Original Article

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Dentistry Section

The Role and Efficacy of Herbal Antimicrobial Agents in Orthodontic Treatment

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ABSTRACT

Aims: To assess the effect of herbal antimicrobial agents on *Streptococcus mutans* count in biofilm formations during orthodontic treatment.

Materials and Methods: We calculated the growth inhibition of oral bacteria in the orthodontic appliances after herbal antibacterial agents were placed in culture media. The Minimum Inhibitory Concentrations (MICs) of these agents on *Streptococcus mutans* growth were determined. After cultivating colonies of *Streptococci* in biofilm medium with these herbal antimicrobial agents and orthodontic attachments, viable cell counting was performed from the bacteria which were attached on them. Scanning electron microscopy (SEM) analysis of morphology was observed on bacterial cells which were attached to orthodontic attachments. The effects of these agents were then evaluated and recommendations were forwarded.

Results: There was an increase in count of *Streptococcus mutans* with respect to the herbal antibacterial agents.

Conclusion: Despite the antibacterial functions of these herbal agents, there was increase in the biofilm formation caused by *Streptococcus mutans* to orthodontic bands, which had occurred most likely through upregulation of glucosyl transferase expression. These extracts may thus play an important role in increased bacterial attachment to orthodontic wires. Thus, this study was corroborative of an amalgamation of Ayurvedic therapy and Orthodontic treatment.

Keywords: Aswagandha, Brahmi, Herbal agents, Minimum inhibitory concentration, SEM, Triphala

INTRODUCTION

The biofilm that forms on the surface of teeth, which is called dental plaque, has the ability to induce some of the most common diseases which afflict the oral cavity, including caries, gingivitis, and periodontitis [1]. *Streptococcus mutans*, which has been implicated as a primary aetiological agent of dental caries in animals and humans, plays an important role in plaque formation and accumulation [2]. The *S. mutans* properties, both acidogenic and aciduric, together with its ability to synthesize extracellular glucans, are considered the major factors for the development and establishment of cariogenic biofilms [2,3]. Glucans, synthesized from dietary sucrose by glucosyl transferases (GTFs), enhances the pathogenic potential of dental plaque by promoting the adherence and accumulation of cariogenic *Streptococci* on the tooth surface, and by contributing to the bulk and structural integrity of plaque [4-6]. *Streptococcus mutans* produces at least three GTFs: B,C and D, which are critical for the adherence of bacteria to the surface of teeth and to each other. Orthodontic band placement which is done during the course of Orthodontic treatment tends to create new surfaces for the accumulation of plaque. It thereby increases the level of microorganisms in the oral cavity. There are elevations in levels of *Streptococci* and lactobacilli. In addition, orthodontic patients with fixed appliances frequently present an abundance of *Streptococcus mutans* in plaque as compared to untreated orthodontic patients [7]. Therefore, prevention of bacterial attachment to orthodontic wires and bands is a critical concern for orthodontists [8]. The three herbal agents are known to have antibacterial effects. Their effects on dental oral biofilms have not been well studied. As *Streptococcus mutans* exists almost exclusively in oral biofilms and it being the primary aetiologic agent of human dental caries, [9] we evaluated the effects of these three herbal agents on biofilm formations caused by *Streptococcus mutans* on orthodontic bands in-vitro.

This study focused on the efficacies of these commonly used antibacterial agents against *Streptococcus mutans*, their counts around orthodontic molar bands, which was the commonest site for bacterial plaque accumulation. Viable cell counting and Scanning Electron Microscope studies were performed to assess the surfaces for microbial attachments.

MATERIALS AND METHODS

We used 20 fresh stainless steel posterior bands of a liberal company to be cultured with the following test agents:

Test Agents

Three herbal antibacterial agents were used: Aswagandha, Triphala and Brahmi. Aswagandha (Withania somnifera), which is commonly referred to as Indian ginseng/ Winter cherry is locally applied to tumours, tubercular glands, carbuncles and ulcers [7]. It has been claimed to have a variety of health promoting effects which range from anti-inflammatory to anti-bacterial to immuno-modulatory to anti-stressor radio-sensitizer to antitumour. Triphala is a mixture of Amalaki (Emblica officinalis), Haritaki (Chebulic myrobalan) and Bhibitaki (Beleric myrobalan), which is used as gastrointestinal tract tonifier and an intestinal cleanser. Brahmi (Bacopa monniera) is used as a mental tonic and it rejuvenates the body. It is a promoter of memory and a nerve tonic.

Bacterial growth and herbal extracts procedure: Effects of three different plant extracts on bacterial biofilm formations on orthodontic bands. Steptococcus mutans was maintained on brain heart infusion (BHI) agar medium and grown under aerobic conditions.The biofilm assay was performed in biofilm medium (BM) which contained 3% sucrose. The three plant extract materials were formulated in DMSO and taken into different concentrations as per the requirements.

Growth inhibition produced by well-diffusion method

Steptococcus mutans was inoculated in BHI broth and incubated for 4–6 hours, to the point when growth was considered to be in the logarithmic phase. The density of the bacterial suspension was adjusted with sterile phosphate buffer saline (PBS) to match the density of McFarland's standard 0.5. The bacterial broth suspension was streaked evenly onto the BHI agar plates with a cotton swab.

After the inoculum had dried, an 8-mm wells were made using a cork borer, 50ul of the plant extracts was added and the plate was kept incubated at 37°C in an aerobic condition. Diameters of the zones were measured at three different points.

Minimum inhibitory concentration determination

Minimum Inhibitory Concentration Determination was done by using tube dilution method (MIC) and the three plant extracts (material), where their concentrations were tested in triplicate at serial dilutions of 0, 1, 2, 4, 8, 16, 32, 64, and 128 mg/ml. Each test tube was filled with 15 ml BHI broth which contained the three plant extracts at different concentrations, which was prepared earlier with 1ml of inoculated broth and incubated overnight at 37°C. To establish the specific MICs, turbidometric measurements were carried out to identify the lowest concentrations of the materials, which inhibited the growth and the turbidity.

Bacterial attachment on orthodontic wire

To evaluate the effects of the three different plant extracts on bacterial biofilm formations, we used sterile orthodontic wire for biofilm formation. *S. mutans* was cultured in a test tube which contained 15 ml of BM-sucrose broth which was soaked with 2-cm wire and sub-MIC of the three extracts after 40- hour incubation at 37°C under aerobic condition, each wire was washed twice in sterile PBS (pH 7.2) and moved to another sterile test tube. For viable cell counting, each band was incubated with *Streptococci* and the three plant extracts in BM-sucrose for 40 hours and was sonicated in 1mL of PBS. The PBS was serially diluted to 1/10,000 and each 100ul was spread on BHI agar plate. After incubation for 2– 3 days, bacterial colonies from each plate were counted and the relative colony-forming units (CFUs) were calculated.

Scanning electron microscope (SEM)

SEM studies were performed to evaluate the surfaces which were treated with the herbal agents.

S.No.	Test	Result
1.	Gram staining	Gram positive cocci in chains
2.	On Mac.Conkey agar	Lactose fermentation observed
3.	Sugar fermentations (TSI)	Ferments – lactose, glucose, sucrose
4.	Catalase	negative

[Table/Fig-1]: Biochemical tests for the sample isolated from the dental swab

Plant Name	Zones of inhibition in mm
Erthromycin	32mm
Triphala	10mm
Aswagandha	nil
Brhami churna	nil

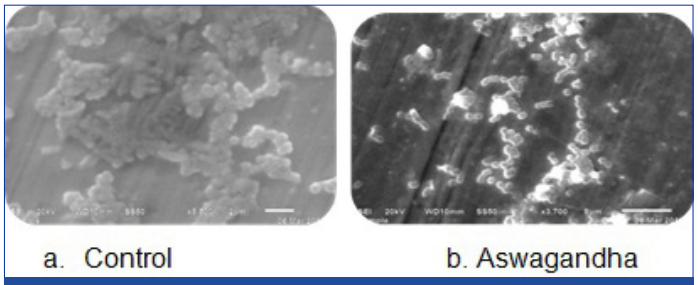
[Table/Fig-2]: Growth inhibition zones against oral bacteria by the three herbal agents

Test Agent	Cell Count
Erthromycin	>0.03 x 105 CFU/ml
Triphala	> 0.55 x 105 CFU/ml
Aswagandha	0.55 x 105 CFU/ml
Brhami	0.35 x 105 CFU/ml

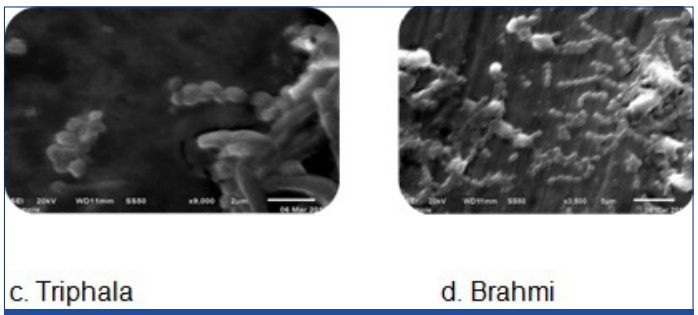
[Table/Fig-3]: Minimum inhibitory concentrations of the herbal agents against *Streptococcus mutans*

RESULTS

Our study showed significant results [Table/Fig-1] shows the various biochemical tests which were carried out for the samples which were isolated from the dental swabs. The organism which was detected showed Gram positivity and lactose fermentation, thus suggesting that it was *Streptococcus mutans*, which was the most important bacterium among those which were involved in early colonization in plaque formation. Growth Inhibition Zones formed against *Streptococcus mutans* by the three herbal agents were observed, as shown in [Table/Fig-2]. The MICs of herbal extracts against *Streptococcus mutans* were determined by using tube dilution method. Erythromycin was considered as the standard drug. It showed the lowest MIC (10 µg/mL). MIC against Triphala was 32-42 mg/mL and those against Aswagandha and Brahmi were greater than 128 mg/ mL [Table/Fig-3]. On measuring the effect of garlic on bacterial attachment to orthodontic band, we interestingly found that the viable cell count [Table/Fig-4] increased continuously, suggesting that more bacterial cells had attached to the orthodontic band in the presence of triphala than in the presence of Aswagandha and Brahmi. The figure shows SEM images of *Streptococcus mutans* on orthodontic band. As compared to those in control, bacterial attachment and aggregation on band had notably increased in the herbal agents. [Table/Fig-5].



[Table/Fig-4]: Viable Cell Count



[Table/Fig-5]: SEM images of the three herbal agents

DISCUSSION

The physical removal of the early *Streptococcal rich* biofilm, as was practised at least as early as in ancient Egyptian civilization, has attained a solid scientific foundation [10].

Herbal agents have wide spectra of antibacterial activities. In this study, we tried to uncover the effects of herbal agents on dental biofilm formations by using *S. mutans*, by analyzing attachment on orthodontic band. Due to their known antibiotic functions, we hypothesized that they would most likely inhibit bacterial attachment to orthodontic bands via their antibacterial effects. Against expectation, however, these agents actually increased bacterial biofilm formations. In agreement with viable counting of the bacterial cells on the bands, SEM image showed clear effects of these agents, especially Triphala, on *S. mutans* growth in terms of increased attachment. This was in contrast to findings of a study, where it showed more potency on *E. faecalis* biofilm. This may be attributed to its formulation, which contains three different medicinal plants in equal proportions. Tannic acid represents the major constituent of the ripe fruits of *T .chebula*, *T.belerica* and *E. officinalis*. Earlier studies have reported that tannic acid was bacteriostatic or bactericidal for some gram positive and gram negative pathogens [11]. A possible explanation could be that these extracts actually contained a biologically active substance which was effective at low doses, for gene activation, prior to bacterial cell growth inhibition.

Bacterial attachment is the initial step in the formation of biofilm communities. *Streptococcus mutans* proliferates as a biofilm on tooth surfaces, where it obtains nutrients and metabolizes

fermentable dietary carbohydrates [12]. The GTFs, in concert with glucan-binding proteins, contribute greatly to initial attachment and to the formation of biofilms. *S epidermidis* biofilm formation is known to be associated with the production of the polysaccharide intercellular adhesin (PIA) and poly-Nacetylglucosamine polysaccharide (PNAG). Recently obtained evidence has indicated that *Staphylococcal* accessory regulator (SarA), a central regulatory element that controls the production of S aureus virulence factors, is essential for the synthesis of PIA/PNAG and that it ensues biofilm development in this species [13]. These results suggested that the enzymes which participated in bacterial biofilm formations were specific for bacteria, and that the increase in bacterial attachments caused by these agents occurred through upregulation of GTF family genes. Their expressions were therefore very specific for *S. mutans* [14].

Previous research done on the regulatory mechanisms of GTF family genes in *S. mutans* showed that biofilm acidifications or excess metabolizable carbohydrates (glucose or sucrose) could induce GTF gene expression [15]. Furthermore, the structure of polysaccharide matrix changes over time, as a result of the action of mutanases and dextranases which are present within plaque. GTFs, at distinct loci, offer chemotherapeutic targets to prevent caries. Nevertheless, agents that inhibit GTFs in solution, frequently have reduced or no effects on adsorbed enzymes. Clearly, conformational changes and reactions of GTFs on surfaces are complex and they modulate the pathogenesis of dental caries in situ [16]. Hence, strategies which target an *S. mutans* density dependent quorum sensing system to attenuate biofilm formation and/or virulence, are currently being used, to develop therapeutic or preventive measures against dental caries [17]. Catabolite Control Protein A (CcpA) has a major role in Carbon Catabolite Repression (CCR) and regulation of gene expression, but it has been revealed that in *S. mutans*, there was a substantial CcpA-independent network that regulated gene expression in response to the carbohydrate source. Based on genetic studies, biochemical and physiological experiments have demonstrated that loss of CcpA impacted the ability of *S. mutans* in transporting and growing on selected sugars [18].

The development of novel technologies and the rapid advances made in dissecting the genetics and physiology of *S. mutans*, have resulted in the emergence of this bacterium as a new Gram positive model organism and they have proved that *S. mutans* was an amyloid-forming organism that contributed to biofilm formations [19]. This study may provide an impetus for understanding the interrelations between the plaque biofilm, tooth tissues and the oral environment, and for the development of procedures to modify the course of caries development [20]. Thus, the progress made in recent decades places *S. mutans* in an interesting position, to further make advances in basic microbiology research done especially on Gram-positive organisms [21].

CONCLUSION

Herbal agents increase bacterial biofilm formations on orthodontic band in a concentration-dependent manner. These agents seem to contain biological materials that promote formation of biofilms via activation of Glucosyl Transferases (GTFs). The present findings may offer fresh insight into herbal agents which induced GTF expression in *S. mutans* at the molecular level and into potential consequences which could occur, so that proper care of orthodontic bands could be undertaken.

LIMITATIONS

In order to verify the changes which occurred in Glucosyltransferase (GTF) expression levels under the effects of these herbal agents, real-time PCR needs to be performed. Other methods like ATP luminescence assay can also be used for easily detecting bacteria.

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