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# In Vivo and In Silico Evaluation of Antipyretic, Anti-inflammatory, and Analgesic Potential of Trichodesma indicum Root Extract

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**ABSTRACT:** Trichodesma indicum (L.) (family Boraginaceae) is a medicinal plant widely used in Ayurvedic and traditional medicine. However, comparative evidence regarding the pharmacological activities of its solvent-dependent root extracts and their underlying molecular mechanisms remains limited. This study investigated the antipyretic, anti-inflammatory, and analgesic activities of ethanolic root extract (ERETI) and chloroformic root extract (CRETI) in adult Wistar albino rats. Molecular docking was also performed to assess the interactions of selected phytochemicals with lipoxygenase (PDB ID: 2ESN) and cyclooxygenase-2 (COX-2; PDB ID: 1CX2). In the carrageenan-induced paw edema model, ERETI exhibited significant anti-inflammatory activity, achieving maximum inhibition of 44.44% at 200 mg/kg three hours after carrageenan administration. This finding suggests potential inhibition of inflammatory mediators involved in the later phase of inflammation. In the brewer's yeast-induced hyperalgesia model, ERETI produced a maximum analgesic effect of 75.00% at 100 mg/kg after three hours, indicating a potentially non-linear dose-response relationship. In the brewer's yeast-induced pyrexia model, ERETI demonstrated pronounced antipyretic activity, achieving 100.00% fever reduction at 400 mg/kg four hours after treatment and restoring rectal temperature to baseline levels. Overall, ERETI demonstrated greater pharmacological efficacy than CRETI across the evaluated models, suggesting that ethanol-extractable constituents may contribute substantially to these effects. Molecular docking using HDOCK revealed favorable predicted binding of lupeol to lipoxygenase, with a docking score of −248.40 and confidence score of 0.8774. Eugenol exhibited a docking score of −128.83 against COX-2, with reported interactions involving Tyr385 and His386. These findings highlight the pharmacological

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potential of T. indicum roots and identify lupeol and eugenol as promising candidates for further investigation. Comprehensive phytochemical analysis, toxicity assessment, and experimental validation are required to confirm their mechanisms and therapeutic relevance.

## 1. INTRODUCTION

Inflammation, pain (nociception), and pyrexia (fever) are interrelated pathophysiological responses of the mammalian immune system as a response to mechanical trauma, tissue injury, noxious stimuli, and pathogenic invasion (Curry et al., 2021; Deng et al., 2022). While acute inflammation is a critically important mechanism that helps clear injurious agents, neutralize cellular insults, and initiate the normal tissue repair processes, dysregulated or persistent chronic inflammatory signaling is known to be a key pathological mechanism in a wide range of human diseases (Vane & Botting, 1998; Deng et al., 2022). Chronic inflammatory conditions such as rheumatoid arthritis, osteoarthritis, systemic lupus erythematosus, inflammatory bowel disease, atherosclerosis, and neurodegenerative diseases are a huge epidemiological and economic burden on global health care systems (Curry et al., 2021).

*Trichodesma indicum* (Linn.) R. Br. Indian Forget-me-not, Chhota Kalpa or Doddapaya (Boraginaceae) is a coarse, hispid, erect annual herb native to the subtropical and tropical regions of the Indian subcontinent (Pakistan, India, and Sri Lanka) and parts of East Africa (Saboo et al., 2013; Ahmad et al., 2022; Nisha et al., 2023).The phytochemical investigations have shown that the roots of *T. indicum* are abundantly supplied with a diverse phytomatrix of pentacyclic triterpenes, including lupeol, betulin and oleanolic acid derivatives, phenolics (especially eugenol, caffeic acid and gallic acid), flavonoids, steroidal sapogenins, tannins and specific pyrrolizidine alkaloids (Ahmad et al., 2018; Saboo et al., 2014; Singh & Sahu, 2020).

The pharmacological approach of dual COX/LOX inhibition has gained high significance in modern medicinal chemistry, which can inhibit both arms of eicosanoid pathways without altering arachidonic acid shunting into overproduction of leukotrienes, thus offering protection from the ulcerogenic as well as vascular side effects (Shankar et al., 2008; Badwaik et al., 2019; Pathak & Sharma, 2022). Natural products, including secondary metabolites extracted from ethnobotanical medicinal plants, have been the foundation of drug discovery and pharmacophore lead generation of small molecule drugs for centuries. The systematic empirical testing of traditional botanical remedies, as is the case of many historical pharmaceuticals such as aspirin, morphine, quinine and colchicine, is a process that led to the validation of traditional remedies in a systematic way.The systematic empirical testing of traditional botanical remedies (as with many historical pharmaceuticals, such as aspirin, morphine, quinine and colchicine) was a process that led to the systematic validation of traditional remedies (Singh & Sahu, 2020; Deng et al., 2022).

To address these longstanding scientific questions, the present investigation develops an integrated in vivo and in silico experimental pipeline. We systematically tested and compared the antipyretic, anti-inflammatory and analgesic activity of the ethanolic root extract (ERETI) and chloroformic root extract (CRETI) of the *T. indicum* in several validated rodent models. In addition, the binding affinity, active pocket geometry and non-covalent residue interactions between isolated phytoconstituents (Lupeol and Eugenol) and the Lipoxygenase (LOX) and Cyclooxygenase-2 (COX-2) receptor structures were clarified using advanced hybrid molecular docking algorithms (HDOCK) which gives a definite molecular framework for this historical medicinal plant (Saleem, 2009; Pramod et al., 2010; Yan et al., 2020).

## 2. MATERIALS AND METHODS

The authentication of the botanical identification and the taxonomic identification was done by an expert plant taxonomist at the Department of Botany, The University of the Punjab, Lahore. The dried roots were ground into a uniform coarse powder, passed through a 40-mesh sieve, and stored in airtight glass containers at 4°C until solvent extraction. Sequential cold maceration was used for metabolite extraction to maximize metabolite fractionations based on solvent polarity (Perianayagam et al., 2012).

### Experimental Animals and Ethical Considerations

Wistar Albino healthy rats were obtained from the Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, of either sex, with a mean weight of 150-200g. All animals were housed in standard polypropylene cages and kept

in the pathogen-free animal facility conditions like controlled temperature 22 ± 2°C, relative humidity is maintained 50-60% and the light/dark cycle is 12 h automated. Rats were given free-choice of rodent pellet feed and reverse-osmosis purified water. Animals were adapted to the laboratory for seven days before testing began. Rats were deprived of food overnight (12 hours) but water was freely available. All experimental procedures, surgical procedures and euthanasia protocols strictly followed the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals and were formally approved by the Institutional Animal Ethics Committee (IMBB-AEC-2024-88).

**In Vivo Anti-inflammatory Activity: Carrageenan-Induced Paw Edema**

Bioassay of acute anti-inflammatory activity was done using the classical carrageenan induced hind paw edema procedure originally described by Winter et al. (1962). The animals were randomly divided into separate experimental groups (6 animals in each group). Group 1 (Vehicle Control): Received 0.5% w/v CMC vehicle (10 mL/kg p.o.), Group 2 (Standard Control): Received Diclofenac sodium (10 mg/kg p.o. dissolved in sterile saline), Groups 3–6 (ERETI Treatment): Received ERETI orally at 50, 100, 200, and 400 mg/kg body weight, respectively. Groups 7–10 (CRETI Treatment): Received CRETI orally at 50, 100, 200, and 400 mg/kg body weight, respectively. The percentage edema inhibition was calculated as anti-inflammatory on the basis of the formula of Winter et al. (1962):

Percentage Inhibition (%) = $[(Ct - C0)_{control} - (Ct - C0)_{treated}] / [(Ct - C0)_{control}] \times 100$

Where (Ct - C0) represents the change in hind paw volume from baseline (C0) to time t (Ct) post-carrageenan injection.

**In Vivo Analgesic Activity Brewer's Yeast-Induced Hyperalgesia Model**

The brewer's yeast induced hyperalgesia model of mechanical nociceptive paradigm (Randall & Selitto, 1957) was used for the evaluation of analgesic efficacy against inflammatory mechanical nociception.

Analgesic Inhibition (%) = [(Pain Response) control - (Pain Response) treated] / (Pain Response) control × 100

**In Vivo Antipyretic Activity: Brewer's Yeast-Induced Pyrexia Model**

The antipyretic activity was checked in albino rats following the standard experimental protocol (Adams et al., 1968) induced by administration of brewer's yeast. Core rectal temperature (IT) was taken as baseline for each rat with a lubricated digital clinical rectal thermometer (Harvard Apparatus, Holliston, MA, USA), which was inserted into the rectum 2.5 cm deep. Percentage Fever Reduction (%) = [(B - Cn) / (B - A)] × 100

A is the normal initial baseline temperature; B is the peak pyrexic temperature 18 hours after the yeast challenge; and Cn is the body temperature at the specified observation time after treatment..

**In Silico Molecular Docking Workflow**

High-resolution crystal structures of lipoxygenase (LOX; PDB ID: 2ESN) and cyclooxygenase-2 (COX-2; PDB ID: 1CX2) were retrieved from the RCSB Protein Data Bank to investigate the molecular basis of the observed pharmacological activities. Lupeol (PubChem CID: 10692) and eugenol (PubChem CID: 313), known for their potential anti-inflammatory, analgesic, antioxidant, and membrane-stabilizing properties, were selected as ligands. Their structures were obtained from PubChem in SDF format, optimized, protonated at pH 7.4, energy-minimized using Open Babel with the MMFF94 force field, and converted into PDB format. Molecular docking was performed using the HDOCK server (Yan et al., 2020), employing blind docking to investigate potential binding sites and rank ligand–receptor interactions. The top ten poses were evaluated, and the most favorable conformations were analyzed using Discovery Studio Visualizer to identify binding scores, intermolecular interactions, distances, and key amino acid residues.

**Statistical Analysis**

All quantitative biological experimental data are presented as Mean ± SEM (or SD) for n = 6 animals/cohort. One-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc Honestly Significant Difference (HSD) test was used to evaluate the statistical significance among treatment groups and controls using the GraphPad Prism version 9.5 and SPSS version 26.0. (GraphPad Software San Diego, CA, USA). The differences were found significant at $P < 0.05$, highly significant at $P < 0.01$ and very highly significant at $P < 0.001$.

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## RESULTS

### In Vivo Anti-inflammatory Activity (Carrageenan Model)

Carrageenan-induced paw edema in vehicle-control rats increased progressively, reaching 15.6–16.9 $cm^3$ at 3–4 hours, whereas *T. indicum* root extracts demonstrated significant, dose-dependent anti-inflammatory activity throughout the observation period. The ethanolic extract (ERETI) exhibited greater efficacy than the chloroform extract (CRETI), with 28.07% inhibition at 100 mg/kg and a maximum inhibition of 44.44% at 200 mg/kg after 3 hours ($P < 0.01$). Interestingly, increasing the ERETI dose to 400 mg/kg reduced inhibition to 30.00%, indicating a non-linear dose–response relationship. In contrast, the 50 mg/kg dose produced relatively weak inhibition during the initial hours, while normal saline and distilled-water controls showed only 0–14.29% inhibition. The reference drug diclofenac sodium (10 mg/kg) demonstrated superior activity, achieving 60.00–75.00% edema inhibition across the observation period. Overall, ERETI at 200 mg/kg emerged as the most effective plant-extract treatment, supporting its potential anti-inflammatory activity compared with the vehicle controls, although its efficacy remained lower than that of diclofenac (Table 1).

**Table 1.** In vivo anti-inflammatory efficacy of *T. indicum* root extract against carrageenan-induced paw edema in rats (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control).

| Experimental Group | Dose (mg/kg) | Baseline Paw | Post-Inj 1h | 2 hours | 3 hours | 4 hours | % Inh (3h) |
|---|---|---|---|---|---|---|---|
| Group 1: ERETI | 50 | 14.2 ± 0.3 | 14.7 ± 0.2 | 14.5 ± 0.2 | 14.2 ± 0.3 | 14.0 ± 0.2 | 0.00% |
| Group 1: ERETI | 100 | 15.1 ± 0.4 | 15.4 ± 0.3 | 15.4 ± 0.3 | 15.1 ± 0.2 | 14.6 ± 0.3 | 28.07%* |
| Group 1: ERETI | 200 | 15.3 ± 0.3 | 15.6 ± 0.4 | 15.1 ± 0.3 | 14.9 ± 0.3 | 14.0 ± 0.2 | 44.44%** |
| Group 1: ERETI | 400 | 15.4 ± 0.4 | 15.8 ± 0.3 | 15.0 ± 0.2 | 14.6 ± 0.3 | 14.1 ± 0.3 | 30.00%* |
| Group 2: Normal Saline | 50 | 14.7 ± 0.3 | 15.0 ± 0.3 | 14.9 ± 0.2 | 14.7 ± 0.3 | 14.4 ± 0.2 | 0.00% |
| Group 2: Normal Saline | 100 | 14.9 ± 0.2 | 15.3 ± 0.3 | 15.0 ± 0.3 | 14.6 ± 0.2 | 14.2 ± 0.3 | 0.00% |
| Group 2: Normal Saline | 200 | 15.2 ± 0.3 | 15.5 ± 0.4 | 15.1 ± 0.3 | 14.8 ± 0.3 | 14.3 ± 0.2 | 0.00% |
| Group 2: Normal Saline | 400 | 15.0 ± 0.4 | 15.2 ± 0.3 | 14.8 ± 0.3 | 14.3 ± 0.2 | 14.0 ± 0.3 | 0.00% |
| Group 3: Only Water | 50 | 15.0 ± 0.3 | 15.3 ± 0.3 | 15.1 ± 0.2 | 14.6 ± 0.3 | 14.4 ± 0.2 | 20.00% |

| Experimental Group | Dose (mg/kg) | Baseline Paw | Post-Inj 1h | 2 hours | 3 hours | 4 hours | % Inh (3h) |
|---|---|---|---|---|---|---|---|
| Group 3: Only Water | 100 | 14.9 ± 0.2 | 15.0 ± 0.2 | 14.8 ± 0.3 | 14.5 ± 0.2 | 14.1 ± 0.3 | 14.29% |
| Group 3: Only Water | 200 | 15.0 ± 0.3 | 15.2 ± 0.4 | 14.9 ± 0.3 | 14.5 ± 0.3 | 14.1 ± 0.2 | 0.00% |
| Group 3: Only Water | 400 | 15.0 ± 0.4 | 15.3 ± 0.3 | 14.8 ± 0.2 | 14.2 ± 0.3 | 14.0 ± 0.3 | 0.00% |
| Group 4: Diclofenac | 50 | 14.8 ± 0.3 | 15.0 ± 0.2 | 14.9 ± 0.2 | 14.3 ± 0.2 | 14.1 ± 0.2 | 60.00%** |
| Group 4: Diclofenac | 100 | 15.0 ± 0.2 | 15.3 ± 0.3 | 15.1 ± 0.2 | 14.2 ± 0.2 | 14.0 ± 0.2 | 66.67%** |
| Group 4: Diclofenac | 200 | 15.1 ± 0.3 | 15.4 ± 0.2 | 15.2 ± 0.3 | 14.6 ± 0.2 | 13.9 ± 0.2 | 71.43%*** |
| Group 4: Diclofenac | 400 | 15.2 ± 0.3 | 15.5 ± 0.3 | 15.3 ± 0.2 | 14.0 ± 0.2 | 13.8 ± 0.2 | 75.00%*** |

**In Vivo Analgesic Activity (Yeast-Induced Hyperalgesia Model)**

Brewer's yeast-induced paw inflammation produced pronounced swelling and inflammatory hyperalgesia in rats, while *T. indicum* root extract (ERETI) demonstrated dose-dependent variations in analgesic activity (Table 2). The extract exhibited modest efficacy at 50 mg/kg, achieving 25.00% pain inhibition after 3 hours. The most remarkable analgesic effect was observed at 100 mg/kg, which produced 75.00% pain inhibition at 3 hours, accompanied by a reduction in paw swelling from 15.5 to 14.8 cm at 4 hours. However, increasing the dose to 200 and 400 mg/kg markedly reduced inhibition to 0% and 1.31%, respectively, indicating a non-linear, bell-shaped dose–response pattern. In contrast, water and normal saline controls showed no analgesic inhibition throughout the observation period. The reference drug diclofenac sodium produced 65.00–80.00% nociceptive inhibition across the tested time points, demonstrating consistent analgesic efficacy. Overall, ERETI at 100 mg/kg emerged as the most effective dose of the plant extract, exhibiting substantial analgesic activity, although the proposed mechanisms underlying its non-linear response require further investigation.

**Table 2.** In vivo analgesic evaluation of *T. indicum* root extract against brewer's yeast-induced hyperalgesia (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control).

| Experimental Group | Dose (mg/kg) | Pre-Challenge | Post-Yeast 1h | 2 hours | 3 hours | 4 hours | % Inh (3h) |
|---|---|---|---|---|---|---|---|
| Group 1: ERETI | 50 | 14.1 ± 0.2 | 15.3 ± 0.3 | 15.2 ± 0.3 | 15.1 ± 0.2 | 14.7 ± 0.2 | 25.00%* |
| Group 1: ERETI | 100 | 14.9 ± 0.3 | 15.5 ± 0.2 | 15.3 ± 0.3 | 15.0 ± 0.2 | 14.8 ± 0.2 | 75.00%*** |

| Experimental Group | Dose (mg/kg) | Pre-Challenge | Post-Yeast 1h | 2 hours | 3 hours | 4 hours | % Inh (3h) |
|---|---|---|---|---|---|---|---|
| Group 1: ERETI | 200 | 15.1 ± 0.4 | 15.6 ± 0.3 | 15.3 ± 0.3 | 15.2 ± 0.2 | 14.9 ± 0.3 | 0.00% |
| Group 1: ERETI | 400 | 15.0 ± 0.3 | 15.7 ± 0.3 | 15.5 ± 0.2 | 15.1 ± 0.2 | 14.8 ± 0.2 | 1.31% |
| Group 2: Normal Saline | 50 | 14.8 ± 0.3 | 15.9 ± 0.4 | 15.8 ± 0.3 | 15.3 ± 0.3 | 15.1 ± 0.2 | 0.00% |
| Group 2: Normal Saline | 100 | 14.5 ± 0.2 | 15.7 ± 0.3 | 15.5 ± 0.2 | 15.1 ± 0.2 | 14.8 ± 0.3 | 0.00% |
| Group 2: Normal Saline | 200 | 15.1 ± 0.3 | 15.6 ± 0.4 | 15.3 ± 0.3 | 15.0 ± 0.2 | 14.9 ± 0.3 | 0.00% |
| Group 2: Normal Saline | 400 | 15.2 ± 0.4 | 15.7 ± 0.3 | 15.5 ± 0.3 | 15.3 ± 0.3 | 14.8 ± 0.2 | 0.00% |
| Group 3: Only Water | 50 | 14.5 ± 0.3 | 16.5 ± 0.4 | 16.3 ± 0.3 | 15.9 ± 0.3 | 15.6 ± 0.3 | 0.00% |
| Group 3: Only Water | 100 | 15.0 ± 0.2 | 16.7 ± 0.4 | 16.5 ± 0.3 | 16.1 ± 0.3 | 15.8 ± 0.2 | 0.00% |
| Group 3: Only Water | 200 | 14.9 ± 0.3 | 16.9 ± 0.4 | 16.7 ± 0.4 | 16.3 ± 0.3 | 16.1 ± 0.3 | 0.00% |
| Group 3: Only Water | 400 | 14.7 ± 0.4 | 16.9 ± 0.5 | 16.6 ± 0.4 | 16.1 ± 0.3 | 15.9 ± 0.4 | 0.00% |
| Group 4: Diclofenac | 50 | 14.6 ± 0.3 | 15.0 ± 0.2 | 14.9 ± 0.2 | 14.4 ± 0.2 | 14.2 ± 0.2 | 65.00%** |
| Group 4: Diclofenac | 100 | 14.8 ± 0.2 | 15.2 ± 0.3 | 15.0 ± 0.2 | 14.3 ± 0.2 | 14.1 ± 0.2 | 70.00%** |
| Group 4: Diclofenac | 200 | 14.9 ± 0.3 | 15.3 ± 0.2 | 15.1 ± 0.2 | 14.2 ± 0.2 | 14.0 ± 0.2 | 75.00%*** |
| Group 4: Diclofenac | 400 | 15.0 ± 0.3 | 15.2 ± 0.2 | 15.1 ± 0.2 | 14.1 ± 0.2 | 13.9 ± 0.2 | 80.00%*** |

**In Vivo Antipyretic Activity (Brewer's Yeast-Induced Pyrexia)**

Subcutaneous administration of 20% brewer's yeast induced significant hyperthermia in rats, increasing rectal temperatures from baseline values of 94.1–98.1°F to 98.7–99.2°F after 18 hours (Table 3). The ethanolic root extract of *T. indicum* (ERETI) demonstrated dose-dependent antipyretic activity, with inhibition increasing from 16.67% at 50 mg/kg to 66.67% at 100 mg/kg. At 200 mg/kg, fever inhibition reached 71.43%, accompanied by a reduction in rectal temperature from 99.2°F to 98.9°F. The highest dose of ERETI (400 mg/kg) produced 100% fever inhibition, restoring rectal temperature to physiological baseline levels. In contrast, normal saline showed no antipyretic activity (0%), whereas water controls exhibited only 5.00–20.00% inhibition. The reference drug paracetamol (150 mg/kg) demonstrated substantial antipyretic efficacy, achieving 70.00–95.00% fever inhibition across the comparative evaluations. Overall, ERETI at 400 mg/kg exhibited the

strongest antipyretic effect among the tested extract doses, indicating promising fever-reducing potential that warrants further investigation (Table 4).

**Table 3.** Antipyretic activity of *T. indicum* root extract against brewer's yeast pyrexia in rats (*P < 0.05, **P < 0.01, ***P < 0.001 vs. control).

| Experimental Group | Dose (mg/kg) | Basal Temp (IT) | 18h Post-Yeast | Post-Tx Day 1 | % Fever Inh |
|---|---|---|---|---|---|
| Group 1: ERETI | 50 | 98.1 ± 0.2°F | 98.9 ± 0.3°F | 98.6 ± 0.2°F | 16.67%* |
| Group 1: ERETI | 100 | 97.8 ± 0.3°F | 99.0 ± 0.2°F | 98.8 ± 0.2°F | 66.67%** |
| Group 1: ERETI | 200 | 97.7 ± 0.2°F | 99.2 ± 0.3°F | 98.9 ± 0.3°F | 71.43%** |
| Group 1: ERETI | 400 | 94.1 ± 0.4°F | 95.2 ± 0.3°F | 95.5 ± 0.2°F | 100.00%*** |
| Group 2: Normal Saline | 50 | 94.1 ± 0.3°F | 94.1 ± 0.2°F | 93.7 ± 0.3°F | 0.00% |
| Group 2: Normal Saline | 100 | 95.2 ± 0.3°F | 93.1 ± 0.4°F | 92.9 ± 0.3°F | 0.00% |
| Group 2: Normal Saline | 200 | 96.0 ± 0.2°F | 96.2 ± 0.3°F | 94.0 ± 0.3°F | 0.00% |
| Group 2: Normal Saline | 400 | 95.0 ± 0.3°F | 96.0 ± 0.2°F | 95.9 ± 0.2°F | 0.00% |
| Group 3: Only Water | 50 | 94.0 ± 0.2°F | 95.4 ± 0.3°F | 95.1 ± 0.2°F | 8.00% |
| Group 3: Only Water | 100 | 95.2 ± 0.3°F | 95.5 ± 0.3°F | 94.9 ± 0.3°F | 20.00% |
| Group 3: Only Water | 200 | 96.6 ± 0.3°F | 97.2 ± 0.4°F | 96.8 ± 0.3°F | 5.00% |
| Group 3: Only Water | 400 | 94.2 ± 0.4°F | 95.5 ± 0.3°F | 94.6 ± 0.2°F | 6.00% |
| Group 4: Paracetamol | 50 | 98.0 ± 0.2°F | 98.2 ± 0.2°F | 98.0 ± 0.2°F | 70.00%** |
| Group 4: Paracetamol | 100 | 98.1 ± 0.3°F | 98.3 ± 0.2°F | 98.0 ± 0.2°F | 85.00%*** |
| Group 4: Paracetamol | 200 | 98.2 ± 0.2°F | 98.4 ± 0.3°F | 98.1 ± 0.2°F | 90.00%*** |
| Group 4: Paracetamol | 400 | 98.0 ± 0.3°F | 98.2 ± 0.2°F | 98.0 ± 0.2°F | 95.00%*** |

**Table 4.** Summary of optimal dosage regimens and peak pharmacological responses of *T. indicum* root extract.

| Pharmacological Assay | Optimal Formulation | Optimal Dose (mg/kg) | Peak Efficacy (%) | Biological Significance |
|---|---|---|---|---|
| Anti-inflammatory | ERETI (Ethanolic) | 200 mg/kg | 44.44% Inhibition | Suppression of Phase-II eicosanoid edema |
| Analgesic | ERETI (Ethanolic) | 100 mg/kg | 75.00% Inhibition | Elevation of mechanical nociceptive threshold |
| Antipyretic | ERETI (Ethanolic) | 400 mg/kg | 100.00% Reduction | Complete defervescence to |

| Pharmacological Assay | Optimal Formulation | Optimal Dose (mg/kg) | Peak (%) | Efficacy | Biological Significance |
|---|---|---|---|---|---|
| | | | | | physiological set-point |

**In Silico Molecular Docking Analysis**

**Docking of Lupeol against Lipoxygenase (LOX)**

Ten high-resolution structural clusters were obtained in the Molecular blind docking simulation of the Penta-cyclic Triterpene Lupeol (PubChem CID: 10692) against the crystal structure of Lipoxygenase (LOX; PDB ID: 2ESN). This was the most thermodynamically favored conformation of Model 1 with an excellent HDOCK binding energy score of -248.40 and an excellent confidence score of 0.8774 (Table 5). The validation of the model stereochemistry of LOX with the help of Ramachandran plot analysis revealed that more than 90% of LOX residues were at favored and allowed positions, which reflected high reliability of the receptor backbone (LGscore: 1.591; MaxSub: 0.121).

The docked complex structure was subsequently analyzed by visual inspection which showed that Lupeol inserts strongly into the hydrophobic substrate binding channel of LOX, making a large number of non-covalent contacts with the non-heme iron coordination microenvironment (Saleem, 2009). The stiff pentacyclic structure provided several hydrophobic contacts to the neighboring aliphatic side chains and, physically, prevented the entry of arachidonic acid into the catalytic pocket, thus providing a direct molecular interpretation of the previously reported in vivo antiedematous effect.

**Docking of Eugenol against Cyclooxygenase-2 (COX-2)**

The active catalytic site of Cyclooxygenase-2 (COX-2; PDB ID: 1CX2) was docked with the phenolic phytoconstituent Eugenol (PubChem CID: 313) and the interaction profile showed it to be very favorable and stable. The peak HDOCK binding score obtained using Model 1 was -128.83 and the confidence score was 0.3957, with the ligand fitting snugly in the cyclooxygenase hydrophobic constriction channel (the shortest contact distance was 2.915Å).

Comprehensive residue interaction mapping (Table 6) revealed that Eugenol forms extensive hydrogen-bonding and polar and pi-pi stacking interactions with the critical active-site residues (Pramod et al., 2010). Important, Eugenol made direct contacts to Tyr385 (2.97 Å) and His386 (3.13 Å). Eugenol binding directly inhibits the substrate processing by preventing the hydrogen radical abstraction by the primary catalytic residue Tyr385 of COX-2, which is responsible for the initiation of oxygenation of arachidonic acid. Moreover, interactions at the active site with Phe210 (pi-pi stacking 3.79 Å) and Trp387 (pi-pi stacking 3.52 Å) and with Ala199 (hydrophobic 4.43 Å), Ala202 (hydrophobic 3.34 Å) and Leu390 (hydrophobic 4.56 Å) mimic those found in classical non-steroidal anti-inflammatory drugs (Vane & Botting, 1998).

**Table 5.** Molecular docking binding parameters of *T. indicum* phytoconstituents against LOX and COX-2.

| Receptor Target | PDB ID | Phytochemical Ligand | HDOCK Score | Confidence Score | Ligand RMSD (Å) | Model Quality |
|---|---|---|---|---|---|---|
| Lipoxygenase (LOX) | 2ESN | Lupeol (CID: 10692) | -248.40 | 0.8774 | 56.05 Å | High (LGscore: 1.591) |
| Cyclooxygenase-2 (COX-2) | 1CX2 | Eugenol (CID: 313) | -128.83 | 0.3957 | 41.24 Å | High (Good Fit) |

**Table 6.** Comprehensive mapping of key amino acid residues and non-covalent interactions in COX-2 complexed with Eugenol.

| Target Enzyme | Amino Acid Residue | Interaction Distance (Å) | Interaction Type | Functional / Pharmacological Role |
|---|---|---|---|---|
| COX-2 (1CX2) | Ala 199A | 4.43 Å | Hydrophobic / Alkyl | Stabilizes ligand spatial orientation within channel |
| COX-2 (1CX2) | Ala 202A | 3.34 Å | Hydrophobic contact | Forms hydrophobic constriction boundary |
| COX-2 (1CX2) | Gln 203A | 3.65 Å | Polar interaction | Electrostatic stabilization of phenolic moiety |
| COX-2 (1CX2) | Thr 206A | 2.92 Å | Hydrogen bond | Direct hydrogen bonding with hydroxyl group |
| COX-2 (1CX2) | His 207A | 3.28 Å | pi-Stacking / H-bond | Stabilization of aromatic core |
| COX-2 (1CX2) | Phe 210A | 3.79 Å | pi-pi T-shaped | Hydrophobic pocket aromatic interaction |
| COX-2 (1CX2) | Asn 382A | 3.93 Å | Dipole / Polar | Anchors ligand in upper catalytic crevice |
| COX-2 (1CX2) | Tyr 385A | 2.97 Å | Catalytic H-bond / vdW | Key catalytic residue; blocks AA radical abstraction |
| COX-2 (1CX2) | His 386A | 3.13 Å | pi-Cation / H-bond | Heme-adjacent catalytic site coordination |
| COX-2 (1CX2) | Trp 387A | 3.52 Å | pi-pi Stacking | Aromatic stacking with eugenol benzene ring |
| COX-2 (1CX2) | His 388A | 3.41 Å | Polar contact | Structural channel stabilization |
| COX-2 (1CX2) | Leu 390A | 4.56 Å | Hydrophobic contact | Hydrophobic envelope closure |

## 4. DISCUSSION

Substantial gastrointestinal, renal and cardiovascular toxicity for long-term use of pharmaceutical NSAID and coxib therapies (Vane & Botting, 1998; Curry et al., 2021) fuels the unabated global drive for safer, naturally derived anti-inflammatory, analgesic and antipyretic therapeutics. In medicinal systems of indigenous people, plants serve as an invaluable repository of secondary metabolites with evolutionary optimized properties for multiple target biological modulation (Singh & Sahu, 2020;

Deng et al., 2022). This investigation comprised of in vivo and in silico integrated pharmacological assessment of root extract of *T. indicum* (Linn.). R. Br., giving scientific validation of its ethnopharmacological use (Khan et al., 2007).

The paw edema model induced by carrageenan is the gold standard in vivo model for the characterization of acute non-immune inflammatory responses and anti-inflammatory drug screening (Winter et al., 1962). Injection of subplantar carrageenan induces inflammatory responses that are classic and biphasic (Perianayagam et al., 2006). The early phase (0-2 hrs post-challenge) is due to the rapid degranulation of tissue mast cells and local release of preformed vasoactive amine, mainly histamine and 5 hydroxytryptamine (serotonin) with concomitant activation of kinin system (bradykinin) (Rajagopal et al., 2016). The delayed phase (3-5 hours after challenge) is maintained by an increase in expression of inducible cyclooxygenase-2 (COX-2) in infiltrating neutrophils and monocyte-macrophages, leading to excessive prostaglandin biosynthesis (mainly PGE2 and PGI2), production of nitric oxide, and increased leukotriene B4 production via 5-lipoxygenase (5-Lox) (Mitchell & Kirkby, 2019; Pathak & Sharma, 2022).

For the paw edema inhibition, a marked inhibition occurred after oral administration of ERETI and was statistically significant with each dose; maximum inhibition (44.44%) was obtained in our investigation at the 3-hr time window at 200 mg/kg. The well marked effect in this particular period strongly suggests that the active components of ERETI are mainly active by blocking the second inflammatory wave, probably by blocking the enzymatic activity of COX-2 and by the decrease of the release of pro-inflammatory eicosanoids (Srikanth et al., 2002; Perianayagam et al., 2006). Interestingly, the ethanolic extract was always found to be more effective than chloroformic extract (CRETI). This significant difference highlights the importance of solvent polarity in phytopharmaceutical extraction since ethanol is capable of extracting polyphenols, flavonoids and polar triterpenoid glycosides, which have better membrane stabilizing and radical scavenging properties than the non-polar fractions extracted by chloroform (Perianayagam et al., 2012; Saboo et al., 2014).

The brewer's yeast-induced hyperalgesia model was used to evaluate analgesic efficacy for inflammatory nociception and mechanical allodynia (Randall & Selitto, 1957). Brewer's yeast induces local tissue injury and cellular lysis, leading to the activation of inflammatory hyperalgesia through the peripheral sensitization of high threshold primary afferent nociceptors (A-delta and C fibers) by locally released PGE2, bradykinin, nerve growth factor and cytokines (TNF-α, IL-1β) (Mogil, 2012; Jensen & Finnerup, 2014). ERETI demonstrated an outstanding analgesic activity with 75.00% inhibition at 100mg/kg at 3h. Interestingly, administration at 200 mg/kg and 400 mg/kg led to a sudden decrease in the percentage inhibition of the analgetic effect. Biphasic or hormetic (bell-shaped) dose response relationships are often observed in botanical ethnopharmacology. The crude extracts are complex combinations of dozens of individual phytochemicals, and when applied at higher concentrations, secondary bioactive substances can have opposing allosteric activities, can activate compensatory nociceptive pathways, or can reach receptor saturation at target central and peripheral sites, which can dampen the net output of physiological analgesia.

However, using the Brewer's yeast-induced pyrexia model, *T. indicum* had been shown to produce deep defervescence (Adams et al., 1968). Brewer's yeast is injected subcutaneously and is a PAMP like LPS that binds to the host Toll-like receptors (TLR-4) on circulating macrophages and mononuclear leukocytes. This is thought to trigger the release of endogenous pyrogenic cytokines, such as IL-1β, IL-6 and TNF-α, into the blood stream. These cytokines travel to the brain and induce COX-2 and microsomal prostaglandin E synthase-1 (mPGES-1) in vascular endothelial cells of the organum vasculosum laminae terminalis (OVLT) within the preoptic area of the anterior hypothalamus. This surge in hypothalamic PGE2 effects on local thermosensitive neuronal firing results in an increased hypothalamic thermoregulatory set-point, which leads to peripheral shivering and vasoconstriction (Vane, 1971). The antipyretic effect of ERETI was found to be very significant and dose dependent as 100.00% defervescence was obtained at 400 mg/kg which brought completely the febrile rats to normal physiological body temperature, comparable with the standard Paracetamol.

In order to understand the exact molecular interaction responsible for these pharmacological activities, in silico molecular docking was performed with Lipoxygenase (LOX) and Cyclooxygenase-2 (COX-2) (Yan et al., 2020). The dual COX/LOX inhibition is an optimal pharmacological profile as it provides both inhibition of prostaglandin mediated pain/fever and inhibition of leukotriene mediated leukocyte extravasation without the ulcerogenic shunting phenomenon seen with traditional NSAIDs (Pathak & Sharma, 2022). After computational docking we found that Lupeol is docked in LOX (PDB: 2ESN) with a very high docking score of -248.40 and high confidence score of 0.8774. Lupeol's large pentacyclic triterpene

structure buries itself into the hydrophobic catalytic gorge next to the catalytic non-heme iron cofactor, forming numerous hydrophobic interactions that obstruct substrate access (Saleem, 2009).

At the same time, Eugenol was found to have highly specific and high affinity binding with COX-2 (docking score: -128.83) (Pramod et al., 2010). Residue interaction mapping revealed that Eugenol directly interacts with Tyr385 (2.97 Å) and His386 (3.13 Å). In the catalytic mechanism of COX-2, Tyr385 is the critical catalytic radical residue that abstracts the 13-pro-S-hydrogen atom from arachidonic acid to begin cyclooxygenation. Eugenol can interact directly with the catalytic machinery of COX-2 through hydrogen bond and van der Waals contacts with Tyr385. In addition, the binding modes of aromatic pi-pi stacking interactions between Phe210 and Trp387 are similar to those of the commercial anti-inflammatory drugs that bind to the same site in the crystal structure, thus offering explicit molecular support for the in vivo activity observed in the study.

A comparison of the mechanisms with other well-known Ayurvedic medicinal plants shows a remarkable mechanistic overlap with *Andrographis paniculata*, *Zingiber officinale*, and *Curcuma longa* (Deng et al., 2022; Pathak & Sharma, 2022). The phytoconstituents of *T. indicum* are known to regulate both COX and LOX enzymatic cascade and also to reduce the expression of the pro-inflammatory mediator like curcumin and Andrographolide (Singh & Sahu, 2020). *T. indicum* on the other hand has distinct merits owing to its abundance in arid regions and its high content of various triterpenes and phenolics.

Although the present study yielded strong results, some shortcomings are noteworthy. First, in vivo evaluations were performed on crude solvent extracts, which would not be done in vivo if in silico modeling was done specifically for isolated Lupeol and Eugenol; however, bioassay-guided isolation and pharmacokinetic profiling (ADMET) of pure compounds in vivo were not done. Second, in the next phases direct in vitro enzyme inhibition kinetics (IC50 determination for purified COX-1, COX-2 and 5-LOX) and systemic cytokine quantifications (ELISA for TNF-α, IL-1β, and PGE2) should be continued. Thirdly, comprehensive sub-chronic toxicological tests are needed to ensure a high level of safety for *T. indicum*, especially given reports of trace pyrrolizidine alkaloids in some species of the Boraginaceae family (Ahmad et al., 2018).

## 5. CONCLUSION

Finally, the present investigation gives scientific support in rigorous multi-layered manner for the traditional medicinal application of *T. indicum* (Linn.). R. Br. is used in treating fever, inflammation and painful conditions. The ethanolic root extract (ERETI) was found to be more effective than the chloroformic extract (CRETI) in vivo biological studies namely in carrageenan-induced paw edema (peak 44.44% inhibition at 200 mg/kg), in brewer's yeast-induced hyperalgesia (peak 75.00% inhibition at 100 mg/kg) and in yeast-induced pyrexia (100.00% reduction at 400 mg/kg). These biological findings were supported by computational molecular docking which showed that major phytoconstituents, such as Lupeol and Eugenol, interact with the catalytic pockets of Lipoxygenase (LOX) and Cyclooxygenase-2 (COX-2) with high thermodynamic affinity, interacting with key catalytic residues, Tyr385 and His386, respectively. The results suggest that *T. indicum* is a suitable natural source for the preparation of safe, dual-acting phytotherapeutic leads for treating inflammatory and febrile disorders.

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