, five fatty acids, four organic acids, four esters, three amino acids, three plant growth hormones, other steroids, anthocyanin, and furan were also found to be present in the DNV formulation.

Development and Optimization of UHPLC Method

Optimization plays a vital role in the analytical method development process. The separation and resolution of chromatographic peaks can be achieved by optimizing sample preparation and column chemistry using the appropriate polarity of solvents. Various combinations of solvents such as methanol:water, 20:80, 50:50, and 80:20 were tried for sample preparation and the optimal diluent which was 80:20, methanol:water was chosen for the final preparation. By previous experience and literature search, we chose to employ reverse phase chromatography to develop a method for phyto-metabolites in DNV. Two different columns Shodex C18-4E (5 µm, 4.6 × 250 mm) and GIST-HP C18 (3 µm, 4 mm × 150 mm) were tested, and Shodex C18 was selected for better separation. Different combinations of mobile phases were also employed to standardize the DNV by ten different polar to mid polar molecules present in the herbs contributed for its preparation. Before finalising the schema of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile and methanol (50:50) ratio (solvent B) at 2% B for 0–3 min, 2–8% B from 3 to 10 min, 8–10% B from 10 to 20 min, 10–28% B from 20 to 40 min, 28–38% B from 40 to 55 min, 38–55% B from 55 to 60 min, 55–90% B from 60 to 65 min, 90–2% B from 65 to 66 and 2% B from 66 to 70 min, with a flow rate of 1.0 mL min^{-1}.

Validation of Optimized UHPLC Method for Quantitative Analysis of Marker Components in DNV

Validation of ten marker components, gallic acid, 5-HMF, protocatechuic acid, magnoflorine, methyl gallate, berberine, rutin, ellagic acid, β-ecdysone and rosmarinic acid were conducted on UHPLC, as per the ICH guidelines. The UHPLC system was first assessed for its suitability by calculating various parameters like relative standard deviations (RSDs) of peak area, tailing factor and theoretical plates. RSDs were found in the range of 0.22 to 1.92%, tailing factors were found minimum for 5-HMF (1.18) and maximum for ellagic acid (1.88) and theoretical plates were above 12,000 for all the marker compounds (Table 2). There was no interference of co-eluting peaks at the same retention time in the chromatograms comparison of DNV sample, reference

Table 1 Mass spectrometric data of identified phyto-metabolites in Divya-Denguenil-Vati (DNV) (Batch# DNV015) by UPLC/MS-QToF in positive and negative mode

S. no	Component name	Peak No as in Fig. 1[A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
Organic acids									
1	D-Gluconic acid	**1**	$C_6H_{12}O_7$	196.0583	195.0516	0.6	0.85	−H	195.0513, 165.0404, 113.0247, 101.0246
2	Malic acid	**2**	$C_4H_6O_5$	134.0215	133.0145	0.2	1.22	−H	133.0144, 115.0037
3	Citric acid	**3**	$C_6H_8O_7$	192.0270	191.0202	0.4	1.41	−H	191.0200, 161.0458, 143.0351, 111.0091
4	Isocitric acid	**4**	$C_6H_8O_7$	192.0270	191.0201	0.3	1.65	−H	191.0199, 161.0446, 111.0091
Alkaloids									
5	*N*-Methylcoclaurine	**16**	$C_{18}H_{21}NO_3$	299.1521	300.1581 298.1444	−1.3 −0.5	10.18 10.20	+H −H	300.1581, 255.1012, 238.0863, 194.0749, 161.0595, 107.0481 298.1449, 239.0710, 197.0458, 161.0454, 147.0443, 121.0291
6	Coclaurine	**19**	$C_{17}H_{19}NO_3$	285.1365	286.1434	−0.3	11.20	+H	286.1431, 255.1009, 243.1028, 161.0591, 107.0481
7	Caffeine	**23**	$C_8H_{10}N_4O_2$	194.0804	195.0858	−1.9	12.43	+H	195.0859, 167.0721, 138.0643, 123.0420, 110.0698
8	Magnoflorine	**31**	$C_{20}H_{24}NO_4$	342.1705	342.1695 340.1559	−0.5 0.4	15.38 15.39	−e −2H	342.1697, 297.1114, 282.0876, 265.0848, 237.0893, 222.0656 340.1556, 310.1087, 282.1131, 252.0427, 224.0479, 196.0523
9	L-Tetrahydrocolumbamine	**38**	$C_{20}H_{23}NO_4$	341.1627	342.1693	−0.6	17.16	+H	342.1699, 314.1749, 297.1117, 282.0892, 265.0851
10	Menisperine	**44**	$C_{21}H_{25}NO_4$	355.1784	356.1854	−0.3	19.17	+H	356.1855, 342.1736, 311.1278, 279.1016, 265.0852
11	*N*-trans-Caffeoyltyramine	**60**	$C_{17}H_{17}NO_4$	299.1158	300.1226 298.1089	−0.4 0.4	25.32 25.35	+H −H	300.1244, 163.0378, 147.0440, 121.0640, 91.0543 298.1090, 190.0510, 135.0450, 125.0243
12	Pseudocarpaine	**64**	$C_{28}H_{50}N_2O_4$	478.3771	479.3850	0.7	26.51	+H	479.3853, 461.3765, 240.1950, 222.1850, 121.1042
13	Dihydroberberine	**66**	$C_{20}H_{19}NO_4$	337.1314	338.1382	-0.5	27.02	+H	338.1385, 307.0843, 279.0886, 265.0733, 250.0861, 236.0703
14	(+)-Carpaine	**70**	$C_{28}H_{50}N_2O_4$	478.3771	479.3849	0.5	28.69	+H	479.3850, 240.1947, 222.1843, 194.1913
15	*N*-cis-Feruloyl typamine	**75**	$C_{18}H_{19}NO_4$	313.1314	314.1383 312.1249	−0.4 0.7	31.20 31.23	+H −H	314.1385, 177.0531, 145.0267, 121.0634, 117.0323 312.1249, 297.1006, 190.0510, 148.0526, 135.0449
16	Palmatine	**77**	$C_{21}H_{21}NO_4$	351.1471	352.1541	−0.2	31.80	+H	352.1543, 336.1226, 320.0914, 292.0960, 278.0798, 263.0573, 248.0701
17	Berberine	**78**	$C_{20}H_{17}NO_4$	335.1158	336.1225	−0.6	31.86	+H	336.1226, 320.0914, 304.0963, 292.0960, 278.0798, 263.0573, 248.0701
18	Solasonin	**82**	$C_{45}H_{73}NO_{16}$	883.4929	884.5012 928.4943	1.0 3.2	34.81 34.81	+H +HCOO	884.5014, 866.4907, 722.4488, 576.3909, 414.3373, 253.1946, 157.1001 928.4947, 720.4324, 574.3730, 317.1428, 161.0457

Table 1 (continued)

S. no	Component name	Peak No as in Fig. 1[A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
19	Solamargine	**84**	$C_{45}H_{73}NO_{15}$	867.4980	868.5070 912.4991	1.7 2.9	36.11 36.10	+H +HCOO	868.5071, 850.4963, 722.4494, 576.3913, 414.3380, 253.1954, 157.1008 912.5008, 720.4319, 489.2812, 179.0521
20	β2-Chaconine	**85**	$C_{39}H_{63}NO_{11}$	721.4401	722.4482 766.4406	0.8 2.3	37.90 37.90	+H +HCOO	722.4483, 704.4378, 576.3905, 414.3380, 313.1992, 253.1944, 157.1001 766.4405, 531.2804, 247.0824, 179.0556
Flavonoids									
21	Gallocatechin gallate	**32**	$C_{22}H_{18}O_{11}$	458.0849	459.0926 457.0775	0.4 −0.1	15.81 15.85	+H −H	459.0946, 315.0521, 289.0723, 153.0180, 139.0376 457.0773, 331.0458, 305.0668, 241.0480, 193.0140, 169.0142, 125.0243
22	Luteolin 7-rutinoside	**33**	$C_{27}H_{30}O_{15}$	594.1585	595.1659 593.1510	0.2 −0.2	16.30 16.32	+H −H	595.1668, 559.1467, 457.1125, 379.0822, 325.0705 593.1513, 503.1198, 473.1092, 383.0773, 353.0668, 297.0759, 206.0824, 164.0716
23	Epigallocatechin gallate	**40**	$C_{22}H_{18}O_{11}$	458.0849	459.0924 457.0782	0.2 0.6	17.47 17.52	+H −H	459.0955, 391.1017, 289.0710, 153.0185, 139.0379 457.0782, 331.0463, 305.0674, 243.0706, 193.0140, 169.0144, 125.0244
24	Manghaslin	**42**	$C_{33}H_{40}O_{20}$	756.2113	757.2193 755.2051	0.7 1.0	18.82 18.85	+H −H	465.1038, 391.0999, 303.0490, 229.0495 755.2052, 300.0271, 271.0242, 119.0502
25	Quercetin 3,4′-dimethyl ether 7-alpha-L-arabinofuranosyl-(1-6)-glucoside	**43**	$C_{28}H_{32}O_{16}$	624.1690	625.1777 623.1617	1.4 0.0	18.98 19.00	+H −H	445.1117, 427.1051, 343.0825, 313.0714, 286.1183 623.1619, 477.1034, 327.0503, 169.0145
26	Clitorin	**48**	$C_{33}H_{40}O_{19}$	740.2164	741.2247 739.2102	1.1 1.1	20.89 20.92	+H −H	595.1674, 449.1097, 287.0543, 213.0534, 153.0186 739.2103, 593.1485, 405.1201, 284.0325, 255.0298
27	Epicatechin gallate	**50**	$C_{22}H_{18}O_{10}$	442.0900	443.0986 441.0833	1.4 0.6	21.41 21.46	+H −H	273.0748, 147.0445, 139.0384, 123.0437 441.0836, 314.0431, 289.0725, 245.0820, 169.0144, 125.0244
28	Rutin	**52**	$C_{27}H_{30}O_{16}$	610.1534	611.1611 609.1464	0.4 0.3	22.05 22.08	+H −H	465.1039, 303.0488, 257.0453, 229.0498 609.1468, 521.0976, 300.0272, 271.0246, 178.9984, 135.0452
29	Lithospermic acid	**53**	$C_{27}H_{22}O_{12}$	538.1111	537.1045	0.6	22.24	−H	493.1151, 387.1098, 295.0622, 267.0668, 159.0451
30	Isoquercetin	**55**	$C_{21}H_{20}O_{12}$	464.0955	465.1029 463.0885	0.1 0.3	22.74 22.77	+H −H	465.1053, 303.0495, 257.0452, 229.0497, 153.0180 463.0888, 421.1149, 301.0340, 300.0281, 271.0249, 255.0300, 151.0035
31	Nicotiflorin	**59**	$C_{27}H_{30}O_{15}$	594.1585	595.1670 593.1512	1.2 0.0	25.09 25.12	+H −H	595.1734, 287.0545, 232.0715 593.1515, 475.1087, 365.0665, 285.0402, 255.0295, 227.0346, 169.0148
32	Astragalin	**62**	$C_{21}H_{20}O_{11}$	448.1006	447.0937	0.4	25.86	−H	447.0942, 284.0332, 255.0300, 227.0350, 169.0138

Table 1 (continued)

S. no	Component name	Peak No as in Fig. 1[A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
33	Cirsilineol	**92**	$C_{18}H_{16}O_7$	344.0896	345.0961 343.0826	−0.8 0.3	42.64 42.64	+H −H	345.0962, 330.0727, 315.0489, 312.0622, 287.0549, 269.0461, 242.0581 343.0825, 328.0591, 313.0361, 268.0372, 241.0498, 214.0276
34	Salvigenin	**94**	$C_{18}H_{16}O_6$	328.0947	329.1008	−1.2	44.82	+H	329.1010, 314.0769, 296.0664, 268.0715, 240.0772, 133.0630
Polyphenols									
35	Gallic acid	**7**	$C_7H_6O_5$	170.0215	169.0146	0.4	3.65	−H	169.0143, 125.0246, 124.0166, 107.0140
36	Syringic acid	**11**	$C_9H_{10}O_5$	198.0528	197.0460	0.4	7.31	−H	197.0455, 179.0340, 169.0137, 151.0374, 135.0449, 123.0451, 109.0291
37	Protocatechuic acid	**13**	$C_7H_6O_4$	154.0266	153.0196	0.2	7.67	−H	153.0192, 137.0250, 108.0219
38	Feruloylsucrose	**14**	$C_{16}H_{20}O_9$	356.1107	355.1043	0.8	8.80	−H	355.1041, 311.1141, 293.1038, 249.1137, 139.0416
39	Neochlorogenic acid	**15**	$C_{16}H_{18}O_9$	354.0951	353.0886	0.7	10.07	−H	353.0877, 179.0554, 135.0450, 125.0240
40	Methyl gallate	**20**	$C_8H_8O_5$	184.0372	183.0302	0.3	11.24	−H	183.0302, 151.0403, 144.0455, 125.0235, 124.0167, 123.0092, 101.0255
41	Isochlorogenic acid	**24**	$C_{16}H_{18}O_9$	354.0951	353.0885	0.7	12.45	−H	353.0880, 191.0559, 175.0378, 161.0214, 115.0034
42	Chlorogenic acid	**25**	$C_{16}H_{18}O_9$	354.0951	353.0888	1.0	12.99	−H	353.0881, 191.0561, 179.0343, 173.0456, 163.0387, 135.0449, 119.0492
43	Phyllanthusiin E	**26**	$C_{13}H_8O_8$	292.0219	291.0152	0.6	13.41	−H	291.0134, 247.0253, 219.0301, 191.0351, 145.0294
44	4-*p*-Coumaroylquinic acid	**29**	$C_{16}H_{18}O_8$	338.1002	337.0933	0.4	15.12	−H	337.1034, 314.1024, 191.0560, 189.0196
45	Urolithin M5	**39**	$C_{13}H_8O_7$	276.0270	275.0203	0.6	17.23	−H	275.0205, 257.0097, 229.0146
46	*o*-Coumaric acid	**45**	$C_9H_8O_3$	164.0473	163.0401	0.1	19.48	−H	163.0401, 119.0502
47	Ferulic acid	**49**	$C_{10}H_{10}O_4$	194.0579	193.0510	0.4	21.34	−H	193.0508, 178.0268, 149.0604, 134.0374, 133.0292, 117.0352
48	Ellagic acid	**51**	$C_{14}H_6O_8$	302.0063	303.0129 300.9996	−0.6 0.6	21.84 21.89	+H −H	303.0133, 285.0024, 275.0195, 257.0087, 247.0251, 229.0133 300.9999, 283.9969, 257.0097, 245.0094, 229.0146
49	Methyl caffeate	**57**	$C_{10}H_{10}O_4$	194.0579	193.0509	0.2	24.54	−H	167.0322, 161.0241, 151.0388
50	Phloridzin	**58**	$C_{21}H_{24}O_{10}$	436.1370	435.1299	0.2	24.79	−H	435.1297, 361.1302, 197.0448, 165.0551
51	(−)-Rabdosiin	**61**	$C_{36}H_{30}O_{16}$	718.1534	717.1465	0.4	25.65	−H	717.1472, 519.0938, 475.1038, 431.1134, 339.0513, 321.0759, 135.0451
52	13,13a-Didehydro-9, 10-dimethoxy-2,3-(ethylenedioxy)-berbine	**67**	$C_{20}H_{19}NO_4$	337.1314	338.1384	−0.3	27.56	+H	338.1385, 322.1069, 294.1118, 279.0887, 265.0729, 236.0697
53	Rosmarinic acid	**68**	$C_{18}H_{16}O_8$	360.0845	359.0775	0.2	27.78	−H	359.0774, 197.0457, 161.0245, 133.0292
54	Aloin A	**69**	$C_{21}H_{22}O_9$	418.1264	417.1195	0.4	28.52	−H	417.1185, 297.0773, 193.0493
55	Aloin B	**72**	$C_{21}H_{22}O_9$	418.1264	417.1191	0.0	30.13	−H	417.1195, 297.0772, 179.0332, 135.0454

Table 1 (continued)

S. no	Component name	Peak No as in Fig. 1[A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
Tannins									
56	6-*O*-galloylglucose	**8**	$C_{13}H_{16}O_{10}$	332.0744	331.0680	1.0	3.97	−H	331.0680, 271.0468, 211.0247, 169.0144, 168.0066, 125.0241
57	Glucogallin	**10**	$C_{13}H_{16}O_{10}$	332.0744	331.0678	0.7	5.59	−H	331.0672, 271.0467, 211.0246, 169.0143, 124.0166
58	1,6-di-*O*-galloyl-glucose	**18**	$C_{20}H_{20}O_{14}$	484.0853	483.0784	0.4	11.11	−H	483.0781, 313.0565, 169.0142
59	2,6-Di-*O*-galloyl-β-D-glucose	**21**	$C_{20}H_{20}O_{14}$	484.0853	483.0781	0.1	11.78	−H	483.0780, 423.0571, 271.0460, 211.0248, 169.0140, 125.0241
60	1,2,4-tri-*O*-galloyl-beta-glucopyranose	**27**	$C_{27}H_{24}O_{18}$	636.0963	635.0889	−0.1	14.19	−H	635.0893, 483.0787, 313.0564, 169.0140
61	Corilagin	**30**	$C_{27}H_{22}O_{18}$	634.0806	633.0728	−0.5	15.31	−H	633.0728, 463.0512, 300.9986, 275.0192, 173.0455
62	1,3,4-tri-*O*-galloyl-beta-glucopyranose	**34**	$C_{27}H_{24}O_{18}$	636.0963	635.0889	−0.1	16.41	−H	635.0894, 465.0677, 313.0565, 169.0141
63	Ellagic acid hexoside	**35**	$C_{20}H_{16}O_{13}$	464.0591	463.0519	0.1	16.94	−H	463.0519, 300.9986, 299.9911
64	3,4,6-Tri-*O*-galloyl-beta-d-glucose	**37**	$C_{27}H_{24}O_{18}$	636.0963	635.0888	−0.2	17.11	−H	635.0893, 465.0668, 313.0595, 169.0138
65	1,2,6-Trigalloylglucose	**41**	$C_{27}H_{24}O_{18}$	636.0963	635.0890	0.0	18.06	−H	635.0893, 465.0682, 313.0566, 169.0143
Terpenoids									
66	Cordifolioside A	**22**	$C_{22}H_{32}O_{13}$	504.1843	549.1821	−0.4	12.23	+HCOO	421.1140, 293.0882, 271.0465, 211.0247, 119.0499
67	Tincordin	**46**	$C_{19}H_{22}O_6$	346.1416	391.1402	0.4	19.61	+HCOO	391.1403, 329.1399, 283.1337, 189.1283, 173.0971, 107.0506
68	Tinospinoside D	**47**	$C_{27}H_{36}O_{13}$	568.2156	567.2081	−0.2	19.91	−H	521.2032, 507.1147, 401.1830, 359.1501, 169.0146
69	Tinocordiside	**65**	$C_{21}H_{32}O_7$	396.2148	419.2038 441.2135	−0.2 0.5	26.66 26.65	+Na HCOO	419.2040, 239.1415, 161.0954 345.1347, 299.1282
70	Tinosponone	**71**	$C_{19}H_{22}O_5$	330.1467	375.1451	0.2	29.91	+HCOO	375.1452, 313.1447, 269.1532, 219.1389, 107.0505
71	Cordifoliside A	**74**	$C_{26}H_{34}O_{11}$	522.2101	545.1996 567.2081	0.3 −0.3	30.46 30.48	+Na +HCOO	545.2002, 462.1917, 365.1366, 311.1280, 265.1230, 203.0517 343.1188, 179.0557
72	Palmarin	**79**	$C_{20}H_{22}O_7$	374.1366	419.1354	0.7	32.66	+HCOO	419.1354
73	Tinosporide	**80**	$C_{20}H_{22}O_7$	374.1366	373.1291	−0.1	33.47	−H	373.1286, 329.1403, 285.1498, 135.0452
74	Cordifoliside B	**81**	$C_{26}H_{34}O_{11}$	522.2101	545.1993 567.2087	0.0 0.4	34.19 34.19	+Na +HCOO	545.1991, 401.1437, 365.1356, 257.1528, 217.1598, 161.0946 567.2082, 195.0666, 179.0555
75	Columbin	**90**	$C_{20}H_{22}O_6$	358.1416	357.1353	1	40.75	−H	357.1353, 313.1077, 269.1190, 173.0970, 157.0656
76	Isocolumbin	**91**	$C_{20}H_{22}O_6$	358.1416	357.1352	0.8	41.38	−H	357.1353, 313.1451, 297.1139, 171.1031, 135.0452
77	Ursolic acid	**96**	$C_{30}H_{48}O_3$	456.3604	455.3536	0.5	53.55	−H	455.3534, 279.2339
78	Betulinic acid	**97**	$C_{30}H_{48}O_3$	456.3604	455.3535	0.5	54.12	−H	455.3531, 407.3286

Table 1 (continued)

S. no	Component name	Peak No as in Fig. 1[A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
Steroids									
79	Ecdysone	**54**	$C_{27}H_{44}O_7$	480.3087	481.3172 525.3076	1.2 0.7	22.50 22.49	+H +HCOO	445.2958, 427.2852, 409.2747, 371.2220, 329.2117, 301.1797, 283.1696 525.3079, 461.2911, 357.1347, 319.1923, 159.1028
80	Makisterone A	**63**	$C_{28}H_{46}O_7$	494.3244	539.3230	0.5	26.06	+HCOO	539.3239, 479.3020, 319.1920
Anthocyanins									
81	Cyanidin-3,5-diglucoside	**28**	$C_{27}H_{30}O_{16}$	610.1534	609.1459	−0.3	14.68	−H	609.1463, 489.1041, 399.0720, 369.0612, 267.0663, 164.0717
Furan									
82	5-hydroxymethylfurfural	**12**	$C_6H_6O_3$	126.0317	127.0374	−1.5	7.56	+H	127.0383, 109.0285, 99.0438, 85.0290
Amino acids									
83	*N*-(1-Deoxy-1-fructosyl)isoleucine	**5**	$C_{12}H_{23}NO_7$	293.1475	294.1540	−0.7	2.27	+H	276.1437, 258.1326, 230.1377, 212.1279, 182.0815, 136.0764
84	*N*-(1-Deoxy-1-fructosyl)leucine	**6**	$C_{12}H_{23}NO_7$	293.1475	294.1539	−0.8	2.45	+H	276.1437, 258.1328, 230.1376, 212.1275, 161.0681
85	Phenylalanine	**9**	$C_9H_{11}NO_2$	165.0790	166.0844 164.0718	−1.8 0.1	4.61 4.63	+H −H	166.0861, 120.0793, 103.0530, 91.0536 164.0720, 147.0446, 123.0444
Fatty acids									
86	Malyngic acid	**86**	$C_{18}H_{32}O_5$	328.2250	351.2140 327.2185	−0.2 0.8	38.86 38.86	+Na −H	351.2145, 275.2014, 257.1947 327.2186, 291.1972, 229.1453, 211.1345, 171.1029
87	2,3,4-Trihydroxy-2,4-octadecadienoic acid	**87**	$C_{18}H_{32}O_5$	328.2250	351.2141 327.2186	−0.1 0.9	39.11 39.10	+Na −H	351.2139, 275.2026, 257.1890 327.2187, 291.1967, 229.1452, 211.1345, 171.1028
88	Tianshic acid	**88**	$C_{18}H_{34}O_5$	330.2406	329.2342	0.8	39.89	−H	329.2343, 311.2226, 293.2130, 229.1453, 211.1346, 171.1029, 139.1130
89	Sanleng acid	**89**	$C_{18}H_{34}O_5$	330.2406	329.2341	0.7	40.03	−H	329.2344, 311.2239, 229.1448, 211.1347, 183.1396, 167.1438, 107.0505
90	Linolenic acid	**95**	$C_{18}H_{30}O_2$	278.2246	279.2314	-0.5	53.09	+H	279.2334, 261.2236, 187.1485, 149.1331, 109.1010
Esters									
91	Trans-isoeugenol-*O*-glucuronide	**17**	$C_{16}H_{20}O_8$	340.1158	339.1091	0.6	10.64	−H	339.1091, 295.1193, 251.1290, 205.1236, 135.0436
92	7,8,9-Trihydroxy-1,2-dihydrocyclopenta[c] isochromene-3,5-dione	**36**	$C_{12}H_8O_6$	248.0321	247.0253	0.5	17.00	-H	247.0254, 219.0302, 191.0352, 173.0245, 145.0295, 135.0450, 117.0348
93	Tinosporine A	**83**	$C_{21}H_{26}O_7$	390.1679	413.1579 389.1612	0.8 0.7	35.25 35.26	+Na −H	413.1588, 359.1507, 323.1304, 313.1455, 296.1042 389.1612, 377.1610, 313.1447, 285.1447, 219.1025, 163.0403, 107.0489

Table 1 (continued)

S. no	Component name	Peak No as in Fig. 1 [A] and [B]	Formula	Neutral Mass (Da)	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts	High energy fragments
94	(2S)-3-{[6-*O*-(α-D-Galactopyranosyl)-β-D-galactopyranosyl]oxy}-2-hydroxypropyl (9Z,12Z,15Z)-9,12,15-o	**93**	$C_{33}H_{56}O_{14}$	676.3670	699.3557 721.3662	− 0.5 1.0	44.72 44.71	+ Na + HCOO	699.3559, 537.3035, 391.2454, 261.2215, 184.0728 642.3636, 415.1462, 397.1359, 313.2393, 277.2175, 235.0827
Plant growth hormones									
95	Gibberellin A1	**56**	$C_{19}H_{24}O_6$	348.1573	393.1554	0.0	24.44	+ HCOO	393.1556, 305.1384, 273.1467, 209.0790, 187.0975
96	(+)-Gibberellic acid	**73**	$C_{19}H_{22}O_6$	346.1416	345.1349	0.6	30.38	− H	345.1348, 327.1240, 283.1341, 239.1440, 225.0906, 137.0249
97	Gibberelline	**76**	$C_{19}H_{22}O_6$	346.1416	345.1352	0.9	31.70	− H	345.1351, 327.1246, 283.1345, 239.1447, 193.0511, 161.0960, 134.0375

standard and diluent blank, at 255, 270 and 325 nm (Fig. 2). Range of testing and along with linearity was ascertained for each reference standard at different concentrations. The observed correlation coefficient (r^2) was within the range of 0.9992 to 0.9999. The range of testing for gallic acid, protocatechuic acid, magnoflorine, methyl gallate, rutin and β-ecdysone was 1.0–100, whereas, for 5-HMF, berberine and rosmarinic acid, it was 0.5–100. For ellagic acid, range was found to be 2–100. LOD and LOQ were determined to define the sensitivity of the developed method. LOD and LOQ with their respective RSDs of peak area for gallic acid (0.5 µg g^{-1}; 4.47% and 1.0 µg g^{-1}; 2.78%), 5-HMF (0.3 µg g^{-1}; 2.18% and 0.5 µg g^{-1}; 1.38%), protocatechuic acid (0.5 µg g^{-1}; 5.19% and 1.0 µg g^{-1}; 8.36%), magnoflorine (0.5 µg g^{-1}; 3.59% and 1.0 µg g^{-1}; 4.16%), methyl gallate (0.5 µg g^{-1}; 3.52% and 1.0 µg g^{-1}; 1.81%), berberine (0.3 µg g^{-1}; 2.49% and 0.5 µg g^{-1}; 2.42%), rutin (0.5 µg g^{-1}; 2.34% and 1.0 µg g^{-1}; 1.26%), ellagic acid (1.0 µg g^{-1}; 6.4% and 2.0 µg g^{-1}; 7.47%), β-ecdysone (0.5 µg g^{-1}; 2.11% and 1.0 µg g^{-1}; 0.77%) and rosmarinic acid (0.3 µg g^{-1}; 2.30% and 0.5 µg g^{-1}; 2.94%), respectively (Table 2). Precision and accuracy of the method were evaluated by calculating RSDs of observed concentrations of analytes, during intraday (0.24–1.62%), interday (0.86–1.67%) and recovery (90.33–102.25%) studies. In intraday and interday study concentration of methyl gallate and berberine were found to be below the limit of quantification. To evaluate the robustness of the developed method, some deliberate changes were made in chromatographic conditions like column temperature and flow rate. %RSDs of area were then calculated for the 18 replicates (Table 2). The flow rate was changed from 1.0 mL min^{-1} to 0.5 and 1.5 mL min^{-1}, and %RSDs were found to be in the acceptable range of 6.88 to 16.00%. The column temperature was set at 38 and 42 °C and %RSDs were found to be at 3.17 to 18.84%. The intermediate precision of the method was also assessed by calculating %RSDs of intraday and interday precision (n = 12), which were found in the range of 2.19 to 8.48% (Table 2).

Optimization of HPTLC Methods

HPTLC method was optimized by selecting mobile phase according to the polarity of molecules. Mobile phases in different combinations were tested before finalizing the schema of, toluene:ethyl acetate:formic acid (5:5:1 v/v/v) for gallic acid and rosmarinic acid; and ethyl acetate:formic acid:acetic acid:water (10:1.1:1.1:2.3 v/v/v/v) for magnoflorine and rutin.

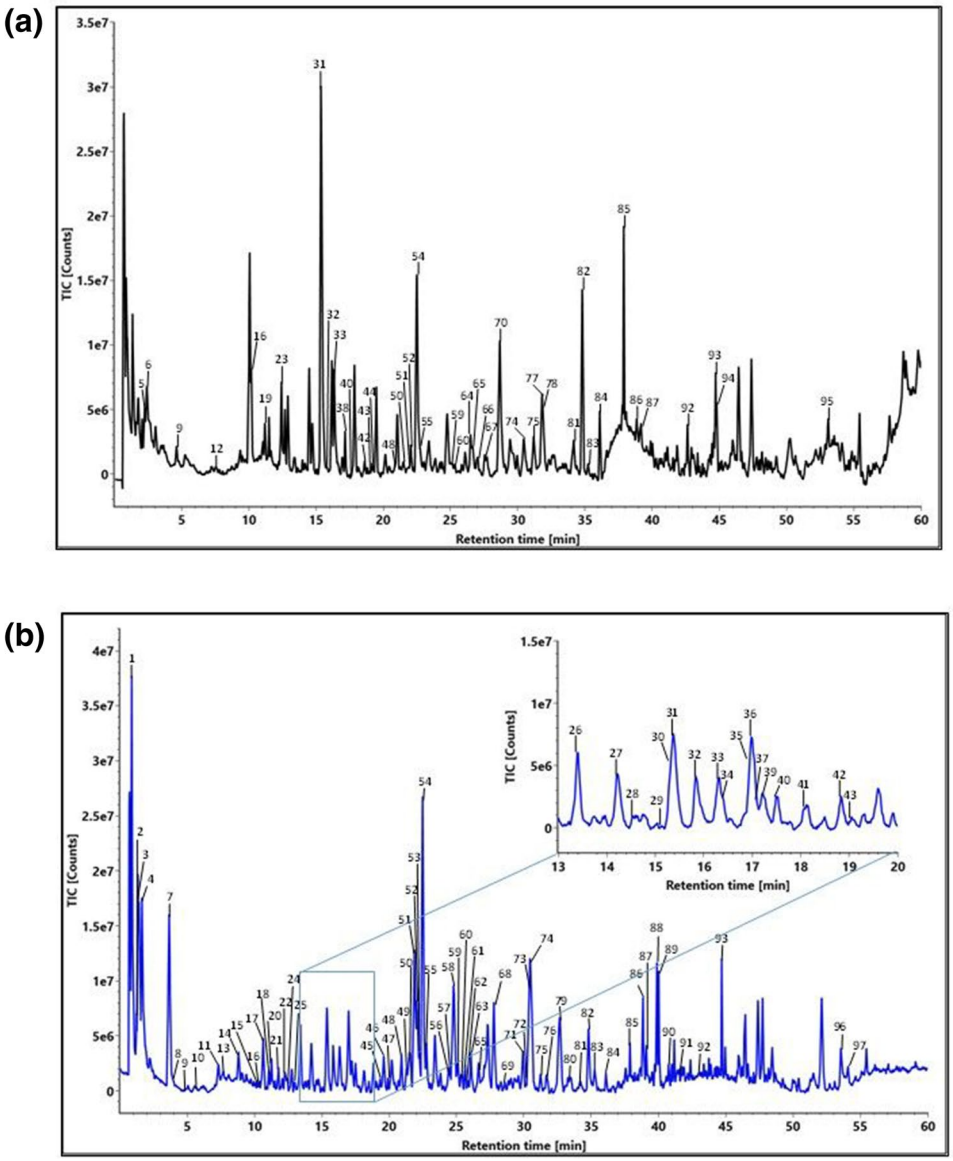

Fig. 1 Total ion chromatogram (TIC) of 97 compounds identified in Divya-Denguenil-Vati (DNV) (Batch# DNV015) using UPLC/MS-QToF (**a**) positive mode (**b**) negative mode. Peak numbers are assigned to identified phyto-metabolites, as per the detailed spectrometric data shown in Table 1

Validation of Optimized HPTLC Method for Quantitative Analysis of Marker Components in DNV

For standardization of HPTLC method for DNV, four signature markers, gallic acid, rosmarinic acid, magnoflorine and rutin were identified on fingerprinting plate and then validated (Fig. 3). Linear regression was plotted between each phyto-constituents concentration and peak area. These plots were found to be linear with co-relation coefficient (r^2) in the range of 0.995 to 0.999. The regression equation and linearity range was found be, $y = 14.20X + 244.3$ and 20–400 (gallic acid), $y = 14.29X + 18.47$ and 50–130 (rosmarinic acid), $y = 3.54X + 69.69$ and 50–600 (magnoflorine), and $y = 4.95X - 4.72$ and 50–180 (rutin) (Table 3). LOD and LOQ of marker components were calculated by the residual plot method [15]. LOD and LOQ, respectively, were observed for gallic acid (20.03, 60.69 µg g^{-1}), rosmarinic acid (0.71, 2.14 µg g^{-1}), magnoflorine (16.22, 49.15 µg g^{-1}) and rutin (0.9, 2.72 µg g^{-1}). RSDs of area calculated for LOD and LOQ were ranged from 1.02 to 2.92% and 1.8 to 3.08%, respectively. Intraday and interday precision at three concentrations, low (80%), medium (100%) and high (120%) of assay were studied, %RSDs found were in the range of 0.58 to 4.75%. Recoveries of four markers were found between 88.97 and 105.4% (Fig. 4).

Quantitative Determination of Targeted Analytes Using UHPLC and HPTLC in DNV

Post-validations of UHPLC and HPTLC analytical methods, 26 commercial batches were analyzed to check

Table 2 Validation parameters for selected marker components of Divya-Denguenil-Vati (DNV) (Batch# DNV015) using UHPLC analysis

Validation parameters	Acceptance criteria		Marker components of DNV									
			Gallic acid	5-HMF	Protocatechuic acid	Magnoflorine	Methyl gallate	Berberine	Rutin	Ellagic acid	Beta-ecdysone	Rosmarinic acid
System suitability												
Area % ($n=6$)	%RSD NMT 2%		1.92	0.74	0.93	0.59	1.14	0.46	0.74	0.22	0.43	1.58
Tailing factor	NMT 2		1.25	1.18	1.37	1.73	1.36	1.74	1.27	1.88	1.27	1.43
Theoretical plates	NLT 5000		12,311	22,250	21,734	24,526	41,348	157,460	229,372	145,734	220,554	154,401
Linearity	Correlation coefficient (r^2) NLT 0.99		0.9992	0.9995	0.9996	0.9993	0.9992	0.9995	0.9997	0.9994	0.9996	0.9999
	Regression equation		$y=0.0191x-0.0462$	$y=0.0418x+0.0399$	$y=0.0155x+0.0092$	$y=0.0265x-0.0211$	$y=0.0246x+0.0334$	$y=0.0373x+0.0261$	$y=0.0178x+0.0159$	$y=0.0334x-0.0782$	$y=0.0118x+0.0120$	$y=0.0261x+0.0094$
	Range (µg mL^{-1})		1–100	0.5–100	1–100	1–100	1–100	0.5–100	1.0–100	2–100	1–100	0.5–100
Precision												
Intraday ($n=6$)	%RSD NMT 5%		0.83	0.88	1.62	1.44	BLQ	BLQ	0.84	1.58	0.24	0.80
Interday ($n=6$)			1.31	1.13	0.93	0.86	BLQ	BLQ	0.96	0.86	0.94	1.67
Recovery (%) ($n=9$)	80–120%	Conc.%	% Recovery									
		80	91.23	99.92	94.72	96.44	91.38	94.59	100.18	97.07	95.91	90.33
		100	95.13	96.67	94.04	94.33	94.49	95.54	98.03	99.75	99.99	98.32
		120	99.66	92.77	98.42	95.50	96.89	94.72	96.38	94.29	100.01	102.25
		Mean	95.34	94.45	95.72	95.42	94.25	94.95	98.19	97.04	98.64	96.97
Intermediate precision ($n=12$)	%RSD NMT 20		3.72	6.88	6.28	8.48	BLQ	BLQ	4.46	4.40	2.92	2.19
Robustness												
Flow rate ($n=18$)			6.88	15.08	12.64	16.00	BLQ	BLQ	8.72	10.77	11.26	7.17
Column temp. ($n=18$)			18.84	12.04	17.55	9.34	BLQ	BLQ	5.80	3.17	11.91	8.21
Limit of Detection (LOD)	%RSD of area NMT 33%		4.47	2.18	5.19	3.59	3.52	2.49	2.34	6.41	2.11	2.30
	LOD (µg g^{-1})		0.5	0.3	0.5	0.5	0.5	0.3	0.5	1.0	0.5	0.3
Limit of quantification (LOQ)	%RSD of area NMT 10%		2.78	1.38	8.36	4.16	1.81	2.42	1.26	7.47	0.77	2.94
	LOQ (µg g^{-1})		1.0	0.5	1.0	1.0	1.0	0.5	1.0	2.0	1.0	0.5

All the parameters are validated as per ICH-Q2 (R1), USP < 1225 > guidelines

BLQ Below limit of quantification, *RSD* Relative Standard Deviation, *NMT* Not more than, *NLT* Not less than

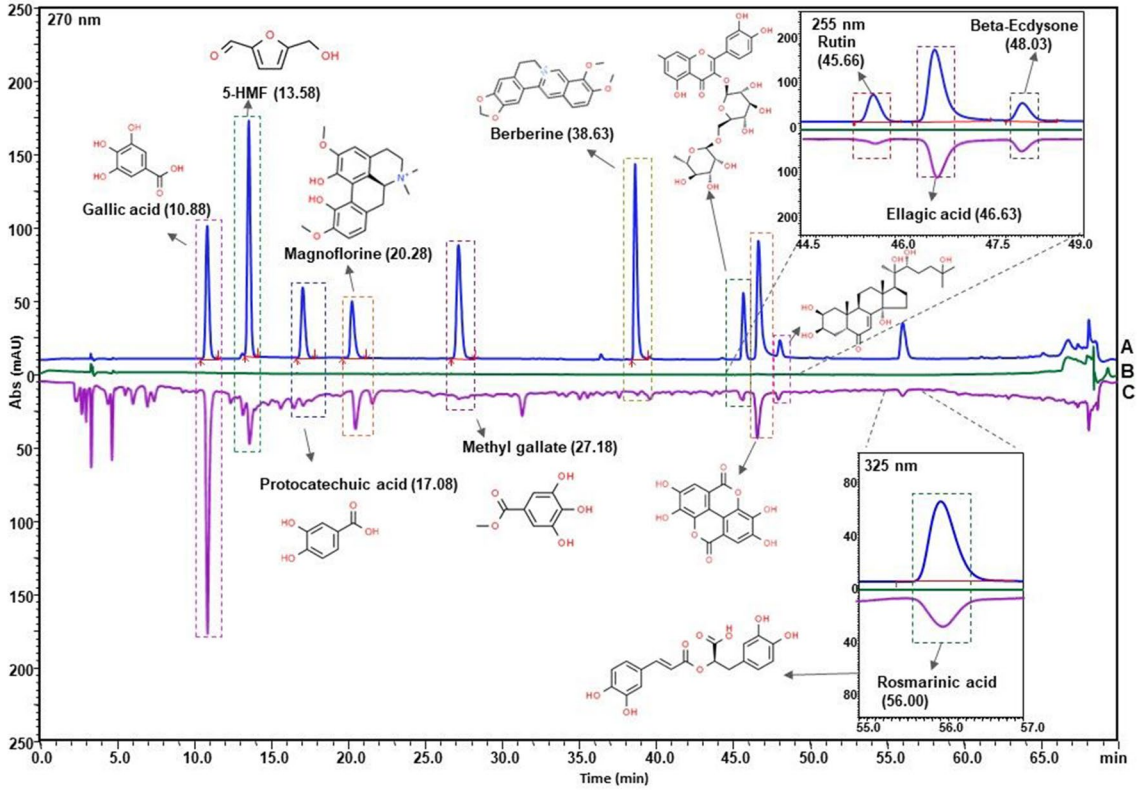

Fig. 2 Overlay chromatograms of UHPLC analysis for Divya-Denguenil-Vati (DNV) (Batch# DNV015), with selected phyto-analytes along with its retention time (in min). [A] Reference standard mix (Blue line), [B] Diluent blank (Green line), [C] DNV sample (Purple line). Chromatograms were recorded at 270 nm for gallic acid (10.88 min), HMF (13.58 min), protocatechuic acid (17.08 min), magnoflorine (20.28 min), methyl gallate (27.80 min), berberine (38.63 min), at 255 nm for rutin (45.66 min), ellagic acid (46.63 min), β-ecdysone (48.03 min) and at 325 nm for rosmarinic acid (56.00 min). Comparison of [a], [b] and [c] demonstrates specificity of method as no interference was observed at the specific retention time of a given analyte (Structures of analytes were sourced from www.chemspider.com (accessed on 18.11.2021))

reproducibility of the methods; and quality consistency of DNV. However, UHPLC quantification of couple of selected markers (methyl gallate and berberine) were observed to be below the limit of quantification (BLQ) in few batches. Therefore, DNV was standardized with eight markers (gallic acid, 5-HMF, protocatechuic acid, magnoflorine, rutin, ellagic acid, β-ecdysone and rosmarinic acid) for suitability of the measurement across the batches. The chromatographic profile of all the tested batches were stacked over one another to see batch to batch consistency in DNV. Content uniformity of DNV was assessed at three wavelengths and also at retention times of eight marker compounds. To avoid complexity, single wavelength (270 nm) chromatograms are displayed in Fig. 5. All the eight tested marker compounds were found to be consistently present in all of the tested batches of DNV, at their respective retention times (Fig. 5). Violin plots of gallic acid, 5-HMF, protocatechuic acid, magnoflorine, rutin, ellagic acid, β-ecdysone and rosmarinic acid were plotted (Fig. 6). Violin plot of marker components shows the distribution of data points, median, first and third quartile. Output of UHPLC and HPTLC techniques were statistically compared for four markers (gallic acid, rosmarinic acid, magnoflorine and rutin) in twenty-six DNV batches (Table ESM_1, Fig. 7). This analysis exhibited a robust correlation between the analytical output measured by UHPLC and HPTLC ($p > 0.05$) for several batches of DNV (Fig. 7).

Discussion

Dengue, also called a backbone fever (*dandaka jwara* in Ayurveda), is an endemic disease in tropical countries such as India, Indonesia, Vietnam, Brazil, and Thailand. After COVID-19 pandemic various countries are struggling with the outbreak of dengue [16]. Un-availability of reliable treatment against dengue is the major challenge that the world is facing in managing this vector borne disease. Alternative medicines of herbal origin may provide a solution to this

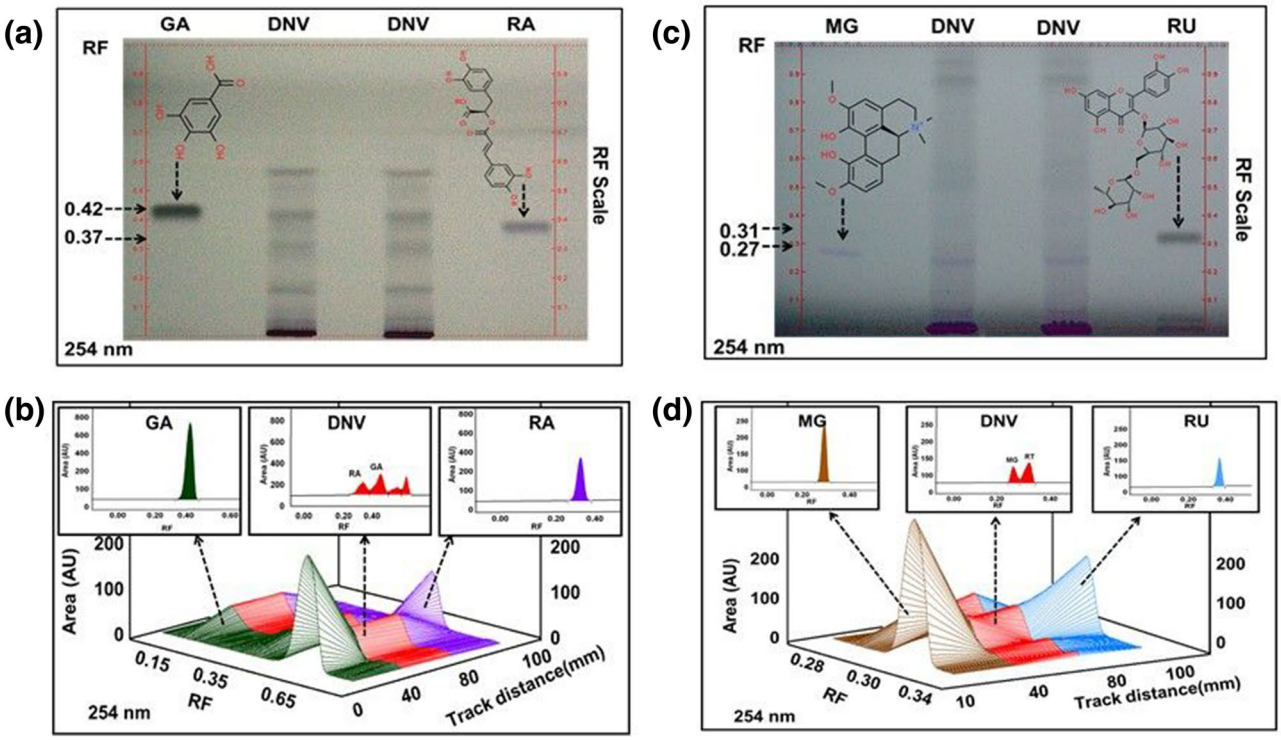

Fig. 3 Phytochemical fingerprints and three dimensional (3-D) chromatograms of Divya-Denguenil-Vati (DNV) (batch# DNV015) acquired from High Performance Thin Layer Chromatographic (HPTLC) analysis. **a**, **c** Chemical fingerprint, **b**, **d** Wireframe spectra of DNV scanned at 254 nm against reference standards of gallic acid (GA), rosmarinic acid (RA) and magnoflorine (MG), rutin (RU), respectively. Bands of gallic acid and magnoflorine were observed both on TLC plate and in scan mode. Whereas, feeble bands of rosmarinic acid and rutin were confirmed by HPTLC profiling (Molecular structures are sourced from www.chemspider.com (accessed on 22.11.2021))

situation, as shown in the cases of other infective diseases, including COVID-19.

Divya-Denguenil-Vati, such herbal formulation, is a blend of stems of *T. cordiofolia*, leaf pulp of *A. barbadensis*, leaves and unripe fruit of *C. papaya*, seeds of *P. granatum* and leaves of *O. sanctum*. These five herbs have also been extensively used as antipyretic, anti-analgesic, and as a blood purifier to reduce fever, joint pains, and to increase hemoglobin and platelet counts [17–20]. Use of *T. cordiofolia* has increased immensely during the COVID-19 pandemic, it has been explored for its immunomodulatory, anti-inflammatory, analgesic and anti-viral activities [8, 21]. The wide-spectrum therapeutic activities of *T. cordiofolia* are due to the presence of numerous key bioactive compounds. *T. cordiofolia* was shown to be responsible for five phyto-constituents out of ten in the current investigation, these are gallic acid, magnoflorine, rutin, β-ecdysone and rosmarinic acid [22–24]. *Aloe barbadensis* or Aloe vera, has been classically used for skin disorders. Several research reports have also revealed anti-bacterial, anti-inflammatory, and immunomodulatory properties [25, 26]. *C. papaya* has been reported for its anti-viral activity against DENV-2 subtype of Dengue virus [27]. Clinically, *C. papaya* extracts have been shown to elevate the platelet count of Dengue patients [28, 29]. DNV also induced similar effects in restoration of reduced platelet counts, in an animal model of Dengue virus infection [7]. *C. papaya* is a rich source of bioactive molecule rutin [30], Vitamins A, C and E [31] which helps the body to gain nourishment, boost immunity and lead to fast recovery [32]. Anti-viral activities of *P. granatum* are well known. Punicalagin as well as other polyphenols (ellagitannins), flavonoids and their derivatives, derived from *P. granatum* have been demonstrated to have anti-DENV properties [33]. Ellagic acid is an ellagitannin that is generated when punicalagin is hydrolyzed and lactonized [36]. DNV contains a substantial level of ellagic acid, which may contribute to its anti-dengue activity. *O. sanctum,* has been considered a holy plant and worshiped for ages. Leaves of basil are also added in tea decoction for the prevention of cold, cough, fever and are also believed to have hepato-protective, anti-inflammatory and anti-viral activities [18]. Leaves extract of tulsi or holy basil contains 88.6% flavonoids, exhibit anti-viral activity against DENV-1 [34, 35]. *O. sanctum* contributes rosmarinic acid, one of the DNV marker components [36]. Another marker compounds of DNV, 5-HMF is likely to be converted from cellulose present in plants. 5-HMF is

Table 3 Validation parameters for selected marker components of Divya-Denguenil-Vati (DNV) (Batch# DNV015) using HPTLC analysis

Validation parameters	Selected phytochemical markers in DNV							
	Gallic acid		Rosmarinic acid		Magnoflorine		Rutin	
Linearity range (μg mL^{-1})	20–400		50–130		50–600		50–180	
Regression equation	$Y=14.20\,X+244.3$		$Y=14.29\,X+18.47$		$Y=3.54\,X+69.69$		$Y=4.95\,X-4.72$	
Correlation coefficient (r^2) NLT (0.99)	0.995		0.999		0.998		0.998	
LOD (μg g^{-1})	20.03		0.71		16.22		0.9	
% RSD (NMT 33%)	1.74		1.02		2.44		2.92	
LOQ (μg g^{-1})	60.69		2.14		49.15		2.72	
% RSD (NMT 10%)	2.51		1.8		3.08		2.27	
Precision	Concentration (μg mL^{-1})	% RSD	Concentration (μg mL^{-1})	% RSD	Concentration (μg mL^{-1})	% RSD	Concentration (μg mL^{-1})	% RSD
Intraday ($n=15$) (%RSD) NMT 5%	20	2.88	50	2.93	50	4.23	50	3.47
	200	0.73	90	0.58	200	1.25	110	2.87
	400	4.23	130	1.92	600	2.65	180	4.02
Interday ($n=30$) (%RSD) NMT 5%	20	3.76	50	4.10	50	4.31	50	4.55
	200	1.62	90	3.31	200	3.30	110	3.73
	400	4.70	130	4.71	600	4.66	180	4.75
Recovery ($n=9$) (80–120%)	Recovery levels	% Recovery	Recovery levels	% Recovery	Recovery levels	% Recovery	Recovery levels	% Recovery
	80	97.13	80	105.04	80	93.83	80	100.7
	100	92.55	100	94.23	100	97.57	100	90.73
	120	101.53	120	97.15	120	88.97	120	96.97

All the parameters are validated as per ICH-Q2 (R1), USP < 1225 > guidelines

RSD Relative Standard Deviation, *NMT* Not more than, *NLT* Not less than

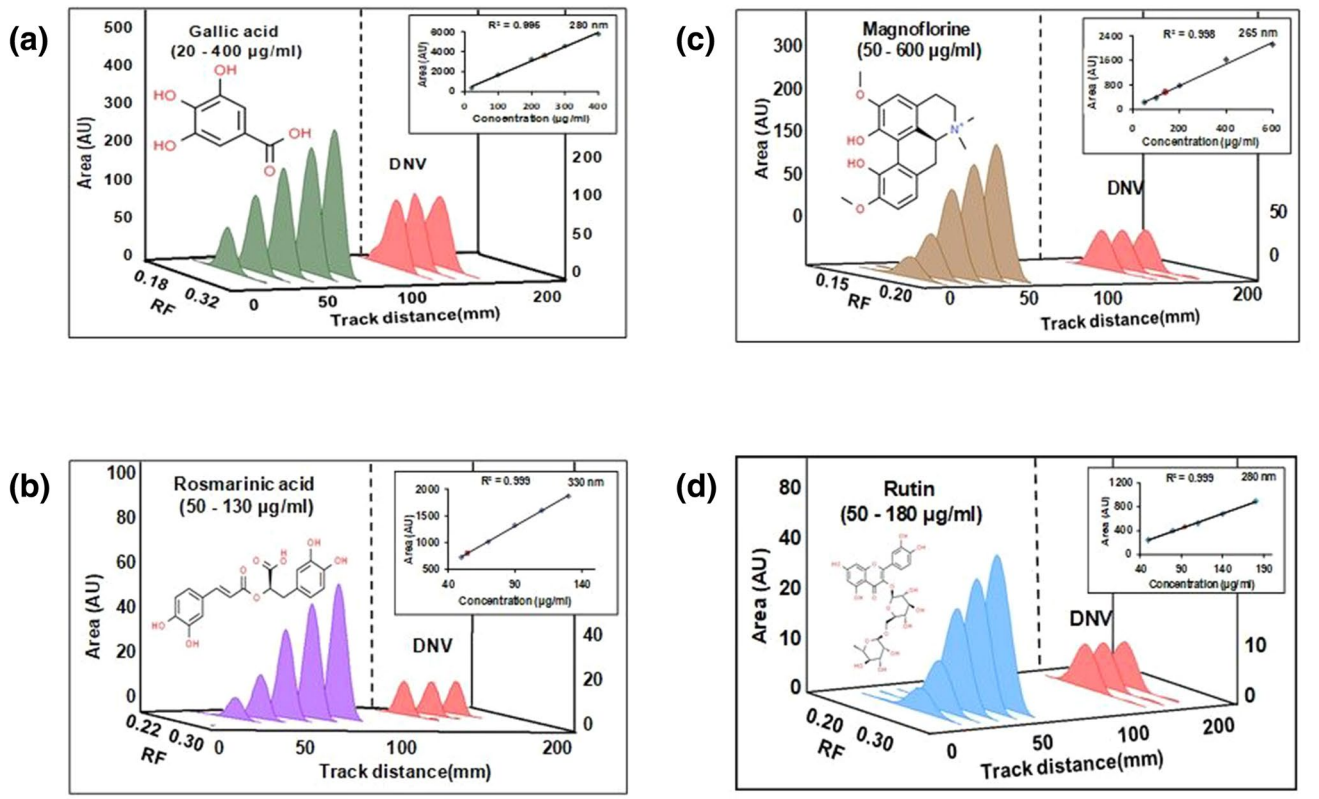

Fig. 4 Three dimensional (3-D) chromatogram and linearity study in Divya-Denguenil-Vati (DNV) by High Performance Thin Layer Chromatography (HPTLC). **a** Gallic acid (GA) with regression equation: $y = 14.20x + 244.3$, correlation coefficient (r^2): 0.995 and linearity range: 20.0–400.0 µg mL^{-1} (green points) at 280 nm. **b** Rosmarinic acid (RA) with regression equation: $y = 14.29x + 18.47$, correlation coefficient (r^2): 0.999 and linearity range: 50.0–130.0 µg mL^{-1} (purple points) at 330 nm. **c** Magnoflorine (MG) with regression equation: $y = 3.54x + 69.69$, correlation coefficient (r^2): 0.998 and linearity range: 50.0–600.0 µg mL^{-1} (brown points) at 265 nm. **d** Rutin (RU) with regression equation: $y = 4.65x - 4.72$, correlation coefficient (r^2): 0.998 and linearity range: 50.0–180.0 µg mL^{-1} (blue points) at 280 nm. Concentration of each marker in DNV is represented by red point on linearity scale (Molecular structures are emulated from www.chemspider.com (accessed on 22.11.2021))

produced by the isomerization of glucose via the fructose pathway post-complex reactions [37]. Taken together, alkaloids, flavonoids, tannins and phenolic acids attributed from five herbs in DNV, identified by UPLC/MS-QToF, developed, validated and standardized by UHPLC and HPTLC are responsible for its anti-oxidative, anti-inflammatory, anti-viral and immunomodulatory properties.

Official monographs like US Pharmacopeia are available for standardization of holy basil, pomegranate and aloe but these methods are developed on single herbs with one or two signature markers [38]. There was no official method available for the standardization of poly-herbal formulation containing herbs used in DNV. Therefore, it becomes necessary to develop analytical method for the quantification of marker components in DNV. The current study aims to identify, develop, optimize, validate and standardize a method using sophisticated, rapid and sensitive techniques like UPLC/MS-QToF, UHPLC and HPTLC. DNV was first screened by UPLC/MS-QToF and 97 phyto-constituents were identified (Table 1). The method was developed and validated for ten markers by UHPLC, four by HPTLC based on their response obtained on respective instruments and availability of reference standards. Results obtained during the validation confirms that the proposed method is specific, sensitive, precise, accurate and rugged for its intended use. UHPLC was checked for system suitability by assessing peak area RSD (NMT 2%), tailing factor (NMT 2) and counting theoretical plates (NLT 5000) (Table 2). All parameters are found to be in acceptability criteria. The method was found to be specific, with no interference of co-eluting peaks from the complex matrix. It was discovered that the peak purity index was larger than or equal to zero. The single point threshold values for all the marker compounds were found to be lower than the peak purity index values, confirming the analytes peak purity. LOD and LOQ of the individual phytochemical marker were calculated by S/N ratio method for UHPLC and residual plot method for HPTLC and %RSDs of $n = 6$ replicates were found within the prescribed limit of less than 5%, which suggests that developed methods are sensitive enough to quantify bioactive markers present in DNV.

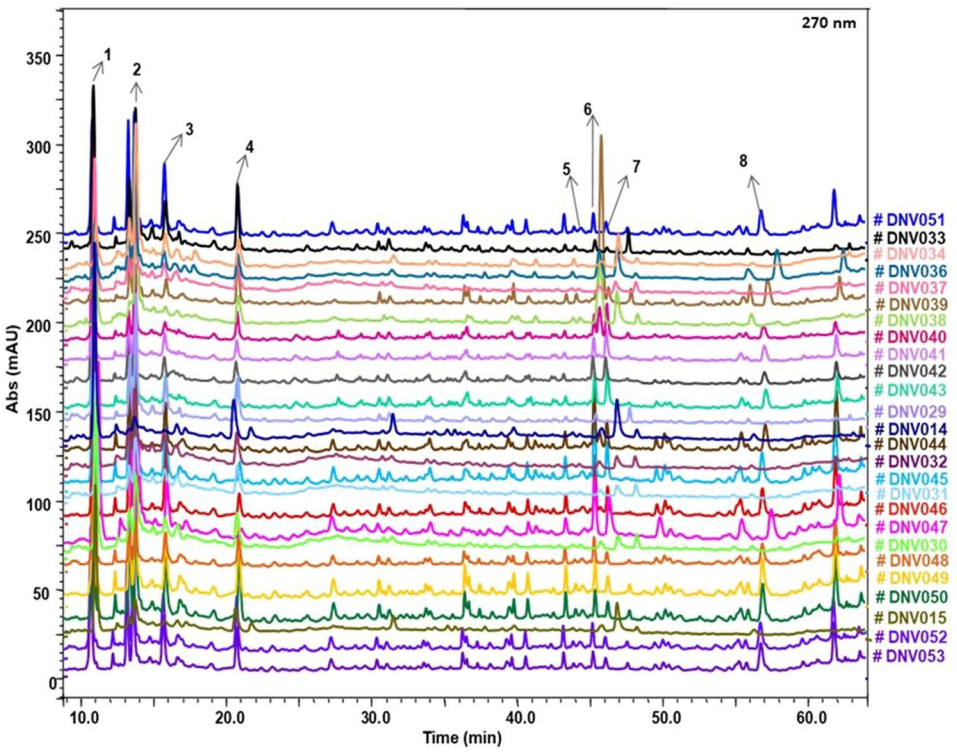

Fig. 5 Overlay chromatograms of Divya-Denguenil-Vati (DNV) Batches# DNV051, 033, 034, 036, 037, 039, 038, 040, 041, 042, 043, 029, 014, 044, 032, 045, 031, 046, 047, 030, 048, 049, 050, 015, 052, 053. Peak numbers are assigned to phyto-constituents in order of their elution which is 1, Gallic acid, 2, 5-HMF, 3, Protocatachuic acid, 4, Magnoflorine, 5, Rutin, 6, Ellagic acid, 7, Beta Ecdysone and 8, Rosmarinic acid. UHPLC fingerprints of 26 batches stacked together demonstrates quality consistency of DNV

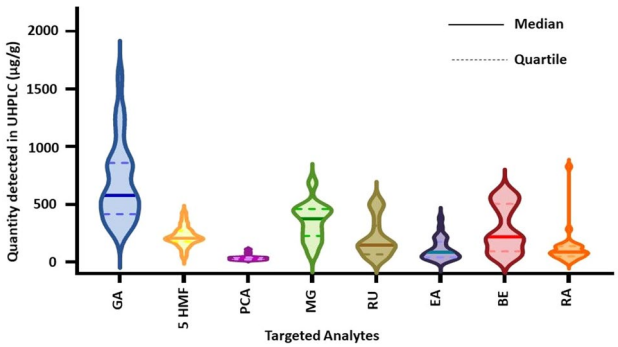

Fig. 6 Violin plot of UHPLC study for 26 batch of Divya-Denguenil-Vati (DNV) with Median, first and third quartile of Gallic acid (GA), 5-Hydroxy methyl furfural (5-HMF), Protocatechuic acid (PCA), Magnoflorine (MG), Rutine (RU), Ellagic acid (EA), β-Ecdysone (BE), Rosmarinic acid (RA). Plot corroborate that 5-HMF, PCA, MG, RU, EA, BE and RA is distributed within 1000 µg g^{-1}, whereas GA was distributed up to 2000 µg g^{-1}. 5-HMF and PCA show one kernel, RU and RA shows two kernel whereas GA, MG, RU and BE shows three kernel distribution

Linearity was plotted between concentration and response of phyto-constituent using equation $Y = MX + C$ for both the analytical techniques. A linearity plot with correlation coefficient (r^2) of NLT 0.99 was found suitable, within the linearity range for the quantification of standards and samples. The calculated sum of squares was greater than the residuals, indicating a good linear relationship. Precision and accuracy of the method were assessed by intraday, interday and recovery study with criteria of %RSDs NMT 5% and from 80 to 120% respectively (Tables 2, 3). Developed analytical methods were reproducible, repeatable, accurate and within the set standards. The intermediate precision of the method was also evaluated by making deliberate changes in flow rate and column temperature for UHPLC analysis and RSDs were observed within the 10% which is much less than the set limit of NMT 20%. Therefore, we can conclude that the method developed to quantify phyto-constituents is fit for its intended use and can be used for other complex herbal formulations like DNV.

At times, standardization of herbal medicine is difficult largely due to unavoidable variations in raw materials and extracts used to prepare poly-herbal medicines. In case of DNV, after development and validation of the analytical methods, 26 commercial batches of DNV were individually analyzed for its quality evaluation. Chromatographic pattern indicates that there is no variation and batch to batch profile were superimposable with each other (Fig. 5). Quantity of all the eight marker components were having similar responses at 255, 270 and 325 nm. All the tested batches were found to have consistent levels of specified compounds. Violin plot of UHPLC data outcome confirms that concentration distribution of marker compounds in DNV falls between the first and third quartile, except a few like gallic acid and rosmarinic acid (Fig. 6). This variation may be attributed due to inherent changes in growing conditions such as seasonal, climate, geographical distribution and stage of plant part used as a raw material in DNV formulation. UHPLC

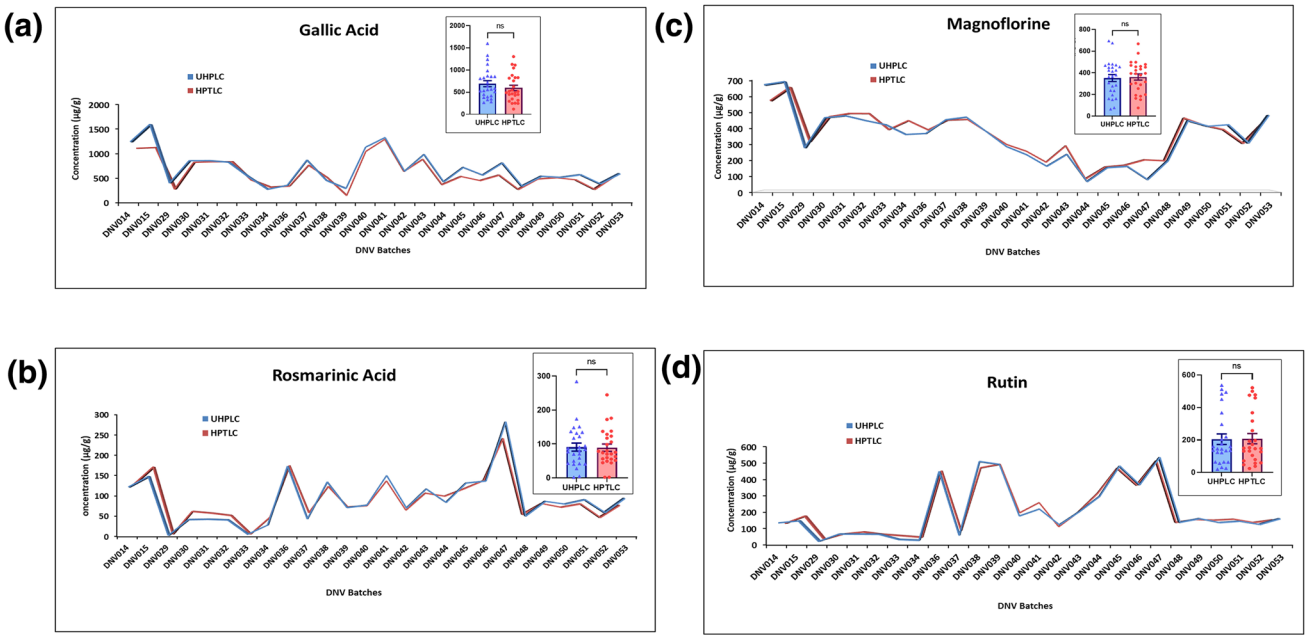

Fig. 7 Comparative UHPLC and HPTLC quantitation of four phyto-constituents, **A** gallic acid, **B** magnoflorine, **C** rutin and **D** rosmarinic acid in 26 batches of Divya-Denguenil-Vati (DNV). Line graph shows uniformity of active markers using both the techniques. Box graph in inset represents distribution of targeted analytes across the batches, standard bars exhibits the variation in concentrations of respective analyte. *p* value was calculated to observe statistical significance. *p* value > 0.05 for all the phyto-constituents indicates that there is no statistical significance (ns) found between UHPLC and HPTLC data

and HPTLC profiling in different batches ensured the consistency of bioactive molecules which may be considered as a good indicator for its quality evaluation. Comparative 3-D line plots of UHPLC and HPTLC reveal that both the techniques are suitable and precise for the selected markers and variation in quantified values is within the acceptance criteria. Box plot in the inset shows no significant variation in both the values, which again proves that analytical techniques are sensitive, effective and comparable for the quantification of marker components in DNV (Fig. 7). Present approach, is first of its kind to identify, develop, validate and standardize using multi-chromatographic analytical tools for simultaneous determination of ten phytochemical markers in DNV, which may be applied for formulations having similar ingredients.

Conclusion

Quality control evaluation and consistency of active molecules are of utmost importance to develop the worldwide acceptability of herbal medicines. In this light, UPLC/MS-QToF was utilized to identify bioactive phyto-constituents in Divya-Denguenil-Vati (DNV). A rapid, sensitive, precise, accurate and rugged method was developed using UHPLC and HPTLC for Divya-Denguenil-Vati. Quality consistency was also evaluated for the standardization of herbal medicine by analyzing 26 batches for gallic acid, 5-HMF, protocatechuic acid, magnoflorine, rutin, ellagic acid, β-ecdysone and rosmarinic acid by UHPLC; and gallic acid, rosmarinic acid, magnoflorine and rutin by HPTLC. Method development, validation, and standardization using combinative analytical strategies like UPLC/MS QTof, UHPLC and HPTLC provide a scientific evidence for quality control evaluation of poly-herbal formulations like DNV.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10337-022-04183-7.

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Author Contributions AB: provided broad direction for the study, ideated the concept, identified the test formulation, generated resources, and gave the final approval for the manuscript. MJ: planning and performed HPTLC experiments. MT: planning and performed UHPLC experiments. SV: planning and performed UPLC/MS-QToF experiments. SG: performed data curing and wrote manuscript. VM: performed statistical analysis and created graphs. JS: supervised the analytical chemistry experiments and reviewed the manuscript. AV: conceptualized and supervised the overall studies and generated resources, critically reviewed, and finally approved the manuscript.

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Declarations

Competing Interests The test article was sourced from Divya Pharmacy, Haridwar, Uttarakhand, India. Acharya Balkrishna holds an honorary trustee in Divya Yog Mandir Trust, which governs Divya Pharmacy, Haridwar. In addition, he holds an honorary managerial position in Patanjali Ayurved Ltd., Haridwar, India. Other than providing the test articles, Divya Pharmacy was not involved in any aspect of this study. Divya Pharmacy, Haridwar India, manufactures and sells many herbal medicinal products, including Divya-Denguenil-Vati. Other authors, Monali Joshi, Meenu Tomer, Sudeep Verma, Seema Gujral, Vallabh Mulay, Jyotish Shrivastava and Anurag Varshney, are employed at Patanjali Research Institute which is governed by Patanjali Research Foundation Trust (PRFT), Haridwar, Uttarakhand, India, a not-for-profit organization. Anurag Varshney is also an adjunct professor in the Department of Allied and Applied Sciences, University of Patanjali, NH-58, Haridwar-249405, Uttarakhand, India; and in the Special Centre for Systems Medicine, Jawaharlal Nehru University, New Delhi-110067, India. All authors declare no conflict of interest.

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