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Microbial Biofilm Inhibition in Dental White Spot Lesions using Crustin Derived Antimicrobial Peptide Crustin (CAMP) and Bio-assisted Sida Acuta Mediated Titanium Nanoparticles (SA_NP)

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Abstract

White spot lesions (WSL), most commonly associated with Streptococcus mutans and Lactobacillus acidophilus are the routinely encountered, drawback in patients undergoing fixed orthodontic therapy due to poor oral hygiene, plaque and biofilm adhesion and retention. Many approaches have been reported against WSL lesions, amid a surge in reports on their antimicrobial resistance. In the present study, we aimed to evaluate two such novel formulations, cysteine-rich crusting antimicrobial peptide (CAMP) and plant-derived nanoparticles (SA_NPs) for the inhibitory activity of dental microbes. CAMP and SA_NP were isolated and characterized, according to the protocol adopted from our previous work. Followingly, the antimicrobial activity was assessed by a well diffusion method and minimum inhibitory concentration (MIC). In situ light microscopy analysis was used to evaluate their respective biofilm inhibition concentration (BIC). The results indicated a dose-dependent relation of CAMP and SA_NP. MIC of CAMP against S. mutans at 75 µg/ml was 17±0.4 mm and against L. acidophilus was 18±0.1mm, where MIC = 75 µg/ml for SA_NP, where in 17±0.1 mm zone of inhibition against S. mutans and 17±0.3 mm against L. acidophilus was noted. Maximum arrest/inhibition of biofilm growth for both S. mutans and L. acidophilus was observed at BIC= 75 µg for both CAMP and SA_NP. Therefore, newer approaches like the incorporation of green synthesized nanoparticles and curated peptide antimicrobials offer a new approach to treating microbial pathogens that are resistant to current treatment practices and for the treatment of pathogenic biofilms.

Keywords: *Antimicrobial Peptide Crustin, Green Synthesis, Nanoparticle, White Spot Lesion.*

Introduction

White spot or incipient lesions (WSL) are characterized as subsurface enamel porosity consequent of enamel demineralization which presents itself as milky-white opacity on enamel surfaces [1]. These decalcifications are often associated with long-term fixed orthodontic therapy and in cases of poor oral hygiene, of which their prevalence has been reported to range from 2%-97%, according to multiple epidemiological studies [2,3]. Poor oral hygiene, impairment in self-cleansing mechanisms and prolonged retention of tenacious plaque and microbial biofilm adherence predominantly, *S. mutans* (MS) and *L. acidophilus* (LA) during orthodontic treatment leads to an acute drop in pH, consequently causing an imbalance in the mineralization-demineralization cycle of enamel, ultimately leading to WSL, cavitation

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and dental caries, which if neglected leads to serious consequences [4, 5]. In various other studies, the incidence of WSL in patients undergoing orthodontic therapy has been 45.8% [4]. The alarming surge in WSL lesions and its debilitating sequelae ranging from mild lesions to large cavitations, necessitates both the patients and primary caregivers to take effective WSL precautionary measures.

Many researchers have worked on natural biosynthetic and synthetically prepared formulations to combat WSL in a multitude of approaches, which include the administration of fluoride products (in various forms like toothpaste, mouthwash, varnish, casein phosphopeptides amorphous calcium phosphate), probiotics, polyols, antiseptics like listerine/chlorhexidine based compounds [6–10]. However, microbial resistance is a predicament commonly encountered even in the dental community in recent times. Therefore, there is a critical need for the identification and evolution of new antibacterial agents against the pathogenic oral microbiome. Two such upcoming approaches to combat oral pathogenic microbiota include the use of antimicrobial peptides and silver/bio-assisted nanoparticle coatings [11]. Multiple studies have reported on the antibacterial properties of various nanoparticles against cariogenic microorganisms, especially *S. mutans* [12,13]. Previous literature reports the use of silver nanoparticles and peptide sequences against medical pathogenic bacteria and has seen a surge in multiple studies in the dental domain targeting cariogenic microbes, especially MS and LA strains [14,15]. These two contributing microorganisms have been widely studied among patients undergoing fixed orthodontic therapy [16]. Therapeutic effects of *Sida acuta* extracts have recently been reported against pathogenic bacteria and cercaria [17,18]. The present study aims to apply the antimicrobial properties in the dental domain by evaluating the antimicrobial and antibiofilm activity of an antimicrobial peptide (AMP), Crustin and a plant-derived (*Sida acuta*) bio-assisted nanoparticle against WSL lesions [11], [19].

Material and Methods

This prospective cohort was conducted in the Department of Orthodontics, Saveetha Dental College and Hospital, Chennai. The subjects undergoing fixed orthodontic therapy were screened for the presence of white spot lesions, based on the classification of WSL by Gorelick *et al* based on visual inspection [5]. Sample size estimation was done using G power software (Version 3.0.10) with a one-tailed mean with a 1:1 allocation ratio, assuming normality (power, 0.95; $\alpha = 0.05$) based on the study by Momeni *et al* and was set to n=10 subjects [20]. Saliva and plaque samples were collected from each subject. 5 ml of unstimulated saliva was collected by the method advocated by Justino *et al* by asking the volunteer to sit still with his/her head up, avoid swallowing and concentrate the saliva on the floor of the mouth before spitting it into a cup container, typically took about an average of 5 mins [21]. The collected saliva was then preserved in Phosphate buffer saline. If an immediate analysis is not available, the samples were stored at −80°C ultra-low temperature freezer. Dental plaque was also collected using a sterile small brush or swab, and then eluted the plaque by scrubbing them in the prepared buffer for about 1 min. Following which, the collected saliva was separated into a 1.5 ml sterile centrifuge tube and centrifuged (10,000–16,000 g, 4°C, 15 min).

Isolation and Characterization of Crustin Derived Antimicrobial Peptide (AMP)

This protocol was adapted from our previous study by Sivakamavalli *et al*, 2014 herein the AMP was isolated and purified from the granular extract from freshly extracted hemolymph from Pacific White leg Shrimp

(*Litopenaeus vannamei*) as mentioned in our previous study protocol.

Preparation of Plant Derived (*Sida acuta*) Nanoparticle (SA_NP)

The plant is collected from a nearby nursery in Chennai (Figure 1). The collected plant leaves were shade-dried for 48h and the dried materials were ground into powder using a motor and pistil. The powder particles were suspended with distilled and filtered water and kept in a water bath at 70^{o}C for 4h. Then the extract was added to 1M titanium dioxide (TiO2) solution and kept in a magnetic stirrer for 1-2h for nanoparticle synthesis.

Figure 1. *Sida acuta* Plant Used for the Preparation of SA_NP

Antimicrobial Assay and Evaluation of Minimum Inhibitory Concentration (MIC) of AMP and SA_NP

The WSL-associated bacterial strains were isolated from the plaque and saliva samples. The antimicrobial assay was conducted on Luria-Bertani (LB) agar plates (in triplicates) under two separate test conditions, one supplemented with SA_NP and the other by AMP in varied concentrations of 25, 50, 75 and 100 µg/ml and control (where no material was added). The plates were incubated in static condition for 24 h at 37◦C. The contents of the wells of the microtiter plates were discarded and gently washed with phosphate-buffered saline (PBS), dried fixed with 2% w/v sodium acetate; wells flooded with crystal violet stain (0.1%, w/v) and incubated in the dark for 30 min. After complete drying, 200 µl of ethanol (95%, v/v) was added to each well and absorbance at 620 nm was measured followed by CV staining and the diameter of the zone of inhibition was measured. The stained cultures were also smeared on slides and viewed under light microscopy ×40 (Nikon, Eclipse, Ti100).

Antibiofilm Assay and Evaluation of Biofilm Inhibitory Concentration (BIC) of AMP and SA_NP

The anti-biofilm activity for the determination of MIC of AMP and SA_NP was conducted in a 24-well microtiter plate (flat bottom, polystyrene). Individual wells of the plates were filled with 180 µl of Mueller-Hinton Broth, to which 10 µl of the test pathogens (OD = 1.0, 600 nm) and both SA_NP and AMP were added in two separate test conditions, following which they were incubated in a static condition for 24 h at 37°C. Consequently, the contents of the wells of the microtiter plates were discarded and gently

washed with phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent bacterial cells. The microtiter plates were then air-dried for 45 min. After drying, adherent "sessile" bacteria in the wells were fixed with 2% w/v sodium acetate and the wells were then flooded with crystal violet stain (0.1%, w/v) and incubated in the dark for 30 min and then thoroughly washed with sterile deionized water until all excess dye was removed. The plates were then air-dried again. After complete drying, 200 µl of ethanol (95%, v/v) was added to each well and absorbance at 620 nm was measured (Multi-scan plate reader, Thermo Fisher Scientific) and viewed under light microscopy × 40 (Nikon, Eclipse, Ti100).

Results

Antimicrobial Assay

The effect of shrimp *L. vannamei* crustin antimicrobial peptide was tested for its antimicrobial activity by a well diffusion method. WSL-associated bacteria, mainly *S. mutans* and *L. acidophilus* were tested against varying concentrations of crustin (25 µg/ml, 50 µg/ml, 75 µg/ml, and 100 µg/ml) that revealed a dose-dependent relationship. Table 1-2 depicts the zone of inhibition (measured in mm) of crustin and SA_NP at varying concentrations against *S. mutans* and *L. acidophilus*, respectively. Results indicate that AMP (Crustin) inhibits bacterial growth with its increasing concentration. Both species of bacteria were inhibited at their respective MIC. Zone of inhibition at Minimum Inhibition Concentration (MIC) of crustin-AMP against *S. mutans* at 75 µg/ml was 17±0.4 mm and against *L. acidophilus* was 18±0.1mm, as illustrated in Figure 2A-B.

Similarly, the evaluation of MIC for SA_NP revealed a dose-dependent relationship, that is higher concentrations of SA_NP exhibited greater diameter of zone of inhibition. Greater than 95% bacterial inhibition was observed at MIC 75 µg/ml, where in 17±0.1 mm zone of inhibition against *S. mutans* and 17±0.3 mm against *L. acidophilus* was noted, as illustrated in Figure 3A-B.

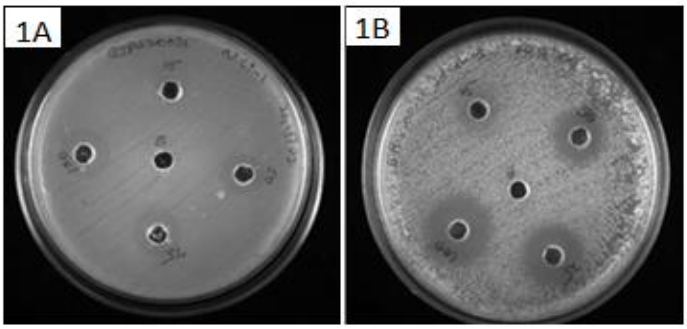

Figure 2 (A-B). Antimicrobial Activity of AMP (Crustin) Against *Streptococcus mutans* and *Lactobacillus acidophilus.*

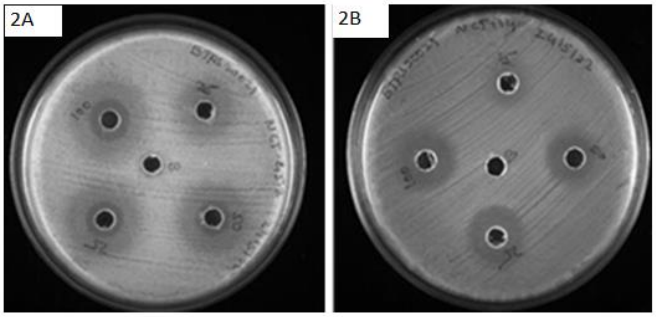

Figure 3 (A-B). Antimicrobial Activity of SA_NPs against *Streptococcus mutans* and *Lactobacillus acidophilus* Respectively.

Table 1. Zone of Inhibition of Shrimp Derived Peptide Crustin Against *S. mutans* and *L. acidophilus*

AMP (Crustin)	**Zone of inhibition**	
Samples	*Streptococcus mutans*	*Lactobacillus acidophilus*
Control	-	-
25 µg/ml	15mm	16mm±0.1
50 µg/ml	15mm±0.1	16mm±0.2
75 µg/ml	17mm±0.4	18mm±0.1
100 µg/ml	18mm±0.1	18mm±0.2

Table 2: Zone of inhibition of SA_NPs against *S. mutans* and *L. acidophilus*

SA_NPs	**Zone of inhibition**	
Samples	*Streptococcus mutans*	*Lactobacillus acidophilus*
Plant extract	-	-
25 µg/ml	15mm±0.1	16mm±0.1
50 µg/ml	15mm±0.4	16mm±0.2
75 µg/ml	16mm±0.2	16mm±0.1
100 µg/ml	17mm±0.1	17mm±0.3

Antibiofilm Assay

In situ microscopy analysis visualized through light and atomic force microscopy, initially the anti-biofilm assays were performed spectrophotometrically on the growth rate of bacterial cultures such as *S. mutans* and *L. acidophilus.* Followingly the purified crustin and SA_NPs was used for biofilm inhibition assay using the different concentrations (25 to 100 µg/ml), as seen in Figure 2-5. The results revealed disrupted biofilm development, with increased disruption with increasing concentrations of both AMP and SA_NP. Complete arrest of biofilm growth for both *S. mutans* and *L. acidophilus* was observed at 75 µg (Biofilm inhibitory concentration: BIC), as viewed on light microscopy as seen in Figure 4-7.

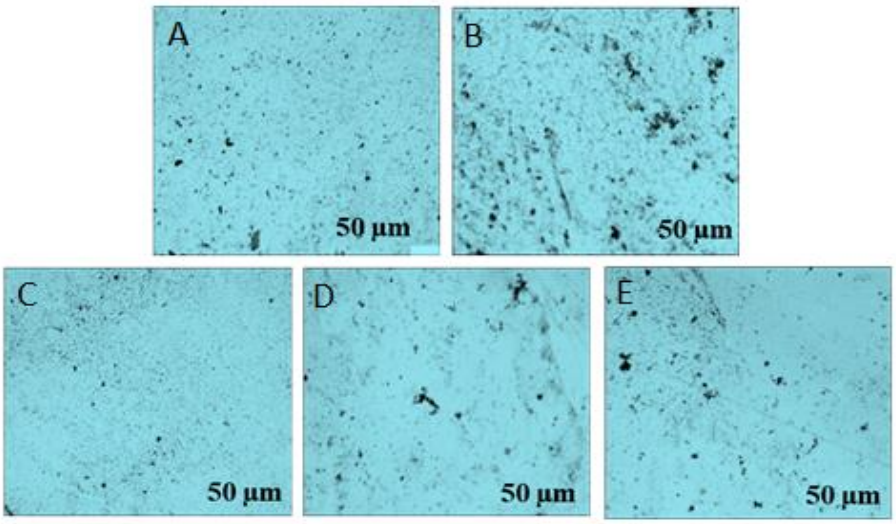

Figure 4 (A-E). Antibiofilm Assay for Different Concentrations of SA_NPs against *S. mutans*

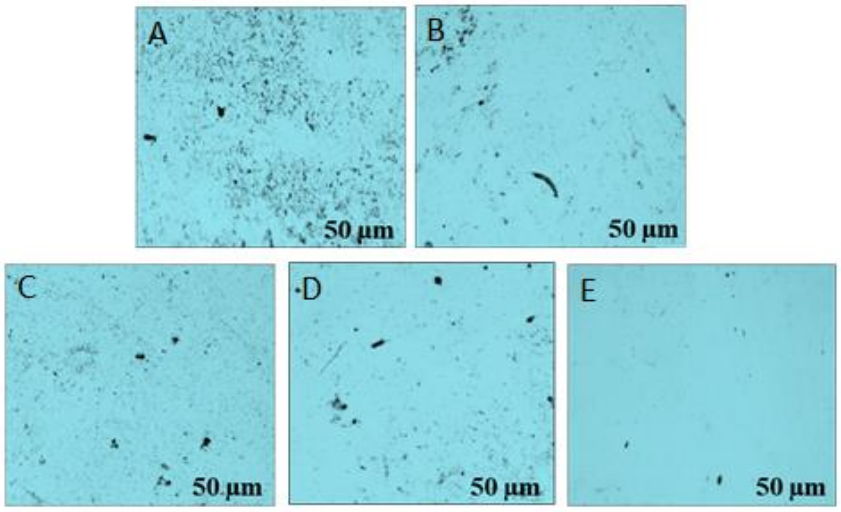

Figure 5 (A-E). Antibiofilm Assay for Different Concentrations of SA_NPs against *L. acidophilus*

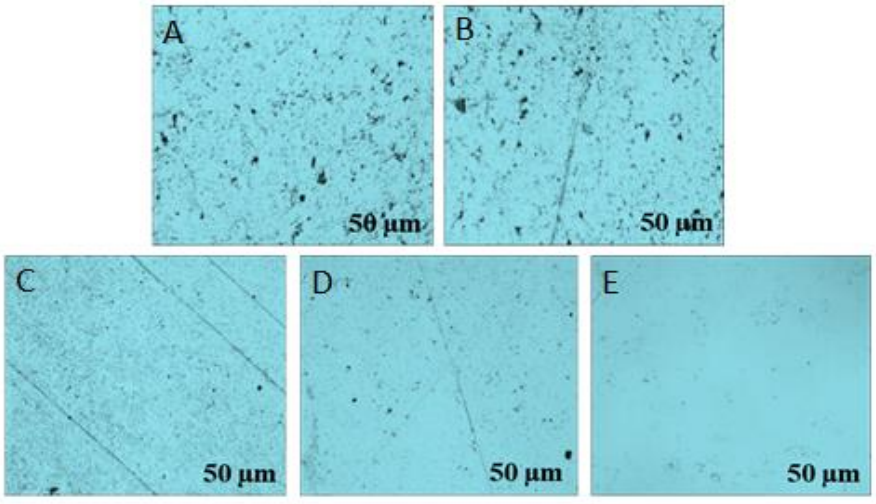

Figure 6(A-E). Antibiofilm Assay for Different Concentrations of AMP against *S. mutans*

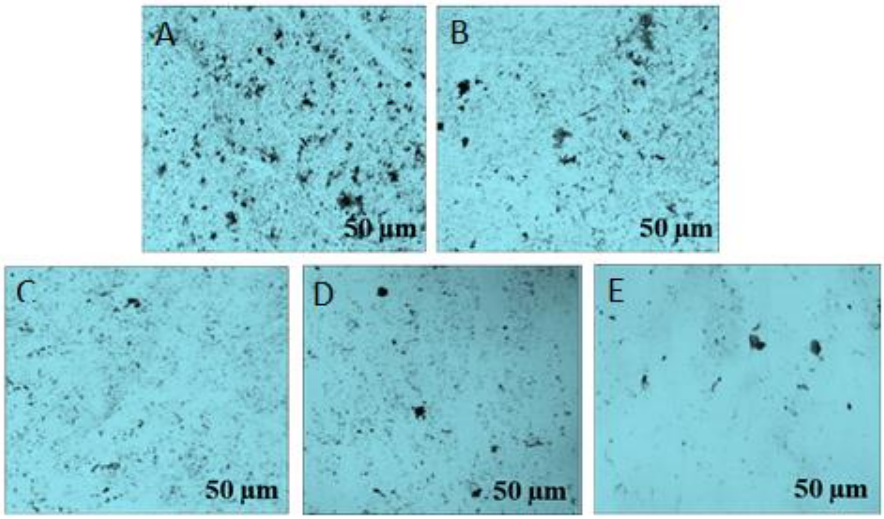

Figure 7 (A-E). Antibiofilm Assay for Different Concentrations of AMP against *L. acidophilus*

Atomic Force Microscopy

Atomic force microscopy was employed to investigate the attachment of bacterial cells to the glass substrate and intercellular attachment forming biofilms, highlighting variations in surface morphology in the captured images. The topography and deflection images acquired for bacterial samples forming biofilms revealed the impact of biofilm inhibition. Detailed analysis of the control and treated biofilms involved studying their average height and surface roughness. The mean surface value was measured relative to the difference in height between the surface's highest and lowest points from the mean plane. Observations from images *of L. vannamei* crustin-treated samples exhibited reduced adherent bacterial presence on the surface in contrast to the control group, which displayed increased thickness and aggregated bacteria in the height profile, as viewed in Figure 8. Under in vitro conditions, the effect of crustacean AMP on bacterial growth inhibition was monitored by examining the binding of acridine orange to adherent cells, providing a direct measure of biofilm effectiveness.

In microtiter wells, S. mutans and L. acidophilus adhered to glass pieces and were subjected to various concentrations of *L. vannamei* crustin and SA_NP treatments. A 24-hour treatment led to a remarkable decrease in biofilm growth, up to 92%, notably with the highest concentration of *L. vannamei* crustin at 75 µg/ml, (Figure 9). These findings suggest that *L. vannamei* crustin hampers the bacterial cell walls of Gram-positive bacteria like S. mutans and L. acidophilus. The interaction between crustacean AMP and bacterial cell walls resulted in bacterial agglutination and the retardation of bacterial growth on glass slides. Bacterial Pathogen-Associated Molecular Patterns (PAMPs) such as lipopolysaccharide (LPS), N-acetyl muramic acid (NAM), N-acetyl glutamic acid (NAG), and peptidoglycan can interact with purified *L. vannamei* crustin and SA_NP, inducing bacterial agglutination and causing inner membrane permeabilization in Gram-positive bacteria.

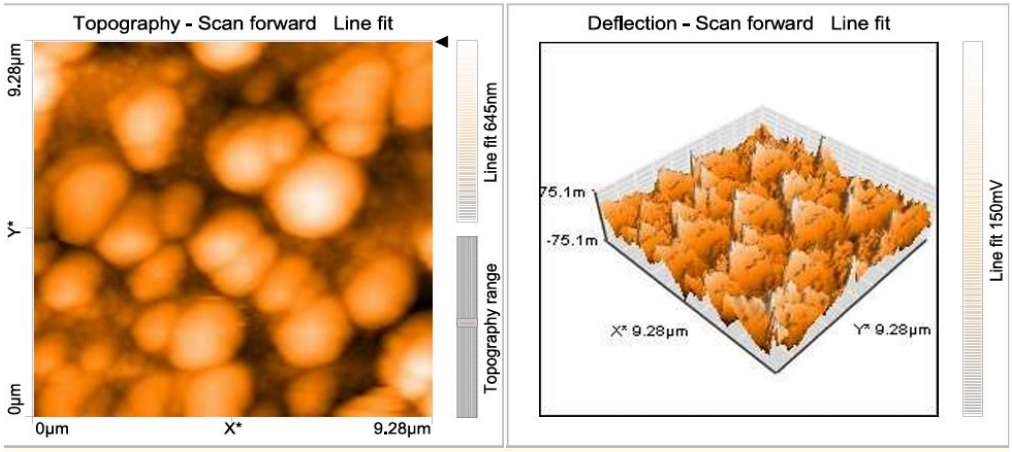

Figure 8. Surface Topography of shrimp *L. vannamei* crustin antimicrobial peptide

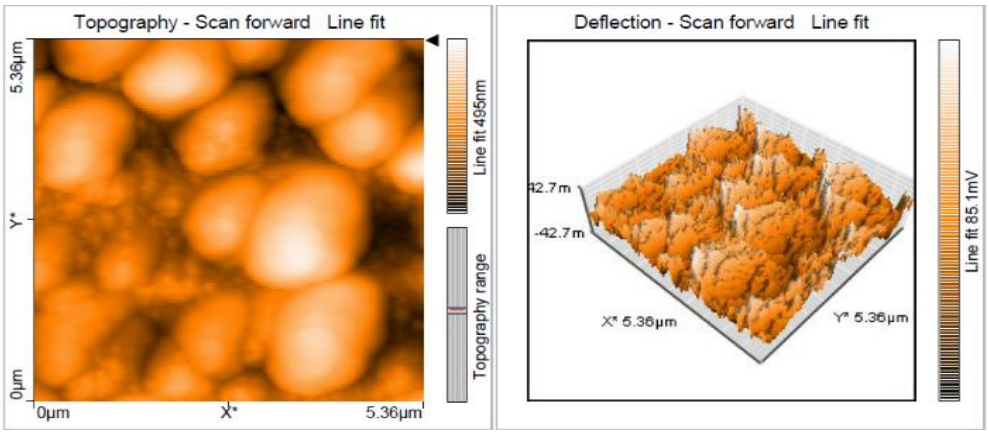

Figure 9. Surface Topography of shrimp *L. vannamei* crustin Antimicrobial Peptide

Discussion

Enamel demineralization, leading to white spot lesions is an inevitable drawback to fixed orthodontic therapy due to more plaque and biofilm retention and impairment of oral self-cleansing mechanism [22]. This iatrogenic effect often is contradictory to the goal of orthodontic treatment, providing the best function and aesthetics in the dentofacial region. Many recent approaches have therefore been developed to combat white spot lesions, which involve a range of preventive pharmaceutical therapeutics to intercept minimally invasive procedures like resin infiltration techniques [6,23]. The most commonly encountered challenge includes oral microbial resistance when subjected to chemical therapeutics like chlorhexidine, and fluoride [24]. Newer strategies involving bioactive compounds have evolved to improve the efficacy of the existing therapeutics or formulate newer natural/synthetic formulations. This study highlights the antimicrobial and antibiofilm properties of two such compounds; marine antimicrobial peptide (crustin- with a molecular weight of 14kDa) derived from *L. vannamei* (pacific whiteleg shrimp) and a plant-derived (*Sida acuta*) nanoparticle, in the hope of overcoming the above-mentioned challenges.

Antimicrobial properties of AMP and SA_NP were assessed by a well diffusion method to measure the zone of inhibition and their respective minimum inhibitory concentration (MIC). Antibiofilm properties of AMP and SA_NP were assessed to measure their respective biofilm inhibitory concentration (BIC) through microscopy technique. The results report a linear dose-dependent relationship between the test bioactive compound (crustin) and plant-derived

nanoparticles (SA_NP). The zone of inhibition at MIC=75 µg/ml of crustin-AMP against *S. mutans* at 40 µg/ml was 17±0.4 mm and against *L. acidophilus* was 18±0.1mm. Greater than 95% bacterial inhibition was observed at MIC=75 µg/ml, where in 17±0.1 mm zone of inhibition against *S. mutans* and 17±0.3 mm against *L. acidophilus* was noted. The antibiofilm assay by light microscopy also revealed a dose-dependent inhibition for the AMP and SA_NP treated samples, where complete arrest of biofilm growth for both *S. mutans* and *L. acidophilus* was observed at 75 µg/ml (BIC).

Due to the recent advances in the field of nanotechnology, there has been enormous research in synthetic and naturally occurring nanoparticles, and their clinical applications in the field of dentistry. Many studies have reported the use of inorganic nanoparticles like selenium, silver, fluoride and their incorporation in various formulations like toothpaste, varnish etc against *S. mutans* [25]. A study by Kulshrestha *et al* reported the anti-cariogenic and anti-biofilm properties of calcium fluoride nanoparticles (CaF_2NPs) by the inhibition of exopolysaccharides against *S. mutans* [26]. Recent studies also report a synergistic effect of various biocompatible polymers like chitosan, alginate and pectin based on their mucoadhesion and slow fluoride-releasing properties [27]. Titanium dioxide, copper and silver nanoparticles incorporated chitosan have been reported to exhibit anti-growth and anti-biofilm properties that promote remineralization [28,29]. Newer biocompatible and mucoadhesive materials like polyethylene glycols (PEG) and poly lactic-co-glycolic acid (PLGA) were reported to exhibit bactericidal and anti-biofilm activity on *S. mutans* [30,31]. However, devoted research towards newer methods by way of green synthesis is gaining momentum, therefore shifting focus on environment-friendly methods by incorporating principles like waste minimization, reduction in pollution and renewable feedstock [32]. These include many plant-derived bioactive nanoparticles (PDNP), which are metal-free and eliminate the concerns around metal toxicity, including hepatic and renal damage [33]. A study on nanoparticles derived from *Curcuma longa* extracts has been reported for its antimicrobial efficacy against multi-drug resistant bacteria by generating reactive oxygen species (ROS) that disrupt cell wall permeability, resulting in DNA and cellular protein malfunction and bacterial cell death [34]. These bioactive compounds have been reported to be safe for humans as oral therapeutics by various researchers [35,36]. Similar results have been reported by metal-free NPs from *Citrus medica* extracts against MDR and biofilm-producing bacteria [37]. Therefore, the current literature strongly suggests and advocates the use of PDNPs as alternative therapies against oral microbiota and their MDR species.

Crustins are now being widely researched. Current literature evidence reports its great antifungal and antimicrobial activity against selected Gram-positive bacterial strains. Several AMPs have been identified and isolated from the haemolymph of different shrimps (*E. tetragonum* [11], *P. monodon* [38], and *F. chinensis* [11,39]. These marine-derived crustins are essentially cysteine rich, negatively charged bioactive compounds with whey acidic domain (WAP) which is reported to attribute to its protease inhibition and antimicrobial activity by membrane disruption of the test pathogens [40,41]. Generally, crustins have been identified to exhibit antibacterial properties against a broad spectrum of Gram-positive and negative bacteria [42]. For AMP derived from *L. vannamei*, previous literature has reported the hydrophilic hemocyanin copper-binding domain to essentially be an immunoglobulin (Ig) containing domain that renders its antibacterial property [43]. A study by Yang et al reported antibacterial activity of *L. vannamei*-derived crustin on *S. aureus, E. coli* and *V. parahaemolyticus*. The current

evidence for its dental implications is low, therefore our aim was to determine its antibacterial and anti-biofilm inhibition. Newer research also highlights its antibiofilm properties against medical pathogens [44]. Recently developed crustin from *Amphibalanus amphitrite, AaCrus1* has been reported to show antibiofilm activity against *S. aureus* and *V. parahaemolyticus* [45]. In another study by Zhang et al on AMPs derived from green tiger shrimp, *Peaneaus semisulcatus* was reported to exhibit antimicrobial activity against Gram-positive (*M. luteus, S. aureus)* and Gram-negative (*E. coli, K. pneumoniae)* bacteria [46]. Mineralisation of artificial substitutes likecalcium carbonate, PRF, nano hydroxyapatite have shown to have clinical benefits across various fields [47-49].

This study remains novel in determining the antimicrobial and anti-biofilm activity of crustin AMP and *Sida acuta-derived* NPs against WSL lesions, laying further emphasis on the green synthesis of bioactive compounds that help combat drug-resistant oral bacteria. According to the results of our study, both crustin AMP and SA_NPs are equally efficacious in antibacterial and antibiofilm activity against WSL lesions. The future scope of this study would be to formulate CAMPs and SA_NPs in dental formulations, which seem promising therapeutic agents against WSL lesions. The limitations, however, of our study includes the lack of randomization in samples. Therefore, higher quality randomized clinical trials are needed to substantiate and validate the results from our study.

Conclusion

Marked antibacterial activity of crustin AMP was observed at MIC= 75 µg/ml and complete arrest of biofilm growth against *S mutans* and *L acidophilu*s was observed at BIC= 75 µg/ml. Marked antibacterial activity of SA_NPs was observed against *S. mutans* and L acidophilus at MIC= 75µg/ml and antibiofilm activity at BIC=75µg/ml. The incorporation of green synthesized nanoparticles and curated peptide antimicrobials offers a new approach to treating microbial pathogens that are resistant to current treatment practices and for the treatment of biofilms.

Conflict of Interest

Nil.

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