



OPEN A ZnO-TiO₂-Amygdalin nanocomposite for bone regeneration and antimicrobial activity

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By decreasing bacterial infection and enhancing the bone repairing/healing process, nanomedicine has recently become an increasingly popular approach for addressing high infection risk and low bony reconstruction. Thus, in our study, we attempted to synthesize ZnO-TiO₂-Amygdalin nanocomposite and investigate its effect against pathogenic microorganisms and also on the growth and differentiation of osteoblast cells. In this work, ZnO-TiO₂-Amygdalin was formulated by co-precipitation. It was characterized by analytical techniques, which revealed the hydrodynamic radius of the nanocomposite to be 115 nm with a nanoflakes structure and Wurtzite hexagonal phase formation. According to the data, the ZnO-TiO₂-Amygdalin nanocomposite surface matrix possesses a strong electrostatic interaction. The antimicrobial effects of ZnO-TiO₂-Amygdalin nanocomposites were investigated in vitro against *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, *S. dysenteriae*, and *C. albicans*, and dose-dependent inhibition of bacterial growth was observed. A time-dependent release of alkaline phosphatase was induced with calcium deposition by incubation ZnO-TiO₂-Amygdalin nanocomposites at different doses in osteoblast-like cells (MG-63) exposed to ZnO-TiO₂-Amygdalin nanocomposites. Ultimately, our data showed that due to its antimicrobial effect, increased osteoblast proliferation, stimulated ALP level, and calcium mineralization potential, ZnO-TiO₂-Amygdalin nanocomposites could be effectively used in orthopedic traumas.

Keywords Nanocomposite, ZnO-TiO₂-Amygdalin, Bone regeneration, ALP, Calcium deposition

The bone is an active connective tissue with the intrinsic capacity for regeneration, which is necessary for growth, skeletal remodeling, and bone injury. Bone is made up of inorganic constituents like calcium, carbonate, sodium, and magnesium, and organic components like collagen, osteocalcin, osteonectin, etc.¹. Bone can be remodeled by the coordinated action of three important types of bone cells: osteoblasts, osteocytes, and osteoclasts. Bone remodeling involves three phases: resorption by osteoclasts (digestion of old bone), reversal by mononuclear cells, formation by osteoblasts, and osteocytes acting as mechanosensors of the bone remodeling process². Dysregulation of bone resorption and generation leads to osteoporosis, and bone injury also leads to various pathological conditions like osteomyelitis, periodontitis, and bone tumors³. Bone repair and remodeling conditions need to be improved by stimulating the proliferation and enhancing the survival of bone cells, which requires a biocompatible scaffold to stimulate tissue differentiation⁴. The common treatment strategy for bone defects involves autograft by removing material from the patient's iliac crest, femur, etc. However, autograft transplant is associated with increased infection, donor site morbidity, and expense. Apart from autografts, other methods like allografts, bone marrow cells, and osteocytes are also involved in the curative process, however, they also cause complications like pain, infection, and bleeding, and sometimes it leads to patient morbidity⁵. Thus, identifying novel compounds via conventional strategies like using herbal-derived phytochemicals that could activate and stimulate bone development in the site of low bone density is highly essential for bone disease remedies. Recently, inorganic nanomaterials have been increasingly explored to understand their preventive effect on bone degeneration, like Zn, Ca, Fe, etc.^{6,7}. Moreover, nanocomposite mixtures consisting of individually prepared nanoparticles with different concentrations were reported to possess similar synergistic effects for biological and biochemical applications⁴. In this study, we synthesized ZnO-TiO₂-Amygdalin nanocomposites to understand their effect on bone regeneration.

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Amygdalin is a D-mandelonitrile- β -gentiobioside found in the apricot kernel and was used for the treatment of various pathological conditions like anemia, tumors, and diabetes. Interestingly, Amygdalin has been reported to regulate bone remodeling of subchondral bone injury and prevent the occurrence of osteoarthritis by transforming growth factor (TGF) β /Smad axis^{8,9} showed that Amygdalin together with hydroxyl safflower yellow A synergistically inhibited IL-1 β -triggered chondrocyte degeneration by inhibiting apoptosis, elevating expression of Aggrecan and Col 2 α 1 and reducing expression of MMP-13. Similarly, Ying et al.¹⁰ demonstrated that Amygdalin enhanced bone fracture recovery by the TGF- β /Smad signalling pathway in tibial fractures induced in C57BL/6 mice.

Previous studies categorized stimulus-responsive carbon-based nanomaterials into carbon nanotubes, carbon nanospheres, and carbon nanofibers based on their morphology. It discusses their applications in probes, bioimaging, tumor therapy, and other fields. The previous study also discusses the advantages and disadvantages of these nanomaterials and their future perspective¹¹. In a cancer study, Elderderly et al. 2022 reported that ZnO-TiO₂-Chitosan-Amygdalin nanocomposites exhibit significant anticancer effects, with an inhibitory concentration (IC₅₀) of 10.34 μ g/ml. These nanocomposites increased the number of early and late apoptotic cells in MOLT-4 leukemia cells. Additionally, they enhanced mitochondrial apoptosis through the Caspase cascade signaling. MOLT-4 cells phosphorylated the Caspase cascade in response to treatment with these nanocomposites. Compared to the control group, cancer cells treated with ZnO-TiO₂-Chitosan-Amygdalin nanocomposites showed significant inhibition of proliferation and induced cleavage of pro-apoptotic proteins, ultimately leading to apoptotic cell death¹².

To the best of our knowledge, much work was not done to understand the role of Amygdalin nanocomposites in bone regeneration, and thus in the current study, we (1) synthesized ZnO-TiO₂-Amygdalin, (2) characterized, (3) studied antibacterial and antifungal effects (4) analyzed mineralization effect in osteoblastic-like cell line MG-63.

Materials and methods

Formulation of zinc oxide-titanium dioxide-amygdalin NCs

ZnO-TiO₂-Amygdalin nanocomposite was synthesized using 0.1 M of Zn (NO₃)₂ in an aqueous medium. 500 mg of TiO₂ solution was mixed with 6H₂O. Furthermore, 50 mg of the phytocomponent Amygdalin was then mixed with ZnO-TiO₂ reagent. The NaOH (0.1 M) was added in a dropwise manner into ZnO-TiO₂-Amygdalin solution until a white residue was collected, and then incubated at 37 °C for 3 h under a magnetic stirrer. The collected nanopowder was washed with ethanol and distilled water, which was centrifuged for 40 min at 15,000 rpm, and finally obtained product nanopowder was dried at 200 °C for 2 h and employed for characterization techniques.

Characterization of the ZTA NCs

The nanocomposite samples of ZnO-TiO₂-Amygdalin were assessed by X-ray diffractometer (XRD) (model: X'PERT PRO PANalytical). The diffraction patterns for ZTA NCs were observed at 2 θ angles between 25° and 80° with CuK α diffraction beam at 1.5406 Å. The Scanning Electron Microscope (Carl Zeiss Ultra-55 FE-SEM) with Energy Dispersive X-ray Spectrometry (EDX) (Inca) was utilized to investigate the ZnO-TiO₂-Amygdalin nanocomposites. The morphological analysis of the ZnO-TiO₂-Amygdalin NCs was performed using the TEM (Tecnai-F20) instrument, which functioned at 200 kV accelerating voltage. The FT-IR spectra were verified in the 400–4000 cm⁻¹ range of wavenumber using the Perkin-Elmer spectrometer. ZnO-TiO₂-Amygdalin nanocomposite absorption spectra were assessed between 200 and 1100 nm using a Lambda-35 spectrometer. The photoluminescence (PL) spectrum was obtained using the spectrometer (Perkin Elmer-LS 14).

Antibacterial activity

The antibacterial effect of ZnO-TiO₂-Amygdalin NCs was examined against gram-positive (*S. aureus* and *S. pneumonia*) and gram-negative (*K. pneumonia* and *S. dysenteriae*) bacterial strains using the well diffusion method. In this study, the Petri dish was seeded with 25 mL of Muller Hinton Agar Medium, gram-positive and negative species. Wells of approximately 10mm were bored using a well-cutter, and ZnO-TiO₂-Amygdalin nanocomposites at different concentrations, 1, 1.5, and 2 mg/mL, were dispersed in a 5% sterilized DMSO and were added to wells, incubated at 37 °C for 24 h overnight. The antibacterial property was observed with a diameter of the zone of inhibition surrounding the well. A positive control, amoxicillin (30 μ g), was used, and the experiments were done in triplicate¹³.

Antifungal activity

The antifungal activity against *Candida albicans* was examined using the agar well diffusion method. The *C. albicans* can be injected into a PDA petri dish by streaking thrice and rotating the plate at a 60 °C angle for every streak. Later, sterile forceps were utilized to put wells, and then diverse dosages (1, 1.5, and 2 mg/mL) of test ZnO-TiO₂-Amygdalin nanocomposites were loaded onto inoculation plates and incubated for 24 h. The inhibitory zone was noted, and a positive control, amoxicillin (30 μ g), was used, and the tests were done in triplicate.

Cell lines

Human osteoblast-like MG-63 cells were acquired from ATCC (Rockville, MD, USA) and were allowed to be cultured in DMEM (Sigma-Aldrich) containing 10% FBS (Gibco), 2.0 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Upon 80% confluences, cells were exposed to ZnO-TiO₂-Amygdalin NCs at varied concentrations of 10 and 20 μ g for 3 h, then used for different experiments as discussed below.

MTT assay

The growth of nanocomposite-treated MG-63 cells was analyzed by the MTT test. The MG-63 cells were cultured in 96 plates at 6×10^3 cells per well and then exposed to 10 and 20 μg of NCs for 24 h at 37 °C. Now, cells were added with 20 $\mu\text{L}/\text{mL}$ of MTT reagent in each well and incubated for 4 h at 37 °C. The resulting purple color dye was determined at 490 nm with the help of micro microplate reader¹³.

Alizarin red S (ARS) staining

The accumulation of calcium in the MG-63 cells was measured using the ARS method based on Gregory et al.¹⁴. The medium was eliminated, and membranes were rinsed with saline and fixed with 3.7% formaldehyde (v/v) at ambient temperature for 15 min. The membranes were rinsed with deionized water and subsequently stained with 2% ARS for 15 min. The membranes were thereafter rinsed thrice with distilled water to eliminate any residual stain. Calcium depositions can be identified by their red coloration under microscopy (Nikon TS-100, Japan). The bound ARS was solubilized in 10% acetic acid for quantification of the staining. The amount of ARS was ascertained using absorbance at 550–570 nm. The ARS amount of the control was subtracted from the ARS amount of the corresponding membranes with MG-63 cells to attain the net mineral amount formed by the MG-63 cells.

Alkaline phosphatase assay by Cayman kit

The control and treated cells were rinsed with saline and dissolved in lysis buffer (glycine (0.1 M), MgCl_2 (1 mM), and Triton X-100 (1%)) for 20 min. Later, 50 μL of the lysate was treated with ALP reagent (150 μL) (Cayman detection kit, Item No. 701710) for 1 h. The absorbance of the suspension was studied at 405 nm.

Statistical analysis

Statistical studies were done using SPSS v.10. The values of cell growth, ALP activity, and mineralization were studied using a nonparametric Mann–Whitney U assay. Variations at $p < 0.05$ were fixed as significant.

Results

Characterization of ZTA NCs

The UV–Vis absorption spectra of ZTA, ZnO, TiO_2 , and Amygdalin nanocomposites are presented in Fig. 1a,b, illustrating the optical properties of the synthesized materials. The UV absorbance edge peak for ZTA nanocomposites is observed at 373, 382, 353, and 264 nm, respectively, indicating their strong light absorption characteristics. Compared to bulk ZnO, which exhibits an absorption peak at 365 nm¹⁵, the ZTA nanocomposites demonstrate a blue shift of 8 nm. This shift suggests a reduction in particle size and possible quantum confinement effects, typically occurring when nanomaterials are synthesized at the nanoscale. The interaction between ZnO, TiO_2 , and Amygdalin in the composite structure may contribute to changes in the electronic band structure, altering the bandgap energy. The observed blue shift also indicates enhanced optical properties, which may improve the photocatalytic activity, charge carrier separation, and overall efficiency of the nanocomposite. Such characteristics are beneficial for applications in photocatalysis and biomedical treatments.

ZTA, ZnO, TiO_2 , and Amygdalin nanocomposites X-ray diffraction patterns are shown in Fig. 2a,b. XRD patterns of ZTA nanocomposites, ZnO diffraction peaks angle (2θ) at 31.49°, 34.45°, 36.19°, 47.56°, 56.58°, 62.62°, 66.46°, 67.94°, 69.06°, 72.64° and 77.01° the respective hkl values for ZnO (wurtzite hexagonal) phase formation were (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0), (1 1 2), (2 0 1), (0 0 4), and (2 0 2) JCPDS card No.36-1451¹⁵. TiO_2 diffraction peak at 25.27°, 30.01°, 35.21°, 43.85°, and 50.02°, exhibiting the anatase/rutile TiO_2 phase (JCPDS card No: 21-1272 and 21-1276)^{16,17}, with TiO_2 doping, the structure of wurtzite hexagonal ZnO does not change. Furthermore, peaks corresponding to TiO_2 phases were identified in the XRD patterns of the composites. This improves the biocidal properties by enhancing photocatalytic activity, charge separation, and reactive oxygen species (ROS) generation. However, 15.93° was reported as the Amygdalin diffraction peak. The ZTA, ZnO, TiO_2 , and Amygdalin nanocomposites FT-IR spectra were illustrated in Fig. 3a,b. Due to the hydroxyl group, the strong (O–H) stretching and bending were noted at 3427 and 1639 cm^{-1} ¹⁸. Nevertheless, the Amygdalin characteristic peaks are symmetric and asymmetric peaks observed at 2924 and 2854 cm^{-1} , due to the $-\text{C}-\text{H}(\text{CH}_2)$ group, at peak 913 cm^{-1} $-\text{HC}=\text{CH}$ out of plane bending peak and C–O stretching peak for 1116 cm^{-1} respectively¹⁹. In addition, for Zn–Ti–O at 718, 740, and 459 cm^{-1} , metal–oxygen stretching vibrations were identified^{20,21}.

The chemical profile and morphology of the ZTA nanocomposites were detected through the FE-SEM/EDAX spectrum, shown in Fig. 4a–c. FE-SEM images with higher and lower magnification revealed that the ZTA nanocomposites display a structure like a nanoflake. The average nanoflake thickness was 55 nm, which corresponds with XRD data. The EDAX spectra of ZTA NCs are revealed in Fig. 4c. For ZTA, on analysis atomic percentage is: 19.91% for (C), 17.56% for (Zn), 14.96% for (Ti), and 44.72% for (O) in the ZTA nanocomposites. TEM/SAED patterns were employed to assess the morphology of the ZTA nanocomposites, as illustrated in Fig. 5. As seen in TEM pictures, Amygdalin is encapsulated in metal oxide ($\text{ZnO}-\text{TiO}_2$). The nanoflake-like structure of ZTA nanocomposites is also noticeable in FE-SEM images. The mean particle size of 50 to 60 nm, as studied by XRD, follows the findings. The formation of ZnO wurtzite hexagonal crystallinity of TiO_2 and Amygdalin nanomaterial was studied using a selected area of electron diffraction (SAED) pattern (Fig. 5c).

The size distribution of the synthesized ZTA NCs was investigated using the DLS technique (Fig. 6). The hydrodynamic size of the particles of ZTA was observed at 115 nm. Thus, the particle size observed is polydispersed, which is studied using a size distribution graph that seems more prominent compared with XRD and TEM. The photoluminescence spectra of ZTA NCs with an excitation wavelength of 325 nm are demonstrated in Fig. 7. The PL emission data were noted at 361, 427, 481, and 483 nm, regardless of the TiO_2 element observed in the ZnO surface matrix. The additional TiO_2 peaks, incorporating TiO_2 into ZnO, follow

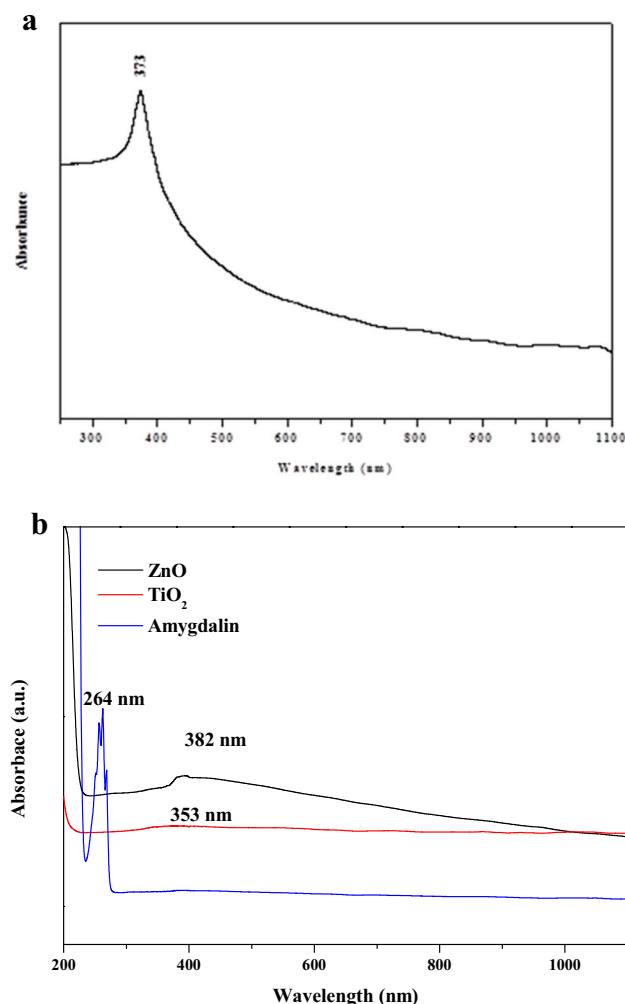


Fig. 1. (a) Characterization of synthesized nanocomposite ZnO-TiO₂-Amygdalin using UV-Vis absorbance spectrum. (b) Characterization of synthesized nanocomposite ZnO, TiO₂, and Amygdalin using UV-Vis absorbance spectrum.

the acquired structural data from the XRD study. The characteristic peak of UV emission is noted at 361 nm, which is attributed to near band edge (NBE) emission due to exciton-exciton collision mechanisms. The violet emission, which was observed at 427 nm, is attributed to the de-excitation from lower vibronic levels in Ti³⁺3d states of the ZnO-TiO₂ lattice to the deep trap levels (acceptor)²². The blue emission bands at 481 and 483 nm correspond to the intrinsic Zn defects²³ or the transition between the shallow donor Zn and deep acceptor V_{Zn} levels²⁴.

Effect of ZTA NCs on bacterial and fungal strains

The potential antimicrobial effect of ZTA nanocomposites was studied by the well diffusion technique using Muller-Hinton agar. This work cultured bacterial strains of *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, *S. dysenteriae*, and *C. albicans* as shown in Fig. 8. The exposure of nanocomposites was in the tested concentration range of 1, 1.5, and 2 mg/mL. The ZTA NCs and conventional antibiotics with amoxicillin exhibit antimicrobial effects (Fig. 8). The antimicrobial activity was determined to be dependent on concentration. Increasing the concentration of ZTA nanocomposites also increased antimicrobial effects against the tested organism.

The study demonstrated that ZTA NCs (ZnO-TiO₂-Amygdalin Nanocomposites) exhibit strong antibacterial activity, with MIC values varying among bacterial strains. After 24 h of incubation under aerobic conditions at 37 °C, turbidity was observed in test tubes containing different bacterial strains treated with ZTA NCs. The minimum inhibitory concentration (MIC) values for tested strains were 0.039–0.156 mg/mL for *E. coli*, 0.039–0.078 mg/mL for *S. aureus*, 0.078–0.312 mg/mL for *P. aeruginosa*, and 0.039–0.078 mg/mL for *B. subtilis*. However, no turbidity was observed at higher concentrations, indicating complete bacterial inhibition. The study concluded that Gram-positive bacteria (*S. aureus* and *B. subtilis*) were more sensitive to ZTA NCs, requiring lower MIC values than Gram-negative bacteria. *P. aeruginosa*, a resistant strain, required higher bactericidal concentrations (≥ 1.25 mg/mL), confirming its stronger defense mechanisms. While at concentrations of 0.625, 1.25, 2.5, and 5 mg/ml, no turbidity was seen, exhibiting inhibition of bacterial growth. The suspension from the

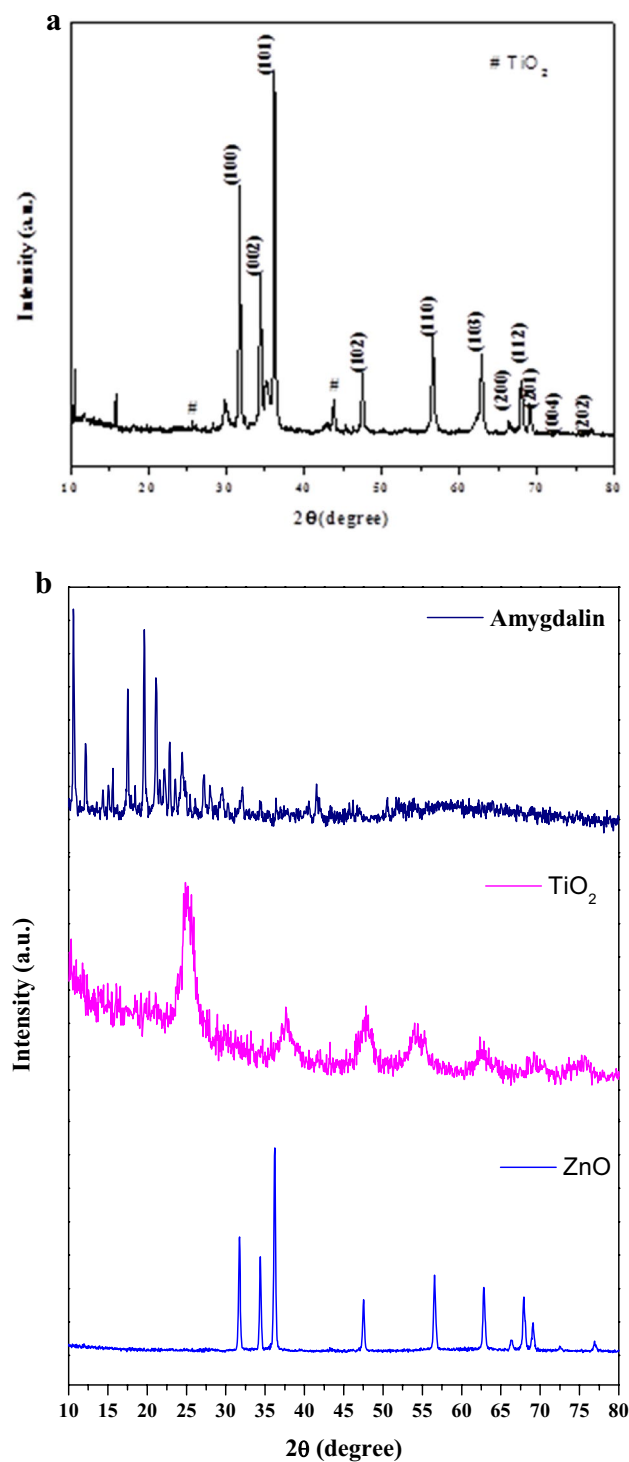


Fig. 2. (a) Characterization of synthesized nanocomposite ZnO-TiO₂-Amygdalin using X-ray diffraction. (b) Characterization of synthesized nanocomposite ZnO, TiO₂, and Amygdalin using X-ray diffraction.

tubes of 0.625, 1.25, 2.5, and 5 mg/ml was inoculated in a BHI agar plate and incubated for 24 h, and no growth of bacteria was observed in all the concentrations, hence confirming it as bactericidal for *E. coli*, *S. aureus*, and *B. subtilis*. *P. aeruginosa* bactericidal activity was exhibited at 1.25, 2.5, and 5 mg/mL in the BHI agar plate and incubated for 24 h.

Effect of ZTA NCs on the cell viability

The result of ZTA NCs on the cell growth of MG-63 cells using MTT cytotoxicity assay and the data is shown in Fig. 9a,b. Nanocomposite incubation at day 1 and day 7 data showed there was a dose-dependent change in

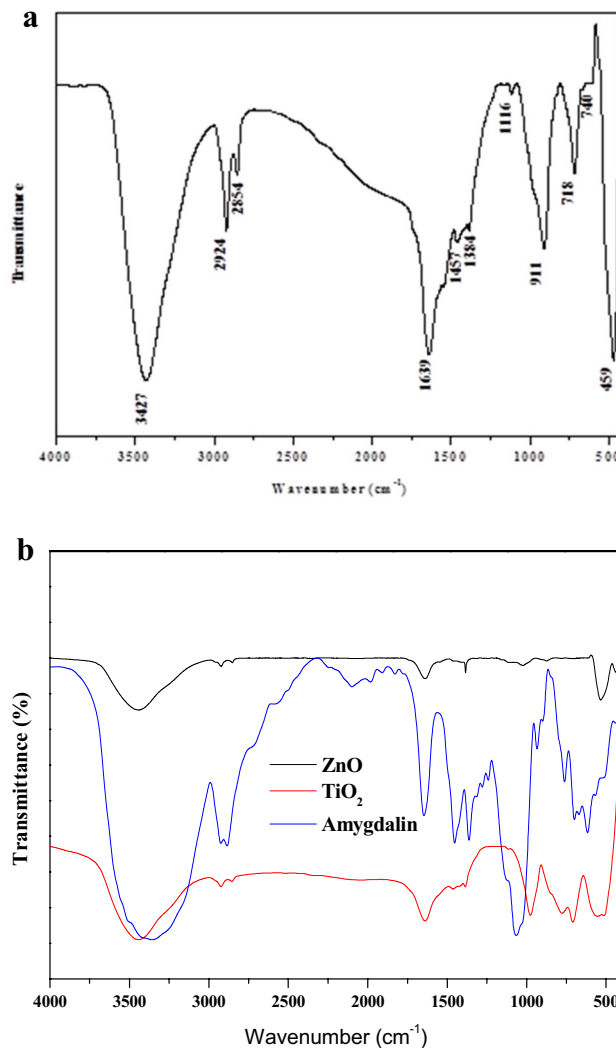


Fig. 3. (a) Characterization of synthesized nanocomposite ZnO-TiO₂-Amygdalin using FT-IR. (b) Characterization of synthesized nanocomposite ZnO, TiO₂, and Amygdalin using FT-IR.

the cell viability when compared to control cells. Nanocomposites at a concentration of 10 μg showed decreased viability at day 1 and day 7 significantly when compared to 20 μg , and thus, increased cell mass was observed at 20 μg of nanocomposite-treated day 7 MG-63 cells significantly. Thus, MG-63 cell growth data showed that nanocomposites enhanced cell growth and improved the multiplication of MG-63 cells in a dose-dependent manner.

Effect of ZTA NCs on calcium deposition

The effect of ZTA nanocomposites on calcium accumulation rate was detected in MG-63 cells using the ARS staining method and the data is shown in Fig. 10. The rate of calcium deposition was enhanced in a dose-dependent manner (10, 20 μg) upon nanocomposite treatment in MG-63 cells in day 1 and day 7. However, pronounced changes in calcium deposition were observed between the 1st and 7th day, and thus suggested that nanocomposites effectively enhanced calcium deposition in a time-dependent manner in MG-63 treated cells.

Effect of ZTA NCs on the ALP activity

The ALP levels in the MG-63 cells were estimated using the ELISA method and shown in Fig. 11. ZTA nanocomposites effectively increased the ALP levels in a dose-dependent manner in MG-63 treated cells. Comparatively, 20 μg of nanocomposite showed a 2.5-fold elevation in the levels of ALP on day 7 when compared to day 1 of MG-63 treated cells.

Discussion

Bone is a connective tissue that possesses self-regenerating capacity during micro-injuries. Nevertheless, several critical conditions like bone damage and fracture that occur due to traumatic and accidental damages worldwide lose their self-regenerating potential and therefore require bone grafts. During the last years, the failure of

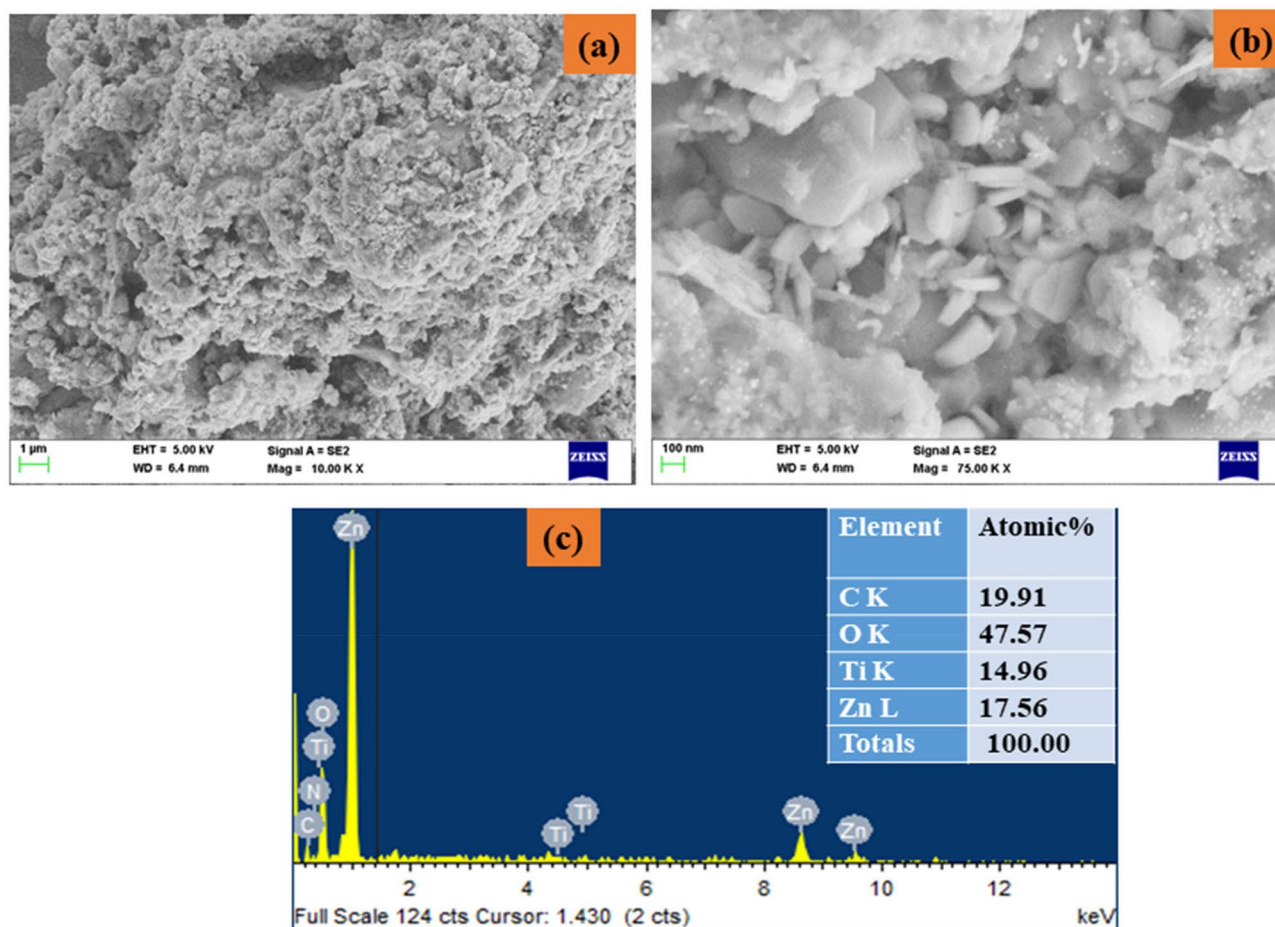


Fig. 4. FE-SEM/EDAX analysis (a, b) Lower and higher magnification FE-SEM images and (c) EDAX spectrum of the ZTA NCs.

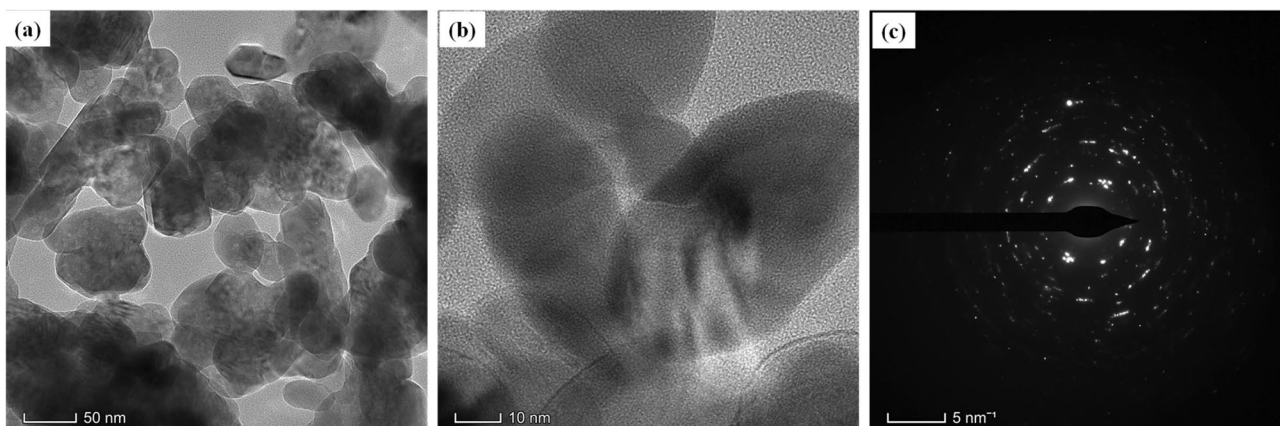


Fig. 5. TEM/SAED analysis (a, b) TEM image and (c) SAED patterns of the ZTA NCs.

currently available treatment strategies has affected the regrowth and repair of injured bone tissue and led to increased economic burden and enhanced rates of morbidity and mortality¹⁰.

Recently, nanomedicine has helped in solving various biological problems through its nanoscale size. Nanocomposites are reported to show various functions like anticancer, antioxidant, and angiogenesis²⁵. Moreover, the development of nanocomposites for the improvement of bone repair/regeneration is currently attracting researchers' attention. In this study, we synthesized a nanocomposite using ZnO, TiO₂, and Amygdalin

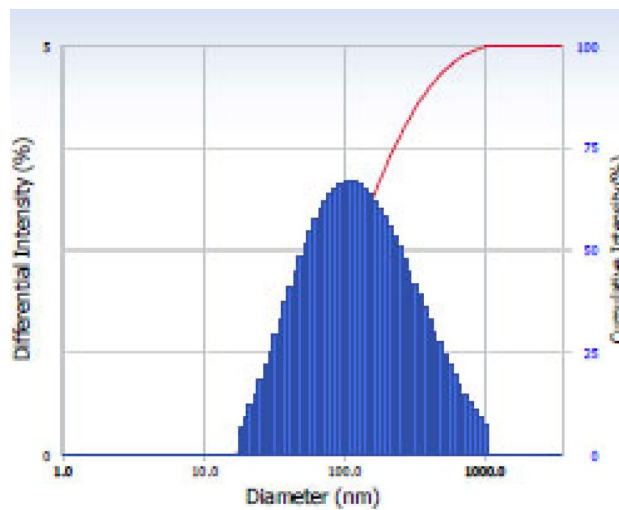


Fig. 6. DLS spectrum analysis of the synthesized ZTA NCs.

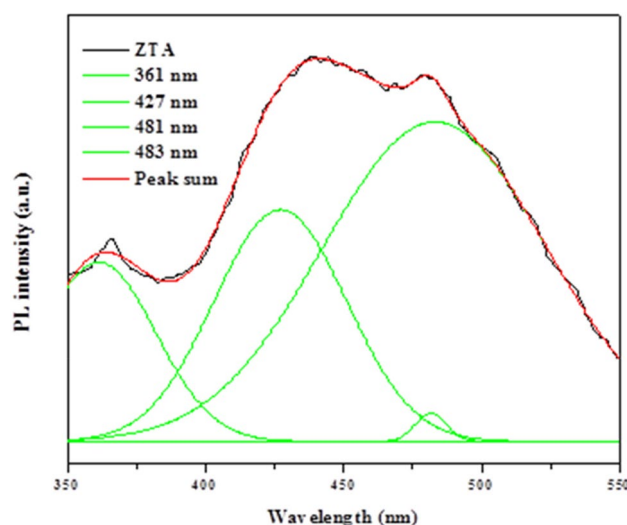


Fig. 7. PL spectrum of the synthesized ZTA NCs.

and analyzed, antimicrobial, proliferation, and calcium mineralization effects on MG-63 cells. An illustration of how ZnO-TiO₂-Amygdalin NCs are synthesized and their applications are shown in Fig. 12.

In our study UV absorbance edge peak for ZTA was shown at 373 nm, which was similar to Yao et al.¹⁵, showing a blue shift of 8 nm compared to bulk ZnO, which peaked at 365 nm. The XRD method offers detailed data on the crystallographic structure and chemical-physical properties of nanocomposites. Our XRD analyses showed that ZnO exhibited a wurtzite hexagonal phase while TiO₂ showed an anatase/rutile phase, and 15.93° was reported to be an Amygdalin diffraction peak. Ali et al. found that because of the ionic radii mismatch of Zn²⁺ and Ti⁴⁺, the XRD peaks were shifted to higher 2θ with reduced crystallite size from ZT15 to ZT35, and also observed tensile lattice strain, with ZT35 exhibiting the highest value. FT-IR analyses help to show the synthesized ZnO-TiO₂-Amygdalin nanocomposite functional groups. In our study, we observed absorption peaks at 459, 740, 718, 911, 1116, 1384, 1457, 1639, 2854, 2924, and 3427 for the nanocomposite. The FT-IR spectrum results confirmed that ZTA nanocomposites and Amygdalin phytochemicals successfully interacted with the ZnO and TiO₂ NPs. These connections were due to the electrostatic interaction between the ZTA nanocomposite surface matrix. The PL emission data were detected at 361, 427, 481, and 483 nm, regardless of the TiO₂ element observed in the ZnO surface matrix. Elderderly et al.¹² reported the absorption peak for ZTA at 394, 418, 442, 457, 473, and 509 nm and suggested that it could be due to the intrinsic band gap in photoluminescence spectra, and also suggested the occurrence of electrostatic connection between ZTA NCs surface matrix. Our FE-SEM data showed that ZTA nanocomposites exhibit a nanoflake-like structure, while Elderderly et al.¹² observed nanorods for ZnO-TiO₂-Chitosan-Amygdalin nanocomposite. The atomic proportions were found to be 19.91% for (C), 17.56% for (Zn), 14.96% for (Ti), and 44.72% for (O) in the ZTA

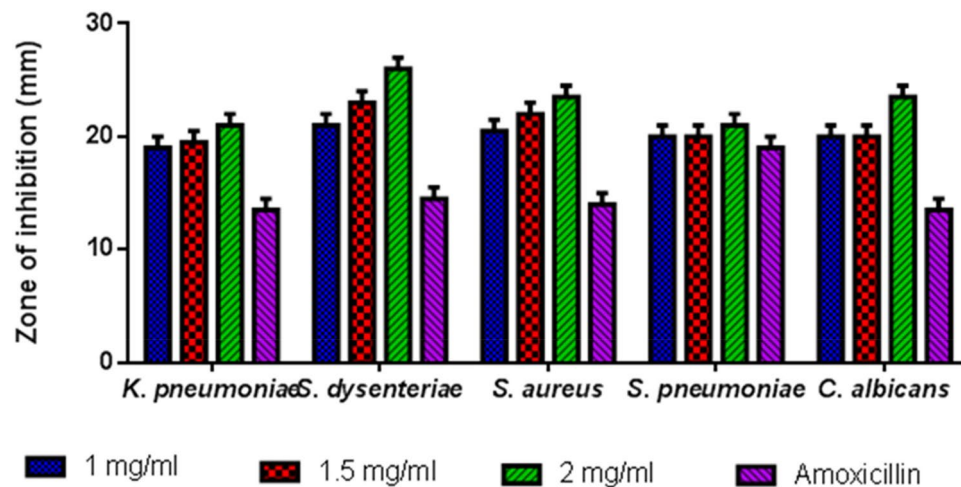


Fig. 8. Zone of inhibition of ZnO-TiO₂-Amygdalin NCs treated with different pathogens showing dose-dependent effects (1, 1.5, and 2 mg/ml).

nanocomposites. The formation of the ZnO wurtzite hexagonal crystalline phase in the TiO₂ and Amygdalin nanomaterial was studied by the SAED pattern. The hydrodynamic size of the particles of ZTA was observed at 115 nm. The size distribution data demonstrates that the particle size is polydisperse and more prominent. Results of the XRD pattern showed that the combination of the ZTA nanocomposite phase's formation is due to both the steric properties and the intermolecular hydrogen bonds between the ZTA surface matrixes. The mean size of the crystals in the ZTA matrix was 55 nm^{26,27}.

Our data showed that increasing the concentration of ZTA nanocomposites significantly enhanced antimicrobial and fungal effects against the tested organisms, including *S. aureus*, *S. pneumoniae*, *K. pneumoniae*, *S. dysenteriae*, and *C. albicans*. The antibacterial mechanism of ZTA nanocomposites suggested that the bacterial membrane damage could have been triggered by ZnO-TiO₂ electrostatic interaction and cell surfaces, production of ROS like H₂O₂ in cells, and cellular internalization of ZnO-TiO₂. The H₂O₂ production by bacteria was confirmed using an oxygen electrode sensor²⁸. ZnO-TiO₂ nanoparticles have been found to have antibacterial properties due to their ability to generate reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals²⁹. These nanoparticles catalyze reactions with water and oxygen, enhancing their photocatalytic activity under UV or visible light. The release of Zn²⁺ ions further enhances antibacterial activity by disrupting bacterial membranes and metabolic functions³⁰. Additionally, because of the elevated surface area of nanocomposites, it might have shown a better connection with pathogenic bacteria to disrupt the bacterial cell wall³¹, and thus, we suggest that the antimicrobial potential of ZTA nanocomposites could help to decrease the risk of bone infection.

The bone repair, regeneration, and remodeling require organized cellular events of osteoblasts, osteoclasts osteocytes. The viability of osteoblast cells is strongly associated with the bone formation mechanism that leads to increased activation of alkaline phosphatase and maturation of mineralization³². In our study, we observed increased viability of osteoblast-like cells (MG-63) while incubating cells with ZTA nanocomposites at the doses of 10 and 20 µg on both day 1 and day 7. Thus, ZTA nanocomposites effectively protected and improved the proliferation of the osteoblast cells till 7 days in a dose-dependent manner. The elevated ALP levels are the markers of osteoblast differentiation phenotype and are necessary for the formation of bone minerals by initiating and enhancing the development of apatite in osteoblast vesicles and helping in the construction of extracellular matrix³³. In our study, ZTA nanocomposites effectively increased the ALP levels in a dose-dependent and time-dependent manner in MG-63 treated cells. The deposition of calcium is a clear indicator of osteoblast differentiation, which would later undergo maturation to produce a mineralized bone matrix. Moreover, the function of ALP is associated with calcium mineralization by disseminating the production of phosphate, which would ultimately lead to the mineralization process^{34,35}. In our study, we observed that the rate of calcium deposition was enhanced in a dose-dependent manner (10, 20 µg) upon treatment of ZTA nanocomposite in MG-63 cells on day 1 and day 7. In a study, it was reported that amygdalin treatment promoted bone fracture recovery by targeting TGF-β/Smad signaling in TGF-βII receptor knockout mice and C3H10 T1/2 murine mesenchymal progenitor cells¹⁰. In a study, it was shown that micro/nanostructured TiO₂/ZnO coating effectively augmented ALP activity, collagen production, and osteopontin, thereby enhancing osteogenic activity in SaOS-2 cells^{36,37}.

Previous study suggests that Rhizoma Drynariae-derived nanovesicles (RDNVs) can potentially reverse postmenopausal osteoporosis by targeting estrogen receptor-alpha and promoting osteogenic differentiation of human bone marrow mesenchymal stem cells³⁸. The earlier study reported that CMC-LYZ-ACP nanogels, containing AgNO₃, TPs, and CHX, exhibit excellent antibacterial properties, inhibiting plaque biofilm growth while maintaining their potent remineralization capabilities, with stable particle sizes within 200 nm³⁹. The earlier study uses neuroscience and biomechanical analysis to optimize attachment points for cable-driven soft

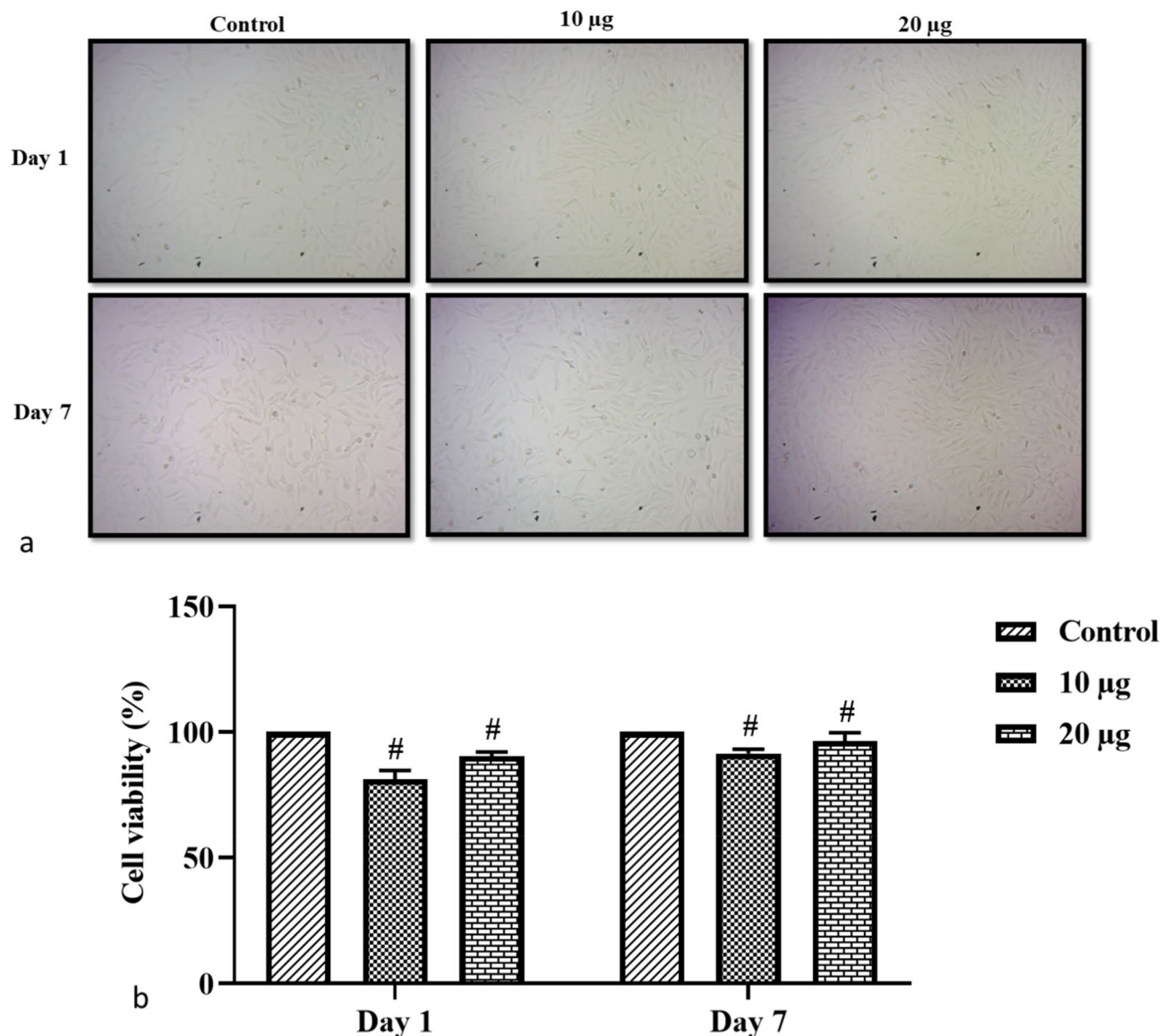


Fig. 9. MTT assay of MG-63 cells. Effect of ZTA NCs on MTT assay of osteoblast-like MG-63 cells on 1st and 7th day (a). Graphical representation of the viability of MG-63 cells (b).

exoskeletons, aiming for natural gait, energy efficiency, and muscle coordination control in human mobility and rehabilitation, contributing significantly to elderly rehabilitation⁴⁰.

Conclusion

In our study, we synthesized ZTA nanocomposite and characterized using UV-Vis spectroscopy, XRD, FT-IR, FE-SEM/EDAX, TEM/SAED, DLS, photoluminescence spectroscopy which showed hydrodynamic radius of 115 nm, nanoflakes structure, wurtzite hexagonal crystalline structure, photoluminescence spectrum at 361, 427, 481, and 483 nm and suggested that ZnO-TiO₂-Amygdalin nanocomposites surface matrix has better electrostatic interaction. Nanocomposites also exerted antimicrobial and fungal activity against several strains, which might be due to free radical generation potential and strong interaction with bacterial cell walls, and could penetrate cells, ultimately leading to cell death. Additionally, our data showed that nanocomposites enhanced the proliferation of osteoblastic-like cells (MG-63), stimulated the levels of ALP, and enhanced calcium mineralization in MG-63 cells, which suggested that nanocomposites effectively protected, proliferated, and stimulated osteoblastic differentiation. Nevertheless, further investigation on the role of nanocomposites in bone regenerating mechanisms in MG-63 cells and in vivo models is highly warranted.

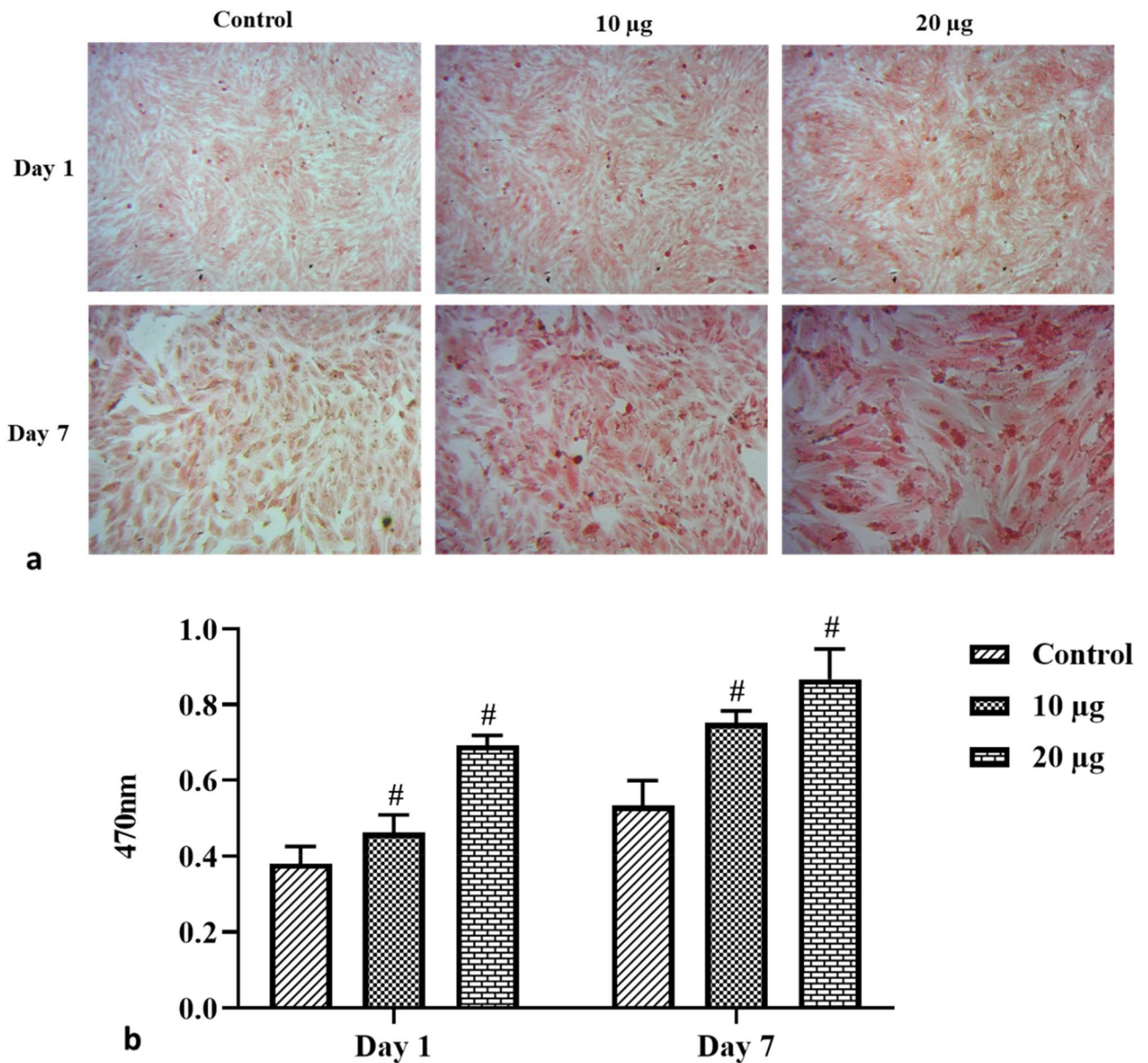


Fig. 10. Effect of ZnO-TiO₂-Amygdalin nanocomposites on calcium mineralization in MG-63 cells using ARS assay (a) showing the graphical representation of calcium mineralization rate on the nanocomposite-treated osteoblast-like MG-63 cells (b).

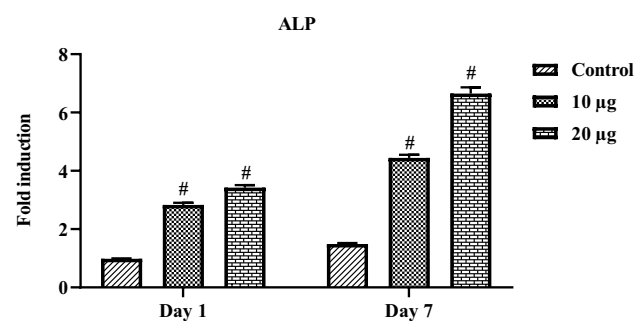


Fig. 11. ALP activity in cells. 10 and 20 µg of ZnO-TiO₂-Amygdalin nanocomposite enhanced alkaline phosphatase in MG-63 cells on 1st and 7th day.

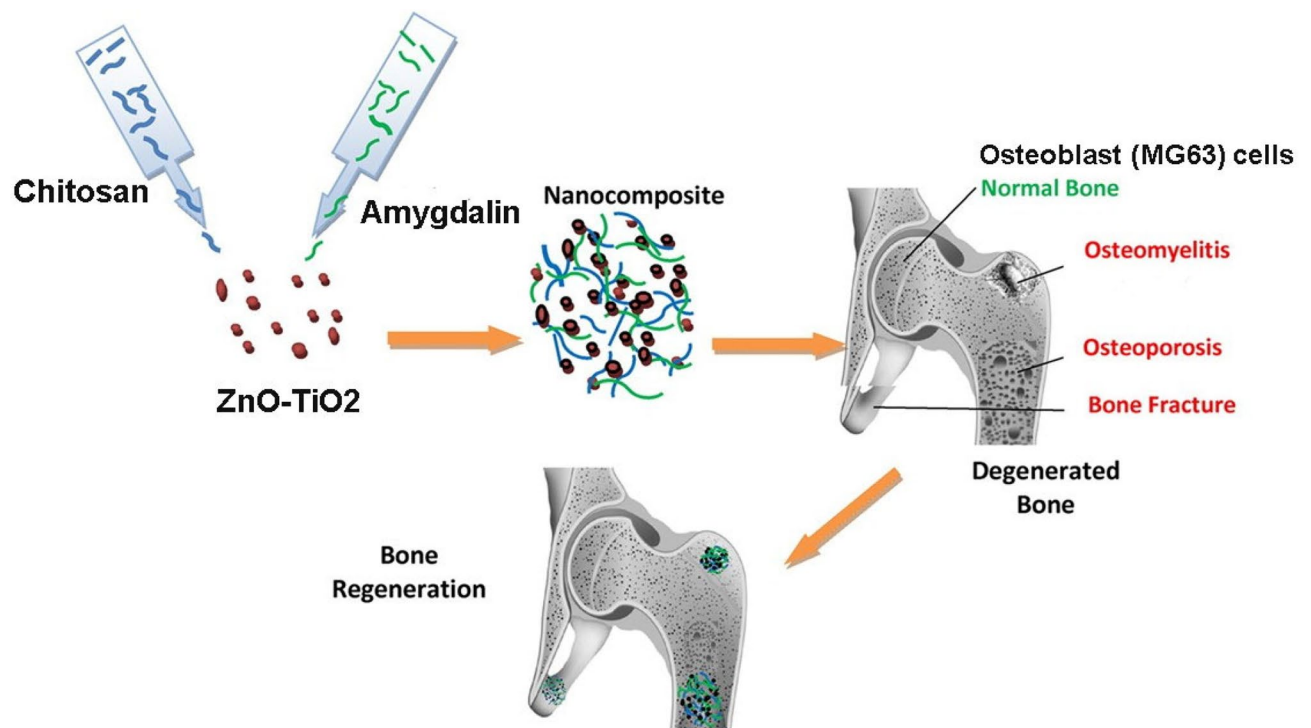


Fig. 12. The schematic representation of the synthesis of ZnO-TiO₂-Amygdalin NCs and its bio application.

Data availability

The corresponding author will provide data supporting the study's findings upon reasonable request.

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Author contributions

Shenghua Guo, Yanchao Cui: Designing and performing the work, Zhi Zhang, Lulu Cao: data collecting, assistance with revising the manuscript, Tao Wu, Binglun Li: interpreting the data and writing the paper.

Declarations

Competing interests

The authors declare no competing interests.

Ethical statement

The study described in this publication was done with the agreement of the institution's animal ethics committee.

Additional information

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