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Original Article

Polymethylmethacrylate-hydroxyapatite antibacterial and antifungal activity against oral bacteria: An in vitro study

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الملخص

أهداف البحث: الحنك المشقوق هو عيب خلقي شائع يتطلب غالبا ترقيع العظام السنخية للحصول على علاج فعال. كان الهدف من هذه الدراسة هو دراسة الأنشطة المضادة للبكتيريا والفطريات لمختلط بولي ميثيل ميثاكريلات-هيدروكسيباتيت ضد الكائنات الحية الدقيقة في الفم. يمكن أن تفيد النتائج الاستخدام المحتمل لـ بولي ميثيل ميثاكريلات-هيدروكسيباتيت كمواد تطعيم عظمية اصطناعية لعلاج عيوب العظام السنخية في حالات الحنك المشقوق.

طريقة البحث: تم تحضير مسحوق هيدروكسيباتيت من مركز السيراميك في إندونيسيا وحبيبات بولي ميثيل ميثاكريلات من مختبرات هاي ميديا بنسبة 20:80 و30:70 و40:60. تم استخدام طريقة الانتشار المضاد للبكتيريا ضد المكورات العنقودية الذهبية، المشعشعة المصاحبة للورم الفطري، وحيدات الخلية البورفيرينية اللثوية و المغزلية الناخرة، في حين تم اختبار طريقة الانتشار المضاد للفطريات ضد المبيضات البيضاء. استخدمت بروتوكولات موحدة للزراعة الميكروبية. تم قياس مناطق التثبيط باستخدام الفرجار الرقمي. وشملت التحليلات الإحصائية اختبارات أنوفا و كروسكال-واليس أحادية الاتجاه، بالإضافة إلى اختبارات "تركي الفرق الكبير بصراحة" اللاحقة.

النتائج: أظهرت سقالة بولي ميثيل ميثاكريلات-هيدروكسيباتيت بنسبة 20:80 أعلى نشاط مضاد للجراثيم ضد المكورات العنقودية الذهبية، المشعشعة المصاحبة للورم الفطري، وحيدات الخلية البورفيرينية اللثوية و المغزلية الناخرة. وتلاها النسب 30:70 و40:60 من حيث النشاط المضاد للبكتيريا. تم تحقيق أهمية إحصائية بالمقارنة مع الضوابط. ومع ذلك، لم تظهر أي من نسب بولي ميثيل ميثاكريلات-هيدروكسيباتيت نشاطا مضادا للفطريات ضد المبيضات البيضاء.

الاستنتاجات: سقالات بولي ميثيل ميثاكريلات-هيدروكسيباتيت لها نشاط مضاد للجراثيم، ولكن ليس لها نشاط مضاد للفطريات.

الكلمات المفتاحية: مضاد للجراثيم؛ مضاد للفطريات؛ الحنك المشقوق؛ هيدروكسيباتيت؛ الدواء؛ بولي ميثيل ميثاكريلات

Abstract

Objective: Reconstruction of alveolar bone defects resulting from aging, trauma, ablative surgery or pathology, remains a significant clinical challenge. The objective of this study was to investigate the antibacterial and antifungal activities of mixed polymethylmethacrylate-hydroxyapatite (PMMA-HA) against oral microorganisms. Our findings could provide valuable insights into the prospective application of PMMA-HA as a synthetic bone graft material to manage alveolar bone defects *via* tissue engineering.

Methods: HA powder was obtained from the Center for Ceramics in Indonesia and PMMA granules were obtained from HiMedia Laboratories; these were prepared in 20:80, 30:70, and 40:60 ratios. The antibacterial diffusion method was then performed against *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum*, while the antifungal diffusion method was used to test against *Candida albicans*. Standardized protocols were used for microbial culturing and inhibition zones were measured with digital

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calipers. Statistical analyses included one-way ANOVA and Kruskal–Wallis tests, supplemented by post-hoc Tukey HSD tests.

Results: A PMMA-HA scaffold with a 20:80 ratio demonstrated the highest antibacterial activity against *S. aureus*, *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum*. This was followed by the 30:70 and 40:60 ratios in terms of antibacterial activity. Statistical significance was achieved with $p < 0.05$ in comparison to controls. However, none of the PMMA-HA ratios showed antifungal activity against *C. albicans*.

Conclusion: PMMA-HA scaffolds have significant activity against bacteria, but not against fungi.

Keywords: Alveolar bone defect; Antibacterial; Antifungal; Hydroxyapatite; Medicine; Polymethylmethacrylate

Introduction

Alveolar bone defects, a common concern due to aging, trauma, ablative surgery or pathology, often necessitate extensive treatment *via* alveolar bone grafting. These defects pose structural challenges within the oral cavity as individuals progress through the stages of growth and development.[1] The absence or inadequacy of alveolar bone integrity, primarily characterized by wide defects, can significantly impact the overall structural and functional aspects of the oral region.[2] Addressing these alveolar bone defects represents a crucial component of comprehensive care, aiming to ensure stability, proper dentition, and the restoration of oral function.

The field of tissue engineering plays a significant role in advancing science and technology and has garnered considerable attention from researchers worldwide, especially those in specialized scientific and research communities. The primary goal of tissue engineering is to restore and activate lost functionalities in malfunctioning body parts and, whenever possible, heal damaged tissue by replacing it with newly grown tissue.[3] Among several methods, autogenous bone grafts from the iliac crest or rib are prominent.[4] However, autografts are considered more invasive as they require a second surgical site and may cause local adverse effects following bone removal. Thus, alloplastic bone grafts have been developed to overcome the limitations of autografts.[5,6] Alloplastic grafts are synthetic bone grafts that mimic the biological properties of natural bone.[7] The primary advantages of using synthetic materials or alloplastic grafts are standardized product quality with no risk of infectious diseases and low antigenicity.[8]

One of the most extensively utilized alloplastic biomaterials for scaffolds is hydroxyapatite (HA). HA is a bioactive material similar to the natural apatite of human bones and teeth, making it biocompatible when interacting with body tissues. As a bone graft material, HA exhibits numerous key properties, including osteoinduction, osteoconduction, osseointegration, and osteogenesis.[9] However, HA has certain weaknesses, including brittleness and poor mechanical characteristics. The potency of HA can be increased by combination with a suitable polymer.[5] Polymethyl methacrylate (PMMA) is a low-toxicity polymer and has been used effectively as a scaffold material in tissue engineering due to its good mechanical stability and cellular adhesion.[10,11] PMMA is also a biocompatible material for human tissue.[12] PMMA, as a polymer, can be widely applied across a diverse range of applications within the field of prosthodontics, such as dental prosthetic purposes, encompassing its utilization in crafting artificial teeth, forming the foundations of dentures, producing complete dentures, fashioning obturators, creating orthodontic retainers, fashioning temporary or provisional crowns, and undertaking repairs of dental prosthetic devices.[13–15]

In a previous study, Saskianti et al. investigated a mixture of PMMA and HA with three composition ratios (20:80, 30:70, and 40:60) as an option for synthetic graft materials in cases with alveolar defects.[16] However, this research was limited to only biomaterial characteristics; no information was generated with regards to the antibacterial and antifungal activity of mixed PMMA-HA as a candidate scaffold material for graft therapy in the treatment of alveolar bone defects. A previous literature review concluded that from a histological point-of-view, new bone growth appears in the alloplastic graft material deposited on PMMA particles.[17] The antibacterial activity of PMMA was detected when combined with other materials such as hydrothermally synthesized anatase TiO_2 nanotubes.[18] Unfortunately, there is no literature relating to the antibacterial and antifungal activity of PMMA alone. Though promising in terms of tissue engineering, PMMA and HA constructs must be able to prevent failure due to bacterial and fungi-related infections since the outcome of alveolar bone grafting is known to be influenced by post-operative infection or inflammation.[19] Previous research has found that post-operative surgical site inflammation and infection are significant factors that contribute to a higher frequency of graft absorption, with a 30% failure rate. Research demonstrated that the typical abundant phyla responsible for these infections were Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Fusobacteria.[20] *Staphylococcus aureus* are bacteria belonging to the phylum Firmicutes and were found at significantly higher levels in patients with CLP than those without CLP. The increase in *S. aureus* occurred due to transmission from the nasal flora to the oral flora.[21] Other bacterial species that have been found to be abundant in subgingival CLP patients are *Aggregatibacter actinomycetemcomitans*, which belongs to the Proteobacteria phylum, and *Porphyromonas gingivalis*, which belongs to the Bacteroidetes phylum. The presence of *A. actinomycetemcomitans* bacteria can induce osseointegration failure due to virulence factors that hinder the tissue healing process.[22] Extensive research, both *in vivo* and *in vitro*, detected a correlation between *P. gingivalis* and increased virulence in other commensal bacteria.[20]

Fusobacterium nucleatum is a member of the Fusobacteria phylum and has the ability to inhibit cellular proliferation, increase the production of pro-inflammatory cytokines from osteoblasts, and reduce the production of pro-inflammatory cytokines from osteoblasts during osteoblast differentiation.[23] Research has also shown that *F. nucleatum* can increase the invasive potential of *P. gingivalis*.[24,25] *Candida albicans* has also been detected at higher levels in CLP patients than in healthy patients.[26] Considering that several bacterial and fungal species may exert deleterious effects on alveolar bone grafting, the aim of the present study was to investigate the antibacterial and antifungal activities of a combination of PMMA-HA against oral bacteria and fungi.

Materials and Methods

Preparation of PMMA-HA scaffolds[16]

In this experiment, we used HA powder obtained from limestone, one of the natural resources of Indonesia; this was sourced from The Center for Ceramics in Indonesia (Balai Besar Keramik Indonesia). The PMMA granules used in our experiments were obtained from HiMedia Laboratories in India. The PMMA-HA scaffolds were prepared in three different ratios: 20:80; 30:70; and 40:60. For the 20:80 ratio, 1 g of PMMA, 2 mL of acetone, and 4 g of HA powder were carefully weighed. Similarly, for a 30:70 ratio, 1.5 g of PMMA, 3 mL of acetone, and 3.5 g of HA powder were combined. For a 40:60 ratio, 2 g of PMMA, 4 mL of acetone, and 3 g of HA powder were combined. PMMA granules were placed in a container, mixed with acetone, and refrigerated at 0 °C for 24 h. Subsequently, HA powder was added to the PMMA solution, and the resulting mixture was stirred using a magnetic stirrer until homogeneity was achieved. The homogeneous mixture was then poured into a mold measuring 5 mm in diameter and 10 mm in height. Further processing involved the freeze-drying procedure.

The antibacterial diffusion method[27]

The culture and preparation of S. aureus

S. aureus (6538, UK) cultures were obtained from a stock using a sterile swab and incubated for 24 h at 37 °C under aerobic conditions in brain heart infusion (BHI) media. The cultures were then adjusted to conform to the well-established McFarland standard, typically denoted as 0.5 or 1.5×10^8 colony-forming units per milliliter (CFU/mL), thus representing a specific range of bacterial densities. If the optical density of the bacterial suspension deviated from the standard, then appropriate corrective measures were employed. Subsequently, the suspension was uniformly spread onto the surface of the nutrient agar medium.

The culture and preparation of A. actinomycetemcomitans

A. actinomycetemcomitans cultures (ATCC43718, UK) were obtained from a stock using a sterile swab and incubated for 24 h at 37 °C in an anaerobic environment using BHI media. The cultures were carefully adjusted to adhere to the McFarland standard of 0.5 or 1.5×10^8 CFU/mL. In cases where the cloudiness of the bacterial suspension did not align with the standard, appropriate dilution was performed.

The culture and preparation of P. gingivalis

P. gingivalis (ATCC33277, UK) cultures were cultured in BHI medium and incubated at 37 °C under anaerobic conditions for a duration of 18–24 h. Subsequently, bacterial colonies were carefully collected using a sterilized probe, heated with a Bunsen burner, and transferred to 3 mL of BHI liquid medium. The collected colonies were then cultured for an additional 18 h at 37 °C. The resulting bacterial suspension was then adjusted to attain a bacterial density of 0.5 or 1.5×10^8 CFU/mL using the well-recognized McFarland standard. The adjusted suspension was then collected with a micropipette and evenly distributed on the surface of nutrient agar media.

The culture and preparation of F. nucleatum

F. nucleatum (ATCC25586, UK) cultures were grown in tryptic soy broth (TSB) medium and incubated under anaerobic conditions at 37 °C for 18–24 h. The resulting bacterial colonies were aseptically collected using a Bunsen burner-heated loop and transferred to 3 mL of liquid BHI medium. The collected colonies were further cultured at 37 °C for 18 h. The resulting bacterial suspension was then adjusted to conform to the standardized McFarland standard of 0.5 or 1.5×10^8 CFU/mL. Subsequently, the adjusted suspension was uniformly spread onto the surface of a nutrient agar medium.

The culture and preparation of C. albicans

C. albicans (10231, UK) was inoculated into a test tube containing liquid Sabouraud dextrose broth (SDB) medium obtained from the stock culture. The inoculated tube was then incubated at 37 °C for 24 h. Subsequently, the turbidity of the culture was assessed to ensure that it adhered to the standardized 0.5 McFarland value (1.5×10^8 CFU/mL). Using the spreading technique, the *C. albicans* fungus was transferred onto three petri dishes containing SDA medium. For each petri dish, six wells with a diameter of 5 mm and a spacing of 24 mm between their centers were carefully created.

Antibacterial and antifungal inhibition zone analysis

PMMA-HA scaffolds at ratios of 20:80, 30:70, and 40:60 (with each ratio replicated five times), showed inhibitory zones against *S. aureus*, *A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, and doxycycline as the positive control. However, no inhibition zone was observed for *C. albicans*. Inhibition zones were measured using a digital caliper (Mitutoyo, Japan) in millimeters and were calculated for each group.

Statistical analysis

The research data were subjected to descriptive and inferential analysis. Descriptive statistics, such as mean and standard deviation, were used to summarize the data, and a bar chart was used for data visualization. Prior to conducting inferential analysis, tests for normality and homogeneity

were conducted, with a significance level set at $p > 0.05$. One-way analysis of variance (ANOVA) was employed for normally distributed and homogeneous data, whereas the Kruskal–Wallis test was used for normally distributed, but non-homogeneous data. Subsequently, post-hoc Tukey honest significant difference (HSD) tests were conducted at a significance level of $p < 0.05$, utilizing the Statistical Package for Social Sciences (SPSS) version 25 for Mac (IBM Corporation, Chicago, IL, USA).

Results

In this study, we aimed to evaluate the antibacterial activity of PMMA-HA scaffolds with varying ratios (20:80, 30:70, and 40:60) and their antifungal efficacy against *C. albicans*. We also included a negative control and a positive control (doxycycline).

Table 1 presents the inhibition zone diameters (in mm) of PMMA-HA scaffolds against *S. aureus*, *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum*. Of these bacteria, *S. aureus* produced the largest inhibition zone in the doxycycline treatment group (28.85 mm), followed by the PMMA-HA scaffold at ratios of 20:80 (18.31 mm), 30:70 (14.44 mm), and 40:60 (10.89 mm) (Figure 1). With regards to *A. actinomycetemcomitans*, the 20:80 scaffold ratio demonstrated the highest inhibition zone (17.10 mm), outperforming the 30:70 (14.38 mm) and 40:60 (8.65 mm) ratios, while doxycycline produced the smallest inhibition zone (26.3 mm) (Figure 2). Similarly, *P. gingivalis* and *F. nucleatum* produced the largest inhibition zones with the 20:80 scaffold ratio (17.35 mm and 17.03 mm, respectively), followed by the 30:70 and 40:60 ratios, with doxycycline consistently yielding the largest inhibition zones (27.2 mm and 27.63 mm, respectively) (Figures 3 and 4).

With regards to antifungal activity, none of the PMMA-HA scaffold ratios exhibited antifungal activity against *C. albicans*, as evidenced by zero inhibition zones (Table 2). In contrast, the doxycycline-treated group exhibited significant antifungal activity, with an inhibition zone of 18.88 mm (Figure 5).

PMMA-HA scaffolds with a ratio of 20:80 demonstrated the highest antibacterial activity against the tested bacteria, although doxycycline remained the most effective antibacterial agent. None of the scaffold ratios exhibited antifungal activity against *C. albicans*, while doxycycline exhibited significant antifungal activity.

Table 1: Diameter of antibacterial inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *A. actinomycetemcomitans*, *S. aureus*, *P. gingivalis*, and *F. nucleatum*.

	20:80	30:70	40:60	Doxycycline	Control Negative
Staphylococcus aureus	18,31	14,44	10,89	28.85	0
Aggregatibacter actinomycetemcomitans	17,10	14,38	8,65	26.3	0
Porphyromonas gingivalis	17,35	14,45	8,39	27.2	0
Fusobacterium nucleatum	17,03	13,83	8,05	27.63	0

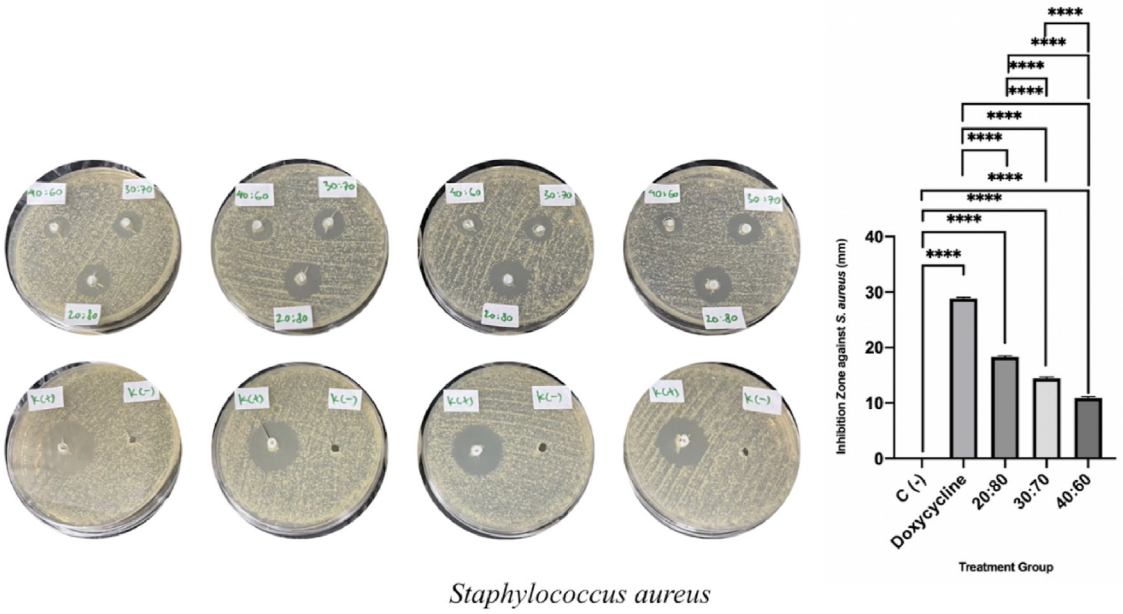
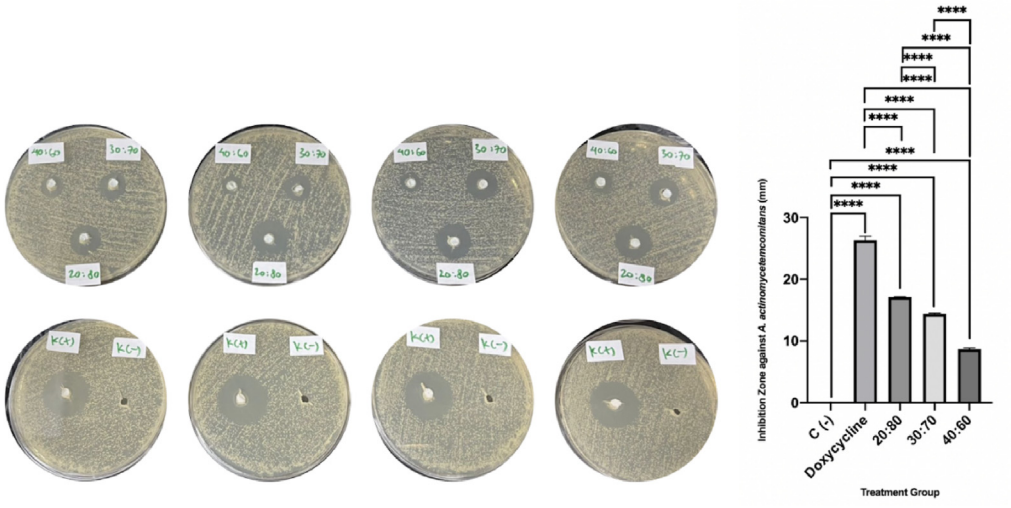
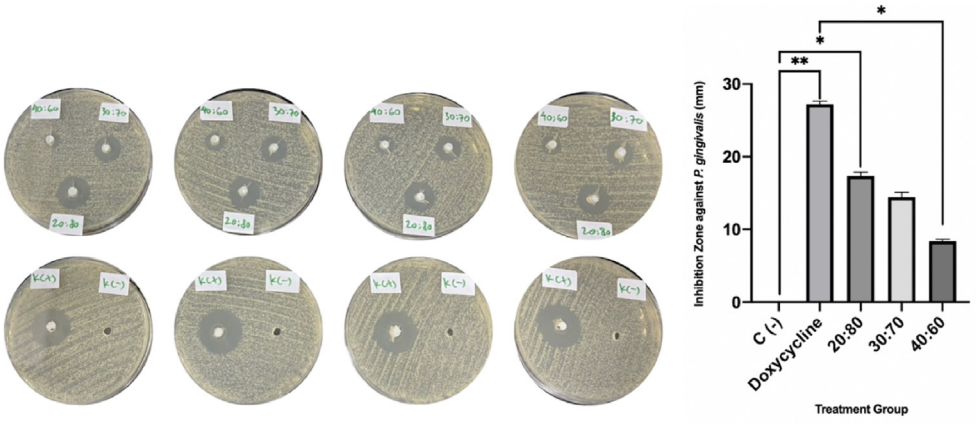


Figure 1: Antibacterial inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *S. aureus*.



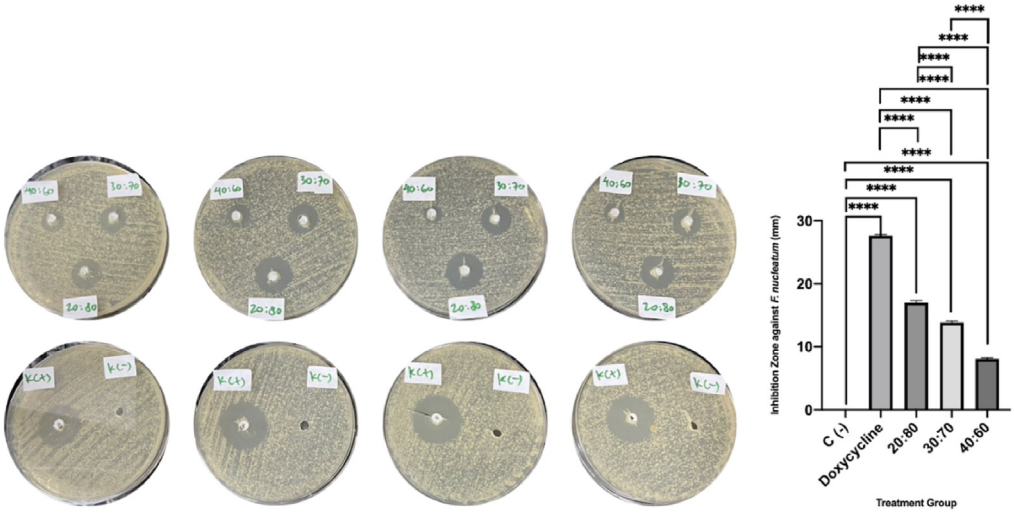
Aggregatibacter actinomycetemcomitans

Figure 2: Antibacterial inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *A. actinomycetemcomitans*.



Porphyromonas gingivalis

Figure 3: Antibacterial inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *P. gingivalis*.



Fusobacterium nucleatum

Figure 4: Antibacterial inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *F. nucleatum*.

Table 2: Diameter of the antifungal inhibition zones of PMMA-HA scaffolds (ratios of 20:80, 30:70, and 40:60) against *C. albicans*.

	20:80	30:70	40:60	Doxycycline	Negative control
Candida albicans	0	0	0	18,88	0

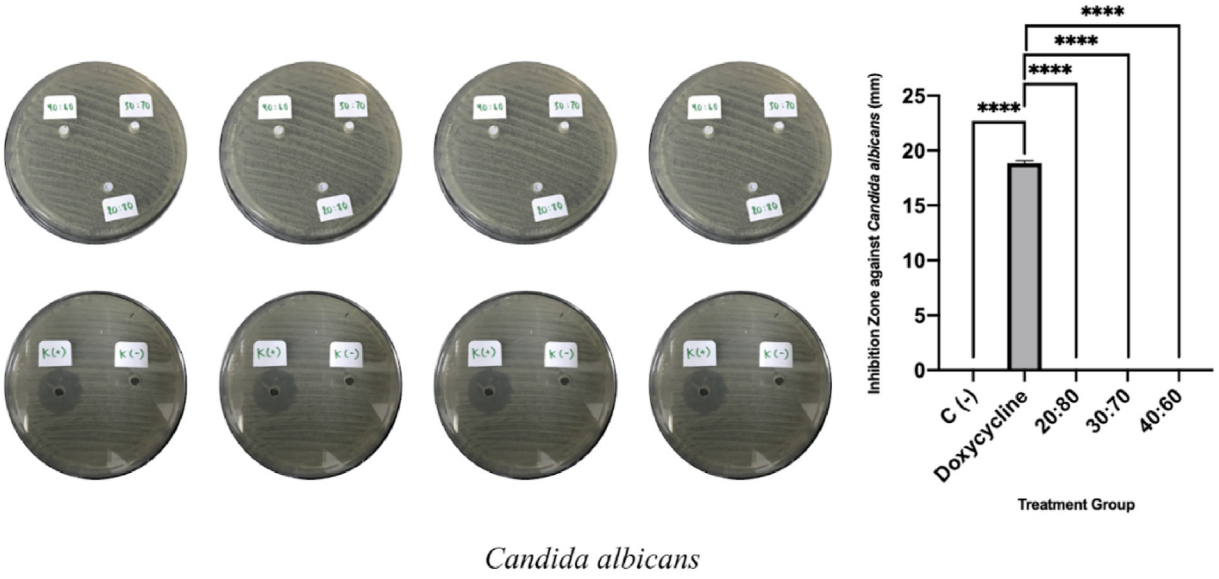


Figure 5: Antifungal inhibition zone of PMMA-HA scaffolds (20:80, 30:70, and 40:60 ratios) against *C. albicans*.

Discussion

Several bacterial and fungal species have become a significant problem for alveolar bone grafting, especially in terms of infection. In this study, PMMA and HA was prepared in three different composition ratios (20:80, 30:70, and 40:60) to analyze its antibacterial and antifungal activity. This experimental study confirms the hypothesis that the combination of PMMA and HA exerts antibacterial activity against *S. aureus, A. actinomycetemcomitans*, *P. gingivalis*, *F. nucleatum*, but not against *C. albicans* in comparison to the control group. This experimental work supports the concept that PMMA-HA can act as a suitable scaffold biomaterial for therapeutic use and exerts antibacterial and antifungal properties to prevent post-operative failure due to bacterial and fungi-related infection or inflammation.

As *S. aureus* is one of the most prevalent bacteria discovered in CLP neonates, an antibacterial test against *S. aureus* was mandatory in this investigation.[28] Our results demonstrated that the 20:80 ratio of PMMA-HA exhibited the highest antibacterial activity due to the highest HA content followed by 30:70 and 40:60, respectively; these results were supported by a previous study by Roy et al.[32] The active components produced from HA, including CaO, SiO_2, and Fe_2O_3 exhibit antibacterial activity against *S. aureus via* several pathways. For example, the interactions of CaO with tisol, amino, and carboxylic protein groups in the cell walls of *S. aureus* can cause destruction of the cell walls.[29] Furthermore, SiO_2 can enter *S. aureus via* its peptidoglycan pores and interact with its cell wall, thus causing damage to the bacterial cytoplasm.[30] In contrast to CaO and SiO_2, Fe_2O_3 increases the production of reactive oxygen species (ROS), which can cause polysaccharide depolymerization, DNA breakage, and the inactivation of bacterial enzymes in *S. aureus*.[31]

Antibacterial tests were also performed on gram-negative bacteria, including *A. actinomycetemcomitans*, *P. gingivalis*, and *F. nucleatum*. The antibacterial activities against these gram-negative bacteria were similar to those obtained for *S. aureus* in that the scaffold ratio composition of 20:80 exhibited the largest inhibition zone followed by the 30:70 and 40:60 ratios. The mechanism underlying the inhibition of activity in gram-negative bacteria was similar to that in gram-positive bacteria due to the active components contained in HA, including CaO, SiO_2, and Fe_2O_3.

The antibacterial action of HA was confirmed by Vijayaraghavan (2022), who demonstrated the strong bactericidal efficacy of hydroxyapatite doped silver nanoparticles (HAp-AgNPs) against both gram-negative and gram-positive bacteria.[32] The ionic state of Ag/HAs disrupts bacterial cell wall and forms bonds with RNA and DNA, thereby impeding bacterial replication. Another research study demonstrated that a HA-TiO_2 composite exhibited enhanced antibacterial activity against gram-positive bacteria due to plasmolysis of the cell walls or separation of the cytoplasm from the cell wall.[33] Unlike TiO_2, which does not generate an inhibitory zone in antibacterial tests, HA consistently produces an inhibitory zone. This discrepancy can be attributed to the bacteria-deactivating properties of HA relying upon an adsorption mechanism. It is widely considered that adherent bacteria are subsequently eliminated as a result of the composites disrupting their cell

membranes. The penetration of the microbial membrane by composite materials has been shown to contribute to such membrane disruption.[34]

In this study, we also investigated the antifungal activity of PMMA-HA against *C. albicans*, a fungus that is commonly found in the oral cavity. *C. albicans* infections are opportunistic and occur when the organism's environment in the body becomes conducive for growth and spread. A person's immune system is usually compromised following any operation, thus leaving them vulnerable to infection.[35] In this investigation, no inhibition zone was observed, thus indicating a clear lack of antifungal activity for PMMA-HA against *C. albicans*. No previous research has reported the antifungal activity of PMMA nor HA against *C. albicans*. This information is crucial for the future development of PMMA-HA as a biomaterial for bone grafts.

Although, we extensively investigated the antibacterial properties of PMMA-HA scaffolds against various bacteria and one species of fungi, this study still has limitations that need to be considered, including the limited scope of the antibacterial and antifungal testing applied in this study. Future studies, need to investigate the efficacy of this scaffold against a broader range of bacterial and fungal strains. In addition, our laboratory-based experiments may not fully represent the *in vivo* activity of the scaffold in the complex and dynamic oral environment. Further research, including *in vivo* studies and clinical trials, is now needed to validate the potential of this scaffold for clinical applications.

Conclusion

Scaffolds prepared from PMMA-HA with at rations of 20:80, 30:70, and 40:60 were demonstrated to exert antibacterial activity against *S. aureus*, *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* but did not exert antifungal activity against *C. albicans*.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

No ethical approval was required as this study did not involve human participants or laboratory animals.

Authors contributions

Conceptualization: TS. Methodology: TS, APN. Validation: TS, APN. Formal analysis: KKW, NF. Investigation: TS, KKW, NF Resources: TS. Data curation: TS, DSE, MK. Writing, Original Draft: KKW, NF. Writing, Review & Editing: TS, AMD, SW. Visualization: KKW, NF. Supervision: TS, AMD, SW, DSE. Project administration: TS. Funding acquisition: TS. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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