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Evaluation of the Anti-inflammatory Effectiveness of Peppermint (*Mentha piperita* L.) Leaf Extract on Carrageenan-Induced Paw Edema in Male Wistar Rats (*Rattus norvegicus*)

Rapidah Azizah Siregar[1], Boyke Marthin Simbolon[2], Suandy Suandy[3]

[1,2]Faculty of Medicine and Dentistry, Universitas Prima Indonesia, Indonesia
[3]Center of Excellence for Phytodegenerative and Lifestyle Medicine, Faculty of Medicine and Dentistry, Universitas Prima Indonesia, Indonesia

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Corresponding Author:
Author Name*: Boyke Marthin Simbolon
Email*: boykemarthins@yahoo.com

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ABSTRACT

Peppermint (*Mentha piperita* L.) contains phytochemical constituents with potential anti-inflammatory activity. This study evaluated the effectiveness of an ethanolic peppermint leaf extract against carrageenan-induced paw edema in male Wistar rats (*Rattus norvegicus*). Thirty rats were randomly allocated to six groups (n = 5): an uninduced normal group, a saline negative control, diclofenac sodium at 2 mg/kg, and oral peppermint extract at 250, 500, or 1000 mg/kg. The extract was prepared by maceration in 96% ethanol and underwent qualitative phytochemical screening. Paw volume and diameter were recorded across six hourly follow-up observations; surface temperature, rubor, peripheral differential leukocytes, and wet paw mass difference were also assessed. Data were analyzed using ANOVA or Kruskal–Wallis tests with exploratory, unadjusted pairwise comparisons. Extraction yielded 60.83 g (20.28%), and all five screened phytochemical classes tested positive. Overall group differences were significant for all evaluated outcomes ($p < 0.001$). Among the tested doses, 1000 mg/kg produced the greatest descriptive local response, with inhibition of 18.54 ± 3.61% for volume, 72.02 ± 2.10% for diameter, 85.00 ± 13.69% for rubor, and 53.75 ± 0.24% for baseline-adjusted wet mass. Mean surface temperature was 36.58 °C at this dose. Neutrophil and monocyte percentages increased to 56.80% and 14.40%, respectively, indicating that the leukocyte differential did not normalize with the local response. The findings support local antiedematous activity of the extract, especially at the highest tested dose, while chemical standardization, mechanistic investigation, and safety evaluation remain necessary.

Keywords: peppermint, *mentha piperita,* carrageenan, paw edema, anti-inflammatory activity, wistar rats

INTRODUCTION

Inflammation is a protective response to tissue injury, infection, and other harmful stimuli. Its vascular and cellular components help contain the initiating insult and support tissue repair, but excessive inflammation can also impair normal function. The classical manifestations are redness, heat, pain, swelling, and loss of function. Vasodilation contributes to redness and heat, whereas increased vascular permeability permits fluid and plasma proteins to enter the interstitial space and produce edema. These changes provide measurable outcomes for evaluating candidate anti-inflammatory agents in experimental animals. The relationship between local tissue changes and immune activation also explains why a reduction in swelling should be considered together with other inflammatory measurements [1], [2].

The burden of joint disease provides a relevant national context for research on inflammatory symptoms. Indonesia's 2018 Basic Health Research survey (Riskesdas) reported physician-diagnosed joint disease in 7.3% of people aged 15 years or older [3]. This population estimate motivates investigation of inflammation and symptom control, while the acute experimental model used here addresses a narrower biological question.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to control inflammatory symptoms. Their inhibition of cyclooxygenase reduces prostaglandin synthesis, but the same pharmacological pathway contributes to gastrointestinal, renal, and cardiovascular adverse effects. Consequently, an effective anti-inflammatory response alone does not establish an acceptable benefit–risk profile [4]. Diclofenac sodium is a useful reference treatment in experimental edema models. Investigations of its effects on serum creatinine in Wistar rats illustrate the importance of evaluating renal outcomes separately from anti-inflammatory efficacy [5]. Similarly, research on breadfruit leaf extract has assessed both suppression of experimental inflammation and gastric safety,

emphasizing that activity and tolerability are distinct questions [6].

Plant extracts offer a broad range of chemical constituents for preclinical investigation. Indonesian studies have evaluated ethanolic extracts of Ambon banana leaves, bitter melon leaves, and Jamaican cherry leaves in carrageenan-induced inflammation [7], [8], [9]. Research on tamarind fruit and leaves has also examined the effects of individual extracts and their combinations [10]. These studies support systematic screening of botanical preparations, but their findings cannot be transferred directly between plant species. Differences in plant material, solvent, extraction conditions, dose, comparator, and measurement schedule can substantially influence the observed response.

Peppermint (*Mentha piperita* L.) is an aromatic member of the Lamiaceae family with established culinary and traditional medicinal uses. Reviews describe a chemically diverse plant containing volatile constituents, including menthol and menthone, together with nonvolatile phenolic acids and flavonoids [11], [12]. Its medicinal use has also been discussed in Indonesian literature on peppermint essential oil [13]. Peppermint leaves are a relevant source material because their glandular structures are associated with essential oil production [14]. Morphological and phytochemical variation across growing environments further indicates that the identity of the plant alone is insufficient to define the chemical characteristics of an experimental preparation [15].

Reported peppermint phenolics include rosmarinic acid, caffeic acid, eriocitrin, hesperidin, and luteolin derivatives. The relative amounts recovered depend on the extraction procedure and the polarity of the solvent [16]. Athanasiadis et al. evaluated cyclodextrin-assisted aqueous extraction and compared the resulting polyphenol recovery with aqueous and hydroethanolic preparations, demonstrating that extraction conditions influence both composition and antioxidant properties [17]. Uribe et al. investigated the effects of vacuum drying temperature on the antioxidant characteristics of peppermint leaves [18]. These observations provide a rationale for reporting extraction conditions and examining the phytochemical classes present before interpreting an in vivo response.

The anti-inflammatory potential of peppermint has been linked in the literature to modulation of inflammatory mediators and oxidative stress. A systematic review discusses pathways involving proinflammatory signaling, cytokine production, and antioxidant responses [19]. These mechanisms are plausible explanations for biological activity, but they require direct confirmation in the preparation and model being tested. Essential oils and ethanolic leaf extracts should also be distinguished: an essential oil is enriched in volatile compounds, whereas a leaf extract can contain a broader mixture of nonvolatile constituents. A comparative investigation of three Mentha essential oils found species-specific chemical profiles and pharmacological responses, underscoring the importance of formulation and botanical identity [20].

Carrageenan-induced paw edema is an established model for observing acute inflammatory changes over a relatively short period. Intraplantar administration initiates a response involving vasoactive mediators, increased permeability, and subsequent inflammatory signaling. Serial paw measurements permit assessment of the progression of swelling and the response to treatment. This model has been used in studies of kecombrang flower gel, alang-alang stem extract, and mekai leaf extract [21], [22], [23]. Its relevance to an acute edema response does not, however, make it a direct model of every chronic inflammatory disorder.

Evaluating several outcomes can clarify whether treatment affects different aspects of the inflammatory response. Paw volume and diameter quantify swelling using different measurement principles. Surface temperature and a redness score describe heat and rubor, while the wet mass difference between the injected and contralateral paws provides a terminal measure associated with local tissue swelling. A peripheral differential leukocyte count adds information about the systemic cellular response. Agreement between these outcomes would strengthen an interpretation of local anti-inflammatory activity; discordance would identify effects requiring further investigation. Neither pain nor loss of function was directly measured in the present experiment.

This study therefore evaluated the anti-inflammatory effectiveness of an ethanolic peppermint leaf extract in male Wistar rats with carrageenan-induced paw edema. The objectives were to compare oral doses of 250, 500, and 1000 mg/kg with saline and diclofenac sodium, determine which tested dose produced the greatest reduction in local inflammatory measurements, and examine the associated peripheral leukocyte differential. The experiment combined phytochemical screening with serial physical observations and terminal tissue and blood measurements to provide a broader assessment than paw swelling alone.

RESEARCH METHODS

Study design and setting

A true experimental laboratory study with a pretest–posttest control group design was conducted at Nusantara Makmur Laboratory in 2026. The experimental unit was the individual rat. Baseline paw measurements were obtained before carrageenan administration, and subsequent measurements were recorded after induction and oral treatment. Six parallel groups were included: an uninduced normal group, a saline negative control, a diclofenac sodium positive control, and three peppermint extract groups.

Animals and sample size

Thirty healthy male Wistar rats (*Rattus norvegicus*), aged 8–10 weeks and weighing 150–200 g, were used. Animals underwent seven days of acclimatization in the laboratory and received adequate

food and drinking water. The use of laboratory rats permits repeated, controlled physical observations, while attention to animal maintenance is important for the quality of experimental work [24]. The rats were randomly divided into six groups of five animals.

The minimum group size was estimated using the Federer criterion, $(n - 1)(t - 1) \geq 15$, where n is the number of animals per group and t is the number of groups. With six groups, the criterion yields $n \geq 4$, corresponding to a minimum of 24 rats. The actual experiment used five rats per group, giving a total of 30 animals. This empirical criterion was used for planning; it was not an outcome-specific statistical power calculation.

Ethical approval

The research protocol received ethical approval from the Health Research Ethics Committee (Komisi Etik Penelitian Kesehatan, KEPK) of Universitas Prima Indonesia, approval no. 031/KEPK/UNPRI/VIII/2026, dated August 15, 2026.

Preparation of peppermint leaf extract

Three hundred grams of dried peppermint leaf material were macerated in 3 L of 96% ethanol for 3 × 24 h with occasional stirring. The macerate was filtered, and the filtrate was concentrated using a rotary evaporator under reduced pressure at 50 °C until a concentrated extract was obtained. The extraction yield was calculated as the mass of the concentrated extract divided by the initial dry material mass, multiplied by 100. Extraction conditions were recorded because solvent choice and processing can influence the recovery of peppermint constituents [16], [17], [18].

Qualitative phytochemical screening assessed flavonoids, alkaloids, tannins, steroid/terpenoid constituents, and saponins. Flavonoids were tested using magnesium powder, concentrated hydrochloric acid, and amyl alcohol. The alkaloid screening listed Mayer, Bouchardat, and Dragendorff reagents. Tannins were screened using 10% ferric chloride, steroid/terpenoid constituents using the Liebermann–Burchard reaction, and saponins using hot distilled water and hydrochloric acid. A positive result indicated a detectable reaction consistent with the corresponding class of constituents. These tests were qualitative and did not determine concentrations or identify individual active molecules.

Carrageenan preparation and treatment allocation

A 1% carrageenan preparation was made by dissolving 1 g of carrageenan in 100 mL of 0.9% sodium chloride solution. The mixture was heated and stirred until homogeneous and then left overnight. All groups except the normal group underwent intraplantar carrageenan induction. Diclofenac sodium was administered at 2 mg/kg, and peppermint extract was administered at 250, 500, or 1000 mg/kg. The treatment groups are summarized in Table 1. The administered extract was an oral leaf preparation.

Table 1. Experimental groups and oral treatments

Group	Carrageenan induction	Oral treatment	n
Normal	No	None	5
Saline negative control	Yes	0.9% NaCl	5
Diclofenac positive control	Yes	Diclofenac sodium, 2 mg/kg	5
Peppermint 250 mg/kg	Yes	Peppermint leaf extract, 250 mg/kg	5
Peppermint 500 mg/kg	Yes	Peppermint leaf extract, 500 mg/kg	5
Peppermint 1000 mg/kg	Yes	Peppermint leaf extract, 1000 mg/kg	5

Induced groups received 1% carrageenan in the right paw. All extract doses are expressed per kilogram of body weight. The normal group was uninduced and untreated.

The paw was marked to standardize the measurement level and cleaned using an alcohol swab before assessment. A baseline measurement was taken before induction. After intraplantar carrageenan administration, a one-hour interval was allowed before the post-induction measurement. The assigned treatment was then administered orally, followed by six hourly observation points. The normal group received neither carrageenan nor an active treatment. The observation-hour labels below refer to the recorded follow-up schedule after the post-induction assessment.

Paw volume and diameter

Paw volume was measured with a mercury plethysmometer, and paw diameter was measured with a caliper. Baseline, post-induction, and hourly follow-up values were recorded for each animal. The same marked level was used for serial volume measurements to reduce variation caused by different immersion depths. The percentage increase in paw volume relative to baseline was calculated using the following expression:

$$\text{Edema (\%)} = \frac{V_h - V_0}{V_0} \times 100$$

Here, V_0 denotes baseline paw volume and V_h denotes the volume at a follow-up observation. The same expression was applied to diameter measurements by replacing volume with diameter. The six hourly percentage increases were averaged for each animal to obtain an animal-level summary for comparison between groups. This arithmetic time average was distinct from a trapezoidal area under the curve. Edema inhibition relative to the negative control was calculated as follows:

$$\text{Inhibition (\%)} = \frac{E_{neg} - E_i}{E_{neg}} \times 100$$

E_{neg} denotes the mean edema percentage in the saline group, and E_i denotes the animal-level edema percentage in a treatment group. Inhibition was

summarized as the group mean and standard deviation across the five animals. Serial measurements from one animal were not treated as independent experimental units.

Paw surface temperature and redness

Paw surface temperature was measured using an infrared thermometer. The available temperature observations were those labeled hours 3, 4, and 6; their arithmetic mean was calculated for each animal. Redness was assessed visually on a modified 0–3 scale: 0, no redness; 1, very slight redness; 2, clearly defined redness; and 3, moderate to severe redness. The tabulated rubor observations were labeled hours 3, 4, and 5. The mean of these observations provided a descriptive animal-level summary, while nonparametric analysis was used for comparisons.

For temperature, rubor, and wet paw mass difference, inhibition was normalized to the difference between the saline and normal groups using the following expression:

$$\text{Inhibition (\%)} = \frac{X_{neg} - X_i}{X_{neg} - X_{norm}} \times 100$$

X_{neg} and X_{norm} denote the negative-control and normal-group means, respectively, while X_i denotes the individual treatment value. This normalization expresses how much of the control-to-normal difference was reduced. For temperature, a value above 100% can occur if the treatment mean is below the normal-group mean; it does not represent more than complete inhibition of every component of inflammation.

Differential leukocyte count

Peripheral blood was assessed at the end of the observation period. Blood smears were prepared, stained with Giemsa, and examined using light microscopy at 1000× magnification. Differential results were expressed as percentages of the 200 classified leukocytes represented in each animal's count. Neutrophils and monocytes were reported separately, and the remaining leukocytes were grouped as other cells, comprising lymphocytes, eosinophils, and basophils. The results describe relative leukocyte composition rather than absolute circulating cell concentrations or leukocyte infiltration within the paw.

Wet paw mass

The right paw subjected to carrageenan induction and the contralateral left paw were excised and weighed using an analytical balance. Wet paw mass difference was calculated by subtracting the left-paw mass from the right-paw mass. The same right-minus-left calculation was applied to the normal animals. The normal-group mean was retained in the baseline-adjusted inhibition calculation because physiological left–right differences need not be exactly zero. Wet mass difference was interpreted as a measure associated with tissue swelling, rather than a chemically specific measurement of inflammatory exudate.

Statistical analysis

Data were processed using SPSS version 25. Continuous animal-level summaries were described using means and standard deviations. The Shapiro–Wilk test assessed distributional assumptions within groups, and Levene's test assessed variance homogeneity. Paw volume, temperature, rubor, and wet mass outcomes were analyzed using the Kruskal–Wallis test followed by pairwise Mann–Whitney tests. Paw diameter and differential leukocyte percentages were analyzed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) procedure.

The significance threshold was $p < 0.05$. Values displayed as 0.000 in the statistical output are reported as $p < 0.001$. Pairwise p values were unadjusted and are presented as exploratory comparisons because multiple groups and outcomes were examined. ANOVA for the other-leukocyte category also required caution: the Shapiro–Wilk result for the 500 mg/kg group was $p = 0.046$, indicating that normality was not supported in every group. Descriptive summaries and normalized inhibition values were calculated at the animal level. Normal-group volume and diameter percentage changes were zero without variation, so a within-group normality test was not applicable to these values.

RESULT AND DISCUSSION

Extraction yield and phytochemical screening

Maceration of 300 g of dried peppermint leaves produced 60.83 g of concentrated extract, corresponding to an extraction yield of 20.28%. The preparation used 3 L of 96% ethanol over a 72-hour maceration period, followed by evaporation at 50 °C. The yield describes the mass recovered under these conditions and does not indicate the concentration or identity of an individual active constituent.

Qualitative screening was positive for all five investigated phytochemical categories: flavonoids, alkaloids, tannins, steroid/terpenoid constituents, and saponins (Table 2). This finding supports the presence of several classes that could contribute to biological activity. It does not establish that the tested batch contained a specified amount of menthol, rosmarinic acid, or any other individual compound discussed in the peppermint literature.

Table 2. Qualitative phytochemical screening of peppermint leaf extract

Phytochemical class	Screening reagent or reaction	Result
Flavonoids	Magnesium, concentrated HCl, and amyl alcohol	Positive
Alkaloids	Mayer, Bouchardat, and Dragendorff reagents	Positive
Tannins	10% ferric chloride	Positive
Steroid/terpenoid constituents	Liebermann–Burchard reaction	Positive
Saponins	Hot distilled water and HCl	Positive

Positive reactions indicate the corresponding phytochemical classes. The screening does not quantify constituents or identify individual marker compounds.

Serial changes in paw volume

Baseline mean paw volume was 4.18 mL in each group, and the normal group maintained this value throughout observation. Mean post-induction volumes in the five induced groups ranged from 9.52 to 9.70 mL. These measurements demonstrate a marked increase after carrageenan induction before the subsequent decline during follow-up. Saline-treated animals also showed spontaneous reduction in swelling, making the contemporaneous negative control important for assessing the treatment-associated response.

At the first hourly observation, mean volumes were 8.64 mL with saline, 8.50 mL with diclofenac, and 8.48, 8.40, and 8.36 mL with peppermint doses of 250, 500, and 1000 mg/kg, respectively. By the sixth observation, the corresponding values were 4.76, 4.44, 4.30, 4.24, and 3.88 mL (Table 3). The highest-dose group had the lowest mean volume among the induced groups at the last observation. Its final mean was also below the common baseline mean, which warrants consideration of measurement variability when interpreting the magnitude of the late response.

Table 3. Mean paw volume before induction and during follow-up (mL)

Group	Baseline	Post	h 1	h 2	h 3	h 4	h 5	h 6
Normal	4.18	4.18	4.18	4.18	4.18	4.18	4.18	4.18
Saline	4.18	9.54	8.64	8.22	7.58	7.26	6.32	4.76
Diclofenac 2 mg/kg	4.18	9.70	8.50	8.20	7.50	7.08	6.24	4.44
Peppermint 250 mg/kg	4.18	9.60	8.48	8.14	7.46	6.64	6.14	4.30
Peppermint 500 mg/kg	4.18	9.58	8.40	8.04	7.40	6.50	6.02	4.24
Peppermint 1000 mg/kg	4.18	9.52	8.36	7.78	7.38	6.42	5.68	3.88

Values are group means (n = 5). Baseline was measured before induction; Post was the post-induction assessment after a one-hour interval and before oral treatment. h 1–h 6 denote the subsequent hourly follow-up observations. The normal group followed the corresponding measurement schedule.

The arithmetic mean edema percentages across the six observations were 70.59% in the saline group, 67.32% with diclofenac, 64.13% with peppermint 250 mg/kg, 61.90% with 500 mg/kg, and 57.51% with 1000 mg/kg. The corresponding inhibition values for the four active-treatment groups were 4.63 ± 3.08%, 9.16 ± 2.41%, 12.32 ± 2.22%, and 18.54 ± 3.61%. Thus, the treatment effect was smaller when expressed as inhibition of the complete six-observation volume summary than a reading of the final volume alone might suggest.

The Kruskal–Wallis analysis indicated an overall group difference ($p < 0.001$). In the reported pairwise comparisons, saline differed from diclofenac at $p = 0.046$ and from each extract group at $p = 0.009$. The 1000 mg/kg group differed from diclofenac and both lower extract doses at $p = 0.009$. Diclofenac versus 250 mg/kg and 250 versus 500 mg/kg were not nominally significant, with $p = 0.074$ for both comparisons. These findings describe exploratory group differences in the summarized outcome; they do not demonstrate a separate significant effect at every observation hour.

Serial changes in paw diameter

Mean baseline paw diameter was 3.54 mm in all groups, whereas post-induction means ranged from 9.52 to 9.70 mm among the induced groups. Diameter decreased during follow-up, with a clearer separation between the extract groups and saline than was observed for volume. At the first observation, mean diameters were 8.32 mm with saline, 7.82 mm with diclofenac, and 6.94, 6.60, and 6.08 mm with the three increasing peppermint doses. At the sixth observation, these values were 4.06, 3.90, 3.80, 3.74, and 3.56 mm, respectively (Table 4).

Table 4. Mean paw diameter before induction and during follow-up (mm)

Group	Baseline	Post	h 1	h 2	h 3	h 4	h 5	h 6
Normal	3.54	3.54	3.54	3.54	3.54	3.54	3.54	3.54
Saline	3.54	9.54	8.32	7.62	8.00	7.40	5.72	4.06
Diclofenac 2 mg/kg	3.54	9.70	7.82	6.82	7.00	5.40	5.26	3.90
Peppermint 250 mg/kg	3.54	9.60	6.94	6.06	5.86	4.28	4.84	3.80
Peppermint 500 mg/kg	3.54	9.58	6.60	5.18	4.88	3.98	4.54	3.74
Peppermint 1000 mg/kg	3.54	9.52	6.08	4.68	4.36	3.74	4.38	3.56

Values are group means (n = 5). Baseline was measured before induction; Post was the post-induction assessment after a one-hour interval and before oral treatment. h 1–h 6 denote the subsequent hourly follow-up observations. The normal group followed the corresponding measurement schedule.

The mean diameter-based edema percentages were 93.61% with saline, 70.48% with diclofenac, and 49.63%, 36.18%, and 26.19% with peppermint 250, 500, and 1000 mg/kg. Inhibition increased from 24.72 ± 5.01% with diclofenac to 46.99 ± 1.79%, 61.35 ± 2.26%, and 72.02 ± 2.10% with increasing extract dose. The highest tested dose therefore produced the greatest descriptive inhibition of diameter-based swelling. ANOVA showed an overall difference ($p < 0.001$), and the reported LSD comparisons were $p < 0.001$ between all groups.

The longitudinal profiles of paw volume and diameter are illustrated in Figure 1. Both measurements favor the highest extract dose descriptively, but their inhibition percentages differ substantially. Plethysmometry measures displaced volume, whereas a caliper measures a single linear dimension. Differences in measurement geometry, placement, and sensitivity mean that the two inhibition percentages should be considered complementary outcomes rather than interchangeable estimates of a single quantity.

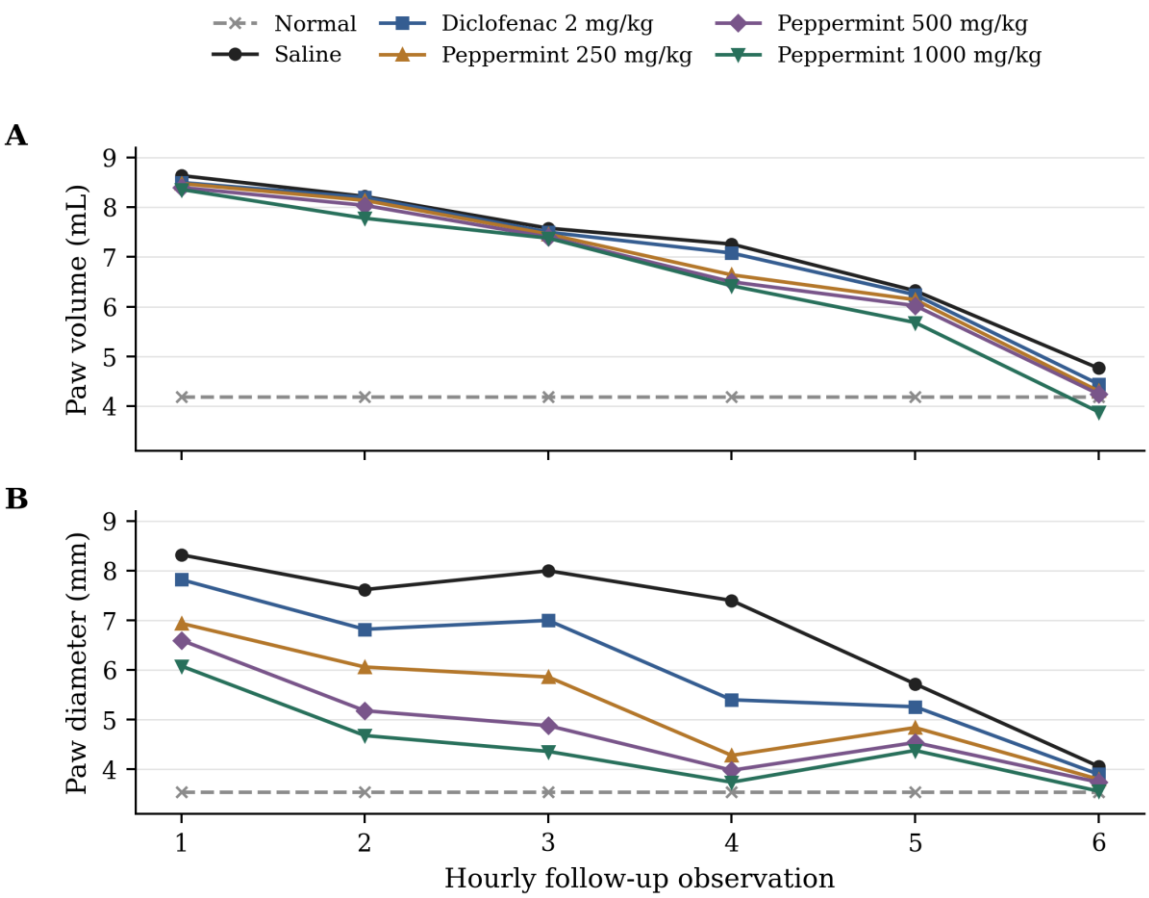

Figure 1. Serial paw volume (A) and diameter (B) during the six hourly follow-up observations. Points represent group means (n = 5). Follow-up labels use the schedule after the post-induction assessment; no time-point-specific statistical significance is implied.

Paw surface temperature and rubor

The normal group's mean surface temperature across the recorded observations was 36.70 °C. The saline group had mean temperatures of 38.46, 37.28, and 36.92 °C at observations 3, 4, and 6, respectively, with an overall arithmetic mean of 37.55 °C. Corresponding overall means were 37.23 °C with diclofenac and 37.06, 36.84, and 36.58 °C with the three increasing extract doses. These temperature outcomes, together with rubor and wet mass summaries, are presented in Table 5.

Temperature inhibition was 37.50 ± 2.76% with diclofenac, 57.81 ± 3.27% with peppermint 250 mg/kg, 83.59 ± 10.84% with 500 mg/kg, and 114.06 ± 3.49% with 1000 mg/kg. The value above 100% resulted from normalization to the saline-to-normal difference, because the highest-dose group had an overall mean below that of normal animals. It should be interpreted as a below-reference temperature response under this calculation, rather than evidence that inflammation was eliminated completely.

The overall temperature comparison was significant by Kruskal–Wallis testing ($p < 0.001$). Saline differed nominally from all active groups, with p values of 0.007–0.008. The highest dose differed from diclofenac at $p = 0.005$, from 250 mg/kg at $p = 0.006$, and from 500 mg/kg at $p = 0.007$. Comparisons with normal animals were not significant for 500 mg/kg ($p = 0.110$) or 1000 mg/kg ($p = 0.180$). These nonsignificant comparisons do not demonstrate physiological equivalence or complete recovery.

Normal animals had no redness at the recorded observations. Mean rubor summaries were 1.33 with saline, 1.13 with diclofenac, and 0.87, 0.60, and 0.20 with peppermint 250, 500, and 1000 mg/kg. Normalized inhibition was 15.00 ± 28.50%, 35.00 ± 28.50%, 55.00 ± 20.92%, and 85.00 ± 13.69% in the active groups, respectively. The large standard deviations for diclofenac and the lowest extract dose indicate substantial individual variation in this ordinal outcome.

The rubor omnibus test was significant ($p < 0.001$). Pairwise comparisons with saline were not nominally significant for diclofenac ($p = 0.522$) or peppermint 250 mg/kg ($p = 0.167$), but were significant for 500 mg/kg ($p = 0.033$) and 1000 mg/kg ($p = 0.008$). The highest dose differed from diclofenac at $p = 0.008$ and from the two lower extract doses at $p = 0.017$ and $p = 0.033$. Its comparison with normal animals was reported as $p = 0.050$, a boundary value that should not be used to claim equivalence to the normal state.

Table 5. Surface temperature, rubor, and wet paw mass difference

Group	Temperature (°C)	Rubor score (0–3)	Right-minus-left wet mass (g)
Normal	36.70 ± 0.15	0.00 ± 0.00	-0.0312 ± 0.0251
Saline	37.55 ± 0.19	1.33 ± 0.53	0.5028 ± 0.1282
Diclofenac 2 mg/kg	37.23 ± 0.02	1.13 ± 0.38	0.3610 ± 0.0314
Peppermint 250 mg/kg	37.06 ± 0.03	0.87 ± 0.38	0.2478 ± 0.0350
Peppermint 500 mg/kg	36.84 ± 0.09	0.60 ± 0.28	0.2440 ± 0.0117
Peppermint 1000 mg/kg	36.58 ± 0.03	0.20 ± 0.18	0.2158 ± 0.0013

Values are mean ± SD of five animal-level summaries. Temperature summarizes observations 3, 4, and 6; rubor summarizes observations 3, 4, and 5. Wet mass was measured at the terminal assessment. A negative normal-group mass difference reflects the left–right imbalance.

Differential leukocyte composition

The blood differential revealed a different pattern from the local physical outcomes (Table 6). Normal animals had mean neutrophil and monocyte percentages of 26.80% and 3.80%. These values were 39.10% and 5.90% with saline and 43.80% and 7.80% with diclofenac. With increasing peppermint dose, neutrophils increased to 48.30%, 52.10%, and 56.80%, and monocytes increased to 9.30%, 12.00%, and 14.40%. Other leukocytes decreased from 69.40% in normal animals to 28.80% at the highest dose. The 250 mg/kg other-leukocyte mean calculated from the individual counts was 42.40%.

Table 6. Peripheral differential leukocyte percentages

Group	Neutrophils (%)	Monocytes (%)	Other leukocytes (%)
Normal	26.80 ± 0.76	3.80 ± 0.91	69.40 ± 1.52
Saline	39.10 ± 1.52	5.90 ± 1.24	55.00 ± 1.58
Diclofenac 2 mg/kg	43.80 ± 1.20	7.80 ± 0.91	48.40 ± 1.88
Peppermint 250 mg/kg	48.30 ± 1.86	9.30 ± 1.44	42.40 ± 1.85
Peppermint 500 mg/kg	52.10 ± 1.08	12.00 ± 0.79	35.90 ± 0.89
Peppermint 1000 mg/kg	56.80 ± 0.91	14.40 ± 0.42	28.80 ± 1.04

Values are mean ± SD ($n = 5$), based on 200 classified leukocytes per animal. Other leukocytes comprise lymphocytes, eosinophils, and basophils. These percentages represent relative composition, not absolute cell concentrations.

ANOVA showed overall differences for neutrophils, monocytes, and other leukocytes (all $p < 0.001$). The LSD comparisons between 1000 mg/kg and saline, diclofenac, or either lower extract dose were $p < 0.001$ for each category. For monocytes, saline versus diclofenac was $p = 0.006$, and diclofenac versus 250 mg/kg was $p = 0.027$. Normal versus saline was $p = 0.003$. The other-leukocyte ANOVA should be considered exploratory because the normality assumption was not satisfied in every group.

These observations show increased relative neutrophil and monocyte representation, not a reduction in those cell types. They therefore cannot be described as normalization of the leukocyte differential toward the normal group. They also do not establish enhanced phagocytosis, because no functional phagocytosis assay was performed. Percentage data are compositional: an increase in one category necessarily reduces the relative contribution of another. Without an absolute leukocyte count and tissue-based measurements, the results cannot distinguish changes in absolute circulating cell numbers from redistribution of leukocyte proportions.

Fachri et al. evaluated yellow passion fruit extract in male Wistar rats and reported a decrease in peripheral monocyte counts [25]. Although that preparation and observation schedule differed, the study illustrates why the direction of a cellular response must be considered separately from a physical reduction in swelling. In the present experiment, the rising monocyte percentage therefore cannot be used as additional evidence that the leukocyte response was suppressed.

Wet paw mass difference

The saline group had a mean right-paw mass of 1.7328 g and a left-paw mass of 1.2300 g, producing a mean difference of 0.5028 g. The corresponding differences were 0.3610 g with diclofenac, 0.2478 g with peppermint 250 mg/kg, 0.2440 g with 500 mg/kg, and 0.2158 g with 1000 mg/kg. Normal animals had a mean difference of −0.0312 g, reflecting a small left–right mass imbalance. This value was retained in the normalized inhibition calculation.

Baseline-adjusted inhibition values calculated from the individual paw masses were 26.55 ± 5.88% with diclofenac, 47.75 ± 6.55% with peppermint 250 mg/kg, 48.46 ± 2.20% with 500 mg/kg, and 53.75 ± 0.24% with 1000 mg/kg (Table 7). These percentages use the same control-to-normal normalization as the temperature and rubor outcomes. They should not be mixed with percentages calculated using saline alone as the denominator.

The Kruskal–Wallis test showed an overall group difference ($p < 0.001$). Saline differed from diclofenac at $p = 0.016$, from the 250 and 500 mg/kg groups at $p = 0.009$, and from the 1000 mg/kg group at $p = 0.008$. The comparison between 250 and 500 mg/kg was not significant ($p = 0.602$), despite the numerical increase in mean inhibition. The highest dose differed from both lower doses and diclofenac at $p = 0.008$. It also differed from normal animals at $p = 0.008$; thus, complete restoration of normal wet paw mass was not demonstrated.

Table 7. Inhibition of local inflammatory outcomes (%)

Outcome	Diclofenac 2 mg/kg	Peppermint 250 mg/kg	Peppermint 500 mg/kg	Peppermint 1000 mg/kg
Paw volume	4.63 ± 3.08	9.16 ± 2.41	12.32 ± 2.22	18.54 ± 3.61
Paw diameter	24.72 ± 5.01	46.99 ± 1.79	61.35 ± 2.26	72.02 ± 2.10
Surface temperature	37.50 ± 2.76	57.81 ± 3.27	83.59 ± 10.84	114.06 ± 3.49
Rubor	15.00 ± 28.50	35.00 ± 28.50	55.00 ± 20.92	85.00 ± 13.69
Wet paw mass difference	26.55 ± 5.88	47.75 ± 6.55	48.46 ± 2.20	53.75 ± 0.24

Values are mean ± SD (n = 5). Volume and diameter inhibition use saline alone as the reference for the six-observation mean edema percentage. Temperature, rubor, and wet mass use the saline-to-normal difference as the denominator. Temperature inhibition above 100% reflects a mean below the normal reference.

Integrated interpretation of the local response

Across the tested extract doses, higher dose was associated descriptively with lower mean paw volume, diameter, surface temperature, rubor, and wet mass difference. The 1000 mg/kg group had the largest inhibition value for each local outcome. Nevertheless, the response was not uniform across measurements. Volume inhibition at the highest dose was 18.54%, whereas diameter inhibition was 72.02%, rubor inhibition was 85.00%, and baseline-adjusted wet mass inhibition was 53.75%. The temperature index exceeded 100% because its denominator was the saline-to-normal temperature difference.

These differences show why a single inhibition percentage cannot represent the complete inflammatory response. They also limit a general claim that peppermint was uniformly superior to diclofenac. The descriptive local outcomes favored 1000 mg/kg, and several unadjusted pairwise comparisons were below 0.05 (Table 8). However, multiplicity was not controlled, only five animals were studied per group, and the systemic leukocyte response did not move toward normal. The most defensible interpretation is evidence of local antiedematous and redness-reducing activity under the experimental conditions, accompanied by a distinct peripheral leukocyte pattern.

Table 8. Exploratory pairwise p values for peppermint 1000 mg/kg

Outcome	vs saline	vs diclofenac 2 mg/kg	vs peppermint 250 mg/kg	vs peppermint 500 mg/kg
Paw volume	0.009	0.009	0.009	0.009
Paw diameter	<0.001	<0.001	<0.001	<0.001
Surface temperature	0.007	0.005	0.006	0.007
Rubor	0.008	0.008	0.017	0.033
Wet paw mass difference	0.008	0.008	0.008	0.008
Neutrophils (%)	<0.001	<0.001	<0.001	<0.001
Monocytes (%)	<0.001	<0.001	<0.001	<0.001
Other leukocytes (%)	<0.001	<0.001	<0.001	<0.001

Values compare the 1000 mg/kg group with the indicated group. Mann–Whitney tests were used for volume, temperature, rubor, and wet mass; LSD comparisons followed ANOVA for diameter and leukocyte percentages. All p values are unadjusted for multiple comparisons and should be interpreted as exploratory.

Phytochemical and mechanistic considerations

The positive flavonoid, tannin, and other screening reactions provide a basis for discussing several possible contributors to the response. Peppermint reviews describe phenolics and flavonoids with antioxidant and anti-inflammatory potential [11], [16], [19]. The chemical diversity of the preparation makes it plausible that more than one constituent contributed. However, broad screening reactions cannot establish which molecule was responsible, whether several constituents acted together, or whether the observed effects depended on the concentration of an unmeasured marker compound.

The cyclooxygenase pathway is relevant because arachidonic acid metabolites contribute to inflammatory vascular changes and because diclofenac acts through prostaglandin inhibition [4], [26]. Docking research on quinic acid and COX-2 provides an example of how individual plant-associated molecules can be examined as candidate interactions [27]. Such theoretical evidence is separate from demonstration of enzyme inhibition in an animal experiment. The present study measured neither COX nor lipoxygenase activity, prostaglandin concentrations, cytokines, NF-κB activation, or oxidative stress markers. Its findings therefore support hypotheses about these pathways rather than direct mechanistic conclusions.

Extraction chemistry is also relevant to comparison with other peppermint work. Athanasiadis et al. demonstrated the influence of extraction conditions on antioxidant polyphenol recovery, while Jamshed and Jabeen characterized peppermint extract in a different experimental application involving urolithiasis [17], [28]. The latter study is informative about the investigation of a chemically complex peppermint preparation, but its renal outcomes are not direct evidence of efficacy in carrageenan-induced paw edema. Similarly, the essential oil studied by Mogosan et al. was chemically distinct from the present ethanolic extract [20].

Menthol-associated cooling and TRPM8 signaling provide a possible context for the temperature response discussed in the peppermint literature [12], [16]. Nevertheless, the extract was administered orally, and neither its menthol concentration nor receptor activity was measured. A surface temperature below the normal-group mean cannot, by itself, identify a menthol-mediated mechanism. Environmental temperature, measurement position, and systemic physiological effects are additional considerations for future standardized assessments.

Ogaly et al. investigated peppermint essential oil in carbon tetrachloride-induced liver fibrosis in rats, providing further evidence that peppermint preparations have been studied in models involving oxidative and inflammatory injury [29]. The organ, injury mechanism, preparation, and observation period differ from the present experiment. Such findings support continued mechanistic investigation, while direct extrapolation to acute paw edema or human treatment remains inappropriate. Chemical fingerprinting and quantitative marker assays would make future comparisons between peppermint studies more informative.

Relationship to previous botanical studies

The present dose ordering is consistent with the rationale for testing several extract doses in experimental edema rather than assuming that one dose is representative. Rahajeng et al. examined alang-alang stem extract at 250, 500, and 1000 mg/kg in male rats, using diclofenac and carrageenan-induced paw swelling as components of the experimental design [22]. The shared dose range provides methodological context, although the plant chemistry and comparator dose differed. Studies of Ambon banana leaves, bitter melon leaves, and Jamaican cherry leaves similarly used botanical extracts in induced inflammation [2], [7], [8], [9].

Recent Indonesian studies cited in the research background provide additional comparisons. Tanjung et al. investigated ethanolic *Impatiens balsamina* leaf extract in carrageenan-induced male rats, while Trisna et al. studied methanolic *Hornstedtia scyphifera* rhizome extract [30], [31]. Akadir et al. evaluated ethanolic *Caesalpinia bonducella* seed extract and reported a dose-related pattern among the tested groups [32]. These investigations support the continued use of induced edema for botanical screening, but their doses and inhibition values cannot be ranked directly against peppermint without matching the protocol and outcome definition.

Preparations and administration routes also differ across the literature. Wardani investigated a

topical kecombrang flower gel, and Widjaja and Firmansyah evaluated a cream containing tamanu and tea tree oils [21], [33]. By contrast, the present study used an oral peppermint leaf extract. Topical and oral studies differ in local exposure and systemic distribution; evidence from one route should not be taken as validation of the other. Badawi et al.'s mekai leaf study further illustrates that edema and inhibition measurements depend on the botanical preparation and experimental comparison [23].

Limitations and future research

The small group size limits precision, sensitivity to distributional assumptions, and the ability to detect modest treatment differences. The Federer criterion supported the selected group size but did not establish power for the individual outcomes. Multiple endpoints and pairwise tests increase the probability of chance findings, so nominal p values should be interpreted as exploratory. The nonsignificant comparisons with normal animals do not demonstrate equivalence. Furthermore, analysis of animal-level arithmetic averages does not model the complete within-animal time course or account for unequal observation intervals in the temperature series.

Methodological standardization should be strengthened in replication studies. The randomization method, allocation concealment, and assessor blinding require explicit documentation, particularly for visually assigned rubor scores. Injection volume, administered oral volume, measurement calibration, sampling times, anesthesia, and terminal tissue collection procedures also require complete reporting. Plant authentication, a voucher specimen, extraction batch characteristics, residual solvent, and quantitative chemical markers would improve reproducibility. Chemical identification of a reference NSAID is another relevant quality-control consideration; analytical methods for distinguishing diclofenac from related NSAIDs have been described [34].

The study did not assess analgesia, functional recovery, tissue histology, absolute leukocyte counts, or biochemical inflammatory mediators. The increased neutrophil and monocyte percentages therefore require follow-up rather than being classified automatically as beneficial immune modulation. Tissue histology and measurements of prostaglandins, cytokines, oxidative stress, and selected signaling pathways would help determine whether the physical improvements and leukocyte changes reflect related or independent processes. Wider dose-ranging studies are needed because the highest tested dose cannot be assumed to be the optimal dose outside the present comparison.

Safety also requires a separate evaluation. A six-hour observational experiment cannot establish the gastrointestinal, hepatic, renal, or longer-term safety of the extract. Future research should include acute and subchronic toxicity studies and comparison with other established anti-inflammatory agents. Work on Wistar rat liver and kidney reference values can inform the selection and interpretation of such assessments [35]. Evidence from diclofenac-related renal testing and botanical gastric safety investigations likewise highlights the distinction between an anti-inflammatory effect and a demonstrated safety advantage [5], [6]. Further preclinical work should establish standardized composition, a reproducible effect, and an acceptable safety profile before clinical investigation.

CONCLUSION

Oral peppermint leaf extract showed a dose-related descriptive reduction in local inflammatory measurements in male Wistar rats with carrageenan-induced paw edema. Among the tested doses, 1000 mg/kg produced the greatest inhibition of the six-observation paw volume and diameter summaries, the lowest mean rubor score and surface temperature, and the smallest wet paw mass difference among the induced groups. The main inhibition values at this dose were 18.54% for volume, 72.02% for diameter, 85.00% for rubor, and 53.75% for baseline-adjusted wet mass. Overall group comparisons were significant, while the pairwise results remained exploratory.

The peripheral differential showed increased neutrophil and monocyte percentages with increasing extract dose, rather than normalization toward the uninduced group. These changes should be investigated separately from the local antiedematous response. The findings support further preclinical evaluation of the extract, but do not establish an optimal clinical dose, a safety advantage, physiological equivalence to normal animals, or confirmed superiority to diclofenac. Quantitative extract standardization, adequately powered experiments, mechanistic measurements, and toxicity testing are needed to clarify its therapeutic potential.

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