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Original Article

Dentistry Section

Comparative Evaluation of Two Antifungal Agents Incorporated in Auto Polymerising Denture Base Resin, Heat Polymerising Denture Base Resin and Permanent Silicone Soft Liner-An In Vitro Study

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ABSTRACT

Introduction: Denture-induced candidiasis is a common disease in elderly denture wearers with *Candida albicans* as the principal causative agent. The problem is aggravated for elderly patients with limited motor skills who fail to follow a strict antifungal drug regime. To overcome this and to avoid the formation of biofilm on denture base resins, several attempts to incorporate antifungal agents/antiseptics into denture base resins, denture reliners and tissue conditioners have been reported. No study has been done to incorporate any herbal extract into polymeric systems and shows its elution and to compare it with commercially available antifungal drug.

Aim: To evaluate the leaching of fluconazole and herbal neem extract incorporated into auto polymerising acrylic resin, heat polymerising acrylic resin and permanent silicone soft liner over a period of 21 days. And comparatively evaluate the effect of the leached antifungal agents on the growth of *Candida albicans* in-vitro.

Materials and Methods: A total of 60 samples; 20 each of auto polymerising acrylic resin, heat polymerising acrylic resin and permanent silicone soft liner, (10 samples in each group incorporated with 10% w/w herbal neem extract and the other 10 with 10% w/w fluconazole), were fabricated using a stainless steel die of specific dimensions (50±1 mm in diameter and 1.0±0.05 mm thickness), as per the American Dental Association (ADA) specification no. 12. They were subsequently checked for leaching of the antifungal agents over a time period of three weeks at the intervals of 2,14 and 21 days using the High Performance Liquid Chromatography (HPLC) apparatus. The eluates were also checked for their anti-candidal activity by measuring the zones of inhibition of each agent in all the three test groups.

Results: Fluconazole exhibited significantly better elution profile and antifungal activity against *Candida albicans* as compared to herbal neem extract. Amongst the materials tested, permanent silicone soft liner exhibited significantly higher elution and better antifungal activity in terms of colony inhibition of *Candida albicans* followed by auto polymerising acrylic resin and heat polymerising acrylic resin.

Conclusion: 1) Fluconazole was established to be more potent than herbal neem extract against *Candida albicans*; 2) Permanent silicone soft liner was established to be the most effective polymeric system for sustained release of antifungal agents up to 21 days.

Keywords: Elution, Fluconazole, Herbal neem extract, Leaching, Zone of inhibition

INTRODUCTION

Acrylic resins and denture reliners find several uses in the speciality of Prosthodontics [1]. Acrylic resins are considered as gold standard for denture fabrication and liners are used to improve the fit of ill-fitting dentures [2,3].

A serious problem with the usage of these materials is colonisation by microorganisms primarily *Candida albicans* [4]. They can induce a chronic inflammatory response in the oral mucosa known as denture stomatitis, which is the most common infectious disease affecting the oral mucosa and is highly prevalent in denture wearers [5-8].

The treatment of Candida-associated denture stomatitis is complex because of its multi factorial aetiology [8]. Denture cleansing and maintenance of oral hygiene by the patient are the preventive measures and the use of antifungal drugs constitutes the therapeutic modality [8]. For elderly and institutionalised patients with debilitating diseases along with limited motor skills, the treatment gets complicated as patients have difficulty in maintaining oral and denture hygiene and in following a strict regimen for antifungal drugs [8].

To overcome these shortcomings and to avoid the formation of biofilm on denture base resin surfaces, several attempts to incorporate antifungal agents/antiseptics into denture base resins, denture reliners and tissue conditioners have been reported [9-14]. The advantage of this process is the possibility of elution of the agents from the denture base materials, thus preventing or reducing bacterial and fungal colonisation [9-15].

Previous studies have shown elution of antifungal drugs when incorporated into auto polymerising acrylic resin and liner materials. However, incorporation of herbal extract into polymeric system, evaluation of its antifungal effect and comparison with commercially available antifungal drug has not been studied.

Neem and its constituents have been demonstrated to exhibit immunomodulatory, anti-inflammatory, antimalarial, antifungal and antibacterial properties [16].

Soluneem is world's first water soluble antimicrobial agent consisting of various limonoids, primarily *azadirachtin-A*. It is derived from the neem seed kernel and produced by a novel patented technology by Vittal Mallya Scientific Research Foundation, Bangalore [16-18].

Soluneem is available as off-white, amorphous powder, which can be stored for more than two years without loss of bioactivity. *Soluneem* has been tested for acute oral and acute dermal toxicity

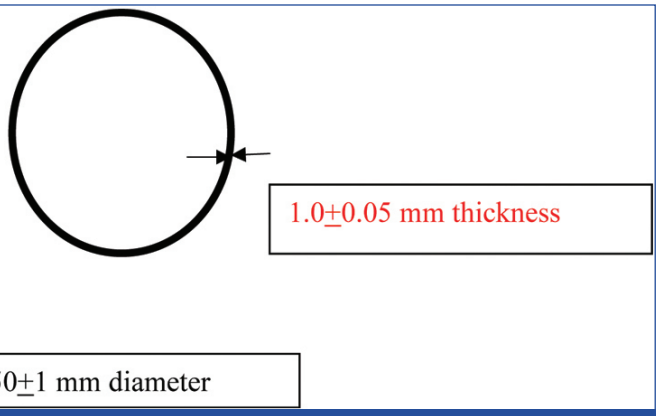
by the Fredrick Institute for Plant Protection and Toxicology (FIPPAT), Chennai, which is an accredited institute following OECD guidelines for testing. The results indicated that *Soluneem* is extremely safe for humans, animals, birds, fishes and beneficial arthropods including honeybees [16-19].

The aim of this study was to incorporate two antifungal agents, fluconazole and *Soluneem* in auto polymerising acrylic resin, heat polymerising acrylic resin and permanent silicone soft liner and to determine their elution in-vitro and subsequent effect of the eluent on the growth of *Candida albicans*.

MATERIALS AND METHODS

The in-vitro study was carried out in DA Pandu Memorial RV Dental college and hospital, Bangalore, Karnataka, India with a total number of 60 samples 20 each of auto polymerising acrylic resin, heat polymerising acrylic resin and permanent silicone soft liner over a period of 21 days in July 2016.

A stainless steel die of dimensions (50±1 mm in diameter and 1.0±0.05 mm thickness, [Table/Fig-1]), as per the American Dental Association (ADA) specification no. 12 was used to fabricate the samples [20].



[Table/Fig-1]: Diagrammatic representation of the die.

Preparation of Samples

The heat polymerising DPI Heat cure™ (DPI, India) acrylic resin and auto polymerising DPI Cold cure™ (DPI, India) acrylic resin material were mixed according to manufacturer's recommended polymer: monomer ratio in a mixing jar. The antifungal agents; fluconazole (Raj Pioneer Laboratories (Indore, Madhya Pradesh, India) and *Soluneem* (Vittal Mallya Research foundation, Bangalore, India) [16] were added to the polymer in the specified ratio (10% w/w) [21,22] and then the mixture of the powders with the liquid monomer were stirred for 15 seconds and left standing for 4 minutes until the dough stage was reached.

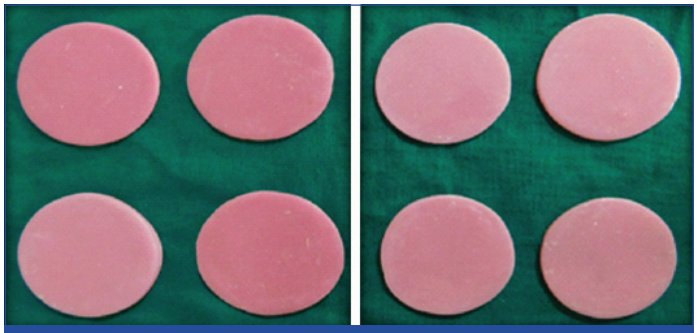
The flasks containing the auto polymerising acrylic resin were then tightened onto the clamp and kept for 15-20 minutes and subsequently cured in a pressure pot (20 psi, 45°C for 25 minutes).

The flasks containing the heat polymerising acrylic resin were held under pressure and were allowed to bench cure at a pressure of 100-150 bars for 30 minutes. Curing was done in an electrically controlled polymerisation unit (Unident Dental acryliser, India). The flasks were placed in water at room temperature and a long curing cycle was followed (74°C for 8 hour with no terminal boiling treatment).

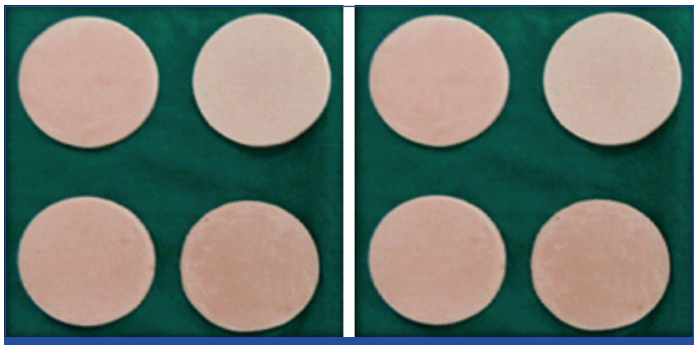
The permanent silicone soft liner material, Ufi Gel SC (VOCO GmbH, Germany) was mixed according to manufacturer's recommended base: catalyst ratio (1:1) on a glass slab with a stainless steel spatula and the antifungal agents, fluconazole and *Soluneem* were added to the mix in the specified ratio of 10% w/w and the homogenous mixture thus obtained was coated into the mould cavity.

The flasks were then tightened onto the clamp and kept for 2-5 minutes and subsequently cured in a pressure pot (20 psi, 45°C for 25 minutes) as per the manufacturer's instructions.

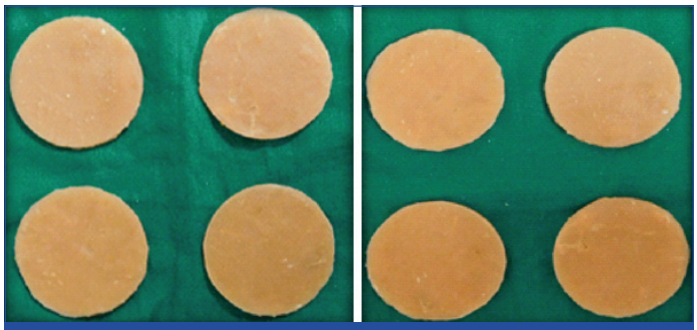
After completing the process, the cured samples were retrieved from the flask by deflasking and were finished and polished according to the manufacturer's instructions [Table/Fig-2a-f]. Twenty samples each of heat polymerising, auto polymerising acrylic resin and permanent silicone soft liner samples were made in a similar manner, 10 each incorporated with fluconazole and *Soluneem*.



[Table/Fig-2a,b]: Auto polymerising acrylic resin samples 10% w/w fluconazole (left) and neem (right).



[Table/Fig-2c,d]: Heat polymerising acrylic resin samples 10% w/w fluconazole (left) and neem (right).



[Table/Fig-2e,f]: Permanent silicone soft liner samples 10% w/w fluconazole (left) and neem (right).

The finished samples were stored in labelled individual containers in distilled water at room temperature in a thermostatically controlled oven (Thermoline Scientific, Australia) at 37°C for a period of three weeks [Table/Fig-3].

At intervals of 2nd day, 14th day and 21st day, the solutions were checked for leaching of the respective antifungal agents.

[Table/Fig-3]: Samples in their respective glass beakers kept in a thermostatically controlled oven at 37°C.

Evaluation of Leaching

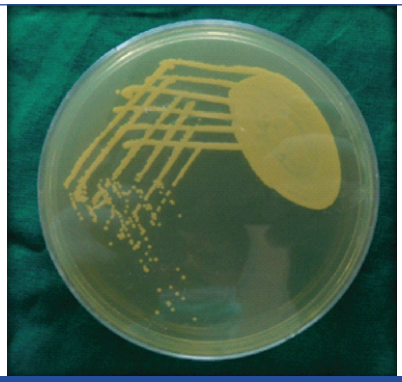
A small amount of the solution was taken from each container at specified time interval in individually labelled plastic containers and taken to the laboratory for the evaluation of leaching using a HPLC apparatus.

Fixed standard volume (20 µL) of the solution from each subgroup was injected onto the HPLC column and a representative chromatogram for each antifungal agent and the amount of antifungal agent leached in mg/L (ppm) in all the three groups was obtained. At each time interval similar procedure was performed and leaching behaviour of the antifungal agents was analysed.

Microbiological Investigation

A standard strain of *Candida albicans* was taken and it was cultured on Sabouraud's dextrose agar medium for 24 hours [Table/Fig-4].

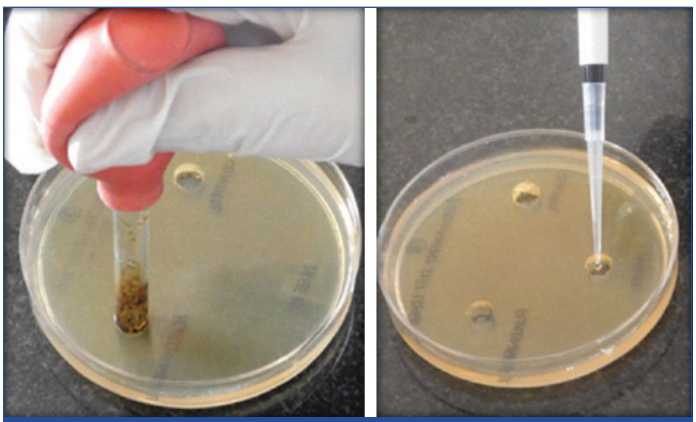
An inoculum was prepared using the growth from 24-hour culture and a suspension was made in a sterile saline solution [Table/Fig-5].



[Table/Fig-4]: *Candida albicans* growth on culture media.

[Table/Fig-5]: a) Inoculum. b) Suspension being prepared.

The *Candida albicans* growth suspension was then poured into Sabouraud's dextrose agar assay plate and allowed to solidify, after which, 3 wells each 4 mm in diameter were cut in the agar. The eluates from each test group were placed into two wells and the third well was used as a control [Table/Fig-6].



[Table/Fig-6]: a) Three wells cut in agar plates; and b) Eluates (50 microlitres) from each test group placed in the wells.

This was done at each of the test intervals i.e., 2, 14 and 21 days for all the three groups and the plates were then incubated at 35°C for 72 hours to check for the inhibition of the growth of *Candida albicans*.

The absence of growth of *Candida albicans*, demonstrated by the zone of low growth/inhibition zone around the wells that contained the drug was interpreted as antifungal activity of the drug. The zone of low growth was also considered as the microorganisms may be partially resistant to fluconazole. The fungicidal efficacy of the drug was expressed by measurement of the diameter of the zone of low growth/ inhibition zone (mm) present around the well.

STATISTICAL ANALYSIS

All the data collected i.e., amount of antifungal agent (mg/L, ppm) leached and the diameter of zone of low growth/inhibition zone was entered in Microsoft excel and statistical analysis was performed using SPSS (ver7.5) software. The statistical test used for comparison was Kruskal-Wallis test followed by Mann-Whitney test.

RESULTS

HPLC Analysis of the Drug Release

Evaluation of leaching of fluconazole and *Soluneem*

From 2nd day to the 14th day the rate of elution of fluconazole increased followed by a reduction in the rate of elution on 21st day for all the subgroups [Table/Fig-7].

Sample Name	2nd day Fluconazole, mg/L (ppm)	14th day Fluconazole, mg/L (ppm)	21st day Fluconazole, mg/L (ppm)
Auto polymerising resin 10% w/w fluconazole(I A)	457.8	540	110.9
Heat polymerising resin 10% w/w fluconazole(II A)	25.8	56	7.7
Permanent soft liner 10% w/w fluconazole (III A)	1105.0	1664	879

[Table/Fig-7]: Table showing amount of fluconazole leached in ppm from the three test materials on three time intervals.

From the 2nd day to the 14th day, the rate of elution of *Soluneem* increased for the samples of subgroup I B and III B followed by a reduction in the rate of elution on the 21st day, whereas for the samples of subgroup II B the rate of elution showed a persistent reduction across all the three time intervals [Table/Fig-8].

Sample Name	2nd day, Neem, mg/L (ppm)	14th day Neem, mg/L (ppm)	21st day Neem, mg/L (ppm)
Auto polymerising acrylic resin, 10% w/w neem (I B)	15.60	16.8	3.7
Heat polymerising acrylic resin 10% w/w neem (II B)	0.56	0.33	<0.5
Permanent soft liner, 10% w/w neem (III B)	7.80	29.2	14.1

[Table/Fig-8]: Table showing amount of neem leached in ppm from the three test materials on three time intervals.

Comparison of leaching behaviour of fluconazole and *Soluneem* amongst the three tested materials

The mean ppm of the fluconazole leached out in the three test materials over the three specified time intervals was compared using the Kruskal-Wallis test followed by Mann-Whitney test.

It was observed that there was a statistically significant difference (p=0.03) in the amount of fluconazole leached (ppm) amongst all the three tested materials with the highest mean ppm for the subgroup III A(1216 ppm) followed by the samples of subgroup I A(369.6 ppm) and least for the samples of subgroup II A(29.8 ppm) [Table/Fig-9a].

The mean ppm of the neem leached out in the three test materials over the three specified time intervals was compared using the Kruskal-Wallis test.

The amount of neem leached (ppm) amongst the three tested materials showed no statistically significant difference (p=0.06). Subgroup III B exhibited the highest mean ppm of leaching (17 ppm) followed by subgroup I B (12 ppm) and least was observed for the subgroup II B (0.5 ppm) [Table/Fig-9b].

Materials	Mean	SD	Min	Max	H	p-value	Sig. diff	p-value
Auto polymerising acrylic resin (I A)	369.6	227.8	110.9	540	7.200	0.03*	1 vs 2	0.04*
Heat polymerising acrylic resin (II A)	29.8	24.4	7.7	56			1 vs 3	0.04*
Permanent silicone soft liner (III A)	1216.0	404.1	879	1664			2 vs 3	0.04*

[Table/Fig-9a]: Table showing comparison of leaching behaviour of fluconazole amongst the three tested materials.

Materials	Mean	SD	Min (ppm)	Max (ppm)	H	p-value
Auto polymerising acrylic resin (I B)	12.0	7.2	3.7	16.8	5.422	0.06
Heat polymerising acrylic rein (II B)	0.5	0.1	0.3	0.6		
Permanent Silicone soft liner (III B)	17.0	11.0	7.8	29.2		

[Table/Fig-9b]: Table showing comparison of leaching behaviour of *Soluneem* amongst the three tested materials.

Evaluation of Anti Candida Effect

Comparison of zone of low growth/inhibition of fluconazole amongst the three tested materials

The zone of low growth/inhibition was highest for the samples of subgroup III A (24.3 mm) followed by the samples of subgroup I A (23.7 mm) and least for the samples of subgroup II A (10 mm). The mean zone of low growth/inhibition of fluconazole in the three tested materials was compared using the Kruskal Wallis test followed by Mann-Whitney test.

It was found out that there was a statistically significant difference in the mean zone of low growth/inhibition over the specified time intervals, amongst the samples of subgroup I A and subgroup II A (p=0.04) and amongst the samples of subgroup II A and subgroup III A (p=0.04) whereas, the difference in mean zone of low growth/inhibition was not statistically significant amongst the samples of subgroup I A and samples of subgroup III A (p>0.05) [Table/Fig-10].

Materials	Mean	SD	Min (mm)	Max (mm)	H	p-value	Sig. diff	p-value
Auto polymerising acrylic resin (I A)	23.7	1.5	22	25	5.468	0.04*	1 Vs 2	0.04*
Heat polymerising acrylic resin (II A)	10.0	8.7	0	16			2 Vs 3	0.04*
Permanent silicone soft liner (III A)	24.3	2.3	23	27			1 vs 3	>0.05

[Table/Fig-10]: Comparison of mean zone of low growth/inhibition (in mm) of fluconazole amongst the three tested materials.

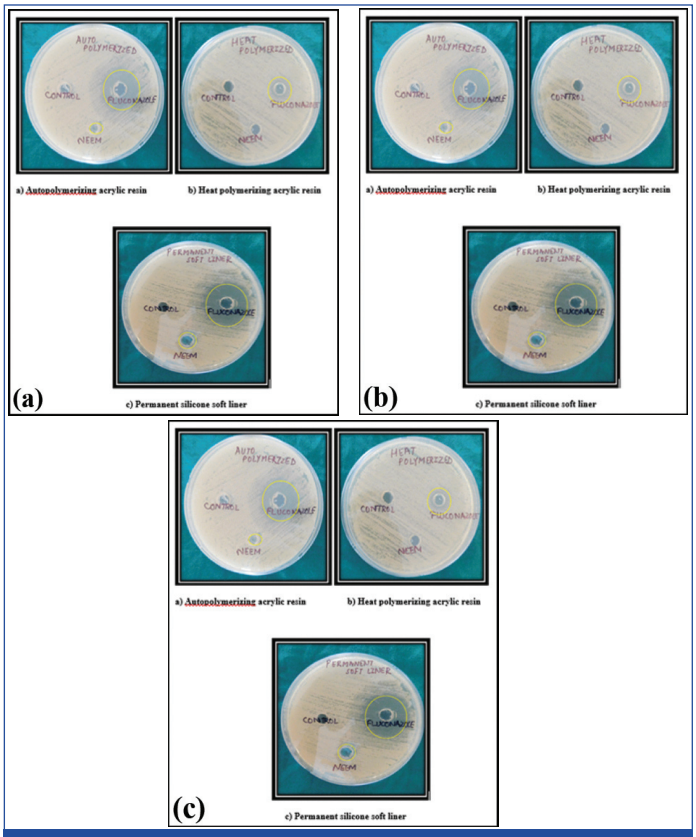
Comparison of mean zone of low growth/inhibition of neem amongst the three tested materials

The mean zone of low growth/inhibition was highest for the samples of subgroup III B (8.0 mm) followed by the samples of subgroup I B (2.7 mm) and least for the samples of subgroup II B (0.0 mm). The mean zone of low growth/inhibition of neem in the three tested materials was compared using the Kruskal Wallis test and it was found that there was no statistically significant difference (p>0.05) amongst the three tested materials [Table/Fig-11].

Mean zone of low growth/inhibition of neem and fluconazole individually amongst the three tested materials [Table/Fig-12].

Materials	Mean	SD	Min (mm)	Max (mm)	H	p-value
Auto polymerising acrylic resin (I B)	2.7	4.6	0	8	3.270	0.20
Heat polymerising acrylic resin (II B)	0.0	0.0	0	0		
Permanent silicone soft liner (III B)	8.0	6.9	0	12		

[Table/Fig-11]: Comparison of mean zone of low growth/inhibition of neem (in mm) amongst the three tested materials.



[Table/Fig-12]: Shows the mean zone of low growth/inhibition of neem and fluconazole amongst the three tested materials individually, and the same was compared using the Mann-Whitney U test. a) 2nd day; b) 14th day; c) 21st day.

It was observed that there was a statistically significant difference in the mean zone of low growth/inhibition amongst the neem and the fluconazole in the samples of Group I and Group III (p=0.04), whereas in the samples of Group II, the mean zone of low growth/inhibition amongst the neem and the fluconazole didn't show a statistically significant difference (p=0.06) over the specified time intervals [Table/Fig-13].

Materials	Group	Mean	SD	Mean Diff	Z	p-value
Auto polymerising acrylic resin (I)	Neem	2.7	4.6	-21.0	-1.993	0.04*
	Fluconazole	23.7	1.5			
Heat polymerising acrylic resin (II)	Neem	0.0	0.0	-10.0	-1.549	0.06
	Fluconazole	10.0	8.7			
Permanent silicone soft liner (III)	Neem	8.0	6.9	-16.3	-2.043	0.04*
	Fluconazole	24.3	2.3			

[Table/Fig-13]: Comparison of mean zone of low growth/inhibition of neem and fluconazole individually amongst the three tested materials.

DISCUSSION

Candida-associated denture stomatitis is a multifactorial condition, commonly affecting elderly denture wearers [4,5]. The use of antifungal medications is the most commonly employed method to prevent the growth of *Candida albicans* on the surface of the prostheses.

The recurrence rate of denture-induced candidiasis is high due to their poor penetration into the microbial biofilm on the porous denture material and also because of their rapid clearance by saliva and tongue movements. [8].

Local drug carriers have been suggested to prolong the efficiency of oral treatment in order to maintain ideal therapeutic drug levels at the site of infection over the required period by release of the drug (Brook IM et al., Brook IM et al., Geerts G et al., [23-25]. These are convenient for the patients as they do not require compliance to frequent application regimes [23-25].

In addition to this, direct delivery of the drug to the site of infection reduces the risk of systemic side effects. Favourable results for incorporation of antifungal agents in different polymeric systems have been reported in various studies done by Amin WM et al., Patel MP et al., Addy M et al., Addy M et al., [14], Ryalat S et al., Darwish RM et al., Geerts G et al., Addy M et al., [10,11,13,14,21,22,25,26]. Therefore, a local delivery system is an alternative option to maintain therapeutic drug levels at the site of pathology [9-11].

A number of effective antifungal agents have been used, either topically or systemically, for management of oral candidiasis. Amphotericin B and nystatin are common topical antifungal agents, whereas azoles such as fluconazole and ketoconazole are available for systemic antifungal treatment [27]. Fluconazole is commonly used to treat denture-induced candidiasis as it has a broad antifungal activity. It is well-tolerated and has few side effects [27,28].

The incorporation of neem extract (*Soluneem*) for this in-vitro study was based on a number of factors. Various preparations of neem obtained from its different parts have been found to have antibacterial, antifungal and antimalarial properties and they have comparatively fewer/no side effects [29].

High Performance Liquid Chromatography is used for a wide range of applications and offers significant advantages in the analysis of pharmaceutical formulations and biological fluids. The detectors used in HPLC are non-destructive and thus facilitate sample recovery [9,10].

The findings of this study revealed that fluconazole had better elution profile than neem as the mean amount (parts per million) of the fluconazole eluted was significantly higher than that of neem in auto polymerising acrylic resin and permanent silicone soft liner, over the three different time intervals at which the observations were made in this study. The highest mean difference between the amount of fluconazole and neem eluted was observed for the samples of permanent soft liner (1199) followed by the samples of auto polymerising acrylic resin (357.5) and least for the samples of heat polymerising acrylic resin (29.4). Previous studies done by Salim N et al., Patel MP et al., Darwish RM et al., have also similarly established that acrylic soft liner and auto polymerising acrylic resins are effective antifungal drug carrier systems [9,11,22]. A study done by Darwish RM et al., demonstrated that fluconazole incorporated into auto polymerising acrylic resin leached out steadily over the time period of 28 days and had significant *Candida albicans* inhibitory activity in terms of colony inhibition [22].

The antifungal activity of the eluents was evaluated by comparing the mean zones of low growth/inhibition and it was observed that there was a statistically significant difference in the mean zone of low growth/inhibition seen with fluconazole and neem, in the samples of auto polymerising acrylic resin and permanent silicone soft liner. The mean zone of low growth/inhibition seen with fluconazole and neem didn't show a statistically significant difference over the specified time intervals with the heat polymerising acrylic resin samples. The mean zone of low growth/inhibition with fluconazole was significantly higher than neem in all the three tested materials over the time interval of three weeks thus proving that fluconazole probably had better anti candidal activity when compared with the *Soluneem*. The antifungal efficacy of fluconazole when incorporated in soft liners has also been demonstrated in studies done by Chopde N et al., Chow CK et al., Truhlar MR et al., [30-32]. Chopde N et al., demonstrated that when two tissue conditioners were combined with nystatin, miconazole and fluconazole. Fluconazole demonstrated maximum zone of inhibition for *Candida albicans* [30].

Amongst the three materials tested, permanent silicone soft liner demonstrated the highest amount of antifungal agent release followed by auto polymerising acrylic resin and least for heat polymerising acrylic resin. The anti candidal activity demonstrated by the eluents was also in accordance with the amount of antifungal agent release with the highest being for permanent silicone soft liner followed by auto polymerising acrylic resin and least for heat polymerising acrylic resin, respectively. This might be due to the fact that the temperature rise during the long curing cycle of heat polymerising acrylic resin and the heat of exothermic polymerisation reaction of auto polymerising acrylic resin might have interfered with the properties of the *Soluneem* thus, limiting its elution and anti candidal activity. This could also be the reason for more elution of the incorporated antifungal agent from the permanent soft liners as there is no heat generated during the polymerisation process of the soft liners. The mean zones of inhibition seen with the liners were also higher corresponding to the higher elution of the antifungal agents.

It was observed that there was an initial high rate of release followed by a sustained release phenomenon over the three week duration. The initial high release is a surface phenomenon where the molecules at the surface are released at the early stage. The later slow diffusion is likely to be due to the diffusion of the drug from the core of the polymer by water cluster formation around the drug particles controlled by concentration dependent diffusion [9,10].

LIMITATION

The results of the present in-vitro study should be corroborated with an in-vivo study to determine the clinical behaviour of the incorporated antifungal agents. In the present study, the leaching of both fluconazole and neem was evaluated for 21 days. The serviceability and durability of the suggested delivery system needs to be investigated to verify its clinical performance by studying the long term release of both the materials. Further the mechanical and physical properties of the impregnated polymer system should not be overlooked and these properties have to be tested prior to implementing its clinical use for the treatment of denture-associated Candidiasis.

CONCLUSION

Within the limitations of this in-vitro study following conclusions were drawn:

Fluconazole exhibited significantly better elution profile than *Soluneem*. The mean zone of inhibition with fluconazole was significantly higher than *Soluneem* thus, establishing that fluconazole had exhibited significantly better antifungal property in-vitro against *Candida albicans* compared to *Soluneem*.

The permanent silicone soft liner demonstrated the highest amount of antifungal agent release followed by auto polymerising acrylic resin and least for heat polymerising acrylic resin.

The mean zones of inhibition seen with the permanent silicone soft liner were also higher corresponding to the higher elution of the antifungal agents and thus, it was established to be the most effective polymeric system as an effective carrier for sustained release of antifungal agents up to 21 days.

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