

Exploring the abundance of de-oiled non-resin analgesic and anti-inflammatory compounds in *Crocus sativus* through polarity-directed quaternary solvent extraction in both diabetic and non-diabetic conditions: Integrating *in silico* and *in vivo* analysis

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ABSTRACT

Prior research indicated that the whole extract of *Crocus sativus* exhibited substantial analgesic and anti-inflammatory effects at a dose of 400 mg/kg. We aimed to examine the anti-inflammatory and analgesic activities of a de-oiled, non-resin fraction of *C. sativus* petals. The 20% *n*-hexane, 60% ethanol, 20% water extract of de-oiled, non-resin fraction of *C. sativus* was administered at 100, 200, and 400 mg/kg in rats. Study was conducted in both diabetic and non-diabetic models. The de-oiled, non-resin fraction of *C. sativus* petal extract imparts significant ($p < 0.05$) anti-inflammatory and analgesic activity at a 200 mg /kg dose, both in diabetic and non-diabetic rats. So, the extract may also contain anti-diabetic compound to ameliorate diabetic condition to demonstrated identical impact both in diabetic and non-diabetic condition. It may be stated that the de-oiled, non-resin fraction of *C. sativus* is a potent source of analgesic and anti-inflammatory compounds in diabetic and non-diabetic conditions.

Keywords: *Crocus sativus*, petal, analgesic, anti-inflammatory, histopathology

INTRODUCTION

Inflammation is the immune system's reaction to detrimental stimuli, such as foreign entities, infections, injured cells, toxic compounds, or chemical radiation¹. NSAIDs are readily accessible, FDA-approved anti-inflammatory and analgesic agents categorized by their chemical structure and selectivity. The USA markets at least 20 OTC NSAIDS and more internationally. While generally safe, effective, and well-tolerated, adverse reactions may include black, tarry stools (melena), coffee ground vomitus, bruising, bleeding, palpitations, and fatigue due to paracetamol and ibuprofen. Ketorolac may induce abdominal pain, cloudy urine, blurred vision, and convulsions, whereas naproxen may result in tinnitus, drowsiness, and convulsions². Prolonged use of anti-inflammatory medications may lead to platelet inhibition, gastric perforation, and ulcers in individuals who consume alcohol excessively and, in the elderly³.

Medicinal plants, being abundant and diverse in numerous bioactive compounds, are globally in high demand for their nutritional, medical, and cosmetic utilities, particularly in North America, Europe, and Asia⁴. Longstanding challenges of high cost and adversities associated with synthetic medications have sparked scientific interest in new medicinal molecules. Next-generation sequencing, genetic, and metabolic engineering may be implemented to obtain the desired

phytoconstituent concentration. A variety of plants have been evaluated for their analgesic and anti-inflammatory properties to date, including *Aloe vera* (Asphodelaceae), *Azadirachta indica* (Meliaceae), *Curcuma longa* (Zingiberaceae), and *Nigella sativa* (Zingiberaceae), among others⁵. They may act as a reservoir of bio-active compounds including resins (α/β boswellic acid, myrrh, asafetida), volatile oils (squalene, safranal, isophorone, 2,6,6-trimethyl-2-cyclohexene-1,4-dione, linalool) flavonoids (e.g. quercetin, apigenin, luteolin, rutin, kaempferol), terpenoids (e.g. curcumin, resveratrol), saponins (astragaloside, glycyrrhizin, diosgenin), tannins (gallic acid, catechin), and alkaloids (e.g. morphine, berberine, capsaicin) that may provide potential analgesic and anti-inflammatory activities (as evinced by relevant previous literature)⁶⁻⁹.

Plant resins are certain inflammatory exudates secreted in response to injury, known to seal wounds by inhibiting inflammatory targets like 5-LOX, COX-1, COX-2, TNF- α , NF- κ B, and NO, or alter crucial mechanisms involving pain transduction, reception, and perception volatile oils, or essential oils, serve as analgesic compounds because their phenylpropanoid, monoterpene, sesquiterpene-like constituents can activate pain control pathways, regulate receptors, inhibit nociceptive activity, modulate neurotransmitter release, or inhibit inflammatory mediators, leading to pain relief¹⁰⁻¹³. Other efficient therapeutic molecules are present within plants, alongside resins and volatile oils, such as glycoside derivatives (crocetin, crocin, picrocrocin), by inhibiting prostaglandin synthesis, suppressing pro-inflammatory mediators, and modulating signaling pathways like NF- κ B and MAPK, carotenoids (lycopene, α/β -carotene, lutein, zeaxanthin) by inhibiting the NF- κ B pathway, reducing cytokines and scavenging ROS; alkaloids, quinones, flavonoids (kaempferol, rutin, quercetin, hesperidin) by inhibiting COX and LOX enzymes, cytokines (IL-1 β , TNF- α), chemokines, and eicosanoids (prostaglandins, leukotrienes); anthocyanins, monoterpenoids (picrocrocin) via cytokine inhibition, di-terpenoids (mangicrocin), aldehydes (vanillin) via reduced NO and prostaglandin production and inhibition of pro-inflammatory cytokines (e.g., IL-1 β , IL-8, IL-6, TNF- α), anthraquinones, fatty acids, esters (di-iso octyl phthalate) etc.¹⁴⁻¹⁸.

The saffron crocus, known scientifically as *Crocus sativus* (Family: Iridaceae), is a highly valued, autumn-flowering, perennial plant native to the Middle East, Southeast Asia, and Western Europe growing up to 60-10 inches. They have been found to include 250 distinct phytoconstituents, including resins, flavonoids, glycosides, volatile and aroma-yielding chemicals, and their

esters^{19,20}. They have enormous potential therapeutic applications, their roles ranging from anti-diabetic, anti-oxidant, anti-inflammatory, analgesic, hepatoprotective, and immunomodulatory properties⁹. A thorough analysis of relevant scientific literatures indicated that this plant contain most of the aforementioned therapeutic compounds, serving as one promising candidate for screening for possible analgesic and anti-inflammatory agents²¹.

Furthermore, our molecular docking simulation studies revealed strong specific receptor-binding potentialities/affinities of squalene, di-iso octyl phthalate with COX-I, while mangicrocin and 3-methyl-2,4,6 triphenyl pyridine with COX-II, with squalene being the only volatile oil constituent among them. So, we our study are focusing on determining the analgesic and anti-inflammatory potential of the plant through compounds other than resins and volatile oils. The current study, therefore, aims to address existing research gaps via the activity assessment of a non-resinated, de-oiled *C. sativus* petal fraction, which includes the three previously mentioned compounds, along with others evaluated in disease-induced rat models through appropriate experimental protocols.

Objective of the study

The principal aim of our present study is to evaluate the potential pain-relieving and anti-inflammatory efficacy of *C. sativus* compounds apart from its resin and volatile oil constituents. We will de-oil and non-resinate the crude methanolic petal fraction and conduct the investigations to identify, ascertain, and assess the potential pharmacologic activity. A specialized solvent system of varying polarity, likely to ensure the yield a wide array of bio-active compounds, is utilized, comprising of 20% *n*-hexane, 60% ethanol, 20% water, where the strongly hydrophobic ones extracted via 20% *n*-hexane, majority of the non-polar/organic/hydrophobic compounds solubilized in ethanol, and the remaining polar, hydrophilic ones dissolved in 20% water.

Literature review

Relevant prior studies have demonstrated that methanolic extracts of the whole plant exhibit considerable therapeutic efficacy at doses of 400 mg/kg²². We conducted suitably designed *in vivo* tests on an appropriate disease-induced rat model utilizing predetermined dosages of 100, 200, and 400 mg/kg, respectively. However, if significant therapeutic activity is observed after the oral administration of 100 or 200 mg/kg, it suggests the presence of powerful medicinal components of *C. sativus* (beyond resins and volatile oils). Thus, the de-oiled, non-resinated fraction is much stronger compared to the whole

extract in terms of its therapeutic efficacy, and can exert desired effects (at a relatively low dosage). Again, if 400 mg/kg produces a noticeable effect, it cannot be stated conclusively whether the compounds within the de-oiled, non-resinated fraction are any stronger than those of the whole extract (containing resins and essential oils). Also, if any substantial activity will not be observed following administration at 400 mg/kg, the chemicals involved are likely of lesser concentration or potency, necessitating a higher dosage to achieve a comparable effect. In that case we have to conclude that the de-oiled, non-resinated fraction is weaker than the whole extract in terms of pharmacological activity.

METHODOLOGY

Drugs, chemicals, and instruments

Healthcare Pharmaceutical Limited provided a gift sample of ibuprofen and aspirin, while acetic acid, ethanol, carrageenan, and alloxan were acquired from Sigma-Aldrich in Germany. The anti-inflammatory and analgesic activities were evaluated using a plethysmometer and analgesia meters.

Plant collection and extract preparation

Fresh petals of *C. sativus* (Family: Iridaceae) were collected from the local market in Dhaka, Bangladesh. Taxonomic identification and authentication of the samples were performed. The plant specimens were preserved by the policies of the National Herbarium of Bangladesh, which generated accession number 92810 on January 15, 2024. The collected petals were gently washed with water to remove contaminants and then thoroughly dried at 45°C temperature to ensure moisture removal. Once dried, the petals were coarsely ground into powder. The powdered petals were carefully placed in an airtight reservoir jar to prevent degradation.

***C. sativus* volatile oil extraction**

The powdered *C. sativus* petals (1 kg), loaded into a glass column, which contained perforations at the bottom for the passage of steam. Steam was generated by boiling water in round round-bottomed flask in a heating mantle. Steam was passed through the packed powder from the bottom of the column, and the column was connected to the condenser, and water passed through it. Volatile oils present in *C. sativus*, along with the steam, were distilled and allowed to evaporate.

De-oiled *C. sativus* powder (DCP)

The remaining *C. sativus* powder in the glass column following volatile oil evaporation via steam distillation was taken out and air-dried.

***C. sativus* water-soluble resin extraction**

C. sativus powder was placed in a round-bottom flask and extracted with water, each time for 2 h, separately by constant shaking on a magnetic stirrer. The identical process of extracting on a stirrer and decanting was repeated 4 times. The solvent obtained was isolated and removed. Again, the remaining *C. sativus* powder following water-soluble resin extraction was collected and air-dried.

***C. sativus* acetone-soluble resin extraction**

The collected *C. sativus* powder was loaded in a glass column, and 1.5 L of acetone was added each time and left in contact for 1h. After 1h, 750 mL of the solvent was collected, and fresh solvent, acetone (150 mL), was added. 6 iterations of the same extraction procedure were conducted. All of the solvent was removed, and the powder was air-dried²³.

Final extraction for obtaining the de-oiled, non-resinated (hydrophilic and hydrophobic) *C. sativus* petal fraction

The remaining *C. sativus* powder, expected to be free of any volatile oil, hydrophobic or hydrophilic resin, was soaked in 2.5 L of 100% ethanol for about 28 days. Following, the mixture was filtered using a cotton plug, the final solution evaporated and concentrated using a rotary evaporator (Buchi, Germany), the dehydrated extract collected and stored carefully under suitable conditions until further analyses.

***In vivo* study design**

Animal model sample size detection

We calculated sample size using the Power “Analysis Method” for every animal model study²⁴. The computation can be done manually with the use of a formula. The formula is displayed below:

Standard Deviation = 3.58

$Z_{\alpha/2} = Z_{0.05/2} = Z_{0.025} = 1.96$ (From Z table) at type 1 error of 5%

$Z_{\beta} = Z_{0.20} = 0.842$ (From Z table) at 80% power

d = effect size = difference between mean values = 7.00

$$\begin{aligned} \text{Sample size} &= 2 \text{SD}^2(Z\alpha/2 + Z\beta)^2/d^2 \\ &= 2 \times (3.58)^2 \times (1.96 + 0.842)^2 / (7.00)^2 \\ &= 4.12 \end{aligned}$$

The rate of attrition was 10%. So the obtained sample size was divided by 0.9.

The final sample size will be = $4.12 \div 0.9 = 4.58 \approx 5$

So, each group contained 5 rats.

Experimental animal handling

55 healthy, adult male Wister rats each weighing between 110-125 gram were obtained from the Department of Pharmacy at Jahangirnagar University in Dhaka, Bangladesh, and housed at the University of Dhaka's Institute of Nutrition and Food Science under a 12-hour light/dark cycle at a constant temperature of 25°C. Regular supplies of a standard pellet diet and pure water were given. Before the inquiry began, the rats were left there to acclimate. The Institutional Animal Ethics Committee's regulations (IEAC) were followed during all rat experiments. According to the standards set forth by the Swiss Academy of Medical Sciences (SAMS) and the Swiss Academy of Sciences (SCNAT), animals were handled and treated humanely.

Every test on rats was carried out in compliance with the guidelines established by the Institutional Animal Ethics Committee (IEAC). Guidelines for the moral care and treatment of animals were established by the Swiss Academy of Sciences (SCNAT) and the Swiss Academy of Medical Sciences (SAMS).

Health status

Determining the rats' health is crucial to guaranteeing the validity and repeatability of the results. To guarantee effectiveness and safety, we documented the health status of and collated all the information into a score sheet for rating results²⁵. We observed the rats' movements, behaviors, loss of weight, and looks. The majority of the rats showed normal scores. Only a small percentage of them scored differently from the others. All measurements and grading schemes were developed in accordance with the recommendations for the housing and care of experimental animals.

Experimental design

After weighing each rat separately, the animals were divided into groups of five rats each, evenly distributed among the rodents based on body weight.

Evaluation of anti-inflammatory activity

Carrageenan-induced acute inflammatory model

The effectiveness of anti-inflammatory medications is frequently evaluated using carrageenan, which prompted scientists to examine rodent paw edema in greater detail. A special kind of instrument called a plethysmometer was used to conduct the anti-inflammatory test. Initially, the volume of each rodent's paw was measured. Next, 1.0% of the freshly made carrageenan solution was injected sub-planarly into the rat's left hind paw at a dose of 0.1 mL per 100 gm body weight to cause edema. It was then granted one hour. After that, rats were given various doses of the test drug and extracts. The paw volume was measured 0–4 hours after the carrageenan infusion using a plethysmometer. Equation 1 was then utilized to determine the rate of edema inhibition²⁶

$$\text{Percentage Inhibition} = \frac{V_{pc} - V_t}{V_{pc}} \times 100 \text{ [Eq 1]}$$

Here,

V_{pc} = volume of animals' paws in positive control group

V_o = volume of animals' paws in treatment group

Table 1 below summarizes the group, group abbreviation, treatment species, abbreviation and the doses administered in each group. Carrageenan was applied to induce inflammation in experimental small animals, to evaluate the anti-inflammatory properties of *C. sativus* extract in low, medium and high doses (100, 200, and 400 mg), respectively, compared to that of the reference medication (ibuprofen-10 mg) in non-diabetic and alloxan-induced diabetic rats, as shown through Tables 1 and 2.

Table 1. Analysis of anti-inflammatory activity in non-diabetic rats, showing group specification, treatment species, group abbreviations and the dose administered in groups 1-5

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
1	Carrageenan Control	N/A	N/A	Car
2	Carrageenan + Ibuprofen	Ibuprofen	10	Car + CS ₁₀
3	Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	100	Car + CS ₁₀₀
4	Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	200	Car + CS ₂₀₀
5	Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	400	Car + CS ₄₀₀

Table 2. Analysis of anti-inflammatory activity in alloxan-induced diabetic rats, showing group specification, treatment species, group abbreviations and the dose administered in groups 6-11

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
6	Alloxan + Carrageenan Control	N/A	N/A	A + Car
7	Alloxan + Carrageenan + Ibuprofen	Ibuprofen	10	A+Car+Ib ₁₀
8	Alloxan + Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	100	A+Car+CS ₁₀₀
9	Alloxan + Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	200	A+Car+CS ₂₀₀
10	Alloxan + Carrageenan + <i>C. sativus</i>	<i>Crocus sativus</i>	400	A+Car+CS ₄₀₀

Evaluation of analgesic activity

Acetic acid-induced writhing test

Peripheral analgesic activity was screened using acetic acid-induced writhing. Acetic acid-induced writhing was used to screen for peripheral analgesic activity. A half-hour before the intraperitoneal infusion of acidic acid, various test samples were given. According to literature review, we injected of 0.9% acetic acid (05 mL/kg) intraperitoneally to induce pain. After that acetic acid was given, the process of calculating the number of writhes (muscular contraction ions) began and lasted for twenty minutes. The percentage of inhibition of writhes was calculated after counting twisting motions for twenty minutes. These movements included contraction of the abdominal muscles, pulling up of the hind limbs into the abdominal walls, stretching of the hind limbs, and periodic arching of the body.

Equation 2 was used to get the proportion of writhes, one of the parameters to show analgesic activity²⁷.

$$\left\{ \frac{A. Control\ mean - Treatment\ mean}{A\ Control\ mean} \right\} \times 100 \quad [Eq\ 2]$$

Where, *T Control* = the mean number of the writhing of each test group

A Control = the mean number of the writhing of acetic acid control group

Tables 3 and 4 below represent group specification, treatment species and the dose administered to evaluate analgesic activity of administered extract employing tail-flick test and acetic acid-induced writhing test.

Table 3. Analysis of analgesic activity in non-diabetic rats using acetic acid-induced writhing test

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
1	Acetic Acid Control	Physiological saline	05 mL/kg	Ace
2	<i>C. sativus</i> + Aspirin	Aspirin	100	As ₁₀₀ +Ace
3	<i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	100	CS ₁₀₀ +Ace
4	<i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	200	CS ₂₀₀ +Ace
5	<i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	400	CS ₄₀₀ +Ace

Table 4. The analgesic efficacy in diabetic rats was examined utilizing the acetic acid induced writhing test

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
1	Alloxan + Acetic Acid Control	Physiological saline	05 mL/kg	A+Ace
2	Alloxan + Aspirin + Acetic Acid	Aspirin	100	A+As ₁₀₀ +Ace
3	Alloxan + <i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	100	A+CS ₁₀₀ +Ace
4	Alloxan + <i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	200	A+CS ₂₀₀ +Ace
5	Alloxan + <i>C. sativus</i> + Acetic Acid	<i>Crocus sativus</i>	400	A+CS ₄₀₀ +Ace

Tail flick method

The tail-flick experiment, a nociceptive assay created by Love and Smith in 1941, is used to assess how animals behave in reaction to unpleasant stimuli²⁸. The latency of the avoidance response to heat stimuli was measured using a UGO BASILE® (Germany) radiant heat programmed tail-flick analgesia meter. The device's nichrome wire was heated with the aid of a heat controller to the appropriate temperature, and after that, a steady current of 4 amps was supplied via the exposed nichrome. In order to induce pain perception in the rats, this method involves placing radiant heat in the middle of their tails. Rats of different groups were treated with respective test specimen 30 minute prior the initiation of test. Then the tai flick time was measured at 0, 15, 30, 45, and 60 minutes.

Tables 5 and 6 have shown the procedure of conducting analgesic activity tests and determining the extract efficacy including the treatment species and dose administered in each group from 1-10, in both experimental non-diabetic and diabetic rat models.

Table 5. Analysis of analgesic activity in non-diabetic rats using tail flick test showing group specification, treatment species, group abbreviations and the dose administered in groups 1-5

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
1	Tail Flick Stress (control)	Physiological saline	10 mL/kg	TFS
2	Aspirin + Tail Flick Stress	Aspirin	100	As ₁₀₀ +TFS
3	<i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	100	CS ₁₀₀ +TFS
4	<i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	200	CS ₂₀₀ +TFS
5	<i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	400	CS ₄₀₀ +TFS

Table 6. Analysis of analgesic activity in diabetic rats using tail flick test, alloxan-induced diabetic rats, showing group specification, treatment species, group abbreviations and the dose administered in groups 6-10

Group Number	Group Specification	Treatment Species	Dose Treatment Species (mg/kg)	Abbreviation of Groups
6	Alloxan + Tail Flick Stress (control)	Physiological saline	10 mL/kg	A+TFS
7	Alloxan + Aspirin + Tail Flick Stress	Aspirin	100	A+As ₁₀₀ +TFS
8	Alloxan + <i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	100	A+CS ₁₀₀ +TFS
9	Alloxan + <i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	200	A+CS ₂₀₀ +TFS
10	Alloxan + <i>C. sativus</i> + Tail Flick Stress	<i>Crocus sativus</i>	400	A+CS ₄₀₀ +TFS

Assessment of serum levels of TNF α, IL-1, and IL-6

In order to estimate TNF α, IL-1, and IL-2 levels in blood samples, the rats were first anesthetized. Next, the blood was drawn from via cardiac puncture. Next, the collected blood samples were centrifuged at 10000 rpm for 10 minutes. Then serum was collected from the blood samples. Prior to usage, the serum samples were kept at -20°C. The levels of TNF α, IL-6, and IL-1α were measured using highly sensitive rat TNF α Elisa kits, rat IL-6 Elisa kits, and rat IL-1α Elisa kits, respectively²⁹.

Histopathological studies

The liver and skin tissues of the experimental rats were carefully removed, and then a standard saline solution was used to clean them. Following that, the samples were kept in 10% buffered neutral formalin for around 48 hours. Before appropriate staining and examination under a fluorescence microscope to evaluate the histopathological alterations in the experimental rats, the specimens undergo procedures for histopathology slides utilizing microtomes³⁰.

Statistical analysis

Using the MS Excel application, we documented and assessed all of our findings (raw data), which we then split into several groups based on different study criteria. Mean SD was used to display the findings of descriptive statistics that were applied to the data. We used the SPSS 1600 program's "One Way Anova Test" option to determine the statistical significance of the inter-group heterogeneity concerning multiple biological variables. A "p" value of less than 0.05 ($p < 0.05$) indicated statistical significance for the events³¹.

Molecular docking, ADMET and toxicity prediction

A literature mining approach was used to create a library of 81 phytoconstituents Table 7 for the in-silico investigation of *Crocus sativa*'s analgesic and anti-inflammatory potential. Utilizing the PubChem database, the compounds' three-dimensional structures were obtained. Avogadro version 1.2.0 was utilized to transform the 2D SDF file into a 3D model for the two-dimensional structures³². The energy of each of the 81 compounds' three-dimensional structures was calculated using Avogadro's steepest descent technique under the forcefield MMFF94, with a convergence value of $10e-7$. PDB files were used to store each compound file.

Two of the most popular protein targets for anti-diabetic effect were chosen: COX-1 (PDB ID: 6Y3C) and COX-2 (PDB ID: 1CX2). The proteins' pdb format files were obtained from the RCSB protein databank Table 7.

The proteins were prepared using the PyMol software program. The Swiss pdb viewer 4.1.0 was used to minimize the energy of the prepared macro molecules in a vacuum using the GROMOS96 force field with 43B1 parameters. The PyRx software's AutoDock Vina module was used for molecular docking³³. By examining the natural ligand's interaction site, the Discovery Studio software package assisted in determining the binding site of the target proteins.

The results of the docking were analyzed with the help of PyMol and Discovery Studio Visualizer 2020 software packages.

The ADMET properties of the top compounds were tested on the Swiss ADME and ProTox-2 servers C³⁴.

Table 7. Protein targets for analgesic and anti-inflammatory drugs

Name of Target	PDB ID	Control Drug	Active Site Residues
Cyclooxygenase-1 (COX-1)	6Y3C	Aspirin	ARG 120, VAL 349, SER 353, TYR 355, LEU 384, TYR 385, TRP 387, MET 522, SER 530
Cyclooxygenase-2 (COX-2)	1CX2		SER 516, TYR 341, ARG 106, SER339, TYR 371, VAL 509, LEU 370, ARG 499, LEU 489, VAL 420

Molecular dynamics simulation studies

To assess the binding stability of compounds and analgesic proteins, 100 ns MD simulations using the Desmond v3.6 Program in Schrödinger (Academic version) were run. The dynamics system was an orthorhombic periodic boundary box with a specific volume and a distance of 10 Å assigned in the predefined TIP3P water model. Na⁺ and Cl⁻, the appropriate ions, were added to this solvated solution at a concentration of 0.15M to achieve equilibrium. Using the OPLS3 force field parameters and the Desmond module & default protocol, the system was minimized. With recording intervals of 100 ps, an NPT ensemble maintained 300 K and one atmospheric (1.01325 bar) pressure. To examine the outcomes, the Desmond module Simulation Interaction Diagram (SID) in the Schrödinger package.

RESULTS and DISCUSSION

Evaluation of anti-inflammatory activity of *C. sativus*

λ-Carrageenan, a high molecular weight colloidal polysaccharide, triggers the release of various inflammatory and pro-inflammatory mediators. The initial stage involves prostaglandins from COX enzymes, histamine, and serotonin (5-HT) until (0–2 hours), followed by an intermediate response involving bradykinin and a later phase involving cytokines like IL-1β, IL-2, IL-6, and IL-1. According to relevant evidence, bradykinin, tachykinins, ROS, and nitrogen species (NO generated by eNOS, nNOS, and rNOS) immediately following subcutaneous carrageenan injection cause edema, hyperalgesia, and erythema^{35,36}. The body's intricate biochemical reaction to infections, irritations, or other traumas, cell damage, and vascularized tissues is called inflammation, and it is essential for both innate and adaptive immunity³⁷. Several studies have strongly linked inflammation to aging, cancer, cardiovascular dysfunction, and other life-threatening disorders³⁸. Plasma and leukocytes infiltrate tissues, causing redness, swelling, discomfort, heat/fever, and functional loss in acute inflammation³⁹. In addition, fluid and plasma protein exudation/extravasation

and leukocyte accumulation at the inflammatory site causes a bi-phasic edema, which modulates the inflammatory cascade's inhibitory molecules⁴⁰. Venous blockage, increased vascular permeability, elevated blood flow, and neutrophil and macrophage infiltration define peripheral edema⁴¹.

Being a well-researched, regularly implemented, highly reproducible, and scientifically-verified technique, the test is usually employed to rapidly evaluate anti-inflammatory and anti-edematous activities/effects of any test compound/natural product⁴². Thus, we assessed paw edema thickness to monitor inflammatory symptoms and paw volume rise by subtracting the initial paw volume (basal) from the paw volume recorded at each time point⁴³. Carrageenan caused paw edema to peak at (0-2) hours, last 5–6 hours, and diminish within 24 hours after subcutaneous injection.

Table 8. Data showing anti-inflammatory activity of *C. sativus* extract and ibuprofen through carrageenan-induced paw edema test in non-diabetic rat models

Group Number	Group Specification	Volume of Paw at Various Time Intervals (µL) and Percentage Inhibition of Edema in Non-diabetic Rats				
		0 min (just before carrageenan injection)	1 hour (just before treatment)	2 hours	3 hours	4 hours
1	Car Control	82.09 ± 3.22	94.51 ± 4.34	109.17 ± 4.07	115.03 ± 3.81	121.34 ± 4.00
2	Car + Ib ₁₀	85.69 ± 4.33	93.41 ± 4.54	106.57 ± 4.05 (2.38%)	106.44 ± 4.14 (7.46%)	102.49 ± 4.48 (15.20%)
3	Car + CS ₁₀₀	83.56 ± 3.07	93.04 ± 4.26	106.52 ± 5.44 (2.45%)	112.29 ± 2.62 (2.38%)	116.91 ± 2.26 (3.65%)
4	Car + CS ₂₀₀	85.01 ± 4.18	94.32 ± 3.97	104.82 ± 7.54 (3.98%)	*107.33 ± 4.01 (6.69%)	*112.78 ± 3.97 (7.05%)
5	Car + CS ₄₀₀	84.47 ± 2.68	92.09 ± 5.35	103.08 ± 4.96 (5.58%)	*105.17 ± 7.24 (8.57%)	*108.26 ± 4.52 (10.77%)

n=5, each value is represented as mean ± SEM

*significance of result

The Table 8 represents data demonstrating the volume of paw edema (µL) at pre-determined time intervals of 0 (before carrageenan administration), the following 1, 2, 3, and 4 hours in non-diabetic experimental rats. From this table, it has been observed that all the non-diabetic groups (Groups 1-5), the paw volume prior carrageenan administration lay within the range of 82-85 µL. The reduction in paw edema volume occurred in a dose-dependent manner and was observed to be more pronounced (117, 113, and 108 µL) in groups that

received comparatively a greater dose, the changes being statistically significant ($p < 0.05$) in Groups 4 (200mg dose) at 4 hours (7.05%) and in Group 5 at both 3 (8.57%) and 4 hours (10.77%). Group 5, which received the highest dose (400mg) of *C. sativus*, demonstrated the greatest reduction (10.77%) in edema volume apart from ibuprofen-treated Group 2 (15.20%), demonstrating that it contains anti-inflammatory substances that function to impart therapeutic activity. Significant changes ($p < 0.05$) in paw volume occurred at 3 hours and also at 4 hours in Group 5 where high dose produced action earlier. However, rats belonged to Group 4 took longer time to work and produced significant ($p < 0.05$) therapeutic activity. Also, rats of Group 3 did not exhibit any significant therapeutic activity. The phenomenon strongly suggesting that the plant possesses considerable potentials in moderately alleviating or even completing eliminating stress-induced inflammation.

Table 9. Data representing anti-inflammatory activity of *C. sativus* extract and ibuprofen through carrageenan-induced paw edema test in alloxan-treated diabetic rat models

Group Number	Group Specification	Volume of Paw at Various Time Intervals (μL) and Percentage Inhibition of Edema in Diabetic Rats				
		0 min (just before carrageenan injection)	1 hour (just before treatment)	2 hours	3 hours	4 hours
6	A + Car Control	82.34 \pm 3.07	97.31 \pm 6.09	110.69 \pm 5.27	114.68 \pm 2.87	119.27 \pm 2.02
7	A + Car + Ib ₁₀	82.40 \pm 3.07	98.11 \pm 6.02	109.16 \pm 3.43 (1.38%)	107.83 \pm 2.59 (5.97%)	103.95 \pm 3.47 (12.84%)
8	A+ Car + C ₁₀₀	84.24 \pm 4.86	98.01 \pm 4.52	107.84 \pm 3.96 (2.57%)	113.38 \pm 2.99 (1.13%)	118.52 \pm 2.86 (0.62%)
9	A + Car + CS ₂₀₀	81.78 \pm 4.21	95.39 \pm 3.29	107.77 \pm 3.89 (2.63%)	113.77 \pm 3.18 (0.79%)	*111.14 \pm 4.19 (6.81%)
10	A+ Car + CS ₄₀₀	82.83 \pm 1.88	96.09 \pm 4.35	105.56 \pm 2.71 (4.63%)	*110.66 \pm 5.00 (3.51%)	*108.53 \pm 5.56 (9.00%)

n=5, each value is represented as mean \pm SEM

*significance of result

The data in Table 9 represents data demonstrating the volume of paw edema (μL) at pre-determined time intervals of 0 (before carrageenan administration), the following 1, 2, 3 and 4 hours in an alloxan-induced diabetic experimental rat model.

The increase in paw volume in the diabetic group following carrageenan administration (Car) and subsequent paw volume reduction following ibuprofen (Ib) and *C. sativus* (CS)-treatment was observed from Table 9, where ibuprofen-treated Group 7 demonstrated a 12.84% reduction in paw edema volume following 4 hours of carrageenan-injection.

From aforementioned data, it may be stated that all diabetic groups (Groups 6-10), the paw volume prior carrageenan administration lay within the range of 82-85 μL . The reduction in final paw edema volume of extract treated groups were occurred in a dose-dependent manner and were observed to be more pronounced (118, 113 and 111 μL) in groups that received comparatively a greater dose, their alterations being statistically significant ($p < 0.05$) in Group 10 at 4 hours (6.81%). Contrarily, all the other groups treated with low, medium and high doses at 1, 2, 3 hours and low, medium dose at 4 hours yielded non-significant changes ($p > 0.05$).

From Tables 8 and 9 we may finally observe that paw edema volume in alloxan-induced (diabetic) rats were slightly greater i.e. in Groups 6-10, when compared to non-diabetic Groups 1-5. The changes occurred by 0.62%, 6.81% and 9.00% in Groups 8-10 and 3.65%, 7.05% and 10.77% in Groups 3-5, respectively. In non-diabetic rats both medium and high doses reduced the paw edema significantly ($p < 0.05$) in 3 and 4 hour. Besides, in diabetic rats high dose reduced the paw edema in 3 and 4 hours as same as non-diabetic groups. But in medium dose the paw edema was significantly reduced only in 4th hour in diabetic rats demonstrating that the same extract was slightly more effective in the non-diabetic groups. Such marginal difference may be caused either by individual physiology or by pre-existing damage caused by alloxan induced elevated inflammatory responses. Nevertheless, severity was not prominently evident in the diabetic groups of this study. This observation suggesting that the plant extract may possess not only analgesic activity but also potential diabetic amelioration activity.

Thus, it may be stated (from the aforementioned observation) that *C. sativus* possesses anti-inflammatory activity. Furthermore, it may also provide anti-diabetic properties; yet, this conclusion cannot be definitively established, since it necessitates a prolonged evaluation period, whereas the determination of anti-inflammatory action was based solely on a brief 4-hour experiment.

Carrageenan sub-plantar injection functions as a local irritant that enhances membrane phospholipase activity, hence initiating the synthesis of several pain and inflammatory cytokines, like as histamines, serotonin (5-HT), bradykinins, leukotrienes, eicosanoids, and prostaglandins, which result in increased

paw size. The initial phase of inflammation commences within one hour of carrageenan injection, characterized by the release of histamine, serotonin, and the activation of cytoplasmic enzymes, followed by a plateau phase sustained by bradykinin-like substances⁴⁴. The anti-inflammatory properties of saffron are likely attributed to its high antioxidant and radical scavenging capabilities, associated primarily with crocetin and crocins⁴⁵. The diverse pharmacological actions of saffron arise from its ability to engage with multiple biological targets and various cell signaling pathways⁴⁶. Compounds such as tyrosol, quercetin, kaempferol, methyl palmitate, methyl linoleate, 1-nonadecene, hexadecane, hexadecenoic acid, hexadecenoic ethyl ester, chalcone, etc., present in *C. sativus*, which may act as inhibitors/antagonists to the aforementioned substances, are likely to be responsible for producing this action⁴⁷.

The COX-2 isoform is mostly expressed in inflammatory cells and is significantly increased during both chronic and acute inflammation; hence, cyclo-oxygenase inhibitors serve as a potent therapeutic target for the treatment of inflammatory diseases. Apart from squalene (C_74) which is one volatile oil, other compounds such as di iso octyl phthalate (C_38), has shown specific receptor-binding potentiality with COX-I enzyme, while mangicrocin (C_13), and 3-methyl-2,4,6 triphenyl pyridine (C_76) with COX-2 enzymes as found from our studies. Further, thorough studies are required to determine whether they are equivalent in therapeutic efficacy with a better safety profile; also, the exact mechanism through which they proceed in anti-inflammatory action needs to be elucidated.

Flavonoid kaempferol functions by inhibiting inflammatory cell activity and the expression of pro-inflammatory cytokines and chemokines, with a mode of action akin to that of the commercial medication ibuprofen and celecoxib⁴⁸. Celecoxib presents much harm over long-term use, including GI distress, MI, stroke and cardiovascular disorders, while no known severe adverse reactions of kaempferol have been evidenced, hence it may be considered to be one safe and effective alternative⁴⁹. Quercetin inhibits the activation of NLRP3 inflammasomes; TLR2/MyD88/NF- κ B and ROS/AMPK pathways while methyl palmitate produces action by regulating the NLRP3–NF- κ B pathway, the agonist of peroxisome proliferator-activated receptor gamma (PPAR- γ)⁵⁰. The compound may cause headache and upset stomach when overdosed, yet it is safer when compared to conventional NSAIDS. Our studies are very similar to the findings of who demonstrated that saffron constituents inhibit pro-inflammatory cytokine production, such as IL-1, by inhibiting NF- κ B activity through the suppression of I kappa B kinase-a (IKK-a) phosphorylation and preventing the nuclear translocation of the NF- κ B p65 subunit^{51,52}. A separate

investigation demonstrated that crocetin treatment in rats may confer anti-inflammatory and antioxidant characteristics⁵³. Both aqueous and ethanolic *C. sativus* extracts, showed anti-inflammatory effects by formalin-induced paw edema in induced chronic inflammation⁵⁴.

Evaluation of analgesic activity of *C. sativus* by acetic acid writhing test

The acetic acid-induced writhing test is one sensitive, predictive and widely-applied evaluation method employed in animal models for acute pain used to assess analgesic effectiveness. It involves injecting irritants such as phenyl-quinone or 0.7% v/v acetic acid solution (volume of injection 10mL/kg) rat or mice model to induce peripheral discomfort (via release of nociceptive mediators such as bradykinins), where analgesic activity of the test compound may be inferred from decrease in the number or frequency of writhings⁵⁵.

Table 10. Data representing the analgesic effect of different doses of *C. sativus* and aspirin by acetic acid writhing test in a non-diabetic rat model

	Group Specification	Number of Writhing	Inhibition %
1	S	93.20 ± 3.56	-
2	S+ As ₁₀₀	74.80 ± 3.70	19.74
3	S+ CS ₁₀₀	89.88 ± 3.34	3.56
4	S+ CS ₂₀₀	*86.89 ± 3.99	6.77
5	S+ CS ₄₀₀	*81.60 ± 3.51	12.45

n=5, each value is represented as mean ± SEM

*significance of result

The results of the acetic acid writhing test carried out in non-diabetic experimental rats have been shown through Table 10, where that the number of writhing is comparatively the highest (93.20 times) in the non-diabetic, non-treated group (Group 1). In the drug-treated group (Group 2), the number was reduced by 19.74%, the lowest writhing among Groups 1-5. While in the CS-treated groups (Groups 3-5), the number of writhings declined by 3.56%, 6.77%, 12.45% respectively. The phenomenon was reduced in a dose-dependent manner, where the medium (200mg) and high dose (400mg) groups (Groups 4 and 5) showed statistically significant ($p < 0.05$) changes. The change in Group 3 was non-significant ($p > 0.05$). This may be due to the reason that higher doses produced better analgesic effects in non-diabetic animals, inhibiting enzymes cascades, reducing pain and therefore decreasing the frequency of writhings.

Table 11. Data evaluating the analgesic effect of different doses of *C. sativus* and aspirin by acetic acid writhing test in alloxan-induced diabetic rat model

	Group Specification	Number of Writhing	Inhibition %
6	A + S	90.20 ± 4.09	-
7	A + S +As ₁₀₀	68.0 ± 3.16	24.61
8	A + S + CS ₁₀₀	87.6 ± 3.28	2.88
9	A + S + CS ₂₀₀	*82.6 ± 2.30	8.42
10	A + S + CS ₄₀₀	*77.8 ± 1.48	13.74

n=5, each value is represented as mean ± SEM

*significance of result

The results of the acetic acid writhing test carried out in alloxan-induced diabetic experimental rats have been shown through Table 11, where the number of writhing observed was slightly lower (13) in the alloxan-induced diabetic rat groups, than their respective non-diabetic counterparts. Yet, the inhibition % in the drug-treated group (Group 7) was 24.61%, with Groups 8, 9, and 10 showing 2.88%, 8.42%, and 13.74% inhibition, respectively. Here, the changes also occurred in a dose-dependent manner with Groups 9 (200mg) and 10 (400mg) demonstrating statistically significant ($p < 0.05$) results while that in Groups 8 being non-significant ($p > 0.05$). As from the aforementioned data, it may be stated that there is not a remarkable variation due to diabetic and non-diabetic conditions in case of analgesic effects.

Compounds such as kaempferol, hexadecane, digentibioside, 4-hydroxydihydro-2(3H)-furanone, etc. present in *C. sativus* that possess analgesic properties may be responsible for producing this action⁵⁶. Kaempferol demonstrates potent inhibition of COX-1 and II enzymes, bearing similarity to both ibuprofen and celecoxib⁵⁷. In addition, compounds in saffron down-regulates the key pro-inflammatory enzymes such as myeloperoxidase (MPO), phospholipase A2, inducible enzymes cyclooxygenase-2 (COX-2), nitric oxide synthase (iNOS), and prostanoids. Helicryoside works by inhibiting of inflammatory enzyme, free-radical scavenging activity and corticoid-like effects⁵⁸. Here, the data obtained from our studies were in conformance with the experiment conducted by where aqueous (0.8 g/kg, i.p.) and ethanolic extracts of *C. sativus* (0.1, 0.2, and 0.4 g/kg, i.p.) intraperitoneal administrated in mice showed antinociceptive activity against acetic acid induced writhing^{59,60}.

Evaluation of analgesic activity of *C. sativus* by tail flick test

The tail-flick test involves briefly restraining the animal with its tail extended on a flat surface. A timer is usually set once a powerful light beam hits the cutaneous skin of the animal's tail, alternatively a hot water bath at temperatures of 52-55°C may be applied⁶¹. The timer stops when the animal flicks away its tail, and latency measures pain threshold. As for example, 5 mg/kg, i.p., opioid analgesics (naloxone/morphine) are known to prolong tail-flick latency, which measures animal nociceptive sensitivity.

Table 12. Data evaluating the analgesic activity of *C. sativus* and aspirin by the tail-flick test method in non-diabetic rat models

Group Number	Group Specification	Basal Reaction	Reaction Time in Seconds			
			after 30 minutes	after 60 minutes	after 120 minutes	after 240 minutes
1	S	2.68 ± 0.65	3.08 ± 0.59	3.22 ± 0.70	3.36 ± 0.47	3.48 ± 0.26
2	S + As ₁₀₀	3.04 ± 0.51	3.30 ± 0.90 (6.67%)	3.50 ± 0.79 (8%)	4.58 ± 0.37 (26.63%)	5.22 ± 0.39 (33.33%)
3	S + CS ₁₀₀	2.88 ± 0.95	2.98 ± 0.96 (-3.24%)	3.40 ± 0.70 (5.29%)	3.54 ± 0.59 (5.08%)	3.84 ± 0.72 (9.38%)
4	S + CS ₂₀₀	3.12 ± 0.67	3.27 ± 0.52 (5.81%)	3.53 ± 0.95 (8.78%)	*4.44 ± 0.48 (24.32%)	*4.62 ± 0.42 (24.68%)
5	S + CS ₄₀₀	3.46 ± 0.87	3.50 ± 0.60 (12%)	3.62 ± 0.69 (11.05%)	*4.52 ± 0.34 (25.66%)	*4.66 ± 0.48 (25.32%)

n=5, each value is represented as mean ± SEM

*significance of results

The result of this test is shown below in Tables 12 and 13; it mainly reflects thermal and mechanical nociception. The pre-determined reaction times starting from basal to 30, 60, 120, and 240 min, respectively were recorded. All the groups showed a gradual increase in their reaction times, as observed through the tabulated data. Group 1 demonstrated a basal reaction time of 2.68 s and 3.48 s at 240 min. 100-mg aspirin treated. Group 2 showed a reaction time of (5.22 ± 0.39) s at 240 min, rising by 33.33% when compared to that of Group 1. Groups 3, 4, and 5 increased the reaction time at 240 min by (9.38%), (24.68%), and (25.32%), respectively, the change occurring observably in a dose-dependent manner. Groups 4 and 5 showed statistically significant results (p<0.05) at 120 and 240 min, respectively, while low dose demonstrated

statistically non-significant ($p > 0.05$) results. The fastest response time among the CS-treated was noted in the high dose (400mg) Group 5 at (4.66 ± 0.48) rising by (25.32%). Here, it may be stated that treatment with *C. sativus* petal extract in Groups 3-5 increased the pain threshold of non-diabetic rats in a dose-dependent manner, however, the effect being lesser when compared to that of the standard drug, 100 mg-aspirin.

Table 13. Data evaluating the analgesic activity of *C. sativus* and aspirin by the tail-flick test method in alloxan-induced diabetic rat models

Group Number	Group Specification	Basal Reaction	Reaction Time in Seconds			
			after 30 minutes	after 60 minutes	after 120 minutes	after 240 minutes
6	A + S	3.36 ± 0.33	3.69 ± 0.40	3.90 ± 0.37	4.34 ± 0.56	4.76 ± 0.66
7	A + S + As ₁₀₀	4.12 ± 0.46	4.44 ± 0.40 (16.89%)	5.08 ± 0.87 (23.23%)	5.38 ± 0.58 (30.77%)	6.12 ± 0.54 (22.22%)
8	A+ S + CS ₁₀₀	3.48 ± 0.37	3.85 ± 0.59 (4.16%)	4.16 ± 0.72 (6.25%)	4.72 ± 0.61 (8.05%)	5.32 ± 0.45 (10.52%)
9	A + S + CS ₂₀₀	3.56 ± 0.49	3.89 ± 0.38 (5.14%)	4.32 ± 0.70 (9.72%)	* 5.40 ± 0.34 (19.63%)	* 5.98 ± 0.71 (20.40%)
10	A + S + CS ₄₀₀	3.62 ± 0.39	3.98 ± 0.25 (7.29%)	4.42 ± 0.42 (11.76%)	* 5.72 ± 0.46 (24.13%)	* 6.22 ± 0.47 (23.47%)

n=5, each value is represented as mean \pm SEM

*significance of results

The basal reaction time of Group 6 was (3.36 ± 0.33) and (4.76 ± 0.66) at 240 min, respectively. Aspirin-treated Group 7 showed (22.22%) rise in reaction time, the changes at 240 of Group 8, 9, and 10 were (10.52%), (20.40%), and (23.47%), respectively. Groups 9 and 10 containing medium (200-mg) and high (400mg) Petal extracts were statistically significant ($p < 0.05$) at both 120- and 240-min intervals for each among alloxan-induced diabetic groups. The highest observed reaction time was seen in Group 10, at (6.22 ± 0.47) s showing a delay of 23.47% at 240 min when treated using 400 mg of *C. sativus* extract, the change was almost identical to the standard (Group 7) with statistical significance ($p < 0.05$).

Excluding squalene (C_74), a volatile oil, other substances in the de-oiled fraction such as diisooctyl phthalate (C_38) exhibit particular receptor-binding potential with the COX-I enzyme, whereas mangicrocin (C_13) and 3-methyl-2,4,6-triphenylpyridine (C_76) interact with COX-2 enzymes, as demonstrated

by our investigations. Additionally, comprehensive studies are necessary to ascertain whether they possess significant therapeutic efficacy as the NSAIDs with an improved safety profile. Furthermore, the precise mechanism underlying the pathway of their analgesic action requires comprehension.

Several compounds such as β -digentibioside, kaemperol, hexadecanoic acid (palmitic acid), hexadecanoic acid ethyl ester, etc. possess analgesic properties, where *n*-hexadecanoic acid acts via competitive inhibition of enzyme phospholipase A2 akin to varespladib where the latter possesses severe side effects such as anemia, hypoxia, thrombocytopenia, acute MI, stroke, etc. while *n*-hexadecanoic acid ethyl ester works via competitive inhibition of COX II enzyme, in a mechanism similar to that of ibuprofen⁶²⁻⁶⁴. There are only minor known side effects such as irritation, nausea, stomach upset, headaches, skin rashes, etc. of the aforementioned phytoconstituents, with no known severities being proven. The studies conducted by indicated the role of crocins or crocetin in acute, inflammatory, or neuropathic pain management, their findings were identical and supported by data obtained from experimental small animal models⁶⁵. It has been observed that both medium and high doses are significantly effective in both non-diabetic and alloxan-induced diabetic models. However, an altered (i.e., lowered) pain perception in diabetic rats would be experienced as medicinal properties. The phenomenon has been demonstrated experimentally, it is similar to diabetic neuropathy symptoms, due to faulty insulin receptor activation in peripheral nerves⁶⁶. Diabetic-induced animal models show hyperalgesia and allodynia, suggesting hyperglycemia and neuronal loss may cause pain perception difficulties⁶⁷. Hyperglycemia and free fatty acids generate oxidative stress and inflammation in type 1 and type 2 diabetes, pro-inflammatory and insulin-resistant diabetic rats exhibit elevated inflammation. These two variables can cause prospective therapeutic substances to have an altered analgesic and anti-inflammatory impact in diabetic rat models. So, the high similarities between the response of both diabetic and non-diabetic rats may indicate the anti-diabetic property of de-oiled and non-resin extract of plant that are ameliorating the diabetic condition of rats⁶⁸. Studies conducted by showed that treatment of diabetic mice using kaempferol (25, 50, and 100 mg/kg, p.o) attenuated the development of diabetic neuropathy and reduced pain sensation^{69,70}. Also, we did not observe noteworthy difference between respective treatment groups of diabetic and non-diabetic groups. So it may infer that anti-diabetic activity may impart additional therapeutic consequences on diabetic groups. Therefore, the medium and high doses of extracts are imparting significant therapeutic activity in both diabetic non non-diabetic groups almost in same pattern.

Measurement of levels of pro-inflammatory cytokines - TNF α , IL 6, and IL 1 levels from blood serum

TNF- α is a key cytokine, implicated in cachexia initiation; it serves as a potent pyrogen, eliciting fever through direct action or by stimulating interleukin-1 secretion; it can also promote cell proliferation and induce cell differentiation under specific conditions. The results of serum levels of different inflammatory markers such as TNF α , IL-2, and IL-6 belonging to Groups (1-10) have been shown in Tables 14 and 15.

Table 14. Data showing the serum pro-inflammatory cytokine – TNF α , IL-2, and IL-6 levels in non-diabetic rat models

Group Number	Group Specification	IL 2 (pg/mL)	IL 6 (pg/mL)	TNF α (pg/mL)
1	Car Control	278.19 \pm 11.72	635.08 \pm 14.38	779.78 \pm 10.94
2	Car + Ib ₁₀	112.14 \pm 07.91	280.86 \pm 08.66	430.20 \pm 10.87
3	Car + CS ₁₀₀	275.93 \pm 07.39	822.59 \pm 11.28	746.49 \pm 09.67*
4	Car + CS ₂₀₀	253.93 \pm 05.32*	578.57 \pm 10.58*	714.01 \pm 12.91*
5	Car + CS ₄₀₀	238.49 \pm 09.94*	547.26 \pm 14.70*	619.17 \pm 11.97*

n=5, each value is represented as mean \pm SEM

*significance of result

The results of serum inflammatory markers in non-diabetic experimental rats have been demonstrated through Table 14, the highest i.e. elevated levels of IL-2 being observed in the carrageenan control group (Group 1). Groups 3, 4, and 5 declined in inflammatory markers in dose dependent manner. In Group 3, IL-2, and IL-6 underwent non-significant statistical variation ($p > 0.05$) at low-dose (000mg), while significant ($p < 0.05$) reductions of IL-2 and IL-6 level occurred in Group 4 and 5. Consequently the TNF α level were significantly ($p < 0.05$) reduced in all treatment groups (low medium and high dose). All the reductions occurred in a dose-dependent manner, where the highest dose (i.e., 400-mg) administered Group 5 showed the maximum reduction.

Table 15. Data representing serum pro-inflammatory cytokines – IL-2, IL-6, TNF α levels in alloxan-induced diabetic rat models

Group Number	Group Specification	IL 2 (pg/mL)	IL 6 (pg/mL)	TNF α (pg/mL)
6	A + Car	285.85 \pm 08.13	673.01 \pm 13.09	814.69 \pm 11.33
7	A + Car + Ib ₁₀	140.85 \pm 07.12	311.08 \pm 10.44	475.69 \pm 18.48
8	A + Car + CS ₁₀₀	283.10 \pm 10.78	637.97 \pm 09.14	773.44 \pm 22.58*
9	A + Car + CS ₂₀₀	276.07 \pm 09.84*	587.11 \pm 14.50*	769.07 \pm 02.74*
10	A + Car + CS ₄₀₀	264.79 \pm 09.23*	562.00 \pm 09.39*	730.88 \pm 80.40*

n=5, each value is represented as mean \pm SEM

*significance of result

The changes in key serum inflammatory marker levels in the alloxan-treated diabetic rats have been demonstrated meticulously through Table 15, where the carrageenan-control group (Group 6) exhibited the highest serum levels of TNF α , while the anti-inflammatory medication ibuprofen-treated group (Group 7) exhibited the lowest plasma levels, in diabetic-induced groups. The reduction was observed to be the highest in the high-dose induced group (10.28%) among the extract treated groups, and reduction in inflammatory markers took place in a dose-dependent manner and the TNF α level in all extract treated groups (8, 9, and 10) were significantly reduced ($p < 0.05$). Also, in case of IL-1 and IL 2 both medium dose and high dose we observed significant restoration. High dose reduce the level of IL-1 and IL 2, respectively 7.37% and 16.49%.

Compounds such as safranal, crocin, crocetin, picrocrocin, quercetin, lycopene, present in *C. sativus* may be responsible for inhibiting the release of inflammatory mediators e.g. IL-1 α , IL-6, MCP-1, VEF-Fa etc. Lycopene, oleic acid and downregulates pro-inflammatory cytokines, including IL-1, IL-1 β , IL-6, and TNF- α . Again, 1-docosane inhibits the release of inflammatory mediators e.g. IL-1 α , IL-6, MCP-1, VEF-Fa etc. while delphinidin-3,5-diglucoside produces pro-inflammatory cytokines TNF-alpha and IL-8 upon demethylation while 1-nonadecane inhibits the release of inflammatory mediators e.g. IL-1 α , IL-6, MCP-1, VEF-Fa etc⁷¹. The anti-inflammatory effect of crocin has been studied by dietary feeding of crocin (100 ppm, 200 ppm for 4 consecutive weeks) significantly suppressed mRNA expression of COX-2, iNOS, NF- κ B,

TNF- α , IL-1 β , and IL-6 in male ICR mice model⁷². Again, aqueous extracts (40 and 80 mg/kg aqueous saffron extracts significantly decreased serum TNF- α and iNOS activity in hippocampus tissue of streptozotocin (STZ)-induced diabetic rats as demonstrated by⁷³. Similarly, reported the efficacy of crocetin ester in the reduction of serum levels of pro-inflammatory cytokines^{74,53}. It has been reported that kaempferol (2 or 4 mg/kg/day, p.o.) can inhibit NF- κ B function by inhibiting the activation of nuclear factor-inducing kinase (NIK)/I κ B kinase (IKK) and MAPKs signal pathways in aged rat kidney, playing anti-inflammatory roles, as showed by^{75,76}. Again, ethanolic and aqueous extracts of *C. sativus* (200 mg/kg, i.p.) reduced neuropathic pain in CCI model through attenuation of pro-inflammatory factors (TNF- α), IL-1 β and IL-6^{59,67}.

Moreover, the aforementioned bio-actives are known exert anti-inflammatory effects by reducing/modulating pro-inflammatory cytokines Hence, any of these molecules either ‘individually’ or ‘synergistically’ may bring about downregulatory effects in the inflammatory cascade, may even play roles in alleviating depression and neuroinflammation⁷⁷.

Histopathological analysis

The following Tables 16 and 17 represent data on inflamed skin area, presence of fluid (edema), and granuloma formation following carrageenan administration collected from non-diabetic and alloxan-induced diabetic groups of the experimental animals. The data had been meticulously organized for further evaluation, analysis, and interpretation.

Table 16. Histopathological evaluation of skin in non-diabetic rat models

Group Number	Experimental Group	Area of Edema	Inflammatory Cell	Inflammatory Exudate	Area of Granuloma	Collagen Thickness
a	Negative control	absent	absent	absent	absent	+
b	Car	+	+	+	absent	++
c	Car + Ib ₁₀	+	absent	+	absent	+
d	Car + CS ₁₀₀	+	absent	absent	absent	+
e	Car + CS ₂₀₀	+	absent	absent	absent	+
f	Car + CS ₄₀₀	+	absent	absent	absent	+

Table 17. Histopathological evaluation of skin in alloxan-induced diabetic rat models

Group Number	Experimental Group	Area of Edema	Inflammatory Cell	Inflammatory Exudate	Area of Granuloma	Collagen Thickness
g	A + Car	+	+	+	absent	+
h	A + Car + Ib ₁₀	absent	absent	+	absent	+
i	A + Car + CS ₁₀₀	+	absent	+	absent	+++
j	A + Car + CS ₂₀₀	+	absent	absent	absent	+
k	A + Car + CS ₄₀₀	absent	absent	absent	absent	+

The histological tissue sections of skin, as illustrated in figure 1 were initially investigated to monitor specific inflammatory markers. Tables 16 and 17 above are representing data regarding the histological evaluation of paw edema, mentioning the presence of inflammatory areas, cells lesions, and exudates in the experimental animals. The negative control group had been treated with physiological saline only and did not exhibit any discernible alteration in terms of acute inflammatory infiltration. Histopathological examinations revealed healthy, intact cellular structures with distinct, visible intracellular compartments indicating no traces of tissue injury or damage.

Animals in the carrageenan control group displayed conspicuous/ infiltration damage as evidenced by the presence of edematous areas and exudate accumulation in both non-diabetic and diabetic models (sample b and g).

In contrast to the control group (Group a), animals that had been treated with a well-known, marketed anti-inflammatory drug (ibuprofen 10 mg/kg) in Group c presented a significant reduction in inflammatory markers in comparison with the positive (carrageenan) control group (Group b). This was apparent from the absence of inflammatory cell, though edematous regions and inflammatory exudates were present in animals from Group c.

Steadily increasing (i.e., low, medium and high) doses of *C. sativus* petal extract were administered along with carrageenan in Groups d, e, and f, respectively, where all three groups demonstrated the presence of edematous regions. Inflammatory cells or exudates were absent in all the CS-treated groups in the non-diabetic model.

The alloxan-treated carrageenan control group (Group g) exhibited the presence of area of edema, inflammatory cell, and exudates. In the ibuprofen-treated group (Group h) only exudates had been observed and the other two parameters were absent. Edematous regions were seen to be present in both low and medium-dose treated groups (Groups i and j), and absent in the high dose treated group (Group k).

In the low-dose group (Group i), edema and exudates were observed to be present, in the medium-dose group (Group j), only edema was seen while none of the inflammatory signs or associated tissue damage were noticed in the high-dose treated group (Group k). This suggests that 400 mg of *C. sativus* extract possesses a strong potential/significant capacity to effectively reverse/alleviate cellular swelling, edema and inflammation.

Granuloma was absent in all the non-diabetic groups, including negative control, carrageenan-induced, ibuprofen, and petal -treated groups. Collagen is one very essential structural protein known to play crucial anti-inflammatory, wound healing, and repairing roles; it is observed to be low in Groups a (control), c, d, e, and f, indicating mild or reduced inflammation, and moderate in Group b indicating the severity of inflammation to be maximum.

Granuloma was absent in all the alloxan-induced diabetic groups too, including negative control, carrageenan-induced, ibuprofen, and petal-treated groups (Groups g-k). Collagen thickness was observed to be low in Groups g, h, j, and k which suggested mild or reduced inflammation, and severe only in Group i indicating that the low-dose petal extract was not very effective in alleviating/reducing tissue inflammation.

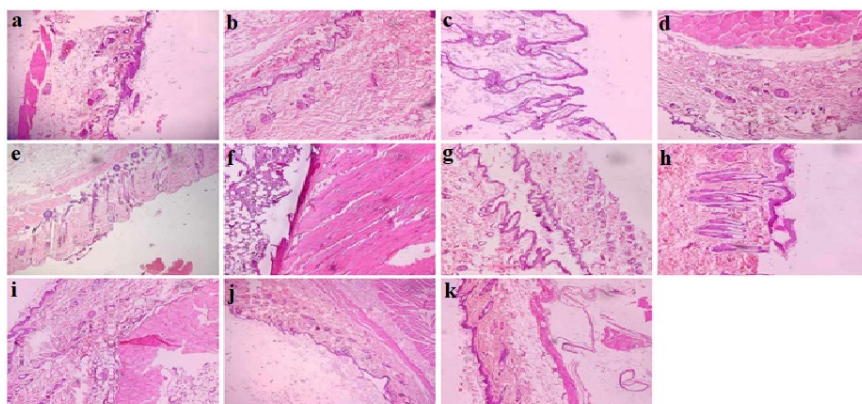


Figure 1. Photomicrographs ($\times 10$) of histopathological analysis of tissue samples taken from the skin of experimental rats of (a) negative control group, (b) disease control (carrageenan-induced) group, (c) Skin tissue of rats treated with carrageenan + ibuprofen 10 mg/kg, (d) Skin tissue of rats treated with carrageenan and low-dose of *C. sativus* (Car + CS₁₀₀), (e) Skin tissue of rats treated with carrageenan and medium-dose of *C. sativus* (Car + CS₂₀₀), (f) Skin tissue of rats treated with carrageenan and high-dose of *C. sativus* (Car + CS₄₀₀), (g) Skin tissue of rats treated with alloxan + carrageenan only (h) Skin tissue of rats treated with alloxan + carrageenan + ibuprofen 10 mg/kg (i) Skin tissue of rats treated with alloxan, carrageenan and low-dose of *C. sativus* (A + Car + CS₁₀₀) (j) Skin tissue of rats treated with alloxan, carrageenan and medium-dose of *C. sativus* (A+ Car + CS₂₀₀) (k) Skin tissue of rats treated with alloxan, carrageenan and high-dose of *C. sativus* (A+ Car + CS₄₀₀)

Following a robust histopathological evaluation, the aforementioned images obtained indicate that the normal rats showed a regular and healthy appearance of the epidermis and dermis without any lesion formation, while paw tissue of carrageenan-injected rats in the control group exhibited vasodilatation along with visible swelling/edema. Inflammatory mediators such as histamine and serotonin cause an increase in vascular leakage. Moreover, the extravasation of the liquid including plasma proteins will cause leukocyte aggregation and adhesion, which are important features of acute inflammation⁷⁸. Our current study showed that carrageenan injection in rat paws induced edema and decreased vascular permeability within 6 hours. Therefore, the inhibition of carrageenan-induced increase in paw weight is regarded as evidence of anti-inflammatory efficacy through reducing vasodilation and hence improving the edematous condition⁷⁹.

The primary goal in managing chronic pain and inflammatory illnesses is the eradication of the underlying cause rather than the temporary alleviation of symptoms.

Currently, prescribed anti-inflammatory drugs including NSAIDs and steroids exert their anti-inflammatory effect mainly by interfering with the biosynthesis of cyclic prostaglandins (such as thromboxane A₂, prostacyclin, etc.)⁸⁰. They are often accompanied by mild or moderate adverse reactions; long-term use in large doses poses severe health treats such as impaired platelet function, gastrointestinal bleeding, nephrotoxicity, etc. Therefore, it is very crucial to comprehend and correlate the mechanisms of acute and chronic inflammation, and to explore and develop inhibitory intervention drugs, in particular, to decline the immunological reactivity and establish them as suitable, safer, and more effective alternatives⁸¹.

According to widespread studies from previous scientific literature, the phytoconstituents such as glycosides, flavonoids, phenolic acids, and terpenoids present in *C. sativus* possess well-known biological properties/activities that serve analgesic, antioxidant, anti-inflammatory, anti-diabetic, anti-atherosclerotic, anti-neoplastic, neuroprotective and immunomodulatory functions. Compounds such as β -digiubiobioside possess analgesic properties; α -crocetin, γ -crocetin, 13-cis-crocetin, α -carotene, β -carotene, lycopene, picrocrocin, quercetin, zeaxanthin, mangicrocin, safranal, benzene ethanol, cis-1,3 octadecanoic acid, hexadecane, squalene, eicosane serves as potent anti-oxidant; while kaempferol served dual roles of analgesic and anti-inflammatory agents^{82,61}. Moreover, a string of relevant recent scientific reports has evinced similar, comparable and noteworthy effects of phytoconstituents in experimental animal models involving rat or mice²².

The expression of inflammatory mediators such as IL-2, IL-6, IL-10 and TNF- α was also downregulated as observed in Groups 3-5 and 8-10, respectively; this may provide some relevant perspective on the probable mechanism of action of the existing plant compounds. To summarize, the majority of anti-inflammatory drugs work (either directly or indirectly) against intercellular stressed conditions by significantly diminishing, reversing, or completely inhibiting mediators such as prostaglandins, enzymes (PLA₂, COX-1, COX-2), or cascade processes⁸³. Lycopene acts by inhibiting NF- κ B, reducing factors such as IL-1, IL-2, L-6, TNF- α , reduces the expression of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), activates nuclear factor-erythroid 2 related factor 2 (Nrf2) pathway, which increases the expression of heme oxygenase-1 (HO-1)⁸⁴.

Furthermore, a few of these plant-derived compounds may possess anti-histaminic, anti-secretory, anti-enzymatic, or receptor-blocker activities, for example.

It may therefore be well-suggested that the antioxidant and anti-inflammatory activities of *C. sativus* result from the presence or be related to the therapeutic action of its aforementioned constituents. Oleic acid (C18-fatty acid) directly regulates the synthesis and modulates the activities of certain antioxidant enzymes, inhibits pro-inflammatory cytokines, and activates anti-inflammatory ones, including sirtuin 1 (SIRT1), helicryoside caused inhibition of inflammatory enzymes, possessed free-radical scavenging activities and corticoid-like effects while fatty acid esters such as methyl palmitate acts by inhibiting NF- κ B⁸⁵⁻⁸⁷.

Organic acids such as palmitic acid/*n*-hexadecanoic acid (C16) result into competitive inhibition of enzyme phospholipase A2 and interleukin IL-6, functioning in much similarity with the drug Varespladib while ethyl ester of the former competitively inhibits enzyme cyclo-oxygenase (COX II), resembling the action of marketed/prescribed anti-inflammatory drug ibuprofen^{88,89}. Hentriacontane may serve as a potent inhibitor of cytokines. The aforementioned similarities may shed light on some of the probable action mechanisms of the constituting plant components⁹⁰.

Naturally occurring polyphenolic flavonoid phytoestrogen, kaempferol (KPF) possesses many beneficial effects; it induces suppression of inflammatory cell activity, inhibition of pro-inflammatory cytokines, and chemokine production/expression⁹¹. It demonstrated potent inhibition of COX-I and COX-II enzymes, functioning in a manner comparable to that of ibuprofen and celecoxib. Originally isolated from petal extracts, these compounds are non-cytotoxic, non-immunogenic, non-mutagenic and non-carcinogenic, moreover, they are abundantly available in nature, convenient, safe, effective and economical from pharmacological perspectives, indicating that they indeed serve to be advantageous over other chemical substances⁶⁹.

Apart from the stated identified compounds, one single plant compound may possess and provides multiple therapeutic purposes, or numerous constituents may offer similar or synergistic pharmacological actions⁵⁹. In addition, compounds that are known to be present but unrecognized for possessing/providing that specific activity may bring about analgesic or anti-inflammatory effects, or entirely novel (which are both unidentified/unelucidated as well as unrecognized) chemical entities may induce pain-relieving and anti-inflammatory actions; their mechanisms of action being not yet discovered/studied/investigated/understood. Our results here have strongly indicated that the CS extracts elicited a marked/notable reduction in the infiltration of the carrageenan-treated hind paws of experimental animal models, according to a stringent histopathological examination⁷⁶.

Table 18. Histopathological evaluation of liver in non-diabetic rat models

Group	Experimental Group	Histopathological Changes								
		Swelling of the Hepatocytes	Cholestasis	Hepatocellular Necrosis	Micro Vesicular Steatosis	Macro Vesicular Steatosis	Lobular Inflammation	Portal Inflammation	Fibrosis	Dilation of Sinusoids
a	Negative control	-	-	-	-	-	-	-	-	-
b	Car	✓	-	✓	✓	✓	✓	✓	-	✓
c	Car + Ib ₁₀	✓	-	✓	✓	✓	✓	✓	-	✓
d	Car + CS ₁₀₀	-	-	-	-	-	-	-	-	✓
e	Car + CS ₂₀₀	-	-	-	✓	-	-	✓	-	✓
f	Car + CS ₄₀₀	✓	-	-	-	✓	✓	✓	-	✓

Table 19. Histopathological evaluation of liver in alloxan-induced diabetic rat models

Group	Experimental Group	Histopathological Changes								
		Swelling of the Hepatocytes	Cholestasis	Hepatocellular Necrosis	Micro Vesicular Steatosis	Macro Vesicular Steatosis	Lobular Inflammation	Portal Inflammation	Fibrosis	Dilation of Sinusoids
g	A + Car	✓	-	✓	✓	✓	✓	✓	-	✓
h	A + Car + Ib ₁₀	-	-	-	-	✓	✓	✓	-	-
i	A + Car + CS ₁₀₀	-	-	-	-	✓	✓	✓	-	✓
j	A + Car + CS ₂₀₀	-	-	-	-	-	-	-	-	✓
k	A + Car + CS ₄₀₀	-	-	-	-	-	✓	-	-	✓

“✓” indicates the presence of a particular histopathological feature; while “-” indicates the absence of a particular histopathological feature.

The liver being one vital, resilient and the largest internal organ plays crucial storage, metabolism, digestion, detoxification, immunological and bio-defensive roles⁹². Histopathological changes were correlated with carrageenan, and symptoms such as necrosis of individual hepatocytes and localized necrotic regions, with associated fibrin thrombi were observed following 12 hours of i.p. infiltration. The alterations in different experimental groups have been summarized through Tables 18 and 19, as well as in Figure 2. Free radicals have long been known to play an important role in the carrageenan-induced acute inflammatory response and in some associated pathophysiological conditions, they have been proposed to mediate cell damage in the liver. As a marker of oxidative damage, lipid peroxidation indicates changes in membrane fluidity and permeability and thus increases in rates of protein degradation, which eventually leads to cell lysis⁹³. One of the consequences of lipid peroxidation can also result in enzyme activity changes.

The histoarchitecture of the negative control group (Group a) exhibited normal liver cells with sound hepatocytes, distinct sinusoidal spaces, well-defined lobular units, and portal tracts. Contrarily, histoarchitecture of the liver samples in the disease control group (Group b) demonstrated significant hepatocellular swelling with intense lobular and portal tract inflammation, severe macro- and micro-vesicular steatosis, and sinusoidal dilation and hepatocellular necrosis. Hepatocytes structures were damaged/disrupted due to the damaging action of free radical species (ROS), peroxy trichloromethyl (OCCl₃) and trichloromethyl (CCl₃) radicals generated during cytochrome P450-dependent metabolic steps, radicals producing covalent bonds with lipoproteins and nucleic acids cause extensive cellular injury⁹⁴. These features have been depicted through Figure 2.

Groups c portrayed a result similar to that of Group b, the changes or damage-reversal being very weak/little, indicating that the low dose CS-extract did not exert much of an effective (quite negligible) treatment on hepatocellular damage. However, the histopathological findings from Group d revealed significantly less damage—sinusoidal dilution only—while cells from Group e also indicated micro-vesicular steatosis and portal inflammation along with sinusoidal damage. This indicates that medium-dose extract was a little more effective in treating/reversing oxidative-stress-induced hepatic damage. The results obtained from histopathological data in experimental Groups d, e showed marked diminution/lessening of hepatic damage, their changes being statistically significant ($p < 0.05$). Yet this reversal was not as potent in Group e as the action of the extract as observed in Group d, the phenomenon has been depicted through photomicrographs in Figure 2.

In the alloxan-treated groups, Group f indicated hepatocyte swelling, macrovascular steatosis, portal and lobular inflammation, and dilution of sinusoids, while Group g showed symptoms of hepatocellular necrosis and microvascular steatosis. Groups h and i revealed macrovascular steatosis, portal and lobular inflammation while Group i also had dilatation of sinusoids. Group j showed only sinusoidal dilation, contrarily, Group k showed both lobular inflammation and sinusoidal dilation. None of the groups (from Group c-k) among non-diabetic or diabetic animals demonstrated observable cholestasis or fibrosis occurring in the experimental animal models.

In summary, the results obtained from histopathological data in experimental Groups j and k showed marked diminution/lessening of hepatic damage, their changes being statistically significant ($p < 0.05$) thereby suggesting strong therapeutic potential of *C. sativus* extract in low, medium doses in non-diabetic groups and medium and high doses in the diabetic-induced groups.

The histopathological alterations occurring in the disease control group were almost entirely reversed by administering varying doses (i.e., low, medium and high doses) of the petal extract, with high dose yielding results nearly similar to that of the standard drug ibuprofen.

The possible mechanism underlying the *in vivo* toxicity of carrageenan and its alleviation by *C. sativus* is discussed in the light of these and other findings. The petal extract contains certain compounds that induce hepatoprotective and anti-oxidant action either directly or indirectly prevent/impede/hinder the process of oxidative stress, interfere with the oxidation process by various mechanisms, such as, reacting with free radicals, chelating free catalytic metals, and acting as oxygen scavengers, etc.⁹⁵. Carotenoids such as lycopene, α - and β -carotenes, leutin and zeaxanthin; flavonoids such as kaempferol, quercetin, rutin, naringin; their mono- di-, tri-glycosidic derivatives, are powerful anti-oxidants.

These may include α -crocetin, trans-crocetin isomer, 13-cis-isomer, α -carotene, β -carotene, tyrosol, zeaxanthin, safranal, vitamin E (α -tocopherol), penta-decylic acid, flavonoids such as picrotoxin, quercetin etc. Helicyoside results in the inhibition of inflammatory enzyme, free-radical scavenging activity, and corticoid-like effects⁹⁶. stated hepatoprotective effects obtained by safranal present in *C. sativus* petal extracts, while also found similar hepatoprotective and anti-oxidative effects by attenuating stress/mitigating inflammation⁹⁷.

Moreover, compounds such as methyl and ethyl palmitate, methyl linoleate, 6-methyl-4-phenylcinnoline, serves as inflammatory inhibitors producing hepato-protective, anti-fibrotic, anti-proliferative activity. The observations in

our studies were consistent/similar from results obtained in the study conducted by, where the aforementioned compounds, e.g. lycopene have showed to possess anti-oxidant properties and play effective roles against hepatitis, fibrosis, cirrhosis, and liver cancer⁹⁷.

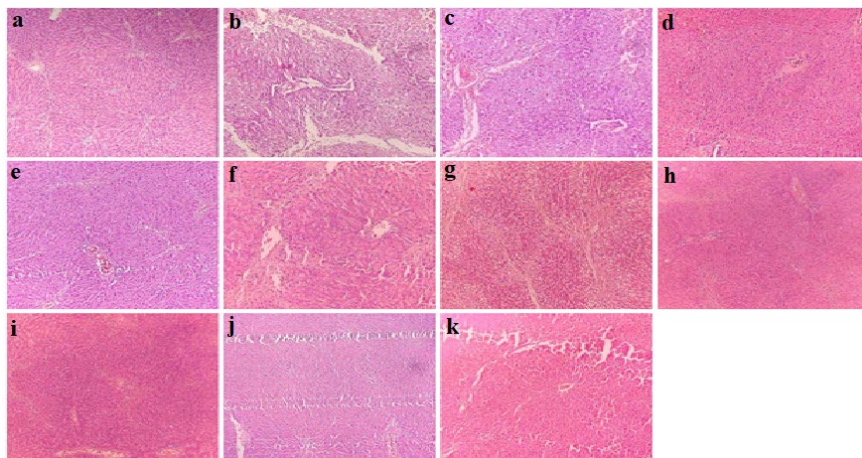


Figure 2. Photomicrographs (×10) of histopathological analysis of liver samples taken from the (a) negative control group, (b) disease control group, (c) Liver architecture of rats treated with carrageenan + ibuprofen 10 mg/kg, (d) Liver architecture of rats treated with carrageenan and low-dose of *C. sativus* (Car + CS₁₀₀), (e) Liver architecture of rats treated with carrageenan and medium-dose of *C. sativus* (Car + CS₂₀₀), (f) Liver architecture of rats treated with carrageenan and high-dose of *C. sativus* (Car + CS₄₀₀), (g) Liver architecture of rats treated with alloxan + carrageenan only, (h) Liver architecture of rats treated with alloxan + carrageenan + ibuprofen 10 mg/kg, (i) Liver architecture of rats treated with alloxan, carrageenan and low-dose of *C. sativus* (A + Car + CS₁₀₀), (j) Liver architecture of rats treated with alloxan, carrageenan and medium-dose of *C. sativus* (A+ Car + CS₂₀₀) and (k) Liver architecture of rats treated with alloxan, carrageenan and high-dose of *C. sativus* (A+ Car + CS₄₀₀)

Molecular docking

COX-1

Out of the 81 compounds screened from the plant, two compounds exhibited higher binding affinities than the control drug aspirin: di iso octyl phthalate (C₋₃₈), with a binding affinity of -7.8 Kj/mol, and squalene (C₋₇₄), with a binding affinity of -8.0 kJ/mol, compared to -6.1 Kj/mol for aspirin. None of these 2 compounds have showed any unfavorable bonds with the target COX-1. The interacting residues formed bonds like van der waals, carbon hydrogen bon and the other conventional pi-sigma bonds with the target for both of the compounds. The interactions are listed and depicted in Table 20 and Figures 3-5, respectively.

Table 20. Protein-ligand interactions between the top two compounds and the control drug with COX -1

Ligand	Binding Affinity	Interaction Type	Interacting Amino Acids	Unfavorable Bond
di iso octyl phthalate (C_38)	-7.8	Van der waals, Carbon hydrogen bond, Pi-sigma, Pi-sulfur, Alkyl, Pi-Alkyl	VAL 116, LEU 359, TYR 355, ALA 527, SER 353, VAL 349, MET 522, LEU 352, ILE 523, GLY 526, LEU 384, TRP 387, TYR 348, TYR 385, PHE 205, PHE 529, PHE 209, GLY 533, ASN 375, ILE 377, GLY 227, VAL 228, PHE 381, VAL344, SER 530, LEU 534, PHE 518, LEU 531, ARG 120,	None
Squalene (C_74)	-8	Van der waals, alkyl, pi- alkyl	SER 530, TYR 385, GLY 526, ILE 523, PHE 518, MET 522, LEU 352, PHE 381, TRP 387, LEU 382, ALA 527, LEU 531, LEU 359, VAL 349, ARG 120, TYR 355, VAL 116, LEU 92, LEU 112, THR 89, LEU 357, TRP 100, LEU 115, SER 353, TYR 348	None
Aspirin	-6.1	Van der waals, conventional hydrogen bond, carbon hydrogen bond, pi-donor hydrogen bond, pi-sigma, pi alkyl	TYR 348, TYR 385, LEU 352, TRP 387, LEU 384, MET 522, GLY 526, ALA 527, ILE 523, PHE 518, SER 353, VAL 349, SER 530,	None

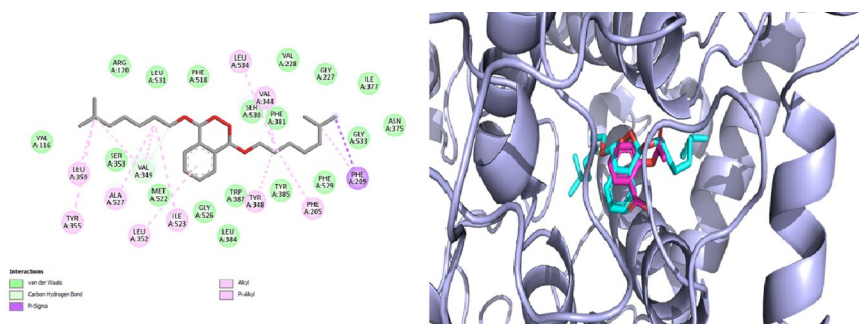


Figure 3. Molecular docking binding pose of Di iso octyl phthalate (C_38) and binding interactions for the target COX-1

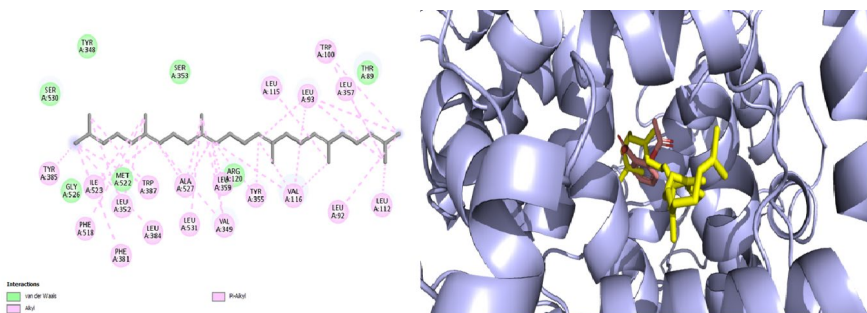


Figure 4. Molecular docking binding pose of Squalene (C₇₄) and binding interactions for the target COX-1

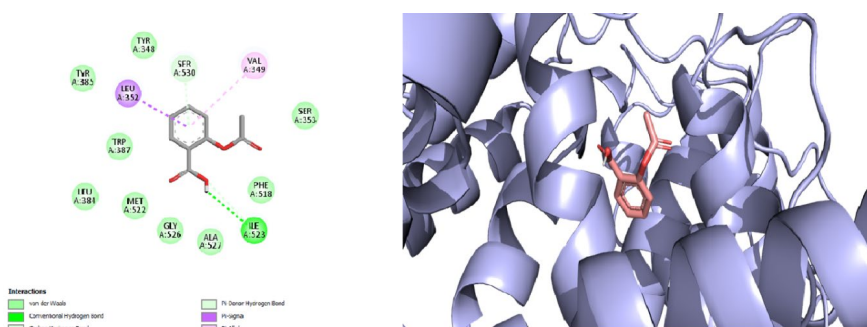


Figure 5. Molecular docking binding pose of control drug (aspirin) and binding interactions for the target COX-1

COX-2

3-Methyl-2,4,6-triphenylpyridine (C₇₆) and Mangicrocin (C₁₃) showed the highest binding affinity towards the target COX-2. But they have different common binding residues when compared with the target drug aspirin. Even though the compound 3-Methyl-2,4,6-triphenylpyridine (C₇₆) has interacted close to where the control aspirin has binding residues, the compound Mangicrocin (C₁₃) has a highly varying binding pocket compared to aspirin (Figure 5). This variety might cause inhibition in a varying scale and might also affect the stability of the total complex. An unfavorable bond between the compound and the HIS 356 residue of the target also accompanies the binding of the compound Mangicrocin (C₁₃), even though it has a binding affinity significantly higher compared to the control drug with a value of -9.7 KJ/mole, whereas in contrast, the compound 3-Methyl-2,4,6-triphenylpyridine (C₇₆) has shown no such unfavorable interaction with the target as well as has a very high binding affinity. The interactions are listed and depicted in Table 21 and Figures 6-8, respectively.

Table 21. Protein-ligand interactions between the top two compounds and the control drug with COX-2

Ligand	Binding Affinity (kJ/mole)	Interaction Type	Interacting Amino Acids	Unfavorable Bond
3-Methyl-2,4,6-triphenylpyridine (C_76)	-10	Van der waals, Pi-Alkyl, Amide pi stacked	GLN 42, ASN 43, LYS 468, LEU 80, ARG 44, PHE 64, GLY 63, THR 76, LYS 79, TYR 122, SER 471, LYS 83, LYS 473, LEU 472, ARG 469	None
Mangicrocin (C_13)	-9.7	Van der waals, conventional, conventional hydrogen bond, pi anion, pi alkyl	PHE 580, ILE 564, ASP 347, SER 579, GLN 350, GLY 354, TYR 355, GLN 192, HIS 351, GLN 565, ASN 581, SER 563, ARG 109, GLU 346, ALA 562, LYS 342, ASN 104, LYS 97	HIS 356
Aspirin	-7.2	Van der waals, conventional hydrogen bond, pi alkyl	GLU 46, ARG 44, PRO 153, LEU 152, ARG 469, CYS 41, GLN 461, GLU 465, ASN 39	None

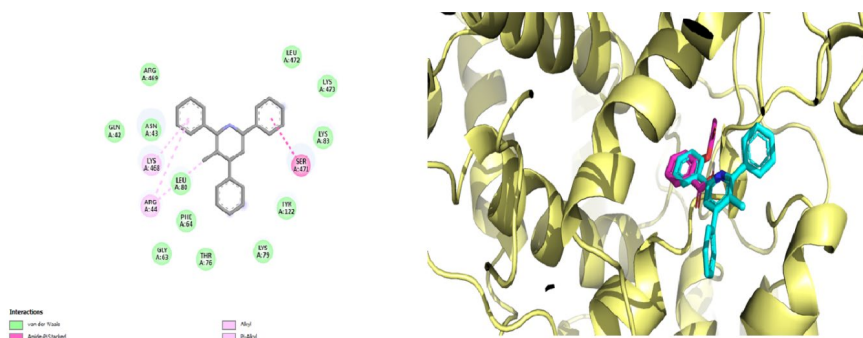


Figure 6. Molecular docking binding pose of 3-methyl-2,4,6 triphenyl pyridine (C_76) and binding interactions for the target COX-2

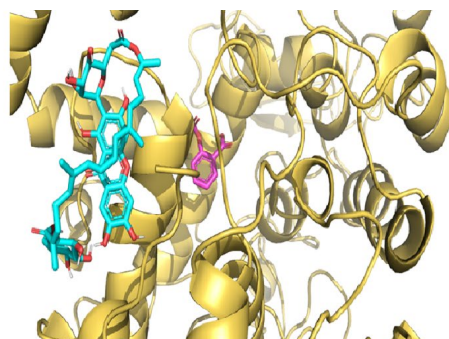
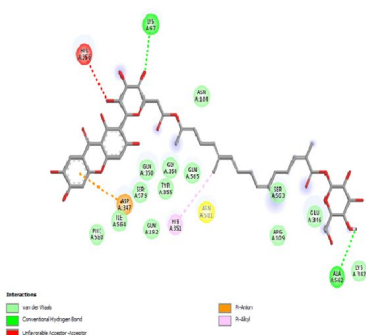


Figure 7. Molecular docking binding pose of Mangicrocin (C_13) and binding interactions for the target COX-2

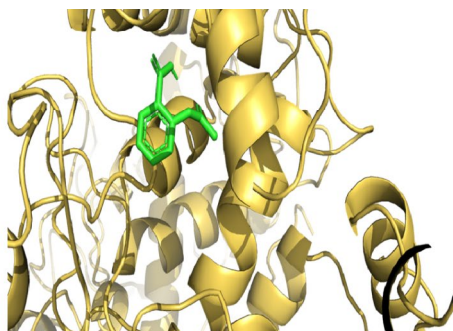
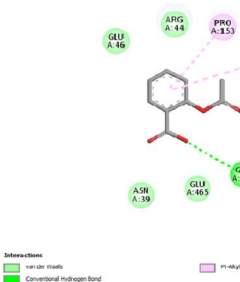


Figure 8. Molecular docking binding pose control drug (aspirin) and binding interactions for the target COX-2

Molecular dynamic simulation

Molecular dynamic simulation studies of top 2 compounds with COX-1 and COX-2

A approach to examine the stability and dynamic behavior of a protein-ligand complex is using molecular dynamic simulation. To ascertain the stability and properties of our filtered top two compounds in a simulated environment, we therefore ran an extended dynamics simulation of 100ns of those compounds associated with COX-1 and COX-2. The top two compounds, di iso octyl phthalate -C38 (Column 1) and Squalene -C74 (Column 2), with COX1 are plotted using RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) data plots in figure 9; Plot indicates the same type of plots for the top compounds, Mangicrocin -C13 (Column 1) and 3-Methyl-2,4,6-triphenylpyridine-C76 (Column 2), with COX-2. Protein ligand stability is typically indicated by small RMSD and RMSF value variations between 1 and 3 angstroms.

As none of the compounds displayed RMSD changes outside of the range of 1 to 3 angstroms, the top 2 compounds, di iso octyl phthalate (C38) and Squalene (C74), have outstanding binding stability with COX-1. No changes are seen above three angstroms, even in the RMSF plots, which exhibit low variability. Although Squalene (C74) had a big spike in RMSF values of residues 1-10, it didn't contain any residues which were in contact with the ligand. Thus, both of the complexes show strong stability and no conformational change.

In Figure 10 we can see that top 2 compounds 3-Methyl-2,4,6-triphenylpyridine (C76) have excellent binding stability with COX-2. as none the compounds showed RMSD fluctuations outside the range of 1 to 3 angstrom. Even the RMSF plots show low variability with no fluctuations over 3 angstroms. Therefore, indicating no conformational change and high stability for the complex. But in case compound Mangicrocin (C13), it showed high RMSD fluctuation around 80 ns and was unstable during the rest of the simulation period Although Mangicrocin-C13 was unstable 3-Methyl-2,4,6-triphenylpyridine-C76 can be an excellent target ligand for the COX-2. complex.

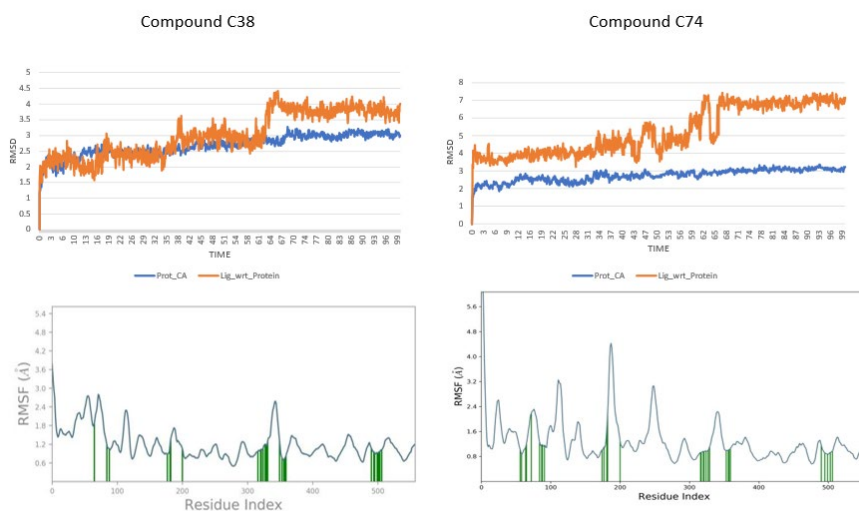


Figure 9. RMSD and RMSF plots of molecular dynamics (MD) of target COX-1 with top 2 hit compounds complexes: Upper row signifies RMSD data and lower row Signifies RMSF plots of di iso octyl phthalate-C38 (column 1) and Squalene-C74(column 2)

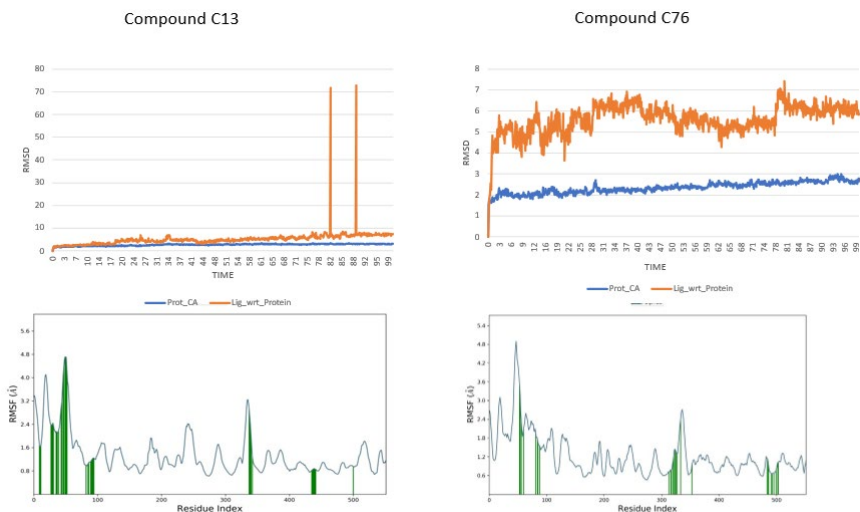


Figure 10. RMSD and RMSF plots of molecular dynamics (MD) of target COX-2 with top 2 hit compounds complexes: Upper row signifies RMSD data and lower row signifies RMSF plots of Mangicrocin, C13 (column 1) and 3-Methyl-2,4,6-triphenylpyridine, C76 (column 2).

ADME, toxicity profile and drug-likeness predictions

ADMET prediction

Among the three selected compounds after the molecular dynamic simulation which showed stability as a complex, no ADMET data for the compound Squalene (C₇₄) could be extracted from the SWISS ADMET site, as the data was not available for the compound. Among the rest of the two compounds di iso octyl phthalate (C₃₈) has shown high GI absorption where 3-Methyl-2,4,6-triphenylpyridine (C₇₆) has low GI absorption. Neither of the compound has BBB permeability where as in case of effect on Pgp substrate both compound has contribution. In the case of effect on the metabolic enzymes di iso octyl phthalate has effect on the CYP2C9 and the CYP2D6 in contrast to 3-Methyl-2,4,6-triphenylpyridine (C₇₆). On the other hand, 3-Methyl-2,4,6-triphenylpyridine (C₇₆) has effect on CY2C19 and CYP3A4 where di iso octyl phthalate (C₃₈) has no effect on these. Both of them has effect on CYP1A2. The log K_p vale of the two compounds range near each other with a value of -3.43 for di iso octyl phthalate(C₃₈) and $_3.88$ for the compound 3-Methyl-2,4,6-triphenylpyridine (C₇₆) Table 22.

Both the compound has the same bio availability score with a value of 0.55. Whereas, di iso octyl phthalate (C₃₈) has lesser drug likeliness violations compared to the compound 3-Methyl-2,4,6-triphenylpyridine(C₇₆) as mentioned in Table 23.

Table 22. ADMET properties of the top two compounds

Compound	ADMET Properties								
	GI Absorption	BBB Permeant	Pgp Substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	log Kp (cm/s)
di iso octyl phthalate (C_38)	High	No	Yes	Yes	No	Yes	Yes	Yes	-3.43
3-Methyl-2,4,6-triphenylpyridine (C_76)	Low	No	Yes	Yes	Yes	No	No	Yes	-3.88

Drug-likeness predictions

Table 23. Drug likeness of the top two compounds

Compounds	Lipinski #violations	Ghose #violations	Veber #violations	Egan #violations	Muegge #violations	Bioavailability Score
di iso octyl phthalate (C_38)	1	1	1	1	1	0.55
3-Methyl-2,4,6-triphenylpyridine (C_76)	1	1	0	1	2	0.55

Toxicity prediction

Both the di iso octyl phthalate (C_38) and the 3-Methyl-2,4,6-triphenylpyridine (C_76) falls under the toxicity class of 4, which is defined as “harmful if swallowed.” But the predicted LD50 value for di iso octyl phthalate is 2000 mg/kg, where for 3-Methyl-2,4,6-triphenylpyridine it is 500 mg/kg. Squalene (C_74) falls under the toxicity class of 5 which is defined as “may be harmful if swallowed”, with a predicted LD50 vale of 5000 mg/kg table 24.

All three compounds showed ecotoxicity at the endpoint toxicity criteria. C-76 has neurotoxicity and di iso octyl phthalate has nephron toxicity and cardiotoxicity. Squalene shows no organ toxicity according to the prediction in Table 25.

Table 24. Toxicity class and LD50 values of the top three compounds

Compounds	Toxicity Class	Predicted LD50 (mg/kg)
di iso octyl phthalate (C_38)	4	2000
Squalene (C_74)	5	5000
3-Methyl-2,4,6-triphenylpyridine (C_76)	4	500

Table 25. Predicted toxicity type of the top three compounds

Toxicity Type		Compounds
Organ toxicity	Hepatotoxicity	-
	Neurotoxicity	3-Methyl-2,4,6-triphenylpyridine (0.65)
	Nephrotoxicity	di iso octyl phthalate (0.54)
	Respiratory toxicity	-
	Cardiotoxicity	di iso octyl phthalate (0.5)
Toxicity end points	Carcinogenicity	-
	Immunotoxicity	-
	Mutagenicity	-
	Cytotoxicity	-
	Ecotoxicity	di iso octyl phthalate (0.54), 3-Methyl-2,4,6-triphenylpyridine (0.82), Squalene (0.62)
	Clinical toxicity	-
	Nutritional toxicity	-

We sought to assess the potential analgesic and anti-inflammatory activity of *C. sativus* petals apart from its resin and volatile oil constituents. Significant therapeutic effectiveness has been demonstrated through prior research at 400 mg/kg, *p.o.* of the whole extract, including pain-relieving and inflammation-reducing effects. However, in our present study, the de-oiled, non-resinated fraction (ethanolic extract) has demonstrated a substantial therapeutic impact

even at a lower dose of 200 mg/kg, *p.o.*, which indicates the presence of suitable bio-active compounds in high concentration. Additionally, compounds such as Mangicrocin (C_13) and 3-methyl-2,4,6-triphenylpyridine (C_76) demonstrated great binding affinity with COX-1, while di iso octyl phthalate (C_38), and squalene (C_74) exhibited strong affinity with COX-2 according to our molecular docking investigations; only squalene being a volatile oil among the four. Hence, by correlating our *in vivo* and *in silico* findings, it may be interpreted that the majority of the rich and diverse array of phytoconstituents found in *C. sativus* that produce analgesic and inflammatory effects are neither volatile oils nor resin components. Also, the similar effect of extract on both diabetic and non-diabetic rats indicates that the plant may possess some anti-diabetic compounds that reduce the impact of diabetes on inflammation and pain.

In future, further thorough and rigorous, in-depth studies, including *in vitro* assays, may be carried out utilizing advanced analytical tools to comprehensively determine the therapeutic efficacy of constituents present in the de-oiled, non-resinated *C. sativus* fraction. Also, appropriate protocols may be implemented to ascertain the *in vivo* therapeutic efficacy in healthy or disease-induced experimental animals. Altogether, *in vivo* pharmacological assessments linked with suitable *in silico* approaches may facilitate the identification and development of safer, cost-effective, naturally occurring bioactive molecules that broaden the horizons of pain and inflammation management.

STATEMENT OF ETHICS

The research project has received ethical approval from the Ethical Review Committee of the Faculty of Pharmacy of University of Dhaka (Ref. no. Fa. Ph. E/062/2025) on January 29 2025.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Designed the protocol: SRA, MRT, MSA, FADMO, NSC.

Wrote the manuscript: NN, RNC, NM, MSHM, MMM.

Worked in the wet lab: MRT, RR, TAA, MAR, MSA (Muhammad Salehuddin AYUBEE), SRA, RA.

Worked in dry lab: NM, RR, RNC, RA, SMB, RA, MSA.

Supervised the project: MSA (Md Shah AMRAN), SK, JAC, AAC, MSR, MRT, SMB, FADMO.

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