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Effect of Extraction Methods on Total Alkaloid Content of *Mimosa pudica* L. Leaves

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ABSTRACT
Mimosa pudica L. leaves contain alkaloids with potential pharmacological activities, and the extraction method may influence alkaloid recovery. This study aimed to analyze the effects of maceration, Soxhlet, and reflux extraction on the total alkaloid content of Mimosa pudica L. leaf extracts. A comparative laboratory experimental design was employed using dried leaf powder extracted with 70% ethanol. Qualitative alkaloid identification was performed using Wagner's and Dragendorff's reagents, while total alkaloid content was determined by UV–Visible spectrophotometry using the bromocresol green (BCG) ion-pair complex method and caffeine as the reference standard. Data were analyzed using the Shapiro–Wilk normality test, Levene's homogeneity test, one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test. Screening confirmed alkaloids in all extracts. The mean total alkaloid contents obtained by maceration, reflux, and Soxhlet extraction were 45.54, 17.45, and 15.23 mg caffeine equivalent per gram of extract (mg CE/g extract), respectively. ANOVA revealed a significant difference among extraction methods ($F = 722.847$, $p < 0.001$). Tukey's HSD test showed that maceration produced significantly higher total alkaloid content than reflux and Soxhlet extraction ($p < 0.001$). However, the difference between reflux and Soxhlet was not statistically significant ($p = 0.102$). In conclusion, maceration yielded the highest total alkaloid content and may be considered a suitable conventional extraction method for obtaining Mimosa pudica L. leaf extracts with higher alkaloid content.
Keywords: *Mimosa pudica L.; maceration; Soxhlet; reflux.*

Introduction

Indonesia possesses abundant medicinal plant resources that have long been utilized in traditional medicine. The utilization of these resources needs to be supported by scientific evidence through standardization processes, including the determination of active compounds obtained from extraction (Marhaeni, 2020; Sakka & Muin, 2023). Among medicinal plants widely used in traditional medicine, *Mimosa pudica* L., commonly known as putri malu, has attracted attention because of its empirical use in the treatment of inflammation, insomnia, pain, diabetes, and wound healing (Prahayu et al., 2024).

Phytochemical investigations have demonstrated that *Mimosa pudica* contains various secondary metabolites, including flavonoids, saponins, tannins, and alkaloids. Alkaloids are important bioactive constituents that have been associated with various pharmacological activities, including analgesic, anti-

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inflammatory, antimicrobial, anticancer, and neuromodulatory (Nayaken et al., 2023). Therefore, the determination of total alkaloid content is an important parameter in the standardization of *M. pudica* leaf extracts and may provide quantitative information regarding the quality of the resulting extract.

The recovery of alkaloids is strongly influenced by the extraction method used. Three conventional extraction methods commonly applied in phytochemical research are maceration, Soxhlet extraction, and reflux extraction. These methods differ in their extraction principles, temperature conditions, solvent contact, and extraction duration. Maceration is generally performed at room temperature, Soxhlet extraction involves continuous solvent circulation, whereas reflux extraction is conducted under heating conditions (Tutik et al., 2022). Differences in these extraction conditions may affect the recovery and stability of secondary metabolites. Previous studies involving other medicinal plants have demonstrated that extraction methods can produce different levels of secondary metabolites. Khasanah and Muslihin (2025) reported that maceration produced the highest total alkaloid content in Papua batik leaves compared with Soxhlet and reflux extraction. Similarly, Olivia Nayaken et al. (2023) found that maceration produced higher alkaloid content than ultrasonic extraction in *Chromolaena odorata*. Maryam et al. (2023) also reported that maceration was more effective than other extraction methods for extracting flavonoids from sapodilla leaves.

Quantitatively, mimosine has been reported as one of the principal alkaloids identified in *Mimosa pudica* L. Nair et al. (2007) reported a mimosine content of approximately 1.96 mg/g (0.2% w/w) using methanol–water extraction followed by reversed-phase high-performance thin-layer chromatography (RP-HPTLC). In addition to serving as a chemical marker, mimosine has been reported to exhibit antimicrobial activity against *Staphylococcus aureus* and dermatophytic fungi, supporting its potential contribution to the biological activity of *M. pudica* L. extracts (Anitha et al., 2005). Similarly, Champanerkar et al. (2010) reported a comparable mimosine content of approximately 1.94 mg/g using acid extraction with 1% HCl followed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Although these studies successfully quantified mimosine, they primarily focused on the determination of a specific alkaloid using more advanced analytical techniques rather than evaluating total alkaloid content under different conventional extraction methods.

Previous research has also confirmed the qualitative presence of alkaloids in *M. pudica* leaves (Prahayu et.al, 2024). However, to the best of our knowledge, limited information is available regarding the quantitative comparison of conventional extraction methods, particularly maceration, Soxhlet extraction, and reflux extraction, for determining the total alkaloid content of *M. pudica* L leaves. This research gap is important because the selection of an appropriate extraction

method is essential for obtaining extracts with optimal alkaloid content and for supporting the standardization of medicinal plant raw materials. In addition, findings from other plant species cannot necessarily be directly applied to *M. pudica* L because differences in plant species, chemical composition, and compound stability may influence extraction efficiency.

To quantitatively evaluate the total alkaloid content, UV–Visible spectrophotometry was selected as the analytical method. This technique has been widely applied in phytochemical research because of its relatively simple procedure, adequate accuracy and precision, and lower analytical cost compared with more advanced instrumental methods (Nayaken et al., 2023). The availability of UV–Visible spectrophotometry in the laboratory also supports its practical application for the quantitative analysis of plant extracts.

The efficiency of alkaloid extraction is fundamentally governed by the applied thermal conditions, extraction duration, and solvent-to-sample dynamics. Alkaloids, including mimosine found in *M. pudica* L, often exhibit varying degrees of thermal sensitivity; prolonged exposure to elevated temperatures can trigger degradation, oxidation, or structural transformations. While conventional methods such as maceration, Soxhlet, and reflux provide distinct mechanical advantages, their varying thermal intensities and operational cycles directly dictate the final quantitative recovery and chemical stability of the extracted secondary metabolites. Consequently, understanding how specific extraction parameters correlate with alkaloid stability remains a critical prerequisite for standardizing herbal extracts (Zhang et al., 2018).

Therefore, this study aimed to analyze the effect of maceration, Soxhlet, and reflux extraction methods on the total alkaloid content of *Mimosa pudica* L. leaf extract using UV–Visible spectrophotometry and to determine the extraction method that produces the highest total alkaloid content. The findings are expected to provide quantitative information regarding the selection of a suitable conventional extraction method and contribute to the standardization of *M. pudica* L. leaf extract as a potential herbal medicinal raw material.

Methods

This study employed a comparative laboratory experimental design to evaluate the total alkaloid content of *Mimosa pudica* L. leaf extracts obtained using maceration, Soxhlet, and reflux extraction methods. The study was conducted at the Research Laboratory of Universitas 17 Agustus 1945 Jakarta, Indonesia, from March to July 2026. As the study involved plant materials and did not involve human participants or experimental animals, ethical approval was not required. The experimental procedures were adapted, with modifications, from previously

published extraction and spectrophotometric methods (Khasanah & Muslihin, 2025; Maryam et al., 2023).

Dried *M. pudica* L. leaf simplicia was obtained from UPT Laboratorium Herbal Materia Medica Batu, East Java, Indonesia, where botanical identification had been performed. The material underwent organoleptic examination, was ground into powder, and was passed through a 40-mesh sieve to obtain a relatively homogeneous particle size. The powdered material was stored in a tightly closed container protected from light and moisture until extraction.

The extraction solvent was 70% ethanol. Analytical reagents included bromocresol green (BCG), caffeine, 2 N sodium hydroxide (NaOH), 2 N hydrochloric acid (HCl), sodium phosphate, phosphoric acid, analytical-grade chloroform, and distilled water. Caffeine was used as the reference standard because a purified, plant-specific alkaloid reference standard was not available for this study. The main instruments included a UV–Visible spectrophotometer, Soxhlet apparatus, reflux apparatus equipped with a condenser, rotary evaporator, water bath, separatory funnel, analytical balance, volumetric flasks, pipettes, pH meter, and supporting laboratory glassware.

For each extraction method, 40 g of powdered *M. pudica* L. leaves was used with 70% ethanol. Although the initial plant-material mass and total solvent volume were standardized at 40 g and 800 mL, respectively, the extraction methods differed in temperature, duration, solvent circulation, and extraction-cycle arrangement.

Maceration. The powdered leaves (40 g) were immersed in 800 mL of 70% ethanol at room temperature (25–30°C) for three consecutive days. The mixture was filtered every 24 h, and the residue was re-extracted with fresh 70% ethanol, giving three extraction cycles in total. The filtrates were combined and concentrated using a rotary evaporator at 40–50°C until a viscous extract was obtained.

Soxhlet extraction. The powdered leaves (40 g) were extracted using 800 mL of 70% ethanol at 70–74°C for approximately 6–8 h, until the solvent in the siphon tube became clear. The resulting extract was collected and concentrated using a rotary evaporator at 40–50°C.

Reflux extraction. The powdered leaves (40 g) were refluxed with 400 mL of 70% ethanol at 30–45°C for 2 h. The mixture was then filtered, and the residue was subjected to a second reflux cycle using another 400 mL of fresh 70% ethanol under the same conditions for 2 h. The filtrates from both cycles were combined. The combined extract was initially concentrated using a rotary evaporator at 40–50°C; concentration was subsequently completed using a water bath when the rotary-evaporation process was discontinued. This additional handling step was recorded because it differed from the concentration procedure used for the other extracts.

The total solvent volume for each extraction method was 800 mL, corresponding to an overall plant-material-to-solvent ratio of 1:20 (w/v). For reflux

extraction, each individual cycle used a ratio of 1:10 (w/v). Differences in temperature, extraction duration, solvent circulation, number of extraction cycles, and post-extraction handling were considered when interpreting the comparative results. Therefore, differences in measured alkaloid content were interpreted in relation to the overall extraction procedures rather than attributed exclusively to temperature. The extraction yield was calculated as the percentage ratio of the concentrated extract mass to the initial mass of dried simplicia (Rizwan et al., 2022).

Qualitative alkaloid identification was performed using Wagner's and Dragendorff's reagents. Approximately 1 mg of each concentrated extract was dissolved in 1 mL of 2 N HCl, diluted with distilled water, and filtered. Two drops of Wagner's reagent were added to the filtrate; the formation of a brown precipitate was interpreted as a positive reaction. In a separate test, two drops of Dragendorff's reagent were added, and the formation of a brick-red precipitate was interpreted as a positive alkaloid reaction (Nayaken et al., 2023). These tests were used for preliminary qualitative screening and were not considered specific identification of individual alkaloid compounds.

A caffeine stock solution of 1,000 ppm was prepared by dissolving 100 mg of caffeine in hot distilled water and diluting it to 100 mL in a volumetric flask. A 100 ppm intermediate solution was prepared by transferring 5 mL of the stock solution into a 50 mL volumetric flask and diluting to volume. Working standard solutions at concentrations of 2, 4, 6, 8, and 10 ppm were prepared by dilution (Nayaken et al., 2023).

The BCG reagent was prepared by dissolving 6.98 mg of BCG in 3 mL of 2 N NaOH and 5 mL of distilled water. The solution was heated at 50–60°C for 15 min and then diluted to 100 mL with distilled water. Phosphate buffer at pH 4.7 was prepared using sodium phosphate and phosphoric acid according to the procedure used in the study (Patel et al., 2015).

The maximum absorption wavelength (λmax) was determined by scanning a 100 ppm caffeine solution over the wavelength range of 200–400 nm using a UV–Visible spectrophotometer (Nayaken et al., 2023). The maximum wavelength obtained was 273 nm.

The operating time of the caffeine–BCG complex was evaluated by measuring absorbance at 5-min intervals for 60 min after reacting 1 mL of 100 ppm caffeine solution with BCG reagent and phosphate buffer at pH 4.7, followed by extraction with 5 mL of chloroform. Based on the operating-time assessment, an operating time of 50 min was selected for subsequent measurements (Nayaken et al., 2023).

For sample preparation, 10 mg of each concentrated extract was dissolved in ethanol and diluted to 10 mL to obtain a nominal concentration of 1,000 ppm. A 100

ppm working solution was prepared by transferring 1 mL of the stock solution into a 10 mL volumetric flask and diluting to volume with ethanol.

For the analysis, 2 mL of the working solution was mixed with 5 mL of phosphate buffer at pH 4.7 and 5 mL of BCG reagent. Subsequently, 5 mL of chloroform was added. The mixture was extracted using a separatory funnel, and the chloroform layer containing the alkaloid–BCG complex was collected in a 10 mL volumetric flask and diluted to volume with chloroform. Absorbance was measured at 273 nm after the selected operating time.

The caffeine calibration curve was constructed using the standard concentrations of 2, 4, 6, 8, and 10 ppm. The regression equation obtained in this study was (y = 0.0165x + 0.0672), with a coefficient of determination ((R^2)) of 0.9805. The sample absorbance values were substituted into the calibration equation to estimate the corresponding caffeine-equivalent concentrations. The results were expressed as milligrams of caffeine equivalent per gram of concentrated extract (mg CE/g extract) (Fazel et al., 2025; Khasanah & Muslihin, 2025).

Caffeine was used as a reference standard rather than as a specific chemical marker for *M. pudica* L. Consequently, the reported values represent operational estimates relative to caffeine under the BCG spectrophotometric procedure and should not be interpreted as the absolute concentrations of all individual alkaloids or of a specific alkaloid such as mimosine.

The analytical assessment reported in this study comprised the caffeine calibration curve and the operating-time assessment. The calibration curve was evaluated using its regression equation and coefficient of determination, while the operating-time assessment was used to select the measurement time for subsequent analyses. However, the study did not establish a complete analytical validation covering precision, accuracy or recovery, limits of detection (LOD), limits of quantification (LOQ), and selectivity in the plant-extract matrix. These characteristics were therefore not claimed as validated performance parameters. The calibration and operating-time results were interpreted as preliminary analytical assessments, and this limitation was considered when interpreting the quantitative findings in accordance with the general principles of analytical-procedure validation (International Council for Hamonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), 2023.

Results were summarized as mean ± standard deviation (SD). Normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was assessed using Levene's test. When the assumptions of normality and homogeneity were satisfied, differences in measured alkaloid content among the maceration, Soxhlet, and reflux groups were evaluated using one-way analysis of variance (ANOVA). When the ANOVA result was statistically significant, Tukey's honestly significant

difference (HSD) post hoc test was used to identify pairwise differences. Statistical significance was set at ($p < 0.05$).

Each extraction method was performed in three independent extraction replicates (n = 3 per method). Each replicate was subsequently analyzed using the same spectrophotometric procedure. The results were expressed as mean ± standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test, while homogeneity of variance was evaluated using Levene's test. Differences in alkaloid content among the three extraction methods were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons when a significant difference was observed. A p-value of < 0.05 was considered statistically significant.

Result and Discussion

1.1 Plant Identification

The botanical identification confirmed that the plant material used in this study was *Mimosa pudica* L. The authenticated simplicia was obtained from UPT Laboratorium Herbal Materia Medica Batu, East Java. Botanical authentication is an important prerequisite for phytochemical research because the reliability of phytochemical analysis depends on the correct identification of the plant material. Misidentification or contamination with other species may affect the chemical profile and compromise the interpretation of analytical results (Ahmad et al., 2012). Therefore, confirmation of the botanical identity provided a basis for evaluating the differences in extraction yield and caffeine-equivalent alkaloid content among the extraction procedures investigated in this study.

1.2 Extraction Yield

The extraction of *M. pudica* L leaves was performed using maceration, Soxhlet extraction, and reflux extraction with 40 g of dry simplicia per method. The extraction yields and qualitative screening results are summarized in Table 1.

Table 1. Extraction Yield of *Mimosa pudica L.* Leaves

Extraction Method	Simplicia Weight (g)	Crude Extract Weight	Yield (%)
Maceration	40	6.5	16.23
Soxhlet	40	6.0	15.00
Reflux	40	0.7	1.75

Extraction yield was calculated as the percentage ratio of the concentrated extract weight to the initial dry simplicia weight. Maceration produced the highest

extraction yield (16.25%), followed by Soxhlet extraction (15.00%), whereas reflux extraction produced the lowest yield (1.75%).

The relatively similar yields obtained from maceration and Soxhlet extraction may be associated with their prolonged contact between the plant material and the extraction solvent. Maceration involved three consecutive extraction cycles over three days, while Soxhlet extraction involved continuous solvent circulation for approximately 6–8 h. Although these methods differ in temperature and extraction mechanisms, both procedures may facilitate the transfer of soluble constituents from the plant matrix into the solvent. However, extraction yield represents the total mass of recovered soluble material and does not necessarily indicate the concentration of a particular class of bioactive compounds (Zhang et al., 2018).

In contrast, the reflux method produced a substantially lower extraction yield than the other two methods. This result differs from the yields reported for reflux extraction of other medicinal plants, including sapodilla leaves (7.7%) (Maryam et al., 2023) and Papua batik leaves (14%) (Khasanah & Muslihin, 2025). Although heating may facilitate solvent penetration and the release of soluble constituents from plant tissues (Zhang et al., 2018), the low yield observed in the present study should not be interpreted as evidence that reflux extraction is inherently less efficient.

An important methodological limitation concerns the post-extraction concentration of the reflux filtrate. The rotary evaporation process was discontinued because of its prolonged duration, and concentration was subsequently completed using a water bath. This handling differed from the concentration procedure used for the other extracts and may have contributed to sample loss during solvent evaporation and transfer. Differences in evaporation efficiency and material retention on laboratory glassware may also have affected the final mass of the concentrated extract (Khasanah & Muslihin, 2025). Consequently, the low reflux yield may reflect both the extraction procedure and the subsequent handling of the extract. This limitation is particularly important when comparing extraction yields across the three methods.

Overall, the extraction yield results demonstrate that the greatest mass of concentrated extract was obtained through maceration, while the lowest was obtained through reflux extraction. Nevertheless, these results must be interpreted alongside the quantitative alkaloid analysis because a higher extraction yield does not necessarily correspond to a higher caffeine-equivalent alkaloid content.

1.3 Qualitative Alkaloid Analysis

Qualitative alkaloid screening was performed as a preliminary assessment of the presence of alkaloids in the extracts obtained using maceration, Soxhlet, and

reflux extraction. Wagner's and Dragendorff's reagents were used, and the observations are presented in Table 2.

Table 2. Qualitative Alkaloid Screening of *Mimosa pudica L.* Leaf Extracts

Extraction Method	Reagent	Observation	Result
Maceration	Dragendorff	Orange to brick-red precipitate	Positive (+)
Maceration	Wagner	Brown precipitate	Positive (+)
Soxhlet	Dragendorff	Orange to brick-red precipitate	Positive (+)
Soxhlet	Wagner	Brown precipitate	Positive (+)
Reflux	Dragendorff	Orange to brick-red precipitate	Positive (+)
Reflux	Wagner	Brown precipitate	Positive (+)

All three extracts produced positive reactions with both reagents. The formation of brown precipitates with Wagner's reagent and orange to brick-red precipitates with Dragendorff's reagent indicated the presumptive presence of alkaloid compounds in the extracts, irrespective of the extraction method employed.

These reactions are based on the formation of precipitates through interactions between alkaloid-containing samples and the reagent components. Wagner's reagent contains iodine and potassium iodide, while Dragendorff's reagent contains bismuth-based complexes that produce characteristic precipitates in the presence of many alkaloids (Nayaken et al., 2023). The positive reactions therefore provide preliminary qualitative evidence of alkaloids in the extracts.

The results are consistent with previous studies reporting positive alkaloid screening in medicinal plant extracts obtained using different extraction procedures. Olivia Nayaken et al. reported positive reactions with Wagner's and Dragendorff's reagents in *Chromolaena odorata* leaf extracts obtained through maceration and ultrasonic extraction (Rizwan et al., 2022). Khasanah and Muslihin (2025) also reported positive alkaloid screening in extracts obtained using maceration, Soxhlet, and reflux methods.

Although all extracts tested positive, the qualitative reactions do not establish that the extracts contain equal amounts of alkaloids. These tests indicate the presumptive presence of alkaloid constituents but do not identify individual compounds or quantify their concentrations. In particular, the positive reactions cannot confirm the presence or concentration of mimosine specifically. Therefore, quantitative analysis was required to compare the caffeine-equivalent alkaloid content of the extracts obtained using the three methods.

1.4 Spectrophotometric Method Optimization (Maximum Wavelength and Operating Time)

1. Maximum Wavelenght Determination

The maximum absorption wavelength (λmax) of the caffeine–Bromocresol Green (BCG) system was determined by scanning the UV–Visible spectrum over the wavelength range of 200–400 nm. The highest absorbance was observed at 273 nm (Ajanal et al., 2012). This wavelength was subsequently used for the calibration curve, operating-time assessment, and quantitative analysis of the extracts.



Figure 1. UV–Visible Spectrum for Maximum Wavelength Determination of the Caffeine–BCG Complex (Pharmaceutical Research Laboratory, Faculty of Pharmacy, Universitas 17 Agustus 1945 Jakarta, Jakarta, Indonesia)

The selection of an appropriate maximum absorption wavelength is important for consistent spectrophotometric measurements. Measuring at the wavelength of maximum absorbance can improve the analytical response to changes in concentration under the selected measurement conditions (Nayaken et al., 2023).

The wavelength obtained in this study was 273 nm, which was close to the value of 275 nm reported by Khasanah and Muslihin (2025) for Papua batik leaf extracts. However, Olivia Nayaken et al. (2023) reported a wavelength of 292 nm for their analytical system involving *Chromolaena odorata* extracts. Differences in the measured wavelength may be associated with variations in the sample matrix, reagent preparation, buffer conditions, solvent system, or instrumental settings.

The wavelength of 273 nm was therefore selected for subsequent measurements under the experimental conditions used in the present study. Nevertheless, wavelength agreement with a previous study should be interpreted as a comparison of analytical conditions rather than as independent confirmation of method selectivity or complete analytical validity. Further validation would be

needed to establish the performance of the method for quantitative determination in the specific plant extract matrix.

2. Operating Time Determination

The operating time was evaluated to determine when the absorbance of the caffeine–BCG system became sufficiently stable for spectrophotometric measurement. Absorbance was measured at five-minute intervals over a total period of 60 min after preparation of the reaction mixture and extraction with chloroform.

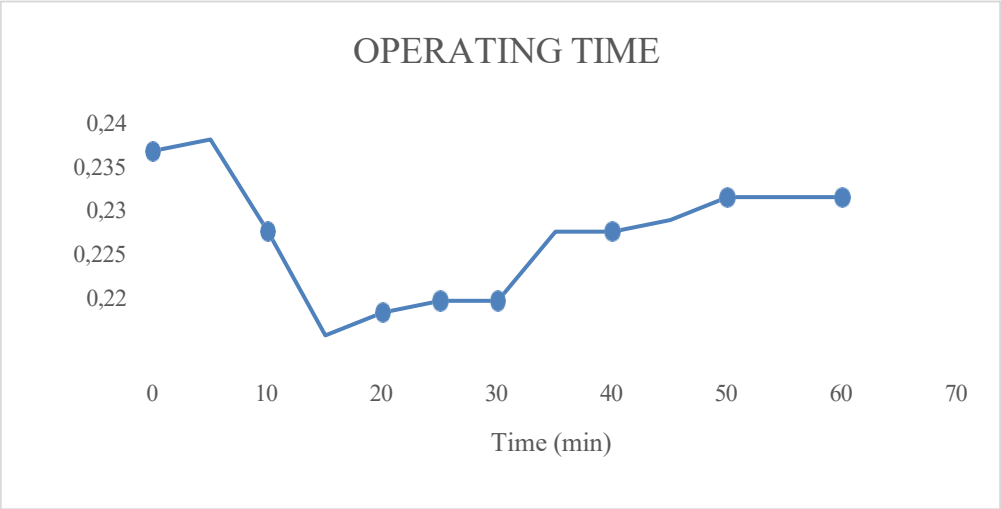


Figure 2. Determination of the Operating Time of the Caffeine–BCG Ion-Pair Complex

The absorbance fluctuated during the initial measurement period but became relatively stable after approximately 50 min, remaining stable through the 60-min observation period. The absorbance at the stable stage was approximately 0.234. Based on this observation, an operating time of 50 min was selected for subsequent measurements.

Determining the operating time is important because measurements taken before the absorbance has stabilized may introduce additional variation into quantitative results. Selecting a consistent measurement time helps standardize the analytical procedure across samples.

The observed stabilization period was broadly comparable to that reported by Olivia Nayaken et al., who found that absorbance for a caffeine–BCG system stabilized within approximately 45–60 min (Nayaken et al., 2023). This similarity supports the use of a 50-min measurement time under the conditions investigated here. However, the operating-time assessment demonstrates absorbance stability over the observation period and should not, by itself, be interpreted as proof of the overall precision, accuracy, or selectivity of the analytical method.

1.5 Calibration Curve

A caffeine calibration curve was prepared using standard solutions at concentrations of 2, 4, 6, 8, and 10 ppm. Absorbance increased with increasing caffeine concentration, indicating a positive relationship between the measured absorbance and the concentration of the reference standard.

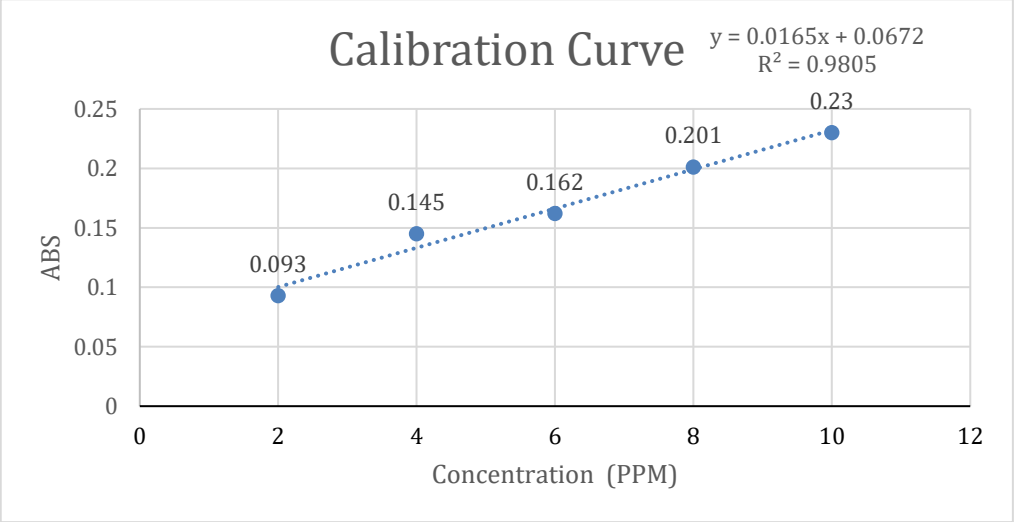


Figure 2. Caffeine Calibration Curve

Linear regression produced the equation:

$$y = 0.0165x + 0.0672$$

with a coefficient of determination (R^2) of 0.9805 and a correlation coefficient (r) of 0.9902. This equation was used to convert sample absorbance into caffeine-equivalent concentrations for the quantitative comparison of the extracts.

The observed positive relationship is consistent with the expected concentration-dependent response in UV–Visible spectrophotometry within a suitable working range (Mayerhöfer et al., 2020). The R^2 value indicates that the calibration data showed a positive linear trend over the tested concentration range. Khasanah and Muslihin reported an R^2 value of 0.9681 for the determination of total alkaloids in Papua batik leaves using a similar analytical approach (Khasanah & Muslihin, 2025).

However, the coefficient of determination alone is insufficient to establish complete analytical validity. The calibration results should be interpreted as evidence of the observed relationship between caffeine concentration and absorbance under the experimental conditions. Additional assessments, such as precision, recovery or accuracy, selectivity, and limits of detection and quantification where appropriate, would be required to characterize the method more comprehensively.

Caffeine was used as the reference standard because a commercially available mimosine standard was not accessible for this study. Accordingly, the

quantitative results were expressed as milligrams of caffeine equivalent per gram of extract (mg CE/g extract). These values are operational estimates based on the response of the sample in the caffeine calibration system. They should not be interpreted as the absolute concentration of mimosine or as the chemically specific concentration of every alkaloid present in *M. pudica* L leaves.

1.6 Total Alkaloid Content of *Mimosa pudica L*. Leaf Extracts

The caffeine-equivalent alkaloid content of the extracts was determined using the UV–Visible spectrophotometric procedure based on the BCG ion-pair system. Sample absorbance was converted into caffeine-equivalent concentration using the calibration equation (y = 0.0165x + 0.0672), and the results were expressed as mg CE/g extract.

Table 3. Caffeine-Equivalent Alkaloid Content of *Mimosa pudica* L. Leaf Extracts

Extraction Method	Replicate	Absorbance	Concentration (µg/mL)	Total Alkaloid Content (mg CE/g extract)
Maceration	I	0.144	4.65	46.55
	II	0.140	4.41	44.12
	III	0.143	4.59	45.94
	Mean	**0.142**	**4.55**	**45.54**
Soxhlet	I	0.094	1.62	16.24
	II	0.093	1.56	15.64
	III	0.090	1.38	13.82
	Mean	**0.092**	**1.52**	**15.23**
Reflux	I	0.097	1.81	18.06
	II	0.096	1.75	17.45
	III	0.095	1.68	16.85
	Mean	**0.096**	**1.75**	**17.45**

Maceration produced the highest mean caffeine-equivalent alkaloid content at 45.54 ± 1.26 mg CE/g extract. Reflux extraction produced a mean of 17.45 ± 0.61 mg CE/g extract, while Soxhlet extraction produced the lowest mean at 15.23 ± 1.26 mg CE/g extract. Thus, the descriptive ranking was maceration, followed by reflux and Soxhlet extraction.

The BCG method provides a spectrophotometric response based on interactions between suitable protonated compounds and the BCG reagent under the specified analytical conditions. A buffer is used to control the reaction environment, and chloroform is used to extract the resulting ion-pair system into the organic phase. Related ion-association approaches have been applied in spectrophotometric analyses of alkaloids and other compounds (Saad et al., 1997).

In the present study, caffeine served as the reference standard because a mimosine standard was unavailable. Consequently, the values reported in Table 3 represent caffeine-equivalent responses rather than compound-specific quantification (Fazel et al., 2008; Khasanah & Muslihin, 2025).

The higher mean value obtained through maceration may be associated with differences in solvent contact, extraction duration, temperature, and the overall handling of the plant material. Maceration was conducted at room temperature over three consecutive extraction cycles, whereas Soxhlet extraction involved continuous solvent circulation at 70–74°C for approximately 6–8 h. Reflux extraction was conducted in two successive heating cycles, with filtration and replacement of the solvent between cycles.

Prolonged heating can potentially affect the stability of heat-sensitive constituents. However, the present study did not directly measure the degradation of individual alkaloids or determine the stability of mimosine during extraction. Therefore, thermal degradation is a possible explanation for the lower mean value obtained with Soxhlet extraction, rather than a demonstrated mechanism. Other factors, including differences in extraction efficiency, solvent circulation, the relative recovery of non-alkaloid constituents, and post-extraction handling, may also have contributed to the observed results.

Although reflux produced a higher mean caffeine-equivalent content than Soxhlet extraction, the difference between these methods was not statistically significant in the reported Tukey HSD analysis ($p = 0.102$). Therefore, the numerical difference between their means should not be interpreted as evidence that reflux extraction yielded a statistically greater alkaloid content than Soxhlet extraction.

The interpretation of the reflux result also requires particular caution because its post-extraction concentration procedure differed from that of the other extracts. The rotary evaporation process was discontinued and completed using a water bath. This difference may have influenced the mass and composition of the final extract and may have affected the measured caffeine-equivalent content. Consequently, the results reflect the performance of the complete extraction and handling procedures used in this study, rather than the isolated effect of the extraction technique alone.

Taken together, the findings indicate that maceration produced the highest measured caffeine-equivalent alkaloid content under the conditions investigated. Nevertheless, the quantitative comparison should be interpreted in light of the reference-standard limitation, the small number of independent replicates, and the differences in extraction and post-extraction procedures.

1.7 Comparison with Previous Studies

The finding that maceration produced the highest measured caffeine-equivalent alkaloid content is consistent with reports from studies investigating

extraction methods in other medicinal plants. Olivia Nayaken et al. (2023) reported a higher alkaloid content in macerated Chromolaena odorata leaves (8.259%) than in samples obtained through ultrasonic-assisted extraction (5.938%). Khasanah and Muslihin (2025) reported the highest total alkaloid content in macerated Papua batik leaves (144.6 mg/g), followed by Soxhlet extraction (48.6 mg/g) and reflux extraction (4.65 mg/g). These studies provide contextual evidence that different extraction procedures can yield different measured alkaloid contents.

However, comparisons across studies should be made cautiously because the plant species, chemical composition, extraction conditions, analytical procedures, and reporting units may differ. The results from *Chromolaena odorata* and *Graptophyllum pictum* cannot be directly generalized to *M. pudica* leaves. Likewise, values expressed as percentages or milligrams per gram are not directly comparable with the caffeine-equivalent values reported in the present study unless the analytical basis and calculation methods are equivalent.

A difference was observed between the relative performance of Soxhlet and reflux extraction in the present study and the findings reported by Khasanah and Muslihin. In the current study, reflux produced a higher mean caffeine-equivalent content than Soxhlet extraction (17.45 versus 15.23 mg CE/g extract), although the difference was not statistically significant. In the Papua batik study, the reported Soxhlet content was higher than the reflux content (Khasanah & Muslihin, 2025). This variation may reflect differences in plant matrices, solvent conditions, extraction duration, temperature, and other procedural factors. It does not establish that one method will consistently outperform the other across different medicinal plant species.

The current Soxhlet procedure used a temperature of 70–74°C for approximately 6–8 h, whereas reflux was performed at 30–45°C in two successive cycles. Differences in thermal exposure may have influenced the recovery or stability of some constituents. Nevertheless, because temperature, duration, solvent circulation, extraction cycles, and subsequent handling differed simultaneously, their individual contributions cannot be isolated from the present experimental design. The lower Soxhlet mean should therefore not be attributed solely to thermal degradation.

Another important observation is that extraction yield was not proportional to measured caffeine-equivalent alkaloid content. Maceration and Soxhlet extraction produced relatively similar extraction yields (16.25% and 15.00%, respectively), but their mean caffeine-equivalent contents differed substantially (45.54 and 15.23 mg CE/g extract). This finding indicates that total extract mass does not necessarily correspond to the relative amount of material contributing to the analytical response used for alkaloid estimation.

Extraction yield includes all recovered soluble constituents, not exclusively alkaloids. Differences in the recovery of non-alkaloid compounds may therefore affect the mass of the final extract without producing a proportional increase in the caffeine-equivalent measurement. Because the present study did not quantify individual alkaloids or non-alkaloid constituents separately, the contribution of these components cannot be determined directly. The results nevertheless demonstrate why extraction yield and caffeine-equivalent alkaloid content should be interpreted as distinct parameters.

The particularly low reflux yield also needs to be considered in relation to the post-extraction concentration procedure. Since concentration was completed using a water bath after rotary evaporation was discontinued, the final yield may have been affected by differences in solvent removal and sample handling. This limitation reduces the extent to which the reflux yield can be compared directly with the yields from maceration and Soxhlet extraction. Future comparisons should standardize the concentration procedure across all methods and include a larger number of independent extraction replicates.

Overall, the present results are broadly consistent with the view that extraction procedures influence the measured recovery of plant constituents. However, the most suitable method should be evaluated for each plant species and analytical objective rather than inferred solely from comparisons with other plants or from extraction yield alone.

1.8 Statistical Analysis

Statistical analysis was performed to evaluate differences in caffeine-equivalent alkaloid content among maceration, Soxhlet, and reflux extraction. Each method was represented by three independent extraction replicates ($n = 3$ per group), provided that each replicate corresponded to a separately conducted extraction process. The small number of replicates was considered when interpreting the statistical results.

The Shapiro–Wilk test was used to assess normality, and Levene's test was used to evaluate homogeneity of variance. The Shapiro–Wilk results were not statistically significant for maceration ($p = 0.465$), Soxhlet ($p = 0.459$), or reflux ($p = 0.991$). Levene's test also indicated no statistically significant evidence of unequal variances (Levene statistic = 1.367, $p = 0.324$). These findings did not indicate a violation of the assessed normality and homogeneity assumptions. However, with only three observations per group, assumption tests have limited ability to detect departures from their underlying assumptions, and their results should be interpreted cautiously.

Table 4. Summary of Statistical Analysis Results for Total Alkaloid Content

Analysis Type / Test	Parameter / Source of Variation	Value / Statistical Output	p-value	Interpretation
Normality Test ***(Shapiro–Wilk)***	Maceration	Statistic = 0.924	0.465	Normal distribution
	Soxhlet	Statistic = 0.922	0.459	Normal distribution
	Reflux	Statistic = 1.000	0.991	Normal distribution
Homogeneity Test ***(Levene's Test)***	Levene Statistic ($df_1 = 2, df_2 = 6$)	1.367	0.324	Homogeneous variances
One-Way ANOVA	Between Groups (Sum of Squares = 1711.894)	F=722.847	< 0.001	Significant difference
Tukey's HSD Post Hoc	Maceration vs. Soxhlet	Mean Diff=30.303	< 0.001	Significant
	Maceration vs. Reflux	Mean Diff=28.083	< 0.001	Significant
	Soxhlet vs. Reflux	Mean Diff=2.220	0.102	Not significant

The post hoc results support the conclusion that maceration produced a higher mean caffeine-equivalent alkaloid content than both heat-assisted methods under the experimental conditions. Although the mean for reflux extraction was numerically higher than that for Soxhlet extraction, the pairwise comparison did not demonstrate a statistically significant difference between these two methods. A non-significant result does not establish that the methods are equivalent; rather, the analysis did not provide sufficient evidence of a difference at the specified significance level.

The pattern observed in the present study is broadly consistent with reports in which maceration produced higher measured secondary-metabolite content than alternative extraction procedures (Maryam et al., 2023). Nevertheless, differences in plant species, experimental design, analytical method, and statistical approach limit direct comparisons across studies. In particular, statistical significance in one study

does not establish that the same pattern will occur under different experimental conditions.

The statistical findings should also be interpreted in the context of the experimental design. Three independent extraction replicates per method provide limited information about variability between extraction runs. Consequently, the estimated means, standard deviations, and significance tests may be sensitive to individual observations, and the findings should not be generalized beyond the conditions investigated without further evidence. Repeated instrumental measurements of the same extract should not be treated as independent extraction replicates.

In addition, the comparison involved differences in extraction temperature, duration, solvent circulation, extraction cycles, and post-extraction concentration. The analysis therefore evaluates differences among the complete procedures rather than the independent effect of temperature or another single factor. The deviation in the reflux concentration procedure is particularly relevant when interpreting both its low extraction yield and its caffeine-equivalent content.

Taken together, the statistical analysis indicates that maceration produced a significantly higher mean caffeine-equivalent alkaloid content than Soxhlet and reflux extraction in this experiment. The difference between Soxhlet and reflux was not statistically significant despite their different descriptive means. Further studies using a larger number of independent extraction replicates, standardized post-extraction processing, and a more fully validated analytical method are needed to confirm the reproducibility and broader applicability of these findings.

Conclusion

This study evaluated the effects of three conventional extraction methods (maceration, Soxhlet, and reflux extraction) on the total alkaloid content of *Mimosa pudica* L. leaves using UV–Visible spectrophotometry. One-way ANOVA revealed a statistically significant difference among the extraction methods ($F = 722.847, p < 0.001$). The mean total alkaloid contents obtained for maceration, reflux, and Soxhlet extraction were 45.54 ± 1.26, 17.45 ± 0.61, and 15.23 ± 1.26 mg caffeine equivalent (CE)/g extract, respectively. Tukey's HSD post hoc test confirmed that maceration produced a significantly higher total alkaloid content than both Soxhlet ($p < 0.001$) and reflux extraction ($p < 0.001$), whereas the difference between Soxhlet and reflux extraction was not statistically significant ($p = 0.102$).

Under the specific experimental conditions evaluated in this study, maceration yielded the highest caffeine-equivalent total alkaloid recovery. However, because the evaluated methods differed simultaneously in extraction temperature, duration, solvent circulation, number of extraction cycles, and post-

extraction concentration procedures, the observed differences reflect the cumulative impact of the complete operational procedures rather than the isolated effect of extraction temperature alone.

Suggestions

1. Procedural Standardization & Parameter Control: Future research should standardize the post-extraction concentration (evaporation) process across all treatment groups and independently control variables such as thermal exposure duration, solvent-to-sample ratio, and post-extraction handling steps to evaluate the isolated effect of each parameter.
2. Method Validation & Compound-Specific Quantification: Further studies should perform comprehensive analytical method validation (evaluating precision, accuracy/recovery, limit of detection, and limit of quantification) using a plant-specific alkaloid reference standard (such as mimosine) via advanced chromatographic techniques (e.g., HPLC or LC–MS/MS).
3. Bioactivity Evaluation: Subsequent investigations should evaluate the biological activities (such as antioxidant, antimicrobial, or anti-inflammatory activities) of the macerated extract to confirm whether the higher caffeine-equivalent alkaloid recovery translates to enhanced pharmacological efficacy.

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