Evaluation of the anti-inflammatory activity of the rhizome essential oil of *Distichochlamys citrea*

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ABSTRACT

Distichochlamys citrea, an endemic plant native to central Vietnam, holds significant ethnomedicinal value in Vietnamese herbal medicine. Although the essential oil derived from the rhizome of *D. citrea* is known for its bioactivities, including antibacterial and antioxidant properties, its anti-inflammatory potential has not been extensively explored yet. In this study, the anti-inflammatory effect of the essential oil has been comprehensively evaluated from *in silico* to *in vivo* studies. A molecular docking study revealed that cineole, neral, and geranial, three main components of the essential oil, were capable of forming promising interactions with 5-lipoxygenase and inducible nitric oxide synthase (ranging from -4.7 to -6.3 kcal/mol). Furthermore, the essential oil exhibited a strong inhibitory effect against protein denaturation (86.67%, 100 µg/mL). In the carrageenan-induced paw edema model, the essential oil (4%) could inhibit 32.83% of paw edema as compared to the NaCl-treated group. Collectively, these findings position the essential oil extracted from rhizomes of *D. citrea* as a novel candidate for the development of anti-inflammatory medicine.

1. Introduction

With 53 genera and over 1,300 species, the Zingiberaceae family (the Ginger family) is the most diverse in the Zingiberales order, predominantly found across tropical regions, especially Southeast Asia (Hajong & Gupta, 2024; Zhou et al., 2018). The species from the Zingiberaceae family are characterized by their aromatic rhizomes and essential oils. Vietnam possesses a highly diverse Zingiberaceae flora, encompassing numerous species valued for their pharmacological and culinary significance, such as ginger, pinecone ginger, galangal, and turmeric (Le et al., 2018). Recently, several new species within this family have been identified, making essential contributions to the country's biodiversity. Among them, *Distichochlamys citrea* stands out as an endemic species of Vietnam. It was first documented in 1995 by the botanist Newman at Bach Ma National Park (Thua Thien Hue Province). It became the first known representative of the *Distichochlamys* genus (also known as "Gung Den" in Vietnamese) (Newman, 1995). The morphological and ecological characteristics of *D. citrea* have been described in previous studies (Newman, 1995; Tran et al., 2022). It is a

perennial, herbaceous, rhizomatous plant primarily distributed in central Vietnamese provinces, including Thua Thien Hue, Quang Binh, Quang Tri, Quang Nam, and Nghe An, typically growing in tropical rainforests or moist forest soils. In Vietnam, *D. citrea* has long been used by ethnic minority groups, such as the Pako people, both as a traditional remedy for infectious diseases and as a culinary spice (Tran et al., 2023).

Recently, the bioactivities of *D. citrea* have been proven and documented in several studies. For instance, according to the study by Nguyen et al. (2023), the hexane extract from *D. citrea* leaves exhibits notable biological activities, including the antioxidant capacity, inhibition of platelet aggregation, and prolonged blood coagulation time via intrinsic pathways. Additionally, the ethyl acetate fractionated extract derived from D. citrea rhizomes exhibited promising antioxidant and anti-α-glucosidase activities (Tran et al., 2022). In addition, its essential oil has become a growing focus of pharmaceutical research. The antimicrobial effects of essential oils extracted using different methods against bacteria and fungi have been demonstrated, with minimum inhibitory concentration values ranging from 200 to 400 µg/mL (Thinh et al., 2022). In a previous study, Tran et al. (2024) reported the presence of 19 chemical constituents in the essential oil of *D. citrea* extracted from the rhizome through GC-MS profiling, of which three compounds - 1,8-cineole, geranial, and neral - were identified as dominant. These major constituents are associated with several therapeutic effects, including anti-cancer, antimicrobial, and anti-inflammatory effects (Masyita et al., 2022). For example, 1,8-cineole has been reported to suppress the growth of liver cancer cells by enhancing cell cycle arrest and senescence (Rodenak-Kladniew et al., 2020). Geranial and neral, isolated from mountain pepper, downregulated the expression of TNF-α and IL-6 (two pro-inflammatory cytokines), as well as pro-IL-1β and inducible Nitric Oxide Synthase (iNOS), two key proteins related to the LPS-induced inflammatory response (Liao et al., 2015).

Although several promising pharmacological properties of *D. citrea* essential oil have been recorded, its anti-inflammatory activity has only been preliminarily explored. Previous research has provided preliminary evidence of its anti-inflammatory potential through *in vitro* nitric oxide inhibition assays and computational molecular docking with cyclooxygenase 1 and 2 (COX-1 and COX-2) (Tran et al., 2024). However, these initial findings lack comprehensive in vivo validation. To address this gap, this study was conducted to supplement and expand upon previous findings by evaluating the anti-inflammatory activity of essential oil *D. citrea* through broader arrays of approaches, including molecular docking simulation targeting iNOS and 5-lipoxygenase (5-LOX), inhibition of protein denaturation assays, and *in vivo* murine models.

2. Materials & methods

2.1. Chemicals

The bovine serum albumin (Himedia, India), dimethyl sulfoxide (DMSO, Fisher Chemicals, USA), saline solution (0.9% NaCl, CPC1 Central Pharmaceutical Joint Stock Company, Vietnam), diclofenac sodium (75 mg/3 mL, Lek Pharmaceutical D. D, Slovenia), and carrageenan (Sigma-Aldrich, St. Louis, MO, USA), and liquid paraffin (Xilong, China) were purchased from the local suppliers.

2.2. The essential oil preparation

In October 2023, fresh rhizomes of *D. citrea* were obtained from their natural habitat in Hue City, Vietnam, and voucher specimens were authenticated through comparison with

reference specimens (Figure 1). After harvesting, the samples were rinsed with tap water, and non-utilizable parts were removed. The essential oil was extracted via hydrodistillation for 240 minutes, and the resulting oil (DCO) was stored in vials containing sodium sulfate to dehydrate the essential oil at 4°C (Tran et al., 2024).

Figure 1

The Fresh Samples of Distichochlamys Citrea

Note. The researcher's photo

2.3. In silico analysis

The structural data from the RCSB Protein Data Bank were utilized in the molecular docking study, which was conducted using three leading software platforms: Discovery Studio Client 2024 (BIOVIA), AutoDock Tools, and PyMOL. The selected ligands were cineole, geranial and neral (three main components of the *D. citrea* essential oil), which were evaluated for their binding affinities to the target proteins such as inducible nitric oxide synthase (iNOS, PDB ID: 3E7G) - a key pro-inflammatory enzyme that functions via the Nitric Oxide (NO) pathway and 5-lipoxygenase (5-LOX, PDB ID: 3O8Y), a key enzyme associated with the leukotriene pathway. The summarized process was as follows: the molecular structures of the target proteins were prepared using BIOVIA by removing water molecules, followed by the addition of hydrogen bonds and the assignment of interactions using AutoDock Tools. Then, the grid box and docking parameters were set. The docking between ligands and the protein was simulated using PyMOL. The results were then visualized and analyzed using BIOVIA to evaluate binding affinities (Rizvi et al., 2013).

2.4. Protein denaturation assay

The anti-inflammatory potential of DCO was assessed by testing the samples' capacity to prevent thermal-induced denaturation of albumin, a predominant serum protein known for its strong binding affinity to Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Albumin is a thermolabile protein that begins to denature at temperatures above 50°C; however, denaturation can be suppressed by low concentrations of NSAIDs. Based on this principle, the protein denaturation assay was performed using the procedure of Tran et al. (2025) with some modifications. Briefly, the reaction mixtures were prepared by combining 0.45mL of 0.5% bovine serum albumin with 0.05mL of diluted DCO or with diclofenac as the positive control. The control mixture contained 0.05mL of saline instead of DCO. The phosphate-buffered saline was added up to 3mL; all samples were then incubated at 37°C for 20 minutes. After a heat-shock period (70°C, 10 min), the mixture was then cooled to room temperature, and the

absorbance at a wavelength of 660nm was subsequently determined. The significant differences in the outcomes between the diclofenac- and essential oil-treated groups with different concentrations were analyzed using Excel software (Student's t-test).

2.5. Carrageenan-induced paw edema model

The anti-inflammatory activity of test samples was evaluated using the carrageenan-induced mouse paw edema model. Carrageenan - a complex polysaccharide comprising polymers of α(1,3)-D-galactose and β-(1,4)-D-galactose extracted from red seaweed - induces inflammation via antigen-antibody immune mechanisms. This agent triggers acute localized inflammation, with peak paw edema occurring 03 - 05 hours post-administration (Borsani et al., 2021). The initial inflammatory phase (0 - 01 hour) involves the release of histamine, serotonin, and bradykinin, while the secondary phase (01 - 05 hours) stimulates prostaglandin synthesis and pro-inflammatory cytokines, including IL-1β, IL-6, IL-10, and TNF-α (Crunkhorn & Meacock, 1971). The carrageenan-induced paw model was established using the procedure of Tran et al. (2025) with some modifications. A total of 20 healthy male Swiss albino mice, weighing approximately 24.0 ± 2.0g, were housed under laboratory conditions for 1 week (24 ± 2°C, 12 hours light/12 hours dark). Then, they were randomly divided into five groups, with four mice in each group: pathological control, positive control, and the essential oil-treated groups with three treatment doses (1%, 2%, and 4% of DCO). In this experiment, the mice in pathological control group were received 50uL of 0.9% NaCl solution on their paws, the positive control group was treated with diclofenac (50uL, 25 mg/mL), a Non-Steroidal Anti-Inflammatory Drug (NSAID), and the test groups were administered the same volume of the essential oils at concentrations of 1%, 2%, and 4% (in liquid paraffin based solution) on their paws. After administering the test solutions for one hour, 1% carrageenan (40µL) was injected into the subplantar region. Paw volumes were measured using a plethysmometer at baseline (before injection) and at 01, 02, 03, 04, and 05 hours post-carrageenan administration. The statistical analysis among the essential oil, reference, and control-treated groups after 05 hours of treatment was performed using Statgraphics software (Multiple range tests).

3. Results and discussion

3.1. Key components of the essential oil exhibited promising interactions with iNOS and 5LOX

Table 1 and Figure 2 showed that the crucial oil constituents-cineole, geranial, and neral-interacted with iNOS, exhibiting moderate binding affinities with docking scores of -4.7. Moreover, both geranial and neral formed a hydrogen bond at the Ser153 residue. In contrast, the positive control, diclofenac, demonstrated a stronger binding affinity (-6.1) and formed three hydrogen bonds at Ser153, Gln192, and Arg195. Similarly, Table 1 and Figure 3 illustrate interactions between the essential oil compounds and 5-LOX, with cineole, neral, and geranial showing docking scores of -6.0, -6.3, and -6.2, respectively. Notably, geranial and neral formed hydrogen bonds at the His367 residue. Diclofenac, while exhibiting a stronger binding affinity (-7.6), did not form any hydrogen bonds. These findings suggest that although the essential oil compounds possess anti-inflammatory potential via iNOS and 5-LOX inhibition, their activity is less pronounced than that of diclofenac. In a previous study, 1,8-cineole has been suggested as a promising PPARγ agonist, a regulator of inflammatory bowel diseases (Venkataraman et al., 2023). Furthermore, all three compounds could interact with COX-1/COX-2 axis with high binding affinities ranging from -5.1 to -6.5 kcal/mol (Tran

et al., 2024). The data from this study align with the existing literature to support the anti-inflammatory potential of DCO constituents, providing a multifaceted insight into this effect through the inhibition of multiple potential pathways.

Table 1

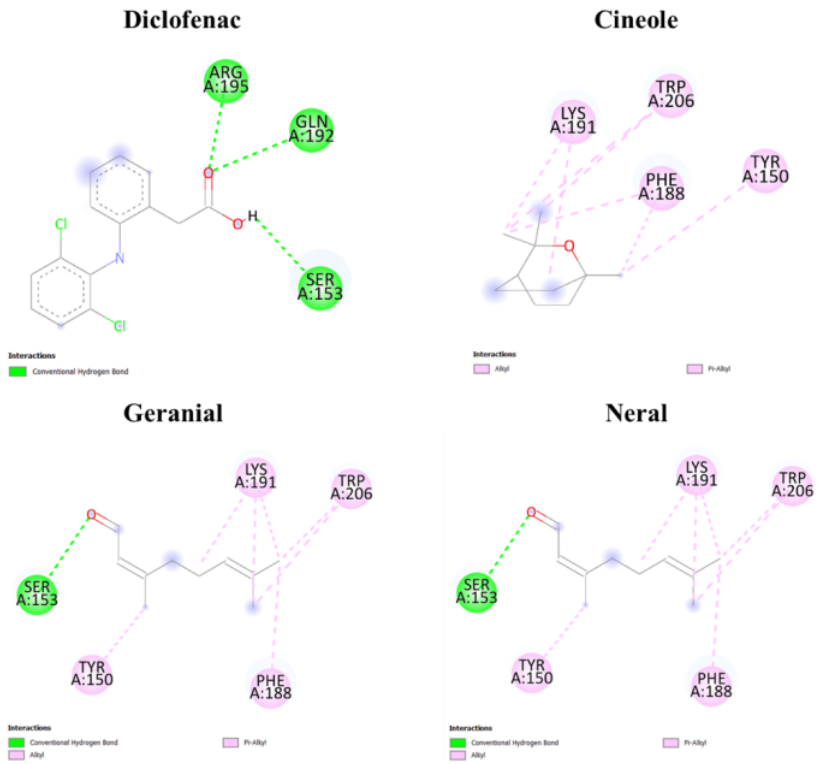
Binding Energies of Three Major Components of DPO with iNOS or 5-LOX

Compound	Docking score (kcal/mol)	No. of H-bonds	Residues
Human iNOS (PDB ID: 3E7G)			
Diclofenac	-6.1	3	Ser 153, Gln 192, Arg 195
Cineole	-4.7	0	-
Geranial	-4.7	1	Ser 153
Neral	-4.7	1	Ser 153
Human 5-LOX (PDB ID: 3O8Y)			
Diclofenac	-7.6	0	-
Cineole	-6.0	0	-
Geranial	-6.3	1	His 367
Neral	-6.2	1	His 367

Note. The researcher's data analysis

Figure 2

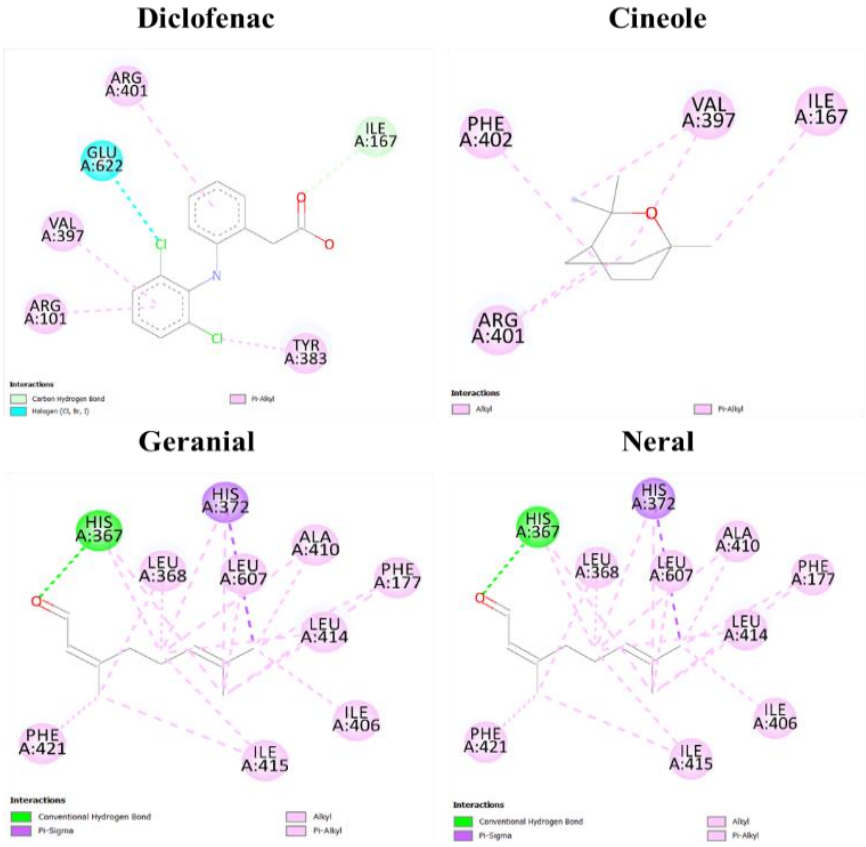
The 2D Scheme Represents the Interactions between Diclofenac, Cineole, Geranial, and Neral with Human iNOS (PDB ID: 3E7G)



Note. The researcher's analysis data

Figure 3

The 2D Scheme Represents the Interactions between Diclofenac, Cineole, Geranial, and Neral with Human 5-LOX (PDB ID: 3O8Y)



Note. The researcher's analysis data

3.2. Anti-inflammatory effect of DCO via preventing albumin denaturation

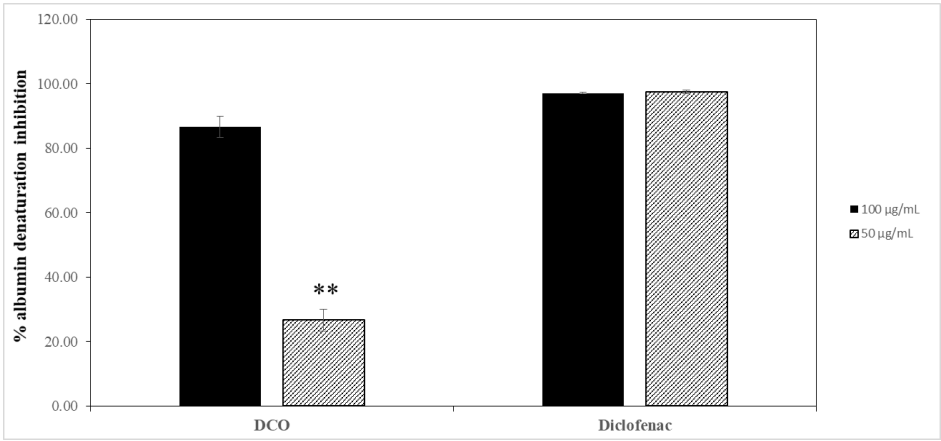
In this study, the anti-inflammatory activity of DCO was evaluated for the first time using the BSA protein denaturation inhibition assay, providing experimental support that complements the *in silico* findings. The results indicated that diclofenac, at both concentrations of 50 and 100 µg/mL, almost completely inhibited BSA denaturation, suggesting a vigorous anti-inflammatory activity. In contrast, the negative control (NaCl) was ineffective in preventing protein denaturation, as evidenced by high turbidity and elevated absorbance values. The essential oil of *D. citrea* demonstrated a dose-dependent inhibition of BSA denaturation ($p < 0.01$) with the highest inhibitory effect (86.67%) at the maximal treatment dose (100 µg/mL). These findings suggest that the essential oil exerts potent anti-inflammatory effects at a concentration of 100 µg/mL. The findings reveal that the essential oil of *Distichochlamys citrea* not only exhibits the ability to inhibit heat-induced BSA denaturation *in vitro* but also suggests a previously unexplored potential in modulating inflammatory responses through the preservation of protein structural integrity. This is the first time such a mechanism has been investigated in this particular species.

According to the theory proposed by Merlin et al. (2020), protein denaturation leads to the production of autoantigens, which can then trigger a type III hypersensitivity reaction - a severe immune response characterized by tissue damage and excessive inflammatory responses. Furthermore, disruption in cell membranes caused by protein denaturation could

trigger phospholipase-mediated conversion of phospholipid to arachidonic acid, which is then catalyzed by cyclooxygenase enzymes (COX1/COX2) to produce pro-inflammatory prostaglandins (Fitriyani & Fatahillah, 2022). Therefore, the capacity of DCO to maintain protein structure may aid in halting the formation of such aberrant antigens, which could further knowledge of its possible anti-inflammatory action. It is worth noting that three key constituents of DCO have strong interactions with COX pathways (Tran et al., 2024). Hence, these results can consolidate the evidence of the anti-inflammatory effect of DCO. Within the *Zingiberaceae* family, protein denaturation inhibition models have been widely employed to evaluate anti-inflammatory activity across multiple species. For example, the methanol rhizome extract from *Curcuma longa*, a member of the *Zingiberaceae* family, exhibited vigorous anti-protein denaturation activity with an efficacy of 67.32% (Anwar et al., 2022). In addition, the ethanol extract from *Curcuma xanthorrhiza* rhizomes also prevented protein denaturation, with an IC50 value of 521.67ppm (Farida et al., 2018). The anti-inflammatory activities of water and ethanol extracts from the rhizomes of two varieties of ginger, Zingiber officinale var. officinarum and rubrum, have also been demonstrated using this assay system (Aksono et al., 2022). These collective findings validate protein stabilization assessment as a robust methodological approach for both screening and elucidating the anti-inflammatory properties of ginger-family species, including *D. citrea*.

Figure 4

The Chart Shows the Percentage Inhibition of BSA Denaturation by D. Citrea Essential Oil and Diclofenac



Note. The researcher's analysis

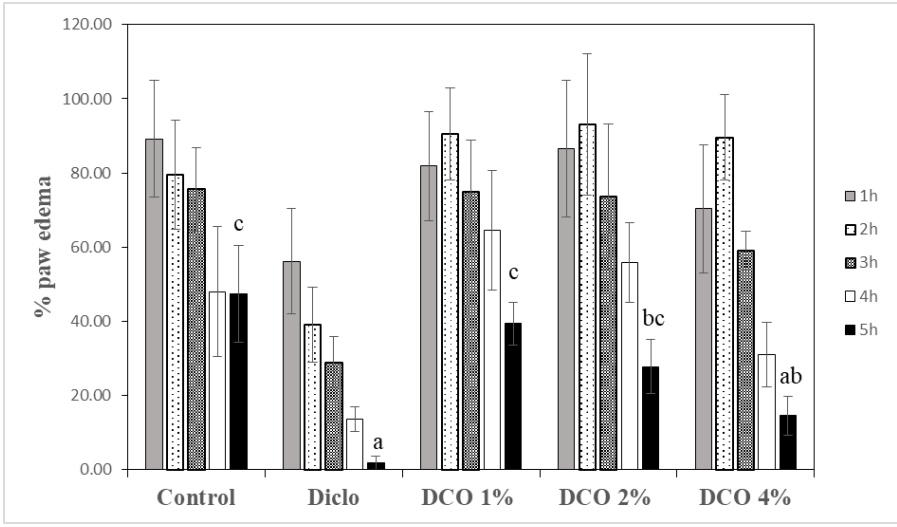
3.3. Anti-inflammatory effect of DCO via carrageenan-induced paw edema

In the pathological control group, paw volume increased sharply after carrageenan injection, peaking within the first hour with a 89.22% swelling (Figure 5). Paw edema eventually declined to a value of 47.41% after 05 hours of treatment. This progression confirms sustained, unmitigated inflammation in untreated subjects. In contrast, diclofenac dramatically decreased paw edema compared to the control. Notably, reference (diclofenac) could reduce over 45% of paw edema after 05 hours of treatment versus saline treatment (1.79 ± 1.79% and 47.41 ± 13.00%, respectively, $p < 0.05$). Anti-inflammatory effects of DCO at concentrations of 1%, 2%, and 4% have been observed (Figure 5). The paw edema of the essential oil-treated groups gradually decreased during the course of treatments (05 hours). As shown in Figure 5, after 05 hours of treatment, only the group treated with DCO 4% showed a significant difference from the pathological control, with a decrease in paw edema of 32.83%

(14.58 ± 5.24% versus 47.41 ± 13.00%, respectively, $p < 0.05$). The paw edema of the DCO 4% treated group was comparable to that of the reference medicine at the 5-hour time point after treatment. These findings provide direct in vivo evidence of the anti-inflammatory effect of *D. citrea* essential oil for the first time. Even though the bioactivities of *Distichochlamys* species, particularly in their anti-inflammatory properties, there is no direct evidence *in vivo* for the anti-inflammatory effect of the essential oil from *D. citrea*. To date, there has been only one study reporting the anti-inflammatory effect of trans-cinnamic acid, an isolated compound from the rhizomes of Distichochlamys benenica, in an animal model (Pham et al., 2021). Some reports also suggested *in vivo* anti-inflammatory effects of other members of the Zingiberaceae family. For instance, the essential oil extracted from *Zingiber ottensii* rhizomes could inhibit the carrageenan-induced paw edema via altering the inflammatory cytokine (TNF-α) and COX-2/PGE-2 (prostaglandin E2) axis (Thitinarongwate et al., 2022). The findings from the present study align with the studies above, highlighting the promising potential of the essential oil of D. citrea, as well as that of species from the Zingiberaceae family, in controlling inflammatory diseases.

Figure 5

The Chart Shows the Percentage Increase in Mouse Paw Volume for the Essential Oil Group and the Control Group



Note. a, b, and c illustrate the statistical significance among essential oil, diclofenac-treated, and pathological control groups after 05 hours of treatment ($p < 0.05$). The data were presented as means ± standard errors. The researcher's analysis data

4. Conclusions

The anti-inflammatory effect of the essential oil extracted from the rhizomes of D. citrea (DCO), an endemic medicinal plant in Vietnam, has been comprehensively evaluated through a range of *in silico* to *in vivo* studies. Three key components exhibited promising binding affinities with iNOS and 5-LOX, with binding energies ranging from -4.7 to -6.3 kcal/mol. In addition, DCO exhibited a strong inhibitory effect in the protein denaturation assay, with the percentage of inhibition reaching 86.67% at the dose of 100 µg/mL. The anti-inflammatory effect *in vivo* of *D. citrea* essential oil has also been reported for the first time employing the carrageenan-induced paw edema model. The data suggest that the essential oil from D. citrea, as well as species from the Zingiberaceae family in general, may be a promising remedy for controlling inflammatory diseases.

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NO CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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