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Molecular Insights into the Anti-Inflammatory Activity via Regulation of Nitric Oxide in LPS-Induced Macrophages and Acute Toxicity Profile of *Pogostemon cablin* Leaf Extract from Kolaka, Indonesia

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ABSTRACT

Pogostemon cablin (patchouli) is a medicinal plant known for its anti-inflammatory potential. This study aimed to investigate the anti-inflammatory potential and safety profile of *Pogostemon cablin* ethanol leaf extract collected from Kolaka (PCEE-K), Southeast Sulawesi, Indonesia. PCEE-K was subjected to phytochemical screening and physicochemical/proximate analysis for the purpose of standardization following standard procedures. Acute toxicity of PCEE-K was evaluated in mice according to OECD guidelines. Anti-inflammatory activity was evaluated *in vitro* via nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. Antioxidant and cytotoxic effects were also evaluated via nitric oxide radical scavenging and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, respectively. Phytochemical screening revealed the presence of flavonoids, terpenoids, alkaloids, saponins, and tannins. Standardization through physicochemical/proximate assessments ensured consistent quality and reproducibility of PCEE-K. Acute oral toxicity testing showed no observable toxicity of PCEE-K at 2000 mg/kg dose, suggesting that the extract is relatively safe on acute oral administration. The extract significantly inhibited NO production induced by LPS in RAW 264.7 macrophages, with an IC_{50} of 31.71 ± 0.55 µg/mL. This effect could be attributed to the downregulation of inducible nitric oxide synthase (iNOS) expression at both the transcriptional and translational levels. In a separate non-cell-based NO scavenging assay, PCEE-K exhibited significant NO radical scavenging activity, with IC_{50} of 1.73 ± 0.41 µg/mL. MTT assay confirmed the extract's cytocompatibility with RAW 264.7 cells, indicating minimal cytotoxicity at bioactive concentrations. These findings support the traditional use of *P. cablin* in the treatment of inflammation-related disorders, and highlight its potential as a safe and effective phytotherapeutic agent.

Keywords: *Pogostemon cablin*, Anti-inflammatory, Acute toxicity, Nitric oxide inhibition, RAW 264.7 macrophage cell line

Introduction

Inflammation is a complex and essential physiological response that enables the body to combat pathogens, repair damaged tissues, and restore homeostasis.[1-3] This process, characterized by redness, swelling, heat, pain, and loss of function, is tightly regulated by a network of immune cells, signaling molecules, and biochemical mediators.[1,3,4] While acute inflammation is generally beneficial and self-limiting, chronic or uncontrolled inflammation can be harmful,[5] contributing to the development of various diseases, including rheumatoid arthritis (RA), inflammatory bowel disease (IBD),[6] diabetes mellitus, cardiovascular diseases (CVD),[7,8] and neurodegenerative disorders such as Alzheimer's disease.[7-10]Among the key molecular mediators involved in inflammation, nitric oxide (NO) plays a pivotal dual role.[11,12] NO is produced primarily by inducible nitric oxide synthase (iNOS) in activated macrophages and other immune cells, acting as a signaling molecule that modulates vascular tone, neurotransmission, and microbial defense.[11,13] However, excessive or prolonged NO production during inflammation can induce oxidative and nitrosative stress, leading to cellular and tissue damage and contributing to the progression of inflammatory diseases.[14,15] Consequently, regulating NO levels and suppressing iNOS expression have emerged as promising strategies in the development of novel anti-inflammatory agents.

Despite the widespread use of synthetic anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, their long-term administration is often associated with serious adverse effects,[2,16,17] including gastrointestinal bleeding, hepatotoxicity, renal impairment, and increased cardiovascular risk.[16-18] These limitations highlight the urgent need for safer and more effective therapeutic alternatives, particularly those derived from natural sources with a history of traditional medicinal use. In this regard,

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medicinal plants represent a valuable reservoir of bioactive compounds with significant pharmacological potential.

Pogostemon cablin (Blanco) Benth., commonly known as patchouli, is a perennial aromatic herb of the Lamiaceae family. It is widely distributed throughout tropical Asia, including Indonesia, and is traditionally valued not only for its distinctive fragrance but also for its medicinal properties.[19-23] In various ethnomedical practices, the leaves of *P. cablin* have been used to treat a wide range of ailments, including fever, colds, headaches, gastrointestinal disturbances, skin infections, and inflammatory disorders.[19,21-23] Phytochemical analyses have revealed that *P. cablin* is rich in secondary metabolites such as sesquiterpenes (notably patchouli alcohol), flavonoids, triterpenoids, phenolic acids, and volatile oils.[19-24] These compounds have been associated with a variety of pharmacological activities, including antioxidant, antimicrobial,[19-23] antiviral,[19,21] antidiabetic,[21,22] and anti-inflammatory effects.[19-23]

Although previous studies have demonstrated the anti-inflammatory activity of *P. cablin* extracts,[19,25,26] comprehensive investigations into its molecular mechanisms, particularly its effect on NO modulation, remain limited. Additionally, there is a notable lack of region-specific studies exploring the biological activity of *P. cablin* sourced from distinct ecological zones. Environmental factors such as soil composition, climate, altitude, and cultivation practices are known to influence the phytochemical profile of medicinal plants, thereby affecting their pharmacological properties.[27,28] Kolaka, a region in Southeast Sulawesi, Indonesia possesses unique environmental conditions including climate, soil type, altitude, and local agricultural practices that may influence the growth and phytoconstituent composition of *P. cablin.*[29] Notably, the region's nickel-rich soil imposes mild abiotic stress, which can stimulate the production of secondary metabolites such as terpenoids and flavonoids as part of the plant's adaptive response.[30,31] These bioactive compounds are often linked to the plant's pharmacological activities, including its anti-inflammatory effects.[19-23] Therefore, *P. cablin* from Kolaka may possess enhanced medicinal value due to its distinctive agrogeochemical environment.

In addition to evaluating pharmacological efficacy, it is essential to establish the safety profile of plant-derived extracts through standardized toxicological assessments. Acute oral toxicity studies, guided by the Organisation for Economic Co-operation and Development (OECD) protocols, serve as a fundamental step in determining the safe dosage range, potential toxic effects, and therapeutic index of natural products. These studies provide critical data for the rational design of dosage regimens and for future preclinical and clinical evaluations.

Therefore, this study aimed to evaluate the anti-inflammatory potential and NO modulation of *P. cablin* ethanol leaf extract collected from Kolaka (PCEE-K) through *in vivo* and *in vitro* approaches. The specific objectives of this research are threefold: (1) to evaluate the *in vitro* modulatory effects of the extract on NO production in lipopolysaccharide (LPS)-stimulated macrophages as a biomarker of inflammatory response; (2) to determine its acute oral toxicity profile *in vivo* using rodent models following OECD 423 guidelines; and (3) to correlate the observed biological activities with the traditional use of *P. cablin* and its phytochemical composition.

By integrating pharmacological and toxicological evaluations, this study seeks to provide novel scientific evidence supporting the therapeutic relevance of PCEE-K as a safe and effective anti-inflammatory agent. The outcomes of this research are expected to contribute valuable insights into the development of regionally sourced phytotherapeutics and promote the sustainable utilization of Indonesia's rich biodiversity for public health and pharmaceutical innovation.

Materials and Methods

Chemicals and biologicals

Distilled water, and 96% ethanol (Bratachem®, Indonesia) were used as the extraction solvents. Reagents used included 0.5% sodium carboxymethyl cellulose (Na-CMC) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma-Aldrich, USA), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 0.04 N hydrochloric acid–isopropanol solution, chloroform, and sulfuric acid (Merck, Germany). Biochemical kits obtained from Erba Mannheim (Germany) included R1 (urease/GLDH), R2 (NADH), R1 (picric acid), R2 (sodium hydroxide), R1 (enzyme reagent), R2 (developer reagent), R1 (ALT substrate), R1 (aspartate substrate), and R2 (4-AA/POD reagent). Other analytical-grade reagents used were bromocresol green reagent (Sigma-Aldrich, USA), total protein monoreagent (Biolabo, France), cholesterol monoreagent (Biolabo, France), triglyceride monoreagent (Biolabo, France), and standard solutions of total protein, triglyceride, albumin, cholesterol, creatinine, and urea (Randox Laboratories, UK). All solvents and reagents used were of analytical grade and used without further purification.

For cell culture, RAW 264.7 cells were obtained from ATCC (USA), while fetal bovine serum (FBS) and 1% penicillin/streptomycin were purchased from Gibco (USA).

Collection and identification of plant material

Fresh leaves of *Pogostemon cablin* (Kolaka chemotype) were collected from Kolaka, Southeast Sulawesi, Indonesia (GPS coordinates: 3.9667° S, 121.5667° E). A voucher specimen (*P. cablin*-KLK-01/12/24-ADR) was prepared and retained by the authors for future reference. Leaves were collected manually using clean pruning tools. The samples were sorted to remove any damaged or contaminated material, thoroughly washed with running water, and air-dried. After drying, the leaves were cut into small pieces to facilitate the extraction process.

Extraction

The dried powdered leaves of *P. cablin* (1.2 kg) were extracted by maceration in ethanol (6 L) at room temperature for 3 × 24 hours. Subsequently, the ethanol extract was filtered through a filter paper and the solvent was evaporated under reduced pressure using a rotary evaporator at 50°C to obtain a crude extract.[2,4]

Phytochemical screening

Phytochemical screening of PCEE-K was conducted to detect the presence of secondary metabolites, including flavonoids, alkaloids, steroids, tannins, and saponins. The screening procedures were adapted from standard methods as described by Harborne, with slight modifications.[32,33]

Extract Standardization

Specific parameters

Standardization was carried out on specific parameters, including organoleptic characteristics, ethanol-soluble extractive content, and water-soluble extractive content, to evaluate the identity and chemical quality of the extract. The analysis was conducted on samples prepared during the study using validated procedures with slight modifications.[34,35]

- *Determination of organoleptic characteristics*

The organoleptic test was conducted by observing the shape, colour, and odour of the extract.

- *Determination of ethanol-soluble extractive content*

One gram of the extract was macerated in 100 mL of 96% ethanol for 24 hours in a closed container. During the first 6 hours, the mixture was shaken occasionally, then allowed to stand for the remaining 18 hours. The mixture was rapidly filtered to prevent evaporation of the ethanol. An aliquot of 20 mL of the filtrate was evaporated to dryness in a pre-weighed dish and then dried at 105°C until a constant weight was achieved. The ethanol-soluble extractive value was calculated using the formula (Equation 1):

$$\text{Ethanol} - \text{soluble extractive content}\ (\%) = \frac{\text{Constant weight} - \text{Empty dish weight}}{\text{Sample weight}} x\ \frac{\text{Total volume}}{\text{Volume used}}\ x\ 100 \ldots \quad \text{(Eq. 1)}$$

- *Determination of water-soluble extractive content*

Similarly, 1 gram of the extract was macerated in 100 mL of chloroform water for 24 hours in a stoppered flask. The mixture was shaken

occasionally during the first 6 hours and then allowed to stand for 18 hours. After filtration, 20 mL of the filtrate was placed in a pre-weighed dish and dried at 105°C until a constant weight was reached. The water-soluble extractive content was calculated using the same formula (Equation 2):

$$\text{Water} - \text{soluble extractive content (\%)} = \frac{\text{Constant weight} - \text{Empty dish weight}}{\text{Sample weight}} x \frac{\text{Total volume}}{\text{Volume used}} x\ 100 \ldots \quad \text{(Eq. 2)}$$

Standardization of non-specific parameters

Standardization of non-specific parameters was carried out to assess the general quality and purity of the extract, including moisture content, loss on drying, specific gravity, total ash, and acid-insoluble ash. The procedures followed were adapted from Syukri *et al*. (2020) and Putri *et al*. (2020) with slight modifications.[34,35]

- *Determination of moisture content*

One gram of extract was accurately weighed into a pre-weighed porcelain dish and dried in an oven at 105°C for 5 hours. The dish was then reweighed and drying was continued until a constant weight was achieved. The percentage of moisture content was calculated using the formula (Equation 3):

$$\text{Moisture content (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Sample weight}} x100 \ldots\ldots\ldots\ldots\ldots \text{(Eq. 3)}$$

- *Determination of loss on drying*

One gram of the extract was placed in a pre-weighed crucible and dried in an oven at 105°C until a constant weight was reached. After cooling in a desiccator, the crucible was reweighed. The weight loss represents the content of volatile matter.

- *Determination of specific gravity*

A clean, dry pycnometer was weighed empty, then filled with distilled water and weighed again to determine the weight of water. The same procedure was repeated with the extract. Specific gravity was calculated by dividing the weight of the extract by the weight of an equal volume of water, multiplied by the specific gravity of water at room temperature (Equation 4):

$$\text{Specific gravity} = \frac{\text{Weight of extract}}{\text{Weight of water}} x\ \text{Specific gravity of water} \ldots\ldots\ldots\ldots\ldots \text{(Eq. 4)}$$

- *Determination of total ash content*

Three grams of the extract were weighed into a previously ignited and tared porcelain dish, then incinerated in a muffle furnace at 500 - 550°C until white ash was obtained. After cooling in a desiccator, the dish was reweighed. The total ash content (%) was calculated using equation 5:

$$\text{Total ash (\%)} = \frac{W_2 - W_1}{W} x\ 100 \ldots\ldots\ldots\ldots\ldots \text{(Eq. 5)}$$

Where;

W_1 = Weight of empty dish, W_2 = Weight of dish with ash, and W = Weight of the sample

- *Determination of Acid-insoluble ash*

The ash obtained was boiled with 25 mL of dilute hydrochloric acid for 5 minutes. The mixture was filtered through ash-free filter paper, the residue rinsed with hot water, dried, and ignited again until carbon-free. It was cooled and weighed. Acid-insoluble ash was calculated using Equation 6:

$$\text{Acid insoluble ash (\%)} = \frac{\text{Weight of residue}}{\text{Weight of sample}} x\ 100 \ldots\ldots\ldots\ldots\ldots \text{(Eq. 6)}$$

Experimental animals

Healthy Swiss albino mice weighing between 25 and 30 g were used in this study. The animals were housed individually in clean acrylic cages under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2°C, and relative humidity 50 - 60%). They were provided with standard rodent pellets and water *ad libitum*. All experimental procedures involving animals were conducted in accordance with the ethical guidelines approved by the Ethical Committee for Animal Experimentation No: 938/UN29.20.1.2/PG/2025, Universitas Halu Oleo, Kendari, Indonesia.

Acute toxicity study

The acute oral toxicity of PCEE-K was evaluated following the guidelines of the Organization for Economic Cooperation and Development (OECD) Guideline 423 (Acute Toxic Class Method), using female Swiss albino mice as the experimental model. A single oral dose of 2000 mg/kg body weight was administered to five animals in a stepwise manner.

The animals were observed individually during the first 30 minutes post-administration, periodically during the first 24 hours, and then daily for 14 consecutive days to monitor clinical signs of toxicity and mortality. No additional doses were given during this period. The 14-day period served solely as the observation phase. The study aimed to evaluate the safety profile of the extract by identifying any sign of acute toxicity and estimating the approximate LD_{50} value. Systemic effects were further assessed by monitoring body weight, analyzing relative organ weights, and conducting histopathological evaluations of vital organs (liver and kidneys) at the end of the observation period.[36,37]

On day 15, blood samples were collected via cardiac puncture under isoflurane inhalational anesthesia delivered through a calibrated vaporizer. Hematological parameters were analyzed using an automated hematology analyzer (Hematology Analyzer Wheisman AC 310®, China). Biochemical parameters, including urea, creatinine, total cholesterol, triglycerides, high-density lipoprotein (HDL), high-density lipoprotein (LDL), Serum Glutamic-Oxaloacetic Transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), total protein, and albumin were measured spectrophotometrically using commercial diagnostic reagent kits.[38]

Nitric oxide radical scavenging assay

The nitric oxide radical scavenging activity of PCEE-K was evaluated using a modified Griess-Ilosvay method as previously described.[39-41] Briefly, 50 µL of the extract at various concentrations (100, 50, 25, 12.5, and 6.25 µg/mL) was mixed with 50 µL of 10 mM sodium nitroprusside (SNP) prepared in phosphate-buffered saline (PBS, pH 7.4) in a 96-well microplate. The mixture was incubated in the dark at room temperature (25°C) for 120 minutes. After incubation, 50 µL of sulfanilamide solution (0.3% in 5% phosphoric acid) was added and allowed to stand for 5 minutes, followed by the addition of 50 µL of N-(1-naphthyl)ethylenediamine dihydrochloride (NED) solution (1%). The reaction mixture was further incubated for 30 minutes at room temperature. The absorbance of the resulting pink chromophore was measured at 540 nm using a microplate reader (Spectrostar Omega, BMG Labtech, Ortenberg, Germany). Ascorbic acid (vitamin C) was used as the positive control at concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/mL. All experiments were performed in triplicate. The percentage of nitric oxide scavenging was calculated using the following equation 7:

$$\text{NO inhibition (\%)} = [(A_0 - A_1)/A_0] \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(Eq. 7)}$$

Where;

A_0 is the absorbance of the control (SNP without extract), A_1 is the absorbance of the sample. The IC_{50} value, representing the concentration of sample required to inhibit 50% of NO production, was calculated using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA).

Cell culture

The RAW 264.7 cell line was obtained from the *American Type Culture Collection* (ATCC), USA. The cell line was cultured in *Dulbecco's Modified Eagle Medium* (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin solution. The culture was maintained at 37°C in a controlled environment incubator

with a 95% humidity and 5% CO_2 atmosphere.[42-44]

MTT assay
The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was conducted to assess the cytotoxic potential of PCEE-K on RAW 264.7 macrophage cells. The cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 100 µL of complete medium and incubated until confluence. Subsequently, the cells were treated with various concentrations of PCEE-K (100, 50, 25, 12.5, 6.25 µg/mL) for 24 hours. The extract was diluted in complete medium prior to administration. After incubation, the medium was removed, and the cells were washed twice with PBS (pH 7.4), followed by the addition of 100 µL fresh complete medium. MTT solution (0.5 mg/mL in PBS, pH 7.4) was added to each well and incubated at 37°C for 4 hours. After removing the MTT solution, the formazan crystals formed were dissolved by adding 100 µL of 0.04 N HCl-isopropanol solution and incubated in the dark at room temperature for 60 minutes. The absorbance was measured at 570 nm using a microplate reader (Spectrostar Omega, BMG Labtech, Ortenberg, Germany). All experiments were conducted in triplicate. The cell viability (CV) of the RAW 264.7 cells was calculated following equation 8:

$$CV\ (\%) = (OD_{sample}/OD_{control}) \times 100 \quad \text{..... (Eq. 8)}$$

Where;
ODsample and ODcontrol were the OD of wells containing the cells that were incubated with the test samples, and that of the wells containing the cells without any treatments, respectively. The test samples were considered to be cytotoxic to the cells if the CV (%) was less than 70%.[42,43]

Nitric oxide production assay
RAW 264.7 macrophage cells were seeded into a 96-well plate at a density of 1×10^4 cells/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. To induce NO production, the cells were stimulated with 1 µg/mL lipopolysaccharide (LPS) and incubated overnight (24 h). After stimulation, the cells were treated with various concentrations of PCEE-K (400, 200, 100, 50, 25, 10, 5, and 1 µg/mL) for an additional 24 hours. Dulbecco's Modified Eagle Medium (DMEM) without LPS was used as a blank control, while the LPS-treated group served as a negative control. Nitrite accumulation in the culture supernatant was measured as an indicator of NO production using the Griess Reagent. A 100 µL aliquot of the culture medium was mixed with an equal volume of Griess Reagent, and the absorbance was measured at 550 nm using a microplate reader (Spectrostar Omega, BMG Labtech, Ortenberg, Germany).[44]

Statistical analysis
All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). The differences between groups were analyzed using the independent *t*-test. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Phytochemical constituents of PCEE-K
Phytochemical screening of PCEE-K revealed the presence of several secondary metabolites, as shown in Table 1. The extract tested positive for flavonoids, alkaloids, steroids/terpenoids, saponins, and tannins.

Table 1: Phytochemical constituents of *Pogostemon cablin* ethanol leaf extract

Phytochemical	Result
Flavonoids	+
Alkaloids	+
Steroids	+
Saponins	+
Tannins	+

'+' indicates a positive result, meaning the compound is present in the extract

These findings confirm the presence of key bioactive compounds in PCEE-K, supporting its potential as an anti-inflammatory agent. The results are consistent with previous studies,[45,46] which also reported the presence of flavonoids, alkaloids, steroids, saponins, and tannins in *P. cablin* leaf extract. The identified secondary metabolites, particularly flavonoids and tannins, are known to modulate pro-inflammatory mediators. These, along with other constituents present in the extract, may collectively underlie its potential anti-inflammatory activity.[47,48]

Standardization of PCEE-K
Standardization of PCEE-K was conducted using both specific and non-specific parameters, as recommended for ensuring the consistency and safety of herbal preparations (Table 2). Organoleptic observations showed that the extract had a thick, viscous texture with a dark green appearance, and a distinctive, strong patchouli aroma, typical of *P. cablin.*[49] The moisture content was found to be 5.00%, which falls below the maximum recommended limit of 10% for crude plant samples, indicating low moisture and a reduced risk of microbial growth or spoilage.[50]

Table 2: Standardization results of specific and non-specific parameters of ethanol extract of *Pogostemon cablin* leaves (PCEE-K)

Parameter	Result	Requirement
Organoleptic	The extract exhibited a dark green colour, a characteristic patchouli-like aroma, and a thick liquid texture.	-
Ethanol-soluble extractive content (%)	25.00 ± 0.10	> 14.8%
Water-soluble extractive content (%)	60.00 ± 0.10	> 13.5%
Moisture content (%)	5.00 ± 0.10	≤ 10
Loss on drying (%)	0.03 ± 0.01	≤ 0.25
Specific gravity (g/mL)	0.88	-
Total ash (%)	3.00 ± 0.10	≤ 15
Acid-insoluble ash (%)	0.30 ± 0.01	< 2

The total ash content was found to be 3.00%, while acid-insoluble ash was 0.30%, both within the permissible limits of ≤15% and <2%, respectively, suggesting minimal inorganic and siliceous contamination.[51] Furthermore, the extractive values using ethanol and water were 25% and 60%, respectively, indicating the solubility of the extract's constituents in different solvents and providing a measure of the quantity of bioactive materials present. The specific gravity of the extract was 0.88 g/mL, which is in accordance with standard parameters for crude plant extract.[52] These results confirm that the extract conforms to the acceptable ranges set by the standard herbal pharmacopeial guidelines. Hence, the PCEE-K extract can be considered to meet the quality requirements necessary for subsequent pharmacological testing and formulation development.

Acute oral toxicity of PCEE-K in mice
The acute oral toxicity of PCEE-K was evaluated using OECD Guideline 423 (Acute Toxic Class Method), employing female Swiss albino mice as experimental subjects. A starting dose of 2000 mg/kg body weight was chosen, as there was no prior acute toxicity data for *P. cablin* leaf extract. This dose aligns with the initial step required by OECD guidelines for untested substances.
The use of male mice was preferred due to their more stable hormonal profile compared to females, who undergo estrous cycles, pregnancy,

and lactation, which may contribute to increased stress sensitivity. The selected animals were 2–3 months old and weighed between 20–30 grams. The mice were observed for clinical signs of toxicity and mortality over a 14-day period. No deaths or overt signs of toxicity were noted in any of the animals. Table 3 presents the detailed observations of toxicity symptoms.

Table 3: Toxicity signs in mice after treatment with ethanol extract of *Pogostemon cablin* leaves (PCEE-K)

Group	Mice	Treatment	Mortality	Toxicity Symptoms					
				1	2	3	4	5	6
Normal	1	None	0	-	-	-	-	-	-
	2		0	-	-	-	-	-	-
	3		0	-	-	-	-	-	-
	4		0	-	-	-	-	-	-
	5		0	-	-	-	-	-	-
Dose 2000 mg/kg	1	2000 mg/kg	0	-	-	-	-	-	-
	2		0	-	-	-	-	-	-
	3		0	-	-	-	-	-	-
	4		0	-	-	-	-	-	-
	5		0	-	-	-	-	-	-

1: Skin and fur abnormalities; **2:** Eye redness or irritation; **3:** Lethargy; **4:** Convulsions; **5:** Tremor; **6:** Diarrhea; (–): No symptoms observed

Throughout the observation period, all animals in the treatment group exhibited healthy physical conditions, normal behavior (such as climbing, digging, and active movement), and no signs of distress or toxicity symptoms such as piloerection, bruised skin, eye redness, lethargy, convulsions, tremors, diarrhea, or clinical indications of pre-mortality. According to OECD 423 criteria, if three or more animals survive at 2000 mg/kg, the LD_{50} is considered to be greater than 2000 mg/kg. This places the extract in GHS Toxicity Category 5 (slightly toxic), with an estimated LD_{50} in the range of >2000–5000 mg/kg.[36] Toxic properties of plant extracts are often associated with their phytochemical constituents. While compounds like alkaloids, flavonoids, phenols, and saponins can be toxic at high doses (e.g., hepatotoxicity or neurotoxicity), the absence of symptoms in this study suggests that the levels present in the *P. cablin* extract are not harmful at the tested dose. This is consistent with previous findings, which also reported the safety of patchouli leaf extracts.[53,54]

Effect of PCEE-K on biochemical profile

The evaluated biochemical parameters included SGOT, SGPT, urea, creatinine, total cholesterol, triglycerides, HDL, LDL, total protein, and albumin, as presented in Table 4. At a dose of 2000 mg/kg BW, the extract-treated group showed SGOT and SGPT levels of 23.2 ± 1.92 µ/L and 15.2 ± 1.30 µ/L, respectively. Although slightly elevated compared to the control group (18.4 ± 2.07 µ/L and 13.2 ± 1.92 µ/L), these values remained within the normal physiological range (SGOT: 3.0–45 µ/L; SGPT: 0–35 µ/L), and the differences were not statistically significant ($p > 0.05$), indicating no signs of hepatotoxicity.

Table 4: Effect of ethanol extract of *Pogostemon cablin* leaves (PCEE-K) on biochemical parameters in mice

Parameter	Group PCEE-K (2000 mg/kg)	Normal	Reference range[58,59]
SGOT (µ/L)	23.2 ± 1.92	12.6 ± 0.55	3.0 – 45
SGPT (µ/L)	15.2 ± 1.30	8.8 ± 0.84	0 – 35
Urea (mg/dL)	26.2 ± 3.27	22.4 ± 0.55	15-45
Creatinine (mg/dL)	1.02 ± 0.16	0.82 ± 0.08	0.7-1.3
Cholesterol (mg/dL)	127 ± 6.96	123.8 ± 4.15	200
Triglyceride (mg/dL)	98.2 ± 0.84	115.4 ± 4.04	150
HDL (mg/dL)	53.8 ± 1.48	41.6 ± 1.14	100
LDL (mg/dL)	76.4 ± 1.14	80.8 ± 0.84	100
Total Protein (g/dL)	6.8 ± 0.10	7.2 ± 0.12	6.0 – 8.0
Albumin (g/dL)	3.78 ± 0.41	4.18 ± 0.08	3.5-5

Values are mean ± standard deviation (SD), n = 5.
SGOT: Serum Glutamic-Oxaloacetic Transaminase; SGPT: Serum Glutamic Pyruvate Transaminase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Total protein and albumin levels in the treatment group were 6.8 ± 0.10 g/dL and 3.78 ± 0.41 g/dL, respectively, which were comparable to the control group (6.6 ± 0.23 g/dL and 3.56 ± 0.31 g/dL) with no significant differences ($p > 0.05$), suggesting preserved liver synthetic function.
Renal function was assessed by measuring urea and creatinine levels. The treatment group showed urea levels of 26.2 ± 3.27 mg/dL and creatinine levels of 1.02 ± 0.16 mg/dL, slightly higher than the control group (23.6 ± 1.94 mg/dL and 0.88 ± 0.14 mg/dL), but still within the normal reference range (urea: 15–45 mg/dL; creatinine: 0.7–1.3 mg/dL). No statistically significant differences were observed ($p > 0.05$), indicating no nephrotoxic effects.
Parameters related to lipid metabolism, total cholesterol, triglycerides, HDL, and LDL were also evaluated, as imbalances in these markers may be indicative of dyslipidemia or metabolic disorders.[55] In the treatment group, total cholesterol was 127.6 ± 9.66 mg/dL, triglycerides were 98.2 ± 0.84 mg/dL, HDL was 53.8 ± 1.48 mg/dL, and LDL was 76.4 ± 1.14 mg/dL. These values were comparable to those in the control group, with no significant differences ($p > 0.05$), and remained within the normal range for mice, suggesting that the extract did not disrupt lipid metabolism after the 14-day observation period following a single oral dose.

Effect of PCEE-K on hematological profile

The administration of a single oral dose of PCEE-K at 2000 mg/kg BW did not induce hematological toxicity in mice after 14 days of observation (Tables 5 and 6). All measured parameters including red blood cell indices, platelet indices, and leukocyte profiles remained within normal physiological ranges, and no significant differences were observed between groups ($p > 0.05$). This indicates that the extract did not adversely affect erythropoiesis, thrombopoiesis, or immune cell function, further supporting its safety at the tested dose.

Table 5: Hematological parameters of mice after 14 days of administration of *Pogostemon cablin* leaf ethanol extract

Parameter	Group PCEE-K (2000 mg/kg)	Normal	Reference range[58,59]
Hemoglobin (g/dL)	15.52 ± 0.28	15.42 ± 0.24	11.0-15.9
Hematocrit (%)	40.54 ± 0.53	51.74 ± 0.48	37-54 %
Erythrocytes ($x10^{12}$/L)	5.30 ± 0.13	4.98 ± 0.16	3.5-5.50
MCV (fL)	82.08 ± 0.76	81.2 ± 0.69	80-100
MCH (pg)	28.46 ± 1.71	28.14 ± 1.09	27-34
MCHC (g/L)	349.6 ± 3.44	344.4 ± 6.97	321-359
RDW-CV (%)	13.38 ± 0.24	15.28 ± 0.24	11.0-16.0
RDW-SD (fL)	38.8 ± 0.73	35.84 ± 0.80	35.0-56.0
Platelets ($x10^9$/L)	287 ± 7.31	156.6 ± 6.27	100-300
MPV (fL)	9.34 ± 0.27	8.4 ± 0.22	6.5-12
PDW (fL)	17.56 ± 0.48	15.78 ± 0.49	10.0 -18.0
PCT (%)	0.14 ± 0.21	0.15 ± 0.008	0.108-0.282
P-LCR (%)	43.02 ± 0.87	29.34 ± 1.29	11.0 – 45.0
P-LCC ($x10^9$/L)	132.4 ± 2.61	130.2 ± 4.55	11-135

Values are mean ± standard deviation (SD), n = 5.
MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RDW-CV: Red Cell Distribution Width – Coefficient of Variation; RDW-SD: Red Cell Distribution Width – Standard Deviation; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Plateletcrit; P-LCR: Platelet Large Cell Ratio; P-LCC: Platelet Large Cell Count.

Nitric oxide radical scavenging activity

NO plays a dual role in biological systems, functioning as a signaling molecule at physiological levels but contributing to oxidative stress and inflammation at high concentrations.[14] The nitric oxide scavenging assay in this study aimed to evaluate the antioxidant potential of PCEE-K by measuring its ability to neutralize NO radicals.

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Table 6: Leukocyte profile of mice after 14 days of administration of *Pogostemon cablin* leaf ethanol extract

Parameter	Group PCEE-K (2000 mg/kg)	Normal	Reference range[58-59]
Leukocyte	9.85 ± 0.08	4.27 ± 0.16	4.0 – 10.0 $x10^9$/L
LYM%	24.36 ± 0.74	23.48 ± 1.06	20 – 40 %
MID %	3.08 ± 0.08	3.6 ± 0.41	3.0 – 14.0 %
GRAN%	62 ± 0.64	55.38 ± 1.78	50 – 70 %
LYM#	3.20 ± 0.29	3.33 ± 0.14	0.8 – 4.0 x10^9/L
MID#	0.29 ± 0.07	0.142 ± 0.024	0.1 – 0.9 x10^9/L
GRAN#	2.54 ± 0.28	2.38 ± 0.30	2.0 – 7.0 x10^9/L
LED	4.6 ± 0.55	13.6 ± 1.40	0-20 mm/hour

Values are mean ± standard deviation (SD), n = 5.
LYM%: Percentage of lymphocytes; **MID%**: Percentage of the combined monocytes, eosinophils, and basophils; **GRAN%**: Percentage of granulocytes; **LYM#**: Absolute count of lymphocytes; **MID#**: Absolute count of the combined monocytes, eosinophils, and basophils; **GRAN#**: Absolute count of granulocytes; **ESR**: Erythrocyte Sedimentation Rate (LED in Indonesian)

As shown in Table 7, PCEE-K exhibited a concentration-dependent scavenging effect on NO. At the highest tested concentration (100 µg/mL), PCEE-K inhibited NO production by 65.72 ± 0.44%, while at the lowest concentration (6.25 µg/mL), the inhibition decreased to 25.76 ± 0.43%. This trend indicates that the scavenging activity increases with the concentration of the extract. In comparison, ascorbic acid, used as a positive control, demonstrated higher scavenging percentages across all concentrations, reaching up to 91.04 ± 0.23% at 100 µg/mL.

Table 7: Percentage Inhibition of nitriv oxide (NO) by *Pogostemon cablin* leaf ethanol extract and Vitamin C

Samples	Concentration (µg/mL)	%Inhibition	IC_{50}
	6.25	59 ± 1.3	
	12.5	65 ± 2.3	
PCEE-K	25	69 ± 0.6	1.73 ±
	50	75 ± 1.1	0.41
	100	77 ± 0.6	
	31.25	64 ± 0.9	
	62.5	66 ± 0.5	
Vitamin C	125	71 ± 1.3	1.07 ±
	250	72 ± 0.2	0.39
	500	74 ± 1.9	
	1000	76 ± 1.0	

Data represent mean ± standard (SD), n = 3.

The IC_{50} value of PCEE-K was found to be 1.73 ± 0.41 µg/mL, compared to 1.07 ± 0.39 µg/mL for the positive control, ascorbic acid. Although slightly higher, the IC_{50} of PCEE-K was not significantly different ($p > 0.05$) from that of ascorbic acid, suggesting comparable antioxidant capacity. This highlights the significant scavenging potential of PCEE-K. The ability of PCEE-K to scavenge NO may be attributed to its phytochemical composition, particularly the presence of phenolic compounds, flavonoids, and other bioactive constituents with known antioxidant properties. Previous studies have reported that *P. cablin* contains patchouli alcohol, flavonoids, and sesquiterpenes,[3] which can donate electrons to neutralize reactive nitrogen species (RNS), including NO.[56,57]
These findings suggest that PCEE-K possesses promising antioxidant potential through its nitric oxide scavenging mechanism. Given that excessive nitric oxide contributes to inflammatory processes, the inhibition of NO by PCEE-K may also support its observed anti-inflammatory effects in both *in vitro* and *in vivo* models. Thus, the extract could offer dual functionality in modulating oxidative stress and inflammation.

Effect of PCEE-K on RAW 264.7 cell viability
Prior to evaluating the anti-inflammatory activity, the cytotoxicity of PCEE-K against RAW 264.7 cell was assessed using the MTT assay to determine safe concentration ranges for subsequent experiments. The extract was tested at concentrations ranging from 6.25 to 100 µg/mL. The results showed no significant reduction in RAW 264.7 cell viability up to 100 µg/mL, with cell viability remaining above 90% compared to untreated controls (Figure 1). This indicates that PCEE-K is non-toxic at these concentrations, suggesting that the extract is suitable for further anti-inflammatory assays.

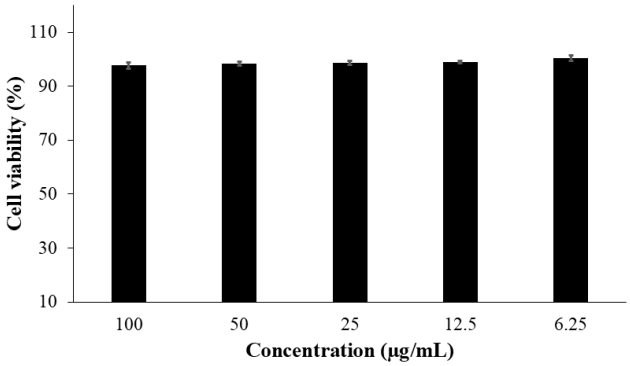

Figure 1: Effect of PCEE-K on RAW 264.7 cell viability
Data represent the mean ± standard deviation (SD), n = 3.

Anti-inflammatory activity: Inhibition of nitric oxide production
The anti-inflammatory potential of PCEE-K was assessed in an *in vitro* model of inflammation using LPS-induced RAW 264.7 murine macrophages. Upon stimulation with lipopolysaccharide (LPS), RAW 264.7 cells exhibit a robust inflammatory response characterized by the upregulation of iNOS, resulting in excessive NO production. NO is a key pro-inflammatory mediator and is commonly used as a biomarker to evaluate the anti-inflammatory effects of test compounds.
Treatment with PCEE-K at concentrations ranging from 1 to 400 µg/mL resulted in a concentration-dependent inhibition of NO production. The extract exhibited a half-maximal inhibitory concentration (IC_{50}) of 31.71 ± 0.55 µg/mL, indicating moderate but significant anti-inflammatory activity. The inhibition of NO production is likely mediated through the suppression of iNOS expression or its enzymatic activity. The results are illustrated in Figure 2. These findings suggest that *P. cablin* may exert anti-inflammatory effects through downregulation of iNOS-mediated NO synthesis in macrophages, thereby mitigating the inflammatory response.

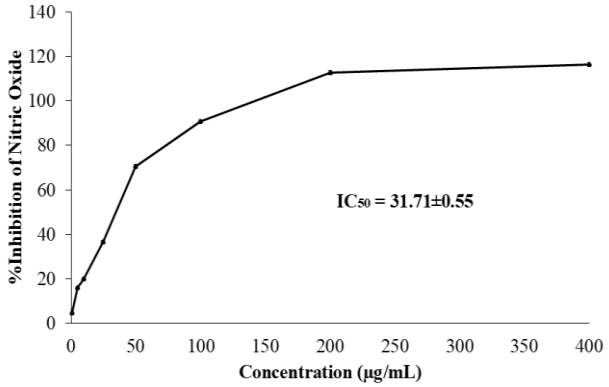

Figure 2: Effect of PCEE-K on Nitric oxide production
Data represent the mean ± standard deviation (SD), n = 3

The data suggest that PCEE-K exerts a potent anti-inflammatory effect through NO suppression and iNOS downregulation, highlighting its potential as a candidate for natural anti-inflammatory therapies, particularly for chronic inflammatory conditions such as arthritis, colitis, and skin disorders.

Given the link between inflammation and oxidative stress, future studies should explore the extract's antioxidant capacity, which may offer dual therapeutic effects. Further *in vivo* investigations and mechanistic studies focusing on NF-κB and MAPK pathways are warranted to elucidate its molecular mode of action. Additionally, metabolomic profiling of *P. cablin* from various regions, including Kolaka, may help identify chemical constituents responsible for its pharmacological activity.

Conclusion

P. cablin ethanol leaf extract significantly inhibits LPS-induced nitric oxide production in RAW 264.7 macrophages, primarily by downregulating iNOS expression at both the transcriptional and translational levels. This potent anti-inflammatory activity is likely mediated by its rich content of terpenoids and flavonoids, enhanced by the unique agroecological environment of Kolaka. The extract also exhibited no signs of toxicity in acute oral toxicity study in mice at doses up to 2000 mg/kg BW, indicating a favorable safety profile. Furthermore, phytochemical screening confirmed the presence of bioactive compounds such as flavonoids, terpenoids, phenolics, and tannins, which are known to possess anti-inflammatory and antioxidant properties. Standardization of the extract using organoleptic, physicochemical, and chromatographic parameters ensures reproducibility and quality consistency for potential therapeutic use. The findings support the traditional use of *P. cablin* in inflammation-related disorders and provide a scientific basis for its development into a novel phytotherapeutic agent with low toxicity and high efficacy. Future studies should focus on evaluating its efficacy in *in vivo* models of acute and chronic inflammation, investigating molecular mechanisms beyond iNOS signaling, and exploring its potential for formulation into standardized herbal or pharmaceutical preparations.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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