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ORIGINAL ARTICLE

An *In-Vitro* Analysis of the Mechanical and Anti-Bacterial Properties of Betel Leaf Extract with Chitosan Coating on Orthodontic Aligners

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ABSTRACT

Objective: To aim at the preparation and characterization of a chitosan and betel leaf extract biopolymer followed by an assessment of mechanical and antibacterial properties. **Material and Methods:** A chitosan-betel leaf stock solution was prepared and dip-coated on thermoformed aligner cubes. The groups (five samples per group) involved were one control (only chitosan coating) and three test groups based on the duration of coating (coating with chitosan and betel extract coated for 5, 10, and 15 minutes, named B5, B10, and B15 respectively). The coating of the samples was confirmed by an FTIR test and anti-microbial properties were assessed by the disc diffusion method. Wear resistance assessment of the coating was carried out by scratch test. **Results:** Coating of the aligners with the extract was confirmed with FTIR. The zone of inhibition against *S. mutans* was noted to be the widest for aligners coated for 15 minutes (7± 0.5mm), and the intergroup difference was statistically significant ($p<0.05$). The samples dip-coated for 15 minutes demonstrated the highest wear resistance (3.2 ±0.17 N) with statistically significant intergroup differences ($p<0.05$). **Conclusion:** Betel leaf extract prepared and incorporated with chitosan biopolymer was successfully coated on thermoformed aligners. A higher antibacterial activity and scratch resistance were evident with aligners dip-coated for 15 minutes, owing to the antibacterial activity of *Piper betel.*

Keywords: Orthodontic Appliances, Removable; Microbiology; Dental Materials; Oral Health.

Introduction

Clear aligner therapy (CAT) involves using clear thermoformed plastic materials called aligners for delivering forces to the dentition for correction of malocclusions by employing composite attachments [1]. For maintaining oral hygiene and reducing the risk of developing caries, patients are often advised to avoid using them while eating and they are advised to maintain good oral hygiene while using them. Therefore, aligners are preferred over braces for patients demanding aesthetic care [2-4]. However, like conventional fixed appliances, aligners do allow plaque biofilm formation, as their surfaces have microcracks and corrugations, leading to bacterial adhesion which in some reports has been lesser than or sometimes comparable to self-ligating systems as well as conventional fixed appliances [5-8]. Previous studies have reported that within two months of the start of clear aligner therapy, growth of *T. forsythia*, *P. intermedia*, *F. nucleatum*, and *P. gingivalis* was noted [3,5]. Regarding salivary counts of *S. mutans* and *L. acidophilus*, earlier studies reported no differences between adolescent patients treated for one month with thermoplastic aligners and those treated with self-ligating appliances [6]. During CAT treatment, aligners were cleaned with mouthwash or toothbrush and toothpaste; these procedures are not always successful in fully removing plaque from the aligner surface [9-11].

The functionalization of the aligner's surfaces to prevent plaque growth has been the focus of recent research on aligner materials. Modified gold nanoparticles were used as coatings on the aligners to cease the increase of *P. gingivalis* and *S. mutans* [9]. Cinnamaldehyde, in the form of an antibacterial agent, has been used as an aligner coating, resulting in a reduction in the growth of Streptococcus bacterial species [12]. However, both these methods were not found to be economically and clinically feasible considering that multiple sets of aligners are required and worn by the patient for up to 14 days during treatment. As a result, it is desirable if the coating of the aligner is created without the need for complicated synthesis processes utilizing readily available materials and straightforward techniques. In addition, we must also consider the risks of leaching of metal oxides from nanoparticle-based coatings, and apply the use of non-toxic bioactive constituents.

The Piperaceae family of plants includes piper betel, which is frequently prescribed in conventional medicine to cure certain common illnesses. Extracts from *Piper betel* leaves are rich in bioactive compounds such as tannins, alkaloids, saponins, and polyphenols which confer anti-inflammatory, detoxification, antioxidant, and anti-mutation properties [1]. Several studies have found a substantial inverse relationship between dental cavities and the amount of *Piper betel* consumption [13-14]. Deacetylating chitin produces chitosan, a non-toxic substance that is biodegradable, biocompatible, and has antifungal and antibacterial mucoadhesion characteristics. These characteristics of chitosan enable its use in the field of dentistry [15].

The current *in vitro* study investigated the utilization of a *Piper betel* leaf extract with a biopolymer covering made of chitosan. This study's objective was to prepare, and coat followed by characterization of a chitosan and betel leaf extract biopolymer over the aligner material and assess its anti-microbial effect as well as its scratch resistance.

Material and Methods

Materials

Aligner materials (0.5 mm Duran sheet, Scheu Dental GmbH, Iserlohn, Germany) were used for this study. Chitosan solution (Sisco Research Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) and coupling agent- Polyethyleneimine (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) were used for the coating. 500 grams of fresh *Piper betel* leaves were procured from the local market. All the reagents and chemicals used in the present study are of Analytical grade (A.R) with 99% purity.

Preparation of the Betel Leaf and Chitosan Biopolymer Coating Solution

Leaf Extract preparation: *Piper betel* leaves were carefully cleaned first under running water. They were then kept in the hot air oven overnight at 50ºC until the moisture content was reduced to 10%. To obtain a fine powder the dried leaves were gathered and then subjected to a high-speed blender followed by sieving with an 80-mesh stainless steel mesh. A magnetic stirrer running at 700 rpm was used to mix 10 grams of *Piper betel* leaf powder into 40 milliliters of ethanol solution constantly for the whole night [16]. The solution was then filtered, and the extract was obtained.

Preparation of Chitosan Solution: 2 wt.% Chitosan solution was prepared in 2 v/v glacial acetic acid.

Preparation of Coating Solution

The lowest inhibitory concentration of the ethanol-based *Piper betel* extract for *V. cholerae*, *S. aureus*, and *E. coli* was found to be 0.625, 0.625, and 0.625 mg/mL, according to research by Khatun and Hoque [17] and Hoque et al. [18]. The coating solution was prepared by mixing 10 ml Piper betel leaf extract and 10 ml chitosan solution in a stirrer to set a MIC of 1 mg/mL.

Thermoformed Aligner Sample Fabrication and Surface Coating

Graphic design software (3ds Max Software, Autodesk) was used to create the template using a platform with a 10 cm diameter and 1x1 cm cubes as were printed (Figure 1). 3D printing of the template was done in resin, and Biostar was used to thermoform the aligner sheet material into cubes (Scheu Dental GmbH, Iserlohn, Germany). After being thermoformed onto the printed blocks, the aligner material sheet cube was separated from the template.

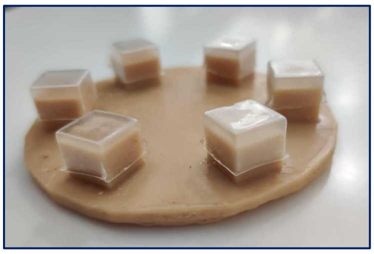

Figure 1. Template for thermoforming the aligner cubes.

A UV chamber was used to surface activate the aligners' surfaces for 20 minutes. After that, they were immersed in a polyethyleneimine crosslinking agent solution for 10 minutes. The cubes were then placed in a hot air oven set to 50 °C for one hour.

Determination of the Antimicrobial Effect of Coating

To analyze the antimicrobial activity of the coated aligners against *S. mutans* and *E. coli*, a disc diffusion test was performed. 100 microliters of test organisms were spread onto nutrient agar plates in sterile conditions. The samples coated for 5, 10, and 15 minutes as well as the controls were subjected to UV sterilization for 30 minutes and discs were punched from the cubes. The discs were then inoculated in nutrient agar for 24 hours at 37ºC and then the zone of inhibition around the discs was measured and recorded using a digital camera. For each sample, a disc diffusion assessment was performed thrice.

Scratch Resistance Assessment

For assessment of scratch resistance the test samples were subjected to a nanoindentation test utilizing a mechanical step surface testing platform (Step 700, Anton Paar Austria GmbH, Graz, Austria) with a Rockwell-type indenter. The samples were subjected to forces of 0–5 N at a rate of 10/ min, held steady for 5 mN for 30 s, and then released, reversing the loading procedure. The scratched surface was next examined under a light microscope to evaluate the scratch resistance qualitatively.

Statistical Analysis

SPSS software package, version 12 (IMB Corp., Armonk, NY, USA) was used to assess the significance level of $p<0.05$ using one way ANOVA method.

Results

As seen in Figure 2, characteristic spectral peaks of aligner material were noted at 1708 cm⁻¹, 1668 cm⁻¹ (stretching mode of the C=O groups, free and H-bonded, respectively); 1527 cm⁻¹ (N–C=O moiety vibrations); 1456 cm⁻¹, 1412 cm⁻¹ (bending modes of C–H bonds); 1242 cm⁻¹ (stretching modes of C–N bonds); 1094 cm⁻¹ and 1012 cm⁻¹ (stretching modes of C–O–C bonds); and 720 cm⁻¹ (bending modes of C–H bonds in aromatic rings).

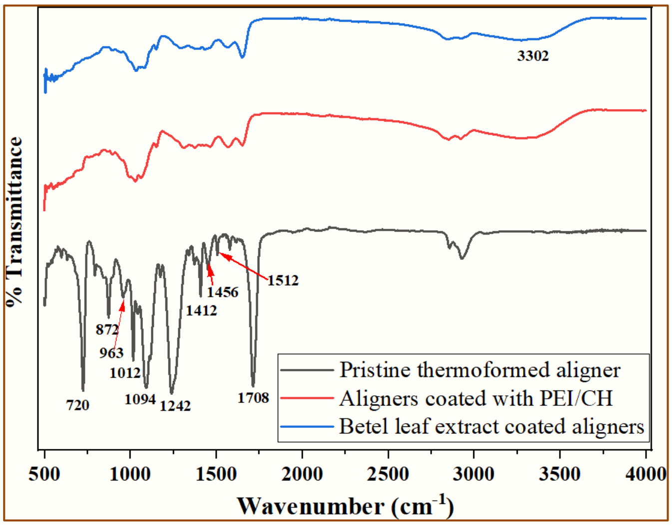

Figure 2. FTIR spectra of betel leaf-coated aligners.

High-density polyurethanes and polyesters majorly constitute the makeup of aligner material [19]. The FTIR spectra observed were also in agreement with that of high-density polyurethanes and polyesters. The aforementioned thermoformed aligners' typical peaks were changed by the coating of betel leaf extract, and a novel peak was found at 3302 cm-1 that corresponded with the alcohol and phenol (O-H) group of *Piper betel* leaf [20]. This demonstrates that the thermoformed aligners were successfully covered with *Piper betel* leaf [16]

All groups showed antibacterial effects against *S. mutans* but no antimicrobial effect against *E. coli*. The diameter of inhibition zones around samples is listed in Table 1. The one-way ANOVA showed a statistically significant intergroup difference ($p<0.05$). B15 showed the widest zone of inhibition followed by the chitosan control and B10 group.

Table 1. Antibacterial efficacy of coated aligners against *S. mutans*.

Groups	No of Samples	Zone of Inhibition in mm	F-value	p-value
Control	5	3 ± 0.5	96.857	<0.001
B5	5	0		
B10	5	3 ± 0.5		
B15	5	7 ± 0.05		

One-way ANOVA test for intergroup differences was statistically significant ($p<0.05$). B15 samples showed the highest scratch resistance, followed by coated for 10 minutes and then 5 minutes. Images of the Nanoindentation test samples are shown in Figure 3. Table 2 shows that as the dip coating time increased, more force was required to dent the coating's surface. This could be attributable to an increase in coating thickness with longer dip coating times.

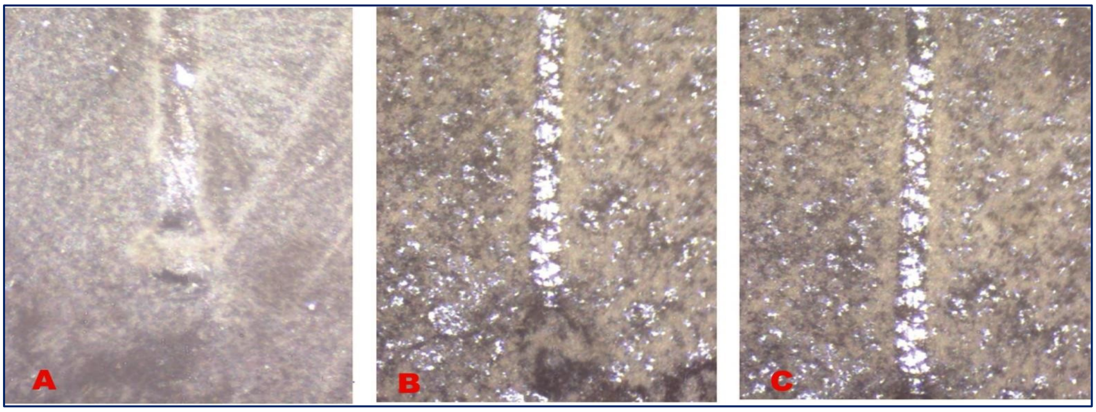

Figure 3. Samples B5 (A), B10 (B), and B15 (C) respectively as seen under the Scanning Electron Microscope at 50X magnification post-scratch test.

Table 2. Results of the nanoindentation test.

Groups	No. of Samples	Force in N	F	p-value
B5	5	1.7 ± 0.09	97.548	<0.001
B10	5	2.9± 0.21		
B15	5	3.2 ±0.17		

Discussion

Thermoforming increases surface roughness of aligner material which in turn increases plaque formation and bacterial adherence [8,21]. There have been no prior descriptions of the use of betel leaf extract-chitosan biopolymer coating on dental aligner material. In the present study, the Betel extract-chitosan biopolymer conjugate exhibited a significant antibacterial effect against *S. mutans*, and the antibacterial activity as well as scratch resistance increased with dip-coating time.

In this study, a concentration of 1 mg/mL of the coating did not have an anti-microbial activity against *E. coli*, although MIC of 0.625 mg/ml of betel leaf extract was reported to display anti-bacterial activity against *E. coli* in previous studies [17]. Surface modification of thermoformed aligners, for reduction in plaque accumulation has previously been reported. Zhang et al. [22] developed a method for coating aligners with ammonium-modified gold nanoparticles and carried out biofilm studies, crystal violet staining assay, and contact antibacterial assay. SEM and confocal microscopy images revealed that the coating had an antimicrobial effect against *Porphyromonas gingivalis* and *Streptococcus mutans* [9,22].

Using isothermal microcalorimetry, Worreth et al. [12] incorporated cinnamaldehyde (trans-cinnamaldehyde 99%) in thermo foil and reported a reduction in the growth rate of *S. epidermidis*, *S. mutans*, and *S. Mitis* [12]. Anitha et al. applied a Zinc oxide nanoparticle coating to thermoformed aligners with antimicrobial activity against *S. mutans* for up to 7 days [23]. Although effective, nanoparticle-based coating carries the risk of metal oxide leaching and is not economically or clinically feasible. An aligner coating of chitosan and gingerol has been previously applied by Vas et al. with antibacterial activity against *S. mutans* [24]. A similar methodology was employed in the present study incorporating gingerol extract into a chitosan biopolymer coating on an aligner surface. The study reported the highest antibacterial activity and scratch resistance of the sample dip-coated for 15 minutes.

Streptococcus mutans, which causes dental cavities, was reported to be resistant to betel leaf extract under 4- Allylpyrocatechol (APC), a phenolic component isolated from *Piper betel* leaves [25,26]. However, Subri et al. reported *Piper betel* leaf extract to be effective at inhibiting the growth of *Streptococcus mutans* at a concentration of 0.25% [25]. The results from the study concur with the results of our study since the coating had the same concentration of extract. According to Phumat et al., the extract is effective in inhibiting the growth of the biofilms of *C. albicans*, *S. intermedius*, and *S. mutans* [26].

Chitosan (CHS), which is typically found in low, medium, and high molecular weights, is a polymeric substance made chemically of N-acetylglucosamine and glucosamine copolymer units [27]. Chitosan demonstrated anti-microbial action against *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* and *S. mutans* [28]. Free amino groups in chitosan allow it to crosslink with the polymers of aligner material, as confirmed by FTIR in this study [29]. This property allows its application as a medium for oral drug delivery, as a scaffold for guided tissue regeneration, and allows it to be coated on dental implants [28]. Magnesium Calcium dip coating has been explored successfully to slow biocorrosion; however, no scratch resistance test was performed [30]. An improvement in scratch resistance was seen in the current study, which was supported by a nano-indentation test, and coating thickness increased with coating time.

Despite its antibacterial effectiveness, a significant drawback of this combination is the staining of the aligner material, which was more evident with an increase in dip coating time. This is due to the pigmentation by carotene, anthocyanins, chlorophyll a, chlorophyll b, and xanthophyll found in the leaf [31]. Future research may attempt to decolorize betel leaf extract using the enzyme Laccase, which has demonstrated promise in the decolorization of synthetic and reactive colors [32,33].

Conclusion

The antimicrobial effect and the scratch resistance of *Piper betel* extract-based aligner coating were investigated in the present study. The FTIR test confirmed crosslinking between the coating and aligner material. The coated aligners displayed anti-bacterial activity against *S. mutans*, according to the findings of the disc diffusion test. Aligners coated for a longer duration had higher scratch resistance which was confirmed by nano-indentation test. As a concluding remark, the *Piper betel* extract might be used as a coating agent in dental applications for microbicide and metal scratch resistance rather than synthetic material-based product usage.

Authors' Contributions

NVV	https://orcid.org/0000-0002-6288-3449	Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft and Writing - Review and Editing.
RKJ	https://orcid.org/0000-0002-7373-3788	Investigation, Writing - Original Draft and Writing - Review and Editing.
KK	https://orcid.org/0000-0003-2603-6733	Writing - Review and Editing, Supervision and Project Administration.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

Financial Support

None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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