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Quantitative Analysis of Swertiamarin Content from *Fagraea fragrans* Leaf Extract using HPLC Technique and its Correlation to Antibacterial Activity

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Abstract

The rise in drug resistance poses escalating challenges for antibacterial medications, leading to an urgent demand for the exploration and innovation of new antibacterial drugs. *Fagraea fragrans* Roxb., belonging to the Gentianaceae family, is one of the common herbal medicines which can be found abundantly in Southeast Asia. The secoiridoid glucoside swertiamarin, one of the major compounds in *F. fragrans* leaf, exhibits antimicrobial effects. To guarantee the medicinal effectiveness of *F. fragrans* leaves, it is essential to identify a standardized analytical method for quantifying the active compound. In this study, the optimized HPLC method following ICH guideline was validated for the quantitative analysis of swertiamarin content in *F. fragrans* leaf in terms of linearity (y = 5733.5x - 369.1; R2 = 0.9999), accuracy (93.57-96.39% recovery), precision (0.91% RSD for repeatability precision; 1.19% RSD for intermediate precision), limit of detection (0.73 µg/mL), limit of quantitation (2.23 µg/mL), specificity (peak purity index = 0.999995) and robustness (% RSD <1).The maximum wavelength of swertiamarin was found to be at 238 nm. The amount of swertiamarin content in *F. fragrans* leaf extract conducted from the validated HPLC method was found to be 0.0259 ± 0.0005 g/100 g crude drug. The leaf extract exhibited antimicrobial activity against clinical isolates of *Enterobacter cloacae*, *Klebsiella pneumonia*, and *Escherichia coli* at 0.125 mg/mL, and *Pseudomonas aeruginosa* at 0.5, showing minimum inhibitory concentration (MIC) values. Whereas swertiamarin exhibited even lower MIC values. The developed HPLC analysis effectively determines swertiamarin content as a chemical marker to ensure the antimicrobial potential of *F. fragrans* leaves.

Keywords**:** *swertiamarin content; antimicrobial activity; herbal medicine; alternative medicine; traditional Thai medicine; secoiridoid glycosides; Fagraea fragrans*

1. Introduction

The growing use of herbal medicines has become essential for primary healthcare as well as an important strategy toward universal health (Benzie, & Wachtel-Galor, 2011). For decades, traditional plant-based medicines have been famous as the effective treatment modality due to their phytochemical properties. There has been a growing exploration of medicinal plants' natural products to enhance human health in combating diverse diseases. Additionally, there is a significant focus on investigating natural products as agents that can modify biological functions due to their varied and multifaceted chemical compositions (Bonam et al., 2018). Therefore, these products continue to serve as valuable therapeutic agents, demonstrating positive applications. Currently, the emerging of bacterial resistance on human health increases in morbidity, mortality, and cost associated with the treatments (Dadgostar, 2019). Chemotaxonomy and pharmacology study reports that swertiamarin is usually found in Gentianaceae family (Jensen, & Schripsema, 2002). It was found that many plants in Gentianaceae family containing a secoiridoid glycoside, swertiamarin possessed antibacterial properties such as *Centaurium* species (Bibi et al., 2006; van der Sluis, 1985). and *Enicostema* species (Perumal et al., 2022; Pillai et al., 2020; Vishwakarma et al., 2004). Moreover, numerous plants containing swertiamarin as the main compound revealed the antimicrobial activity (Niroj et al., 2022). Therefore, this leads to the interest of seeking the new natural plant-based medicine to kill or inhibit bacteria infectious diseases from another plant species in Gentianaceae family.

Fagraea fragrans Roxb., commonly known as Kankrao in Thai, is a plant species in the Gentianaceae family. It is a large evergreen tree native to Southeast Asia (Motley, 2004). Different medicinal properties derived from various parts of *Fagraea fragrans* have been widely used in traditional and folk medicine among many countries. The ethnopharmacological survey in Cambodia reports that the bark of *F. fragrans* is employed in the treatment of both fever and malaria (Jonville et al., 2010). An ethnic community native to Malaysia utilizes *F. fragrans* leaves and bark for addressing pancreatitis and gastric pains. (Kamarudin, & Latiff, 2002). The infusion made from the leaves serves as a solution for dysentery. It is employed in the treatment of malaria, with a decoction derived from either the leaves, bark, or root (Kamarudin, & Latiff, 2002). In traditional Thai medicine, the leaves are utilized to address asthma, nourish the bodily elements, and treat skin conditions. The stem, characterized by its astringent and bitter taste, serves as a nourishing agent for the body, acting as an elixir for longevity. It is employed in the treatment of various conditions such as fever, tremors, asthma, cough, hemorrhoids, dropsy, diarrhea, chest tightness, spleen nourishment, blood enrichment, and remedy for blood disorders (Pripdeevech, & Saansoomchai, 2013). Additionally, the ethnobotany survey in the southern part of Thailand reports that the plant has been traditionally used to treat bacterial and fungal infectious diseases as well as inflammation (Neamsuvan et al., 2015).

Previous studies have shown that swertiamarin is a major compound in *F. fragrans*. The use of a TLC-densitometer was developed to quantify the swertiamarin content in the leaves of *F. fragrans*, indicating that this secoiridoid glucoside is the major phytochemical constituent in *F. fragrans* leaves (Bangprapai et al., 2016). Moreover, earlier investigations of other plants in Gentianaceae family have revealed the presence of swertiamarin in various parts of distinct species, including the aerial parts of *Enicostemma axillare* (Rana et al., 2015), the whole plant of *Enicostemma littorale* (Ahamad et al., 2014), and the whole plant of *Centaurium erythraea* (Gubar et al., 2020). The quantification of swertiamarin content in these botanical specimens was achieved through method validations employing the HPLC-PDA technique (Gubar et al., 2020; Rana et al., 2015) and HPLC-UV method (Ahamad et al., 2014). Based on the findings of prior researches, the utilization of the HPLC technique for quantifying swertiamarin content in *F. fragrans* leaf is deemed appropriate for implementation in this study. Unfortunately, studies on the antibacterial activities of *F. fragrans* leaves are lacking. Thus, the relationship between the amount of swertiamarin content in the leaves and antibacterial effect should be evaluated based on the aforementioned studies that plants containing swertiamarin exhibited antimicrobial activity. Additionally, quantitative analysis of swertiamarin content can be used as a chemical marker and a tool to strengthen the quality control of herbal medicine. As a consequence, the development of reliable analytical method for swertiamarin quantification

in the leaves of *F. fragrans* was investigated using HPLC technique.

2. Objectives

The aims of the present study were to: 1) develop and validate a new HPLC standard method for determination of swertiamarin content in *Fagraea fragrans* leaf extract, and 2) evaluate the correlation between the quantification analysis of swertiamarin in the extract and its antimicrobial activities.

3. Materials and methods

3.1 Plant collection and extraction

The mature leaves of *F. fragrans* were collected at Mae Fah Luang University Botanical Garden, Chiang Rai, Thailand. The collected plants were authenticated by the botanical expert and the voucher specimens were deposited at the School of Integrative Medicine, Mae Fah Luang University, Thailand. The leaves underwent a meticulous examination, and any leaves showing signs of fungus damage, infection, or aging were eliminated. The plant material was thoroughly washed with distilled water two to three times. Subsequently, the cleaned leaves were finely powdered using a sterile electronic blender, and the resulting powder was stored airtight for 15 days during shade drying. Five grams of the sample were exhaustively extracted with 95% ethanol by Soxhlet apparatus. The solution underwent filtration, and the resulting filtrate was evaporated until dryness using a water bath set at 50-60 °C. The yield of leaf extract in 95% ethanol was 15% (g/100 g crude drug). Extracts from the process were saved for antibacterial activity. For HPLC analysis, the extract was dissolved in one milliliter of methanol to achieve the desired final concentration. Ultimately, the methanol extract was diluted to different concentrations and subjected to further HPLC analysis after filtration through a 0.45 µm PTFE membrane syringe filter.

3.2 HPLC method validation

3.2.1 Chemical and reagent for HPLC method validation

The standard swertiamarin was purchased from Sigma-Aldirch, USA (BCBW3521, ≥95%). HPLC grade chemicals were used in this analysis such as ultrapure water and methanol.

3.2.2 Instrumentation and equipment for HPLC method validation

The utilized equipment included the Shimadzu HPLC LC-20A, auto-sampler (SIL-20A HT), HPLC column oven (CTO-20AC), HPLC degasser (DGU-20A3), HPLC LC-20A system, HPLC photodiode array (PDA) detector (SPD-M20A), HPLC system controller (CBM-20A), HPLC two-solvent delivery unit (LC-20AD) from Shimadzu, Japan. Additionally, an HPLC guard column (5 µm, 2.1 x 50 mm) (CN-3) was used from Inertsil, GL Sciences Inc., Japan, along with a water purification system from Brinkmann, USA.

3.2.3 Standard swertiamarin preparation for HPLC method validation

The swertiamarin stock was created by dissolving 1 mg of the standard swertiamarin in 1 ml of methanol. The solution underwent filtration using a 0.45 µm PTFE membrane syringe filter.

3.2.4 HPLC Chromatographic condition for HPLC method validation

Shimadzu LC Solution software was used to carry out the HPLC system control and data analysis. The reversed-phrase C18 column coupled with HPLC guard column was used to conduct the chromatographic separation. The chromatographic condition of HPLC was shown in table 1. All of the sample was analyzed in triplicate.

In the HPLC method validation for swertiamarin, several key parameters were assessed according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH guideline) (Borman & Elder, 2017). The parameters evaluated included the limit of quantification (LOQ), limit of detection (LOD), linearity, precision, accuracy, specificity, and robustness.

Linearity was established using a calibration curve with swertiamarin concentrations of 3.13, 6.25, 12.50, 25.00, and 50.00 µg/ml, measuring peak area against standard concentrations. LOD and LOQ were calculated from the calibration curve using formulas: LOD = 3.3 (residual standard deviation)/slope (S) and LOQ = 10 (residual standard deviation)/S.

Table 1 Chromatographic condition of HPLC for swertiamarin quantification

Parameter	Condition
Column (Stationary phase)	Reverse phase C_{18} Solvent: Water: Methanol (20:80) Mode: Isocratic elution
Flow rate	0.5 ml/min
Injection volume	10 µl
Column oven temperature	35 °C
Detector	PDA detector, λ_{max} 238 nm

Accuracy was assessed by the percent recovery method from spiked samples at varying concentrations, using the formula: % recovery = [A/(B+C)] x 100, where A is the swertiamarin identified in the spiked sample, B is detected in the un-spiked sample, and C is the standard added. Precision involved calculating the relative standard deviation (% RSD = (SD/Mean) x 100) across three concentrations and replicates both within the same day and across different days. Specificity was determined by comparing absorbance spectra of swertiamarin at peak start, apex, and end positions. Robustness was tested by adjusting the column oven temperature from 39 to 41 °C and observing the effect on the % RSD.

3.3 Antimicrobial activity

3.3.1 Bacteria isolates and growth conditions

Without any preference for particular strains, anonymized clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *and Enterobacter cloacae* were obtained from a repository held at the Pathogen Hunters Research Laboratory and kindly donated by Dr. Dhammika Leshan Wannigama. The bacterial isolates were cultured on Müller–Hinton agar plates at 37°C and cryopreserved at –80°C in tryptic soy broth with 15% (v/v) glycerol until subsequent use in these studies.

3.3.2 Antimicrobial agents

Ceftazidime (Sigma-Aldrich) was used to prepare stock solutions <24 h before use. All agents were dissolved in cation-adjusted Müller–Hinton II broth (MHIIB) (Sigma-Aldrich), and the solutions were sterilized through a syringe filter with a membrane nominal pore size of 0.22 µm. Serial dilutions of the antibiotic, *Fagraea fragrans* leaf extract, and swertiamarin stocks were prepared in MHIIB immediately before use.

3.3.3 Susceptibilities to F. fragrans leaf extract, swertiamarin and ceftazidime

We established the susceptibility of planktonic cultures of the various strains to antibiotics using standard techniques (broth microdilution to determine minimal inhibitory concentrations, MICs) as described previously (Luk-in et al., 2021; Phuengmaung et al., 2020; Shein et al., 2021; Shein et al., 2022; Shein et al., 2023; Srisakul et al., 2022; Wannigama et al., 2014; Wannigama et al., 2020; Wannigama et al., 2019; Wannigama et al., 2023), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST criteria for Enterobacteriaceae only) (European committee on antimicrobial susceptibility testing, 2016) and U.S. Clinical and Laboratory Standards Institute (CLSI) guidelines (Weinstein, & Lewis, 2020). We used *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 for quality control. Susceptibility testing was performed for all clinical isolates.

3.8 Data analysis

For HPLC quantitative analysis, the area under peak chromatogram of standard swertiamarin and samples was calculated using the software from Shimadzu.

4. Results and discussion

4.1 Development of HPLC method for swertiamarin content

Phytochemical constituents are typically regarded as chemical markers, and their measurement often serves as an indicator of the quality of plant materials based on their therapeutic properties (European Medicines Agency, 2008). The validated HPLC coupled with a PDA detector in this current study was optimized for the quantitative assessment of swertiamarin content in *F. fragrans* leaves. This validation encompassed

aspects such as accuracy, precision, linearity, LOD, LOQ, specificity, and robustness.

The absorbance range between 200–800 nm was examined to identify the presence of a standard swertiamarin. The detected maximum wavelength of swertiamarin was at 238 nm, which was consistent with the previous report of swertiamarin content in the aerial parts of *Enicostemma axillare* using HPLC technique (Virendra et al., 2012). On the other hand, some reports on swertiamarin content in *Enicostemma littorale*, *Bacopa monnieri* and *Chromatographia* using HPLC techniques exhibited different maximum base line separation and quantification of swertiamarin at 227 nm and 254 nm, respectively (Alam et al., 2009; Bhandari et al., 2006).

For linearity determination, the calibration curve of swertiamarin content in the range of 3.125-50 µg/mL was obtained from the calculation of regression equation of y = 5733.5x - 369.1 (Figure 1). The coefficient of determination (R^2) for standard swertiamarin was determined to be 0.9999, suggesting the acceptability of the analytical method. The standard swertiamarin peak was clearly separated with a retention time of 9.08 min as displayed in Figure 2. Similarly, Figure 3 displays the well separated peak in HPLC chromatogram of swertiamarin in the *F. fragrans* leaves extract. The peak purity index of swertiamarin in *F. fragrans* leaf extract was 0.999995 (Figure 4). This indicates that the approach method utilized in this study demonstrates selectivity and specificity in quantifying the swertiamarin content within the *F. fragrans* leaf extract.

The accuracy was verified using a recovery method involving the introduction of standard swertiamarin spikes into the sample (*F. fragrans* leaf extract) at three distinct concentrations (8, 24, and 40 µg/mL). The outcomes presented in table 2 are deemed acceptable (1.29-0.40 for repeatability precision; and 1.99-0.28 for intermediate precision), meeting the established criteria for acceptability, which typically falls within the range of 80-120% (Borman, & Elder, 2017). Utilizing identical spiked concentrations, the mean values for both intermediate precision and repeatability precision were 1.19% and 0.91% RSD, respectively. The RSD percentage achieved in this current investigation was deemed satisfactory as it did not exceed 15%, thereby meeting the stipulated FDA requirement (U.S. Food and Drug Administration, 2018).

The limits of detection (LOD) and limits of quantification (LOQ), derived from the residual standard deviation of the regression line and the slope of the calibration curve, were determined to be 0.73 and 2.23 µg/mL, respectively. By altering the column temperature within the range of 39-41°C, the findings suggested that there were negligible variations displayed as % RSD less than 1 in the area under the standard curve and the retention time of swertiamarin. The robustness test affirmed the method's dependability by demonstrating its resilience to minor variations in the method parameters (Borman & Elder, 2017). Therefore, the developed HPLC was valid for quantification of swertiamarin in *F. fragrans* leaf crude drug. Moreover, the quantification of swertiamarin in one gram of the extract was found to be 0.26 g (Table 4).

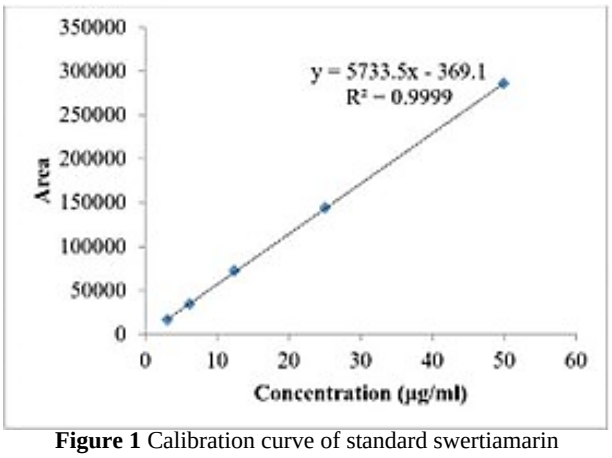

Figure 1 Calibration curve of standard swertiamarin

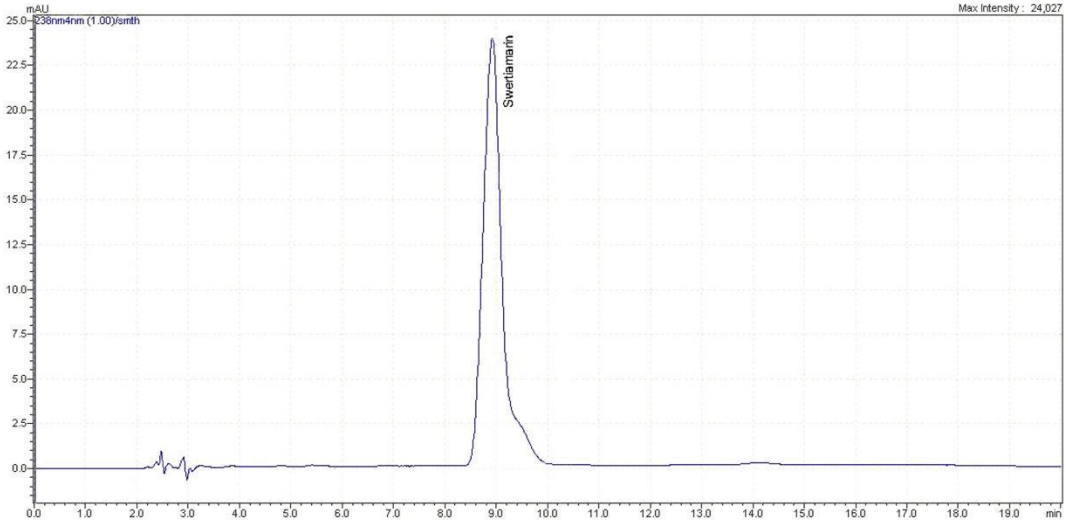

Figure 2 HPLC chromatogram of standard swertiamarin at 238 nm

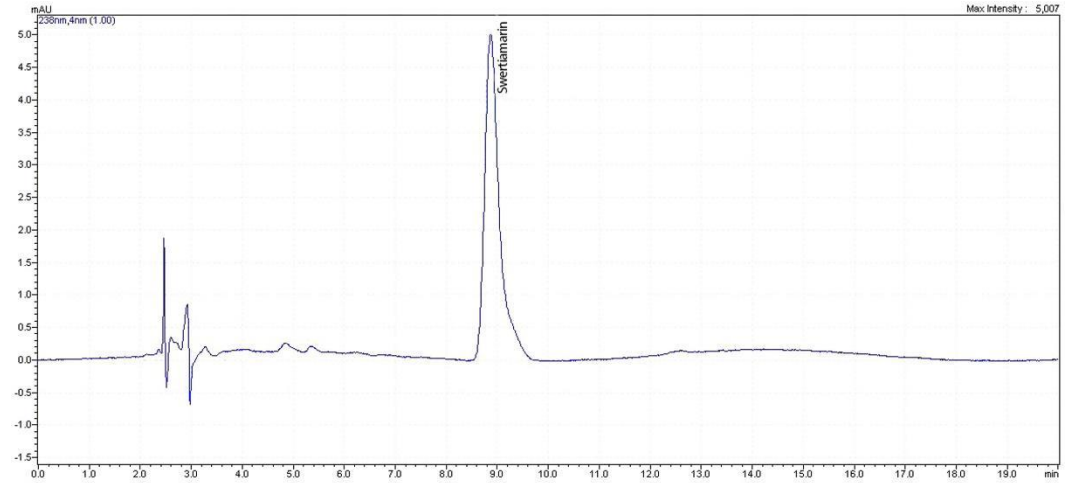

Figure 3 HPLC chromatogram of swertiamarin in *Fagraea fragrans* leaf extract

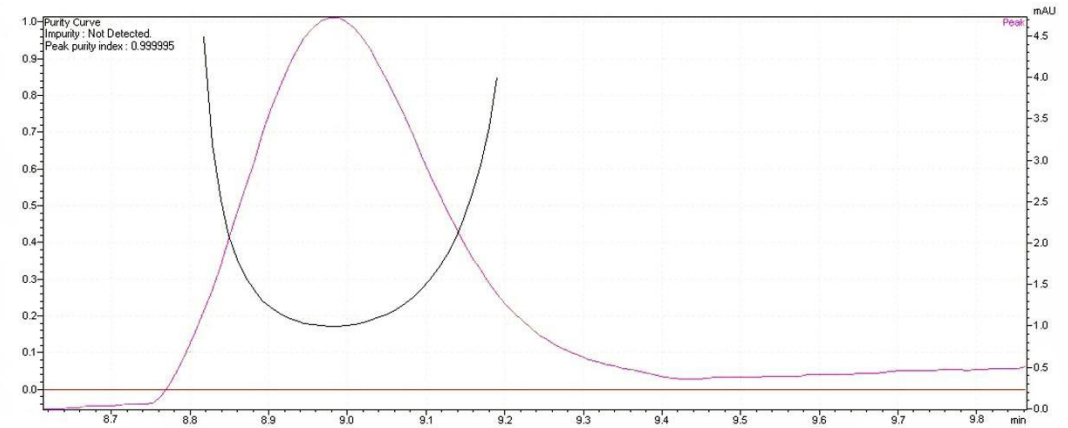

Figure 4 Peak purity of swertiamarin in *Fagraea fragrans* leaf extract (Peak purity index: 0.999995)

Table 2 Accuracy and precision of swertiamarin in *Fagraea fragrans* leaf extracts

Level of spike extract	%Recovery	%RSD	
		Repeatability precision	Intermediate precision
Low	94.56	1.06	1.99
Medium	96.39	0.88	1.03
High	93.57	0.40	0.28

Table 3 Robustness of swertiamarin in *Fagraea fragrans* leaf extracts

Temperature	Area	Retention time
39°C	132,725	8.95
40°C	135,070	8.93
41°C	134,004	8.87
Mean ± SD	133,933 ± 1,174	8.92 ± 0.04
%RSD	0.88	0.47

Table 4 Swertiamarin content in *Fagraea fragrans* leaf extracts

Sample	Yield of extract (g/100g crude drug)	Swertiamarin in extract (g/g) (n=3)	Swertiamarin in crude drug (g/100g crude drug) (n=3)
F. fragrans leaf extracts	15.00	0.26 ± 0.01	3.87 ± 0.11

Table 5 MIC values (mg/mL) of the leaf extract and swertiamarin

Clinical isolates	*F. fragrans* leaf extract MIC (mg/mL)	Swertiamarin MIC (mg/mL)	Ceftazidime MIC (mg/mL)
Escherichia coli (n=20)	0.125	0.02	> 64
Pseudomonas aeruginosa (n=16)	0.500	0.04	> 128
Klebsiella pneumoniae (n=26)	0.125	0.02	> 128
Enterobacter cloacae (n=14)	0.125	0.02	> 64

Minimal inhibitory concentrations (MIC, mg/mL)

4.2 Antibacterial activity

We further investigated the antimicrobial efficacy of *F. fragrans* leaf extract and swertiamarin against multidrug resistance clinical isolates of *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Enterobacter cloacae*. The Minimum Inhibitory Concentrations (MIC) were determined for each substance. Notably, *F. fragrans* leaf extracts exhibited MIC values of 0.125 mg/mL for *Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae*, and 0.02 mg/mL for swertiamarin. In contrast, ceftazidime displayed higher MIC values, as all these bacteria isolates resistance to ceftazidime (> 64 mg/mL for *Escherichia coli* and *Enterobacter cloacae*, and > 128 mg/mL for *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*). The results of swertiamarin against *Escherichia coli, Pseudomonas aeruginosa* and *Enterobacter cloacae* were consistent with the previous report with MIC values between 0.01 – 0.02 mg/mL (Šiler et al., 2010). These findings suggest the potential of *F. fragrans* leaf extracts and swertiamarin as antimicrobial agents against the tested ceftazidime resistance clinical isolates. In addition, Jonville and co-workers (Jonville et al., 2008) reported that swertiamarin can be found in various parts of *F. fragrans* such as the stem bark, roots and fruits. Numerous compounds isolated from *F. fragrans* stem bark extract in combination with the antibiotic erythromycin, demonstrated robust antimycobacterial effects against *Mycobacterium smegmatis* (Madmanang et al., 2016). Hence, specific compounds derived from plants, including swertiamarin sourced from *F. fragrans*, have the potential to be utilized as enhancers of antibiotics for managing infections.

5. Conclusion

Application of reversed-phase HPLC with the photodiode array (PDA) detector in this study

was valid for the determination of swertiamarin content in the ethanolic extract of *F. fragrans* leaves. Thus, the established HPLC method is applicable for defining the specifications of this raw medicinal substance by referencing its chemical marker. Additionally, the swertiamarin and the extract exhibited antibacterial activity. Nevertheless, further investigations, encompassing both in vitro and in vivo examinations, are necessary to establish their effectiveness against microbes.

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