INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
23–0136/2023/30–2–145–153
DOI: 10.17957/IJAB/15.2069
http://www.fspublishers.org

Full Length Article

Development and Validation of Simultaneous Analysis Method of Brazilin and 6-Gingerol in the Combined Extracts of Sappan Wood (*Caesalpinia sappan*) and Ginger Rhizome (*Zingiber officinale*) Using RP-HPLC

Debora Ekaputri Gondokusumo[1*], Abdul Mun'im[1], Delly Ramadon[1], Febrina Amelia Saputri[1], Rizka Soraya[2] and Hilli Silfian[2]

[1]*Faculty of Pharmacy, Universitas Indonesia, Depok 16424, West Java, Indonesia*
[2]*Department of Research and Development, PT Phytochemindo Reksa, Bogor, West Java-Indonesia*
[*]For correspondence: Debora.e@ui.ac.id; Debora.gondokusumo@gmail.com
Received 25 April 2023; Accepted 05 July 023; Published 10 August 2023

Abstract

Brazilin and 6-gingerol, bioactive compounds found in sappan wood (*Caesalpinia sappan* L.) and ginger rhizome (*Zingiber officinale* Rosc.) extracts, offer various potential pharmacological benefits. The combination of these extracts has shown promising antithrombotic and antihyperlipidemic properties, suggesting the potential use of this combination in herbal products. Quantitative analysis is required to ensure the quality control of herbal products. However, the simultaneous quantification of Brazilin and 6-gingerol using an HPLC method is currently unavailable. To address this gap, this study aimed to develop and validate a simultaneous quantification method for Brazilin and 6-gingerol in combined extracts of sappan wood and ginger using RP-HPLC. Chromatographic analysis was performed using a reverse-phase C18 Inertsil ODS3 column (4.5 × 250 mm; particle size 5 μm) at room temperature, with detection at 282 nm using a UV detector. The mobile phase consisted of acetonitrile (A) and water containing 0.1% acetic acid (B), with gradient elution optimized as follows: 0–12 min 15% A: 85% B; 12–16 min 30% A: 70% B; 16–21 min 45% A: 55% B; 21–35 min 60% A: 40% B, at a flow rate of 1 mL/min with an injected volume of 20 μL. The developed method demonstrated acceptable system suitability (peak resolution, tailing factor, theoretical plate number, selectivity) and validated parameters. Both Brazilin and 6-gingerol displayed linear calibration curves ($R^2 > 0.999$), high intraday and interday precision (%RSD < 2%) and accuracy (93–106%). This study successfully developed and validated a rapid RP-HPLC method for simultaneous quantifying Brazilin and 6-gingerol in combined extracts of sappan wood and ginger rhizome. This method provides a reliable means for quality control analysis and could facilitate the development of herbal products incorporating these bioactive compounds.

Keywords: RP-HPLC; *Caesalpinia sappan*; Simultaneous quantification; *Zingiber officinale*; Method validation; Herbal extracts

Introduction

Sappan wood (*Caesalpinia sappan* L.) and ginger rhizome (*Zingiber officinale* Rosc.) are herbal plants that are commonly used as traditional medicines. Ginger rhizome extract, known for its main active compound 6-gingerol (Zhong *et al.* 2022), has been reported to have various biological capabilities such as anti-platelet (Nurtjahja-Tjendraputra *et al.* 2003), antioxidant, antitussive, hypotensive, analgesic, anti-inflammatory, anti-cancer and anti-gastric ulcer (Saputri *et al.* 2017). Sappan wood extract, containing Brazilin as its main compound (Yan *et al.* 2015), has been studied for its anti-inflammatory, antibacterial, hypoglycemic, anti-allergic, antioxidant, hepatoprotective (Nirmal *et al.* 2015), cytotoxic (Haryanti *et al.* 2018) and neuroprotective properties (Wan *et al.* 2019).

In Indonesia, the combination of sappan wood and ginger rhizome is commonly used in traditional medicinal drinks and these drinks are believed to provide various health benefits such as lower blood pressure (Sari and Suhartati 2016; Setyowati *et al.* 2023). *In-vivo* studies have shown that the combination of sappan wood and ginger rhizome extracts containing 6-gingerol and Brazilin exhibits an antithrombotic effect (Saputri *et al.* 2017) and can lower cholesterol and triglyceride level in hyperlipidemic-induced rats (Izzatinisa 2022). Although the number of available pharmacological studies is limited, the combination of ginger and sappan extracts shows potential to be developed

To cite this paper: Gondokusumo DE, A Mun'im, D Ramadon, FA Saputri, R Soraya, H Silfian (202x). Development and validation of simultaneous analysis method of brazilin and 6-gingerol in the combined extracts of sappan wood (*Caesalpinia sappan*) and ginger rhizome (*Zingiber officinale*) using RP-HPLC. *Intl J Agric Biol* 30:145–153

into herbal dosage forms with antithrombotic and antihyperlipidemic benefits.

Ensuring the quality, efficacy and safety of herbal medicines is crucial. One important aspect of quality control is the identification and analysis of bioactive compounds in extracts or herbal dosage forms, which can be achieved using accurate, rapid, sensitive and precise High-Pressure Liquid Chromatography (HPLC) (Chaudhari *et al*. 2020). Several quantification methods using HPLC have been developed for separate analyses of Brazilin and 6-gingerol. Theoretically, simultaneous quantification of Brazilin and 6-gingerol using Reversed Phase HPLC (RP-HPLC) is feasible. Both Brazilin (with a Log P of +1.3 and a Hydrophobic Index of 5.0) (Dapson and Bain 2015) and 6-gingerol (with a log P of +3.56 and is insoluble in water) (Ley-Martínez *et al*. 2022), are both semi-polar compounds and have similar wavelength detection at 282 nm in the UV-VIS spectrum (Nirmal *et al.* 2015; Cafino *et al*. 2016). Simultaneous analysis methods offer advantages over separate quantification, including time-saving, cost-effectiveness, higher throughput and simplification. However, there is currently a lack of references for the simultaneous quantification of Brazilin and 6-gingerol in extract samples or finished herbal products using RP-HPLC. In this research, a simultaneous quantification method for Brazilin and 6-gingerol in combined extracts of sappan wood and ginger rhizome using RP-HPLC has been developed and validated. The method developed in this study can serve as a standardization tool for herbal extracts containing Brazilin and 6-gingerol.

Materials and Method

Materials and extraction

Plant materials: The dried sliced ginger rhizome was collected from the local market in Malang and sappan wood was collected from the local market in Blora and both are in East Java, Indonesia. Sample authentication was conducted by the Research and Development Division of PT. Phytochemindo Reksa, Bogor, Indonesia using Thin Layer Chromatography (TLC) and comparing the samples to Brazilin and 6-gingerol markers. The samples were ground to a size of 3 mm using a grinder (Rong Tsong, China) and stored in a sealed container, protected from sunlight until they were used in the study.

Plants extraction: The Extraction process followed a high-pressure method, as described in the previous study (Hu *et al*. 2011) but with some modifications. In this research, the extraction was conducted simultaneously on the ginger rhizome and sappan wood. Two hundred grams of each ground ginger rhizome and ground sappan wood were immersed in one liter of 70% ethanol with low agitation at 230 ppm (IKA® RW 20 Digital Stirrer, Germany) at room temperature for 30 min. The mixture of ground samples and solvent were then put in a filter bag and placed into a lab-scale high-pressure extraction machine (Hydrotech, Indonesia). A pressure of 200 bar was applied for 30 min at room temperature. After the compression period, the system was decompressed to normal atmospheric pressure and the filtrate was discharged through the bottom valve and collected in a container. The filtrate was further evaporated at 45°C for three hours using a vacuum rotary evaporator (Buchi, Switzerland) with a pressure of -50 cmHg until a thick liquid of extract was obtained. The thick liquid extract was collected and transferred to dark glass vials, completely covered with aluminum foil and stored at 4°C in the refrigerator until analysis.

Reagents: Brazilin and 6-gingerol standards were purchased from Sigma, United States, and used for the system suitability test and validation of the method. Methanol, Acetic Acid and Acetonitrile (ACN) of HPLC grade were purchased from Sigma, United States and used as the mobile phase.

Instrument: During this research, an HPLC system (Waters™ Alliance e2695) equipped with a quaternary low-pressure mixing pump, autosampler and Photodiode Array (PDA) detection system (PDA 2998 Waters™) was used. This system used Empower3™ software to control the instrument parameters. A calibrated weighing balance (Sartorius BSA2245-CW, Germany) was used to accurately weigh all the standards.

Selections of chromatographic conditions

The development of a simultaneous quantification method of Brazilin and 6-gingerol levels was performed based on previously established analytical methods for separate quantification of Brazilin (Settharaksa *et al.* 2019; Wan *et al.* 2019) and 6-gingerol (Sharif and Bennett 2016; Simon-Brown *et al.* 2016). These previously developed methods shared similar conditions such as the mobile phase, detection wavelength, temperature, and column type, but differed in mobile phase ratios: a more polar phase for Brazilin and a more non-polar phase for 6-gingerol. In this study, optimization of various mobile phase ratios and gradient adjustments was performed to achieve proper separation, system suitability, and good validation, accuracy, and precision. Chromatographic separation of Brazilin and 6-gingerol was developed using a reverse-phase Inertsil ODS3 C-18 column (250 mm × 4.6 mm; 5 μm particle size). The mobile phase consisted of a mixture of Acetonitrile (A) as the organic phase and HPLC-grade water containing 0.1% w/v acetic acid (B) as the aqueous phase, employing a gradient elution mode with the following profile: 0–12 min 15% A: 85% B; 12–16 min 30% A: 70% B; 16–21 min 45% A: 55% B; 21–35 min 60% A: 40% B. The mobile phase was filtered through a 0.45 μm membrane filter and the flow rate was set at 1 mL/min. The injection volume of 20 μL was used. The column temperature was maintained at room temperature, the UV detection wavelength was set at 282 nm and the total run time was targeted to be less than 35 min.

Standard and extract sample solution preparation

Primary standard solutions of Brazilin and 6-gingerol at a concentration of 1000 μg /mL were prepared by dissolving 5 mg of standard Brazilin in 5 mL methanol in a volumetric flask and similarly, 5 mg of standard 6-gingerol was dissolved in 5 mL methanol in another volumetric flask. These standard solutions were stored in dark glass vials, completely covered by aluminium foil and stored in a refrigerator at 4°C until analysis. For the sample preparation, 50 mg of the thick extract obtained from the simultaneous extraction was dissolved in methanol (25 mL). The extract sample solution was placed in an ultrasonic bath (ROHS, China) with a frequency of 40KHz for 15 min. All extract sample solutions were filtered through a 0.45 μm micropores membrane and stored in dark vials covered with aluminium foil. These vials were stored in a refrigerator until further analysis.

System suitability test

The system suitability procedures and acceptance criteria were performed in accordance with the guidelines provided by USP and AOAC (USP-NF 2022; AOAC 2012) to ensure that the chromatographic system was appropriate for the desired analysis. To conduct the test, three replicate injections of a standard solution containing 100 μg/mL of Brazilin and 100 μg/mL of 6-gingerol were used. Additionally, the extract sample solutions were also injected. System suitability parameters, including peak resolution, tailing factor, theoretical plate number, and selectivity, were determined using the Empower3™ software.

Method validation

The validation parameters of the proposed method followed guidelines provided by ICH (International Conference on Harmonization 2022); this includes linearity, sensitivity, specificity, interday and intraday precision, accuracy and recovery.

Linearity: A linearity study was conducted by preparing calibration curves using Brazilin and 6-gingerol standard solutions at various concentrations. The primary standard solution was serially diluted to obtain concentrations of 25, 50, 75, 100 and 150 μg/mL for both Brazilin and 6-gingerol standard solutions. Each standard solution was then injected three times using the developed analytical method. The resulting peak area was then plotted against the corresponding concentrations to construct calibration curves for Brazilin and 6-gingerol using linear regression. The linearity of the calibration curves was assessed by evaluating the correlation coefficient (R^2) values.

Sensitivity: The sensitivity of the analysis method was determined from the calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ) shown below (International Conference on Harmonization 2022).

$$LOD = \frac{3.3\ Sy}{S}$$

$$LOQ = \frac{10\ Sy}{S}$$

$$Sy = \sqrt{\frac{\sum(y_i - \bar{y})^2}{n-2}}$$

S is the slope of the calibration curve.

Accuracy/Percent recovery: In this study, the accuracy test was conducted using a spiking study: adding a standard solution of known concentration to a sample solution. The accuracy was reported in the form of a recovery percentage of several standards added (addition/spike) to the test sample with a total sample of a minimum of three repetition series of three standard solution concentration levels, resulting in a minimum of nine test points (International Conference on Harmonization 2022). Three standard solutions added to the sample solution were 25, 37.5 and 50 μg/mL and% recovery is calculated using the equation below.

$$\%Recovery = \frac{Cf - Cu}{Ca}\, x\ 100\%$$

Cf is the total concentration of the sample solution after the addition of the standard, *Cu* is the concentration of the analyte in the sample solution and *Ca* is the concentration of the standard solution (AOAC 2012).

Specificity: In this study, the chromatogram of the extract sample solution containing Brazilin, 6-gingerol, and other unspecified compounds was compared to the chromatogram of mixed standard solutions of Brazilin and 6-gingerol. The presence of other unidentified impurities in the extract should not interfere with the peak area of Brazilin and 6-gingerol. The results between the chromatogram of the standard solution and the extract sample solution must show identical peaks with the same retention time. Additionally, the chromatogram of the blank solution should exhibit no visible disturbances in the area around the retention time. A spiking study was also conducted, involving the addition of a standard solution to the extract sample solution (Raut and Shaji 2021). The chromatography results should only display the enlarged peak areas and should not show any additional peaks in the retention areas of Brazilin and 6-gingerol.

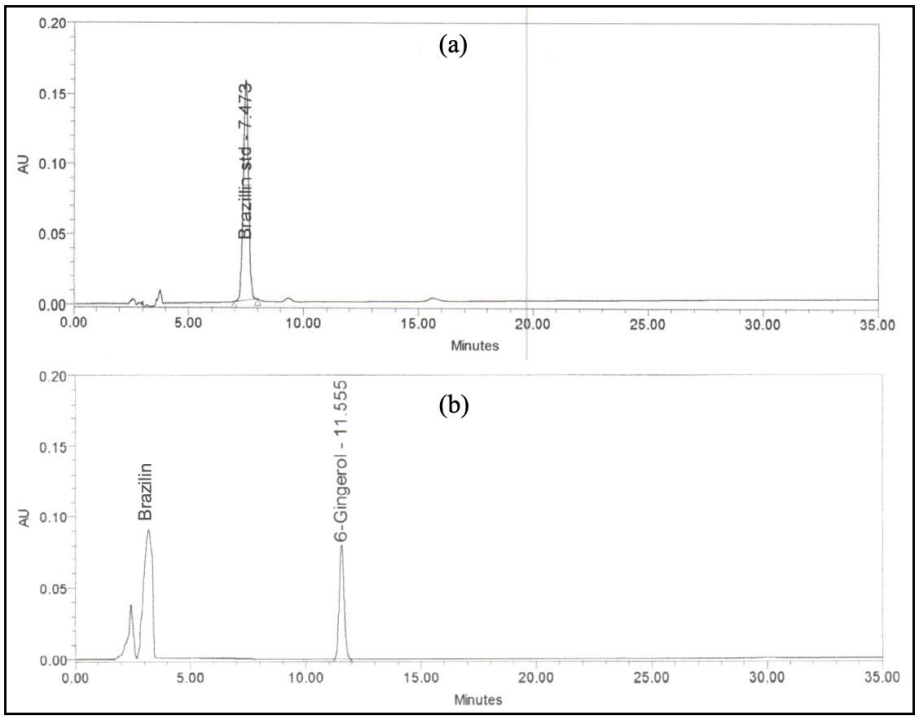

Fig. 1: Chromatogram results of 100 μg/mL Brazilin and 6-gingerol in mixed standard solutions. (**a**) Using isocratic elution mode in a more polar mobile phase (20% A: 80% B), only the Brazilin peak was observed, while the 6-gingerol peak was absent. (**b**) In isocratic elution mode (50% A: 50% B), a peak corresponding to 6-gingerol was visible, along with a shoulder peak of Brazilin

Precision: Precision calculations were assessed from two test categories: repeatability (inter-day precision) and intermediate/intraday precision. Repeatability (inter-day precision) was assessed using a minimum of 3 concentrations with three replicates each within the range of use of the analytical method or 6 data with 100% concentration on the same day (International Conference on Harmonization 2022). All measurements are carried out by the same analyst in the same test laboratory, with test intervals that are close enough.

The intermediate/interday precision test was performed on different days, environmental conditions, analysts, or equipment to know the effect of different environments on the performance of the analytical method. This research assessed intraday and intermediate precision by injecting three standard solution concentration levels: 25, 100 and 150 μg/mL (with 3 replicates each) across three different days.

Precision was characterized by the relative standard deviation (RSD), which should be within the specification limits of < 2% (USP-NF 2022). RSD was calculated using the equation:

$$\%RSD = \frac{100}{\bar{y}}\ x\ \sqrt{\frac{\sum(y_i - \bar{y})^2}{n-1}}$$

Statistical Analysis

The % RSD was calculated to assess the accuracy and precision of the method, with the specification limits set at < 2%. Linearity was determined using linear regression analysis. Both ANOVA and linear regression analyses were calculated using SPSS Statistics v. 25 software, with a confidence level of 95%.

Results

Method Development

In this study, various mobile phase ratios and gradient adjustments were optimized through several trials. The chromatogram result of Brazilin and 6-gingerol standard solution in a more polar mobile phase (20% A – 80% B, Fig. 1a) showed only the Brazilin peak while the 6-gingerol peak did not appear during the 35 min chromatogram run. The ratio was then changed to a more non-polar phase (50% A – 50% B, Fig. 1b). The chromatogram result showed a sharp 6-gingerol peak but only a broad Brazilin peak. Since the isocratic elution methods (20% A – 80% B and 50% A – 50% B) could not produce sharp peaks for both Brazilin and 6-gingerol, the elution was then changed to gradient mode.

A gradient elution mode (0–8 min 15% A: 85% B; 8–11 min 30% A: 70% B; 11–15 min 45% A: 55% B; 15–30

Table 1: System Suitability Test Parameters and Values in Standard Solutions

Analytes	Retention time (min)	Resolution (R)	Theoretical Plate (N)	Tailing Factor (T)	Selectivity (α)
Brazilin	12.68 ± 0.04	-	5539.01 ± 94.71	0.96 ± 0.01	-
%RSD	0.31%		1.71%	1.10%	
6-Gingerol	29.02 ± 0.01	26.50 ± 0.11	45385.84 ± 349.79	1.07 ± 0.01	2.34 ± 0.01
%RSD	0.28%	0.40%	0.77%	0.70%	0.35%

Expressed in mean value ± SD (n = 6)

Table 2: System Suitability Test Parameters and Values in Extract Sample Solutions

Analytes	Retention time (min)	Resolution (R)	Theoretical Plate (N)	Tailing Factor (T)	Selectivity (α)
Unknown peak 1	11.48 ± 0.4		5235.24 ± 236.88	1.01 ± 0.03	
%RSD	3.50%		4.52%	2.85%	
Brazilin	12.86 ± 0.41	1.99 ± 0.09	4868.57 ± 109.68	0.96 ± 0.02	1.13 ± 0.01
%RSD	3.15%	4.55%	2.25%	2.18%	0.51%
Unknown Peak 2	14.44 ± 0.43	2,03 ± 0.01	5294.62 ± 296.58	1,03 ± 0.04	1.13 ± 0.01
%RSD	3.01%	0.28%	5.60%	3.69%	0.01%
6-Gingerol	29.09 ± 0.05	20,60 ± 0.76	37317.08 ± 1629.02	1.07 ± 0.01	2.05 ± 0.06
%RSD	0.17%	3.68%	4.37%	0.54%	3.13%

Expressed in mean value ± SD (n = 3)

Table 3: % Recovery at different concentrations of added standard solution

Analytes	Nominal standard concentration (µg/mL)	Actual added concentration* (µg/mL) + SD	% recovery* + SD	%RSD
Brazilin	25	25.16 ± 0.16	100.63 ± 0.63%	0.63
	37.5	39.9 ± 0.10	106.41 ± 0.27%	0.25
	50	53.05 ± 0.09	106.09 ± 0.18%	0.17
6-Gingerol	25	23.44 ± 0.33	93.76 ± 1.30%	1.39
	37.5	39.01 ± 0.19	104.03 ± 0.51%	0.49
	50	49.82 ± 0.14	99.64 ± 0.28%	0.28

*mean value (n = 3)

Table 4: Accuracy and Intraday Precision at different concentration of validated method

Analytes	Nominal standard concentration (µg/mL)	Actual concentration* (µg/mL) + SD	% Accuracy* + SD	Intraday Precision (%RSD)
Brazilin	25	24.52 ± 0.26	96.90 ± 1.04%	1.07
	75	74.92 ± 0.06	99.9 ± 0.08%	0.08
	150	147.99 ± 0.23	98.66 ± 0.15%	0.15
6-Gingerol	25	25.41 ± 0.03	101.63 ± 0.11%	0.11
	75	74.26 ± 0.31	99.01 ± 0.42%	0.42
	150	150.31 ± 0.66	100.20 ± 0.44%	0.44

*mean value (n = 3)

min 60% A: 40% B) successfully displayed both peaks of Brazilin and 6-gingerol with good resolutions and selectivity using standard solution (Fig. 2a). However, this method failed to achieve efficient chromatographic separation between Brazilin and the adjacent peaks in the extract sample solution (Fig. 2b). Therefore, the gradient method then was modified to use a slightly longer elution time and a higher ratio of the aqueous phase (0–12 min 15% A: 85% B; 12–16 min 30% A: 70% B; 16–21 min 45% A: 55% B; 21–35 min 60% A: 40% B). With this modified method, the retention time for Brazilin and 6-gingerol in standard solution was observed at 12.7 and 29.0 min, respectively (Fig. 3a). This method also achieved efficient separation between Brazilin and the adjacent unknown peaks in the extract sample solution (Fig. 3b).

System suitability

The resolution between Brazilin and 6-gingerol peaks in the standard solution chromatogram was reported as 25.65 (Table 1). Furthermore, the chromatogram of the extract sample solution showed average peak resolution values greater than 1.99 between Brazilin, 6-gingerol and other adjacent peaks (Table 2). Other parameters such as the tailing factor, theoretical plate number, and selectivity were found to be 0.96–1.07, 4868–45385 and 1.13–2.34, respectively, in both the standard solution and extract sample solution (Table 1 and 2).

Method validation

Linearity: The regression equation of the Calibration curve for Brazilin, with a concentration range between 25–150 µg/mL, was y = 27493.5x -56238.7, yielding a correlation coefficient (R^2) of 0.9997. For 6-gingerol, the regression equation was y = 11285.2x + 4207.8 with a correlation coefficient (R^2) of 0.9998. Both regression models had p values < 0.000 at a confidence level of 95%.

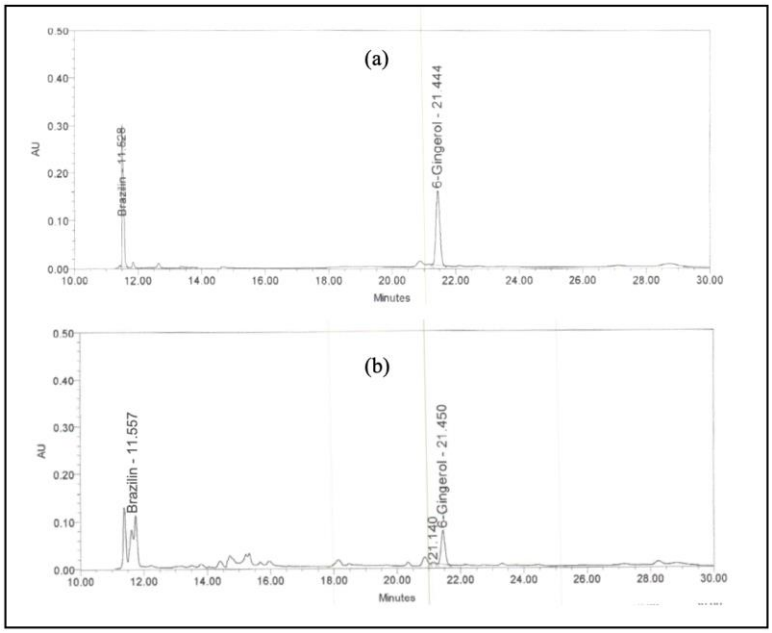

Fig. 2: Chromatogram result of Brazilin and 6-gingerol (**a**) in mixed standard solution, 100 μg/mL each and (**b**) in the extract sample solution using gradient elution mode (0-8 min 15% A: 85% B; 8-11 min 30% A: 70% B; 11-15 min 45% A: 55% B; 15-30 min 60% A: 40% B)

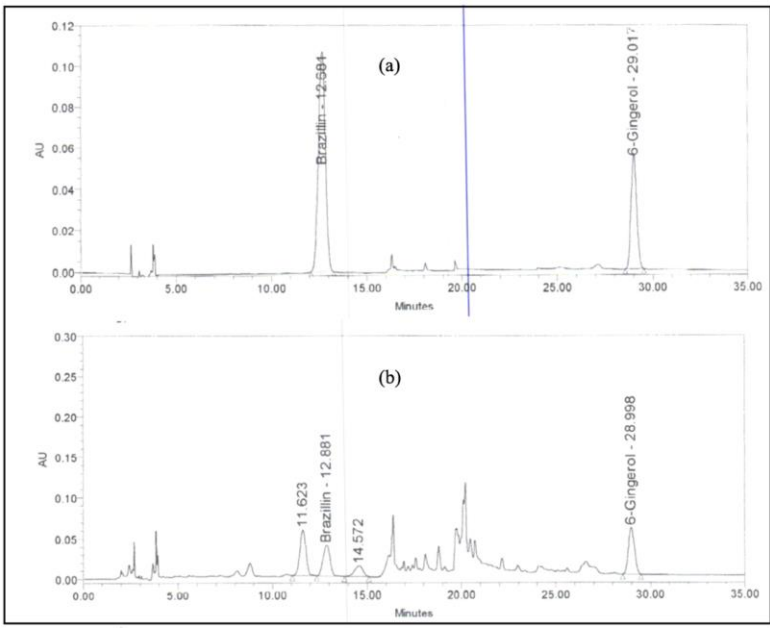

Fig. 3: Chromatogram of Brazilin and 6-gingerol (**a**) in mixed standard solutions, 100 μg/mL each and (**b**) in the extract sample solution using gradient elution mode (0–12 min 15% A: 85% B; 12–16 min 30% A: 70% B; 16–21 min 45% A: 55% B; 21–35 min 60% A: 40% B)

Sensitivity: LOD and LOQ of this analysis method for Brazilin were 3.31 and 10.02 μg/mL, respectively, while LOD and LOQ for 6-gingerol were 2.67 and 8.08 μg/mL, respectively.

Accuracy: The accuracy level for this method was demonstrated by the percent recovery of the spiked sample with the addition of 25, 37.5 and 50 μg/mL Brazilin and 6-gingerol standard solutions. % recovery for 6-gingerol was 93–99% while for Brazilin was 100–106% (Table 3). RSD% was found to be 0.17–1.39% (Table 3).

Specificity: Results of the chromatogram of the standard solution (Fig. 4b) and the extract sample solution (Fig. 4c)

Table 5: Inter-day Precision at different concentrations of a validated method

		Brazilin	6-Gingerol	Brazilin	6-Gingerol	Brazilin	6-Gingerol
	Nominal Standard Conc.	25 μg/mL		100 μg/mL		150 μg/mL	
Day 1	Actual Conc + SD (n = 3)	24.22 ± 0.26	25.41 ± 0.03	103.66 ± 0.31	99.98 ± 0.16	147.99 ± 0.23	150.31 ± 0.66
	% Accuracy	96.90 ± 1.04	101.63 ± 0.11	103.66 ± 0.31	99.98 ± 0.16	98.66 ± 0.15	100.20 ± 0.44
	Intraday Precision (%RSD) (n = 3)	1.07%	0.11%	0.30%	0.16%	0.15%	0.44%
Day 2	Actual Conc + SD (n = 3)	23.89 ± 0.16	23.93 ± 0.32	97.26 ± 0.11	98.18 ± 0.10	145.88 ± 0.69	146.94 ± 0.42
	% Accuracy	96.20 ± 0.64	101.63 ± 0.11	97.26 ± 0.11	99.01 ± 0.42	97.26 ± 0.46	97.96 ± 0.28
	Intraday Precision (%RSD) (n = 3)	0.67%	0.11%	0.11%	0.42%	0.47%	0.29%
Day 3	Actual Conc + SD (n = 3)	24.47 ± 0.14	24.35 ± 0.44	103.43 ± 1.04	98.2 ± 0.56	154.24 ± 0.35	150.75 ± 0.70
	% Accuracy	97.89 ± 0.57%	97.39 ± 1.75%	103.43 ± 1.04	98.2 ± 0.56	102.82 ± 0.24%	98.20 ± 0.56%
	Intraday Precision (%RSD) (n = 3)	0.58%	1.80%	1.01%	0.57%	0.23%	0.57%
	Interday Precision (%RSD)	1.03%	2.91%	3.15%	0.95%	2.53%	1.26%

*mean value (n = 3)

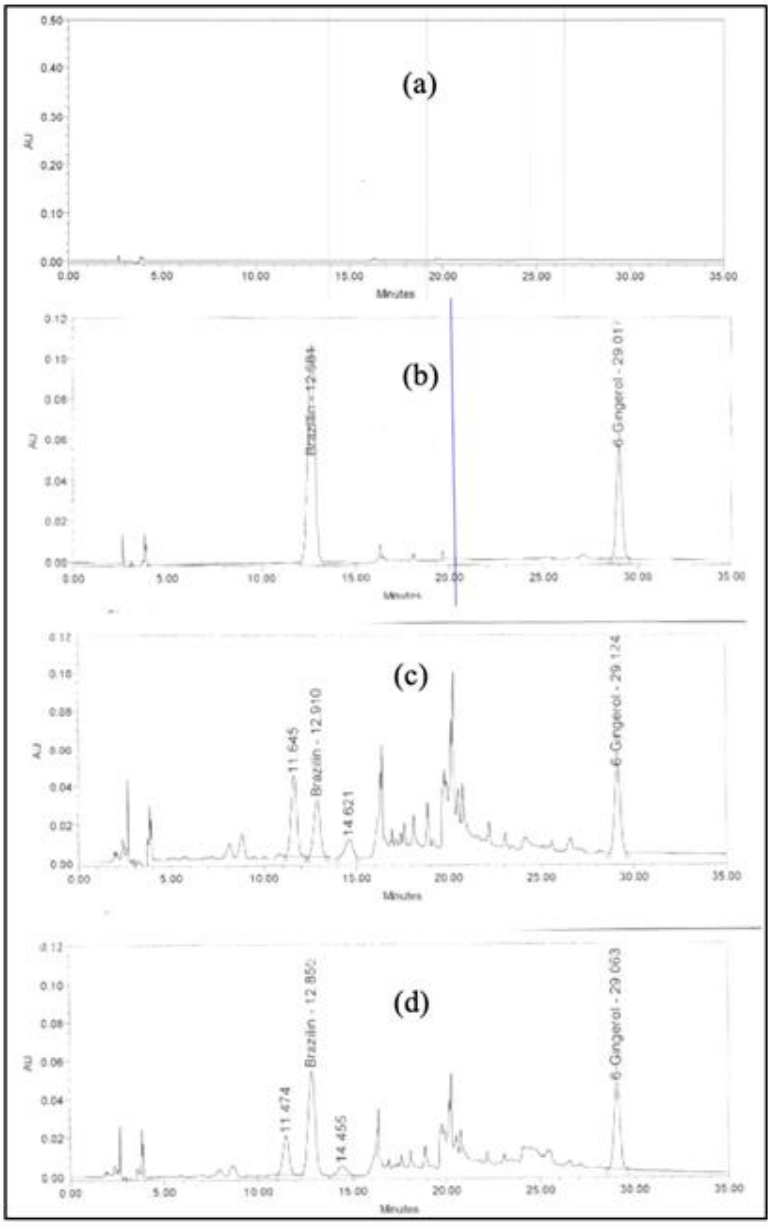

Fig. 4: Chromatogram of the blank sample (**a**), standard solution (**b**), extract sample solution without the addition of standard (**c**) and extract sample solution with the addition of standard (**d**)

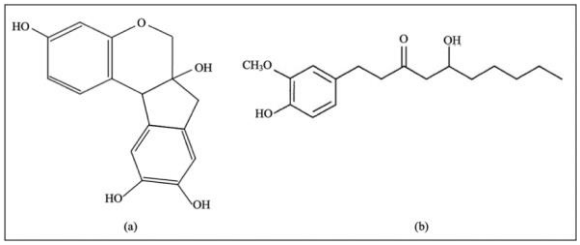

Fig. 5: Chemical structural representation of (**a**) Brazilin and (**b**) 6-gingerol

showed identical peaks with the same retention time. Additionally, there were no visible disturbances in the chromatogram of the blank solution in the area around the retention time of Brazilin and 6-gingerol (Fig. 4a). Furthermore, the addition of the standard solution to the sample solution was also performed, and the chromatography results showed no additional peaks in the retention areas of Brazilin and 6-gingerol (Fig. 4d).

Intraday and Interday Precision: Repeatability (intraday precision) results were presented in Table 4, with % RSD values ranging from 0.08 to 1.07% for Brazilin and 0.11 to 0.44% for 6-gingerol. Interday injections demonstrated % RSD values of 1.03–3.15% for Brazilin and 0.95–2.91% for 6-gingerol (Table 5).

Discussion

The development of an RP-HPLC method for the simultaneous quantification of Brazilin and 6-gingerol began with the optimization of mobile phase ratios using isocratic elution while other conditions were maintained constant. The criteria for selecting the optimum mobile phase ratio were as follows: 1) meeting the acceptance criteria for system suitability in terms of peak resolution between the adjacent peaks, tailing factor, theoretical plate number and selectivity; 2) enabling the elution of Brazilin and 6-gingerol with sharp peaks and 3) achieving the shortest retention time for Brazilin and 6-Gingerol in both standard and extract sample solutions (Cafino *et al*. 2016). However, the isocratic elution mode did not meet these criteria, leading to a change in the mobile phase to the gradient mode/ the mobile phase then was changed to gradient mode. Brazilin (Fig. 5a) (log p +1.3) is more polar than 6-gingerol (Fig. 5b) (log p is +3.56) (Dapson and Bain 2015; Ley-Martínez *et al*. 2022); Brazilin is less hydrophobic and has less affinity to the reversed-phase column than 6-gingerol. During gradient elution mode (0–12 min 15% A: 85% B; 12–16 min 30% A: 70% B; 16–21 min 45% A: 55% B; 21–35 min 60% A: 40% B), as Brazilin has more affinity to the more polar mobile phase, it was eluted first. 6-gingerol has more affinity to the column; therefore, the strength of hydrophobic interaction needed to be reduced to elute 6-gingerol faster. This was done by adjusting the mobile phase composition to a more organic phase during the later stage of gradient elution mode. The proposed gradient mode successfully eluted both Brazilin and 6-gingerol, with retention times of 12.7 and 29.0 min, respectively (Fig. 3a). This simultaneous analysis within 30 min simplifies routine quality control procedures and makes the overall process time-efficient and cost-effective.

The proposed method met the required criteria for system suitability. The peak resolution between Brazilin and 6-gingerol in standard solutions was 26.5 on average (Table 1), while the average peak resolution between Brazilin and adjacent peaks in extract samples was 1.99 (Table 2). A resolution of at least 1.5 is necessary for good peak separation (Papadoyannis and Samanidou 2004; AOAC 2012). The extract sample solution was expected to show more peaks due to the presence of other compounds besides Brazilin and 6-gingerol. A suitable method should be not only able to separate the markers but also be able to separate all other compounds present in the extracted sample. Other system suitability parameters, such as theoretical plate number (4868–45385), separation factor (α) values (1.13–2.34) and tailing factor (A_S) values of 0.96–1.07 demonstrated efficient column efficiency, good selectivity and symmetrical peaks. The criteria for a well-accepted theoretical plate number are > 2000, the tailing factor (A_S) value should be within the range of 0.8–1.8 and the separation factor (α) is > 1 (USP-NF 2022).

The calibration curves for both Brazilin and 6-gingerol standard solutions, with a concentration range between 25–150 μg/mL, showed linearity with correlation coefficient (R^2) values of 0.9997 for Brazilin and 0.9998 for 6-gingerol. The p-values of < 0.000 for both regression models were statistically significantly good fits for the data. The percent recovery for 6-gingerol ranged from 93 to 99% and for Brazilin it ranged from 100 to 106% (Table 3). The RSD values were 0.17–1.39%. The acceptance criteria for accuracy, represented by the percent recovery, in biological samples are at 90–110% (Papadoyannis and Samanidou 2004; AOAC 2012). Additionally, the relative standard deviation (RSD) should be lower than 2%. These results demonstrate that the proposed method was accurate (close to true values).

The limit of detection (LOD) and limit of quantification (LOQ) for Brazilin were 3.31 and 10.02 μg/mL, respectively, while for 6-gingerol, the LOD and LOQ were 2.67 and 8.08 μg/mL, respectively. These values indicate that the method was sensitive for effective detection and quantification of Brazilin and 6-gingerol in the concentration range between 25–150 μg/mL. The specificity study showed that the proposed method is specific to Brazilin and 6-gingerol. Intraday precision demonstrated % RSD values of 0.08–1.07% for Brazilin and 0.11–0.44% for 6-gingerol, while interday precision showed %RSD values of 1.03 to 3.15% for Brazilin and 0.95 to 2.91% for 6-gingerol. The method demonstrated repeatability and precision in both intraday and interday analyses.

Conclusion

The analytical method for simultaneous quantification of Brazilin and 6-gingerol in the extract sample was successfully developed and validated following ICH and USP guidelines. The system suitability test parameters, including resolution, selectivity, theoretical plate number, tailing factor, as well as validation parameters, met the acceptance criteria with good linearity, high selectivity, specificity, accuracy, and precision. Therefore, this developed and validated method can be applied for quality control analysis of Brazilin and 6-gingerol in the combined extracts of sappan wood and ginger rhizome. Further studies are needed to test this analysis method and quantify the level of Brazilin and 6-gingerol in herbal dosage forms containing extracts of sappan wood and ginger rhizome. These studies will provide valuable information on the content of Brazilin and 6-gingerol in the final products and ensure their quality and consistency.

Acknowledgments

The first author would like to express gratitude to PT Phytochemindo Reksa, who partially funded this research, and to Prof. Abdul Mun'im, M.Si., Apt, Mr. Delly Ramadon, M. Farm., Ph.D., Apt and Dr. Febrina Amelia Saputri, M.Farm., Apt from the Faculty of Pharmacy at Universitas Indonesia for their continuous support, guidance, insights, and expertise that assisted the completion of this research.

Author Contributions

DEG planned the experiments, made write up, statistically analysed the data and made graphs; AM, DR and FAS provided guidance, insights, and expertise; RA performed HPLC injections, data acquisition, and technical support; HS prepared plant materials and performed plant extractions.

Conflict of Interest

Authors declare no conflict of interest.

Data Availability

Data presented in this study will be available to all authors.

Ethics Approval

Not applicable to this paper.

References

AOAC (2012). Appendix K: guidelines for dietary supplements and botanicals. guidelines for dietary supplements and botanical. *In: AOAC Official Methods of Analysis*, pp:1–9

Cafino EJV, MB Lirazan, EC Marfori (2016). Simple HPLC method for the analysis of [6]-gingerol produced by multiple shoot culture of ginger (*Zingiber officinale*). *Intl J Pharmcogn Phytochem Res* 8:38–42

Chaudhari VS, RM Borkar, US Murty, S Banerjee (2020). Analytical method development and validation of reverse-phase high-performance liquid chromatography (RP-HPLC) method for simultaneous quantifications of quercetin and piperine in dual-drug loaded nanostructured lipid carriers. *J Pharm Biomed Anal* 186:113325

Dapson RW, CL Bain (2015). Brazilwood, sappanwood, brazilin and the red dye brazilein: From textile dyeing and folk medicine to biological staining and musical instruments. *Biotech Histochem* 90:401–423

Haryanti S, Y Widiyastuti, N Rahmawati (2018). Cytotoxic and MMPs inhibitory activities of Sappan Wood (*Caesalpinia sappan* L.): various extracts on 4T1 breast cancer cell line. *Health Sci J Ind* 9:51–56

Hu J, Z Guo, M Glasius, K Kristensen, L Xiao, X Xu (2011). Pressurized liquid extraction of ginger (*Zingiber officinale* Roscoe) with bioethanol: An efficient and sustainable approach. *J Chromatogr A* 1218:5765–5773

International Conference on Harmonization (2022). ICH guideline Q2(R2) on validation of analytical procedures. *Eur Med Agency* 2:1–39

Izzatinisa R (2022). *Effect of Red Ginger Extract (Zingiber officinale var. Rubrum) and Secang* (*Caesalpinia sappan* L.) *Hyperlipidemic Rat Model on Lipid Profile*. Faculty of Pharmacy Universitas Indonesia

Ley-Martínez JS, JE Ortega-Valencia, O García-Barradas, M Jiménez-Fernández, E Uribe-Lam, CI Vencedor-Meraz, J Oliva-Ramírez (2022). Active Compounds in *Zingiber officinale* as Possible Redox Inhibitors of 5-Lipoxygenase Using an *In Silico* Approach. *Intl J Mol Sci* 23:6093–6106

Nirmal NP, MS Rajput, RGSV Prasad, M Ahmad (2015). Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: A review. *Asian Pac J Trop Med* 8:421–430

Nurtjahja-Tjendraputra E, AJ Ammit, BD Roufogalis, VH Tran, CC Duke (2003). Effective anti-platelet and COX-1 enzyme inhibitors from pungent constituents of ginger. *Thromb Res* 111:259–265

Papadoyannis IN, FV Samanidou (2004). Validation of HPLC Instrumentation. *J Liq Chromatogr Relat Technol* 27:753–783

Raut R, J Shaji (2021). HPLC method validation for quantification of tetrahydrocurcumin in bulk drug and formulation. *Future J Pharm Sci* 7:1–6

Saputri FS, N Nabila, A Mun'im (2017). Combination of ginger and sappan wood extract effect on *in vivo* antithrombotic activity test. *J Young Pharm* 9:230–233

Sari R, S Suhartati (2016). Secang (*Caesalpinia Sappan* L.: Tumbuhan herbal kaya antioksidan. *J Penelit Sos Ekon Kehut* 13:57–67

Settharaksa S, C Monton, L Charoenchai (2019). Optimization of *Caesalpinia sappan* L. heartwood extraction procedure to obtain the highest content of brazilin and greatest antibacterial activity. *J Integr Med* 17:351–358

Setyowati N, Masyhuri, JH Mulyo, Irham, B Yudhistira (2023). The hidden treasure of wedang uwuh, an ethnic traditional drink from Java, Indonesia: Its benefits and innovations. *Intl J Gastron Food Sci* 31:100688

Sharif MF, MD Bennett (2016). The effect of different methods and solvents on the extraction of polyphenols in ginger (*Zingiber offici*nale). *J Teknol* 78:49–54

Simon-Brown K, KM Solval, A Chotiko, L Alfaro, V Reyes, C Liu, B Dzandu, E Kyereh, AG Barnaby, I Thompson, Z Xu, S Sathivel (2016). Microencapsulation of ginger (*Zingiber officinale*) extract by spray drying technology. *Lwt – Food Sci Technol* 70:119–125

USP-NF (2022). ⟨621⟩ Chromatography. *In: The United States Pharmacopeia/ National Formulary*

Wan YJ, L Xu, WT Song, YQ Liu, LC Wang, MB Zhao, Y Jiang, LY Liu, KW Zeng, PF Tu (2019). The ethanolic extract of *Caesalpinia sappan* heartwood inhibits cerebral ischemia/reperfusion injury in a rat model through a multi-targeted pharmacological mechanism. *Front Pharmacol* 10:1–15

Yan Y, YC Chen, YH Lin, J Guo, ZR Niu, L Li, SB Wang, LH Fang, GH Du (2015). Brazilin isolated from the heartwood of *Caesalpinia sappan* L. induces endothelium-dependent and-independent relaxation of rat aortic rings. *Acta Pharmacol Sin* 36:1318–1326

Zhong L, R Wang, QH Wen, J Li, JW Lin, XA Zeng (2022). The interaction between bovine serum albumin and [6]-,[8]- and [10]-gingerol: An effective strategy to improve the solubility and stability of gingerol. *Food Chem* 372:131280