Validation of quantitative RP-HPLC-DAD method and extraction optimization of 4-methoxycinnamyl *p*-coumarate and *trans*-4-methoxycinnamaldehyde in *Etlingera pavieana* rhizomes

Klaokwan Srisook[1,2†] (iD), Chartchai Malapong[3†], Petchrat Sawai[1], Ekaruth Srisook[1,4*] (iD)

[1]Department of Biochemistry and Research Unit of Natural Bioactive Compounds for Healthcare Products Development, Faculty of Science, Burapha University, Chonburi, Thailand.

[2]Center of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi, Thailand.

[3]Science Innovation Facility, Faculty of Science, Burapha University, Chonburi, Thailand.

[4]Department of Chemistry and Research Unit of Natural Bioactive Compounds for Healthcare Products Development, Faculty of Science, Burapha University, Chonburi, Thailand.

ABSTRACT

Etlingera pavieana is known as a spice and medicinal plant. According to previous reports, *E. pavieana* extracts exhibit various biological activities. However, the content of the primary phenylpropanoids of the *E. pavieana* rhizomal extract, 4-methoxycinnamyl *p*-coumarate (MCC) and *trans*-4-methoxycinnamaldehyde (MCD), has not been determined. This study was designed to validate a reversed-phase high-performance liquid chromatography method that quantified MCC and MCD from *E. pavieana* extracts. The method was validated based on the international council for harmonization (ICH) guidelines. The method displayed acceptable validation parameters, such as an excellent correlation coefficient higher than 0.999. The linearities of the response range of MCD and MCC were 1.00–20.00 and 2.5–60.00 µg/ml, respectively. The limit of detection and limit of quantitation were found to be 4.16 and 12.48 ng/ml, respectively, for MCD and 10.42 and 31.26 ng/ml, respectively, for MCC. The percentage relative standard deviation (RSD) of repeatability and intermediate precision were 1.03%–5.80% and 3.14%–6.84%, respectively. The ethyl acetate extracts prepared by the maceration and reflux method yielded the largest amount of MCD and MCC. This study confirmed that the high-performance liquid chromatography method for the simultaneous analysis of MCD and MCC was accurate, sensitive, and reproducible. This method can be used to quantitate the content of MCD and MCC in *E. pavieana* extracts.

INTRODUCTION

     Etlingera pavieana (Pierre ex Gagnep) R.M.Sm., belonging to the family Zingiberaceae, is used as a food and medicinal herb in Southeast Asia (Poulsen & Phonsena, 2017). *Etlingera pavieana* rhizome extracts exhibit anti-inflammatory (Srisook *et al.*, 2017, 2020), antioxidant (Srisook *et al.*, 2018),

antimicrobial (Naksang *et al.*, 2020; Tachai & Nuntawong, 2016), and cytotoxic effects (Iawsipo *et al.*, 2018; Tachai & Nuntawong, 2016). Thus, *E. pavieana* is believed to have health benefits.

     Various phenylpropanoids are isolated from *E. pavieana* rhizomes (EPE) (Srisook *et al.*, 2017). Among them, 4-methoxycinnamyl *p*-coumarate (MCC) and trans-4-methoxycinnamaldehyde (MCD) are the primary phytoconstituents. They exhibit potent anti-inflammatory activity by suppressing inflammatory mediators and cytokines as well as endothelial adhesion molecules (Mankhong *et al.*, 2019; Srisook *et al.*, 2019, 2020). Thus, *E. pavieana* can be a new source of MCC and MCD for functional foods and dietary supplements for the prevention of inflammation-related diseases. However, the quantity of these active compounds in *E. pavieana* rhizome

Corresponding Author
Ekaruth Srisook, Department of Biochemistry and Research Unit of Natural Bioactive Compounds for Healthcare Products Development, Faculty of Science, Burapha University, Chonburi, Thailand.
E-mail: ekaruth @ go.buu.ac.th
[†]These authors contributed equally to this work.

extracts has not been described. A sensitive and reliable method for determining MCC and MCD is necessary to control the quality of the herbal products containing *E. pavieana* extract.

The present study aimed to validate a high-performance liquid chromatography (HPLC) method to quantify MCC and MCD extracted from EPE according to the International Council for Harmonization (ICH) guidelines. In addition, the validated method was applied to determine the compound contents in *E. pavieana* extracts prepared with different solvents and methods to identify the optimal extraction procedure of the plant rhizomes.

EXPERIMENTALS AND METHODS

Materials

HPLC-grade methanol (Honeywell Burdick & Jackson, Seoul, Korea) was purchased and deionized water was prepared with a water purification system (MicroPure UV Thermo Scientific, Budapest, Hungary). MCC and MCD were extracted as described by Srisook *et al.* (2017). The purity of MCD (Supplementary Figure S1) and MCC (Mankhong *et al.*, 2019) determined by HPLC was more than 97%.

Plant extraction

The rhizomes of *E. pavieana* (Pierre ex Gagnep.) R.M.Sm. were dehydrated in a hot air dryer at 50°C and finely ground. The plant powder was extracted with different organic solvents, ethanol, 70% ethanol, 40% ethanol, and ethyl acetate, using the maceration or reflux method. For maceration extraction, 20 g of plant powder was macerated in 200 ml of extraction solvent at room temperature with continuous shaking for 6 hours and further placed for another 18 hours. The process was performed in triplicate. For reflux extraction, 20 g of the plant powder was soaked in 200 ml of solvent, refluxed for 1 hours. The extract solvent was filtered and evaporated using a rotary evaporator.

Stock standard solution and working standard solution

Five milligrams of MCC and MCD each was transferred to a microtube and dissolved in methanol to prepare a 5 mg/ml standard solution. The standard solutions were stored at −20°C. The working solution was freshly prepared by diluting the standard solution with methanol and filtered through a 0.45-µm membrane filter.

Chromatographic conditions

MCC and MCD were separated using the HPLC technique as described by Srisook *et al.* (2020). The HPLC system consisted of a quaternary pump system, an autosampler, and a diode array detector on the HPLC Agilent 1260 Infinity II platform (Agilent Technology, Wood Dale, IL). The column Phenomenex Luna C18 (4.6 × 250 mm, 5-µm) was used and maintained at 35°C. The mobile phase of methanol: water (70:30 *v/v*) was applied during isocratic elution. The flow rate was set to 1.0 ml/minute. The injection volume was 10µl. The detector wavelength was set at 320 nm. The total run time was 30 minutes.

Method performance characteristic

The analytical characteristics of the proposed methods for MCD and MCC were investigated for optimum conditions. Each method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy following the ICH of Technical Requirements for Pharmaceuticals for Human Use (ICH, 2005).

Linearity

The linearity was determined using the standard working solutions of MCD at 0.05–200 µg/ml and MCC at 0.125–200 µg/ml. The concentration at each standard solution was analyzed in triplicate. The linearity was obtained from the plot between the peak area (*y*-axis) and the concentration of the MCD and MCC standard solutions.

Precision

The method's precision was determined at the low, middle, and high concentrations in the calibration curve. The repeatability was determined by analyzing and calculating the relative standard deviation (RSD) 10 times within 1 day. The intermediate precision was determined by analyzing and calculating the RSD for nine consecutive days. The precision was expressed by the percent relative standard deviation (%RSD) as follows:

$$\%RSD = \frac{SD}{\bar{x}} \times 100$$

Accuracy

The accuracy of the proposed method was expressed in terms of recovery. Recovery was studied by spiking MCD and MCC at low, middle, or high concentrations into 5 mg of crude extract and performing the analysis in triplicate (*n* = 3). The percent recovery was calculated as follows:

$$recovery = \frac{C_{Sample\ +\ Standard} - C_{Sample}}{C_{Standard}} \times 100$$

LOD and LOQ

A standard solution was diluted to the lowest concentration by methanol. The signal-to-noise (S/N) ratios of 3 and 10 were used to determine the LOD and LOQ, respectively.

Preparation of crude extract sample

Five milligrams of dried crude extract were dissolved in methanol. After filtrating with a 0.45-µm membrane filter, the extract solution was injected into the HPLC system.

Statistical analysis

All data were expressed as the means ± standard deviations of triplicate measurements. A factorial design was applied to the study with two parameters, solvent types and extraction methods. The results were analyzed using the Minitab software version 17.1. The experimental groups were compared using one-way analysis of variance followed by Tukey's multiple comparison tests; the differences with a statistical significance of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Method validation

HPLC was used to analyze MCD and MCC in *E. pavieana* rhizome extracts. MCD and MCC were separated in

reversed-phase high-performance liquid chromatography and detected at the maximum absorbance of 320 nm. According to the MCD and MCC chromatograms under the optimal condition (Fig. 1A), symmetric and well-resolved peaks for MCD and MCC were yielded under this condition. The retention times (R_t) of MCD and MCC were 4.5 and 13.6 minutes, respectively. On the other hand, the chromatogram of the ethanol-extract EPE was shown (Fig. 1B). The specificity of the validated method was established by comparing the R_t and the absorption spectra of the peaks of the *E. pavieana*-extracted MCD and MCC.

The analytical characteristics of the proposed method were determined following the ICH guidelines. The regression equation was summarized in Table 1. The calibration curve was obtained by plotting the integrated peak area and concentration of MCD and MCC (Supplement data Figure S-2). The calibration curves of MCD and MCC were found to be linear in the ranges of 1.0–20.00 and 2.0–60.00 µg/ml, respectively (Table 1). The correlation coefficient (R^2) was more than 0.999 for MCD and MCC.

The LOD is defined as the lowest concentration of detection, and the LOQ is defined as the lowest quantifiable amount of analyte in the sample. Both values were obtained using the S/N ratio method. The standard solution was further diluted to a known low concentration and injected into HPLC for determination of the S/N ratio. The S/N ratios of 3 and 10 were used to estimated LOD and LOQ, respectively. The LOD and LOQ were determined to be 4.16 and 12.48 ng/ml, respectively, for MCD and 10.42 and 31.26 ng/ml, respectively, for MCC (Table 1). These data confirmed the sensitivity of the method.
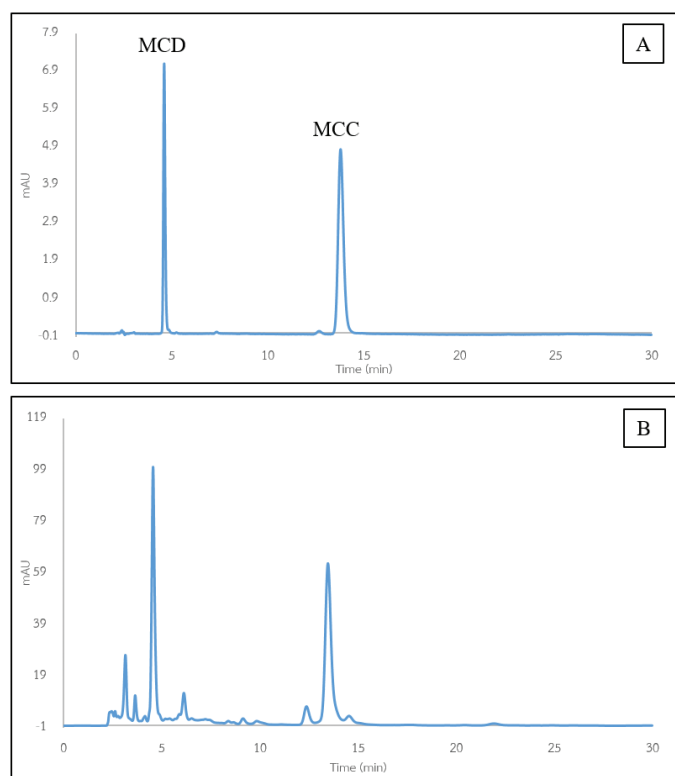
Precision was studied by measuring the intraday (repeatability) and interday or intermediate precision. The precision was expressed by the percentage relative to the standard deviation. The percentage RSD of repeatability and intermediate precision were 1.03–%5.80% and 3.14%–6.84%, respectively (Table 2), at an acceptable level according to the AOAC (2016) manual.

The accuracy of the proposed method was tested by adding a standard solution at three concentrations to the *E. pavieana* crude extract. The recovery of MCD and MCC was between 91.94% and 100.44% and 99.08% and 102.75%, respectively, at an acceptable level according to the AOAC (2016) manual.

Extraction optimization of MCC and MCD from *E. pavieana* rhizome

Several studies suggest that the solubility of phenolic compounds is dependent on the polarity of the solvent used in the extraction (Boeing *et al*., 2014; Iloki-Assanga *et al*., 2015; Lopes *et al*., 2018). Therefore, we determined the effect of different solvents and extraction methods on the content of MCD and MCC in the *E. pavieana* rhizome extracts. The contents of MCD and MCC in the extract calculated from the calibration curve produced the relationship between the concentration of analyte and the peak area obtained from the validated HPLC technique. The results revealed that ethyl acetate extraction yielded the largest amount of MCD and MCC, followed by ethanol, 70% ethanol, and 40% ethanol, respectively (Fig. 2). A similar trend was observed for the MCD and MCC contents in the extracts by the maceration and reflux extraction methods. There was no significant difference ($p > 0.05$) in the contents of MCD and MCC in the extracts by the maceration and reflux

Figure 1. HPLC chromatograms. (A) MCD and MCC. (B) Ethanol-extracted MCD and MCC from EPE.

Table 1. The performance characteristics of the proposed HPLC method.

Characteristics	Analyte	
	MCD	**MCC**
Regression equation	$y = 48.835x − 11.983$	$y = 40.607x − 26.737$
Correlation coefficient (r^2)	0.9993	0.9993
Linearity Range (µg/ml)	0.25–200.00	2.50–200.00
Calibration curve (µg/ml)	1.00–20.00	2.50–60.00
LOQ (ng/ml)	12.48	31.26
LOD (ng/ml)	4.16	10.42
Recovery (%)	91.94–100.44	99.08–102.75

Table 2. The repeatability and intermediate precision of the proposed HPLC method.

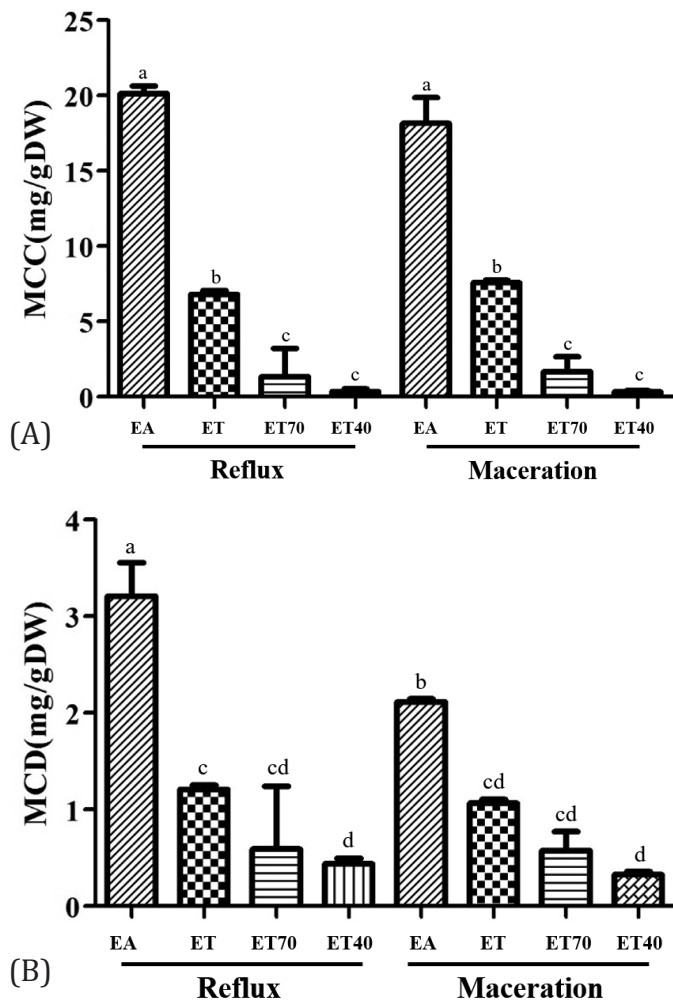| Analyte	Concentration (µg/ml)	Repeatability (%RSD, $n = 10$)	Intermediate precision (%RSD, $n = 9$ days)
MCD	2.0	5.80	6.84
	10.0	1.80	6.05
	20.0	1.79	4.43
MCC	2.5	3.46	3.14
	10.0	3.41	6.07
	40.0	1.03	3.70

Figure 2. Contents of MCD (A) and MCC (B) of *E. pavieana* rhizome extracts using different solvents and different methods, maceration or reflux. Values with different letters are significantly different ($p < 0.05$). EA, EPE extracted by ethyl acetate. ET, extraction by ethanol. ET70, extraction by 70% ethanol. ET40, extraction by 40% ethanol.

methods using the same solvent except ethyl acetate. A higher MCD content was found in the ethyl acetate extract by the reflux method than the maceration method. Since a phenol group of MCD or MCC is protected by a methyl group in the methoxy group, MCD and MCC are less polar than their corresponding phenolic compounds. Therefore, ethyl acetate, which is less polar than ethanol or aqueous ethanol, is the best solvent for extracting MCC and MCD from EPE. The data from the present study indicated that the polarity of a solvent affected the extraction of both MCD and MCC, and the extraction method affected the yield of MCD.

CONCLUSION

The HPLC method for the simultaneous determination of MCD and MCC in *E. pavieana* was validated. The proposed method was demonstrated to be sensitive, precise, and accurate. It can be used for quantifying MCD and MCC in EPE, a potentially significant source of active ingredients for natural health products.

This method would be beneficial for the quality control of raw materials and extracts of *E. pavieana*.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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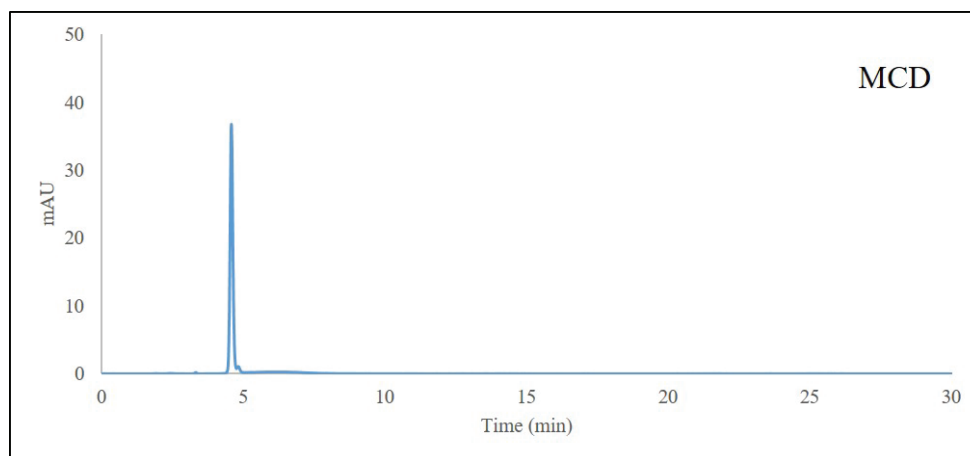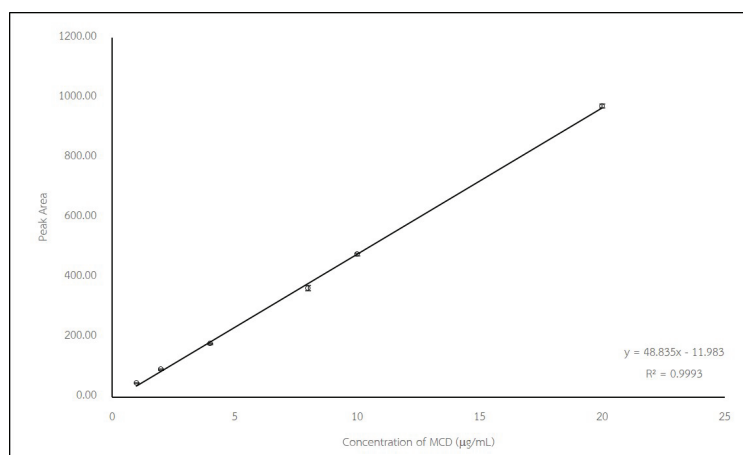
SUPPLEMENTARY FIGURE

Figure S1. High performance liquid chromatography chromatogram of *trans*-4-methoxycinnamaldehyde (MCD) detected at 320 nm.
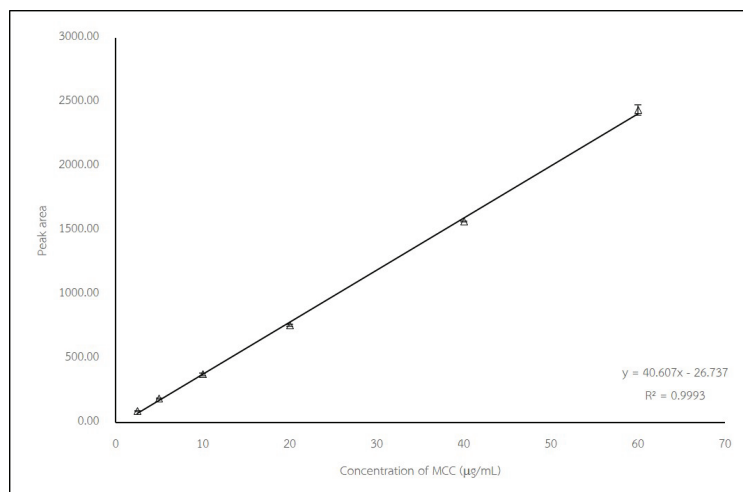
(A)

(B)

Figure S2. Calibration Curve of MCD (A) and MCC (B).