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 Balkrishna1,2 · Monali Joshi1 · Meenu Tomer1 · Sudeep Verma1 · Seema Gujral1 · Vallabh Prakash Mulay1 · Jyotish Srivastava1 · Anurag Varshney1,2,3

Received: 22 December 2021 / Revised: 6 June 2022 / Accepted: 13 June 2022 / Published online: 20 July 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

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, patients 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
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 cordifolia (Heart-leaved moonseed, Giloy, Saptāśirikā aromapatrā), Aloe barbadensis (Aloe vera, Majjapatrakam aṅgārasumam), Carica papaya (Papaya, Kumbhakarṇatiṅa kṛṣṇerūtḥ), Punica granatum (Pomegranate, Dādīmakam dantabījam) and Ocimum sanctum (holy basil, Tulsi, Sūmaṇjariṅkā rāṃṭā). 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], Ukransan [11] and SJTDI-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 solvents 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), Degasging 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 (ATS4), TLC scanner 4, TLC Visualizer was used for the analysis. HPTLC was performed on a pre-coated silica gel 60 F₂₅₄ (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, $\beta$-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. | Formula | Neutral Mass (Da) | Observed m/z | Mass error (mDa) | RT (min) | Adducts | High energy fragments |
|-------|----------------|-------------------|---------|------------------|--------------|------------------|----------|---------|----------------------|
|       | /-Gluconic acid | 1                 | C6H12O7 | 196.0583         | 195.0516     | 0.6              | 0.85     | - H    | 195.0513, 165.0404, 113.0247, 101.0246 |
|       | Malic acid      | 2                 | C4H6O5  | 134.0215         | 133.0145     | 0.2              | 1.22     | - H    | 133.0144, 115.0037 |
|       | Citric acid     | 3                 | C6H8O7  | 192.0270         | 191.0202     | 0.4              | 1.41     | - H    | 191.0200, 161.0458, 143.0351, 111.0091 |
|       | Isocitric acid  | 4                 | C6H8O7  | 192.0270         | 191.0201     | 0.3              | 1.65     | - H    | 191.0199, 161.0446, 111.0091 |
|       | Organic acids   |                   |         |                  |              |                  |          |         |                      |
|       | N-Methylcoclaurine | 5             | C18H21NO3 | 299.1521       | 298.1444     | - 1.3            | 10.18    | + H    | 300.1581, 255.1012, 238.0863, 194.0749, 161.0595, 107.0481 |
|       | Coclaurine      | 6                 | C17H19NO3 | 285.1365       | 286.1434     | - 0.3            | 11.20    | + H    | 286.1431, 255.1009, 243.1028, 161.0591, 107.0481 |
|       | Caffeine        | 7                 | C8H10N4O2 | 194.0804       | 195.0858     | - 1.9            | 12.43    | + H    | 195.0859, 167.0721, 138.0643, 123.0420, 110.0698 |
|       | Magnoflorine    | 8                 | C20H24NO4 | 342.1705       | 342.1695     | - 0.5            | 15.38    | - H    | 342.1697, 297.1114, 282.0876, 265.0848, 237.0893, 222.0656, 340.1556, 310.1087, 282.1131, 252.0427, 224.0479, 196.0532 |
|       | /-Tetrahydrocolumbamine | 9          | C20H23NO4 | 341.1627       | 342.1693     | - 0.6            | 17.16    | + H    | 342.1699, 314.1749, 297.1117, 282.0892, 265.0851 |
|       | Menisperine     | 10                | C21H25NO4 | 355.1784       | 356.1854     | - 0.3            | 19.17    | + H    | 356.1855, 342.1736, 311.1278, 279.1016, 265.0852 |
|       | N-trans-Caffeoyl tyramine | 11        | C17H17NO4 | 299.1158       | 300.1226     | - 0.4            | 25.32    | + H    | 300.1244, 163.0376, 147.0440, 121.0640, 91.0543 |
|       | Pseudocarpaine  | 12                | C28H50N2O4 | 478.3771       | 479.3850     | 0.7              | 26.51    | + H    | 479.3835, 461.3765, 240.1950, 222.1850, 121.1042 |
|       | Dihydroberberine | 13               | C20H19NO4 | 337.1314       | 338.1382     | - 0.5            | 27.02    | + H    | 338.1385, 307.0843, 279.0886, 265.0733, 250.0861, 236.0703 |
|       | (+)-Carpaine    | 14                | C28H50N2O4 | 478.3771       | 479.3849     | 0.5              | 28.69    | + H    | 479.3850, 240.1947, 222.1843, 194.1913 |
|       | N-cis-Feruloyl tyramine | 15         | C19H21NO4 | 313.1314       | 314.1383     | - 0.4            | 31.20    | + H    | 314.1385, 177.0531, 145.0267, 121.0634, 117.0323 |
|       | Palmitine       | 16                | C21H25NO4 | 351.1471       | 352.1541     | - 0.2            | 31.80    | + H    | 352.1543, 336.1226, 320.0914, 292.0960, 278.0798, 263.0573, 248.0701 |
|       | Berberine       | 17                | C20H17NO4 | 335.1158       | 336.1225     | - 0.6            | 31.86    | + H    | 336.1226, 320.0914, 304.0963, 292.0960, 278.0798, 263.0573, 248.0701 |
|       | Solasonin       | 18                | C21H27NO10 | 883.4929      | 884.5012     | 1.0              | 34.81    | + H    | 884.5014, 866.4907, 722.4488, 576.3909, 414.3373, 253.1946, 157.1001 |

Identification, Validation and Standardization of Bioactive Molecules Using UPLC/MS-QToF
| 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     | 1.7             | 36.11    | +H     | 868.5071, 850.4963, 722.4494, 576.3913, 414.3380, 253.1954, 157.1008 |
| 20    | β2-Chaconine                    | 85                               | C_{43}H_{63}NO_{11} | 721.4401        | 722.4482     | 0.8             | 37.90    | +H     | 722.4483, 704.4378, 576.3905, 414.3380, 313.1992, 253.1944, 157.1001 |
|       |                                 |                                  |             |                  | 766.4406     | 2.3             | 37.90    | +HCOO  | 766.4405, 531.2804, 247.0824, 179.0556 |
|       | Flavonoids                       |                                  |             |                  |              |                 |          |         |                                         |
| 21    | Gallocatechin gallate            | 32                               | C_{22}H_{18}O_{11} | 458.0849        | 459.0926     | 0.4             | 15.81    | +H     | 459.0946, 315.0521, 289.0723, 153.0180, 139.0376 |
| 22    | Luteolin 7-rutinoside            | 43                               | C_{27}H_{30}O_{15} | 594.1585        | 595.1659     | 0.2             | 16.30    | +H     | 595.1668, 559.1467, 475.1125, 379.0822, 325.0705 |
| 23    | Epigallocatechin gallate         | 30                               | C_{27}H_{30}O_{15} | 624.1585        | 625.1659     | 0.2             | 17.47    | +H     | 625.1668, 589.1467, 489.1125, 393.0822, 338.0733 |
| 24    | Manghasalin                      | 42                               | C_{27}H_{40}O_{20} | 740.2164        | 741.2247     | 1.1             | 20.89    | +H     | 741.2254, 695.1409, 593.0822, 497.0578, 444.0323 |
| 25    | Quercetin 3,4ʹ-dimethyl ether 7-alpha-L-arabinofuranosyl-(1-6)-glucoside | 53                               | C_{28}H_{32}O_{16} | 432.1585        | 433.1659     | 0.2             | 22.05    | +H     | 433.1674, 387.0972, 303.0486, 245.0706, 178.0012 |
| 26    | Clitorin                         | 48                               | C_{27}H_{30}O_{15} | 594.1585        | 595.1659     | 0.2             | 18.82    | +H     | 595.1668, 559.1467, 475.1125, 379.0822, 325.0705 |
| 27    | Epicatechin gallate              | 50                               | C_{22}H_{18}O_{10} | 442.0900        | 443.0986     | 1.4             | 21.41    | +H     | 443.0992, 284.0332, 255.0304, 169.0148 |
| 28    | Rutin                           | 52                               | C_{27}H_{40}O_{15} | 610.1534        | 611.1611     | 0.4             | 22.05    | +H     | 611.1624, 555.1483, 497.0542, 444.0323, 390.0286 |
| 29    | Lithospermic acid                | 53                               | C_{27}H_{40}O_{15} | 538.1111        | 539.1191     | 0.6             | 22.24    | +H     | 539.1193, 483.0453, 427.0115, 369.0276, 313.0497 |
| 30    | Isoquercetin                     | 55                               | C_{27}H_{40}O_{15} | 464.0955        | 465.1029     | 0.1             | 22.74    | +H     | 465.1042, 409.0345, 353.0267, 297.0189, 247.0081 |
| 31    | Nicotiflorin                     | 59                               | C_{27}H_{40}O_{15} | 594.1585        | 595.1670     | 1.2             | 25.09    | +H     | 595.1682, 539.1494, 489.1206, 433.0918, 377.0731 |
| 32    | Astragalin                       | 62                               | C_{21}H_{20}O_{12} | 448.1006        | 449.0986     | 0.4             | 25.86    | +H     | 449.0992, 284.0332, 255.0304, 169.0148 |
| 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    | 0.3             | 42.64    | + H    | 345.0962, 330.0727, 315.0489, 312.0622, 287.0549, 269.0461, 242.0581 |
| 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_{7}H_{6}O_{5}  | 170.0215        | 169.0146    | 0.4             | 3.65     | − H    | 169.0143, 125.0246, 124.0166, 107.0140 |
| 36    | Syringic acid   | 11                              | C_{9}H_{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_{18}H_{16}O_{7} | 154.0266        | 153.0196    | 0.2             | 7.67     | − H    | 153.0192, 137.0290, 108.0219 |
| 38    | Feruloylsucrose | 14                              | C_{18}H_{16}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_{18}H_{16}O_{9} | 354.0951        | 353.0886    | 0.7             | 10.07    | − H    | 353.0877, 179.0554, 135.0450, 125.0240 |
| 40    | Methyl gallate  | 20                              | C_{6}H_{6}O_{7}  | 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_{18}H_{16}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_{18}H_{16}O_{8} | 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_{8}O_{7} | 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_{18}H_{16}O_{7} | 338.1002        | 337.0933    | 0.4             | 15.12    | − H    | 337.1034, 314.1024, 191.0560, 189.0196 |
| 45    | Uroliithin M5   | 39                              | C_{16}H_{18}O_{9} | 276.0270        | 275.0203    | 0.6             | 17.23    | − H    | 275.0205, 257.0097, 229.0146 |
| 46    | o-Coumaric acid | 45                              | C_{7}H_{6}O_{5}  | 164.0473        | 163.0401    | 0.1             | 19.48    | − H    | 163.0401, 119.0502 |
| 47    | Ferulic acid   | 49                              | C_{18}H_{16}O_{8} | 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_{18}H_{16}O_{8} | 302.0063        | 301.0209    | 0.6             | 21.84    | + H    | 303.0133, 285.0024, 257.0195, 257.0087, 247.0251, 229.0133 |
| 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_{10}H_{10}O_{7} | 436.1370        | 435.1299    | 0.2             | 24.79    | − H    | 435.1297, 361.1302, 197.0448, 165.0551 |
| 51    | (−)-Rabdosiin  | 61                              | C_{20}H_{19}N_{2} | 337.1314        | 338.1384    | − 0.3           | 27.56    | + H    | 338.1385, 322.1069, 294.1118, 279.0887, 265.0729, 236.0697 |
| 52    | 13,13a-Didehydro-9, 10-damethoxy-2,3-(ethylenedioxy)-berbine | 67                        | C_{20}H_{19}N_{2} | 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 |
|-------|----------------------------------------|---------------------------------|---------|------------------|--------------|------------------|----------|---------|-----------------------|
|       |                                        |                                 |         |                  |              |                  |          |         |                       |
| 56    | 6-O-galloylglucose                      | 8                               | C₁₈H₂₂O₁₀ | 332.0744         | 331.0680     | 1.0              | 3.97     | − H    | 331.0680, 271.0468, 211.0247, 169.0144, 168.0066, 125.0241 |
| 57    | Glucogallin                             | 10                              | C₁₈H₂₂O₁₀ | 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                              | C₁₃H₁₆O₁₀ | 484.0853         | 483.0784     | 0.4              | 11.11    | − H    | 483.0781, 313.0565, 169.0142 |
| 59    | 2,6-Di-O-galloyl-β-glucose             | 21                              | C₁₃H₁₆O₁₀ | 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                              | C₁₈H₂₀O₁₄ | 636.0963         | 635.0889     | − 0.1            | 14.19    | − H    | 635.0893, 483.0787, 313.0564, 169.0140 |
| 61    | Corilagin                               | 30                              | C₁₈H₂₀O₁₄ | 636.0963         | 635.0889     | − 0.1            | 15.31    | − H    | 633.0728, 463.0512, 300.9986, 275.0192, 173.0455 |
| 62    | 1,3,4-tri-O-galloyl-beta-glucopyranose | 34                              | C₁₃H₁₆O₁₀ | 504.1843         | 549.1821     | − 0.4            | 12.23    | + HCOO | 421.1140, 293.0882, 271.0465, 211.0247, 119.0489 |
| 63    | Ellagic acid hexoside                   | 46                              | C₁₉H₂₂O₆  | 346.1416         | 391.1402     | 0.4              | 19.61    | + HCOO | 391.1403, 329.1399, 283.1337, 189.1283, 173.0971, 107.0506 |
| 64    | 3,4,6-Tri-O-galloyl-beta-D-glucose      | 47                              | C₂₇H₂₄O₁₃ | 568.2156         | 567.2081     | − 0.2            | 16.41    | − H    | 463.0519, 300.9986, 299.9911 |
| 65    | 1,2,6-Trigalloylglucose                 | 41                              | C₂₇H₂₄O₁₃ | 636.0963         | 635.0890     | 0.0              | 18.06    | − H    | 635.0893, 465.0682, 313.0566, 169.0143 |
|       |                                        |                                 |         |                  |              |                  |          |         |                       |
| 66    | Cordifoliside A                         | 22                              | C₂₂H₃₂O₁₃ | 504.1843         | 504.1806     | − 0.4            | 12.23    | + HCOO | 421.1140, 293.0882, 271.0465, 211.0247, 119.0489 |
| 67    | Tincordin                              | 46                              | C₁₉H₂₂O₁₃ | 396.2148         | 396.2148     | − 0.2            | 16.41    | − H    | 463.0519, 300.9986, 299.9911 |
| 68    | Tinospinoside D                         | 47                              | C₁₉H₂₂O₁₃ | 375.1451         | 375.1451     | 0.2              | 29.91    | + HCOO | 357.1452, 313.1447, 269.1532, 219.1389, 107.0505 |
| 69    | Tinocordiside                          | 71                              | C₁₉H₂₂O₁₃ | 522.2101         | 522.2101     | 0.3              | 30.48    | + HCOO | 343.1188, 179.0557 |
| 70    | Tinosponone                            | 74                              | C₁₉H₂₂O₁₃ | 545.1996         | 545.1996     | 0.3              | 30.48    | + HCOO | 343.1188, 179.0557 |
| 71    | Cordifoliside A                        | 74                              | C₁₉H₂₂O₁₃ | 374.1366         | 374.1366     | 0.7              | 32.66    | + HCOO | 419.1354 |
| 72    | Palmarin                               | 80                              | C₁₉H₂₂O₁₃ | 374.1366         | 374.1366     | 0.1              | 33.47    | − H    | 373.1286, 329.1403, 285.1498, 135.0452 |
| 73    | Tinosponide                            | 81                              | C₁₉H₂₂O₁₃ | 545.1993         | 545.1993     | 0.0              | 34.19    | + HCOO | 545.1991, 401.1437, 365.1356, 257.1528, 217.1998, 161.0946 |
| 74    | Cordifoliside B                        | 90                              | C₁₉H₂₂O₁₃ | 358.1416         | 358.1416     | 1                | 40.75    | − H    | 357.1353, 313.1077, 269.1190, 173.0970, 157.0506 |
| 75    | Columbin                               | 91                              | C₁₉H₂₂O₁₃ | 357.1352         | 357.1352     | 0.8              | 41.38    | − H    | 357.1353, 313.1451, 297.1139, 171.1031, 135.0452 |
| 76    | Isocolumbin                            | 96                              | C₁₉H₂₂O₁₃ | 455.3536         | 455.3536     | 0.5              | 53.55    | − H    | 455.3534, 279.2339 |
| 77    | Ursolic acid                           | 97                              | C₁₉H₂₂O₁₃ | 455.3535         | 455.3535     | 0.5              | 54.12    | − H    | 455.3531, 407.3286 |
| 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 |
|-------|----------------|---------------------------------|---------|------------------|-------------------|-----------------|--------|---------|-----------------------|
| 79    | Ecdysone       | 54                              | C\(_{27}\)H\(_{44}\)O\(_7\) | 480.3087          | 481.3172          | 0.1             | 22.50  | + H     | 445.2958, 427.2852, 409.2747, 371.2220, 329.2117, 301.1197, 283.1696 |
|       |                 |                                 |         |                   | 525.3076          | 0.7             | 22.49  | + HCOO  | 525.3079, 461.2911, 357.1347, 319.1923, 159.1028 |
| 80    | Makisterone A  | 63                              | C\(_{28}\)H\(_{46}\)O\(_7\) | 494.3244          | 495.3230          | 0.5             | 26.06  | + HCOO  | 539.3239, 479.3020, 319.1920 |
| 81    | Cyanidin-3,5-diglucoside | 28 | C\(_{27}\)H\(_{30}\)O\(_{16}\) | 610.1534          | 610.1459          | −0.3            | 14.68  | − H     | 609.1463, 489.1041, 399.0720, 369.0612, 267.0663, 164.0717 |
| 82    | 5-hydroxymethylfurfural | 12 | C\(_{6}\)H\(_{6}\)O\(_3\) | 126.0317          | 127.0374          | −1.5            | 7.56   | + H     | 127.0383, 109.0285, 99.0438, 85.0290 |
| 83    | N-(1-Deoxy-1-fructosyl)isoileucine | 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\(_{8}\)H\(_{11}\)NO\(_2\) | 165.0790          | 166.0844          | −1.8            | 4.61   | + H     | 166.0861, 120.0793, 103.0530, 91.0536 |
|       |                 |                                 |         |                   | 164.0718          | 0.1             | 4.63   | − H     | 164.0720, 147.0446, 123.0444 |
| 86    | Malnygic acid  | 86                              | C\(_{18}\)H\(_{32}\)O\(_5\) | 328.2250          | 329.2342          | 0.8             | 39.89  | − H     | 329.2344, 311.2239, 229.1448, 211.1347, 183.1396, 167.1438, 107.0505 |
| 87    | 2,3,4-Trihydroxy-2,4-octadecadienoic acid | 87 | C\(_{18}\)H\(_{32}\)O\(_5\) | 328.2250          | 329.2341          | 0.9             | 39.11  | + Na    | 351.2145, 275.2014, 257.1947 |
|       |                 |                                 |         |                   | 327.2186          | 0.8             | 39.10  | − H     | 327.2187, 291.1972, 229.1453, 211.1345, 171.1024 |
| 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 |
| 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\(_{10}\)H\(_{16}\)O\(_6\) | 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          | 389.1612          | 0.7             | 35.25  | + Na    | 413.1588, 359.1507, 323.1304, 313.1455, 296.1042 |

Table 1 (continued)
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 (10:1:1:1:2.3 v/v/v/v) for magnoflorine and rutin.
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     | %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 | y = 0.0418x | y = 0.0155x | y = 0.0265x | y = 0.0246x | y = 0.0373x | y = 0.0178x | y = 0.0334x | y = 0.0118x | y = 0.0261x |
|                       | Range (μg mL⁻¹)      | 1–100       | 0.5–100 | 1–100      | 1–100         | 1–100        | 0.5–100     | 1–100 | 0.5–100| 1–100         | 0.5–100      |
| Precision              | %RSD NMT 5%         | 0.83        | 0.88    | 1.62       | 1.44          | BLQ          | BLQ         | 0.84  | 1.58  | 0.24         | 0.80         |
|                       | Conc.% % Recovery    | 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 | %RSD NMT 20         | 3.72        | 6.88    | 6.28       | 8.48          | BLQ          | BLQ         | 4.46  | 4.40  | 2.92         | 2.19         |
| Robustness             | %RSD NMT 33%        | 6.88        | 15.08   | 12.64      | 16.00         | BLQ          | BLQ         | 8.72  | 10.77 | 11.26         | 7.17         |
|                       | LOD (μg g⁻¹)        | 18.84       | 12.04   | 17.55      | 9.34          | BLQ          | BLQ         | 5.80  | 3.17  | 11.91         | 8.21         |
|                       | %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         |
| Limit of Detection (LOD)| LOQ (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 (μg g⁻¹)       | 2.78        | 1.38    | 8.36       | 4.16          | 1.81         | 2.42        | 1.26  | 7.47  | 0.77         | 2.94         |

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

Divya-Denguemoil-Vati, such herbal formulation, is a blend of stems of *T. cordifolia*, 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. cordifolia* 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. cordifolia* are due to the presence of numerous key bioactive compounds. *T. cordifolia* 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* 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

**Fig. 3** Phytochemical fingerprints and three dimensional (3-D) chromatograms of Divya-Denguemoil-Vati (DNV) (batch# DNV015) acquired from High Performance Thin Layer Chromatographic (HPTLC) analysis. a. Chemical fingerprint, b. 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))
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 (r²) 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             | % RSD 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
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. 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: $y = 4.66x - 4.72$, 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)).
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...
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.

**Acknowledgements** The authors are thankful to Ms. Priyanka Kandpal, Mr. Tarun Rajput, Mr. Gagan Kumar, and Mr. Lalit Mohan for their swift administrative supports.

**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.

**Funding** This research work has been conducted using internal research funds from Patanjali Research Foundation Trust, Haridwar, India. It did not receive any external funding.
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.

References

1. Gupta N, Srivastava S, Jain A, Chaturvedi UC (2012) Dengue in India. Indian J Med Res 136:373–390
2. World Health Organisation (2014) Dengue and severe dengue. WHO Fact Sheet 117:1–4
3. Yung CF, Lee KS, Thein TL et al (2015) Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, Singapore. Am J Trop Med Hyg 92:999–1005. https://doi.org/10.4269/ajtmh.14-0628
4. Jain J, Dubey SK, Shrinet J, Sunil S (2017) Dengue chikungunya co-infection: a live-in relationship?? Biochem Biophys Res Commun 492:608–616. https://doi.org/10.1016/j.bbrc.2017.02.008
5. WHO Dengue and severe dengue. https://www.who.int/news-room/fact-sheets. Accessed 6 June 2022
6. Kala CP, Dhyani PP, Sajwan BS (2006) Developing the medicinal plants sector in northern India: challenges and opportunities. J Ethnobiol Ethnomed 2:1–15. https://doi.org/10.1186/1746-4269-2-32
7. Balkrishna A, Solleti SK, Verma S, Varshney A (2020) Validation of a novel zebrafish model of dengue virus (DENV-3) pathology using the pentaherbal medicine denguenil vati. Biomolecules 10:1–24. https://doi.org/10.3390/biom10070971
8. Balkrishna A, Tomer M, Verma S et al (2021) Phytometabolite profiling of Coronil, a herbal medicine for COVID-19, its identification by mass-spectroscopy and quality validation on liquid chromatographic platforms. J Sep Sci. https://doi.org/10.1002/jssc.202100499
9. Balkrishna A, Verma S, Sharma P et al (2021) Comprehensive and rapid quality evaluation method for the ayurvedic medicine Divya-Swastari-vati using two analytical techniques: UPLC/QToF MS and HPLC-DAD. Pharmaceuticals 14:1–22. https://doi.org/10.3390/ph14040297
10. Balkrishna A, Sharma P, Joshi M et al (2021) Development and validation of a rapid high-performance thin-layer chromatographic method for quantification of gallic acid, cinnamic acid, pipericine, eugenol, and glycyrrhizin in Divya-Swastari-Vati, an ayurvedic medicine for respiratory ailments. J Sep Sci 44:3146–3157. https://doi.org/10.1002/jssc.202100096
11. Kim YJ, Jeong S, Seo C et al (2018) Simultaneous determination of the traditional herbal formula Ugkansan and the in vitro antioxidant activity of ferulic acid as an. Molecules 23:1–15. https://doi.org/10.3390/molecules23071659
12. Ladva BJ, Mahida VM, Kantaria UD, Gokani RH (2014) Marker based standardization of polynuclear formulation (SJT-DI-02) by high performance thin layer chromatography method. J Pharm Bioallied Sci 6:213–219. https://doi.org/10.4103/0975-7406.135249
13. ICH Q2 (R1) (2005) Validation of analytical procedures: text and methodology. In: International conference on harmonization. 1994:1–17. https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf. Accessed on 18th Nov 2021
14. USP (2021) Validation of compendial procedures. United States Pharmacopoeia XXXXVII Natl. Formul. XXXII 2007. http://www.usp.org/topics.jsp?uspsn=292400&uspsnf240=0_c1225.html. Accessed on 18th Nov 2021
15. Shrivastava A, Gupta V (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles Young Sci 2:21–25. https://doi.org/10.4103/2229-5186.79345
16. Lu X, Bambrick H, Pongsumpun P et al (2021) Dengue outbreaks in the COVID-19 era: alarm raised for Asia. PLoS Negl Trop Dis 15:1–6. https://doi.org/10.1371/journal.pntd.0009778
17. Kumar A, Agarwal K, Maurya AK, Shanker K, Bushr U, Sudeep Tandon DUB (2015) Pharmacological and phytochemical evaluation of Ocimum sanctum root extracts for its antiinflammatory, analgesic and antipyretic activities. Pharmacogn Mag 11:217–224. https://doi.org/10.4103/0978-1296-157743
18. Lim SYM, Chieng JY, Pan Y (2021) Recent insights on anti-dengue virus (DENV) medicinal plants: review on in vitro, in vivo and in silico discoveries. All Life 14:1–33. https://doi.org/10.1080/26895293.2020.1856192
19. Colombo E, Sangiovanni E, Dell’Agli M (2013) A review on the anti-inflammatory activity of pomegranate in the gastrointestinal tract. Evid-based Complement Alter Med 2013:1–11. https://doi.org/10.1155/2013/247145
20. Hussain L, Akash MSH, Ain NU et al (2015) The analgesic, anti-inflammatory and anti-pyretic activities of Tinospora cordifolia. Adv Clin Exp Med 24:957–964. https://doi.org/10.17219/acem/27909
21. Panchabhai TS, Kullarai UP, Rege NN (2008) Validation of therapeutic claims of Tinospora cordifolia: a review. Phyther Res 22:544–549. https://doi.org/10.1002/ptr.2347
22. Khan T, Iqshita A, Mazumdar R et al (2020) Bioactive polyphenol profiling and in-vitro antioxidant activity of Tinospora cordifolia Mirers ex Hook F and Thoms: a potential ingredient for functional food development. Bangladesh J Sci Ind Res 55:23–34. https://doi.org/10.3329/hjsar.v55i1.46729
23. Ghosh S, Saha S (2012) Tinospora cordifolia: one plant, many roles. Anc Sci Life 14:1–33. https://doi.org/10.4103/0257-7491.107344
24. Abiramasmundari G, Mohan Gowda CM, Sreepriya M (2018) Selective estrogen receptor modulator and protrastimulatory effects of phytosterogen β-ecdysone in Tinospora cordifolia on osteoblast cells. J Ayurveda Integr Med 9:161–168. https://doi.org/10.1016/j.jaim.2017.04.003
25. Cock IE (2015) The Genus Aloe: phytochemistry and therapeutic uses including treatments for gastrointestinal conditions and chronic inflammation. Prog Drug Res 70:179–235. https://doi.org/10.1007/978-3-0348-0927-6_6
26. Shellenb RMM (1991) Aloe vera, its chemical and therapeutic properties. Int J Dermatol 30:679–683. https://doi.org/10.1111/j.1365-4362.1991.tb02607.x
27. Paul A, Vibhuti A, Raj VS (2021) Evaluation of antiviral activity of Andrographis paniculata and Tinospora cordifolia using in silico and in vitro assay against DENV-2 Evaluation of antiviral activity of Andrographis paniculata and Tinospora cordifolia using in silico and in vitro assay. J Pharmacogn Phytochem 10:486–496. https://doi.org/10.22271/phryo.2021.1012f.13847
28. Sathasivam K, Ramanathan S, Mansor SM et al (2009) Thrombocyte counts in mice after the administration of papaya leaf suspension. Wien Klin Wochenschr 121:19–22. https://doi.org/10.1007/s00508-009-1229-0
29. Prakash Kala C (2012) Leaf juice of Carica papaya L.: a remedy of dengue fever. Med Aromat Plants 01:4–6. https://doi.org/10.4172/2167-0412.1000109
30. Khor BKK, Chear NJY, Azizi J, Khaw KY (2021) Chemical composition, antioxidant and cytoprotective potentials of Carica papaya leaf extracts: a comparison of supercritical fluid and conventional extraction methods. Molecules 26:1–18. https://doi.org/10.3390/molecules26051489
31. Santana LF, Filiú WFO, Pott A et al (2019) Nutraceutical potential of Carica papaya in metabolic syndrome. Nutrients 11:1-19A. https://doi.org/10.3390/nu11071608
32. Subenthiran S, Choon TC, Cheong KC et al (2013) Carica papaya leaves juice significantly accelerates the rate of increase in platelet count among patients with dengue fever and dengue haemorrhagic fever. Evid-Based Complement Altern Med 2013:1–8. https://doi.org/10.1155/2013/616737
33. Loaiza-Cano V, Monsalve-Escudero LM, Filho CdSMB et al (2021) Antiviral role of phenolic compounds against dengue virus: a review. Biomolecules 11:1–28. https://doi.org/10.3390/biom11010011
34. Tang LIC, Ling APK, Koh RY et al (2012) Screening of anti-dengue activity in methanolic extracts of medicinal plants. BMC Complement Altern Med 12:3. https://doi.org/10.1186/1472-6882-12-3
35. Ling APK, Khoo BF, Seah CH, et al (2014) Inhibitory activities of methanol extracts of Andrographis paniculata and Ocimum sanctum against Dengue-1 Virus. 47–52. https://doi.org/10.15242/iiebc.e814013
36. Shanmuga Sundaram R, Ramanathan M, Rajesh R et al (2012) LC-MS quantification of rosmarinic acid and ursolic acid in the Ocimum sanctum Linn. leaf extract (Holy Basil, Tulsi). J Liq Chromatogr Relat Technol 35:634–650. https://doi.org/10.1080/10826076.2011.606583
37. Menegazzo F, Ghedini E, Signoretto M (2018) 5-Hydroxymethylfurfural (HMF) production from real biomasses. Molecules 23:1–18. https://doi.org/10.3390/molecules23092201
38. USP-NF (2015) Dietary supplements compendium. US Pharma-cop. https://www.usp.org/products/dietary-supplements-compendium

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.