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RESEARCHARTICLE

COMPARATIVE EVALUATION OF THE EFFECT OF CLOTRIMAZOLE AND FLUCONAZOLE INCORPORATED INTO TWO AUTO-POLYMERIZING ACRYLIC BASED DENTURE LINERS ON THE GROWTH AND COLONIZATION OF CANDIDA ALBICANS

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ABSTRACT

Statement of problem: Adherence and candidal colonization on resilient denture liner materials plays an important role in the pathogenesis of denturestomatitis. It has been reported that combining anti-fungal agents into these denture relining materials might be used in the treatment and prevention of denture stomatitis.
Purpose of study: The aim of the study was to compare and evaluate the effectiveness of incorporation of two antifungal agents- Clotrimazole (C) andFluconazole (F) into the two auto-polymerized acrylic based resilient denture liners (Permasoft & Soft-liner) on the growth and colonization of candida albicans and to suggest a better antifungal agent out of them used in the study.
Materials and Methods: Acrylic based resilient denture liners Permasoft and Soft-liner were incorporated with Clotrimazole (C) and Fluconazole (F)drugs both at 1% wt/wt concentration and experimental circular disc shaped specimen were fabricated using brass die with cross-section (20x4) mm. For fungal growth assessment, they were inoculated with C.albicans suspension prepared according to Clinical Laboratory and Standards Institute (CLSI) guidelines. The discs were incubated at 37^0 C and after incubation they were rinsed and centrifuged in sterile water to remove surface organisms. The drug treated and control circular disc shaped specimens were stored in distilled water for 30, 60 and 90 days and were wiped daily with wet cotton for 1 minute. Assessment of colony forming units (CFU) was carried out using McFarland turbidity standards as per CLSI guidelines. The data were compared using One-way analysis of variance (ANOVA), Repeated measure analysis of variance (ANOVA) and Paired 't' Test.
Results: The mean colony forming units CFU($x10^6$/ml) at different time intervals differ significantly among groups; (Permasoft & Soft-liner) linermaterial ($p<0.05$). The values also differ highly significantly among subgroups; (Control, Clotrimazole and Fluconazole) and with both groups & subgroups ($p<0.001$).
Conclusion: The addition of Clotrimazole and Fluconazole into acrylic based resilient denture liners Permasoft and Soft-liner significantly reduced thegrowth and colonization of C.albicans. The decrease in the mean colony forming units (CFU$x10^6$/ml) after 30 days followed by increase in them after 60 and 90 days interval was observed as compared to that of control ($p<0.001$). Combination of SOFT-LINER with Clotrimazole is found to be more effective than PERMASOFT with Clotrimazole and combination of PERMASOFT with Fluconazole is found to be more effective than SOFT-LINER with Fluconazole after subsequent time intervals.

Key words:Permasoft, Soft-liner, Clotrimazole, Fluconazole, Denture stomatitis.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen in the oral cavity resulting in candidal infections. Chronic atrophic candidiasis, also known as denture stomatitis, is a common form of oral candidiasis, associated with the adherence of candida albicans to denture base surfaces. The etiology is multifactorial and it is found most commonly in maxilla and usually in elderly denture wearers (Akiba *et al*., 2005). Prevalence has been reported at 11-67% in complete denture wearers (Bulad *et al*., 2004 and Doracka-Bobkowska *et al*., 2007). Candida albicans colonization of the fitting surface of the prosthesis is promoted because of continuous denture wearing i.e. not removing the dentures at night that facilitates denturestomatitis by increasing the local injury and the time of mucosal exposure to denture plaque. Resilient denture lining materials are used to limit such injury (Bulad *et al*., 2004). Liners exhibit porous surfaces that are favourable for the growth of micro organisms such as C albicans. To keep the tissue surface free of micro organisms and debris, meticulous denture hygiene has to be maintained. Conventional methods of denture cleansing include mechanical and chemical cleansing. Mechanical cleansing using a brush is not indicated for a relined denture because of the possible damage to the resilient liner. Chemical cleansing of the relined denture also affects the properties of the relined materials such as resiliency, hence not advisable. Topical application of antifungal agents has not been encouraged, as the medicine gets washed away by the saliva, leaving an insufficient concentration at the site of action. Systemic administration requires large doses of drugs with a serious risk of side effects. To overcome these

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disadvantages, antifungal agents have been incorporated into the denture liners. The incorporation of antifungal agents into tissue conditioners has been shown to be effective and viable in some in vitro and in vivo studies (Urban *et al*., 2006). Antifungal agents such as Zinc undecylenate, Copper sulphate, Chlorhexidine, Amorolfine antifungal varnish, Amphotericin B (AMB), Nystatin, 5-Fluorocytosine, Miconazole, Ketoconazole and Clotrimazole have been incorporated to the resilient liners.

Chlorhexidine produces staining and altered taste perception. Nystatin has a bitter taste which reduces patient compliance. Amphotericin B usually reserved for intravenous treatment of life threatening systemic fungal infections. Ketoconazole administration for more than 2 weeks causes idiosyncratic liver toxicity and harmful drug interactions which may produce life threatening cardiac arrythmias. Chlorhexidine, Clotrimazole, Fluconazole and Nystatin can be released from tissue conditioner matrix and it had also been found that mixing these antifungal agents into tissue conditioners did not alter the mechanical and physical properties beyond acceptable limits (Vojdani *et al*., 2009). Ease of use of these azoles drugs also increases the likelihood of good compliance (Chow *et al*., 1998).The rationale for choosing Clotrimazole and Fluconazole for this study is that both drugs are one of the most commonly used broad spectrum antifungal agents and the most common alternative to topicalnystatin and are commonly given to immunocompromised patients (Chow *et al*., 199 and Falah-Tafti *et al*., 2010). Fluconazole is the agent of first choice for all forms of oral candidiasis apart from chronic erythematous candidiasis and also has a good safety profile when given systemically, with few contraindications and side effects (Williams *et al*., 2011). Clotrimazole, which is a synthetic substituted imidazole derivative as opposed to nystatin, has a very stable chemical structure and is not inactivated by heat, light or acid[19].The aim of the study was to study the relative effectiveness of two antifungal drugs- Clotrimazole and Fluconazole, with two types of auto-polymerizing acrylic based denture liners and to investigate whether incorporation of these antifungal drugs into the above mentioned resilient liners is a clinically effective method of drug delivery to inhibit fungal growth.

MATERIALS AND METHODS

Specimen fabrication

A total of 180 circular (disc shaped) test specimens of 2 auto-polymerizing acrylic based resilient denture liners were fabricated using a brass die, as shown in figure-1, with cross section (20x4) mm, and divided into two groups; Long-term liners (L)- Permasoft (Dentsply, Austenal USA) and Short-term liners (S)- Soft-liner (GC Corporation Tokyo, Japan) containing, 90 specimens each. These two groups were further divided into three subgroups (LI,LII, LIII) & (SI,SII, SIII) of 30 specimens each, of which one subgroup acted as control and the rest two subgroups were incorporated with two antifungal agents; Clotrimazole and Fluconazole. Auto-polymerizing acrylic based liner materials; PERMASOFT and SOFT-LINER, are available in powder and liquid monomer form. The powder of each liner material was hand mixed with the antifungal agents at wt/wt% i.e. 1%wt/wt (0.99g powder of PERMASOFT/SOFT-LINER + 0.01g Clotrimazole/ Fluconazole. Then, the liquid monomer was added to the mixed powder of liner and antifungal agent, and was mixed according to manufacturer's instructions. For Permasoft, available in powder li quid form system, the ratio was kept 2:1; whereas for Soft- liner powder liquid ratio was kept 4:1.

Figure 1. Brass die used for fabricating test specimens

Then, the mixture of powder and the liquid monomer was stirred for 15 seconds and left standing for 4 minutes until plastic dough was formed. It was then packed in a specially designed brass die. Brass die was pressed in a clamp and processing was done in a water bath at 75^0 C for Permasoft for 15 minutes while for SOFT-LINER, for the completion of polymerization, process room temperature was maintained for 5 minutes. Specimens were removed from the die and excess material was trimmed. Sealer was then applied on the surface of the specimens and left for drying. 180 circular specimens, 90 of each type of liner, of dimension (20x4) mm were prepared in this manner out of which 30 acted as control, 30 contained Clotrimazole and 30 contained Fluconazole.

Figure 2. Candidal colonies in active growth

InoculationandincubationwithCandida Albicans

A lyophilized culture of Candida albicans MTCC 227 (Microbial type cell culture) was obtained. 5 mm of Sabouraud's broth was placed into each of 180 test tubes and autoclaved 24 hours before placing the discs in the test tubes, the broth was inoculated with inoculum equivalent to a 0.5 McFarland standard i.e. containing yeast colonies equivalent to

100 CFU(x10^6/ml) as per the Clinical Laboratory and Standards Institute Guidelines (CLSI) guidelines to ensure that the organisms are in active phase of growth when the broth was added to the discs as shown in figure-2. Then, all the 180 circular disc specimens were placed in test tubes which were incubated at 37^0C for 24 hours.

Storage of specimens

After incubation, the broth was removed with a sterile digital micropipette. The discs were rinsed thoroughly with sterile water to remove the loosely adherent C.albicans. After that, discs were placed in sterile test tubes that contained sterile saline and centrifuged for 5 minutes to remove surface organisms. Thereafter, these 180 circular disc specimens were stored in distilled water for 30, 60 and 90 days and were wiped daily with wet cotton for 1 minute.

Table 1. Repeated Measure Anova Test of within subject effects

	Df	f VALUE	p VALUE
TIME	2	3.46	0.032*
TIME AND GROUP	2	0.014	0.986
TIME AND SUBGROUP	4	0.677	0.608
TIME, GROUP AND SUBGROUP	4	0.011	1.000

*p<0.05; Significant

Table 2. Repeated Measure Anova - Test of inter-subject effects

SOURCE	df	f VALUE	p VALUE
GROUPS (LONG TERM AND SHORT TERM)	1	4.225	0.041*
SUB GROUPS (CONTROL, CLOTRIMAZOLE & FLUCONAZOLE)	1	213.84	<0.001**
GROUP AND SUBGROUPS	2	21.23	<0.001**

*p<0.05; Significant; **p<0.001; Highly significant

Table 3. Comparison of Control (C), Clotrimazole (CL) & Fluconazole (FL) in long term (Permasoft) & short term (Soft-Liner) Materials, One way Anova

GROUP	TIME	f VALUE	p VALUE	COMPARISON	p VALUE
LONG TERM LINER MATERIAL (PERMASOFT)	AT 30	46.714	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	0.994
	AT 60	34.914	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	0.994
	AT 90	32.094	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	0.977
SHORT TERM LINER MATERIAL (SOFT-LINER)	AT 30	102.811	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	<0.001**
	AT 60	90.075	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	<0.001**
	AT 90	84.521	<0.001**	C/CL	<0.001**
				C/FL	<0.001**
				CL/FL	<0.001**

**P<0.001; Highly significant

Table 4. Comparison of long term (Permasoft) & short term (Soft-Liner) Materials, One way Anova

TIME	SUBGROUP	F VALUE	P VALUE
AT 30	CONTROL	0.209	0.649
	CLOTRIMAZOLE	3.196	0.079
	FLUCONAZOLE	31.772	<0.001**
AT 60	CONTROL	0.051	0.822
	CLOTRIMAZOLE	3.156	0.081
	FLUCONAZOLE	33.025	<0.001**
AT 90	CONTROL	0.059	0.809
	CLOTRIMAZOLE	3.226	0.078
	FLUCONAZOLE	27.829	<0.001**

**P<0.001; Highly significant

Table 5. Comparison of different time intervals using Paired 't' test

GROUP	SUBGROUP	COMPARISON	't' VALUE	P VALUE
LONG TERM LINER MATERIAL (PERMASOFT)	CONTROL LI	At30 - At60	0.147	0.884
		At 30- At 90	0.320	0.752
		At 60- At 90	1.795	0.083
	CLOTRIMAZOLE LII	At30 - At60	1.140	0.264
		At 30- At 90	2.262	0.031*
		At 60- At 90	2.693	0.012*
	FLUCONAZOLE LIII	At30 - At60	0.867	0.393
		At 30- At 90	1.690	0.102
		At 60- At 90	2.693	0.012*
SHORT TERM LINER MATERIAL (SOFT-LINER)	CONTROL SI	At30 - At60	0.367	0.717
		At 30- At 90	0.000	1.000
		At 60- At 90	0.414	0.682
	CLOTRIMAZOLE SII	At30 - At60	0.384	0.704
		At 30- At 90	0.787	0.438
		At 60- At 90	0.474	0.639
	FLUCONAZOLE SIII	At30 - At60	0.740	0.465
		At 30- At 90	1.682	0.103
		At 60- At 90	2.971	0.006*

*P<0.05; Significant

Statistical analysis

Assessment of colony forming units (CFU) was carried out on all 180 circular (disc shaped) test specimens of auto-polymerizing acrylic based liner materials acting as control and containing Clotrimazole and Fluconazole after 30, 60 and 90 days respectively, using McFarland turbidity standards. The measurements were subjected to statistical analysis to draw conclusions from the experimental data.

Descriptive statistical measures such as mean, standard deviation, standard error of mean, 95% confidence interval, lower confidence limit, upper confidence limit were computed for all the colony forming units CFU (x10^6/ml) for all the study groups.

The colony forming unit- CFU(x10^6/ml) variations among the Control, Clotrimazle and Fluconazole specimens were also evaluated with One-way analysis of variance (ANOVA), Repeated measure analysis of variance (ANOVA) and Paired 't' test (Table 1-5). These statistical computations are presented:

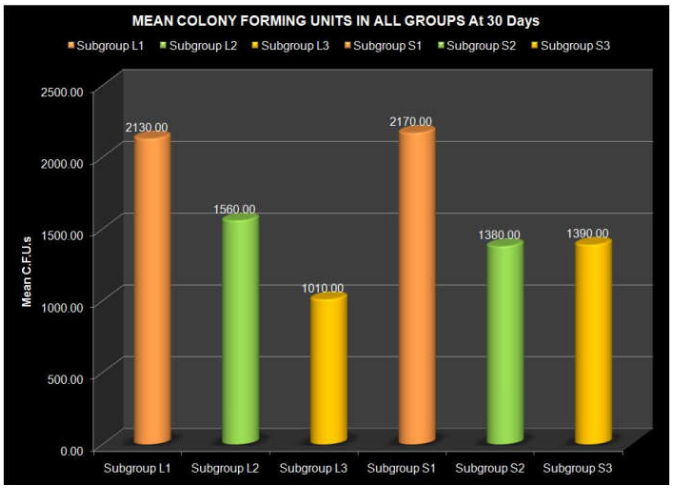

Figure 3. Comparison of means of Colony Forming Units CFU(x10^6/ml) of the study subgroups after 30 days interval

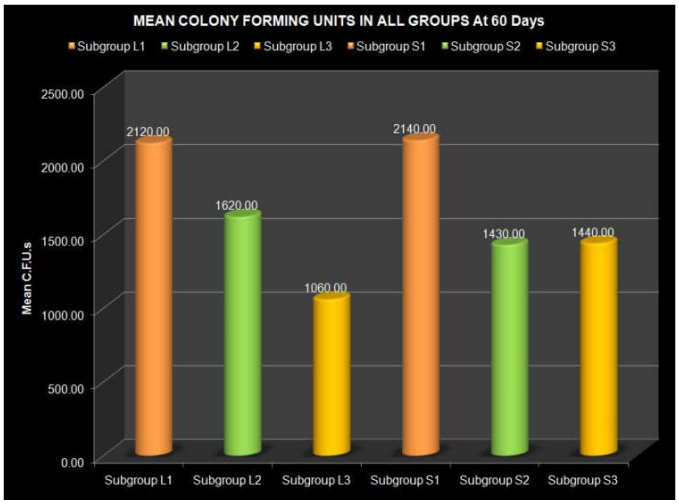

Figure 4. Comparison of means of Colony Forming Units CFU(x10^6/ml) of the study subgroups after 60 days interval

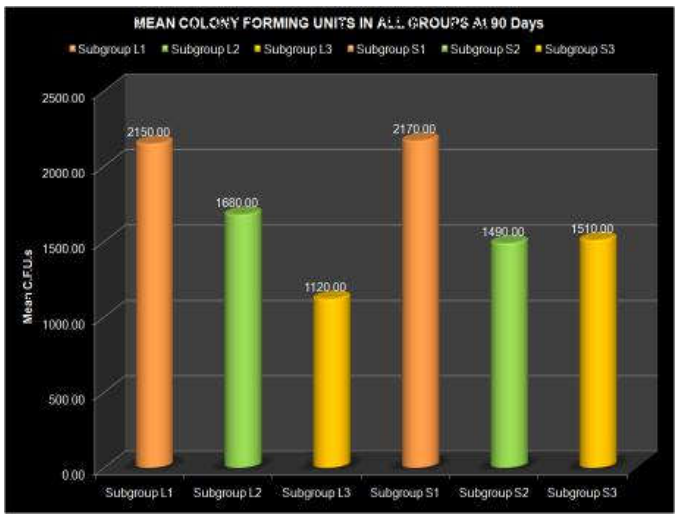

Figure 5. Comparison of means of Colony Forming Units CFU(x10^6/ml) of the study subgroups after 90 days interval

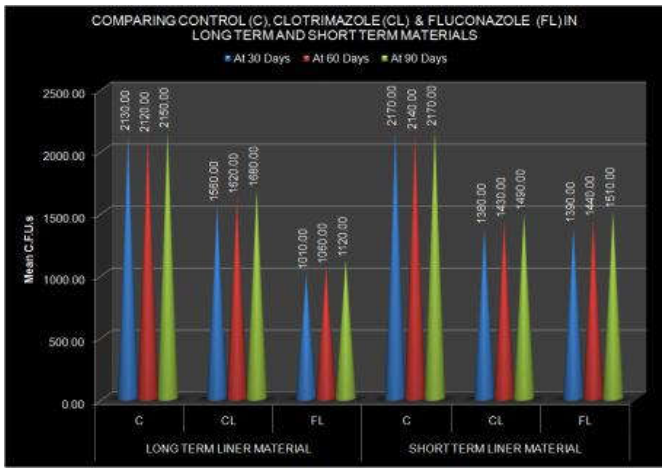

Figure 6. Comparison of means of Colony Forming Units CFU(x10^6/ml) comparing study subgroups in long term (Permasoft) & short term (Soft-Liner) material at 30, 60 & 90 days interval

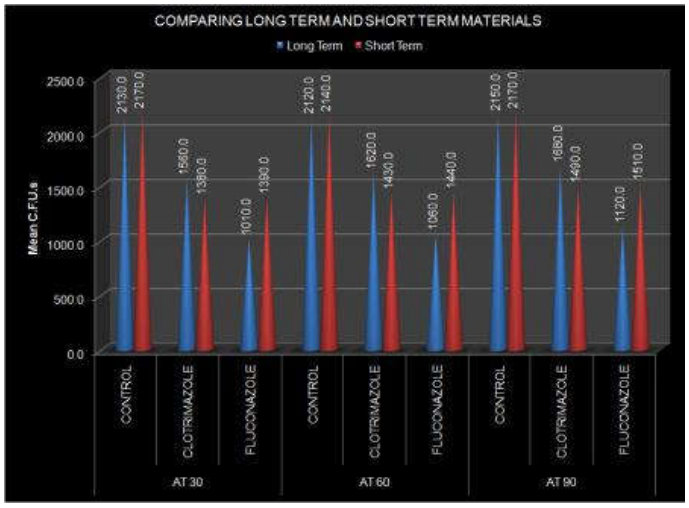

Figure 7. Comparison of means of Colony Forming Units CFU(x10^6/ml) of the study groups long term (Permasoft) & short term (Soft-Liner) material at 30, 60 & 90 days interval

Figure 8. Comparison of means of Colony Forming Units CFU(x10^6/ml) at different time intervals

DISCUSSION

Denture relining materials are commonly used for the recovery of mechanically abused oral tissues, but fungal growth colonization on the tissue conditioners is of common occurrence which is known to cause irritation of the oral mucosa.Various methods have been used in the past to keep the tissue surface of the denture free of micro organisms including conventional methods of denture cleansing i.e. mechanical and chemical cleansing. Since 1973, the incorporation of antifungal agents into tissue conditioners has been shown to be an effective and viable method in some in-vitro and in-vivo studies (Gupta *et al*, 2011; Urban *et al*., 2006). Polyene agents (eg, nystatin, amphotericin B) and azole antifungals (eg, miconazole, ketoconazole, fluconazole and itraconazole) are the drugs most commonly used. In this study, the rationale for using Clotrimazole and Fluconazole is that both the drugs have an excellent safety record and ease of use increases the likelihood of good compliance. Direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug interactions. The present study was conducted to compare the effect of Clotrimazole and Fluconazole incorporation into two auto-polymerizing acrylic based denture liners; PERMASOFT and SOFT-LINER on the growth and colonization of candida albicans. The results revealed that adding Clotrimazole and Fluconazole in both lining materials initially showed a decrease in the mean colony forming units CFU($x10^6$/ml) after 30 days followed by increase in them after 60 and 90 days interval as compared to that of control. Mean colony forming units-CFU($x10^6$/ml) present on the auto-polymerizing acrylic based resilient lining material (PERMASOFT), depending upon the time period of storage and type of antifungal agents used, was found to be maximum for control subgroup after 90 days (2150.00CFU($x10^6$/ml)) and minimum for Fluconazole subgroup after 30 days (1010.00 CFU($x10^6$/ml)).

Mean colony forming units-CFU($x10^6$/ml) present on the auto-polymerizing acrylic based resilient lining material (SOFT-LINER), depending upon the time period of storage and type of antifungal agents used, was found to be maximum for control subgroup after 30 and 90 days (2170.00 CFU($x10^6$/ml)) and minimum for Clotrimazole subgroup after 30 days (1380.00 CFU($x10^6$/ml)). Among the two liners and drug combination used, the lowest value of mean CFU($x10^6$/ml) (1010.00) was seen in Fluconazole subgroup of PERMASOFT after 90 days storage in distilled water and the highest value of mean CFU($x10^6$/ml) (2170.00) was observed in the control group of SOFT-LINER after 30 days storage in distilled water. The data obtained were subjected to one way ANOVA, where the comparison among different subgroups is done. The comparison showed highly significant difference ($p<0.001$) among all the subgroups. In the present study, the initial decrease in the mean colony forming units CFU($x10^6$/ml) after 30 days followed by increase in them after 60 and 90 days interval as compared to that of control shows that the liner material was able to maintain the drug concentrations initially followed by a decrease because of leaching of drugs from the liner material when the specimens were stored in distilled water.

Both the azoles used had inhibited the growth of candida albicans in both the autopolymerizing acrylic based liner materials (PERMASOFT) and (SOFT-LINER) in the similar manner. Among the two drugs, the decrease in mean CFU($x10^6$/ml) was more in Fluconazole with PERMASOFT (1010.00 CFU($x10^6$/ml)) and Clotrimazole with SOFT-LINER (1380.00 CFU($x10^6$/ml)). Combination of SOFT-LINER with Clotrimazole is found to be more effective than PERMASOFT with Clotrimazole and combination of PERMASOFT with Fluconazole is found to be more effective than SOFT-LINER with Fluconazole after subsequent time intervals. For PERMASOFT, the values of colony forming units CFU($x10^6$/ml) differ significantly at 30-90 days time interval in the Clotrimazole subgroup and at 60-90 days time interval in both the Clotrimazole and Fluconazloe subgroup. For SOFT-LINER, the values of colony forming units CFU($x10^6$/ml) differ significantly at 60-90 time interval in the Fluconazole subgroup. In the present study, the combination of SOFT-LINER with Clotrimazole is found to be more effective than PERMASOFT with Clotrimazole and combination of PERMASOFT with Fluconazole is found to be more effective than SOFT-LINER with Fluconazole after subsequent time intervals. However, as only two types of resilient liners were used in the study, further investigations are required to analyse the effect of antifungal agents on the growth and colonization of other available acrylic based and silicon-based resilient liners. Because it is not possible to completely simulate clinical conditions and reproduce the oral environment in the laboratory, so clinical investigations are also required to be carried out before reaching the final conclusion.

Conclusion

Incorporation of antifungal agents (Clotrimazole and Fluconazole) into two auto-polymerizing acrylic based resilient lining materials (PERMASOFT and SOFT-LINER) inhibited the growth and colonization by candida albicans. Incorporation of Fluconazole to short term (SOFT-LINER) & long term (PERMASOFT) liner materials showed highly significant differences among colony forming units CFU($x10^6$/ml) at 30,60 and 90 days time intervals ($p<0.001$).

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