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Original article

Effects of the natural compounds embelin and piperine on the biofilm-producing property of *Streptococcus mutans*

Deepak Dwivedi [a, *], Vinod Singh [b]

[a] Minor Forest Produce Processing and Research Center, Bhopal, Madhya Pradesh, India
[b] Department of Microbiology, Barkatullah University, Bhopal, Madhya Pradesh, India

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ABSTRACT

We aimed to evaluate the effects of the natural compounds embelin and piperine on the biofilm-formation property of *Streptococcus mutans*. A total of 30 clinical isolates were identified as *S. mutans* and screened for biofilm formation using the microtiter plate method. The strongest biofilm producer (SM03) was used for identifying both minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC). We subsequently used this concentration against each of the strong biofilm producer isolates at $A_{492} < 0.5$ optical density (OD). Of the 30 isolates screened for biofilm formation, 18 isolates showed strong biofilm formation, 09 isolates showed moderate formation, and 03 isolates showed poor/nonbiofilm formation. The MIC of embelin for the strongest biofilm producer (SM03) was 0.55 ± 0.02, whereas that of piperine was 0.33 ± 0.02. The MBIC of embelin was 0.0620 ± 0.03, whereas that of piperine was 0.0407 ± 0.03, which was lower than that of embelin. At $OD_{492} < 0.5$, the MBIC of both compounds significantly inhibited biofilm formation of all the 18 strong biofilm-forming isolates. The results of this study demonstrate a significant antibiofilm effect of the natural compounds embelin and piperine, which can contribute towards the development of a database for novel drug candidates for treating oral infections caused by *S. mutans*.

1. Introduction

Microorganisms exist as free-floating cells or, more often, in a community of cells attached to a substrate. This sessile form of life is referred to as a *biofilm*. By definition, a biofilm is a community of cells attached to either a biotic or an abiotic surface enclosed in a complex exopolymeric substance.[1] Biofilms allow microorganisms to trap nutrients and withstand hostile environmental conditions by quorum sensing (QS). QS is a widespread and well-known cell-to-cell communication phenomena that regulates biofilm formation and virulence behaviors.[2–4] QS also involves chemical communication among bacteria including formation, secretion, detection, and reaction to molecules known as *autoinducers*. Several serious infections are reported to be a result of biofilm formation, which leads to chronic diseases in most cases. These persistent infections are a challenge for public health on a global scale, because they reduce the effectiveness of treatments and increase morbidity, mortality, and health-care costs.[5]

Streptococcus mutans is an impotent pathogen and is a common cause of oral infections such as dental caries. *S. mutans* effectively utilizes dietary sucrose to synthesize large amounts of exopolysaccharides, which play an important role in the accumulation, adhesion, and plaque matrix formation of microorganisms. These processes in most cases lead to serious infections. The ability of a microorganism to form a biofilm on a host tissue surface is an important step in the development of infection.[6] Because of poor hygiene, various pathogenic microorganisms cause infections. At present, a number of antibiotics are used for treating these infections. However, because these antibiotics are associated with significant side effects, there is increased attention toward using natural, biologically active herbal compounds as an alternative medicine.[7,8] Both embelin and piperine are natural compounds that are found in *Embelia ribes* and *Piper longum*, respectively. *E. ribes* and *P. longum* species are widely distributed across India. They are reputed medicinal herbs and their various parts have been used as a traditional cure in the Asian system of medicine (Indian, Chinese, and Malaysian) for treating a variety of disease conditions.[9–12]

* Corresponding author. Minor Forest Produce Processing and Research Center, Barkheda Pathani, Bhopal 462021, Madhya Pradesh, India.
E-mail address: microbio.deep@gmail.com (D. Dwivedi).
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The objective of this study was to identify the effects of natural compounds such as embelin and piperine on biofilm formation.

2. Material and methods

2.1. Isolation and identification of bacteria

S. mutans was isolated from dental caries or plaque from patients in the Outpatient Department of Peoples Dental Academy (Bhopal, Madhya Pradesh, India). The bacterium was isolated from both male and female patients (mean age, 20 years). The standard strain isolated was *S. mutans* ATCC 25175. Bacterial samples were cultured on the following media: brain heart infusion (BHI) agar medium (HiMedia Laboratories, India) in a 5% CO_2-enriched atmosphere and Mutans-Sanguis agar medium (HiMedia Laboratories, India). Biochemical tests were performed to identify the bacterial strains. Among the 20 samples from patients having dental caries, 30 isolates were identified as *S. mutans.*

2.2. Preparation of natural compounds

The natural compounds embelin and piperine were purchased from Natural Remedies (India). The compounds were dissolved in dimethyl sulfoxide (DMSO, Mark, Germany) at a concentration of 10 mg/mL.

2.3. Screening of S. mutans for biofilm formation

2.3.1. Microtiter plate method

Quantification of *S. mutans* isolates' biofilm formation was carried out using the microtiter plate method. To assay biofilm formation of the *S. mutans* isolates, an overnight culture of each isolate was grown in BHI broth (HiMedia Laboratories, India) for 18–20 hours at 37°C. Approximately 1 mL of each overnight culture was transferred to 10 mL of sterile BHI broth with the addition of 1% sucrose for biofilm production. The suspensions were adjusted using the same BHI medium to 0.5 on the McFarland turbidity standard as measured by absorbance (0.08–0.1 at 625 nm) in a spectrophotometer (Shimadzu, Australia), corresponding to approximately 10^2 CFU/mL. Then, from each culture, a 250-μL volume was transferred into the wells of a microtiter plate (HiMedia Laboratories).[13] Blank wells contained only the broth. Plates were made in triplicate and incubated at 37°C for 24 hours. After 24 hours, the planktonic suspension and nutrient solution were aspirated and each well was washed three times with 300 μL of sterile physiological saline. The plates were strongly shaken to remove all nonadherent bacteria. The remaining attached bacteria were fixed with 250 μL of 96% ethanol/well and, after 15 minutes, the plates were emptied and left to dry. Each well was then stained for 5 minutes with 200 μL of 2% crystal violet (CV Gram stain, Merck, Germany). The stain was rinsed off by placing the plates under running tap water. After drying the stained plates, biofilms were visible as purple rings on the sides of each well. The quantitative analysis of biofilm formation was performed by adding 200 μL of 33% (v/v) glacial acetic acid (Merck) per well. The optical density (OD) of the stain was then measured at 492 nm using an enzyme-linked immunosorbent assay reader (Lisa, Germany) as described previously.[13] Biofilm formation was scored as follows: nonbiofilm forming ($A_{492} \leq 1$); +, weak ($1 \leq A_{492} \leq 2$); ++, moderate ($2 < A_{492} \leq 3$); and +++, strong ($A_{492} > 3$). Microtiter assay was performed in triplicate.

2.4. Microscopic analysis using the coverslip method

The biofilm of *S. mutans* clinical isolates was grown as follows: individual sterile culture dishes were filled with 2.5 mL of BHI broth with 1% sucrose. A sterile 18-mm diameter glass coverslip was added to cover each culture dish. Each sample was inoculated with a defined volume of overnight culture. The dishes were incubated microaerobically at 37°C for 48 hours. Glass cover slips containing the attached biofilm were removed from the dishes, rinsed briefly with phosphate-buffered saline, and stained for 5 minutes with 0.5% crystal violet. The stained biofilms were observed under a microscope.[14]

2.5. Determination of the minimum inhibitory concentration of embelin and piperine

The minimum inhibitory concentration (MIC) of the natural compounds embelin and piperine was evaluated on all the isolates by the broth dilution method. The natural compounds were dissolved in DMSO (initial concentration, 2–0.0078 mg/mL). The initial test concentration was serially diluted twofold. Each well was inoculated with 5 μL of suspension containing 10^8 CFU/mL of bacteria. The plates with bacteria were incubated at 37°C for 24 hours. After incubation, 5 μL of tested broth was placed on the sterile BHI plates and incubated at respective temperature (37C). The MIC for bacterial isolates was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures on the agar plate. Triplicates were maintained.[15,16]

2.6. Biofilm-inhibition assay in the presence of embelin and piperine

Only those isolates of *S. mutans* that were classified as strong biofilm producers were used in the biofilm-inhibition assay. Test compounds were dissolved in DMSO (10 mg/mL), and twofold dilutions were prepared to obtain a final concentration ranging from 2 mg/mL to 0.0078 mg/mL in the wells after the addition of the freshly diluted BHI broth culture containing 10^6 CFU of the strong biofilm-forming isolates per well. After incubation at 37°C for 24 hours, the microtiter plate was washed, fixed, and biofilms were stained and visualized as explained earlier. The inhibitory effect of the compounds on biofilm production was calculated by subtracting the media control. The biofilm inhibitory concentration (BIC) is the concentration of the natural compound at which the biofilm formation was reduced to an absorbance (A_{492}) < 0.5 OD. Each assay for BIC determination was performed in triplicate.

2.7. Statistical analysis

Calculations and statistics were performed using GraphPad 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). The results were analyzed using one-way analysis of variance. Significance was defined as $p < 0.05$. Results are presented as mean ± the standard error of the mean.

3. Results

3.1. Screening of S. mutans for biofilm formation

The 30 *S. mutans* isolates were screened for biofilm formation by the microtiter plate method and the results are shown in Fig. 1. All 30 isolates were classified based on their biofilm-forming potential as follows: 18 were strong biofilm producers, nine isolates were moderate producers, and three were poor/nonbiofilm producers. The *S. mutans* ATCC 25175 was included as an assay control and identified to be a moderate biofilm producer. This observation confirms that the magnitude and intensity of biofilm formation of the 18 isolates were significantly greater than those of the poor/nonbiofilm producers. Fig. 1 also shows the quantitative evaluation for identifying and demarcating strong biofilm-producing *S. mutans*