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## **An in-vitro study of effect of incorporation of antifungal drugs upon growth of candida and surface hardness in permanent soft liners**

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**Abstract**---Introduction: - Long term soft liners (acrylic and silicone based) are used to prevent damage to the abused mucosa, but they have the disadvantage of becoming porous with time leading to a suitable environment for opportunistic infections. This study was aimed to examine the inhibitory effect of incorporation of antifungal drug clotrimazole into long term denture soft liners on the growth of *Candida albicans* in vitro and evaluate the change in the surface hardness of soft liners. Materials and Method: The present study was done for two different long term soft liners, acrylic based (Permasoft) and silicone-based (Mollosil), by addition of clotrimazole drug in their minimum inhibitory concentration. The properties –zone of inhibition and surface hardness (Shore A) were evaluated at time intervals of 1,7,14,21,35 and 42 days. A total of 224 samples were prepared. The properties were analyzed and inter group and intra group data was analyzed statistically by using One way ANOVA, Tukey post hoc-test

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and Paired t test. Result: The results showed significant creation of zone of inhibition for samples containing clotrimazole with the zone of inhibition decreasing with time. The surface hardness of all the samples increases with time. Conclusion: Antifungal clotrimazole can be added into permanent soft liner to provide antifungal effect and as mode of drug delivery system. There is gradual release of the antifungal from the soft liner and the drug release decreases with time. Addition of antifungal into the soft liner increases the surface hardness of the soft liner, but the values are within clinically acceptable limits.

**Keywords**---permanent soft liners, clotrimazole, acrylic soft liners, silicone soft liners, zone inhibition, surface hardness.

## Introduction

The removable dental prosthesis seats on the oral tissues, especially the alveolar ridges and hard palate providing adequate support to the prosthesis. With time the alveolar ridges and oral tissues undergo changes and remodeling which affects the adaptation of the prosthesis to the tissues a challenge. Patients suffering from diabetes, vitamin deficiency, and immunocompromised diseases have friable nature of the supporting mucosa. Thus areas of force concentration or misfit of the denture base, can result in tissue trauma and sore spots. This demands for either a new prosthesis fabrication with soft liners or alteration of the current prosthesis. Soft liners act as shock absorber and are mostly used as a therapeutic measure for patients who cannot tolerate stresses induced by dentures. They act by providing a cushioning effect to the underlying tissues<sup>1,2,3</sup>. They can be short term or long term depending on their usage. Depending on their composition they can be silicone based or acrylic based.

However, despite their vast clinical benefits, there are some drawbacks of the material. The plasticizers leach out of the material over a period of time making it more porous in structure, the water sorption also increases with time. This will provide a niche for growth of bacteria and fungus like *Candida albicans*. This might lead to candidiasis which is an opportunistic fungal infection. Therefore, prevention of the growth of *C. albicans* has focused on the use of antifungal medications.

Topical therapies are more effective on the fungi that invade superficial tissues. But are frequently associated with poor patient cooperation. Systemic therapy has been reserved for oral infections that are unresponsive to topical agent. due to the incidence of serious side-effects<sup>4</sup>.

The topical drugs are less palatable, leading to discomfort and uncertain application by the patients. Thus studies have been carried out by incorporating the antifungal agents directly into the soft liner. The composition of the material is altered; this might lead to alterations in the surface properties of the material such as hardness, roughness, water absorption, elasticity<sup>5,6,7,8,9,10</sup>.

Among the several properties to be evaluated, hardness is regarded as one of the fundamental properties<sup>[8,13]</sup> because it represents a simple method to determine its modulus of elasticity. The hardness provides information about the quality of the material, because a rigid material is not suitable to be used as a base for resilient relining removable dentures.

The incorporation of antifungal agents to temporary soft lining materials has demonstrated to be effective and viable in order to extend their longevity and reduce biofilm accumulation<sup>[11,12,13]</sup>. The literature lacks data regarding the incorporation of antifungal agents in long term soft liner<sup>[14,15,16]</sup>. Thus this study was carried out.

### **Aims and objectives**

This study was aimed:

1. To examine the inhibitory effect of incorporation of antifungal drug clotrimazole into long term denture soft liners on the growth of *Candida albicans* in vitro.
2. To evaluate the change in the surface hardness and roughness of long term denture soft liners by addition of antifungal clotrimazole.

### **Objectives**

1. To study if addition of antifungal clotrimazole at minimum inhibitory concentration into the denture liners is an effective antifungal therapy against *Candida albicans* or not to treat and prevent oral candidiasis.
2. To study the time duration upto which the antifungal drug is effective.
3. To study the addition of antifungal upto the effective concentration which maintains the properties like surface hardness and roughness of the material along with additional benefit of antifungal therapy.

### **Material and Methodology**

The study was conducted in Department of Prosthodontics and Microbiology at Karnavati school of Dentistry. All the samples used were fabricated by the same operator to prevent any inter operator bias. Two stainless steel dies of dimensions 13cm x 6cm x 0.6cm and 13cm x 6cm x 1cm were fabricated. Both the dies consisted of a solid base and lid sheet of 0.2cm thickness. The first die included a center 0.2 cm thick plate with 10 cylindrical cut out of 2cm diameter each.(figure 1) The second die included 0.6 cm thick plate with 10 cubical cut out of 2cm width each.(figure 2). The material Permasoft was mixed in P/L ratio 2.5:1 and allowed to set in the stainless steel die for 10 minutes at room temperature and then in warm water bath for 2 minutes. For fabrication of samples of mollosil, the material was mixed in a ratio of ( 1:1 ) and was transferred into the dies and allowed to set for 13-15 minutes. For fabrication of samples of group 2 and 4 antifungal drug clotrimazole was incorporated at the minimum inhibitory concentration of 5µg/ml while mixing. Thus the drug was weighed in the weighing scale -0.6mg clotrimazole/60 gm of powder. Thus total of 4 groups were formed. Group 1- Permasoft control, Group 2- Permasoft with clotrimazole, Group

3- Mollosil control, Group 4- Mollosil with clotrimazole. A total of 56 samples/group (48 discs and 8 blocks) were fabricated and a total of 224 samples were fabricated. The samples were stored in 10 ml salivary at 37°C to simulate the conditions of oral cavity. (figure 3 and 4). The circular discs were used to check the zone of inhibition and the blocks for measuring the surface hardness.

### Evaluation of antifungal activity

0.5 McFarland concentration of *Candida albicans* (ATCC10231) was prepared. (figure 5,6) Swabs were taken and lawn culture was done on petri dishes containing Sabouraud dextrose agar medium. 8 samples from each group were used to check the drug efficacy and antifungal activity. 1 sample from each group was placed in the center of the petri dish and the petri dish was incubated at 37°C for 24 hrs. The visible zone of inhibition created by the drug was measured in mm on the petri dish with a metallic ruler. The same procedure was repeated at time intervals of 1, 7, 14, 21, 35 and 42 days and results were noted down. (figure-7)

### Evaluation of surface hardness

The sample blocks were used for calculating the surface hardness. 8 samples from each group were tested at a time. Shore A durometer (American ASTM D2240) was used to calculate the surface hardness. The depth indicator was set to 0 and placed on the block. Pressure was applied for 30 seconds with the index finger on the indenter. The hardness value displayed on the hardness tester was noted. 3 different readings on 3 different sites were recorded and the mean of the readings was taken for statistical analysis. (figure-8) The same procedure was repeated at time intervals of 1, 7, 14, 21, 35 and 42 days and results were noted down.

### Statistical analysis

The data collected were tabulated and subjected to statistical analysis by using SPSS software (v22 for windows, Philadelphia, PA, USA). The data was analyzed statistically by using One way ANOVA, Tukey post hoc-test and Paired t test. P-value < 0.005 was considered statistically significant.

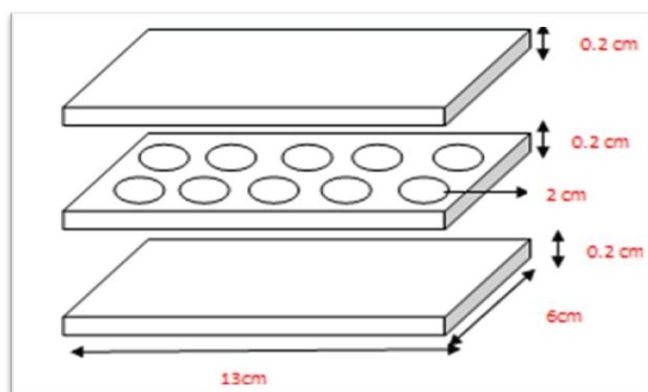


Figure 1: Schematic diagram of dimensions of die used for disc

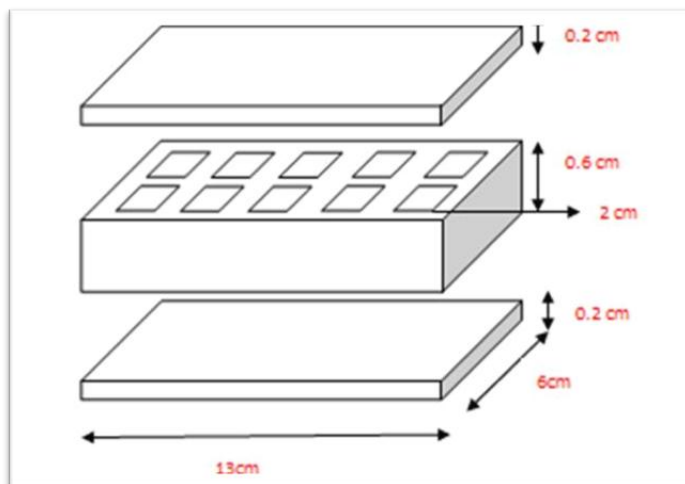


Figure 2: Schematic diagram of dimensions of die used for block



Figure 3: Weighing clotrimazole drug for incorporation at mic



Figure 4: Sample of disc and block

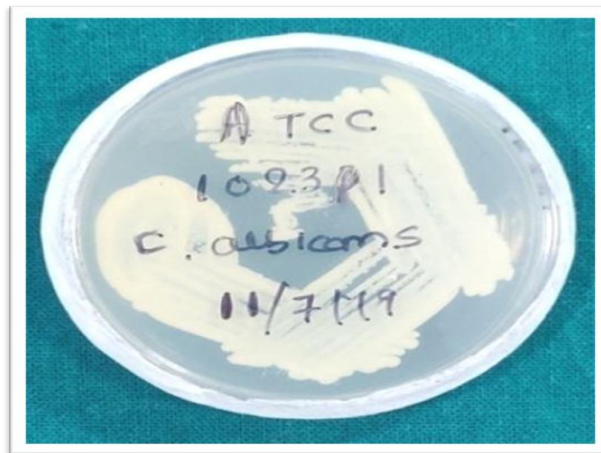


Figure 5: Radiograph Atcc Strain 10231



Figure 6 : Densitometer For Mc Farland Standard

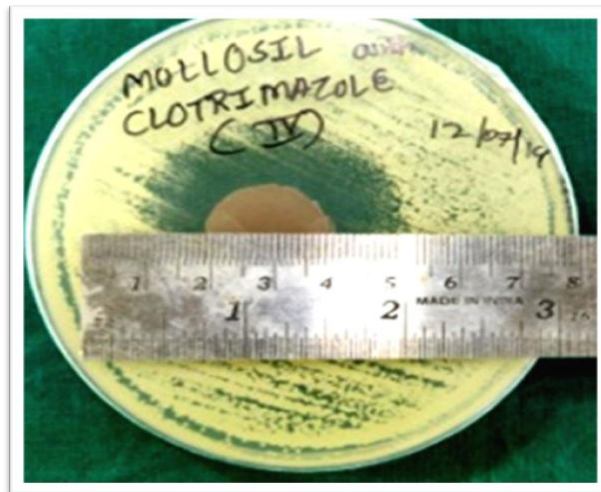


Figure 7: Measuring the zone of inhibition after incubation for 24 hrs



Figure 8: Shore A Hardness Value (Shore A Hardness Number) Being Checked Using Durometer

## Results

The mean value comparison of zone of inhibition of 4 groups of long term soft liners (Permasoft control, Permasoft with clotrimazole, Mollosil control and Mollosil with clotrimazole) the values of reading were measured in mm and mean value was obtained (Table 1). The mean value comparison of surface hardness of 4 groups of long term soft liners (Permasoft control, Permasoft with clotrimazole, Mollosil control and Mollosil with clotrimazole) the values of reading were measured and mean value was obtained. (Table 2)

Readings for samples of Permasoft Control (Group 1) at all time intervals was statistically insignificant ( $p > 0.01$ ). For Permasoft with Clotrimazole (Group 2) in present study had statistically significant  $p$  value ( $p < 0.01$ ) at time intervals of 1, 7, 14, 21, 35 and 42 days when compared to each other except for the reading between day 7 and 14 ( $p$  value  $> 0.01$ ). Samples of Mollosil with Clotrimazole (Group 4) had a statistically significant value with  $p < 0.01$  at time intervals of 1, 7, 14, 21, 35 and 42 days except for the reading between day 35 and 42 ( $p$  value  $> 0.01$ ) which suggests that there was an increase in zone of inhibition value from day 1 to day 35; the zone of inhibition remained constant thereafter. The comparison of four groups with respect to differences in zone of inhibition at different time interval was done using Tukey post hoc test. There was no relative change in zone of inhibition between group 1 and 3 ( $P > 0.001$ ) suggesting that the zone of inhibition remains constant. Comparison of zone of inhibition in between groups and within groups by One way ANOVA. ( $p < 0.001$ ) statistically significant.

For Permasoft control group, the hardness value gradually increased from day 1 to day 42, ( $p < 0.001$ ). For Permasoft with clotrimazole (Group 2) surface hardness increased from day 1 to 14 ( $p < 0.001$ ) but the value did not change significantly from 14th to 35th day ( $p > 0.01$ ). For Mollosil control (Group 3) there was increase in surface hardness from day 1 to day 42 ( $p < 0.001$ ) but the increase in surface hardness was gradual. For Mollosil with Clotrimazole (Group 4), there was increase in surface hardness from day 1 of reading to day 42 ( $p < 0.001$ ).

Table 1- Comparison Of Zone Of Inhibition (In Mm) Of 4 Groups Of Long Term Soft Liners

	day 1	day 7	day 14	day 21	day 35	day 42	Group
Mean value	19.625	19.625	19.625	19.625	19.625	19.625	1
Mean value	39.5	35.5	35.5	28	25.5	24.75	2
Mean value	19.625	19.625	19.625	19.375	19.625	19.625	3
Mean value	45	41.75	40.375	34.875	31.5	31.5	4

Table 2- Comparison Of Surface Hardness (Shore A Value) Of 4 Groups Of Long Term Soft Liners Liners

Sr no	day 1	day 7	day 14	day 21	day 35	day 42	Group
Mean value	25.91	26.81	27.32	27.95	27.9	29.11	1
Mean value	24.82375	25.8625	26.9	27.9	27.775	29.875	2
Mean value	29.16	29.165	29.165	29.165	29.165	29.165	3
Mean value	13.25	18.1625	19.9	21.825	22.75	23.2	4

## Discussion

Studies are done by incorporating different antifungals such as nystatin, amphotericin B, miconazole and ketoconazole combined with different tissue conditioner<sup>[12,17,18,19,20]</sup>. Clotrimazole and nystatin are the most commonly used drugs for topical application to treat oral candidiasis<sup>[21]</sup>. But the release of nystatin from the tissue conditioner into the oral cavity is gradual and the release also diminishes in few days.<sup>[22]</sup> Since this study was done on long term soft liners, the use of nystatin as antifungal agent was not suitable.<sup>[18]</sup> Hence only clotrimazole was incorporated into the long term permanent soft liner.<sup>[23]</sup> No studies have been done to check the effect of addition of clotrimazole on Permasoft and Mollosil.

The MIC is determined by preparing solutions of the chemical in vitro at increasing concentrations, incubating the solutions with separate batches of cultured bacteria, and measuring the results using agar dilution or broth micro dilution. The study done for ATCC 10231 for the minimum inhibitory concentration shows minimum inhibitory concentration for this strain when clotrimazole is used is 0.6 µg/ml for incubation of 24 hrs.<sup>[24]</sup> Thus this is the minimum amount of drug required for the antifungal property of clotrimazole used with the soft liner. The zone of inhibition is the visible zone around the test sample where there is no growth of the fungal or microbial species. It gives an idea about the range upto which the effect of antifungal agent will persist

The hardness provides information about the quality of the material, because a rigid material is not suitable to be used as a base for resilient relining removable dentures. The greater the amount of plasticizers in the material, the softer it becomes. In this context, a major disadvantage of soft lining materials is the rapid loss of plasticizer, leading to gradual hardening.<sup>[25]</sup>

The present study was done for two different long term soft liner, acrylic based (Permasoft) and silicone based (Mollosil) by addition of clotrimazole drug in their minimum inhibitory concentration. The properties –zone of inhibition, surface hardness and surface roughness were evaluated at time intervals of 1,7,14,21,35 and 42 days. A total of 224 samples were prepared. The change in values of zone of inhibition, surface hardness and surface roughness were analyzed and inter group and intra group data that was obtained. The data was analyzed statistically by using One way ANOVA, Tukey post hoc-test and Paired t test. The data obtained in relation to zone of inhibition and surface hardness was evaluated using one way analysis of variance (ANOVA) test, Tukey's post hoc analysis using SPSS software (SPSS 12.0; SPSS, INC, CHICAGO,III)

## Zone of Inhibition

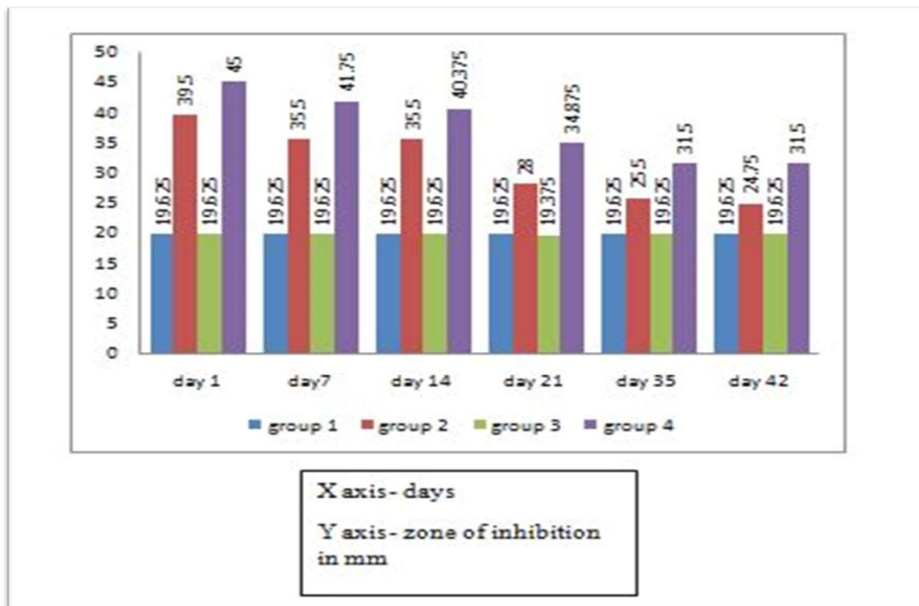
The values of zone of inhibition were analysed and readings plotted on graph.(graph 1). Paired t test carried out for Change in zone of inhibition of all groups at time interval of day1,7,14,21,35 and 42 showed statistically significant reading of P value>0.001 for group 1 and 3. This implies no change zone of inhibition. Thus the long term soft liner do not have antifungal properties of their own. Readings for samples of Permasoft with Clotrimazole (Group 2) in

present study had statistically significant p value ( $p < 0.01$ ) at time intervals of 1,7,14,21,35 and 42 days when compared to each other except for the reading between day 7 and 14 ( $p \text{ value} > 0.01$ ) which suggests that there is increase in zone of inhibition value from day 1 to day 7; the zone of inhibition remained constant from day 7 to 14 and then it again decreased thereafter upto 42 days. Thus the drug releasing and antifungal property in Permasoft with clotrimazole (group 2) rapidly decreased within the first 7 days and then decreased gradually thereafter. could be due to the excessive release of plasticizers from the Permasoft as it is acrylic based soft liner.<sup>[25,26,27]</sup>

The drug releasing property of Mollosil with clotrimazole (group 4) was relatively more constant as compared to group 2 with gradual loss of zone of inhibition and reached a plateau level at day 35. The results are similar to the studies done by M. Addy et al.<sup>[28]</sup>, Marta Radnai et al.<sup>[29]</sup>, Thomas et al.<sup>[13]</sup>, Vojdanim et al.<sup>5</sup> for different soft liners and different antifungal drugs. Silicone soft liners as opposed to acrylic ones are stable in composition. They do not contain plasticizer or soluble material that leach out in water during storage periods and are more stable as compared to acrylic based soft liners. Hence the ability to retain the antifungal drug and the slow release of the antifungal is better than that of Permasoft.<sup>[2]</sup>

The change in the values and change in statistical significance between the groups suggests that the addition of clotrimazole to the long term soft liners makes them a suitable carrier for the drug releasing property. The comparison of four groups with respect to differences in zone of inhibition at different time interval was done using Tukey post hoc test, There was no relative change in zone of inhibition between group 1 and 3 ( $P > 0.001$ ) suggesting that the zone of inhibition remains constant. This means that they don't have antifungal property of their own. The antifungal properties were solely due to addition of clotrimazole to the soft liner. These results are in co-ordination with the study done by Griiber et al. showed that silicone and methacrylate soft denture liners would support the growth of *C. albicans*.<sup>[9]</sup>

Comparison of zone of inhibition in between groups and within groups by One way ANOVA. ( $p < 0.001$ ) statistically significant indicates that zone of inhibition constantly decreases over time. If the antifungal stays in contact with the mucosa for a longer period of time there is a possibility of development of resistant strains. The antifungal activity of the drug diminishes after 42 days thus the possibility of development of resistant strains is less. After this time period the soft liner with clotrimazole will function similar to a soft liner control group and will serve all the purpose except for the antifungal property. The drug releasing or diffusing properties amongst the four groups can be compared as follows from maximum to minimum.



Graph1 - Comparison Of Zone Of Inhibition Values(In Millimetres) For All Groups At Various Time Intervals(In Days)

**Mollosil with clotrimazole>Permasoft with clotrimazole>Mollosil control=Permasoft control**

### Surface Hardness

The soft lining materials are susceptible to hardness changes. The more hard the material, lesser is the cushioning ability of it. Maintaining a satisfactory hardness is one of the most complicated factors for acrylic liners because they are not stable in an aqueous medium, where as for silicone based liners the materials relatively more stable in aqueous medium. The values of surface hardness were analysed and plotted on a graph(graph 2).ForPermasoft control group, the hardness value gradually increased from day 1 to day 42,( $p < 0.001$  For Permasoft with clotrimazole (Group2) surface hardness increased from day 1 to 14 ( $p < 0.001$ ) but the value did not change significantly from 14th to 35<sup>th</sup> day ( $p > 0.01$ ). The value again increased after day 35( $p < 0.001$ ). thus from start to end of the study, due to the leaching out of plasticizers and ethanol content of the acrylic soft liner, the hardness value increased gradually. This does not go in co ordinance with the studies done by Mirianet al.<sup>[11]</sup> The probable reason why the surface hardness of the Permasoft with clotrimazole (Group 2)increased can be due to the low molecular weight of the clotrimazole. The low molecular weight of clotrimazole will allow greater diffusion into the medium in which it is immersed, leading to more hardness of the material itself.

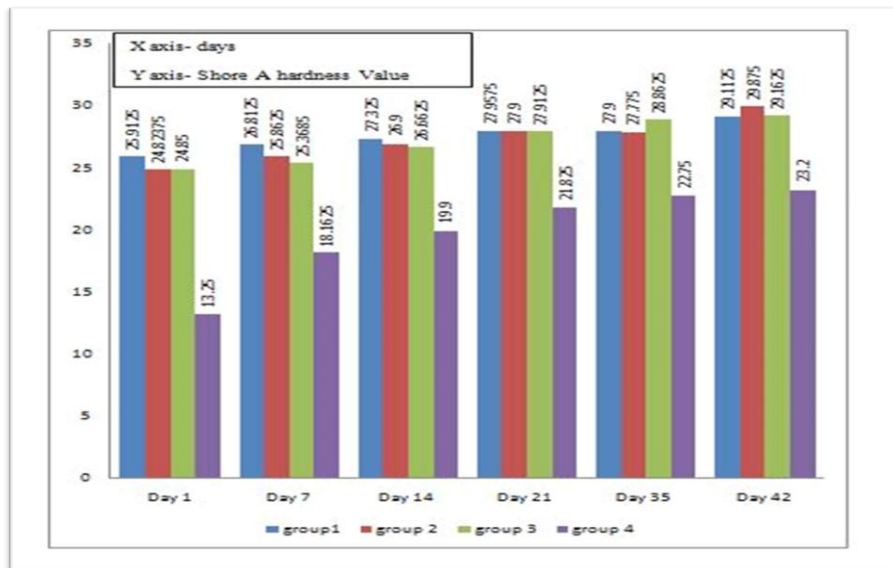
For Mollosil control (Group 3) there was increase in surface hardness from day1 to day42( $p < 0.001$ ) but the increase in surface hardness was gradual. As there are no plasticizers present in the silicone soft liners, there is no possibility of the material being leached out and becoming stiffer, the reason for gradual increase

in the hardness can be due to the cross linking of the elastomeric chains and reaction making it more stiffer due to the bonding of elastomers.

For Mollosil with Clotrimazole (Group 4), there was increase in surface hardness from day 1 of reading to day 42 ( $p < 0.001$ ) but there was not significant rise in the value of hardness from day 35 to 42. ( $p > 0.001$ ) Thus hardness became constant after day 35. The reason for very low values for surface hardness on day 1 and 7 is due to addition of clotrimazole, the particles of drug will prevent the complete elastomeric chain. Due to the low molecular weight of clotrimazole, the drug will be constantly dissolved into the salivary substitute. As the drug particles will be removed from their positions the elastomeric reaction will continue and thus the hardness value increased. But the hardness value did not increase than that of the Mollosil control as the antifungal particles were still present within the matrix formed by the silicone soft liner, which avoided the complete elastomeric chain reaction. This justifies why the surface hardness values increased for each group from day 1 to day 42.

On comparison of four groups with respect to differences in surface hardness at different time interval by Tukey post hoc test. Suggests, addition of clotrimazole to Permasoft decreased the surface hardness initially upto 14 days ( $p < 0.001$ ) due to hindrance in polymerization chain reaction due to the presence of clotrimazole molecules in between but did not adversely affect the surface hardness of the material over a long period of time within limits. There was significant difference in hardness value of Mollosil control (Group 3) and Mollosil with clotrimazole (Group 4) from day 1 to day 42. Thus addition of clotrimazole to mollosil lead to decrease in surface hardness of the material ( $p < 0.001$ ) within the clinically acceptable limits<sup>[23]</sup> of the hardness value. Hence it did not adversely affect the property of the material.

Thus from the present study, values of surface hardness from day 1 to day 42, the hardness values increased constantly within the clinically acceptable limits of the long term permanent soft liners. The minimum to maximum values of surface hardness were as follows at day 1.



Graph 2- Comparison Of Surface Hardness Values (Shore A Hardness Value) For All Groups At Various Time Intervals (In Days)

### **Mollosil with clotrimazole << Mollosil control = Permasoft with clotrimazole < Permasoft control**

The minimum to maximum values of surface hardness were as follows at day 42.

### **Mollosil with clotrimazole < Permasoft control < Mollosil control = Permasoft with clotrimazole**

The comparison of inter property relation within a group i.e. comparison of zone of inhibition, surface hardness and the change in the values relative to each other showed a statistically significant value of  $P < 0.001$ . thus all the properties are related to each other. And change in one property will lead to change in the other.

### **Conclusion**

Within the limitations of the laboratory testing conditions and materials the following conclusions can be drawn. Permasoft and Mollosil do not have any antifungal property per se. Addition of clotrimazole at minimum inhibitory concentration in the soft liner can prove to be an effective way of drug release for a long period of time. The addition of antifungal into the soft liner does change the properties of the material but the change is within the clinically acceptable limits. The antifungal releasing property of Mollosil i.e. Silicone based soft liner is better than that of Permasoft i.e. Acrylic based soft liner. The zone of inhibition of both the materials decreases over a period of time indicating that the drug constantly leaches out of the soft liner. The addition of clotrimazole drastically affects the hardness of Mollosil initially but the hardness values increase and reaches upto the clinically acceptable limits at day 42. The addition of clotrimazole does not drastically change the hardness values of the Permasoft.

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