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Spectrophotometric Analysis of Caffeine Content in Coffee Mistletoe (*Dendrophthoe petandra* L.)

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Abstract: Caffeine, a well-known alkaloid presents in plants such as tea, coffee, and kola nuts, was thoroughly analyzed in coffee mistletoe. Our study successfully extracted caffeine using dichloromethane and chloroform, incorporating qualitative testing and robust method validation with a visible spectrophotometer. Notably, dichloromethane yielded the highest caffeine extract at 0.042 grams. The qualitative tests distinctly confirmed the presence of caffeine, evidenced by a color change with Parry's reagent and a maximum absorption wavelength of 273 nm. We also pinpointed the caffeine complex's maximum wavelength at 640 nm, further validating our findings against a standard solution. Our method validation showed impressive linearity (r = 0.9974), with limits of detection at 1.81 ppm and quantitation at 6.02 ppm. The accuracy ranged between 90.0% and 97.5%, while the precision values were consistently around 98%. Additionally, we determined the water content of the coffee mistletoe leaf powder to be 8.733%. This comprehensive analysis establishes a strong foundation for the caffeine content in coffee mistletoe.

Keywords: Caffeine, Coffee mistletoe, Spectrophotometric, Visible.

INTRODUCTION

Mistletoe from the *Dendrophthoe petandra* species grows on various host plants, including mango, jackfruit, water apple, chocolate, tea, and coffee [1]. Phytochemical studies have shown that coffee mistletoe contains flavonoids, alkaloids, steroids, phenolics, and saponins [2]. Analysis of cocoa mistletoe revealed the presence of flavonoids, phenolics, terpenoids, and saponins in different extracts [1]. Additionally, tea mistletoe, known as *Scurrula oortiana*, has been found to contain caffeine, catechin, phytol, and flavonoid glycosides [3]. Caffeine, a common alkaloid, can be derived from various plants, including mistletoe [4]. While the caffeine content in coffee mistletoe has not been extensively studied, some preliminary screenings have been conducted.

This research aims to analyze caffeine using a visible spectrophotometer with Parry's reagent, and to extract caffeine from coffee mistletoe leaves using dichloromethane and chloroform. According to [5], dichloromethane has been found to yield better results for this purpose. The validity of the method will be evaluated based on parameters such as linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision [6].

EXPERIMENTAL

Instrumentation

The equipment used in this study included a visible spectrophotometer (model 721), an OHAUS analytical balance, a spray bottle, cuvettes, a pipette ball, a Mohr pipette, various glassware, aluminum foil, filter paper, a separating funnel, a watch glass, a vacuum distillation setup, a water bath, a blender, and a 60-mesh sieve.

Chemicals

The sample used in this study was coffee mistletoe (*Dendrophthoe petandra* (L.) Miq.), sourced from the plantations in Sidomulyo Village, Silo District, Jember Regency. The specific part used for analysis was the leaves of the coffee mistletoe. The chemicals used in the study included caffeine (Merck), aquades, cobalt chloride ($CoCl_2$, Merck), ammonium hydroxide (NH_4OH), dichloromethane (Merck), chloroform (Merck), calcium carbonate ($CaCO_3$, Merck), magnesium sulfate ($MgSO_4$, Merck), and methanol.

Sample Preparation

A total of 55.221 grams of coffee mistletoe leaves (*Dendrophthoe petandra* (L.) Miq) were cut into small pieces and then dried at room temperature for approximately six days. The dried leaves were ground and sieved through a 60-mesh sieve to obtain uniform-sized particles, which increases the contact area with the solvent [7].

Parry's Reagent Preparation

A 0.5 g $CoCl_2$ powder was placed into a 50 mL glass beaker and dissolved in a small quantity of methanol. This solution was then transferred to a 50 mL measuring flask and diluted with methanol up to the mark. Following this, 2 mL of the $CoCl_2$ solution was taken and further diluted in a 25 mL measuring flask, again filling until the mark with methanol to prepare a 0.04 M $CoCl_2$ solution.

For the preparation of the ammonium hydroxide solution, 6.6 mL of a 30% NH_4OH solution was transferred to a 50 mL measuring flask. It was then diluted with distilled water until the mark to make a 1 M NH_4OH solution.

Qualitative test of Caffeine

The filtered mistletoe powder was weighed 100 mg and dissolved in 25 mL of hot water in a 50 mL beaker (note that the solubility of caffeine in 100°C water is 67 g per 100 mL). After dissolving, the mixture was filtered. Next, 8 mL of the coffee mistletoe water extract was transferred into a clean beaker,added with 1 mL of cobalt(II) chloride ($CoCl_2$), followed by 0.4 mL of 1 M ammonium hydroxide (NH_4OH). The solution is considered to contain caffeine if it turns blue-green [8].

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Caffeine extraction

The mistletoe powder (1 g) was added to a 150 mL glass beaker with 125 mL of distilled water and heated for 30 minutes while stirring. Then, 1.5 g of $CaCO_3$ was added to release caffeine from its matrix. The mixture was then filtered to separate the liquid (filtrate) from the solid residue. The filtrate was cooled to 15-20 °C, and 5 mL was placed in a separating funnel. This extract was then subjected to three extractions, each with 5 mL of dichloromethane. The dichloromethane phase was separated from the water phase and collected in a 100 mL glass beaker. To each dichloromethane extract, MgSO4 was added and allowed to sit for 10 minutes before filtering [9].

The dichloromethane was removed by vacuum distillation and left caffeine remained. This process was repeated with another 5 mL of the mistletoe leaf extract, extracting three times with 5 mL of chloroform to yield a white caffeine precipitate.

The caffeine precipitate was obtained and dissolved in a vacuum distillation flask with 15 mL of distilled water at 100°C until fully dissolved. This ensured that all precipitates in the flask could be completely taken during vacuum distillation.

From the caffeine solution, 8.6 mL was transferred into a 10 mL volumetric flask. 1 mL of 0.04 M $CoCl_2$ was added to this solution, followed by 0.4 mL of 1 M NH_4OH. Distilled water was then added, resulting in a blue-green solution.

A blank solution was prepared by taking 8.6 mL of distilled water in a separate 10 mL volumetric flask, followed by the addition of 1 mL of 0.04 M $CoCl_2$ and 0.4 mL of 1 M NH_4OH, with distilled water added to the mark. The blue-green caffeine solution was then placed in a cuvette to measure its absorbance using a visible spectrophotometer at a wavelength of 640 nm. The analysis was repeated five times.

Caffeine analysis using Spectrophotometer visible

Standard caffeine solutions were prepared by diluting 0.5 to 4.5 mL of a 500ppm caffeine standard to achieve final concentrations of 10 to 100 ppm. For each solution, take 8.6 mL and add it to a 10 mL flask with 1 mL of 0.04 M $CoCl_2$ and 0.4 mL of 1 M NH_4OH, then fill to the mark with distilled water. This yields blue-green solutions with concentrations ranging from 8.6 to 86.0 ppm. The blank solution was prepared by mixing 8.6 mL of distilled water with 1 mL of $CoCl_2$ and 0.4 mL of NH_4OH in a 10 mL flask, filling to the mark with water. Measure the absorbance of each solution at 640 nm with a visible spectrophotometer. Use the linear regression from this data, based on Lambert-Beer law, to calculate caffeine concentration in coffee mistletoe leaf samples.

Method validation

Linearity

The linearity test was conducted by preparing standard caffeine solutions with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ppm, ensuring each concentration was triplicate. We measured 8.6 mL for each solution into a 10 mL volumetric flask, then added 1 mL of 0.04 M $CoCl_2$ and 0.4 mL of 1 M NH_4OH, filling the flask with distilled water up to the 10 mL mark, resulting in a blue-green solution. A blank solution was prepared by adding 1 mL of 0.04 M $CoCl_2$ and 0.4 mL of 1 M NH_4OH to 8.6 mL of distilled water in a 10 mL volumetric flask and similarly filled it to the mark with distilled water. Subsequently, the colored standard solutions were placed in a cuvette to measure their absorbance using a visible spectrophotometer set to 640 nm. We applied the equation (y = a + bx) for linear regression analysis. The slope (b), intercept (a), and correlation coefficient (r) provided insights into the linear relationship. A correlation coefficient greater than 0.9970 indicates an acceptable relationship, as noted by [6]. The ideal r value approaches +1 or -1, depending on the direction of the line. Should the regression value fall below 0.9970, we plan to adjust the concentrations of the standard solutions to achieve an acceptable regression, as suggested by [10].

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The detection and quantitation limits were determined by measuring the absorbance of the blank solution at a wavelength of 640 nm, with six replicates conducted for accuracy. The blank solution was prepared by combining 8.6 mL of the base solution with a 10 mL measuring flask. Subsequently, 1 mL of 0.04 M CoCl2 and 0.4 mL of 1 M NH_4OH were added, and distilled water was used to fill the flask to the designated mark. The linear equation derived from the calibration curve was then utilized to calculate the detection and quantitation limits. The detection and quantitation limit were counted by following equation:

$$\boldsymbol{LOD} = \frac{\boldsymbol{3x\,Sb}}{\boldsymbol{Slope}} \qquad \boldsymbol{LOQ} = \frac{\boldsymbol{10\,x\,Sb}}{\boldsymbol{Slope}} \quad \text{..... (1)}$$

Accuracy

One gram of mistletoe powder was mixed with 125 mL of distilled water in a 150 mL beaker and heated for 30 minutes while stirring. Afterward, 1.5 g of calcium carbonate ($CaCO_3$) was added to the solution. The mixture was filtered, and this process was repeated six times to obtain six samples. The theoretical caffeine concentrations (40, 60, and 80 ppm) were prepared in 5 mL portions. The mistletoe extract was cooled, and 5 mL portions were extracted three times with 5 mL of dichloromethane. The dichloromethane was separated, treated with magnesium sulfate (MgSO4), and filtered. It was then evaporated to yield caffeine powder dissolved in 15 mL of hot distilled water at 100 °C. From this, 8.6 mL was combined with 1 mL of 0.04 M cobalt chloride (CoCl2) and 0.4 mL of 1 M ammonium hydroxide (NH4OH) in a 10 mL measuring flask to create a blue-green solution. A blank was prepared similarly with distilled water. The resulting solution was measured using a UV-Vis spectrophotometer at 640 nm. The recovery data obtained using this equation:

$$\%\boldsymbol{Recovery} = \frac{\boldsymbol{mf-mA}}{\boldsymbol{mA*}}\ \boldsymbol{x}\ \boldsymbol{100}\% \quad \text{..... (2)}$$

Precision

Standard caffeine solutions were prepared at concentrations of 40, 60, and 80 ppm. For each concentration, 8.6 mL of the solution was transferred into a 10 mL measuring flask. Then, 1 mL of 0.04 M CoCl2 and 0.4 mL of 1 M NH4OH were added, followed by distilled water added to the boundary mark to obtain a blue-green solution. A blank solution was also prepared by taking 8.6 mL of distilled water, placing it into a 10 mL measuring flask, and adding 1 mL of 0.04 M CoCl2 and 0.4 mL of 1 M NH4OH, with distilled water added to the boundary mark. The blue-green standard solutions were placed in a cuvette to measure their absorbance using a visible spectrophotometer at a wavelength of 640 nm. This analysis was repeated six times, and the similarity of the results was evaluated using the standard deviation (s), and 2/3 CV Horwitz (Coefficient Variance Horwitz).

RESULT AND DISCUSSION

Optimum solvent for extracting caffeine from coffee mistletoe

The extraction of caffeine from coffee mistletoe leaves is a focused process that effectively isolates caffeine compounds from other constituents present in the leaves. A pivotal factor in successful extraction is the selection of the right solvents. The chosen solvent must efficiently extract the desired substance based on the strength of the interactions between the solvent and the solute. Stronger interactions lead to a higher yield of solute and a more rapid extraction process. To identify the optimal solvent, we can compare the mass of caffeine extracted from different solvents through three repetitions. The result shows that the average mass of caffeine extracted from three coffee mistletoe leaf powder samples using dichloromethane was 0.042 × 10^{-2} grams. At the same time, extraction with chloroform produced a lesser yield of 0.023 × 10^{-2} grams. Consequently, the average caffeine concentration from the dichloromethane extraction stood at 0.042%, whereas the chloroform extraction yielded only 0.023%. These results decisively demonstrate that dichloromethane is the superior solvent for extracting caffeine compared to chloroform. This conclusion aligns with research by [5], which established dichloromethane as the optimum solvent for caffeine extraction. Because caffeine is polar, it effectively dissolves in polar solvents, adhering to the principle of "like dissolves like." Dichloromethane possesses a higher dielectric constant than chloroform. The solvent's increased dielectric constant and polarity stem from a greater number of functional groups and a reduced number of carbon atoms in the solvent [11].

Qualitative result of caffeine in the extracts

This qualitative caffeine test involves adding a specific reagent to an extract of coffee mistletoe leaf powder and then comparing the results with those obtained from adding Parry's reagent to a standard caffeine solution. Parry's reagent is derived from cobalt chloride ($CoCl_2$) and ammonium hydroxide (NH_4OH). When caffeine is present, it reacts positively with Parry's reagent, resulting in a blue-green color [8], a as shown in Fig 1. The reaction between caffeine and Parry's reagent as below:

$$CoCl_{2(s)} + 2CH_3OH_{(aq)} \rightarrow Co(OH)_{2(aq)} + 2CH_3Cl_{(aq)}$$

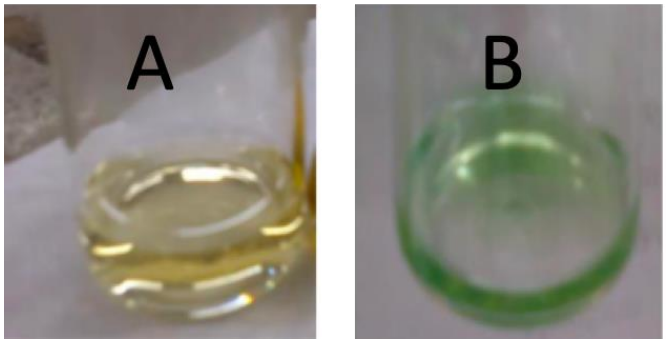

Figure 1. Qualitative result of mistletoe extracts with Parry reagent: A. Before the reagen addition B. After the addition of reagent

Method Validity

Linearity

Linearity refers to the ability of an analytical method to provide a proportional response to the concentration of the analyte in a sample. The purpose of the linearity test is to evaluate the effectiveness of the calibration curve in linking the response (y) with the concentration (x) [12]. To determine linearity, a calibration curve is created based on the analysis results of several standard solutions with known concentrations. The minimum linearity is established using five different concentrations [13]. In this linearity test, ten standard solutions are utilized with concentration variations of 8.6, 17.2, 25.8, 34.4, 43.0, 51.6, 60.2, 68.8, 77.4, and 86.0 ppm. The linearity is assessed by the correlation coefficient (r) derived from the regression equation of the standard calibration curve, as illustrated in Figure 2. The correlation coefficient obtained from the standard solution calibration curve results is 0.9974. This value indicates that the standard calibration curve is linear. It meets the established criteria, as a correlation coefficient greater than 0.9970 is considered to demonstrate linearity [6].

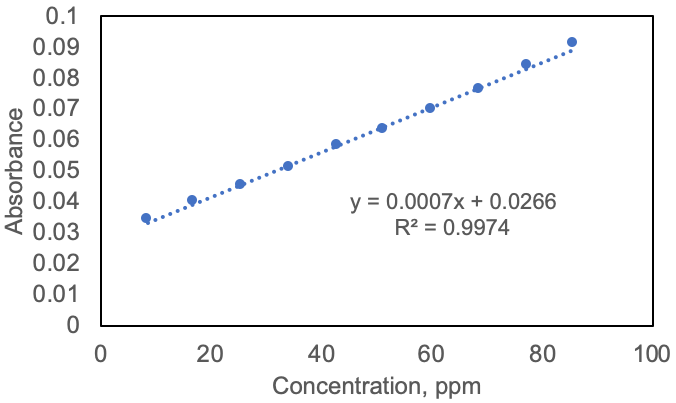

Figure 2. Calibration curve of caffeine

Additionally, the intercept value (a) of the regression equation reflects the influence of the matrix on the solution being analyzed. In this case, the intercept is close to zero, at 0.0266, suggesting that the sample matrix does not significantly impact the determination of caffeine levels. The slope value (b) of the line indicates the sensitivity of the method.

Accuracy

Accuracy is a measure that indicates how closely the analysis results align with the actual levels of the analyte. It can be determined using the standard addition method, which involves adding a caffeine standard with a known concentration to several samples. The accuracy is expressed as the percent recovery of the added analyte. Initially, samples without standard solutions are analyzed, followed by the addition of several standard solutions with known concentrations. These mixtures are then reanalyzed. In this study, standard caffeine concentrations of 40, 60, and 80 ppm are added to the dichloromethane extract of coffee mistletoe leaves. The results from the two analyses are compared to the actual levels. A recovery value is considered acceptable if it falls within the range of 90-107% [14].

The recovery value obtained ranges from 90.0% to 97.5%. This result meets the established requirements, indicating that the visible spectrophotometry method demonstrates a good level of accuracy and precision. The percent recovery value may be less than or exceed 100% due to several factors in the analysis process. This discrepancy can arise from a suboptimal extraction process or the use of inappropriate tools, which may adversely affect the recovery value [10].

Table 1. Percent recovery of sample and standards

Standard addition	%recovery
Sample+ 40 ppm standard	90.0 %
Sample+ 60 ppm standard	90.0%
Sample+ 80 ppm standard	97.5 %

Precision

The relative standard deviation (RSD) for the standard solution with a concentration of 40 ppm is 1.66%, indicating a precision value of 98.34%. The 2/3 CV Horwitz for this concentration is 6.21, which shows that the error rate across six measurements is 1.66%. For the standard solution with a concentration of 60 ppm, the RSD is 1.84%, leading to a precision value of 98.16% and a 2/3 CV Horwitz of 6.17, resulting in an error rate of 1.84% over six measurements. Additionally, the RSD for the standard solution with a concentration of 80 ppm is 1.74%, yielding a precision value of 98.34% and a 2/3 CV Horwitz of 5.96, which corresponds to an error rate of 1.741%.

These results, as shown in table 2, demonstrate that the method employed meets the necessary requirements, specifically that %SBR is less than or equal to 2/3 CV Horwitz (detailed calculations can be found in Appendix 4.7). According to ICH (2006), an accepted relative standard deviation value is less than 2.00%. A lower RSD signifies better agreement between the test results. The standard deviation values derived from the three concentrations of the standard caffeine solution conform to these requirements, suggesting that the method used is accurate and suitable for analyzing caffeine extracts in coffee mistletoe leaves.

Table 2. Precision and accuracy of standard analysis

Concentration (ppm)	RSD (%)	Precision (%)	2/3 CV Horwitz
40	1.66	98.34	6.21
60	1.84	98.16	6.17
80	1.74	98.26	5.96

Caffeine content

The caffeine content in coffee mistletoe leaves was determined using the visible spectrophotometry method. The caffeine content in the coffee mistletoe leaf extract has an average caffeine concentration of 0.042%. Since this is the first study to assess the caffeine content in coffee mistletoe leaves, there is no comparative data available. Therefore, half-old Robusta coffee leaves were used as a reference for comparison. The results show that the caffeine content in coffee mistletoe leaf powder is relatively low compared to the half-old Robusta coffee leaves analyzed by [15], which contain 0.12% caffeine. The lower caffeine content in coffee mistletoe can be attributed to its growth habits; coffee mistletoe feeds and survives by attaching itself to the stem of the coffee plant, whereas coffee leaves are an integral part of the coffee plant itself. Consequently, the caffeine concentration in coffee leaves is higher than that in coffee mistletoe.

CONCLUSION

This study found that dichloromethane is a better solvent than chloroform for extracting caffeine from coffee mistletoe leaves (*Dendrophthoe petandra* (L.) Miq.). Using dichloromethane, we extracted 0.042 × 10-2 g of caffeine, while chloroform yielded only 0.023 × 10-2 g. We analyzed the caffeine in the leaves using visible spectrophotometry, which met all required standards. The method showed a strong relationship between concentration and measurement, with a correlation coefficient (r) of 0.9974. The limit of detection (LOD) was 1.81 ppm, and the limit of quantitation (LOQ) was 6.02 ppm. The method's accuracy ranged from 90.0% to 97.5%, based on recovery tests. We checked precision with standard solutions at concentrations of 40, 60, and 80 ppm, resulting in precision values of 98.34%, 98.16%, and 98.26%. The 2/3 CV Horwitz values for these concentrations were 6.21, 6.17, and 5.96. In conclusion, the caffeine content in the coffee mistletoe leaf powder (*Dendrophthoe petandra* (L.) Miq.) was found to be 0.042% when extracted with dichloromethane.

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