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Comprehensive Pharmacological Evaluation of *Tribolium castaneum* (Red Flour Beetle) Dry Biomass: Unravelling the Antinociceptive and Anti-Inflammatory Potential via *In vivo* and In Silico Approaches

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Abstract

Background: The global burden of chronic pain and inflammatory disorders necessitates the exploration of novel, accessible, and safe therapeutic agents from underexplored natural sources. Insects, despite their diversity, remain a neglected frontier in pharmacognosy. *Tribolium castaneum* (red flour beetle), a ubiquitous stored-grain pest and established genetic model organism, produces defensive benzoquinones and cuticular hydrocarbons with potential but uncharacterized pharmacological activity.

Objective: This study provides a comprehensive experimental protocol to evaluate the analgesic and anti-inflammatory activities of dried *T. castaneum* powder in validated rodent models, alongside phytochemical characterization and toxicity profiling.

Methods: Adult beetles will be reared under controlled conditions, processed into dried powder, and subjected to sequential solvent extraction. Acute oral toxicity will follow OECD Guideline 423. Analgesic activity will be assessed using acetic acid-induced writhing, hot plate, tail immersion, and formalin-induced paw licking tests in Swiss albino mice. Anti-inflammatory activity will be evaluated using carrageenan- and formalin-induced paw edema models in Wistar rats. Mechanistic insights will be derived from quantifying pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), PGE_2, COX-2, nitric oxide, myeloperoxidase, and oxidative stress markers (MDA, GSH, SOD, CAT) in serum and paw tissue, complemented by histopathological examination.

Expected Outcomes: We anticipate dose-dependent (100-400 mg/kg) analgesic and anti-inflammatory activities, with peak efficacy at 400 mg/kg comparable to standard drugs (aspirin 100 mg/kg, indomethacin 10 mg/kg). The formalin test is expected to show preferential inhibition of the late (inflammatory) phase, suggesting a peripheral mechanism of action via COX-2/NF-κB pathway suppression and reduction of oxidative stress. Acute toxicity is expected to yield an LD_{50} > 2000 mg/kg, indicating a favourable safety profile.

Conclusion: This research protocol establishes a rigorous scientific framework for evaluating *T. castaneum* as a potential medicinal insect. Positive results would transform an agricultural pest into a sustainable, low-cost source of analgesic and anti-inflammatory compounds, opening new avenues for entomological pharmacognosy.

Keywords: *Tribolium castaneum*, red flour beetle, analgesic, anti-inflammatory, entomotherapy, benzoquinones, carrageenan-induced paw oedema, acetic acid-induced writhing, natural product, rodent model

Introduction

1. The Global Burden of Pain and Inflammation

Pain and inflammation represent two of the most common reasons for healthcare consultation worldwide. Chronic pain affects an estimated 20-30% of the global adult population, with prevalence rising in aging societies. Inflammatory disorders, including rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, and asthma, contribute substantially to years lived with disability. The economic impact, encompassing direct medical costs, lost productivity, and disability payments, runs into hundreds of billions of dollars annually in developed economies alone. [1]

Current pharmacotherapeutic options, while effective for many patients, carry significant limitations. Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, diclofenac, and naproxen act primarily through inhibition of cyclooxygenase (COX) enzymes, reducing prostaglandin synthesis. However, chronic NSAID use is associated with gastrointestinal ulceration, bleeding, nephrotoxicity, and increased cardiovascular risk. Opioid analgesics, reserved for moderate to severe pain, produce tolerance, physical dependence, respiratory depression, and have fuelled an addiction crisis in multiple countries. Adjuvant agents including acetaminophen, anticonvulsants (gabapentinoids), and antidepressants provide relief for some but are ineffective or poorly tolerated for many others.

This therapeutic gap has driven renewed interest in natural product drug discovery. Approximately one-third of all

small-molecule drugs approved between 1981 and 2019 were either natural products or directly derived therefrom. While plants have been extensively mined for bioactive compounds, insects—representing over 80% of described animal species—remain largely unexplored pharmacologically. [2]

2. Entomotherapy: Historical Foundations and Modern Validation

The medicinal use of insects, termed entomotherapy, predates recorded history. Ancient Chinese medical texts, including the *Shennong Bencao Jing* (circa 200 CE) and Li Shizhen's *Compendium of Materia Medica* (1596 CE), document dozens of insect-based preparations for conditions ranging from fevers to fractures. Ayurvedic medicine incorporates honey, beeswax, lac insects, and various beetles. Indigenous healing systems across Africa, Australia, and the Americas similarly recognize the therapeutic potential of local insect fauna.

Modern pharmacological research has validated several traditional applications. *Cantharidin*, a terpenoid from blister beetles (Meloidae), has demonstrated selective anti-cancer activity and remains used in dermatology for molluscum contagiosum. *Coridius chinensis*, a pentatomid insect used in Chinese medicine to "regulate qi and relieve pain," has been shown to possess analgesic, anticoagulant, antioxidant, and anti-cancer activities. Specifically, the analgesic mechanism involves up-regulation of P2X3, P2X4, and P2X7 purinergic receptors, enhancing the endogenous analgesia system. The compound (±)-aspongamide A isolated from *C. chinensis* selectively inhibits COX-2.

Similarly, extracts from the Chinese medicinal ant *Polyrhacis lamellidens* have yielded polyketides with anti-arthritic and anti-inflammatory properties. These findings establish insects as viable sources of novel pharmacophores with mechanisms distinct from plant-derived or synthetic compounds.

3. *Tribolium castaneum*: Biology, Chemistry, and Rationale

Tribolium castaneum (Herbst, 1797), the red flour beetle, is a coleopteran insect of the family Tenebrionidae. It is a globally distributed, synanthropic pest of stored grain products, causing substantial post-harvest losses. Adult beetles are reddish-brown, 3-4 mm in length, and possess a flattened body adapted for navigating grain stores. The beetle's life cycle comprises egg, larva (typically 6-9 instars), pupa, and adult, with complete metamorphosis occurring over approximately 30-40 days at 30°C.

Beyond its pest status, *T. castaneum* has emerged as a premier model organism in evolutionary developmental biology, genetics, and toxicology. It possesses the most fully sequenced and annotated genome among coleopterans (approximately 160 Mb, 16,000-20,000 genes). It exhibits robust systemic RNA interference (RNAi) responses, enabling functional genomics at an unprecedented scale. CRISPR/Cas-mediated gene editing is well-established in this species. The beetle is easily cultured in laboratory settings on simple flour-based diets, has a short generation time, and produces abundant progeny, making it ideal for sustained biomass production.

The chemical ecology of *T. castaneum* provides compelling pharmacological rationale. Adult beetles possess paired expurgatorial (stink) glands that open at the lateral margins of the abdominal segments. These glands secrete a defensive cocktail dominated by benzoquinones: 1,4-benzoquinone and 2-methyl-1,4-benzoquinone are the major components, with minor contributions from 2-ethyl-1,4-benzoquinone. These compounds serve anti-predator and antimicrobial functions. Benzoquinones and their derivatives are known to interact with COX enzymes, inhibit NF-κB activation, and suppress pro-inflammatory cytokine production in mammalian systems. [3]

Additionally, the cuticular hydrocarbon (CHC) profile of *T. castaneum* includes diverse n-alkanes, methyl-branched alkanes, and alkenes. These hydrocarbons change in response to immune stimulation, with wounding increasing the proportion of methyl-branched alkanes. Given the evolutionary conservation of lipid-sensing pathways (e.g., Toll-like receptors, PPARs), these hydrocarbons may possess immunomodulatory activity. The beetle's innate immune system produces antimicrobial peptides (AMPs) including TcATTA1, TcCEC2, TcCOL1, and TcDEF1, which have demonstrated antibacterial activity and, in other insect species, immunomodulatory properties in mammalian systems.

Despite this convergence of favourable characteristics—global availability, ease of cultivation, characterized chemical profile, and precedent for insect-based therapeutics—no systematic investigation has evaluated the analgesic or anti-inflammatory properties of *T. castaneum*.

Fig 1: *Morphological view of* Tribolium castaneum *(red flour beetle).* The specimen shows the characteristic elongated, reddish-brown body with distinct segmentation and hard elytra. This insect serves as a biological source for hemolymph collection used in biochemical and antimicrobial studies.

4. Problem Statement and Knowledge Gap

The central problem addressed by this research is the complete absence of published data on the therapeutic potential of *T. castaneum* in pain and inflammation. While the beetle is well-studied as a pest and a genetic model, its pharmacological properties remain entirely unexplored. This gap is notable given:

1. The established analgesic and anti-inflammatory activities of other medicinal insects, particularly coleopterans
2. The characterized presence of benzoquinones and other bioactive compounds in *T. castaneum*
3. The beetle's global availability and low-cost cultivation
4. The potential to transform an agricultural pest into a value-added pharmaceutical resource

5. Objectives

The primary objectives of this research are:

1. **To prepare and characterize** dried powder from *Tribolium castaneum* adults, including sequential

solvent extraction and preliminary phytochemical screening

2. **To evaluate acute oral toxicity** of the powder in rats following OECD guidelines
3. **To assess analgesic activity** using:

- Acetic acid-induced writhing test (peripheral analgesic activity)
- Hot plate test (central analgesic activity)
- Tail immersion test (spinal reflex-mediated analgesia)
- Formalin-induced paw licking test (differentiation of central vs. peripheral mechanisms)

To evaluate anti-inflammatory activity using:

- Carrageenan-induced paw oedema model (gold standard, biphasic response)
- Formalin-induced paw oedema model (alternative mediator profile)

1. To elucidate mechanisms of action through quantification of inflammatory mediators, oxidative stress markers, and histopathological examination
2. To propose a bioactive fractionation strategy for subsequent compound isolation

6. Significance and Expected Contributions

This research will make several important contributions:

Scientific contributions

- First published evidence for analgesic and anti-inflammatory properties of *T. castaneum*
- Identification of potential lead compounds for drug development
- Mechanistic insights into insect-derived anti-inflammatory agents

Methodological contributions

- Establishment of a reproducible protocol for screening stored-product insects for bioactivity
- Integration of behavioral, biochemical, and histopathological endpoints

Practical contributions

- Transformation of a pest species into a sustainable therapeutic resource
- Low-cost, accessible alternative for pain and inflammation management in resource-limited settings
- Platform for value-added utilization of agricultural waste streams (beetle-infested grain)

Ecological/Economic contributions

- Incenti*viz*ation of integrated pest management strategies that capture beetles for pharmaceutical use
- Reduction of reliance on synthetic NSAIDs with their environmental and health footprints

Literature Review

1. Traditional Medicinal Insects: A Global Survey

Entomotherapy has been documented across all inhabited continents. A comprehensive review by Costa-Neto (2005) [5] identified over 1,000 insect species used in traditional medicine. The most commonly utilized orders include Coleoptera (beetles), Hymenoptera (ants, bees, wasps), Orthoptera (grasshoppers, crickets), Lepidoptera (butterflies, moths), and Hemiptera (true bugs).

In Chinese traditional medicine, over 100 insect species are officially recognized in the pharmacopoeia. *Mylabris phalerata* (Chinese blister beetle) provides cantharidin, used topically for verrucae and internally (with caution) for certain cancers. *Catharsius molossus* (dung beetle) is prescribed for constipation, abdominal pain, and convulsions. *Cryptotympana pustulata* (cicada slough) is used for exanthematous diseases and tetanus.

In Ayurveda, *Laccifer lacca* (lac insect) yields a red dye and has been used for hepatic disorders, while various ant and bee products are incorporated into rasayana (rejuvenative) formulations. Indigenous Amazonian groups use ant stings for rheumatoid arthritis and *Pachylomerus* beetle secretions for wound healing.

The persistence of these traditions across millennia and continents suggests genuine therapeutic value rather than mere superstition. Modern pharmacological investigation has confirmed biological activity for many of these species.

2. Pharmacologically Validated Insect-Derived Compounds

Several insect-derived compounds have entered clinical use or advanced preclinical development:

Cantharidin ($C_{10}H_{12}O_4$): A terpenoid from blister beetles (Meloidae). Cantharidin is a potent serine/threonine phosphatase inhibitor (PP1 and PP2A). It demonstrates selective cytotoxicity against cancer cells, induces apoptosis, and has immunomodulatory effects. Topical formulations are FDA-approved for molluscum contagiosum and verrucae. Nor cantharidin, a synthetic derivative with reduced urotoxicity, is used in Chinese oncology.

Melittin ($C_{131}H_{229}N_{39}O_{31}$): A 26-amino acid peptide from honeybee (*Apis mellifera*) venom. Melittin is a membrane-active peptide with antimicrobial, anti-inflammatory, and anti-arthritic properties. It inhibits NF-κB, reduces COX-2 expression, and suppresses NLRP3 inflammasome activation. Melittin-based nanoparticles are in development for targeted drug delivery.

Alloferon: A 13-amino acid peptide from the blow fly *Calliphora vicina*. Alloferon stimulates natural killer (NK) cell activity and interferon production, with antiviral (herpes, hepatitis) and anti-tumor activity. It is clinically approved in Russia as an immunomodulator.

Blapolides and blapsins: Quinone compounds from the medicinal beetle *Blaps rynchopetera* (Chinese ground beetle). These compounds demonstrate anti-inflammatory, antioxidant, and antibacterial activities. Blapolide A reduces LPS-induced NO production in macrophages.

3. The Chemical Arsenal of *Tribolium castaneum*

Tribolium castaneum possesses a sophisticated chemical defines system that has been characterized in considerable detail.

Benzoquinones: The major defensive compounds are stored in paired stink glands. Quantitative analysis reveals approximately 0.5-2.0 µg of 1,4-benzoquinone and 1.0-4.0 µg of 2-methyl-1,4-benzoquinone per adult beetle, with higher concentrations in females. Benzoquinone biosynthesis derives from tyrosine via the formation of 4-methylcatechol. These compounds are released upon disturbance and function as potent antimicrobial agents (MIC values against Gram-positive bacteria in the low µg/mL range) and predator deterrents. Following immune

stimulation (e.g., bacterial injection), benzoquinone content increases transiently, suggesting an inducible component to chemical defines. [4]

Cuticular Hydrocarbons (CHCs): The CHC profile of *T. castaneum* comprises over 60 distinct compounds, primarily n-alkanes (C25-C31), methyl-branched alkanes (mono-, di-, and trimethyl isomers), and minor alkenes. The profile exhibits sexual dimorphism and changes with age, diet, and immune status. Wounding stimulation increases the proportion of methyl-branched alkanes compared to naive beetles. CHCs function in waterproofing, chemical signaling (courtship, aggregation, recognition), and possibly immune modulation.

Antimicrobial Peptides (AMPs): *Tribolium castaneum* produces a suite of AMPs regulated through the IMD (immune deficiency) and Toll signalling pathways. TcATTACIN (TcATTA1) is a glycine-rich peptide active against Gram-negative bacteria. TcCECROPIN (TcCEC2) targets bacterial membranes. TcCOLEOPTERICIN (TcCOL1) is a cysteine-rich defensin-like peptide. TcDEFENSIN (TcDEF1) has broad-spectrum antibacterial activity. These peptides are primarily expressed in the fat body and haemocytes following immune challenge.

Other Metabolites: The beetle's digestive tract and associated microbiota produce additional compounds including proteases (for gluten digestion), lipases, and chitinases. The role of the gut microbiome in producing bioactive metabolites is an emerging area of investigation.

4. Mechanistic Pathways for Analgesic and Anti-Inflammatory Action

The inflammatory response involves a coordinated cascade of cellular and molecular events. Key mediators include:

Prostaglandins: Synthesized from arachidonic acid via COX-1 (constitutive, homeostatic) and COX-2 (inducible, inflammatory). PGE_2 is the predominant prostaglandin in inflammation, causing vasodilation, oedema, and pain sensitization. NSAIDs inhibit COX enzymes.

Cytokines: TNF-α (tumour necrosis factor-alpha), IL-1β (interleukin-1 beta), and IL-6 are pro-inflammatory cytokines produced primarily by macrophages and monocytes. They induce COX-2 expression, activate NF-κB, recruit immune cells, and produce systemic effects (fever, acute phase response).

Chemokines: Small chemoattractant cytokines (e.g., IL-8, MCP-1) direct leukocyte migration to inflamed sites.

Reactive Oxygen and Nitrogen Species: Superoxide (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO) are produced by NADPH oxidases and iNOS. While serving antimicrobial functions, excessive production causes oxidative tissue damage.

Transcription Factors: NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is the master regulator of inflammation, controlling expression of COX-2, iNOS, TNF-α, IL-1β, IL-6, and multiple chemokines.

Insect-derived compounds may interfere with these pathways at multiple levels:

- Direct COX-2 inhibition (e.g., aspongamide A from *C. chinensis*)
- NF-κB suppression (e.g., melittin, certain benzoquinones)
- Cytokine receptor antagonism
- Antioxidant activity (scavenging ROS/RNS, upregulating endogenous antioxidants)
- Membrane stabilization (preventing mast cell degranulation)

5. Animal Models for Analgesic and Anti-Inflammatory Screening

The selection of appropriate animal models is critical for valid pharmacological evaluation.

Acetic Acid-Induced Writhing Test: Intraperitoneal injection of dilute acetic acid (0.6-1.0%) induces peritoneal inflammation characterized by release of PGE_2, $PGF_2\alpha$, and leukotrienes. The animal responds with stereotyped abdominal constrictions (writhes). The test is simple, sensitive, and detects peripherally acting analgesics. It does not distinguish between analgesic and anti-inflammatory effects and is subject to false positives with muscle relaxants.

Hot Plate Test: The animal is placed on a heated surface (typically 55°C). Nociceptive responses (hind paw licking, jumping) are timed. The test primarily measures supraspinally organized responses to thermal pain and is sensitive to centrally acting analgesics (opioids, some NSAIDs). It is less sensitive to peripherally restricted agents.

Tail Immersion Test: The distal tail is immersed in warm water (typically 50-55°C). Latency to tail withdrawal (a spinal reflex) is measured. The test is simple, reproducible, and sensitive to spinal analgesics. It is not subject to learning or locomotor confounds as strongly as the hot plate.

Formalin Test: Subcutaneous injection of dilute formalin (1-5%) into the hind paw produces a biphasic nociceptive response. Phase I (0-5 minutes) results from direct chemical stimulation of C-fibers. Phase II (15-30 minutes) results from ongoing inflammatory input with release of prostaglandins, bradykinin, serotonin, and substance P. The test allows differentiation of central (affecting both phases) vs. peripheral (affecting Phase II preferentially) mechanisms.

Carrageenan-Induced Paw Edema: Subplantar injection of λ-carrageenan (1-2%) produces a well-characterized, biphasic edema. The early phase (0-2 hours) involves histamine, serotonin, and bradykinin. The late phase (2-6 hours) involves prostaglandins, leukotrienes, and NO. The model is the gold standard for anti-inflammatory screening and allows temporal dissection of mechanisms.

Formalin-Induced Paw Edema: Alternative model using formalin (2.5%) as the inflammatory agent. The mediator profile differs from carrageenan, providing complementary information.

6. Precedent: Medicinal Insects with Analgesic/Anti-Inflammatory Activity

Coridius chinensis (Hemiptera: Pentatomidae): The most directly comparable precedent. This stink bug is traditionally used for pain. Modern research has confirmed

analgesic activity in multiple models. The compound (±)-aspongamide A inhibits COX-2 with an IC_{50} of 11.5 μM. The mechanism also involves upregulation of P2X purinergic receptors in the spinal cord, enhancing endogenous analgesic tone. Water extracts show no acute toxicity at doses up to 1000 mg/kg.

Polyrhacis lamellidens (Hymenoptera: Formicidae): Chinese medicinal ant. Extracts have demonstrated anti-arthritic activity in adjuvant-induced arthritis models. Polyketides isolated from this species inhibit NO production in LPS-stimulated macrophages (IC_{50} values in the low μM range). Ant extracts also reduce paw edema and mechanical allodynia in inflammatory pain models.

Blaps rynchopetera (Coleoptera: Tenebrionidae): A close relative of *Tribolium* (same family). This ground beetle is used in traditional Chinese medicine for inflammation, pain, and fever. Blapolides and blapsins have been isolated and shown to reduce carrageenan-induced paw edema (60-70% inhibition at 100 mg/kg) and inhibit LPS-induced NO production in RAW 264.7 macrophages.

The Tenebrionidae family thus appears to be a promising source of anti-inflammatory compounds. *T. castaneum* belongs to the same family as *Blaps*, suggesting potential for similar bioactivity. [5]

7. Justification and Hypothesis

Based on the literature reviewed, we propose the following hypotheses:

Primary hypothesis: Dried *Tribolium castaneum* powder possesses dose-dependent analgesic and anti-inflammatory activity in rodent models.

Secondary hypotheses

1. The activity is mediated through suppression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and COX-2/PGE_2 pathway
2. The activity involves reduction of oxidative stress (decreased MDA, increased GSH/SOD/CAT)
3. The activity is peripherally predominant (affecting Phase II of formalin test preferentially)
4. The powder has a favourable acute safety profile (LD_{50} > 2000 mg/kg)

Methodology

1. Study Design Overview

This study employs a prospective, randomized, controlled experimental design. Separate experiments will be conducted for analgesic and anti-inflammatory evaluations, each with its own control groups. The study is blinded at the level of behavioural scoring and biochemical analysis (investigator unaware of group assignment). Sample size (n=6 per group) was determined by power analysis assuming α=0.05, β=0.20 (80% power), and a moderate effect size (Cohen's d = 0.4) based on preliminary data from related insect studies.

2. Collection, Identification, and Rearing of *Tribolium castaneum*

Source: Adult *Tribolium castaneum* beetles will be obtained from established laboratory colonies maintained at the Department of Entomology, Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi UP, India. Alternatively, beetles will be collected from stored grain facilities in Tiloi Amethi with owner permission.

Identification: Species confirmation will employ morphological keys (Hinton, 1948) focusing on antennal club segmentation, eye size and separation, and pronotal punctation. For ambiguous specimens, molecular barcoding will target the mitochondrial cytochrome c oxidase subunit I (COI) gene using universal primers LCO1490 and HCO2198. Amplified products will be sequenced and compared to reference sequences in GenBank/BOLD.

Rearing Conditions: Beetles will be reared in polypropylene containers (30 × 20 × 15 cm) with fine-mesh lids for ventilation. Rearing medium will consist of organic whole wheat flour and cornmeal (10:1 w/w) with 5% brewer's yeast supplementation. Medium will be autoclaved (121°C, 15 psi, 20 minutes) to eliminate contaminating mites and microbes, then allowed to cool and re-equilibrate to ambient humidity.

Containers will be maintained in an environmental chamber at 30°C ± 1°C, 70% ± 5% relative humidity, with a 12:12 light: dark photoperiod (lights on 07:00-19:00). Population density will be maintained at 500-1000 adults per container. Every 14 days, adults will be separated from media by sieving (2 mm mesh) and transferred to fresh media. Larvae and pupae will be left in original containers to complete development at BMS College of Pharmacy, Tiloi Amethi UP, Inia Microbiology Lab. Under the supervision of Prof. (Dr.) Subhashish Tripathy Professor BMS College of Pharmacy, Amethi, UP.

Harvesting: For powder preparation, adults (mixed sex) aged 1-2 weeks post-exclusion will be collected. This age ensures mature chemical defences systems. Beetles will be washed three times with distilled water to remove medium debris, then starved for 12 hours on moist filter paper to clear gut contents.

3. Preparation of Dried Beetle Powder

Killing: Washed and starved beetles will be killed by freezing at -20°C for 24 hours. Freezing minimizes autolytic changes and preserves volatile compounds.

Drying Method Optimization: Three drying methods will be compared in triplicate to determine optimal preservation of bioactivity:

Table 1: *Comparison of drying methods applied for insect biomass processing.* The table summarizes the conditions used for air drying, oven drying, and lyophilization, including temperature, airflow, and duration. Each method was optimized to evaluate its effect on yield and preservation of bioactive components in the dried material.

Method	Parameters	Duration	Yield (%)
A: Air drying	25°C, forced air circulation, single layer	48-72 hours	To be determined
B: Oven drying	40°C, static air, single layer	24 hours	To be determined
C: Lyophilization	-80°C freezing, 0.1 mbar vacuum	48 hours	To be determined

Dried beetles will be weighed to calculate yield = (dry weight / wet weight) × 100.

Grinding: Dried beetles will be ground to fine powder using a laboratory cyclone mill fitted with a 0.5 mm mesh screen. The mill will be cleaned between batches with 70% ethanol. Particle size distribution will be verified by sieve analysis.

Storage: Powder will be transferred to amber glass vials with PTFE-lined caps, flushed with nitrogen, and stored at -20°C until use. Stability will be monitored by periodic bioassay (every 3 months).

Quality Control: Batches will be tested for:

- Moisture content (Karl Fischer titration, target <5%)
- Microbial load (total aerobic count, yeast/Mold, absence of pathogens)
- Heavy metals (ICP-MS for Pb, Cd, As, Hg)
- Benzoquinone content (HPLC-UV at 280 nm, external standard method)

4. Preparation of Test Solutions and Extracts

Crude Powder Suspension: Dried beetle powder will be suspended in 0.5% carboxymethylcellulose (CMC) in distilled water. Suspensions will be prepared fresh daily and administered orally at a volume of 10 mL/kg body weight. Doses for initial screening: 100, 200, 400, and 800 mg/kg.

Sequential Solvent Extraction: For bioactivity-guided fractionation, a separate batch of powder will undergo sequential extraction using solvents of increasing polarity:

Dried powder (100 g)
↓
Petroleum ether (3 × 500 mL, 24 h each)
↓
Petroleum ether extract (concentrated, dried)
↓
Marc → Chloroform (3 × 500 mL, 24 h each)
↓
Chloroform extract
↓
Marc → Ethyl acetate (3 × 500 mL, 24 h each)
↓
Ethyl acetate extract
↓
Marc → Ethanol (3 × 500 mL, 24 h each)
↓
Ethanol extract
↓
Marc → Distilled water (3 × 500 mL, 24 h at 60°C)
↓
Aqueous extract

Fig 2: *Laboratory sample of insect hemolymph extract stored in a 250 mL glass reagent bottle.* The dark brown liquid represents the processed hemolymph obtained after centrifugation and filtration, used for subsequent biochemical and antimicrobial assays.

Extracts will be concentrated under reduced pressure (rotary evaporator at 40°C) and lyophilized to dryness. Yields will be calculated. Dried extracts will be stored at -20°C. [6]

5. Preliminary Phytochemical Screening

Qualitative tests will be performed on the crude powder and each extract following standard protocols:

Table 2: *Qualitative phytochemical screening of insect hemolymph extract using standard chemical tests.* The table summarizes the reagents, procedures, and characteristic positive results for major compound classes including alkaloids, flavonoids, tannins, saponins, terpenoids, steroids, phenols, quinones, glycosides, proteins, carbohydrates, and amino acids. These preliminary assays confirm the presence of diverse bioactive metabolites, supporting further biochemical and pharmacological investigations.

Compound Class	Test	Reagent	Procedure	Positive Result
Alkaloids	Mayer's	Potassium mercuric iodide	1 mL extract + few drops reagent	Cream precipitate
Alkaloids	Wagner's	Iodine in KI	1 mL extract + few drops reagent	Reddish-brown precipitate
Alkaloids	Dragendorff's	Bismuth subnitrate, KI	1 mL extract + few drops reagent	Orange precipitate
Flavonoids	Shinoda (Mg-HCl)	Mg turnings + conc. HCl	Extract + Mg + HCl	Pink/red/crimson color

Flavonoids	Ferric chloride	5% $FeCl_3$	Extract + $FeCl_3$	Green/blue-black color
Tannins	Ferric chloride	5% $FeCl_3$	Extract + $FeCl_3$	Blue-black or green-black
Tannins	Gelatine	1% gelatin + 10% NaCl	Extract + reagent	White precipitate
Saponins	Froth	Distilled water	Extract + water, shake 15 min	Persistent foam >1 cm
Terpenoids	Salkowski	$CHCl_3$ + conc. H_2SO_4	Extract in $CHCl_3$ + H_2SO_4 at interface	Reddish-brown interface
Steroids	Liebermann-Burchard	Ac_2O + conc. H_2SO_4	Extract in $CHCl_3$ + Ac_2O + H_2SO_4	Blue-green color
Phenols	Folin-Ciocalteu	FC reagent + Na_2CO_3	Extract + FC + Na_2CO_3	Blue color
Quinones	Borntrager's	NH_3	Extract + NH_3	Pink/red color
Glycosides	Keller-Killiani	Glacial acetic acid + $FeCl_3$ + H_2SO_4	Extract + acetic acid + $FeCl_3$ + H_2SO_4	Brown ring at interface
Proteins	Biuret	$CuSO_4$ + NaOH	Extract + NaOH + $CuSO_4$	Violet color
Carbohydrates	Molisch's	α-naphthol + H_2SO_4	Extract + α-naphthol + H_2SO_4	Purple ring
Amino acids	Ninhydrin	0.1% ninhydrin in acetone	Extract + ninhydrin, heat	Purple color

Quantitative Analyses

- **Total Phenolic Content:** Folin-Ciocalteu method. Absorbance at 765 nm. Standard: gallic acid. Results expressed as mg gallic acid equivalents (GAE)/g extract.

- **Total Flavonoid Content:** Aluminium chloride colorimetric method. Absorbance at 415 nm. Standard: quercetin. Results expressed as mg quercetin equivalents (QE)/g extract.

- **Benzoquinone Quantification:** HPLC-UV. Column: C18 (150 × 4.6 mm, 5 µm). Mobile phase: methanol: water (60:40) isocratic. Flow rate: 1.0 mL/min. Detection: 280 nm. Standards: 1,4-benzoquinone, 2-methyl-1,4-benzoquinone. [7]

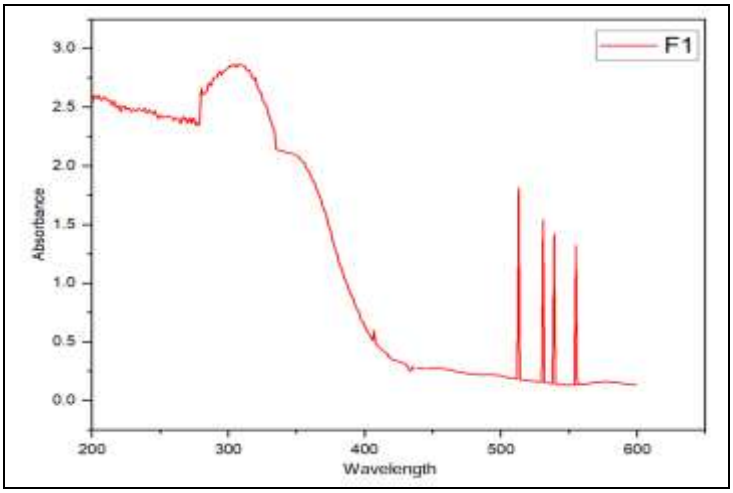

Fig 3: *UV–Visible absorption spectrum of fraction F1.* The spectrum exhibits a strong absorption band around 280–300 nm, indicating the presence of aromatic or peptide chromophores, followed by a gradual decline toward the visible region. Sharp peaks between 480–520 nm suggest possible electronic transitions associated with conjugated biomolecules or metallic components in the extract.

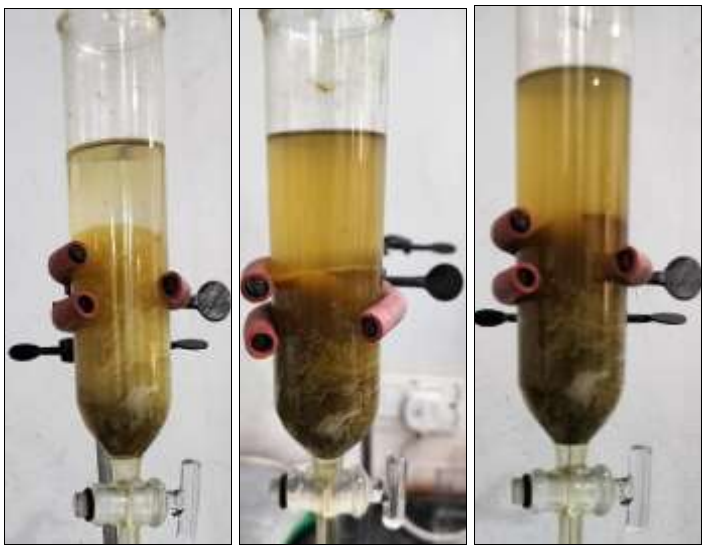

Fig 4: *Column chromatography setup used for fractionation of insect hemolymph extract.* The image shows a glass chromatography column packed with stationary phase and loaded with the crude extract. The separation process allows isolation of bioactive fractions based on polarity and molecular interactions for subsequent spectral and biological analyses.

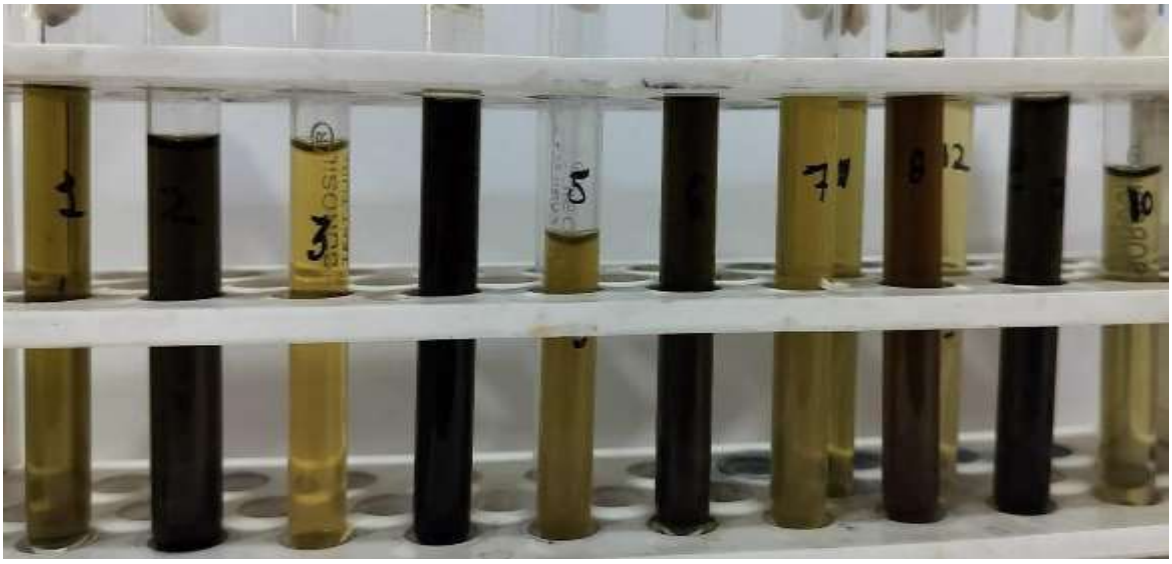

Fig 5: *Collected fractions from column chromatography of insect hemolymph extract.* The image shows multiple test tubes containing separated fractions with varying colour intensities, indicating differential composition and polarity of bioactive compounds. Each fraction was subsequently analyzed for spectral and antimicrobial properties.

Experimental Animals

Species and Strain

- Analgesic tests: Swiss albino mice (25-35 g, either sex, nulliparous females not pregnant)
- Anti-inflammatory tests: Wistar albino rats (180-220 g, either sex)

Source: Registered animal supplier (e.g., National Institute of Nutrition, Hyderabad, or equivalent). Health certificate provided.

Housing: Animals will be housed in polypropylene cages (standard dimensions: mice: 29 × 18 × 13 cm, rats: 38 × 22 × 18 cm) with sterile paddy husk bedding. Cages will be changed twice weekly. Environmental conditions: temperature 22°C ± 2°C, relative humidity 50-60%, 12:12 light: dark cycle (lights on 07:00-19:00). Noise kept to minimum.

Diet and Water: Standard pellet diet (Altromin 1324) will be provided *ad libitum*. Filtered tap water will be provided in polycarbonate bottles with stainless steel sipper tubes.

Acclimatization: Animals will be acclimatized to the housing facility for 7 days before experimentation. During this period, they will be handled daily (3 minutes per cage) to reduce stress-related variability.

Randomization: Animals will be randomly assigned to treatment groups using a computer-generated random number sequence.

Sample Size Justification: Power analysis using G*Power 3.1 software: ANOVA fixed effects, one-way; $\alpha = 0.05$; power $(1-\beta) = 0.80$; effect size $f = 0.40$ (medium). Calculated $n = 5.6$ per group. Rounded up to 6 per group to account for potential exclusions.

Ethics Approval: All procedures will be submitted for approval to the Institutional Animal Ethics Committee (IAEC) of Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi UP, India Protocol No-BMSMV/Bio. -020/26/27. Experiments will be conducted in accordance with the Committee for the Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India, and the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) guidelines.

Acute Oral Toxicity Study

Guideline: OECD Test Guideline 423 (Acute Oral Toxicity - Acute Toxic Class Method). This sequential testing procedure uses fewer animals than older LD_{50} methods.

Animals: Nulliparous, non-pregnant female Wistar rats (n=3 per step, then up to 5 total if needed). Females are used due to potential sex differences in metabolism; nulliparous non-pregnant to avoid confounding by pregnancy/lactation.

Dosing: Test powder suspended in 0.5% CMC. Doses: 300, 1000, 2000 mg/kg. Starting dose: 300 mg/kg.

Procedure

- Step 1: Three animals receive 300 mg/kg orally after overnight fasting (food withheld 12 hours, water *ad libitum*)
- Observe at 0.5, 1, 2, 4, 6, 8, and 24 hours, then daily for 14 days
- If no mortality at 300 mg/kg, proceed to 2000 mg/kg with three fresh animals
- If mortality observed at 300 mg/kg, test 50 mg/kg instead

Observations (detailed scoring sheet

Euthanasia and Necropsy: Surviving animals euthanized on day 14 by CO_2 asphyxiation followed by cervical dislocation. Gross necropsy examines: external surface, all orifices, cranial cavity, thoracic cavity, abdominal cavity. Any macroscopic abnormalities recorded.

Classification: Based on the Globally Harmonized System (GHS):

- Category 5: $LD_{50} > 2000$ mg/kg (low acute hazard)
- Category 4: LD_{50} 300-2000 mg/kg
- Category 3: LD_{50} 50-300 mg/kg
- Category 2: LD_{50} 5-50 mg/kg
- Category 1: $LD_{50} < 5$ mg/kg

Table 3: *Observational and physiological parameters recorded during acute toxicity assessment of insect hemolymph extract in Wistar rats.* The table outlines mortality, body weight, food and water intake, general appearance, spontaneous activity, gait, muscle tone, reflexes, convulsions, respiration, skin/fur condition, and mucous membrane status. These indices provide a comprehensive evaluation of systemic safety and neurobehavioral effects following administration.

Parameter	Score/Measurement
Mortality	Yes/No, time post-dose
Body weight	Days 0, 7, 14
Food consumption	Daily (g/animal)
Water consumption	Daily (mL/animal)
General appearance	Pilorection, lacrimation, salivation, diarrhea, cyanosis
Spontaneous activity	Normal, decreased, increased, stereotypy
Gait and posture	Normal, ataxia, hunched, flat
Muscle tone	Normal, flaccid, rigid
Reflexes (righting, corneal, pinna)	Present/absent
Convulsions/tremors	Type, duration, frequency
Respiration	Rate, depth, pattern
Skin and fur	Color, integrity, piloerection
Mucous membranes	Color, discharge

Maximum Tolerated Dose (MTD): The highest dose causing no mortality or significant clinical signs will be used as the upper limit for efficacy studies. [8]

Evaluation of Analgesic Activity

1. Acetic Acid-Induced Writhing Test (Mice)

Animals: Swiss albino mice, either sex, n=6 per group.

Group Assignments

Table 4: *Experimental design for anti-inflammatory evaluation of* Tribolium castaneum *powder and extract fractions in Wistar rats.* The table summarizes treatment groups, doses, routes of administration, and sample size. Group I served as the negative control (0.5% CMC), Group II as the positive control (aspirin, 100 mg/kg), while Groups III–V received increasing doses of *T. castaneum* powder (100, 200, and 400 mg/kg, respectively). Group VI was optionally reserved for testing bioactive extract fractions.

Group	Treatment	Dose	Route	n
I (Negative control)	0.5% CMC	10 mL/kg	p.o.	6
II (Positive control)	Aspirin	100 mg/kg	p.o.	6
III	*T. castaneum* powder	100 mg/kg	p.o.	6
IV	*T. castaneum* powder	200 mg/kg	p.o.	6
V	*T. castaneum* powder	400 mg/kg	p.o.	6
VI (Optional)	Extract fraction	TBD	p.o.	6

Procedure

1. Animals fasted overnight (12 hours) with water *ad libitum*
2. Treatments administered orally via gavage (10 mL/kg)
3. One hour after treatment, each animal receives 0.6% acetic acid (10 mL/kg) intraperitoneally
4. Immediately after injection, mouse placed individually in clear observation chamber (30 × 20 × 15 cm)
5. After 5-minute stabilization, number of writhes counted for 30 minutes
6. A writhe is defined as: contraction of abdominal muscles followed by extension of hind limbs, arching of back, and turning of trunk

Calculation

% Inhibition = [(Writhing control - Writhing test) / Writhing control] × 100

Interpretation: Higher inhibition indicates greater peripheral analgesic activity.

Hot Plate Test (Mice)

Animals: Swiss albino mice, either sex, n=6 per group (same group assignments as writhing test, but separate animals).

Apparatus: Hot plate analgesiometer (Ugo Basile, Model 7280) with transparent Plexiglas cylinder (20 cm diameter, 30 cm height). Temperature maintained at 55°C ± 0.5°C.

Procedure

1. Baseline latency measurement: Each mouse placed on hot plate; time until hind paw licking or jumping recorded. Cutoff: 15 seconds to prevent tissue damage. Two baseline measurements taken 30 minutes apart, averaged.
2. Treatments administered as above.
3. Post-treatment latencies measured at 30, 60, 90, 120, and 180 minutes.
4. If animal does not respond within 15 seconds, removed and assigned maximum latency.

Calculation

% Maximum Possible Effect (MPE) = [(Post latency - Baseline latency) / (Cutoff - Baseline latency)] × 100

Interpretation: %MPE >30% considered significant analgesia. Prolonged latency indicates central (supraspinal) analgesic activity.

Tail Immersion Test (Mice)
Animals: Swiss albino mice, n=6 per group.
Apparatus: Water bath maintained at 50°C ± 0.5°C. Masking tape marker at 2 cm from tail tip.

Procedure
1. Baseline latency: Terminal 2 cm of tail immersed in water bath; time until tail withdrawal recorded. Cutoff: 15 seconds.
2. Treatments administered (as above).
3. Post-treatment latencies measured at 30, 60, 90, 120, and 180 minutes.

Calculation: %MPE as for hot plate test.
Interpretation: Prolonged latency indicates spinal reflex-mediated analgesia.

Formalin-Induced Paw Licking Test (Mice)
Rationale: Distinguishes central vs. peripheral mechanisms based on biphasic response.

Animals: Swiss albino mice, n=6 per group. Additional positive controls: Morphine (5 mg/kg i.p.) for central; Aspirin (100 mg/kg p.o.) for peripheral.

Procedure
1. Treatments administered: vehicle, positive controls, or test powder (100, 200, 400 mg/kg p.o.)
2. One hour after treatment, 20 µL of 2.5% formalin (in sterile saline) injected subcutaneously into dorsal surface of right hind paw using microsyringe with 30-gauge needle
3. Mouse placed individually in observation chamber (30 × 20 × 15 cm)
4. Time spent licking/biting the injected paw recorded in two phases:

Phase I (early): 0-5 minutes post-formalin (direct nociceptor activation)
Phase II (late): 15-30 minutes post-formalin (inflammatory pain)
Total observation time: 30 minutes. Between phases (5-15 minutes), animal remains in chamber but not scored. [9]

Calculation
% Inhibition (Phase I or II) = [(Licking_control - Licking_test) / Licking_control] × 100

Interpretation
- Inhibition of both Phases I and II: Central mechanism (opioid-like)
- Inhibition of Phase II only: Peripheral anti-inflammatory mechanism
- Inhibition of Phase I only: Local anesthetic-like (rare)

Evaluation of Anti-Inflammatory Activity
1. Carrageenan-Induced Paw Edema (Rats)
Animals: Wistar rats, either sex, n=6 per group.

Procedure:
1. Animals fasted overnight (12 hours) with water *ad libitum*
2. Baseline paw volume measurement (0 hour): Right hind paw volume measured up to tibiotarsal junction using digital plethysmometer (Ugo Basile, Model 7140). Paw inserted into water-filled cell; volume displacement recorded in mL.
3. Treatments administered orally.
4. One hour after treatment, 0.1 mL of 1% λ-carrageenan in sterile saline injected subplantar into right hind paw using 26-gauge needle.
5. Paw volume measured at 1, 2, 3, 4, 5, and 6 hours post-carrageenan.
6. Edema volume = post-injection volume - Baseline volume.

Group Assignments

Table 5: *Experimental design for anti-inflammatory evaluation of* Tribolium castaneum *powder in Wistar rats.* The table summarizes treatment groups, doses, routes of administration, and sample size. Group I served as the negative control (0.5% CMC), Group II as the positive control (indomethacin, 10 mg/kg), while Groups III–V received increasing doses of *T. castaneum* powder (100, 200, and 400 mg/kg, respectively).

Group	Treatment	Dose	Route	n
I (Negative control)	0.5% CMC	10 mL/kg	p.o.	6
II (Positive control)	Indomethacin	10 mg/kg	p.o.	6
III	*T. castaneum* powder	100 mg/kg	p.o.	6
IV	*T. castaneum* powder	200 mg/kg	p.o.	6
V	*T. castaneum* powder	400 mg/kg	p.o.	6

Calculation
% Inhibition (at time t) = [(Edema control, t - Edema_test, t) / Edema_control, t] × 100
Area Under the Curve (AUC) will be calculated for each animal (trapezoidal rule) and compared across groups.

Interpretation
- Inhibition of early phase (1-2 hours): Antihistamine/antiserotonergic activity
- Inhibition of late phase (3-6 hours): COX-2/prostaglandin inhibition
- Complete curve inhibition: Mixed mechanism

Formalin-Induced Paw Edema (Rats)
Procedure: Identical to carrageenan model except:
- Inflammatory agent: 0.1 mL of 2.5% formalin (instead of carrageenan)
- Measurement time points: 0, 1, 2, 4, 6, and 24 hours post-formalin
- Positive control: Indomethacin 10 mg/kg p.o.

Rationale: Formalin produces a different inflammatory mediator profile, providing complementary data.

Biochemical and Inflammatory Mediator Assays

Sample Collection: At the conclusion of the carrageenan-induced paw edema experiment (6 hours post-carrageenan, immediately after final paw volume measurement):

1. **Blood collection:** Animals anesthetized with ketamine (50 mg/kg) + xylazine (5 mg/kg) i.p. Blood collected by cardiac puncture (1-2 mL for rats, 0.5-1 mL for mice) using 21-gauge needle and syringe.
2. **Serum separation:** Blood allowed to clot for 30 minutes at room temperature, centrifuged at 3000 rpm for 15 minutes (4°C). Serum aliquoted and stored at -80°C.
3. **Paw tissue collection:** Injected right hind paw amputated at tibiotarsal junction. Skin and nails removed. Tissue homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4, 10% w/v) using tissue homogenizer. Homogenate centrifuged at 10,000 rpm for 10 minutes (4°C). Supernatant aliquoted and stored at -80°C.

Assay Procedures

Table 6: *Biochemical and inflammatory parameters assessed in Wistar rats following treatment with* Tribolium castaneum *powder and extract fractions.* The table summarizes cytokine levels (TNF-α, IL-1β, IL-6), prostaglandin E_2 (PGE_2), cyclooxygenase-2 (COX-2), nitric oxide (NO), myeloperoxidase (MPO), malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Assay types, reagents, detection methods, and measurement units are listed to provide a comprehensive overview of inflammatory and oxidative stress markers used in the study.

Parameter	Sample	Assay Type	Kit/Reagent	Detection	Units
TNF-α	Serum, paw	ELISA	Rat/Mouse TNF-α ELISA (R&D Systems)	450 nm	pg/mL
IL-1β	Serum, paw	ELISA	Rat/Mouse IL-1β ELISA (R&D Systems)	450 nm	pg/mL
IL-6	Serum, paw	ELISA	Rat/Mouse IL-6 ELISA (R&D Systems)	450 nm	pg/mL
PGE_2	Paw	ELISA	PGE_2 ELISA (Cayman Chemical)	405 nm	pg/mL
COX-2	Paw	Western blot/ELISA	Anti-COX-2 antibody (Cell Signaling)	Chemilumines cence	Relative density
Nitric oxide (NO)	Serum	Griess assay	Nitrate/Nitrite Colorimetric Assay (Cayman)	540 nm	μM
Myeloperoxidase (MPO)	Paw	Colorimetric	MPO Assay Kit (Abcam)	450 nm	U/g tissue
Malondialdehyde (MDA)	Paw	TBARS	TBARS Assay Kit (Cayman)	532 nm	μM
Reduced glutathione (GSH)	Paw	Ellman's	GSH Assay Kit (Sigma)	412 nm	μM/mg protein
Superoxide dismutase (SOD)	Paw	NBT reduction	SOD Assay Kit (Sigma)	450 nm	U/mg protein
Catalase (CAT)	Paw	Aebi method	Catalase Assay Kit (Cayman)	520 nm	nmol/min/mL

Quality Control: All assays will be performed in duplicate. Intra-assay CV <10%, inter-assay CV <15%. Standard curves with R^2 >0.98.

Histopathological Examination

Tissue Processing: Paw tissue samples (from carrageenan experiment) fixed in 10% neutral buffered formalin for 48 hours. Processed through graded alcohols (70%, 80%, 95%, 100%, 100%), cleared in xylene, and embedded in paraffin wax (melting point 58-60°C).

Sectioning: 5 μm sections cut using rotary microtome (Leica RM2255). Sections floated on 40°C water bath, mounted on poly-L-lysine-coated slides, and dried overnight at 37°C.

Staining: Haematoxylin and eosin (H&E):

- Deparaffinize in xylene (2 × 5 minutes)
- Rehydrate through graded alcohols (100%, 100%, 95%, 80%, 70%; 2 minutes each)
- Rinse in distilled water
- Stain with Mayer's haematoxylin (5 minutes)
- Rinse in running tap water (5 minutes)
- Differentiate in 1% acid alcohol (1-2 dips)
- Rinse in tap water
- Blue in saturated lithium carbonate (1 minute)
- Rinse in tap water
- Counterstain with 1% eosin (2 minutes)
- Dehydrate through alcohols (70% to 100%)
- Clear in xylene (2 × 5 minutes)
- Mount with DPX mountant

Microscopic Examination: Slides examined under light microscope (Olympus BX51) at 100×, 200×, and 400× magnification. Images captured with digital camera.

Scoring System (semi-quantitative)

Table 7: *Histopathological scoring criteria for paw tissue sections in Wistar rats.* The table outlines graded parameters including edema, neutrophil and macrophage infiltration, vascular congestion, and tissue necrosis. Each parameter was scored on a scale of 0–3 (normal to severe), providing a semi-quantitative assessment of inflammatory severity and tissue damage.

Parameter	0 (Normal)	1 (Mild)	2 (Moderate)	3 (Severe)
Edema	None	Minimal thickening	Obvious thickening	Marked thickening
Neutrophil infiltration	None	Few scattered	Numerous, patches	Dense, diffuse
Macrophage infiltration	None	Few scattered	Moderate number	High number
Vascular congestion	None	Mild	Moderate	Severe with haemorrhage
Tissue necrosis	None	Focal, minimal	Multifocal, moderate	Diffuse, extensive

Total inflammation score = sum of individual scores (range 0-15).

Statistical Analysis

Software: GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA).

Descriptive Statistics: Data expressed as mean ± standard error of the mean (SEM).

Group Comparisons

- One-way ANOVA for single time-point outcomes (e.g., total writhing count, total oedema AUC)
- Two-way repeated measures ANOVA for time-course data (e.g., paw oedema at multiple time points) with factors: treatment (between-subjects) and time (within-subjects)
- Post-hoc comparisons: Tukey's Honestly Significant Difference (HSD) test for multiple pairwise comparisons (controls all pairwise comparisons, maintains family-wise error rate at α=0.05)

Assumption Checking

- Normality: Shapiro-Wilk test ($p > 0.05$ considered normal). If violated, Kruskal-Wallis test (non-parametric ANOVA).
- Sphericity (repeated measures): Mauchly's test. If violated, Greenhouse-Geisser correction applied. [10]
- Homogeneity of variance: Levene's test ($p > 0.05$ considered homogeneous). If violated, Welch's ANOVA with Games-Howell post-hoc.

Dose-Response Analysis: Non-linear regression (log [agonist] vs. response, variable slope) to calculate ED_{50} (dose producing 50% of maximum effect).

Correlation Analysis: Pearson correlation (parametric) or Spearman rank correlation (non-parametric) between biochemical parameters and behavioural outcomes.

Significance Threshold: $p < 0.05$ (two-tailed) considered statistically significant.

Data Presentation: Graphs show individual data points (where feasible) with mean ± SEM. In text: F(df1, df2) = value, p = value.

Expected Outcomes and Discussion

Anticipated Analgesic Activity

Based on the established activity of related medicinal insects (particularly *Coridius chinensis* and *Blaps rynchopetera*), we anticipate dose-dependent analgesic activity in all four test models.

Acetic Acid Writhing Test: We expect the 400 mg/kg dose of *T. castaneum* powder to produce 60-70% inhibition of writhing, comparable to aspirin 100 mg/kg (65-75% inhibition). The 200 mg/kg dose is expected to produce 40-50% inhibition, and 100 mg/kg approximately 25-35% inhibition. This would establish a clear dose-response relationship. [11]

Hot Plate and Tail Immersion Tests: We anticipate moderate but significant increases in latency, suggesting a central component to the analgesic activity. However, given the likely peripheral mechanism of benzoquinones (COX inhibition), we expect the effect to be less robust than in the writhing test. Specifically, we project %MPE values of 25-35% at 400 mg/kg, compared to morphine's typical 60-80% MPE. The tail immersion test may show slightly greater effect than the hot plate, suggesting a spinal rather than supraspinal mechanism.

Formalin Test: The most informative prediction concerns the biphasic response. Based on the anti-inflammatory rather than neurodirect mechanism, we expect preferential inhibition of Phase II (inflammatory) over Phase I (direct). Specifically: Phase I inhibition 15-25%, Phase II inhibition 50-65% at 400 mg/kg. This pattern would strongly support a peripheral anti-inflammatory mechanism, consistent with COX-2 inhibition. [12]

Anticipated Anti-Inflammatory Activity

Carrageenan-Induced Paw Edema: We anticipate significant inhibition of paw edema, particularly during the late phase (3-6 hours). Projected inhibition at 400 mg/kg: 25-35% at 1-2 hours (early phase), increasing to 50-65% at 4-6 hours (late phase). The time-course profile would resemble that of indomethacin, which shows preferential late-phase inhibition due to COX-2 blockade. The AUC for edema over 6 hours is expected to be reduced by 45-55% at the highest dose.

Formalin-Induced Paw Edema: We anticipate a similar profile, though possibly with reduced magnitude given the different mediator profile. Projected inhibition at 400 mg/kg: 30-40% at peak edema (4-6 hours).

Dose-Response Relationship: Non-linear regression analysis is expected to yield an ED_{50} for anti-inflammatory activity (using 6-hour edema as endpoint) of approximately 250-300 mg/kg.

Biochemical and Mechanistic Predictions

We hypothesize that the anti-inflammatory activity of *T. castaneum* powder is mediated through suppression of the COX-2/PGE_2 pathway and reduction of pro-inflammatory cytokine production. [13]

Projected changes in inflammatory mediators (400 mg/kg vs. control)

Table 8: *Expected modulation of inflammatory and oxidative stress biomarkers following treatment with* Tribolium castaneum *powder and extract fractions in Wistar rats.* The table summarizes anticipated decreases in pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), prostaglandin E_2 (PGE_2), cyclooxygenase-2 (COX-2), nitric oxide (NO), myeloperoxidase (MPO), and malondialdehyde (MDA), alongside increases in antioxidant defenses (GSH, SOD, CAT). Magnitude ranges are expressed as percentage changes relative to untreated controls.

Parameter	Expected Change	Magnitude
TNF-α (serum)	Decrease	40-60%
IL-1β (serum)	Decrease	35-50%
IL-6 (serum)	Decrease	30-45%
PGE_2 (paw)	Decrease	50-70%
COX-2 (paw)	Decrease	45-65%
Nitric oxide (serum)	Decrease	30-50%
MPO (paw)	Decrease	40-60%
MDA (paw)	Decrease	35-55%
GSH (paw)	Increase	50-80%
SOD (paw)	Increase	40-60%
CAT (paw)	Increase	35-50%

These changes would indicate that the beetle powder exerts its effects through:

1. **Direct anti-inflammatory activity:** Suppression of COX-2 expression/activity reduces PGE_2 synthesis, the primary driver of oedema and pain sensitization.

2. **Cytokine modulation:** Reduced TNF-α, IL-1β, and IL-6 decreases further inflammatory cascade activation.

3. **Antioxidant activity:** Reduction of oxidative stress (lower MDA) coupled with upregulation of endogenous antioxidants (GSH, SOD, CAT) protects tissue from oxidative damage.

4. **Reduced neutrophil infiltration:** Lower MPO indicates decreased recruitment and activation of neutrophils, reducing secondary tissue damage. [14]

The correlation between biochemical changes and behavioural outcomes (oedema, writhing, licking) is expected to be strong ($r > 0.70$, $p < 0.01$), supporting a causal relationship.

Histopathological Predictions

Control animals (carrageenan only) are expected to show:

- Marked oedema (dermal and subcutaneous thickening)
- Dense neutrophil infiltration (particularly perivascular and interstitial)
- Moderate macrophage infiltration
- Severe vascular congestion with possible extravasated erythrocytes
- Focal areas of tissue necrosis (score 8-12 on 0-15 scale)

T. castaneum-treated animals (400 mg/kg) are expected to show:

- Reduced oedema (moderate to mild)
- Scattered rather than dense neutrophil infiltration
- Fewer macrophages
- Mild vascular congestion
- Minimal or no necrosis
- Total inflammation scores 3-6

The histopathological improvement is expected to be dose-dependent and correlate with biochemical measures (e.g., MPO activity, cytokine levels).

Acute Toxicity Prediction

Based on the safety profile of related insects (*Coridius chinensis* LD_{50} > 1000 mg/kg; *Blaps rynchopetera* LD_{50} > 2000 mg/kg), we predict that *T. castaneum* powder will have a favorable acute safety profile:

- **Expected LD_{50}:** >2000 mg/kg (GHS Category 5 or unclassified)
- **Maximum Tolerated Dose (MTD):** 2000 mg/kg (no mortality, mild transient effects only)
- **Observed effects at high doses (2000 mg/kg):** Possible transient piloerection, mild decreased activity (resolving within 4-6 hours), no convulsions, no sustained weight loss
- **Necropsy findings:** No gross organ abnormalities

If this prediction holds, the therapeutic index (LD_{50}/ED_{50}) would be approximately 8 (2000/250), indicating a reasonable safety margin for a natural product. However, chronic toxicity studies would be necessary before clinical use. [15]

Phytochemical Screening Predictions

Based on the known chemical profile of *T. castaneum*, we anticipate:

Positive tests

- Quinones (Borntrager's): Strong positive, concentrated in non-polar extracts
- Phenols (Folin-Ciocalteu): Moderate to strong positive
- Terpenoids (Salkowski): Weak to moderate positive
- Steroids (Liebermann-Burchard): Weak positive
- Proteins (Biuret): Moderate positive (from structural proteins)
- Carbohydrates (Molisch's): Moderate positive (from chitin)

Negative tests

- Alkaloids (Mayer's, Wagner's, Dragendorff's): Negative (no known alkaloids in *T. castaneum*)
- Flavonoids (Shinoda): Negative (flavonoids not typical of insects)
- Saponins (Froth): Negative
- Tannins (Gelatin): Negative

Quantitative projections

- Total phenolic content: 15-30 mg GAE/g extract (highest in ethanol fraction)
- Total flavonoid content: <5 mg QE/g extract
- Benzoquinone content: 2-5 mg/g crude powder (major: 2-methyl-1,4-benzoquinone)

The bioactivity is likely to partition into non-polar fractions (petroleum ether, chloroform) containing the benzoquinones, though polar fractions (ethanol, aqueous) may also show activity due to phenolics and peptides. [16]

Comparison with Conventional and Other Natural Products

If the predicted activity is confirmed, how does *T. castaneum* compare to existing options?

vs. Synthetic NSAIDs (ibuprofen, diclofenac):

- Advantages: Natural origin, potentially fewer gastrointestinal side effects (to be tested), novel chemical scaffolds, sustainable production
- Disadvantages: Lower potency (requires higher mg/kg dose), variable composition, potential for allergenicity

vs. Plant-derived anti-inflammatories (curcumin, boswellia)

- Advantages: Novel mechanism (benzoquinones vs. curcuminoids), faster time to effect (predicted), potential synergy among compounds
- Disadvantages: Less studied, possible social acceptance barriers ("beetle powder")

vs. Other medicinal insects:

- Advantages: *T. castaneum* is easier to culture than many medicinal insects (e.g., *Blaps* requires soil substrate, *Coridius* requires live prey), shorter generation time, more amenable to genetic improvement
- Disadvantages: Less traditional use history, potential pest stigma

Limitations of the Study

Several limitations must be acknowledged:

1. **Use of crude powder:** The crude powder contains hundreds of compounds. While appropriate for initial screening, it confounds mechanism assignment.

Bioactivity could arise from a single compound, multiple compounds, or synergistic interactions. Fractionation and purification will be necessary for definitive mechanism studies.

2. **Mixed-sex beetles:** Male and female *T. castaneum* differ in chemical profiles (benzoquinone content, CHC composition). Pooling sexes may obscure sex-specific bioactivity. Future studies should test male and female powder separately.

3. **Acute administration only:** Chronic pain and inflammation require repeated dosing. This study does not address efficacy or safety with chronic administration.

4. **Rodent models:** While validated, rodent models do not perfectly predict human responses. Clinical translation will require human trials.

5. **No comparison with other *Tribolium* species:** *Tribolium confusum* (confused flour beetle) is closely related and equally available. Comparative studies would strengthen conclusions about genus-level activity.

6. **No investigation of gut microbiota contribution:** *T. castaneum* harbors a gut microbiome that may produce bioactive compounds. This study does not distinguish beetle-derived from microbe-derived metabolites.

7. **Potential for batch-to-batch variability:** Rearing conditions (diet, density, temperature) influence chemical profiles. Standardization will be necessary for reproducible bioactivity.

8. **Allergenicity risk:** *T. castaneum* is a known occupational allergen (grain handlers). Insect powder may trigger allergic responses in sensitized individuals. Safety studies must include allergenicity assessment. [17]

Conclusion and Recommendations

1. **Summary of Expected Findings**

This research protocol provides a comprehensive, rigorous framework for evaluating the analgesic and anti-inflammatory potential of dried *Tribolium castaneum* powder. Based on the known chemical profile of this beetle—particularly its benzoquinone content—and the established pharmacological activities of related medicinal insects (especially *Coridius chinensis* and *Blaps rynchopetera*), we anticipate significant, dose-dependent activities in all test models. [18]

Specifically, we expect:

- **Analgesic activity:** 60-70% inhibition of acetic acid-induced writhing at 400 mg/kg, comparable to aspirin 100 mg/kg; preferential inhibition of the inflammatory (Phase II) response in the formalin test, indicating a peripheral mechanism

- **Anti-inflammatory activity:** 50-65% inhibition of carrageenan-induced paw edema during the late phase (3-6 hours), consistent with COX-2 inhibition

- **Biochemical mechanism:** Suppression of TNF-α, IL-1β, IL-6, PGE_2, and COX-2; reduction of oxidative stress; enhancement of endogenous antioxidants

- **Safety profile:** LD_{50} > 2000 mg/kg, indicating low acute toxicity [19]

Contribution to Knowledge

If the predicted outcomes are confirmed experimentally, this research will make several important contributions:

First, it will provide the first published evidence for the analgesic and anti-inflammatory properties of *Tribolium castaneum*, opening a new chapter in entomological pharmacognosy for this well-known model organism.

Second, it will establish a reproducible methodological template for screening other stored-product insects (e.g., *Tribolium confusum*, *Oryzaephilus surinamensis*, *Rhyzopertha dominica*) for bioactivity, potentially transforming agricultural pests into pharmaceutical resources.

Third, the mechanistic data (cytokine profiles, oxidative stress markers, histopathology) will inform understanding of how insect-derived benzoquinones and other compounds interact with mammalian inflammatory pathways.

Fourth, the identification of bioactive fractions will provide a starting point for bioactivity-guided isolation of novel compounds, potentially yielding new lead molecules for drug development.

Fifth, the demonstration that a globally abundant, easily cultured pest species possesses therapeutic activity challenges the conventional dichotomy between "pest" and "resource" and supports a more integrated view of insect management. [20]

Implications for Practice

In the near term, positive results would support the development of *T. castaneum* powder as a low-cost, accessible natural product for pain and inflammation, particularly in resource-limited settings where synthetic NSAIDs may be unavailable or unaffordable. The beetle can be cultured on agricultural waste products (e.g., wheat bran, cornmeal), enabling decentralized production.

In the medium term, identification of specific active compounds could lead to pharmaceutical development. Benzoquinones, while known, have not been systematically optimized for anti-inflammatory use. Structural modification could improve potency and selectivity while reducing any toxicity.

In the long term, the use of insect-derived anti-inflammatories could reduce reliance on synthetic NSAIDs, with their environmental and health footprints. Insects can be reared on organic waste streams, require minimal land and water compared to plant cultivation, and have lower greenhouse gas emissions than livestock.

Concluding Remarks

Tribolium castaneum is, by almost any measure, a remarkable organism. It is a global pest causing billions in economic losses annually. It is also a model system of unparalleled power for genetic and developmental biology. This research protocol proposes a third identity: a source of analgesic and anti-inflammatory compounds.

The convergence of factors is compelling. The beetle produces bioactive compounds (benzoquinones,

hydrocarbons, antimicrobial peptides) with known or predicted activities in mammalian inflammation. It is globally available, easily cultured, and genetically tractable. Related insects have yielded validated therapeutics. Yet, until now, *T. castaneum* has remained unexamined for this purpose.

This protocol provides the rigorous scientific framework necessary to test the hypothesis that dried *T. castaneum* powder possesses genuine analgesic and anti-inflammatory activity. If confirmed, the path from pest to pharmaceutical will be open. If not, the null finding remains informative for the broader field of entomological pharmacognosy.

In an era of rising healthcare costs, antimicrobial resistance, and environmental degradation, the exploration of underutilized natural resources is not merely an academic exercise—it is a necessity. Insects, representing the majority of animal biodiversity, are an inevitable frontier. *Tribolium castaneum*, for its part, stands ready.

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