

ANTI-ARTHRITIC AND ANTI-INFLAMMATORY ACTIVITIES OF *POGOSTEMON CABLIN* FROM NORTH KOLAKA: PRO-INFLAMMATORY CYTOKINE SUPPRESSION IN AN ACUTE INFLAMMATION MODEL AND GC-MS/MS-BASED METABOLITE PROFILING

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Manuscript received: August 2026

Abstract

Rheumatoid arthritis is a chronic autoimmune disorder marked by persistent inflammation and progressive joint destruction, while current therapies may cause adverse effects and limited tolerability. This study evaluated the metabolite profile and anti-inflammatory/anti-arthritis activities of *Pogostemon cablin* leaves from North Kolaka. Ethanolic extract was fractionated using n-hexane, ethyl acetate, and water. Volatile constituents were tentatively identified by GC-MS/MS via library matching, without confirmation by reference standards. Anti-inflammatory activity was evaluated using HRBC membrane stabilization and BSA denaturation assays, followed by a xylene-induced ear edema model in mice (n = 3/group), with plasma TNF- α , IL-1 β , and IL-6 measured by ELISA. Anti-arthritis activity was separately assessed using a CFA-induced arthritis model, evaluating effects on paw edema progression. Seven compounds were tentatively identified, including eucalyptol, γ -terpinene, p-cymene, caryophyllene, α -guaiene, seychellene, and patchouli alcohol. The extract and fractions showed concentration-dependent anti-inflammatory activity *in vitro*. In the acute model, treatment significantly reduced edema and cytokine levels (p < 0.05); in the CFA model, paw edema progression was significantly attenuated (p < 0.05). These findings support *P. cablin* from North Kolaka as a source of bioactive compounds for inflammatory and arthritic disorders.

Rezumat

Poliartrita reumatoidă este o boală autoimună cronică, caracterizată prin inflamație persistentă și distrugere articulară progresivă. Terapiile disponibile în prezent pot avea însă reacții adverse și o tolerabilitate limitată. Acest studiu a evaluat profilul metaboliților, precum și activitatea antiinflamatoare și antiartritică a frunzelor de *Pogostemon cablin* din Kolaka de Nord. Extractul etanolic a fost fracționat cu n-hexan, acetat de etil și apă. Conținutul volatil au fost identificați prin GC-MS/MS, prin compararea cu bibliotecile de spectre, fără confirmare cu standarde de referință. Activitatea antiinflamatoare a fost evaluată inițial prin testele de stabilizare a membranei eritrocitare umane (HRBC) și de inhibare a denaturării albuminei serice bovine (BSA). A fost realizat un model de edem auricular indus de xilen la șoarece (n = 3/grup), în care nivelurile plasmatice de TNF- α , IL-1 β și IL-6 au fost determinate prin ELISA. Activitatea antiartritică a fost evaluată separat pe un model de artrită indusă cu adjuvant Freund complet (CFA), urmărindu-se efectul asupra evoluției edemului labei. Au fost identificați șapte compuși: eucaliptol, γ -terpinen, p-cimen, cariofilen, α -guaien, seychellen și alcool de patchouli. Extractul și fracțiile au avut *in vitro* o activitate antiinflamatoare dependentă de concentrație. În modelul de inflamație acută, tratamentul a redus semnificativ edemul și nivelurile citokinelor (p < 0,05). În modelul CFA, tratamentul a încetinit semnificativ evoluția edemului labei (p < 0,05). Aceste rezultate susțin că *P. cablin* din Kolaka de Nord este o sursă de compuși bioactivi utili în tratarea afecțiunilor inflamatorii și artrite.

Keywords: *Pogostemon cablin*; rheumatoid arthritis; cytokine inhibition; phytochemical profiling; GC-MS/MS

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder that inflames the synovium, degrades cartilage, and destroys joints over time. Immune dysregulation drives the disease, including synovial cells overproducing proinflammatory cytokines, and

oxidative stress builds up in the joint microenvironment. Tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) inflame the synovium, activate osteoclasts, and damage tissue (1-3).

Synthetic disease-modifying antirheumatic drugs and biologics that target these cytokines have

improved outcomes for RA patients, but long-term use carries adverse effects, high costs, and a greater risk of immunosuppression. Researchers have turned to plant-derived compounds as alternative or complementary treatments, especially those with structurally diverse secondary metabolites that act on multiple inflammatory and immune targets (1, 2). Medicinal plants are complex chemical systems in which biological activities are intrinsically linked to the qualitative and quantitative profiles of secondary metabolites (4). Terpenoids, phenolic compounds, flavonoids, and other low-molecular-weight constituents are widely acknowledged as major contributors to anti-inflammatory and immunomodulatory effects (5, 6). Identifying and characterizing these metabolites reveals the therapeutic potential of medicinal plants and grounds their traditional uses in scientific evidence. Comprehensive phytochemical profiling supports quality control and chemotaxonomic assessment, and it clarifies the molecular mechanisms behind observed pharmacological activities (7, 8).

Pogostemon cablin (Blanco) Benth. (*Lamiaceae*) is an aromatic medicinal plant that Asian traditional medicine has long used to treat inflammation, gastrointestinal disorders, infections, and pain (9, 10). The plant produces a wide range of secondary metabolites, including sesquiterpenes, monoterpenes, and other volatile compounds. Several of these show anti-inflammatory, antioxidant, and immunoregulatory effects. Patchouli alcohol and related terpenoids, for instance, modulate inflammatory pathways and cytokine production, which supports the plant's pharmacological relevance (9, 10). Geographical origin, environmental conditions, harvest timing, and post-harvest processing all shape the chemical composition of *P. cablin*, and this variability can produce marked differences in biological activity (10).

Indonesia has some of the largest natural populations of *P. cablin*, yet researchers have focused their phytochemical and pharmacological studies on plants from only a few regions. North Kolaka, in Southeast Sulawesi, remains largely unstudied despite ecological and climatic conditions that likely shape its secondary metabolite profile (11, 12). No study has yet combined comprehensive phytochemical profiling of *P. cablin* from North Kolaka with an experimental evaluation of its potential against rheumatoid arthritis. Previous studies have reported anti-inflammatory effects of *P. cablin* extracts and have characterized samples collected from other Indonesian regions, including North Konawe and Kolaka. However, the chemical profile and anti-arthritic activity of *P. cablin* collected from North Kolaka have not been clearly distinguished from those reported previously. In the present study, leaves collected from North Kolaka were subjected to chromatographic profiling,

fractionation, *in vitro* anti-inflammatory screening, and *in vivo* evaluation using xylene-induced inflammation and CFA-induced arthritis models. This design aimed to determine whether the North Kolaka material and its fractions showed anti-inflammatory and anti-arthritic activity and to identify the experimental evidence needed for subsequent bioactivity-guided standardization.

Therefore, this study aimed to characterize the secondary metabolite profile of *P. cablin* from North Kolaka using GC-MS/MS analysis and to evaluate its anti-rheumatoid arthritis activity through experimental pharmacological approaches. Additionally, the modulatory effects of the extract on key proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6, were investigated to elucidate its anti-inflammatory relevance.

Materials and Methods

Materials

The materials used in this study included dried leaves of *P. cablin* (Blanco) Benth. collected from North Kolaka, Indonesia. Analytical-grade reagents and solvents were used throughout the study. Ethanol 96% (Bratachem®, Indonesia) served as the extraction solvent. Sodium carboxymethyl cellulose (Na-CMC, 0.5%), diclofenac sodium, EDTA, sodium citrate, and heparin were obtained from Sigma-Aldrich (USA). All chemicals and reagents were of analytical grade and used without further purification.

Sample collection and preparation

Leaves of *P. cablin* (Blanco) Benth. (*Lamiaceae*) were collected from cultivated plants in Tambuha Village, North Kolaka, Southeast Sulawesi, Indonesia, at -3.271211, 121.013826, on May 26, 2025. Botanical identification was performed by Dian Munasari Solo at the Research Laboratory, Faculty of Pharmacy, Halu Oleo University. A voucher specimen (*P. cablin*-KLU-27/05/25-ADR) was prepared and retained by the authors for future reference. Only mature leaves were used. The leaves were wet-sorted, cleaned, air-dried at room temperature away from direct sunlight, milled into coarse powder, and stored in airtight containers until extraction.

Extraction and fractionation of *P. cablin*

Dried *P. cablin* leaves were extracted by maceration using ethanol as the solvent. A total of 1,500 g of dried leaves was macerated using ethanol at a plant-to-solvent ratio of 1:14 (w/v), corresponding to 7,000 mL of fresh ethanol *per* maceration cycle. Maceration was performed for three successive cycles of 24 h at room temperature with occasional stirring. After each 24-h cycle, the macerate was filtered through Whatman No. 1 filter paper, and the plant residue was re-macerated with a fresh 7,000 mL portion of ethanol under the same conditions. The

filtrates from the three cycles were combined and concentrated under reduced pressure using a rotary evaporator at 50°C, then dried to constant weight to obtain 78.88 g of crude extract (5.26% yield, w/w). The dried crude extract was stored in an airtight container in a refrigerator at 4-8°C until further analysis (10, 13, 14).

The crude extract (20 g) was dissolved in 100 mL of ethanol. Liquid-liquid partitioning was performed in a separatory funnel using n-hexane; the mixture was shaken until two layers formed, and distilled water (50 mL) was added when the phases failed to separate. The upper (n-hexane) layer was collected, and the lower layer (residue) was re-partitioned with fresh n-hexane; this process was repeated 2-3 times until the solvent layer appeared clear. The final residue was then partitioned in the same manner using ethyl acetate, again repeated 2-3 times until the solvent layer appeared clear. The residue remaining after both partitioning steps constituted the aqueous fraction. Each fraction (n-hexane, ethyl acetate, and aqueous) was collected separately, concentrated under reduced pressure, and dried to constant weight. Yields were: n-hexane fraction, 2.84 g (14.2%); ethyl acetate fraction, 3.38 g (16.9%); aqueous fraction, 6.21 g (31.05%) (w/w, relative to 20 g crude extract partitioned; total recovery 62.17%). Each fraction was stored in an airtight container in a refrigerator at 4-8°C until further analysis (14).

GC-MS/MS Analysis of P. cablin

The ethanolic extract of *P. cablin* was analyzed by GC-MS/MS to identify its volatile and semi-volatile constituents. Briefly, 1 g of the dried extract was dissolved in 2 mL of distilled water and shaken for 10 min. Ethyl acetate (10 mL) and anhydrous sodium sulfate (10 g; Merck®, Germany) were added to remove residual moisture, followed by sonication for 10 min. The mixture was then filtered to remove insoluble materials, and the organic phase was evaporated to dryness. The residue was re-dissolved in 1 mL of n-hexane (Merck®, Germany) and transferred into a sealed GC vial. Analysis was carried out using an Agilent 8890 gas chromatograph coupled with a Waters Xevo® TQ-GC mass spectrometer (UK), operated in single-stage full-scan acquisition mode. Separation was achieved on a DB-5MS capillary column (30 m × 250 µm × 0.25 µm) with helium as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was programmed from 110°C (held for 3.5 min) to 200°C at 10°C/min (held for 1 min), followed by an increase to 280°C at 5°C/min (held for 12 min). The injector temperature was set at 280°C in splitless mode, and 1 µL of sample was injected. The mass spectrometer operated in electron ionization (EI) mode over a mass range of 50-500 m/z, with the ion source and transfer line temperatures set at 200°C and 250°C, respectively. Solvent delay and detector voltage parameters were not recorded

during data acquisition and are therefore not reported. Compound identification was performed by comparing mass spectra with the NIST Chemistry WebBook (NIST Standard Reference Database Number 69, 2025/2026 update) using spectral match, reverse match, and probability scores.

A solvent blank (n-hexane) was analyzed under identical GC-MS/MS conditions to identify potential solvent- or laboratory-derived artifacts. Peaks detected in the sample chromatogram at retention times overlapping with peaks present in the solvent blank (retention time range 3.0 - 8.0 min) were excluded from compound reporting, as they could not be reliably distinguished from background contamination. Only compounds with retention times not corresponding to blank peaks (10.6 - 20.0 min) are reported in Table I. No internal standard or n-alkane series (C7-C30) was co-injected; therefore, retention indices were not calculated, and compound identification relies solely on library spectral matching. Relative abundance is reported as library match, reverse match, and probability scores (%) rather than percentage relative peak area (10).

In vitro anti-inflammatory activity

Human Red Blood Cell (HRBC) Membrane Stabilization Assay

The anti-inflammatory activity of the extract and fractions was evaluated using the human red blood cell (HRBC) membrane stabilization assay. As a preliminary study, fresh blood (10 mL) was collected via venipuncture from a single healthy volunteer (44 years old, male) after obtaining informed consent, with approval from the Health Research Ethics Committee of the Institute for Research and Community Service (LPPM), Universitas Halu Oleo, Indonesia (approval no. 3342/UN29.20.1.2/PG/2024). The donor had no history of disease or medication use; exclusion criteria included use of NSAIDs/anti-inflammatory drugs within the preceding 2 weeks, history of hematological disorders, or hemolytic conditions. Blood was transferred into heparinized tubes and centrifuged at 855 × g for 10 min at 25°C, and the erythrocyte pellet was washed three times with isotonic saline solution. The washed cells were resuspended in isotonic saline to obtain a 10% (v/v) erythrocyte suspension.

Stock solutions of the extract and fractions were prepared at 1,000 µg/mL in ethanol and serially diluted with isotonic saline to obtain final test concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL, corresponding to final ethanol concentrations of approximately 0.14%, 0.28%, 0.56%, 1.11%, and 2.22% (v/v) in the reaction mixture, respectively. The reaction mixture consisted of 0.5 mL of erythrocyte suspension, 1 mL of phosphate-buffered saline (PBS, pH 7.4), 2 mL of hyposaline solution, and 1 mL of the extract or fractions at the concentrations above. Diclofenac sodium, the reference standard, was

tested at the same concentrations: 6.25, 12.5, 25, 50, and 100 µg/mL. A vehicle-only control (isosmotic saline containing 2.22% v/v ethanol, corresponding to the highest ethanol concentration tested, without extract) was included in parallel to account for any intrinsic effect of ethanol on erythrocyte membrane stability.

The mixtures were incubated in a water bath at 56°C for 30 min to induce hemolysis under heat stress conditions, followed by centrifugation at $2376 \times g$ for 10 min. The absorbance of the supernatant, corresponding to released hemoglobin, was measured at 560 nm using a UV-Vis spectrophotometer. All assays were performed using blood from this single donor as a preliminary assessment, with three technical replicates *per* concentration. The percentages of hemolysis and membrane stability were calculated using Equations [1] and [2] (1, 13).

$$\% \text{hemolysis} = \frac{\text{Abs test}}{\text{Abs control}} \times 100 \quad [1]$$

$$\% \text{stability} = 100 - \% \text{hemolysis} \quad [2]$$

Where Abs test is the absorbance of HRBC suspension with the test samples, while Abs control is the absorbance of HRBC suspension without the test samples.

BSA denaturation inhibition assay

The *in vitro* anti-inflammatory activity of the extract and fractions was evaluated using the protein denaturation inhibition assay, which assesses the ability of test samples to stabilize bovine serum albumin under stress conditions. Briefly, 50 µL of each sample solution was mixed with 5 mL of 0.2% (v/v) bovine serum albumin prepared in Tris-buffered saline (TBS, pH 7.4). The mixture was incubated at 25°C for 30 min to allow interaction between the sample and the protein. Protein denaturation was induced by heating the mixture at 72°C for 1 min, followed by cooling at room temperature (25°C) for 25 min. The degree of turbidity, reflecting the extent of protein denaturation, was measured at 660 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). All assays were performed with three technical replicates *per* concentration. The percentage inhibition of protein denaturation was calculated using Equation [3] (1, 13, 15).

$$\% \text{inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad [3]$$

Where Abs control is the absorbance of the BSA reaction mixture without the test samples, while Abs sample is the absorbance of the BSA reaction mixture with the test samples.

Ethical approval

Animal care procedures were carried out at the Animal Care Laboratory, Faculty of Pharmacy, Universitas Halu Oleo, Indonesia. All experimental procedures involving animals were reviewed and approved by the Health Research Ethics Committee of

the Institute for Research and Community Service (LPPM), Universitas Halu Oleo, Indonesia, under approval number 3343/UN29.20.1.2/PG/2024, and were conducted in accordance with international guidelines for the care and use of laboratory animals. *In vivo anti-inflammatory activity of extract and fractions*

Experimental animals

18 male BALB/c mice (8-10 weeks old; body weight 20-30 g) were obtained from the Experimental Animal Laboratory, Faculty of Medicine, Halu Oleo University. The animals were maintained under standard laboratory conditions (12 h light/dark cycle, temperature $22 \pm 2^\circ\text{C}$, relative humidity 60-70%) with free access to food and water. All mice were allowed to acclimatize for at least 7 days prior to the experiment. Sample size ($n = 3$ *per* group) was selected based on prior studies from our research group using the same extract and model system (10), consistent with sample sizes commonly used in preliminary/exploratory *in vivo* pharmacological screening of plant-derived extracts. Humane endpoints, including signs of severe distress, greater than 20% loss of body weight, or inability to access food or water, were monitored throughout the study in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, 2011); no animals met these criteria during the study period.

Induction and evaluation of acute inflammation

Acute inflammation was induced by topical application of xylene (20 µL) to both the anterior and posterior surfaces of the right ear of each mouse, a therapeutic (post-induction) protocol. Fifteen minutes after xylene administration, the animals received oral treatments consisting of 0.5% Na-CMC (negative control), diclofenac sodium (6.5 mg/kg BW, positive control), or ethanolic extract of *P. cablin* at doses of 25, 50, and 100 mg/kg BW. All treatments were suspended or dissolved in 0.5% Na-CMC as the vehicle and administered at a fixed dosing volume of 10 mL/kg BW (0.01 mL/g BW). This time-course design follows the protocol established in prior validated studies from our research group using the same model. The normal group received neither xylene nor treatment. Ear thickness was measured once *per* animal at each time point using a digital vernier caliper (accuracy: 0.01 mm) at 15, 30, and 45 min after xylene application. The percentage of inflammation was calculated using the following equation [4] (10):

$$\% \text{inflammatory} = [(D_t - D_0)/D_0] \times 100 \quad [4]$$

where D_t represents ear thickness at each observation time, and D_0 represents baseline thickness before induction. The percentage of inflammation inhibition was determined as follows, according to equation [5]:

$$\% \text{inhibition} = [(a - b)/a] \times 100 \quad [5]$$

where *a* is the percentage of inflammation in the xylene-induced vehicle (negative control) group, and *b* is the percentage of inflammation in the treated group. The doses of 25, 50, and 100 mg/kg BW were selected based on a previous report demonstrating dose-dependent anti-inflammatory efficacy of *P. cablin* ethanolic extract at the same dose range in an identical xylene-induced ear edema model (10).

Determination of Pro-inflammatory Cytokine Levels
Following the final ear thickness measurement at 45 min post-induction, the animals were euthanized by cervical dislocation. Blood samples (1 mL) were collected via intracardiac puncture into EDTA-coated tubes and centrifuged at $855 \times g$ for 15 min to obtain plasma. Plasma samples were stored at -80°C until further analysis. Plasma levels of TNF- α , IL-1 β , and IL-6 were quantified using commercial ELISA kits (Elabscience®, Houston, TX, USA; Cat. No. E-EL-M0037, E-EL-M0039, and E-EL-M0046, respectively), following the manufacturer's protocols. The vehicle-treated group served as the negative control, while diclofenac sodium-treated animals were used as the positive control for cytokine inhibition (10).

Anti-rheumatoid arthritis activity using complete freund's adjuvant-induced arthritis model

Induction of arthritis

Rheumatoid arthritis (RA) was induced using the complete Freund's adjuvant (CFA) model. A total of twenty-four (24) male BALB/c mice, weighing 20–30 g, were acclimatized under standard laboratory conditions for 7 days with free access to food and water. Arthritis was induced by a single intraplantar injection of 0.1 mL CFA (Freund's Complete Adjuvant, Modified, *Mycobacterium butyricum*, Cat. No. 344289, CAS 9007-81-2, Sigma-Aldrich, USA), composed of 85% mineral oil (Drakeol 5NF), 15% mannide monooleate (Arlacel A), and 0.1% heat-killed *M. butyricum* dry cells (w/w) into the right hind paw of each mice, except for the normal group. Paw edema was recorded from day 0 to day 16 to confirm the successful establishment of arthritis. Treatment was subsequently administered from day 17 to day 31 post-induction, as illustrated in the study timeline (Figure 1) and described in the following sections (1, 9).



Figure 1.

Study design timeline for the complete Freund's adjuvant (CFA)-induced arthritis model. Arthritis was induced by a single intraplantar injection of 0.1 mL CFA into the right hind paw on day 0. Paw edema was monitored from day 0 to day 16 to confirm successful establishment of arthritis. Oral treatment (extract, fractions, methotrexate, or vehicle, according to the experimental group) was administered once daily for 15 consecutive days, from day 17 to day 31 post-induction, with the study endpoint on day 31

Experimental groups

Animals were randomly divided into eight groups ($n = 3$ per group). Group I served as the normal control and received no CFA or treatment. Group II was the negative control (C-), consisting of CFA-induced mice treated with 0.5% Na-CMC. Group III served as the positive control (C+), consisting of CFA-induced mice treated with methotrexate *per os* (2.5 mg/kg BW, thrice weekly; Sigma-Aldrich, USA), consistent with standard anti-arthritic dosing regimens for methotrexate in murine models. Groups IV-V consisted of CFA-induced mice treated orally with the ethanolic extract of *P. cablin* (EEPC) at doses of 50 and 100 mg/kg BW, respectively. Groups VI-VIII consisted of CFA-induced mice treated orally with the ethyl acetate, n-hexane, and aqueous fractions of EEPC, respectively, each administered at a dose of 100 mg/kg BW. All

treatments were suspended or dissolved in 0.5% Na-CMC as the vehicle and administered orally at 09:00 AM at a fixed dosing volume of 10 mL/kg BW (0.01 mL/g BW). Na-CMC, the ethanolic extract, and the fractions were administered once daily for 15 consecutive days (day 17 to day 31 post-induction), while methotrexate was administered thrice weekly (approximately six doses in total) over the same period. Animals were monitored visually throughout the treatment period for overt signs of toxicity (*e.g.*, lethargy, piloerection, abnormal posture); no mortality was observed (1, 9).

Measurement of paw edema

Paw edema thickness was measured once per animal at each time point from day 17 to day 31 in CFA-induced mice using the same digital ultrasonic thickness gauge (Benetech GM100, 5 MHz probe; measuring range 1.2–225 mm; accuracy $\pm(1\%H + 0.1)$

mm; resolution 0.1 mm) used for ear thickness measurement. Paw thickness changes were used as an indicator of arthritis. The percentage of rheumatoid

arthritis inhibition (%RA inhibition) for each treatment group was calculated using the following equation [6] to evaluate therapeutic efficacy (1, 9).

$$\% \text{inhibition} = \frac{\text{Edema thickness of (C-) group} - \text{edema thickness of treatment group}}{\text{edema thickness of (C-) group}} \times 100\% \quad [6]$$

Data analysis

All experimental data were analyzed using IBM SPSS Statistics software (version 27). For single-timepoint, between-group comparisons, statistical comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify significant differences between groups. For longitudinal ear-edema data (15, 30, and 45 min post-treatment), a two-way repeated-measures ANOVA (Time x Treatment) was used, followed by Tukey's post hoc test where appropriate; Mauchly's test was used to assess sphericity, with Greenhouse-Geisser correction applied where violated. Normality of data was assessed using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test, prior to all parametric analyses. Here, n refers to the number of individual animals *per* group (biological replicates), not technical replicates, as each animal was measured once *per* time point. A *p* value < 0.05 was considered statistically significant. All data are presented as mean ± standard deviation (SD).

Results and Discussion

GC-MS/MS identification of *P. cablin* from North Kolaka

Chromatographic analysis of the ethanolic leaf extract resulted in the tentative detection of seven volatile or semi-volatile constituents, as presented in Table I. The assignments were based on retention time and comparison of mass spectra with the NIST library. Because several compounds showed low match probabilities and authentic standards were not used, these identifications should be regarded as tentative. Relative peak areas and retention indices should be added before major constituents are ranked.

Among the tentatively identified constituents, patchouli alcohol exhibited the longest retention time (19.996 min), whereas eucalyptol eluted earliest (10.589 min) among the constituents retained after exclusion of six early-eluting peaks (retention time 3.1- 9.1 min) that overlapped with solvent blank signals and were therefore considered potential laboratory or solvent-derived artifacts rather than genuine plant metabolites. Seven terpenoid constituents were tentatively identified, namely eucalyptol, γ -terpinene, *p*-cymene, caryophyllene, α -guaiane, sychellene, and patchouli alcohol. Compound identification was performed based on comparison with mass spectral library databases, yielding similarity match probabilities ranging from 3.34% to 19.4%,

with the highest value corresponding to eucalyptol and the lowest to caryophyllene. Given the relatively low similarity indices obtained, these assignments should be regarded as tentative rather than conclusive, as compound identities were not verified against authentic reference standards. A comprehensive GC-MS/MS profile, encompassing retention times, molecular weights, similarity scores, and previously reported biological activities, is presented in Table I.

This study provides additional pharmacognostic and pharmacological insights into *P. cablin* by integrating phytochemical characterization with an evaluation of its anti-rheumatoid arthritis (RA) activity. Beyond demonstrating anti-inflammatory effects, the findings provide experimental evidence that sesquiterpene-enriched fractions of *P. cablin* collected from North Kolaka modulate the inflammatory and immune responses involved in RA pathogenesis. The strength of this study lies in its integrated experimental design, which combines GC-MS/MS-based phytochemical profiling, fraction-based pharmacological assessment, RA-relevant *in vitro* assays, immune-mediated *in vivo* modeling, and cytokine analysis. To date, no previous study has comprehensively investigated the anti-rheumatoid arthritis potential of *P. cablin* from North Kolaka using a complete Freund's adjuvant (CFA)-induced RA model. These findings emphasize the relevance of geographical origin and fractionation in determining the biological activity of plant-derived materials.

The identification of a fraction with consistent activity supports further bioactivity-guided isolation and underscores its potential for standardization, both of which are critical steps in the rational development of phytopharmaceutical products. GC-MS/MS profiling of the volatile and semi-volatile fraction of the ethanolic extract tentatively identified seven constituents after exclusion of solvent blank-associated peaks, comprising sesquiterpenes (caryophyllene, α -guaiane, sychellene, patchouli alcohol) and monoterpenes (eucalyptol, γ -terpinene, *p*-cymene). It should be noted that GC-MS/MS is inherently limited to volatile and thermally stable compounds; since the sample analyzed was an ethanolic extract rather than an essential oil, a substantial portion of the extract's mass, including polar and non-volatile constituents, was not amenable to detection by this method. Therefore, this profile represents only a subset of the extract's overall chemical composition. Patchouli alcohol, a compound widely recognized as a chemical marker of *P. cablin*, was among the

constituents tentatively identified in the present study. This finding suggests that region-specific ecological conditions in North Kolaka may influence secondary metabolite biosynthesis, thereby contributing to variations in pharmacological activity (10, 13, 16).

Earlier studies on *P. cablin* have focused mainly on essential oil composition in relation to perfumery, antimicrobial, or antioxidant applications, with relatively little emphasis on immunological relevance

(17, 18). The results of the present study support the hypothesis that oxygenated sesquiterpenes are involved not only in antioxidant mechanisms but also in modulating immune responses. Moreover, the detection of multiple bioactive constituents suggests that the observed anti-arthritic effects are more likely due to synergistic interactions among compounds than to the action of a single predominant component.

Table I

Chemical constituents identified in the ethanolic leaf extract of *Pogostemon cablin* collected from North Kolaka, as determined by gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis

No.	Retention Time	Molecular Weight (MW)	Match	Reverse Match	Probability (%)	Compounds name	Biological activity	Suggested Identification
1.	10.589	154	660	665	19.4	Eucalyptol (C ₁₀ H ₁₈ O)	Anti-inflammatory, antimicrobial, and analgesic (19,20)	Tentative
2.	11.578	136	612	649	5.38	γ -Terpinene (C ₁₀ H ₁₆)	Antioxidant, Anti-inflammatory (21,22)	Tentative
3.	12.190	134	684	739	10.2	p-Cymene (C ₁₀ H ₁₄)	Anti-inflammatory, antibacterial, analgesic (23)	Tentative
4.	16.796	204	705	707	3.34	Caryophyllene (C ₁₅ H ₂₄)	Anti-inflammatory, analgesic, Antioxidant (24)	Tentative
5.	16.952	204	733	751	8.51	α -Guaiene (C ₁₅ H ₂₄)	Antimicrobial/antifungal (25)	Tentative
6.	17.242	204	737	750	5.59	Seychellene (C ₁₅ H ₂₄)	Antimicrobial, Antioxidant (26)	Tentative
7.	19.996	222	628	629	17.3	Patchouli alcohol (C ₁₅ H ₂₆ O)	Anti-inflammatory, antimicrobial (10)	Tentative

HRBC membrane stabilization activity of *P. cablin* extract and fractions

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets satisfied these assumptions ($p > 0.05$). The HRBC membrane stabilization assay demonstrated that the ethanolic extract and fractions of *P. cablin* leaves exhibited concentration-dependent anti-inflammatory activity, as reflected by decreased hemolysis and increased erythrocyte membrane stability (Figure 2). As shown in Figure 2A, the ethanolic extract of *P. cablin* progressively reduced the percentage of hemolysis with increasing concentrations (6.25 - 100 μ g/mL), accompanied by a corresponding increase

in membrane stabilization. At the highest concentration tested, the extract showed substantial protective effects against erythrocyte membrane lysis, although its activity remained lower than that of the positive control, sodium diclofenac. The IC₅₀ of sodium diclofenac could not be calculated for this assay, as its percentage of membrane stabilization exceeded 87% across the entire tested concentration range, leaving no data points bracketing the 50% response level. The ethyl acetate fraction (Figure 2B, Table II) also demonstrated a concentration-dependent reduction in hemolysis and an increase in membrane stabilization. However, its overall potency was lower than that of the other tested *P. cablin* samples based on its higher IC₅₀ value (14.62 $\pm$ 0.05 μ g/mL).

Table II

Half-maximal inhibitory concentration (IC₅₀) values of *P. cablin* extract and its solvent fractions against HRBC membrane stabilization. Values are presented as mean $\pm$ standard deviation (SD) of three independent determinations (n = 3)

No.	Samples	IC ₅₀ (μ g/mL)
1.	<i>P. cablin</i> Extract	8.72 $\pm$ 1.08
2.	Ethyl acetate fraction of <i>P. cablin</i>	14.62 $\pm$ 0.05
3.	n-hexane fractions of <i>P. cablin</i>	8.14 $\pm$ 0.10
4.	Aqueous fraction of <i>P. cablin</i>	9.73 $\pm$ 0.06

Na-Dic IC₅₀ could not be determined (>87% membrane stabilization at all tested concentrations, 6.25–100 μ g/mL).

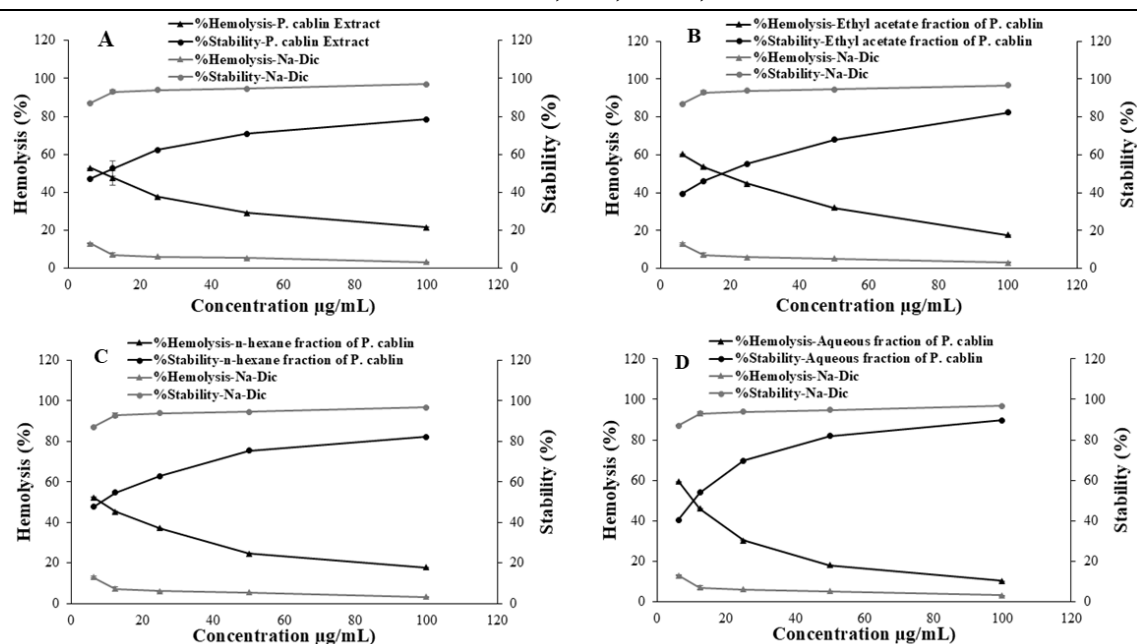


Figure 2.

Hemolysis inhibition and erythrocyte membrane stabilizing activity (HRBC assay) of (A) crude extract, (B) ethyl acetate fraction, (C) n-hexane fraction, and (D) aqueous fraction of *P. cablin* leaves collected from North Kolkata, tested at concentrations ranging from 6.25 to 100 µg/mL. Sodium diclofenac served as the reference standard. Values represent the mean $\pm$ standard deviation of triplicate determinations ($n = 3$)

Similarly, the n-hexane fraction (Figure 2C, Table II) showed a concentration-dependent decrease in hemolysis and enhancement of membrane stability. Based on the IC_{50} values, the n-hexane fraction exhibited the strongest membrane-stabilizing activity among the tested *P. cablin* samples, with the lowest IC_{50} value (8.14 ± 0.10 µg/mL). The aqueous fraction (Figure 2D, Table II) also exhibited anti-hemolytic activity, with a clear trend of decreasing hemolysis and increasing membrane stabilization as concentration increased. Its IC_{50} value (9.73 ± 0.06 µg/mL) indicated moderate membrane-stabilizing potency compared with the n-hexane fraction and crude extract.

Protein denaturation inhibitory activity of *P. cablin* extract and fraction

Anti-denaturation activity of the *P. cablin* extract and its fractions was assessed by the bovine serum albumin assay. As shown in Figure 3, all tested samples exhibited concentration-dependent inhibition in the range of 6.25-100 µg/mL.

The IC_{50} values are summarized in Table III. Sodium diclofenac exhibited the lowest IC_{50} value (37.20 ± 1.05 µg/mL), followed by the aqueous fraction (59.83 ± 0.96 µg/mL), n-hexane fraction (60.93 ± 0.34 µg/mL), crude extract (63.04 ± 0.96 µg/mL), and ethyl acetate fraction (79.51 ± 1.56 µg/mL).

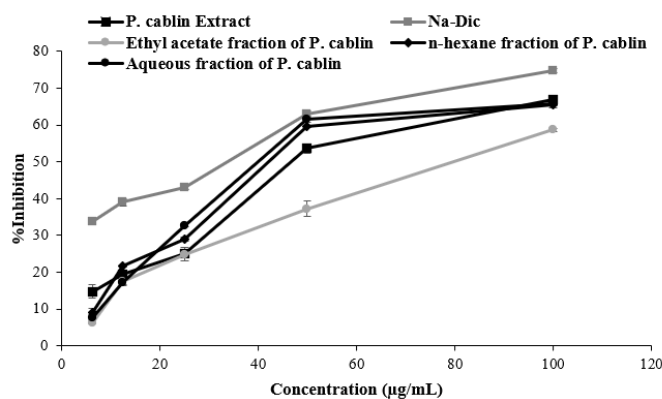


Figure 3.

Inhibitory effect of sodium diclofenac (Na-Dic), *P. cablin* extract, and its ethyl acetate, n-hexane, and aqueous fractions on protein denaturation across tested concentrations. Values represent the mean $\pm$ standard deviation of triplicate determinations ($n = 3$)

Table III

Half-maximal inhibitory concentration (IC₅₀) values of *P. cablin* extract, its solvent fractions, and sodium diclofenac against protein denaturation. Values represent the mean $\pm$ standard deviation of triplicate determinations (n = 3)

No.	Samples	IC ₅₀ (μg/mL)
1.	Na-Dic	37.20 $\pm$ 1.05
2.	<i>P. cablin</i> Extract	63.04 $\pm$ 0.96*
3.	Ethyl acetate fraction of <i>P. cablin</i>	79.51 $\pm$ 1.56*
4.	n-hexane fractions of <i>P. cablin</i>	60.93 $\pm$ 0.34*
5.	Aqueous fraction of <i>P. cablin</i>	59.83 $\pm$ 0.96*

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets satisfied these assumptions ($p > 0.05$). One-way ANOVA revealed significant differences among treatment groups ($p < 0.05$). Post hoc Tukey analysis showed that sodium diclofenac had significantly higher inhibitory activity than the extract and all fractions ($p < 0.05$). No significant difference was observed among the aqueous fraction, n-hexane fraction, and crude extract ($p > 0.05$), whereas the ethyl acetate fraction showed significantly lower activity ($p < 0.05$).

Interestingly, the activity rankings differed between the HRBC membrane stabilization and BSA protein denaturation assays. In the HRBC assay, the n-hexane fraction showed the lowest IC₅₀ value, indicating the highest membrane-stabilizing potency among the *P. cablin* samples, whereas the ethyl acetate fraction showed the highest IC₅₀ value. In contrast, in the BSA assay, the aqueous fraction exhibited the lowest IC₅₀ among the *P. cablin* samples, while the ethyl acetate fraction again showed the weakest activity. These differences suggest that the fractions may contain different proportions of constituents that interact with erythrocyte membranes or proteins through distinct mechanisms. Therefore, activity in the HRBC membrane stabilization assay does not necessarily correspond directly to activity in the protein denaturation assay.

Membrane destabilization and protein denaturation both contribute to the amplification of inflammatory responses and the generation of autoantigens in rheumatoid arthritis (27). The differential activity profiles observed across the two assays point to multiple biological mechanisms and phytochemical constituents driving the anti-inflammatory and anti-arthritis effects of *P. cablin* (10, 13).

These *in vitro* models cannot capture immune cell signaling, cytokine networks, or the metabolic transformation of phytochemicals *in vivo*. The HRBC membrane stabilization and protein denaturation results therefore support the *in vivo* anti-inflammatory and anti-rheumatoid arthritis findings without proving therapeutic efficacy on their own.

In vivo anti-inflammatory activity of P. cablin extract

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets

satisfied these assumptions ($p > 0.05$). Xylene induction produced a marked increase in ear edema thickness in the negative control group. A two-way repeated-measures ANOVA (Time $\times$ Treatment) revealed significant main effects of Treatment ($F(4,10) = 36.42$, $p < 0.0001$) and a significant Time $\times$ Treatment interaction ($F(8,20) = 4.21$, $p = 0.004$); the main effect of Time alone was not significant after Greenhouse-Geisser correction ($p = 0.191$). Follow-up simple-effects analyses (one-way ANOVA with Tukey's post hoc) at each time point showed that the positive control significantly reduced ear edema compared to the negative control at 15, 30, and 45 min ($p < 0.05$ at all time points), as shown in Figure 4. Treatment with *P. cablin* extract (EEPC-KU) at 100 mg/kg significantly reduced ear edema thickness compared to the negative control from 15 min onward ($p < 0.05$), while the 25 and 50 mg/kg doses reached statistical significance from 30 min onward ($p < 0.05$). The effect of the extract at 100 mg/kg at 45 min was statistically comparable to the positive control ($p > 0.05$). The reduction in edema was time- and dose-dependent.

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets satisfied these assumptions ($p > 0.05$). The percentage inhibition of inflammation showed that the positive control produced the highest inhibitory effect ($p < 0.05$ vs. negative control), as shown in Figure 5. Administration of *P. cablin* extract (EEPC-KU) resulted in a significant, dose-dependent increase in percentage inhibition compared to the negative control ($p < 0.05$). The inhibition observed at 100 mg/kg was comparable to the positive control ($p > 0.05$), whereas the 25 and 50 mg/kg doses showed lower inhibitory effects ($p < 0.05$).

The *in vivo* ear edema model was employed as an initial assessment of acute inflammatory responses, reflecting early vascular permeability changes and inflammatory mediator release. Treatment with *P. cablin* extract and its fractions significantly reduced ear edema formation, indicating effective suppression of acute inflammatory processes. Although this model does not involve immune sensitization, it provides important preliminary evidence that *P. cablin* can interfere with early inflammatory events that may precede chronic inflammation. These findings

are consistent with previous reports describing the general anti-inflammatory activity of *P. cablin*,

while extending its relevance beyond transient symptomatic inflammation (10, 13, 28).

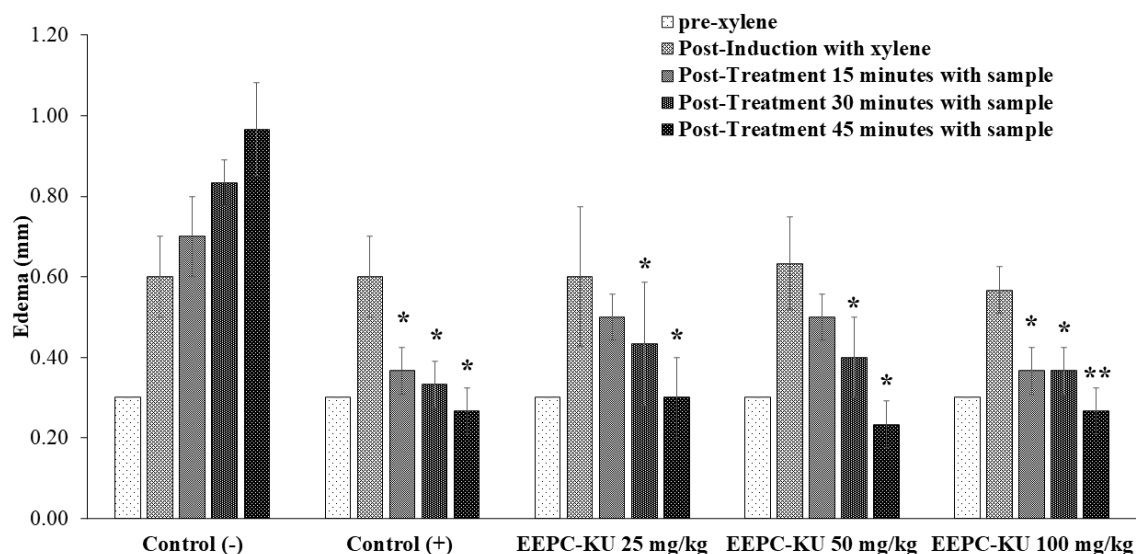


Figure 4.

Anti-inflammatory effect of *Pogostemon cablin* leaf extract (EEPC-KU) on xylene-induced ear edema in mice, assessed at 15, 30, and 45 min post-induction. Values represent the mean $\pm$ SD of $n = 3$ animals *per* group

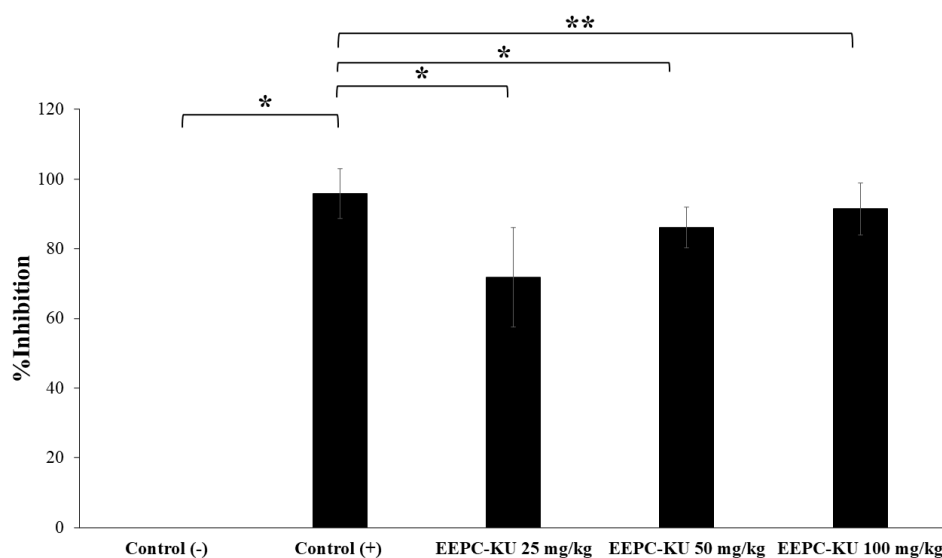


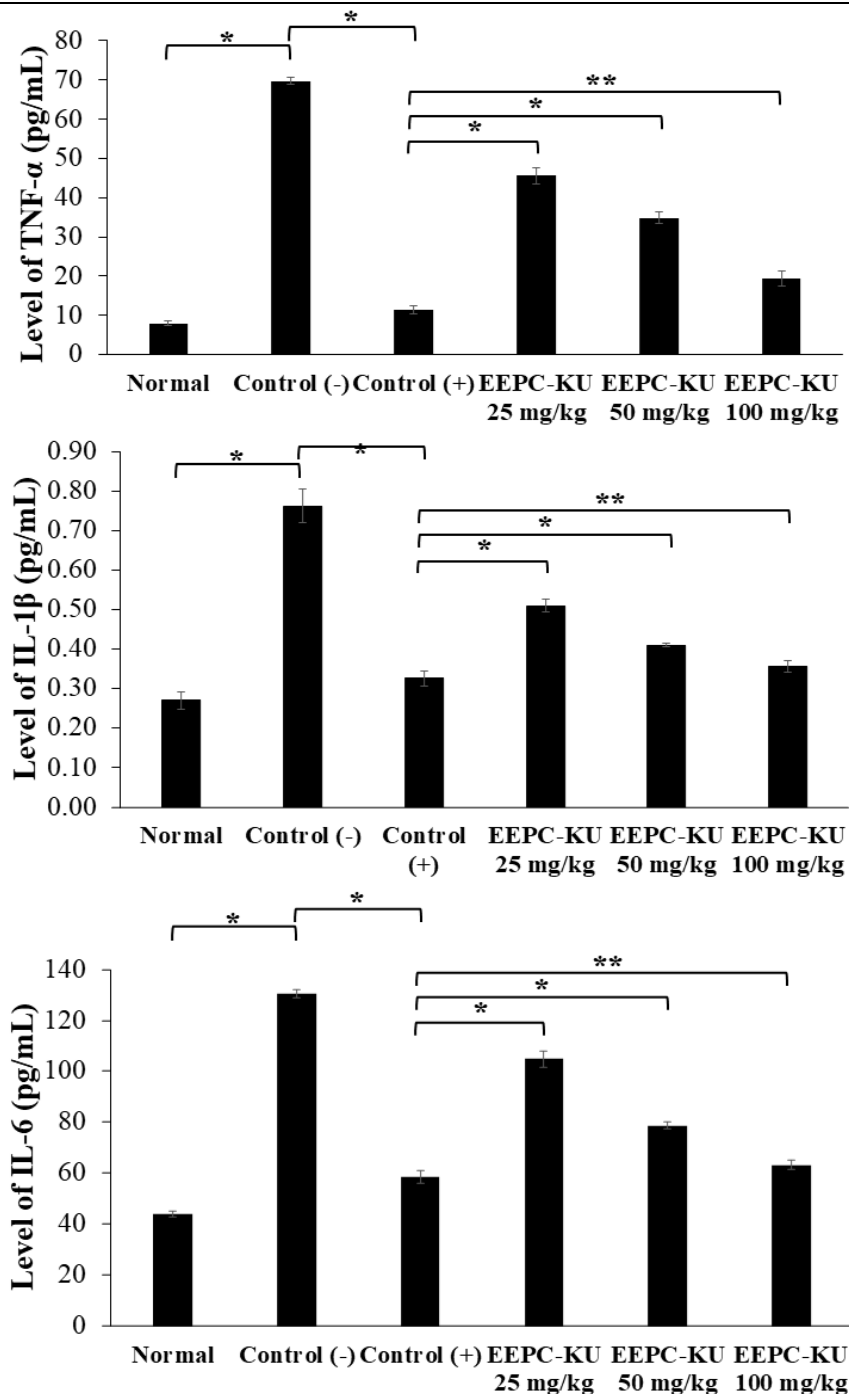
Figure 5.

Inhibitory effect of *P. cablin* ethanolic extract (EEPC-KU) on xylene-induced ear edema in mice, expressed as percentage inhibition relative to the control group. Values represent the mean $\pm$ SD of $n = 3$ animals *per* group

Effect of *P. cablin* extract on proinflammatory cytokine levels

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets satisfied these assumptions ($p > 0.05$). Xylene induction significantly increased TNF- α , IL-1 β , and IL-6 levels in the negative control group compared with the normal group ($p < 0.05$), as shown in Figure 6A-C. The positive control significantly reduced these proinflammatory cytokine levels compared with the negative

control ($p < 0.05$). Treatment with *P. cablin* extract (EEPC-KU) at doses of 25, 50, and 100 mg/kg significantly decreased TNF- α , IL-1 β , and IL-6 levels compared to the negative control ($p < 0.05$). The reductions were dose-dependent, with the greatest decrease observed at 100 mg/kg. At this dose, cytokine levels were comparable to those of the positive control ($p > 0.05$), whereas the 25 and 50 mg/kg doses showed significantly higher cytokine levels than the positive control ($p < 0.05$).

**Figure 6.**

Effects of *P. cablin* extract (EEPC-KU) on proinflammatory cytokine levels in experimental animals: (a) TNF- α , (b) IL-1 β , and (c) IL-6. Values are presented as mean $\pm$ standard deviation (SD), $n = 3$ per group

Plasma levels of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 were measured to clarify the mechanism behind these effects. These three mediators link acute inflammation to chronic immune disorders such as rheumatoid arthritis, driving synovial inflammation, activating osteoclasts, and degrading cartilage (29, 30). Treatment suppressed all three cytokines simultaneously, a pattern consistent with modulation of an upstream signaling pathway rather than inhibition of a single mediator. Nuclear factor

kappa B (NF- κ B) stands out as a candidate, since it coordinates proinflammatory cytokine production in rheumatoid arthritis (31).

Prior studies demonstrate that patchouli alcohol suppresses TNF- α in macrophage models (32, 33), but *in vivo* evidence for coordinated modulation across multiple cytokines has remained scarce. These findings address part of that gap and suggest a broader immunomodulatory role for *P. cablin*. Cytokines were measured only in plasma; plasma levels may

not reflect conditions within the local joint microenvironment.

Anti-rheumatoid arthritis activity

Arthritis was induced in mice via intraplantar injection of Complete Freund's Adjuvant (CFA) on day 0, and animals were monitored for 16 days for the development of arthritic manifestations, including progressive paw swelling and erythema. On day 17, paw edema thickness was measured to quantify inflammation severity. As shown in Figure 7, CFA-injected mice exhibited a significant increase in paw thickness relative to pre-induction baseline values ($p < 0.05$), confirming successful establishment of the adjuvant-induced arthritis model.

Pronounced paw swelling was observed in the negative control group, in contrast to the normal group, which displayed no visible signs of inflammation. Treatment with the positive control markedly reduced paw edema. Mice treated with *P. cablin* extract (EEPC-KU) at doses of 50 and 100 mg/kg, as well as those receiving the ethyl acetate, n-hexane, and aqueous fractions (100 mg/kg), showed visibly reduced paw swelling compared to the negative control. The reduction in paw edema in the treated groups was comparable to that observed in the positive control.

Normality and homogeneity of variance were assessed (Shapiro-Wilk and Levene's tests, respectively) prior to one-way ANOVA; all datasets satisfied these assumptions ($p > 0.05$). The percentage

inhibition of rheumatoid arthritis showed that the positive control produced the highest inhibitory effect ($p < 0.05$ vs. negative control), as shown in Figure 8. Treatment with *P. cablin* extract (EEPC-KU) at doses of 50 and 100 mg/kg, as well as the ethyl acetate, n-hexane, and aqueous fractions (100 mg/kg), resulted in a significant increase in RA inhibition compared to the negative control ($p < 0.05$). The inhibitory effects observed in the EEPC-KU-treated and fraction-treated groups were comparable to the positive control ($p > 0.05$).

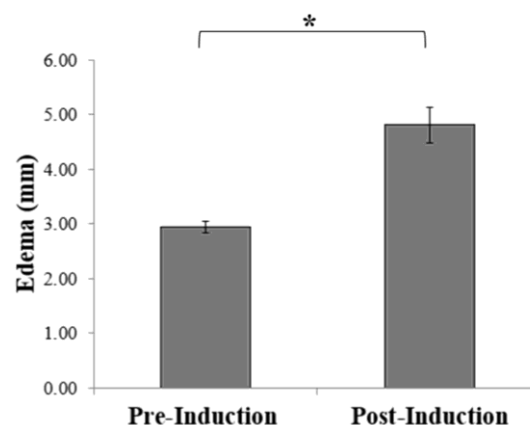


Figure 7.

Effect of complete Freund's adjuvant (CFA) injection on paw thickness in mice. Values are expressed as mean $\pm$ SD ($n = 3$)

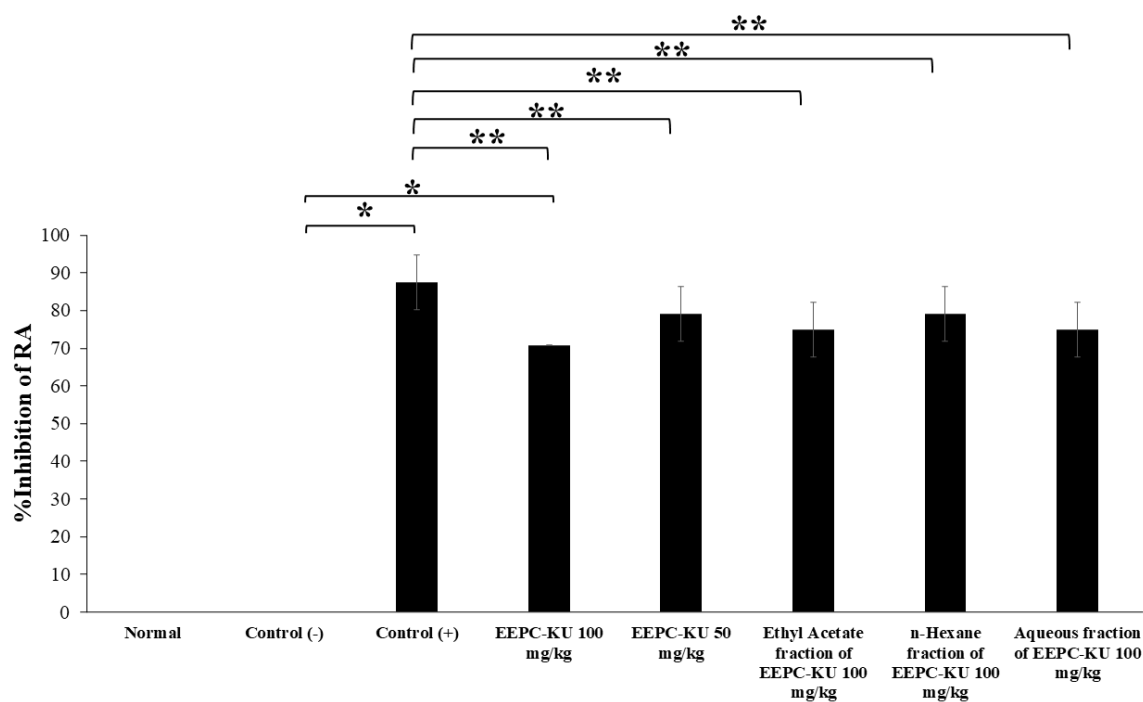


Figure 8.

Inhibitory effect of *Pogostemon cablin* extract (EEPC-KU) and its derived fractions on rheumatoid arthritis progression, expressed as percentage inhibition. Values represent the mean $\pm$ standard deviation (SD) of three independent replicates ($n = 3$)

Based on the observed anti-edematous and cytokine-modulating effects, the complete Freund's adjuvant (CFA)-induced arthritis model was employed to evaluate the efficacy of *P. cablin* under conditions that more closely mimic human rheumatoid arthritis. Carrageenan-induced paw edema, the model used in most prior *P. cablin* studies, captures acute inflammation only. The CFA model differs, including macrophage activation, T-cell responses, and cytokine dysregulation that persist over time, so it better approximates autoimmune arthritis (34, 35). When the extract and its fractions reduced paw edema in this model, they suppressed a chronic inflammatory process, not just the acute mediators that carrageenan tests measure. This points to immune-regulatory activity beyond simple anti-inflammatory action.

Paw edema alone cannot establish disease modification. This study did not assess synovial hyperplasia, cartilage loss, or bone erosion, so the extract's effect on the arthritic disease course remains unconfirmed. The xylene-induced model produced a distinct pattern, such as TNF- α , IL-1 β , and IL-6 concentrations decreased after treatment, confirming anti-inflammatory activity in an acute setting. These cytokines, however, were measured in a different model under different conditions. Extending the finding to CFA-induced arthritis would require plasma or joint-tissue cytokine measurements in the arthritic animals themselves, which this study did not perform. Together, the results offer preliminary evidence of anti-inflammatory and anti-arthritic potential for the extract and its fractions. A specific anti-rheumatoid mechanism has not been established.

Several limitations qualify these findings. Fraction partitioning (n-hexane, ethyl acetate, aqueous) was performed as a single step rather than repeated to exhaustion, which may have left separation by polarity incomplete. The HRBC assay relied on blood from a single donor, so inter-individual variability could not be assessed; serial dilution from an ethanolic stock also produced varying vehicle concentrations across test doses (0.14 - 2.22% v/v), and a contribution of ethanol to the observed effects cannot be fully excluded despite the highest-concentration vehicle control. The small *in vivo* sample size (n=3/group) limits reliable normality assessment and constrains the robustness of parametric tests. The 45-min timepoint chosen for plasma cytokine measurement precedes the typical peak kinetics of TNF- α and IL-6; these findings should be treated as preliminary pending studies with additional timepoints (e.g., 1, 2, 4 h). The short interval between xylene induction and treatment, as early as 15 min, follows an established protocol from prior work (10) but still warrants cautious interpretation; an extended observation window (2-6 h) would strengthen future studies. Cytokines were measured only in the acute ear-edema model, not in CFA-arthritic animals, so paw

edema reduction alone does not demonstrate disease modification, and these findings should not be extrapolated to arthritis without direct tissue or serum measurement. Collectively, the results constitute preliminary evidence of anti-inflammatory and anti-arthritic potential rather than definitive proof of an anti-rheumatoid mechanism.

Conclusions

The ethanolic leaf extract and fractions of *P. cablin* from North Kolaka exhibited preliminary anti-inflammatory activity *in vitro* and reduced inflammation in mice models. The extract decreased xylene-induced ear edema and lowered plasma TNF- α , IL-1 β , and IL-6 levels in the acute inflammation model, while the extract and fractions reduced paw edema in CFA-induced arthritis. These findings support further investigation of *P. cablin* as a source of anti-inflammatory constituents. Additional studies using authenticated plant material, validated chemical quantification, adequately powered animal experiments, joint histopathology, and cytokine analysis in the arthritis model are required before therapeutic or disease-modifying claims can be made.

Acknowledgement

The authors thank the Directorate of Research and Community Service, Directorate General of Higher Education, Research and Technology, Ministry of Higher Education, Research and Technology, Republic of Indonesia, for financial support through the 2025 Regular Fundamental Research scheme under the main contract number 068/C3/DT.05.00/PL/2025 and the derivative contract number 06/UN29.20/PG/2025.

Funding

This study was supported by the 2025 Regular Fundamental Research scheme under the main contract number 068/C3/DT.05.00/PL/2025 and the derivative contract number 06/UN29.20/PG/2025.

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Animal care procedures were carried out at the Animal Care Laboratory, Faculty of Pharmacy, Universitas Halu Oleo, Indonesia. All experimental procedures involving animals were reviewed and approved by the Health Research Ethics Committee of the Institute for Research and Community Service (LPPM), Universitas Halu Oleo, Indonesia, under approval number 3343/UN29.20.1.2/PG/2024, and

were conducted in accordance with international guidelines for the care and use of laboratory animals.

AI use disclosure

The authors used Grammarly to improve the language and readability of the manuscript. The authors reviewed and edited the content and take full responsibility for its accuracy. No images were generated or manipulated using AI tools.

Conflict of interest

The authors declare no conflict of interest.

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