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Effect Of Silorane Composite On Adhesion, Survival And Growth Of Oral Microflora.

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ABSTRACT

The microflora around the restorative material plays a crucial role in its survival. The study was performed to assess the antimicrobial property of the composites in the presence of Streptococcus mutans, Lactobacillus acidophilus, and Candida albicans. Agar disc diffusion and Broth macro dilution methods were used on silorane and methacrylate based composite to study the microbes along with adhesion and penetration ability. Student's t-test was performed to analyze antimicrobial activity and chi-square test (Fishers exact) to analyze adhesion and penetration assay, at a 5% level of significance. The materials tested were effective against *S. mutans* and *C. albicans*, with resistance to *L. acidophilus*. The silorane group presented better antimicrobial property in comparison to the methacrylate composite. Surface adhesion and penetration were reported to be higher in the methacrylate group than the silorane composite, however without a statistical significance. The antimicrobial effect and low adhesion capacity of the silorane based composite can be attributed to yttrium fluoride in the composition, which provides hydrophobicity to the surface. The antimicrobial property and hydrophobicity of the surface may enhance the durability of the esthetic restoratives clinically by an effective reduction in secondary caries.

Keywords: adhesion capacity, antimicrobial activity, methacrylate based composite, penetration potential, silorane based composite

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INTRODUCTION

The micro flora surrounding the restorative materials is a contributing factor leading to its early failure. The presence of microorganisms in the oral cavity left after treatment or their intrusion through a micro crack that can occur sometimes between the tooth and its filling results in secondary caries [1,2]. Therefore antimicrobial activity is an essential property of dental restorative material. Bacterial adhesion promotes secondary caries and periodontal diseases [3,4]. Therefore, restorative materials are known to succumb to the adherent microflora resulting in the early failure in service.

On adherence to the tooth structure, Mutans streptococci initiate caries. Elevated levels of lactobacilli invoke carious milieu resulting in demineralization of carious lesion [5,6]. The formation and preservation of oral microbes are associated with interbacterial coaggregation and its interaction with yeasts like Candida albicans. Candida albicans is known to promote oral candidosis on its adhesion to the restorative materials [7]. Hence antimicrobial parameters were tested in this study.

The dental composite containing methacrylate is extensively used as an esthetic restoration. They present with particular disadvantage like polymerization shrinkage leading to microleakage. The marginal integrity of restoration can be improved by altering the setting mechanism from free radical addition polymerization to ring-opening cationic polymerization by employing a silorane based composites. Filtek P90 is a silorane based material consisting of silorane resin, fillers, an initiator system, preservative, and colorants. Silorane is a combination of siloxane and oxirane structural moieties. These composites not only claims for less polymerization shrinkage because of the presence of oxirane, but also other properties like hydrophobicity, water sorption due to siloxane [8-12]. The biocompatibility of Filtek P90 was analysed as a whole without segregating the specific ingredients.

The hydrophobicity and the antimicrobial property of silorane composite make it durable and anti-cariogenic. The positive results of the study on silorane based composite would suggest its efficacy as an esthetic restorative material. The study was performed to analyze the antibacterial property, antifungal potential, and susceptibility to adherence and penetration by oral microbes of silorane and methacrylate based composites. The study also aimed to investigate the concentration/ amount of bacteria and fungus on the surface of these composites.

MATERIALS AND METHODS

Materials used in the study

Two varieties of composites resins, different in composition were selected for the study. Hence forth, Silorane based micro hybrid composite - Filtek P90 (3M/ESPE, St. Paul, MN, USA) will be abbreviated as 'SBC' and methacrylate based hybrid composite- Z100 (3M/ESPE, St. Paul, MN, USA) will be abbreviated as 'MBC'. The details of the compositions are presented in Table 1.

Table 1: Composites used in the study

Name	Shade	Organic matrix	Inorganic fillers	Filler content [vol.%]
Filtek P90	**A2**	3,4-Epoxycyclohexylethyl cyclopolymethylsiloxane, bis-3,4-poxycyclohexylethylphenylmethylsilane	Silanized quartz, yttrium fluoride	55
Z100	**A2**	Bis-GMA and TEGDMA	Zirconium, silica	66

Sample preparation of silorane and methacrylate based composites

A brass mold of dimension 10 mm diameter x 2 mm thickness was employed for the fabrication of disc samples packed between clear matrix strip and a glass slide. The mold was packed in excess with as received composite material. After elimination of the flash on the application of mild force, the mold containing the composite was light-cured through the top and bottom of the glass slide for the duration specified by the manufacturer. The intensity of the curing unit was tested before every sample polymerization with a Hilux curing

light meter. On setting, the retrieved samples were conditioned at 100% relative humidity at 37 °C for 24 hours. The sample fabrication was performed by a single operator to lessen inconsistency in handling.

Evaluation of antimicrobial activity

Streptococcus mutans (*S. mutans*), gram-positive facultative anaerobic cocci shaped bacteria); Lactobacillus acidophilus (*L. acidophilus*), gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria; and Candida albicans (*C.albicans*) a diploid fungus that grows both as yeast and filamentous cells were selected as test organisms. Agar disc diffusion and Broth macro dilution (MIC) were used to evaluate the antimicrobial potential against these organisms on the test specimens, SBC and MBC, in the present study.

From strains of Candida albicans

The readily available strain of Candida albicans (ATCC 2091) was employed experimental purpose. Before the experiment 100ml, stationary phase culture of Candida was prepared in SDB. Cells harvested using centrifugation (750g, 20min), were washed thrice in sterile phosphate buffered saline (PBS), pH 7.2 and resuspended in PBS to a 2 McFarland which corresponds to a cell concentration of $1.5\pm0.3x\ 10^{-7}$ cells/ml.

From Strains of Streptococcus mutans and Lactobacillus acidophilus

The readily available strains of *S. mutans* (ATCC 25175) and *L. acidophilus* (ATCC 4356) were chosen as test strain for the study. After frozen (-60 °C) precultures were established, the bacteria were exposed on Brain Heart Infusion agar Media (BHI) for *S. mutans* and Rogosa agar for *L. acidophilus* and incubated at 37 °C for 48 hours. Single colonies were cultivated in sterile trypticase soy broth supplemented with yeast extract for 16 hours at 37 °C. A day before the test, 1 ml of each bacterial suspension were inoculated with 250ml of the sterile trypticase soy broth. The set up was incubated for 12 hours at 37 °C followed by centrifugation for 5 min at 2000 rpm at 18 °C. This was later rinsed twice by PBS. A spectrophotometer was used employed to regulate the optical density of the suspensions to 0.3 at 540nm [13]. The cells of *L. acidophilus* were suspended in thioglycolate broth and incubated anaerobically for 2 days.

Antimicrobial activity by agar disc diffusion method

The lawn culture of the organisms was grown on the agar plates. Uniform perforations 10 mm diameter was made at equidistant points in agar (SDB for *C. albicans*, tryptic soy broth for *S. mutans* and rogosa agar for *L. acidophilus*) using a sterile loop, to receive the testing materials. The agar plates were inoculated at 37 ºC for 24- 48 hours for the growth of microorganisms. Following incubation, the mean diameter of the inhibition zones formed about the wells was measured. All the assays were done in triplicates, under aseptic conditions, and the mean was reported. Student's t-test, at a significance level of 5%, was performed using SPSS software (SPSS Inc. SPSS for Windows, Version 15.0. Chicago, SPSS Inc.) for statistical analysis.

Antimicrobial activity by broth macro dilution method (determination of MIC)

The procedure for the carry out of the study was adopted, as explained by Andrews 2001 [14].

Preparation of bacterial inoculums

Three to four isolated colonies on blood agar was picked using straight wire and inoculated into BHI broth, followed by incubation at 37 °C for 4-6 hours. The turbidity was adjusted to Mc Farlands 0.5 standard. The turbidity matched broth culture was diluted 1 in 10 in BHI broth to get bacterial inoculums having bacterial concentration approximately $1.5x\ 10^{8}$cfu/ml.

Broth Macro dilution test

Ten test tubes, each containing 2ml sterile BHI broth was arranged in a rack. Two ml of SBC / MBC (test solution) (in broth) were added to the first tube and thus mixed and transferred into the next tube in 2 ml quantities till 9^{th} tube to get doubling dilutions (1 in 4 to 1 in 1024). The tenth tube served as growth control. Standardized bacterial inoculums were added (0.02ml) to each tube and incubated at 35 °C for 18 hours before

the examination. The minimum concentration (higher dilution) of the SBC / MBC that inhibits bacterial growth was considered the minimum inhibitory concentration (MIC).

Adhesion and penetration assay

Adherence test

SBC and MBC were pre-sterilized and separately placed in the sterile bottles with a magnetic stirrer. Microbial suspensions around 600µl and 1ml of BHI broth in case of *C. albicans* and *S. mutans* and thioglycolate broth in case of *L. acidophilus* was poured into the bottles were sealed tightly. A pilot study revealed that it takes two hours approximately to saturate the surface of the restorative material with bacterial adhesion. Hence, the suspension was stirred at a constant speed for two hours at 37 °C. The restorative plates were removed and washed with a copious quantity of distilled water. This was followed by fixing the plates with 2.5% glutaraldehyde at 4 °C for 30 min, and staining with 1% acridine orange for 30 min. A fluorescence microscope with 40X magnification was used to count adhered bacteria directly. 20 random fields of view were chosen on each plate. The numbers of bacteria were counted randomly in each restorative plate and divided by the area of the view. The mean and standard deviation were calculated for the obtained data and statistically analyzed using the chi-square test (Fishers exact) at 5% level of significance.

Penetration test

A clamp was used to position the dried test specimens horizontally. The sections obtained were submerged in 0.03% acridine orange for a minute, washed in distilled water, dried parallel with the cut section placed topmost. The sections were observed under fluorescence microscopy with 1000X magnification and oil immersion. The ensure complete viewing of the sample the objective was centered on one corner of the section and moved down accordingly.

This process was replicated thrice:

Level one - once with the objective positioned on the topmost outer layer of the sample,
Level two- once in the center of the sample and
Level three- once close to the lowermost layer of the sample.

The penetration of microorganism into SBC/ MBC was calculated by counting the number of cells visible within every microscopic field. The counted cells were then summed up for each level, and the mean was reported. Chi-square test (Fishers exact) was used to statistically analyze the obtained data, at a 5% level of significance.

RESULTS

Evaluation of antimicrobial activity

The antimicrobial effect was determined by two established measures: the agar disc diffusion method and broth macro dilution method (determination of MIC).

Antimicrobial activity by agar disc diffusion method

Effect of the composite SBC and MBC on the growth of the microorganisms was assessed by agar disc diffusion method, in triplicate, then compared using Student's t-test. Both SBC and MBC displayed bactericidal activity against *S. mutans* and *C. albicans*. SBC and MBC were resistant to *L. acidophilus* (no zone of inhibition), as shown in Fig 1. Although the inhibition halo was not observed for *L. acidophilus* growth was not observed over the SBC or MBC disks either. The antimicrobial activity of SBC was significantly higher in both *C. albicans* ($p=0.0108$) and *S. mutans* ($p=0.0042$). The antimicrobial activity was higher in SBC with a mean zone of inhibition of 18 ±1.2 mm in *C. albicans* species and 15 ±1.3mm in *S. mutans*, as shown in Fig 1. In MBC, the zone of inhibition was a mean zone of inhibition of 13±1.5mm in *C. albicans* species and 9±1.2mm in *S. mutans*, as observed in Fig 1. In conclusion, SBC and MBC showed antibacterial effect against *C. albicans* and *S. mutans*. Higher antimicrobial effect was seen with SBC than MBC against both the species, *L. acidophilus* was resistance.

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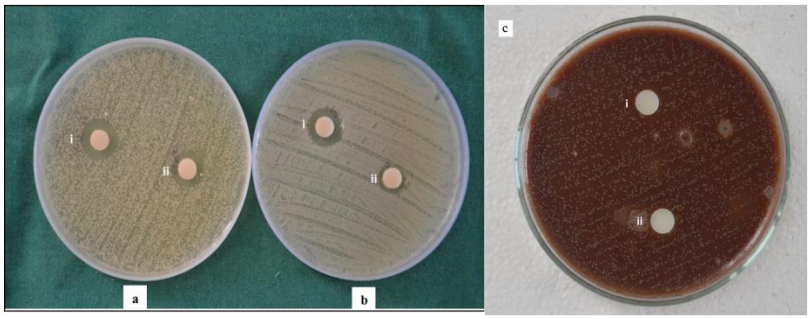
Fig 1. Culture sensitivity of (a) C. albicans and (b) S. mutans: i. SBC ii. MBC (c) L. acidophilus i. SBC ii. MBC



Antimicrobial activity by broth macro dilution method (determination of MIC)

The broth macro dilution method determined the minimum growth inhibition (MIC) of 0.2µg/ml when assessed against *S. mutans* and *L. acidophilus* and 1.6µg/ml when tested against *C. albicans*. Both the composites SBC and MBC showed inhibitory action against all the tested microorganisms.

Adhesion and penetration assay

Fig 2. (a) Adhesion and (b) Penetration of S. mutans on SBC; (c) Adhesion and (d) Penetration of L. acidophilus on SBC; (e) Adhesion and (f) Penetration of C. albicans on SBC

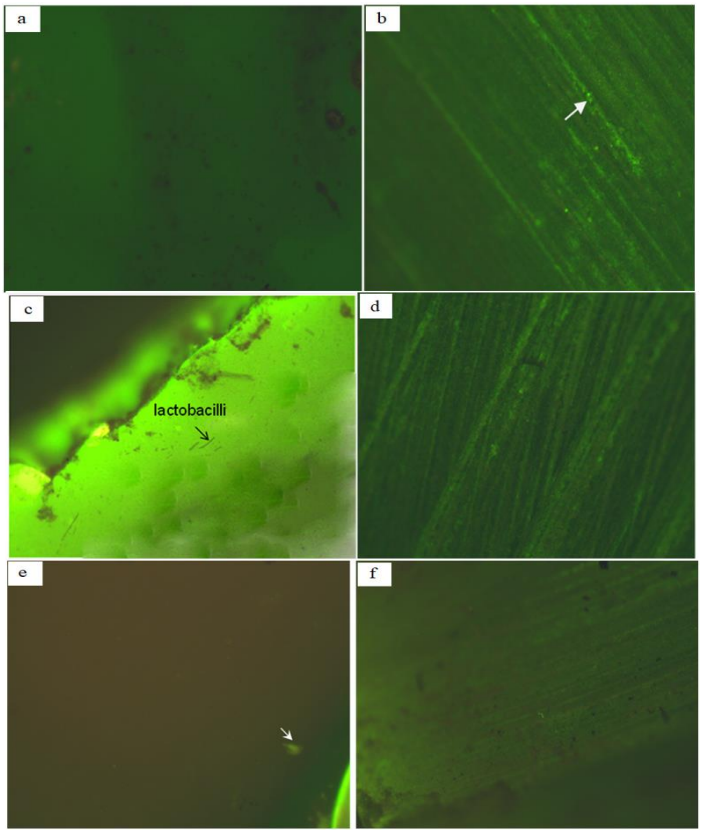


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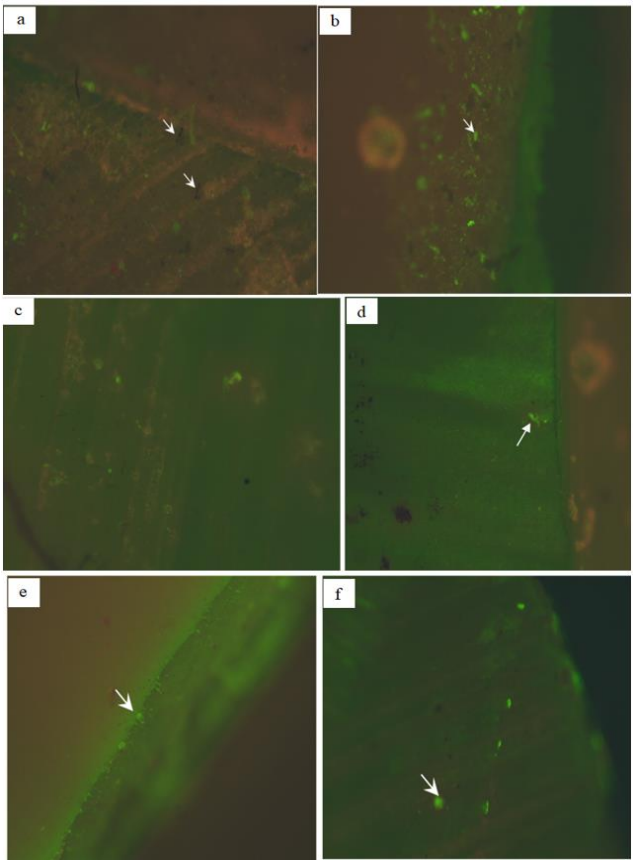
Fig 3. (a) Adhesion and (b) Penetration of S. mutans on MBC; (c) Adhesion and (d) Penetration of L. acidophilus on MBC; (e) Adhesion and (f) Penetration of C. albicans on MBC



The microorganisms showed surface adherence and penetration to MBC more than SBC. There was no statistical difference in the adhesion and penetration between the two materials (as observed using fishers exact chi-square test) in *C. albicans* (p=0.462), *L. acidophilus* (p=0.061) and *S. mutans* (p=1.00) species. SBC had a reduced number of adhering bacteria in comparison with MBC. The different sections of the discs were tested after 24 - 48 hours of incubation (aerobic incubation for *C. albicans*, facultative anaerobic for *S. mutans* and anaerobic incubation for *L. acidophillus*). It was observed that SBC had very less adherence activity seen, which is about the penetration as represented from Fig 2 and Fig 3. Among the microorganism, irrespective of the materials, surface adherence and penetration of *C. albicans* was seen more, comparative to *S. mutans* and *L. acidophilus*, though the data was not statistically significant.

DISCUSSION

Evaluation of antimicrobial activity

S. mutans and *L. acidophilus* are regarded as one of the causative factors of caries, periodontal diseases, and added infections. The equilibrium of oral microflora can be altered by them to create a favorable environment for adhesion of opportunistic bacteria and fungi, to the periodontium and prosthesis [7,15].

C. albicans is a frequent colonizer in oral biofilm with a capacity to produce organic acids and collagenolytic enzymes. It is well associated with the formation of caries. The organic acid causing demineralization and the hyphae on the cells of *C. albicans* invading the dentinal tubules demonstrates its ability to damage organic and inorganic oral tissues [16]. In vitro [17] and in vivo studies [18] have reported that plaque build-up is more commonly attributed to resin composites compared to other restoratives or structures like enamel. Presence of antimicrobial agents as one of the ingredients in the composition is known to prevent biofilm formation. Antimicrobials may be restrained into the resin component or may be added as a filler, based on its physical and chemical characteristics.

Agar disc diffusion method is used to assess the antimicrobial effect of polymerized composite resin [19]. *S. mutans* and *C. albicans* showed an inhibition zone in SBC significantly greater than MBC, as shown in Fig 1. There was no antibacterial effect against *L. acidophilus* seen according to our results in which no inhibition halo was induced by SBC and MBC, as shown in Fig 1. Broth macro dilution method (determination of MIC) was performed in this investigation to assess the antibacterial property. Minimum inhibitory concentration (MIC) is the least concentration of an antimicrobial which will prevent the observable growth of a microorganism with overnight incubation. MIC is used by analytical research laboratory mostly to verify resistance and as a means to govern the in vitro activity of recent antimicrobials [14]. The results of MIC demonstrated that SBC could significantly inhibit the growth of microorganisms: *S. mutans, L. acidophilus*, and *C. albicans*. The SBC is said to have yttrium fluoride (76%) in its composition [20,21] and fluoride in any form are said to have antimicrobial action [22-24]. Fluoride reduces the acid tolerance of the bacteria and the cariogenicity of plaque. The composite MBC lacked fluoride in its composition and hence, can be considered as the reason for the lower level of antimicrobial potential when compared to SBC (Table 1). Also, the cytotoxic profiles of MBC due to the release of TEGDMA or other residual toxic monomers should not be neglected. These have been reported in previous literature [25-27].

Adhesion and penetration assay

The present study was performed to estimate the surface adhesion and penetration of microorganisms, *S. mutans, L. acidophilus, and C. albicans* to SBC and MBC.

Microbial adhesion and penetration to the composite surface is an important etiological factor in secondary caries formation. Studies have revealed that Streptococcus mutans and Lactobacillus acidophilus are the main organisms obtained from plaque samples from different surfaces at early carious stage [28]. Candida albicans also are known to possess high adhesion capacity to a material surface in a similar way to the dental tissues and forms biofilm [29]. A low adhesive material reduces or delays the development of biofilm, thereby preventing the development of dental plaque [30]. These low adhesive restorative materials not only have a protective effect against secondary caries but also have anti-bacterial action due to the fluoride releasing ability [19,31]. This fluoride ion has both an inhibition effect on cariogenic bacteria metabolism and remineralizing potential on the hard tissues of the tooth. Nevertheless, the correlation between the fluoride release rate, the antibacterial action, and adhesion potential is not equivocally demonstrated in literature [32]. Therefore the surface characteristics of the material may influence the probability of bacterial infection, in turn controlling biofilm formation and host infection. The available literature states that aspects like free energy, surface roughness, hydrostatic forces, hydrophobicity, characteristic of the material, water sorption, alter the nature of adherence and penetration behavior of microorganisms [33-35]. Intra oral structures having rough surfaces retain more plaque than do smoother surfaces [33].

Surface roughness has a positive effect on microbial adhesion [36,37]. In the present study, the conventional MBC yielded the highest surface roughness in comparison to SBC. This could be associated with a higher amount of larger hybrid fillers compared to SBC with its micro-hybrid filler particles present in SBC. Surface hydrophobicity also plays a vital part in microbial adhesion to dental tissues [36,37]. It is established that the surfaces with water contact angles higher than 90° are hydrophobic and angles lower than 90° are hydrophilic. Generally, SBC shows considerably greater surface hydrophobicity [20,21]. It can be associated with its hydrophobic siloxane backbone, resulting in increased hydrophobicity in comparison to MBC. Our findings also agreed with the results reported in previous investigations [38-40]. The decreased bacterial adhesion and penetration can be associated with increased hydrophobicity of SBC with MBC in the present study.

CONCLUSIONS

Thus from the results of the study and the discussion it can be concluded that the antimicrobial activity of SBC was significantly higher in both *C. albicans* and *S. mutans* when compared to MBC, whereas it was resistance against *L. acidophilus* with no zone of inhibition. Among the micro organism, irrespective of the materials, surface adherence, and penetration of *C. albicans* were seen more, comparative to *S. mutans* and *L. acidophilus*. The SBC revealed a significantly lower susceptibility to adhere to all the three microorganisms tested (*S. mutans, L. acidophilus, C albicans*) when compared to conventional MBC.

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