Influence of zingiberene on the biofilm formation of 
_Streptococcus mutans_

Z. Mohamed Noufal¹, R. Gayathri²*, V. Vishnu Priya²

ABSTRACT

**Aim:** The aim is to study the antimicrobial effect of zingiberene species biofilms and the biocompatibility of this modified dental material. **Materials and Methods:** _Streptococcus mutans_ UA159 were cultured in brain heart infusion with the condition of 37°C anaerobically (5% CO₂, 90% N₂, and 5% H₂). The final inoculum for biofilm formation was composed of bacteria (10,000,000 colony-forming unit/ml each) in 2 ml medium with 50 mM piperazine-1,4-bisethane sulfonic acid and 0.2% sucrose in 24-well plates with specimens placed at 37°C aerobically for 72 h, and the media were changed every 24 h. **Results:** Minimum inhibitory concentration of zingiberene was found to be 1 mg/mL, where it exhibits 20% inhibition as compared to the moisture control vancomycin. **Conclusion:** Zingiberene exhibited an antimicrobial activity in biofilm formation analyzed using _S. mutans_.

**KEY WORDS:** Zingiberene, Biofilm, Streptococcus Mutans

INTRODUCTION

The inhibitory effect of CR extract and ME fraction of _Zingiber officinale_ on biofilm formation by _Streptococcus mutans_ was performed using the protocol described elsewhere. Briefly, 50 μL of overnight culture of _S. mutans_ (105–106 Colony-forming unit [CFU] ml⁻¹) was inoculated into 150 μL of brain heart infusion (BHI) with 5% (w/v) sucrose containing various concentrations of CR extract and ME fraction with respective controls. After incubation at 37°C for 24 h, media and unbound cells were decanted from the microtiter plates. The remaining planktonic cells were removed by gently rinsing with sterile water. The wells with attached cells (biofilms) were fixed with formalin (37%, diluted 1:10) plus 2% sodium acetate. Each well was stained with 200 μL of 0.1% crystal violet for 15 min at room temperature.

After two rinses with sterile water, bound dye was removed from the cells with 100 μL of 95% alcohol. Plates were then set on a shaker for 10 min to allow full release of the dye. Biofilm formation was then quantified by measuring optical density of the suspension at 600 nm by a microplate reader (BIORAD iMark TM Microplate reader, India). Separate biofilms were formed in the presence of extracts for time-dependent effect at 6, 12, 20, and 24 h.

Dental caries is a complex and multifactorial condition which causes demineralization and progressive destruction of the dental enamel.⁶ _S. mutans_, a member of endogenous oral microflora, has long been implicated to play a key role in the pathogenesis of this disease.²,³ _S. mutans_ survival depends strictly on a biofilm lifestyle in its natural ecosystem, i.e., dental plaque.⁴ Dental plaque formation is important for its persistence since biofilms do not allow easy penetration of chemotherapeutic agents, permitting to cause resistance against antibiotics, immune factors, and host-derived antibacterial agents.⁵ The ability of this bacterium to produce (acidogenic) and tolerate (aciduric) acids coupled with its property of synthesizing extracellular glucans allows its effective colonization in the oral cavity leading to the establishment of highly cariogenic dental biofilms.⁶

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Extracellular glucans which are synthesized from sucrose by glucosyltransferases (GTFs) play a critical role in the adhesive interactions of \textit{S. mutans} and contributes to the structural integrity of dental plaque.\cite{7} \textit{S. mutans} expresses three different GTFs, namely GTF B, GTF C, and GTF D. The insoluble and soluble glucan is mostly synthesized by GTF B and GTC C, respectively. However, GTF C is known to synthesize a mixture of soluble and insoluble glucans.\cite{8} These virulence properties thus provide a unique microenvironment for unobstructed survival of \textit{S. mutans} in the oral cavity.\cite{9} Therefore, approaches to inhibit various factors governing the virulence properties of \textit{S. mutans} could be an alternative to prevent dental caries.\cite{10}

**MATERIALS AND METHODS**

\textit{S. mutans} UA159 was cultured in BHI with the condition of 37°C anaerobically (5% CO\textsubscript{2}, 90% N\textsubscript{2}, and 5% H\textsubscript{2}).\cite{11} The final inoculum for biofilm formation was composed of bacteria (10,000,000 CFU/ml each) in 2 ml medium with 50 mM piperazine-1,4-bisethane sulfonic acid and 0.2% sucrose in 24-well plates with specimens placed at 37°C aerobically for 72 h, and the media were changed every 24 h.\cite{12}

**CFU**

After culture, specimens with 72 h biofilms were washed twice with PBS buffer to remove microbes then moved to culture dishes with 2 mL phosphate buffer saline (PBS) buffer in advance. Biofilms were collected using sterilized blades to remove microbes from the specimen and then vortexed thoroughly.\cite{13} BHI broth agar plates and Sabouraud’s Dextrose Agar (SDA) were prepared for culturing the microbes which were gradient diluted in PBS previously. Then, BHI and SDA agar plates were cultured aerobically at 37°C for 2 days for CFU counting.\cite{14}

**Minimum Inhibitory Concentration**

To examine the inhibitory effect of test agents on the biofilm growth, modified crystal violet assay was carried out. Zingiberene (ZBN) was dissolved in sterile distilled water to a shock concentration of 200 mg/ml. The positive control for bacterial cultures was ciprofloxacin. Controls were prepared at a stock concentration of 0.01 mg/ml. Briefly, 100 mL of sterile distilled water was aliquoted into all the wells of the microtiter plate. The ZBN was then pipetted into the wells A1–A12 of the plate. 100 mL of the bacterial culture showing maximum cell count at 590 OD was treated with test agent. The plates were incubated at 37°C for 24 h for all bacterial strains. Water was used as a negative control. Following incubation, the crystal violet assay was performed to assess the biomass of the attached cells. The percentage of biofilm inhibition was calculated using the following equation.\cite{14,15}

\[
\text{% of Inhibition} = \frac{\text{OD 590 of cells treated with test agent}}{\text{OD 590 of non-treated control}} \times 100
\]

**RESULTS AND DISCUSSION**

**Inhibition of a Preformed Biofilm**

Zingiberene exhibited inhibition of \textit{S.mutans} biofilm formation in a dose dependent manner [Table 1 and Figure 1]. It is evident from the table that with a concentration of 5 mg/mL, Zingiberene, exhibited 76.6% inhibition. There is always a need for the replacement of antibiotics with phytochemical constituents of herbal extract. Zingiberene a phytoconstituent of ginger proves to be a good inhibitor of biofilm formation. Organisms when grown as a biofilm, in oral cavity always presents a worst case scenario and leads to other clinical complications as well. It is always evident that biofilms are more difficult to get eradicated. However, Zingiberene when tested invitro was able to act and inhibit the biofilm formation of \textit{S.mutans}.\cite{14,15}

**CONCLUSION**

Zingiberene exhibited an antimicrobial activity in biofilm formation analyzed using \textit{S. mutans}.  

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**Table 1: This table depicts the Minimum Inhibitory Concentration of the ZBN**

<table>
<thead>
<tr>
<th>Conc (mg/ml)</th>
<th>Absorbance Mean OD ± SD</th>
<th>% of Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZBN (1.0)</td>
<td>0.411 ± 0.25*</td>
<td>20.0</td>
</tr>
<tr>
<td>ZBN (2.0)</td>
<td>0.365 ± 0.24*</td>
<td>28.9</td>
</tr>
<tr>
<td>ZBN (3.0)</td>
<td>0.264 ± 0.11*</td>
<td>48.6</td>
</tr>
<tr>
<td>ZBN (4.0)</td>
<td>0.178 ± 0.09*</td>
<td>65.3</td>
</tr>
<tr>
<td>ZBN (5.0)</td>
<td>0.120 ± 0.17*</td>
<td>76.6</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.514 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.032 ± 0.01*</td>
<td>93.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM expressed as (n=3); MIC of ZBN is 1.0mg/ml. *p<0.001 statistically significant as compared with Negative control

**Fig 1:** Biofilm mass that formed during incubation period was stained using crystal violet. NC: 2-ZBN(1.0); 3-ZBN(2.0); 4-ZBN(3.0); 5-ZBN(4.0); 6-ZBN(5.0); 7-Vancomycin

1-[OD 590 of cells treated with test agent/OD 590 of non-treated control].100
REFERENCES


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