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DEVELOPMENT AND CHARACTERIZATION OF POLY(METHYL METHACRYLATE)/HYDROXYAPATITE BIOCOMPOSITE TREATED WITH AN ANTIMICROBIAL AGENT AS A BONE ANALOGUE MATERIAL

RAZVOJ IN KARAKTERIZACIJA BIO KOMPOZITA IZ POLIMETIL METAKRILATA IN HIDROKSIAPATITA, OBDELANEGA Z ANTIMIKROBNIM SREDSTVOM, UPORABNEGA ZA ORTOPEDSKE REKONSTRUKCIJE KOSTI

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Bones play a vital role in physical activities and protection of organs. Small bone injuries normally heal as a result of bone's inherent ability to repair damaged tissues. However, larger bone defects continue to pose a challenge to clinicians. In the field of bone tissue engineering, biocomposite materials that stimulate and promote the regeneration of broken-bone tissue have become a focus of research, providing temporary artificial environments, specifically designed to support bone growth. This research aims to fabricate a biocomposite material of PMMA and hydroxyapatite (HA) combined with various concentrations of curcumin and EGDMA used for bone tissue scaffold engineering. A characterization of fabricated samples was performed using X-ray diffraction (XRD), field-emission scanning electron microscopy (FESEM), atomic force microscopy (AFM), as well as the measurements of their mechanical properties including the tensile fracture and compressive strength. In addition, two of the most common bacteria that cause osteomyelitis, *Staphylococcus aureus* and *Escherichia coli*, were tested with an agar diffusion assay to develop antibacterial biomaterials capable of effectively treating and preventing osteomyelitis. Our results strongly indicate that the integration of HA and curcumin nanoparticles into the PMMA polymer matrix with reinforced EGDMA for the fabrication of biocomposite scaffolds offers promising potential for bone tissue applications, possibly increasing the performance and effectiveness of such scaffolds. This composite (PMMA/HA/curcumin) exhibits excellent antibacterial properties in addition to its mechanical and morphological properties, thus having the potential to substitute bone tissue.

Keywords: bone tissue engineering, biomaterial, PMMA, EGDMA, hydroxyapatite (HA), curcumin

Kosti igrajo vitalno vlogo pri človekovi fizični aktivnosti in zaščiti organov. Majhne poškodbe kosti se normalno pozdravijo same zaradi samosvoje naravne sposobnosti ozdravitve poškodovanega tkiva. Vendar pa so večje poškodbe kosti zahteven izziv za zdravnike. Na področju inženiringa kostnih tkiv biokompozitni materiali lahko stimulirajo in pospešijo regeneracijo zlomljenih kostnih tkiv. Zato je to postal glavni fokus najnovejših raziskav na področju, ki zagotavlja začasno specifično oblikovano (dizajnirano) umetno okolje v katerem se pospešuje rast oziroma celjenje kostnih tkiv. V tem članku avtorji opisujejo izdelavo biokompozitnega materiala iz polimetil-metakrilata (PMMA) in hidroksiapatita (HA) kombiniranega z različnimi koncentracijami kurkumina in etilen-glicol dimetakrilata (EGDMA) kot podpora inženiringu kostnih tkiv. Avtorji so izdelane vzorce okarakterizirali z uporabo rentgenske difrakcije (XRD), vrstične elektronske mikroskopije na emisijo polja (FESEM) in mikroskopije na atomsko silo (AFM). Določili so tudi mehanske lastnosti vzorcev (natezno in tlačno trdnost ter trdoto). Dodatno so avtorji izvedli teste na dve najbolj pogosti bakteriji, ki povzročata osteomielitis (Staphylococcus aureus in Escherichia coli). Za to so uporabili difuzijsko infiltracijo z želatinsko snovjo agar in s tem razvili antibakterijski biomaterial, ki učinkovito preprečuje in tudi zdravi osteomielitis. Rezultati raziskave so dokazali, da izdelava bikompozitnih skeletov s kombinacijo HA in nanodelcev kurkumina v PMMA polimerni matrici ojačani z EGDMA ponuja obetajoče se področje uporabe za zdravljenje poškodovanih kosti. Pri tem je še potreben nadaljnji napredek pri izboljšanju kvalitete in učinkovitosti te vrste kompozitnih skeletov. Ta kompozit (PMMA/HA/Curcumin) ima odlične antibakterijske, morfološke in mehanske lastnosti in kot tak lahko služi kot potencialna zamenjava za poškodovane kosti.

Ključne besede: inženiring kostnih tkiv, biomaterial, polimetil-metakrilat, etilen-glicol dimetakrilat, hidroksiapatit $Ca_5(PO_4)_3(OH)$, kurkumin

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1 INTRODUCTION

Bones play a vital role in the functioning of the human body, providing structural support and physical protection for vital organs as well as a number of other functions. In addition, they can change and adapt to pressure or mechanical forces.[1] Infections, tumors, and trauma are most commonly responsible for bone defects. Recent advances in tissue engineering have been used to evaluate alternative scaffolding materials that combine biological and engineering characteristics to fabricate short-term artificial environments specifically designed to promote the growth of bone tissue.[2] As a treatment option for severe bone defects, bone tissue engineering holds immense potential for addressing the problem, while avoiding the effects of complications associated with conventional allogeneic or autologous bone replacements.[3] A variety of calcium phosphate bioceramics, including hydroxyapatite (HA) nanoparticles, have been mainly used to replace bones in orthopedics and dentistry. Additionally, polymers can be used for hard tissue prostheses that carry load due to their physical properties, their ability to form strong structures, inertness and excellent stability in body fluids.[4] Among the many applications of poly(methyl methacrylate) (PMMA), bone cement is commonly used in total joint replacement surgery as well as bone fillers and bone substitutes, due to its affordability, biocompatibility, and processability. Although PMMA exhibits a high level of bone regeneration efficiency, it is limited by its low level of bioactivity and poor osseointegration.[5] As an osteoconductive material, HA shows a porosity matching the natural bone, stimulating bone tissue growth within cavities or on their surfaces. When HA degradation occurs, phosphate and calcium ions are released, contributing to osteoinductive and osteogenic activities.[6]

It has been reported that synthetic polymer matrices, such as PMMA, can be functionalized by incorporating bioactive nanoparticles, such as HA, which may improve the properties of hybrid matrices.[7–9] Additionally, HA scaffolds are brittle and weak, which makes them highly susceptible to fractures. HA-based samples have been effectively hindered from being used for these applications due to their low strength and brittle nature.[10] PMMA biomaterial is widely considered one of the most sensitive to microbiological contamination. It also exhibits a considerable percentage of pores and it is considered to be an inert material, unlikely to stimulate anchoring of bones to PMMA bone cements, as well as being prone to contamination by bacteria.[11,12] The perennial herb turmeric contains curcumin, which has received considerable attention, has been shown to be beneficial in treating a wide range of tumors.

A number of studies have also indicated that curcumin is capable of inhibiting osteosarcoma cell proliferation in vitro and treating bone defects in vitro. Thus, curcumin may be promising for treating osteosarcoma.[13] In vitro and in vivo tests showed that curcumin enhanced the bone mineral density and bone structure. Besides exhibiting anti-inflammatory properties, curcuma longa is also an antioxidant. In addition, curcumin has antibacterial properties, and studies have been conducted to determine the effects of a curcumin-containing bone cement on a variety of bacteria.[14–17] As a result, the primary focus of bone scaffold research has shifted from achieving the required level of biocompatibility to improving the mechanical characteristics, adding osteoinductivity to the scaffold, and building a porous structure with low density and antimicrobial properties. Ethylene glycol dimethacrylate (EGDMA) acts as a cross-linking agent; it is incorporated into PMMA to enhance the material's crack resistance; its greatest advantage is its resistance to small surface cracks, as reported in previous studies.[18–20] This study investigates the ability of HA, polymethyl methacrylate (PMMA) and EGDMA, combined with curcumin, to form biocomposite materials. This is done by synthesizing a biocomposite and determining its structural and morphological characteristics. In addition to the mechanical properties of the biocomposite, the antibacterial properties are also investigated as they would allow its application in bone tissue engineering.

2 MATERIALS AND METHODS

2.1 Materials

The biocomposite was fabricated from self-curing acrylic resin (PMMA powder, Maarc dental, India), mixed with methyl methacrylate monorner (MMA) (Maarc dental, India) and ethylene glycol dimethacrylate (EGDMA) as the crosslinking agents (Sigma-Aldrich, USA). HA nanoparticles of APS 20 nm, with a nodular structure, 96 % purity and 502.31 molar mass were supplied by MK Nano, Toronto, Canada. Curcumin was purchased from Fluka (Switzerland, diferuloylmethane, Natural Yellow 3, molecular formula: $IC_{21}H_{20}O_6$).

2.2 Methods

A ball mill was used to mix different amounts of PMMA, HA, and curcumin over ten minutes to produce composites of different weight fractions. Smooth mixtures were achieved, without any conglomerates. **Table 1** illustrates the percentage composition of each sample. To prepare the samples, each composite material was dissolved in a liquid solvent (MMA, EGDMA) in a ratio of 2:1. Once a mixture was pasty, it was placed in a silicone mold specially designed for this study. The process of sample fabrication is illustrated in **Figure 1**. As the final stage of fabrication, hardening or setting was conducted for at least 30 minutes at room temperature. The samples were ready for characterization five days after their fabrication.

Table 1: Sample compositions

Group	Composition (w/%)			Liquid content	
	Powder content				
	PMMA	HA	Curcumin	MMA	EGDMA
S1	100 %	0 %	0 %	100 %	0 %
S2	80 %	20 %	0 %	95 %	5 %
S3	80 %	20 %	0 %	90 %	10 %
S4	80 %	20 %	0 %	85 %	15 %
S5	77 %	20 %	3 %	95 %	5 %
S6	77 %	20 %	3 %	90 %	10 %
S7	77 %	20 %	3 %	85 %	15 %
S8	75 %	20 %	5 %	95 %	5 %
S9	75 %	20 %	5 %	90 %	10 %
S10	75 %	20 %	5 %	85 %	15 %
S11	60 %	40 %	0 %	95 %	5 %
S12	60 %	40 %	0 %	90 %	10 %
S13	60 %	40 %	0 %	85 %	15 %
S14	57 %	40 %	3 %	95 %	5 %
S15	57 %	40 %	3 %	90 %	10 %
S16	57 %	40 %	3 %	85 %	15 %
S17	55 %	40 %	5 %	95 %	5 %
S18	55 %	40 %	5 %	90 %	10 %
S19	55 %	40 %	5 %	85 %	15 %

2.3 Characterizations of the fabricated samples

To determine the X-ray diffraction patterns of fabricated samples, an XRD unit (Haoyuan Instrument Co., Dx-2700BH) was used in a 2θ range of 5–80°. A field emission scanning electron microscope (FEI Quanta 450, USA) was employed to examine the morphology of biocomposite specimens. For accurate analysis and preservation of sample integrity, the samples were coated with a thin coating of gold under vacuum to prevent electrostatic charges and heat accumulation. The surface topography (surface roughness) was observed using an atomic force microscope (model TT-2 AFM). To evaluate bone and scaffold failure, tensile fracture strength is considered an important parameter. For the evaluation of the fracture strength of a sample, the diametrical compression test (Brazilian test) was used. Each sample was tested with an Instron Tinius Olsen (H50 KT). Tests were conducted at 0.5 mm/s at a constant speed rate. A crack occurred in the vertical direction, passing through the center of the sample. A compression force was gradually increased until the fracture was completed. This diametral tensile strength was calculated using the following equation:[9]

$$\sigma t = \frac{2F}{\pi Dt}$$

A cylindrical test specimen with a diameter of 1.2 cm and height of 2.5 cm was used to calculate the compressive strength of the composite material as shown in **Figure 2**. To calculate the compressive strength, the load was applied axially and the following equation was used:[21]

$$\sigma c = \frac{2F}{\pi(D/2)}$$

where:

σt – the tensile fracture strength

σc – the compressive strength

F – the load applied

D, t – the diameter and thickness of the sample, respectively

π – 3.14 (a constant)

2.4 Antibacterial activity

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*), representative of Gram-positive and Gram-negative pathogens involved in orthopedic infections, as reported in previous studies,[22,23] were tested for

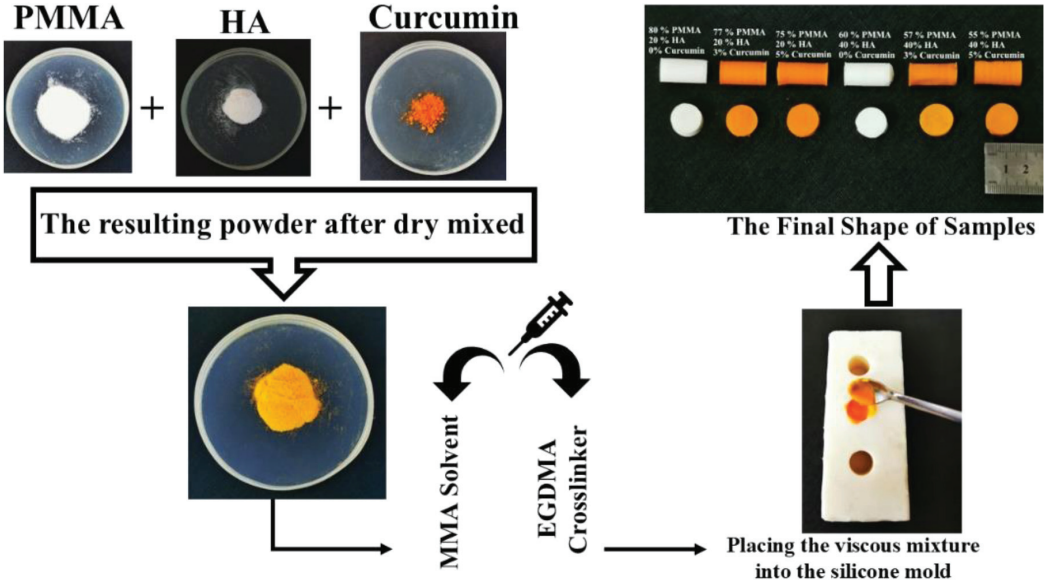

Figure 1: Schematic diagram of the fabrication of a 3D biocomposite scaffold made of PMMA, HA, and curcumin

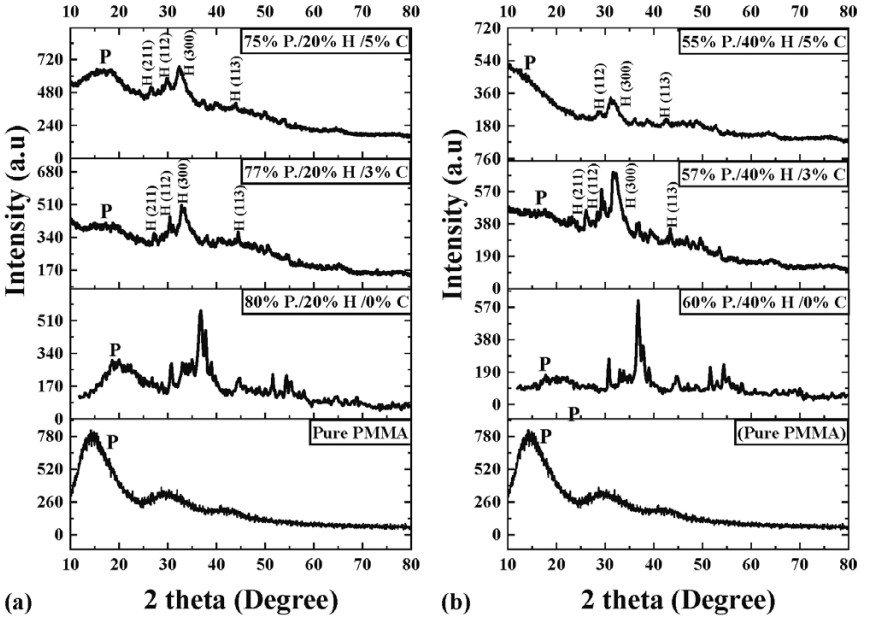

Figure 2: XRD spectra of biocomposite PMMA/HA/curcumin with: (a) 80 PMMA/20 HA, (b) 60 PMMA/40 HA; different P, H, C concentrations are assigned to PMMA, HA and curcumin, respectively

the antibacterial activity on the fabricated samples. By adding 38 grams of Muller-Hinton powder to 1 L of distilled water, then mixing and warming the mixture, one can prepare the Muller-Hinton (M-H) solution. It is necessary to autoclave M-H for 15 minutes at 121 °C for sterilization. After cooling it to 50 °C, it was poured into a petri dish and allowed to solidify for 15 minutes before being flipped upside down and kept at 4 °C in a freezer. Muller-Hinton (MH) agar was aseptically poured into sterile Petri dishes, and 20 mL of each prepared sample 2, 5, 8, 11, 14, 17 was applied to assess antibacterial activity against Gram-positive and Gram-negative bacteria. With a wire loop, the bacteria types were collected from their standardized cultivations. An agar plate was bored with 6 mm-diameter holes with a sterile tip after the cultivation. After incubating the experimental bacteria for 24 h at 37 °C, the mean diameter of inhibiting zones was determined and measured.

3 RESULTS AND DISCUSSION

3.1 XDR analysis

An XRD analysis was performed to further elucidate the structure of the biocomposite (PMMA/HA/Curcumin) and establish a better understanding of the phase structure of the fabricated samples. Based on the XRD patterns of selective samples, as shown in **Figure 2**, it can be seen that the diffraction peaks at 27.1802°, 30.3502°, 33.3957° and 44.5282° indicate the presence of HA phase in accordance with the standard data set (JCPDS No. 74-0566).[24] Since the PMMA/HA biocomposite contains a low concentration of curcumin, no curcumin peak appears in the diffraction pattern. There is an increase in the HA peak intensity when the curcumin content is increased, while in the 60 PMMA/40 HA group, this peak becomes less intense when the curcumin content is increased. This indicates that curcumin is evenly distributed within the matrix of the biocomposite scaffold. As a result, curcumin in the XRD pattern is dominated by its biocomposite structure, as reported in a previous study.[25] The results of the XRD analysis clearly show that the composite materials were mixed to a high degree of homogeneity, as evidenced by smooth peaks and phase stability. As a result, the additive particles are evenly distributed throughout the polymeric matrix.

3.2 Morphology observation

3.2.1 FE-SEM analysis

The FE-SEM technique was used for the analysis of sample surface morphologies and the outcomes are shown in **Figure 3**, which displays different weights of HA and curcumin along PMMA in the investigated samples. FE-SEM images demonstrate that HA particles are uniformly distributed on the composite surfaces, directly enhancing the mechanical properties of the biocomposite scaffolds. Furthermore, the HA particles adhere uniformly to the composite matrix, indicating a successful fabrication, as well as a high degree of adhesion of the HA particles to the composite matrix. A composite (55PMMA/40HA/5curcumin) induced fibrous structure can be observed in **Figure 3f** as well as a fibrous organization identical to that of natural bones. This fibrous structure indicates significant cell contact, making it more likely that the tissue adheres to itself and heals.[26] Based on morphological results, homogeneous arrangements of particles and fibrous structures are detected, which are potentially useful scaffolds for bone tissue engineering.

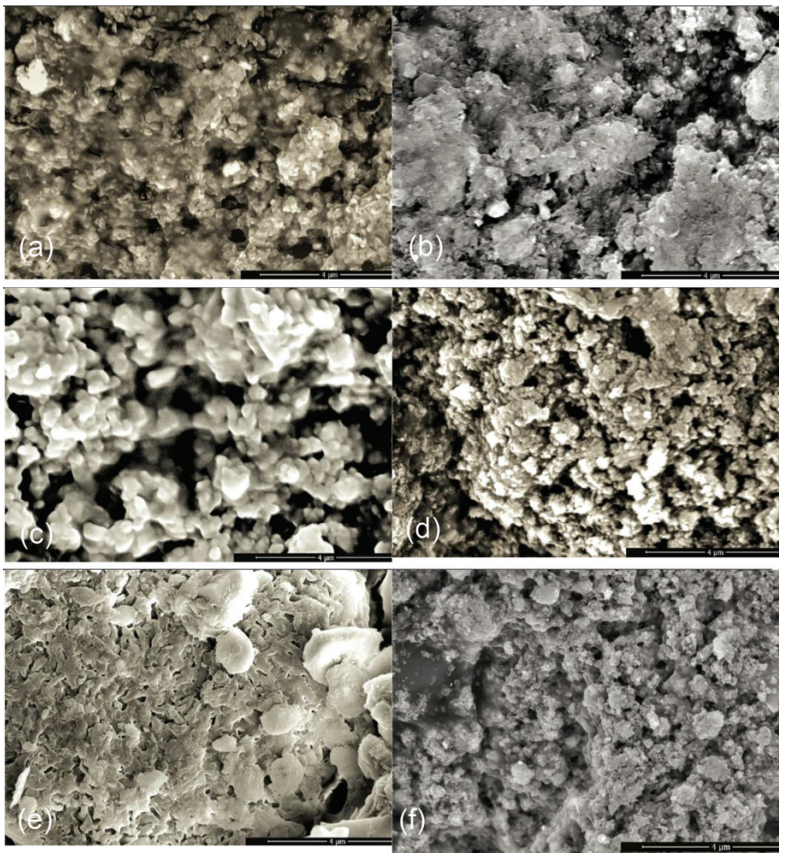

Figure 3: Typical FE-SEM images of the fabricated biocomposite: a) S2, b) S5, c) S8, d) S10, e) S14, f) S17

3.2.2 AFM analysis

AFM was used to examine the sample surface morphologies and identify surface characteristics. Microstructure arrangements of the PMMA and HA samples with a varying curcumin concentration are shown in **Figure 4**. Homogeneous distributions can be observed in the sample microstructures, and the interconnections between HA and curcumin, which can be seen in the PMMA pattern, contribute useful information for improving the mechanical characteristics of the material. As shown in **Table 2**, variations in the roughness of the sample surfaces with different compositions were found to be significant. The maximum surface roughness was found for 55PMMA/40HA/5curcumin, i.e., a roughness of 10.7 nm, while 77PMMA/20HA/3curcumin yielded a roughness of 5.55 nm. The increased surface roughness of cells was also linked to the increased differentiation and proliferation of cells.[27–29] Recruitment, proliferation, and differentiation of mesenchymal progenitors are required during bone formation. As part of the ideal scaffold for bone tissue engineering, growth factors should be present, in addition to the other factors that facilitate cell adhesion, differentiation, and migration.[30,31]

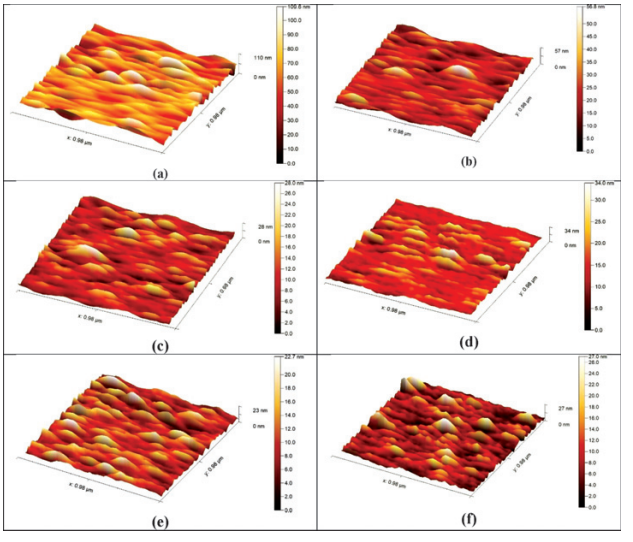

Figure 4: Surface characteristics of biocomposite PMMA/HA/curcumin, selected based on the AFM 3D surface structure: a) S2, b) S5, c) S8, d) S10, e) S14, f) S17

Table 2: AFM parameters varying with the sample surface roughness

Sample codes	Mean roughness (nm)	RMS (nm)	Maximum peak height (nm)
S2	4.66	5.76	66.8
S5	4.78	5.55	66.7
S8	4.88	5.98	71.4
S10	5.08	6.6	77.7
S14	6.4	8.9	108.66
S17	8.3	10.7	125.07

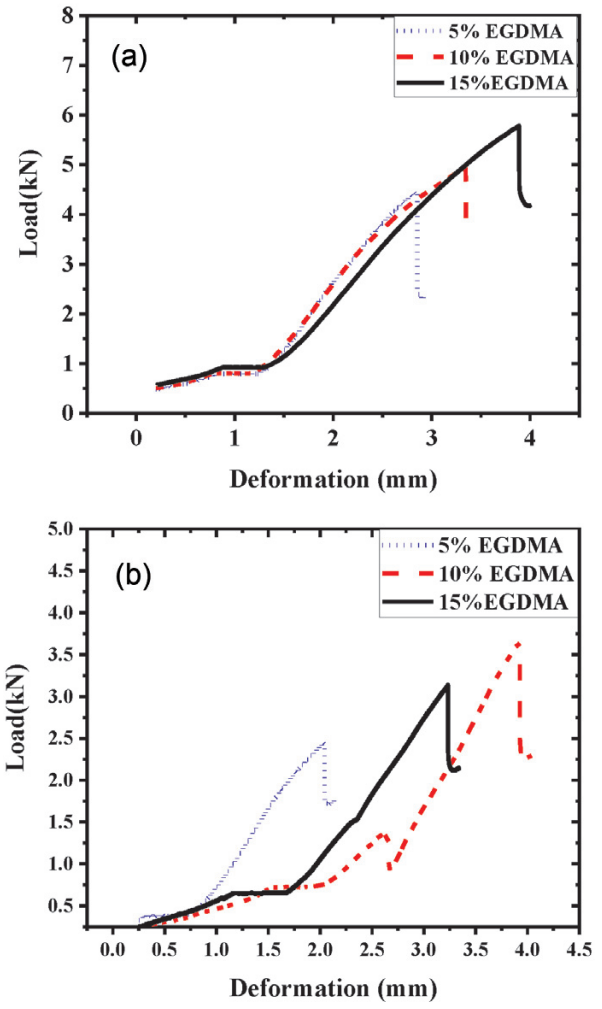

Figure 5: Load-deformation curves for tensile fracture: a) 20 % HA matrix, b) 40 % HA matrix with different concentrations of PMMA and EGDMA

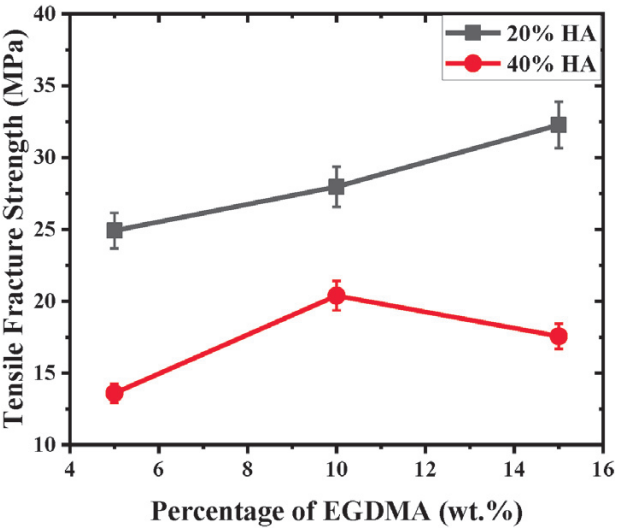

Figure 6: Variation in the tensile fracture against different percentages (*w*/%) of EGDMA for (20, 40) *w*/% HA

3.3 Mechanical characteristics

3.3.1 Tensile fracture and compressive strength

Brazilian tests (diametral compression tests) were used for the evaluation of the tensile fracture strength of the biocomposite. To assess the fracture strength of the samples, which is particularly important when biocomposite scaffolds are subjected to bearing loads (**Figure 5**), a comparison was made between the *w*/% of EGDMA and the tensile fracture strength (**Figure 6**), considering various weight percentages of PMMA and HA. As the *w*/% of EGDMA was increased, the fracture strength also increased. It was observed that within the 20 % HA sample group, the highest values were obtained under 32 MPa, and the minimum values were obtained under 24 MPa. In contrast, within the 40 % HA sample group, the highest values were determined for samples under 21 MPa, and the lowest values were measured for samples under 13 MPa.

Several previous investigations confirmed the beneficial effect of EGDMA on the mechanical properties of

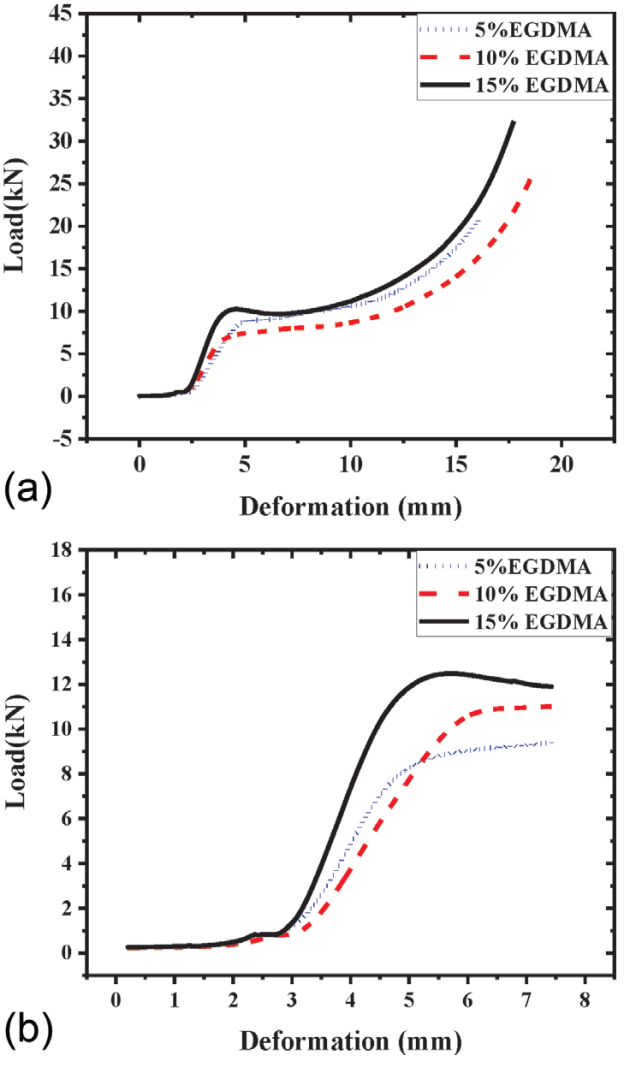

Figure 7: Load-deformation curves for compressive strength: a) 20 % HA matrix, and b) 40 % HA matrix with different concentrations of PMMA and EGDMA

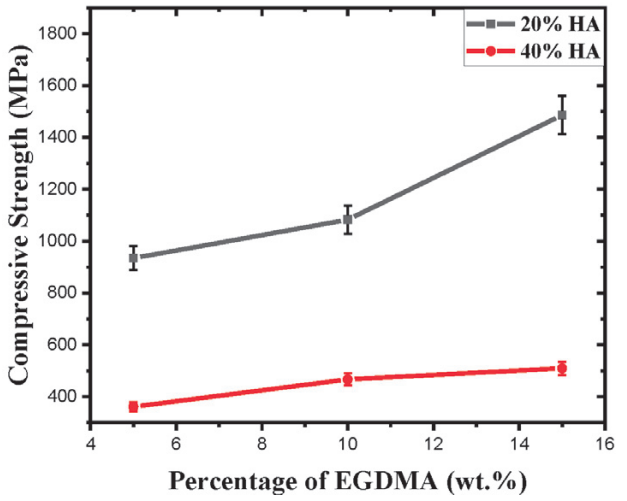

Figure 8: Variation in compressive strength against different percentages (*w*/%) of EGDMA for (20, 40) *w*/% HA

polymer composites.[18,19] In **Figures 7** and **8** the compressive strength properties of composites with and without EGDMA are shown. It was found that the incorporation of EGDMA into the system increased the compressive strength of composites. The tensile fracture and compressive strength were enhanced. Additionally, the high mechanical characteristics may be explained with the fact that the synthetic bone produced by the proposed system was employed for a long time, thus providing reasonable evidence of the material's biological stability and reduced degradation rate. Therefore, the material is ideal for bone replacement applications because of its high mechanical properties.

3.4 Antibacterial activity

The results revealed that an obvious antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* is observed for the prepared PMMA/HA/curcumin biocomposites, as shown in **Figure 9**. According to the inhibition zone, the greater the concentration of curcumin in the biocomposite, the greater the inhibition zone in the samples; therefore, the antimicrobial effects increase with the concentration. An additional quantitative antibacterial test carried out on 55PMMA/40HA/50curcumin showed that its rate of antibacterial activity against both *S. aureus* and *E. coli* was as high as that of the composite with 0 % curcumin. In this regard, curcumin can be viewed as an inexpensive component that can not only provide antimicrobial properties, but also enhance PMMA bioactivity when applied to bone regeneration. Furthermore, by increasing the curcumin percentage, both intercellular attachment and bone formation are enhanced, without compromising the bone quality, as reported in a reference.[14] Based on the results of this study, curcumin nanoparticles, combined with biocomposite may be able to control bacterial infections around artificial bone tissue. Our findings indicate that the composite material presented in this study is well-suited for bone tissue engineering due to its integrated multifunctionality, suggesting significant potential for clinical applications.

4 CONCLUSIONS

In this study, PMMA/HA/biocomposite samples containing different curcumin contents were fabricated using

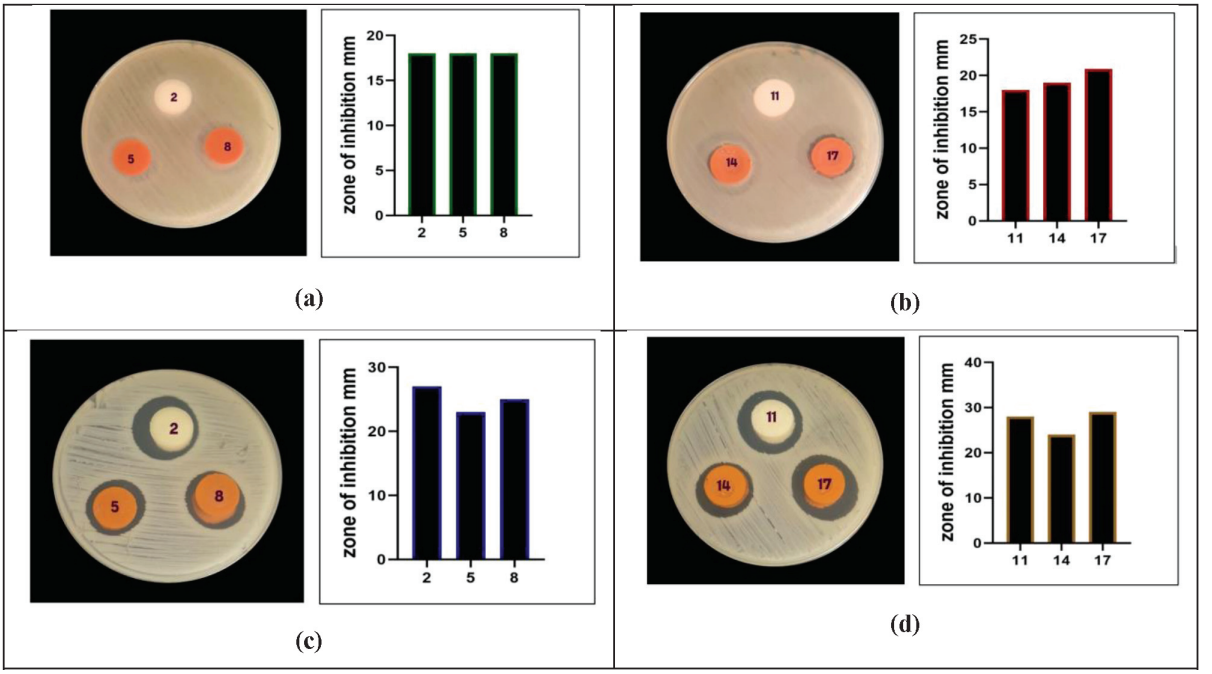

Figure 9: Inhibition zones of pristine PMMA/HA/curcumin biocomposites for the *Escherichia coli* test: a) S2, S5, S8; b) S11, S14, S17; and *Staphylococcus aureus* test: c) S2, S5, S8; d) S11, S14, S17

an MMA/EGDMA solvent. Their structural phases, morphological characteristics, and antibacterial properties were examined. According to the findings, the smooth peaks of the composite elements are indicative of homogeneous mixing. The FE-SEM morphological analysis confirmed that the components were uniformly distributed throughout the fibrous structure. The AFM analysis also revealed that the highest surface roughness (8.3 nm) was found in the 55%PMMA/40%HA/5%curcumin sample with 15 % EGDMA and 85 % MMA. Cell differentiation and proliferation are affected by the roughness of the sample surface. As a result of adding 10–15 % of EGDMA to the polymer networks and crosslinked composites, the compressive strength and tensile fracture strength of the biocomposite were increased. The biocomposite inhibited both gram-positive and gram-negative bacteria growth. Due to its excellent antibacterial properties, it effectively inhibited bacterial colonization, thus reducing the use of antibiotics. Therefore, this biocomposite is an appropriate candidate for bone grafting. We recommend that future studies include an assessment of its bioactivity using in vitro experiments. Furthermore, a significant amount of work remains to be conducted in vivo to assess the impact of the biocomposite on the bone cell activity, thus evaluating its ability to stimulate the regeneration of broken bone tissues.

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