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Original article

## Development and validation of UPLC-PDA method for concurrent analysis of bergenin and menisdaurin in aerial parts of *Flueggea virosa* (Roxb. ex Willd.)

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## ABSTRACT

Bergenin and menisdaurin are biologically active components which are found in plant *Flueggea virosa* (Phyllanthaceae). Bergenin has pharmacological actions such as chemopreventive and antihepatotoxic while menisdaurin has an anti-viral activity which needs its evaluation by an analytical method (UPLC-PDA method) that can be applied to the quality control of pharmaceutical preparations. The developed UPLC-PDA method was applied for identification and quantification of standards bergenin and menisdaurin in the methanol extract of *F. virosa* (FVME). The analysis was carried out using Eclipse C18 (4.6 × 100 mm, 3.5 μm) UPLC column. The optimized chromatographic condition was achieved at 0.16 mL/min flow rate using gradient system with acetonitrile and water as mobile phase. Both biomarkers were measured at λ<sub>max</sub> 235 nm in PDA detector at ambient temperature. The developed method furnished sharp and intense peaks of menisdaurin and bergenin at Rt = 2.723 and 3.068 min, respectively along with r<sub>2</sub> > 0.99 for both. The recoveries of bergenin and menisdaurin were found in the range of 99.37–101.49% and 98.20–100.08%, respectively. With other validation data, including precision, specificity, accuracy, and robustness, this method demonstrated excellent reliability and sensitivity. The separation parameters i.e. retention, separation, and resolution factors for resolved standards (bergenin and menisdaurin) were >1, which showed good separation. The quantity of bergenin and menisdaurin in the FVME sample was found as 15.16 and 3.28% w/w, respectively. The developed UPLC-PDA method could be conveniently adopted for the routine quality control analysis.

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## 1. Introduction

*Flueggea virosa* (Roxb. ex Willd.) Royle (Fig. 1) belongs to family Phyllanthaceae is a deciduous shrub or small tree with many erect or arching branches and widely distributed in the Middle East, tropical Africa, Australia, tropical Asia, Japan, and Polynesia; usually grows up to 4 m, but exceptionally to 7 m while it can also

be grown domestically. The plant has several ethnobotanical uses in the treatment of fever, pain, diabetes and as a contraceptive. Its various uses have been recorded in Chinese herbal medicine (Al-Rehaily et al., 2015). Several bioactive phytoconstituents have been reported from *F. virosa* such as 9(10 → 20)-abeo-ent-podocarpene (terpenoid) having activity against hepatitis-C virus and anticancer activity against Huh7.5-cells (Chao et al., 2016); Flueggether A and Virosinine A (alkaloids) having anti-HIV property (Zhang et al., 2015), Flueggin-B (alkaloid) having growth inhibitory activity against MCF-7 and MDA-MB-231 human breast cancer cells (Zhao et al., 2011), and bergenin having trypanocidal activity (Nyasse et al., 2004).

Bergenin (Fig. 2A) is a C-glycoside of 4-O-methyl gallic acid. It has been isolated from several plant species and widely explored molecule for its diverse pharmacological effects such as antianxiety (Singh et al., 2016), antimalarial (Liang et al., 2014), cancer chemoprevention (Zhang et al., 2013), antidiabetic (Kumar et al.,

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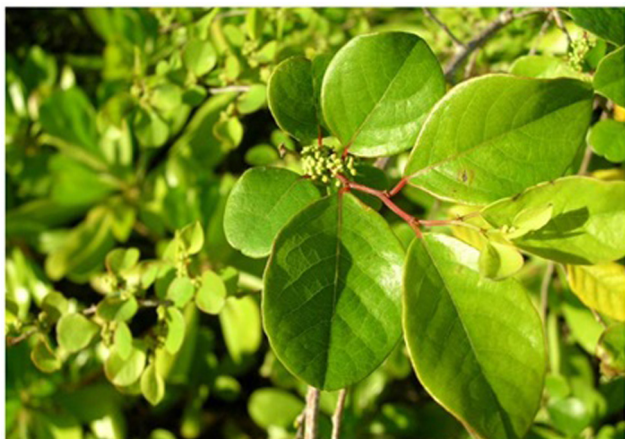


Fig. 1. Picture of *F. virosa* (Roxb. ex Willd.) Royle.

2012) and antihepatotoxic (Kim et al., 2000). Menisdaurin (Fig. 2B) is a cyclohexylideneacetonitrile derivative and found in several plants. Similar to bergenin, it is also having many other pharmacological actions such as anti-Hepatitis B virus (HBV) activity (Yi et al., 2015; Geng et al., 2012), antimicrobial and antifungal activity (Muhammad and Sirat, 2013a), and anti-inflammatory activity (Muhammad and Sirat, 2013b). Due to extensive pharmacological actions of both the marker (bergenin and menisdaurin), several chromatographic methods have been developed for their analysis in plant extracts and blood plasma samples. Bergenin has been quantified in plant extracts using HPLC (Hendrychova et al., 2015), in human plasma using HPLC-MS/MS (Wang et al., 2009), LC-MS (Yu et al., 2009) and the metabolites of bergenin in rat plasma were analyzed using HPLC-QTOF mass spectrometry (Song et al., 2013). Moreover, both the standards; bergenin and menisdaurin have not been quantified simultaneously before in *Flueggea virosa* methanol extract (FVME) using UPLC-PDA method. In this study, authors first time report the identification and quantification of bergenin and menisdaurin in FVME using a developed and validated UPLC-PDA method. The developed and validated UPLC-PDA method can be used in quality evaluation of pharmaceutical preparation.

## 2. Materials and methods

### 2.1. Plant material and extract preparation

The aerial part of *F. virosa* was gathered from the southern region of Saudi Arabia and identified by field taxonomist “Dr. Mohammed Yusuf of Medicinal Plant Collection and Survey Unit, Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh.” The voucher specimen was deposited in College of Pharmacy, King Saud University. The collected aerial parts were dried, crushed and powdered then sieved through 0.75 mm sieve. Ultrasonication extracted around 50 g of the powdered material in *Transsonic-460/H* ultrasonic cleaner (*ELMA*, Germany, having 20 kHz frequency, 100 W power) using methanol as a solvent for 30 min. Since both the markers were found highly soluble in methanol, therefore, methanol has been used as a solvent for extraction. The obtained methanolic extract (ME) was centrifuged for 20 min at 5,000 rpm and filtered through Whatman filter paper. Finally, collected ME of *F. virosa* (FV) was concentrated through solvent evaporation technique and dried out using rotary evaporator (R-210, BUCHI) at reduced pressure. The calculated yield of FVME was found approximately 9.62%, w/w.

### 2.2. Chemicals and reagents

Acetonitrile and methanol (HPLC grade) were obtained from Fisher Scientific UK. Millipore Milli-Q® (Bedford, MA, USA) assembly was used to get the highly pure water. Millipore-Millex-HV® filter unit with a membrane filter (0.45 µm pore size) was used for the filtration of solvents, and syringe filter of 0.22 µm was used for sample preparation. Bergenin and Menisdaurin standard were purchased from Sigma-Aldrich, USA. All other used solvents were of HPLC grade and reagents were of analytical grades in the present study.

### 2.3. Stock and standard working solutions

The accurately weighed (2.5 mg) of each reference materials (Bergenin and Menisdaurin) were separately dissolved in 5 mL methanol in a 50 mL volumetric flask then the volume was made up to 50 mL with methanol to get 50 µg/mL concentration of the

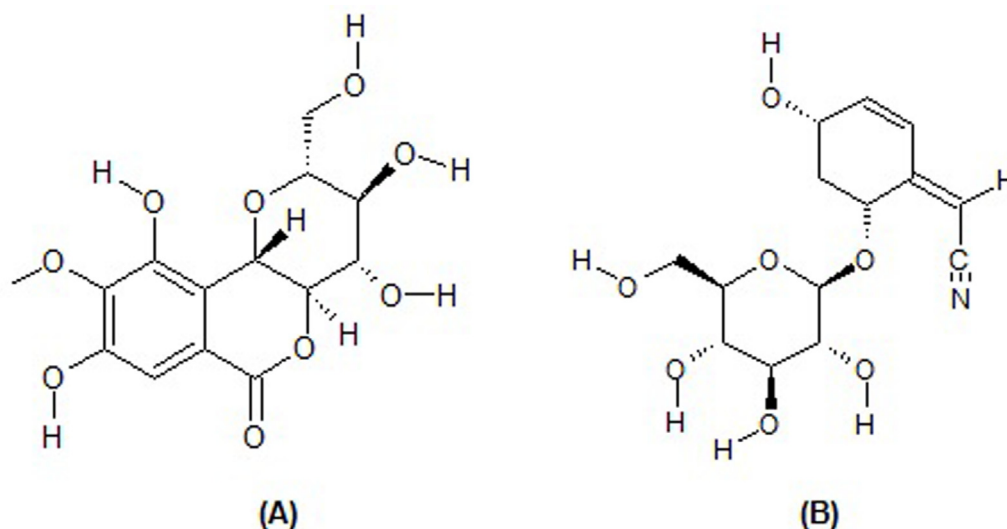


Fig. 2. Chemical structures of Bergenin (A) and Menisdaurin (B).

stock solution. Further dilution of the solutions was done with the mobile phase to get 1 µg/mL concentrations. A six-point calibration plot of different concentration ranging from 1 to 50 µg/mL was made to quantify the bergenin and menisdaurin in *FVME* separately. A mixture of both the biomarker was prepared with a concentration 5 µg/mL each. The mixture of both the biomarkers was used in UPLC-PDA analysis for the identification. The prepared samples were kept at 4 °C until used in the experiment.

#### 2.4. Sample solutions

About 400 mg of *FVME* was dispersed in 10 mL of methanol and sonicated for its complete and quick dissolution. Subsequently, in a 10 mL capacity volumetric flask, exactly 1250 µL of the obtained solution was transferred, and the volume was made up to 10 mL with the binary mobile phase to get a final known concentration of 5 mg/mL. The prepared samples were kept at 4 °C until used in the experiment.

#### 2.5. Instrumentation and chromatographic conditions

The Ultra-Performance Liquid Chromatography (UPLC) coupled with photodiode array detector (PDA) was used for the analysis of bergenin and menisdaurin in *FVME*. The UPLC-PDA Agilent Technologies (1290 Infinity) machine was equipped with Infinity Binary Pump (G4220A), Autosampler (G4226A), Thermostat Column Compartment (G1316C) and Photodiode Array Detector (G4212A). Chem Station software programmes the above configuration of UPLC-PDA. The chromatographic condition such as flow rate, injection volume, column temperature, wavelength and mobile phase ratio was optimized for the proper elution of bergenin and menisdaurin. The elution of bergenin and menisdaurin was performed on Eclipse C<sub>18</sub> analytical column (4.6 × 100 mm, 3.5 µm) (Agilent, California, USA). The column temperature was kept constant 25 ± 1 °C. The most suitable chromatographic condition was attained at 0.16 mL/min flow rate, with an injection volume of 1.0 µL in gradient system at wavelength 235 nm in PDA detector. The mobile phase consisted of solvent A which is 100% acetonitrile and solvent B which is 100% water. The stepwise gradients were 10–90% B (0 min), 30–70% B (0–2 min), 35–65% B (2–5 min), 40–60% B (5–7 min) and 100% B (7–10 min) and the post time was 2 min.

#### 2.6. Method validation

The proposed chromatographic method for the quantification of bergenin and menisdaurin in the *FVME* was validated as per International Conference on Harmonization (ICH) Guideline 2005 (ICH, 2005). The method was validated to determine the parameters such as specificity, linearity, precision, sensitivity, accuracy, and recovery. System suitability was tested at the beginning of the quantitative analysis.

##### 2.6.1. Specificity

Specificity is the ability of a method to discriminate between the study analyte(s) and other components in the sample. Specificity of the developed UPLC method was confirmed by elution order and separation of desired analytes from other potential constituents and natural endogenous materials, impurities, degradants and the matrix in the used methanol extract. The specificity was established by running a procedural blank, where 2 mL of methanol was diluted up to 100 mL adding mobile phase and use for analysis. Also, the identification of the peaks of biomarkers that could be present in the *FVME* was determined by comparing the chromatograms of the reference standard and sample solutions. The resolution was computed and estimated through the Chemstation software. The method was quite specific as can be seen

from Fig. 2. The retention times of both the biomarkers were almost similar during three set of experiments (n = 3). There was no effect of the presence of impurities or other constituents on the retention times and peak shapes of these biomarkers. These findings indicated the excellent specificity of the reported method.

##### 2.6.2. Linearity

The linearity of calibration curves (peak area vs. concentration) for both the biomarkers was confirmed using three calibration curves; six different concentration solution ranging from 1 to 50 µg/mL of both the standard of bergenin and menisdaurin, were used for calibration. The values of the peak area and concentration of the analytes were subjected to the linear regression analysis using MS-Excel software 2010.

##### 2.6.3. Limit of detection (LOD) and limit of quantification (LOQ)

The sensitivity of the developed method was recognized in terms of detection and quantification of the analytes, which was examined from the calibration curves of the bergenin and menisdaurin. The LOD and LOQ were calculated using the following equations:

$$LOD = 3.3 \left( \frac{SD}{S} \right) \quad (1)$$

$$LOQ = 10 \left( \frac{SD}{S} \right) \quad (2)$$

where “SD” was the standard deviation of the responses and “S” was the slope of the calibration curve.

##### 2.6.4. Accuracy

The accuracy of the developed UPLC method was determined by recovery assay using different concentrations of the reported biomarkers. The assay of the biomarkers was carried out in triplicate (n = 3); adding known amounts (10, 20 and 40 µg/mL of both bergenin and menisdaurin) of standard solutions into the initial concentration of the sample. The accuracies were determined by interpolation of three replicates peak areas of these biomarkers.

##### 2.6.5. Precision

The precision of the method was considered on repeatability, intermediate precision (inter-day variation) and reproducibility by determination of standard solution at 100% of the test concentration. To assess the intra-day precision (repeatability) of the method, and the sample was injected three times within a day. The inter-day precision was determined with the sample assayed on different days and by another analyst. Reproducibility was assessed by injection of the sample three times and consequently comparing the results in two separate laboratories. Precision was expressed as the relative standard deviations (% RSD) of the concentrations of each biomarker.

##### 2.6.6. Recovery

Recovery of the analyte by using the developed UPLC method was retrieved by analyzing the obtained peak areas of three determinations at four different concentration levels (20, 30, 40, and 50 µg/mL, for both bergenin and menisdaurin). The variations in the recovered amount were stated in terms of percentage (%) of the obtained concentrations of the standards as well as in terms of “relative standard deviations” (%RSD).

##### 2.6.7. Robustness

Robustness of the method was determined by varying the wavelength parameter from 233 nm to 237 nm, by using columns from different suppliers and by changing the flow rate from 0.15 to 0.17 mL/min, for that three sample solutions were prepared and analyzed under the conditions established.

## 2.7. Statistical analysis

The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test for the estimation of total variation in a set of data. Results were communicated in terms of mean values with  $\pm$ SD where the probability ( $p < 0.05$ ) of getting the result was considered as significant.

## 2.8. Analysis of the FVME sample

The developed and validated UPLC-PDA method was applied for the identification and quantitation of bergenin and menisdaurin in the methanolic extract of *Flueggea virosa* (FVME). Identification of the bergenin and menisdaurin biomarker was confirmed by comparing the retention times; Quantification was performed by linear calibration plots of the PDA absorption peak area at 235 nm against concentration.

## 3. Results

### 3.1. Chromatographic condition optimization

The chromatographic condition such as flow rate, injection volume, column temperature, wavelength and mobile phase ratio was

**Table 1**  
Retention time and linear regression data for the calibration curve and sensitivity parameters for bergenin and menisdaurin.

| Parameters                           | For bergenin           | For menisdaurin       |
|--------------------------------------|------------------------|-----------------------|
| Linearity range ( $\mu\text{g/mL}$ ) | 1–50                   | 1–50                  |
| Regression equation                  | $Y = 1.2805x + 1.0123$ | $Y = 2.5983x + 1.326$ |
| Correlation coefficient ( $r^2$ )    | $0.9971 \pm 0.0004$    | $0.9962 \pm 0.0004$   |
| Retention time ( $R_t$ )             | 3.068 min              | 2.723 min             |
| Slope $\pm$ SD                       | $1.2805 \pm 0.012$     | $2.5983 \pm 0.038$    |
| Intercept $\pm$ SD                   | $1.0123 \pm 0.011$     | $1.326 \pm 0.015$     |
| LOD ( $\mu\text{g/mL}$ )             | 0.032                  | 0.048                 |
| LOQ ( $\mu\text{g/mL}$ )             | 0.099                  | 0.148                 |

**Table 2**

Intra- and inter-day precision of the developed UPLC method for the analysis of bergenin and menisdaurin (mean  $\pm$  SD,  $n = 3$ ).

| Analyte     | Nominal concentration ( $\mu\text{g/mL}$ ) | Intra-day precision                                  |         | Inter-day precision                                  |         |
|-------------|--|--|---------|--|---------|
|             |  | Concentration detected ( $\mu\text{g/mL}$ ) $\pm$ SD | RSD (%) | Concentration detected ( $\mu\text{g/mL}$ ) $\pm$ SD | RSD (%) |
| Bergenin    | 10.00                                      | $10.004 \pm 0.135$                                   | 1.353   | $9.373 \pm 0.125$                                    | 1.337   |
|             | 20.00                                      | $19.348 \pm 0.324$                                   | 1.678   | $18.638 \pm 0.304$                                   | 1.634   |
|             | 40.00                                      | $39.048 \pm 0.725$                                   | 1.856   | $38.585 \pm 0.705$                                   | 1.827   |
| Menisdaurin | 10.00                                      | $9.984 \pm 0.138$                                    | 1.383   | $9.945 \pm 0.118$                                    | 1.188   |
|             | 20.00                                      | $20.060 \pm 0.321$                                   | 1.601   | $19.945 \pm 0.311$                                   | 1.559   |
|             | 40.00                                      | $39.134 \pm 0.686$                                   | 1.755   | $38.942 \pm 0.676$                                   | 1.738   |

\* RSD: "relative standard deviation".

**Table 3**

Recovery of bergenin and menisdaurin for the accuracy of the developed method (mean  $\pm$  SD,  $n = 3$ ).

| Percentage of bergenin added (%)    | Theoretical concentrations of bergenin ( $\mu\text{g/mL}$ )             | Concentrations of bergenin found ( $\mu\text{g/mL}$ ) $\pm$ SD    | % RSD | % Recovery |
|-------------------------------------|---|---|-------|------------|
| 0.0                                 | 20.0  | $19.876 \pm 0.29$   | 1.481 | 99.37      |
| 50.0                                | 30.0  | $30.163 \pm 0.70$   | 2.332 | 100.54     |
| 100.0                               | 40.0  | $39.818 \pm 0.83$   | 2.091 | 99.54      |
| 150.0                               | 50.0  | $50.746 \pm 1.04$   | 2.063 | 101.49     |
| Percentage of menisdaurin added (%) | Theoretical concentrations of menisdaurin ( $\mu\text{g/mL}$ ) $\pm$ SD | Concentrations of menisdaurin found ( $\mu\text{g/mL}$ ) $\pm$ SD | % RSD | % Recovery |
| 0.0                                 | 20.0  | $20.016 \pm 0.45$   | 2.252 | 100.08     |
| 50.0                                | 30.0  | $29.594 \pm 0.75$   | 2.536 | 98.64      |
| 100.0                               | 40.0  | $39.282 \pm 1.10$   | 2.803 | 98.20      |
| 150.0                               | 50.0  | $49.621 \pm 1.45$   | 2.924 | 99.24      |

optimized for the proper elution and resolution of bergenin and menisdaurin in both the standard mixture and FVME. Eclipse  $C_{18}$  analytical column ( $4.6 \times 100$  mm,  $3.5 \mu\text{m}$ ) (Agilent, California, USA) was used for the separation and identification. The mobile phases were a gradient of water and acetonitrile. The optimized gradient was solvent A which was 100% acetonitrile and solvent B which was 100% water. The stepwise gradients were 10–90% B (0 min), 30–70% B (0–2 min), 35–65% B (2–5 min), 40–60% B (5–7 min) and 100% B (7–10 min) and the post time was 2 min. The different flow rate (0.10–0.20 mL/min) were tried to get the complete resolution and separation. The best separation was achieved at 0.16 mL/min. Moreover, the wavelength was also optimized to get the absorption maxima ( $\lambda_{\text{max}} = 235$  nm) in PDA detector. The chromatograms of the standard individual biomarker were also obtained using the same chromatographic conditions and used for calibration plot. The chromatograms of both standards in the standard mixture (bergenin and menisdaurin) and samples solution (Plant extract) were given in Fig. 2a and b, respectively.

### 3.2. Method validation

#### 3.2.1. Linearity

Linear calibration curves were obtained over a broad concentration range of 1–50  $\mu\text{g/mL}$  for bergenin and menisdaurin. Calibration curves exhibited linear regression with correlation coefficient ( $r^2$ ) values 0.9971 and 0.9962 for bergenin and menisdaurin, respectively. The parameters related to linearity are described in Table 1.

#### 3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

The lowest amount of an analyte in any sample that can be noticed and identified but not substantially quantified is termed as LOD of any analytical method. The lowest amount of an analyte in any sample that can be calculated accurately (quantify) with appropriate precision is termed as LOQ of an analytical method. The LOD/LOQ ( $\mu\text{g/mL}$ ) obtained (Table 1) through the developed



UPLC-PDA method were found as 0.032/0.099 and 0.048/0.148 for bergenin and menisdaurin, respectively.

### 3.2.3. Precision

The numerical values obtained for the intra-day and inter-day precision were documented in Table 2. The relative standard deviation (RSD) values for repeatability of intra-day and inter-day precision studies were below 2.0%, which was very well matched with the International Conference on Harmonization guidelines, exhibited that developed method was found to be precise.

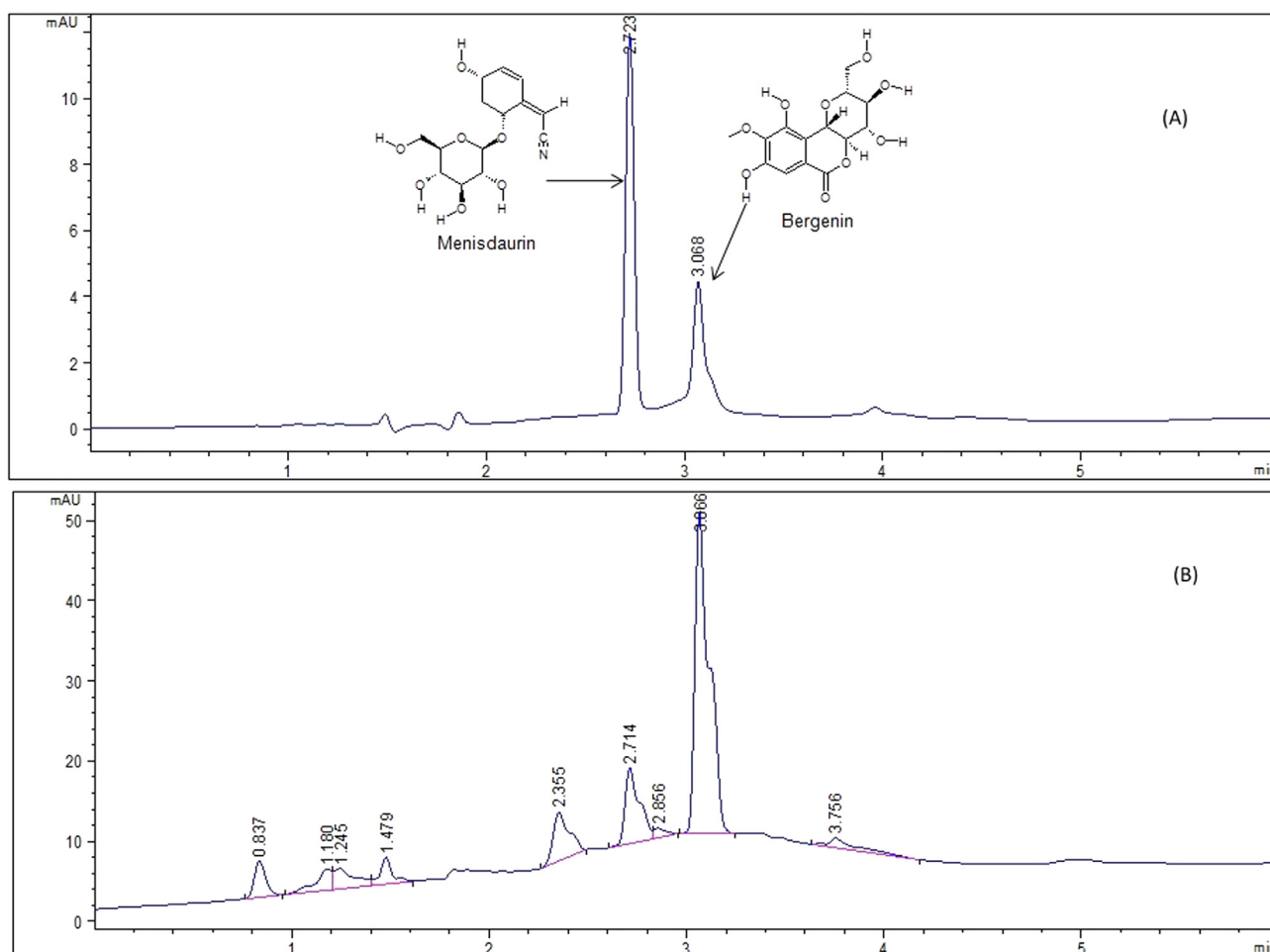
### 3.2.4. Recovery as accuracy

The accuracy of the developed UPLC-PDA method was appraised at four (0, 50, 100 and 150%) concentration levels (viz. 20, 30, 40 and 50 µg/mL) in triplicate ( $n = 3$ ). The recoveries (%) and %RSD were calculated from the numerical values of the  $y$ -intercept and slope of the obtained calibration curve. The calculated recovery values (Table 3) were found in the range of 99.37–101.49% and 98.20–100.08%, for bergenin and menisdaurin, respectively confirming the accuracy of the developed method.

**Table 4**

Robustness of the developed UPLC method (20 µg/mL, of bergenin and menisdaurin, mean ± SD,  $n = 3$ ).

| Optimization conditions                                 | Peak area for 20 µg/mL of bergenin |         | Peak area for 20 µg/mL of menisdaurin |         |
|---|------------------------------------|---------|---------------------------------------|---------|
|   | Mean ± SD                          | RSD (%) | Mean ± SD                             | RSD (%) |
| <b>Wavelength (<math>\lambda_{max} = 235</math> nm)</b> |                                    |         |                                       |         |
| (233 nm)  | 160.352 ± 4.391                    | 2.738   | 259.868 ± 6.691                       | 2.574   |
| (235 nm)  | 161.462 ± 4.567                    | 2.828   | 262.679 ± 6.957                       | 2.648   |
| (237 nm)  | 158.628 ± 4.458                    | 2.810   | 258.492 ± 6.857                       | 2.652   |
| <b>Mobile phase flow rate (0.16 ± 0.1)</b>              |                                    |         |                                       |         |
| (0.15 mL/min)   | 134.356 ± 3.528                    | 2.626   | 253.193 ± 6.617                       | 2.613   |
| (0.16 mL/min)   | 150.441 ± 4.366                    | 2.902   | 263.378 ± 7.054                       | 2.678   |
| (0.17 mL/min)   | 128.187 ± 2.669                    | 2.082   | 245.851 ± 6.173                       | 2.511   |



**Fig. 3.** Representative chromatogram of bergenin and menisdaurin estimation in the *Flueggea virosa* methanolic extract (FVME) [Conditions: Eclipse XDB 80 Å C18 column (4.6 × 100 mm, 3.5 µm); mobile phase, acetonitrile: water (gradient system); flow rate, 0.16 mL/min;  $\lambda_{max} = 235$  nm at temperature (25 ± 1 °C)]. (A) Representative chromatogram of menisdaurin and bergenin showing retention time at 2.723 and 3.068 min, respectively; (B) Representative chromatogram of *F. virosa* methanolic extract (FVME) showing menisdaurin at Rt = 2.714 min and bergenin at Rt = 3.066 min.

**Table 5**Detailed analysis report of bergenin and menisdaurin in methanolic extract of *Flueggea virosa*.

| Theoretical concentration of extracts (µg/mL) | Concentration of reference compound found (µg/mL) ± SD | Drug content (%) | Retention time (R <sub>t</sub> ) (min) |
|---|--|------------------|--|
| <i>Bergenin</i>                               |  |                  |  |
| 500.0   | 76.149 ± 2.188   | 15.229           | 3.059                                  |
| 1000.0  | 151.92 ± 4.302   | 15.192           | 3.066                                  |
| 5000.0  | 753.54 ± 15.317  | 15.071           | 3.068                                  |
| <i>Menisdaurin</i>                            |  |                  |  |
| 500.0   | 15.961 ± 0.485   | 3.192            | 2.714                                  |
| 1000.0  | 32.387 ± 0.891   | 3.238            | 2.725                                  |
| 5000.0  | 169.871 ± 4.892  | 3.397            | 2.761                                  |

**Table 6**

Retention capacity (k), Selectivity (α) and Resolution (Rs) of Bergenin and Menisdaurin in the standard and sample solution of FVME.

| Sample name                               | k1   | k2   | α   | Rs    |
|---|------|------|-----|-------|
| <i>Markers in standard sample mixture</i> |      |      |     |       |
| Bergenin - Menisdaurin                    | 1.17 | 1.45 | 1.2 | 1.145 |
| <i>Markers in sample solution of FVME</i> |      |      |     |       |
| Bergenin - Menisdaurin                    | 2.2  | 2.6  | 1.1 | 1.0   |

### 3.2.5. Robustness of the developed UPLC-method

When the wavelength was deliberately changed from 233–237 nm, almost no marked effect was detected in the chromatogram, and no significant difference was found in peak area as well as in retention time (R<sub>t</sub>). However, a little variation in retention time (R<sub>t</sub>) was observed when the flow rate was varied from 0.15 to 0.17 mL/min. The low calculated values of standard deviations and %RSD with practically unaffected R<sub>t</sub> values for bergenin and menisdaurin subsequently with small careful and deliberate changes as mentioned above indicated the robustness of the developed UPLC-method (Table 4).

### 3.3. Analysis of bergenin and menisdaurin in FVME

The chromatographic characteristic features such as retention times and λ max (UV–vis absorption) for the standards were established, and the identical experimental conditions were applied for determining corresponding components in the samples. The typical UPLC chromatograms of the mixture of standards solution and sample solution demonstrate the complete resolution of bergenin and menisdaurin (Fig. 3a and b). Quantitative analyses of bergenin and menisdaurin in the FVME sample was performed using linear calibration plot and found to be 15.16 and 3.28% w/w, respectively (Table 5).

## 4. Discussion

The UPLC separation parameters, i.e., retention (k), separation (α), and resolution (Rs) factors were calculated for the resolved biomarkers and given in Table 6. It indicates that the values of capacity, separation and resolution factors for bergenin and menisdaurin were >1, resulting to the complete separation. The values of capacity factor and selectivity factor for both the biomarkers in standard and sample (FVME) were same while the values of resolution factor of bergenin and menisdaurin in a standard and sample (FVME) were slightly different; it may be because of the presence of the other constituents in the sample solution. The sharp peaks of the chromatograms of standards and sample mixtures showed that the chromatographic conditions were optimized. The prominent presence of bergenin (15.16% w/w) in *F. virosa* could be a new finding for the development of herbal formulations because of its antiplasmodial (Khan et al., 2016; Uddin

et al., 2014), antioxidant and immunomodulatory actions (Siddiqui et al., 2015). Moreover, the lesser presence of menisdaurin (3.28% w/w) has been detected which shows the specificity and sensitivity of the developed method. Thus, the developed UPLC-PDA method would be very helpful for the identification and quantification of bergenin and menisdaurin either as a mixture or individual.

## 5. Conclusion

The reported UPLC-PDA method was developed and validated by an extensive experiment for the analysis of bergenin and menisdaurin in FVME. The UPLC-PDA method was optimized and found to be economical, reproducible, accurate, linear, precise, and robust. The developed and validated method was used for the separation, identification, and quantitation of these biomarkers bergenin and menisdaurin in FVME. According to the qualitative and quantitative results, the method could be applied to the quality control of pharmaceutical preparations and the standardization of the herbal products.

## Conflict of interest

The authors declare that they do not have any conflict of interest.

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