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In Vitro Assessment of Antifungal Property of Azadirachta Indica (Neem) Modified Heat-Cured Polymethyl Methacrylate Resin Denture Base Material After Water Storage

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ABSTRACT

Objective: The study's objective was to compare the antifungal properties of conventional and 2.5wt% neem-impregnated heat-cured PMMA through an adherence assay performed after 48 hours and 30 days storage time at room temperature in distilled water. **Study Design:** Laboratory-based experiment. **Settings:** Acrylic resin specimens were prepared in the Dental Materials Laboratory, Peshawar Dental College, and their antifungal tests were performed at the Department of Microbiology, Peshawar Medical College, Peshawar Pakistan. **Duration:** The study was completed within six months from 01-05-2021 to 30-10-2021. **Methods:** For this study, a total of 20 samples were made using a stainless-steel master Mold (10 x 10 x 2mm) for the fabrication of test specimens for antifungal properties prepared according to ISO 179A1:2005 specifications. Control groups were prepared according to manufacturer instructions with no neem extract. Experimental groups were reinforced with 2.5% neem extract. The antifungal property was assessed through adherence assay. Statistical analysis was done using one-way ANOVA and Post hoc Tukey's test. $P \leq 0.05$ was considered significant. **Results:** The incorporation of neem to acrylic denture base resin significantly decreased the number of colonies, as evidenced by one-way ANOVA that shows a significant result ($P = 0.011$). The highest number of colonies were found in the control group for 30 days of water storage followed by 48 hours of water storage samples. **Conclusion:** Antifungal property was significant with 2.5% neem extract, incorporated in PMMA after water storage for 48 hours and 30 days.

Keywords: *Denture base polymethyl methacrylate, Neem extract, Antifungal property, Adhesion assay, Water storage.*

INTRODUCTION

Polymethyl methacrylate (PMMA) is most commonly used to fabricate denture bases because of numerous advantages such as low cost, simple processing, stability in oral setting and acceptable aesthetics.[1] However, it is not a perfect material due to compromised properties such as poor flexural and tensile strength, making it highly prone to fracture and eventual clinical failure.[2]

Denture-related stomatitis (DRS) refers to inflamed mucosa under a denture. It became more common with advancing age, particularly in people with weakened medical conditions.[3] A fungus commonly found in the oral cavity called Candida albicans has been linked to bloodstream infections and mucosal infections .[4]

The treatment of candidiasis includes meticulous oral & denture hygiene regime, systemic administration, or topical application of antifungal agents.[5] Candida albicans can penetrate the acrylic resin (to a depth of 631µm,) and can survive disinfection. The depth of candida albicans penetration and its ability to remain viable have major implications on the management of patients with denture stomatitis. The porosity and

fissures inherent in acrylic resin facilitate the attachment and colonization of candida by the microcolony and biofilm formation, which, therefore, augment retention in the resin.[6]

The reduced saliva secretion and patient noncompliance to maintain denture hygiene could affect topical medicine administration in the oral cavity. Thus, rendering it ineffective against deep penetration of microorganisms.[7] To overcome denture-related stomatitis, attempts have been made to incorporate antifungal properties into acrylic resin.

According to Bansal *et al*., 2019, neem's antibacterial and antifungal properties were comparable to chlorhexidine. Neem powder possesses antifungal properties because it decreases the ability of candida albicans to adhere to PMMA acrylic resin and denture base materials.[8] With the minimum attachment concentration at a maximum weight percentage of 2.5wt%, the bacterial count reduced as the neem concentration increased. Still, their work did not assess such properties at different time durations. Hamid *et al*. (2019) supports the concept of using natural products such as neem as an antifungal agent in acrylic resin as it decreases the accumulation of candida albicans in the denture base materials.[9]

Thus, the literature supports the antibacterial or antifungal nature of neem, but their efficacy at different time periods needs to be explored. Therefore, the goal of this study was to test the antifungal properties of acrylic resin when neem extract 2.5% was added to acrylic resin denture base material and to compare the antifungal properties at 48 hours and after 30 days of water storage.

METHODS

Approval of the project from BASR (Board of Advanced Studies and Research) at Peshawar Dental College under RIPHAH University supervision was obtained (Prime/IRB/2021-308). Neem powder was stirred continuously for 24 hours in 250ml of 10% methanol solution. The solution was filtered and concentrated at 40°C in an oven until completely evaporated. Neem extract (2.5wt%) was added to PMMA liquid and mixed at 400rpm for 10 minutes.[20]

Acrylic resin specimens (control and test) were prepared in a customized stainless-steel Mold of 10x10x2mm to fabricate test specimens for antifungal properties.[10] Wax patterns were invested in curing the flask using the standard technique, as shown in Figure 1.

After setting the dental stone (type 3), the wax was removed in a hot water bath. The investment was bench-cooled, and a mixture of sodium alginate (cold mold seal) was applied to the mold surface. Conventional heat-cure acrylic resin (as supplied) and modified heat-cure acrylic resin with 2.5wt% neem extract were made by mixing PMMA powder to liquid in a ratio of 2.5:1 (21gm PMMA powder and 8.4ml of monomer) in accordance with the manufacturer instructions. The acrylic was packed in a dough state under a hydraulic press[11] (9.8MPa) for five minutes. Heat curing was performed by immersing the flask in water at room temperature and raising the temperature gradually to reach 100°C over one and a half hours. The temperature was maintained for 30 minutes. The acrylic bath was gradually cooled down to room temperature. All specimens were recovered from Mold and finished using 200, 300, and 600 grit sandpaper.

The defect-free specimens were immersed in distilled water at room temperature for 24 hours and disinfected with NaOCl and 70% methanol.[12]

Strains of candida albicans (Atcc 10231) were provided by the Department of Microbiology, Peshawar Medical College. On Sabouraud dextrose agar (SDA), stock culture was grown for 48 hours in the incubator at 37°C. The fungus suspension was made from 48-hour cultures in saboraud dextrose broth (SDB), and using the McFarland standard chart, the broth's concentration was standardized to 1x106 CFU/ml. Sterile normal saline was then used to obtain the turbidity of 0.5 McFarland standards. The samples were incubated at 37 °C for 1 hour before being rinsed with normal saline for 5 seconds. Spinning for 20 seconds at 3000 rpm eliminated adhered cells from acrylic resin samples. Threefold serial dilutions of the suspension were done, the diluted suspension was inoculated on SDA (incubated for 48 hours at 37 C°), and candida albicans were quantified by using the colony count method.

Resin specimens for antifungal properties were stored in distilled water in a sealed container for 48 hours and 30 days at room temperature (Galav *et al*., 2017).[13] After 48 hours and 30 days the specimen were desiccated and disinfected before subjecting to microbial tests. Adherence assay test was performed after water storage for 48 hours and 30 days.

Figure 1: Wax pattern for Antifungal test of 10x10x2 mm invested in the flask using dental stone

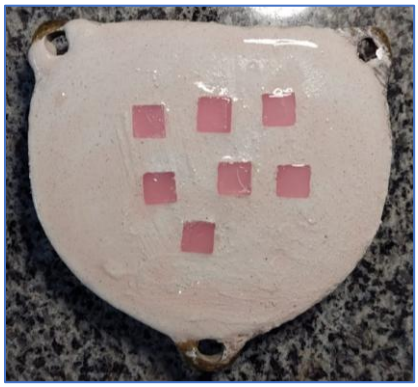

Figure 2a: Control group

Figure 2b: Experimental group

Mean and ± standard deviation values for antifungal test were computed. Statistically significant differences between the experimental groups were determined by using One-way ANOVA and Post hoc (Tukey Test) at p≤ 0.05 was considered significant, using the SPSS statistical software package version 26.0.

RESULTS

Table 1 shows the means and standard deviations of the heat-cured PMMA specimens for the antifungal test.

The addition of neem extract to heat cure acrylic resin shows that with the adherence assay method, the number of colonies of C. albicans in experimental groups decreased as compared with the control group. The highest decrease was shown in the experimental group with water storage for 48hrs which shows the lowest colonies formation of C. albicans (p= 0.014) and then followed by 30 days of water storage (*p*= 0.021). The highest number of colonies were found in the control group for 30 days of water storage followed by 48 hours of water storage samples. According to one-way ANOVA, the results were significant as $p < 0.05$ (table 4.2).

According to Post Hoc (Tukey test) shown in Table 3, comparing control 48 hours group with experimental 48 hours, control 48 hours shows a high number of colonies of C. albicans with a mean value of 5.2x106 CFU/ml as compared to the experimental 48 hours which shows less number of colonies of C. albicans with a mean value of 1.2 x106 CFU/ml. However, this difference was statistically insignificant (p=0.513). Comparing the control 30 days group with the experimental 30 days, the control 30 days shows the highest number of colonies of C. albicans with a mean value of 1.12 x107 CFU/ml as compared to the experimental 30 days with a mean value of 1.8 x106 CFU/ml with a highly significant difference (p= 0.021).

Table 1: Mean value and standard deviations of the heat-cured PMMA specimens for Antifungal Test

Anti-Fungal Test	N	Mean (CFU/ml)	Std. Deviation ±	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
48 hours control	5	5.2×10^6	1.49×10^6	8.63×10^6	9.53×10^6	3×10^6	1.10×10^7
30 days control	5	1.12×10^7	1.22×10^6	9.83×10^6	2.14×10^7	3×10^6	2×10^7
48 hours experimental	5	1.2×10^6	1.47×10^5	6.44×10^7	1.75×10^6	1×10^6	2×10^6
30 days experimental	5	1.8×10^6	1.36×10^5	7.61×10^5	2.83×10^6	1×10^6	3×10^6
Total	20	4.8×10^6	1.79×10^6	2.13×10^6	7.56×10^6	1×10^6	2×10^7

Table 2: One-way ANOVA for Anti-fungal Test

	Sum of Squares (CFU/ml)	Df	Mean Square (CFU/ml)	F	Sig.
Between Groups	3.15×10^{14}	3	1.05×10^{13}	5.204	.011
Within Groups	3.23×10^{14}	16	2.02×10^{13}		
Total	6.38×10^{14}	19			

Table 3: Multiple Comparisons of all groups of denture base resin with and without neem extract addition, Tukey test

Anti-fungal Test /Tukey HSD					
(I) Group	(J) Group	Mean (CFU/ml) Difference (I-J)	Sig.	95% Confidence Interval	
				Lower Bound	Upper Bound
48 Hours Control	30 Days Control	-6 x10^6	.192	-1.41 x10^7	2.13 x10^6
	48 Hours Experimental	4 x10^6	.513	-4.1 x10^6	1.21 x10^7
	30 Days Experimental	3.4 x10^6	.638	-4.73 x10^6	1.15 x10^7
30 Days Control	48 Hours Control	6.0 x10^6	.192	-2.13 x10^6	1.41 x10^7
	48 Hours Experimental	1.0 x10^7	**.014**	1.86 x10^6	1.81 x10^7
	30 Days Experimental	9.4 x10^6	**.021**	1.26 x10^6	1.75 x10^7
48 Hours Experimental	48 Hours Control	-4 x10^6	.513	-1.21 x10^7	4.13 x10^6
	30 Days Control	-1.00 x10^6	**.014**	-1.81 x10^7	-1.86 x10^6
	30 Days Experimental	-6.00 x10^5	.997	-8.73 x10^6	7.53 x10^6
30 Days Experimental	48 Hours Control	-3.40 x10^6	.638	-1.75 x10^7	-1.26 x10^6
	30 Days Control	-9.8 x10^6	**.021**	-1.83 x10^7	-1.27 x10^6
	48 Hours Experimental	6 x10^5	.997	-7.53 x10^6	8.73 x10^6

DISCUSSION

Denture stomatitis is the most prevalent disease in denture wearers, where salivation is reduced and manual dexterity is compromised due to aging or debilitating diseases.[13] Although apparently this disease has been reported in the literature as more of a local complication of ill-fitted dentures, if not treated timely, it can lead to fatal systematic complications. While reviewing the literature on antifungal additives in denture bases, it was obvious that none have been successful to the extent of being commercially available for common use.[14]

Neem extract was added to denture base materials in the current research to explore therapeutic release for denture stomatitis patients. The test group's average number of C. albicans colonies dramatically decreased as compared to unmodified/ control PMMA, which has been conventionally heating polymerized. The null hypothesis of this study was rejected, stating that adding neem extract (2.5wt%) to heat-cured PMMA resin will not impart antifungal properties after water storage for 48 hours and 30 days.

A review of the literature agrees that neem is a potent antifungal source that can effectively cut down on candida growth when used in various percentages.[15-19] However, compromise in various mechanical and physical aspects of acrylic has opened the potential for research to optimize its concentration to avail the best of antifungal and antimicrobial properties without compromising other aspects of denture base.[19]

In accordance with Hamid *et al*., who worked on the antibacterial nature of neem powder, they advocated 2.5wt% as an optimal level to be used to get good antimicrobial activity. The present study used the recommended percentage level and vindicated its antifungal capability in Neem powder extract form.[9] In the current study, instead of using neem powder in PMMA, neem extract was prepared, then homogenized with monomer liquid and added to the PMMA denture base powder. The extraction was done with the motive of reducing the potential agglomeration of the modifier.

According to Polaquini *et al*., who studied the antifungal effects of neem powder on dental composite resin, reported that neem has antifungal effects on C. albicans adhesion and colonization as well as biofilm development.[15] This finding is consistent with the findings of the current investigation. However, the current study extended this view by working on neem extract.

In this research, the addition of neem extract improved the antifungal properties. After 48 hours and 30 days of water storage, there were low C. albicans colonies count on the plates with 2.5% neem concentrations. These results are in line with those of Nawasrah *et al*. findings. His work advocates natural products in addition to PMMA as it imparts antifungal effects. Al-Thobity *et al*., 2017, also worked on plant extracts (including henna and thymoquinone) that decreased C. albicans adhesion.[16-18] But the fact their work was on natural products other than

neem and it was powder form rather than extract makes comparison difficult. The present work has adapted neem extraction from work done by Raghavendra & Balsaraf.[20]

For extract formation, methanol solution was used to facilitate neem extract easy homogenization with acrylic monomer and expected less or no discoloration of acrylic denture base due to modification with direct addition of neem in powder form. They advocated the 2% neem extract capable and having a comparative antifungal effect better than 3% NaOCl and 2% chlorhexidine against the candida albicans strain. But the extract, when added in 2.5% to monomer, imparted a dark greenish brown hue to the resultant acrylic specimens compromising the esthetics of the denture base.[14]

Heat cure acrylic is more stable as compared to self-cure resin, with less monomer leaching. Chincholikar *et al*. studied neem powder efficacy when added to tissue conditioner compared to acrylic polymer. Their finding advocated the use of natural additives in tissue conditioners as they show more release, hence effective antifungal properties.[19] Contrarily, every patient seeking treatment does not require tissue conditioners, and such linings add to the cost of treatment as well.

Although the present study neem extract was adapted from Raghavendra & Balsaraf[20] study their work lacks on antifungal activity. No literature could be reported nor compared with present study findings to comment on neem methanol base extract antifungal activity over a prolonged period of time.

CONCLUSION

The experimental groups show a significant increase in antifungal properties. The antifungal property was improved with water storage for 48 hrs and 30 days.

LIMITATIONS

The neem extract in the present study was prepared with a motive to minimize adverse effects on the physical properties of PMMA denture base material when natural additives are added. Contrarily, the neem extract had a dark shade, due to which its addition had to be kept to a minimum. An increase in the percentage would affect the optical properties of PMMA denture base material.

SUGGESTIONS / RECOMMENDATIONS

Although the objective of this study was achieved, there are variables like the effect of different aging procedures like varying pH, long-term storage in saliva, and temperature changes that should be explored. It is also necessary to investigate the impact of neem on the physical characteristics, such as the optical & mechanical properties of the acrylic resin denture base materials.

CONFLICT OF INTEREST / DISCLOSURE

None.

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