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### **ORIGINAL ARTICLE**

# Development and validation of a simple, sensitive and reproducible method for simultaneous determination of six polyphenolic bioactive markers in *Dendrobium* plants



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### KEYWORDS

Dendrobium; UPLC; Phenolic compounds; Orchid **Abstract** *Dendrobium* is a large genus of orchid plants containing several bioactive polyphenols, including bibenzyls, phenanthrenes, and flavanones that have been used as antioxidants in nutraceutical and pharmaceutical products. Several polyphenols including (2*S*)-eriodictyol, (2*S*)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin are primarily found in *Dendrobium* species and can serve as bioactive markers of orchid plants. In the present study, a simple

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UPLC-UV method for the simultaneous determination of six polyphenols was developed for evaluating the distribution of bioactive phytochemicals in orchid plants and validated according to the ICH Q2 (R1) guidance. The sample was prepared by extracting dendrobium powder with methanol and the supernatant was analyzed using the Waters ACQUITY UPLC<sup>TM</sup> H-Class system on an ACQUITY UPLC<sup>TM</sup> BEH C18 column ( $2.1 \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) with gradient elution of water and acetonitrile, containing 1% v/v trifluoroacetic acid (TFA) each, at a flow rate of 0.2 mL/min and UV detection at 280 nm. The chromatographic condition provided good peak shape and resolution. The method was linear over the specific ranges with the coefficient of determination ( $r^2$ ) > 0.995. Accuracy of the method expressed as %recovery ranged from 80 to 110%. The precision of the method demonstrated as %/CV was < 7.3%. The method is simple, accurate, precise and robust and is recommended for routine quality control analysis of orchid plants containing the six polyphenols as the main principles in the herb. The proposed method was successfully applied for simultaneous quantification of the 6 polyphenolic compounds in 11 samples from 3 *Dendrobium* species, suggesting that the method was suitable for quality assessment of orchid herbal raw materials.

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#### 1. Introduction

Folk medication with natural herbal products has been traditionally used in the Asia health care system to treat acute and chronic diseases including degenerative ailments. They have been used to prevent and treat several diseases because they consist of various bioactive phytochemicals (Lam et al., 2015). Nowadays, polyphenolic compounds are fascinating phytochemicals as potential candidates for the treatment of several diseases. Oxidative stress from cell aging, food intake and environmental pollution, which can induce deteriorated cell growth resulting in a pathological condition, is the main target hit of polyphenols (Minh et al., 2016). Antioxidant activity from polyphenolic compounds plays not only a crucial role in the traditional health care system but is also commonly applied for the combination treatment with other substances in modern medicine, skincare, and nutraceutical products.

Dendrobium species, belonging to the family Orchidaceae, are polyphenol-rich plants often used in traditional medicine for human disorder treatments such as cancer, autoimmune disorders and atherosclerosis (Handique and Baruah 2002). It is also a source of tonic, astringent, analgesic, and anti-inflammatory substances. Polyphenols in orchid plants are naturally synthesized from secondary metabolisms. These secondary metabolites do not essentially function in growth promotion, but their actions involve alternative roles such as insect-repellant, disease protection, or plant hormone<sup>4</sup>. The natural polyphenols, including bibenzyls, flavanones, and phenanthrenes, are major components in orchids of the genus Dendrobium and possess various bioactivities (Phechrmeekha et al., 2012, Chimsook 2016). Among those polyphenols, (2S)-eriodictyol, (2S)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin (Fig. 1) are primarily found and can serve as bioactive markers for evaluating phytochemical distribution of orchid plants.

Several published articles reported the phytochemical distribution and biological activities of the six polyphenols in *Dendrobium* spp (Song et al., 2010; Song et al., 2012; Cao et al., 2019; Thongin et al., 2019; Deng et al., 2020; Liang et al., 2020; Guo et al., 2022; Cardile et al., 2020; Hossasin, 2011; Fischer et al., 2019; Guo et al., 2022; Majumder and Chatterjee, 1989; Paudel et al., 2019; Cai, 2021). Chysotoxine isolated from *D. aurantiacum* inhibited 6-hydroxydopamineinduced apoptosis via mitochondria protection and NF- $\kappa$ B modulation and possessed neuroprotection effects (Song et al., 2010; Song et al., 2012). Eriodictyol isolated from *D. officinale* and *D. ellipsophyllum* was reported with various biological activities including neuroprotective, cardioprotective, hepatoprotective, antidiabetic and antioxidant effects (Cao et al., 2019; Thongin et al., 2019; Deng et al., 2020; Liang et al., 2020; Guo et al., 2022). Moscatilin found in *D. moschatum*, *D. aurantiacum* and *D. loddiesii* demonstrated an apoptosis induction in melanoma cells as well as other pharmacological properties such as antiplatelet, anti-inflammatory and antioxidant effects (Cardile et al., 2020; Hossasin, 2011). The neuroprotective effect exhibited by homoeriodictyol was isolated from *D. densifiorum* (Fischer et al., 2019; Guo et al., 2022). Crepidatin purified from *D. crepidatum* was demonstrated to possess antimitotic and antioxidant activities (Majumder and Chatterjee, 1989; Paudel et al., 2019). Finally, gigantol isolated from *D. aurantiacum* was shown to have anticancer activity (Cai, 2021).

Previous studies showed that the constituents in *Dendrobium* plants are diverse not only amongst species but even in the same species. The contents of the investigated compounds have demonstrated considerable variation because of environmental differences and varied cultural practices. Therefore, determining various profiles of biologically active phenolic compounds in *Dendrobium* species can explain the accumulation of the secondary metabolites derived from different environmental circumstances and aid in determining the optimal harvesting period. Furthermore, systematic data analysis provides helpful information on finding novel sources of naturally occurring bioactive natural products as lead compounds (Yang et al., 2006, Choonong et al., 2019).

High-performance liquid chromatography (HPLC) has been widely utilized for quality control of pharmaceuticals and medicinal herbs (Xu et al., 2010). The HPLC coupled with a diode array detector (DAD) approach is a suitable tool for determining natural compounds such as phenolic compounds in plants and their extracts. Its simplicity and precision facilitate comprehensive quantitative analysis (Yang et al., 2006, Yang et al., 2007, Choonong et al., 2019). For medicinal plant analysis, identification and quantification of bioactive and marker compounds in complex matrices are realized using the HPLC-DAD method even with structurally similar natural products, based on their chromatographic retention time, spectral characteristics, and quantitative information (Yang et al., 2006). Recently, ultraperformance liquid chromatography (UPLC) with better separation efficiency, shorter analytical run time, and less solvent consumption compared to HPLC has increased considerable attention (Swartz and Technologies 2005, Xu et al., 2010). Here, we describe our efforts in the first publication of a simple, sensitive, specific, and robust UPLC method to determine six polyphenolic constituents simultaneously in orchid plants. The developed method was also validated according to the Q2(R1) ICH guidelines (The International Conference on Harmonization, 2005).

In the present study, we therefore further developed and validated a simultaneous determination of 6 markers in *Dendrobium* spp. to achieve a shorter analysis time and robust analytical method with com-



Fig. 1 Six polyphenolic bioactive markers in *Dendrobium* plants.

parable sensitivity. The validated method was applied to determine the markers in 11 samples from 3 *Dendrobium* spp. to demonstrate the method's applicability for routine analysis purposes.

### 2. Materials and methods

### 2.1. Sample collection

*D. stricklandianum* was collected and authenticated by Dr. Boonchoo Sritularak and Mr. Yanyong Punpreuk. *D.* crumenatum, D. *friedericksianum* and *D. officinale* were purchased from local markets in Bangkok, Thailand. The voucher specimens were deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

### 2.2. Material and reagents

(2S)-Eriodictyol, (2S)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin were provided from the Depart-

ment of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. They were isolated from orchid plants and confirmed their structures using infrared spectrophotometry (IR), nuclear magnetic resonance spectroscopy, and high-resolution spectrometry (Chanvorachote mass et al., 2013: Tanagornmetar et al., 2014; Klongkumnuankarn et al., 2015). Chromatographic purity of the isolated compounds was determined by UPLC coupled with a photodiode array (PDA) detector to confirm their purity of > 98.0% and used as standards in the validation and assay. Chromatographic purities of (2S)-Eriodictyol, (2S)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin are 99.8, 98.3, 98.2, 99.3, 100.0, and 98.6 respectively. Reagent grade trifluoroacetic acid was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). HPLC gradient grade acetonitrile and methanol were obtained from Fisher (Seoul, Korea). High purity water was prepared in-house using Ultra-high quality (UHQ) water purification system (Milli-Q, Millipore Corporation, Bedford, MA, USA).

### 2.3. Instruments and chromatographic conditions

The Waters ACQUITY UPLC<sup>TM</sup> H-Class system (Waters Corporation, Milford, MA, USA) consisted of a quaternary pump, a column oven, an autosampler and a PDA detector was used for method development and validation. The Empower 3 software was used for instrument control and data acquisition process. Chromatographic separation was achieved on an ACQUITY UPLC<sup>TM</sup> BEH C18 1.7  $\mu$ m, 2.1  $\times$  50 mm column (Waters Chromatography Ireland Limited, Dublin, Ireland). The mobile phase consisting of water (A) and acetonitrile (B), containing 1 %v/v TFA each, was delivered at a flow rate of 0.2 mL/min. The gradient elution program was optimized as follows: initial isocratic A-B of 68:32 from 0.0 to 1.0 min; linear gradient A-B of 35:65 from 1.0 to 7.0 min; linear gradient A-B of 68:32 from 7.0 to 7.3 min; isocratic A-B of 68:32 from 7.3 to 11.0 min. All analytes and interferences were achieved with a complete chromatographic separation under the total chromatographic run time of 11 min. The column oven temperature and detection wavelength were set at 30 °C and 280 nm, respectively. The injection volume was 2 μL.

### 2.4. Preparation of a system suitability solution

Six standard stock solutions of (2*S*)-eriodictyol, (2*S*)homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin were separately prepared by dissolving 5 mg of each compound with methanol in 25-mL volumetric flasks (200  $\mu$ g/mL). A 3 mL of the standard stock solution was transferred to a 10-mL volumetric flask and diluted to volume with methanol to obtain a working standard solution of each compound at the concentration of 60  $\mu$ g/mL.

A system suitability solution was then prepared by cospiking 1 mL of (2S)-eriodictyol, (2S)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin working standard solutions to obtain a solution mixture at a concentration of 10  $\mu$ g/mL. The system suitability solution was filtered through a 0.22  $\mu$ m nylon membrane filter prior to analysis.

#### 2.5. Calibration curve preparation

A five-point calibration curve of each compound was constructed in the range of 8–80, 1–40, 5–100, 0.5–50, 1–50 and 1–40  $\mu$ g/mL for (2*S*)-eriodictyol, (2*S*)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin, respectively. Calibration samples were prepared in a dilution of the standard stock solution of each compound. The solution was filtered through a 0.22 µm nylon membrane filter before analysis.

### 2.6. Matrix and sample preparation

#### 2.6.1. Matrix preparation

The stem of *D. stricklandianum* was separately cut into small pieces and dried in an oven at 60 °C until the constant weight was obtained, approximately 3 h. The dried plants were powdered using a kitchen grinder and passed through a 40 mesh screen before extraction. The powdered plant of each species (250 mg) was transferred into a 15-mL screw-cap test tube.

After that, 5 mL methanol was added, and the sample was extracted by heat-maceration at 50 °C for 60 min. Then, the mixture was subsequently sonicated for 45 min and centrifuged at 5,500 rpm, 20 °C for 10 min. The supernatant was filtered through a 0.22  $\mu$ m nylon membrane filter before analysis.

### 2.6.2. Sample preparation

The samples of *D. crumenatum*, *D.friedericksianum and D. officinale* were prepared as above described in section 2.6.1.

### 2.7. Method validation

The method was validated according to the ICH Q2 (R1) guidance for the validation of analytical procedures in the aspect of assay procedures (The International Conference on Harmonization, 2005). The validation parameters including system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and robustness, were assessed under the appropriately designed validation experiments.

### 2.7.1. System suitability

The system suitability test was performed to verify the repeatability of injection and the performance of the chromatographic system prior to analysis. For the repeatability of injection and chromatographic performance tests, the system suitability solution at a concentration of 10  $\mu$ g/mL was injected in five replicates. The system reproducibility was evaluated on the variations (%CV  $\leq$  2) of retention time and peak area to indicate the precision of injections (Center for Drug Evaluation and Research, 1994). The performance of the chromatographic system was assessed as the resolutions (R  $\geq$  2) between two adjacent peaks to demonstrate the efficiency of chromatographic separation, the USP tailings of six phenolic compounds (T < 2) and the number of theoretical plates (N > 2,000).

### 2.7.2. Specificity

Specificity was investigated by separately injecting the diluent (methanol) and working standard solutions of (2*S*)eriodictyol, (2*S*)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin at a concentration of 20  $\mu$ g/mL. The standard mixture of six polyphenolic components at 10  $\mu$ g/mL was injected to indicate the selectivity of the chromatographic column. *D. stricklandianum* was selected and used as the surrogate matrix in the validation experiment.

### 2.7.3. Linearity and range

Calibration curves of authentic standard solutions of six phenolic compounds (n = 3) at 5 concentrations ranging from 8 to 80, 1–40, 5–100, 0.5–50, 1–50 and 1–40 µg/mL for (2*S*)-eriodictyol, (2*S*)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin, respectively were constructed to determine the linear relationship. Linearity was assessed from the calibration curves plotted between the peak response (y) and the concentration (x) using the linear least square model (Danzer and Currie 1998). The percent deviation of the mean back-calculated concentrations of six phenolic compounds should be within  $\pm$  10% (Jurado et al., 2017). The linearity is acceptable if the coefficient of determination ( $r^2$ ) is higher than 0.995 (Ip et al., 2018). The regression analysis of the resid-

ual plot is often used to determine whether the slope and yintercept are significantly different from zero at a 95% confidence interval. In the case of a *p*-value below 0.05, a significant difference of zero can be observed in the slope and y-intercept (Barwick 2003). The linear relationship between the peak response (y) and the concentration (x) is assessed from the *F* value when  $F_{cal}$  is greater than  $F_{ANOVA}$  (Danzer and Currie 1998).

## 2.7.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The sensitivity of the method was evaluated using the limit of detection (LOD) and limit of quantification (LOQ). The LOD was assessed in terms of a signal-to-noise (S/N) ratio of  $\geq$  3 with a %CV of six replicate injections of  $\leq$  15. The LOQ was estimated by determining the S/N ratio of  $\geq$  10. The estimated LOQ was confirmed by six replicate injections of the

spiked sample to achieve 80–110 % recovery with %CV of  $\leq 11$ .

### 2.7.5. Accuracy and precision

Accuracy and precision were determined by analyzing the spiked powdered plant samples at 8 (LOQ), 40 and 80  $\mu$ g/mL for (2S)-eriodictyol, 1 (LOQ), 10 and 40  $\mu$ g/mL for (2S)-homoeriodictyol and crepidatin, 5 (LOQ), 20 and 100  $\mu$ g/mL for moscatilin, 0.5 (LOQ), 25 and 50  $\mu$ g/mL for gigantol and 1.0 (LOQ), 10 and 50  $\mu$ g/mL for chrysotoxine. Each concentration level was prepared and analyzed in triplicate on the same day and for two different days representing intra-day (within–run) and inter-day (between–run) analyses, respectively. Accuracy was assessed using the percent recovery. The percent recovery should be in the range of 80 – 110%. The intra-day and inter-day precision were assessed as %CV of percent recovery.



Fig. 2 The UV spectra of six detected polyphenols: (2S)-eriodictyol, (2S)-hyomoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin.

### 2.7.6. Robustness

Robustness is a validation parameter to determine whether the method remains tolerable or unaffected when slight variations in chromatographic parameters were deliberately changed. In this study, the robustness of the method was determined as the suitability of the system under the slight variation of the



**Fig. 3** Representative chromatograms of (A) the overlaid working standard solutions of each polyphenol standard at a concentration of 20  $\mu$ g/mL, (B) the system suitability solution containing six polyphenols at a concentration of 10  $\mu$ g/mL, (C) the diluent (methanol), and (D) the matrix extract of *D. stricklandianum*.

method parameters including the change of the acid content in the mobile phase and column temperature. The percentage of TFA was changed from the original chromatographic condition of 1% (v/v) TFA ( $\pm 0.1\%$ ), and the column temperature was varied from the method condition of 30 °C ( $\pm 1$  °C). The precision values of the peak response and retention time for six phenolic compounds were determined after a slight variation in the method parameters. The results were evaluated using the system suitability parameters as mentioned in Section 2.7.1 to ensure the efficiency of the method under minor modifications.

### 3. Results and discussion

Currently, there are several quantitative methods of chemically bioactive constituents in *Dendrobium* spp (Yang et al., 2006; Yang et al., 2007; Xu et al., 2010; Choonong et al., 2019). Yang et al., (2006) developed qualitative and quantitative determination of 11 phenolic constituents in Dendrobium species using HPLC-DAD with a total analysis time of about 55 min (Yang et al., 2006). Sample pre-treatment was achieved by 80% (v/v) methanol maceration and ultra-sonication. The LOD and LOD ranged from 0.04 to 0.61 and 0.13-1.83 µg/ mL, respectively. Later on, they reported simultaneous analysis of 9 phenolic compounds using the similar previously reported HPLC-DAD method (Yang et al., 2007). SPE was used to remove matrix interferences. The LOD and LOQ ranged from 0.04 to 0.6 and 0.1-1.8 µg/mL, respectively. The study also employed HPLC-MS for structural confirmation of unknown compounds found in Dendrobium spp. A few vears later, the UPLC method was developed for simultaneous determination of 5 markers in *Dendrobium* spp. with a total analysis time of about 6.5 min (Xu et al., 2010). Sample preparation was performed by pressurized liquid extraction (PLE) under 1500 psi using 80% methanol (v/v) at 140 °C for 15 min. The LOD and LOQ ranged from 0.03 to 2.44 and 0. 08-7.32 µg/mL, respectively. Recently, we developed the reversed-phase HPLC-UV to determine 7 bioactive compounds in 33 orchid species with a total analysis time of about 60 min (Choonong et al., 2019). Samples were treated with methanol and then processed via ultrasonic-assisted extraction for 15 min for 3 times. The LOD and LOQ ranged from 0.10 to 1.57 and 0.32-5.23 µg/mL, respectively.

It is of note that Yang et al., (2007) implemented an HPLC-MS technique in the analysis of *Dendrobium* spp. in addition to the HPLC-DAD method. The HPLC coupled with MS was applied to identify the components in the plant as a qualitative measurement. The structural confirmation by MS provided additional information on the markers that are not available as reference or working standards. For example, in such study, moscatilin, gigantol, moscatin and coumarin were structurally characterized by HPLC-MS due to the lack of standard materials. In our current study, all six polyphenolic compounds were isolated, purified and characterized to serve as standard materials. However, the MS technique can be further implemented if unknown markers are applied.

### 3.1. Method development

The analytical method was developed for simultaneous determination of six polyphenolic markers in orchid plants. The compounds contain phenolic groups that can ionize in the mobile phase. Separation of polyphenolic analytes on reverse-phase chromatography can be achieved by an ionsuppression technique using organic acids such as formic acid, acetic acid, or TFA (Lian et al., 2005). Formic acid is most often chosen as a modifier for ion suppression due to its ecofriendly with no pungent smell like glacial acetic acid. However, formic acid is insufficient to completely suppress the ionization of polyphenolic functional groups. A stronger acid such as TFA at 1 %v/v is usually preferred because of its ability to control pH of the mobile phase and suppress ionization of phenolic groups. Buffers such as acetate and phosphate at higher pH can cause peak splitting, especially long-eluting peaks. An ACQUITY UPLC<sup>TM</sup> BEH C18 column was chosen because it can tolerate the low pH of the mobile phase and provide symmetrical peaks. In contrast, some phenolic compounds cannot retain on the C8 column and co-elute with matrix interferences. Alternatively, a phenyl column can make much more retention of phenolic analytes, resulting in a longer analysis time. Chromatographic separation of the six polyphenolic compounds was achieved at a flow rate of 0.2 mL/min with the gradient elution program varying the ratio of aqueous (1% v/v TFA in water) and organic (1% v/v TFA in acetonitrile) phases. Initially, the column condition was equilibrated at a 32% organic composition for 1 min. Then, the organic phase was slowly gradient up to 65 % to achieve the complete separation of all analytes within 7 min. It was noted that too fast ramping of an organic composition might make the peak coelution. After that, the mobile phase composition was gradient to the initial composition within 0.3 min. Conditioning the column should be performed with sufficient time to ensure column re-equilibration. An attempt to use methanol was unsuccessful due to peak broadening and long analytical run time. As the UV spectra of six detected polyphenols shown in Fig. 2, the detection wavelength was selected at 280 nm repre-

| <b>Table 1</b> Summary of the system suitability results. |                            |               |                      |            |                           |                 |  |  |  |  |
|---|----------------------------|---------------|----------------------|------------|---------------------------|-----------------|--|--|--|--|
| Compound  | $\% \mathrm{CV} \ (n = 5)$ |               | Retention Time (min) | Resolution | <b>USP Tailing Factor</b> | USP Plate Count |  |  |  |  |
|   | Retention time             | Peak response |                      |            |                           |                 |  |  |  |  |
| (2S)-Eriodictyol  | 0.13                       | 0.22          | 1.35                 | _          | 1.1                       | 2,104           |  |  |  |  |
| (2S)-Homoeriodictyol                                      | 0.22                       | 0.28          | 2.21                 | 6.1        | 1.1                       | 2,913           |  |  |  |  |
| Moscatillin   | 0.23                       | 0.24          | 2.98                 | 4.5        | 1.0                       | 4,252           |  |  |  |  |
| Gigantol  | 0.19                       | 0.23          | 3.39                 | 2.3        | 1.0                       | 5,830           |  |  |  |  |
| Chrysotoxine  | 0.12                       | 0.24          | 4.63                 | 8.1        | 1.0                       | 21,233          |  |  |  |  |
| Crepidatin  | 0.11                       | 0.19          | 4.95                 | 2.5        | 0.9                       | 25,061          |  |  |  |  |

| Compound             | Nominal conc. (µg/mL)        | Back-calculated                      | l conc. (µg/ | /mL)          | mean back-calculated | %Deviation | %CV |
|----------------------|------------------------------|--------------------------------------|--------------|---------------|----------------------|------------|-----|
| -                    |                              | Day 1 Day 2 Day 3                    |              | conc. (µg/mL) |                      |            |     |
| (2S)-Eriodictyol     | 8.0                          | 8.5                                  | 8.7          | 8.5           | 8.6                  | 6.9        | 1.9 |
|                      | 20.0                         | 19.3                                 | 19.6         | 20.5          | 19.8                 | -1.0       | 3.4 |
|                      | 40.0                         | 37.3                                 | 40.4         | 39.5          | 39.1                 | -2.3       | 4.1 |
|                      | 60.0                         | 58.6                                 | 60.8         | 59.6          | 59.7                 | -0.5       | 1.8 |
|                      | 80.0                         | 79.9                                 | 80.6         | 81.5          | 80.7                 | 0.8        | 1.0 |
|                      | r <sup>2</sup>               | 0.9986                               |              |               |                      |            |     |
|                      | $F_{\rm cal}$                | 8388.13                              |              |               |                      |            |     |
|                      | $F_{ANOVA}$                  | $1.17 \times 10^{-19}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of slope     | $1.17 \times 10^{-19}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of intercept | 0.064                                |              |               |                      |            |     |
| (2S)-Homoeriodictyol | 1.0                          | 1.0                                  | 1.0          | 1.0           | 1.0                  | -0.4       | 1.0 |
|                      | 5.0                          | 4.5                                  | 4.5          | 4.7           | 4.6                  | -9.0       | 2.0 |
|                      | 10.0                         | 10.1                                 | 10.2         | 9.6           | 10.0                 | -0.2       | 2.9 |
|                      | 20.0                         | 20.7                                 | 20.5         | 21.3          | 20.8                 | 4.1        | 2.0 |
|                      | 40.0                         | 39.3                                 | 40.5         | 39.1          | 39.6                 | -0.9       | 1.8 |
|                      | r <sup>2</sup>               | 0.9984                               |              |               |                      |            |     |
|                      | $F_{\rm cal}$                | 8205.09                              |              |               |                      |            |     |
|                      | FANOVA                       | $1.35 \times 10^{-19}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of slope     | $1.35 \times 10^{-19}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of intercept | 0.300                                |              |               |                      | <i>.</i>   |     |
| Moscatilin           | 5.0                          | 5.3                                  | 5.5          | 5.1           | 5.3                  | 6.0        | 3.1 |
|                      | 10.0                         | 9.5                                  | 10.8         | 10.0          | 10.0                 | 1.1        | 6.5 |
|                      | 20.0                         | 20.4                                 | 19.6         | 19.4          | 19.8                 | -1.1       | 2.6 |
|                      | 50.0                         | 47.8                                 | 49.8         | 51.1          | 49.6                 | -0.8       | 3.3 |
|                      | 100.0                        | 99.5                                 | 101.1        | 100.1         | 100.2                | 0.2        | 0.8 |
|                      | r-                           | 0.9995                               |              |               |                      |            |     |
|                      | F <sub>cal</sub>             | 25/46.57                             |              |               |                      |            |     |
|                      | F <sub>ANOVA</sub>           | $8.06 \times 10^{-23}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of slope     | $8.06 \times 10^{-23}$               |              |               |                      |            |     |
| C'                   | <i>p</i> -value of intercept | 0.098                                | 0.6          | 0.0           | 0.6                  | 1.5        | 2.7 |
| Gigantol             | 0.5                          | 0.6                                  | 0.6          | 0.6           | 0.6                  | 1.5        | 2.7 |
|                      | 1.0                          | 1.1                                  | 1.1          | 1.1<br>5.1    | 1.1<br>5.1           | 1.4        | 0.1 |
|                      | 5.0                          | 5.0<br>24.9                          | 2.3<br>22.9  | 5.1<br>24.6   | 5.1<br>24.4          | 2.4        | 2.9 |
|                      | 25.0                         | 24.8                                 | 23.8         | 24.0          | 24.4                 | -2.5       | 2.1 |
|                      | -2<br>-2                     | 0.0004                               | 49.4         | 50.2          | 50.5                 | 0.5        | 1.0 |
|                      | Г<br>Е                       | 0.9994                               |              |               |                      |            |     |
|                      | r <sub>cal</sub>             | 204/8.00<br>2.57 × 10 <sup>-22</sup> |              |               |                      |            |     |
|                      | r <sub>ANOVA</sub>           | $3.37 \times 10^{-22}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of slope     | $3.37 \times 10$                     |              |               |                      |            |     |
| Chrysotovino         | <i>p</i> -value of intercept | 0.570                                | 1.0          | 1.0           | 1.0                  | 26         | 0.1 |
| Chirysotoxine        | 5.0                          | 5.2                                  | 1.0          | 5.1           | 5.1                  | -5.0       | 2.0 |
|                      | 10.0                         | 10.0                                 | 4.9          | 0.0           | 10.0                 | 0.1        | 2.9 |
|                      | 25.0                         | 25.5                                 | 24.7         | 9.9<br>24.6   | 24.0                 | -0.1       | 2.0 |
|                      | 50.0                         | 20.0<br>40.3                         | 50.0         | 24.0<br>50.7  | 50.0                 | -0.5       | 2.0 |
|                      | $r^{2}$                      | 49.5                                 | 50.0         | 50.7          | 50.0                 | 0.1        | 1.4 |
|                      | I<br>F                       | 37025.83                             |              |               |                      |            |     |
|                      | I cal                        | $6.51 \times 10^{-24}$               |              |               |                      |            |     |
|                      | <i>n</i> -value of slope     | $6.51 \times 10^{-24}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of intercept | 0.274                                |              |               |                      |            |     |
| Creniatin            | 10                           | 11                                   | 11           | 11            | 11                   | 91         | 0.5 |
| creptutin            | 5.0                          | 49                                   | 5.0          | 5.0           | 5.0                  | -0.4       | 1.2 |
|                      | 10.0                         | 9.8                                  | 9.9          | 9.8           | 9.8                  | -17        | 0.9 |
|                      | 20.0                         | 19.8                                 | 20.4         | 20.1          | 20.1                 | 0.5        | 1.6 |
|                      | 40.0                         | 39.7                                 | 40.3         | 40.0          | 40.0                 | 0.0        | 0.8 |
|                      | $r^2$                        | 0 9998                               | 40.5         | 40.0          | 10.0                 | 0.0        | 0.0 |
|                      | Faal                         | 61788 47                             |              |               |                      |            |     |
|                      | FANOVA                       | $2.73 \times 10^{-25}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of slope     | $2.73 \times 10^{-25}$               |              |               |                      |            |     |
|                      | <i>p</i> -value of intercent | 0.079                                |              |               |                      |            |     |
|                      | p runde of intercept         | 0.075                                |              |               |                      |            |     |

**Table 2** Mean inter-day back-calculated standard and calibration curve results (n = 3).

senting the maximum absorption wavelength of the analytes  $(\lambda_{(2S)-\text{Eriodictyol}} = 287; \lambda_{(2S)-\text{Homoeriodictyol}} = 287; \lambda_{Moscatilin} = 280; \lambda_{Gigantol} = 280; \lambda_{Chrysotoxine} = 279 and \lambda_{Crepidatin} = 279$ ). The developed chromatographic condition provided sufficient separation among all analytes and other matrix interferences.

### 3.2. System suitability

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Suitability was verified during method validation and sample analysis to ensure the validity of the chromatographic condition. The system suitability solution containing (2*S*)eriodictyol, (2*S*)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin at a concentration of 10  $\mu$ g/mL for each compound was prepared by mixing 1 mL of each working standard solution (60  $\mu$ g/mL). Fig. 3A and B represent the overlaid chromatograms of each working standard solution and the typical chromatogram of the system suitability solution. The retention times of (2S)-eriodictyol, (2S)homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin were 1.35, 2.21, 2.98, 3.39, 4.63 and 4.95, respectively. System performance was achieved with sufficient resolution between the adjacent analytes ( $\mathbf{R} > 2.2$ ), tailing factor ( $\mathbf{T} < 1.1$ ) and theoretical plate ( $\mathbf{N} > 2,104$ ). Five-replicate injection of the system suitability solution demonstrated that the system was reproducible with low variation of peak area, retention time, resolution, tailing factor and theoretical plate. The overall system suitability results summarized in Table 1 suggest that the developed chromatographic condition is suitable for further validation of simultaneous analysis of the six phenolic compounds.

### 3.3. Specificity

Specificity is crucial in method development to separate analytes from solvent, matrices, and potential impurity. Fig. 3C and D represent blank chromatograms of methanol and matrix extract of *D. stricklandianum*, respectively. Compared with the chromatograms of polyphenolic analytes as shown in Fig. 3A and B, the chromatograms of methanol and the

| Compound             | Limit of Detection $(n = 6)$ |                    |                         | Limit of Quantitation $(n = 6)$ |                         |                    |     |  |
|----------------------|------------------------------|--------------------|-------------------------|---------------------------------|-------------------------|--------------------|-----|--|
|                      | Nominal conc.<br>(μg/mL)     | Precision<br>(%CV) | $\mathbf{S}/\mathbf{N}$ | Nominal conc.<br>(μg/mL)        | Accuracy<br>(%Recovery) | Precision<br>(%CV) | S/N |  |
| (2S)-Eriodictyol     | 1.00                         | 1.6                | 6                       | 8.00                            | 92.0                    | 0.9                | 45  |  |
| (2S)-Homoeriodictyol | 0.25                         | 1.9                | 4                       | 1.00                            | 89.2                    | 6.5                | 18  |  |
| Moscatillin          | 0.50                         | 1.3                | 5                       | 5.00                            | 98.9                    | 1.8                | 42  |  |
| Gigantol             | 0.25                         | 1.7                | 6                       | 0.50                            | 88.3                    | 6.4                | 15  |  |
| Chrysotoxine         | 0.25                         | 1.8                | 6                       | 1.00                            | 102.7                   | 3.8                | 23  |  |
| Crepidatin           | 0.25                         | 1.2                | 6                       | 1.00                            | 104.2                   | 1.5                | 22  |  |

|--|

| Compound         | Nominal conc. | Intra-day $(n = 3)$                   |                          |                    | Inter-day $(n = 6)$                   |                          |                    |  |
|------------------|---------------|---------------------------------------|--------------------------|--------------------|---------------------------------------|--------------------------|--------------------|--|
|                  | (µg/mL)       | Mean back-calculated<br>conc. (µg/mL) | Accuracy (%<br>Recovery) | Precision<br>(%CV) | Mean back-calculated<br>conc. (µg/mL) | Accuracy (%<br>Recovery) | Precision<br>(%CV) |  |
|                  | 8.00          | 7.41                                  | 92.6                     | 0.8                | 7.36                                  | 92.0                     | 0.9                |  |
| (2S)-Eriodictyol | 40.00         | 39.36                                 | 98.4                     | 1.4.               | 37.84                                 | 94.6                     | 4.7                |  |
|                  | 80.00         | 74.88                                 | 93.6                     | 0.2                | 78.68                                 | 98.4                     | 5.4                |  |
|                  | 1.00          | 0.94                                  | 94.4                     | 1.0                | 0.91                                  | 89.2                     | 6.5                |  |
| (2 <i>S</i> )-   | 10.00         | 9.31                                  | 93.1                     | 2.2                | 8.98                                  | 89.8                     | 4.4                |  |
| Homoeriodictyol  |               |                                       |                          |                    |                                       |                          |                    |  |
|                  | 40.00         | 36.31                                 | 90.8                     | 2.6                | 36.09                                 | 90.2                     | 1.9                |  |
|                  | 5.00          | 4.96                                  | 99.3                     | 2.6                | 4.94                                  | 98.9                     | 1.8                |  |
| Moscatillin      | 20.00         | 18.64                                 | 93.2                     | 0.9                | 19.48                                 | 97.4                     | 4.9                |  |
|                  | 100.00        | 95.14                                 | 95.1                     | 0.6                | 95.90                                 | 95.9                     | 1.5                |  |
|                  | 0.50          | 0.45                                  | 90.6                     | 8.4                | 0.44                                  | 88.3                     | 6.4                |  |
| Gigantol         | 10.00         | 9.71                                  | 97.1                     | 1.7                | 9.19                                  | 91.9                     | 6.3                |  |
| -                | 50.00         | 46.39                                 | 92.8                     | 0.1                | 45.31                                 | 90.6                     | 2.6                |  |
|                  | 1.00          | 1.06                                  | 105.7                    | 2.8                | 1.03                                  | 102.7                    | 3.8                |  |
| Chrysotoxine     | 10.00         | 8.60                                  | 86.0                     | 3.1                | 8.61                                  | 86.1                     | 2.1                |  |
|                  | 50.00         | 48.40                                 | 96.8                     | 1.2                | 47.84                                 | 95.7                     | 1.5                |  |
|                  | 1.00          | 1.05                                  | 105.1                    | 0.9                | 1.04                                  | 104.2                    | 1.5                |  |
| Crepidatin       | 10.00         | 9.16                                  | 91.6                     | 0.9                | 9.04                                  | 90.4                     | 2.1                |  |
| -                | 40.00         | 37.13                                 | 92.8                     | 1.4                | 37.85                                 | 94.6                     | 2.6                |  |

matrix extract of *D. stricklandianum* revealed no co-eluting peaks at the retention times of the polyphenolic analytes. The specificity result suggests that the chromatographic system is sufficient to revolve the analyte peaks from other interference peaks.

### 3.4. Linearity and range

The linear relationships between analyte concentrations and signal responses were evaluated at the concentration range of 8–80, 1–40, 5–100, 0.5–50, 1–50 and 1–40 µg/mL for (2S)-eriodictyol, (2S)-homoeriodictyol, moscatilin, gigantol, chrysotoxine and crepidatin, respectively. The calibration curves were constructed by plotting between the concentrations versus the peak responses of analytes. Using the linear least square model, the calibration curve was linear over the tested concentration range ( $r^2 > 0.995$ ). The percent deviations of the mean back-calculated concentrations and actual concentrations of (2S)-eriodictyol, (2S)-homoeriodictyol, mos-

catilin, gigantol, chrysotoxine and crepidatin were in the ranges of -2.3-6.9, -9.0-4.1, -1.1-6.0, -2.3-2.4, -3.6-1.6, and -1.7-9.1, respectively. The %CV (n = 3) of the back-calculated concentrations was < 6.5%. The calibration curve results summarized in Table 2 indicate that the ranges of the analytical procedure possess suitable levels of precision, accuracy and linearity.

The residual plots and regression generated by one-way analysis of variance demonstrated that the *F* values ( $F_{ANOVA}$ ) of all regression lines were significantly less than the calculated *F* values ( $F_{cal}$ ), indicating an excellent linear relationship between the peak response (*y*) and the concentration of analyte (*x*). The *p*-value is the regression parameter used to indicate whether the slope and y-intercept are significantly different from zero at a 95% confidence interval. The *p*-values of slope and y-intercept are summarized in Table 2. The *p*-values of slopes were < 0.05, indicating a significant difference from zero. The *p*-values of intercepts exceeded 0.05, indicating that the intercepts of all regression lines were insignificantly differ-

| Compound         | Parameter                               | $\% \mathrm{CV} \ (n = 5)$ |                  | Retention time | Resolution | USP               | USP            |
|------------------|---|----------------------------|------------------|----------------|------------|-------------------|----------------|
|                  | change                                  | Retention time             | Peak<br>response | (min)          |            | Tailing<br>Factor | Plate<br>Count |
| (2S)-Eriodictyol | No change                               | 0.13                       | 0.22             | 1.35           | -          | 1.1               | 2,104          |
| (2 <i>S</i> )-   | -                                       | 0.22                       | 0.28             | 2.21           | 6.1        | 1.1               | 2,913          |
| Homoeriodictyol  |   |                            |                  |                |            |                   |                |
| Moscatillin      |   | 0.23                       | 0.24             | 2.98           | 4.5        | 1.0               | 4,252          |
| Gigantol         |   | 0.19                       | 0.23             | 3.39           | 2.3        | 1.0               | 5,830          |
| Chrysotoxine     |   | 0.12                       | 0.24             | 4.63           | 8.1        | 1.0               | 21,233         |
| Crepidatin       |   | 0.11                       | 0.19             | 4.95           | 2.5        | 0.9               | 25,061         |
| (2S)-Eriodictyol | Column temperature                      | 0.22                       | 0.72             | 1.37           | _          | 1.1               | 2,122          |
| (2 <i>S</i> )-   | (29 °C)                                 | 0.14                       | 0.75             | 2.24           | 6.2        | 1.1               | 2,979          |
| Homoeriodictyol  |   |                            |                  |                |            |                   |                |
| Moscatillin      |   | 0.14                       | 0.71             | 2.99           | 4.5        | 1.0               | 4,396          |
| Gigantol         |   | 0.15                       | 0.74             | 3.42           | 2.2        | 1.0               | 6,160          |
| Chrysotoxine     |   | 0.11                       | 0.72             | 4.64           | 8.1        | 1.0               | 21,281         |
| Crepidatin       |   | 0.11                       | 0.72             | 4.96           | 2.5        | 0.9               | 25,140         |
| (2S)-Eriodictyol | Column temperature                      | 0.15                       | 0.77             | 1.34           | _          | 1.1               | 2,053          |
| (2 <i>S</i> )-   | (31 °C)                                 | 0.09                       | 0.75             | 2.18           | 6.0        | 1.1               | 2,855          |
| Homoeriodictyol  |   |                            |                  |                |            |                   |                |
| Moscatillin      |   | 0.12                       | 0.80             | 2.96           | 4.4        | 1.0               | 4,159          |
| Gigantol         |   | 0.11                       | 0.79             | 3.35           | 2.3        | 1.0               | 5,575          |
| Chrysotoxine     |   | 0.08                       | 0.77             | 4.60           | 8.0        | 1.0               | 20,498         |
| Crepidatin       |   | 0.07                       | 0.76             | 4.93           | 2.4        | 0.9               | 24,248         |
| (2S)-Eriodictyol | Percent of acid (0.9% TFA)              | 0.11                       | 0.27             | 1.35           | _          | 1.1               | 2,041          |
| (2 <i>S</i> )-   | × , , , , , , , , , , , , , , , , , , , | 0.12                       | 0.27             | 2.19           | 6.3        | 1.1               | 2,860          |
| Homoeriodictyol  |   |                            |                  |                |            |                   |                |
| Moscatillin      |   | 0.12                       | 0.32             | 2.96           | 4.2        | 1.0               | 4,019          |
| Gigantol         |   | 0.08                       | 0.28             | 3.36           | 2.2        | 1.0               | 5,479          |
| Chrysotoxine     |   | 0.06                       | 0.27             | 4.61           | 8.2        | 1.0               | 19,563         |
| Crepidatin       |   | 0.08                       | 0.27             | 4.93           | 2.4        | 0.9               | 23,029         |
| (2S)-Eriodictyol | Percent of acid (1.1% TFA)              | 0.09                       | 0.69             | 1.33           | _          | 1.1               | 2,090          |
| (2 <i>S</i> )-   | · · · · · · · · · · · · · · · · · · ·   | 0.13                       | 0.63             | 2.15           | 6.2        | 1.1               | 2,921          |
| Homoeriodictyol  |   |                            |                  |                |            |                   |                |
| Moscatillin      |   | 0.15                       | 0.69             | 2.92           | 4.3        | 1.0               | 4,184          |
| Gigantol         |   | 0.10                       | 0.67             | 3.32           | 2.3        | 1.0               | 5,492          |
| Chrysotoxine     |   | 0.03                       | 0.67             | 4.58           | 8.2        | 1.0               | 20,693         |
| Crepidatin       |   | 0.04                       | 0.71             | 4.90           | 2.3        | 0.9               | 24,453         |

 Table 5
 Robustness tests under the variations of column temperature and %TFA in the aqueous phase of the mobile phase.

ent from zero. The linearity results suggest that the calibration curve within the specified range can be applied for the routine analysis of the six polyphenolic compounds, and the singlepoint standard can also be used for the single-point assay.

### 3.5. LOD and LOQ

LOD and LOQ of six polyphenolic analytes were experimentally determined, and the results are summarized in Table 3. The LOD of analytes showed the S/N ratio ranging from 4 to 6 with a %CV of six replicate injections of  $\leq$  1.9. The LOQ of analytes showed the S/N ratio ranging from 15 to 45. The accuracy and precision at the LOQ of all analytes were achieved with percent recovery ranging from 88.3 to 104.2 % and precision (%CV) of  $\leq$  6.5.

### 3.6. Accuracy and precision

The accuracy and precision of the matrix extract spiked with individual polyphenolic standards were determined at three



Fig. 4 Typical UPLC chromatograms of methanolic extracts of (A) D. crumenatum, (B) D. friedericksianum and (C) D. officinale.

| Sample              | Sample Code | Content (µg/g | g)              |            |          |             |            |
|---------------------|-------------|---------------|-----------------|------------|----------|-------------|------------|
|                     |             | Eriodictyol   | Homoeriodictyol | Moscatilin | Gigantol | Chysotoxine | Crepidatin |
| D. crumenatum       | DC01        | -             | -               | 3,484      | 355      | -           | 1,064      |
|                     | DC02        | _             | -               | 2,444      | 1,796    | _           | 2,468      |
|                     | DC03        | _             | _               | 2,026      | 1,404    | -           | 9,311      |
|                     | DC04        | _             | -               | _          | 2,272    | _           | 11,021     |
|                     | DC05        | _             | -               | 545        | 245      | -           | 7,582      |
| D. friedericksianum | DF01        | 172           | 171             | _          | -        | 595         | _          |
|                     | DF02        | 144           | 173             | _          | _        | 974         | _          |
|                     | DF03        | 7             | 165             | _          | -        | 939         | -          |
|                     | DF04        | 163           | 95              | _          | -        | 391         | -          |
|                     | DF05        | 173           | 182             | -          | _        | 701         | _          |
| D. officinale       | DO01        | 27            | 4               | 9.4        | -        | 925         | -          |

| <b>Table 0</b> The content of six polyphenone compounds in stems of three <i>Denarobian</i> sp | Table 6 | The content | of six | polyphenolic | compounds in | stems o | of three | Dendrobium | spp |
|--|---------|-------------|--------|--------------|--------------|---------|----------|------------|-----|
|--|---------|-------------|--------|--------------|--------------|---------|----------|------------|-----|

concentration levels. The accuracy and precision results are summarized in Table 4. The intra-day accuracy and precision exhibited percent recoveries ranging from 86.0 to 105.7% with relative standard deviations of %CV between 0.1 and 8.4. The inter-day accuracy and precision showed percent recoveries ranging from 86.1 to 104.2% with relative standard deviations of %CV between 0.9 and 6.5. The results demonstrated satisfactory accuracy and precision of the proposed method.

### 3.7. Robustness

The system suitability solution was used to assess the robustness of the method under slight variations of a column temperature and a TFA concentration in the mobile phase. The robustness results are summarized in Table 5. The retention times of six polyphenolic analytes were insignificantly different among the modified chromatographic conditions. In addition to the retention time, the slight variations in the column temperature and TFA concentration had no impact on the reproducibility of injections. The %CV values of the retention time and peak response were within the limit criteria with % CV < 2. The variations in the column temperature and TFA concentration had a subtle effect on the peak separation but the chromatographic resolutions between two adjacent analytes remained > 2. The slight changes in tested chromatographic factors did not affect the system's performance as measured by the tailing factor (T < 1.1) and the number of theoretical plates (N > 2,041).

## 3.8. Method applications for determination of six polyphenolic bioactive markers in Dendrobium spp.

The content of six phenolic compounds (moscatilinm gigantol, crepidatin, chrysotoxine, eriodictyol and homoeriodictyol) in *Dendrobium* spp. was quantified using the developed and validated UPLC-UV method. Typical UPLC chromatograms of methanolic extracts of *D. crumenatum*, *D. friedericksianum* and *D. officinale* are depicted in Fig. 4. The content of six phenolic compounds in stems from the three *Dendrobium* plants is summarized in Table 6. The results show that only a few markers were found in each *Dendrobium* spp. with different amounts. These observations are in agreement with previous reports (Yang et al., 2006; Yang et al. 2007; Xu et al., 2009). For example, the HPLC method developed by Yang et al.

(2006) for determination of 11 compounds in *Dendrobium* spp. demonstrated that only three primary detected markers were found including gigantol, moscatilin, and moscatin. In addition, Yang et al. (2007) reported that only 4 phenolic compounds including moscatilin, gigantol, moscatin, and coumarin were detected in which gigantol was observed as the primary bioactive marker (Yang et al., 2007). Xu et al. (2009) also found only gigantol and moscatilin in most orchid hybrids. In our study, moscatilin, gigantol and crepidatin were mainly found in *D. crumenatum*. However, eriodictyol, homoeriodictyol and chysotoxine were primarily located in *D. friedericksianum* and *D. officinale*.

### 4. Conclusions

To the best of our knowledge, there is no currently available UPLC method for simultaneous determination of six bioactive phenols including eriodictyol, homoeiodictyol, moscatilin, gigantol, chrysotoxine, crepidatin. Because the UPLC approach can provide a fast, sensitive and robust method compared to HPLC, UPLC is therefore more suitable for qualitative analysis of multiple biomarkers in plants obtained from various sources with complex matrices. Simplified sample preparation can also facilitate routine phytochemical screening. Our developed UPLC method proposed in the present study had a total analysis run time of about 11 min using simple methanolic extraction as sample pre-treatment. The developed method was achieved in the complete validation according to the ICH Q2 (R1) guidance for the validation of analytical procedures. The validation results indicate sufficient accuracy, precision and sensitivity, suggesting that the proposed UPLC method is applicable for routine analysis of the six phenolic constituents in identifying Dendrobium plants.

### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### CRediT authorship contribution statement

Worathat Thitikornpong: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. Ponsiree Jithavech: Writing – original draft, Writing – review & editing. Somphob Thompho: Investigation. Yanyong Punpreuk: Investigation. Hasseri Halim: Writing – review & editing. Boonchoo **Sritularak:** Conceptualization, Writing – review & editing, Supervision. **Pornchai Rojsitthisak:** Conceptualization, Writing – review & editing, Project administration, Resources, Supervision, Funding acquisition.

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### References

- Barwick, V.J., 2003. Preparation of calibration curves: a guide to best practice, In LGC/VAM/2003/032; LGC.
- Cai, Y., Hao, Y., Chen, K., 2021. Gigantol inhibits cell proliferation and induces apoptosis by regulating DEK in non-small cell lung cancer. Exp. Ther. Med. 22, 1317. https://doi.org/10.3892/ etm.2021.10752.
- Cao, H., Ji, Y., Li, S., et al, 2019. Extensive metabolic profiles of leaves and stems from the medicinal plant *Dendrobium officinale* Kimura et Migo. Metabolites 9, 215. https://doi.org/10.3390/ metabo9100215.
- Cardile, V., Avola, R., Graziano, A.C.E., et al, 2020. Moscatilin, a bibenzyl derivative from the orchid Dendrobium loddigesii, induces apoptosis in melanoma cells. Chem. Bio. Interact. 323,. https://doi. org/10.1016/j.cbi.2020.109075 109075.
- Center for Drug Evaluation and Research (CDER), 1994. Reviewer Guidance: Validation of Chromatographic Methods. 1-30.
- Chanvorachote, P., Kowitdamrong, A., Ruanghirun, T., et al. 2013. Anti-metastatic activities of bibenzyls from *Dendrobium pulchellum*. Nat. Prod. Commun. 8, 115-118. <u>https://doi.org/</u>10.1177% 2F1934578X1300800127.
- Chimsook, T., 2016. Phytochemical screening, total phenolic content, antioxidant activities and cytotoxicity of Dendrobium signatum leaves. MATEC Web Conf. 62, 03005. https://doi.org/ 10.1051/matecconf/20166203005.
- Choonong, R., Sermpradit, W., Kitisripanya, T., et al, 2019. The contents of bibenzyl derivatives, flavonoids and a phenanthrene in selected *Dendrobium* spp. and the correlation with their antioxidant activity. Sci. Asia 45, 245–252. https://doi.org/10.2306/scienceasia1513-1874.2019.45.245.
- Danzer, K., Currie, L.A., 1998. Guidelines for calibration in analytical chemistry. part I. fundamentals and single component calibration (IUPAC Recommendations 1998). Pure Appl. Chem. 70, 993–1014. https://doi.org/10.1351/pac199870040993.
- Deng, Z., Hassan, S., Rafiq, M., et al, 2020. Pharmacological activity of eriodictyol: The major natural polyphenolic flavanone. Evid. Based Complement. Alternat. Med. eCAM 2020, 6681352. https:// doi.org/10.1155/2020/6681352.
- Fischer, W., Currais, A., Liang, Z., et al, 2019. Old age-associated phenotypic screening for Alzheimer's disease drug candidates identifies sterubin as a potent neuroprotective compound from Yerba santa. Redox Biol. 21, https://doi.org/10.1016/j.redox.2018.101089 101089.

- Guo, P., Zeng, M., Wang, S., et al, 2022. Eriodictyol and Homoeriodictyol improve memory impairment in Aβ<sub>25-35</sub>-induced mice by inhibiting the NLRP3 Inflammasome. Molecules 27, 2488. https:// doi.org/10.3390/molecules27082488.
- Handique, J.G., Baruah, J.B., 2002. Polyphenolic compounds: an overview. React Funct Polym. 52, 163–188. https://doi.org/ 10.1016/S1381-5148(02)00091-3.
- Hossain, M.M., 2011. Therapeutic orchids: traditional uses and recent advances- An overiew. Fitoterapia. 82, 102-140. https://doi.org/ 10.1016/j.fitote.2010.09.007.
- Ip, K., Shan, A., Carvalho, M., et al, 2018. Physicochemical stability of extemporaneously prepared oral suspension of fluconazole 50 mg/mL in SuspendIt<sup>™</sup>. Pharm. Technol. Hosp. Pharm. 3, 101–112. https://doi.org/10.1515/pthp-2018-0007.
- Jurado, J.M., Alcázar, A., Muñiz-Valencia, R., et al, 2017. Some practical considerations for linearity assessment of calibration curves as function of concentration levels according to the fitnessfor-purpose approach. Talanta 172, 221–229. https://doi.org/ 10.1016/j.talanta.2017.05.049.
- Klongkumnuankarn, P., Busaranon, K., Chanvorachote, P., et al, 2015. Cytotoxicity and antimigratory activities of phenolic compounds from *Dendrobium brymerianum*. Evid. based Complement Alternat. Med. 2015, https://doi.org/10.1155/2015/350410 350410.
- Lam, Y., Ng, T.B., Yao, R.M., et al, 2015. Evaluation of chemical constituents and important mechanism of pharmacological biology in dendrobium plants. Evid. Based Complement. Alternat. Med. eCAM 2015, https://doi.org/10.1155/2015/841752 841752.
- Lian, H., Li, D., Wu, X., et al, 2005. Determination of phenolic preservatives in gelatin and vacant capsules for medicine use by ion-suppression reversed-phase high performance liquid chromatography. J. Pharm. Biomed. Anal. 37, 369–372. https://doi. org/10.1016/j.jpba.2004.11.001.
- Liang, C.Y., Rengasamy, K.P., Huang, L.M., et al, 2020. Assessment of violet-blue color formation in Phalaenopsis orchids. BMC Plant Biol. 20, 212. https://doi.org/10.1186/s12870-020-02402-7.
- Majumder, P.L., Chatterjee, S., 1989. Crepidatin, A bibenzyl derivative from the orchid Dendrobium crepidatum. Phytochem. 28, 1986–1988. https://doi.org/10.1016/S0031-9422(00)97904-4.
- Minh, T.N., Khang, D.T., Tuyen, P.T., et al, 2016. Phenolic compounds and antioxidant activity of phalaenopsis orchid hybrids. Antioxidants 5. https://doi.org/10.3390/antiox5030031.
- Paudel, M.R., Chand, M.B., Pant, B., 2019. Assessment of antioxidant and cytotoxic activities of extracts of *Dendrobium crepidatum*. Biomolecules 9 (478). https://doi.org/10.3390/biom9090478.
- Phechrmeekha, T., Sritularak, B., Likhitwitayawuid, K., 2012. New phenolic compounds from Dendrobium capillipes and Dendrobium secundum. J. Asian Nat. Prod. Res. 14, 748–754. https://doi.org/ 10.1080/10286020.2012.689979.
- Song, J.X., Shaw, P.C., Sze, C.W., et al, 2010. Chrysotoxine, a novel bibenzyl compound, inhibits 6-hydroxydopamine induced apoptosis in SH-SY5Y cells via mitochondria protection and NF-κB modulation. Neurochem. Int. 57, 676–689. https://doi.org/10.1016/ j.neuint.2010.08.007.
- Song, J.X., Shaw, P.C., Wong, N.S., et al, 2012. Chrysotoxine, a novel bibenzyl compound selectivity antagonizes MPP<sup>+</sup>, but not rotenone, neurotoxicity in dopaminergic SH-SY5Y cells. Neurosci. Lett. 521, 76–81. https://doi.org/10.1016/j.neulet.2012.05.063.
- Swartz, M.E., 2005. UPLC<sup>™</sup>: an introduction and review. J. Liq. Chromatogr. Relat. Technol. 28, 1253–1263. https://doi.org/ 10.1081/JLC-200053046.
- Tanagornmetar, K., Chaotham, C., Sritularak, B., et al, 2014. Cytotoxic and anti-metastatic activities of phenolic compounds from *Dendrobium ellipsophyllum*. Anticancer Res 34, 6573–6580.
- The International Conference on Harmonization (ICH), 2005. Harmonized Tripartite Guideline. Validation of Analytical Procedures: Text and methodology Q 2(R1), 1–13.
- Thongin, S., Teerapattarakan, N., Benya-Aphikul, H., et al, 2019. Eriodictyol attenuates hydrogen peroxide-induced cell death in

neuronal SH-SY5Y cells. Thai J. Pharm Sci. 43, 131–137 http:// www.tjps.pharm.chula.ac.th/ojs/index.php/tjps/article/view/22.

- Xu, J., Zhao, W.M., Qian, Z.M., et al, 2010. Fast determination of five components of coumarin, alkaloids and bibenzyls in *Dendrobium* spp. using pressurized liquid extraction and ultra-performance liquid chromatography. J. Sep. Sci. 33, 1580–1586. https://doi.org/ 10.1002/jssc.201000034.
- Yang, L., Wang, Z., Xu, L., 2006. Simultaneous determination of phenols (bibenzyl, phenanthrene, and fluorenone) in *Dendrobium*

species by high-performance liquid chromatography with diode array detection. J. Chromatogr. A 1104, 230–237. https://doi.org/10.1016/j.chroma.2005.12.012.

Yang, L., Wang, Y., Zhang, G., et al, 2007. Simultaneous quantitative and qualitative analysis of bioactive phenols in *Dendrobium aurantiacum* var. *denneanum* by high-performance liquid chromatography coupled with mass spectrometry and diode array detection. Biomed Chromatogr. 21, 687–694. https://doi.org/ 10.1002/bmc.801.