

In vitro investigation of anti-inflammatory activity of propolis/saffron extract/curcumin-loaded ZIF8 nanoparticles and their potential application for treating osteoarthritis

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Background: Osteoarthritis (OA) poses a significant healthcare challenge globally, necessitating the development of effective therapeutic interventions. It is crucial to develop novel drug delivery systems for OA treatment.

Aims: This study explores the potential of propolis, saffron extract, and curcumin-loaded zeolitic imidazolate framework-8 (ZIF8) nanoparticles as a treatment modality for OA. The anti-inflammatory and chondroprotective properties of these natural compounds make them promising candidates for OA management.

Methods: Through comprehensive in vitro investigations, including scanning electron microscopy (SEM), MTT assays, anti-inflammatory assays, cell migration assays, Fourier transform infrared (FTIR) spectroscopy, and release assays, we evaluated the physicochemical and biological characteristics of propolis, saffron extract, and curcumin-loaded ZIF8 nanocarriers.

Results: Our findings demonstrate that these nanocarriers effectively encapsulated the bioactive compounds, exhibited sustained release profiles, and displayed significant anti-inflammatory properties. Notably, propolis-loaded ZIF8 nanocarriers exhibited superior anti-inflammatory activity compared to other formulations. The encapsulation of propolis, saffron extract, and curcumin within ZIF8 nanoparticles holds promise for enhancing their therapeutic efficacy and ensuring targeted delivery to affected joints in OA treatment.

Conclusion: This study highlights the potential of nanotechnology-based delivery systems in harnessing the therapeutic benefits of natural compounds for OA management.

Keywords: *osteoarthritis, zeolitic imidazolate framework, nanoparticles, drug delivery, propolis, saffron extract, curcumin*

1. Introduction

Osteoarthritis (OA) is a chronic, degenerative joint disease characterized by the progressive deterioration of articular cartilage, leading to pain, stiffness, and impaired mobility [1]. Despite being one of the most prevalent musculoskeletal disorders globally, effective therapeutic interventions for OA remain limited, often focusing on symptomatic relief rather than disease modification [2]. In recent years, there has been growing interest in utilizing natural compounds with known anti-inflammatory and chondroprotective properties as potential treatments for OA [3]. Among these natural compounds, propolis [4], saffron extract [5], and curcumin [6] have shown promise due to their

diverse pharmacological activities and favorable safety profiles.

Propolis, a resinous substance collected by honeybees from various plant sources, has been extensively studied for its antioxidant, anti-inflammatory, and antimicrobial properties [7]. It contains a complex mixture of polyphenols, flavonoids, and other bioactive compounds that exhibit protective effects against cartilage degradation and inflammation associated with OA [8]. Saffron, derived from the stigma of *Crocus sativus* flowers, is renowned for its rich content of carotenoids, crocins, and safranal, which possess potent antioxidant, anti-inflammatory, and analgesic properties [9]. Studies have demonstrated saffron's ability to attenuate cartilage damage and alleviate OA symptoms through multiple mechanisms [10]. Curcumin, the principal bioactive

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component of turmeric (*Curcuma longa*), has garnered considerable attention for its diverse pharmacological effects, including anti-inflammatory, antioxidant, and chondroprotective actions [11]. Numerous preclinical and clinical studies have highlighted curcumin's potential as a therapeutic agent for OA by targeting various molecular pathways involved in disease pathogenesis [12].

Despite their promising therapeutic effects, the clinical translation of propolis, saffron extract, and curcumin is often hindered by poor bioavailability and rapid metabolism, necessitating the development of innovative drug delivery systems to enhance their efficacy and ensure sustained release at the target site [13]. Metal-organic frameworks (MOFs), particularly the zeolitic imidazolate framework-8 (ZIF8), have emerged as promising nanocarriers for encapsulating and delivering bioactive compounds due to their high surface area and tunable pore size [14]. These nanoparticles possess inherent magnetic properties facilitating targeted drug delivery and imaging. Their high surface area-to-volume ratios enhance drug loading capacity, improving efficacy in drug delivery systems [15]. Moreover, ZIF8 nanoparticles demonstrate chemical stability under physiological conditions, ensuring structural integrity within biological environments [16]. Furthermore, ZIF8 nanoparticles serve theranostic purposes by concurrently carrying drugs and contrast agents [17]. However, these advantages come with potential cytotoxicity concerns. ZIF-8 nanoparticles have the capacity to elevate reactive oxygen species (ROS) generation across different cell types such as human osteoclasts and vascular smooth muscle cells. This could potentially stimulate apoptosis pathways and modify cellular functions [18]. However, research findings demonstrated that ZIF-8 doses below 45 mg/kg administered over a 24-hour period did not result in notable tissue damage to organs such as the heart, liver, spleen, lung, and kidney in mice [19]. Therefore, it appears that the toxic effects of these nanocarriers are dose-dependent and can be mitigated at lower doses. Studies should therefore prioritize the utilization of low doses of these nanocarriers for biomedical applications.

By encapsulating propolis, saffron extract, and curcumin separately into ZIF8 nanoparticles, it is possible to overcome their inherent limitations and harness their full therapeutic potential for treating OA. In the present research, our aim is to develop a delivery system for propolis, saffron extract, and curcumin by loading them into ZIF8 nanocarriers, thereby creating a potential treatment modality for OA.

2. Methods and materials

2.1. Preparation of propolis, saffron extract, and curcumin-loaded ZIF nanocarriers

ZIF8 nanoparticles devoid of content were subjected to a pretreatment at 600°C for an hour to eliminate any moisture residing within the zeolite pores. Subsequently, these nanoparticles were employed to encapsulate bioactive substances. The process involved mixing solutions of propolis, saffron extract, and curcumin, each prepared at a concentration of 10 g per 150 mL of water (sourced from Barij Essence, Kashan, Iran), with ZIF8 nanoparticles (20 g). The mixture was then magnetically stirred for a duration of 24 hours. The resulting drug-loaded ZIF8 nanoparticles were collected through centrifugation at $15,000 \times g$ for 30 minutes, followed by a washing step with deionized water and subsequent centrifugation. The resultant solutions were then subjected to lyophilization for 2 days to obtain the drug-loaded ZIF8 nanoparticles. These ZIF8 nanocarriers, loaded with propolis, saffron extract, and curcumin, were respectively denoted as PROZIF, SAFROZIF, and CURCUZIF. Drug-free ZIF8 nanoparticles were named ZIF.

2.2. Scanning electron microscopy imaging

PROZIF, SAFROZIF, ZIF and CURCUZIF were coated with gold for 250 seconds and then imaged using a scanning electron microscopy (SEM) device at 25 kV accelerating high voltage.

2.3. MTT assay

L929 cells, a murine fibroblast cell line obtained from ATCC, USA, were cultured in 96-well plates at a density of 4000 cells per well for 5 days. Various concentrations (1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 100 $\mu\text{g/mL}$, and 1000 $\mu\text{g/mL}$) of PROZIF, SAFROZIF, and CURCUZIF nanocarriers were used. Cell viability assays were conducted on days 1, 3, and 5 using the MTT assay kit from Sigma Aldrich, USA. The assay involved replacing the culture media with 200 μL of MTT solution (0.5 mg/mL) and allowing the cells to incubate with the MTT reagent for 4 hours. This led to the conversion of MTT into formazan crystals by metabolically active cells, indicating their metabolic activity and overall health. After incubation, 200 μL of dimethyl sulfoxide (DMSO) solution was added to each well to dissolve the formazan crystals. Thorough mixing ensured complete solubilization, enabling accurate absorbance measurements at a wavelength of 570 nm.

2.4. Anti-inflammatory assay

To assess the anti-inflammatory properties of PROZIF, SAFROZIF, and CURCUZIF in vitro, the RAW264.7 macrophage cell line was utilized. RAW264.7 cells, a murine-derived macrophage cell line obtained from ATCC, were cultured in well plates at a density of 500,000 cells per well. These cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine. Inflammation was induced by exposing the RAW264.7 macrophages to lipopolysaccharide (LPS) at a concentration of 1 $\mu\text{g/mL}$. Following LPS exposure, various concentrations (1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, and 1000 $\mu\text{g/mL}$) of PROZIF, SAFROZIF, ZIF and CURCUZIF nanocarriers were introduced to the culture wells containing the stimulated RAW264.7 macrophages. After co-culturing with the nanocarriers for 24 hours, the culture medium underwent centrifugation to remove cellular debris. The resulting supernatant was analyzed using an enzyme-linked immunosorbent assay (ELISA) kit from Abcam, USA, to accurately measure the

levels of pro-inflammatory cytokines, specifically tumor necrosis factor-alpha ($\text{TNF-}\alpha$), interleukin-1 beta ($\text{IL-1}\beta$), and interleukin-6 (IL-6) present in the culture medium.

2.5. Cell migration assay

L929 cells were plated in a 24-well plate and allowed to grow until they reached 80% confluence. Subsequently, a straight incision was created at the center of the cell monolayer using a 200- μL pipette. The cellular debris was then eliminated by rinsing with phosphate-buffered saline (PBS). Following this, the cells were treated with a concentration of 100 $\mu\text{g/mL}$ (determined from the results of the antiinflammatory assay) of each nanocarrier for a duration of 72 hours. The reduction in wound size was examined using a light microscope on days 0, 1, and 3, and image analysis software was employed to calculate the extent of wound size reduction.

2.6. Fourier transform infrared spectrum

The functional groups of PROZIF, SAFROZIF, and CURCUZIF nanocarriers were analyzed using FTIR (Bruker). FTIR is a powerful analytical technique used to identify chemical bonds and functional groups present in a sample by measuring the absorption of infrared radiation. The FTIR assay was performed within the spectral range of 400–4000 cm^{-1} , covering both the fingerprint region and functional group-specific regions of the infrared spectrum.

2.7. Release assay

The release of propolis, saffron extract, and curcumin from ZIF nanocarriers was investigated using UV-visible spectroscopy at wavelengths of 356 nm, 371 nm, and 412 nm, respectively. Initially, 200 mg of each nanocarrier was loaded into 20 mL of phosphate buffer solution and maintained at 37°C for 7 days. At various time intervals, 0.5 mL of the phosphate buffer solution was sampled and replaced with an equal volume of fresh buffer solution. Subsequently, the optical density of the sampled solution was measured, and the values obtained were correlated with the standard curve of

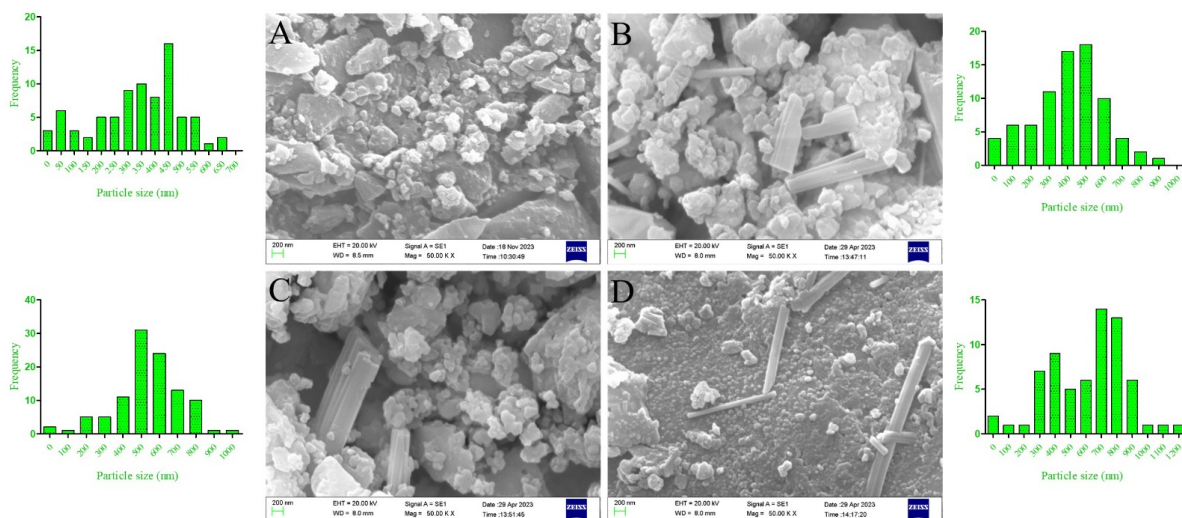


Fig. 1. Representative SEM imaging results for (A) ZIF, (B) PROZIF, (C) SAFROZIF, and (D) CURCUZIF nanocarriers and their size distribution

each drug. Ultimately, the cumulative drug release was determined.

2.8. Statistical analysis

The data was analyzed using GraphPad Prism software, employing Student's t-test and one-way analysis of variance (ANOVA) methods.

3. Results

3.1. SEM imaging results

Results (Fig. 1) showed that ZIF, PROZIF, SAFROZIF, and CURCUZIF nanocarriers were composed of irregularly shaped particles. The size distribution of the nanocarriers was relatively wide, and the particles exhibited a rough surface. Particle size measurements revealed that ZIF, PROZIF, SAFROZIF, and CURCUZIF nanocarriers had approximate sizes of 338.123 ± 18.29 nm, 412.72 ± 22.26 nm, 536.87 ± 17.29 nm, and 614.23 ± 29.64 nm, respectively.

3.2. MTT assay results

Results (Fig. 2) showed that ZIF, PROZIF, SAFROZIF, and CURCUZIF nanocarriers exhibited a dose-dependent effect on the viability of L929 cells. In all groups, at all the studied time

points, cells incubated with 100 and 1000 $\mu\text{g/mL}$ nanocarriers displayed significantly lower viability compared to other groups. It appears that concentrations of 1 and 10 $\mu\text{g/mL}$ imparted significantly lower toxicity towards cells, as evidenced by the lack of significant differences in optical density between these concentrations and the control group.

3.3. Anti-inflammatory assay results

Results (Fig. 3) showed that RAW264.7 macrophage cells cultured with a concentration of 10 $\mu\text{g/mL}$ had significantly lower levels of proinflammatory cytokines compared to the other concentrations. In Figure 4, the antiinflammatory activity of ZIF, PROZIF, SAFROZIF, and CURCUZIF was compared. Across most of the concentrations studied, PROZIF exhibited stronger anti-inflammatory activity compared to the other groups.

3.4. Cell migration assay

Results (Fig. [5]) showed that on the third day, L929 cells cultured with PROZIF exhibited a significantly higher percentage of in vitro wound closure compared to the other groups. The percentage of wound closure in the ZIF group was significantly lower than in the PROZIF, SAFROZIF,

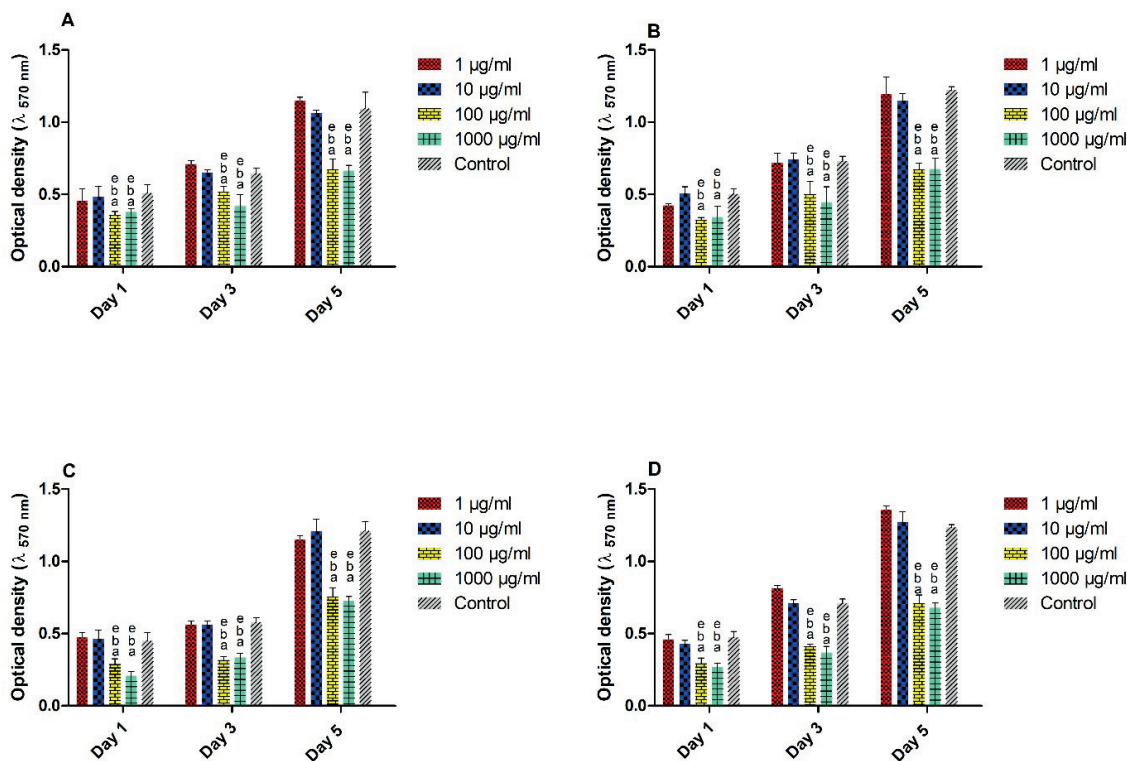


Fig. 2. MTT assay with L929 cells cultured with different concentrations of (A) PROZIF, (B) SAFROZIF, (C) CURCUZIF, and (D) ZIF nanoparticles, a, b, and e show p -values < 0.05 relative to 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and control groups, respectively

and CURCUZIF groups. On the second day, no significant statistical difference was found between all groups.

3.5. FTIR assay results

Figure 6 shows the FTIR spectra of different nanocarrier systems. As shown, in the ZIF spectra, one of the prominent features is a strong peak observed in the region of $1550\text{--}1600\text{ cm}^{-1}$, indicative of the C=N stretching vibration originating from the imidazole ring. This vibrational mode contributes significantly to the overall spectrum and serves as a distinguishing marker for ZIF compounds. Additionally, a medium-intensity peak typically appeared between 1400 and 1500 cm^{-1} , corresponding to the C-N stretching vibration within the imidazole ring. This feature further elucidates the presence and configuration of the imidazole moiety within the framework. Furthermore, a weaker peak in the range of $800\text{--}900$

cm^{-1} is discernible, attributed to the C-H bending vibration within the imidazole ring. Concurrently, PROZIF exhibited additional peaks or modifications in peak intensities within this range, reflecting interactions between propolis constituents and the ZIF matrix. Furthermore, characteristic peaks of propolis components, such as flavonoids and phenolic compounds, appeared in the FTIR spectra. These include peaks at approximately $1650\text{--}1750\text{ cm}^{-1}$, corresponding to C=O stretching vibrations of flavonoids, and peaks around $1200\text{--}1300\text{ cm}^{-1}$, attributed to C-O stretching vibrations of phenolic compounds. In the FTIR spectra of saffron-loaded ZIF nanocarriers, several peaks indicative of specific functional groups present in saffron constituents were identified. These included peaks observed around $2900\text{--}3000\text{ cm}^{-1}$, representing C-H stretching vibrations, which are abundant in saffron's organic compounds, including crocin, picrocrocin, and safranal. Carbonyl stretching vibrations, typically found in compounds like

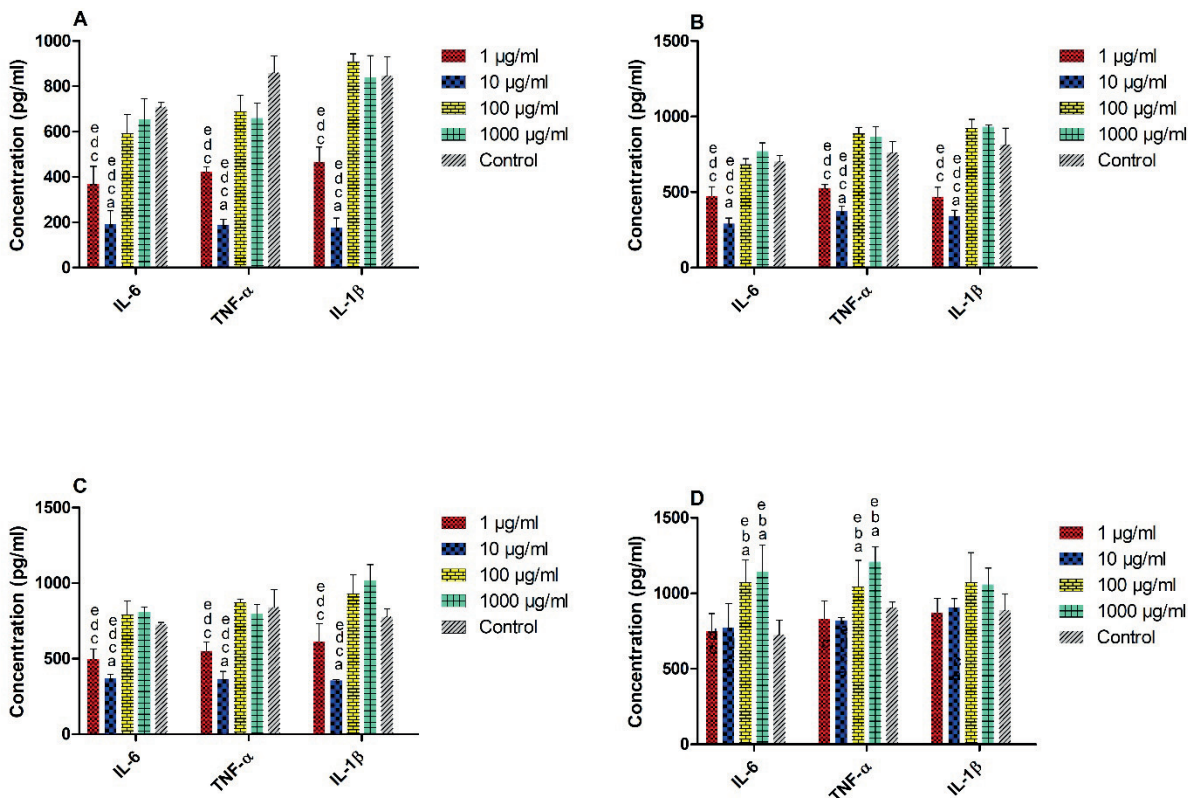


Fig. 3. Histograms comparing the anti-inflammatory activity of (A) PROZIF, (B) SAFROZIF, (C) CURCUZIF, and (D) ZIF nanoparticles at different concentrations, a, b, c, and d show p-value < 0.05 relative to 1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, and 1000 $\mu\text{g/mL}$ concentrations, respectively

crocin and safranal, were detected in the range of 1700–1800 cm^{-1} . Additionally, peaks around 1100–1200 cm^{-1} corresponded to C–O stretching vibrations, prevalent in saccharide molecules present in crocin, the principal carotenoid in saffron. Bending vibrations of C–H bonds, indicative of aromatic compounds such as crocin and safranal, were observed near 800–900 cm^{-1} . Furthermore, peaks in the fingerprint region (below 1500 cm^{-1}) provided insights into the overall molecular structure and composition of saffron extract, including various functional groups present in its constituents. In the FTIR spectra of CURCUZIF particles, several characteristic peaks indicative of both the ZIF framework and curcumin constituents were identified. Among these features, specific peaks associated with curcumin molecules were discernible. For instance, curcumin contains functional groups such as aromatic rings and conjugated double bonds, which give rise to characteristic

peaks in the FTIR spectra. Peaks associated with aromatic C=C stretching vibrations were observed around 1600–1650 cm^{-1} . Furthermore, peaks corresponding to C=O stretching vibrations in the β -diketone moiety of curcumin appeared around 1650–1700 cm^{-1} . Additionally, peaks in the range of 1300–1400 cm^{-1} were attributed to the C–O stretching vibrations in the methoxy groups of curcumin.

3.6. Release assay results

Results (Fig. 7) showed that propolis, saffron extract, and curcumin were released from the ZIF structure in a sustained manner. In the initial hours, there was a burst release profile that was followed by a sustained release phase that continued for more than 7 days. At the end of 7th day, the cumulative release percentage of propolis, saffron extract, and curcumin was measured to be around

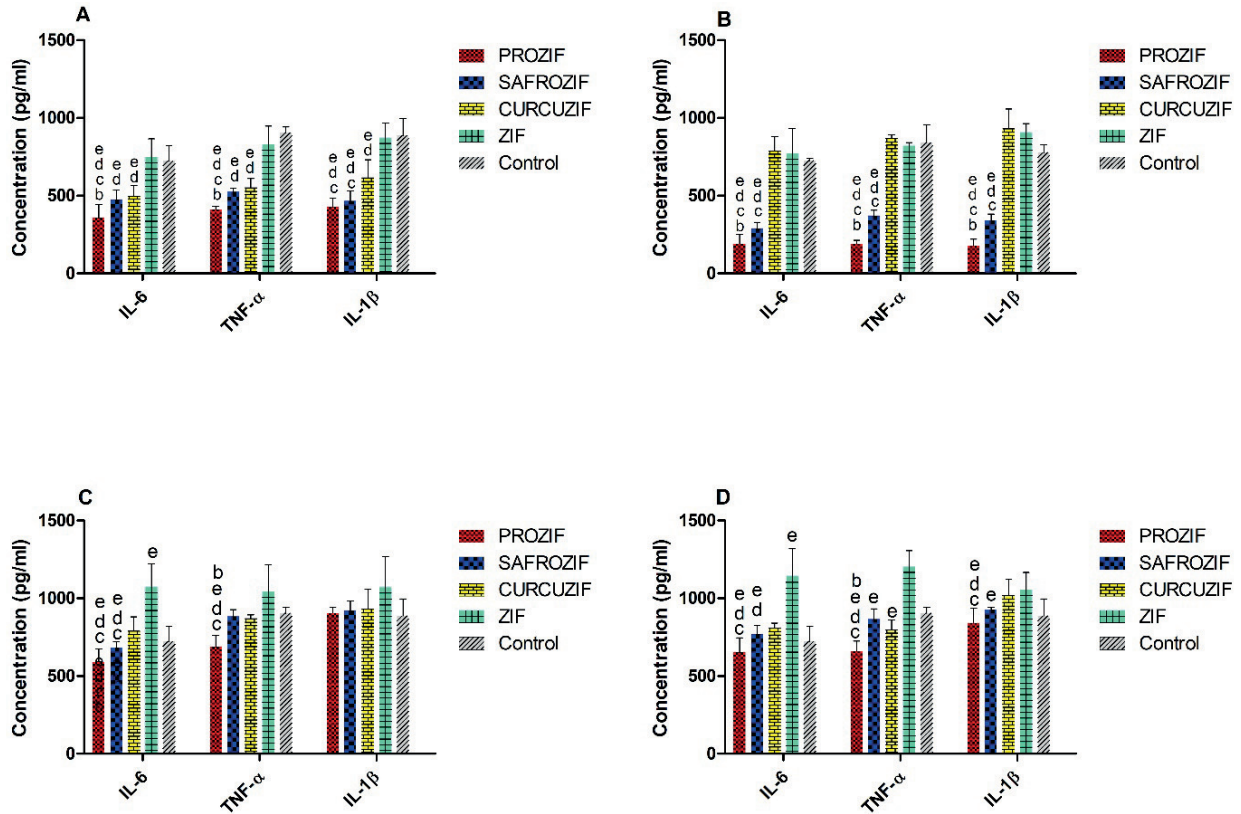


Fig. 4. Histograms comparing the anti-inflammatory activity of ZIF, PROZIF, SAFROZIF, and CURCUZIF at (A) 1 μg/mL, (B) 10 μg/mL, (C) 100 μg/mL, and (D) 1000 μg/mL concentrations

82.71 ± 7.27%, 95.30 ± 2.60%, 84.60 ± 5.31%, respectively.

4. Discussion

ZIF nanocarriers represent a promising avenue for OA treatment. These nanocarriers offer a unique combination of biocompatibility, high surface area, and tunable drug release kinetics [14]. By encapsulating therapeutic agents within their porous structure, ZIF nanocarriers enable targeted delivery to affected joints, minimizing systemic side effects. Their stability under physiological conditions ensures sustained drug release, providing prolonged relief from OA symptoms [20]. In the current research, we loaded three different anti-inflammatory substances into ZIF nanoparticles and assessed their various physicochemical and biological properties. Our microstructure analysis results showed a submicron size

of the developed nanocarriers. The small size of these nanocarriers enables them to efficiently penetrate into the joints, accessing inflamed areas more effectively than larger particles. Additionally, their rough surface promotes adherence to cartilage and nearby tissues, extending their presence within the joint and improving the delivery of drugs to specific treatment sites. The MTT assay revealed dose-dependent toxicity of our developed nanocarriers. At elevated concentrations, their toxic effects may stem from heightened exposure to reactive surfaces and components, potentially inducing cytotoxicity. Furthermore, the nanocarriers' small size and rough surface enhance interaction with cell membranes, potentially causing physical damage or permeabilization [21]. Additionally, surface metal ions or reactive groups on ZIFs may catalyze reactive oxygen species (ROS) generation upon contact with cellular environments [22]. Our anti-inflammatory

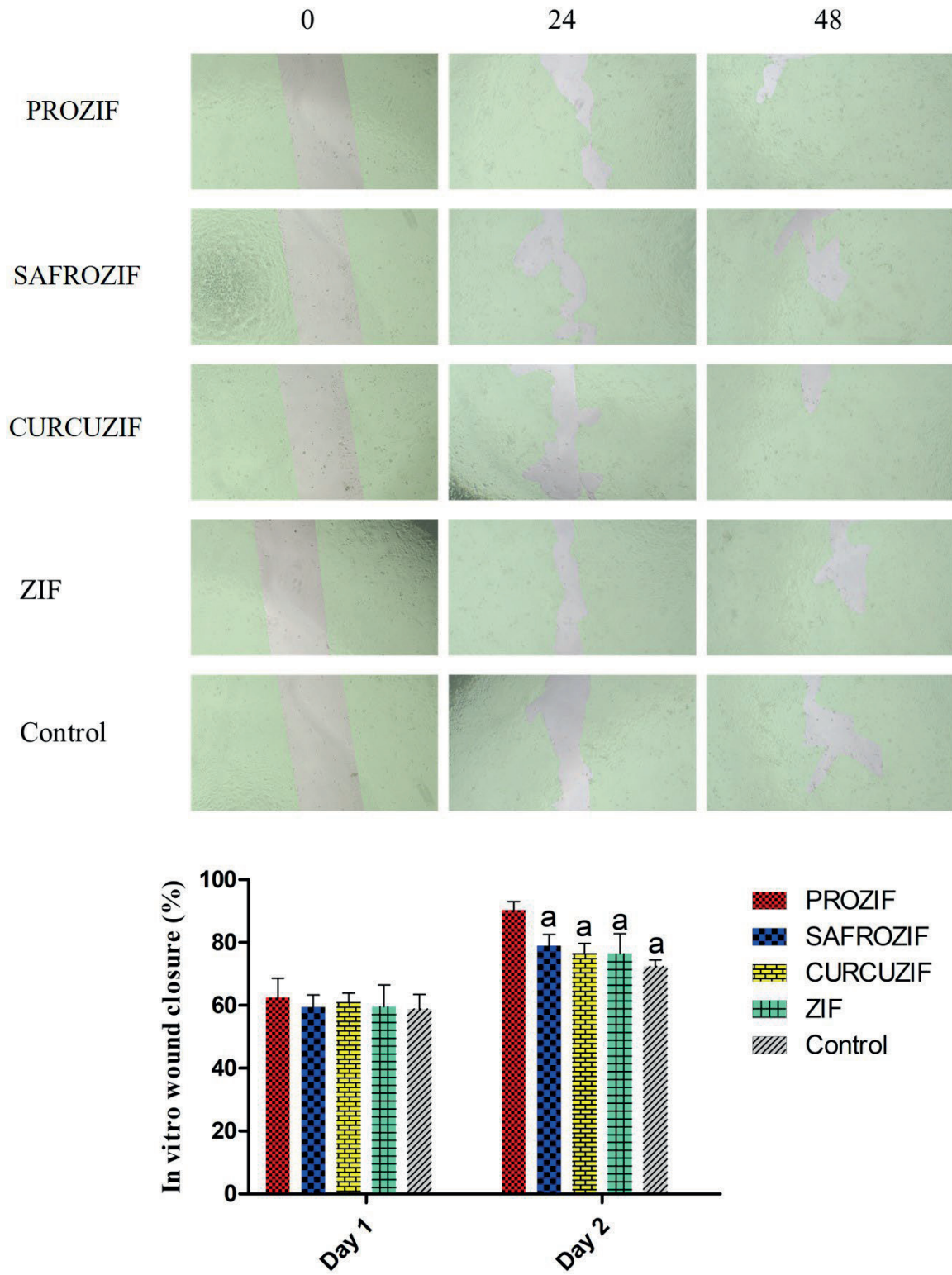


Fig. 5. Cell migration assay with L929 cells cultured with different nanocarriers on day 0 (left panel), day 1 (middle panel), and day 2 (right panel), a shows p -value < 0.05 relative to PROZIF group

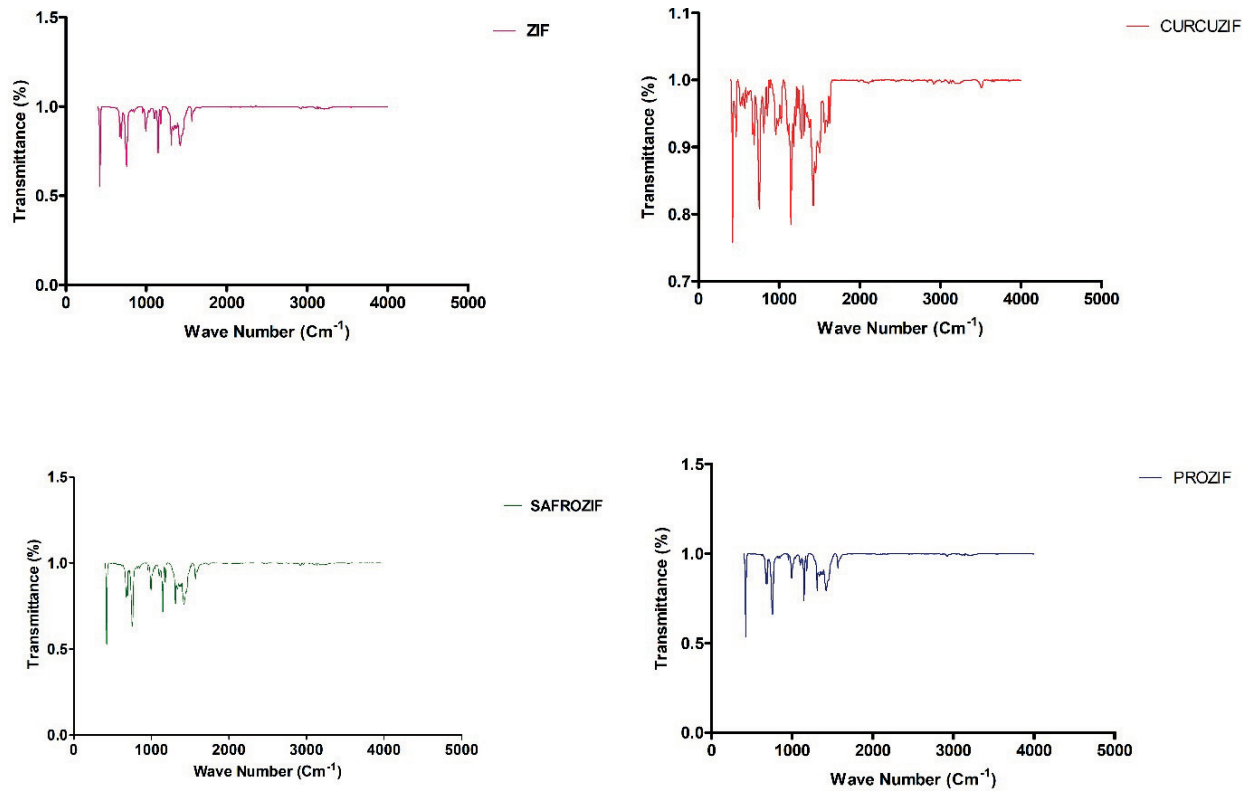


Fig. 6. FTIR spectra of ZIF, PROZIF, SAFROZIF, and CURCUZIF nanoparticles

assay results indicated that the optimal dosage for all nanocarriers to modulate inflammation was 10 $\mu\text{g/mL}$. In comparing the anti-inflammatory activity of ZIF, PROZIF, SAFROZIF, and CURCUZIF nanocarriers, it was evident that PROZIF particles exhibited notably greater anti-inflammatory potential than the other particles. Propolis exhibits multifaceted anti-inflammatory properties [23]. It inhibits the production and secretion of proinflammatory cytokines such as IL-1 β , IL-6, IFN- γ , and TNF- α while simultaneously stimulating the production of anti-inflammatory cytokines like IL-10 [24]. Additionally, propolis and its compounds inhibit the activity of cyclooxygenase (COX) enzymes involved in the inflammatory pathway, reducing the synthesis of proinflammatory prostaglandins [25]. Furthermore, propolis diminishes nitric oxide production, a crucial inflammatory mediator. It also hampers inflammatory cell migration, particularly macrophages and neutrophils, possibly through the downregulation of chemokines CXCL9 and CXCL10 [26]. Moreover,

propolis modulates NF- κB and NLRP3 inflammasome pathways, impeding their activation and thus exerting a comprehensive anti-inflammatory effect [27]. The cell migration assay results revealed that when cultured at equivalent concentrations, L929 cells exposed to PROZIF nanocarriers exhibited significantly increased migration activity compared to other groups. Polyphenolic substances found in propolis, including flavonoids and caffeic acid phenethyl ester (CAPE), have the capacity to trigger signaling pathways such as MEK/ERK and PI3K/Akt. These pathways, once activated, promote both fibroblast migration and proliferation [28]. Our FTIR assay validated the encapsulation of propolis, saffron extract, and curcumin within the ZIF nanocarriers. Furthermore, our release assay demonstrated that these substrates exhibited a sustained release profile over time. Drug release from ZIF nanocarriers involves diffusion through pores, degradation-induced liberation, ion exchange with the environment, and external stimuli-triggered transformations.

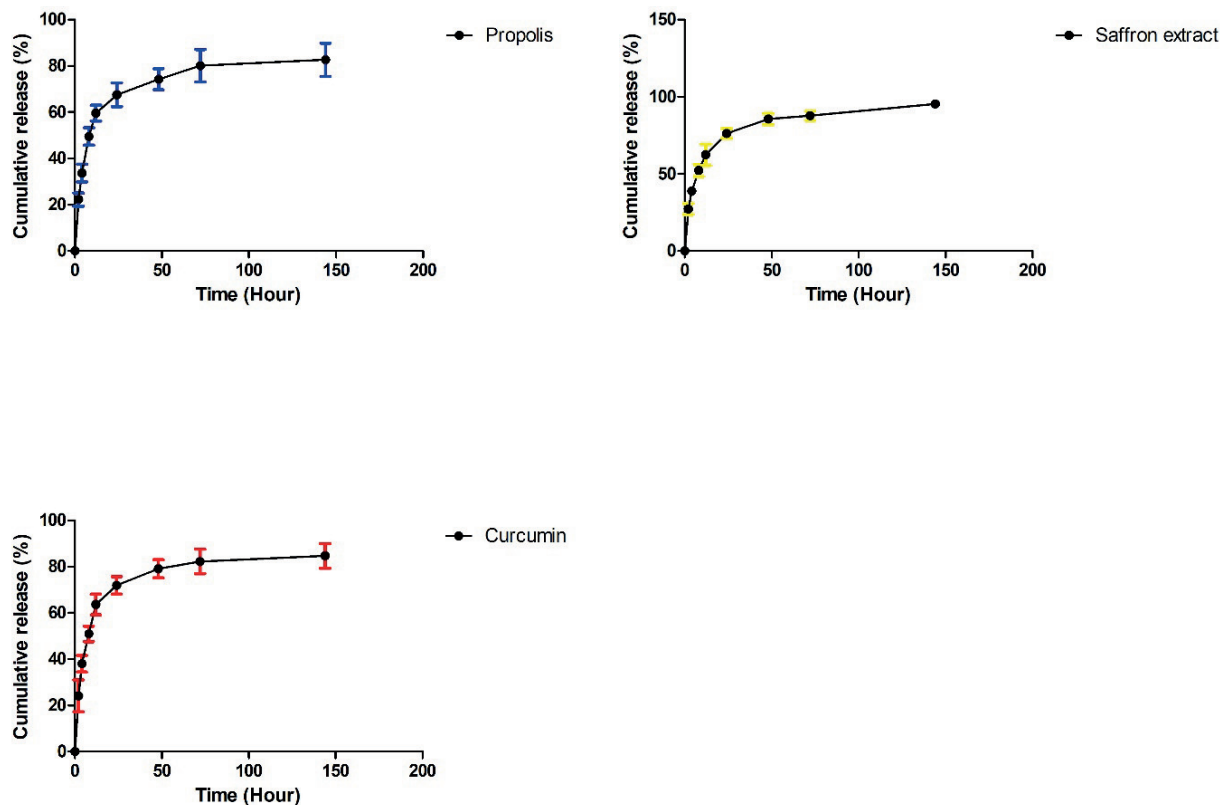


Fig. 7. Cumulative release profile of propolis, saffron extract, and curcumin from PROZIF, SAFROZIF, and CURCUZIF during the course of 7 days in PBS

5. Conclusion

In conclusion, the development of propolis, saffron extract, and curcumin-loaded ZIF nanocarriers presents a promising avenue for treating OA. Through various *in vitro* analyses, including SEM imaging, MTT assays, antiinflammatory assays, cell migration assays, FTIR spectroscopy, and release assays, we demonstrated the feasibility and potential therapeutic efficacy of these nanocarriers. Our findings indicate that these nanocarriers effectively encapsulated the bioactive compounds, exhibited sustained release profiles, and displayed significant anti-inflammatory properties. Notably, PROZIF nanocarriers showed superior anti-inflammatory activity and enhanced cell migration compared to other formulations. Propolis possesses multifaceted anti-inflammatory mechanisms, further supporting its potential as an effective agent for OA treatment when delivered through ZIF nanocarriers. Overall, this study underscores

the promise of utilizing nanotechnology-based delivery systems for enhancing the therapeutic outcomes of natural compounds in OA management.

Conflicts of interests

None

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