Comparative Evaluation of Anti-Inflammatory Activity of Nanocomposites Derived from Nano-Hydroxyapatite (Ha), Chitosan and Vitamin K2

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**Abstract:** Inflammation, orchestrated by the immune system, is a response to various stimuli in vascularized tissue. Nanotechnology has gained prominence in biomedicine, offering the potential to target and modulate inflammatory processes using nanoparticles. Nano-hydroxyapatite (nHAP), chitosan, and Vit K2 Possess unique properties that could make them effective anti-inflammatory agents when combined into a nanocomposite.We prepared a novel nanocomposite by combining nHAP, chitosan, and Vit K2and evaluated its anti-inflammatory activity. The nanocomposite was synthesized through a stepwise process, and its components were characterized. To assess anti-inflammatory activity, a Bovine Serum Albumin (BSA) assay was conducted, with various concentrations of the nanocomposite tested alongside individual components and controls. The results revealed concentration-dependent effects. Vit K2displayed moderate inhibitory activity at lower concentrations but decreased at higher concentrations. nHAP exhibited increased inhibitory activity up to a certain concentration, followed by a slight decline. The nanocomposite demonstrated enhanced anti-inflammatory activity compared to individual components, particularly at concentrations of 30 µL and 50 µL. A synergistic effect between chitosan, nHAP, and Vit K2 was evident in the nanocomposite's superior anti-inflammatory efficacy.The synthesized nanocomposite (K2+Chito+nHAP) exhibited potent anti-inflammatory properties, potentially attributed to a synergistic interaction among its components. This nanocomposite holds promise for diverse biomedical applications, including targeted drug delivery and tissue regeneration. Further investigations into the molecular mechanisms and in vivo applications are warranted to fully realize the clinical potential of this innovative nanocomposite.

**Keywords:** inflammation, nanocomposite, nano-hydroxyapatite, chitosan, vitamin K2, anti-inflammatory activity, targeted drug delivery, tissue regeneration,Dental, Health

# Introduction

Inflammation represents the immune system's response to stimuli of physical, chemical, or biological origin. This reaction is self-regulating and typically resolves promptly through counteractive mechanisms involving anti-inflammatory cytokines and lipid mediators that curb proinflammatory signaling [(Chen et al., 2018)](https://paperpile.com/c/nA8t14/pKc1D). In recent years, nanotechnology has emerged as a pioneer field in biomedicine. [(Ajay et al., 2023; Chokkattu et al., 2023; Padarthi et al., 2023)](https://paperpile.com/c/nA8t14/SQOAl+gihga+dbJDA) Nanoparticles, due to their unique properties, offer potential as anti-inflammatory agents by targeting and modulating inflammatory processes at the molecular level, opening avenues for innovative therapeutic strategies in targeted drug delivery, tissue engineering, and disease management [(Afzal et al., 2022; *[No Title]*, n.d.)](https://paperpile.com/c/nA8t14/y3muH+jE593). Promoting the advancement of nanocomposites featuring anti-inflammatory characteristics holds promise for augmenting their functional attributes and efficacy, particularly within tissues vulnerable to infections [(Khan et al., 2021)](https://paperpile.com/c/nA8t14/uAqkM).Hydroxyapatite (HA), HA (Ca10(OH)2(PO4)6) exhibits chemical and structural resemblance to the inorganic constituent of the bone matrix, abundantly present in the structural framework of human bones and dental hard tissues [(Feng, 2009; Ramesh et al., 2008)](https://paperpile.com/c/nA8t14/SCLQg+LPdJv), making it an exceptional candidate for applications in orthopedic and dental implantology. When synthesized at the nanoscale as nano-hydroxyapatite (nHAP), they exhibit heightened bioactivity and biocompatibility, making it an enticing choice for devising innovative bone tissue regenerative strategies and highly proficient drug delivery systems in the realm of orthopedic and dental implant surgeries.[(Dharman et al., 2023; S. Sindhu et al., 2023; Sreenivasagan et al., 2023)](https://paperpile.com/c/nA8t14/WhVFH+bYyRp+SwTQe)Chitosan, derived from chitin, is a biocompatible, biodegradable polysaccharide possessing antimicrobial qualities and remarkable film-forming characteristics. Its unique biological characteristics make it an ideal matrix for encapsulating therapeutic agents and facilitating targeted drug release. [(Ramakrishnan et al., 2023; Shenoy & Maiti, 2023; J. S. Sindhu et al., 2023)](https://paperpile.com/c/nA8t14/5i2NH+2dWFM+Sv1c2) Chitosan exhibits certain immunological functions, encompassing the suppression of pro-inflammatory cytokines, facilitation of tissue granulation through fibroblast mobilization [5], and synthesis of type III collagen [6]. Collectively, these findings imply the potential utilization of chitosan as a multifaceted agent, displaying antimicrobial, anti-inflammatory, and wound-healing acceleration attributes.[(Kasabwala et al., 2021; Rajeshkumar & Lakshmi, 2021; Varghese et al., 2023)](https://paperpile.com/c/nA8t14/fw9TZ+Ia91l+SJopD) Nevertheless, despite its acquisition of a polycationic charge within a mildly acidic milieu, such as the epidermal environment, chitosan exhibits limited solubility in physiological solvents, thus constraining its clinical application to the present time [7].Vitamin K2 (Vit K2) plays a vital role in the regulation of calcium metabolism and bone health. Beyond its traditional role in coagulation, emerging research has highlighted its anti-inflammatory and immunomodulatory potential, making it a valuable component in the development of nanocomposite biomaterials with enhanced therapeutic capabilities, Vitamin K2 exerts an impact on the expression of proinflammatory cytokines in the context of inflammation [(Li et al., 2018)](https://paperpile.com/c/nA8t14/qwrjO). However, the combined synergistic effect of these compounds in combating inflammation remains to be elucidated. Therefore, the aim of the current study was to formulate a novel nanocomposite material by combining nHAP, chitosan, and Vit K2and evaluate its anti-inflammatory activity. This will enable us to further investigate the potential of the material as a therapeutic agent for inflammatory disorders and tissue regeneration.

# Materials and methods

The experimental design encompassed the preparation of distinct solutions, each containing specific combinations of Vit K2, chitosan, and nHAP. The preparation of each test solutions were given as follows;

## Preparation of Test Compounds

Preparation of Vit K2 solution: 150 mg of Vit K2 was dispersed in 15 mL of distilled water, followed by mixing to achieve complete dissolution of Vit K2 in the water.Preparation of Vit K2+ Chitosan Solution: 5 mL of the prepared Vit K2 solution was combined with 5 mL of chitosan solution, and were mixed well to obtain a homogeneous mixture of Vit K2 and chitosan.Preparation of nHAP + Chitosan Solution: 0.5 g of nHAP was added to 5 mL of chitosan solution, and the solution was thoroughly mixed to ensure the uniform dispersion of nHAP in the chitosan matrix.Preparation of nHAP + Chito + Vit K2 Solution: The Vit K2+ Chitosan solution (5 mL) was combined with the nHAP + Chitosan solution, and the solutions were mixed well to create the final nHAP+Chito+K2 nanocomposite.

## Anti-inflammatory Activity and Spectrophotometric Measurement

0.45 mL of Bovine Serum Albumin was mixed with 0.05 mL of the prepared test solutions, Vit K2+ Chitosan, nHAP + Chitosan, and nHAP + Chito + Vit K2 at different concentrations (10, 20, 30, 40 and 50 µg/mL). The pH of the mixtures was adjusted to 6.3 to ensure uniformity. After cooling the samples in the test tube to room temperature, they were incubated in a water bath set at 55°C for 30 minutes to induce protein denaturation.After incubation, the absorbance of each sample was measured at 660 nm using a spectrophotometer. The percentage of protein denaturation (% inhibition) was calculated to assess the anti-inflammatory activity of the prepared solutions.

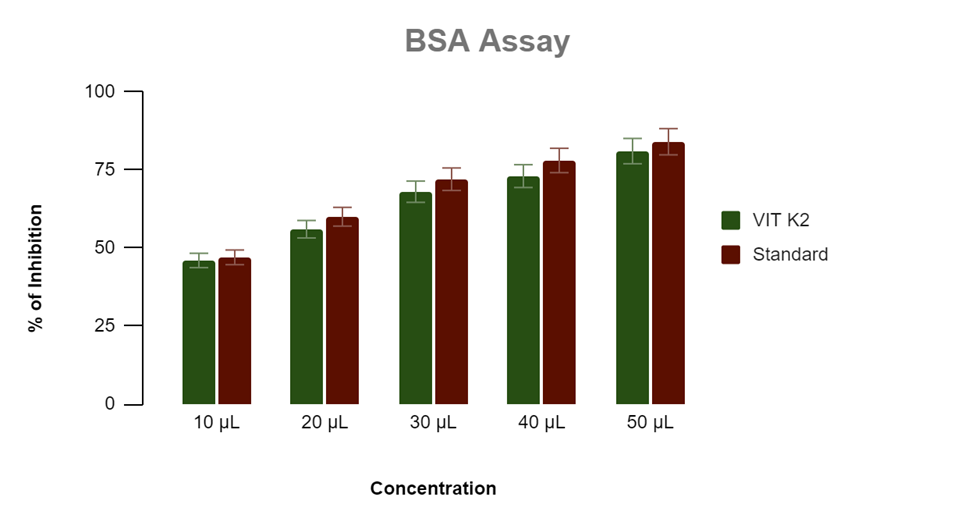
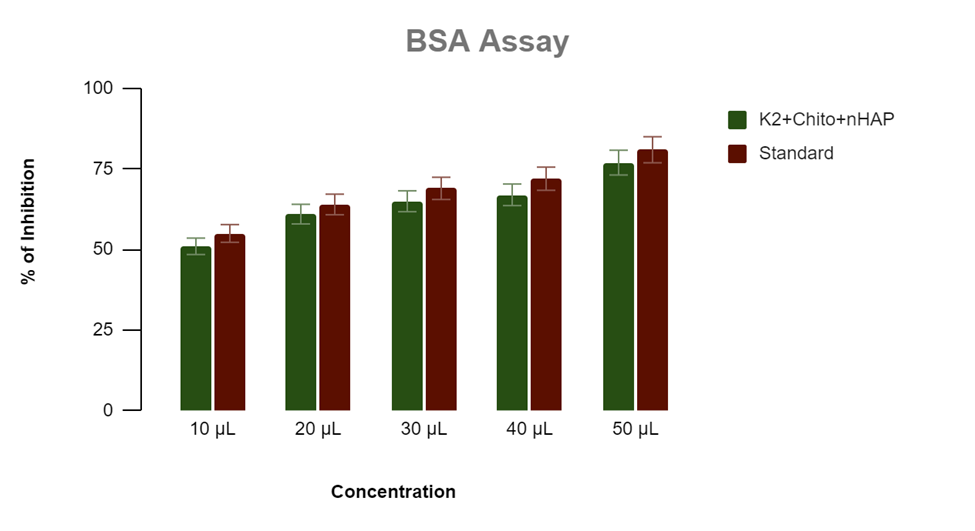
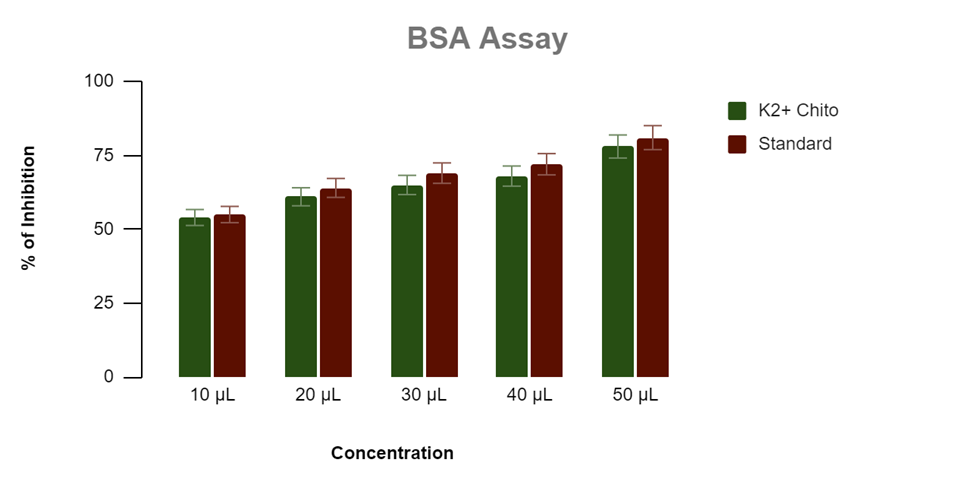
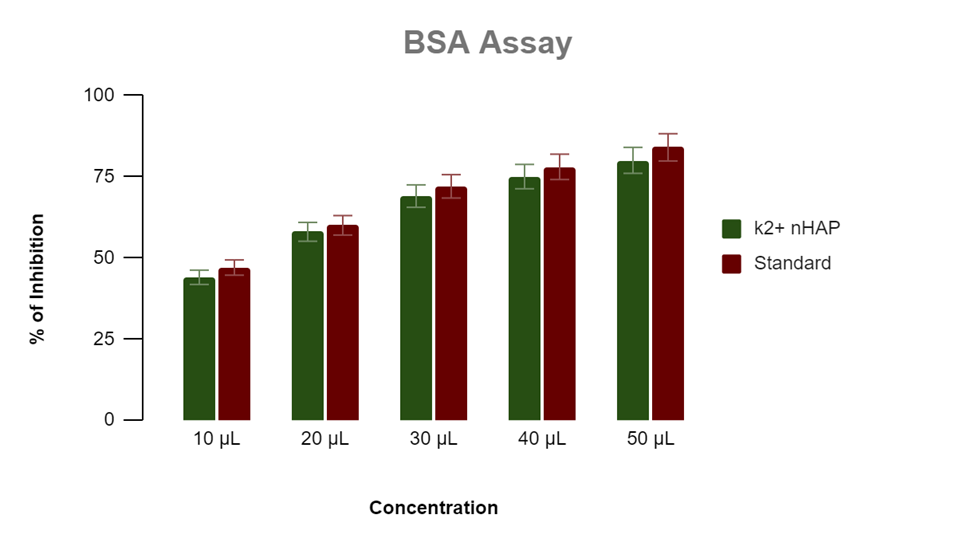
% inhibition = Absorbance of control- Absorbance of sample×100

Absorbance of control

The percentage of protein denaturation (% inhibition) was calculated using a well-established formula. To ensure the reliability of the findings, both positive and negative controls were integrated into the experimental setup. The positive control employed diclofenac sodium, a known anti-inflammatory agent, while the negative control utilized dimethyl sulfoxide (DMSO).

# Results

The values obtained from the assay represent the degree of inhibition of Bovine Serum Albumin binding to the inflammatory markers. The results illustrated in figure 1 showed that both Vit K2 and nHAP exhibited a concentration-dependent effect on the BSA assay. At lower concentrations of 10 µL and 20µL, VIT K2 showed moderate inhibitory activity with values of 0.588 and 0.116, respectively. However, at higher concentrations (30 µL, 40 µL, and 50 µL), the inhibitory effect decreased, with values of 0.136, 0.152, and 0.123, respectively. For nHAP, the inhibitory activity increased with increasing concentrations, reaching a maximum value of 0.631 at a concentration of 30 µL, and then decreased slightly at 40 µL and 50 µL.



A

B

C

D

**Figure 1:** (A)-(D) Anti-inflammatory effects of Vitamin K2 in conjunction with A: nHAP, B: Vit K2 with chitosan, C: Vit K2 with chitosan and nHAP, D: Vit K2. X-axis represents the concentration dose of the test compounds while the Y-axis represents the % of zone of inhibition.

Chitosan and Vit K2 (K2+Chito) was tested at the same concentrations as before(Saadh et al., 2024). The results in figure 2 demonstrated a significant improvement in anti-inflammatory activity compared to individual components(Almatrafi et al., 2024). At 10 µL and 20 µL concentrations, the nanocomposite exhibited inhibitory values of 0.296 and 0.557, respectively were higher than the corresponding individual component values. At 30 µL and 50 µL concentrations, the nanocomposite showed even more potent inhibitory effects, with values of 0.956 and 1.076, respectively. However, at the highest concentration of 40 µL, the nanocomposite showed a reduced inhibitory effect with a value of 0.239.

# DISCUSSION

Inflammation serves as a defensive reaction in response to infections, burns, toxins, allergens. During inflammation, various chemical mediators are released including cytokines, chemokines and prostaglandins. These signaling molecules exert their effects on blood vessels, inducing vasodilation, heightened permeability, and the attraction of immune cells to the afflicted site. The release of enzymes such as proteases leads to the degradation of extracellular matrix proteins and other structural proteins in the affected tissues.[(Keerthana & Ramesh, 2021; Murugesan, 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/nA8t14/WLIi5+6oanX+W0NkV)[(Keerthana & Ramesh, 2021; Murugesan, 2021; Subramanian et al., 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/nA8t14/WLIi5+6oanX+W0NkV+yioZt) Chronic wounds frequently exist in a state of halted inflammation, impeding their advancement in the healing process. This phenomenon is associated with the prevalence of pro-inflammatory macrophages (M1-type macrophages) at the injury site [(Jain et al., 2019; Vitali, 2023)](https://paperpile.com/c/nA8t14/u1aJS+Cfz59). This excessive proteolysis disrupts the normal tissue architecture, leading to tissue damage and loss of function. Protein denaturation has been well correlated with the occurrence of the inflammatory response [(Mizushima, 1966)](https://paperpile.com/c/nA8t14/Oo2Q3). Tissue damage sustained through inflammation could potentially be attributed to the denaturation of proteins, where they lose functional and structural integrity, rendering them less effective or even non-functional [(Opie, 1962)](https://paperpile.com/c/nA8t14/7jpVY). Therefore, the ability of a substance to inhibit denature of the protein may imply its efficacy for eliciting anti-inflammatory effects. Monitoring the levels of denatured proteins in tissues could provide a valuable means of assessing the extent and progression of inflammation [(*Journal of Complementary Medicine Research*, n.d.)](https://paperpile.com/c/nA8t14/srtyS).Considering the relationship between protein denaturation and inflammation, substances capable of preventing protein denaturation could be promising candidates for the development of anti-inflammatory drugs [(Banerjee et al., 2014; Chaithanya et al., 2021)](https://paperpile.com/c/nA8t14/GapT3+ULfbN) . This study assessed the anti-inflammatory effects of Vit K2, Vit K2 + Chitosan, nHAP + Chitosan, and nHAP + Chito + Vit K2 using bovine serum albumin as a model protein due to its structural resemblance to human serum albumin. [(*Evaluation Composite Restoration Posterior Teeth Proanthocyanidin Pretreatment Liner Using Fédération Dentaire Internationale Criteria: Split-Mouth Randomized Controlled Trial*, n.d.; Pranati et al., 2021; Sakthi, 2021)](https://paperpile.com/c/nA8t14/TOvss+YKiFH+4UgNc)) We observed concentration-dependent effects for both Vit K2 + nHAP, with Vit K2 demonstrating moderate inhibition at lower concentrations but a reduced effect at higher concentrations [figure 4]. Conversely, nHAP exhibited increased inhibitory activity up to a certain concentration, after which the effect slightly declined. Vit K2 is one of the two most common types of VK in the human diet [(Fujii et al., 2015)](https://paperpile.com/c/nA8t14/nUEGO). Vit K2 has demonstrated a multifaceted impact on the inflammatory response. Primarily, it seems to exert influence on the expression of inflammatory cytokines; IL-17A, IL-10, and tumor necrosis factor α (TNF-α) [(Zhang et al., 2021)](https://paperpile.com/c/nA8t14/CKNgz) Research suggests that Vit K2may have the capability to modulate both the influence the function of these cytokines, implying a potential function in the control of inflammatory pathways [(Kołakowski et al., 2021; Shea et al., 2008)](https://paperpile.com/c/nA8t14/bjJVp+qRzP6). Furthermore, the anti-inflammatory capacity observed in this study could be justified by studies where Vit K2 was demonstrated to attenuate arachidonic acid levels [(Kaźmierczak-Barańska & Karwowski, 2022)](https://paperpile.com/c/nA8t14/EjIvS), a prominent proinflammatory fatty acid, within HepG2 cells [(Kołakowski et al., 2021)](https://paperpile.com/c/nA8t14/bjJVp). This reduction in arachidonic acid content suggests that Vit K2 Could possess the ability to mitigate the inflammatory cascade. Moreover, Vit. K2 appears to potentiate the synthesis of anti-inflammatory mediators, thereby potentially augmenting its anti-inflammatory efficacy [figure 1] [(Kołakowski et al., 2021; Mukundh Chaithanya et al., 2022)](https://paperpile.com/c/nA8t14/bjJVp+4vu4I).The combination of Vit K2+ chitosan in our study also showed moderate anti-inflammatory activity [figure 2]. We postulate that the combination of chitosan and Vit K2 could potentially evoke a synergistic interaction. Several research investigations have explored the potential anti-inflammatory and pro-inflammatory effects of chitosan [(Jhundoo et al., 2020; Kerch, 2015)](https://paperpile.com/c/nA8t14/L5skt+4ULRZ). Anti-inflammatory properties of chitosan with varying molecular weights, namely high (115 kDa) and low (5.2 kDa) weights. Both samples exhibited enhanced stimulation of the anti-inflammatory cytokine IL-10 along with the inhibition of the progression of colitis [(Kim, 2018)](https://paperpile.com/c/nA8t14/TRJqq). Chitosan's principal role in eliciting anti-inflammatory effects arises from distinctive structural attributes inherent in its molecular composition. When chitosan is subjected to immune system activation prompted by bacterial endotoxins, they adeptly subdued the ensuing inflammatory reaction triggered by lipopolysaccharides (LPS) [(Tao et al., 2022)](https://paperpile.com/c/nA8t14/8085G), culminating in a substantial four-fold decrease in proinflammatory cytokine TNFα levels.[(G. & Ganapathy, 2022; Kumar & Ramesh, 2021)](https://paperpile.com/c/nA8t14/OXslE+nQAyo)) The key determinant underpinning chitosan's anti-inflammatory efficacy was ascribed to the structural constituents intrinsic to its molecular configuration, a trait demonstrated consistently across varying molecular weights [(Duraisamy, 2021a)](https://paperpile.com/c/nA8t14/vTuy9).A combination of Vit K2and chitosan (K2+Chito) without nHAP was tested. The results showed that this combination had moderate anti-inflammatory activity at all concentrations. However, the anti-inflammatory effect was less pronounced compared to the nanocomposite, indicating the importance of nHAP in enhancing the overall anti-inflammatory properties of the formulation. nHAP stands out as a prudent choice due to its chemical composition mimicking that of bone. It is osteoconductive, osteoinductive capacity for drug loading and adhesion of cellular structure affinity, all of which profoundly contribute to its biocompatibility and therapeutic potential in bone-related applications [(Jiang et al., 2021; Schmitz & Hollinger, 1986)](https://paperpile.com/c/nA8t14/63KmH+LXRWH). nHAP coatings can help modulate the inflammatory response and promote a more balanced and controlled healing process. The integration of nHAP into implant coatings has the potential to reduce the release of pro-inflammatory cytokines while fostering the secretion of anti-inflammatory factors. This collectively fosters a less inflammatory milieu at the implantation site.Moreover, nHAP has demonstrated a positive impact on the behavior of macrophages, pivotal immune cells engaged in both inflammatory reaction and tissue regeneration [(Duraisamy, 2021b)](https://paperpile.com/c/nA8t14/Q2Aqd). Research indicates that nHAP coatings possess the ability to shift macrophages towards a more anti-inflammatory phenotype, thereby fostering tissue regeneration and mitigating unfavorable responses. When coupled with the aforementioned osteoinductive and osteoconductive attributes, the inclusion of nHAP as implant surface coatings exhibits significant potential. By fostering a favorable immune response, nHAP coatings could substantially contribute to enhanced tissue integration and overall implant success, concurrently lowering the likelihood of complications linked to chronic inflammation.In this study, we have successfully demonstrated the remarkable potential of the synthesized nanocomposite (K2+Chito+nHAP) as a robust anti-inflammatory agent. Our findings reveal that this nanocomposite exhibited the highest anti-inflammatory activity when compared to the other formulations, particularly at concentrations of 30 and 50 [figure 3]. This enhanced effect could be due to their intrinsic antiinflammatory activities of attributes of chitosan and nHAP, which, when combined with the potent anti-inflammatory capability of vitamin K2, yielded a multiplicative impact [(Duraisamy, 2021)](https://paperpile.com/c/nA8t14/XxnAi). The significance of this synergistic enhancement is noteworthy, as it implies that the combined utilization of these components has the potential to revolutionize anti-inflammatory interventions across a range of applications.Considering the results of our study, it is essential to acknowledge the limitations of this work, such as the need for further exploration into the underlying mechanisms driving this synergistic effect and potential variations in response based on different contexts. Additionally, the in vitro nature of our experiments calls for careful consideration when extrapolating these findings to in vivo situations.

# Conclusion

The nanocomposite (K2+Chito+nHAP) reveals a synergistic anti-inflammatory effect resulting from the combined action of chitosan, nHAP and Vit K2. The observed anti-inflammatory activity demonstrates the potential of this nanocomposite as a robust therapeutic agent in managing inflammatory responses. By effectively inhibiting protein denaturation and modulating inflammatory pathways, this nanocomposite holds promise in diverse biomedical applications, ranging from targeted drug delivery to tissue regeneration.

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