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Analysis of Flavonoid Contents in Coffee Mistella (*Dendrophthoe pentandra* (L.) Miq.) Using Thin Layer Chromatography-Densitometry Techniques

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Abstract: Flavonoids, a class of phenolic compounds widely distributed in plants, are present in the coffee mistletoe *Dendrophthoe pentandra* (L.) Miq. The extraction of flavonoids from *Dendrophthoe pentandra* (L.) Miq leaves involve the use of methanol as a solvent in maceration extraction methods. The presence of flavonoids was ascertained through color changes from green to yellow-green, yellow, and yellow-orange when subjected to $AlCl_3$ 5%, NaOH 10%, and Mg-HCl, respectively. Thin-layer chromatography using aluminum plates coated with silica gel F_{254} as the stationary phase and methanol-chloroform 4:1 (v/v) as the mobile phase was employed to separate the flavonoids. The method validation demonstrated strong linearity in the concentration range of 60-130 ppm for flavonoid standard solutions, with a correlation coefficient (r) of 0.998. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were determined to be 182.5 ng and 608.3 ng, respectively. Precision, expressed as Relative Standard Deviation (RSD), was found to be 5.1%, 2.8%, and 2.5% for concentrations of 80, 90, and 100 ppm, respectively. These RSD values were also less than 2/3 CV Horwitz, which were 5.5, 5.4, and 5.4. The method's accuracy was assessed through percent recovery values for each concentration (80, 90, and 100 ppm), which were found to be 102.46 ± 1.78%, 86.14 ± 1.74%, and 89.89 ± 1.44%. The flavonoid content in the dried coffee mistletoe powder was determined to be $1.404x10^{-2}$ ± 0.0007 mg per gram, with a water content of 8.7%.

Keywords: Flavonoids, coffee mistletoe, TLC, densitometry, validation.

INTRODUCTION

Coffee mistletoe (*Dendrophthoe pentandra (L.)* Miq) is a parasitic plant that can grows by attaching itself to other plant. Coffee mistletoe is a plant known to be harmful as it can damage its host. It is traditionally used to treat degenerative diseases such as flu, coughs, and diarrhea, and is known for its anti-cancer and anti-allergy properties [1]. The leaves of *Dendrophthoe pentandra (L.)* Miq contain alkaloids, phenolics, saponin, terpenoids and flavonoids. Flavonoids are plant compounds that provide red, purple, blue, and yellow colors and commonly used for medicinal purposes [2, 3]. They are very valuable groups of plant origin compounds of great interest in.

Various chromatographic methods exist to analyze flavonoids in mistletoe, including HPLC, GC, and TLC. However, the Thin Layer Chromatography-Densitometry method was used in this study because it is simpler and requires less sample extraction preparation [4]. Densitometry is an instrumental analysis method based on the interaction of electromagnetic radiation with analytes spotted on TLC (Mulja & Suharman, 1995). However, in this study, the Thin Layer Chromatography-Densitometry (TLC-Densitometry) method was used because it is more straightforward than gas chromatography or high-performance liquid chromatography, which requires longer analysis time and sample extraction preparation with large amounts of chemicals [4].

The TLC-Densitometry method is a method of analyzing flavonoid levels in coffee and parasites. It can be used for qualitative and quantitative analysis relatively quickly and is easier to apply. Densitometry is an instrumental analysis method based on the interaction of electromagnetic radiation with analytes spotted on TLC [5].

Validation of the TLC-Densitometry analytical method needs to be carried out to find out or ensure that the analytical method that has been carried out is suitable to be applied as a method for analyzing flavonoid levels in coffee mistletoe leaves. Based on test results on validation parameters, which include linearity, LOD (limit of detection), LOQ (limit of quantitation), precision, and accuracy [6].

METHODS

Chemical and Instrumentation

Mistletoe coffee leaves were collected from Sidomulyo Village, Silo District, Jember, Indonesia. The chemicals used including flavonoids (quercetin) from Merck, as well as chloroform, ethyl acetate, methanol, distilled water, tissue, filter paper, aluminum foil, plastic, and silica gel TLC documented as F254. A range of instruments was utilized, such as a blender, glass wares, porcelain cup, glass funnel, 60 mesh sieve, spray bottle, hair dryer, stir bar, pipette ball, dropper pipette, micropipette, ultraviolet lamp, 10×5×15 cm chamber, analytical balance (Pioner), and Camag 3 Densitometric Scanner.

Sample Preparation

The coffee mistletoe sample were cleaned and air-dried for ten days without exposure to sunlight. The dried leaf samples were ground using a blender and sieved using a 60-mesh sieve. The delicate leaves were weighed, each weighing 2 grams in triplicate [7].

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The water content was determined by drying a sample of 1.00 grams of mistletoe coffee leaf powder in an oven for 3-5 hours at a temperature of 105°C. Afterward, the sample was cooled in a desiccator for 30 minutes and weighed until constant weight, according to [8] in 2015.

Maceration

Two grams of coffee mistletoe (*Dendrophthoe pentandra* (L.) Miq.) leaf powder was macerated using 20 mL of methanol (p.a). The maceration process should be carried out for 4x24 hours, every 1x24 hours; after the first 24 hours, the extract should be filtered using a Buchner funnel, and the remaining residue should be macerated again with new methanol. This process should be repeated thrice [9].

Qualitative test of Flavonoids

Three test tubes were filled with 3 mL of methanol extract of coffee mistletoe leaves (*Dendrophthoe pentandra* (L.) Miq.). To test for flavonoids, tube one was added with 10% NaOH, and if a yellowish-green-brownish-yellow solution was formed, it was considered positive for flavonoids. Tube two was added with $AlCl_3$, and if a yellow color formed, it was considered positive. Tube 3 was added with concentrated HCl and Mg powder, and if an orange to red color formed, it was considered positive for containing flavonoids [10].

Optimization of Eluent Composition

A solution containing 90 parts per million (ppm) of flavonoids was applied onto an activated TLC plate made of F254 silica gel. The solution was placed using a micropipette at a distance of 1 cm from the bottom of the plate, with a 1 cm distance between each point. The TLC plate was then subjected to elution using a mixture of methanol and chloroform with different volume ratios of 1:1, 4:1, 3:2, 2:3, and 1:4. After the elution process, the F254 Silica Gel TLC plate was removed and aired, and then analyzed using densitometry at quercetin λmax. For qualitative analysis, the TLC plate was sprayed with 5% $AlCl_3$ spray reagent. The optimization process was repeated three times (triple) for each variation in eluent volume ratio.

Method Validation

Linearity range

Flavonoid standard solutions were tested with varying concentrations ranging from 40 ppm to 130 ppm. The testing was done using TLC-densitometry with a mixture of methanol and chloroform (4:1) at a quercetin λmax of 369 nm. The results were measured in terms of area (AU) and mass of the standard solution (ng), which were then plotted on a calibration curve using the equation y = bx + a.

Limit of Detection and Limit of Quantitation

The detection limit and quantitation limit were determined using statistical methods from the results of the calibration curve obtained from linearity measurements [11]. The formula for the calculation is as follows:

$$Limit\ of\ Detection\ (LOD) = \frac{3 \times Sb}{SI}$$

$$Limit\ of\ Quantitation\ (LOQ) = \frac{10 \times Sb}{SI}$$

Precision

We conducted a TLC-Densitometry analysis on Flavonoid standard solutions with concentrations of 80, 90, and 100 ppm. The analysis was performed using a mixed eluent of methanol: chloroform (4:1) at quercetin λmax. To detect the flavonoids, we used a 5% $AlCl_3$ spray reagent. We repeated the experiment six times for each concentration of the flavonoid standard solution. We measured the similarity using standard and relative standard deviations with the following formula:

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

Accuracy

Standard flavonoids solutions with concentrations of 80, 90, and 100 ppm were subjected to TLC-Densitometry testing using a mixed eluent of methanol and chloroform (4:1) at quercetin λmax. The plate was then sprayed with a 5% $AlCl_3$ reagent. This process was repeated six times for each concentration of the flavonoid standard solution. Similarity was measured using standard and relative standard deviations with the formula below:

$$\%recovery = \frac{m_f - m_a}{m_a} \times 100\%$$

Flavonoids analysis Using TLC-Densitometric

A solution of flavonoids with a concentration of 90 parts-per-million was prepared. 40 mL of each sample was taken and placed on an activated Silica Gel TLC plate. The sample was spotted at a distance of 1 cm from the bottom of the plate, and 1 cm from the right and left sides of the plate. A punching distance of 1 cm was kept between each spot. The plate was then eluted with a mixture of methanol and chloroform (4:1) in a 10 x 10 x 5 cm chamber. After elution, the plate was removed and aired to dry. The dried plate was then tested using a densitometer at quercetin λmax. Qualitative analysis of the sample was carried out using TLC plates identified using densitometry and treated with 5% $AlCl_3$ spray reagent. The analysis was done three times to ensure accuracy.

RESULT AND DISCUSSION

Flavonoids Extracts from Coffee Mistletoe

To extract samples of coffee mistletoe leaves, the maceration extraction method was applied, resulting in a dark green solution. During this process, pressure differences between the cell walls and membranes caused the breakdown of the cells, leading to the dissolution of secondary metabolite compounds, particularly flavonoids, in methanol. Flavonoids dissolved in methanol due to the formation of hydrogen, dipole-dipole, and van der Waals bonds with methanol, which contains a polar hydroxy group (-OH) and a non-polar methyl group (-CH3) [11, 12].

The water content of a material can be expressed as a percentage of its wet weight (wet basis) and dry weight (dry basis). The presence of water in a sample might impact the quality and quantity of active compounds present, such as in coffee mistletoe. In this research, the water content of coffee mistletoe leaf powder was found to be 8.7% based on a 1.0-gram sample of air-dried leaves.

Qualitative Flavonoid Test Results

The study found that the methanol extract of coffee mistletoe contains flavonoids. The presence of flavonoids was confirmed through qualitative tests, which showed a change in color from green to greenish-yellow when 10% NaOH was added, from green to yellow when 5% $AlCl_3$ was added, and from green to orange-yellow when HCl-Mg was added. These color changes match the ones produced by a standard solution of flavonoids (quercetin). Comparing the color changes in the samples with literature also suggested that the methanol extract contains flavonoid compounds of the flavanone, flavone, flavonol, isoflavone, and aurone groups.

The study used quercetin as a standard compound for flavonoids. The maximum absorption of quercetin was found to be at a wavelength of 369.0 nm with an absorbance value of 0.831. This shows that the quercetin molecule can absorb light energy from UV light at 369 nm for electron transitions. The electron transition occurs from bonding orbitals with a lower energy level to antibonding orbitals with a higher energy level. The appropriate transition type is the n-σ* transition for the ether group, the π-π* transition for benzene, alkene, and carbonyl groups, and the n-π* transition for carbonyl and hydroxyl groups.

Optimum Eluent Composition for TLC

Eluent optimization was a process that determine the best eluent for achieving the desired separation. The selection of the optimal eluent based on the number of spots on the TLC or the number of peaks produced by the densitogram data, and the standard deviation value. The 4:1 methanol-chloroform eluent composition had the highest number of peaks (seven peaks), compared to the other eluent compositions, such as the 1:1 eluent (five peaks), the 1:4 eluent (three peaks), the 2:3 eluent (four peaks), and the 3:2 eluent (five peaks). This indicated that the 4:1 eluent composition has the highest value to separate the components in the extract.

The selection of eluent composition optimization results was also influenced by the standard deviation (SD) value. The standard deviation value of the 4:1 methanol-chloroform eluent composition was 0.001, smaller than the standard deviation value of the other eluent compositions. This means that the separation of analytes for the 1:1, 1:4, and 2:3 methanol-chloroform eluents, was not good enough. Although the 3:2 methanol-chloroform eluent has almost the same Rf value as the 4:1 eluent composition, its standard deviation value was larger. Therefore, the 4:1 methanol-chloroform eluent was chosen as the eluent to produce accurate measurements.

Method Validation

Linearity was a measure of a method's ability to yield test results that were directly proportional to the concentration of the analyte in the sample, within a given concentration range. In the TLC-Densitometry method, linearity can be assessed by examining the correlation coefficient of the calibration curve of the analyte (x-axis) to the area (y-axis). To determine the standard area of quercetin, the analyte was scanned using a densitometer, allowing its mass to be measured. The data obtained were then plotted on a calibration curve that correlates the mass of the analyte (ng) and the densitogram (AU) area. The results of the linearity test are presented in Figure 1.

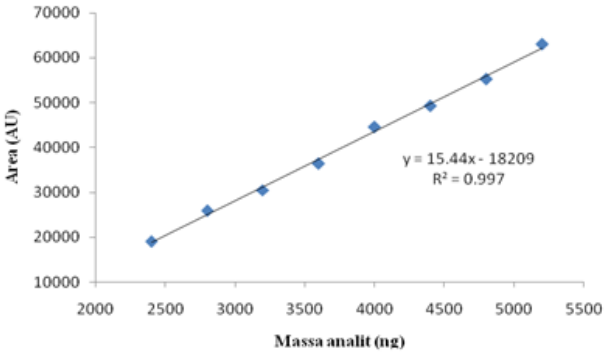

Figure1. Linear curve of quercetine

The calibration curve used in this study has a high level of accuracy, with an R2 value of 0.997 and a correlation coefficient (r) of 0.998. This correlation coefficient value indicates a strong linear relationship between the mass of quercetin and an area of 0.998. The correlation coefficient value is based on the requirement that the correlation coefficient value (r) must be equal to or greater than 0.997 [6].

To determine the detection and quantitation limits of the TLC-Densitometry method, linear area calibration curve equation data analysis was used. This method was based on the standard deviation of the response, calculated from the calibration curve's slope. The results showed that the method can detect quercetin concentrations as low as 4.6 ppm (LOD) and can accurately measure concentrations of quercetin as low as 15.2 ppm (LOQ).

The lowest limit of the linear region of the calibration curve and the mass of quercetin in coffee mistletoe leaves was found to be 60 ppm. These results demonstrate that the TLC-Densitometry method was highly effective for analyzing the flavonoid levels in coffee mistletoe.

A precision test was conducted to assess the consistency of examination results by measuring six standard flavonoid solutions with the same concentration and under the same conditions. The test results are summarized in Table 1.

Table 1. Precision test in RSD and 2/3 Horwitz

Concentration (ppm)	RSD (%)	2/3 CV Horwitz (%)
80	5.1	5.5
90	2.8	5.4
100	2.5	5.4

The following is the precision test data for standard flavonoid solutions with three different concentrations. Based on the research results, the %RSD value for the precision test was 5.1% for 80 ppm, 2.8% for 90 ppm, and 2.5% for 100 ppm. The precision test results meet the acceptance criteria with an SBR value of ≤ 7.3%. Moreover, the RSD (%) obtained from the experiment should be smaller than the Horwitz CV (%) for the precision of a method to meet the requirements. Since the RSD value from the six experimental results is lower than the CV Horwitz value, the analysis of flavonoids in coffee mistletoe leaves using the TLC-Densitometry method can be considered precise.

In this study, the accuracy test was conducted to evaluate the proximity of the analysis results to the actual analyte levels in the

sample. The accuracy is indicated in percent recovery using the standard addition method. The accuracy test results are provided in Table 2.

Table 2. Recovery test for 3 different concentration

Addition standard	% Recovery
Sample + 80 ppm	102.46±1.78
Sample + 90 ppm	86.14±1.74
Sample + 100 ppm	89±1.44

Based on the research results, the % recovery value of flavonoids for each concentration using the TLC-Densitometry method was 102.46 ± 1.78%, 86.14 ± 1.74, and 89.89 ± 1.44%. The percent recovery value is within the range of accepted flavonoid analysis validation test criteria for an analyte concentration of 0.001%, namely 80-110% [14].

Flavonoids Content in Coffee Mistletoe

The TLC-densitometry method was used to analyze flavonoids in coffee mistletoe leaves. The result of this analysis was a densitogram that show the area (y-axis) with different units of AU (Absorbance Unit) and Rf (x-axis) for each sample. Usually, the densitogram results in 3-6 peaks because the analyzed compounds are less specific, which means there might be other compounds that are non-flavonoids. To ensure that the stain was from flavonoids, a 5% $AlCl_3$ spray reagent was used. When the TLC plate was sprayed using the 5% $AlCl_3$, yellow spots become visible, indicating that coffee mistletoe leaf extract was likely to contain flavonoids [12]. This data was then used as a reference to determine which peaks are likely to be flavonoid compounds. The results of the flavonoid analysis using TLC-Densitometry can be seen in Table 3.

The flavonoid levels present in air-dried coffee mistletoe powder samples were found to be $1.404x10^{-2} \pm 0.0007$ mg per gram of sample. This result has the higher value than previous research by [13], with the result of 2.48%.

Table 3. Flavonoids content in the coffee mistletoe

Repetition	Flavonoids content in coffee mistletoe (mg/g)
1	$1.421 \times 10^{-2} \pm 0.0007$
2	$1.327 \times 10^{-2} \pm 0.0007$
3	$1.462 \times 10^{-2} \pm 0.0007$
Average	$1.404 \times 10^{-2} \pm 0.0007$

CONCLUSION

The best eluent composition for separation of flavonoids from mistletoe coffee leaves was a mixture of methanol and chloroform in the ratio of 4:1, with. The TLC-Densitometry validation method indicated that it provided linear results with a correlation coefficient of 0.998, in the concentration range of 60-130 ppm. The LOD and LOQ values were 182.5 ng and 608.3 ng, respectively. The precision of the method was 2.5 to 5.1%, while and the accuracy was 86.14 to 102.46%. The measurement results show that the flavonoid content of mistletoe coffee leaves was $1.404x10^{-2} \pm 0.0007$ mg/g sample.

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