Natural Product Sciences
21(3) : 192-195 (2015)

Quantitative HPLC Analysis and Extraction of 2,6-dimethoxy-1,4-benzoquinone from *Ficus foveolata* Stems

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Abstract – An antibacterial benzoquinone, 2,6-dimethoxy-1,4-benzoquinone, isolated from *Ficus foveolata* stems was used as a standard marker for establishment of quantitative HPLC analysis for the stem extracts of *F. foveolata* . The method utilized a TSK-gel ODS-80Ts column (5 μm, 4.6 × 250 mm) with the mixture of methanol and 5% acetic acid in water (24:76, v/v) as the mobile phase at a flow rate of 1 mL/min, and quantitative detection at 289 nm. The parameters i.e. linearity, intraday and interday precision, accuracy, specificity and sensitivity of the method were evaluated for method validation. The recoveries of the method were 99.5 - 103.6% and good linearity ($R^2 \geq 0.9999$) was obtained. A high degree of specificity, sensitivity as well as repeatability and reproducibility (RSD less than 2 and 5%, respectively) were also achieved. Chloroform was served as the most suitable solvent for extraction of 2,6-dimethoxy-1,4-benzoquinone. The optimised sample preparation and HPLC method can be practically used in the routine quality control process of *F. foveolata* stem extracts.

Keywords – *Ficus foveolata*, 2,6-Dimethoxy-1,4-benzoquinone, HPLC, Antibacterial, Method validation, Extraction

Introduction

Ficus foveolata Wall (syn *Ficus sarmentosa* Buch.-Ham. Ex Sm) is a scandent shrub belonging to Moraceae family, and widely distributed in Asia[1]. In Thailand, it is commonly known as "Ma Kra Thuep Rong". The stems of this plant are traditionally used as a rejuvenating agent and a tonic[2]. Our previous investigations reported an antibacterial benzophenone compound, 2,6-dimethoxy-1,4-benzoquinone (Fig. 1) isolated from the crude ethyl acetate extract of *F. foveolata* stems. This compound exhibited strong antibacterial activities against Gram-positive bacteria, i.e. *Streptococcus pyogenes*, *S. mitis*, *S. mutans*, *Bacillus subtilis*, *Staphylococcus aureus*, and Gram-negative bacteria, i.e. *Salmonella typhimurium* and *Escherichia coli*[3-4]. In addition, this compound showed inhibitory effect against Ehrlich ascites tumor cell propagation[5-6]. Thus 2,6-dimethoxy-1,4-benzoquinone can be used as a standard marker for the quality control of *F. foveolata* stem extracts.

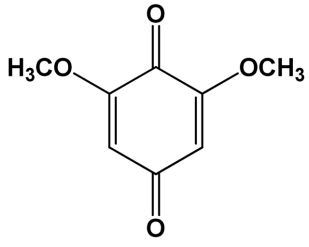

Fig. 1. Chemical structure of 2,6-dimethoxy-1,4-benzoquinone.

In this study, we report a rapid HPLC method for the quantitative analysis of 2,6-dimethoxy-1,4-benzoquinone in F. *foveolata* stem extracts. The parameters of linearity, accuracy, precision, specificity, limit of detection, and limit of quantitation of the HPLC method were defined to validate the HPLC method. The developed HPLC method was successfully applied for investigating the extraction conditions, which yielded a high level of 2,6-dimethoxy-1,4-benzoquinone in the stem extracts of *F. foveolata*.

Experimental

General experimental procedures – HPLC-grade (methanol) and analytical-grade solvents (hexane, chloro-

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form, ethyl acetate, methanol and acetic acid) were from Labscan limited (Songkhla, Thailand). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Standard 2,6-dimethoxy-1,4-benzoquinone had been previously purified[3]. *F. foveolata* stems were collected from Nakhon Sri Thammarat Province, Thailand, in June 2011. A voucher specimen (SKP 117 06 06 01) was deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The stems were washed and dried at 50 ºC in a hot air oven for 24 h, then ground and passed through a sieve no. 45 to obtain a fine powder.

HPLC apparatus and chromatographic conditions – HPLC analysis was carried out using the Waters 1500 series equipped with a Waters 2998 Photodiode-array detector (PDA) and Waters 2707 autosampler. Data analysis was performed using Waters Chemstation for Empower software. Separation was achieved at 25 ºC on a TSK-gel ODS-80Ts column, 5 µm, 250 × 4.6 mm i.d. (Tosho Bioscience, Japan). The mobile phase consisted of methanol and 5 % acetic acid in water (26:74, v/v). The mobile phase flow rate was 1 mL/min. Sample injection volumes were 20 µL, and quantitative detection was by UV at a wavelength of 289 nm.

Preparation of standard solutions – A stock solution of the reference standard, 2,6-dimethoxy-1,4-benzoquinone was made in mobile phase, and subsequently diluted to provide a series five concentrations of the standard solutions ranging from 9.75 - 156.00 µg/mL for use in constructing a calibration curve for 2,6-dimethoxy-1,4-benzoquinone.

Preparation of samples – *F. foveolata* stem powder (10 g) was extracted with chloroform (250 mL) under reflux conditions (RE) for 1 h. The extract was then filtered, and the solvent was evaporated to dryness at 40 ºC under reduced pressure. An aliquot of the dried extract (5 mg) was reconstituted and adjusted to 10 mL with the mobile phase. Samples were filtered through a 0.45 µm membrane filter, and analysed immediately in order to avoid possible chemical degradation. All assays of samples were performed in triplicate.

Validation of the method – The method was validated according to the Guidelines of the International Conference on Harmonization of Technical Requirement for the Registration of Pharmaceuticals for Human Use[7]. Calculating data for the linearity, accuracy, intra-day and inter-day precision, specificity, limit of determination (LOD) and limit of quantitation (LOQ) were used to validate the HPLC method.

Linearity – The calibration curves of 2,6-dimethoxy-1,4-benzoquinone were constructed on three consecutive days by analysis of the standard solutions at five different concentrations, and by plotting peak areas against the concentrations of the reference standard. The linearity of the detector response for the standard was determined by means of linear regression. The coefficient of determination (R^2) of the regression line should be not less than 0.999.

Accuracy – Sample portions were fortified with known quantities of the standard 2,6-dimethoxy-1,4-benzoquinone in order to check the accuracy of the data. Prior to standard 2,6-dimethoxy-1,4-benzoquinone fortification, the background level of 2,6-dimethoxy-1,4-benzoquinone in *F. foveolata* stem extracts was determined so as to calculate actual recoveries. The amounts of 2,6-dimethoxy-1,4-benzoquinone were determined in triplicate and the percentage recoveries were then calculated.

Precision – Precision experiments were conducted to ascertain any intraday and interday variability. The solution of one sample of *F. foveolata* stem extracts was used to check the intraday precision (repeatability). Six separate injections of this sample were carried out on the same day. The data were used to calculate % RSD for intraday precision (less than 2%). The interday precision (reproducibility) was validated by repeating the extraction procedure on the same sample of *F. foveolata* stems. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the extraction in triplicate on 3 different days with a freshly prepared mobile phase and sample. The data was used to calculate % RSD for interday precision (less than 5%).

Specificity – Peak identification was carried out using the standard 2,6-dimethoxy-1,4-benzoquinone, and scanning the UV absorption spectrum of the peak using the photodiode-array detector. The UV absorption spectra were taken at various points of the peaks, at least three points, to check peak homogeneity.

LOD and LOQ – Serial dilutions of 2,6-dimethoxy-1,4-benzoquinone were performed with the mobile phase, and were then analysed by the HPLC method. LOD and LOQ were the concentrations that give a signal to noise ratio equal to 3 and 10, respectively.

Determination of extraction conditions – Different extraction conditions including types of solvent and dried powder to solvent ratios were evaluated to maximise the yield of 2,6-dimethoxy-1,4-benzoquinone in the stem extract of *F. foveolata*.

Types of solvents – *F. foveolata* dried stem powders (10 g) were separately extracted with hexane, chloroform, ethyl acetate and methanol (250 mL) under reflux conditions for 1 h.

Dried powder to solvent ratios – *F. foveolata* dried stem powders (10, 15, 20 and 25 g) were extracted with chloroform (250 mL) under reflux conditions for 1 h. After the extraction step, the filtered solutions were evaporated to dryness under reduced pressure at 40 °C. The residue was reconstituted and the volumes adjusted to 10 mL with the mobile phase. These samples were filtered through a 0.45 μm membrane filter and 20 μL volumes were subjected to HPLC analysis. All experiments were performed in triplicate.

Statistical analysis – Values are expressed as a mean ± SD. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$).

Result and Discussion

The optimal conditions of quantitative HPLC determination of 2,6-dimethoxy-1,4-benzoquinone in *F. foveolata* stem extracts was accomplished using a reverse phase isocratic HPLC system. A mixture of methanol and 5% aqueous acetic acid were investigated as the mobile phase, and the ratios were changed until the compound was satisfactorily resolved at the baseline in 12 minutes (Fig. 2). The most suitable ratio of methanol and 5% aqueous acetic acid to afford a good resolution of 2,6-dimethoxy-1,4-benzoquinone was 24:76, v/v. This HPLC method is simple and fast.

Defining the specificity, sensitivity, linearity, accuracy, intraday- and interday-precision validated the HPLC method. Specificity of the HPLC method was determined using the UV-absorption spectra produced by the photo-diode array detector. The UV-absorption spectra were taken at three points of the peak for 2,6-dimethoxy-1,4-benzoquinone detected in the sample. When they were compared with that of the standard 2,6-dimethoxy-1,4-benzoquinone, all UV-absorption spectra of both peaks were identical. The LOD and LOQ were determined by serial dilutions of 2,6-dimethoxy-1,4-benzoquinone with mobile phase. It was found that the HPLC method was very sensitive for detecting 2,6-dimethoxy-1,4-benzoquinone with the LOD and LOQ of 0.97 and 3.90 μg/mL, respectively.

Linearity was evaluated using standard 2,6-dimethoxy-1,4-benzoquinone over five calibration points (9.75 - 156.00 μg/mL) with six measurements for each calibration point. The calibration curve of standard 2,6-dimethoxy-1,4-benzoquinone had linearity over the evaluated ranges with a linear equation of $Y = 50293X + 7899.3$ ($R^2 \geq 0.9999$) (Fig. 3). The accuracy of the method was determined by

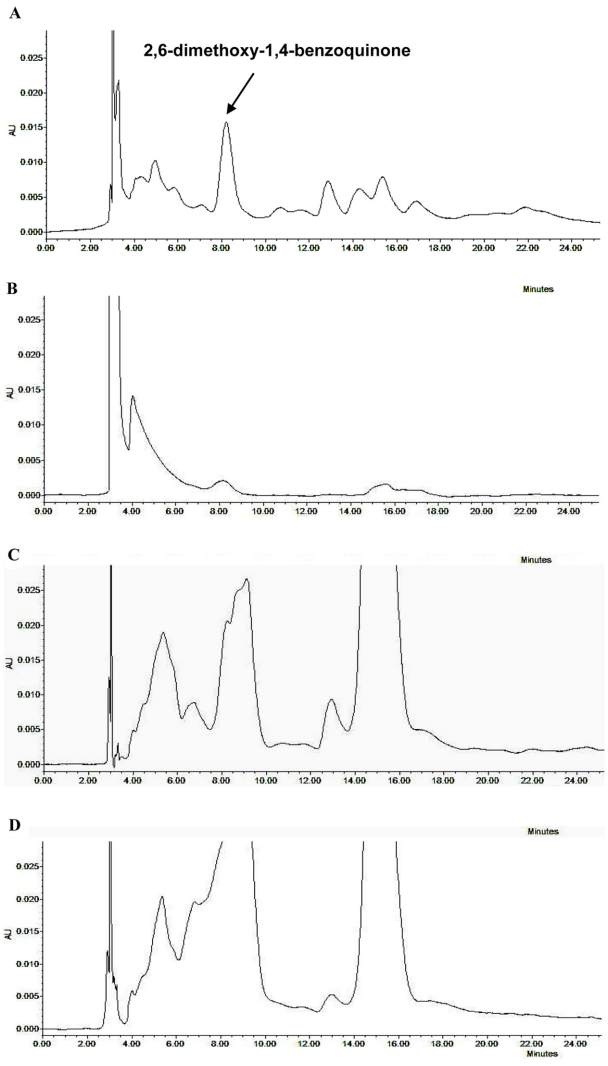

Fig. 2. HPLC chromatograms of (**A**) chloroform, (**B**) hexane, (**C**) ethyl acetate, and (**D**) methanol extracts of *F. foveolata* stems.

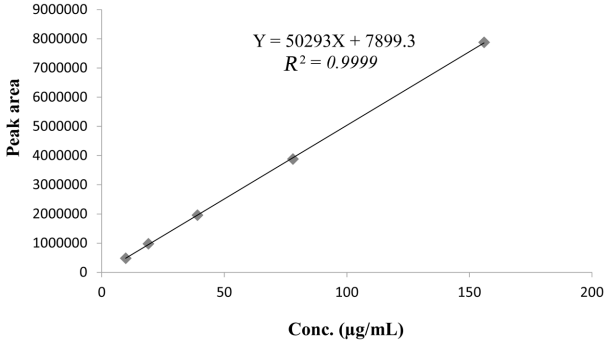

Fig. 3. Calibration curve of 2,6-dimethoxy-1,4-benzoquinone.

analysing *F. foveolata* stem extracts fortified with known quantities of the standard analyte. Recoveries in the range of 99.5 - 103.6% were observed for 2,6-dimethoxy-1,4-benzoquinone. Intraday-precision was evaluated by the

Table 1. Effect of extraction solvents on 2,6-dimethoxy-1,4-benzoquinone content of the dried extracts

Solvents	Yield of dried extracts (% w/w; Mean ± SD)	2,6-Dimethoxy-1,4-benzoquinone content (% w/w; Mean ± SD)
Hexane	0.18 ± 0.01*	n.a.
Chloroform	0.43 ± 0.01	1.45 ± 0.10
Ethyl acetate	0.94 ± 0.01*	n.a.
Methanol	7.64 ± 0.01*	n.a.

* Significant difference ($P < 0.05$) when compared with chloroform for extraction in the same column. n.a. = not analyse due to lower than limit of quantitation or poor resolution of the analyte peak.

Table 2. Effect of dried powders to solvent ratios on 2,6-dimethoxy-1,4-benzoquinone content of the dried extracts

Dried powder (g)	Yield of the dried extracts (mg; Mean ± SD)	2,6-Dimethoxy-1,4-benzoquinone content (% w/w; Mean ± SD)
10	47.20 ± 0.55	1.37 ± 0.16
15	61.03 ± 0.90*	1.60 ± 0.34
20	80.46 ± 0.64*	1.97 ± 0.06*
25	95.96 ± 0.80*	1.98 ± 0.23*

* Significant difference ($P < 0.05$) when compared with 10 g of dried powder in the same column.

relative standard deviation of six measurements for 2,6-dimethoxy-1,4-benzoquinone. Analysis of three independently prepared samples of *F. foveolata* stem extracts determined the interday-precision. The relative standard deviation values for both intraday- and interday analysis of 2,6-dimethoxy-1,4-benzoquinone were 1.28 ± 0.04 and 4.03 ± 0.13%, respectively.

Various extraction solvents were applied to determine the solvent that produced the *F. foveolata* stem extracts contained maximum content of 2,6-dimethoxy-1,4-benzoquinone. On the basis of the HPLC analysis, the extracts that were produced by chloroform extraction gave the highest content of 2,6-dimethoxy-1,4-benzoquinone up to 1.45% w/w (Table 1). The hexane extracts showed the peak of 2,6-dimethoxy-1,4-benzoquinone with under the limit of quantitation (Fig. 2). This implies that the polarity of hexane is too low to extract 2,6-dimethoxy-1,4-benzoquinone. In contrast, the polarities of both ethyl acetate and methanol are too high resulted in extraction of other polar compounds, which interfered the determination of 2,6-dimethoxy-1,4-benzoquinone (Fig. 2). Chloroform was therefore considered as the most suitable solvent used for sample preparation of *F. foveolata* stem extracts for quantitative HPLC analysis. In order to determine the extraction capacity of chloroform, the dried powders to solvent ratios were evaluated. An increase in the ratios of dried powders to solvent up to 20 g/250 mL resulted in an increase in the 2,6-dimethoxy-1,4-benzoquinone content in the dried stem extracts of 1.97% w/w (Table 2). However, a higher ratio of dried powders to solvent of 25 g/250 mL gave no further increase in the level of 2,6-dimethoxy-1,4-benzoquinone in the dried extracts. Therefore, the suitable ratio of dried stem powders to volume of chloroform in this extraction conditions was 20 g/250 mL.

Acknowledgments

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The authors would also like to acknowledge Dr. Brian Hodgson for assistance with the English.

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Received April 23, 2015
Revised May 15, 2015
Accepted May 17, 2015