Phytochemical Isolation of Scopoletin from Cassia fistula Pods and its Anti-Inflammatory, Analgesic Activities: In Vivo and Molecular Docking Evidence for COX-2 Inhibition

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Phytochemical Isolation of Scopoletin from *Cassia fistula* Pods and its Anti-Inflammatory, Analgesic Activities: *In Vivo* and Molecular Docking Evidence for COX-2 Inhibition

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Abstract

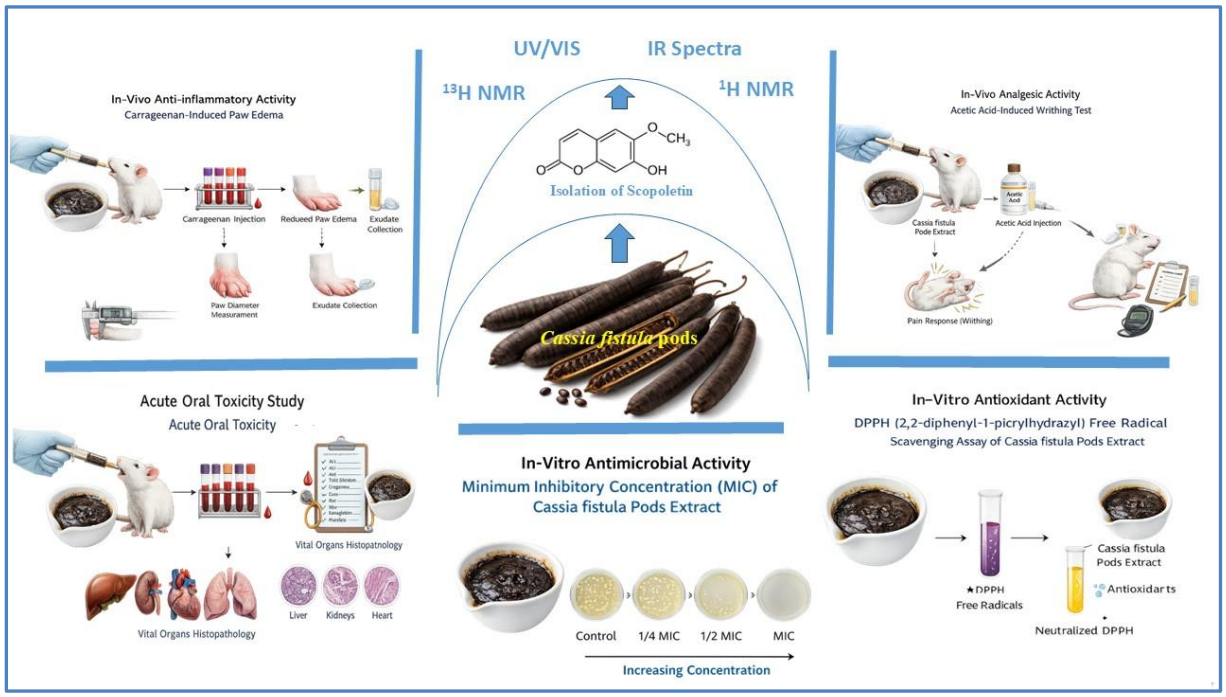
Aim of proposed study was to scientifically validate traditional use of *Cassia fistula* pods for treatment of inflammation and pain and to identify bioactive compound responsible for these effects, with particular emphasis on the COX-2 inhibitory activity of scopoletin. The study evaluated the ethanolic extract of *Cassia fistula* pods for its antioxidant and antimicrobial potential. It also assessed its acute oral toxicity, an anti-inflammatory potential with the carrageenan-induced paw edema animal model, and further its analgesic potential was conducted by acetic acid-induced writhing performance. Scopoletin extraction through silica gel column chromatography and followed with UV and IR and $^{1}H/^{13}C$ NMR spectroscopy for compound identification. Molecular docking executed against the cyclooxygenase-2 (COX-2) enzyme which accessed through the PDB ID: 3NT1. The extract demonstrated high antibacterial effectiveness which achieved 99% pathogen suppression at concentration of 15.63 µg/mL and shows increasing antioxidant strength which reached 56.48 ± 0.12% at 1000 µg/mL. The testing showed no toxic effects at the higher dose of 5000mg/kg. The treatment group achieved 71.27% reduction in paw swelling which reached its peak after 5 hours at the 1000 mg/kg dosage. The analgesic test showed that 56.99% of writhing was eliminated through treatment while naproxen achieved 69.85% of this effect. The docking studies demonstrated that scopoletin formed a strong interaction with COX-2 through a binding energy measurement of -7.3 kcal/mol. These findings identify scopoletin as a major bioactive constituent of *Cassia fistula* pods, supporting its traditional therapeutic use and potential for further pharmaceutical development.

Keywords: **S**copoletin, analgesic, anti-inflammatory, COX-2, antioxidant, antimicrobial

Highlights

- Scopoletin was successfully isolated and characterized from *Cassia fistula* pods; no prior reports exist.
- Acute oral toxicity and organ histopathology confirmed safety up to 5000 mg/kg.
- Ethanolic extract exhibited dose-dependent analgesic and anti-inflammatory activity.
- Molecular docking revealed favorable scopoletin binding to COX-2 (–7.3 kcal/mol).

Graphical Abstract



1. Introduction

Inflammation and pain are fundamental physiological responses to tissue injury, infection, or harmful stimuli and are classified as acute or chronic depending on duration and progression (Soliman and Barreda 2022). While acute inflammation is protective, chronic and uncontrolled inflammation contributes to serious disorders such as arthritis, atherosclerosis, gastritis, musculoskeletal diseases, and cytokine storm syndromes, often accompanied by persistent pain and reduced quality of life (Khumalo et al. 2022; Wang et al. 2021). Pain, particularly chronic musculoskeletal and inflammatory pain, affects a significant percentage of the worldwide populace and is strongly associated with functional disability, frailty, and psychological conditions such as anxiety and depression, especially among the elderly (El-Tallawy et al. 2021). Conventional therapies, including NSAIDs, SAIDs, glucocorticoids, and opioid analgesics, are effective for symptomatic relief but are limited by major adverse effects viz GI injury, renal and hepatic damage, cardiovascular complications, and dependency (Bindu et al. 2020; Vonkeman and van de Laar 2010). Consequently, there is an increasing need for safer and more effective alternatives. Medicinal plants have long been and remain very important sources for analgesic and anti-inflammatory drugs, such as morphine isolated from *Papaver somniferum* and salicylates from *Salix alba* (Licciardi and Underwood 2011), as more than 80% of the population uses plant medicine (Akubugwo et al. 2022). Their rich repertoire of bioactive secondary metabolites underpins multi-targeted therapeutic effects with improved safety profiles, supporting continued exploration of medicinal values plants for allignment of inflammation and pain (Chen et al. 2020).

In India, leaves of plant were traditionally used to treat inflammatory conditions, the flowers were employed as purgative, and fruits were utilized for their antipyretic, anti-inflammatory, abortifacient, purgative, demulcent, and refrigerant charectristics; additionally, the plant was used in traditional healthcare practices for the management of chest complaints,

eye disorders, influenza, and cardiovascular and hepatic ailments. *Cassia fistula Linn*. or the "Golden Shower Tree" is one of the highly researched plant owing to the wide array of its pharmacological properties (Awasthi et al. 2022). It owned by the *Fabaceae* family and is one of the plants utilized in Unani and Ayurveda medicine as a laxative, antipyretic and for its an analgesic and anti-inflammatory potential. It is widely conventionally used in conventional treatment for the management of pain and inflammatory (Duraipandiyan and Ignacimuthu 2007). Such prevalence of inflammatory and pain-associated disorders increases with advancing age. The pods of *Cassia fistula* contain secondary metabolites namely phenolic acids, flavonoids, anthraquinones, coumarins and other phytochemicals associated with a range of beneficial properties (Bhagwati Prashad Sharma et al.). Recent pharmacological studies have validated its therapeutic potential, especially in also oxidative stress–linked pathologies (Siddiqua et al. 2018). Animal models showed that *Cassia fistula*–containing herbal preparations significantly elicit anti-inflammatory, anti-arthritic, and analgesic activities (Yoon and Lee 2017). Moreover, it was also confirm that the ethanolic extracts of *Cassia fistula* fruit pulp significantly decreased collagen-induced arthritis through the inhibition of pro-inflammatory cytokines (TNF-α, IL-1β, IL-17 and IL-6) and the restoration of oxidative balance through modulation of NO, MDA, GSH, and catalase levels (Mishra et al. 2025). These led to marked improvement in joint microarchitecture and disease severity. In another work also found that the ethanolic leaf extract has anti-arthritic properties through inhibition of NF-κB signaling, confirming involvement of sennoside B in countering inflammation and oxidative stress (Mishra et al. 2026). Furthermore, the pod extracts of *Cassia fistula* demonstrated pronounced anti-inflammatory, antibacterial, and antifungal properties, which were proven through GC-MS confirmation of active compounds such as thymine, oleic acid, α-sitosterol, and Vitamin E, along with the attenuation of TNF-α and NO in LPS-stimulated models (Zubairi et al. 2024). Beside its inflammation inhibitor, other hepatoprotective and antioxidant properties

of *Cassia fistula* fruit extracts have been explained based on Keap1 pathways (Ahmed et al. 2025), and recent nanotechnology and molecular docking analyses further support its versatility. Although this information is promising, no study to date has critically examined the analgesic, anti-inflammatory, and antioxidant effects along with its acute, subacute, and specific organ toxicity of the ethanol extract of *Cassia fistula* pods with the reporting of it isolate namely scopoletin. This absence of integrated toxicological and pharmacodynamic data thus underlines the imperative for detailed in-vivo evaluation of the toxicity level of the *Cassia fistula* pod ethanolic extracts. In addition, the importance of scopoletin, a coumarin compound found in the plant *Cassia fistula*, has been established by earlier phytochemical screening. Coumarin compounds, including scopoletin, have been isolated and structurally characterized from the aril of *Cassia fistula* bark using spectroscopic techniques (Ching-Kuo Lee et al. 2001), while LC–MS/MS profiling studies have further confirmed exitance of scopoletin in leaf extracts of the plant (Abdellatif et al. 2024). Although scopoletin is recognized as a major bioactive compound with broad pharmacological potential and is abundantly reported in the leaves and bark of *Cassia fistula*, direct experimental evidence confirming its isolation and mechanistic role from the pods remains limited.

In view of this gap, this study designed to isolate and analyze scopoletin from *Cassia fistula* pod extract, followed by evaluation of its antimicrobial, antioxidant, analgesic and anti-inflammatory potential. Furthermore, molecular docking analysis occurred to elucidate probable mechanism of action by examining interaction of scopoletin with cyclooxygenase-2 (COX-2) enzyme, key molecular target involved in inflammatory pain. Plant extract shown anti-inflammatory and analgesic effects, scientifically investigated and validated through comparison with the commonly used NSAID naproxen. The basic idea behind this integrative method is to establish scientific rationale for use of the pods of *Cassia fistula* and to explore

ways for ensuring the safety and utilization of plant drugs for the relief of pain and inflammation.

2. Materials and Methods

2.1 Materials

Key chemicals utilized in current study were carrageenan (minimum of 98%, Sigma-Aldrich) for carrying out paw edema, glacial acetic acid (minimum of 99.7%, Merck) for carrying out the writhing tests, and diclofenac sodium (minimum of 99%, Sigma-Aldrich) for standardization of the anti-inflammatory and analgesic activity. Carboxymethyl cellulose (CMC; analytical grade, HiMedia) was used as a suspending agent, while normal saline (0.9% NaCl, IP grade) served as the vehicle for drug administration. Ethanol (≥99.5%, Merck) was employed for the preparation of the *Cassia fistula* Pod ethanolic extract (CFPPE) used in the AOT study conducted as per OECD guideline 425. All chemicals used were of AR grade and procured from reputed commercial suppliers.

The antimicrobial activity was evaluated against *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778), and *Candida albicans* (NCIM 3466) utilizing 24-hour-old cultures. The bacterial and fungal strains were obtained from authenticated culture collections and were maintained by subculturing in nutrient broth under aseptic conditions prior to use in the study.

2.2 Extraction of Pods of Cassia fistula Plant

Mature pods of *Cassia fistula* were collected from Wai Forest (Lat 18.03523°, Long 73.963596°), India, and authenticated by a qualified botanist. Pods were washed, shade-dried, and pulverized into fine powder. Extraction was carried out using Soxhlet apparatus with analytical-grade ethanol (95%) for 24 h to ensure through extraction of bioactive constituents. The rotary evaporator was employed to remove excess the solvent under reduced pressure which produced a dark brown viscous extract which was kept at 4 °C until required. For the

purpose of comparative antimicrobial evaluation, additional extracts for the microbial study were made using petroleum ether, ethyl acetate, and water.

2.3. Antimicrobial Activity through Minimum Inhibitory Concentration (MIC)

Antimicrobial (antifungal and antibacterial) activities of CFPPE were evaluated against selected microorganisms, including *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778), and *Candida albicans* (NCIM 3466). Antimicrobial activity was assessed through the broth dilution technique as outlined by Clinical and Laboratory Standards Institute guidelines (PA 2002). Stock solutions of each extract were prepared at a strength of 1000 µg/mL, followed by two-fold serial dilutions to get final concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, 7.81 µg/mL, 3.91 µg/mL, and 1.95 µg/mL. Fresh 24 h cultures of the test organisms were used to prepare the inoculum, which was standardized to obtain turbidity equivalent to 0.08–0.1 absorbance units, corresponding to the recommended microbial density, prior to inoculation. Each test tube having 3 mL of sterile broth medium were inoculated with the standardized microbial suspension, followed by the addition of respective extract concentrations, and then incubated at exact 37 °C for continuous 24 h. Then after, microbial growth and % inhibition were quantified spectrophotometrically by considering absorbance at 600 nm (Nugraha et al. 2019). The MIC was defined as lower strength of the plant extract at which no any visible microbial growth was observed.

2.4 Antioxidant Activity

To assess antioxidant potential of the test sample, DPPH free radical scavenging technique was estimated, which estimate ability of compounds to donate hydrogen atoms and neutralize free radicals (Silva et al. 2024). DPPH reagent was prepared by dissolving the reagent in ethanol and protected from light to prevent photo-degradation until use. Test sample solutions of CFPPE were prepared in solvent namely ethanol at fixed strength of 1000, 500, and 250 µg/mL. For comparison, standard antioxidant solutions of ascorbic acid were prepared at the

same concentrations. In clean test tubes, DPPH solution in amount of 1.0 mL was dissolved with 1.0 mL of each concentration of CFPPE test sample and standard, vortexed gently, and mixture was incubated in the dark environment at ambient temperature for continuous 30 min to allow reaction to proceed. Control or blank solution containing 1.0 mL of DPPH solution and 1.0 mL of ethanol (without test sample or standard) was prepared following the reported method (Chavan et al. 2020). Absorbance was determined at 517 nm by UV/Visible spectrophotometer, with ethanol utilized as a blank. Absorbance values of the control and test samples were used to estimate percentage inhibition of DPPH radicals.

2.5 In-Vivo Activity

2.5.1 Experimental Animals

This investigation was conducted using healthy adult Wistar albino rats of either sex with total body weights from 150 to 200 g. They were kept in cages made of polypropylene with sterile rice husk bedding under controlled laboratory set conditions, including set temperature of 26 ± 1 °C, relative humidity (RH) of 45.0–55.0%, and 12 h light and dark condition. All selected animals were supplied with quality pellet diet and water Ad-libitum throughout experimental period. Prior to initiation of experiment, animals were acclimatized for minimum a week to minimize stress and ensure uniform physiological conditions.

All the experimental procedures were strictly executed in compliance with set guidelines recommended by CCSEA, India of Experiments on Animals, Government of India, otherwise known and referred to as CPCSEA guidelines. The protocol of this study has been assessed and authorized by Institute Animal Ethics Committee (IAEC) formed under BVCP, Near Chitranagari, Kolhapur, Maharashtra 416013, India, and experiments were conducted in compliance with the approved protocol number BVCPK/CCSEA/IAEC/24/2450.

2.5.2 Acute Oral Toxicity Study

An acute study protocol based on OECD Test Guideline 425 was followed. The nulliparous, non-pregnant albino female rats 150–200 g in weight and having 6–8 weeks aged were selected. Animals were acclimated under laboratory conditions for a period of a week before the start of this experiment. In the limit test, selected animals were fasted for complete a one day with access to water prior to administration of single oral dose of test substance at 5000 mg/kg total body weight. Then after completion of treatment, the animals were carefully examined during first four hour and then daily for continuous 14 days after application, mortality, clinical signs of toxicity, and changes in behavioral pattern; the skin and fur, eye, circulation, respiration, autonomic and CNS activity, and motor activity were studied, with special emphasis on the following symptoms: diarrhea, salivation, tremors, convulsions, lethargy, and coma. Body weight was measured on days 0, 7, and 14 for assessment for general health and signs of systemic toxicity. Following the completion of the treatment period, blood samples were received under an anesthetic for determination of hematological and serum biochemical parameters such as liver function tests (LFT i.e. AST, ALT, ALP, and total bilirubin), kidney function parameters (creatinine, urea, and BUN), and blood parameters (RBC, WBC, Hemoglobin, and Platelets) respectively. Following euthanasia, vital organs namely liver, heart, kidneys, and lungs were excised, preserved in 10% formalin, processed, and further stained with hematoxylin (H) and eosin (E) for histopathological examination to detect any treatment-related structural or cellular abnormalities.

2.5.3 In-Vivo Anti-inflammatory Activity

2.5.3.1 Paw Edema Method

For determination of anti-inflammatory potential, healthy rats were further randomly classified into total five cluster, each group consisting of 6 rats, and inflammation was induced with 0.1 mL of carrageenan solution with 1% concentration injected into the subplantar zone of right hind paw in all treated groups.

Group I received carrageenan and served as control group. The animals in Group II received oral administration of naproxen (10 mg/kg) as body weight, completely dissolved in distilled water, and considered as the standard group. Groups III, IV, and V were treated as test sample orally with CFPPE at fixed doses of 1000 mg/kg as high dose category, 500 mg/kg as medium dose category, and 250 mg/kg considered as low dose category, respectively, followed by carrageenan induction. All treatments were administered orally 1h prior to induction of inflammation. Paw volume was measured using a plethysmometer made by UGO Basile, USA after carrageenan injection (0 h) and subsequently at one, two, three, four, and five h. Mean paw volume for each group was calculated and then compared with the carrageenan control and standard animal groups to evaluate anti-inflammatory activity. Percentage inhibition of edema was noted based on the reduction in paw volume by comparing to the control animal. Percentage inhibition of edema was noted using following ***Eq. I***:

$$\%\ \textit{inhibition} = \frac{T0 - Tt}{T0} * 100 \ldots\ldots\ldots\ldots\ldots (Eq.\ I)$$

Where T_t indicates the paw volume of rats receiving the test extract at a given time, and T_o indicates the paw volume of control rats at the same time interval.

2.5.4 In-Vivo Analgesic Activity

2.5.4.1 Writhing test

Peripheral analgesic efficacy of CFPPE was estimated utilizing the acetic acid–induced writhing test (Hyesook Lee et al. 2025). Experimental animals for acetic induced writhing test were randomly clustered into five groups, each group having six rats. Group I considered as the control group and received acetic acid alone. Groups II, III, and IV were treated with test sample at oral dosage of 250 mg/kg, 500 mg/kg, and 1000 mg/kg total body weight, respectively, All selected rats were fasted 12 h (overnight) with free access to water before experiment to ensure uniform metabolic conditions. The dose of the test extract or standard drug for each rat was calculated according to its body weight. All treatments were administered orally one hour

before the induction of pain. Nociception was induced by intraperitoneal injection of freshly prepared acetic acid solution in DW at dosage of 10 mL/kg total body weight. Following acetic acid administration, animals were carefully observed, and the number of writhing responses characterized by abdominal constrictions, pelvic rotation, and extension of hindlimbs was noted for total time of 10 min, starting 5 min after injection. Analgesic activity of extract was estimated by comparing mean number of writhes in treated rat groups with that of the control sample received group, and inhibition of writhing was determined utilizing the appropriate ***Eq. II***.

$$\%\ inhibition = \frac{N\text{-}Nt}{N} * 100 \ldots\ldots\ldots\ldots\ldots (Eq.\ II)$$

Where ***N*** represents average of writhes observed in control treated group, and ***Nt*** represents average of writhes observed in test or standard treatment group. Following the experiment, all animals were monitored for total period of fourteen days to observe any delayed effects or signs of toxicity.

2.6 Isolation by Column Chromatography

The ethanolic extract of *Cassia fistula* pods was successfully subjected to column chromatography for separation of Scopoletin. Defatting was first performed using n-hexane & ethanol (1:1 v/v), and the defatted layer was used for chromatographic separation. The chromatographic column was sealed with 60–120 mesh silica gel by wet packing using solvent as a chloroform, and crude extract was dissolved in chloroform:methanol (90:10 v/v) before being carefully loaded onto column. Elution was carried out utilizing a gradient solvent system of chloroform and methanol in increasing polarity, specifically 100% chloroform, followed by 98:2, 96:4, and 90:10 chloro-form:methanol ratios (Rashid et al. 2017).

2.6.1 Characterization of the isolated Compound

The UV-Visible spectrum of CFPPE was recorded using a double-beam UV-Vis spectrophotometer (Model No: 31-0197-00-0104). The spectrophotometric analysis was carried out in the range of 200 to 800 nm in absorbance mode (Abs), with a spectral bandwidth of 2.00 nm and scan interval of 5.00 nm. The FTIR spectrum of the sample was noted using an FT/IR-4100 type A, Serial No: B137461016. Instrument employed a standard light source with a TGS (Tri-Glycine Sulfate) detector to capture the infrared absorption characteristics of the material. To confirm the presence of isolated compound, Scopoletin, Nuclear Magnetic Resonance (NMR) spectroscopy was performed. Both proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded. The isolated Scopoletin was completely dissolved in deuterated dimethyl sulfoxide (DMSO-d_6) at an appropriate concentration. The 1H NMR spectrum was obtained by using a Bruker spectrometer at 400 MHz, while the NMR (^{13}C) spectra were noted in a same equipment at 100 MHz. The chemical shifts were noted in unit as ppm with respect to internal standard TMS.

2.7 Molecular Docking Study

The molecular docking process was aimed at assessing the interaction of Scopoletin with the cyclooxygenase-2 (COX-2) enzyme. Three-dimensional structure of COX-2 complexed with a naproxen molecule (PDBID: 3NT1) was received from Protein Data Bank (RCSB). Selected protein structure was prepared by utilizing Auto Dock Tools by subtracting all the water molecules and heteroatoms and adding Kollman charges and polar hydrogens to obtain a prepared structure. Chemical structure of Scopoletin was received from PubChem in SDF format and changed to PDBQT format by utilizing Open Babel and AutoDockTools, followed by molecular docking via AutoDockVina. A grid box was generated to define the active site of the protein, with center coordinates set at X: –46.2074, Y: –56.0396, Z: –24.4278, and dimensions of X:41.2566, Y: 33.1457, and Z: 25.0000. The docking simulation was run to predict binding affinity and orientation of Scopoletin within COX-2 binding pocket. Post-

docking analysis and visualization of the ligand–protein interactions were carried out using PyMOL and Discovery Studio Visualizer.

2.8 Statistical Analysis

Experimental results were shown as the average ± standard error mean (SEM), with six rats (n = 6) used in each selected group. Statistical analysis was executed using suitable statistical software tool (e.g., GraphPad Prism). Comparisons among different groups were carried out by applying one-way ANOVA, followed by Tukey's post hoc multiple comparison to verify statistically significant differences selected between groups. Results with (*) $p < 0.05$ were considered statistically significant.

3. Results

3.1 MIC

MIC values were found to be the lowest concentration of each extract without any turbidity observed after the incubation period. The values of MIC of each extract were found to be between 250-1000 µg/ml for different test organisms. The untreated control without bacteria did not exhibit inhibition of growth, while standard drug, fluconazole, exhibited an inhibition of 80% for all four different pathogens. The ethanolic extract displayed concentration-dependent inhibitory activity. When tested in small concentrations (1.95 and 3.91 µg/ml), there was little inhibitory activity (1.20–11.52%) on all strains. Moderate inhibitory activity was detected at 7.81µg/ml, where B. cereus was most sensitive (33.25 ± 0.95 %) compared to E. coli (23.54 ± 0.98 %) and C. albicans (13.54 ± 0.52 %). It can be noted that with the higher strength of the ethanolic extract (15.63 µg/mL), there was a marked antimicrobial activity with a subsequent inhibition of about 99% for all pathogens in this study (**Table 1**). Therefore, there are clear indications that the antimicrobial activity of CFPPE ethanolic extract can be very effective at a higher concentration similar to standard compound mentioned above.

Table 1: MIC of the CFPPE

Sample	% Inhibition against Candida albicans	% Inhibition against Escherichia coli	% Inhibition against Bacillus cereus
Only Bacteria	--	--	--
Fluconazole	80.50 ±1.25 %	81.56 ± 2.25 %	81.78 ± 2.45 %
CFPPE (1.95 µg/ml)	1.99 ± 0.25 %	1.20 ± 0.10 %	1.45 ± 0.15 %
CFPPE (3.91 µg/ml)	1.52 ± 0.12 %	9.05 ± 0.52 %	11.52 0.65 %
CFPPE (7.81 µg/ml)	13.54 ± 0.52 %	23.54 ± 0.98 %	33.25 ± 0.95 %
CFPPE (15.63 µg/ml)	98.98 ± 2.15 %	99.02 ± 1.54 %	99.54 ± 2.14 %

3.2 Antioxidant Activity

Antioxidant potential of CFPPE was evaluated using standard DPPH radical scavenging technique in a 96-well plate format, and the results are presented in **Table 2**. The DPPH radical scavenging activity of ascorbic acid (considered as standard antioxidant), increased in a concentration-dependent manner, showing 34.04 ± 6.01% inhibition at 250 µg/mL, which increased to 47.65 ± 1.74% at 500 µg/mL and reached 60.74 ± 0.63% at 1000 µg/mL. Similarly, Sample CFPPE demonstrated notable free radical scavenging activity with 40.08 ± 6.24 % inhibition at 250 µg/mL, 45.15 ± 0.17% at 500 µg/mL, and 56.48 ± 0.12% at 1000 µg/mL, indicating clear dose-dependent response. At lower (250 µg/mL) concentration, Sample CFPPE showed higher antioxidant activity than the standard, whereas at higher concentrations, ascorbic acid exhibited slightly greater activity. The results suggest that CFPPE possesses significant antioxidant potential, comparable to the standard antioxidant, and may act as an effective free radical scavenger, likely due to existence of phenolic and flavonoid constituents.

Table 2: Effect of CFPPE by using antioxidant activity by DPPH (96 well method)

Sample	Concentration (µg/mL)	% Inhibition (Mean ± SD)
Control	—	—
Ascorbic acid (Standard)	250	34.04 ± 6.01
	500	47.65 ± 1.74
	1000	60.74 ± 0.63
CFPPE	250	40.08 ± 6.24
	500	45.15 ± 0.17
	1000	56.48 ± 0.12

3.3 Toxicity Study

3.3.1 Body Weight Observation:

The total body weight of rats in vehicle treated control and test groups was observed on days 1, 7, and 14 to examine any significant changes due to the administration of CFPPE. As shown in (**Table 3**), the CFPPE Test-I group (2000 mg/kg) showed a comparable weight increase from 210.105±1.7 g to 218.1917±2.6 g, while the CFPPE Test-II group (5000 mg/kg) exhibited a slight but non-significant reduction in weight gain (210.13±2.3 g to 216.1983±2.1 g).

Table 3: Comparative assessment of rat body weight in control and treated (CFPPE Test-I and CFPPE Test-II) rats during the 14th days study period

Groups	**Average weight of rats (grams)**		
	1st day (Mean±SD)	**7th day (Mean±SD)**	**14th day (Mean±SD)**

Control	210.06±2.1	214.1867±1.6	220.2017±2.3
CFPPE Test-I	210.105±1.7	214.155±2.2	218.1917±2.6
CFPPE Test-II	210.13±2.3	212.155±2.5	216.1983±2.1
Note: *Control: Normal group vehicle (with CMC Sodium 0.5%) treated control, CFPPE Test-I: Group treated with pod extract of CA with 2000 mg/kg, CFPPE Test-II: Group treated with pod extract of CA animals treated with dose 5000 mg/kg. No any further signs of toxicity and mortality were noted.*			

Observed study no weight loss in selected groups, indicating that the CFPPE ethanolic extract did not induce adverse metabolic or systemic effects. The results confirm the safety of *Cassia fistula* fruit pod ethanolic extract at both tested doses, as no any observed signs of toxicity or mortality were seen during fourteen days study period shown in (**Table 3**).

3.3.2 Toxic symptoms in the primary test:

No group showed any indications of poisoning, death, or aberrant behavior during the course of the investigation. Parameters such as mortality, itching, tremors, convulsions, coma, sleep disturbances, locomotor activity, urination, salivation, fecal consistency, fur, skin, and eye conditions remained normal, confirming the safety of CFPPE shown in **Supplementary Table 1**.

3.3.3 Biochemical Enzyme level:

Table 4 presents the comparison of average biochemical enzyme levels between control animal group sample animal treated groups (CFPPE Test-I and CFPPE Test-II) of rats during the experimental period. The serum SGOT levels were comparable across all groups, with values of 103.08±0.2 in the control group, 102.75±0.2 in Test-I, and 102.92±0.2 in Test-II,

indicating no significant alteration in hepatic transaminase activity. Similarly, SGPT levels showed only marginal variations, with values of 40.22±0.1 in the control group, 41.60±0.09 in CFPPE Test-I, and 40.84±0.3 in CFPPE Test-II, suggesting that the treatment did not induce hepatocellular damage. Alkaline phosphatase (AP) levels were also found to be consistent among the groups, ranging from 126.41±0.1 in the control to 127.28±0.2 and 127.38±0.2 in Test-I and Test-II, respectively.

The levels of total bilirubin remained in the normal physiological ranges in all groups with a value of 0.08±0.01 in the Control group, 0.05±0.01 in CFPPE Test-I, and 0.09±0.01 in CFPPE Test-II, showing that the bilirubin biosynthesis was normal with an absence of cholestatic injury. The renal function indices, viz creatinine and urea remained unaltered in the Control and Test groups with a marginal decrease in CFPPE Test-I and CFPPE Test-II, measuring 18.39 ± 0.2 and 19.33 ±0.1 mg/dl respectively, compared to the Control (22.13±0.1 mg/dl), but the values of creatinine remained nearly equal, ranging between (0.33 ± 0.009 to 0.41 ± 0.002). The biochemical parameters indicate that the test treatment did not produce adverse effects on liver or kidney function, suggesting that the administered dose was biochemically safe during the study period. No significant alterations in biochemical enzyme levels were noted in any control and treated animals. Liver function and kidney function markers shown in **Table 4**, remained within normal ranges, indicating that CFPPE at 2000 (lower) and 5000 mg/kg (higher) caused no hepatic or renal toxicity.

Table 4: Comparison of mean Biochemical enzyme level of the control animal group and that of tested animal (CFPPE Test-I and CFPPE Test-II) group of rats

Groups	**SGOT**	**SGPT**	**AP**	**Total bilirubin**	**Urea**	**Creatinine**
Control	103.08±0.2	40.22±0.1	126.41±0.1	0.08±0.01	22.13±0.1	0.33±0.009

CFPPE Test-I	102.75±0.2	41.60±0.09	127.28±0.2	0.05±0.01	18.39±0.2	0.34±0.007
CFPPE Test-II	102.92±0.2	40.84±0.3	127.38±0.2	0.09±0.01	19.33±0.1	0.41±0.002
Note: *Control: Normal vehicle (with CMC Sodium 0.5%) treated control group, CFPPE Test-I: Group treated with pod extract of CA with 2000 mg/kg, CFPPE Test-II: Group treated with pod extract of CA group treated with dose of 5000 mg/kg. No any further signs of toxicity and mortality were seen*						

3.3.4 Hematological Parameters

Table 5 shows the comparison of average hematological parameters between control animal group and treated animal groups (Test-I and Test-II) of rats during the experimental period. Hemoglobin (Hb) levels were found to be 15.11 ± 0.07 g/dL in the control group, while a slight reduction to 14.96 ± 0.12 and 14.22 ± 0.08 g/dL was observed in both CFPPE Test-I and CFPPE Test-II groups, respectively; however, these values remained within the normal physiological range, indicating no treatment-related anemia. Hematocrit values were identical (37.53 ± 0.4, 37.62 ± 0.2, 37.28 ± 0.4 for control, CFPPE Test I and CFPPE Test II, respectively) across all groups, suggesting that the packed cell volume was unaffected by the test treatment.

Total white blood cell (WBC) counts showed a mild increase in the treated (CFPPE Test I and CFPPE Test II) groups (4.16±0.07 and 4.13±0.19 $\times 10^3/\mu L$) compared with control group ($3.93\pm0.1\times10^3/\mu L$), which may indicate a normal physiological or immunological response rather than pathological alteration. Red blood cell (RBC) counts were comparable, with values of $7.93 \pm 0.01\times10^3/\mu L$ in the control and CFPPE Test-I groups 7.85 ± 0.02 and a slight increase to $8.09 \pm 0.01\times10^3/\mu L$ in CFPPE Test-II. Platelet counts were higher in the treated groups

(753.68 and 796.73 $\times 10^3/\mu L$ in CFPPE Test-I and CFPPE Test-II, respectively) compared to the control (663.25 $\times 10^3/\mu L$), but these differences were not indicative of any adverse hematological effect.

Red cell indices, including mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), showed marginal increases in the treated groups (MCV: 54.14±0.01; MCH: 18.83±0.2 pg in CFPPE Test 1; MCV: 54.27±0.02; MCH: 18.65±0.1 pg in CFPPE Test 1I) compared to the control group (MCV: 51.64±0.01 fl; MCH: 17.24±0.1 pg), reflecting normal erythrocyte morphology and hemoglobin content. The hematological profile demonstrates that the test treatment did not produce any significant or detrimental alterations in blood parameters, confirming its hematological safety during the study period. Hematological analysis showed no significant changes between sample treated and control animal groups. Parameters in **(Table 5)**, remained within normal limits, indicating no hematotoxic effects. The results suggest that CFPPE at 2000 and 5000 mg/kg is hematologically safe.

Table 5: Comparison of mean Hematological Parameters of control anima group and that of the tested animal (CFPPE Test-I and CFPPE Test-II) group of rats

Groups	**Hb (g/dl)**	**Hematocrit (%)**	**WBC ($10^3/\mu l$)**	**RBC ($10^3/\mu l$)**	**Platelets ($10^3/\mu l$)**	**MCV (fl)**	**MCH (Pg)**
Control	15.11±0.07	37.53±0.4	3.93±0.1	7.93±0.01	663.25±0.01	51.64±0.01	17.24±0.1
CFPPE Test-I	14.96±0.12	37.62±0.2	4.16±0.07	7.85±0.02	753.68±0.1	54.14±0.01	18.83±0.2
CFPPE Test-II	14.22±0.08	37.28±0.4	4.13±0.19	8.09±0.01	796.73±0.1	54.27±0.02	18.65±0.1

Note*: Control: Normal vehicle (CMC Sodium 0.5%) treated control group, Test-I: Group treated with pod extract of CA with 2000 mg/kg, Test-II: Group treated with pod extract of CA group treated with dose of 5000 mg/kg. No any further signs of toxicity and mortality were observed.*

3.3.5 Observation results of organs

Observation based on (**Supplementary Table 2**), carried out no abnormalities in color, surface texture, or consistency across control and treated groups. Organs maintained their normal appearance without signs of congestion, discoloration, or structural damage, indicating that CFPPE caused no visible toxicity.

3.3.6 Relative Organ Weight Analysis:

The relative organ weights of heart kidneys, spleen, liver, and lungs, exhibited no any significant differences between control and treated groups. The weights remained found in (**Table 6**), within normal physiological limits, with *p*-values > 0.05 for all organs, indicating no statistically significant changes or toxicity at 2000 and 5000 mg/kg doses of CFPPE.

Table 6: Observations on relative organ weights

Organs	**Relative organ weight (%)**		
	Control	**Test-I**	**Test-II**
Liver	3.76 ± 0.35	4.82 ± 1.46	4.20 ± 0.5
Heart	0.82 ± 0.24	0.83 ± 0.07	0.82 ± 0.1
Spleen	0.80 ± 0.21	0.80 ± 0.07	0.82 ± 0.09
Lungs	1.86 ± 0.42	2.01 ± 0.07	1.87 ± 0.05
Right Kidney	0.79 ± 0.4	0.77 ± 0.03	0.76 ± 0.04

Left Kidney	0.80 ± 0.31	0.81 ± 0.03	0.80 ± 0.04
Note*: Control: Normal vehicle (CMC Sodium 0.5%) treated control, Test-I: Group treated with pod extract of CA with 2000 mg/kg, Test-II: Group treated with pod extract of CA group treated with 5000 mg/kg. No found weight loss.*			

3.3.7 Organ histopathology

This research examined the histopathological differences in kidney, liver, heart, lung and spleen organs after acute oral CFPPE administration through histopathological examinations of vital organs. Microscopic evaluation of tissues required H&E staining after processing and sectioning through standard methods. The liver sections obtained from groups receiving 2000 mg/kg and 5000 mg/kg doses revealed normal hepatocellular arrangements and no hepatotoxic indicators such as inflammation or necrosis or fatty changes. The kidney sections displayed healthy glomeruli and renal tubules without signs of cellular damage or degeneration or fibrosis because no nephrotoxic effects were detected for evaluation. No cardiac tissue abnormalities were found on histological examination of the heart which demonstrated both normal myocardial fibers and the absence of inflammatory infiltration or necrosis thus ruling out cardiotoxicity caused by the extract (**Figure 1**).

An evaluation of lung tissues revealed normal alveolar spaces together with intact bronchioles and no congestion signs or hemorrhage or inflammation of inflammatory cells thus demonstrating pulmonary safety. The spleen maintained normal distributions of white and red pulp areas while avoiding both lymphoid depletion and pathological modifications which indicated unaffected immune function. Organ histopathological examinations matched biochemical and hematological test results which proved that the CFPPE did not harm major organs both morphologically and functionally. The absence of tissue abnormalities in all

specimens validates the secure utilization of this extract for pharmaceutical research and utilization.

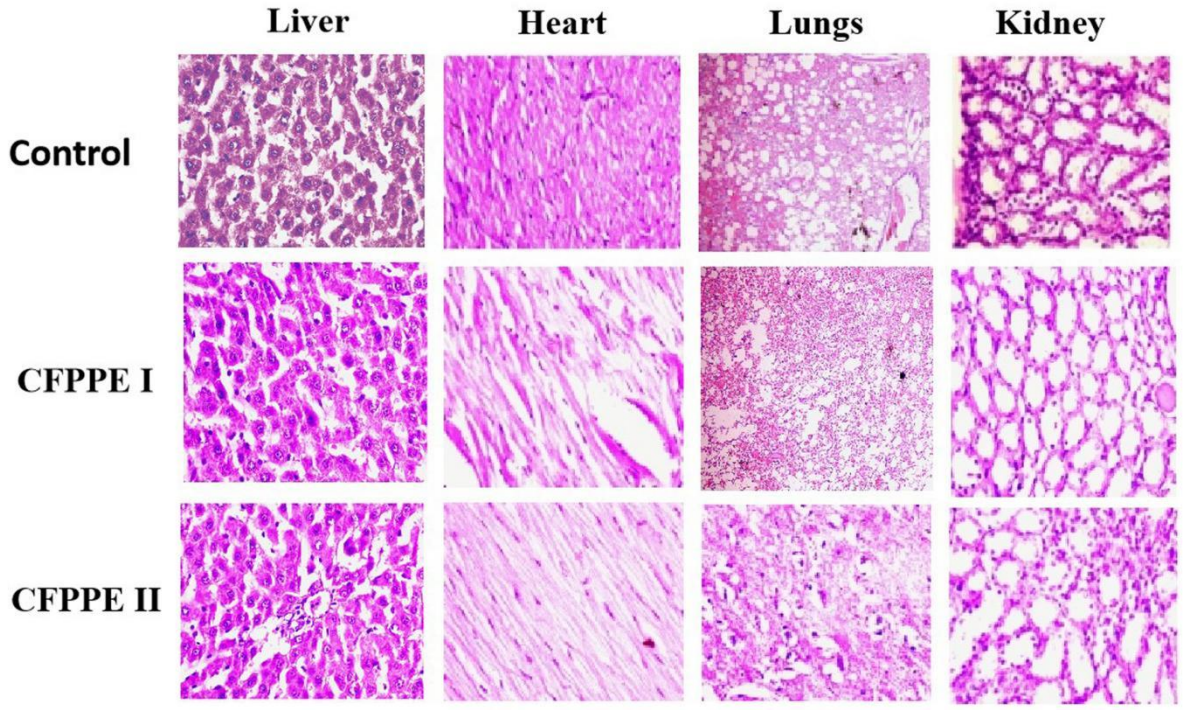


Figure 1: Representative histopathological sections of the examined organs stained with hematoxylin and eosin (H&E). **C:** Normal control animals group receiving 0.5% CMC-Na; **CFPPE I:** CFPPE-treated group (2000 mg/kg body weight); **CFPPE II:** CFPPE-treated group (5000 mg/kg body weight).

3.4 In-Vivo Anti-Inflammatory Activity

3.4.1 Paw Edema

CFPPE reduced paw edema in concentration-dependent patten in inflamed paw edema model using carrageenan. In control animal group, paw edema was allowed to progress to a maximum of 3.83 mL, which was recorded 5 hours later, and increased in volume continuously during the exposure time. At the 250 mg/kg extract dose rate, CFPPE I, Response 1.5a recorded a moderate decrease in volume where the 5 hour value was 1.81 ml. This was comparable to a 52.14% decrease relative to control. In CFPPE II at the maximum of 500 mg/kg which was an escalation with no intermediate results, produced a far greater active response with volume to

1.14 ml at 1 hour and to 1.16 ml at the 5 hour mark amounting to an overall decrease of 17.98 and 69.71% relative to control (**Table 7**). Of the extract treated groups the 1000 mg/kg CFPPE III had the greater proportion of anti-inflammatory effect with the 5 hour pace recorded at 1.1 and a decrement of 1 ml at the 1 hour mark. Inhibition during exposure of 28.05 relative to control and 71.27 % at the end of the exposure.

The standard drug naproxen (10 mg/kg) effectively reduced paw edema at all-time points, with paw volumes decreasing from 0.57 ml at 1 hour to 1.00 ml at 5 hours. **Table 8** summarizes the effect of *Cassia fistula* pod ethanolic extract (CFPPE) on carrageenan-induced edema in rats, relative to standard (10 mg/kg), for the period of 5 hours. The standard group showed a pronounced and time-dependent inhibition of paw edema, with inhibition values of 58.99 ± 1.39% at 1 hour, progressively increasing to 73.89 ± 0.33% at 5 hours, confirming its strong anti-inflammatory efficacy.

Among the extract-treated groups, CFPPE I (250 mg/kg) showed mild inhibition at the initial phase (7.19 ± 0.06 % at 1 hour), Followed by gradual enhancement anti-inflammatory activity, reaching 52.14 ± 1.52% inhibition at 5 hours. CFPPE II (500 mg/kg) demonstrated moderate and more consistent inhibition, with 17.98 ± 1.37 % at 1 hour, increasing to 43.09 ± 1.48 % at 2 hours and achieving a marked inhibition of 69.71 ± 0.52% at 5 hours. The most intense effect within the extract-treated groups was achieved by the CFPPE III at a dose of 1000 mg/kg, which demonstrated 28.05 ± 0.69 % at 1 hour and a time-dependent increase to reach 71.27 ± 0.97 % at the 5th hour.

The results indicate a clear dose- and time-dependent anti-inflammatory activity of CFPPE. Although the standard drug showed superior inhibition throughout the experimental period, the higher doses of CFPPE (500 and 1000 mg/kg) exhibited comparable effects at later phases of inflammation, particularly at the 5th hour. The results indicate that CFPPE exerts significant, progressively increased anti-inflammatory activity with increasing dose, with higher dose approaching the efficacy of the standard drug naproxen. This means the extract

works better when the inflammation from carrageenan is ongoing, possibly due to inhibition of prostaglandin-mediated pathways, thereby supporting with its potential considering as natural anti-inflammatory agent.

Table 7: Action of pod extract of CFPPE at different doses (250 and 500 mg/kg, and 1000 mg/kg) and Naproxen 10 mg/kg as compared to carrageenan induced control rat group at 1, 2, 3, 4 and 5 h in carrageenan-induced paw edema animal model using Plethysmometer

Groups	**Treatment (mg/kg) p.o.**	**Paw edema volume (ml)**				
		1 hr.	**2 hr.**	**3 hr.**	**4 hr.**	**5 hr.**
Control (0.1 ml of 1% w/v Carrageenan)	-	1.39±0.03	2.39±0.03	3.71±0.07	3.27±0.04	3.83±0.02
Standard (Naproxen) (0.1 ml of 1% w/v Carrageenan)	10	0.57±0.05	0.89±0.07	1.19±0.07	1.01±0.05	1.00±0.03
CFPPE 1 (0.1 ml of 1% w/v Carrageenan)	250	1.29±0.09	1.54±0.03	2.50±0.07	1.93±0.07	1.81±0.09
CFPPE 2 (0.1 ml of 1% w/v Carrageenan)	500	1.14±0.03	1.36±0.06	2.11±0.09	1.21±0.05	1.16±0.06
CFPPE 3 (T-III + 0.1 ml of 1% w/v Carrageenan)	1000	1.00±0.04	1.24±0.05	1.98±0.08	1.10±0.04	1.10±0.07

Table 8: Inhibition of paw edema in extract treated rats and carrageenan-treated rats

Groups	% of Inhibition of paw edema				
	1 Hour	**2 Hour**	**3 Hour**	**4 Hour**	**5 Hour**
Standard (10mg/kg)	58.99±1.39	62.76±1.06	67.92±0.39	69.11±0.24	73.89±0.33
CFPPE I (250mg/kg)	7.19±0.06	35.56±0.95	32.61±0.93	40.97±1.40	52.14±1.52
CFPPE II (500mg/kg)	17.98±1.37	43.09±1.48	43.12±1.54	41.89±1.41	69.71±0.52
CFPPE III (1000mg/kg)	28.05±0.69	48.11±0.76	46.63±1.34	46.17±1.82	71.27±0.97

3.5 In-Vivo Analgesic Activity

3.5.1 Writhing test

CFPPE produced a dose-dependent reduction in writhing response in Wistar rats. In the control group, animals exhibited an average of 37.69 ± 0.62 writhes in 10 minutes. Rats treated with the CFPPE at 250 mg/kg (CFPPE I) noted 33.16 ± 0.52 writhes, corresponding to 12.12% inhibition. At 500 mg/kg (CFPPE II), the number of writhes decreased to 28.13 ± 0.47, with 25.36% inhibition. Highest dose of 1000 mg/kg (CFPPE III) noted in a further reduction to 16.21 ± 0.41 writhes (**Table 9**), achieving 56.99% inhibition. The standard drug naproxen (10 mg/kg) exhibited the most pronounced effect, with 11.36 ± 0.66 writhes and 69.85 % inhibition. These data clearly indicate that there's a dose-dependent reduction in total number of writhes with an increase in concentration of CFPPE.

Table 9: Effect of pod extract of CFPPE on Acetic Acid Induced Writhing Response in Wistar Rat

Groups	Treatment (mg/kg) p.o.	Number of writhing in 10 min	% inhibition of writhing
Control	-	37.69±0.62	-
Standard	10	11.36±0.66	69.85±0.74
CFPPE I	250	33.16±0.52	12.12±0.45
CFPPE II	500	28.13±0.47	25.36±0.66
CFPPE III	1000	16.21±0.41	56.99±0.75

3.6 Isolation by Column Chromatography

The ethanolic extract of *Cassia fistula* pods was successfully separated using column chromatography, and different fractions of varying solvent polarities were obtained. After the defatting procedure using n-hexane:ethanol (1:1), the defatted extract was charged to a column chromatography (preparative) using silica gel (60-120 mesh), where $CHCl_3$ served as the chromatographic solvent. This led to four major fractions after sequential elution was executed with gradient system consisting of chloroform:methanol in the ratio 100:0, 98:2, 96:4, and 90:10 v/v. The fraction isolated was collected, concentrated, and further structural confirmation done using UV, IR, and NMR spectroscopic analyses.

3.7 Characterization of isolated Compound

The UV-Visible spectroscopy analysis of the isolated Scopoletin was tested with a UV/vis spectrophotometer instrument across selected wavelength range started from 200 to ended with 800 nm. Result shows that there were three major peaks at 345 nm, 230 nm, and 215 nm. The major peak that appeared at 345 nm shows that it contains a conjugated aromatic system, indicating that it is a coumarin compound, such as Scopoletin (**Figure 2a**).

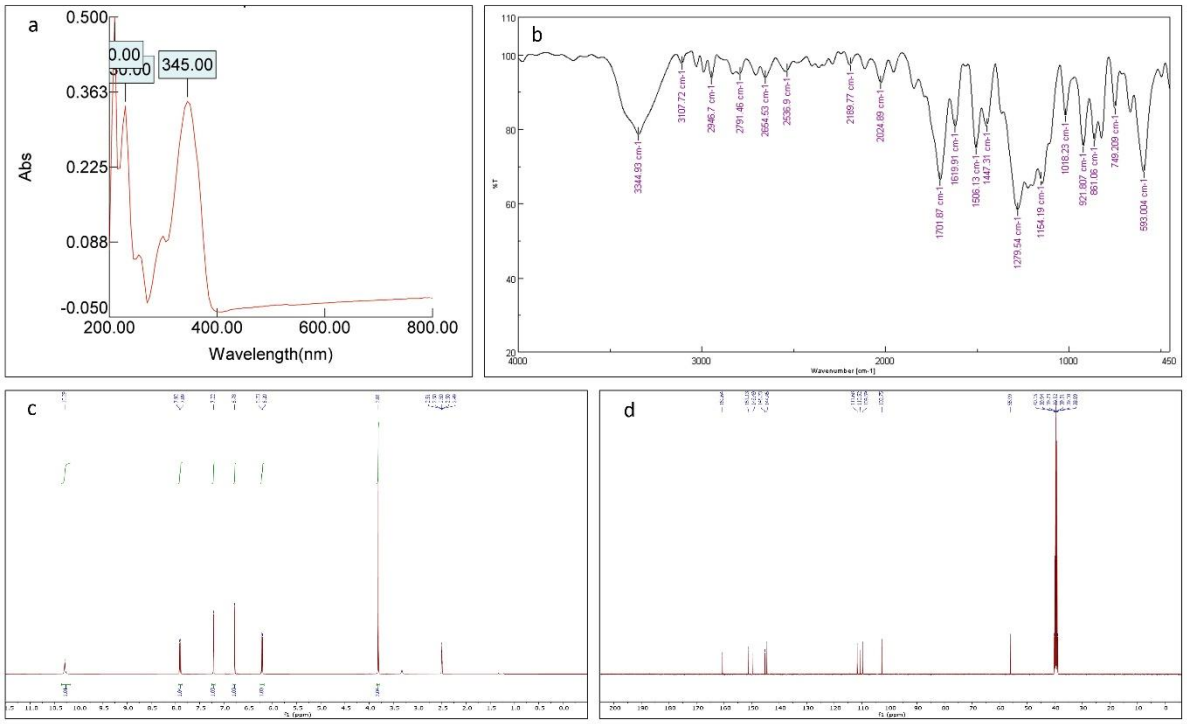


Figure 2. Spectroscopic characterization of isolated scopoletin. **(a)** UV–Visible spectrum of isolated scopoletin recorded in band of 200–800 nm, showing major absorption maxima at 345, 230, and 215 nm, characteristic of a conjugated aromatic coumarin system. **(b)** FT-IR spectrum of isolated scopoletin displaying characteristic absorption bands corresponding to phenolic O–$H_{stretch}$, aromatic and aliphatic C–$H_{stretch}$, and methoxy-related vibrations, confirming key functional groups of scopoletin. **(c)** ^{1}H NMR spectrum of isolated scopoletin showing diagnostic proton signals, including aromatic and olefinic protons and a methoxy singlet, in close agreement with standard scopoletin and reported literature data, confirming its structural framework. **(d)** ^{13}C NMR spectrum of isolated scopoletin exhibiting ten distinct carbon resonances corresponding to aromatic, olefinic, carbonyl, and methoxy carbons of the coumarin scaffold, consistent with reported ^{13}C NMR data and confirming the identity and purity of scopoletin.

The IR spectra of isolated compounds showed characteristic absorption peaks verifying the functional groups related to scopoletin, broad spectra at 3344.93 cm^{-1} were ascribed to O-

H bonds of phenolic functional groups. Aromatic C–$H_{stretch}$ was observed at 3107.72 cm^{-1}, while bands at 2946.70, 2791.46, and 2654.53 cm^{-1} indicated aliphatic C–$H_{stretch}$ and overtone vibrations associated with methoxy substituents (**Figure 2b**). Strong absorption at 1701.87 cm^{-1} confirmed presence of conjugated lactone C=O group, characteristic of the coumarin nucleus. The band at 1619.91 cm^{-1} represented aromatic C=$C_{stretch}$, further supporting the conjugated system. Peaks at 1279.54 cm^{-1} and 1154.19 cm^{-1} corresponded to C–O–C and C–O stretching **(Figure 2b)**, confirming presence of a methoxy and phenolic ether functionalities.

The structural elucidation of Scopoletin (7-hydroxy-6-methoxy coumarin) was carried out using proton and carbon-13 NMR (^{1}H and ^{13}C NMR) spectroscopy. Spectra were observed in DMSO-d_6, and the chemical shifts are noted in parts per million (δ ppm).

A comparative analysis of the ^{1}H NMR spectral data of scopoletin obtained in the present study with that of the standard scopoletin and previously reported data by (Bhatt Mehul et al. 2011). The ^{1}H NMR spectrum of the isolated fraction exhibited characteristic proton signals consistent with the scopoletin framework. Singlet peak noted at δ 6.78 (1H, s) corresponds to H-2, closely matching the chemical shifts reported for the standard (δ 6.79) and Bhatt's isolated scopoletin (δ 6.79). The proton at position H-5 appeared as a singlet at δ 7.22 (1H), showing good agreement with the standard (δ 7.14) and literature values (δ 7.13) (**Figure 2c**). The aromatic protons H-7 and H-8 showed diagnostic doublet signals at δ 7.91 (1H, d, J = 9.5 Hz) and δ 6.21 (1H, d, J = 9.4 Hz), respectively, indicating ortho coupling and corroborating the reported data for standard scopoletin and Bhatt's work. Additionally, the methoxy protons (H-11) resonated as a singlet at δ 3.81 (3H), which is in close proximity to the standard and literature values (δ 3.93). Overall, the close correspondence of chemical shifts and coupling constants confirms the identity and structural integrity of scopoletin isolated in the present investigation.

The ^{13}C NMR spectrum of Scopoletin, consistent with its molecular formula $C_{10}H_8O_4$, reveals ten distinct carbon resonances corresponding to the expected carbon environments

present within the coumarin scaffold, including aromatic, olefinic, carbonyl, and methoxy functionalities. The comparative ^{13}C NMR spectral data of scopoletin isolated in the present study with the reported ^{13}C NMR data from Shah et al. 2014 (Shah et al. 2014). The ^{13}C NMR spectrum (100 MHz) of the isolated fraction displayed carbon resonances characteristic of the scopoletin skeleton and showed close agreement with the literature values. The aromatic and olefinic carbons appeared at δ 110.52 (C-1) and δ 109.59 (C-2), which correspond well with the reported values of δ 111.4 and δ 107.4, respectively. The oxygenated aromatic carbons were observed at δ 149.49 (C-3) and δ 145.23 (C-4), closely matching Shah's data (δ 149.6 and δ 143.2) (**Figure 2d**). Signal at δ 102.75 was allocated to C-5, while the downfield resonance at δ 151.13 corresponded to C-6, indicating the presence of hydroxyl substitution. Further aromatic carbon signals were recorded at δ 144.45 (C-7) and δ 111.68 (C-8), showing good conformity with the reported literature values. The lactone carbonyl carbon (C-9) appeared at δ 160.64, which is consistent with the characteristic carbonyl resonance of scopoletin. Additionally, the methoxy carbon (C-11) resonated at δ 55.99, in close agreement with Shah's reported value of δ 56.3. The close correspondence of the ^{13}C NMR chemical shifts confirms the structural identity and purity of scopoletin isolated in the present study. Collectively, chemical shifts and coupling patterns of both ^{1}H and ^{13}C NMR spectra match the reported data for scopoletin, confirming the identity, structural integrity, and high purity of the isolated compound.

3.8 Molecular Docking:

Docking study revealed the binding behavior of Scopoletin with the high-resolution crystal structure of naproxen-bound COX-2 enzyme (PDB ID: 3NT1). Docking was performed using a predefined grid box centered at coordinates X: –46.2074, Y: –56.0396, Z: –24.4278, with grid dimensions of X: 41.2566, Y: 33.1457, and Z: 25.0000. Scopoletin demonstrated a favorable binding affinity of –7.3 kcal/mol, indicating stable accommodation within the active site

(Figure 3a). The ligand established significant van der Waals interactions with residues SER A:353, TYR A:355, PHE A:518, MET A:522, and SER A:530, which play crucial role in stabilizing ligand within the catalytic pocket. Additional Pi-sigma bonding was found for VAL A:349 (5.48 Å), ALA A:527 (3.48 Å), and LEU A:325 (3.74 Å). These bonds add to the hydrophobic complementarity of the binding pocket (**Figure 3b**). Additionally, an amide-π stacking interaction with GLY A:526 (3.94 Å) added to the stabilization of the ligand. Taken together, these bonds indicate that Scopoletin is a potent binder to the naproxen-binding site of COX-2 and may have selective COX-2 inhibitory potential.

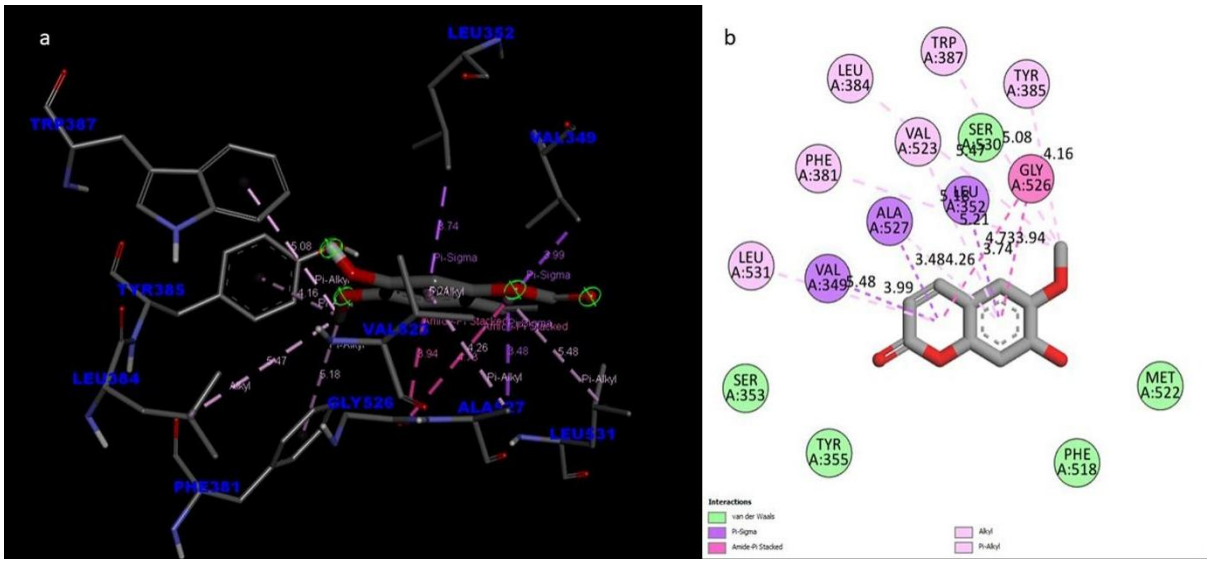


Figure 3: Scopoletin exhibited a promising binding with 3NT1 receptor with affinity of –7.3 kcal/mol **(a)** 2D interaction image of Scopoletin binding with 3NT1 **(b)**

4. Discussions:

Cassia fistula pods contain abundant phytochemicals and bioactive constituents with diverse pharmacological properties, making them valuable in management of numerous disorders and diseases (DK Sharma et al. 2022). The composition and characterization of the alcoholic extract of *Cassia fistula* pods were examined, and its antioxidant, anti-inflammatory, antimicrobial, and analgesic activities were assessed. Phenolic compounds extracted using an alcoholic solvent showed a yield of 15.52 %. Methanol has been identified as an optimal solvent

for phenolic extraction due to its inhibitory effect on polyphenol oxidases involved in polyphenol degradation (Lihu Yao et al. 2004). CFPPE exhibit highest proanthocyanidin, total phenolic, and flavonoid contents among all plant parts, showing strong correlations with antioxidant potential as measured by FRAP and TEAC assays, thereby highlighting the pods as the most potent antioxidant-rich reproductive organs (Luximon-Ramma et al. 2002). Phytochemical evaluation of *Cassia fistula* Linn pods using different extraction solvents revealed the presence of several therapeutically important secondary metabolites. Methanolic, aqueous, and fresh juice extracts of young pods were studied to contain flavonoids, saponins, tannins, glycosides, and alkaloids, although variations in phytochemical presence were observed depending on the solvent used. These results have emphasized the role of extraction solvent on phytochemical content and have also proved the medical value of pods of *Cassia fistula* for alignment management.

The current research gives a full chemical and pharmacology basis to the analgesic activity of the extract via successful isolation and computer simulation verification of scopoletin. In addition, the analysis done using the GC–MS technique was able to identify various compounds present within the pod extract, which includes thymine, furancarboxaldehyde, valeric acid, vitamin E, n-hexadecanoic acid, oleic acid, myo-inositol derivatives, and α-sitosterol, giving credence to the pods' pharmacological values and their use within functional teas (Zubairi et al. 2024). Although earlier the study has shown various pharmacological values, the study still has some limitation that should be noted. First, the study has been done on an *in vitro* level; therefore, the anti-inflammatory, antibacterial, and antifungal properties shown within the CFPPE might not necessarily address the issue within the *in vivo* and clinical level, which might have been its original aim if done on that level. In fact, the very less literature is available for the pods.

Earlier work had already shown that the pods of *Cassia fistula* contained very high amounts of polyphenols, proanthocyanidins, and flavonoids (Luximon-Ramma et al. 2002), which had a very good positive correlation with the antioxidant value of the plant extract. These types of phytochemicals are very well known to show anti-inflammatory properties through the mechanism of free radical scavenging activities; therefore, the very high content of these types of phytochemicals in the pods of *Cassia fistula* can be related to its anti-inflammatory properties or activities. These findings were supported earlier research, identified other phytoconstituents in methanolic, aqueous, or fresh extracts of pods including flavonoids, saponins, tannins, glycosides, and alkaloids, thus reaffirming their medical utility. Cumulatively, these works provide sound phytochemical justification for the anti-inflammatory and analgesic potentials of *Cassia fistula* pods, but the literature to date is filled with various works on phytochemical screening and *in vitro* assessments. More significantly, no proper *in vivo* studies have been conducted so far to confirm these pharmacological activities; neither are there any systematic toxicity studies that present information on organ specific safety or histopathological changes. The absence of *in vivo* efficacy data and detailed toxicological profiling, including organ toxicity and histopathology, represents a significant research gap. Therefore, future studies should focus on *in vivo* anti-inflammatory and *in vivo* analgesic models alongside acute and chronic toxicity assessments to establish the safety, efficacy, and translational potential of *Cassia fistula* pod extracts.

Investigating the phytopharmacological potential of CFPPE especially for its antimicrobial, antioxidant, anti-inflammatory, and analgesic characteristics, current study found the antioxidant potential of the CFPPE to be moderate. The CFPPE exhibited a dose-dependent scavenging effect during the DPPH free radical scavenging assay, likely owing to presence of bio-phytochemicals viz. flavonoids, phenolic compounds, and other secondary metabolites which are known to possess free radical scavenging properties (Zahra et al. 2024).

Presence of mentioned above bioactive phytochemicals suggests that the extract could alleviate oxidative stress that is implicated in numerous chronic inflammatory and degenerative conditions.

MIC testing for anti-microbial activity showed that all the solvent extracts of *Cassia fistula* pods, pet-ether, ethyl-acetate, water, and ethanol, had the ability to inhibit the microbial growth depending on the concentration. The most potent of all was the alcoholic extract. MIC values were 250 µg/ml to 1000 µg/ml, indicating that extract is capable of inhibiting growth of both Gram +Ve and Gram -Ve bacterial strains as well as fungi, thus showing its potential as broad-spectrum antimicrobial agent.

Carrageenan-induced edema animal model, ethanolic extract exhibited a considerable anti-inflammatory effect that was dependent on the dose. Extract at selected doses of 250 mg/kg, 500 mg/kg, and 1000 mg/kg caused a gradual decrease in volume of the paw over the 5 hours of observation, with the 1000 mg/kg dosage having a similar effect to that of naproxen (10 mg/kg), the standard drug. The observed findings pointing that extract could strongly inhibit acute inflammation, probably due to inhibition of release of pro-inflammatory mediators during process of edema caused by carrageenan. On the other hand, writhing test, CFPPE extract exerted protective analgesic response depending on the doses. Based on 56.99% inhibition at 1000 mg/kg, a decrease in total number writhes exhibited by higher doses of the extract relative to the 69.85% inhibition of naproxen. It indicates that the analgesic component of this extract acts peripherally by affecting the inflammation mediator, prostaglandin, which mediates pain. The findings present preclinical evidence for the CFPPE for its antimicrobial, antioxidant, anti-inflammatory, pain relieving properties, and other pharmacological potential.

Column chromatography: Scopoletin came out as one of the prominent biological constituents of the plant by column chromatography, whose identity was unambiguously established by ^{1}H and ^{13}C NMR spectra, implying a high degree of similarity with standard samples and earlier

literature values. The presence of typical peaks of aromatic, oxygenated, and lactone carbons of the separated coumarin confirms its integrity.

The noted analgesic potential of the CFPPE extract can be strongly correlated with presence of scopoletin, a compound extensively reported for its antinociceptive and anti-inflammatory characteristic. As highlighted by Gao et al., 2024, scopoletin mediates analgesic effects primarily through suppression of inflammatory mediators namely prostaglandins, nitric oxide, and pro-inflammatory cytokines, along with attenuation of oxidative stress–induced nociceptive signaling (Gao et al. 2024). These selected mechanisms are mainly relevant in peripheral pain models, where cyclooxygenase-2 (COX-2) plays crucial role in prostaglandin biosynthesis and pain sensitization.

Substantial experimental evidence demonstrates that scopoletin effectively inhibits inflammation in various *in vivo* models, including mouse ear swelling caused by 12-O-tetradecanoylphorbol-13-acetate, ethyl phenylpropiolate, carrageenan, croton oil, and 2,4-dinitrochlorobenzene, in addition to models that show inflammation in the paw and skin. These anti-inflammatory effects are closely associated with reduced production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and nitric oxide, which collectively contribute to inflammatory pain. At the cellular level, scopoletin significantly suppresses COX-2 and inducible nitric oxide synthase (iNOS) in human gingival fibroblasts and RAW 264.7 macrophages that have been stimulated with lipopolysaccharide (LPS), resulting in reduced levels of TNF-α, IL-6, and Nitric Oxide (Gao et al. 2024). Mechanistic studies further revealed that scopoletin inhibits phosphorylation and degradation of IκBα, thereby blocking nuclear factor-κB (NF-κB) activation and downstream inflammatory gene transcription (Moon et al. 2007). Scopoletin is used to stop the making of inflammatory cytokines by blocking the IkappaB/NF-kappaB signal pathway in human mast cell line called HMC-1 (Leventhal et al. 2006). Furthermore, scopoletin suppresses leukocyte infiltration and

macrophage activation in monosodium urate crystal–induced inflammation by modulating mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways (Xiujuan Yao et al. 2012). Similar inhibition of NF-κB and p38 MAPK signaling has been noted in pleurisy and central nervous system inflammation models, further confirming its multi-target anti-inflammatory mechanism (Pereira dos Santos Nascimento et al. 2016).

To further substantiate the mechanistic basis of analgesic activity, molecular docking analysis was performed against the high-resolution crystal structure of the naproxen-bound COX-2 enzyme (PDB ID: 3NT1). As depicted in **Figure 3a**, scopoletin showed promising binding affinity of –7.3 kcal/mol, indicating a stable and energetically favorable interaction within the COX-2 catalytic pocket. The docking pose revealed interactions with key amino acid residues involved in substrate recognition and enzyme inhibition, comparable to those observed with established non-steroidal anti-inflammatory drugs such as naproxen. This supports a direct COX-2 inhibitory mechanism, which aligns well with the reported suppression of prostaglandin synthesis and inflammatory pain mediators.

These in silico findings strongly complement the experimental analgesic observations and align with the conclusions of the recent literature by Gao *et al.* (2024), which emphasizes COX-2 inhibition and NF-κB modulation as central pathways underlying scopoletin-mediated antinociceptive and anti-inflammatory action. Furthermore, reported reduction of TNF-α, IL-1β, and NO levels by scopoletin-rich extracts in osteoarthritis and other inflammatory conditions supports its clinical relevance (Wan Osman et al. 2019).

Taken together, the evidence collected through chromatographic isolation and identification, spectral analysis, biological data, molecular docking results, and already known and proven pathways confirms that scopoletin can be considered the major constituent in anti-inflammatory and analgesic action possessed by plant extract. The consistency in data from studies and recent pharmaceutical reviews on the pharmacological activity of scopoletin

indicates the importance and further development potential of scopoletin as a natural dual target COX-2 selective analgesic and anti-inflammatory agent.

5. **Conclusions:**

The CFPPE has yielded variable degrees of pharmacological effects against differing biological models. The extract possessed good antimicrobial activity (Minimum Inhibitory Concentration: 3.91 µg/ml), appreciable antioxidant property (56.48 % inhibition at 1000 µg/ml), in addition to good anti-inflammatory (71.27 % inhibition of edema) and analgesic action (56.99 % inhibition of writhing). These findings not only may confirm the use of herb in traditional medicine but also its use as major source of natural anti-inflammatory and analgesic drugs, and further, it can be stated that it is owing to the combination of chromatographic isolation, spectroscopic analysis, evidence of bioactivity, docking studies, and existing mechanism of action that there is conclusive evidence of scopoletin playing an important role in analgesic and anti-inflammatory properties of this extract. Future studies will emphasize mechanistic analyses to uncover signaling pathways involved and to identify novel bioactive compounds with pharmaceutical relevance.

CRediT authorship contribution statement

Jayant C. Thorat: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing **Sonali V. Dhamal:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing, Supervision, **Somnath D. Bhinge:** Writing – original draft, Writing – review & editing. **Asha S. Jadhav:** Writing – original draft, Writing – review & editing.

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Declaration of Competing Interest

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References:

Abdellatif N. A., Eltamany E. E., El-Shenawy N. S., Nafie M. S., Hassan Y. M., Al-Eisa R. A., Badr J. M., Abdelhameed R. F. (2024). *Cassia fistula* leaves extract profiling and its emphasis on induced ulcerative colitis in male rats through inhibition of caspase 3 and cyclooxygenase-2. Arabian Journal of Chemistry, 17(4):105672.

Ahmed B. F., Islam A., Faruque M., Mahmud A.-S., Islam M. M., Seidel V., Siraj M. A. (2025). Hepatoprotective effects of a *cassia fistula* fruit extract and molecular insights into its phytoconstituents' interactions with keap1. Pharmacological Research-Natural Products:100341.

Akubugwo E. I., Emmanuel O., Ekweogu C. N., Ugbogu O. C., Onuorah T. R., Egeduzu O. G., Ugbogu E. A. (2022). Gc-ms analysis of the phytochemical constituents, safety assessment, wound healing and anti-inflammatory activities of cucurbita pepo leaf extract in rats. Scientia Pharmaceutica, 90(4):64.

Awasthi P., Kesharwani V., Kabra S. (2022). Golden shower tree: Emerging medicinal properties composed of phytochemistry. International Journal of Pharmacognosy, 9(11):170-85.

Bhatt Mehul K., Dholwani Kishor K., Saluja Ajay K. (2011). Isolation and structure elucidation of scopoletin from ipomoea reniformis (convolvulaceae). J Appl Pharm Sci, 1:138-144.

Bindu S., Mazumder S., Bandyopadhyay U. (2020). Non-steroidal anti-inflammatory drugs (nsaids) and organ damage: A current perspective. Biochemical pharmacology, 180:114147.

Chavan R. R., Bhinge S. D., Bhutkar M. A., Randive D. S., Wadkar G. H., Todkar S. S., Urade M. N. (2020). Characterization, antioxidant, antimicrobial and cytotoxic activities of green synthesized silver and iron nanoparticles using alcoholic blumea eriantha dc plant extract. Materials Today Communications, 24:101320.

Chen M., Fu Q., Song X., Muhammad A., Jia R., Zou Y., Yin L., Li L., He C., Ye G. (2020). Preparation of resveratrol dry suspension and its immunomodulatory and anti-inflammatory activity in mice. Pharmaceutical biology, 58(1):8-15.

Duraipandiyan V., Ignacimuthu S. (2007). Antibacterial and antifungal activity of *cassia fistula* l.: An ethnomedicinal plant. Journal of ethnopharmacology, 112(3):590-594.

El-Tallawy S. N., Nalamasu R., Salem G. I., LeQuang J. A. K., Pergolizzi J. V., Christo P. J. (2021). Management of musculoskeletal pain: An update with emphasis on chronic musculoskeletal pain. Pain and therapy, 10(1):181-209.

Gao X.-Y., Li X.-Y., Zhang C.-Y., Bai C.-Y. (2024). Scopoletin: A review of its pharmacology, pharmacokinetics, and toxicity. Frontiers in pharmacology, 15:1268464.

Khumalo G. P., Van Wyk B. E., Feng Y., Cock I. E. (2022). A review of the traditional use of southern african medicinal plants for the treatment of inflammation and inflammatory pain. Journal of Ethnopharmacology, 283:114436.

Lee C. K., Lee P. H., Kuo Y. H. (2001). The chemical constituents from the aril of *cassia fistula* l. Journal of the Chinese Chemical Society, 48(6A):1053-1058.

Lee H., Han J.-H., Jeong R. G., Kang Y. J., Choi B. H., Kim S. R., Cheon C. K., Hur J., Lee S. Y. (2025). Oral trehalose improves histological and behavior symptoms of mucopolysaccharidosis type ii in iduronate 2-sulfatase deficient mice. Scientific Reports, 15(1):4882.

Leventhal L., Brandt M. R., Cummons T. A., Piesla M. J., Rogers K. E., Harris H. A. (2006). An estrogen receptor-β agonist is active in models of inflammatory and chemical-induced pain. European journal of pharmacology, 553(1-3):146-148.

Licciardi P. V., Underwood J. R. (2011). Plant-derived medicines: A novel class of immunological adjuvants. International immunopharmacology, 11(3):390-398.

Luximon-Ramma A., Bahorun T., Soobrattee M. A., Aruoma O. I. (2002). Antioxidant activities of phenolic, proanthocyanidin, and flavonoid components in extracts of cassia fistula. Journal of agricultural and food chemistry, 50(18):5042-5047.

Mishra A. U., Sen S., Sahu M., Devi M., Singh D. P., Gupta A., Rai A. K., Ch R., Shukla A. K., Bawankule D. U. (2025). *Cassia fistula* fruit pulp extracts mitigate collagen-induced arthritis via regulation of oxidative stress and inflammation. Inflammopharmacology, 33(6):3143-3156.

Mishra A. U., Sen S., Devi M., Rai A. K., Ch R., Bawankule D. U., Mani D. N. (2026). Anti-arthritic potential of *cassia fistula* leaf extracts and sennoside b via nf-κb inhibition: *In vivo* and *in vitro* pre-clinical study. Inflammopharmacology, 34(1):779-796.

Moon P.-D., Lee B.-H., Jeong H.-J., An H.-J., Park S.-J., Kim H.-R., Ko S.-G., Um J.-Y., Hong S.-H., Kim H.-M. (2007). Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the iκb/nf-κb signal cascade in the human mast cell line hmc-1. European Journal of Pharmacology, 555(2-3):218-225.

Nugraha S. E., Achmad S., Sitompul E. (2019). Antibacterial activity of ethyl acetate fraction of passion fruit peel (passiflora edulis sims) on staphylococcus aureus and escherichia coli. Indonesian Journal of Pharmaceutical and Clinical Research, 2(1):07-12.

PA W. (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts, approved standard. CLSI document M27-A2.

Pereira dos Santos Nascimento M. V., Arruda-Silva F., Gobbo Luz A. B., Baratto B., Venzke D., Mendes B. G., Fröde T. S., Geraldo Pizzolatti M., Dalmarco E. M. (2016). Inhibition of the nf-κb and p38 mapk pathways by scopoletin reduce the inflammation caused by carrageenan in the mouse model of pleurisy. Immunopharmacology and Immunotoxicology, 38(5):344-352.

Rashid R., Hajam G. N., Wani M. H., Hussain A. (2017). Extraction method and isolation of phytoconstituents from the chloroform extract of plant solanum nigrum l. By colunm chromatography. International Journal of Advance Research, Ideas and Innovations in Technology, 3(6):1-5.

Shah M. R., Shamim A., White L. S., Bertino M. F., Mesaik M. A., Soomro S. (2014). The anti-inflammatory properties of au–scopoletin nanoconjugates. New Journal of Chemistry, 38(11):5566-5572.

Sharma B. P., Choudhari S. S., Reagan S. K., Mishra S. The golden shower (cassia fistula): Ornamental, ethnomedicinal, pharmacological, and ecological significance. MEDICINAL TREES OF INDIA:31.

Sharma D., Sharma S., Saini S., SINGLA S., BHARDWAJ E. (2022). Antioxidant and antibacterial activity of aquaethanolic extract of leaves of cassia fistula. Journal of Veterinary Pharmacology and Toxicology/June, 21(1/38):42.

Siddiqua A., Zahra M., Begum K., Jamil M. (2018). The traditional uses, phytochemistry and pharmacological properties of cassia fistula. Journal of Pharmacy and Pharmacology Research, 2(1):15-23.

Silva F., Veiga F., Cardoso C., Dias F., Cerqueira F., Medeiros R., Paiva-Santos A. C. (2024). A rapid and simplified dpph assay for analysis of antioxidant interactions in binary combinations. Microchemical Journal, 202:110801.

Soliman A. M., Barreda D. R. (2022). Acute inflammation in tissue healing. International journal of molecular sciences, 24(1):641.

Vonkeman H. E., van de Laar M. A. (2010). Nonsteroidal anti-inflammatory drugs: Adverse effects and their prevention. Seminars in arthritis and rheumatism Elsevier. (pp 294-312).

Wan Osman W. N., Che Ahmad Tantowi N. A., Lau S. F., Mohamed S. (2019). Epicatechin and scopoletin rich morinda citrifolia (noni) leaf extract supplementation, mitigated osteoarthritis via anti-inflammatory, anti-oxidative, and anti-protease pathways. Journal of food biochemistry, 43(3):e12755.

Wang Y., Chen S., Du K., Liang C., Wang S., Boadi E. O., Li J., Pang X., He J., Chang Y.-x. (2021). Traditional herbal medicine: Therapeutic potential in rheumatoid arthritis. Journal of ethnopharmacology, 279:114368.

Yao L., Jiang Y., Datta N., Singanusong R., Liu X., Duan J., Raymont K., Lisle A., Xu Y. (2004). Hplc analyses of flavanols and phenolic acids in the fresh young shoots of tea (camellia sinensis) grown in australia. Food Chemistry, 84(2):253-263.

Yao X., Ding Z., Xia Y., Wei Z., Luo Y., Feleder C., Dai Y. (2012). Inhibition of monosodium urate crystal-induced inflammation by scopoletin and underlying mechanisms. International immunopharmacology, 14(4):454-462.

Yoon W. H., Lee K. H. (2017). Anti-inflammatory, anti-arthritic and analgesic effect of the herbal extract made from bacopa monnieriis, *cassia fistula* and phyllanthus polyphyllus. Natural Product Sciences, 23(2):108-112.

Zahra M., Abrahamse H., George B. P. (2024). Flavonoids: Antioxidant powerhouses and their role in nanomedicine. Antioxidants, 13(8):922.

Zubairi M., Afzal M., Akhtar S., Noman A., Musarrat A., Fayyaz H., Sultan M., Rao N., Khalid M., Saeed W. (2024). Phytochemicals analysis via gc-ms, assessment of anti-inflammatory, anti-bacterial, and anti-fungal properties of *cassia fistula* pod-based functional tea. J Popul Ther Clin Pharmacol, 31:1021-1031.

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