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Chitosan – A naturally occurring viable alternative to Nystatin as an anti-fungal agent incorporated in heat polymerised denture base resin

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ABSTRACT:

Aims and Objectives: To evaluate and compare the antifungal efficacy of low molecular weight chitosan and nystatin by their inhibitory action on candida biofilm formation on Triplex heat cure denture base after 7 days and 14 days.

Materials and Methods: A total of 10 specimens were fabricated using the Triplex heat cure PMMA acrylic resin by the conventional compression molding technique.Candida albicans biofilm was allowed to form over a period of 72 hours on all the specimens after pre-treatment with artificial saliva. Two groups of 5 specimens each were established. Group A was treated with low molecular weight chitosan suspension and group B was treated with nystatin suspension, both on the 4^{th} day after biofilm formation. The treated specimens were now stored in a yeast nitrogen base broth medium to complete the time period of 7 days for initial maturation of the biofilm. The CFU count of candida colonies was recorded on the 7^{th} day in both the groups.Specimens were treated again on the 11^{th} day using chitosan and nystatin suspension for the assigned groups and the residual biofilm was allowed to completely mature

till the 14th day, when another CFU count of the candida colonies was recorded for respective groups. The results obtained were compared and subjected to statistical analysis.

Results: The results showed that there was a significant reduction in the number of candida colonies in group A (Chitosan) from the 7th to 14th day of biofilm formation. (p value = 0.04). On the other hand, the mean count of candida colonies showed insignificant reduction from 7th to 14th day in group B (nystatin) with a p value of 0.20. On comparison of CFU count between Group A and B on 7th day, insignificant reduction was witnessed (p value = 0.26) but a markedly significant decrease in the number of colonies was seen on the 14th day (p value = 0.02).

Conclusion: It was concluded thatChitosan possesses noticeably better antifungal properties as compared to nystatin, especially when it comes to inhibitory effect on mature and adherent candida albicans biofilm formation on heat cured PMMA acrylic resin denture bases after 14 days.

Key words: chitosan, nystatin, candida biofilm, acrylic denture base, antifungal

INTRODUCTION:

The natural polysaccharide, Chitin known to be the structural element in the exoskeleton or shells of crabs and shrimps is the second most abundant material after cellulose that is also found in the fungal cell walls. Research on chitin was limited until the 1980s due to its complex structure, difficult extraction and insolubility in aqueous solutions. [1]

Chitosan is a contemporary bioresource and the only known alkaline polysaccharide obtained by thorough purification process of the raw material chitin, which consists of cycle of depolymerization, demineralization, deproteination, decoulourisation finally followed by chemical deacetylation. [1, 2]

Chitosan is an eco-friendly antioxidant with unique antifungal and antibacterial properties in addition to non-cytotoxicity and biocompatibility. [1, 2]

Chitosan can only be dissolved when the pH is less than 6.5 which limits its antifungal activity. Hence, various derivatives of chitosan have been created that could improve its solubility and antifungal properties. Studies have shown that the antifungal activity increases as the degree of deacetylation increases and the molecular weight of chitosan reduces. (41.2 kDa and 18kDa). [2, 3]

Increase in geriatric population has witnessed a rise in the requirement of removable complete dentures since 7-69% of the adult population is known to comprise of those rendered edentulous, internationally. [4]

Candida albicans is a commensal of oral cavity. The prevalence of candida increases 60-100% in denture wearers due to reduced flow of oxygen and saliva to underlying tissues producing a local acidic and anaerobic microenvironment favoring yeast overgrowth. Moreover, almost 67% of existing denture wearers are thought to have candida associated denture stomatitis. [4] Candida biofilm formation takes place in 3 phases. The initial adhering of candida and formation of microcolonies by expressing glycoproteins is seen in the first phase within 1-11 hours. The second stage, called as the intermediate stage occurs from 12-30 hours forming a bilayer of yeast, germ tubes and young hyphae in extracellular matrix. The final stage of maturation completes in 38-72 hours. [4]

Polymethyl methacrylate (PMMA) resin is the most commonly used denture base material which acts as the main substrate for candida albicans by promoting initial attachment followed by colonization on denture base and oral mucosa of patients to develop an adherent biofilm. [5]

Inefficient cleaning of denture surfaces promotes inhabitation of microorganisms, specifically candida albicans. Steam sterilization of dentures is not adviced as an appropriate hygiene protocol due to the thermal instability and low ebullition temperature of the monomer in PMMA resins. Use of chemical disinfectants like gluteraldehyde is internationally accepted but has shown to carry a high toxic potential causing irritation of the skin and mucous membrane that can later lead to allergies or inflammation. [5,6]Both mechanical and chemical means for cleaning of denture prosthesis have been suggested that includes soaking and brushing with commercially available denture cleansers like sodium bicarbonate, sodium hypochlorite , chlorhexidine and alkaline peroxide solutions, the most effective proven to be 0.5% sodium hypochlorite solution. But soaking of dentures in higher concentration of NaOCl solution for more than 10 minutes may lead to certain unwanted outcomes like soft tissue dissolution, unfavorable taste and smell and increased roughness on denture surfaces. [7,8]

Mechanical cleansing of dentures appears to be inefficient against candida species. Despite the availability of several chemical denture cleansers for daily use, maintaining adequate oral and denture hygiene has become difficult. A time-dependent effect or possible recolonization in a complete mature biofilm is observed after the 14th day from the first day of initial colonization.[5,7,8]Several antifungal agents like amphotericin B, fluconazole and nystatin have been tested against the candida biofilm on denture acrylic (PMMA) that adheres to it either directly or via an indirect intermediate layer of plaque forming bacteria. [9]

Nystatin is considered as a standard antifungal agent with 70-80% of inhibitory effect on the candida biofilm. Yet, in spite of antifungal treatment for denture stomatitis, infection is found to recur as the therapy stops. This shows that denture plaque may provide a protected reservoir for the growth of Candida albicans. [9, 10]Also, it has been found that candida species are developing resistance against nystatin.

Therefore, this study is designed to evaluate the inhibitory effect of low molecular weight chitosan on candida biofilm formation in comparison with the standard antifungal agent, nystatin on heat polymerizing PMMA denture base resin after 7 days and 14 days.

MATERIALS AND METHODOLOGY:

A sample size of 10 acrylic specimens was chosen, with 5 for treatment with LMW chitosan and 5 with nystatin. An in vitro study was performed to evaluate and compare the antifungal efficacy of these two materials on candida biofilm formation after 7 and 14 days.

Preparation of Acrylic specimens

A cuboid-shaped metal mold with dimensions 50mm x 30mm x 2-3mm[10]thickness was used to obtain wax plates of uniform thickness simulating the thickness of conventional dentures. It wasthenacrylized into heat cured acrylic resin using conventional short curing cycle followed by sequential finishing and polishing of the retrieved specimens.

Before use, the specimens are ultrasonically sterilized using sterile ultrapurified water and 70% alcohol for 20 minutes.[4]

Chronological sequence of steps followed in the study:

1. Preparation of Candida culture

Candida albicans was cultured and incubated for 24 hours at 37 degree Celsius in yeast nitrogen base (YNB) containing 50mM of glucose. Cells were harvested, washed with Phosphate buffered solution (PBS, pH 7.2) and standardized to 1 x 10^7 cells/ml.[7]

2. Pre-treatment of specimens in artificial saliva

The specimens were submerged in artificial saliva and incubated at 37 degree Celsius for 2-3 hours in an incubator for salivary acquired pellicle formation prior to be used for biofilm formation.[7]

3. Biofilm formation on specimen surfaces

80 ul quantity of standardized Candida albicans cell suspension was applied on the surface of the specimens placed in petri dish and was left there for 90 minutes at 37 degree celsius as per the adhesion time of the yeast cells.The non-adhered cells were then gently washed off with 5mL PBS solution.Now the specimens were immersed in new sterile YNB containing 50mM glucose for allowing biofilm formation over a period of 72 hours. [7] (This medium was changed daily for every 3 days)

4.Preparation of antifungal suspensions

a) LMW Chitosan suspension

7.75mg/ ml Chitosanpowder[2] in 4 mL of Glacial Acetic acid

b) Nystatin suspension

7.75 mg/ml of nystatin powder in 4ml of YNB broth[7]

5. Testing of specimens on immersion in the prepared antifungal suspensions.

The specimens were removed from the growth medium and washed in PBS

Immersion of specimens:

Specimens were immersed in each suspension for a period of 10 minutes, once every week for two weeks. [9]

GRP A (n=5) was placed in LMW chitosan suspension on the 4th day after biofilm formation, for 10 minutes.

GRP B (n=5) were placed in nystatin suspension on the 4th day after biofilm formation, for 10 minutes.

Specimens were removed after 10 minutes of immersion and gently washed with PBS solution and again transferred into a sterile YNB medium containing 50mM glucose along with artificial saliva for two days. (This medium was changed every day for 2 days)

6. Quantitative measurement of biofilms and their microscopic evaluation to compare the inhibitory effect of antifungal suspensions. [4]

- The treated specimens were removed from the YNB medium and washed with 3ml sterile PBS on the 7th day for biofilm assay. The Candida biofilms were removed from the specimens using a sterile micro brush from a 2 x 2 cm area. The microbrush was inserted into microcentrifuge tubes containing sterile saline solution (1 ml) and vortexed for 30 seconds followed by sonication at 20W with 3 pulses of10 seconds each.[9]Serial dilutions of sonicated solutions in PBS solutions were carried out and 20ul aliquots were plated on Sabouraud's dextrose agar.

  The plates were incubated at 37 degree Celsius under aerobic conditions for 48 hours.

- The samples were viewed under a microscope for counting of Colony forming units (CFU) in the biofilm and results were obtained in CFU/substrate area after a time period of 7 days.
- The treated specimens were now suspended into a YNB + glucose medium after immersion in artificial saliva as mentioned before over a period of next one week and were again immersed in the antifungal suspensions on the 11th day as mentioned earlier. The specimens were removed from the growth medium and gently washed in 3 ml sterile PBS on the 14th day and biofilm collection and analysis using the CFU counting method was done as before.

Figure1: Photograph of the metal mold used for acrylic specimen fabrication:

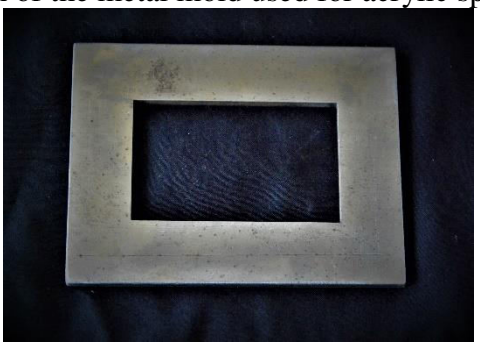

Figure 2: Photograph of wax pattern fabricated and flasked for processing:

Figure 3: Photograph of acrylic specimens ready for biofilm formation:

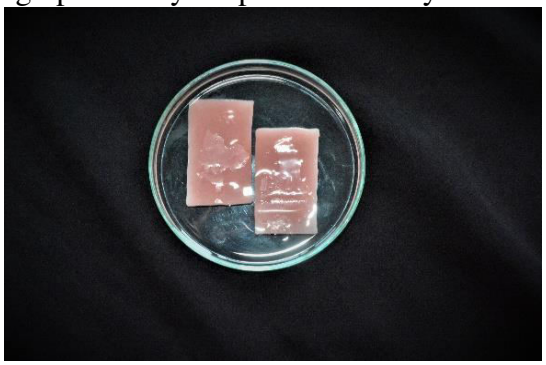

Figure 4: Photograph of pre-treatment of specimens with saliva followed by incubation:

Figure 5: Photograph of candida cell suspension and biofilm formation:

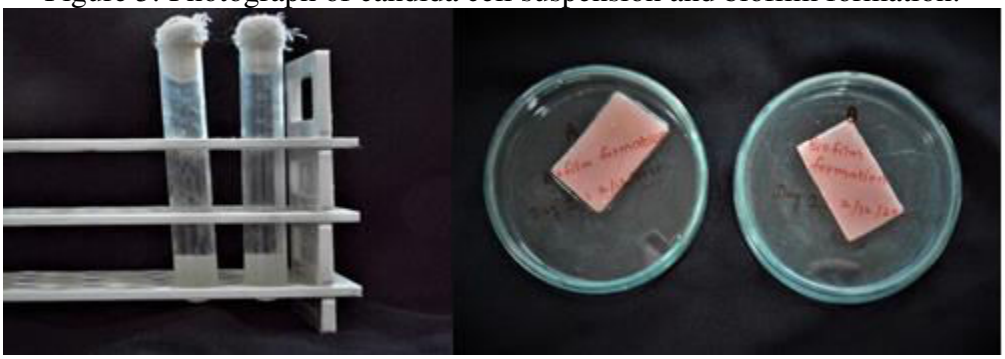

Figure 6: Photograph of Preparation of antifungal suspensions. (Chitosan- left & Nystatin-right)

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Figure 7: Photograph of Immersion in antifungal suspensions on 4th Day (Similarly on 11th day)

Figure 8: Photograph of Plating and inoculation of the sample on SDA plates

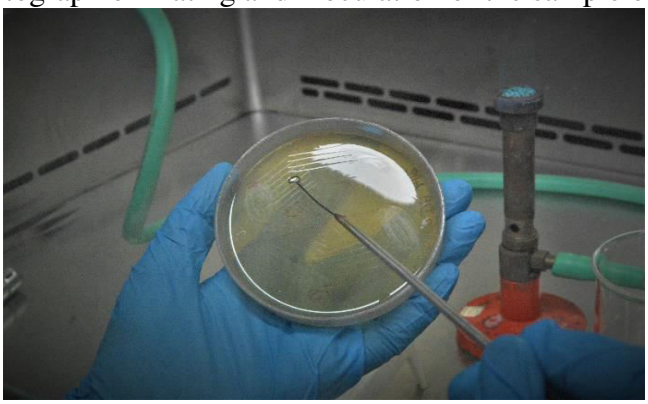
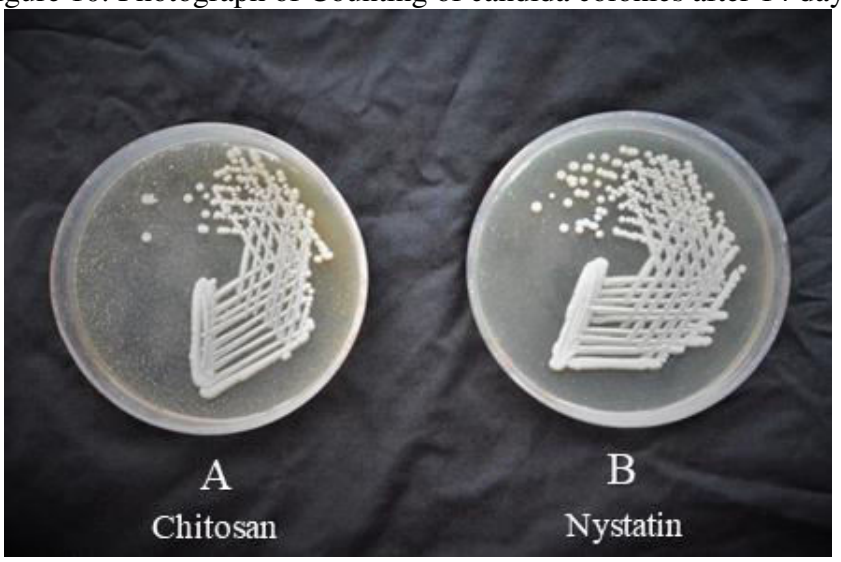


Figure 9: Photograph of Counting of candida colonies after 7 days

Figure 10: Photograph of Counting of candida colonies after 14 days



RESULTS:

In this study candida biofilm formation was carried out on all the 10 samples over a period of 72 hours. These were divided into two groups of 5 specimens each where Group A was treated with chitosan suspension after 7 days and 14 days of biofilm formation and Group B was treated with nystatin suspension after 7 days and 14 days of biofilm formation to test and compare the antifungal efficacy of both the materials used.

The CFU count of candida colonies was recorded on the 7th day and the 14th day for Group A (Chitosan) treated specimens and a mean count was calculated. (Table 1 and Graph 1)

Similarly, the mean count was calculated from the CFU count of colonies for Group B (Nystatin) (Table 2 and Graph 2)

Testing of data analysis: Paired- t test

Comparison of CFU count within Group A on 7th and 14th Day:

It was found that a significant reduction in the CFU of candida colonies was shown in Group A from 7th day to 14th day from a mean count of 64000 to 82. A p value of 0.04 suggested that chitosan showed a significant reduction in the number of candida colonies from 7th day to 14th day. (Table 3)

Comparison of CFU count within Group B on 7th and 14th Day:

The mean count of colonies had reduced in Group B from 280000 to 6400 from 7th day to 14th day but a p value of 0.20 showed that nystatin did not show a significant amount of reduction in candida count from 7th day to 14th day. (Table 4)

Comparison of CFU count between Group A and Group B on 7th and 14thDay :

Difference between the chitosan group and nystatin group was calculated and compared at the 7th and 14th day. A p value of 0.26 obtained on the 7th day of comparison suggested that no significant difference is observed between Chitosan and Nystatin in candida count at 7th day but

a p value of 0.02 on the 14th day showed that a significant difference is observed between Chitosan and Nystatin in candida count on the 14th day. (Table 5 & Graph 3)
Though statistically insignificant reduction is seen in the candida counts on the 7th day between chitosan and nystatin, a markedly significant reduction in the count on the 14th day is observed in chitosan group as compared to that of nystatin group suggesting better antifungal action of chitosan against candida biofilm than nystatin.

Table no.1- Descriptive data of mean count of candida in Chitosan group on 7th and 14th day

	N	Minimum	Maximum	Mean	Std. Deviation
Chitosan 7th day	5	10000	100000	64000.00	49295.030
Chitosan 14th day	5	10	100	82.00	40.249

Table no.2- Descriptive data of mean count of candida in Nystatin group on 7th and 14th day

	N	Minimum	Maximum	Mean	Std. Deviation
Nystatin 7th day	5	100000	1000000	280000.00	402492.236
Nystatin 14th day	5	1000	10000	6400.00	4929.503

Table no.3- Difference within the Chitosan group for candida count between 7th to 14thday

	Mean	t-value	Significance (p)
Chitosan 7th day	64000.00	2.90	**0.04***
Chitosan 14th day	82.00		

Significance at p<0.05

Table no.4- Difference within the Nystatin group for candida count between 7th to 14thday

	Mean	t-value	Significance (p)
Nystatin 7th day	280000.00	1.52	0.20
Nystatin 14th day	6400.00		

Tableno.5- Difference between the Chitosan and Nystatin group at 7th and 14th day with respect to candida count

	N	Mean	Mean difference	t-value	Significance (p)
Chitosan	7th day	64000.00	216000	1.19	0.26
Nystatin		280000.00			
Chitosan	14th day	82.00	6318	2.87	**0.02***
Nystatin		6400.00			

Significance at p<0.05

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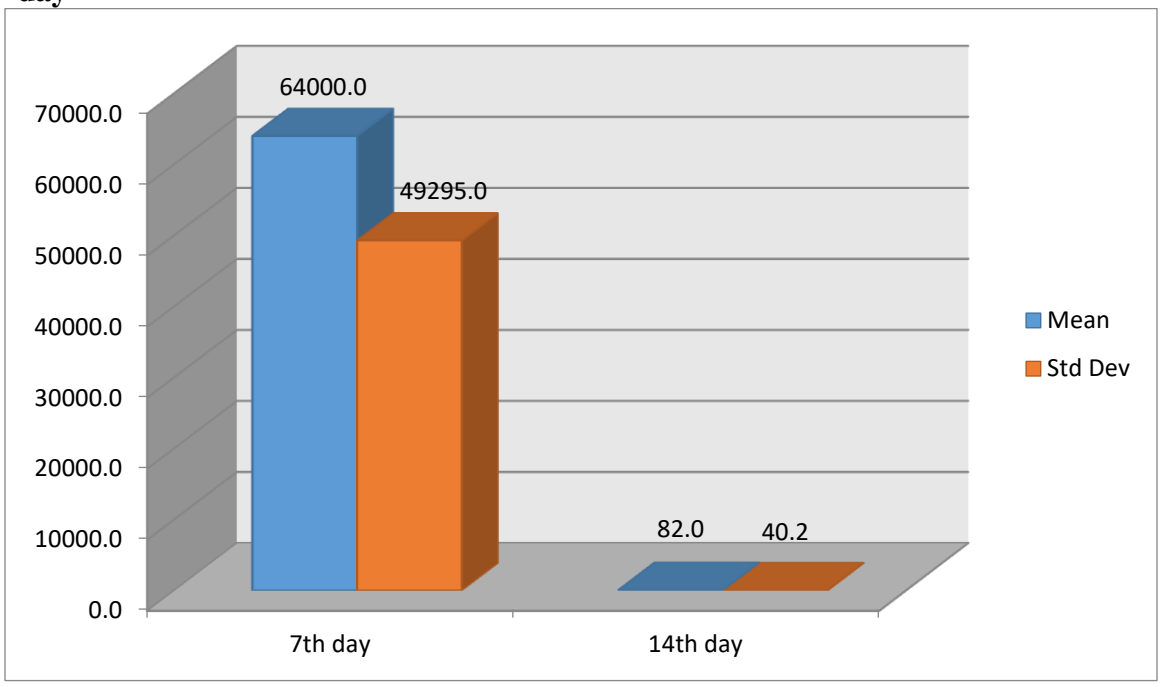
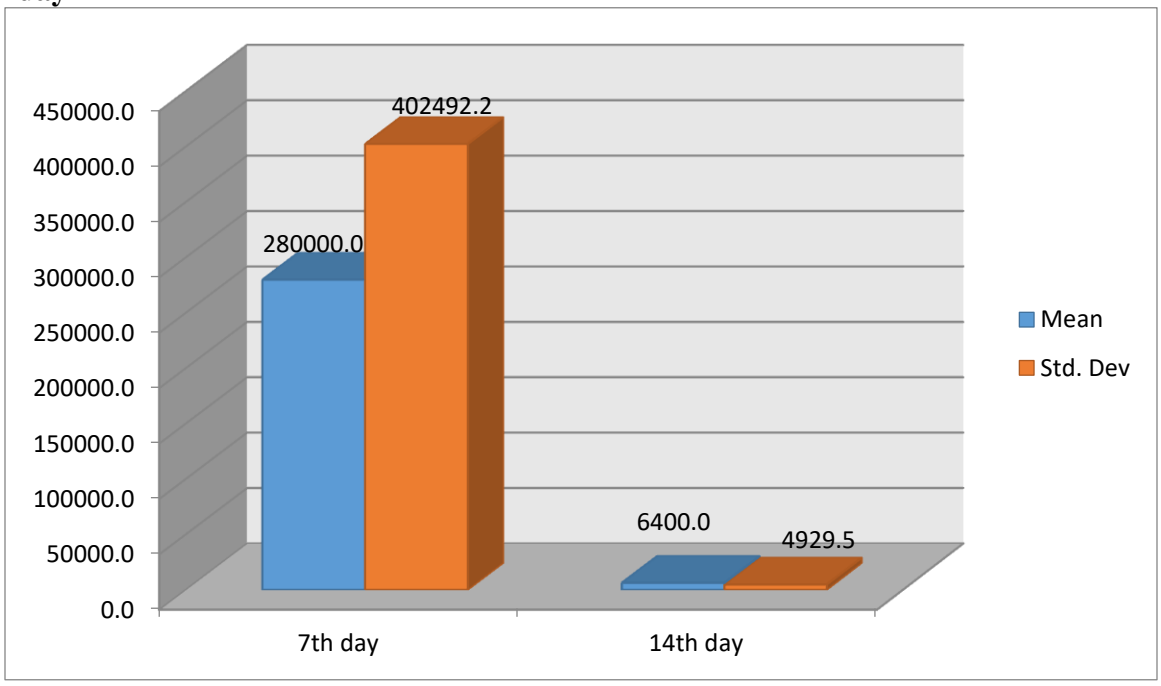
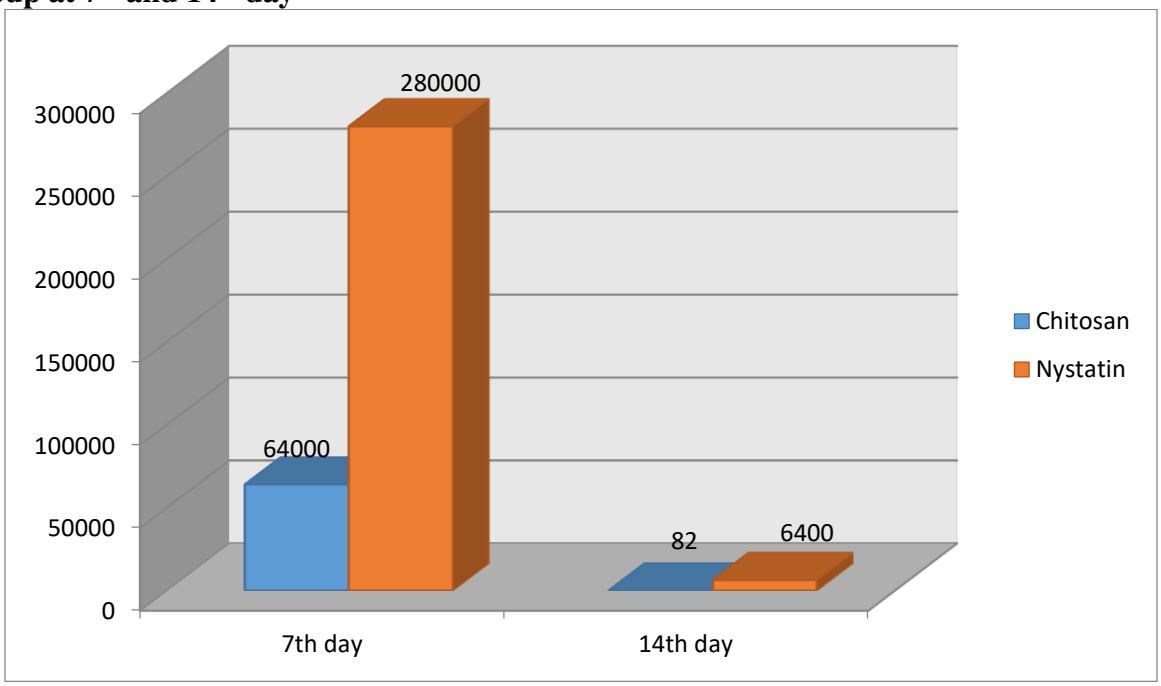
Graph no.1- Bar diagram representing mean candida count in Chitosan group at 7th and 14th day



Graph no.2- Bar diagram representing mean candida count in Nystatin group at 7th and 14th day



Graph no.3- Bar diagram representing mean candida count in Chitosan and Nystatin group at 7th and 14th day



DISCUSSION:

The longevity and functionality use of any removable oral prosthesis depends on how well it is maintained and taken care of by the patient, pertaining to hygiene maintenance protocols for complete dentures in specific.

In the present study, a simple in-vitro model of biofilm was developed using an inoculum of 1×10^7 cells/ml with an adhesion time of 90 minutes over a period of 72 hours supplemented with glucose and yeast nitrogen base broth medium for optimum growth.

The two time points chosen in this study, 7th and 14th day represent the initial and final maturation of the colonies in the biofilm respectively. [9]

This in vitro study aimed to analyze the antifungal efficacy of a contemporary biodegradable material, chitosan against the candida biofilm formation on heat polymerized PMMA acrylic resin and compare it with the gold standard, nystatin.The recent problem with using nystatin as the antifungal agent is that, candida species is developing cross resistance against it and many other standard antifungal materials. The suggested mechanism for the distinct antifungal behavior of chitosan involves a permeable chitosan film formation on the cell surface which interferes with the fungal growth and activates several defense processes that leads to inhibition of DNA/RNA synthesis and disruption of protein synthesis. [2, 14]

The current study showed that the specimens treated with chitosan showed a considerable reduction in the mean count of candida from the 7th to 14th day dropping from 64000 to 82 as shown in table 3.

The mean count of colonies reduced in specimens treated with nystatin on 14th day as compared to 7th day from 280000 to 6400 but this reduction is seen to be clinically insignificant as given in table 4.
When the CFU was compared between chitosan and nystatin on the 7th day, the p value obtained was 0.26 indicating that the antifungal efficacy of both the materials on the 7th day did not show any significant difference. This is because, initial colonization consists of immature candida cells and the action of both chitosan and nystatin relatively, closely inhibited the biofilm formation on the 7th day.
When the CFU count was compared on the 14th day, the p value obtained was 0.02, suggesting a significant difference in reduction of the candida count by the antifungal action of chitosan and nystatin, with chitosan showing better results as shown in table 5.
Certain limitation of this research include the requirement of a better control of contamination and cross contamination of candida colonies.
Therefore, the study conducted resulting in a favourable response and anti-candida property of chitosan, opens up pathways for further research to be performed to exploit its essential qualities upto maximum potential to be used as an alternative treatment for generalized or candida-associated denture stomatitis.

CONCLUSION:

It was found that chitosan significantly reduced the candida count on the 14th day from the 7th day and also showed remarkably better results as compared to nystatin, indicating that the antifungal efficacy of chitosan is superior to that of nystatin when dealing with the mature and adherent cells of candida albicans in specific, colonizing the surface of acrylic specimens made of heat cured PMMA resins.
Further research with evidence in the use of chitosan for maintaining complete denture hygiene needs to be carried out to generate more effective means to enhance the eradication of adherent biofilms on denture surfaces in order to minimize the occurrence of candida associated denture stomatitis.
The current research does show promising results and hence can form a stable premise to extend the scope of it to be applied in denture wearing patients in future to ensure optimum oral and general health.

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