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Original Research Article

Correlation Analyses of the Oral Biofilm Growth Inhibition towards Hydrophobicity Reduction of Oral Pathogenic Bacteria

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ABSTRACT

Bacteria have a tendency to form multicellular biofilms, which adhere to surfaces and contain extracellular polymeric substances (EPS). This tendency is accompanied by hydrophobic interactions, which are vital in the biofilm attachment process. Oral biofilms contribute to a range of oral health issues, including gingivitis, dental caries, and halitosis. Therefore, this research aimed to investigate the correlation between oral biofilm growth inhibition and hydrophobicity reduction in *Streptococcus mutants*, *Streptococcus sanguinis*, *Lactobacillus acidophilus*, and *Actinomyces viscosus*. Essential oil-derived compounds, namely eugenol, C-10 massoia lactone, thymol, cinnamaldehyde, and zerumbone, were applied in the experiment. A microdilution assay using crystal violet staining evaluated the oral microbes' biofilm growth. The adhesion of microbes to hexadecane was measured to assess hydrophobicity reduction, which was analyzed correlatively using PAST *(Paleontological Statistics)* software and the Principal Component Analysis (PCA) method. The results showed a positive correlation of the hydrophobicity reduction towards the biofilm formation inhibition for all tested microbes (graphical angle <45°). The Principal Component (PC) analysis, based on the eigene values, showed that PC1 and PC2 accounted for 54.149% and 25.652% of the total variation, respectively. These two components explained 79.801% of the total variation, indicating a significant level of variability. This finding supported the notion that a greater reduction in microbial hydrophobicity was associated with stronger inhibitory activity against planktonic growth. The hydrophobicity reduction assay may indicate a potential of bioactive compounds against biofilm growth inhibition of oral microbes.

***Keywords*:** biofilm growth inhibition, correlation analyses, essential oil, hydrophobicity, oral microbes

Introduction

Bacteria are naturally inclined to form complex and diverse multicellular biofilms.[1] These biofilms are created when bacteria adhere to a surface and bind together, surrounded by Extracellular Polymeric Substances (EPS).[2] Several oral health issues, such as dental caries, gingivitis, and halitosis, are attributed to oral biofilms.[3,4] This process occurs when facultative bacteria like *Streptococcus sanguinis*, *Streptococcus mutans*, *Streptococcus oralis*, and *Streptococcus mitis* attach to tooth surfaces coated with the acquired pellicle. The initial colonization of these bacteria leads to their aggregation on the tooth surface.

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Subsequently, other Gram-positive bacteria like *Actinomyces viscosus*, *Actinomyces israelii*, and *Actinomyces gerencseriae*, as well as Gram-negative bacteria like *Veillonella parvula*, join the biofilm community.[5] The supragingival dental plaque predominantly hosts oral bacterial species such as Lactobacilli, Streptococci, and Bifidobacterial.[6–9] These bacteria and many other commensal species form a complex biofilm community in the oral cavity. While most of these species are commensal, some act as low-level opportunistic pathogens and contribute to the development of dental caries. [5,10–14] Several factors influence the composition and growth of the oral microbiota, including drug treatment, dietary habits, systemic diseases, endogenous nutrients, and the host's immune system.[4,15–17] Saliva plays a crucial role in providing nutrients for the oral microbial ecosystem. However, a decrease in saliva production can increase opportunistic pathogens, such as fungi and non-pathogenic bacteria. Systemic diseases that elevate glucose levels in saliva also affect bacterial nutrition. These changes in the oral ecosystem directly impact the dysbiosis of bacterial biofilms and contribute to dental deterioration.[16,18]

The ability of bacteria to adhere to a surface is crucial for forming biofilms. In the case of oral bacteria, their attachment to the tooth surface is determined by the interaction between bacterial cells and the surface.[19] This initial attachment is facilitated by hydrophobic interactions, which play a significant role in the process. The success of this interaction depends on factors such as the structure of the bacterial

cell surface and the physicochemical properties of the substrate to which they are attaching. [20,21]

The effectiveness of mouthwash in controlling dental plaque accumulation on the tooth surface is widely acknowledged. This is attributed to several mechanisms, including the inhibition of bacterial attachment, the disruption of bacteria colonization on the surface, and the modification of the oral ecology. By targeting these processes, mouthwash helps prevent the buildup of dental plaque and promotes oral health.[14,22]

Many essential oils derived compounds have been reported as potent antibiofilm towards a wide range of microbes. Listerine is one of the several compounds used in commercial and hygiene products. Other include eugenol and thymol, which have been found effective against biofilms of *Streptococcus sanguinis*, *Lactobacillus acidophilus, Actinomyces viscosus,* and *Streptococcus mutans, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. [23,24] C-10 massoialactone can degrade polymicrobial biofilms such as *Streptococcus mutans, Lactobacillus acidophilus, Streptococcus sanguinis* and *Actinomyces viscosus*. In addition, zerumbone showed an antibiofilm effect on *Streptococcus sanguinis* and *Lactobacillus acidophilus*. [25]

The objective of this research was to investigate the relationship between the inhibition of oral biofilm growth by essential oil compounds and the reduction of hydrophobicity *S. mutans, S. sanguinis, L. acidophilus* and *A. viscosus* assessed by the adhesion to hexadecane method. The result is expected to widen the opportunity for further research on oral biofilm inhibition by targeting bacterial hydrophobicity reduction.

Materials and Methods

Materials

The essential oil compounds used in this research, including thymol, cinnamaldehyde, C-10 massoialactone, and eugenol, were sourced from Sigma Aldrich (Germany). The media utilized were Brain Heart Infusion (BHI) from Oxoid and Muller Hinton Agar (MHA) from Oxoid (UK). Furthermore, dimethyl sulfoxide (DMSO) (Merck, Germany), hexadecane (Merck, Germany), Cristal violet (Himedia, India), and 95% ethanol pro analyses (Merck, Germany) were used as solvents for assay. A commercial mouthwash product, "L," was used as a control. A commercial mouthwash product labelled as "L" served as the control, containing Camelia Sinensis (Green tea) leaf extract, thymol, menthol, methyl salicylate, and sodium fluoride. Other materials used were 24 well flat-bottom polystyrene microplates and 96 well flat-bottom polystyrene microplates (Iwaki, Japan), anaerogen gas pack (Oxoid, UK) and a coverslip.

Equipments

The equipments used in this research were Laminar air flow, micropipette pipetman 20-200 µL (Gilson, France), micropippette micropipettes with volumes of 2-10 µL, 20-200 µL, and 100-1000 µL (Socorex, Swiss), autoclave (Sakura, Japan), multichannel micropipette (Socorex, Swiss), Spectrophotometry (Geneys 10 UV Scanning, 335903) (Thermo Scientific, USA), microtiter plate reader (optic Ivymen System 2100-C, Spain), and incubator (IF-2B) (Sakura, Japan).

Bacterial strains

Bacterial strains used in this research were obtained from *A. viscosus* ATCC 15987, *S. sanguinis* ATCC 10566, *S. mutans* ATCC 25175, *S. mutans* ATCC 25175, *L. acidophillus* ATCC 4356. They were re-cultured from the stock culture in Brain Heart Infusion (BHI) medium and incubated in an incubator at 37°C for 24h. The optical density (OD_{600}) of each microbial cultures was measured to achieve respective values as follows, *A. viscosus* has OD λ 600= 0.5 ($1.3x10^8$ CFU/mL) [26], *S. mutans* has OD λ 600 = 0.2 ($2 x 10^8$), [27] *Streptococcus sanguinis* has OD λ 600 = 0.1 ($1.3x10^8$ CFU/ml) (28), and *L. acidophilus* has OD λ 600= 0.4 ($3.3x10^8$ CFU/mL) [29]

Biofilm Formation Inhibition Assay in vitro

In this experiment, four bacterial strains, namely *Streptococcus mutans, S. sanguinis, L. acidophilus, and A. viscosus,* were inoculated into BHI broth media initially prepared in a 96 micro-well plate. [30] The test compounds C-10 massoialactone, thymol, eugenol, cinnamaldehyde, and zerumbone were prepared in dilution series of 1.0, 0.5, 0.25, and 0.125% v/v, respectively. In addition, the final volume in each well was 100 µL. Bacteria and BHI were used as negative controls, while a commercial product labelled L with a concentration of 1% v/v served as the positive control.

The following steps were used to evaluate the inhibition of biofilm formation. First, a bacterial suspension was prepared in the Brain Heart Infusion (BHI) medium. Subsequently, 2 % sucrose and the test compound were added to each well of a microplate containing the bacterial suspension. Furthermore, the mixtures were incubated under anaerobic conditions at 37°C for 24h to promote biofilm formation and for intermediate phase observation. Afterwards, the culture medium was removed, and the attached biofilm was rinsed with sterile distilled water. To visualize the biofilm, it was stained with 1% (v/v) crystal violet solution and then rinsed with water. The formed biofilm was quantified by adding 200 µL of 95% ethanol to each well, and the absorbance was measured at 595 nm using a microplate reader. Finally, the percentage of inhibition was calculated using a specific formula. Each assay was performed at least in triplicates. (1)

$$\% \text{ inhibition} = \frac{OD\ growth\ control - OD\ sample}{OD\ growth\ control}\ x\ 100 \ldots\ldots (1)$$

Hydrophobicity assay

The hydrophobicity of the test compounds was determined by using the following procedures. First, a sterile test tube filled with 600 µL aqua dest sterile was used as a negative control. A series of dilutions of the test compounds were prepared and added to the separate sterile test tubes. Furthermore, each of these test tubes was then inoculated with 600 µL of bacterial suspension. The tubes were vigorously shaken and vortexed for one minute and left undisturbed at room temperature for 15 minutes. After the incubation period, measurements were taken using a UV-Vis spectrophotometer at a wavelength of 550 nm. The initial absorbance value (At) was recorded for each test compound without hexadecane. The bacterial suspension was returned to the test tube, and 40 µL of hexadecane was added. The tubes were shaken vigorously with a vortex for one minute and left at room temperature for 15 minutes. Finally, the absorbance value (Au) was measured for each test compound in the presence of hexadecane. The hydrophobicity of hexadecane was calculated using the following formula: (2)

$$\text{Ab} = \frac{(\text{At} - \text{Au})}{\text{At}}\ x\ 100\% \ldots\ldots (31)\ (2)$$

Ab = hydrophobicity of bacterial cells to hexadecane

At = optical density absorbance against total bacterial cell suspension before the addition of hexadecane

Au = optical density to total bacterial cell suspension after the addition of hexadecane

Data Analysis

The present research used PAST (*Paleontological Statistics*) statistical software, specifically version 14.10 for Windows 10, to conduct correlation analysis between dependent variables. In addition, multivariate analysis was carried out using the PCA (Principal Component Analysis) method. This research mainly focused on examining the antibiofilm effect of each tested compound against different microbes and its hydrophobicity to hexadecane. The findings indicated that eugenol, thymol, zerumbone, C-10 Massoialactone, and cinnamaldehyde were responsible for reducing cell surface hydrophobicity.

Results and Discussion

Cell surface hydrophobicity has a notable impact on bacterial adhesion to various surfaces.[32] In addition, the auto-aggregation of cell surface hydrophobicity contributes to the topography, shape, and maturation of the climax biofilm community.[33] Based on the principles of thermodynamics, thymol, eugenol, C-10 Massoialactone, zerumbone, and cinnamaldehyde increases the tension between the cells and the surrounding liquid medium.[34] These compounds have the potential to reduce intercellular surface tensions. [35]

Table 1: Data multivariate analysis between hydrophobicity test and 24-hr biofilm formation inhibitory activity

	PA 24	PP 24	HD
C10NFSS	52.90	32.90	36.80
C10NFSM	61.60	40.00	26.50
C10NFLA	60.20	44.40	15.20
C10NFAV	55.30	38.40	42.70
EUGFSS	87.60	49.50	21.07
EUGFSM	93.00	82.30	17.73
EUGFLA	87.90	43.90	27.65
EUGFAV	73.90	40.30	25.40
TIMFSS	62.40	53.50	29.87
TIMFSM	51.80	87.30	50.14
TIMFLA	40.30	65.70	27.73
TIMFAV	59.80	45.90	47.40
SINNFSS	77.30	13.90	30.00
SINNFSM	81.60	26.10	28.54
SINNFLA	60.90	7.60	27.13
SINNFAV	91.90	57.40	33.92
ZERNFSS	50.20	24.50	34.26
ZERNFSM	25.10	21.10	29.57
ZERNFLA	18.30	24.40	26.38
ZERNFAV	19.70	26.00	8.77

Caption: PA 24 : planktonic growth inhibition test; PP 24: 24-hr biofilm formation inhibition test; PP 48 : 48 hr biofilm formation inhibition test; HD: hydrophobicity test; C10NFSS : C-10 non-phenolic C-10 Massoialactone *S. sanguinis;* C10NFSM : C-10 non-phenolic C-10 Massoialactone *S. mutans;* C10NFLA : C-10 non-phenolic C-10 Massoialactone *L. acidophilus;* C10NFAV : C-10 non-phenolic C-10 Massoialactone *A. viscosus;* EUGFSS : *S. sanguinis* phenolic eugenol; EUGFSM : *S. mutans* phenolic eugenol; EUGFLA: phenolic eugenol *L. acidophilus;* EUGFAV : Eugenol phenolic A. *viscosus;* TIMFSS : Phenolic thymol *S. sanguinis;* TIMFSM : thymol phenolic *S. mutans;* TIMFLA : thymol phenolic *L. acidophilus;* TIMFAV : phenolic thymol *A. viscosus;* SINNFSS : non-phenolic cinnamaldehyde *S. sanguinis;* SINNFSM : non-phenolic cinnamaldehyde *S. mutans;* SINNFLA : non-phenolic cinnamaldehyde *L. acidophilus;* SINNFAV : non-phenolic cinnamaldehyde *A. viscosus;* ZERNFSS : non-phenolic zerumbone *S. sanguinis;* ZERNFSM : zerumbone non phenolic *S. mutans;* ZERNFLA : zerumbon non phenolic *L. acidophilus;* ZERNFAV : non-phenolic zerumbone *A. viscosus*

Table 2: PC summary data/eigenvalue

PC	*Eigenvalue*	% Variance
1	900.967	54.149
2	426.507	25.652
3	221.992	13.352
4	89.1222	5.3602
5	18.7282	1.1264
6	5.65865	0.34034

In addition to their impact on cell surface hydrophobicity, thymol, eugenol, C-10 Massoialactone, zerumbone, and cinnamaldehyde were observed to inhibit biofilm formation.[36–39] This suggests that the phenolic contents, specifically in eugenol and thymol, were essential in inhibiting biofilm formation and Streptococcal aggregation. It was also discovered that the cell surface hydrophobicity influenced the formation of oral streptococci biofilms on hard surfaces.[32] These findings emphasized the multifaceted nature of cell surface hydrophobicity in biofilm development as well as its potential as a target for addressing biofilm-related challenges in oral health.

The outcome of the multivariate analysis conducted in this research is shown in Table 1. The use of multivariate analysis was essential because it allowed for the examination of multiple variables and their collective influence on one another. The research specifically investigated the effects of administering the test compound to oral bacteria on both the 24-hour biofilm formation inhibition and hydrophobicity tests.

The correlation analyses indicate a positive relationship between the inhibition of biofilm growth and the reduction in hydrophobicity of the tested microbes against hexadecane. This correlation was observed after adding certain compounds shown in Figure 1. The result showed that the angle formed between the hydrophobicity test and the biofilm formation assay was less than 45°C. [40] Based on the eigene values, PC1 and PC2 exhibited variable variation of as much as 54.149%; and 25.652%, respectively, as shown in Table 2. Therefore, these two principal components collectively explained 79.801% of the variation in the data.

The multivariate analysis using the PCA method showed that eugenol exhibited the highest potential for inhibiting planktonic growth among the tested compounds. On the other hand, thymol exhibited the greatest potential for inhibiting biofilm formation across all mono-microbial cultures. Previous research has also supported the strong antimicrobial activity of eugenol and thymol against polymicrobial biofilm cultures of oral microbes. [30] Both compounds belong to the phenolic group. It has been previously established that the presence of the phenolic group is essential for disrupting the extracellular polymeric substances (EPS) forming the structural matrix of biofilms.[23]

In this research, various physicochemical aspects were considered as potential factors influencing the antibiofilm activity of the compounds. These aspects included molecular weight, Log P (partition coefficient), and the presence of a phenolic group. The essential oil compounds used had relatively small molecular weights, less than 300 g/mol. This characteristic facilitates its passage through the pores of the extracellular polymeric substances (EPS) present in biofilms. Furthermore, an increase in the Log P value makes it easier for the compound to penetrate the bacterial membrane and access the intracellular space, ultimately reducing the hydrophobicity of the cell surface.[41] The compound zerumbone, with a Log P value of 3.9, exhibited favorable characteristics when passing through the bacterial membrane. The presence of a phenolic moiety was found to be relevant for antibacterial and antibiofilm activities, as proven by the highest activity observed in this research for the two phenolic-containing compounds, eugenol and thymol. The results indicated that the more active compounds belonged to the phenolic groups.

Conclusion

In conclusion, the positive correlation between the reduction in hydrophobicity and the inhibition of biofilm formation in all tested microbes provided strong evidence of a synergistic relationship. A larger reduction in the hydrophobicity of the microbes was associated with greater inhibitory activity against planktonic growth. Therefore, the hydrophobicity assay indicated the effectiveness of bioactive compounds in inhibiting the biofilm growth of oral microbes. This finding holds significant value for further research to develop new antibiofilm agents for oral hygiene products. Expanding the scope of this research to include a wider range of compounds with different structures would be an interesting approach. This aids in confirming the correlation between the physicochemical properties of a compound and its antibiofilm and antibacterial effects. Further research involving clinical isolates associated with oral health problems would be valuable. This would provide additional confirmation regarding the manipulation of microbial hydrophobicity and its impact on the biofilm-forming capability of oral microbes.

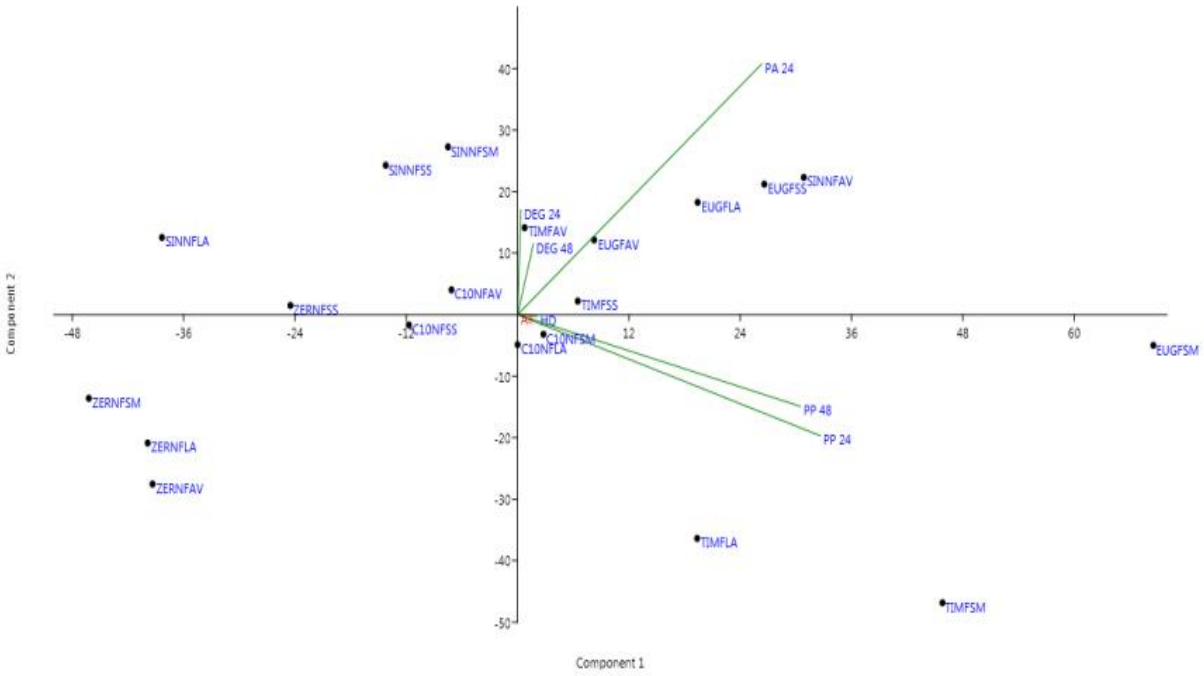

Figure 1: Biplot graph of the hydrophobicity test and biofilm growth inhibition assay using PAST statistical software; HD: hydrophobicity assay; PA24: planktonic growth inhibition assay 24h; PP24: biofilm growth inhibition assay 24h; PP48: biofilm growth inhibition assay 48h; DEG24: biofilm degradation assay 24h; DEG48: biofilm degradation assay 48h; C10NFSS: C-10 Massoialactone against *S. sanguinis*; C10NFSM: C-10 Massoialactone against *S. mutans*; C10NFLA: C-10 Massoialactone against *L. acidophilus*; C10NFAV: C-10 Massoialactone against *A. viscosus*; EUGFSS: Eugenol against *S. sanguinis*; EUGFSM: Eugenol against *S. mutans*; EUGFLA: Eugenol against *L. acidophilus*; EUGFAV: Eugenol against *A. viscosus*; TIMFSS: Thymol against *S. Sanguinis*; TIMFSM: Thymol against *S. mutans*; TIMFAV: Thymol against *A. viscosus*; SINNFSS: Cinnamaldehyde against S. sanguinis; SINNFSM: Cinnamaldehyde against *S. mutans*; SINNFLA: Cinnamaldehyde against *L. acidophilus*; SINNFAV: Cinnamaldehyde against *A. viscosus*; ZERNFSS: Zerumbone against *S. sanguinis*; ZERNFSM: Zerumbone against *S. mutans*; ZERNFLA: Zerumbone against *L. acidophilus*; ZERNFAV: Zerumbone against *A. viscosus*

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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