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Antimicrobial Properties of Acrylic Resin Incorporated with Propolis Nanoparticles

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A B S T R A C T

Objectives: One of the main problems with polymethyl methacrylate (PMMA) used for the fabrication of oral removable appliances is plaque accumulation due to surface porosities. Incorporation of antimicrobial agents in this material might help tackle this problem. The aim of this study was to evaluate the antimicrobial activity of PMMA acrylic resin incorporated with propolis nanoparticles (PNPs).

Materials and Methods: Antimicrobial properties of acrylic resin incorporated with PNPs were assessed against *Streptococcus mutans* (*S. mutans*), *Streptococcus sanguinis* (*S. sanguinis*), *Lactobacillus acidophilus* (*L. acidophilus*) and *Candida albicans* (*C. albicans*). Acrylic discs were fabricated in four groups: A control group without PNPs and three experimental groups containing 0.5%, 1% and 2% concentrations of PNPs. Disc agar diffusion (DAD) test was performed to determine the antimicrobial effects of PNPs by measuring the microbial growth inhibition zones on Muller-Hinton agar plates. The eluted components test evaluated the viable counts of microorganisms in liquid medium after 24 and 72h. Finally, biofilm inhibition test assessed the efficacy of PNPs for inhibition of biofilm formation. $P<0.05$ was considered significant.

Results: The acrylic discs failed to produce microbial inhibition zones in the DAD test. Discs containing 1% and 2% nanoparticles showed anti-biofilm effects on all four microbial species. The colony counts of all microorganisms significantly decreased following exposure to liquids containing nanoparticles after 24 and 72h in eluted component test.

Conclusion: PMMA acrylic discs incorporated with PNPs presented some antimicrobial properties against *S. mutans*, *S. sanguinis*, *L. acidophilus*, and *C. albicans*.

Keywords: Anti-Infective Agents; Nanoparticles; Orthodontics; Polymethyl Methacrylate; Propolis

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INTRODUCTION

Polymethyl methacrylate (PMMA) continues to serve as the most common material used for the fabrication of dentures and baseplates of orthodontic appliances due to its favorable properties such as affordability, low weight, and acceptable esthetics [1].

One of the main complications of using oral

acrylic appliances including dentures and orthodontic appliances is plaque accumulation due to the surface porosities, and subsequent increase in the bacterial and fungal load in the oral cavity, and development of enamel demineralization, gingivitis, and stomatitis [2-4].

The microbial plaque formed on acrylic appliances is routinely removed by mechanical and chemical procedures which basically depend on patients' compliance and may be compromised in children and disabled patients [5-7]. Consequently, direct incorporation of antimicrobial agents into oral acrylic appliances has been suggested to overcome this problem and contribute to oral hygiene maintenance [8].

Nanotechnology as a milestone in material science has successfully produced materials with improved biological and mechanical properties [9,10]. Therefore, an increasing number of researches have been conducted to apply this technology in dentistry. Nano-coating of orthodontic archwires, and incorporation of nanoparticles in orthodontic adhesives and elastomeric ligatures are common examples of the application of nanotechnology in orthodontics [11-15]. In this regard, much consideration has been recently directed towards evaluation of the antimicrobial and mechanical properties of different types of nanoparticles incorporated into acrylic oral appliances.

Propolis, a material produced by the honey bees to protect their hives, is a glue-like substance composed of plant resins, bee waxes, and pollens [16]. This material has shown a wide range of antimicrobial and anti-inflammatory properties [17,18]. Therefore, it has been used in mouthwashes and toothpastes not only to prevent dental caries, but also as a therapeutic agent to alleviate denture ulcers and treat root sensitivity [17-23]. Considering the strong antimicrobial properties of propolis, the primary purpose of this study was to evaluate the antimicrobial activity of propolis nanoparticles (PNPs) incorporated in a conventional acrylic resin (SR-Ivocap Triplex Hot, Ivoclar, Vivadent, Liechtenstein) against *Streptococcus mutans* (*S. mutans*), *Lactobacillus acidophilus* (*L. acidophilus*), *Streptococcus sanguinis* (*S. sanguinis*) and *Candida albicans* (*C. albicans*).

MATERIALS AND METHODS

Preparation of PNPs:

Twenty grams of raw propolis was dissolved in 100ml of distilled ethanol. The solution was kept under stirring for 7 days at room temperature. The extracted propolis was then filtered using a regular filter paper to remove the impurities. After that, it was added to distilled water in a ratio of 1:10 for precipitation of pure propolis particles. Afterwards, the suspension was bath sonicated for 20-30min. This process produced PNPs in a liquid colloid state. Scanning electron microscopic observations (ZEISS, Oberkochen, Germany) confirmed the nano-sized particles at ×65000 magnification.

The colloid nano-propolis was centrifuged at 9000 rpm (HEROLAR; Unicem M, Herolab GmbH, Stuttgart, Germany) for 20 min. After that, it was filtered by a filter paper (Wattman-40 Ashless, Germany). Finally, the filtered material was treated by freeze-drying (Martin Christ, Osterode am Harz, Germany) for 24h at –70°C. After 24h, powdered PNPs were obtained.

Preparation of acrylic discs:

This study used 108 round acrylic discs with 10 mm diameter and 4mm thickness (Triplex; Ivoclar Vivadent, Schaan, Lichtenstein). This study compared four groups of acrylic samples including 0% (control group), 0.5%, 1% and 2% PNPs. The powder and liquid were mixed in 3 to1 ratio based on the manufacturer's instructions. The mixture was then poured into custom-made molds with 11mm diameter and 5mm thickness. The molds were placed on a glass slap, and mild pressure was applied on each mold by a thin glass slide. The setting time of the samples with 2% PNPs considerably increased, as they were not completely set even after 20h. After complete polymerization, the samples were removed from the molds by finger pressure from both sides. The discs were then fine-finished and reached a final dimension of 10mm×4mm. Finally, all specimens were gamma-sterilized with a minimum dose of 25kGy. Each group was divided into four subgroups depending on

the microorganisms that were evaluated in this study.

Preparation of microbial specimens:

L. acidophilus (ATCC314), *S. mutans* (ATCC35668), *S. sanguinis* (ATCC10556), and *C. albicans* (ATCC14053) were cultured on MRS agar, mutans valinomycin mitis salivarius, MM10 sucrose, and Sabouraud dextrose agar, respectively for 24h at 37°C. *L. acidophilus*, *S. mutans*, and *S. sanguinis* were incubated in an anaerobic jar while *C. albicans* was incubated in aerobic conditions in all phases of the study.

After culture of microorganisms in their specific media, *L. acidophilus*, *S. mutans*, *S. sanguinis*, and *C. albicans* were rehydrated in brain heart infusion (BHI) broth (Difco, Sparks, MD, USA) and incubated at 37°C according to the growth conditions of each microorganism as mentioned above. The logarithmic growth phase of each micro-organism was adjusted at a concentration of 1.5×10^8 colony forming units (CFUs)/ml, as verified by optical density measured at 600nm wavelength (0.08-0.13) by a spectro-photometer (Eppendorf BioSpectrometer® fluorescence, Hamburg, Germany).

Antimicrobial tests:

(a) Disk agar diffusion test (DAD)

DAD test reveals the ability of antimicrobial agents to diffuse within agar which is recognized by microbial inhibition zones. For this purpose, 0.5 McFarland standard concentration of each microbial suspension containing 1.5×10^8 CFUs/ml of each microorganism was spread on the surface of Mueller Hinton agar (HiMedia, Mumbai, India) by a sterile swap. Afterwards, the acrylic discs with different concentrations of PNPs were placed on the surface of agar with 2 cm distance from each other. After incubation at 37°C for 24h under appropriate growth conditions for each microorganism, the results were recorded by measuring the growth inhibition zones.

(b) Eluted component test:

The acrylic discs were immersed in tubes containing 1ml of sterile saline. After 24 and 72h, 50µL of the liquid media was removed from each tube and added separately to the tubes containing 50 µL of each microorganism at a concentration of 1.5×10^8 CFUs/ml. The tubes were vibrated in a shaking incubator at 300 rpm for 24h at 37°C. Eventually, 10 µL of the diluted content of each tube was spread on BHI agar using the spread technique with a L-shaped bar. The plates were incubated at 37°C for 24h, and the number of CFUs/ml was counted using the method described by Miles et al [24].

(c) Biofilm inhibition test:

Acrylic discs were placed in tubes containing 1.5×10^8 CFUs/ml of each microorganism and incubated at 37°C for 48h for biofilm formation on their surfaces. Afterwards, the discs were removed with sterile forceps and gently washed with sterile saline to remove the non-adherent planktonic microorganisms. They were then placed in the tubes containing 1ml of sterile saline and sonicated at a speed of 50Hz with 150W to detach the biofilms from the discs; 10µL of the tubes was cultured on BHI agar, and incubated for 24h at 37°C and the colony count of the test wells was calculated as mentioned earlier (reported as CFUs/ml).

This study was approved by the ethic committee of Tehran University of Medical Sciences.

Statistical analysis:

ANOVA and Tukey's HSD test were used for statistical analyses. $P<0.05$ was considered statistically significant.

RESULTS

(a) DAD test:

None of the discs containing 0%, 0.5%, 1% and 2% PNPs displayed inhibition zones in any of the microbial cultures.

(b) Eluted component test:

Eluted component test after 24h:

The eluted component test revealed that the *C. albicans* and *S. mutans* colony counts in all experimental groups were significantly lower than those in the control group after 24h ($P<0.05$). There was no statistically significant difference between the study groups except between 2% and 0.5%, such that the 0.5% group revealed higher bacterial count ($P=0.009$).

Table 1. Mean of colony counts (CFUs/ml) of microorganisms in eluted component test after 24h

Microorganism	CFU/ml±Standard Deviation ×10^5				P
	0%	0.5%	1%	2%	
Streptococcus mutans	31 ± 6^{a}	19 ± 3^{b}	$14\pm3^{b,c}$	5 ± 2^{c}	<0.001
Streptococcus sanguinis	34 ± 3^{d}	19 ± 3^{e}	14 ± 3^{e}	5 ± 2^{f}	<0.001
Lactobacillus acidophilus	63 ± 18^{g}	26 ± 5^{h}	17 ± 2^{h}	12 ± 2^{h}	0.001
Candida albicans	71 ± 12^{i}	43 ± 5^{j}	$24\pm7^{j,k}$	15 ± 2^{k}	<0.001

*Similar letters are not significantly different

All study groups presented significantly lower *L. acidophilus* colony count than the control group (P<0.05), while the difference between the study groups was not statistically significant (P>0.05). *S. sanguinis* colony count was also significantly different between the study groups and the control group (P<0.05). However, the difference between the 1% and 0.5% groups was not statistically significant (P=0.174), while the colony count in 2% group was significantly lower than both 1% (P=0.018) and 0.5% (P=0.001) groups.

The mean colony counts for each microbial strain in the four groups after 24 and 72h is shown in Tables 1 and 2, respectively.

(c) Biofilm inhibition test:

The results of biofilm inhibition test for *S. mutans* and *S. sanguinis* revealed that application of PNPs at 0.5%, 1% and 2% concentrations significantly inhibited biofilm formation by these bacteria. Meanwhile, increasing the concentration of PNPs from 0.5% to 2% did not lead to any significant decrease in biofilm formation.

Table 2. Mean of colony counts (CFUs/ml) of microorganisms in eluted component test after 72h

Microorganism	CFU/ml±Standard Deviation ×10^5				P
	0%	0.5%	1%	2%	
Streptococcus mutans	33.3 ± 3^{a}	11.6 ± 2^{b}	$10.6\pm2^{b,c}$	4.3 ± 1^{c}	<0.001
Streptococcus sanguinis	39.3 ± 4^{d}	14 ± 2^{e}	11.3 ± 2^{e}	11.3 ± 2^{e}	<0.001
Lactobacillus acidophilus	67 ± 15^{f}	24 ± 5^{g}	16.3 ± 2^{g}	10.3 ± 1^{g}	<0.001
Candida albicans	74 ± 5^{h}	33 ± 6^{i}	19.3 ± 3^{j}	12 ± 2^{j}	<0.001

*Similar letters are not significantly different

Eluted component test after 72h:

The results of eluted component test for *S. mutans* after 72h followed the same pattern as 24h. The colony counts of *L. acidophilus* and *S. sanguinis* after 72h showed similar trends, as the experimental groups did not have any significant difference with each other (P>0.05), while all of them showed significantly lower colony count than the control group (P<0.05).

C. albicans showed significantly lower colony count in the experimental groups compared with the control group (P<0.05). The difference between 1% and 2% concentrations was not statistically significant (P>0.05). Also, *C. albicans* and *L. acidophilus* showed similar trends such that addition of 0.5% PNPs to acrylic resin could not decrease biofilm formation.

Table 3. Mean of colony counts (CFUs/ml) of microorganisms based on biofilm inhibition test

Microorganism	CFU/ml±Standard Deviation				P
	0%	0.5%	1%	2%	
Streptococcus mutans	$30\pm16\times10^{4a}$	$3\pm1\times10^{4b}$	$6\pm3\times10^{3b}$	$1\pm1\times10^{2b}$	0.007
Streptococcus sanguinis	$19\pm8\times10^{4c}$	$5\pm2\times10^{4d}$	$11\pm3\times10^{3d}$	$3\pm1\times10^{2d}$	0.003
Lactobacillus acidophilus	$30\pm5\times10^{4e}$	$23\pm4\times10^{4e}$	$23\pm4\times10^{3f}$	$14\pm2\times10^{2f}$	<0.001
Candida albicans	$31\pm6\times10^{4g}$	$27\pm6\times10^{4g}$	$28\pm3\times10^{3h}$	$16\pm3\times10^{2h}$	<0.001

*Similar letters are not significantly different

However, acrylic blocks containing 1% and 2% PNPs inhibited *C. albicans* and *L. acidophilus* biofilm formation with no significant difference between them. The mean colony count for each microbial strain in the four groups is shown in Table 3.

DISCUSSION

In the recent years, various types of nanoparticles such as titanium dioxide/ silicon dioxide, silver and platinum have been incorporated in acrylic resin in order to induce antimicrobial activity [4,25-27]. Silver nanoparticles added to the acrylic plate of retainers showed strong antimicrobial activity against *S. mutans* [28]. However, since such metal agents have raised some biological concerns, we tried to incorporate a natural organic product with proven antimicrobial effects [29,30]. Since incorporation of nanoparticles has been suggested to enhance the antimicrobial activity of acrylic resins, and considering the previous studies that reported successful application of propolis in inhibition of dental caries and gingivitis, this study aimed to evaluate the antimicrobial effects of PNPs incorporated in PMMA [4,31-33]. The current study evaluated the antimicrobial properties of acrylic resin containing 0.5%, 1% and 2% concentrations of PNPs against *S. mutans*, *S. sanguinis*, *L. acidophilus*, and *C. albicans*.

The reason for choosing these microbial species is due to their major role in biofilm formation, and development of caries, gingivitis, and denture stomatitis in patients wearing oral acrylic appliances.

The present study showed that the antimicrobial effect of PNPs against *S. mutans* was stronger than that against *C. albicans* which was in accordance with the results of Ghahremanloo and Movahedzadeh [34] who showed that PMMA loaded with silver nanoparticles had stronger antimicrobial rather than antifungal effects.

Regarding the results of the eluted component test, acrylic discs containing different concentrations of PNPs showed antimicrobial activity against *S. mutans*, *S. sanguinis*, *L. acidophilus*, and *C. albicans*. In other words, this test proved that PNPs can present antimicrobial properties in liquid media such as saliva. Therefore, saliva may act as an appropriate carrier of PNPs to the tooth surfaces, gingiva, and oral mucosa which are the main targets for antimicrobial activity. Nevertheless, the results of DAD test showed that PNPs did not produce any growth inhibition zone in the culture of tested microorganisms. This test revealed that PNPs cannot diffuse in a liquid medium and reach adjacent areas. Hence, areas with direct contact with the acrylic appliance would not take advantage of antimicrobial activity, unless they are exposed to saliva. This would be more important in patients suffering from xerostomia because they would be deprived of the sufficient flow of the saliva containing PNPs. No growth inhibition zone in DAD test was also observed in other studies. Sodagar et al, [35] and Aydin Sevinc et al. [36] showed that despite the optimal antimicrobial activity of curcumin nanoparticles and zinc nanoparticles added to composite resins, they did not present a growth inhibition zone in the disc diffusion test. Similarly, Mirhashemi et al. [37] could not induce growth inhibition zones by adding up to 5% chitosan/zinc oxide nanoparticles to composite resin. Therefore, other nanoparticles are not superior to propolis in terms of direct diffusion in liquid media. In biofilm inhibition test, unlike the eluted component test, samples with 0.5% PNPs could not provide a significant antimicrobial effect against *L. acidophilus* and *C. albicans* biofilms. It might be due to the fact that biofilms are much more resistant to antimicrobial agents in comparison with the planktonic form of bacteria [38].

Consequently, if we aim to provide antimicrobial effects against both planktonic and biofilm forms of all four types of tested microorganisms, we need to add at least 1% of PNPs to the acrylic resin. The results of the present study indicated that samples containing 2% PNPs did not show any significant reduction in any microbial colony count except in *S. sanguinis* colony count after 24h in eluted component test. Since *S. sanguinis* is associated with non-cariogenic plaque and competes with *S. mutans* since

these two streptococci are in equilibrium, a percentage of PNPs that reduces *S. sanguinis* count and does not affect *S. mutans* is unfavorable [35,39,40]. Therefore, there would be no advantage in increasing the percentage of PNPs from 1% to 2%. Regarding the long and clinically unacceptable setting time of acrylic resin containing 2% PNPs, and taking into account that the biofilm inhibitory effect against *L. acidophilus* and *C. albicans* is not provided by samples including 0.5% PNPs, it seems that incorporation of PNPs in 1% concentration would provide optimal antimicrobial effect for clinical application.

CONCLUSION

The results of the present study recommend incorporation of 1% PNPs to PMMA for oral acrylic appliances in order to induce antimicrobial activity.

CONFLICT OF INTEREST STATEMENT

None declared.

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