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Determination Levels of Caffeine Isolated from Robusta Coffee Beans from Pagar Alam in Wistar Rat Blood

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ABSTRACT

Coffee provides a genuine source of caffeine. Caffeine can have a variety of pharmacological effects on both central and peripheral nervous systems and most of these effects are related to the antagonistic mechanism at adenosine receptors. The most prominent effect of caffeine remains stimulation of brain function, improving mood and physical performance. Robusta coffee obtains an intense level of caffeine and Pagar Alam City is one of the cities in Indonesia that produces robusta coffees. This study aims to identify and characterize caffeine isolated from Pagar Alam robusta coffee beans using thin-layer chromatography methods, UV-Visible spectrophotometry and to determine caffeine levels in wistar rat blood using the ELISA method. This method is cost-effective, familiar to perform and has a considerable degree of accuracy and reproducibility. The isolated samples have similar characteristics to standard caffeine with an Rf value of 0.77, a maximum wavelength at 273 nm with the absorbance of 1.3 and from caffeine profile in the blood sample we obtained the maximum sample concentration in blood 0.582 ± 0.249 µg/ml and the time needed to reach the maximum concentration is 30 minutes.

Keywords: caffeine, TLC, UV-Vis spectrophotometry, ELISA, robusta coffee beans, pagar alam.

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INTRODUCTION

Caffeine is an alkaloid derivative of xanthine (purine base) that is naturally abundant in coffee (Suwiyarsa et al., 2018). Indonesia is one of the largest coffee producers in the world after Brazil, Vietnam, and Colombia. Coffee can be easily found in Indonesia, from low-quality coffee to the best quality (Zarwinda & Sartika, 2019). There are generally two types of coffee, *Coffea arabica* and *Coffea canephora* (Goldemberg et al., 2014). The selection of robusta coffee samples is due to robusta coffee (*Coffea canephora* var. Robusta) having higher levels of bioactive components such as caffeine, and Pagar Alam robusta coffee is the best robusta coffee in Indonesia (Goldemberg et al., 2014) (Pabendon et al., 2017).

In its pure form, caffeine is a white crystalline powder that is fairly soluble in water and various types of organic solvents such as ethanol, ethyl acetate, methanol, benzene, and others. If taken from its natural source and purified, caffeine will have a white powdery form (Gebrewold & Geletu, 2018). At submillimolar concentrations, caffeine can affect various physiological functions with different effects on each organism and has long been known to cause several things, including inhibition of phosphodiesterase, thereby increasing intracellular cAMP levels, directly affecting intracellular calcium concentrations, indirectly affecting intracellular calcium concentrations through membrane hyperpolarization and antagonizing adenosine receptors (Mohammed & Al-Bayati, 2009). Through these mechanisms, caffeine consumption can trigger alertness, excitement, improve mood and cause the release of catecholamines, which will have beneficial effects on human behavior (Andrén-Sandberg, 2016). Caffeine is often added in a certain amount to drinks. Excessive caffeine consumption can cause nervousness, agitation, tremors, insomnia, hypertension, nausea, and seizures (Maramis et al., 2013). According to SNI 01-7152-2006, the maximum limit for caffeine in food and drinks is 150 mg/day and 50 mg/serving (Zarwinda & Sartika, 2019).

Caffeine is also widely used in ergogenic supplement preparations consumed during exercise/sports. The ergogenic effect of caffeine may be related to several factors, such as increased substrate utilization, delayed fatigue and staying awake (Andrén-Sandberg, 2016). Absorption and Distribution. Caffeine can be given through several routes, but oral is the most commonly used route (Alsabri et al., 2018). Caffeine is rapidly absorbed from the digestive tract (O'callaghan et al., 2018). Although caffeine is a water-soluble compound, it also has hydrophobic characteristics (log P = 0.85) that make it easy to penetrate all biological membranes (Alsabri et al., 2018). Metabolism and Excretion. Research shows that most of the caffeine consumed (>80%) is metabolized in the liver. More than 80% of the consumed caffeine is metabolized by cytochrome-450 enzyme into paraxanthine, 16% into theophylline, and 4% into theobromine (Alsabri et al., 2018). The remaining caffeine is excreted mainly through urine (O'callaghan et al., 2018).

The methods commonly used to measure the caffeine content in biological specimens are gas chromatography and high-performance liquid chromatography, which take a significant amount of time and are quite expensive. Carregaro et al., (2001) compared gas chromatography and ELISA methods to determine caffeine levels in blood. The statistical analysis of the data showed that both methods showed good correlation. The ELISA examination technique is very sensitive and selective for both quantitative and qualitative analysis of antigens, including protein compounds, peptides, nucleic acids, hormones, and secondary metabolites from plants (Sakamoto et al., 2018). The aim of this study is to isolate caffeine from robusta coffee beans and identify caffeine using thin-layer chromatography, UV-Vis spectrophotometry, and observe the caffeine content in animal test subjects over time using the ELISA method.

MATERIALS AND METHODS

The research was conducted at the Faculty of Medicine, Universitas Sriwijaya and the Balai Besar Laboratorium Kesehatan (BBLK) Palembang from December 2020 to March 2021. Extraction, isolation and characterization with thin-layer chromatography method were carried out in the Biotechnology Laboratory, characterization with UV-Vis spectrophotometry method was conducted in the Biochemistry Laboratory, in vivo tests and blood sample collection from rats were performed in the Animal House Laboratory at the Faculty of Medicine, Universitas Sriwijaya. Blood serum was centrifuged and collected at the Biotechnology Laboratory, Faculty of Medicine, Universitas Sriwijaya. The determination of caffeine levels in blood serum was done with ELISA method in the Immunology Laboratory at the Balai Besar Laboratorium Kesehatan Palembang.

Materials and Eqiupment

This research uses a descriptive experimental design that provides an overview of the characteristics of caffeine isolated from Robusta coffee beans from Pagar Alam, South Sumatra, and an overview of the caffeine level in animal test subjects' blood using the ELISA method. Identification of caffeine was performed using Thin Layer Chromatography (TLC), UV-Visible spectroscopy. In the examination of caffeine levels, the amount of blood serum used in each mixture was 5 µl (Carregaro et al., 2001).

The materials used in this research: Pagar Alam Robusta coffee beans (completed with a determination certificate), pure caffeine (Brataco), sodium carbonate/Na_2CO_3 (Merck), 70% ethyl acetate (Brataco), pure ethyl acetate (Merck), pure ethanol (Merck), pure chloroform (Merck), pure acetone (Merck), aqua dest (Brataco), Al Silica Gel GF 254 TLC Plate (Merck), Caffeine Elisa kit (Biovision, USA), blood samples taken using a 1 cc syringe.

The tools used: Hot plate (Thermo Scientific), double-beam UV spectrophotometer (Shimadzu UV-1800), analytical balance (Matrix AJ302B), analytical balance (Shimadzu AUW 220), filter paper, tissue, rotary evaporator (IKA RV 10), TLC plate (silica gel F254) (Merck), and supporting glassware such as: Erlenmeyer flask (Iwaki), stir bar, dropper pipette (Iwaki), funnel (Iwaki), measuring glass (Iwaki), separating funnel (Iwaki), thermometer (Alla France), microscope (Olympus), micropipette (Ecopipette), microtube (Axygen), Elisa Reader (Infinite F50). This study was approved by the Research Ethics Committee of the Faculty of Medicine, Sriwijaya University, Palembang, South Sumatra, Indonesia.

Obtainment of Robusta Coffee Beans Sample

The robusta coffee beans from Pagar Alam were obtained from coffee farmers in Pagar Alam and were determined by the Agriculture Department of Pagar Alam. The coffee beans were dried and stored in an airtight container in the Biotechnology laboratory.

Caffeine Extraction

Sample 200 g of coffee beans obtained from Pagar Alam were dried, ground, and then added to an Erlenmeyer flask along with 1000 ml of aquadest. The mixture was heated until boiling, then allowed to cool and filtered using filter paper. 20 g of sodium carbonate was added to the filtrate and heated until half of the mixture, then cooled and filtered. The filtrate was then transferred to a separating funnel. The extraction of the liquid was performed using 100 ml of semipolar solvent ethyl acetate for 3 repetitions. The ethyl acetate layer was collected and evaporated using a rotary evaporator to obtain the fractionated liquid extract.

Purification of Caffeine

Ten milliliters of acetone was added to the dried fraction, and then filtered with filter paper. The acetone solution was carefully evaporated at 56°C, and slowly, the acetone will evaporate and form long needle-like caffeine crystals at the bottom of a porcelain dish (Sarfraz et al., 2012), the obtained caffeine crystals were collected (Wilantari, 2018).

Identification by TLC

A sample of caffeine and standard caffeine in ethyl acetate solvent were taken. It was spotted on GF254 plate and placed into a chamber saturated with chloroform-ethanol (99:1) mobile phase. The chromatogram was then viewed under UV light at a wavelength of 254 nm (Suwiyarsa et al., 2018).

Identification by UV-Visible Spectroscopy

Ten milligrams of standard caffeine and ten milligrams of caffeine sample were weighed. Each was dissolved in 10 ml of distilled water to obtain a stock solution with a concentration of 1000 ppm. From the stock solution, 1 ml was pipetted and made into a solution with a concentration of 100 ppm. Then, a working solution was made with a series of concentrations of 1 ppm, 3 ppm, 5 ppm, 7 ppm, and 9 ppm. The ultraviolet light spectrum was recorded using a UV-Visible spectrophotometer in the range of wavelengths 200–400 nm. The determination of the maximum wavelength and the absorbance values of the sample and standard at the maximum wavelength were carried out (Zahra et al., 2019).

The In Vivo Study

This study uses 24 healthy male Wistar rats with a body weight of 150-200 grams, aged 2-3 months. The animals are kept in the Laboratory Animal House of the Faculty of Medicine at the University of Sriwijaya in Palembang. The room temperature is maintained within the range of a controlled room temperature (25 ± 2 ° C), with controlled light and dark conditions (12:12 hours) and given unlimited food and water pellets. The experiment was conducted after a 1-week acclimatization and before treatment, the test animals were fed for one night (Singh et al., 2012). In all test animals, a negative control test was carried out first by taking a 1 ml blood sample taken through the caudal vein as a blank.

The recommended dose of caffeine per serving for humans is 50 mg (Zarwinda & Sartika, 2019). This dose is then converted using the Laurence Bacharach Dosage Conversion Table (Martina et al., 2019). Test animals in group 1 were given 1 ml of distilled water as a negative control, while test animals in group 2 were given a caffeine sample dissolved in distilled water with a dose of 0.9 mg. The test drug and distilled water as a negative control were administered orally with the aid of an orogastric tube (Singh et al., 2012). Blood was then taken 1 ml at various times, i.e. at minutes: 15, 30, 45, 60, 120, 180, 240, 360, and 480 through the caudal vein using a 1 cc syringe and collected in a microtube. Before blood was taken, the rat was given topical anesthesia. The serum was obtained through the centrifugation process, then placed in a new microtube and stored at -20oC. After the experiment was over, the rats were euthanized using chloroform, and then the rats were buried (Bahrami et al., 2016).

Serum Sample Preparation

Add 20 μl of serum solution to 180 μl of serum in an Eppendorf tube, then vortex. Incubate at 37oC for 45 minutes, incubate the sample again at 85-90oC for 10 minutes. Dilute the sample 40 times using the Sample diluent (mix 5 μl of serum sample with 195 μl of Sample diluent). Use 50 μl per well for use in the examination (Dilution factor: 40) (Carregaro et al., 2001).

Caffeine Assay using ELISA

Method The ELISA used is a competitive ELISA. Prepare each reagent, standard, and sample. Add 50 μl of standard or sample per well. Then add 50 μl of conjugate working solution and 50 μl of antibody to all wells containing sample and standard. Close the microtiter plate using a plate sealer and mix well. Incubate the plate at room temperature (25°C) for 45 minutes. Clean all reagents and wash each well 4 times: add 250 μl of 1 X Wash Buffer and incubate for 30 seconds. Discard the 1 X Wash Buffer well before moving to the next washing step (the washing step is very important for accurate results). Repeat this step 3 times. Add 100 μl of TMB substrate to each well. Tap or shake the plate to ensure proper mixing. Measure OD at 650 nm for wells without caffeine

(S0). When the readings range from 0.8 to 1.0 (usually between 5-30 minutes after adding TMB substrate), add 50 μl of stop solution and gently tap the plate to ensure proper mixing. The substrate will turn blue with the presence of HRP. Measure OD at 450 nm for standards and samples using a microwell reader. The color intensity is inversely proportional to the drug/substance concentration in the sample. Samples without drugs will have a bright blue color, while samples containing drugs/substances will have a light blue color or even no color with increasing caffeine concentration in the sample (Carregaro et al., 2001).

RESULTS AND DISCUSSION

Extraction, Fractionation, and Isolation

The sample is robusta coffee powder (*Coffea canephora* var. Robusta) from Pagar Alam, South Sumatra Province. Extraction is a method used to separate organic compounds from a mixture of compounds. This technique selectively dissolves one or more compounds into an appropriate solvent. The solubility of caffeine in water is 22 mg/ml at 25°C, 180 mg/ml at 80°C and 670 mg/ml at 100°C (Chaugule et al., 2019). Therefore, in this study, the extraction of caffeine was performed with boiling water because caffeine dissolves well in boiling water (dePaula & Farah, 2019). In the water extract, not only caffeine is contained but also other compounds are also extracted, especially tannins. Tannins are phenolic compounds that are acidic so tannins are first converted into their salts using sodium carbonate which is basic (Chaugule et al., 2019). By converting tannins into their salts, tannins will change into phenolic anions that are soluble in water but not soluble in ethyl acetate (Wilantari, 2018). The use of ethyl acetate in fractionation is because caffeine is soluble in ethyl acetate and ethyl acetate has non-toxic properties (Ramalakshmi & Raghavan, 1999). Caffeine purification was performed by changing temperature and evaporating the acetone solvent to obtain caffeine crystals which were then identified using thin layer chromatography and UV-Vis spectroscopy.

The results of TLC showed that the caffeine sample spot was parallel to the standard caffeine and the Rf value between the sample was almost the same as the standard, meaning that the sample under investigation contained caffeine (Table 1). The results obtained were slightly different from the experiments conducted by Suwiyarsa et al., 2018. This is likely due to the differences in the solvent used to dissolve the material to be spotted on the TLC plate. In the experiments conducted by Suwiyarsa et al., (2018), the solvent used to dissolve caffeine was chloroform, while in this study, the solvent used to dissolve caffeine was ethyl acetate. However, the results of both studies still fall within the good TLC range value of 0.2-0.8 (Suwiyarsa et al., 2018).

Table 1. Results of Identification by Thin Layer Chromatography (TLC) Method

No	Subtances	Rf Value			
1	Sample	0.78	0.76	0.78	0.77
2	Standard	0.78			0.78

The Ultraviolet Visible (UV-Vis) Spectrophotometry method utilizes light in the ultraviolet and visible regions in the form of an electromagnetic spectrum which is used to analyze samples in the form of molecular and complex ion compounds (Hammado & Illing, 2013). In determining the maximum wavelength, 5 ppm was used as the concentration because 5 ppm is the middle value from a series of concentrations 1, 3, 5, 7 and 9 ppm (Zahra et al., 2019). The results of the identification using the UV-Vis spectroscopy method showed that the caffeine sample had a spectrum similar to the caffeine standard and the maximum wavelength of the sample was the same as the caffeine standard, at 273 nm (Table 2) (Figure 1). This is in line with literature that states that the maximum wavelength of caffeine solutions in water is 272-273 nm (dePaula & Farah, 2019). Therefore, it can be concluded that the isolated sample is caffeine. The determination of caffeine

levels in animal test blood was performed using the competitive ELISA method. The advantage of competitive ELISA is that it has high sensitivity and can detect the presence of a substance, whether antigen or antibody, even in small amounts (Aydin, 2015). In line with the study performed by Carregaro et al., (2001), the R2 value obtained from the standard curve was > 0.99.

Table 2. Results of Identification by UV-Vis Spectroscopy

No	Subtances	λ_{maks} (nm)	Absorbance
1	Sampel	273 nm	1.30
2	Standar	273 nm	1.41

From the graph, it can be seen that the peak caffeine level in the blood was achieved at 30 minutes after oral administration and the maximum concentration of caffeine in the sample blood was 0.582 ± 0.249 μg/ml (Figure 2). This is consistent with the study conducted by Iwano (2015) . Caffeine is rapidly absorbed after oral administration and the peak level in blood is usually reached within 30 minutes (Roy & Das, 2015). Molecules with very low concentrations such as peptides/proteins, hormones, vitamins, and drugs exhibit high levels of specificity to antibodies or antigens specifically developed for them. Thus, it is almost impossible for an antibody to bind to another molecule besides its antigen (Aydin, 2015). Although simple, ELISA consists of several steps. For a better understanding of this method, such as the steps for preparing the wash solution, analysis, determining the result interpretation, and others are explained in each ELISA kit. Therefore, it is important to read and understand the protocol in the ELISA kit before starting the experimental procedure with the ELISA method.

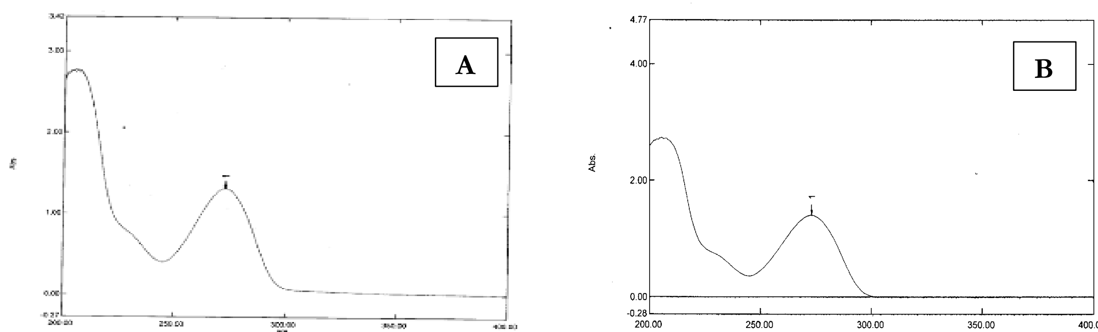

Figure 1. Spectrum of Caffeine Sample (**A**) Spectrum of Standard Caffeine (**B**)

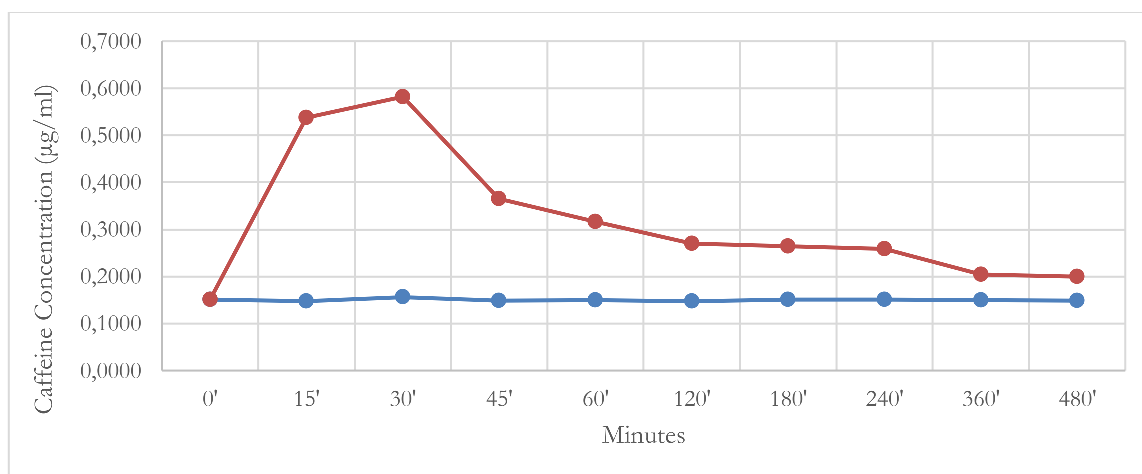

Figure 2. Graph of caffeine concentration in rat blood serum. Negative Control (blue line); Caffeine Sample (red line)

CONCLUSION

Identification and characterization of caffeine isolated from robusta coffee beans (*Coffea canephora* var. Robusta) in Pagar Alam, South Sumatra, Indonesia using thin layer chromatography and UV-Vis spectrophotometry. The results of the identification showed that the obtained compound was caffeine and an in vivo study showed a picture of the concentration of caffeine isolated in the blood of test animals measured using ELISA method.

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