Section A-Research paper
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ANTIMICROBIAL EFFECT OF INCORPORATION OF CARVACROL OIL, OZONATED OIL AND POVIDONE IODINE IN DENTURE BASE SOFT LINER- AN IN VITRO COMPARATIVE STUDY

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Abstract

Introduction: Functional soft liners have come in to practice with the incorporation of antimicrobial agent to reduce the microbial load. The present study was aimed to analyze and compare three different anti microbial agents i.e Carvacrol Oil, Ozonated Oil and Povidone iodine against Staphylococcus Aureus and Candida Albicans at three different time intervals of 24 hrs, 14 days and 1 month.

Material & methods: A total of two hundred and forty soft liner discs were prepared using 3D printed circular rings of 10mm x 2mm dimensions. Sixty discs were made for each group (n=60) which were further subdivided into the subgroup (a) and subgroup (b), (n=30) to be tested against *Candida albicans* and *Staphylococcus aureus* for the study Group C (Control Group) was devoid of any antimicrobial agent, Group 1(Carvacrol oil) was incorporated with 20 micron litres of carvacrol oil using a micro pipette, Group 2 (Ozonated oil) was incorporated with 10 micron litres of 5 % ozonated oil using a micro pipette and Group 3 (Povidone Iodine) was incorporated with 20 micron litres of 5 % Povidone Iodine using a micro pipette.

Results: All the three antimicrobial agents against Staphylococcus Aureus, Povidone Iodine after 24 hrs had the highest zone of inhibition i.e 3.805+/- 1.205 cm which declines rapidly during the follow up whereas Carvacrol Oil had moderate zone of inhibition formation after 24 hrs i.e 2.6200 +/- 1.2127 cm which relatively stays constant in the follow up period after 1 month i.e 2.3500 +/- 0.91196 cm. Ozonated oil has the poorest antimicrobial response in the all three time frames.

Conclusion:. The results show that against both Staphylococcus Aureus and Candida Albicans, Carvacrol Oil has the most profound and lasting antimicrobial action followed by Povidone Iodine and Ozonated oil. However, due to limited literature further research is required to study the in vivo response of these antimicrobial agents.

Keywords: antimicrobial, denture, soft liner

Introduction

Prosthodontic treatment planning plays an important role to achieve results that satisfy both the patients and the clinicians. In order to successfully rehabilitate edentulous patients, constant follow up for evaluation of the denture and underlying tissues is utmost important. Denture-lining materials are often used to reline the surfaces of complete dentures and to help condition traumatized tissues. They are classified into short term or long term. They are also used for provisional or diagnostic purposes, temporary relining of immediate dentures or immediate surgical splints, relining cleft palate speech aids, tissue conditioning during implant healing and for functional impression materials whereas long- term/permanent soft liners are mostly used as a therapeutic measure for patients who cannot tolerate the stresses induced by dentures providing an interim or permanent cushion like effect. Tissue conditioners or short-term soft liners usually consist of poly ethyl methacrylate powder, aromatic esters and alcohol. The tissue conditioners should only ever be used as temporary materials, even if they can be used for a few days to a week. Long-term silicone soft liners are usually used for up to 1 year.[1]

Soft lining materials are easily contaminated in the oral environment and are difficult to clean or brush effectively. For creating an efficient denture disinfection, the hygiene of denture soft liners play a significant role. Studies have reported that the microorganisms can enter porous spaces within the denture liner and that their colonization may reduce the intra-oral life of the material. Hence a soft liner incorporated with antimicrobial agent may also benefit physically compromised patients who are unable to perform routine denture care [2]. Though there have been studies showing antimicrobial effect of incorporation of various agents in soft liners, but to the best knowledge of investigators there is limited evidence on antimicrobial effect of soft liners incorporated with a natural herb oil i.e. Carvacrol oil, Ozonated oil and Povidone Iodine.

Plant based Essential oils (EOs), can be a natural source of antimicrobial agents.. Origanum syriacum Oil has two main chemo-types: Thymol and Carvacrol. In the oil extracted from lebanese plants, the amounts of thymol and carvacrol range between 2.3% and 74.4% for thymol, and 17.6% and 78.4% for carvacrol. The high contents of these phenolic oxygenated monoterpenes confer to this Essential Oil a strong and powerful antimicrobial capacity. Ozone therapy is becoming more popular due to its great healing outcomes, straightforward use, lasting benefits, and harmless nature. Considering its effectiveness against both Gram-positive and Gram-negative bacteria, viruses and fungi as per literature [3] , Ozonated oil is considered to be incorporated into the soft denture base liner to evaluate its antimicrobial effect in the present study. Elemental iodine or its derivatives polyvinylpyrrolidone-iodine complex (Povidone-I) is the most broad-spectrum and potent antiseptics available.[4] In the present study, povidone iodine is used and incorporated into soft denture liner to evaluate whether this addition would have an antimicrobial effect on Candida Albicans and Staphylococcus Aureus. With this in mind, the present study is aimed to evaluate and compare the antimicrobial effect of incorporation of Carvacrol oil, Ozonated oil and Povidone iodine in denture base soft liner.

Material & Methods

The study was conducted in the Department of Prosthodontics, Crown and Bridge, Maxillofacial prosthodontics and Oral Implantology to evaluate and compare the antifungal and antibacterial effect of incorporation of Carvacrol oil, Ozonated oil and Povidone iodine in denture base soft liner on specific fungal and bacterial strains i.e. *Candida albicans* and *Staphylococcus aureus* respectively. To standardize the size of the soft liner discs 10 ring shaped moulds were made with light cured acrylic resin to standardize the procedure. Design of the rings of size 10mm x 2mm was made digitally in STL format which was then sent to the lab for 3D printing. A total of two hundred and forty soft liner discs were prepared. Sixty discs were made for each group i.e Group C (control group), Group1 (Carvacrol oil), Group 2 (Ozonated oil) and Group 3 (Povidone Iodine). The sixty soft liner discs in each group were further subdivided into groups of thirty to be tested against Candida albicans and Staphylococcus aureus for the study.

Freeze dried samples of micro-organisms were obtained from Microbial Type Culture Collection & Gene bank (MTCC) , CSIR – Institute of Microbial Technology , Chandigarh for the purpose of the study. The strain for *Staphylococcus aureus* was MTCC 3160 and for *Candida albicans* was 3017 which was procured in the powder form in the glass vials. Sabouraud Dextrose broth (SDB) and Brain heart infusion (BHI) broth was freshly prepared in test tubes for incubation of *Candida albicans* and *Staphylococcus aureus* respectively. The glass vials containing microorganisms were added in to the test tubes containing SDB and BHI and test tubes were incubated at 37 degree Celsius for 24 hrs. MIC of all the test agents i.e. Carvacrol oil, Ozonated oil and Povidone iodine which was to be added in the soft liner was determined after comprehensive literature search. The MIC of Carvacrol is 20 micron litres for 10 soft liner discs, 10 micron litres of 5% ozonted oil and 20 micron litres of 5% povidone iodine.

One measure of powder (3gm) was dispensed into the dappen dish, and one measure of liquid (2 ml) was added into it, which was then mixed for 30 seconds (at room temperature) under biosafety cabinet to obtain 10 discs of soft liner. After 2-3 mins from the starting of the mixing the material was then transferred into the ring shaped moulds placed on a clean glass slab to provide flat base with the help of mixing spatula. After that material was allowed to set, (i.e 7-8 mins from the start of the mixing) the soft liner discs were retrieved from the moulds and were placed in artificial saliva to simulate oral environment to finally produce samples for Group C (Control Group).

Similarly, one measure of powder (3gm) was dispensed into the dappen dish, and one measure of liquid (2 ml) along with 20 micron litres of carvacrol oil was added to it using a micro pipette and same method was used to prepare samples for **Group I (Soft liner incorporated with carvacrol oil).** In same manner, one measure of powder (3gm) was dispensed into the dappen dish, and one measure of liquid (2 ml) along with 10 micron litres

of 5 % ozonated oil was added to it using a micro pipette to prepare samples for **Group II (Soft liner incorporated with ozonated oil).** Finally, one measure of powder (3gm) was dispensed into the dappen dish, and one measure of liquid (2 ml) along with 20 micron litres of 5 % Povidone Iodine was added to it using a micro pipette to produce **samples for Group III (Soft liner incorporated with Povidone iodine n=60)**

One measure of powder (3gm) was dispensed into the dappen dish, and one measure of liquid (2 ml) along with 20 micron litres of 5 % Povidone Iodine was added to it using a micro pipette. The material was then mixed for 30 seconds (at room temperature) using mixing spatula under biosafety cabinet to obtain 10 discs of soft liner. After 2-3 mins from the start of the mixing, 3D printed rings were placed on the glass slab to provide a flat surface and Povidone Iodine impregnated soft liner was transferred into the rings to obtain standardized discs of soft liner. After that the material was allowed to set for 4-5 mins (i.e 7-8 mins from the start of the mixing) and soft liner discs of 10mm diameter and 2 mm were retrieved from the rings and were inspected for the uniformity of size(Figure 1). They were then placed in artificial saliva to simulate oral environment. Petridishes containing sabouraud dextrose agar (SDA) and nutrient agar media (NAM) were made for *Candida albicans* and *Staphylococcus aureus* respectively.Using cotton swab sticks, colonies of Candida was spread on SDA and Staph aureus was spread on NAM agar plates, respectively (Figure 2).20 impregnated soft liners discs from each of the group was taken after immersion in artificial saliva for 24 hrs and 10 each were placed individually on separate petridishes containing SDA and NAM respectively.These discs were then again placed in to the incubator for 24 hrs at 37 degree Celsius for colonies to form.After 24 hr, 14 days and 1 month time intervals, formation of zone of inhibition was measured which was done by measuring the diameter of the clear zone using ruler and a divider (Figure 3). Data was then tabulated in excel sheet and statistically analyzed.

Results:

Intragroup comparison of zone of inhibition against S aureus was done using Repeated measures of ANOVA. Among Gr 1, the mean zone of inhibition didn't change significantly. While among Gr 2 & Gr 3, the mean zone of inhibition decreased significantly from 24 hr to 14 days and then further from day 14 to 1 month. Intergroup comparison of zone of inhibition against S aureus was done using one way ANOVA test, and post hoc Tukey's test. A statistically significant difference was found in ZOI exhibited by different test agents. After 24 hrs, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Group 2. After 14 days, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Gr 1. After 1 month, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Gr 3, which was further significantly lower than that among Gr 1(Table 1)

Intragroup comparison of zone of inhibition against C albicans was done using Repeated measures of ANOVA. Among Gr 1, the mean zone of inhibition didn't change significantly. While among Gr 2 & Gr 3, the mean zone of inhibition decreased significantly from 24 hr to 14 days and then further from day 14 to 1 month. Intergroup comparison of zone of inhibition against C albicans was done using one way ANOVA test, and post hoc Tukey's test. A statistically significant difference was found in ZOI exhibited by different test agents. After

24 hrs, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Gr 3. After 14 days, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Gr 1. After 1 month, the mean zone of inhibition produced by Gr 2 was significantly lower than that produced by Gr 3, which was further significantly lower than that among Gr 1 (Table 2)

Discussion:

In the intragroup comparison of Group 1 (Carvacrol oil) on the basis of time duration against Staph Aureus, zone of inhibition did not change significantly with time ($p = 0.824$) depicting a constant antimicrobial effect throughout the month. In the intragroup comparison of Group 2 (Ozonated oil) against Staph Aureus, the decrease in zone of inhibition was significant ($p= 0.019$). This shows that Ozonated oil shows a high initial antimicrobial activity but it decreases constantly during the span of 1 month which can be due to the leaching out of oil in the saliva. Similarly, in the intra group comparison of Group 3 (Povidone Iodine) against Staph Aureus, antimicrobial effect however decreases after 14 days and after 1 month. The intragroup results for Group 3 (Povidone Iodine) is also significant with p value of 0.01. These statistics infer that along the span of 1 month, antimicrobial properties Povidone Iodine also diminishes after its application.

In the intragroup comparison against Candida Albicans, a high initial antibacterial response is seen for the Group 1(Carvacrol oil) after 24 hrs with the mean zone of inhibition as wide as 4.54 cm and standard deviation of 1.41 cm. However, zone of inhibition slightly decreases after 14 days and further decreases after 1 month rendering the change non significant. The results of our study are in accordance with the similar study conducted by Tuba Baygar et al. in 2017 where zone of inhibition of 38.33 +/- 1.15mm was seen against Candida Albicans. The soft liner discs where incorporated with Carvacrol oil and zone of inhibition was seen against Candida Albicans after incubating the discs for 24-48hrs. Dalleau et al. (2008) reported that Carvacrol Oil inhibited Candidal biofilm by 75% when used at 0.03% concentration. Lima et al. (2013) suggested that carvacrol oil is able to act by altering the cell membrane structure of the fungal cell. Denture lining materials combined with carvacrol has shown great in vitro antimicrobial activity against microorganism including oral microbes. According to the hopeful results of the present study, required in vitro and in vivo studies will be required for clinical use of carvacrol incorporated denture soft-liner.[5] The significant change in the result denotes that with time zone of inhibition significantly decreases rendering a poorer antimicrobial effect in Group 2. Similarly for Group 3 (Povidone Iodine) also gradual decline is seen in the antifungal response from 24 hrs to 1 month.

In the intergroup comparison between Group 1 (Carvacrol oil) and Group 2 (Ozonated oil) against Staph Aureus , it was found that Carvacrol oil has potent and lasting antimicrobial effect on Staphylococcus Aureus when compared to Ozonated oil. In the intergroup comparison between Group 2 (Ozonated oil) and Group 3 (Povidone Iodine), a significant result with antimicrobial effect of Group 3 i.e Povidone Iodine being more potent than Group 1 (Ozonated oil) against Staphylococcus Aureus. Whereas, when Group 1 (Carvacrol oil) and Group 3 (Povidone Iodine) are compared, leaching out of Povidone Iodine is more than Carvacrol

whereas latter has more prolonged and lasting results in the follow up period rendering it more effective than Povidone Iodine and Ozonated oil against Staphylococcus Aureus. The intergroup comparison between the three antimicrobial agent shows significant result with Povidone Iodine having high initial antimicrobial action and Carvacrol Oil having constant antimicrobial effect throughout the study period.

Intergroup comparison of Group 1 (Carvacrol Oil) And Group 2 (Ozonated oil) against Candida Albicans, Group 1 i.e Carvacrol oil has more potent antifungal effect than Ozonated oil. In the Intergroup comparison between Group 2 and Group 3, results shows statistically significant result ($p<0.001$) denoting that antifungal properties of Povidone Iodine is more efficacious than Ozonated oil, however the effect of both reduces with time. Intergroup comparison against Candida Albicans between Carcvacrol oil (Group 1) and Povidone Iodine (Group3) shows statistically significant result favouring Carvacrol oil. This comparison states that Carvacrol Oil's antifungal properties are more potent and lasting when compared to Povidone Iodine and Ozonated Oil. There is limited literature available on the incorporation of Povidone Iodine and Ozonated oil into soft liner to study its antimicrobial property. The results of study conducted by Tuba Baygar et al in 2017 in which antibiofilm and antimicrobial properties of Carvacrol oil incorporated in soft liner are in accordance with the present study. The zone of inhibition noted by Tuba Baygar (2017) against Candida and Staphylococcus Aureus after 24-48 hrs was 38.33 +/- 1.15mm and 26.67 +/- 1.53mm respectively. However, due to the lack of follow up in the previous researches, the results obtained after 14 days and 1 month in the present study cannot be verified.

The limitations of present study include that an in vitro test may not always reflect dynamic intraoral conditions and cannot absolutely predict clinical knowledge; they are still useful in gaining and understanding basic knowledge of materials and their properties. There are studies available on antimicrobial properties of Carvacrol and Povidone Iodine against Staphylococcus Aureus and Candida Albicans, however only one study is available to show antimicrobial and antibiofilm response of soft liner incorporated with carvacrol oil against the said microorganisms. And as per the best knowledge of the investigators no literature is available on antimicrobial effect of Povidone Iodine and Ozonated Oil when it is incorporated in to soft liner. Moreover, there is also lack of follow up studies when antimicrobial agents are incorporated in to soft liner. The diminishing antimicrobial response of all the three agents with time in the present study can be hypothesized to leaching out of agent in the saliva but due to lack of evidence and literature further research is required in the field.

Conclusion:

To overcome the problems associated with microbial contamination of soft liners, various antimicrobial agents have been researched to be added in the soft liner such as silver zeolite, nystatin , fluconazole etc. In the present study antimicrobial effect of Carvacrol Oil, Ozonated oil and Povidone Iodine was being tested against Staphylococcus Aureus and Candida Albicans after 24 hrs, 14 days and 1 month. Of the three tested agents in the present study, Carvacrol oil has shown to have highest and lasting antimicrobial response against both the microorganisms during all the three time frames.

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Table 1: Intragroup & Intergroup comparison of zone of inhibition against S aureus

Zone of Inhibition against S aureus							
	After 24 hrs		After 14 days		After 1 month		
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	P value of Intragroup comparison[c]
Gr 1	2.3200	1.21271	2.6200	.75836	2.4500	.91196	0.824, NS
Gr 2	2.2000	1.11455	1.6800	.64601	.9700	.52926	0.019, S
Gr 3	3.8500	1.20577	2.2400	.81813	1.5800	.43153	0.001, S
P value of intergroup comparison[a]	0.007, S		0.029, S		<0.001, S		
Post hoc pairwise comparison[b]	Gr 2 < Gr 3		Gr 2 < Gr 1		Gr 2 < Gr 3 < Gr 1		

[a] One way ANOVA, [b] Post hoc Tukey's test, [c] Repeated measures of ANOVA

Table 2: Intragroup & Intergroup comparison of zone of inhibition against C albicans

Zone of Inhibition against C albicans							
	After 24 hrs		After 14 days		After 1 month		
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	P value of Intragroup comparison
Gr 1	4.5400	1.41044	4.0800	.82435	3.2200	1.09118	0.065, NS
Gr 2	3.0200	.70993	1.7800	.71616	1.2100	.64369	<0.001, S
Gr 3	3.3600	.82219	2.7800	.74207	1.5000	.67330	<0.001, S
P value of intergroup comparison	0.007, S		<0.001, S		<0.001, S		
Post hoc pairwise comparison	Gr 2, Gr 3 < Gr 1		Gr 2 < Gr 3 < Gr 1		Gr 2, Gr 3 < Gr 1		

[a] One way ANOVA, [b] Post hoc Tukey's test

Figure 1: Fabrication of soft liner discs of 10mm x 2mm dimension

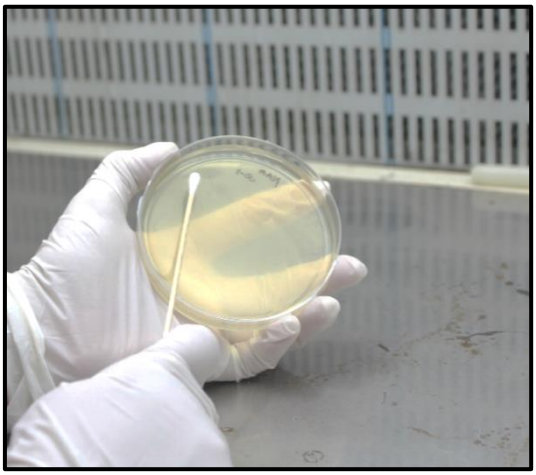
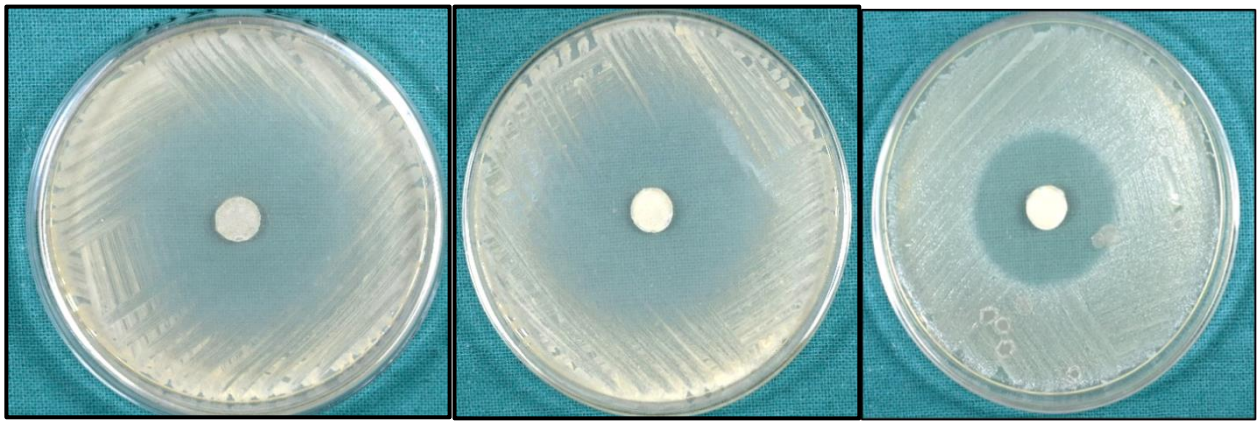


Figure 2: Spreading of microorganisms using cotton swab stick



a) After 24 hrs b)after 14 days c) after 1 months

Figure 3: Zone of inhibition at different time intervals