Simultaneous Quantification of Biomarkers Bergenin and Menisdaurin in the Methanol Extract of Aerial Parts of Flueggea virosa by Validated HPTLC Densitometric Method

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A simple, sensitive high-performance thin-layer chromatography (HPTLC) method was developed for the simultaneous quantification of biomarker bergenin and menisdaurin in the methanol extracts of aerial parts of Flueggea virosa (FVME). Chromatography was performed on glass-backed silica gel 60F254 HPTLC plates using dichloromethane: methanol as mobile phase. Scanning and quantification was done at UV absorption maxima of 260 nm. The system was found to give compact spot for bergenin and menisdaurin at Rf = 0.29 ± 0.01 and 0.16 ± 0.01, respectively. The linearity ranges for bergenin and menisdaurin were found to be the same (100–800 ng/spot) with correlation coefficients (R2 values) of 0.997 and 0.999, respectively. The limit of detection for bergenin and menisdaurin was found to be 27 and 36.2 ng, respectively. Intra- and interday precisions (n = 6) for bergenin and menisdaurin were found to be 1.41–1.71 and 1.65–1.87%, and 1.68–1.89 and 1.75–1.93%, respectively. The percent recoveries were found to be 98.7–99.4 and 99.5–99.9%, respectively, for bergenin and menisdaurin. The percentage of bergenin and menisdaurin was found to be 15.25 and 4.22% (w/w), respectively, in FVME. The developed method permitted the simultaneous quantification of bergenin and menisdaurin and showed good resolution and separation from other constituents of extract; hence, the method can be used to standardize herbal formulations as well as bulk drugs for bergenin and menisdaurin.

Keywords: Flueggea virosa, Bergenin, Menisdaurin, HPTLC, Validation

Introduction

Flueggea virosa belongs to the family Euphorbiaceae and grows wild in tropical Africa and most parts of the world, but can also be domesticated. Different parts of the plant have been claimed to have many folkloric usages for treatment of many diseases like diabetes, HIV-related infections, arrhythmia, fever, malaria, epilepsy and hepatitis (1, 2). It has been used as medicinal plant for a number of biological activities such as antioxidant (3), antiplasmodial, trypanocidal and antiarrhythmic (4). Phytochemical studies of F. virosa showed the presence of triterpenoids (5), steroids, flavonoids and saponins (6), resins, steroids (7), alkaloids (8), cardiac glycosides and anthraquinones (9).

Bergenin (Figure 1) is a trihydroxybenzoic acid glycoside, isolated from several plants (22–25). It has been reported to be active as antimicrobial (26), anti-inflammatory (27), antithepatitis-B (23) and as antitumor agent (28).

In recent years, because of its several unique features like low operation cost, high sample throughput and need for minimum sample clean-up, the high-performance thin-layer chromatography (HPTLC) has become a conventional analytical approach for the quality control (QC) of herbal drugs (29). It is widely used for the identification, assay and the testing for purity, stability, dissolution or content uniformity of raw materials (herbal and animal extracts, fermentation mixtures, drugs and excipients) and formulated products (pharmaceuticals, cosmetics and nutrients) (30). Bergenin has been quantified in several plant extracts by the use of HPTLC like in different Bergenia species (B. ligulata, B. ciliata and B. stracheyi) (31), in B. ligulata (32) and Mallotus philippensis (33), but a complete validated HPTLC method has not been reported yet for simultaneous quantification of bergenin and menisdaurin in the aerial parts of F. virosa. Therefore, the present study was planned to develop and validate HPTLC densitometric method for analysis of bergenin and menisdaurin. The same method was used for the simultaneous quantification of bergenin and menisdaurin in methanol extract of aerial parts of F. virosa. The proposed method was validated as per International Conference on Harmonization (ICH) guideline (34).

Experimental

Materials

The aerial parts of F. virosa were collected from the southern region of the Kingdom of Saudi Arabia and authenticated by Dr Mohammed Yusuf, field taxonomist, Medicinal Plant Collection and Survey Unit, Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia. Specimen of the plant was deposited in the Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia.

Apparatus and reagents

Bergenin (standard) was obtained from Sigma-Aldrich and menisdaurin (standard) from ChemFaces. Analytical grade reagents and solvents (dichloromethane and methanol) were purchased from WINLAB and BDH (UK). Glass-backed silica gel 60F254 HPTLC plates (10 × 10 cm) were purchased from Merck (Darmstadt, Germany). Methanol solution of standards and extract was applied to chromatographic plates bandwise, by means of a CAMAG automatic TLC sampler-4 (CAMAG, Muttenz, Switzerland)
The aerial parts of *F. virosa* were air-dried and pulverized. One hundred grams of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol (95%) for 72 h. Thereafter, methanol extract was filtered through Whatman paper no. 42, and the obtained extract was concentrated under reduced pressure and finally vacuum-dried. The yield of *F. virosa* methanol extract (FVME) was found to be 9.76% (w/w). Marker compounds were found to be soluble in methanol hence methanol was used for the extraction of sample.

**Preparation of standard stock solution**

Stock solution of standards (bergenin and menisdaurin) (1 mg/mL) was prepared in methanol. One milliliter of the stock solution was again diluted with 9 mL of methanol to make the concentration 100 ng/μL. For calibration, bergenin and menisdaurin standard solutions (1, 2, 3, 4, 5, 6, 7 and 8 μL) were applied on an HPTLC plate to furnish concentration in the range 100, 200, 300, 400, 500, 600, 700 and 800 ng/band.

**Method validation**

Method validation was carried out as per the ICH guidelines for linearity range, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ) and recovery. Precision (inter- and intraday) and accuracy of the assay were evaluated by performing replicate analyses (*n* = 6) of samples at three quality control (QC) levels low, medium and high of 150, 300 and 600 ng/band, respectively, for bergenin and 200, 400 and 600 ng/band, respectively, for menisdaurin. Interday precision and accuracy were determined by repeating the intraday assay on three different days. Precision was expressed as % CV of measured concentrations for each calibration level whereas accuracy was expressed as percentage recovery.

Robustness was studied in triplicate at 300 ng/band by making small changes to mobile phase composition, mobile phase volume and duration of mobile phase saturation. The results were examined in terms of relative standard deviation (RSD %) and standard error of peak areas. Mobile phases prepared from dichloromethane : methanol (8.5 : 1.5, v/v) in different proportions (8.3 : 1.7, v/v; 8.2 : 1.8, v/v; 8.6 : 1.4, v/v) were used for chromatography. Mobile phase volume and duration of saturation investigated were 20 ± 2 mL (18, 20 and 22 mL) and 20 ± 10 min (10, 20 and 30 min), respectively. The plates were activated at 110°C for 30 min before chromatography.

The LOD and LOQ were calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approaching the LOD according to the formulae: [LOD = 3.3 (SD/S) and LOQ = 10 (SD/S)]. The SD of the response was determined based on the SD of y-intercepts of regression lines.

Recovery was studied by applying the method to drug samples to which known amounts of marker corresponding to 50, 100 and 150% of the bergenin and menisdaurin had been added. Each level was analyzed in triplicates. This was to check the recovery of bergenin and menisdaurin at different levels in the extracts.

**Results**

**Method development**

Chromatogram was developed for bergenin and menisdaurin under chamber saturation conditions using solvents dichloromethane: methanol (8.5 : 1.5, v/v) as solvent system (Figure 3A and B). The same mobile phase was also employed for the separation of sample FVME (Figure 4). The developing chamber was saturated with 25 mL of mobile phase and optimized saturation time was observed as 20 min. The densitometric analysis was performed at 260 nm in the absorbance mode. Compact, sharp, symmetrical and high-resolution bands of bergenin and menisdaurin were observed.
obtained at \( R_f 0.29 \pm 0.001 \) and \( 0.16 \pm 0.001 \), respectively (Figure 5). The developed method was found to be quite selective with good baseline resolution. The identity of the bands of compounds in the sample extracts was confirmed by overlaying their absorption spectra with those of the standards (Figure 6). The findings of this experiment proposed maiden procedure to quantify bergenin and menisdaurin simultaneously in *F. virosa* by an economic, accurate, rapid and cost-effective, validated HPTLC method.

**Method validation**

Linearity of compounds bergenin and menisdaurin was validated by the linear regression equation and correlation coefficient. The eight-point calibration curve for bergenin and menisdaurin was found to be linear in the range of 100–800 ng/spot. Regression equations and \( R^2 \) values for the reference compound bergenin and menisdaurin were observed as \( Y = 6.459X + 53.046 \) and \( 0.997 \) and \( 5.723X + 62.801 \) and \( 0.999 \), respectively, which revealed a good linearity response for developed method (Table I). The mean value with ±SD of the slope and intercept was \( 6.459 \pm 0.004 \) and \( 53.046 \pm 0.011 \) for bergenin and \( 5.723 \pm 0.003 \) and \( 62.801 \pm 0.013 \) for menisdaurin. No significant difference was observed in the slopes of standard plots \((P > 0.05)\).

Table II presents intra- and interday precision and accuracy of the assay for bergenin and menisdaurin at triplicate QC levels (150, 300 and 600 ng/band and 200, 400 and 600 ng/band, respectively, for bergenin and menisdaurin). Both intra- and interday precision was determined in terms of percent of coefficient variation (% CV). Intra- and interday precisions \((n = 6)\) for bergenin and menisdaurin were found to be 1.41–1.71 and 1.65–1.87%, and 1.68–1.89 and 1.75–1.93%, respectively, which demonstrated the good precision of proposed method. However,
intra- and interday accuracy of bergenin and menisdaurin were observed as 97.2–98.1 and 97.7–98.5% and 98.6–99.4 and 99.2–99.8%, respectively. These results indicated the accuracy of the proposed method. In Table III, the SD and % RSD were calculated at 300 ng/band concentration level of bergenin and menisdaurin. The low values of SD and % RSD obtained after introducing small deliberate changes in the method indicate that the method was robust. LOD and LOQ were found to be 27 and 81 ng/band and 36.2 and 108 ng/band, respectively, for bergenin and menisdaurin (Table I). This indicated that the proposed method exhibits a good sensitivity for the quantification of above compounds. Good recoveries were obtained by the fortification of the sample at three QC levels of bergenin and menisdaurin. It is evident from the results that the percent recoveries for bergenin and menisdaurin after sample processing and applying were in the range of 98.7–99.4 and 99.5–99.9%, respectively, as shown in Table IV.

**HPTLC analysis of FVME**

The utility of the proposed method was evaluated by applying this method for the quantification of bergenin and menisdaurin...
in FVME (Figure 7). The content of bergenin and menisdaurin in the FVME was found to be $15.25 \pm 0.03$ and $4.22 \pm 0.05\%$ (w/w), respectively. It is for the first time, a simple, accurate and rapid HPTLC method has been developed for the simultaneous quantification of bioactive markers bergenin and menisdaurin in *F. virosa* collected from Saudi Arabia.

**Discussion**

The HPTLC method developed is precise, accurate and specific for the simultaneous determination of bergenin and menisdaurin. The method is proved to be reproducible with the help of statistical analysis and is also selective for the analysis of bergenin and menisdaurin in crude drug as well as herbal formulations. The quantity of bergenin found in *F. virosa* (15.25%) is the largest quantity ever reported earlier on any plant. It has been reported that bergenin is a very good antioxidant and immunomodulatory compound and this might be due to its chemical nature of c-glycosides, which are more stable and acid-resistant than other glycosides and probably its therapeutic action is due to the attached free OH groups on the molecule. Being present in higher amount, *F. virosa* can be exploited in herbal formulations monitored by proposed HPTLC method. Menisdaurin is a non-cyanogenic cyanoglycoside, which does not liberate HCN after enzymatic or acid hydrolysis, which indicate that the physiological activity of menisdaurin is either due to intact glycoside or corresponding lactone menisdaurilide formed by cyclization when treated with acid. The use of menisdaurine in hepatitis B and in cancer suppression suggests that it is a medicinally useful glycoside hence its presence in *F. virosa* (4.22%) supports folkloric use of *F. virosa* in the treatment of hepatitis (1). Therefore, proposed method of HPTLC can be taken into consideration for the quantification of menisdaurin in raw material as well as in formulations by using the proposed HPTLC method.

**Conclusion**

The outcomes of this study may help in providing optimized use of bergenin and menisdaurin as well as other acid and cyanoglycosides in pharmaceutical formulations and make them more efficacious and safe. As the given method separated the drugs from other constituents present in the extract, it can be employed as quality control method for in process as well as finished products in the market. It is suggested for the further study of degradation kinetics of bergenin and menisdaurin and its determination in plasma and other biological fluids.

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**References**

10. Dhalwal, K., Shinde, V.M., Biradar, Y.S., Mahadik, K.R.; Simultaneous quantification of bergenin, catechin, and gallic acid from *Bergenia ciliata* and *Bergenia ligulata* by using thin-layer chromatography; *Journal of Food Composition and Analysis*, (2008); 21: 496–500.