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Optimization Method and Stability Test to Determinate Luteolin, Quercetin, Apigenin, and Sinensetin Levels in Herbal Medicines Using TLC-Densitometry

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Abstract

Background*: Nephrolithiasis is a condition in which there are one or more kidney stones in the pelvis or calyces. Luteolin, quercetin, apigenin, and sinensetin are marker compounds in the extracts of Plantago major, Sonchus arvensis, Strobilanthes crispus and Orthosiphon stamineus which have nephrolithiasis activity. To control the quality of herbal medicines, a TLC-Densitometry method was developed in this study using luteolin, quercetin, apigenin, and sinensetin as phytochemical markers.* ***Objective****: The present work aimed to develop optimal conditions for analyzing luteolin, quercetin, apigenin, and sinensetin.* ***Methods****: Determination of optimal conditions for analysis is carried out by determining the composition of the mobile phase, chamber saturation time, and analysis wavelength. Silica gel 60 F_{254} was used as the stationary phase. Stability tests were carried out by analyzing standards and samples at 0, 4, 8, and 24 hours.* ***Results****: The best separation that produces symmetrical peaks of herbal medicine was achieved under isocratic conditions using the composition of the mobile phase chloroform : acetone: dichloromethane : acetonitrile : formic acid (6 : 2: 2 : 0,05 : 0.05 v/v/v/v/ v) with a wavelength of 335 nm with a saturation time of 30 minutes.* ***Conclusion****: In this study, the optimal conditions for the analysis of luteolin, quercetin, apigenin, and sinensetin. Luteolin, quercetin, apigenin, and sinensetin are unstable during 8 hours of storage. Therefore, standard solutions and samples must be made fresh to maintain stability.*

Keywords*: TLC-densitometry, luteolin, quercetin, apigenin, sinensetin*

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INTRODUCTION

Over the decades, herbal plants and their active constituents have been developed and used to treat various ailments. The increasing development and use of herbal medicines are based on several reasons, namely: not all modern medicines can efficiently overcome/cure all human pathologies, there is increasing interest and attention to the guarantee and safety of synthetic medicines, and many natural products are proven to have better quality than synthetic medicines. *Plantago major*, *Sonchus arvensis*, *Strobilanthes crispus*, and *Orthosiphon stamineus*, which are empirically used to treat or prevent nephrolithiasis have been developed in many countries. Extracts of leaves, stems, and roots have long been used as a treatment for kidney stones, antifungal, bladder, antioxidant, gastrointestinal infections, diabetes, and anticancer (Kartini & Azminah, 2012; Hossain & Ismail, 2016). Several studies have proven that *Plantago major*, *Sonchus arvensis*, *Strobilanthes crispus*, and *Orthosiphon stamineus* have nephrolithiasis effects (Singh *et al.,* 2009; Alkreathy *et al.,* 2014; Chun *et al.,* 2020; Ghiasian *et al.,* 2021). Jimoh *et a*l. (2021) proved that luteolin, quercetin, apigenin, and sinensetin compounds play a role in controlling the crystallization process of kidney stones (jimoh *et al.,* 2021). Hossain & Ismail, (2012) also proved that luteolin, quercetin, apigenin, and sinensetin compounds could inhibit electrolyte reabsorption in the loop of Henle so that it has a diuretic effect (Hossain & Ismail, 2016). Diuretics effectively reduce urinary calcium excretion and kidney stone recurrence in nephrolithiasis patients by controlling urine supersaturation (Ahmed *et al.,* 2018). The structure of luteolin, quercetin, apigenin, and sinensetin can be seen in Figure 1.

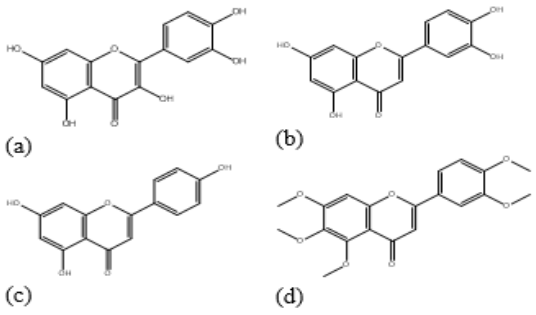

Figure 1. Structure of luteolin (a), quercetin (b), apigenin (c) dan sinensetin (d)

In developing polypharmacy herbal medicines, the selection of marker compounds and methods of identification/quantification of marker compounds play an important role in ensuring quality control of herbal medicines. The analytical method commonly used to identify marker compounds is TLC-Densitometry (Kartini & Azminah, 2012; Kim *et al.,* 2010; Bertrams *et al.,* 2013; Attarde *et al.,* 2017). Identification and quantitation of extracts in polypharmacy herbal medicines is a challenge. Therefore, developing and validating the TLC-Densitometry method are essential to obtain a simple and fast procedure applied to a quality control laboratory. The research that has been carried out is limited to determining marker compounds in single herbal plants and has not been applied to the formulation of herbal medicinal preparations using the TLC-Densitometry method (Abdullah *et al*., 2012; Hossain & Ismail, 2016; Kuppusamy *et al*., 2017). Several analytical methods for determining luteolin, quercetin, apigenin, and sinensetin have been developed using the HPLC method (Kuppusamy *et al*., 2017; Grek *et al.,* 2019; Ghiasian *et al.,* 2021). however, this instrument is expensive and require expertise specialized in instrument operation.

This study aimed to obtain an alternative method of TLC-densitometry which is cheaper and more straightforward for the simultaneous analysis of luteolin, quercetin, apigenin, and sinensetin in herbal medicines containing extracts of *Plantago major*, *Sonchus arvensis*, *Strobilanthes crispus*, and *Orthosiphon stamineus*.

MATERIALS AND METHODS

Materials

Luteolin standard (Sigma Aldrich), quercetin standard (Sigma Aldrich), apigenin standard (Sigma Aldrich), sinensetin standard (Sigma Aldrich), and Herbal medicinal products obtained from the pharmaceutical industry in Indonesia (PT. Interbat, Sidoarjo), ethanol (Merck, US/Canada), formic acid (Merck, US/Canada), chloroform (Merck, US/Canada), dichloromethane (Merck, US/Canada), acetone (Merck, US/Canada), acetonitrile (Merck, US/Canada).

Tools

Silica gel TLC plate 60 F_{254} aluminum sheet 20 x 20 cm (E, Merck, Darmstadt, Germany), Camag vessel 10 x 10 cm (Camag), TLC Scanner 3 with UV detector (Camag), winCATS software version 1.4.8.2012 (Camag).

Method

Preparation of standard solution

Standards (1.0 mg) were each dissolved in 10 mL of ethanol in a measuring flask. The stock standar solutions were then diluted to working solutions of 20 - 100 ppm.

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Table 1. Variation of mobile phase composition tested to obtain optimal conditions

No.	Mobile phase composition	Ref.
1.	Chloroform - dichloromethane - ethyl acetate (7:4:1, *v/v/v*)	(Kartini *et al.*, 2020)
2.	Isopropyl alcohol - n-butanol (5:5, *v/v*)	(Choudhary *et al.*, 2020)
3.	Toluene - ethyl acetate - formic acid (6:4:0,15, *v/v/v*)	(Attarde *et al.*, 2017)
4.	Chloroform - acetone - dichloromethane - acetonitrile - formic acid (6:2:2:0,05:0,05, *v/v/v/v/v*)	(Singh *et al.*, 2009)

Table 2. Variations in the composition of the mobile phase composition of a mixture of luteolin, quercetin, apigenin, and sinensetin

Mobile phase	Compound	Retardation factor (Rf)	Rs	Peak
1	Luteolin	0.19	0.94	*Fronting*
	Apigenin	3.34	-	
	Sinensetin	0.82	2.4	
2	Luteolin	0.34	-	*Fronting*
	Quercetin	0.42	1.2	
	Apigenin	0.51	1.4	
	Sinensetin	0.61	2.1	
3	Luteolin	0.18	-	*Fronting*
	Quercetin	0.21	-	
4	Luteolin	0.23	2.3	*Symmetric*
	Quercetin	0.24	2.2	
	Apigenin	0.34	1.8	
	Sinensetin	0.60	3.7	

Sample preparation

Herbal medicine was weighed 1 g and dissolved 20 ethanol in a measuring flask. The solution was sonicated for 30 minutes filtered through a 0.45 µm membrane filter and spotted on a TLC plate.

Preparation of mobile phase

For optimization of the mobile phase, the mobile phase is made in several compositions as shown in Table 1.

Optimization of the analytical conditions

Optimization of analytical conditions is done by changing the composition of the mobile phase, the saturation time of the vessel, and determining the maximum wavelength. The development method uses various mobile phase processes. The mobile phase was sonicated for 15 minutes before use. Parameters observed in selecting optimal conditions are the retardation factor (Rf), and the best resolution (Rs).

Stability test of the test solution

The stability test was carried out by dividing the standard solution and the sample into four different tubes. Each tube is labeled 0 hours, 4 hours, 8 hours, and 24 hours. The test solution was analyzed according to the time specified on the label. All tubes were stored at 4^{o}C.

RESULTS AND DISCUSSION

The data in Table 2 are based on experiments conducted on variations in the composition of the mobile phase.

The composition of the mobile phase 1 shows a resolution < 1.5 so that the peaks of luteolin, quercetin, apigenin, and sinensetin are not entirely separated from the peaks of impurities. In addition, the peak is also fronting so mobile phase 1 is not selected. The composition of phase 2 also showed poor resolution between luteolin, quercetin, apigenin, and sinensetin, namely < 1.5. This could affect the analysis results, so mobile phase 2 was not selected. The composition of mobile phase 3 was unable to completely separate luteolin, quercetin, apigenin, and sinensetin, which showed a resolution < 1.5 and had a fronting peak, so the composition of mobile phase 3 was not chosen. The composition of the mobile phase 4 shows the most optimal resolution of > 1.5 and an asymmetrical peak, so this mobile phase was selected and used for further analysis. The optimal conditions obtained in this study can be used to test the stability of the pre-validation stage method. The composition of the mobile phase 4 shows the most optimal resolution of > 1.5 and an asymmetrical peak, so this mobile phase was selected and used for further analysis. The optimal conditions obtained in this study can be used to test the stability of the pre-validation stage method.

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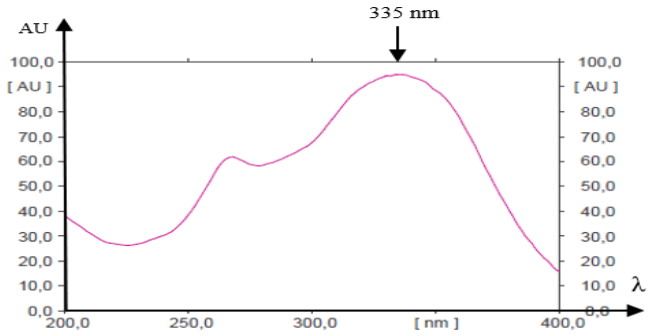

Figure 2. The Spectrum of apigenin compounds and its maximum wavelength using solvents chloroform - acetone - dichloromethane - acetonitrile - formic acid (6:2:2:0.05:0.05, *v/v/v/v/v*)

Table 3. Sample load optimization results

Concentration (ng/ µl)	Sample load (µl)	Compound	Area	Peak
12,18	4	Luteolin	6686.1	*Symmetrical*
		Quercetin	20802.7	
		Apigenin	12071.5	
		Sinensetin	9858.0	
12,18	8	Luteolin	7563.3	*Symmetrical*
		Quercetin	2187.7	
		Apigenin	1398.3	
		Sinensetin	9987.0	
12,18	14	Luteolin	10987.3	*Fronting*
		Quercetin	26234.6	
		Apigenin	16345.3	
		Sinensetin	13345.6	

The maximum wavelength of the selected analysis was observed from the peak, which gave the largest area value in the spectra of luteolin, quercetin, apigenin, and sinensetin. Besides, it did not cause interference peaks that could affect the analysis results of luteolin, quercetin, apigenin, and sinensetin compounds. At 335 nm, the maximum wavelength of apigenin compounds produced by high peaks of luteolin, quercetin, apigenin, and sinensetin and the resulting interference peak is very small, so the selected wavelength used is 335 nm (Figure 2).

Complete saturation of the vessel is required so that the elution and separation process can run smoothly good. The results of the sample load optimization are presented in Table 3. Based on the results obtained, the sample loads that give a symmetrical peak shape are 4 µl and 8 µl. At a sample load of 14 µl, the peak experienced fronting. This could be because the injection volume is too large. The optimal separation in thin-layer chromatography will be obtained if the sample is spotted with the smallest and narrowest possible spot size. As in other chromatographic procedures, the resolution will decrease if too many samples are used (Chun *et al.*, 2020).

The saturation time of the vessel has been optimized in this study. The optimization results are listed in Table 4. From the optimization results, it is found that the saturation time of the vessel can affect Rf. The optimal vessel saturation time in this study is at least 30 minutes because the area and Rf are stable after 30 minutes.

Table 4. Optimization of apigenin saturation time

Saturation time (minutes)	Area	Rf
15	956,6	0,19
30	1098,8	0,23
60	1095,0	0,23

Stability of the test solution

A stability test on standard solutions and samples is one of the tests carried out at the pre-validation stage. This stability test is carried out to evaluate the stability of the test solution at a certain storage time. Parameters observed in the selection of optimal conditions are the retardation factor (Rf), and the best resolution (Rs); a good Rf value indicates a good separation is in the range of 0.2-0.8, and a good separation if the Rs value meets the requirements, namely > 1.5 (AOAC, 2015). The results of the stability test of the test solution at several times of measurements can be seen in Table 5 and Table 6.

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Table 5. The results of stability tests of standard solutions in the range 0 - 24 hours

Observation Time (hours)	Standard	Average Area	Area Difference (%)	Rf	Difference Rf (%)
0	Luteolin	76861	-	0.23	-
	Quercetin	218027	-	0.24	-
	Apigenin	13071.5	-	0.34	-
	Sinensetin	9958	-	0.6	-
4	Luteolin	7543.2	1.86	0.23	-
	Quercetin	21513.5	1.33	0.24	-
	Apigenin	13042.8	0.22	0.34	-
	Sinensetin	9843.4	1.15	0.61	1.64
8	Luteolin	7435.9	3.26	0.23	-
	Quercetin	20978.1	2.49	0.25	4.00
	Apigenin	12878.2	3.78	0.35	2.86
	Sinensetin	9634.7	1.48	0.62	3.23
24	Luteolin	6928.9	9.85	0.22	4.55
	Quercetin	19913.3	8.67	0.24	-
	Apigenin	11976.3	8.38	0.34	-
	Sinensetin	8967.9	9.94	0.61	1.64

Table 6. The results of stability tests of sample solutions in the range 0 - 24 hours

Observation Time (hours)	Sample	Average Area	Area Difference (%)	Rf	Difference Rf (%)
0	Luteolin	8542.2	-	0.23	-
	Quercetin	7128.3	-	0.24	-
	Apigenin	7893.4	-	0.34	-
	Sinensetin	19077	-	0.6	-
4	Luteolin	8512.1	0.35	0.23	-
	Quercetin	7098.3	0.42	0.24	-
	Apigenin	7856.9	0.46	0.34	-
	Sinensetin	18893.5	0.97	0.6	-
8	Luteolin	8467.9	0.88	0.23	-
	Quercetin	6983.2	2.08	0.25	4.00
	Apigenin	7398.4	6.69	0.35	2.86
	Sinensetin	18349.1	3.97	0.61	1.64
24	Luteolin	8119.3	5.21	0.22	4.55
	Quercetin	6723.3	6.02	0.24	-
	Apigenin	7239.9	9.03	0.34	-
	Sinensetin	18122.1	5.27	0.62	3.23

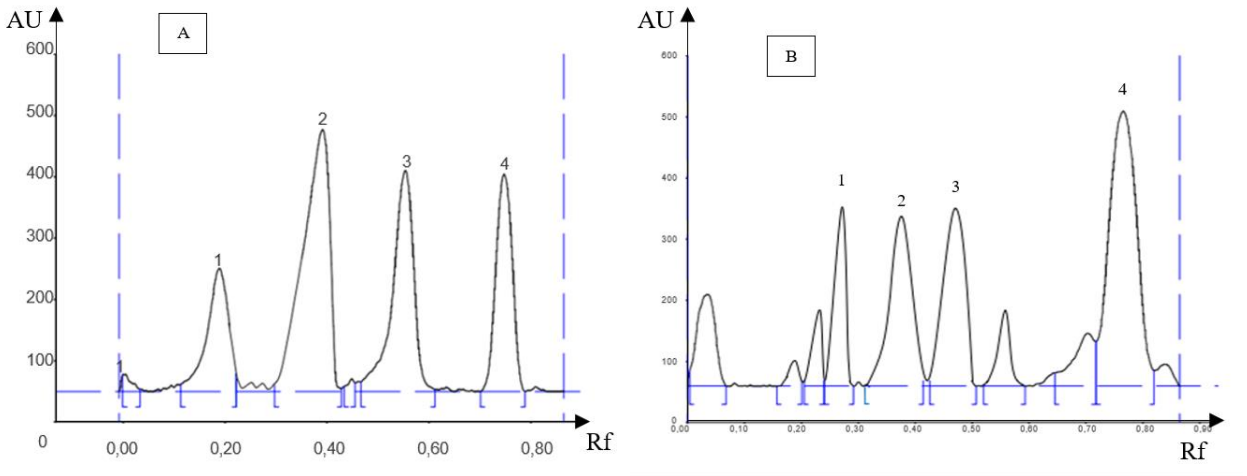

Figure 3. Chromatogram of standard (1) luteolin (2) quercetin (3) apigenin and (4) sinensetin (A) and herbal drug sample (B) with mobile phase composition of chloroform : acetone : dichloromethane : acetonitrile : formic acid (6 : 2 : 2 : 0.05 : 0.05 *v/v/v/v/v*)

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Based on the data obtained, the test solution is known to be unstable after 8 hours of storage. Then an analysis was carried out using SPSS to determine whether there was a significant difference between the area and factor retardation at each observation time. The data obtained showed a normal and homogeneous distribution so two-way ANOVA tested it. The results of the analysis show that there are significant differences in the area mean and Rf at each time of observation. This indicates that the longer the standard solution is stored, the lower the concentration will be (Figure 3).

CONCLUSION

The optimal conditions obtained in this study for philanthropic analysis were the composition of the mobile phase chloroform: acetone: dichloromethane: acetonitrile: formic acid (6 : 2 : 2 : 0.05 : 0.05 *v/v/v/v/v*), with saturation time 30 minutes, wavelength analysis 335 nm. The stability of the solution was tested in this study as a pre-validation stage. The stability of the test solution decreased with storage for a certain time, indicated by a decrease in area and a retardation shift in the chromatogram. It is recommended that the test solution be made fresh when the analysis is carried out. The optimal conditions obtained can be continued with method validation for further research.

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AUTHOR CONTRIBUTIONS

Conceptualization, M. H., M. Y., R. P.; Methodology, M. H., M. Y.; Software, M. H.; Validation, M. H., M. Y., R. P.; Formal Analysis, M. H., M. Y., R. P.; Investigation, M. H., M. Y., R. P.; Resources, M. H., M. Y., R. P.; Data Curation, M. H.; Writing - Original Draft, M. H.; Writing - Review & Editing, M. H., M. Y., R. P.; Visualization, M. H., M. Y., R. P.; Supervision, M. H., M. Y., R. P.; Project Administration, M. H., M. Y., R. P.; Funding Acquisition, M. H., M. Y., R. P.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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