

Total Alkaloid Determination in Ethanolic Extract of Cocoa (*T. cacao*) and Robusta Coffee (*C. canephora*) Beans using TLC-Densitometric Method

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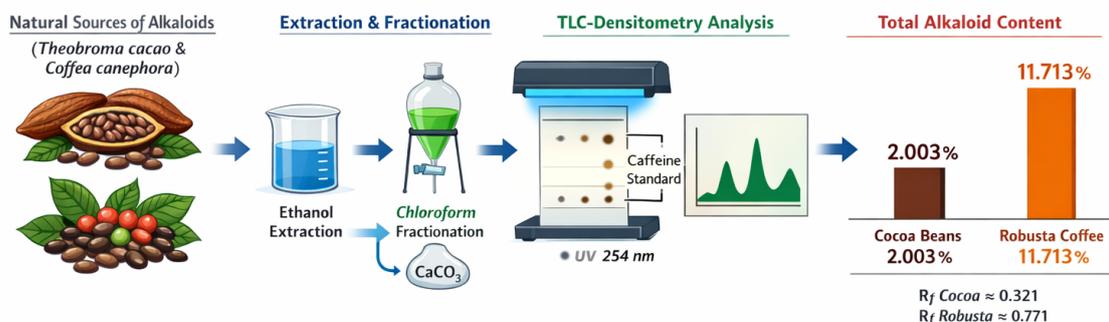
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GRAPHICAL ABSTRACT



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ABSTRACT

Alkaloids are a major class of bioactive secondary metabolites commonly found in various natural products, including cocoa (*Theobroma cacao* L.) and robusta coffee (*Coffea canephora*). These compounds have been widely reported to exhibit important pharmacological activities, including antioxidant, antidiabetic, antifungal, and antibacterial effects, making them valuable candidates for the development of pharmaceutical and functional herbal products. This study aimed to provide reliable quantitative data on the total alkaloid content of ethanolic extracts from cocoa (*Theobroma cacao* L.) and robusta coffee (*Coffea canephora*) beans using the TLC-densitometric method, thereby supporting the standardization, quality control, and potential pharmaceutical applications of these natural materials. However, alkaloid was extracted and fractionated from cocoa and robusta coffee beans using ethanol and chloroform. The total alkaloids in the ethanolic extracts of cocoa and robusta coffee beans were identified by TLC-Densitometry, with average Retardation factors (R_f) of 0.321 and 0.771, respectively. Caffeine was used as a standard. The quantification of total alkaloid in the ethanolic extract of cocoa and robusta coffee beans was 2.003% per 50 mg and 11.713% w/w per 10 mg extract, respectively. In summary, cocoa and robusta coffee beans show promising potential as herbal medicines because they contain substantial levels of pharmacologically active alkaloid compounds.

1. INTRODUCTION

Cocoa and coffee remain major agricultural commodities in Indonesia: for example, in 2023, the country produced about 632,100 tons of cocoa beans and over 700,000 tons of coffee beans, with coffee cultivation spanning hundreds of thousands of hectares nationwide [1]. Cultivation and national research on cocoa and coffee are established at the Indonesian Coffee and Cocoa Research Institute (CCRI) in Jember, East Java [2, 3]. Cocoa (*Theobroma cacao* L.) beans are reported to contain chemical compounds, known as phytochemicals, including flavonoids, tannins, terpenoids, saponins, and alkaloids that are beneficial as antioxidants [4], antifungal [5], antibacterial [6, 7], and antidiabetic [8]. In line, coffee beans are known to have similar phytochemical content [9] and have various benefits to the human body, including antioxidant, antibacterial, and central nervous system stimulation [10-12]. Robusta coffee (*Coffea canephora*) beans contain several compounds, including alkaloids, flavonoids, tannins, saponins, steroids, volatiles (aldehydes, furfural, ketones, alcohols, esters, formic acid, and acetic acid), phenolics (chlorogenic acid), and caffeine [13].

Alkaloids are a type of secondary metabolite compound containing nitrogen atoms (N) that can be found in plant and animal tissues. Alkaloids are present in various plant parts, including flowers, seeds, leaves, twigs, roots, and bark [14]. Caffeine is a compound derived from the secondary metabolism of the alkaloid group in the coffee plant and has a bitter taste [15]. Alkaloid compounds can be obtained using an extraction method that separates a mixture of solvent and solute. Various extraction methods are available, including maceration [16], Soxhlet extraction [17], percolation [18], and modern methods such as ultrasonic and microwave extraction. Following extraction, fractionation can be performed to separate the main compounds of interest and eliminate impurities in the extract [19].

Quantification of alkaloid can be conducted using the caffeine (Figure 1) as standard solution with various analysis methods, such as Ultraviolet-Visible spectrophotometry (UV-VIS) method [20], High Performance Liquid Chromatography (HPLC) method [21], gravimetric method [22], Thin Layer Chromatography Digital picture method (TLC-Digital pic) [23], TLC-GCMS method [24], and TLC-Fluorescence [25]. Moreover, identification and determination of alkaloids in herbs can be performed using TLC-densitometry, which is developed based on the TLC pharmacopoeia. The method has several advantages, such as the ability to quantify directly on suspension or turbid samples, simultaneous analysis, ease with simple tools, and results that are valid and reliable [26]. The TLC-densitometric method is based on chromatographic principles, in which a mixture is separated into two phases, namely a mobile phase and a stationary phase [27].

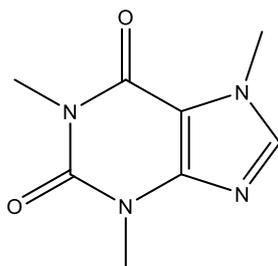


Figure 1. Structural formula of caffeine

Based on the background above, the total alkaloid content of cocoa and robusta coffee beans needs to be determined, as the abundant alkaloids can be utilized in the pharmaceutical industry as raw materials for traditional medicine. For example, the plant *Catharanthus roseus* has been used for centuries in traditional medicine for conditions such as diabetes, hypertension, and infections, and modern pharmacology has successfully isolated its alkaloids (e.g., Vincristine and Vinblastine), which are used as clinically important anticancer drugs [28]. Thus, this study aimed to quantify the total alkaloid content in ethanolic extracts of cocoa and robusta coffee beans using the TLC-densitometric method. This method was selected because it offers several advantages over other analytical techniques, including cost-effectiveness, ease of operation, and the ability to separate and quantify compounds in complex plant matrices simultaneously. Unlike direct spectrophotometric methods, TLC-densitometry provides chromatographic separation prior to quantification, thereby

improving specificity and accuracy, while requiring less sophisticated instrumentation than high-performance liquid chromatography (HPLC). Moreover, previous studies have demonstrated that TLC-densitometric methods can achieve good linearity, precision, and accuracy when properly validated, making them suitable and reliable for routine quantitative analysis of alkaloids in plant extracts [29, 30].

2. EXPERIMENTAL METHODS

2.1. Materials

Cocoa (*Theobroma cacao* L.) and robusta coffee (*Coffea canephora*) beans were obtained from the Indonesian Coffee and Cocoa Research Institute (CCRI), Jember, East Java, Indonesia. They were taxonomically determined at the State Polytechnic of Jember with the number 54/PL17.8/PG/2023. The chemicals used in this study include analytical-grade ethanol 96% (Merck), CaCO₃ (Merck), chloroform (Merck), Caffeine standard (Sigma-Aldrich, batch number 0000181333), Silica gel 60 F254 plate (Merck, analytical grade), and methanol (Merck). The equipment included an oven (Mettler), a TLC chamber, a densitometer (Camag), a rotary evaporator (Heidolph), glassware (Pyrex), a UV254 Lamp, and an analytical balance (Ohaus).

2.2. Methods

2.2.1 Extraction and fractionation process

Cocoa and robusta coffee beans were extracted using the maceration method. Herein, 500 grams of bean samples were placed in a glass jar, and 2 liters of 96% ethanol were added until the samples were submerged, with no replication. The maceration process was carried out at room temperature (25–30 °C) for 5 days, protected from light, with occasional stirring to optimize extraction [31]. Filtration was performed to separate the pulp from the filtrate. The obtained filtrate was concentrated using a waterbath at 50 °C. Subsequently, the residue obtained from the initial filtration was remacerated with 1 liter of 96% ethanol, soaked for 24 hours at room temperature, and filtered and concentrated on a water bath [32].

The concentrated extract was fractionated using a liquid-liquid extraction method. Fifty milligrams of cocoa and 10 mg of robusta coffee bean concentrated extract were added to a 1.5% w/v CaCO₃ solution in distilled water for filtration. Calcium carbonate (CaCO₃) was added to the concentrated extract to raise the pH of the aqueous solution, facilitating conversion of alkaloid salts to their free base forms and enhancing their partitioning into the organic phase during liquid-liquid extraction, a strategy supported by recent studies on pH-modified extraction of alkaloids and similar compounds [33]. The filtrate was extracted using 25 mL of chloroform in a separatory funnel. The chloroform phase was re-extracted twice and collected. Subsequently, the chloroform phase was evaporated (in a vaporizer cup) on a waterbath until all chloroform disappeared and formed a thick extract. Each of the thick cocoa and robusta coffee bean extracts was dissolved in 96% ethanol as a sample solution for downstream analysis [34].

2.2.2. Preparation of standard solution

Caffeine was used as a standard because it is a xanthine alkaloid with a similar structure to the compound to be tested (total alkaloid) [35, 36]. Fifty milligrams of caffeine was dissolved in chloroform to obtain a caffeine stock solution at a concentration of 2000 ppm. A serial dilution of a caffeine standard was prepared at concentrations ranging from 200 to 2000 ppm to accommodate differences in alkaloid content between cocoa and robusta coffee bean extracts and to increase the accuracy of the results [37]. In TLC-densitometric quantification, constructing a calibration curve from multiple standard concentrations improves analytical accuracy by reducing regression uncertainty and ensuring the analyte responses fall within the instrument's linear range. This approach has been successfully applied in validated methods for alkaloids and other compounds, with high correlation coefficients ($R^2 \approx 0.98$ – 0.9998) and acceptable recovery values obtained [38].

2.2.3. Identification and determination of alkaloid using TLC

The presence of alkaloids in the ethanolic extract of cocoa and robusta coffee beans was identified using TLC. The TLC used a silica gel 60 F₂₅₄ plate as the stationary phase and chloroform

: ethanol (99 : 1 v/v) as the mobile phase. The mobile phase composition of chloroform : ethanol (99 : 1 v/v) was selected because it provided optimal separation of alkaloids on the silica gel 60 F₂₅₄ plate; chloroform effectively mobilizes moderately polar alkaloids, while the small proportion of ethanol increases the elution strength sufficiently to prevent spot tailing and improve resolution, resulting in clear and well-defined chromatographic bands suitable for densitometric analysis. In this study, each solution (caffeine standards at various concentrations, cocoa bean extract, and robusta coffee extract) was applied to the silica gel plate at a constant volume of 20 μ L per spot. Using a consistent volume for each spot ensures that the amount of analyte deposited on the stationary phase is controlled, which facilitates comparison of spot intensity and retention factor (R_f) values across standards and samples. Such uniform application is standard practice in caffeine TLC procedures to minimize variability caused by differing spot sizes or solvent effects during development, and to optimize the resolution of analyte bands on the plate [39]. The alkaloid was allowed to separate from the sample solutions until the mobile phase reached the upper limit of the plate. The alkaloid spot was identified under UV light at 254 nm and determined by the retardation factor (R_f) value. The R_f value is the ratio of the distance traveled by the compound to the distance traveled by the mobile phase. A reliable R_f value on TLC ranges from 0.2 - 0.8 cm [37].

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by mobile phase}}$$

2.2.4. Quantification of total alkaloid

Analysis of total alkaloid content in cocoa bean and robusta coffee bean extracts was performed using thin-layer chromatography coupled with densitometry (TLC-densitometry). Densitograms obtained from caffeine standard solutions at various concentrations were used to construct a calibration curve. The relationship between the densitometric peak area and the amount of caffeine applied per spot was evaluated using linear regression analysis, a widely accepted quantitative approach in TLC-densitometric studies. Therefore, detailed reporting of all densitometer operating conditions is necessary for other laboratories to replicate the analytical procedure and validate the results [40]. The total alkaloid mass in the extract was calculated by considering the total extract volume and dilution factor, using the formula:

$$\text{Total alkaloid mass (mg)} = \frac{x \times V \times DF}{1000}$$

where:

- x = amount of alkaloid per spot (μ g),
- V = total volume of extract (mL),
- DF = dilution factor (if any),
- 1000 = conversion factor from micrograms to milligrams.

Expressing results as total alkaloid content in terms of caffeine equivalents is consistent with validated quantification practices in densitometric studies of caffeinated samples and complex matrices [41, 42].

3. RESULTS AND DISCUSSIONS

Cocoa (*Theobroma cacao* L.) and robusta coffee (*Coffea canephora*) beans are a source of phytochemicals. One of the most abundant phytochemicals in their beans is alkaloids in the form of caffeine. Qualitative analysis of total alkaloids is required to determine their abundance, as they have potential as a source of raw material in traditional medicine. Alkaloids from cocoa and robusta coffee beans were obtained through extraction and fractionation using 96% ethanol and chloroform, respectively. Maceration was selected as the extraction method because it is practical, simple, and conducted at room temperature without the need for a heating step. In extraction methods performed at room temperature, caffeine and other co-extracted alkaloids are not at risk of heat-induced degradation, because typical processing temperatures are far lower than the onset of thermal transformation/degradation [43, 44].

The identification of alkaloids in the cocoa and robusta coffee bean extracts was carried out using TLC, which was based on the R_f value. The TLC was chosen as a reliable method for alkaloid

quantification based on TLC analysis [45-47]. Moreover, direct quantification of the compound in the suspension sample can be performed simultaneously using TLC [48]. The TLC used silica gel F₂₅₄ as the stationary phase, which contains silanol and siloxane groups. Prior to use, the TLC plates were activated using an oven at 110°C for 30 minutes to remove remaining solvent from washing and activating the silanol and siloxane groups for optimal interaction with the analyte [40]. Moreover, TLC used chloroform : ethanol at a ratio of 99 : 1 v/v for improved separation of polar alkaloid in the extract to reach R_f value in the range of 0.2 - 0.8. The result is in agreement with a previous study, which reported that the separation of alkaloid-containing compounds from pandan leaf extract using a chloroform–ethanol mobile phase yielded an average R_f value of 0.4 [49]. The TLC separation results of cocoa and robusta coffee bean ethanolic extract are presented in Figure 2.

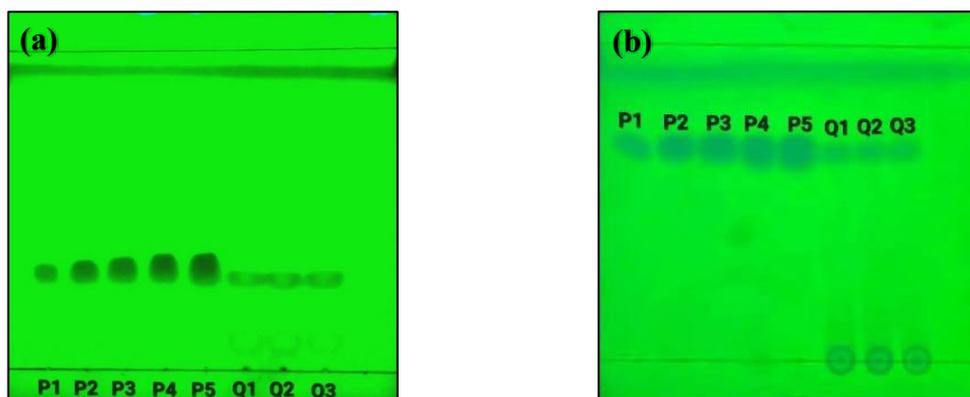


Figure 2. Thin-layer chromatographic (TLC) profiles of (a) cocoa bean extract and (b) robusta coffee bean extract developed on silica gel F₂₅₄ plates with chloroform : ethanol (99 : 1, v/v) as the mobile phase and visualized under UV light at 254 nm. Caffeine standards (P1 – P5) at concentrations of 200 – 1000 ppm for cocoa and 500 – 2000 ppm for robusta coffee, along with extract samples (Q1 – Q3) at 2000 ppm, were spotted at 20 μ L per spot. The chromatographic separation was assessed based on the R_f values of the detected spots.

Figure 2a shows the TLC chromatographic profile of the cocoa bean ethanolic extract alongside caffeine standard solutions (P1 – P5) developed on silica gel F₂₅₄ using chloroform : ethanol (99 : 1, v/v) as the mobile phase. The caffeine standards produce distinct, well-defined spots with increasing intensity as the concentration increases, indicating good linearity between concentration and spot intensity. The extract samples (Q1–Q3) exhibit spots at R_f values comparable to those of the caffeine standards, suggesting the presence of alkaloid compounds, predominantly caffeine or caffeine-related alkaloids, in the cocoa bean extract. The clear separation and minimal tailing demonstrate that the selected mobile phase provides adequate resolution for alkaloid analysis in cocoa beans. Figure 2b presents the TLC profile of the robusta coffee bean ethanolic extract together with caffeine standards (P1–P5). The standards exhibit consistent migration behavior and increasing spot intensity with increasing concentration, confirming the suitability of the chromatographic system for densitometric quantification. The extract samples (Q1–Q3) exhibit intense spots with R_f values closely matching those of the caffeine standards, indicating a higher alkaloid content in robusta coffee beans than in cocoa beans. The stronger signal intensity observed in the extract lanes reflects the naturally higher caffeine concentration in robusta coffee, supporting the reliability of TLC-densitometry for comparative alkaloid analysis.

The detection and determination of alkaloids in the cocoa and robusta coffee bean extracts were carried out using the stain alignment method and R_f calculation, respectively [50]. Stain alignment was performed by spot alignment of the caffeine standard and the ethanolic extract during mobile phase optimization (unpublished data). The distance-based similarity between the standard and the sample indicates that the sample contains phytochemicals similar to those in the standard [51].

TABLE I. The R_f value calculation of cocoa bean ethanolic extract

Vial	R_f Value	Correlation
Caffeine ₂₀₀	0.319	0.997766
Caffeine ₄₀₀	0.326	0.997766
Caffeine ₆₀₀	0.328	0.998540
Caffeine ₈₀₀	0.330	0.999541
Caffeine ₁₀₀₀	0.333	0.999923
Cocoa ₁	0.335	0.998492
Cocoa ₂	0.300	0.998177
Cocoa ₃	0.297	0.997952

TABLE II. The R_f value calculation of robusta coffee bean ethanolic extract

Vial	R_f Value	Correlation
Caffeine ₅₀₀	0.768	0.997236
Caffeine ₁₀₀₀	0.771	0.997236
Caffeine ₁₂₅₀	0.777	0.999958
Caffeine ₁₅₀₀	0.789	0.999930
Caffeine ₂₀₀₀	0.793	0.999641
Robusta coffee ₁	0.769	0.998933
Robusta coffee ₂	0.755	0.997696
Robusta coffee ₃	0.752	0.996434

The R_f value indicated the degree of separation between samples on TLC, ranging from 0.2 to 0.8. The calculated R_f values for the caffeine standard at all concentrations tested and for the ethanolic extract across all replications were within the accepted range. The calculation of the R_f value is shown in Table 1 for cocoa bean ethanolic extract and Table 2 for robusta coffee bean ethanolic extract. The results showed that cacao and robusta coffee beans contain alkaloids, as they produced R_f values in a similar range on TLC analysis. The difference in R_f value between cacao ethanolic extract and robusta coffee ethanolic extract might be due to variations in their bean alkaloid content, which required adjustment of the caffeine standard concentration to improve quantitative data accuracy. These differences resulted in the spot shifting of the caffeine standard and all the sample spots. The observed difference in R_f values between the ethanolic extracts of cacao and robusta coffee may be attributed to differences in their alkaloid compositions. Cocoa extract is predominantly rich in theobromine, whereas coffee robusta contains a higher proportion of caffeine. Because these methylxanthine alkaloids interact differently with the polar silica gel stationary phase due to their structural and polarity differences, the migration behavior on TLC plates differs, resulting in distinct R_f values under identical chromatographic conditions [52].

Prior to quantification of total alkaloids in cocoa and robusta coffee bean extracts, optimization was required to determine the concentrations of the standards and their detection wavelengths. After several preliminary experiments (unpublished data), caffeine standard solution stock was prepared at a concentration of 2000 ppm from caffeine isolate, followed by its serial dilution at concentrations of 200, 400, 600, 800, and 1000 ppm for the cocoa and at concentrations of 500, 1000, 1250, 1500, and 2000 ppm for robusta coffee bean. Both ethanolic extracts were prepared at a concentration of 2000 ppm, and 3 replicate samples were analyzed to achieve optimal accuracy and consistency [21].

Additionally, to optimize wavelength detection of alkaloids in the extract, a serial dilution of a caffeine standard was scanned, yielding a wavelength of 275 nm (Figure 3), indicating improved detection sensitivity [21]. Based on the alkaloid structure, they have conjugated double-bond groups that absorb UV light at 275 nm, a wavelength characteristic of chromophore compounds [51]. In agreement, this method yielded the same wavelength as other methods used to determine the optimal detection wavelength, such as HPLC and HPTLC [21, 27].

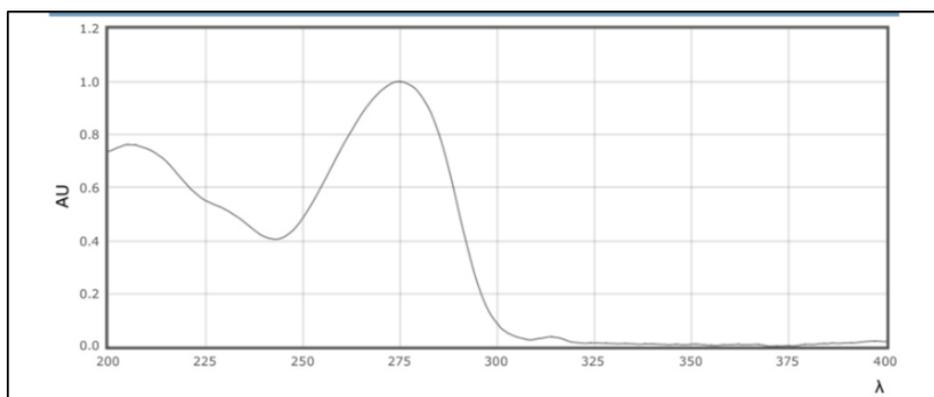


Figure 3. Optimization of wavelength detection in cocoa beans and robusta coffee bean ethanolic extract exhibited the most optimal detection of caffeine in the extract to be at 275 nm.

To determine the total alkaloid content in cocoa and robusta coffee bean extracts [53], the densitometry method was employed by scanning the TLC plate with a densitometer, producing a peak on the densitogram shown in Figure 4.

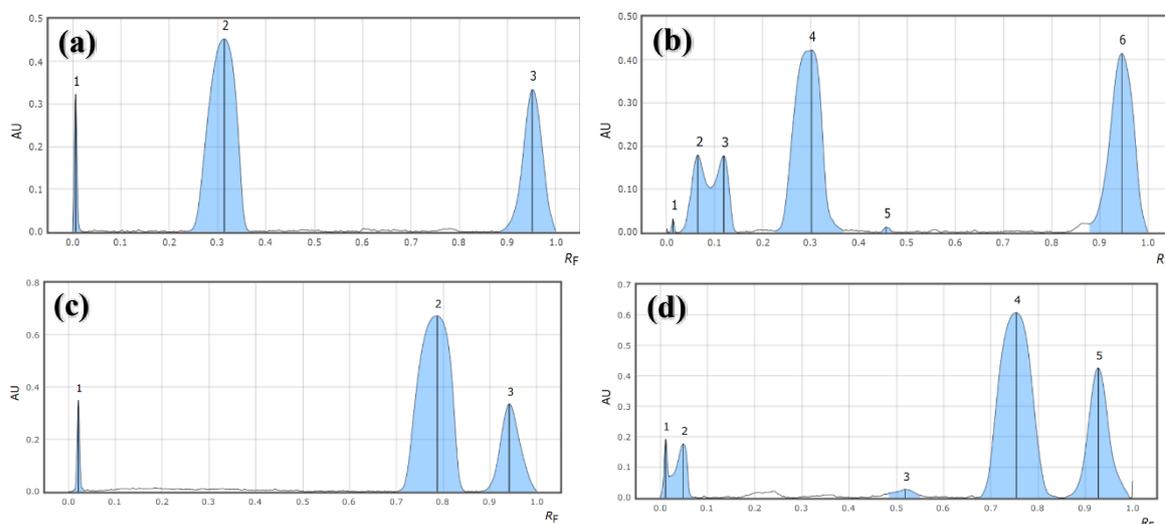


Figure 4. Representative densitogram of (a) caffeine standard at concentration of 200 ppm (b) caffeine in cocoa bean ethanolic extract, (c) caffeine standard at concentration of 500 ppm (d) caffeine in robusta coffee bean ethanolic extract.

The area of the densitogram peak was calculated and plotted against concentration using linear regression to produce a standard curve for caffeine, as shown in Figure 5.

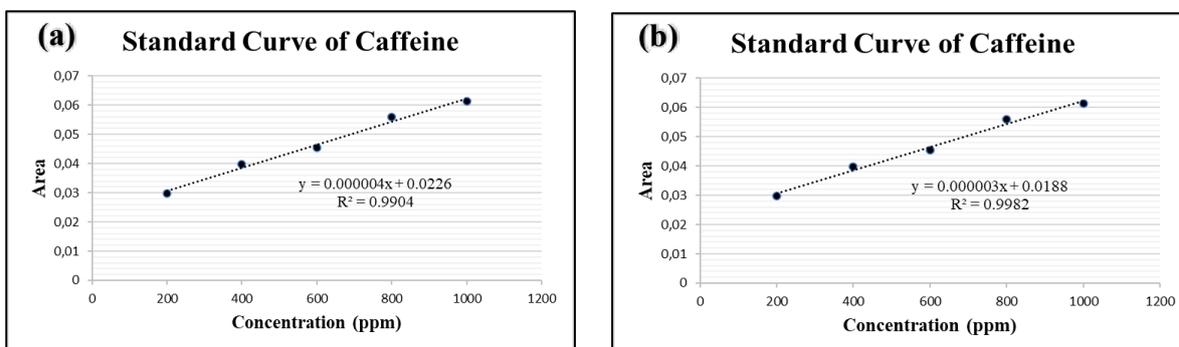


Figure 5. Standard curve of caffeine for quantification of total alkaloid in (a) cocoa bean and (b) robusta coffee bean ethanolic extract.

Subsequently, the total alkaloid content of the cocoa and robusta coffee bean ethanolic extracts was determined by plotting the sample area on the caffeine standard curve [53]. The results are shown in Tables 3 and 4. On average, the total alkaloid content of 50 mg of cocoa bean ethanolic extract is

0.02003 mg, or about $2.003\% \pm 0.001$ b/b, with a relative standard deviation (RSD) of 4.99%. This result is consistent with a previous study, which showed that the total alkaloid content in cocoa beans is between 0.8 and 3.7%, leading to antibacterial activity by damaging the bacterial peptidoglycan [54].

TABLE III. Total alkaloid in cocoa bean ethanolic extract

Sample	Area	Concentration mg/mL	% Total Alkaloid Content (w/w)	Average % Total Alkaloid Content (w/w)	%RSD
Cocoa ₁	0.03939	1.049	2.098 %	2.003 % \pm 0.001	4.99 %
Cocoa ₂	0.03851	0.994	1.988 %		
Cocoa ₃	0.03799	0.962	1.924 %		

RSD : relative standard deviation

The total alkaloid in robusta coffee bean ethanolic extract is about 0.117133 mg in 10 mg extract or as much as $11.713\% \pm 0.005$ w/w with RSD of 4.87%. These results are higher than those reported in a previous study by Wulandari and Marpaung [55], who used a single robusta coffee extract containing total alkaloid levels of 0,776% mg. The differences in total alkaloid content among the extracts could be attributed to variations in analytical detection methods, such as the use of UV - Vis spectrophotometry, as well as differences in sample sources, including robusta coffee beans cultivated in different geographical areas and at varying altitudes.

TABLE IV. Total Alkaloids in robusta coffee bean ethanolic extract

Sample Name	Area	Concentration mg/mL	% Total Alkaloid Content (w/w)	Average % Total Alkaloid Content (w/w)	% RSD
Robusta coffee ₁	0.03253	1.144	11.440 %	11.713% \pm 0.005	4.87 %
Robusta coffee ₂	0.03289	1.174	11.740 %		
Robusta coffee ₃	0.03316	1.196	11.960 %		

RSD : relative standard deviation

Together, this study showed that cocoa and robusta coffee beans from CCRI, Jember, East Java, contain caffeine, which represents the total alkaloid content in the ethanolic extracts. The total alkaloid content of these ethanolic extracts can be determined by TLC-densitometry with high precision, as indicated by low RSD values in repeat measurements. Further research is required to develop herbal medicine, as it contains considerable amounts of phytochemicals, including alkaloids with pharmacological activity [13].

4. CONCLUSIONS

The total alkaloid content in cocoa and robusta coffee beans is on average 2.003% and 11.713% in 50 mg and 10 mg ethanolic extracts, respectively. Optimum optimization in this study used chloroform : ethanol mobile phase with different ratios in each sample. Cocoa and coffee beans were analyzed using a chloroform : ethanol (99 : 1, v/v) mobile phase. Standard concentrations of caffeine in cocoa beans and robusta coffee beans were 200, 400, 600, 800, and 1000 ppm, respectively, with a volume of 20 μ L. The sample concentrations in cocoa beans and robusta coffee beans were 2000 ppm. The wavelength used in the study was 275 nm. The results for total alkaloid content in cocoa beans were 2.003%, and in robusta coffee beans, 11.713%.

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