Kurdistan Journal of Applied Research (KJAR)
Print-ISSN: 2411-7684 | Electronic-ISSN: 2411-7706

Original Article

Assessment of the Antifungal Activity of PMMA-MgO and PMMA-Ag Nanocomposite

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Submitted: 8 May 2024
Revised: 17 May 2024
Accepted: 26 May 2024

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Keywords: PMMA, MgO, Ag, Nanoparticles, Antifungal property, Candida albicans.

How to cite this paper: A. N. Arf, F. A. kareem, Y. K. Khdir, and M. S. Zafar, "Assessment of the Antifungal Activity of PMMA-MgO and PMMA-Ag Nanocomposite, KJAR, vol. 9, no. 1, pp. 66–76, Jun. 2024, doi: 10.24017/science.2024.1.6.

Abstract: Orthodontic acrylic resin is used in the construction of orthodontic appliances. It lacks antimicrobial properties and is prone to microbial infection. So, the infection associated with it can be reduced via modification of orthodontic acrylic resin with nanoparticles (NPs) incorporation. The study directed to evaluate the antifungal properties of modified orthodontic acrylic resin incorporated with magnesium oxide (MgO)-NPs and silver (Ag)-NPs. NPs were mixed with polymethylmethacrylate (PMMA) in ethanol-assisted mixing method. Disc samples (10 mm in diameter and 2 mm thick) of PMMA-MgO, PMMA-Ag nanocomposites and PMMA alone (as control) were prepared. Then, *C. albicans* was isolated and identified clinically through taking swabs from acrylic denture base orthodontic appliances, cultured on a Sabouraud Dextrose Agar medium, followed by transferring on HiCrome™ candida Differential agar which is a selective and differential medium to distinguish distinct Candida species. The polymerase chain reaction was performed and the amplicon was separated by 2% gel electrophoresis and then visualised by ethidium bromide. DNA sequencing was performed on the sample at Sanger sequencing/ ABI 3500. Antifungal activity of PMMA-MgO and PMMA-Ag (1%, 3% and 5% of NPs) was conducted through disc diffusion assay and colony forming unit counts. The result showed a decrease in the number of adhered *Candida albicans* (*C. albicans*) of all concentrations of both nanocomposite and the decrease was statistically significant ($P<0.05$) in all experimental groups except MgO-NPs 1% and 3%. Increasing the concentration of NPs was associated with decrease in the adhered *C. albicans*. It was concluded that PMMA-MgO and PMMA-Ag nanocomposites showed anti-adherence activities against clinically isolated *C. albicans* in concentration dependent manner.

1. Introduction

The increasing demand for orthodontic treatments has created a greater necessity for utilizing orthodontic acrylic resins in the construction of removable orthodontic appliance and retainers [1]. One significant challenge associated with the use of these materials is the buildup of microorganisms on acrylic resins. Two contributing factors to this issue are difficulty of maintaining oral hygiene measures with orthodontic appliances and the presence of surface porosities in the appliances. These factors

result in the retention of residual food particles and microorganisms like *Streptococcus mutans* and *Candida albicans* (*C. albicans*) on the acrylic resins. The accumulation of these microorganisms increases the risk of developing cavities and oral diseases, posing a threat to the effectiveness of orthodontic treatments [2]. This is particularly crucial for self-cure acrylic resins, as they exhibit greater porosity compared to heat-cure acrylic resins [3].

Self-cure acrylic resins such as polymethyl methacrylate (PMMA) are commonly used in preparing removable prostheses, orthodontic retainers, and functional appliances [4,5]. In contrast to natural teeth, acrylic resin appliances attract microbial biofilm over a larger surface area, contributing to the emergence of cariogenic oral flora [6] and fungal infections in the oral cavity [7]. To combat this, antifungal medicaments are often added to these materials. *C. albicans* is identified as the most contagious among various candida species. The differing virulence levels among these species can be attributed to variations in the microorganisms' capability to attach to epithelial cells and denture surfaces compared to other candida species. The adhesion of candida to denture surface material is regarded as the initial stage of colonization [8].

Reducing biofilm or its growth on the oral acrylic appliance can be achieved via mechanical cleaning, especially with adjunctive antimicrobial solutions containing Nitradine, Cetylpyridinium chloride, or chlorhexidine. However, these kinds of adjunctive measures mainly depend on the patient's cooperation, which can be problematic in young children and subjects with disabilities [9]. The use of denture cleaning agents may result in the degradation of the denture base, resulting in a coarser surface and ultimately increasing the chances for candida attachment [10]. Hence, using preventive methods that do not need patient cooperation can be useful [11], such as the fabrication of orthodontic acrylic resin incorporated with antimicrobials [12].

Orthodontic stomatitis associated with orthodontic appliances can be avoided by halting the proliferation of *C. albicans* beneath the appliance by integrating antifungal medications into the appliance. Consequently, many studies investigated the incorporation of NPs' into removable oral acrylic materials [13].

Incorporating antimicrobial agents into dental materials has proven to be an effective method for enhancing their antimicrobial characteristics [14]. However, it must be ensured that this modification has no significant negative effect on the physical or mechanical properties [15]. Utilizing nanoscale materials is preferable for reducing the activity of the microorganisms due to an enhanced surface area for antimicrobial effectiveness, and possess a higher surface-to-volume ratio [16] which subsequently exert antimicrobial properties on a wide scale [17]. Studies have indicated that NPs exhibit superior physical, chemical, mechanical, and optical characteristics compared to microparticles. Consequently, they can be employed in the development of dental materials with enhanced mechanical properties and more effective antimicrobial effects [18].

The antimicrobial properties of Magnesium oxide nanoparticles (MgO-NPs) have been investigated for various biomedical applications [19] and a very little inherent risk of cellular toxicity [20]. Furthermore, the United States Food and Drug Administration regarded the MgO-NPs as a safe material for biomedical applications [21]. Similarly, silver nanoparticles (Ag-NPs) are known for their antimicrobial activity and can eliminate pathogenic microorganisms [22]. In addition to antimicrobial properties, Ag-NPs are nontoxic to humans [23] and have been investigated for various biomedical applications [24]. To the best of our knowledge, there are limited evidence suggesting that microorganisms can develop resistance to MgO-NPs and Ag-NPs. This study aims to develop a modified orthodontic acrylic resin with antifungal activity against clinically isolated *C. albicans* by incorporating MgO-NPs and Ag-NPs.

2. Materials and Methods

2.1. Materials

The materials used in this study are as follows:

- Clear orthocryl (cold cure acrylic) PMMA (powder: polymethylmethacrylate, REF 160-300-00 and liquid: methylmethacrylate, REF 161-150-00) (DENTAURUM. Ispringen, Germany).
- Ag-NP (20 nm, Spherical, 99.99%, metal basis CAS No.:7440-22-4, Hongwu International Group Ltd. Guangzhou, China).

- MgO-NP (99.9%, 10–30 nm, Sky Spring Nanomaterials, Inc. Houston, TX, USA).
- Absolute ethanol (CAS No: 64-17-5, Darmstadt, Germany).
- Sabouraud dextrose agar (SDA). HiMedia Laboratories Pvt. Ltd. Mumbai, India.
- HiCrome™ candida Differential agar HiMedia Laboratories Pvt. Ltd. Mumbai, India.
- Sabouraud dextrose Broth (SDB). HiMedia Laboratories Pvt. Ltd. Mumbai, India.
- Mueller Hinton agar (MHA). HiMedia Laboratories Pvt. Ltd. Mumbai, India.

2.2. Methods

C. albicans was isolated and identified clinically. The antifungal activity of PMMA-MgO and PMMA-Ag nanocomposite was then assessed using two methods which were agar disc diffusion assay and anti-adherence assessment through CFU counts, as described in the following sections.

2.2.1. Isolation and Identification of C. albicans

Swabs were collected from 10 acrylic denture base orthodontic appliances, cultured on a SDA medium and incubated at 37°C for 48 hrs. Cream-coloured, circular and convex colonies were detected and transferred on HiCrome™ candida Differential agar which is a selective and differential medium and incubated at 37°C for 48 hrs to distinguish distinct candida species based on colour (green colonies were *C. albicans*). The PCR was performed to amplify intergenic spacer regions (ITS) of the gene encoding 5.8 S rDNA using forward ITS1-F (5′- TCCGTAGGTGAACCTGCG-3′) and reverse ITS4-R (5′-TCCTCCGCTTATTGATATGC -3′) primers. The amplicon was separated by 2% gel electrophoresis and then visualised by ethidium bromide. DNA sequencing was performed on the sample at Sanger sequencing/ ABI 3500, Macrogen Genome Center, Republic of Korea and sequence manipulating was done by BioEdit software (V7.0.5.3) and NCBI database. The DNA sequencing for 5.8S rRNA gene by PCR confirmed the genetically identical *C. albicans* ATCC 10231 by 100% (Figure 1).

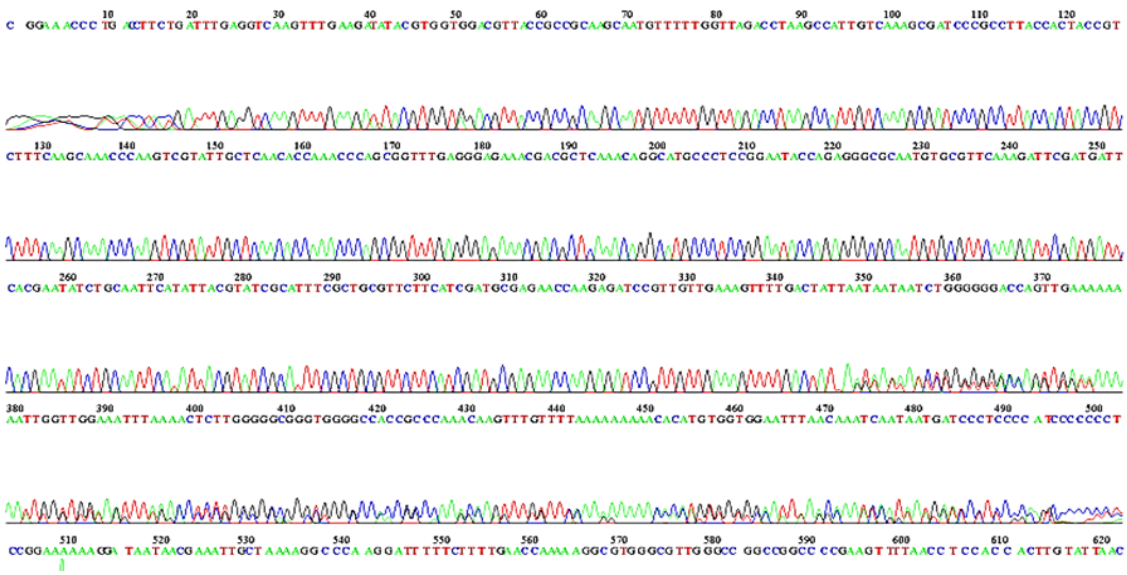

Figure 1: 5.8S rRNA gene sequence graph of the purified colonies of *C. albicans* done in Macrogen - South Korea.

2.2.2. Sample Preparation

MgO-NPs and Ag-NPs were mixed with PMMA powder using an ethanol-assisted procedure with the aid of 100% ethanol to form 1%, 3%, and 5% PMMA-MgO and PMMA-Ag nanocomposite, as shown in Table 1. A viscous solution was formed by adding determined amounts of each NP (using sensitive balance), which were dissolved in 10mL of 100% ethanol and sonicated for 5 minutes [25] using a UP100H ultrasonic processor (Hielscher Ultrasound Technology). After the ethanol had evaporated, the mixed powder was crushed with a mortar and pestle at a temperature of 50°C for two hours while

magnetic stirring was taking place at 400 rpm. To ensure that the solvent had evaporated, the powder was then put back in the oven for four hours at 50 °C [26].

Table 1: Percentages and amounts of PMMA powder, monomer and MgO-NPs and Ag-NPs.

MgO and Ag PMMA groups	MgO and Ag-NPs weight	PMMA powder weight	MMA liquid volume
1%	0.353 g	24.647 g	10 ml
3%	1.082 g	23.918 g	10 ml
5%	1.842 g	23.158 g	10 ml
Control	Zero	25 g	10 ml
Ethanol-cotrol	Zero	25 g	10 ml

MgO: Magnesium oxide, Ag: silver, PMMA: polymethylmethacrylate and MMA: Methylmethacrylate.

Using a stainless-steel mould (manufactured by Hongniu fiber laser cutting machine), three-disc samples (10 mm in diameter and 2 mm thick) were fabricated for each study group [27]. After the mold had been inserted between two glass pads, a cold mould seal separating media was applied to it. As directed by the manufacturer, a 2.5:1 ratio of PMMA powder to monomer was combined. Next, the discs were put into a 1-liter Bu¨ Chiglasuster polyclave reactor that was filled with water, and the polyclave was then allowed to sit at 50°C and 2.2 bar of pressure for 25 minutes [28] (Figure 2).

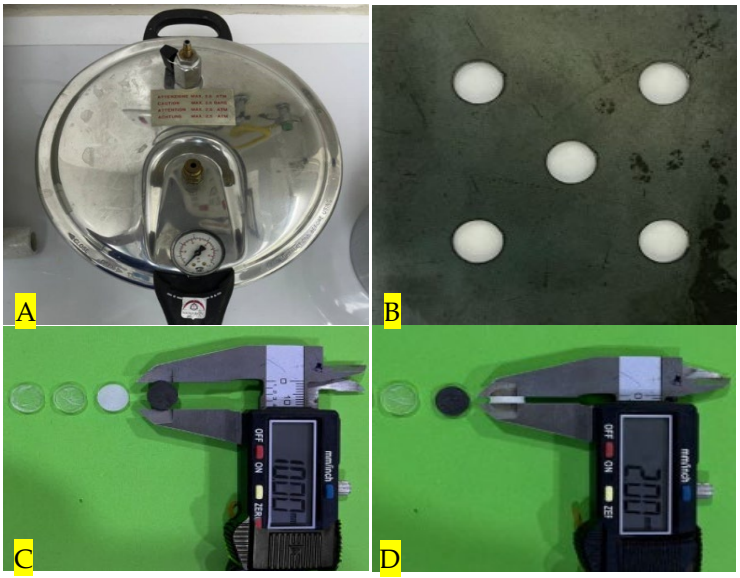

Figure 2: (A) Polyclave, (B) Stainless steel mould, (C) Diameter of disc, and (D) Thickness of disc.

2.2.3. Agar Disc Diffusion Assay for PMMA-MgO and PMMA-Ag Nanocomposite

Agar disc diffusion assay was used to check the antifungal activity of PMMA-MgO and PMMA-Ag nanocomposite [29]. MHA with aid of cotton buds used to prepare a petri plate, 50 µl broth stock suspension of 0.5 McFarland *C. albicans* was inoculated on MHA plates. The samples immersed for 20 mins in 70% alcohol and washed by sterilized distilled water before conducting the antifungal tests [30]. Later, the nanocomposite disc samples with controls (PMMA alone, ethanol-control and positive control which was filter paper infiltrated with 0.5% Clotrimazole) were placed onto inoculated MHA plates. After that, the agar plates were incubated for 48 hours at 37°C, and the sizes of the inhibition zones that formed around the specimens were measured using a digital caliper three times. Every assay was run in triplicate.

2.2.4. PMMA-MgO and PMMA-Ag Nanocomposite Anti-adherence Assessment through CFU Counts

Anti-adherence activity of the PMMA-Ag as well as PMMA-MgO nanocomposite was evaluated by colony forming unit (CFU) [27]. The grit sandpapers of 150, 300, and 600 were used to polish the disc specimens, and the excessive monomer was removed via storage of the specimens in distilled water at 37°C for 48 hrs. A profilometer (SRT 6200, Figure 3 A) and digital caliper were used to check the quality of polishing and measuring the specimens' size for standardization, respectively [31]. The discs with roughness between 2.7 and 3.1 were selected because a strong positive correlation was found between the surface roughness of the specimens and the amount of adhered *C. albicans*. Before the antifungal features were evaluated, the specimens were washed with sterile distilled water and submerged in 70% (v/v) alcohol for 20 minutes [30] to ensure that the samples are sterile and free from contaminants, thereby allowing for accurate and reliable testing. Subsequently, the samples were cultured for two days at 37°C in a 5 mL sterile test tube with 2 mL of SDB 0.5 McFarland *C. albicans* (Figure 3 B). The specimens were then removed, and three PBS washes (pH 7.2) were performed to eliminate any non-adherent cells. Later, the samples were transferred into 10 mL sterile tubes with 1 mL of SDB (Figure 3 C), and they were vortexed for 5 minutes at 3000 rpm. The accumulated fungal suspensions were cultured on SDA plates for 48 hours after being two-fold serially diluted eight times (Figure 3 D). The number of colonies and the dilution factor were used to compute the total CFU/mL on each disc. [32]. All experiments were carried out in triplicates.

Figure 3: (A) Profilometer, (B) 5 mL sterile test tube containing nanocomposite discs with 2mL of SDB 0.5 McFarland *C. albicans*, (C) 10mL sterile test tube containing nanocomposite discs with 1mL of SDB and (D) Plates inoculated with 8 times two-fold serial dilution.

2.2.5. Statistical Analysis

Mann-Whitney and Kruskal-Wallis tests were applied to determine the impacts of different types of NPs in PMMA-NPs nanocomposites on anti-adherence activity against clinically isolated *C. albicans*. SPSS statistical software (SPSS, Chicago, Il, USA, V.25) with the level of significance of $p \leq 0.05$ was used to carry out the statistical analysis.

3. Results

3.1. Agar Disc Diffusion Assay of PMMA-MgO and PMMA-Ag Nanocomposite

None of the examined concentrations (1%, 3% and 5% NPs) of PMMA-MgO and PMMA-Ag nanocomposite showed any inhibition zone against clinically isolated *C. albicans* (Figure 4).

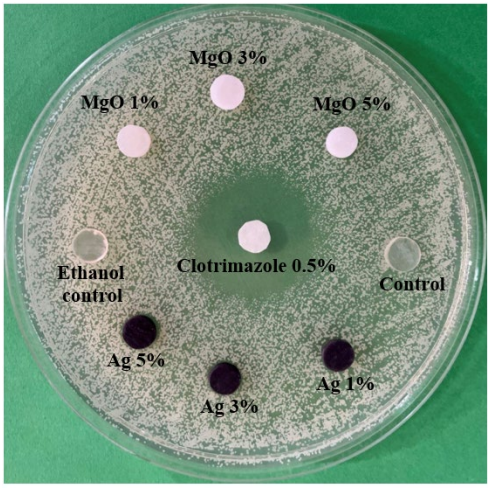

Figure 4: Agar nanocomposite discs diffusion assay.

3.2. PMMA-MgO and PMMA-Ag Nanocomposite Anti-adherence Assessment through CFU Counts

The anti-adherence activities of both PMMA-MgO and PMMA-Ag nanocomposites using 1%, 3%, and 5% concentrations were examined by CFU count (Figure 5).

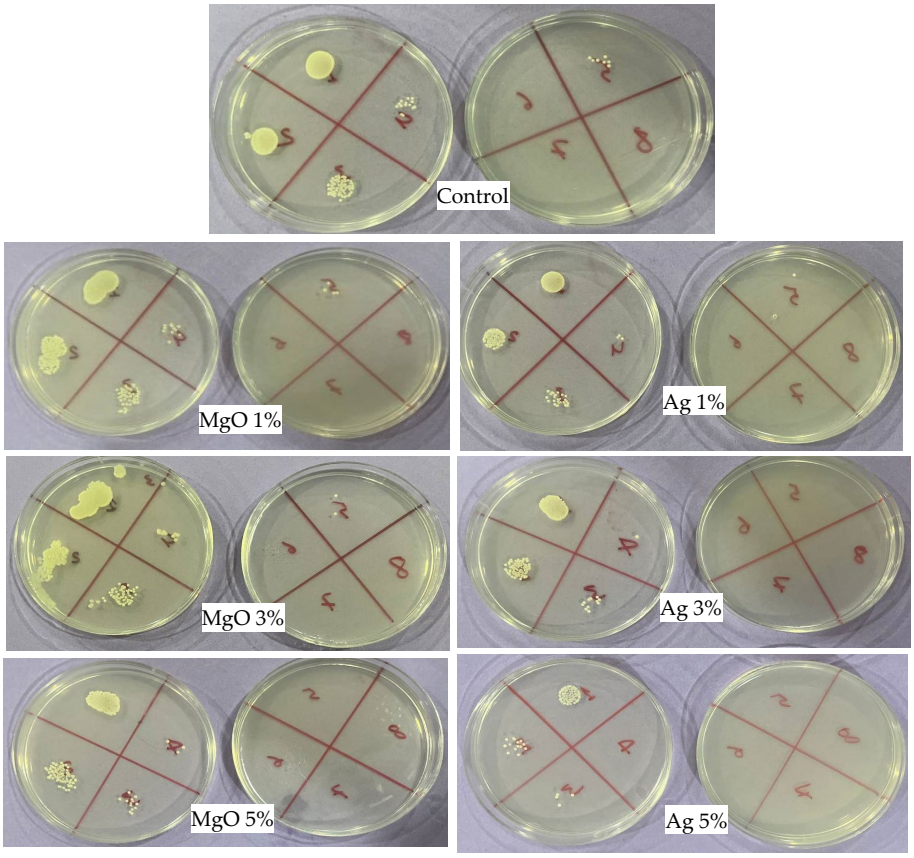

Figure 5: CFU/mL of control and tested groups on SDA.

The results showed a decrease in the number of adhered *C. albicans* between the control and the experimental groups, with all of the experimental groups showing a significant reduction of adhered

C. albicans except MgO 1% and 3%. Furthermore, increasing the concentration of NPs was associated with a decrease in the adhered *C. albicans*. Lastly, Ag-NPs showed better anti-adherence activities compared to MgO-NPs (Figure 6 and Table 2).

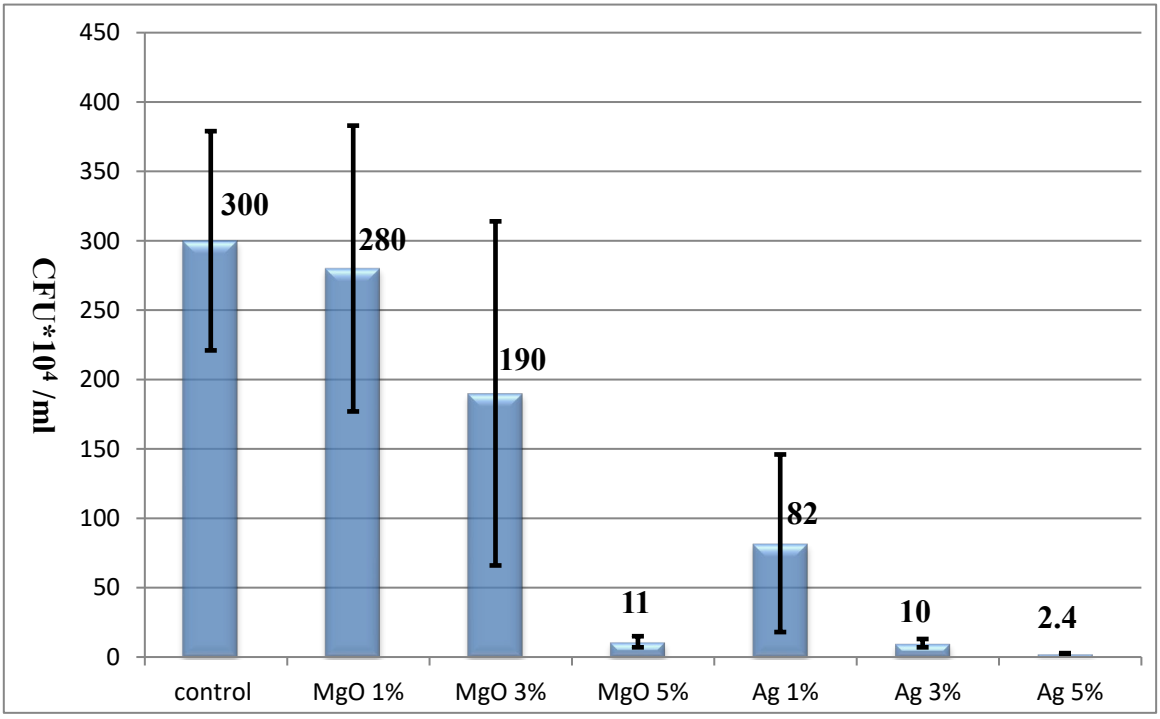

Figure 6: Mean and SD of CFU/mL of control and tested groups.

Table 2: Comparison between control and PMMA-NPs nanocomposite groups with different percentage of NPs and between PMMA-MgO and PMMA-Ag nanocomposite groups at the same concentration.

Tested groups (Mean ± SD (×104))		P value*	P value**
Control (300±79)	MgO1% (280±103)	0.527	**0.0001**
Control (300±79)	MgO3% (190±124)	0.058	
Control (300±79)	MgO5% (11±4)	**0.002**	
Control (300±79)	Ag 1% (82±64)	**0.002**	
Control (300±79)	Ag 3% (10±3)	**0.002**	
Control (300±79)	Ag 5% (2.4±0.4)	**0.002**	
MgO1% (280±103)	Ag 1% (82±64)	**0.002**	
MgO3% (190±124)	Ag 3% (10±3)	**0.002**	
MgO5% (11±4)	Ag 5% (2.4±0.4)	**0.002**	

SD: Standard deviation, MgO: Magnesium oxide, Ag: silver, NPs: Nanoparticles, *: Mann-Whitney, **: Kruskal-Wallis. The mean difference is significant at the 0.05 level.

4. Discussion

Self-cure acrylic resin is frequently used in the construction of temporary removable prosthetics, orthodontic devices, and the repair of fractured denture bases [33]. The oral cavity is a good habitat for various microorganisms, including bacteria, viruses, and fungi [34]. Intraoral devices such as acrylic dentures can act as a reservoir for microorganisms [35, 36].

Removable appliances in orthodontic treatment have shown to increase the colonization of candida species within the oral cavity. *C. albicans* is the predominant species observed in individuals with

removable orthodontic appliances [7] and it has been reported to be the causative pathogen for denture stomatitis (DS) [37]. To address DS, different techniques such as surface coating, immersion in denture cleansers, or antimicrobial filler incorporation have been examined [38–40], because a denture base exhibiting antifungal properties could serve as a preventive measure against DS and offer assistance to denture wearers facing challenges or disabilities in maintaining effective denture hygiene measures [41]. This study evaluated the antifungal and anti-adherence activity of PMMA-Ag and PMMA-MgO nanocomposite with 1%, 3%, and 5% NPs concentration against clinically isolated *C. albicans* through disc diffusion assays and CFU.

In the present study, MHA was used for disc diffusion assay because it has a relatively low agar content, which facilitates the uniform diffusion of antimicrobial agents through the agar medium [42]. None of the examined concentrations (1%, 3%, and 5% NPs) of PMMA-Ag and PMMA-MgO nanocomposite discs showed any inhibition zone against clinically isolated *C. albicans*, which is similar to the result of some other studies [29, 43, 44]. This result was due to the Ag-NPs not being released from the PMMA discs to enable the nanoparticles embedded in the PMMA to act as an agent to inhibit the growth of microorganisms on the surface of the discs. Therefore, finding the inhibition zones on agar plates is challenging. Meanwhile, the anti-adherence activities of PMMA-MgO and PMMA-Ag nanocomposites of 1%, 3%, and 5% concentrations showed a decrease in the number of adhered *C. albicans* for all concentrations of both nanocomposites. The decrease in *C. albicans* count in all experimental groups was statistically significant except for MgO 1% and 3%. Furthermore, increasing the concentration of NPs was associated with decrease in the adhered *C. albicans*. Lastly, Ag-NPs showed better anti-adherence activities compared to MgO-NPs due to the release of silver ions (Ag+), which can interact with microbial cell membranes, proteins and DNA.

The findings are commensurate with the result of the Altaee and Al-Ali study [45] which indicated that the control group, using pure acrylic (without the addition of MgO-NPs), exhibited the highest levels of *C. albicans* adhering to the material. However, the other three groups, incorporating MgO-NPs at the concentrations of 1.25%, 2.5%, and 5%, displayed a decline in *C. albicans* colonies attached to the acrylic specimens. Notably, the group with a 5% concentration showed the most substantial reduction (proven to be statistically significant), followed by the 2.5% concentration and then the 1.25% concentration. This trend suggests that antifungal efficacy increases by increasing the concentration of MgO-NPs.

Kanathila *et al.* [46], investigated the efficacy of various MgO-NPs concentrations (1%, 3%, 5% and 7%) when combined with tissue conditioners. This study reported an increased antifungal effectiveness increasing the MgO-NPs concentrations. These findings are in agreement with the results of the present study. Further, incorporating 3% of MgO-NPs into the denture soft liner notably enhanced its ability to combat *C. albicans* and *S. aureus* for a duration of up to six months [47]. On the other hand, soft liner with 0.1%, 0.2%, and 0.3% of MgO-NPs revealed a significant reduction in *C. albicans* CFU count, however, there was no inhibition zone around the specimens for disc diffusion assay, which is similar to the result of the present study [44].

The above results could be linked to the antimicrobial traits of MgO-NPs in PMMA nanocomposite influencing the enhancement of surface properties in PMMA resin. For instance, the surface roughness decreased from 2.08 μm to 1.7 μm after the addition of MgO-NPs [48]. In addition, electrostatic interaction represents one of the mechanisms behind the antimicrobial impact of MgO-NPs. For example, microorganisms possess negatively charged surfaces, thereby attracting NPs with an opposite charge, causing further harm through the leakage of microbial cell contents [49]. Furthermore, MgO-NPs can produce reactive oxygen species (ROS) [50]. These ROS can dismantle and deactivate essential biomolecules like DNA, proteins, and lipids. As the NP concentration rises, the quantity of ROS increases, potentially accounting for the increase in the antimicrobial effects observed with higher concentrations of NPs [51].

The antifungal activity PMMA-Ag nanocomposite is commensurate with the result of Kurt *et al*. [52], which indicated that PMMA material containing different percentages of Ag-NPs (0.25%, 0.5%, and 1%) showed significant antifungal activity against *C. albicans* in terms of CFU. Moreover, PMMA resin specimens with ZnO NPs and Ag NPs incorporation (0.5, 2.5, 5, 10, and 20%) significantly

decreased the population of *C. albicans* through CFU count with stronger antifungal effect in the latter than the former one (P<0.001), and this result is consistent with current data [53].

Ag-NPs' antibacterial property can be triggered by two different processes: either the released Ag ions from the substance under test or the production of reactive oxygen species. This procedure is dependent on several variables, including the production process, Ag-NP dispersion, and Ag proportion in the PMMA matrix [29]. Research has shown that Ag-NPs have antibacterial ability against a variety of candida species as well as antifungal effects against Gram-positive and Gram-negative microbes [54]. The reason that NPs have an antibacterial effect is because of their crystals' chemical reactivity, which can be significantly impacted by their shape. The structure of surface atoms and surface energy is closely related to this form [55]. Moreover, adding Ag-NPs to the acrylic resin matrix reduces surface roughness, which inhibits *C. albicans* adhesion and colonization [56].

The study has some limitations, such as the lack of antifungal activity of PMMA- NPs nanocomposite after the ageing of the material [57]. In addition, the surface roughness of the modified nanocomposites has not been examined, while it is well known that surface smoothness has an impact on the colonization of microbes.

5. Conclusions

PMMA-MgO and PMMA-Ag nanocomposites of 1%, 3%, and 5% concentrations exhibited effective anti-adherence activities against clinically isolated *C. albicans* for all concentrations. Furthermore, increasing the concentration of NPs was associated with a decrease in the adhered *C. albicans*. Lastly, Ag-NPs demonstrated better anti-adherence activities compared to MgO-NPs.

Authors contributions: Awder Nuree Arf: Methodology, data Curation, Formal Analysis. **Fadil Abdullah Kareem:** Investigation, Supervision, Writing – original draft. **Younis Khalid Khdir:** Investigation, Supervision, Methodology**. Muhammad Sohail Zafar:** Methodology, Writing – review & editing.

Data availability: Data will be available upon reasonable request.

Conflicts of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding: The authors did not receive support from any organization for the submitted work.

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