



VALIDATED RP-HPLC METHOD FOR QUANTIFICATION OF GASTRODIN IN ETHANOLIC EXTRACT FROM THE PSEUDOBULBS OF *Grammatophyllum speciosum* Blume

(Validasi Kaedah FT-KCPT bagi Kuantifikasi Gastrodin di dalam Ekstrak Etanolik dari
Pseudobulbs *Grammatophyllum speciosum* Blume)

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Abstract

A reversed phase-high performance liquid chromatography-diode array detector (RP-HPLC-DAD) method was developed to determine the amount of gastrodin, the major active component of *Grammatophyllum speciosum* pseudobulbs. A previous study suggested that the ethanolic extract of *G. speciosum* had a potential to encourage stemness of keratinocytes. Therefore, in order to determine the quality of *G. speciosum* ethanolic extract, the quantitative analysis of gastrodin should be validated. The optimized RP-HPLC condition was achieved within 18 min, using a column, Inertsil ODS-3 (4.6 x 150 mm, 5 μ m). The mobile phase consisted of a mixture of water: acetonitrile with a gradient from 1:99 to 100:0 in 14 minutes and keep constant at 1:99 for 4 minutes. The flow rate was 1.7 mL/min with the monitored UV wavelength of 220 nm. Gastrodin was eluted at the retention time of 6.5 minutes. The calibration curve of gastrodin at the concentration of 10 - 100 μ g/mL showed good linearity ($r^2 = 0.999$). In addition, the intraday and interday precisions of gastrodin were 1.05 and 0.38%, respectively. The percentage recovery was within 99.3 - 101.5%. Average gastrodin contents in 3 samples were 55.52 - 58.12 mg/g. The results indicate that this method can be utilized to control the quality of *G. speciosum* extract.

Keywords: gastrodin, *Grammatophyllum speciosum*, reversed-phase high performance liquid chromatography, quantification

Abstrak

Kaedah fasa terbalik-kromatografi cecair berprestasi tinggi-pengesan susunan diod (FT-KPCT-PSD) telah dibangunkan untuk menentukan jumlah kandungan gastrodin, komponen aktif utama bagi pseudobulbs *Grammatophyllum speciosum*. Kajian sebelumnya mencadangkan ekstrak etanolik terhadap *G. speciosum* mempunyai potensi menguatkan stem keratinocytes. Oleh demikian, bagi menentukan kualiti ekstrak etanolik *G. speciosum*, analisis kuantitatif terhadap gastrodin perlu ditentusahkan. Keadaan optimum FT-KCPT telah dicapai pada minit ke 18, menggunakan turus Inertsil ODS-3 (4.6 x 150 mm, 5 μ m). Fasa bergerak mengandungi campuran air:asetonitril dengan cerunan dari 1:99 hingga 100:0 bagi tempoh 14 minit dan kekal malar pada 1:99 selama 4 minit. Kadar aliran ialah 1.7 mL/min dengan pemerhatian panjang gelombang UV pada 220 nm. Gastrodin terelusi pada masa tahanan ialah minit ke 6.5. Lengkung kalibrasi gastrodin pada aras kepekatan 10 - 100 μ g/mL telah

menunjukkan kelinearan yang baik ($r^2 = 0.999$). Kejituan intra-hari dan inter-hari telah dicapai masing-masing pada 1.05 dan 0.38%. Peratusan perolehan semula berada di dalam julat 99.3 - 101.5%. Purata kandungan gastrodin di dalam 3 sampel adalah 55.52 - 58.12 mg/g. Keputusan ini menunjukkan kaedah boleh digunakan untuk mengawal kualiti *G. speciosum* yang diekstrak.

Kata kunci: gastrodin, *Grammatophyllum speciosum*, fasa terbalik kromatografi cecair berprestasi tinggi, kuantifikasi

Introduction

Grammatophyllum speciosum Blume or Waan-Phet-Cha-hueng (Thai name) is a plant in Orchidaceae family. It is also known as giant orchid, tiger orchid, sugar cane orchid or queen of the orchid. This gigantic orchid is typically found in the tropical rainforests of Southeast Asia such as Thailand, Malaysia, Indonesia, and Philippines [1, 2]. In Thai traditional medicine, its root is utilized as a treatment for centipede and scorpion venom, while its trunk, when blended with alcohol, is utilized to treat sores. The previous research found that the liquid extract of *G. speciosum* at the concentration of 0.706 mg/mL was effective in treating scorpion venom (*Heterometrus laoticus*) with a 23% cure rate [3]. Phytochemical investigation by Sahakitpichan et al. [2], reported new compounds isolated from the methanolic extract of the fresh pseudobulbs of *G. speciosum* which included glucosyloxybenzyl derivatives of (R)-2-benzylmalic acid and of (R)-eucomic acid, grammatophyllosides A-C, and a new phenolic glucoside, grammatophylloside D, cronupapine, vandateroside II, gastrodin, vanillolloside, orcinolglucoside, and isovitexin.

Gastrodin (Figure 1), a phenolic glycoside [p-hydroxymethylphenyl- β -D-glucopyranoside] found as a major active component in *G. speciosum*, is a potent antioxidant. Gastrodin was also found to function as anti-apoptotic activities in brain areas [4], a memory enhancer [5, 6], as well as having anti-convulsant [7], anti-cell death [8], anti-Alzheimer's disease [9], anti-Parkinson's disease activities [10], as well as protective effects against osteoporosis linking to a reduction in reactive oxygen species [11]. In addition, *G. speciosum* ethanolic extract was reported to have a potential to increase stem cell phenotypes of human keratinocytes. Moreover, the extract also had an ability to protect the cells against superoxide anion-induced cell death [12]. As gastrodin is a major active compound in the *G. speciosum* ethanolic extract, this study selected gastrodin as an analytical marker to control the quality of *G. speciosum* ethanolic extract. It was also used as an analytical marker for quality evaluation of *Gastrodiae rhizoma* extracts using RP-HPLC [13, 14, 15].

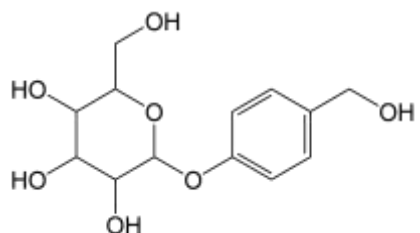


Figure 1. Basic structure of gastrodin

Therefore, in this study, RP-HPLC technique was developed to analyze the quantity of gastrodin in *G. speciosum* extract. The method was developed and validated according to the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [16]. The quantitative analysis of gastrodin, the analytical marker in *G. speciosum* extract by RP-HPLC technique can be utilized to monitor the quality of the plant's raw materials and may also be applied to analyze *G. speciosum* extract in future cosmetic formulations.

Materials and Methods

Extract preparation

Fresh pseudobulbs of *G. speciosum* were collected from the Khao Hin Sorn Royal Development Study Center's Area, Chachoengsao province. Dry pseudobulbs of *G. speciosum* were ground up and macerated 3 times for 3 days in ethanol (1:9 w/v), at 25 °C. The extract was then filtered and evaporated under vacuum pressure at a temperature

below 40 °C. The extract of *G. speciosum* were prepared in 3 different batches (batch 1: January, batch 2: May, and batch 3: September, year 2016). Each batch was collected 4 months apart.

LC-MS analysis

The *G. speciosum* extract was analyzed using a Dionex Ultimate TM 3000 HPLC (Germering, Germany) coupling with Bruker Amazon SL mass spectrometer (Bremen, Germany). Acclaim 120-C18 column (2.1 x 150 mm, 3 µm, Thermo Fisher Scientific, Germany) was used as a stationary phase. Analysis was performed in a gradient mode using 0.1% formic acid-acetonitrile ratio at 99:1 (0-3 minutes), gradient from 99:1 to 90:10 (3-5 minutes), gradient from 90:10 to 75:25 (5-8 minutes), gradient from 75:25 to 50:50 (8-10 minutes), gradient from 50:50 to 1:99 (10-11 minutes), isocratic at 1:99 (11-16 minutes), and re-equilibrate at 99:1 for 5 minutes. The column was maintained at 25 °C with a flow rate of 0.15 mL/min and the injection volume was 5 µL. The mass spectrometer was equipped with an ESI ion source and a quadrupole-ion trap. LC-MS evaluation was performed using full scan in negative mode recorded on a mass range of m/z 100-1,000. Capillary voltage was set at 4,500 V, and drying gas temperature was 200 °C with a flow rate of 7.0 L/min. Nebulizer pressure was set at 2 bars. Gastrodin was recognized by comparing the parent and fragment ions with the reference standard using the multiple reactions monitoring (MRM) method. Data were processed by Compass 1.3 SR2 program.

Instrumentation and chromatographic conditions

Chromatographic analysis was performed using Agilent high performance liquid chromatographic system (Agilent Technologies, California, United States of America) with a quaternary pump (G1311A). An auto-sampler (Agilent G1313A) and column oven (G1316A) were used with a photodiode array detector (G 1315A). Results were acquired and evaluated by ChemStation software (Agilent Technologies, California, United States of America). Chromatography was performed under gradient conditions with a total run time of 18 minutes. The mobile phase was filtered through a 0.22-µm membrane filter prior to use. The mobile phase consisted of solvent A (water) and solvent B (acetonitrile). The gradient started with 99% solvent A for 3 minutes, was adjusted to 90% solvent A for 2 minutes, to 75% solvent A for 3 minutes, to 50% solvent A for 2 minutes, to 100% solvent B for 4 minutes, and finally to 99% solvent A for 4 minutes. The flow rate of the system was 1.7 mL/min with the column temperature controlled at 25 °C. The injection volume was set at 20 µL. DAD was adjusted at 200-380 nm with a monitoring wavelength of 220 nm. The RP-HPLC column used was an Inertsil ODS-3 C18 (4.6 x150 mm, 5 µm) column. Gastrodin was found to elute at the retention time of 6.5 minutes. Gastrodin was identified by comparing the chromatogram and retention time of *G. speciosum* extract with gastrodin standard (% purity > 98%) (Chengdu Biopurify Phytochemicals, Sichuan, China).

Calibration and method validation

An amount 5 mg of gastrodin standard was dissolved in 5 mL of ethanol (20% w/w) and diluted with ethanol (20% w/w) corresponding to test ranges. Gastrodin standard solution was diluted with ethanol (20% w/w) to obtain final concentrations of 10, 20, 30, 40, 50, 70 and 100 µg/mL. After filtering through a 0.22-µm syringe filter, 20 µL of each standard solution was injected into the RP-HPLC system. The calibration curve was constructed by plotting peak areas against the corresponding concentration of gastrodin. Each concentration was analysed in triplicate. The slope, y-intercept and r^2 were calculated using the least squares regression method. Limits of detection (LOD) and quantification (LOQ) were calculated from the calibration line at the low concentration. LOD and LOQ were calculated from the following formulas in equation 1 and 2 [17]:

$$\text{LOD} = 3.3 \text{ Sy/S} \quad (1)$$

$$\text{LOQ} = 10 \text{ Sy/S} \quad (2)$$

where Sy is the standard deviation of the response curve and S is the slope of the calibration curve.

Specificity was examined by separately injecting the gastrodin standard solution at concentration of 50 µg/mL and *G. speciosum* extract solution using RP-HPLC to obtain gastrodin peak response.

The precision of the developed method was evaluated through intraday and interday variations of gastrodin at concentration of 50 µg/mL. The intraday precision analysis was performed in 6 replicates on samples prepared on a single day, while interday precision analysis was performed in 6 replicates on 3 different days. Accuracy was evaluated by determining the average % recovery of analytes. Three different concentrations were prepared by accurately weighing 50 mg of *G. speciosum* extract solution into well-sealed glass containers, after which 80 ml of ethanol (20% w/w) was added and the solution was sonicated for 5 minutes. A gastrodin standard solution was added to each *G. speciosum* extract solution sample to obtain final concentrations of 45, 50 and 55 µg/mL. Ethanol (20% w/w) was then added to acquire a volume of 100 ml. Each concentration was analyzed in triplicate. Average % recovery and % relative standard deviation (%RSD) values were calculated to determine the accuracy and precision of the system.

Sample analysis

An amount 5 mg of *G. speciosum* extract was dissolved in 5 mL of ethanol (20% w/w). The extract mixture was then filtered through 0.22-µm syringe filter, and 20 µL of the resulting filtrate was injected into the RP-HPLC system. Every sample solution was analyzed in triplicate.

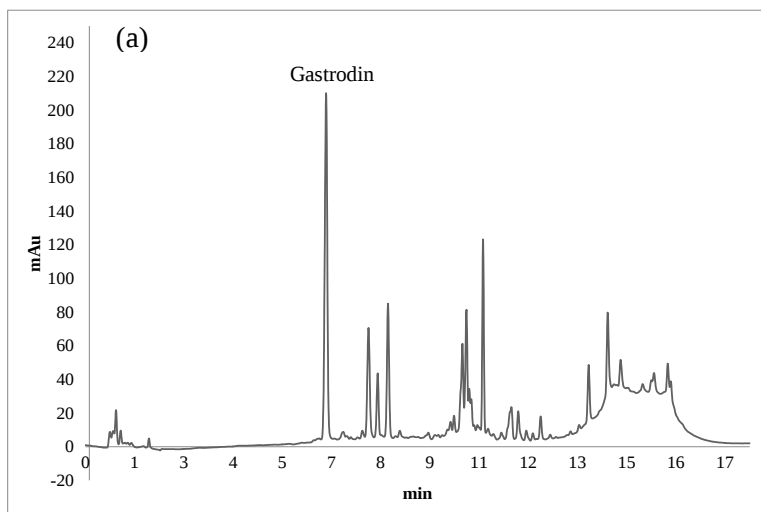
Statistical analysis

Results were expressed as the mean values $\pm$ standard deviation (SD) from at least three independent experiments. The statistical comparisons were carried out using one-way ANOVA with post hoc test (SPSS, SPSS Inc., Chicago, IL, USA). Statistical significance was accepted within the 95% confidence limit ($p < 0.05$).

Results and Discussion

To optimize the RP-HPLC condition, the tests were done under various conditions. The mobile phases were prepared using multiple proportions including water-acetonitrile or water-methanol. Among several C-18 reversed-phase columns, column Inertsil ODS-3 (4.6 x 150 mm, 5 µm) was selected as the most suitable condition because of its relatively shorter running time.

For specificity, gastrodin peaks were identified with retention times comparable with the gastrodin standard and confirmed for its characteristic spectra by diode array detection (range 200-380 nm). The retention time of *G. speciosum* extract samples and standard solutions were found to elute at 6.483 and 6.494 minutes, respectively (Figure 2). In addition, the UV pattern of the extract at the retention time of 6.483 minutes was similar to the gastrodin standard (Figure 3).



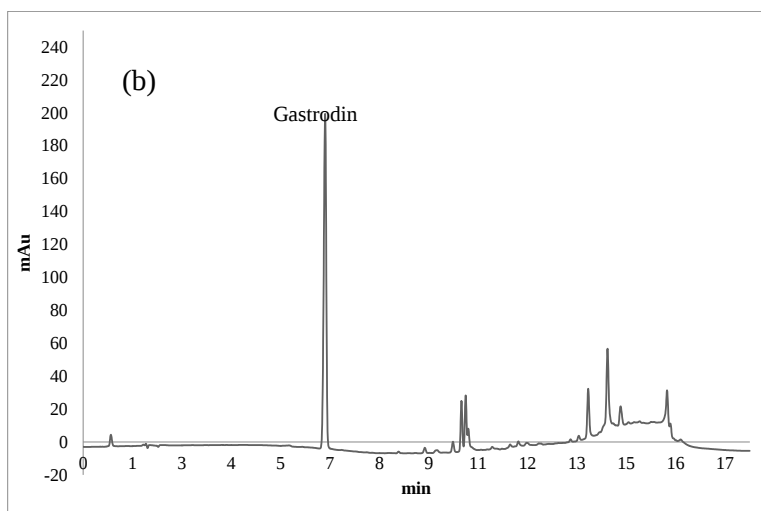


Figure 2. (a) A typical HPLC chromatogram of the *G. speciosum* pseudobulb ethanolic extract and (b) gastrodin standard HPLC chromatogram

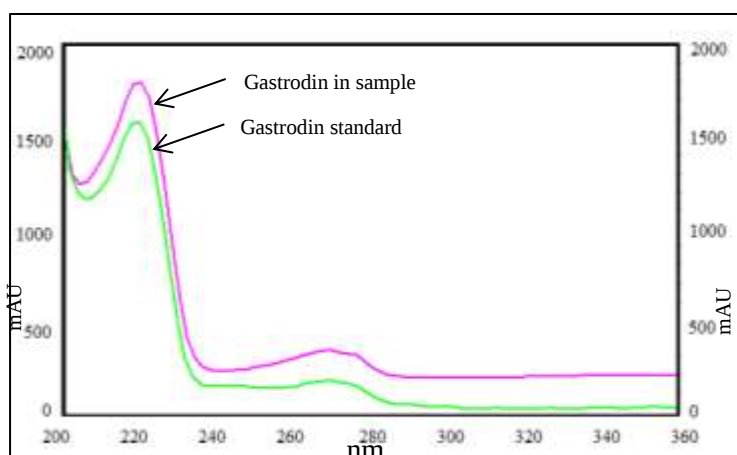


Figure 3. LC-UV absorption patterns of gastrodin in the *G. speciosum* Pseudobulb ethanolic extract (upper line) and gastrodin standard (lower line)

Moreover, the identification of gastrodin, an analytical marker of *G. speciosum* ethanolic extract, was confirmed using LC-MS technique. The mass transition was monitored in the reference and sample solutions to guarantee specificity. The molecular mass at 331.1 m/z ($[M-H]^-$ 331.1) of the *G. speciosum* extract was comparable to the gastrodin standard using formic acid addition in negative ion mode. The fragmentation pattern mass spectrum was found at 285.0, 206.8, 160.8 and 122.9 m/z which was consistent with the previous study [14] (Figure 4). Linearity parameters of the system are shown in Table 1. The results showed that gastrodin exhibited good linearity within specified ranges with coefficient of determination (r^2) of 0.9999. These results complied with the AOAC Guidelines, which stated that the coefficient of determination should be >0.99 for acceptable linearity. The regression equation was $y = 20.0029x + 0.9758$, where y is the peak area and x is the corresponding concentration of gastrodin standard. The linear range was 10 - 100 $\mu\text{g/mL}$. The calculated LOD and LOQ of gastrodin were 0.1244 and 0.3769 $\mu\text{g/mL}$, respectively.

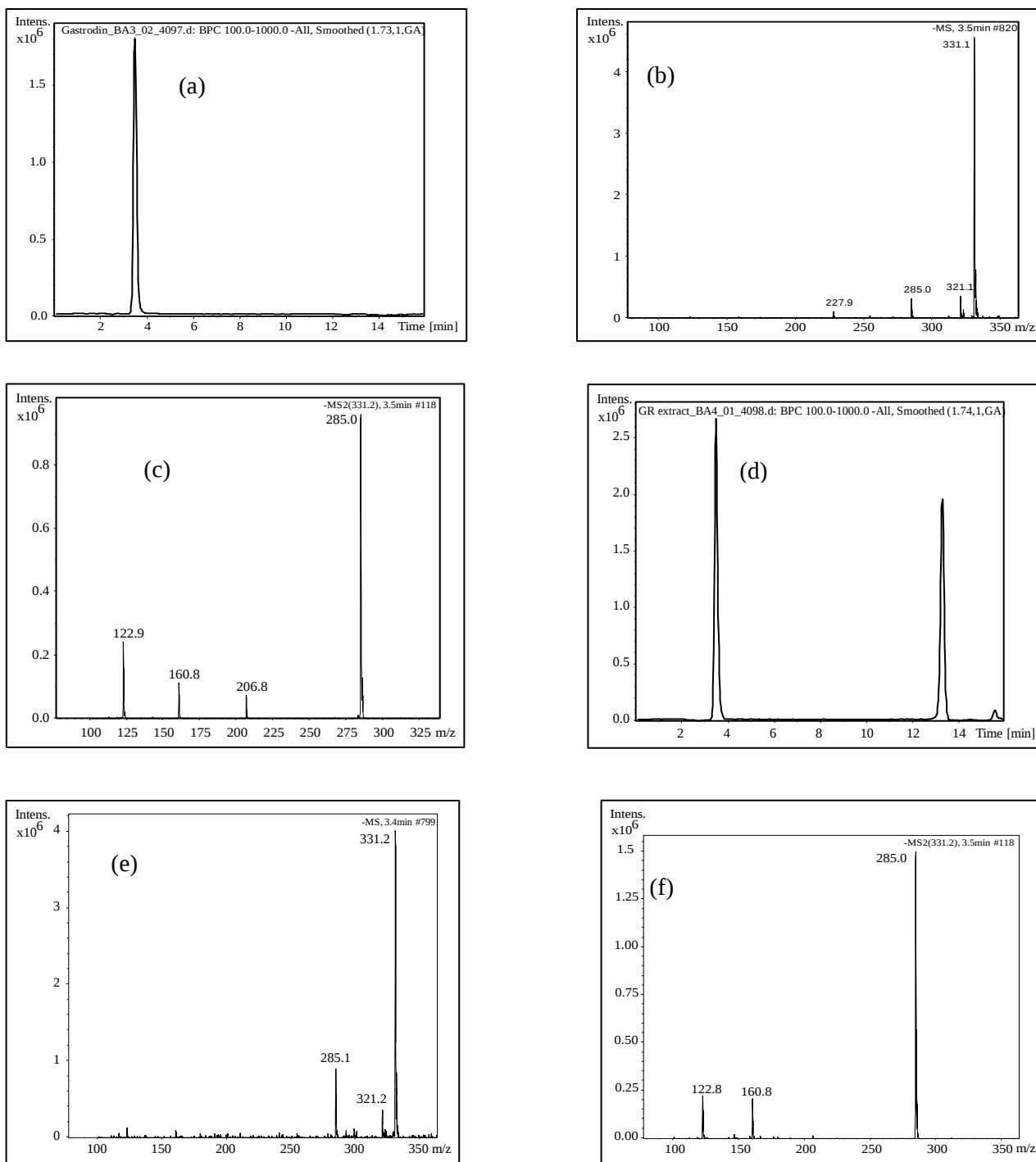


Figure 4. Typical chromatogram and MS spectra of the gastrodin (a) Base peak chromatogram of gastrodin standard (Rt = 3.5 min) (b) MS spectra of gastrodin standard ion [M+HCOOH-H]⁻ (c) MS2 spectra of gastrodin standard ion [M+HCOOH-H]⁻ at m/z 331.1 (d) Base peak chromatogram of *G. speciosum* extract (e) MS spectra of *G. speciosum* extract ion at 3.5 min (f) MS2 spectra of *G. speciosum* extract ion at m/z 331.2

Repeatability precision of the system is shown as a %RSD values in Table 1. The intraday and interday precisions were 1.05 and 0.38%, respectively. The repeatability results for gastrodin analytical markers showed %RSD values of <1.5, which complied with the AOAC Guidelines [16]. The accuracy of the system is shown in Table 1. The average % recovery was found 100.14, 100.54 and 101.25 for 80%, 100% and 120% concentrations of gastrodin, respectively. The average %recovery of gastrodin complied with the AOAC Guidelines, which stated that this value should be between 95-102%. These results indicate that the developed HPLC method was accurate and precise in the determination of gastrodin in ethanolic extracts of *G. speciosum*.

Table 1. Validation data of gastrodin

Parameters	Gastrodin
Linearity	
Range (µg/mL)	10 - 100
Coefficient of determination (r^2)	0.9999
Equation	
Intercept	0.9758
Slope	20.0029
Precision (50 µg/mL)	
Intra-day (%RSD)	1.05
Inter-day (%RSD)	0.38
Accuracy (%Recovery)	
80% (45 µg/mL)	100.14
100% (50 µg/mL)	100.54
120% (55 µg/mL)	101.25
Limit of quantitation (µg/mL)	0.3769
Limit of detection (µg/mL)	0.1244

The amount of gastrodin in 3 different batches of *G. speciosum* ethanolic extract was analyzed using a validated method. The results showed that the amount of gastrodin from batch 1, batch 2, and batch 3 contained 58.12±4.6, 57.24±2.5, and 55.50±1.4 mg/g, respectively (Table 2), which was considered to be not significantly different from one another ($p>0.05$). The result thus suggested that gastrodin levels were not significantly different among batches.

Table 2. Gastrodin contents of *G. speciosum* pseudobulb at different batches (n = 5)

Batch	1	2	3
Gastrodin (mg/g)	58.12±4.6	57.24±2.5	55.50±1.4

Conclusion

This developed HPLC method agreed well with AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals which can be effectively used for the quality control of *G. speciosum* extracts and can be applied for quantification of gastrodin in future cosmetic products. Further studies on the determinations of gastrodin contents from all varieties and sources of *G. speciosum* will be worthwhile in establishing a quality control database.

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