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EFFECT OF POLYHERBAL COMBINATIONS AND ESSENTIAL OILS AGAINST BIOFILM OF *STREPTOCOCCUS MUTANS*

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Keywords:

Streptococcus mutans, Antiadherence activity, Polyherbal extracts, Plate count method, Essential oils

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ABSTRACT: Background: Herbal extracts have been used in dental products for many years owing to their anti-adherence effect on *Streptococcus mutans* (*S. mutans*) in the biofilm formation. Dental caries are developed by the colonization of oral bacteria on the surface of teeth and adherence is the first step in the colonization process. **Objective:** The objective of the present study was to explore the anti-biofilm effect of the various combinations of herbal extracts and essential oils against *S. mutans* which play a central role in causing dental caries. **Methods:** Hydroalcoholic extracts of *Terminalia chebula* (*T. chebula*), *Psidium guajava* (*P. guajava*), *Azadirachta indica* (*A. indica*) and *Pongamia pinnata* (*P. pinnata*) were prepared separately and dried. Various combinations of herbal extracts, as well as essential oils Syzygium aromaticum (clove) and Mentha piperita (Peppermint oil), were tested for anti-biofilm potential on the glass surface. The number of adhering bacteria (CFU/ml) was determined by the plate count method. **Results:** It was found that all extract combinations and essential oils have shown anti-biofilm activity. The 2:2:1:1 of extracts and 2:2 ratio of essential oils has shown less bacterial count compared to all other tested ratios. Furthermore the herbal extract ratio of 2:2:1:1 has shown significant ($P<0.01$) anti-biofilm activity when compared to standard chlorhexidine mouthwash. **Conclusion:** These findings suggest that the active constituents present in the combined extracts could synergize the anti-biofilm activity owing to the reinforcement effect of constituents present in the combined mixture.

INTRODUCTION: The human mouth with its diverse nature and environmental change are well known for its unrestricted growth and formation of natural biofilms comprising a heterogenous microbial population among which vast variety of organisms are bacteria.

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Among the microbes *Streptococcus mutans* (*S. mutans*) have been implicated as a primary causative organism of dental caries [1]. Dental caries and gingivitis are the most prevalent oral infectious diseases of humans and are due to the accumulation of the dental plaque (a microbial biofilm) to the tooth surface and at the gingivitis margin respectively [2]. Strains of *S. mutans* adhere by hydrophobic bonds to enamel surface and ferment dietary carbohydrates, notably sucrose [3].

Sucrose metabolism promotes the firm adherence and cellular aggregation (biofilm) of bacteria to the tooth surface using glucan produced by the

cooperative action of glucosyltransferase (gtf) [4]. The oral microflora of biofilm produces acids by carbohydrate fermentation and initiates the dissolution of the tooth enamel and accumulation of acids in the dental plaque, subsequently leading to localized decalcification, cavitation and breakdown of calcified dental tissue [5]. Therefore, inhibition of biofilm is one of the effective strategies to prevent dental caries. Herbal products have become the choice of interest for thousands of years in folk medicine for various purposes worldwide. Several plant products have been successfully incorporated into dentifrices (or) mouthwashes in many countries as an anti-biofilm agent [6]. The objective of the present study was to evaluate anti-biofilm (anti-adherence) activity of various combinations of plant extracts and essential oils against *S. mutans*.

MATERIALS AND METHODS: Ethanol, sucrose, dimethylformamide (DMF), disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride (NaCl), chlorhexidine mouthwash were purchased from Merck, Mumbai. The brain heart infusion (BHI) broth was procured from Himedia, Mumbai. The essential oils of clove and peppermint were procured from essential oil manufacturers (PSC aromatics) Ooty, Tamil Nadu. All chemicals used in the present study were of AR grade.

Collection and Authentication of Plant Material: Four medicinal plants, *Terminalia chebula (T. chebula), Psidium guajava (P. guajava), Azadirachta indica (A. indica), Pongamia pinnata (P. pinnata)* and two essential oils, clove *Syzygium* aromaticum (clove oil), *Mentha piperita* (peppermint oil) were used as test samples in the present study. Fruits of *T. chebula* (068) were purchased from the local commercial market. The leaves of *P. guajava* (081), twigs of *A. indica* (0125) and *P. pinnata* (001) were collected from the institutional medicinal garden, Bharat Institute of Technology, Hyderabad. All the collected plant materials were authenticated by the Department of Botany, Osmania University, Hyderabad and voucher specimens were submitted in the same place.

Preparation of Hydroalcoholic Extracts of Plants: Selected plant materials were shade dried and powdered separately. Each plant material was extracted separately using an equimolar ratio of ethanol and water (50:50) by using a Soxhlet extraction method. The hydroalcoholic extracts were concentrated using a rotary evaporator and the dried extracts were preserved at 4^{o}C for further use.

Culture Collection: *S. mutans* (MTCC 49) used for the present study was procured from the Centre for Cellular and Molecular Biology (CCMB, Tarnaka, Hyderabad). A stock culture of *S. mutans* (MTCC 49) was prepared in glycerol and preserved at 0 °C until further use.

Preparation of Test Samples: The effective concentration of four herbal extracts and two essential oils for anti-adherence activity was taken from our previous gtf inhibition studies which play a key role in bacterial adherence. Herbal extracts of *T. chebula* (A), *A. indica* (B), *P. guajava* (C), *P. pinnata* (D) were used to prepare different polyherbal combinations such as 1:1:1:1, 2:1:1:1, 2:2:1:1. In a similar way three different ratios 1:1, 1:2, 2:1 of two essential oils, clove oil (E) and peppermint oil (F) were prepared. The prepared test samples were subjected to anti-biofilm studies against *S. mutans*.

Anti-biofilm Assay of Test Samples against *S. mutans*: The test tubes containing 0.37 gm of dehydrated culture media (BHI broth + 10% sucrose) and 9 ml of each of test solutions were inserted with standardized glass specimens (diameter= 2 mm, length= 5 cm) and submitted to sterilization. To this mixture 1ml of standardized *S. mutans* suspension (1.5×10^8 cells/ml) was inoculated and then incubated for 90 min at 37 °C. After the period of incubation, the glass specimens were transferred to tubes containing buffered phosphate saline (pH 7.2) and submitted to agitation. From this initial suspension, dilutions of 10^{-1} and 10^{-2} were obtained in sterilized NaCl (0.85%) saline solution. Then, aliquots of 0.1 ml of each dilution were plated in duplicate on BHI agar and incubated for 48 h at 37 °C. The numbers of colonies were counted by the plate count method and the value of log CFU/ml was calculated [7].

Statistical Analysis: The experiments were repeated thrice and the values were expressed as a mean ± standard deviation. Data were analyzed

using one way ANOVA using Graph pad prism, version 8. The results were analyzed using Dunnet's multiple comparison test at $P<0.0001$.

RESULTS AND DISCUSSION: Dental biofilms of the oral cavity are playing a critical role in the development of various infectious periodontal diseases. In spite of the various mechanisms, the interference of bacterial adhesion to the tooth surface became the novel strategy to reduce the development of dental caries. Previous studies have been reported to study the anti-adherence activity of various medicinal plants. The study of the combination of herbal extracts and essential oils is unusual in literature. In the present study log CFU/ml values for polyherbal combinations and essential oils were calculated and furnished with **Table 1** and **2** respectively to determine the anti-biofilm activity.

The results were more significant compared to the anti adherence activity of plant-based stimulants such as coffee-chicory combinations reported earlier [8]. In our study, the polyherbal combinations have shown significantly less bacterial count when compared to the control group and essential oils.

TABLE 1: ANTIADHERENCE ACTIVITY OF POLYHERBAL COMBINATIONS

S. no.	Polyherbal Combinations (A: B: C: D)	log CFU/ml			
		Trial 1	Trial 2	Trial 3	Mean ± SEM
1	1:1:1:1	3.810	3.800	3.795	3.802±0.004
2	2:1:1:1	3.401	3.396	3.385	3.394±0.005
3	2:2:1:1	3.006	3.002	3.004	3.004±0.001*$
4	Standard	3.535	3.528	3.520	3.528±0.004
5	Control	3.998	3.998	3.985	3.994±0.004

* Values are expressed in mean ± SEM. Values are significant at p<0.0001; * *vs.* standard, $ *vs.* control

TABLE 2: ANTIADHERENCE ACTIVITY OF ESSENTIAL OILS

S. no.	Essential oil ratios (E:M)	log CFU/ml			
		Trial 1	Trial 2	Trial 3	Mean ± SEM
1	1:1	3.748	3.738	3.755	3.747±0.005
2	1:2	3.358	3.345	3.365	3.356±0.006*$
3	2:1	3.735	3.746	3.726	3.736±0.006
4	Standard	3.535	3.528	3.520	3.528±0.004
5	Control	3.998	3.998	3.985	3.994±0.004

*Values are expressed in mean ± SEM. Values are significant at p<0.0001; * *vs.* standard, $ *vs.* control.

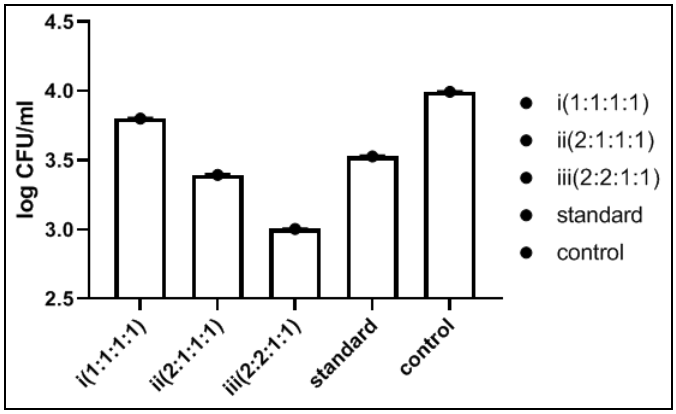

FIG. 1: ANTIADHERENCE ACTIVITY OF POLYHERBAL COMBINATIONS

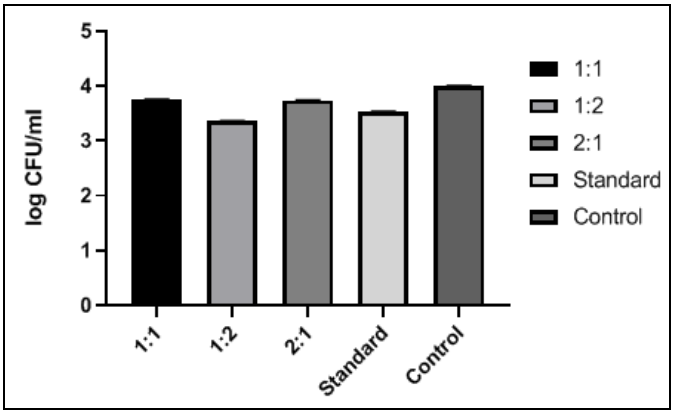

FIG. 2: ANTIADHERENCE ACTIVITY OF ESSENTIAL OILS

Nevertheless, from the **Table 1** and **Fig. 1** it has been confirmed that the combination of 2:2:1:1 has shown a significant reduction in the bacterial count (3.004 ± 0.001) compared to other combinations. As well as a significant reduction in the bacterial count was observed when compared to commercial chlorhexidine mouthwash (standard). The results of **Table 2** and **Fig. 2**, confirmed that 1:2 ratio of clove and peppermint oil exhibited a significant reduction in the bacterial count (3.356 ± 0.006) when compared to control, which is a measure of anti-biofilm effect. Several studies were reported previously about the anti-cariogenic activity of *T. chebula* (A), *A. indica* (B), *P. guajava* (C) and *P.*

pinnata (D) and Clove oil (E), Peppermint oil *(*F) [9, 10, 11, 12, 13, 14]. However, this is the first report on the anti-biofilm activity of combinations of these extracts and essential oils against *S. mutans.*

CONCLUSION: The results obtained from the present study suggested that both polyherbal extracts of *T. chebula*, *A. indica*, *P. guajava*, *P. pinnata* and essential oils of clove and peppermint could inhibit the adherence of *S. mutans in-vitro* on the glass surface, but with different potential. Moreover, herbal extracts have been proved to exhibit potent anti-biofilm activity rather than essential oils. Further, *in-vivo* studies are necessary to formulate this combination in the future as a potential agent to combat dental caries.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

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