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Original Article

Evaluation of Anti-inflammatory and Analgesic Effects of the n-hexane Fraction from *Piper Betle* Plant in Experimental Models

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Abstract: Background: *Piper betle* L. plant has been reported to possess analgesic and anti-inflammatory properties; however, evidence regarding the *in vivo* pharmacological activity of their non-polar fraction remains limited. This study evaluated the analgesic and anti-inflammatory effects of the n-hexane fraction obtained from an ethanol fraction of *Piper betle* L. plant (PB-Hex) in experimental animals. Methods: The analgesic activity of PB-Hex was evaluated using the acetic acid-induced writhing test in *Swiss* albino mice. Animals received vehicle, aspirin at 100 mg/kg, or PB-Hex at 100 or 300 mg/kg orally once daily for five consecutive days. Anti-inflammatory activity was assessed in *Wistar* rats using carrageenan-induced paw edema and carrageenan–formaldehyde-induced peritonitis. Rats received vehicle, aspirin at 150 mg/kg, or PB-Hex at 50 or 150 mg/kg using the same treatment schedule. The evaluated outcomes included the number of writhing responses, changes in paw volume, percentage inhibition of paw edema, peritoneal exudate volume, protein concentration, and total leukocyte count. Results: PB-Hex-treated mice showed lower numbers of acetic acid-induced writhing responses than the vehicle-treated model control group, with a more consistent numerical effect at 100 mg/kg than at 300 mg/kg. In the carrageenan-induced paw-edema model, PB-Hex attenuated the increase in paw volume, particularly at 50 mg/kg, whereas the 150 mg/kg dose showed its greatest apparent effect at an earlier time point. In the peritonitis model, both PB-Hex doses were associated with lower peritoneal exudate volume, protein concentration, and total leukocyte count than the model control treatment. No clear dose-dependent response was observed across the experimental endpoints. Conclusion: PB-Hex showed preliminary analgesic and anti-inflammatory effects in the experimental models employed.

Keywords: Piper betle L., n-hexane fraction, analgesic, anti-inflammatory, writhing test, paw edema.

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1. Introduction

Pain and inflammation are common pathological manifestations associated with tissue injury, infection, immune responses and many acute or chronic diseases. Inflammation is a protective biological response; however, excessive or prolonged inflammation may contribute to tissue damage, functional impairment and reduced quality of life. Pain is also one of the most important clinical symptoms accompanying inflammatory processes. Therefore, the search for agents with both anti-inflammatory and analgesic effects remains an important direction in experimental pharmacology.

Non-steroidal anti-inflammatory drugs and corticosteroids are commonly used for the management of pain and inflammation. Although these drugs are effective, their long-term or inappropriate use may be associated with adverse effects, including gastrointestinal, renal, cardiovascular or metabolic complications. For this reason, medicinal plants and plant-derived extracts have attracted increasing attention as potential sources of safer anti-inflammatory and analgesic agents.

Piper betle L., belonging to the family Piperaceae, is a medicinal plant widely distributed and traditionally used in many Asian countries, including Vietnam. The plant of *Piper betle* have been reported to contain various biologically active constituents, especially phenolic compounds, phenylpropanoids and other secondary metabolites. Previous studies have suggested that *Piper betle* plant possess several pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory and enzyme-inhibitory effects [1-4]. These biological properties provide a scientific basis for further investigation of the pharmacological activities of different extracts and fractions obtained from *Piper betle* plant.

Among different solvent fractions, the n-hexane fraction represents a non-polar fraction that may contain lipophilic constituents contributing to biological activity. However, experimental evidence regarding the anti-inflammatory and analgesic effects of the n-hexane fraction from *Piper betle* plant remains limited. Therefore, evaluation of this fraction using established animal models is necessary to provide scientific evidence for its potential pharmacological application. [1, 4] This study aimed to evaluate the analgesic and anti-inflammatory effects of PB-Hex in experimental animals by: i) Assessing anti-nociceptive activity in the acetic acid-induced writhing test in mice; ii) Assessing acute local anti-inflammatory activity in the carrageenan-induced paw-edema model in rats; and iii) Assessing effects on peritoneal exudate volume, protein concentration, and total leukocyte count in the carrageenan–formaldehyde-induced peritonitis model in rats.

2. Materials and Methods

2.1. Materials

2.1.1. Study Extract

The study material was the *n*-hexane fraction from plant of *Piper betle* L. (Piperaceae), hereafter referred to as PB-Hex.

Fresh plant of *Piper betle* L. were collected from Dong Hy, Thai Nguyen, Vietnam in January 2024. The plant material was taxonomically identified by the National Institute of Medicinal Materials, Hanoi, Vietnam. A voucher specimen coded (DV-260124) was deposited at the National Institute of Medicinal Materials, Hanoi, Vietnam for future reference.

The collected plants were washed thoroughly under running water, shade-dried at room temperature for 07 days and ground into a fine powder. The dried powder was stored in airtight containers, protected from light and moisture, until extraction.

Dried powdered plants of *Piper betle* L. were extracted with 96% ethanol at 70°C. The extraction was carried out three times, each for 3 hours, using solvent-to-material ratios of (6:1, v/w) respectively. The combined extracts were

filtered and concentrated under reduced pressure to obtain the crude ethanol extract. The crude extract was then suspended in distilled water and successively partitioned with *n*-hexane. The *n*-hexane fractions were collected, combined and evaporated under reduced pressure to obtain PB-Hex. The extraction yield of the n-hexane fraction from the initial dry medicinal material was 3.14%. The fractions was stored at 4°C until use.

Before administration to animals, PB-Hex was suspended in an appropriate vehicle and administered orally at the required doses.

2.1.2. Chemicals and Equipment

The main chemicals and reagents used in this study included aspirin, acetic acid, carrageenan, formaldehyde, sodium chloride 0.9%, distilled water, ethanol 96% and other standard laboratory reagents.

The main equipment included oral gavage needles, analytical balance, surgical instruments, micropipettes, graduated tubes, cell-counting equipment, protein assay reagents/equipment and other standard experimental pharmacology equipment.

2.1.3. Experimental Animals

Swiss albino mice, 4-6 weeks old, of both sexes, weighing 20 ± 2 g, were used for the acetic acid-induced writhing test.

Wistar rats, 8-10 weeks old, of both sexes, weighing 180 ± 20 g, were used for the carrageenan-formaldehyde-induced peritonitis model and carrageenan-induced paw edema model.

Animals were supplied by the National Institute of Hygiene and Epidemiology (NIHE), Vietnam. They were acclimatized under standard laboratory conditions for at least 5 days before the experiment. Animals were housed in clean cages with free access to standard food and water. All experimental procedures were performed in accordance with institutional guidelines for the care and use of laboratory animals.

The in vivo animal study was approved by Animal Research Ethics Advisory Council of the Institute of Medicinal Materials, Hanoi, Vietnam with No 416/VDL-HĐTVĐĐTĐV.

2.2. Methods

2.2.1. Acetic Acid-induced Writhing Test

The analgesic effect of PB-Hex was evaluated using the acetic acid-induced writhing test in mice according to the method of Koster et al., [5].

Swiss albino mice were randomly divided into four groups, with 6 mice in each group:

- Group 1: Control group, receiving vehicle.
- Group 2: Positive control group, receiving aspirin at 100 mg/kg/day.
- Group 3: PB-Hex-treated group, receiving PB-Hex at 100 mg/kg/day.
- Group 4: PB-Hex-treated group, receiving PB-Hex at 300 mg/kg/day.

Mice were orally administered vehicle, aspirin or PB-Hex once daily in the morning for 5 consecutive days. On the fifth day, 1 hour after the final administration, each mouse was intraperitoneally injected with 0.2 mL of 1% acetic acid solution to induce abdominal constriction.

The number of writhing responses, characterized by abdominal constriction and stretching of the hind limbs, was counted every 5 minutes for 30 minutes after acetic acid injection. The percentage inhibition of writhing was calculated using the following formula:

Inhibition of writhing (%) = [(Xc - Xt) / Xc] × 100

where Xc is the mean number of writhes in the control group and Xt is the mean number of writhes in the treated group.

2.2.2. Carrageenan-formaldehyde-induced Peritonitis Model

The acute anti-inflammatory effect of PB-Hex was evaluated in rats using a carrageenan-formaldehyde-induced peritonitis model, adapted from established experimental inflammation models [6, 7].

Wistar rats were randomly divided into four groups, with 6 rats in each group:

- Group 1: Control group, receiving vehicle.
- Group 2: Positive control group, receiving aspirin at 150 mg/kg.

- Group 3: PB-Hex-treated group, receiving PB-Hex at 50 mg/kg.
- Group 4: PB-Hex-treated group, receiving PB-Hex at 150 mg/kg.

Rats were orally administered vehicle, aspirin or PB-Hex once daily in the morning for 5 consecutive days. On the fifth day, 1 hour after the final administration, peritoneal inflammation was induced by intraperitoneal injection of a carrageenan/formaldehyde mixture.

The inflammatory mixture was prepared by dissolving carrageenan 0.05 g and formaldehyde 1.4 mL in distilled water to a final volume of 100 mL. Each rat received 2 mL of this mixture per 100 g body weight by intraperitoneal injection.

Twenty-four hours after induction, the animals were sacrificed, the abdominal cavity was carefully opened, and the peritoneal exudate was collected. The anti-inflammatory effect was evaluated based on the volume of inflammatory exudate, total white blood cell count in 1 mL of exudate, and protein concentration in the peritoneal exudate.

2.2.3. Carrageenan-induced Paw Edema Model

The acute anti-inflammatory effect of PB-Hex was evaluated using the carrageenan-induced paw edema model in rats.

Wistar rats were randomly divided into four groups, with 6 rats in each group:

- Group 1: Control group, receiving vehicle.
- Group 2: Positive control group, receiving aspirin at 150 mg/kg.
- Group 3: PB-Hex-treated group, receiving PB-Hex at 50 mg/kg.
- Group 4: PB-Hex-treated group, receiving PB-Hex at 150 mg/kg.

The rat doses of PB-Hex were adjusted from the corresponding mouse doses according to interspecies dose conversion used in the experimental design.

Rats were orally administered vehicle, aspirin or PB-Hex once daily in the morning for 5 consecutive days. On the fifth day, 1 hour after the final administration, acute inflammation was induced by subplantar injection of 0.1 mL of 1% carrageenan suspension in normal saline into the right hind paw of each rat.

The paw volume was measured using a plethysmometer before carrageenan injection and at 2, 4, 6 and 24 hours after carrageenan injection. The percentage increase in paw volume was calculated as follows:

$\Delta V\ (\%) = [(Vt - V0) / V0] \times 100$

where V0 is the paw volume before carrageenan injection and Vt is the paw volume at each time point after carrageenan injection.

The percentage inhibition of paw edema was calculated using the following formula:

Inhibition of edema (%) $= [(\Delta Vc - \Delta Vt) / \Delta Vc] \times 100$

where ΔVc is the mean percentage increase in paw volume in the control group and ΔVt is the mean percentage increase in paw volume in the treated group.

2. 2.4. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism version 10.5.0. Time-course outcomes were analyzed using two-way repeated-measures ANOVA with treatment group as the between-subject factor and time as the within-subject factor, followed by Dunnett-adjusted comparisons against the vehicle control at prespecified time points. Greenhouse–Geisser correction was applied when the sphericity assumption was violated. Single-time-point outcomes were analyzed using one-way ANOVA followed by Dunnett's multiple-comparisons test. When model assumptions were not met, the prespecified non-parametric alternative was the Kruskal–Wallis test followed by Dunn's test. Adjusted two-sided $p < 0.05$ was considered statistically significant

3. Results

3.1. Analgesic Effect of PB-Hex in the Acetic Acid-induced Writhing Test

The time-course profile of writhing responses is shown in Figure 1. The vehicle-

treated control group maintained the highest mean number of pain responses throughout the 30-minute observation period. Aspirin and PB-Hex-treated groups showed a visible reduction in writhing responses compared with the control group, indicating a peripheral analgesic effect of PB-Hex in the acetic acid-induced abdominal constriction model.

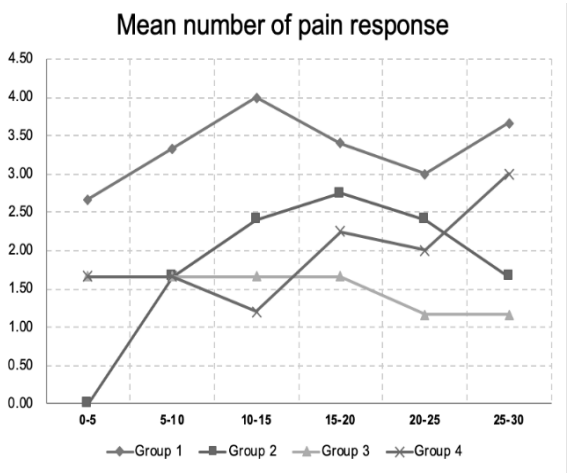


Figure 1. Time-course of mean pain responses in the acetic acid-induced writhing test.

PB-Hex at 100 mg/kg showed a relatively consistent reduction in nociceptive responses throughout the observation period. At 300 mg/kg, the reduction was observed mainly during the early and intermediate intervals, whereas nociceptive responses increased toward the final interval. Thus, the 100 mg/kg dose exhibited a more consistent effect than the 300 mg/kg dose, although no clear dose-dependent response was observed.

As shown in Figure 2, the vehicle-treated model control group exhibited the highest total number of nociceptive responses. Aspirin at 100 mg/kg reduced the total response count compared with the model control group, indicating that the experimental model was responsive to the reference analgesic. The PB-Hex-treated groups also showed lower response counts than the model control group, with the greatest numerical reduction observed at 100 mg/kg. However, these findings should be interpreted as a trend unless statistically significant differences are demonstrated. In addition, the lower apparent effect at 300 mg/kg indicates that no clear dose-dependent response was observed.

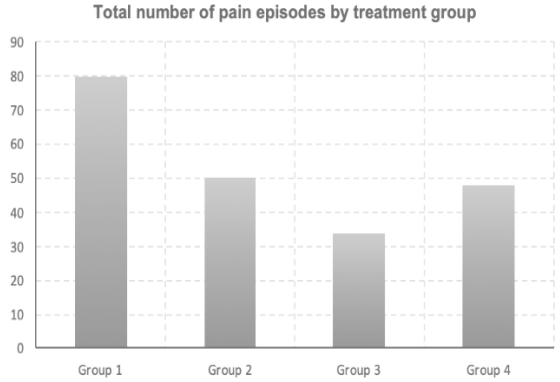
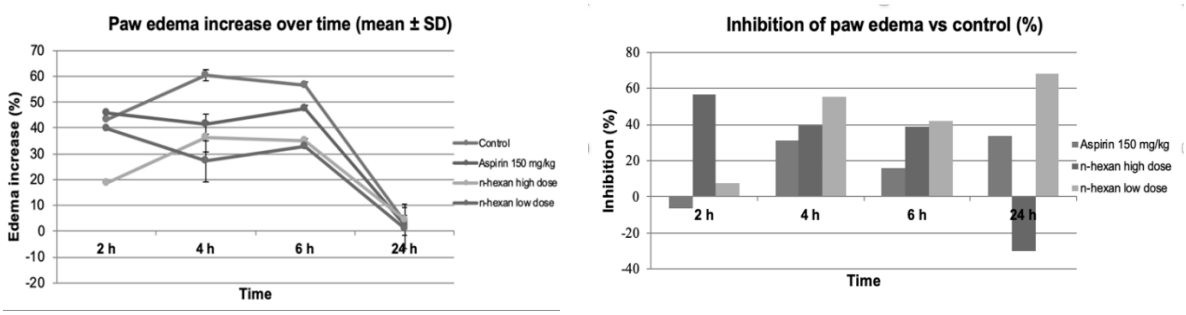


Figure 2. Effect of PB-Hex on the total number of acetic acid-induced writhing responses in mice.

3.2. Anti-inflammatory Effect of PB-Hex in the Carrageenan-induced Paw Edema Model

As shown in Figure 3A, paw edema in the vehicle-treated model control group increased during the first 4 hours after carrageenan injection, reached a maximum at 4 hours, and remained elevated at 6 hours. Aspirin at 150 mg/kg produced a lower paw-edema response than the model control treatment, particularly from 4 hours onward. The PB-Hex-treated groups also showed numerically lower edema responses, with the 50 mg/kg group exhibiting a more consistent reduction across the observation period than the 150 mg/kg group.

The descriptive inhibition values shown in Figure 3B were generally higher in the 50 mg/kg PB-Hex group at 4, 6, and 24 hours. PB-Hex at 150 mg/kg showed its greatest numerical inhibition at 2 hours, but the apparent effect was weaker at the later time points. These graphical trends suggest a potential anti-inflammatory effect of PB-Hex; however, this interpretation should be considered preliminary unless supported by statistically significant between-group differences. No clear dose-dependent response was observed.



A B

Figure 3. Effects of PB-Hex on carrageenan-induced paw edema in rats.
(A) Effect of n-hexane fraction on carrageenan-induced paw edema over time;
(B) Percentage inhibition of paw edema by n-hexane fraction compared with the control group.

3.3. Effect of PB-Hex on Peritoneal Exudate Parameters in the Experimental Peritonitis Model

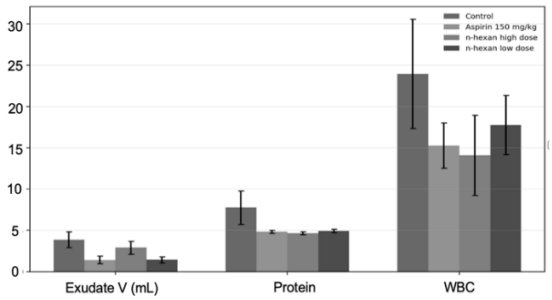


Figure 4. Effect of n-hexane fraction on peritoneal exudate parameters in the experimental peritonitis model.

As shown in Figure 4, the vehicle-treated model control group exhibited the highest peritoneal exudate volume, protein concentration, and total leukocyte count. Aspirin at 150 mg/kg produced lower values for all three parameters compared with the model control group. The PB-Hex-treated groups also showed numerically lower exudate volume, protein concentration, and total leukocyte count. PB-Hex at 50 mg/kg showed the greatest numerical reduction in exudate volume, whereas PB-Hex at 150 mg/kg showed a more pronounced numerical reduction in total leukocyte count. These trends are consistent with a possible reduction in vascular leakage and leukocyte recruitment. However, the findings should be interpreted cautiously unless supported by statistically significant differences. No clear dose-dependent response was observed across the evaluated parameters.

4. Discussion

The present study evaluated the analgesic and anti-inflammatory effects of PB-Hex, an n-hexane fraction obtained from an ethanol extract of *Piper betle* L. plant, using three complementary *in vivo* models. PB-Hex was associated with lower acetic acid-induced writhing responses in mice, attenuation of carrageenan-induced paw edema in rats, and reductions in several peritoneal inflammatory-exudate parameters. Collectively, these findings indicate a potential pharmacological effect of PB-Hex on nociceptive and inflammatory responses. However, the observed effects were not consistently dose dependent, and the findings should therefore be interpreted as preliminary until supported by complete statistical analysis, chemical characterization, and mechanistic investigation.

The acetic acid-induced writhing test is commonly used to evaluate peripheral anti-nociceptive activity. Intraperitoneal administration of acetic acid produces

abdominal constrictions through local irritation and the release of endogenous mediators involved in nociceptive sensitization, including prostaglandins, bradykinin, histamine, serotonin, and other products of arachidonic acid metabolism [5, 9]. In the present study, the vehicle-treated model control group exhibited the highest number of writhing responses, whereas aspirin reduced the response count, supporting the responsiveness of the experimental model to a reference peripheral analgesic.

PB-Hex also produced lower writhing-response counts than the model control treatment. The numerical reduction was more consistent at 100 mg/kg than at 300 mg/kg, indicating that increasing the dose did not enhance the apparent analgesic effect. This finding does not support a conventional monotonic dose-response relationship. Similar non-linear responses may occur with chemically complex plant fractions because multiple constituents may differ in bioavailability, pharmacological potency, or biological interactions. However, other explanations, including biological variability, limited sample size, differences in extract dispersion or absorption, and measurement variability, cannot be excluded. Consequently, antagonistic interactions among constituents should not be proposed as the primary explanation without supporting pharmacokinetic or chemical data.

The apparent anti-nociceptive effect observed in the present study is broadly consistent with previous investigations of *Piper betle* L. plant preparations. Alam et al. reported analgesic and anti-inflammatory activities of a methanolic leaf extract [1], while Arambewela et al. demonstrated anti-nociceptive activity of aqueous and ethanol extracts of *Piper betle* L. plant [3]. Reddy et al., also reported analgesic activity of a hydro alcoholic extract in experimental animals [8]. In addition, De et al., suggested that an ethanolic leaf extract reduced nociception through modulation of pathways associated with arachidonic acid metabolism [9]. Nevertheless, these previous studies used extracts with different solvent compositions and chemical profiles. Their findings therefore support the biological plausibility of the present observations but do not establish that PB-Hex acts through the same mechanisms.

Carrageenan-induced paw edema is a widely used model of acute local inflammation. The inflammatory response is generally described as a multiphasic process. Early edema formation is associated mainly with the release of histamine, serotonin, and bradykinin, whereas the later phase involves prostaglandins, nitric oxide, pro-inflammatory cytokines, increased vascular permeability, and leukocyte recruitment [6, 10]. In the vehicle-treated model control group, paw edema increased during the first four hours after carrageenan injection, reached its highest level at approximately 4 hours, and remained elevated at 6 hours. This time course is consistent with the expected development of carrageenan-induced inflammation.

Aspirin produced a lower paw-edema response than the model control treatment, particularly during the later observation period. This pattern is compatible with the known pharmacological action of aspirin on cyclooxygenase-dependent prostaglandin synthesis. PB-Hex-treated groups also showed lower edema responses, with the 50 mg/kg dose producing a more consistent numerical reduction across the observation period. In contrast, the 150 mg/kg dose showed its greatest apparent inhibition at 2 hours, followed by a weaker effect at later time points. These findings suggest that PB-Hex may influence more than one component of the inflammatory response; however, the current experimental design does not identify the specific mediators or pathways involved.

The absence of a clear dose-dependent effect in the paw-edema model is consistent with the pattern observed in the writhing test. The lower dose was not uniformly inferior to the higher dose and appeared more consistent across several time points. This non-monotonic pattern should be acknowledged as an important finding rather than interpreted as evidence that the lower

dose is definitively more effective. Confirmation would require additional dose levels, larger group sizes, appropriate repeated-measures analysis, and preferably assessment of the area under the edema–time curve for each animal.

The carrageenan–formaldehyde-induced peritonitis experiment provided additional information on acute exudative inflammation. Formation of peritoneal exudate is associated with changes in vascular permeability and movement of fluid and plasma proteins into the peritoneal cavity. Increased total leukocyte count reflects recruitment of inflammatory cells to the site of stimulation. In the present study, the model control group exhibited the highest numerical values for exudate volume, protein concentration, and total leukocyte count. Aspirin and PB-Hex treatment were associated with lower values for these parameters.

PB-Hex at 50 mg/kg showed the greatest apparent reduction in exudate volume, whereas PB-Hex at 150 mg/kg showed a more pronounced numerical reduction in total leukocyte count. Reductions in exudate volume and protein concentration are consistent with, but do not directly demonstrate, decreased vascular leakage. Similarly, a reduction in total leukocyte count may reflect lower inflammatory-cell recruitment, but this interpretation would be strengthened by differential leukocyte counting and direct assessment of chemotactic or adhesion-related pathways. The different response patterns across exudate volume and leukocyte count may indicate that these outcomes represent partly distinct components of the inflammatory process. However, no single PB-Hex dose was consistently superior across all peritonitis endpoints.

Several classes of biologically active constituents have been reported in *Piper betle* L. plant, including phenolic compounds, phenylpropanoids, and volatile or lipophilic constituents such as hydroxychavicol, chavibetol, eugenol, and related derivatives [4, 11]. Because PB-Hex represents a relatively non-polar fraction, its activity may be associated with lipophilic compounds. Nevertheless, the chemical composition of the PB-Hex batch used in the present study was not determined. It is therefore not possible to attribute the observed effects to any specific constituent.

Previous mechanistic studies provide possible explanations for the biological activity of *Piper betle* L. preparations. Ganguly et al. reported that an ethanol extract of *Piper betle* L. reduced nitric oxide production [2]. Other studies have shown that polar extracts of *Piper betle* L. may modulate inflammatory mediators and signaling pathways associated with oxidative stress, NF-κB, MAPK, iNOS, and COX-2 [12]. These findings support the general pharmacological plausibility of anti-inflammatory activity within the species. However, they were obtained using extracts with different chemical compositions, often in cell-based systems, and should not be considered direct evidence that PB-Hex acts through these pathways.

The consistency of the numerical trends across the writhing, paw-edema, and peritonitis experiments suggests that PB-Hex may act on both nociceptive and inflammatory processes. In conclusion, PB-Hex showed preliminary analgesic and anti-inflammatory effects in acetic acid-induced writhing, carrageenan-induced paw edema, and carrageenan–formaldehyde-induced peritonitis models. The effects were generally more consistent at the lower tested doses, but no clear dose-dependent relationship was demonstrated. Further studies should include chemical profiling and standardization of PB-Hex, additional dose levels, appropriate sex-balanced analysis, toxicity assessment, and measurement of relevant inflammatory mediators. Such investigations are necessary to confirm the pharmacological activity of PB-Hex and to identify the constituents and mechanisms responsible for its potential analgesic and anti-inflammatory effects.

5. Conclusion

PB-Hex showed that the 100 mg/kg dose exhibited a more consistent effect than the 300

mg/kg dose, although no clear dose-dependent response was observed. The PB-Hex-treated groups also showed lower response counts than the model control group, with the greatest numerical reduction observed at 100 mg/kg. Aspirin at 150 mg/kg produced a lower paw-edema response than the model control treatment, particularly from 4 hours onward. The PB-Hex-treated groups also showed numerically lower edema responses, with the 50 mg/kg group exhibiting a more consistent reduction across the observation period than the 150 mg/kg group.

Regarding anti-inflammatory activity, PB-Hex was associated with at 150 mg/kg showed its greatest numerical inhibition at 2 hours, but the apparent effect was weaker at the later time points. The PB-Hex-treated groups also showed numerically lower exudate volume, protein concentration, and total leukocyte count. PB-Hex at 50 mg/kg showed the greatest numerical reduction in exudate volume, whereas PB-Hex at 150 mg/kg showed a more pronounced numerical reduction in total leukocyte count.

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