




Research Article

# An Initial Investigation of the Potential of Robusta Coffee, Arabica Coffee, and Caffeine in Asthma Treatment through the Evaluation of 5-Lipoxygenase Inhibition Activity

Tegar Achsendo Yuniarta <sup>1</sup>   

Rosita Handayani <sup>2,3\*</sup>   

<sup>1</sup> Department of Pharmacy, Universitas Surabaya, Surabaya, East Java, Indonesia

<sup>2</sup> Department of Pharmacy, Universitas Airlangga, Surabaya, East Java, Indonesia

<sup>3</sup> Department of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia

\*email: [rosita.handayani@ff.unair.ac.id](mailto:rosita.handayani@ff.unair.ac.id);  
phone: +6282246930441

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## Abstract

Numerous studies have documented the potential of coffee to aid in asthma prevention. Nevertheless, research into how coffee influences asthma management has not been available. One known mechanism by which asthma medications work involves inhibiting 5-lipoxygenase (5-LOX) activity. This study aims to determine the potency of *Coffea canephora* var. Robusta extract (CRE), *Coffea arabica* extract (CAE), and caffeine are the primary isolates against 5-LOX activity. Extraction was performed by a reflux procedure using 96% ethanol with a sample-total solvent ratio of 1 : 10, an extraction time of 1 hour, and the extraction was conducted in triplicate. Fractionation was carried out by liquid-liquid partition using a chloroform-water system. Caffeine further purification was performed by the sublimation method, and the inhibition of 5-LOX activity was evaluated using the spectrophotometric method at  $\lambda = 234$  nm. Apigenin was used as a positive control. From the experiment conducted, the  $IC_{50}$  of the CRE, CAE, caffeine, and apigenin against 5-LOX was  $32.2 \pm 1.4$ ,  $42.1 \pm 2.3$ ,  $14.3 \pm 1.6$ , and  $7.4 \pm 1.7$   $\mu\text{g/mL}$ , respectively. Continued efforts to isolate bioactive compounds from coffee extract led to the discovery of caffeine, which exhibited a more potent inhibitory effect on 5-LOX. The inhibition of 5-LOX activity by caffeine occurs in a non-competitive manner.

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## INTRODUCTION

Respiratory disorders are one of the most prevalent diseases in the world. According to the World Health Organization (WHO), 262 million people worldwide suffer from asthma, and this number will increase to 400 million in 2025, where 80% of deaths from asthma occur in developing countries<sup>1</sup>. Asthma is characterized by bronchoconstriction, mucus hypersecretion, and airway inflammation<sup>2</sup>. Products from the 5-lipoxygenase (5-LOX) activity, such as leukotrienes B<sub>4</sub> and 5-hydroxy-eicosatetraenoic acid (5-HETE), are responsible for triggering bronchoconstriction and excessive mucus secretion in response to inflammation<sup>3</sup>. One approach used in the treatment of asthma is through the inhibition of 5-LOX activity. Lipoxygenase (LOX) is an oxidative enzyme that contains non-heme iron (Fe) in its active site. This enzyme initiates inflammatory reactions by triggering the formation of proinflammatory mediators known as leukotrienes. Lipoxygenase catalyzes the addition of oxygen (O<sub>2</sub>) to poly-unsaturated fatty acids (PUFAs) such as arachidonic acid and linoleic acid<sup>4,5</sup>.

5-lipoxygenase is known to have an essential role in acute inflammation and trigger cardiovascular disorders through increased leukocyte chemotaxis, blood vessel inflammation, and increased permeability of the respiratory tract membrane<sup>6</sup>. 5-lipoxygenase is also known to play an essential role in triggering asthma responses. Activated immune cells will first

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produce arachidonic acid as a result of a reaction catalyzed by phospholipase A2 in the plasma membrane then, followed by the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by 5-LOX to produce further leukotrienes, which have strong potential to cause bronchoconstriction via binding to the cysteinyl leukotriene receptor 1<sup>7,8</sup>.

Zileuton (trade name Zyflo) is the only 5-LOX inhibitor available for over 25 years<sup>9,10</sup>. Along with research assessing the effectiveness of 5-LOX inhibitors in treating asthma, research to find potential 5-LOX inhibitor candidates is ongoing, especially research on natural compounds derived from plant extracts<sup>11,12</sup>. Coffee is one of the exciting plants to be further developed as a 5-LOX inhibitor. Coffee, a significant agricultural product, yields over seven million tons of green beans annually and is the second most traded commodity worldwide. The two main species cultivated throughout the tropical world are *Coffea arabica* and *Coffea canephora* var. Robusta represents 70% and 30%, respectively, in world production<sup>13</sup>. Coffee has a positive effect on reducing the inflammatory reaction that triggers asthma. Coffee consumption has an inverse relationship with mortality due to respiratory disorders<sup>14,15</sup>.

Coffee studies in treating asthma have been carried out at the extract level. Coffee extract (CE) provides a weak bronchodilation effect and reduces muscle fatigue in the airways. Coffee extract from Ethiopia, Kenya, and Brazil inhibited LOX activity with EC<sub>50</sub> values of 2750 to 2940 µg/mL<sup>16</sup>. The compound responsible for producing the anti-asthma effect in CE is predicted to be caffeine, the dominant compound in coffee. Chemically, caffeine is similar to theophylline used as an asthma medication. One of the results of caffeine metabolism in the body is also theophylline. From this relationship, it is predicted that caffeine can provide anti-asthma effects like theophylline<sup>17</sup>. Clinical trials on 55 patients showed that caffeine consumption at doses of less than 5 mg/kg BW could improve lung function for 2 hours after use. Based on the study, caffeine in CE can inhibit the NFκB signaling pathway, vital in producing various proinflammatory cytokines and chemokines (TNF-α and IL-6) by suppressing cyclooxygenase-2 (COX2) expression<sup>18</sup>. Meanwhile, studies on caffeine activity in inhibiting LOX have never been done.

Although clinically proven, the mechanism of action of CE in the treatment of asthma is still being studied. We tested *C. canephora* var. Robusta extract (CRE), *C. arabica* extract (CAE), and caffeine as bioactive compounds on 5-LOX activity in this study. This investigation aims to determine whether CE and caffeine can hinder the creation of leukotrienes that initiate airway inflammation by targeting LOX activity. This study is intended to serve as preliminary research, contributing to our understanding of how caffeine, as the primary component in CE, is involved in anti-asthmatic effects, particularly concerning the suppression of 5-LOX activity.

## MATERIALS AND METHODS

### Materials

The materials and instruments used in this research include soybean 5-LOX (Sigma Aldrich-L7395, US), apigenin (Sigma Aldrich-10798, US), linoleic acid (Sigma Aldrich-1376, US), demineralized water (Brataco, Indonesia), 96% ethanol (Brataco, Indonesia), distilled water (Brataco, Indonesia), Robusta Gold and Arabica Gold coffee powders (commercially available product from Indonesia), microplate reader (BioTek ELX800), and thin-layer chromatography (TLC) scanner (CAMAG).

### Methods

#### *Coffee extraction and caffeine isolation*

One hundred g of coffee powder (Robusta and Arabica) were extracted with 400 mL of 96% ethanol for 2 hours using the reflux method. The extract was concentrated using a rotary vacuum evaporator at 60±5°C and dried in a vacuum oven for 6 hours to form a thick extract. This extract was used as a sample in the LOX activity assay. The remained thick extract of Robusta coffee was then added with 50 mg of MgO and 300 mL of distilled water, heated for 1 hour at 90±10°C, and filtered. The residue was boiled again for 1 hour with 500 mL of distilled water; this process was repeated two times, and then the obtained extract was filtered using a Buchner funnel. The filtrate obtained was added with 50 mL of 10% H<sub>2</sub>SO<sub>4</sub> and then concentrated until the volume was reduced to 250 mL. The liquid-liquid extraction was added with 250 mL of chloroform into the aqueous filtrate. The chloroform layer was taken and washed with 40 mL of 1% NaOH and shaken with 40 mL of hot water. The transparent-colored chloroform layer is evaporated to obtain a concentrated filtrate. The sublimation was

then carried out to the filtrate at a temperature of 180-200°C to obtain caffeine isolate in white needle crystals<sup>19</sup>. The caffeine isolate was used in the LOX activity assay as a sample.

#### Examination of isolate purity using TLC-densitometry

The isolate was dissolved in 96% ethanol until a concentration of 1000 µg/mL was obtained and then eluted with the mobile phase *n*-hexane : ethyl acetate : ethanol (2.5 : 1.5 : 0.4). The spot was compared to standard caffeine. The purity level was assessed from the number of visible spots under UV 254 and 366 nm and the instrument calculations<sup>20</sup>.

#### Melting point determination

The melting point of the isolate was determined using the Fisher-Johns melting point apparatus with corrected temperature<sup>21</sup>. Isolate crystals obtained from the sublimation process were put in the capillary tube. The temperature when the crystals melt for the first time until they melt entirely was observed using a thermometer on the instrument. The melting point was then compared to the caffeine standard.

#### 5-LOX inhibitory activity assay

5-LOX inhibitory activity assay was carried out based on previous studies<sup>22-24</sup> with slight modifications. As much as 50 µL of each CRE, CAE, caffeine isolate, and apigenin solution was put into the vial, then added with 1650 µL of 0.2 M borate buffer solution pH 9 and 1000 µL of 300 µM linoleic acid as substrate solution. The mixture was vortexed and then incubated for 10 minutes at 25°C. Then 300 µL of 1000 U/mL 5-LOX solution was added to the mixture, then incubated for 15 minutes at 25°C. After incubation, 1000 µL methanol was added to the mixture and vortexed. The absorbance of the solution was measured using a UV spectrophotometer under 234 nm.

#### Enzyme kinetics assay

The kinetics of the inhibitory enzyme activity was carried out by varying the concentration of the linoleic acid as substrate (final concentration = 50, 75, 100, and 125 µM) with constant inhibitor concentration (final concentration of caffeine = 18.75 µg/mL). The concentration of caffeine used for the enzyme kinetics assay was determined by the concentration of caffeine that could inhibit 50% of the enzyme activity (IC<sub>50</sub>). To determine the type of inhibition of 5-LOX activity, an enzyme kinetics assay of 5-LOX inhibition by caffeine was performed using Michaelis–Menten kinetics, as shown in **Equations 1 to 3**, in which  $V_i$  was the initial velocity of an enzymatic reaction,  $V_{max}$  was the maximal velocity of the enzymatic reaction,  $K_m$  was Michaelis constant, and  $[S]$  was substrate concentration.

$$V_i = \frac{V_{max} [S]}{K_m + [S]} \quad [1]$$

$$\frac{1}{v_i} = \frac{K_m + [S]}{V_{max} [S]} \quad [2]$$

$$\frac{1}{V_i} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad [3]$$

#### Data analysis

Statistical analysis was performed using one-way ANOVA, and further multiple comparison between groups was analyzed using the Tukey Post Hoc Method.

## RESULTS AND DISCUSSION

The coffee plant contains various bioactive compounds with antioxidant properties, specifically phenolic compounds such as caffeic acid, chlorogenic acid, coumaric acid, ferulic acid, and cinnamic acid<sup>25</sup>. In addition to phenolic compounds, coffee contains methylxanthine alkaloid compounds such as caffeine, theophylline, and theobromine. Both groups of phenolic compounds and methylxanthine alkaloids have good solubility in organic solvents<sup>26</sup>. Thus, to produce a yield with a high content of bioactive compounds, the solvent chosen for the extraction of coffee bean powder is ethanol—extraction of *C. canephora* var. *Robusta* and *C. arabica* using ethanol yielded 9.84% and 8.12% samples, respectively. The resulting extract had a dark brown color and emitted a coffee aroma. The subsequent isolation process was performed in CRE following the alkaloid extraction principle, involving adding a base and extraction from the organic solvent layer (chloroform). This

process yielded fractions containing multiple compounds. The crude fraction was then subjected to purification through sublimation, producing pure caffeine in the form of needle-like crystals, with a yield of 0.11% relative to the total ground sample (**Table I**). The purity of the caffeine crystals formed from the sublimation process was confirmed by comparing the melting point of the crystal to caffeine standards, observing the number of spots visible on TLC, and observing the spot purity level by TLC-densitometry.

**Table I.** Extraction yield.

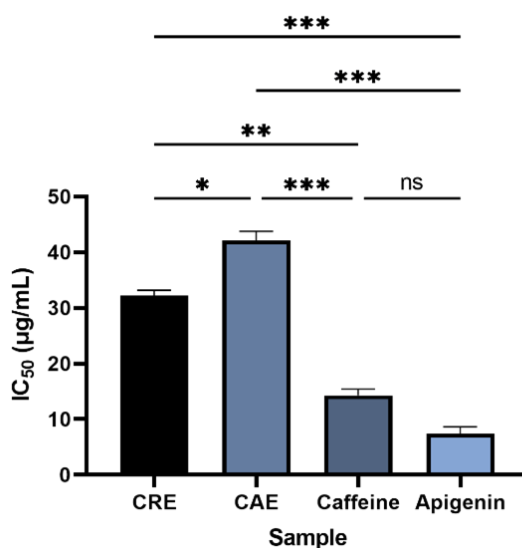
Component	Weight (g)	Yield (%)
Total sample weight	100	-
<i>Coffea canephora</i> var. Robusta extract	9.84	9.84
<i>Coffea arabica</i> extract	8.12	8.12
Caffeine	0.11	0.11

The melting point test results found that the caffeine crystals isolated from CRE had a narrow melting point range between 234-236°C, indicating a high purity level. The melting point range of isolated caffeine was similar to the caffeine standard (**Table II**). The chromatographic profile was evaluated using the TLC technique. The isolated compound produced a single spot under UV 254 nm with the same color, R<sub>f</sub>, and spectral profile as standard caffeine. Examination of the purity level using TLC-densitometry showed a purity level of 98.6%. The results indicated that the isolated crystal was pure and confirmed as caffeine.

**Table II.** Evaluation of the melting point range of isolated and standard caffeine.

Component	Melting point range (°C)
Isolated caffeine	234-236
Standard caffeine	234-237.5

All CRE, CAE, and caffeine isolate samples were tested for 5-LOX inhibitory activity. The assay was carried out in triplicate. The IC<sub>50</sub> values of CRE, CAE, and caffeine against 5-LOX were 32.2 ± 1.4, 42.1 ± 2.3, and 14.3 ± 1.6 µg/mL respectively. The standard compound used as a positive control was apigenin, which showed an IC<sub>50</sub> value of 7.4 ± 1.7 µg/mL. In **Figure 1**, it can be observed that there is a significant difference in activity between CRE, CAE, and caffeine. As a single compound isolated from coffee extract, caffeine demonstrates an inhibitory activity of 2.3 times stronger than CRE and three times stronger than CAE, as indicated by the smaller IC<sub>50</sub> values. Interestingly, caffeine's inhibition of 5-LOX activity is not significantly different (ns) compared to apigenin, used as the positive control. CRE's activity is superior to CAE; we hypothesize that this difference is due to variations in caffeine content in the samples. Robusta coffee consistently shows higher caffeine levels in various studies compared to Arabica coffee (±2.54% vs ±1.22%)<sup>27,28</sup>.

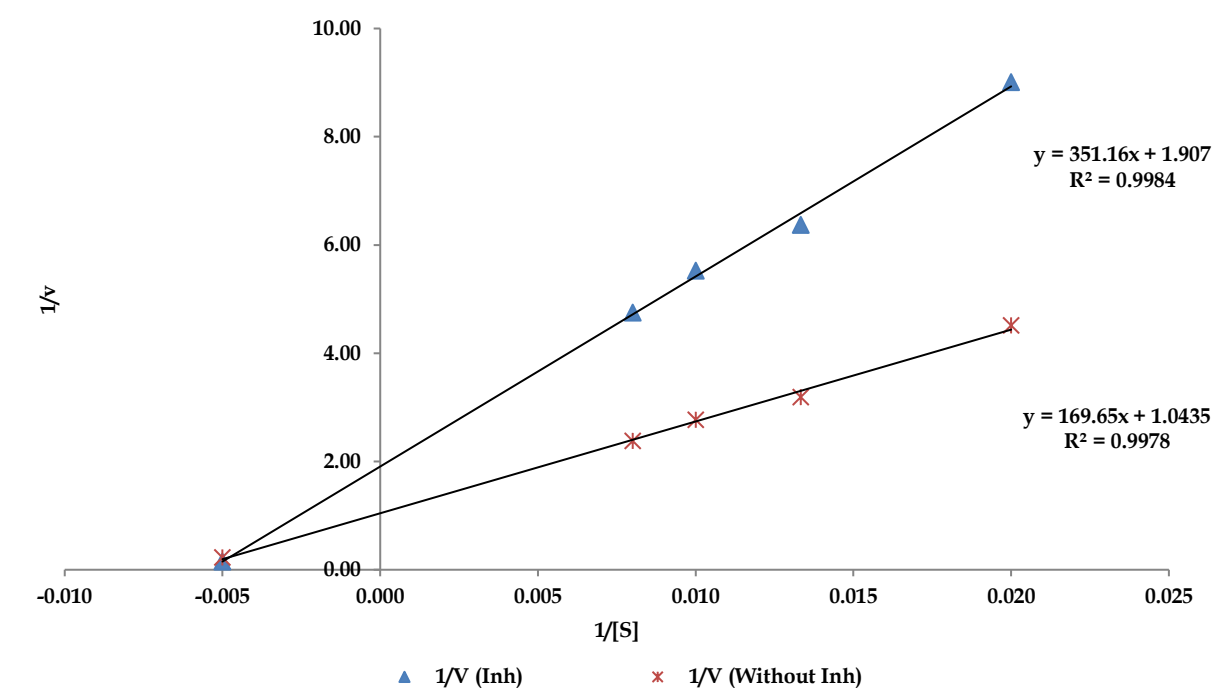


**Figure 1.** Inhibition of 5-LOX activity by CRE, CAE, caffeine, and apigenin represented by the IC<sub>50</sub> value.

Data were obtained in the enzyme kinetics assay, as shown in **Table III**, and the Lineweaver-Burke graph shows the intersection on the  $x$ -axis (**Figure 2**). The results indicate that the type of inhibition is non-competitive. Non-competitive inhibition occurs when the inhibitor (Inh) binds to the enzyme at a different site than the substrate (S). There is no competition between the inhibitor (caffeine) and the substrate in non-competitive inhibition. The presence of non-competitive inhibitors decreases the  $V_{\max}$  value with a relatively stable  $K_m$  value.

**Table III.** Enzyme kinetic measurement data.

Parameter	With inhibitor (Caffeine)	Without inhibitor
a	1.907	1.0435
b	351.16	169.65
r	0.9992	0.9989
$V_{\max}$	0.524	0.958
$K_m$	184.143	162.578



**Figure 2.** Enzyme kinetic profile of 5-LOX activity inhibition by inhibitors (caffeine).

Lipoxygenase is a metalloenzyme that has pro-oxidation and pro-inflammatory properties. Lipoxygenase generally catalyzes the oxidation of unsaturated fatty acids. In the human body, LOX metabolizes arachidonic acid to leukotrienes (a potent inflammatory mediator), making LOX a critical enzyme in the inflammatory pathway. Based on the relative oxidation position in the arachidonic acid structure, LOX is classified into 5-LOX, 12-LOX, and 15-LOX<sup>29</sup>. Inhibition of LOX activity can reduce oxidation and inflammation, which can trigger asthma. The studies for effective LOX inhibitors from plants are still ongoing. Compounds from plants that are known to inhibit LOX activity are phenolics and alkaloids, both of which are found in coffee<sup>30</sup>. It is known that coffee extract also contains alkaloid compounds of the methylxanthines type: caffeine (1,3,7-trimethyl xanthine), theophylline (3,7-dimethyl xanthine), and theobromine (1,3-dimethyl xanthine). Coffee also has theobromine and theophylline despite concentrations 20 times lower than caffeine<sup>31</sup>. The caffeine content in one cup of coffee depends on the variety of coffee plants used. The average caffeine content in brewed coffee grounds is 57 mg/100 mL<sup>32</sup>. Other research stated that coffee contains caffeine  $\pm$  1177 mg/g as the main compound<sup>33</sup>.

Our study proves that caffeine plays a role in inhibiting LOX. The inhibitory potency of caffeine in inhibiting 5-LOX cannot be compared with Zileuton (a standard drug approved by the FDA as a 5-LOX inhibitor) due to access limitation; thus, in this study, apigenin was used as a positive control. However, from other studies, it was known that using a similar colorimetric method, Zileuton showed an  $IC_{50}$  of 2.08  $\mu$ M or 32 times more potent in inhibiting 5-LOX activity compared to caffeine in this study. Zileuton is the ligand that inhibits iron binding to the LOX and has a weak potential reduction<sup>34</sup>.

Although caffeine has a weaker 5-LOX inhibitory potency than Zileuton, caffeine in coffee may be used as an alternative in relieving asthma because of its pharmacokinetic profile. A comparative study showed that caffeine has a more rapid onset of action, lower fluctuations in plasma concentrations, a longer half-life, and fewer peripheral side effects compared to other methylxanthines<sup>32,35,36</sup>. In oral administration, gastrointestinal absorption of caffeine is rapid and complete, achieving almost 100% bioavailability. After reaching the bloodstream, caffeine binds to albumin and is distributed to all tissues by simple diffusion or carrier-mediated transport<sup>32,37</sup>.

This study's *in vitro* enzymatic activity assay results might be used as preliminary data to assess the caffeine potency in inhibiting 5-LOX activity. For future research, more selective and stable methods might be developed, one of which is the colorimetric method using thiocyanate ions (SCN<sup>-</sup>) to form a red ferric thiocyanate (FTC) complex measured at  $\lambda$  480 nm. This colorimetric method is considered more selective and stable for measuring products formed from the enzymatic activity of 5-LOX<sup>38</sup>.

## CONCLUSION

Coffee exhibits potential inhibition activity against 5-LOX, with IC<sub>50</sub> values of  $32.2 \pm 1.4$   $\mu\text{g/mL}$  for CRE,  $42.1 \pm 2.3$   $\mu\text{g/mL}$  for CAE,  $14.3 \pm 1.6$   $\mu\text{g/mL}$  for caffeine, and  $7.4 \pm 1.7$   $\mu\text{g/mL}$  for apigenin. Further bioactive compound isolation of coffee extract produced caffeine with a more substantial inhibitory potential against 5-LOX. Caffeine inhibits 5-LOX activity in a non-competitive manner.

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## AUTHORS' CONTRIBUTION

**Conceptualization:** Tegar Achsendo Yuniarta, Rosita Handayani

**Data curation:** Tegar Achsendo Yuniarta, Rosita Handayani

**Formal analysis:** Tegar Achsendo Yuniarta, Rosita Handayani

**Funding acquisition:** Rosita Handayani

**Investigation:** Tegar Achsendo Yuniarta, Rosita Handayani

**Methodology:** Tegar Achsendo Yuniarta, Rosita Handayani

**Project administration:** Rosita Handayani

**Resources:** Tegar Achsendo Yuniarta, Rosita Handayani

**Software:** -

**Supervision:** -

**Validation:** Tegar Achsendo Yuniarta, Rosita Handayani

**Visualization:** Tegar Achsendo Yuniarta, Rosita Handayani

**Writing - original draft:** Tegar Achsendo Yuniarta

**Writing - review & editing:** Rosita Handayani

## DATA AVAILABILITY

None.

## CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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