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Original Research Article

Correlation Between *Citrus hystrix* Peel Essential Oil and its Analgesic, Antipyretic, and Anti-inflammatory Activities in an Experimental Mouse Model

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ABSTRACT

Citrus hystrix DC. peel essential oil, rich in bioactive compounds, is traditionally used for pain, fever, and inflammation. This study evaluated its dose-dependent effects in mice following oral administration at 50, 100, and 150 mg/kg. The negative control group received distilled water, while positive controls included tramadol (5 mg/kg, p.o.) for analgesia, paracetamol (150 mg/kg, p.o.) for antipyresis, and indomethacin (10 mg/kg, p.o.) for inflammation. Analgesic activity was assessed via hot plate and tail-flick tests at 30, 60, and 90 minutes post-treatment. EOCHP150 significantly increased latency time in the hot plate test ($p < 0.01$) and achieved 72.5% inhibition in the tail-flick test. Yeast-induced pyrexia (20% Brewer's yeast, 10 mL/kg, s.c.) was used to assess antipyretic activity, with EOCHP150 reducing rectal temperature by 4.0% at 4 hours, comparable to paracetamol. Carrageenan-induced paw edema (1% carrageenan, 50 µL, s.c.) showed a 46% inhibition in paw swelling at 4 hours with EOCHP150, accompanied by significant suppression of COX-2, PGE2, TNF-α, IL-1β, and IL-6 levels ($p < 0.01$). Strong correlations were observed between EOCHP dose and pharmacodynamic responses. These findings support EOCHP as a promising multi-target natural agent for the management of pain, fever, and inflammation.

Keywords: Analgesic activity, Antipyretic effect, *Citrus hystrix*, Essential oil.

Introduction

Pain, fever, and inflammation are among the most common clinical symptoms encountered in medicine and public health, often indicating underlying pathological conditions and significantly impairing patients' quality of life.[1] Although conventional therapeutic agents, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, remain widely used, they are associated with numerous adverse effects, including gastrointestinal irritation, renal dysfunction, tolerance, and the risk of dependence.[2] These limitations have intensified interest in safer, plant-based alternatives with comparable pharmacological efficacy and improved safety profiles.

Citrus hystrix DC., a citrus species belonging to the genus Citrus in the family Rutaceae, is widely distributed across Southeast Asian countries, including Thailand, Indonesia, Malaysia, and Vietnam. Locally, it is known as "kaffir lime" in English and "Chanh Sa" or "Chanh Thai" in Vietnamese. The essential oil extracted from *Citrus hystrix* peel (EOCHP) is a rich source of volatile compounds, primarily monoterpenes and sesquiterpenes.[3,4] According to Long *et al.*,[5] EOCHP contains 22 volatile constituents accounting for 99.98% of its total composition.

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The major components include monoterpene hydrocarbons (83.52%) such as limonene, β-pinene, and sabinene, along with monoterpenoids (15.78%) like terpinen-4-ol and citronellal. Although sesquiterpenes such as caryophyllene, germacrene D, and copaene are present in smaller amounts (approximately 0.68%), they are considered biologically potent.[5] Numerous studies have reported the pharmacological activities of these constituents, especially in Citrus species. For example, limonene, one of the primary monoterpenes, has demonstrated anti-inflammatory, antioxidant, and analgesic effects in both *in vitro* and *in vivo* models.[6] Caryophyllene, a sesquiterpene, exhibits anti-inflammatory and analgesic activity via selective binding to the CB2 cannabinoid receptor, without affecting the central nervous system.[7] Similarly, terpinen-4-ol and citronellal have shown notable antibacterial and anti-inflammatory properties.[5]

Due to its diverse chemical composition and promising bioactivities, *Citrus hystrix* has garnered increasing attention in the development of natural health and medicinal products. However, the direct relationship between the phytochemical profile of its peel essential oil and specific pharmacological effects, particularly analgesic, antipyretic, and anti-inflammatory actions, remains underexplored, especially in in vivo models. Therefore, this study aims to clarify the correlation between the chemical composition of *Citrus hystrix* peel essential oil and its analgesic, antipyretic, and anti-inflammatory activities in a murine experimental model. The findings are expected to strengthen the scientific basis for the use of *Citrus hystrix* in traditional medicine and support its potential application in the development of herbal medicines, functional foods, and natural cosmetics.

Materials and Methods

Sample collection and preparation of Citrus hystrix peel essential oil

In June 2024, mature fruits of *Citrus hystrix* DC. were collected from Tinh Bien District, An Giang Province, Vietnam. The plant species (voucher specimen number: CH040624VST) was identified by a taxonomist (Minh Thao Nguyen) and deposited at the Biotechnology Laboratory, Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City, Vietnam. Only healthy, naturally

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ripened fruits were selected for processing. Following thorough washing, the peels were manually separated, shade-dried for 3-5 days, and ground into small fragments. A total of 500 g of dried peel was subjected to steam distillation for 4 hours to obtain the essential oil (EOCHP), which yielded 3.1% (v/w). The oil was collected and stored in airtight amber glass bottles at 4°C, shielded from light, until further experimental analysis.

Phytochemical characterization of Citrus hystrix peel essential oil

In this research, the phytochemical constituents of *Citrus hystrix* DC. peel essential oil (EOCHP) were characterized based on the chemical profile previously described by Long *et al.*[5] Their analysis demonstrated that EOCHP primarily contains volatile compounds, notably monoterpenes, which consist of monoterpene hydrocarbons, oxygenated monoterpenes, as well as sesquiterpenes. Importantly, bioactive components such as germacrene D, camphene, and caryophyllene were highlighted, each known for exhibiting diverse biological effects. Utilizing the chemical composition outlined by Long *et al.* ensures accurate phytochemical consistency and provides a foundation for exploring the pharmacological potential of EOCHP.[5]

Experimental animals

The experiment was conducted on male Swiss albino mice (weighing 28–30 g), obtained from the Pasteur Institute, Ho Chi Minh City, Vietnam. The animals were acclimatized for 7 days in glass cages lined with rice husk bedding treated with biological agents for odor control. Environmental conditions were maintained at 24 ± 2°C, with 55-60% humidity and a 12-hour light/dark cycle. Standard rodent chow and filtered water were provided ad libitum. Each experimental model consisted of five to six groups: a normal control group, a negative control group induced with either 20% Brewer's yeast (10 mL/kg, s.c.) for the antipyretic test or 1% carrageenan solution (50 µL, s.c.) for the anti-inflammatory test, a positive control group treated with a standard reference drug (tramadol, paracetamol, or indomethacin), and three experimental groups administered EOCHP at oral doses of 50, 100, and 150 mg/kg.[8] At the end of each experiment, animals were euthanized, and blood and tissue samples were collected for biochemical analysis. Inflammatory biomarkers, including COX-2, PGE2, TNF-α, IL-1β, and IL-6, were evaluated at 4 hours post-induction to assess the acute-phase response. All animal handling and experimental procedures complied with the Basel Declaration and the International Guiding Principles for Biomedical Research Involving Animals, ensuring ethical treatment and welfare throughout the study.[9] Ethical approval for the study was obtained from the Institutional Animal Ethics Committee of the University.

Experiment design

Assessment of analgesic activity

Twenty-five mice, fasted for 16 hours with free access to water, were randomly assigned to five groups (n = 5). The control group orally received saline (10 mL/kg), while the standard group (TRM) was given tramadol at 5 mg/kg. EOCHP was administered orally at 50, 100, and 150 mg/kg (EOCHP50, EOCHP100, EOCHP150), suspended in 1% Tween 80.

Tail-flick test: To evaluate centrally mediated analgesia, the tail immersion test was performed by dipping the distal 2–3 cm of each mouse's tail into hot water maintained at 55 ± 2°C. The latency to tail flick was recorded before treatment and at 30, 60, 90, and 120 minutes post-administration as previously described, with a cutoff time of 25 seconds to prevent tissue damage. Increased latency indicated analgesic activity. The percentage of pain inhibition (PIT) was calculated as:[10,11]

$$\text{PIT (\%)} = \frac{\text{Test latency} - \text{Control latency}}{25 - \text{Control latency}} \times 100 \quad \text{(Eq. 1)}$$

Hot plate test: Mice were tested on a heated surface to measure reaction times to pain, indicated by paw licking or jumping. Recordings were taken at 0, 15, 30, 45, and 60 minutes post-treatment as already described, with a cutoff time of 45 seconds. The maximum analgesic effect (MAH) was determined using the formula:[10,11]

$$\text{MAH (\%)} = \frac{\text{Test reaction time} - \text{Control reaction time}}{45 - \text{Control reaction time}} \times 100 \quad \text{(Eq. 2)}$$

Assessment of antipyretic activity

A yeast-induced hyperthermia model was used to evaluate the antipyretic effects of EOCHP. Mice were divided into six groups (n = 5). The normal control group received sterile saline (10 mL/kg) without yeast induction. The fever control group was injected subcutaneously with 20% Saccharomyces cerevisiae suspension (10 mL/kg) but received no treatment. The positive control group (Yeast + PCM) orally received paracetamol (150 mg/kg) after yeast induction. The remaining groups (Yeast+EOCHP50, Yeast+EOCHP100, Yeast+EOCHP150) were orally treated with EOCHP at 50, 100, and 150 mg/kg, respectively, diluted in 1% Tween 80, following yeast-induced fever.

Temperature monitoring: Mice were fasted overnight with free access to water. Baseline rectal temperatures were recorded using a digital thermometer (Omron Healthcare, Japan). Fever was induced by yeast injection, and 18 hours later, animals showing a temperature increase of 0.3-0.5°C were selected. Rectal temperatures were then measured at 1, 2, and 3 hours post-treatment. The percentage of fever reduction (PFR) was calculated using:[11]

$$\text{PFR (\%)} = \frac{\text{Post-fever temperature} - \text{Temperature at each time point)}}{\text{Post-fever temperature} - \text{Normal temperature}} \times 100$$

(Eq. 3)

Cyclooxygenase-2 (COX-2) and Prostaglandin E2 (PGE2) determination: Blood was collected via retro-orbital puncture and centrifuged at 12,000 rpm for 5 minutes to isolate serum. Levels of COX-2 and PGE2 were quantified using ELISA kits (Absolute Biotech Co., Ltd.). Samples and standards were added to antibody-coated wells, followed by horseradish peroxidase (HRP) conjugate and incubation at 37°C for 1 hour. After washing, reagents A and B were added and incubated for 15-30 minutes, then stopped with the stop solution. Absorbance was measured at 450 nm, and concentrations were determined based on a standard curve.[11]

Assessment of anti-inflammatory activity

Swiss albino mice (30-32 g) were randomly divided into six groups (n = 5). The normal control group received sterile saline (10 mL/kg) orally without inflammation. Inflammation was induced in the remaining groups by subcutaneous injection of 50 µL of 1% carrageenan into the right hind paw. The carrageenan-only group (CGN) received no further treatment. The positive control group (CGN+IND) was treated with indomethacin (10 mg/kg, orally), while the experimental groups (CGN+EOCHP50, CGN+EOCHP100, CGN+EOCHP150) received EOCHP orally at doses of 50, 100, and 150 mg/kg, respectively.

Paw edema measurement: Baseline paw circumference was recorded using a digital caliper (Mitutoyo, Japan) before inflammation. Edema was assessed at 1, 2, 3, and 4 hours post-carrageenan injection by measuring paw circumference. The percentage inhibition of paw edema (PIP) was calculated as:[12]

$$\text{PIP (\%)} = \frac{\text{Edema in control} - \text{Edema in test group}}{\text{Edema in control}} \times 100 \quad \text{(Eq. 4)}$$

Cytokine quantification

Levels of pro-inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and interleukin-1 beta (IL-1β), were determined using ELISA from mice subjected to the anti-inflammatory screening. Plates pre-coated with specific antibodies were incubated with samples overnight, followed by the addition of biotinylated antibodies and enzyme-conjugated streptavidin. Colorimetric detection was performed at 450 nm. Cytokine concentrations were calculated from standard curves and expressed as ng/mg tissue.[12]

Dose-response correlation analysis

The dose–response relationship of EOCHP (50–150 mg/kg) was evaluated by analyzing key pharmacological indicators, including: latency time and percentage of pain inhibition (analgesic effect); rectal temperature, percentage of fever reduction (PFR), and levels of COX-2 and PGE2 (antipyretic effect); paw circumference, percentage inhibition of paw edema (PIP), and concentrations of TNF-α, IL-1β, and IL-6 (anti-inflammatory effect). Pearson's correlation coefficients were calculated to assess the relationship between EOCHP dose and these

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parameters. For non-normally distributed data, Spearman's rank correlation was applied. A significance level of $p < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism (latest version).[13]

Data presentation

Scatter plots were used to illustrate statistically significant correlations ($r > \pm 0.7$, $p < 0.05$) between EOCHP doses (X-axis) and efficacy parameters (Y-axis). Each plot included a linear regression line along with the corresponding r and p values. Column charts were employed to compare the effectiveness of different EOCHP doses, particularly for biochemical markers that did not exhibit a clear linear relationship. A summary table of correlation coefficients was also prepared, displaying all r and p values for each dose–response pair, serving as the basis for selecting appropriate charts and interpreting the dose–response relationship.[14]

Statistical analysis

Data were expressed as mean ± standard deviation (Mean ± SD). Differences between groups were analyzed using one-way ANOVA, followed by Tukey's post hoc test when significant. Correlations between EOCHP doses and pharmacological parameters were assessed using Pearson's correlation coefficient for normally distributed data or Spearman's rank correlation for non-normal distributions. A p-value < 0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 10.4.2.

Results and Discussion

Characteristics of Citrus hystrix peel essential oil (EOCHP)

This study investigates the analgesic, antipyretic, and anti-inflammatory properties of *Citrus hystrix* DC. peel essential oil (EOCHP) in mice, using the phytochemical profile reported by Long *et al.* as a reference.[5] EOCHP is composed of 22 volatile compounds, making up 99.98% of its content. The oil is rich in monoterpenes (99.3%), primarily monoterpene hydrocarbons (83.52%) and oxygenated monoterpenes (15.78%), along with a smaller proportion of sesquiterpenes (0.68%) such as copaene, caryophyllene, and germacrene D. Notable bioactive components, including germacrene D (0.15%), camphene (0.17%), and caryophyllene (0.22%), are associated with diverse pharmacological effects. This chemical composition provides a strong foundation for assessing the therapeutic potential of EOCHP in biological models.

The phytochemical composition of EOCHP provides a strong rationale for its observed analgesic, antipyretic, and anti-inflammatory effects in experimental models. EOCHP is rich in monoterpenes (99.3%), primarily monoterpene hydrocarbons such as limonene, β-pinene, sabinene, and camphene, which have been extensively reported for their pharmacological properties. Limonene, in particular, has been shown to exert anti-inflammatory and antinociceptive effects by inhibiting pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and modulating the NF-κB signaling pathway.[15,16] The methanolic leaf and root extracts of *Gomphocarpus purpurascens* have demonstrated peripheral analgesic effects in the formalin-induced pain model in mice, through interference with inflammatory mediators.[17] In addition, the presence of oxygenated monoterpenes (15.78%) such as terpinen-4-ol and citronellal further supports EOCHP's therapeutic potential. Terpinen-4-ol has been reported to reduce the production of nitric oxide and pro-inflammatory cytokines in lipopolysaccharide (LPS)-stimulated macrophages,[18] while citronellal exhibits anti-inflammatory and antipyretic properties in carrageenan-induced inflammation and yeast-induced fever models, respectively.[19] Although sesquiterpenes represent a smaller fraction (0.68%), their biological relevance is considerable. Caryophyllene - a known cannabinoid 2 (CB2) receptor agonist - exerts strong anti-inflammatory and analgesic effects without central nervous system involvement, as demonstrated in models of neuropathic pain and inflammation.[20] Germacrene D, although less studied, has shown the ability to suppress leukocyte migration and reduce oxidative stress in inflammation models.[3] Together, these findings support the hypothesis that the major and minor constituents of EOCHP synergistically contribute to its pharmacological effects. The correlation between its chemical composition and therapeutic outcomes observed in this study is consistent with previously reported data and reinforces the potential of *Citrus hystrix* peel essential oil as a natural alternative for managing pain, fever, and inflammation.

Correlation between Citrus hystrix peel essential oil doses and analgesic efficacy

The results presented in Tables 1 and 2 reveal a strong and statistically significant dose-dependent correlation between EOCHP administration and analgesic responses in both the hot plate and tail-flick test models. In the hot plate test, foot-licking latency increased progressively with EOCHP dose at all measured time points, 15, 30, 45, and 60 minutes, compared to the negative control group, with correlation coefficients ranging from $r = 0.976$ to $r = 0.994$ and $p < 0.01$. The strongest correlation was observed at 30 minutes ($r = 0.994$), indicating maximal sensitivity of this time point to dose variation. Similarly, the maximum analgesic effect (MAH) also increased significantly in EOCHP-treated groups versus the negative control, with correlation coefficients between $r = 0.969$ and $r = 0.992$ across all time points ($p < 0.01$).

In the tail-flick test, EOCHP significantly prolonged latency and enhanced the percentage of pain inhibition (PIT) at 30 and 60 minutes compared to the negative control group. Tail-flick latency showed strong positive correlations with dose ($r = 0.981$ at 30 min; $r = 0.979$ at 60 min; $p < 0.01$), while PIT also demonstrated consistent correlation ($r = 0.976$ at both time points; $p < 0.01$). These findings collectively support a clear and statistically significant dose-dependent analgesic effect of EOCHP in central (hot plate) and spinal (tail-flick) nociceptive pathways.

Figures 1 and 2 illustrate the dose-dependent effects of *Citrus hystrix* peel essential oil (EOCHP) on analgesic parameters in the hot plate and tail-flick test models. Statistically significant increases ($p < 0.01$) were observed in all EOCHP-treated groups (50, 100, and 150 mg/kg) when compared to the negative control group. In the hot plate test (Figure 1), panel A shows that foot-licking latency (reaction time) increased significantly from 2.55 ± 0.12 s in the 50 mg/kg group to 3.62 ± 0.08 s in the 150 mg/kg group ($p < 0.01$ vs. control). The Pearson correlation coefficient across time points ranged from $r = 0.976$ to 0.994, indicating a strong, consistent dose-dependent relationship. Panel B reveals that the maximum analgesic effect (MAH) increased from 0.175% at 50 mg/kg to a peak of 0.255% at 100 mg/kg ($p < 0.01$), followed by a slight reduction to 0.215% at 150 mg/kg. Despite this decline, the correlation remained significant ($r = 0.969$ to 0.993, $p < 0.01$), suggesting that the analgesic efficacy of EOCHP may plateau beyond 100 mg/kg. In the tail-flick test (Figure 2), panel A demonstrates a significant increase in latency time from 17.45 ± 0.15 s (50 mg/kg) to 21.55 ± 0.11 s (150 mg/kg) relative to the negative control ($p < 0.01$), with a correlation coefficient of $r = 0.979$-0.981. Panel B shows a corresponding dose-dependent enhancement in the percentage of pain inhibition (PIT), rising from 43.6% to 73.8% across the same dose range ($p < 0.01$), also with a strong positive correlation ($r = 0.976$). These results confirm the robust analgesic potential of EOCHP in both central and spinal pain models.

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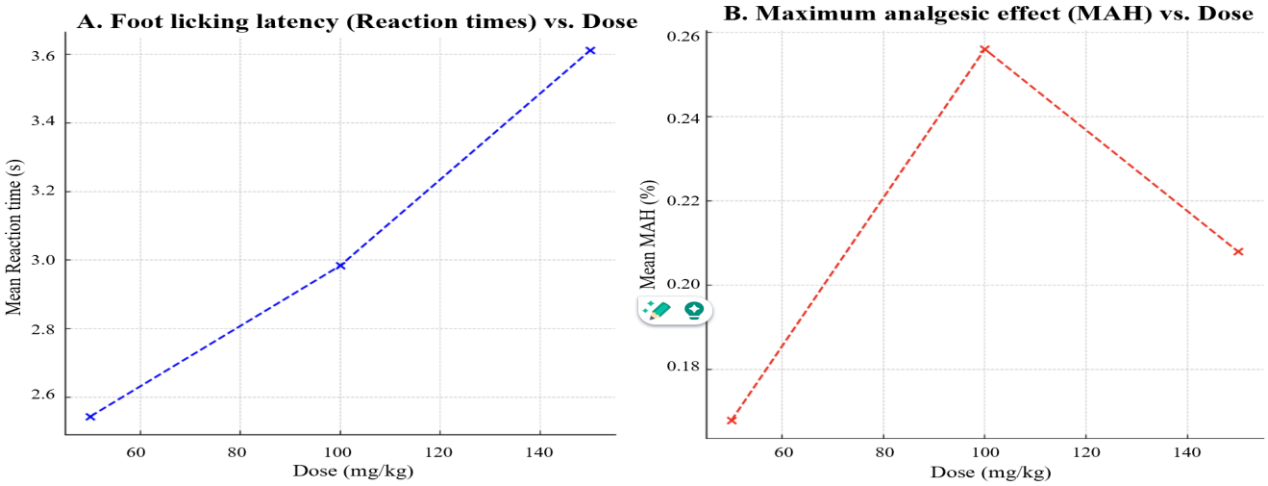

Figure 1: Scatter plots showing the correlation between *Citrus hystrix* peel essential oil (EOCHP) dose and analgesic parameters in the hot plate test. (A) Foot-licking latency (reaction time); (B) Maximum analgesic effect (MAH). Data are presented as mean ± SD (n = 5).

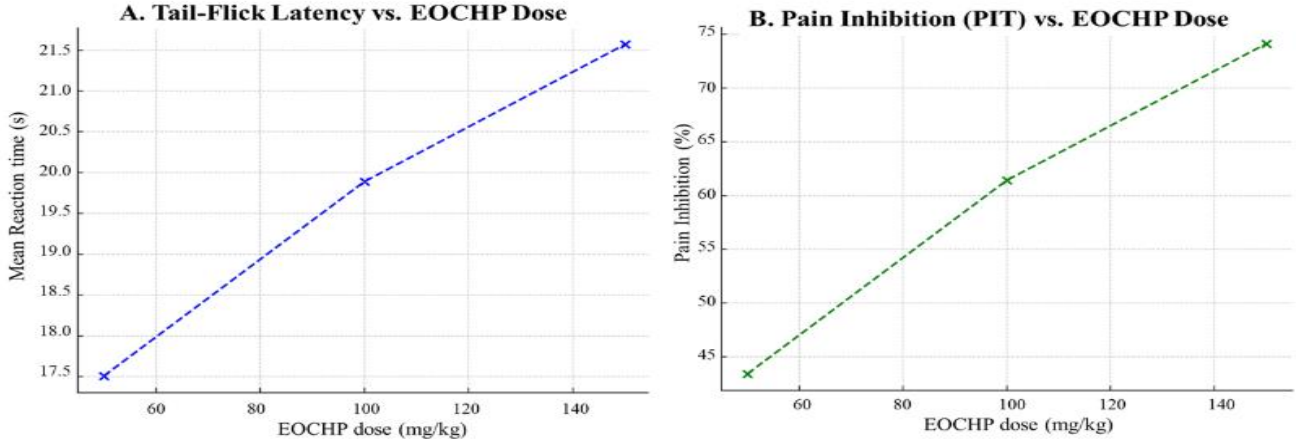

Figure 2: Scatter plots showing the linear correlation between *Citrus hystrix* peel essential oil (EOCHP) doses and analgesic parameters in the tail immersion test. (A) Tail-flick latency (reaction time); (B) Percentage of pain inhibition (PIT). Data are presented as mean ± SD (n = 5).

Figure 3 illustrates the time- and dose-dependent analgesic effects of *Citrus hystrix* peel essential oil (EOCHP) at doses of 50, 100, and 150 mg/kg in murine pain models. In panel A (hot plate test), the maximum analgesic effect (MAH) increased significantly over time and across treatment doses. At 15 minutes post-administration, all groups displayed low MAH values (≤ 1.0%) with no statistically significant difference between them. However, by 30 minutes, the EOCHP150 group exhibited a significantly higher MAH (approximately 3.1%) compared to EOCHP100 (2.4%) and EOCHP50 (0.8%) ($p < 0.05$). This pattern was further amplified at 60 minutes, where the EOCHP150 group reached the highest MAH (10.5%), significantly exceeding EOCHP100 (8.2%) and EOCHP50 (2.4%) ($p < 0.05$, letters $a \neq b \neq c$ indicate significance). In panel B (tail-flick test), a comparable dose-dependent trend was observed in the percentage of pain inhibition (PIT). At 30 minutes, PIT increased significantly from 16.8% (EOCHP50) to 31.5% (EOCHP100) and 39.2% (EOCHP150). At 60 minutes, the EOCHP150 group exhibited the highest inhibition (72.5%), followed by EOCHP100 (60.3%) and EOCHP50 (45.8%) ($p < 0.05$, between all groups). These results confirm the significant, graded analgesic effects of EOCHP, with higher doses producing markedly greater efficacy over time.

This study demonstrates a clear, dose-dependent relationship between EOCHP and its analgesic efficacy in mouse models. Central (hot plate) and peripheral (tail-flick) pain assessments consistently showed enhanced analgesic responses, with increased latency times and analgesic indices at higher EOCHP doses. Statistical analyses confirmed strong positive correlations at all tested time points, with the maximum analgesic effects observed at 150 mg/kg, supporting EOCHP's dose-dependent pharmacodynamics. EOCHP's analgesic properties result primarily from its bioactive constituents, including monoterpenes and sesquiterpenes. Phytochemical analysis identifies camphene, limonene, *β*-caryophyllene, and germacrene D as major components,[5] each independently linked to antinociceptive, anti-inflammatory, and antioxidant activities. *β*-Caryophyllene acts as a selective CB2 receptor agonist, mediating peripheral analgesia and inflammation reduction,[21] while germacrene D modulates opioid and TRPV1 receptor pathways crucial in pain transmission.[22] Similar dose-dependent analgesic profiles have been reported in Eucalyptus essential oils, highlighting shared phytochemical mechanisms across essential oils rich in monoterpenes.[23] Interestingly, peak efficacy was observed at the highest EOCHP dose, followed by a subtle plateau, indicating pharmacodynamic saturation, where increasing doses yield diminishing returns. Recognizing this phenomenon aids in defining the optimal therapeutic dose range for balancing efficacy, safety, and cost-effectiveness. Furthermore, EOCHP exhibits concurrent anti-inflammatory and antipyretic activities, due to overlapping biochemical pathways involving prostaglandin synthesis, cytokine modulation, and oxidative stress responses. Overall, these findings affirm EOCHP's dose-responsive analgesic potential and highlight its broader pharmacological value as a natural therapeutic agent.

Correlation between doses of Citrus hystrix peel essential oil and antipyretic effects

The correlation analysis presented in Table 3 confirms a statistically significant, dose-dependent antipyretic effect of *Citrus hystrix* peel essential oil (EOCHP) in the yeast-induced pyrexia model. Compared

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to the negative control group (yeast-induced fever without treatment), EOCHP-treated groups (50, 100, and 150 mg/kg) exhibited a progressive reduction in rectal temperature over time, with a moderate negative correlation between EOCHP dose and rectal temperature values at 15, 30, 45, and 60 minutes ($r = -0.188$ to -0.464, $p < 0.05$). This trend indicates that higher doses of EOCHP are associated with a greater reduction in body temperature. In contrast, the percentage of fever reduction (PFR) showed a significant positive correlation with EOCHP dose at all time points, increasing from 15 to 60 minutes ($r = 0.419$ to 0.647, $p < 0.01$), suggesting a strong dose-responsive antipyretic effect. Although correlations between EOCHP dose and COX-2 ($r = 0.122$) and PGE2 ($r = 0.194$) levels were statistically significant ($p < 0.01$), their relatively weak coefficients imply that EOCHP's antipyretic action may not primarily result from direct inhibition of these downstream mediators. Instead, its effects may involve broader upstream anti-inflammatory mechanisms or central thermoregulatory pathways, potentially mediated via cytokines such as IL-1β or TNF-α, or through the synergistic interaction of its bioactive constituents.

Table 1: Correlation coefficients between *Citrus hystrix* peel essential oil (EOCHP) doses and analgesic parameters in the hot plate test

	Foot-licking latency latency				Maximum analgesic effect (MAH)			
Parameter	**15 min**	**30 min**	**45 min**	**60 min**	**15 min**	**30 min**	**45 min**	**60 min**
Correlation with EOCHP dose	r = 0.976	r = 0.994	r = 0.991	r = 0.991	r = 0.969	r = 0.993	r = 0.992	r = 0.992
Significance (p-value)	p < 0.01				p < 0.01			

Table 2: Correlation coefficients between *Citrus hystrix* peel essential oil (EOCHP) doses and analgesic parameters in the tail-flick test

Parameter	Tail-flick latency		Percentage of pain inhibition (PIT)	
	30 min	**60 min**	**30 min**	**60 min**
Correlation with EOCHP dose	r = 0.981	r = 0.979	r = 0.976	r = 0.976
Significance (p-value)	p < 0.01		p < 0.01	

Table 3: Pearson correlation coefficients between *Citrus hystrix* peel essential oil (EOCHP) doses and antipyretic parameters (rectal temperature, PFR, COX-2, and PGE2) in the yeast-induced pyrexia model.

	Rectal temperature (°C)				Percentage of fever reduction (PFR)					
Parameter	**15 min**	**30 min**	**45 min**	**60 min**	**15 min**	**30 min**	**45 min**	**60 min**	**COX2**	**PGE2**
Correlation with EOCHP dose	r = -0.188	r = -0.279	r = -0.435	r = -0.464	r = 0.419	r = 0.532	r = 0.598	r = 0.647	r = 0.122	r = 0.194
Significance (p-value)	p < 0.05				p < 0.01				p < 0.01	

Note: PFR - percentage of fever reduction, COX-2 - cyclooxygenase-2, PGE2 - prostaglandin E2

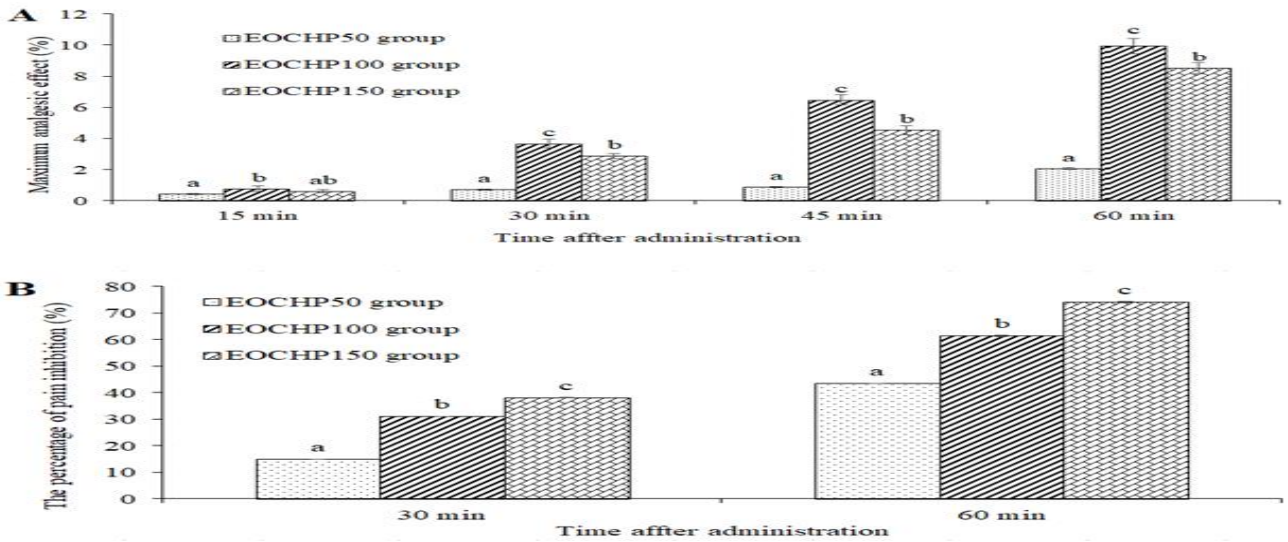

Figure 3: Comparison of analgesic effects of *Citrus hystrix* peel essential oil (EOCHP) at various doses in yeast-induced fever model. (A) Maximum analgesic effect (%) in the hot plate test; (B) Percentage of pain inhibition in the tail-flick test. Data are expressed as mean ± SD (n = 5). Different letters (a, b, c) denote significant differences between groups ($p < 0.05$).

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Table 4 presents the Pearson correlation analysis between *Citrus hystrix* peel essential oil (EOCHP) doses and anti-inflammatory parameters in the carrageenan-induced paw edema model. Compared to the negative control group (injected with 1% carrageenan without treatment), EOCHP-treated groups (50, 100, and 150 mg/kg) showed a significant dose-dependent reduction in paw swelling. Paw circumference negatively correlated with EOCHP dose at all time points from 1 to 4 hours ($r = -0.98$ to -1.06, $p < 0.05$), indicating that higher doses of EOCHP were associated with greater suppression of edema formation.
Conversely, the percentage inhibition of paw edema (PIP) exhibited a strong positive correlation with EOCHP dose, particularly at 2, 3, and 4 hours post-carrageenan injection ($r = 0.96$-0.98, $p < 0.01$), confirming a dose-dependent anti-inflammatory effect. Additionally, EOCHP administration significantly suppressed the levels of key pro-inflammatory cytokines. Strong negative correlations were observed between EOCHP dose and TNF-α ($r = -0.98$), IL-1β ($r = -0.98$), and IL-6 ($r = -0.98$) ($p < 0.01$ for all), suggesting that EOCHP mitigates inflammation not only by reducing edema but also by modulating key inflammatory mediators. These data support the conclusion that EOCHP exerts potent anti-inflammatory activity via both local and systemic pathways.
Figure 5 illustrates the time-dependent antipyretic effects of *Citrus hystrix* peel essential oil (EOCHP) at doses of 50, 100, and 150 mg/kg in the yeast-induced pyrexia model. At 1 hour post-administration, all EOCHP-treated groups exhibited comparable and mild fever reduction, with percentage of fever reduction (PFR) values close to 1.0%. No statistically significant differences were observed among the three groups ($p > 0.05$), indicating the absence of a dose-dependent effect during the early phase. At 2 hours, PFR increased modestly to approximately 2.0% across all groups, yet the intergroup differences remained statistically non-significant ($p > 0.05$), suggesting that EOCHP requires time for systemic absorption and pharmacological activation. By 3 hours post-administration, a significant dose-dependent antipyretic effect emerged. The EOCHP150 group exhibited a significantly higher PFR (~4.0%) compared to both the EOCHP100 and EOCHP50 groups (~3.0%) ($p < 0.05$), as indicated by differing superscript letters (a vs. b). These findings suggest that while EOCHP exerts a delayed-onset antipyretic effect, higher doses (≥150 mg/kg) are necessary to achieve maximal efficacy, likely due to threshold-dependent modulation of endogenous pyrogens such as PGE2 or pro-inflammatory cytokines.
The antipyretic effect of EOCHP is closely related to its phytochemical constituents and dose-dependent biological activity. In the yeast-induced pyrexia model, EOCHP administration effectively decreased fever parameters at higher doses, demonstrating enhanced activation of antipyretic pathways. EOCHP is abundant in bioactive monoterpenes and sesquiterpenes such as limonene, β-pinene, sabinene, camphene, and β-caryophyllene, which have established anti-inflammatory and antipyretic properties.[5] These compounds modulate critical molecular targets involved in fever pathogenesis, notably through inhibition of cytokines IL-1β, IL-6, and TNF-α, and suppression of prostaglandin E2 (PGE2) synthesis.[8,24] EOCHP constituents reduce cytokine production by downregulating key signaling pathways such as NF-κB and MAPK, and decreasing nitric oxide (NO) and cyclooxygenase-2 (COX-2) expression, pivotal in PGE2 formation. Consequently, EOCHP attenuates inflammatory signals responsible for elevating the hypothalamic thermoregulatory set-point during fever.[25,26] These pharmacological effects intensify with increasing EOCHP concentrations, establishing a clear dose-response relationship. Collectively, current findings supported by existing literature confirm EOCHP's therapeutic potential as a natural antipyretic agent, aligning modern pharmacological evidence with traditional medicinal use.[27,28]

Correlation between doses of Citrus hystrix peel essential oil and anti-inflammatory effects

Table 4 presents the correlation between EOCHP dose and anti-inflammatory parameters in the carrageenan-induced paw edema model. Compared to the carrageenan-only control group, EOCHP-treated groups (50, 100, and 150 mg/kg) exhibited a significant and consistent reduction in paw swelling. A strong inverse correlation was observed between EOCHP dose and paw circumference at 1, 2, 3, and 4 hours post-carrageenan injection ($r = -0.98$ to -1.06, $p < 0.05$), confirming the dose-dependent attenuation of acute inflammation by EOCHP. Similarly, the percentage inhibition of paw edema (PIP) demonstrated a strong positive correlation with EOCHP dose at 2, 3, and 4 hours ($r = 0.96$ to 0.98, $p < 0.01$), indicating that anti-edematous efficacy increases with dose and time. In contrast, the correlation at 1 hour was weak ($r = 0.21$, not significant), suggesting that the anti-inflammatory effects of EOCHP emerge during the later stages of the inflammatory cascade, possibly reflecting time-dependent bioavailability and tissue accumulation of its active compounds. Additionally, EOCHP dose was significantly and negatively correlated with pro-inflammatory cytokine levels—TNF-α, IL-1β, and IL-6 ($r = -0.98$ for all, $p < 0.01$). These cytokines are central to the propagation of inflammation; thus, their consistent downregulation supports the hypothesis that EOCHP exerts systemic anti-inflammatory effects via modulation of molecular inflammatory mediators.

Table 4: Pearson correlation coefficients between *Citrus hystrix* peel essential oil (EOCHP) doses and anti-inflammatory parameters (paw circumference, PIP, and cytokines) in the carrageenan-induced inflammation model

Parameter	**Paw circumference (mm)**				**Percentage inhibition of paw edema (PIP)**				**Cytokines**		
	1 h	**2 h**	**3 h**	**4 h**	**1 h**	**2 h**	**3 h**	**4 h**	**TNF-α**	**IL-1β**	**IL-6**
Correlation with EOCHP dose	r = -0.99	r = -0.98	r = -0.99	r = -1.06	r = 0.21	r = 0.98	r = 0.96	r = 0.98	r = -0.98	r = -0.98	r = -0.98
Significance (p-value)	p < 0.05				p < 0.01				p < 0.01		

Note: PIP - percentage inhibition of paw edema

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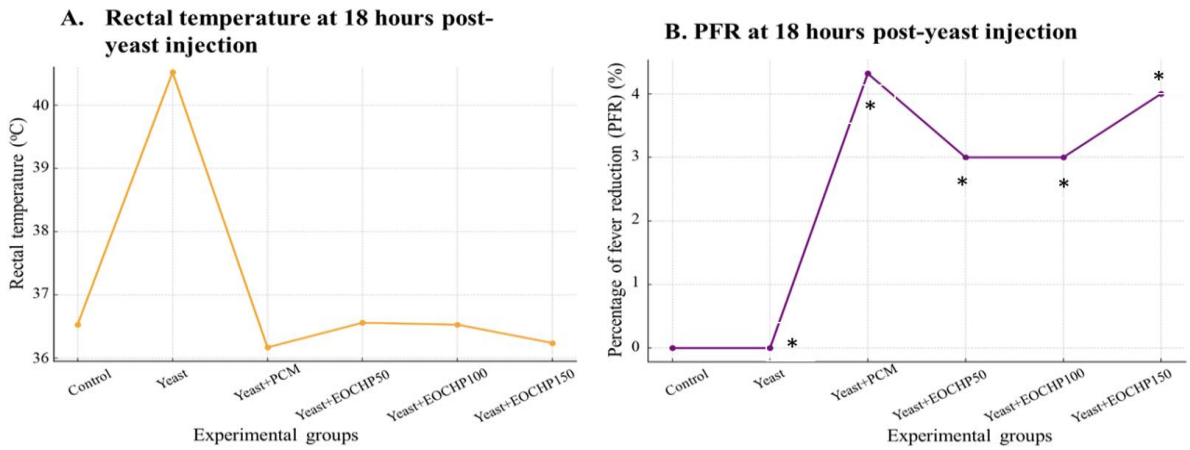

Figure 4: Effects of *Citrus hystrix* peel essential oil (EOCHP) on (A) rectal temperature and (B) percentage of fever reduction (PFR) at 18 h post-yeast injection. Data are presented as mean ± SD (n = 5). Asterisks (*) indicate significant differences compared to the yeast group ($p < 0.05$).

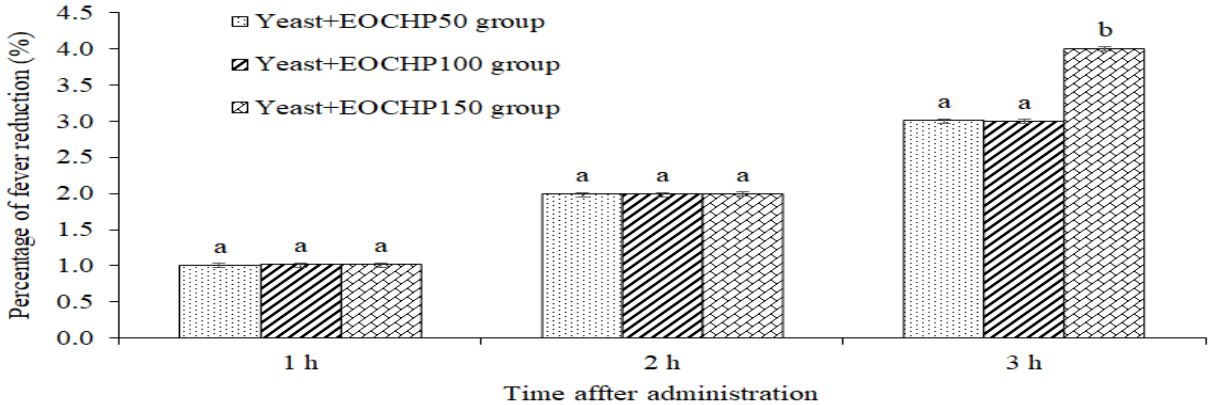

Figure 5: Antipyretic effects of *Citrus hystrix* peel essential oil (EOCHP) at different doses over time. Data are presented as mean ± SD (n = 5). Different superscript letters (a, b) indicate significant differences among groups at each time point ($p < 0.05$).

Figure 6 depicts the anti-inflammatory effects of *Citrus hystrix* peel essential oil (EOCHP) at different doses (50, 100, and 150 mg/kg), assessed by paw circumference (Figure 6A) and the percentage inhibition of paw edema (PIP) (Figure 6B) at 4 hours post-carrageenan (CGN) injection. In Figure 6A, the CGN-only group showed a substantial increase in paw circumference (approximately 42 mm) in comparison with negative control, confirming acute inflammatory edema compared to the normal control group (23 mm). Treatment with indomethacin (CGN + IND group) significantly reduced paw circumference to ~23 mm ($p < 0.01$ vs. CGN group). Likewise, all EOCHP-treated groups (CGN + EOCHP50, 100, and 150) showed significant reductions in paw size (23.0-24.5 mm), statistically different from the CGN group ($p < 0.05$), and approaching values seen in the IND and normal control groups. These findings indicate a robust anti-edematous effect of EOCHP, particularly at higher doses. Supporting this, Table 4 reports a strong negative correlation between EOCHP dose and paw circumference at 4 hours ($r = -1.06$, $p < 0.05$), confirming the inverse relationship between EOCHP concentration and inflammation severity. In Figure 6B, the PIP values further reflect this trend. The EOCHP150 group achieved a PIP of ~46%, nearly equivalent to the IND group, while the EOCHP50 and EOCHP100 groups exhibited PIP values of ~45.5% and ~43%, respectively. Although the difference among EOCHP doses was not statistically significant ($p > 0.05$), the EOCHP150 group tended to show the highest inhibition. These data align with the correlation reported in Table 4 ($r = 0.98$, $p < 0.01$), suggesting that EOCHP exerts a dose-responsive anti-inflammatory effect, particularly in the later phase of carrageenan-induced edema.

Figure 7 compares the time-dependent anti-inflammatory efficacy of *Citrus hystrix* peel essential oil (EOCHP) at doses of 50, 100, and 150 mg/kg, based on the percentage inhibition of paw edema (PIP) in a carrageenan-induced inflammation model. At 1 hour post-administration, all EOCHP-treated groups exhibited modest yet statistically significant differences in PIP: 5% (EOCHP50), 7% (EOCHP100), and 9% (EOCHP150). Superscript letters (a, b, c) indicate significant pairwise differences among all doses ($p < 0.05$), suggesting an early-phase, dose-dependent anti-inflammatory response. At 2 hours, PIP increased uniformly across all groups (~17–18%), but no significant differences were detected between doses ($p > 0.05$, all groups labeled "a"), indicating convergence of effect as the inflammatory response progressed. At 3 hours, PIP continued to rise, reaching ~30–31%, with the EOCHP150 group demonstrating slightly higher inhibition (31%) than EOCHP100 (30%) and EOCHP50 (29%). A statistically significant difference was observed between the EOCHP150 and EOCHP50 groups ($p < 0.05$, indicated by different superscripts a vs. b), supporting a sustained dose-dependent effect during the intermediate phase. By 4 hours, all EOCHP-treated groups reached high levels of inhibition (~45–47%). Notably, the EOCHP100 group showed a slightly lower PIP (45%) than EOCHP50 and EOCHP150 (~47%), with statistical analysis indicating significant differences between EOCHP100 and the other two groups ($p < 0.05$, as reflected by superscript a vs. b). This pattern suggests a potential non-linear dose-response at the resolution phase, possibly due to differential tissue distribution or saturation effects of EOCHP constituents.

The anti-inflammatory activity of EOCHP demonstrates clear dose dependence and involves well-characterized biochemical pathways. In the carrageenan-induced inflammation model, increased EOCHP doses effectively suppressed paw edema and inhibited the production of key pro-inflammatory cytokines. EOCHP contains significant amounts of

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bioactive monoterpenes and sesquiterpenes, such as limonene, sabinene, and *β*-caryophyllene, which are well-documented for their anti-inflammatory properties.[5] These constituents modulate inflammation through multiple mechanisms: early-phase vascular mediator attenuation (e.g., histamine and serotonin) and late-phase suppression of prostaglandin synthesis and cytokine release.[28] This dual-phase mechanism explains the enhanced anti-edematous responses observed following EOCHP administration. At the molecular level, limonene and *β*-caryophyllene downregulate crucial inflammatory signaling pathways, notably NF-κB and MAPK, central regulators of inflammatory gene expression. Specifically, limonene inhibits the transcription of inflammatory cytokines TNF-*α*, IL-1*β*, and IL-6, limiting the propagation of the inflammatory cascade.[29] *β*-Caryophyllene, a selective agonist of the CB2 cannabinoid receptor, reduces leukocyte infiltration and cytokine secretion, facilitating systemic resolution of inflammation.[21] The improved efficacy observed at higher EOCHP doses likely results from the accumulation of these lipophilic compounds in inflamed tissues, enhancing their synergistic effects over time.[30] Collectively, this study provides a clear mechanistic basis for the dose-dependent anti-inflammatory activity of EOCHP. By concurrently modulating vascular permeability, cytokine synthesis, and immune signaling, EOCHP demonstrates multi-layered anti-inflammatory actions consistent with existing literature, strongly supporting its therapeutic potential as a natural anti-inflammatory agent.

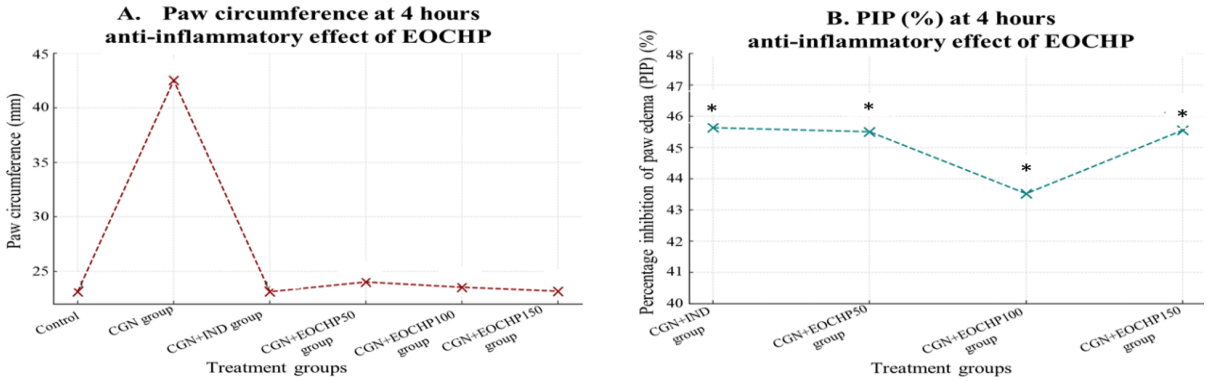

Figure 6: Correlation diagram showing the effects of *Citrus hystrix* peel essential oil (EOCHP) on (A) paw circumference and (B) PIP (%) at 4 hours post-carrageenan injection. Data are presented as mean ± SD (n = 5). Asterisks (*) indicate significance compared to the CGN group ($p < 0.05$).

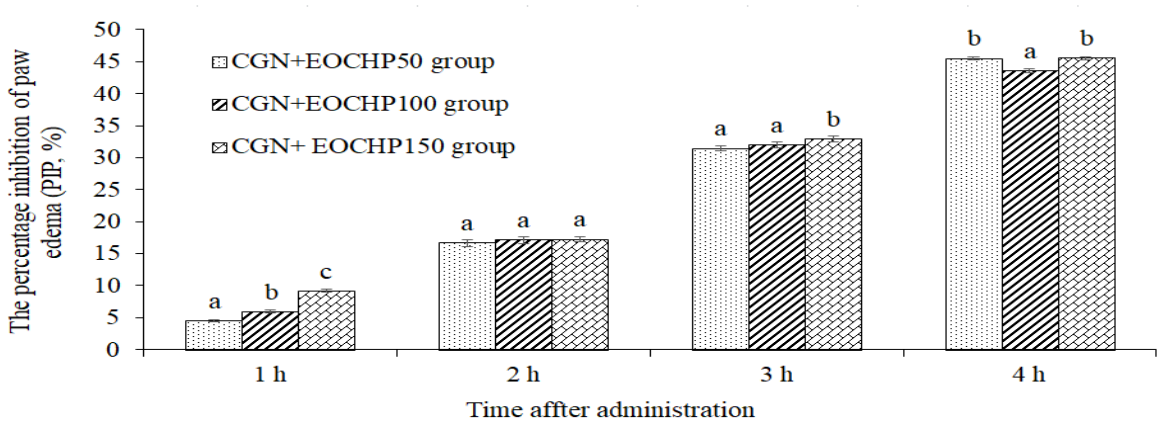

Figure 7: Time-course comparison of PIP (%) in *Citrus hystrix* peel essential oil (EOCHP) -treated mice following carrageenan-induced inflammation. Data are expressed as mean ± SD (n = 5). Different superscript letters (a, b, c) represent statistically significant differences between groups ($p < 0.05$).

Conclusion

This study provides comprehensive evidence supporting a strong, dose-dependent correlation between *Citrus hystrix* peel essential oil (EOCHP) and its analgesic, antipyretic, and anti-inflammatory activities in experimental mouse models. EOCHP significantly enhanced pain threshold, reduced rectal temperature, suppressed paw edema, and inhibited pro-inflammatory cytokines in a manner that intensified with increasing doses. These pharmacological effects are consistent across behavioral, physiological, and molecular endpoints and are likely mediated through the modulation of key inflammatory pathways, including prostaglandin synthesis and cytokine signaling. Together, the findings highlight EOCHP as a promising natural agent with multi-targeted therapeutic potential for the management of pain, fever, and inflammation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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