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In Vitro Anti-Inflammatory Potential Of *Tribolium Castaneum* (Red Flour Beetle) Extract Using Human Red Blood Cell Membrane Stabilization And Heat-Induced Hemolysis Assays

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ABSTRACT

Inflammation is a fundamental biological response that protects the body against infection and tissue injury; however, persistent or excessive inflammation contributes to the development of numerous chronic disorders. The search for safer, naturally derived anti-inflammatory agents (plants and animals extract) has therefore become an important area of biomedical research. The present study investigated the *in vitro* anti-inflammatory activity of *Tribolium castaneum* (red flour beetle) extract using human red blood cell (HRBC) membrane stabilization and heat-induced hemolysis assays. A hydroethanolic extract of *T. castaneum* was prepared and evaluated at concentrations ranging from 10 to 50 µg/mL, with diclofenac sodium used as the reference anti-inflammatory drug. The extract exhibited concentration-dependent membrane-protective activity in both assays. In the HRBC membrane stabilization assay, the percentage inhibition of hemolysis increased from 7.85% at 10 µg/mL to 43.98% at 50 µg/mL, whereas diclofenac sodium achieved 65.45% inhibition at the highest concentration. Similarly, in the heat-induced hemolysis assay, the extract demonstrated inhibition ranging from 9.79% to 45.36%, compared with 65.46% for the standard drug. These findings indicate that *T. castaneum* extract possesses significant *in vitro* anti-inflammatory activity, likely through stabilization of erythrocyte membranes and protection against inflammatory damage. This study provides preliminary evidence supporting the therapeutic potential of insect-derived bioactive compounds as natural anti-inflammatory agents. Further studies are required to isolate the active constituents, elucidate their molecular mechanisms, and validate their efficacy and safety through *in vivo* and clinical investigations.

Keywords: Inflammation, Diclofenac Sodium, Erythrocyte, Hemolysis, *In Vivo*, *T. Castaneum*

Introduction

Inflammation is a highly regulated physiological process that constitutes a fundamental component of the innate immune system, protecting the host against pathogenic microorganisms, tissue injury, toxic substances, and other harmful stimuli (Chavda et al., 2024). It is initiated through the coordinated activation of immune cells and signaling pathways that facilitate the elimination of damaging agents while promoting tissue repair and restoration of homeostasis. During the inflammatory response, numerous biochemical mediators, including cytokines, chemokines, histamine, prostaglandins, leukotrienes, and nitric oxide, are released to regulate vascular permeability, recruit immune cells, and orchestrate the healing process (Gusev and Zhuravleva, 2022). Although acute inflammation is generally beneficial and self-limiting, persistent or uncontrolled inflammation can disrupt normal physiological functions and contribute to progressive tissue injury.

Chronic inflammation is now recognized as one of the principal underlying mechanisms driving the development and progression of numerous human diseases. Sustained activation of inflammatory pathways promotes oxidative stress, mitochondrial dysfunction, cellular damage, and immune dysregulation, thereby contributing to the pathogenesis of autoimmune diseases, cardiovascular disorders, diabetes mellitus, metabolic syndrome, neurodegenerative diseases, and several forms of cancer (Furman et al., 2020; Wiya et al., 2020; Pham et al., 2021). Furthermore, excessive production of inflammatory mediators has been strongly associated with rheumatoid arthritis, severe infectious diseases, cardiovascular complications, and neurological disorders,



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emphasizing the urgent need for effective therapeutic strategies capable of controlling excessive inflammatory responses without causing significant adverse effects (Chaudhary et al., 2023; Holub et al., 2024; Abdulkhaleq et al., 2018). Consequently, the discovery of novel anti-inflammatory compounds remains a major priority in pharmaceutical and biomedical research.

Currently available anti-inflammatory drugs, including steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), effectively reduce inflammation by suppressing cyclooxygenase-mediated prostaglandin synthesis. Despite their widespread clinical use, prolonged administration of these medications is frequently associated with serious adverse effects, including gastrointestinal ulceration, renal toxicity, cardiovascular complications, and hepatic dysfunction (Anyasor and Ijitiyi, 2018). These limitations have intensified global interest in identifying safer therapeutic alternatives from natural sources. Bioactive compounds derived from plants, microorganisms, marine organisms, and animals have demonstrated considerable therapeutic potential because of their antioxidant, immunomodulatory, antimicrobial, and anti-inflammatory activities. Many naturally occurring compounds suppress inflammatory responses by inhibiting cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nitric oxide production, and pro-inflammatory cytokines, thereby reducing tissue damage and restoring immune homeostasis (Mueller et al., 2010). Therefore, natural products continue to represent one of the most promising resources for the development of next-generation anti-inflammatory therapeutics.

Among natural resources, insects have recently emerged as an exceptionally valuable yet comparatively underexplored reservoir of biologically active compounds with remarkable pharmaceutical potential. Representing the most diverse group of animals on Earth, insects have evolved highly efficient innate immune systems that enable them to survive in environments rich in pathogenic microorganisms. As a consequence, they produce a wide variety of antimicrobial peptides, proteins, enzymes, lipids, and secondary metabolites that exhibit antimicrobial, antioxidant, antiviral, immunomodulatory, anticancer, wound-healing, and anti-inflammatory activities (Kim et al., 2011; Wu et al., 2015). In recent years, insect-derived biomolecules have attracted increasing attention as promising candidates for drug discovery because of their structural diversity, biological specificity, biodegradability, and relatively low toxicity. Moreover, edible and medicinal insects have been recognized as sustainable sources of bioactive peptides capable of modulating inflammatory signaling pathways and reducing oxidative stress, making them attractive alternatives for the development of novel therapeutic agents (Rumpold et al., 2013; Zielińska et al., 2017).

The pharmaceutical significance of insects extends beyond their nutritional value. Numerous insect-derived peptides have demonstrated the ability to regulate immune responses by suppressing inflammatory mediators, including tumor necrosis factor-alpha (TNF- α), interleukins, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nitric oxide production. These findings suggest that insects represent an untapped source of multifunctional therapeutic molecules with potential applications in inflammatory, infectious, and metabolic diseases. Consequently, the exploration of insect-derived bioactive compounds has become an emerging research frontier in natural product chemistry, pharmacology, and biotechnology, offering new opportunities for the discovery of safer and more effective anti-inflammatory agents.

Tribolium castaneum (Herbst), commonly known as the red flour beetle, is a cosmopolitan stored-product insect belonging to the family Tenebrionidae. Although it is widely recognized as an economically important pest of stored grains and cereal



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products, recent investigations have revealed that this species possesses a sophisticated innate immune system capable of producing numerous biologically active molecules, particularly antimicrobial peptides involved in host defense (Bertrams et al., 2021). Most previous studies on *T. castaneum* have primarily focused on its genetics, developmental biology, immunity, pest management, and antimicrobial properties. In contrast, information regarding its anti-inflammatory potential remains extremely limited. Considering its rich repertoire of immune-associated bioactive molecules, *T. castaneum* may represent a promising but largely unexplored source of natural anti-inflammatory compounds. Investigating the biological activities of this insect could therefore contribute not only to the discovery of novel therapeutic agents but also to expanding our understanding of insect-derived biomolecules in biomedical research.

Therefore, the present study was designed to evaluate the in vitro anti-inflammatory activity of *T. castaneum* extract using the human red blood cell (HRBC) membrane stabilization assay and the heat-induced hemolysis assay. Diclofenac sodium was employed as the reference anti-inflammatory drug to compare the membrane-protective effects of the extract. By investigating the anti-inflammatory potential of *T. castaneum*, this study provides preliminary evidence supporting the utilization of insect-derived bioactive compounds as promising natural alternatives for future anti-inflammatory drug development and establishes a scientific foundation for subsequent phytochemical, molecular, and in vivo investigations.

Materials and Methods

Chemicals and Equipment

The chemicals and materials used during the study included; ethanol (90%), distilled water, DMSO (1%), phosphate-buffered saline (PBS, pH 7.4), hypotonic saline, isotonic saline, EDTA tubes, Falcon tubes, Eppendorf tubes, sterile syringes, and blood suspension. Diclofenac sodium was used as a standard anti-inflammatory drug. The equipment used included a grinder, shaker, water bath, centrifuge, incubator, micropipette, analytical balance, and spectrophotometer.

Extract Preparation

The beetles were collected from the sampling area and carried to Entomology lab of Abdul Wali Khan University Mardan. Where they were crushed to form a fine powder by use of a grinder. The crushed beetles were then put in a mixture of solvents, which had 10ml of ethanol and 90ml of distilled water. The mixture was then put in a shaker for 48 hours in order to facilitate the extraction of bioactive compounds. Following the extraction, the resulting mixture was filtered with the use of filter paper to obtain the extract away from any solid components. This process was carried out in order to maximize the yield of the extract. The extracted material was then evaporated in a water bath for about three days before it was stored in a refrigerator.

Experimental Design

The anti-inflammatory activity of *T. castaneum* extract was evaluated using two in vitro methods: human red blood cell membrane stabilization assay (HRBC assay) and heat-induced hemolysis assay. These methods were used to assess the ability of the extract to protect erythrocyte membranes against damage. Diclofenac sodium was used as a standard drug, while untreated samples were considered as control groups. The anti-inflammatory activity was determined by calculating the percentage inhibition of hemolysis through spectrophotometric measurement at 540 nm.



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Membrane Stabilization Assay

The HRBC membrane stabilization assay was performed to evaluate the protective effect of *T. castaneum* extract against erythrocyte membrane lysis. Extract and diclofenac sodium solutions (0.05 mg/ml) were prepared in distilled water and sonicated for 20 minutes to ensure complete dissolution. Blood was taken from a healthy male/ female who was not under any steroids for 15 days prior to the experiment. The blood was centrifuged at 3000 rpm for 15 minutes in EDTA tubes. An erythrocyte pellet was obtained after washing the cells thrice in isotonic solution to prepare a 10% RBC suspension.

The reaction mixture containing PBS (pH 7.4), hypotonic saline, isotonic saline, extract solution, and RBC suspension was prepared and incubated at 37°C for 30 minutes. Similar mixtures containing diclofenac sodium were prepared as standard treatment, while the control group received no extract or standard drug. After incubation, samples were centrifuged at 3000 rpm for 15 minutes, and the absorbance of the collected supernatant was measured at 540 nm using a spectrophotometer. The percentage inhibition of hemolysis was calculated using the formula:

$$\% \text{ inhibition} = (A_{\text{ctrl}} - A_{\text{spl}}) / A_{\text{ctrl}} * 100$$

RBC Hemolysis Assay

Heat-induced hemolysis test was conducted to determine the membrane protecting activity of the extract of *T. castaneum*. Solutions of extract and diclofenac sodium were prepared in distilled water, which were used for treatments. Blood sample was collected from a healthy individual and centrifuged at 3000 rpm for 15 minutes. Pellet of red blood cell was suspended in isotonic saline and the preparation of RBC suspension (10%) was done.

Reaction mixture was made up of PBS (pH 7.4), RBC suspension and various concentrations of the extract (10-50 µg/ml). Sodium diclofenac was taken as standard drug while PBS and RBC suspension as control. Incubation of the samples was done at 30 minutes at 54°C to induce thermal hemolysis. Samples were then cooled, centrifuged at 5400 rpm for 5 minutes and the absorbance of supernatant was noted at 540 nm. Percentage hemolysis was determined using the equation:

$$\% \text{inhibition} = A_{\text{control}} - A_{\text{test}} / A_{\text{control}} * 100$$

Results**In vitro anti-inflammatory effect of extract from *T. castaneum***

The in vitro anti-inflammatory activity of *T. castaneum* extract was evaluated using two complementary assays, namely the human red blood cell (HRBC) membrane stabilization assay and the heat-induced hemolysis assay. Both methods are widely employed to assess the membrane-protective capacity of natural compounds, which is considered an important indicator of anti-inflammatory potential. Diclofenac sodium was used as the standard anti-inflammatory drug for comparison.

HRBC Membrane Stabilization Assay

The membrane stabilization assay demonstrated that *T. castaneum* extract exhibited concentration-dependent anti-inflammatory activity. The extract effectively protected erythrocyte membranes against hypotonic solution-induced hemolysis, with membrane stabilization increasing progressively as the extract concentration increased from 10 to 50 µg/mL. At the lowest concentration (10 µg/mL), the extract produced 7.85% inhibition



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of hemolysis. Increasing the concentration to 20 $\mu\text{g/mL}$ enhanced the inhibition to 15.18%, while concentrations of 30 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$ resulted in inhibition values of 24.61% and 35.60%, respectively. The highest concentration (50 $\mu\text{g/mL}$) exhibited the maximum membrane stabilization, producing 43.98% inhibition of hemolysis (Figure 1). Diclofenac sodium showed greater membrane stabilization at all tested concentrations. The standard drug produced inhibition values of 28.27%, 36.65%, 45.55%, 57.59%, and 65.45% at concentrations of 10, 20, 30, 40, and 50 $\mu\text{g/mL}$, respectively. Although the activity of *T. castaneum* extract was lower than that of diclofenac sodium, the extract consistently exhibited dose-dependent membrane-protective effects throughout the experiment. The progressive increase in membrane stabilization observed with increasing extract concentration indicates that *T. castaneum* contains biologically active constituents capable of protecting erythrocyte membranes against osmotic stress. Since erythrocyte membranes share structural similarities with lysosomal membranes, stabilization of these membranes is considered an important mechanism underlying anti-inflammatory activity. Therefore, the observed membrane-protective effect suggests that the extract possesses promising in vitro anti-inflammatory properties.

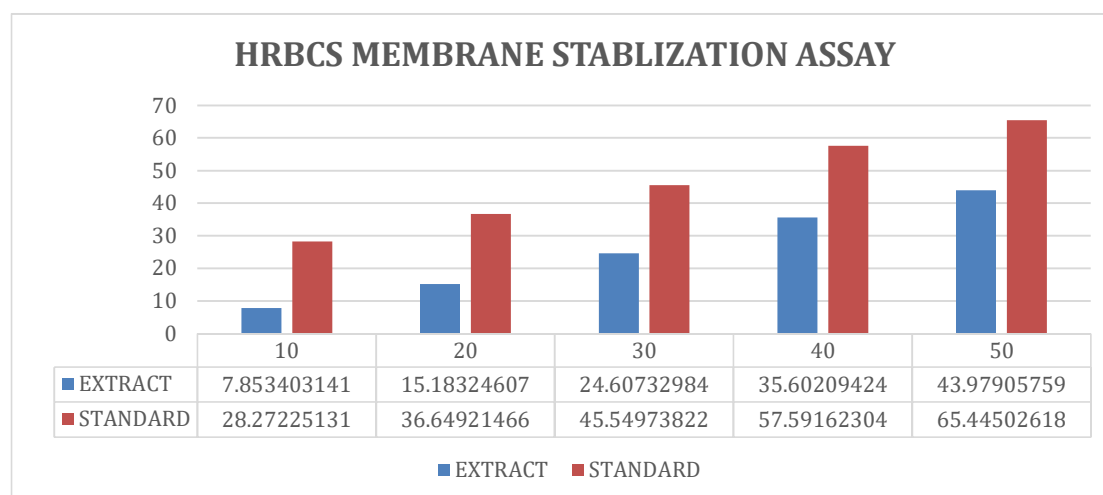


Figure 1: Effect *T. castaneum* Extract on HRBCS membrane stabilization assay

Heat-induced hemolysis assay

The anti-inflammatory activity of *T. castaneum* extract was further evaluated using the heat-induced hemolysis assay. Similar to the HRBC membrane stabilization assay, the extract demonstrated a clear concentration-dependent inhibition of thermal hemolysis, indicating its ability to protect erythrocyte membranes from heat-induced cellular damage. The extract produced 9.79% inhibition at 10 $\mu\text{g/mL}$, which increased to 19.59% at 20 $\mu\text{g/mL}$. Further increases in extract concentration resulted in inhibition values of 24.74% at 30 $\mu\text{g/mL}$, 38.66% at 40 $\mu\text{g/mL}$, and a maximum inhibition of 45.36% at 50 $\mu\text{g/mL}$ (Figure 2). These findings demonstrate a gradual enhancement in membrane protection with increasing extract concentration. The reference drug, diclofenac sodium, exhibited significantly higher inhibition than the extract across all concentrations. The standard produced inhibition values of 20.62%, 29.38%, 42.27%, 56.19%, and 65.46% at concentrations ranging from 10 to 50 $\mu\text{g/mL}$. Nevertheless, *T. castaneum* extract consistently reduced heat-induced hemolysis, confirming its membrane-stabilizing capacity under thermal stress conditions. Overall, the results obtained from both experimental models demonstrated similar response patterns. In both assays, increasing



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concentrations of *T. castaneum* extract produced progressively greater inhibition of erythrocyte membrane lysis, indicating a concentration-dependent anti-inflammatory effect. Although the activity remained lower than that of diclofenac sodium, the extract exhibited substantial membrane stabilization at higher concentrations, suggesting the presence of bioactive constituents capable of reducing inflammatory damage. Collectively, these findings provide strong preliminary evidence that *T. castaneum* extract possesses significant in vitro anti-inflammatory activity. The consistent dose-dependent response observed in both the HRBC membrane stabilization and heat-induced hemolysis assays supports the potential of this insect as a promising natural source of anti-inflammatory compounds. These results provide a scientific basis for future investigations aimed at isolating and characterizing the active constituents responsible for the observed biological activity and evaluating their therapeutic efficacy using in vivo experimental models.

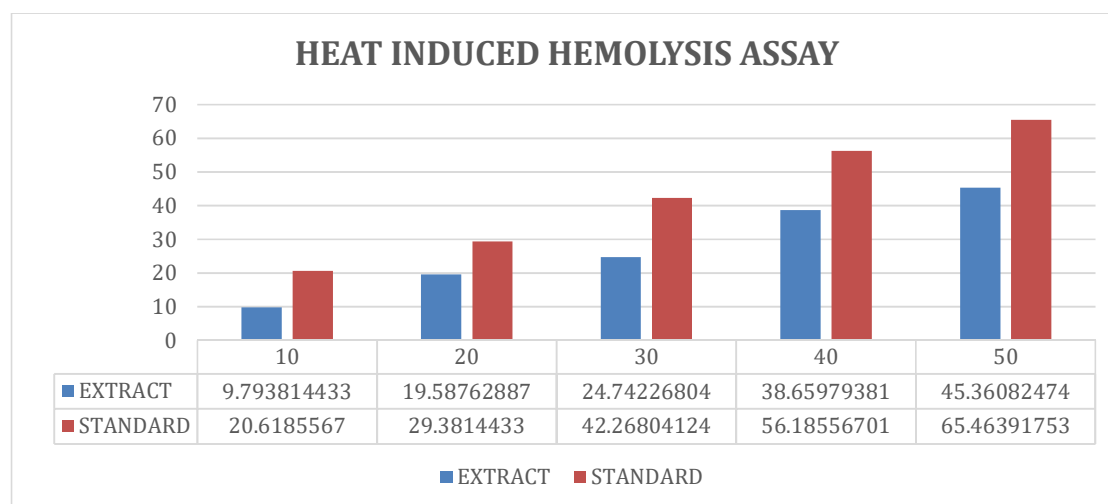


Figure 2: Potential of extract on heat induced hemolysis assay

Discussion

The present study demonstrated that *T. castaneum* extract exhibits concentration-dependent in vitro anti-inflammatory activity in both the human red blood cell (HRBC) membrane stabilization assay and the heat-induced hemolysis assay. The percentage inhibition increased progressively with increasing extract concentration, reaching maximum membrane stabilization values of 43.97% in the HRBC membrane stabilization assay and 45.36% in the heat-induced hemolysis assay at 50 µg/mL. Although the anti-inflammatory activity of the extract was lower than that of the standard drug diclofenac sodium, the observed membrane-protective effect suggests the presence of bioactive constituents capable of mitigating inflammatory responses.

The HRBC membrane stabilization assay is a widely accepted method for evaluating anti-inflammatory activity because the erythrocyte membrane closely resembles the lysosomal membrane in structure and composition. Stabilization of the erythrocyte membrane prevents membrane disruption and inhibits the release of lysosomal enzymes and inflammatory mediators responsible for tissue injury during inflammation (Sarveswaran et al., 2017). Membrane stabilization is therefore considered an important mechanism underlying anti-inflammatory activity, as it limits the release of inflammatory mediators and lysosomal enzymes that contribute to the inflammatory process (Debnath et al., 2013; Joshi et al., 2016). Consequently, the membrane-protective effect observed



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in the present study provides evidence supporting the anti-inflammatory potential of *T. castaneum* extract.

The findings of the present study are consistent with previous reports on insect-derived bioactive compounds exhibiting anti-inflammatory properties. Kim et al. (2020) reported that the antimicrobial peptide periplanetasin-5, isolated from *Periplaneta americana*, significantly suppressed nitric oxide (NO) production and down regulated the expression of inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF- α), and interleukin-6 (IL-6), through inhibition of the NF- κ B and MAPK signaling pathways. Similarly, Kim et al. (2011) demonstrated that papiliocin, isolated from *Papilio xuthus*, inhibited NO production and reduced the expression of pro-inflammatory cytokines via regulation of the TLR4/NF- κ B signaling pathway. Comparable anti-inflammatory effects have also been reported for other naturally derived bioactive compounds. For example, Yang et al. (2014) found that questinol, isolated from the fungus *Eurotium amstelodami*, significantly inhibited NO production and suppressed the expression of iNOS, COX-2, TNF- α , and IL-6 in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. Although these investigations employed cell-based inflammatory models rather than membrane stabilization assays, they collectively support the anti-inflammatory potential of naturally occurring bioactive compounds.

The membrane-stabilizing activity of *T. castaneum* extract may be attributed to the presence of bioactive peptides, proteins, and secondary metabolites. Insect-derived peptides have attracted considerable attention because of their diverse biological activities, including antimicrobial, antioxidant, immune-modulatory, and anti-inflammatory effects (Koczulla & Bals, 2003). For instance, SibaCec, a cecropin-like peptide isolated from the salivary glands of black flies, exhibited potent anti-inflammatory activity by suppressing the production of inflammatory mediators and attenuating lipopolysaccharide-induced inflammatory responses (Wu et al., 2015). Similarly, bioactive peptides isolated from edible insects have demonstrated significant anti-inflammatory potential in both in vitro and in vivo studies (Zielińska et al., 2017; Teixeira et al., 2023). These findings suggest that similar bioactive molecules may contribute to the membrane-protective activity observed in *T. castaneum* extract.

Previous studies on *T. castaneum* have primarily focused on its antimicrobial peptides, innate immune defense mechanisms, and applications in pest management. For example, Bertrams et al. (2021) reported that defensin-1 isolated from *T. castaneum* exhibited potent antimicrobial activity against *Moraxella catarrhalis*, highlighting the biological significance of this insect as a source of functional bioactive molecules. However, investigations specifically addressing the anti-inflammatory properties of *T. castaneum* extracts remain limited. Therefore, the present study provides preliminary evidence supporting the anti-inflammatory potential of this species and expands current knowledge regarding its pharmacological applications.

Overall, the findings suggest that *T. castaneum* extract may serve as a promising natural source of anti-inflammatory compounds. Nevertheless, further investigations are required to isolate and characterize the active constituents responsible for the observed biological activity, elucidate their underlying molecular mechanisms, and evaluate their efficacy and safety through in vivo, preclinical, and clinical studies. Such studies will be essential for determining the therapeutic potential of *T. castaneum*-derived bioactive compounds in the development of novel anti-inflammatory agents.



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Conclusion

The identification of safe and effective natural anti-inflammatory agents remains an important objective in biomedical research due to the adverse effects associated with long-term use of conventional anti-inflammatory drugs. In this study, the in vitro anti-inflammatory activity of the hydroethanolic extract of *T. castaneum* was evaluated using HRBC membrane stabilization and heat-induced hemolysis assays, with diclofenac sodium as the reference drug. The extract exhibited concentration-dependent membrane-protective activity in both assays, achieving maximum inhibition of 43.98% and 45.36%, respectively, at 50 µg/mL. Although its activity was lower than that of diclofenac sodium, the consistent results obtained from both assays confirmed the presence of bioactive constituents capable of stabilizing erythrocyte membranes, an important mechanism of anti-inflammatory action. These findings demonstrate the pharmacological potential of *T. castaneum*, traditionally regarded as a stored-product pest, as a promising source of natural anti-inflammatory compounds. This study provides a scientific foundation for future isolation and characterization of the active constituents, mechanistic studies, and in vivo investigations to further validate its therapeutic potential.

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