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Nature's Remedy: Evaluating the Combined *in Vitro* and *in Vivo* Anti-inflammatory Potential of *Citrus limon* Peel Extracts

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ABSTRACT

Chronic inflammation contributes to many disorders, encouraging the search for natural agents with safer therapeutic potential. *Citrus limon* is highly recognised for its plant chemical richness as well as its capacity to modulate oxidative and inflammatory pathways. It is frequently used in traditional medicine. This study aimed to characterise the phytochemical constituents of *C. limon* peel extracts in both methanolic and water-based forms and to compare their anti-inflammatory effects through *in vitro* and *in vivo* approaches. Phytochemical analysis revealed that the methanolic extract contained higher levels of total polyphenols (92.07 ± 1.22 mg GAE/g) and flavonoids (65.37 ± 2.9 mg QE/g) than the aqueous extract (53.01 ± 1.16 mg GAE/g and 46.79 ± 2.3 mg QE/g). The extract's effects on inflammation were explored through tests for protein denaturation (bovine serum albumin [BSA]and egg albumin) and erythrocyte membrane stabilisation. The methanolic extract exhibited lower IC_{50} values for BSA denaturation (53.97 ± 5.56 µg/mL), egg albumin (84.95 ± 1.77µg/mL), and membrane stabilisation (65.99 ± 4.66 µg/mL) compared with the aqueous extract (94.51 ± 0.98, 142 ± 2.48 , and 114.6 ± 1.35µg/mL). For the *in vivo* test, paw edema was induced by carrageenan. The methanolic extract (200 mg/kg) significantly reduced edema progression at the sixth hour to 5.46 ± 0.36%, compared with 11.04 ± 0.37% for the aqueous extract, approaching the efficacy of diclofenac (10 mg/kg, 6.62 ± 0.29%). Overall, the methanolic peel extract of *C. limon* demonstrated superior anti-inflammatory activity, supported by lower IC_{50} values and stronger inhibition of edema.

Keywords: *Citrus limon*, Peel Extract, Polyphenols, Flavonoids, Anti-Inflammatory Activity.

Introduction

Over the last 30 years, oxidative stress-related research has been a major concern, with a large number of studies addressed to attenuate its deleterious effects. Oxidative stress is a complex player in human health and is heavily associated with inflammatory processes.[1] Inflammation serves as a natural and advantageous reaction to cellular damage, playing a vital part in the recovery process. Nonetheless, when inflammation becomes chronic, it transforms into a harmful mechanism which can result in various unwanted disease conditions such as diabetes, cardiovascular diseases, and cancer.[2,3]Reactive oxygen species (ROS) are major contributors to inflammation and functionally mediate the processes of signal conduction and gene transcription. Excess ROS can disrupt macromolecular integrity, affecting nucleic acids, proteins, and lipids, or induce inflammation in mammalian cells.[4] At the same time, in addition to ROS, nitric oxide (NO) is also an important signalling molecule in immune defence and inflammation.

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NO regulates blood pressure, induces vasodilation, and suppresses platelet aggregation under physiological conditions. However, overactivated NO production is toxic, serving as a pro-inflammatory mediator and promoting the occurrence of inflammatory diseases.[5] During inflammation, NO is enzymatically produced using nicotinamide adenine dinucleotide phosphate (NADPH) by various endothelial cells and tissues.[6] Artificial antioxidants and nonsteroidal anti-inflammatory drugs (NSAIDs) have been employed to control oxidative stress and inflammation; however, prolonged administration may frequently lead to side effects such as gastrointestinal and renal toxicity.[7,8] Consequently, there is an increasing demand for natural therapeutic alternatives which can offer comparable efficacy with fewer side effects.[8] Accordingly, studies have investigated the pathways mediating the protective activity of these plant metabolites.[1] Phenolic compounds from products of the *citrus* industry have been targeted because of their potential applications in food technology, cosmetics, and pharmaceutical industries.[9] Epidemiological studies have shown that increased fruit and vegetable consumption correlates with a decreased incidence of cardiovascular diseases and cancer.[9]

The Rutaceae family contains the *Citrus* genus, well known for its high levels of secondary metabolites.[10] Fruits, peels, and leaves of different *Citrus* species are popular in food, spices, fresh fruit, and traditional medicine.[10] *Citrus limon* (lemon) is particularly abundant in bioactive molecules such as flavonoids, vitamins, and prominent phenolic acids, including pcoumaric, caffeic, ferulic, sinapic, and chlorogenic acids.[11] Its polyphenolic profile is dominated by flavonoids, particularly hesperidin, eriocitrin, and diosmin, which exhibit notable antioxidant, anti-inflammatory, and anticancer properties, with hesperidin being the major flavonoid in lemon.[12] In this sense, this study represents a pharmacological evaluation of methanolic and aqueous extracts of *Citrus limon* fruit peels. The main goal of this research is to

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investigate their anti-inflammatory potential through a complementary set of experimental models: *in vitro* assays including erythrocyte membrane stabilisation (hemolysis inhibition), bovine serum albumin (BSA) denaturation, and egg albumin denaturation, as well as an *in vivo* carrageenan-induced paw edema model in mice.This work introduces a novel approach by systematically combining complementary cell-based assays and animal model studies to investigate the inflammation-modulating potential of *Citrus limon* peel preparations. This integrated strategy provides a robust, multi-level assessment of inflammation-suppressing effects, linking protein structural changes, membrane stabilisation, and modulation of acute inflammatory responses, and highlights the relevance of peel-derived bioactive compounds in a unified experimental framework.[13]

The choice of these methods is highly pertinent: *In vitro* assays are well-established, cost-effective, and predictive of key anti-inflammatory mechanisms, whilst paw edema induced by carrageenan in mice continues to serve as an established animal model for testing acute anti-inflammatory substances.[14] Importantly, this work contributes to the valorisation of lemon peels, a major by-product of the citrus juice industry which is currently underutilised and often discarded as waste. By demonstrating their pharmacological potential, our study supports the development of sustainable, high-value applications for citrus waste, which could generate significant economic opportunities in the nutraceutical, cosmetic, and pharmaceutical sectors.

Materials and Methods

Chemicals

Various chemicals, including quercetin, gallic acid, BSA (≥ 96%), diclofenac sodium, HPLC-grade methanol (≥ 99.9%), and carrageenan, were purchased from Sigma-Aldrich (St. Louis, MO, USA), along with Folin–Ciocalteu reagent and aluminium trichloride ($AlCl_3$). Disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogenphosphate (KH_2PO_4), and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany), whilst all other reagents and solvents were of analytical grade.

Animals

To evaluate the anti-inflammatory effects, adult male Swiss albino (NMRI) mice, aged between 8 and 12 weeks and weighing 25–30 grams, were employed. These mice were sourced from the Pasteur Institute of Algiers (Algeria) and kept under controlled laboratory conditions, which included a 12-hour light/dark cycle and free access to food and water.

They were housed in a pathogen-free setting to reduce the risk of infection. All experimental procedures complied with the guidelines set by the Institutional Animal Care Committee of the Algerian Ministry of Higher Education and Scientific Research, following Executive Decree No.10–90, which modifies Executive Decree No.04–82 concerning animal welfare. Ethical approval was granted by the Algerian Association of Experimental Animal Sciences (Agreement Number 45/DGLPAG/DVA.SDA.14).

Plant Material Collection

In January 2024, fruit samples were obtained from local farms situated in the Chlef region of Algeria at the geographical coordinates 36°17'06.0"N 1°19'29.0"E, with an elevation of 369.53 meters. The species is commonly cultivated and described in the reference collections of the Technical Institute of Fruit Arboriculture and Viticulture, which is recognized for its expertise in *Citrus* species. Its identity was verified by a voucher specimen with the Algerian number CAS09/01/23.

After being washed with distilled water, the skins were manually peeled and left to dry naturally in a dark place at room temperature to preserve the integrity of their phytochemicals and then ground to powder with the aid of an electric grinder (Bomann, Germany). The powder was stored in sealed containers at ambient temperature pending use.

Preparation of Plant Extracts

Following the method described by[15] with slight modifications, 20 g of *Citrus limon* peel powder was extracted using 200 mL of either methanol or distilled water in a 1:10 (w/v) ratio. After 24 hours of orbital agitation at ambient temperature, the extracts were centrifuged (3000 rpm, 10 min, 4°C). The clear supernatants were filtered using Whatman No.1 paper and subsequently concentrated at 40°C under reduced pressure with a rotary evaporator (Heidolph, Hei-VAP Value, Schwabach, Germany). The resulting dried extracts were properly stored until subsequent analyses.

Determination of Total Polyphenol Content

The total phenolic content (TPC) was assessed using a modified Folin–Ciocalteu colorimetric assay.[16]Specifically, 1.5 mL of diluted Folin–Ciocalteu reagent (1:10 v/v) was introduced to the extract, and the reaction was incubated for 5 minutes at 22°C in darkness.

Then 1.5 mL of a $NaHCO_3$ solution (60 g/L) was added, and the mixture was incubated at room temperature for 90 min. Absorbance was assessed at 725 nm using a UV-Vis spectrophotometer (Optizen Pop, Korea), and TPC values were computed from a gallic acid calibration curve, expressed as mg gallic acid equivalents (GAE) per gram of extract. The measurements were performed in triplicate.

Quantification of Total Flavonoid Content

The quantity of total flavonoid content (TFC) was determined via an aluminium chloride colorimetric technique with minor adjustments.[17] In this procedure, 500 µL of the extract was combined with 500 µL of a 2% $AlCl_3$ solution. Following a 15 min incubation at room temperature, absorbance was measured at 420 nm relative to a reagent blank.

The total flavonoid content (TFC) was determined employing a quercetin standard curve at concentrations of 20, 40, 60, 80, 100, and 120 µg/mL and represented as mg QE per gram of extract. The measurements were performed in three trials.

In Vitro Anti-Inflammatory Activity

Assay for Inhibition of Egg Albumin Denaturation

In vitro inflammation-modulating potential of the extracts was assessed following the methods previously reported by[18,19], with slight modifications. The assay system consisted of 2.8 mL of phosphate-buffered saline (PBS; pH 6.4), 0.2 mL of fresh egg albumin, and 2 mL of either methanolic or aqueous peel extracts of *Citrus limon*, constituting a final volume of 5 mL.

The extracts were tested at doses varying from 31.25 to 500 µg/mL, with diclofenac sodium employed as the standard inflammation-modulating drug, prepared at equivalent concentrations. Distilled water served as the negative control.

The assay mixture, in which contents were gently mixed, incubated at 37°C for 15 min and subsequently incubated in a water bath at 70°C for 5 min. After it was allowed to cool to room temperature, absorbance values were recorded at 660 nm. Data collection was measured in triplicate.

The extent of protein denaturation inhibition was determined using the following formula (equation 1):

$$\% \textbf{ Inhibition } = \frac{\textbf{Ac}-\textbf{At}}{\textbf{At}} \times \textbf{100} \quad (1)$$

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where Ac represents the absorbance of the control and At that of the test sample.

Assay for Inhibition of BSA Denaturation

A slightly modified version of the procedure described by [20] was employed to conduct the experiment. The reaction mixture (0.5 mL, pH 6.3) consisted of 0.45 mL of a 5% (w/v) aqueous solution of BSA and 0.05 mL of either methanolic or aqueous *Citrus* extract at concentrations from 31.25 to 500 µg/mL.

The mixtures were incubated at 37°C for 30 min and then placed in a water bath at 57°C for 5 min. Once cooled to room temperature, 2.5 mL of PBS (pH 6.3) was added, and the solution's turbidity was determined using a spectrophotometer at 600 nm. For the control group, 0.45 mL of BSA with 0.05 mL of distilled water was employed. Triplicate tests were performed.

The percentage of protein denaturation inhibition was then calculated as follows (equation 2):

$$\text{Percentage of protein denaturation inhibition} = \frac{\text{Ac-At}}{\text{At}} \times 100 \quad (2)$$

where Ac is the absorbance of the control, and At is the absorbance of the treated sample.

Evaluation of the Protective Effect on Human Erythrocyte Membranes

The membrane-stabilising capacity of *Citrus limon* peel extracts was tested using the human red blood cell (HRBC) assay, adhering to established protocols with minor adjustments.[21,22] Heparinised blood from a healthy donor was centrifuged at 3000 rpm for 5 min, and the erythrocytes were washed thrice with 0.9% NaCl before being reconstituted in isotonic phosphate buffer (10 mM, pH 7.4) to achieve a 10% suspension.

The reaction mixture included 1 mL of phosphate buffer, 2 mL of hypotonic saline, 0.5 mL of extract (31.25–500 µg/mL), and 0.5 mL of RBC suspension. Diclofenac sodium at comparable doses functioned as the standard anti-inflammatory agent. A control was established under the same circumstances without the extract.

Following incubation at 54°C for 30 min, the samples underwent centrifugation at 3000 rpm for 5 min, and the absorbance of the supernatant was measured at 560 nm. Each measurement was conducted in triplicate. The proportion of protein denaturation inhibition was then computed using equation 2.

In Vivo Anti-Inflammatory Activity

In NMRI mice, paw edema was induced by administering 0.1 mL of a 1% (w/v) carrageenan solution in saline into the subplantar region of the right hind paw, as described in methods.[23,24] The experimental groups were pre-treated orally 60 min before the administration of carrageenan, with the aqueous or methanolic extracts of *C. limon* having 200 mg/kg, diclofenac 50 mg/kg, or normal saline 10 mL/kg. Paw thickness was measured at different times over 6 hours using a digital vernier caliper (Mitutoyo, Japan). The percentage increase in paw edema thickness (%AUG) was calculated.for all groups of mice. The measurements were repeated six times.

The equation is given below (equation 3):[25]

$$\%\,\mathbf{AUG} = \frac{\mathbf{Dn-D0}}{\mathbf{D0}} \times \mathbf{100} \quad (3)$$

Dn is the diameter of the paw after n hours carrageenan injection, and D_0 is the paw diameter prior to carrageenan injection.

Statistical Analysis

All statistical treatments were conducted using GraphPad Prism version 10.5.0 (GraphPad Software Inc., San Diego, CA, USA). Differences amongst groups in the *in vitro* anti-inflammatory assays were examined through a two-factor ANOVA, followed by Tukey's post-analysis procedure, and IC_{50} values were calculated from the dose–response curves. For the *in vivo* carrageenan-induced paw edema model, data were processed using two-factor ANOVA combined with Dunnett's comparison test to evaluate differences between the treated groups and the control. Results are presented as mean ± SD, and a significance threshold was set at $p < 0.05$.

Results and Discussion

Quantification of Total Phenolic and Flavonoid Contents

Chemical analysis of *Citrus limon* peel revealed a rich composition of bioactive compounds, particularly phenolics and flavonoids. The methanolic extract contained higher TPC of 92.07 ± 1.22 mg GAE/g compared with the aqueous extract (53.01 ± 1.16 mgGAE/g), indicating that methanol acts as a notably efficient medium for isolating these phytochemicals. Similarly, the TFC was significantly greater in the methanolic extract (65.37 ± 2.9 mg QE/g) than in the aqueous extract (46.79 ± 2.3 mg QE/g). Such outcomes agree with earlier observations reporting the superior efficiency of methanol for phenolic and flavonoid extraction.[26]

Polyphenol concentrations in *Citrus* peels are reported to be 2–4 times higher than in the pulp, supporting the relevance of the peel as an abundant reservoir of functional metabolites.[27] Compared with other *Citrus* species, the TPC of *C. limon* peel observed in this study is higher than those reported for *C. aurantifolia*, *C. nobilis*, *C. grandis*, and *C. reticulata*.[28] Variations may result from species differences, genetics, plant age, environmental factors, or extraction methods.[29] The flavonoid content of the methanolic extract is also amongst the highest reported in *Citrus* peels. Previous studies indicate TFC values ranging from 25 to 80 mg QE/100 g, with the highest levels typically found in *C. limon*.[29] Hesperidin, a major flavanone in *Citrus* peel, has been widely documented for its pharmacological activities, including its roles in modulating inflammatory pathways and neutralising oxidative stress.[29,30] It has been reported that plant extracts rich in phenolics and flavonoids show strong antioxidant activity, reflecting their broad protective effect.[31] Overall, the elevated levels of phenolics and flavonoids in the methanolic fraction emphasise its relevance as a promising reservoir of health-promoting molecules. These observations highlight the value of choosing an extraction solvent to maximise the yield of phytochemicals and the necessity of optimising extraction conditions during plant screening for bioactive constituents.

Protein Denaturation Inhibition Test

Protein denaturation seems to be a large part of the pathophysiology of inflammatory diseases. It generates new antigens and enhances the immune response in chronic inflammatory disorders, including rheumatoid arthritis.[32,18]In the course of this research, the methanolic extract of *Citrus limon* peel demonstrated a clear concentration-dependent inhibition of egg albumin denaturation (Figure 1). It exhibited the lowest IC_{50} value (84.95 ± 1.77 µg/mL), indicating a markedly stronger anti-denaturation effect compared with the aqueous extract (142 ± 2.48 µg/mL). The methanolic extract also showed greater inhibitory activity than the reference drug diclofenac sodium, which presented an IC_{50} of 114 ± 2.22 µg/mL. Significant variations were noted across the tested groups ($p < 0.0001$).

This activity aligns with the recognised role of phenolic and flavonoid compounds in stabilising protein structures under thermal stress.[19] Plant extracts rich in phenolics and flavonoids have been reported to protect protein conformation and prevent aggregation, highlighting their broad protective potential.[33]

BSA Structural Integrity Preservation

The ability of *Citrus limon* peel extracts to preserve BSA structural integrity provides additional evidence of their anti-inflammatory potential. In this assay, the methanolic extract showed the strongest protective effect, presenting the lowest IC_{50} value (53.97 ±5.56), followed by diclofenac sodium IC_{50} (62.71 ± 4.01), whereas the aqueous extract exhibited a higher IC_{50} (94.51 ± 0.98) (Figure 2). Highly

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significant variations were observed amongst all treatments ($p < 0.0001$).

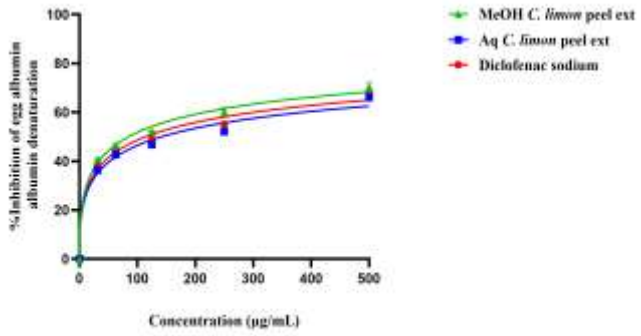

Figure 1: IC_{50} of *Citrus limon* peel extracts and diclofenac: inhibitory effect on egg albumin denaturation. MeOH: methanolic extract; Aq: aqueous extract; ext: extract. Data represent mean ± SD (n = 3).

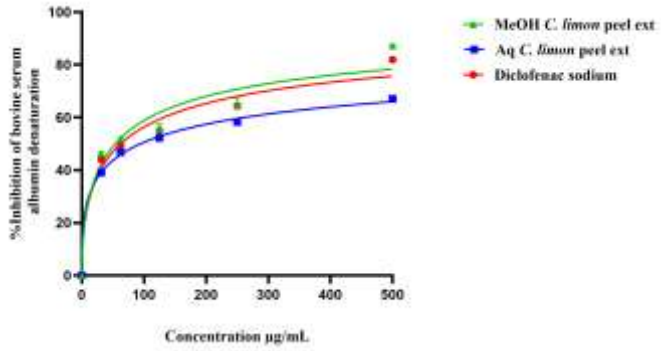

Figure 2: IC_{50} of *Citrus limon* peel extracts and diclofenac: protection against bovine serum albumin (BSA) denaturation. MeOH: methanolic extract; Aq: aqueous extract; ext: extract. Data represent mean ± SD (n = 3).

The protective effect observed for the methanolic extract may be linked to its high content of phenolic and flavonoid compounds, which can preserve the native conformation of BSA and limit the formation of protein aggregates. This effect is supported by previous studies demonstrating that polyphenol-rich plant extracts can maintain protein integrity and reduce structural perturbations, reinforcing their potential to protect protein function under stress conditions.[33,34,35] Such protective activity may also involve interactions between phenolic compounds and the protein surface, which can restrict unfolding events and maintain functional regions, consistent with reports on methanolic extracts of *Moringa oleifera* and *Olea parvifolia*, which exhibited similar BSA preserving effects.[22,18]

Membrane Stabilisation Assay (Heat-Induced Hemolysis)
The anti-inflammatory properties of HRBC were evaluated *in vitro* by examining hemolysis triggered by heat. Erythrocyte membranes are heat-labile, leading to hemolysis and a release of intracellular components in a manner analogous with a lysosomal membrane rupture observed during inflammation.[36]

Considering the different extracts, the methanolic preparation presented the strongest membrane-stabilising activity, with the lowest IC_{50} (65.99 ± 4.66), surpassing the protective effect of the reference drug diclofenac sodium IC_{50} (72.88 ± 4.59). The aqueous extract also conferred significant protection IC_{50} (114.6 ± 1.35), although to a lesser extent (Figure 3). The observed variations amongst the extracts were extremely significant ($p < 0.0001$).

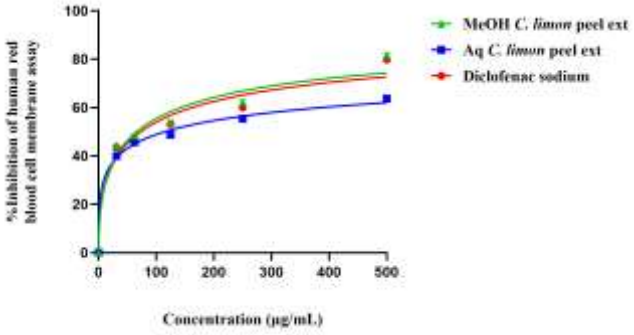

Figure 3: IC_{50} of *Citrus limon* peel extracts and diclofenac: membrane protective effect on human red blood cells. MeOH: methanolic extract; Aq: aqueous extract; ext: extract. Data represent mean ± SD (n = 3).

The methanolic extract of *Citrus limon* peel effectively protected HRBC from heat-induced hemolysis. This effect is attributed to its flavonoids, as well as phenolic acids, which interact with membrane proteins and phospholipids, reducing fluidity and permeability whilst scavenging reactive oxygen species. Similar protective activity has been reported for *Moringa oleifera* and *Olea parvifolia*, highlighting the anti-inflammatory potential of phenolic-rich plant extracts.[18]

In Vivo Evaluation of Anti-Inflammatory Effects
The extracts' ability to modulate inflammation was examined using the carrageenan-induced paw edema model, a method widely recognised for its reliability in acute inflammatory studies.[37] This model exhibits a biphasic inflammatory response: an early phase (1–3h) mediated by histamine, serotonin, and bradykinin, and a late phase (3–6h) dominated by prostaglandin production and COX-2 induction.[37]

In the nontreated control group, carrageenan induced a progressive and marked increase in paw edema throughout the experimental period, consistent with established data.[38] Diclofenac sodium (10 mg/kg), used as the reference drug, produced a rapid and sustained reduction in edema, with values of 12.63 ± 0.63% at 1 h, 27.1 ± 0.71% at 3 h, and 6.62 ± 0.29% at 6 h. This inhibition was highly significant compared with the control group at all time points ($p < 0.0001$). The methanolic extract (200 mg/kg) showed strong anti-inflammatory activity, with edema values of 10.23 ± 0.52% (1 h), 24.67 ± 0.52% (3 h), and 5.46 ± 0.36% (6 h). The reduction in edema was highly significant versus the control ($p < 0.0001$) at all measured times. This suggests activity across both phases of inflammation, likely due to polyphenols and flavonoids, known to suppress mast cell degranulation and pro-inflammatory signalling.[39]

The aqueous extract (200 mg/kg) also significantly reduced edema, with values of 14.09 ± 0.64% (1 h), 34.35 ± 0.54% (3 h), and 11.04 ± 0.37% (6 h). This effect was also highly significant compared with the control group ($p < 0.0001$). Although slightly less potent than the methanolic extract, its activity indicates a contribution from hydrophilic compounds such as tannins and saponins.[40] Figure 4 illustrates the time course of paw edema (%) in all experimental groups. Overall, both extracts demonstrated highly significant anti-inflammatory effects ($p < 0.0001$), with the methanolic extract being the most effective. Their activity across both inflammatory phases potentially involving modulation of nitric oxide and other mediators supports a multimodal mechanism mediated by flavonoids, terpenoids, alkaloids, and coumarins, as previously reported[39]

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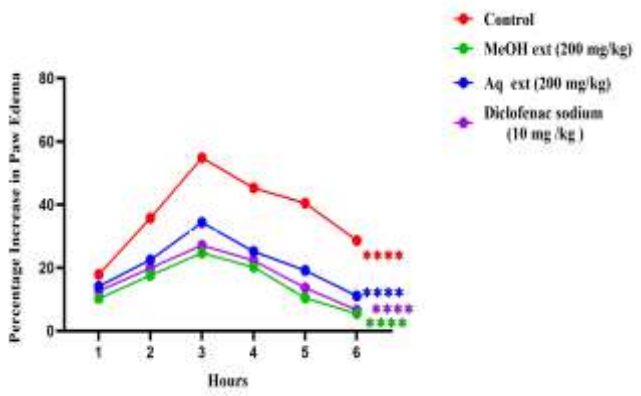

Figure 4: Effect of *C. limon* peel extract at dose of 200mg/kg p.c on carrageenan-induced mice paw oedema. Values are mean ± SEM $n = 6$. *, **, ***, and **** indicate a significant difference respectively at $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ compared to the control (ANOVA, posthoc Dunnett test).MeOH: methanolic extract; Aq: aqueous extract; ext: extract.

Conclusion

The findings of the present investigation demonstrated that both methanolic and aqueous extracts exhibit significant anti-inflammatory properties, as evidenced by both *in vivo* and *in vitro* assays. Importantly, the methanolic extract demonstrated a superior anti-inflammatory effect, surpassing not only the aqueous extract but also the reference drug, diclofenac. These findings highlight the promising therapeutic potential of these extracts, particularly in the management of inflammation associated with oxidative stress. They also confirm the growing interest in natural phytochemicals, which are considered safer alternatives or complementary approaches to conventional anti-inflammatory drugs, often associated with long-term adverse effects. In essence, this work provides concrete evidence of the anti-inflammatory efficacy of these extracts, with marked effects in acute inflammation and potential benefits in certain chronic inflammatory conditions. Further bio-guided fractionation appears to be a relevant strategy for identifying the specific active ingredients responsible for thres effects. Additional research will be needed to clarify the mechanisms of action and confirm the therapeutic relevance of these compounds. Beyond demonstrating efficacy, this work highlights the importance of exploring *Citrus*-derived metabolites within modern pharmacological research. In this context, bio-guided fractionation emerges as a particularly relevant strategy to isolate, purify, and evaluate the key molecules contributing to the observed anti-inflammatory activity.

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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