










## Antibacterial and Antiadhesion Effects of *Psidium guajava* Fractions on a Multispecies Biofilm Associated with Periodontitis

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### ABSTRACT

**Objective:** To assess the antibacterial activity of *Psidium guajava* fractions and their effects on adhesion of a multispecies biofilm consisting of *Streptococcus gordonii*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* *in vitro*. **Material and Methods:** Guava leaves were obtained from the mountains of northern Peru, where they grow wild and free of pesticides. The antimicrobial activity of 25 mg/mL petroleum ether, 25 mg/mL dichloromethane and 25 mg/mL methanol fractions of *P. guajava* was evaluated by measuring inhibition halos, as well as the effect on the adhesion of multispecies biofilms at 4, 7 and 10 days of growth by measuring the optical density. In addition, antimicrobial susceptibility was compared using the Kruskal-Wallis test and its multiple comparison tests, and differences in mean biofilm adhesion between each fraction were assessed by repeated measures analysis and the Tukey multiple comparison test. **Results:** The rank-based Kruskal-Wallis test highlighted differences in the effects of the fractions on the zone of inhibition for each oral bacterium, including *S. gordonii* (p=0.000), *F. nucleatum* (p=0.000), and *P. gingivalis* (p=0.000), the Tukey test showed that the group treated with 0.12% chlorhexidine exhibited the least amount of adhesion, followed by the group treated with the 1.56 mg/mL methanol fraction. **Conclusion:** The methanol fraction of *P. guajava* had an antibacterial effect on *S. gordonii* and *P. gingivalis*, and the 1.56 mg/mL methanol fraction decreased biofilm adhesion.

**Keywords:** Psidium; Biofilms; Gram-Negative Bacteria.

## Introduction

The constant colonization and bacterial growth on tooth surfaces lead to the formation of oral biofilms, the bacterial composition of these biofilms, initially dominated by cocci and small bacilli, begins to change towards a spirochete-dominated flora, accompanied by the appearance of gingivitis over two to three weeks [1-3]. This transition of the bacterial flora appears to be the key process in the induction of periodontitis at a later stage. While *Streptococcus sp.* and *Actinomyces sp.* are recognized as dominant species in the healthy oral flora and their role as early colonizers of oral biofilms. The sequence of events responsible for the changes from biofilms dominated by these early colonizers to the completely altered consortium detected in the pockets is associated with bacteria capable of impairing the host immune response and increasing the pathogenic potential of the entire biofilm [4,5].

The presence of periodontal pathogens indisputably characterizes periodontal disease [6,7]. *Fusobacterium nucleatum* and *Porphyromonas gingivalis* are among the bacteria that are usually isolated from patients with periodontal disease, and the latter is the most common bacterium associated with periodontitis [8-10]. Therefore, the most effective strategy for periodontal disease prevention is the elimination of pathogenic biofilms, which is challenging. In fact, the high incidence of periodontal disease represents a main public health problem that must be overcome [10].

Guava (*Psidium guajava*) trees are grown for their nutritious fruits, characterized by a high content of minerals and vitamins [11]. However, other parts (the leaves, bark, and root) of guava trees are used in traditional medicine to treat various diseases. Different guava leaf extracts show strong biological activities, such as anti-inflammatory, antipyretic, neuroprotective, antihypertensive, hypolipidemic, antiobesity, cardioprotective, antioxidant, hepatoprotective, antidiarrheal, anticancer, immunostimulant, antiadrenal, antimicrobial, antiviral, and antimicrobial plaque actions [12,13]. In addition, several chemical studies have identified various vitamins (A, C, B, E, and K), carbohydrates, tannins, triterpenoids, flavonoids, benzophenones, and phenols [13,14]. Many essential oils (EO) compounds can be extracted from guava leaves worldwide, especially terpenoids such as limonene,  $\alpha$ -pinene, eucalyptol, caryophyllene isomers,  $\alpha$ -humulene,  $\gamma$ -murolene, selinene isomers,  $\beta$ -bisabolene, caryophyllene oxide, and epi- $\beta$ -cubanol [15,16].

Some studies have assessed the effects of these extracts on oral microorganisms. For example, Millones-Gómez et al. [17] evaluated the antimicrobial activity and antiadhesion effects of the crude organic extract (COE) and three fractions (aqueous, butanolic, and chloroform) of *P. guajava* (guava) leaves in a cariogenic biofilm model. The authors found that the COE and the chloroform fraction have antibacterial activity against *Streptococcus gordonii* and a significant effect on biofilm adhesion, sustained throughout the seven days of evaluation [17]. Similarly, Shetty et al. [18] demonstrated that guava extracts are potential therapeutic agents for periodontitis because they show significant activity against *Aggregatibacter actinomycetemcomitans* and *P. gingivalis*.

Although *P. guajava* has been used as an antimicrobial agent over the years, it is important to know if there is variation in its effect using different solvents, either in its more rudimentary form or in modern research; its action against bacteria associated with periodontal disease is not a fully known. Considering the knowledge gap in this line of research and the remarkable potential of *P. guajava*, we propose to analyze the microbiological activity of *P. guajava* fractions on three standard bacterial strains and their effect on biofilm adhesion of *S. gordonii* ATCC 51656, *F. nucleatum* ATCC 10953 and *P. gingivalis* ATCC 33277 *in vitro*.

## Material and Methods

### Study Design

This experimental *in vitro* study was conducted at the Bacteriology Laboratory of the College of Science of the Cayetano Heredia University (Universidad Peruana Cayetano Heredia – UPCH).

### Sample Collection

Ten kilograms of leaves of *P. guajava* were collected during November 2018 in rural areas of the city of Oxapampa, Peru, using latex gloves and taking into account the principles of biosafety. First, pruners were used to cut the branches, which were put inside a plastic bag. Then, the best leaves that were intact and clean were selected. Next, all the leaves were placed carefully inside cardboard boxes lined with Kraft paper. Last, the leaves were packed to be transported to the Chemistry Laboratory of the National University of Engineering in Lima, Peru.

### Extraction of Guava Fractions from the Chloroform Residue

A previous evaluation of the crude extract and the fractions of guava leaves [17] showed that the guava chloroform residue had the strongest inhibitory effect on *Streptococcus gordonii* biofilm adhesion at 1, 4, and 7 days of growth. Therefore, this residue was fractionated using a Sephadex LH-20 column with a stationary phase and the solvents petroleum ether, dichloromethane, and methanol as mobile phases (Figure 1).

The guava chloroform residue was solubilized with petroleum ether. Then, open column chromatography with a stationary phase was performed on a Sephadex LH-20 (100 g) column in petroleum ether. The solubilized sample was added to the column and eluted with petroleum ether (450 mL), followed by dichloromethane (350 mL), and finally methanol (500 mL), thereby collecting the petroleum ether, dichloromethane, and methanol fractions. These fractions were dried in a fume hood [19].

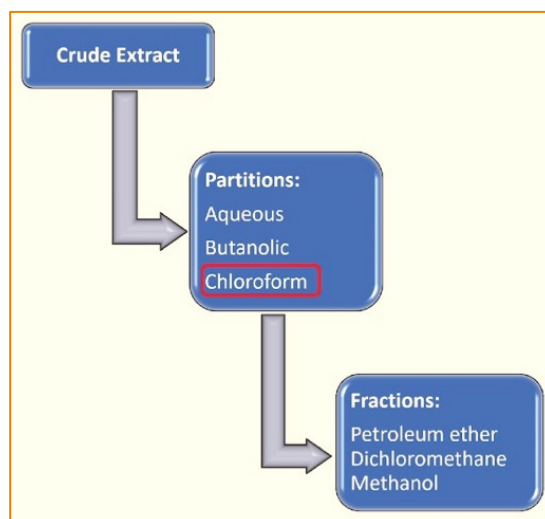


Figure 1. Preparation process of *P. guajava* fractions from crude extract and partitions [17].

### Antimicrobial Susceptibility Test of the Methanol Fraction of *P. guajava*

Based on the antimicrobial susceptibility tests of the three fractions, the methanolic fraction presented the greatest zones of inhibition on *Streptococcus gordonii*, the microorganism responsible for adherence of the biofilm. Therefore, only the methanol fraction was used in further analyses.

The following strains were used: *Streptococcus gordonii* ATCC 51656, *Fusobacterium nucleatum* ATCC 10953, and *Porphyromonas gingivalis* ATCC 33277 [20]. To assess the antibacterial effect, brain heart infusion (BHI) agar plates were prepared for *S. gordonii*, BHI agar plates supplemented with 5% sheep blood plus menadione and vitamin K were prepared for *F. nucleatum*, and BHI agar plates were supplemented with horse blood plus menadione and vitamin K were prepared for *P. gingivalis*. All plates were controlled for 24 hours to check their sterility.

Inoculum preparation: The three strains were grown in BHI broth for 24 hours; subsequently, turbidity was calculated to a 0.5 McFarland standard. For comparison, a swab was soaked with the previously prepared inoculum, streaked on the surface of agar plates four times, and left to rest for 5 minutes. Subsequently, 6-mm-wide qualitative filter paper circles (Whatman®, Grade 3) impregnated with 10 µL of the natural extract and controls were placed. This procedure was replicated five times, considering a maximum difference of 15 mm between fractions of *Psidium guajava*, with a standard deviation of 5 mm and a type I error of 5%, reaching a power of 93.9% [21]. Using 0.12% chlorhexidine as a positive control and a 1% DMSO solution plus Milli-Q water (1:1) as a negative control, all plates were incubated with the natural extract and controls at 37°C for 48 hours under anaerobic conditions. After 48 hours of incubation, the plates were read, and the zones of inhibition were measured using a caliper graduated in mm.

#### Determination of the Minimum Inhibitory Concentration (MIC) of Guava for the Three Oral Bacteria

The method used was the broth microdilution method using 96-well microtiter plates. BHI broth was used as a culture medium, in anaerobiosis, at 37 °C. Tryptic soy broth (TSB) (140 µL) was added to the wells of 96-well microtiter plates; then, 140 µL of natural guava extract was added to one well, followed by a transfer of 140 µL to the next well with a micropipette and homogenization and repeating the same procedure well by well; the final 140 µL was discarded. Subsequently, 20 µL of the culture of strains of *S. gordonii*, *F. nucleatum*, and *P. gingivalis* was added to the wells, calibrating to a 0.5 McFarland standard. The microtiter plates were incubated at 37 °C for 48 hours under anaerobic conditions with 0.12% chlorhexidine as a positive control and the 1% DMSO + Milli-Q water (1:1) solution as a negative control. The procedure was replicated five times [17].

The reading of the minimum inhibitory concentration of the propolis was determined according to the concentration of the well where no development was observed (turbidity). To verify bacterial viability, 5 µL of each well of culture medium of each bacterium was seeded. The minimum bactericidal concentration was considered to be the one where there was no colony growth.

#### Biofilm Formation of the Three Species

The oral bacteria *S. gordonii* ATCC 51656, *F. nucleatum* ATCC 10953, and *P. gingivalis* ATCC 33277 were used to form a biofilm on an 8-well Nunc Lab-Tek Chamber Slide™ system. To start the model, each strain was separately inoculated with 15 mL of TSB at 37 °C under anaerobic conditions until reaching the exponential growth phase; *S. gordonii* was incubated for 4 hours and 30 minutes, *F. nucleatum* was incubated for 8 hours, and *P. gingivalis* ATCC 33277 was incubated until reaching an optical density (OD) of 0.125 nm with 150 x 10<sup>6</sup> cells/mL. The surface of Lab Tek slides was coated with 30 µL of poly-L-lysine, and the slides were incubated at room temperature for 30 minutes, washed with 30 µL of PBS, and then left to dry at 37°C for 24 hours under sterile conditions. Then, 300 µL of artificial saliva [17] was added to each well, which was then incubated at 4 °C for 16 hours. Subsequently, the artificial saliva was removed, and cells were washed twice

with 300  $\mu\text{L}$  of PBS (1X). After washing, 250  $\mu\text{L}$  of BHI broth + 20  $\mu\text{L}$  of 2.5% sucrose was added before inoculating 10  $\mu\text{L}$  of *S. gordonii*, *F. nucleatum*, or *P. gingivalis* on each slide and incubating for 24 hours at 37 °C under anaerobic conditions [17,20]. To test the biofilm formation of the three species, DNA concentration was quantified in Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA), and using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA).

#### Assessment of the Effect of Guava on Biofilm Adhesion

The first dose was administered 24 hours after biofilm formation by carefully removing the supernatant from each well and then washing twice with 300  $\mu\text{L}$  of PBS (1X). Subsequently, 300  $\mu\text{L}$  of the guava fraction was administered, and the wells were incubated for 1 minute at room temperature. After removing the extract, the surface of each well was washed twice with 300  $\mu\text{L}$  of PBS (1X), subsequently adding 300  $\mu\text{L}$  of sterile culture medium, consisting of BHI broth + 2.5% saccharose, and incubating at 37 °C for 24 hours under anaerobic conditions. This procedure was repeated at 4 (time 1), 7 (time 2), and 10 (time 3) days; 1% DMSO + Milli-Q water (1:1) was used as a negative control, and 0.12% chlorhexidine was used as a positive control [17].

To determine the absorbance, the biofilm was removed from the anaerobic jar, carefully discarding the supernatant from each well. While avoiding turbulence, each well was washed three times with 300  $\mu\text{L}$  of PBS (1X), pH 7.0 (heated to 25-30 °C), for 10 seconds using a Pasteur pipette (to remove the remaining culture medium and unattached bacteria). Then, 300  $\mu\text{L}$  of trypsin was added, and the plate was rocked for 5 minutes to remove all adhering cells from well surfaces. Subsequently, 100  $\mu\text{L}$  of the content of each well was obtained and placed in a 96-well microplate to measure the OD at 595 nm on a Smart Spectrophotometer plus reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) [17].

#### Statistical Analysis

The data were processed with SPSS version 26 using the nonparametric Kruskal-Wallis test and its multiple comparisons tests to compare the antimicrobial susceptibility of the *P. guajava* fractions and their positive and negative controls based on ranges of diameter measurements of the zone of inhibition.

The effects of guava on biofilm adhesion were assessed by repeated measures analysis, which also included analysis of variance (ANOVA) and the Tukey multiple comparison test of the means of each fraction. Differences were considered significant at a p-value of 0.05.

## Results

After measuring the zones of inhibition at 24 hours of incubation, the results of each oral bacterium under study were compared, as outlined in Table 1.

**Table 1. Antimicrobial susceptibility of three oral bacteria to *P. guajava* fractions.**

<i>Psidium guajava</i> Fraction	<i>S. gordonii</i> Mean (SD)	<i>F. nucleatum</i> Mean (SD)	<i>P. gingivalis</i> Mean (SD)
Methanol Fraction	15.62 $\pm$ 0.28 <sup>d</sup>	0 $\pm$ 0 <sup>ab</sup>	9.44 $\pm$ 0.30 <sup>b</sup>
Dichloromethane Fraction	9.32 $\pm$ 0.63 <sup>bc</sup>	8.24 $\pm$ 0.40 <sup>c</sup>	0.0 $\pm$ 0.0 <sup>a</sup>
Petroleum Ether Fraction	7.24 $\pm$ 0.38 <sup>ab</sup>	0.0 $\pm$ 0.0 <sup>ab</sup>	0.0 $\pm$ 0.0 <sup>a</sup>
0.12% Chlorhexidine	13.14 $\pm$ 0.30 <sup>cd</sup>	11.16 $\pm$ 0.25 <sup>c</sup>	11.80 $\pm$ 0.47 <sup>b</sup>
1% DMSO + Milli-Q Water	0.0 $\pm$ 0.0 <sup>a</sup>	0.00 <sup>a</sup>	0.00 <sup>a</sup>
p-value <sup>#</sup>	0.000	0.000	0.000

<sup>#</sup>Kruskal-Wallis, multiple comparisons (a, b, and c).

The 1% DMSO + Milli-Q water group showed no zone of inhibition for any bacterial strain. Additionally, the methanol residue showed no zone of inhibition for *F. nucleatum* ATCC 10953, the dichloromethane residue showed no zone of inhibition for *P. gingivalis* ATCC, and the petroleum ether residue showed no zone of inhibition for *F. nucleatum* ATCC 10953 and *P. gingivalis* ATCC (Figures 2, 3 and 4).

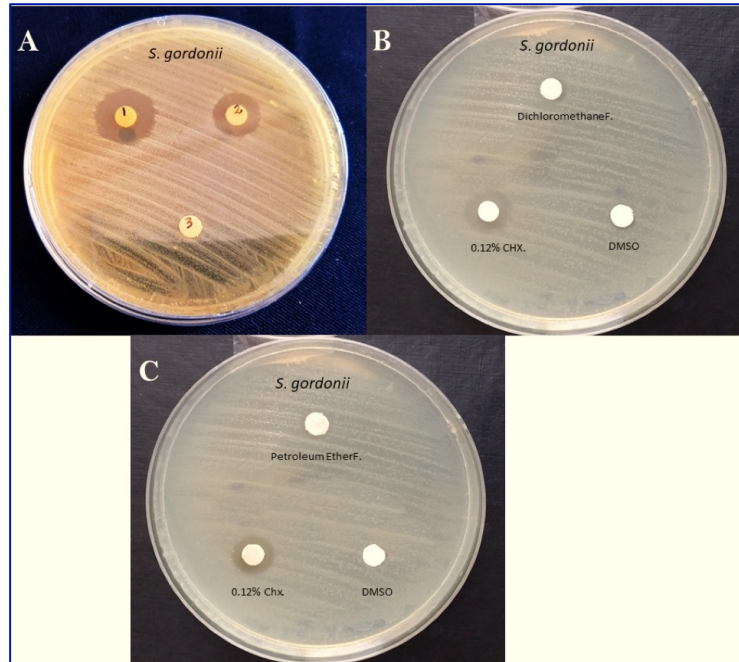


Figure 2. Agar plates containing *S. gordonii* and the methanol (A), dichloromethane (B), and petroleum ether (C) fractions of 25 mg/mL guava extracts and the controls, including 0.12% chlorhexidine and 1% DMSO with Milli-Q water.

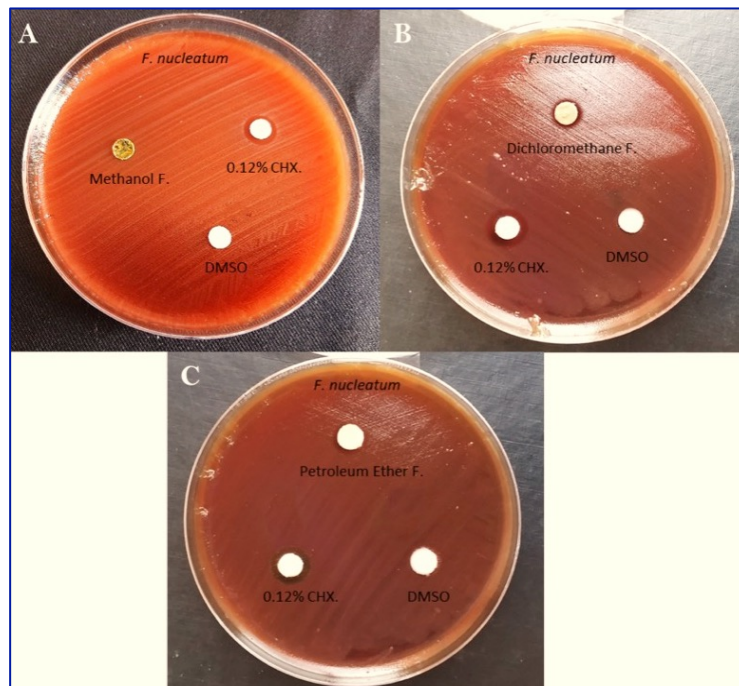
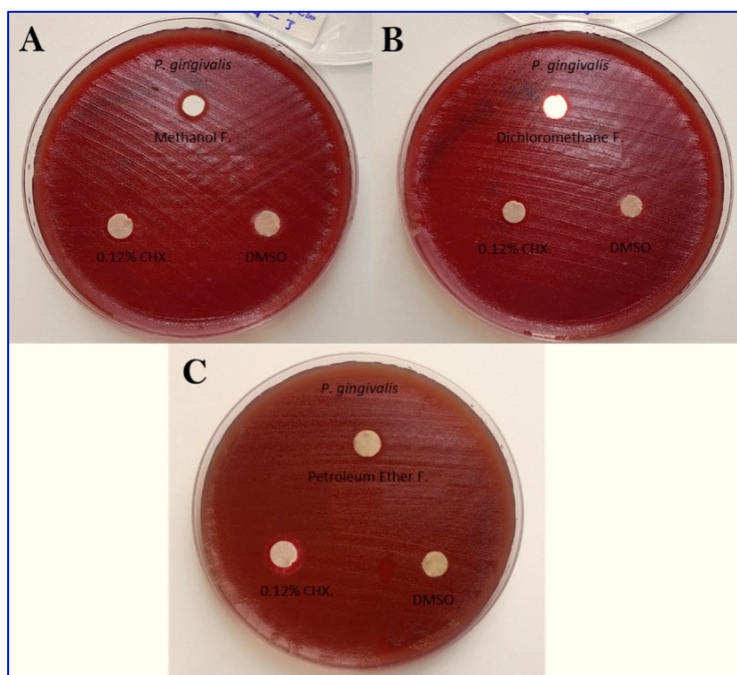


Figure 3. Agar plates containing *F. nucleatum* and the methanol (A), dichloromethane (B), and petroleum ether (C) fractions of 25 mg/mL guava extracts and the controls, including 0.12% chlorhexidine and 1% DMSO with Milli-Q water.



**Figure 4.** Agar plates containing *P. gingivalis* and the methanol (A), dichloromethane (B), and petroleum ether (C) fractions of 25 mg/mL guava extracts and the controls, including 0.12% chlorhexidine and 1% DMSO with Milli-Q water.

The rank-based Kruskal-Wallis test highlighted differences between fractions regarding their effects on the zone of inhibition of the oral bacteria *S. gordonii* ATCC 51656 ( $p=0.000$ ), *F. nucleatum* ATCC 10953 ( $p=0.000$ ), and *P. gingivalis* ATCC ( $p=0.000$ ). In addition, 0.12% chlorhexidine showed the greatest *F. nucleatum* ATCC 10953 ( $11.16 \pm 0.25$ ) and *P. gingivalis* ATCC ( $11.80 \pm 0.47$ ) growth control, albeit without reaching significant differences from the dichloromethane ( $8.24 \pm 0.40$ ) and methanol ( $9.44 \pm 0.30$ ) fractions, respectively. Furthermore, the methanol fraction showed the highest *S. gordonii* ATCC 51656 ( $15.62 \pm 0.28$ ) growth control but without significant differences from 0.12% chlorhexidine ( $13.14 \pm 0.30$ ).

The two (methanol and dichloromethane) fractions that demonstrated the strongest antibacterial effect in the antimicrobial susceptibility test were used to test the MIC of guava. In addition, considering the MIC results, the following concentrations were used in the adhesion test: 1.56 mg/mL for the methanol fraction and 0.78 mg/mL and 3.12 mg/mL for the dichloromethane fraction (Table 2).

**Table 2.** MIC of *P. guajava* on *S. gordonii*, *F. nucleatum* and *P. gingivalis*.

Fraction	<i>S. gordonii</i>	<i>F. nucleatum</i>	<i>P. gingivalis</i>
Methanol	1.56 mg/mL	---	1.56 mg/mL
Dichloromethane	0.78 mg/mL	3.12 mg/mL	

The effects of guava on biofilm adhesion are shown in Table 3, which indicates differences in means between treatments ( $F=4026.24$ ,  $p=0.000$ ). In addition, the Tukey test demonstrated that the group treated with 0.12% chlorhexidine showed the lowest adhesion ( $0.07 \pm 0.53$ ), followed by the group treated with the 1.56 mg/mL methanol fraction ( $0.23 \pm 0.05$ ), with greater adhesion in the groups treated with the other extracts.

**Table 3.** Effects of *P. guajava* fractions on biofilm adhesion.

Extract	Fraction	4 Days	7 Days	10 Days	Mean <sup>#</sup>
Guava	Methanol (1.56 mg/mL)	$0.24 \pm 0.01$	$0.27 \pm 0.06$	$0.18 \pm 0.02$	$0.23 \pm 0.05^b$
	Dichloromethane (0.78 mg/mL)	$0.62 \pm 0.01$	$0.86 \pm 0.05$	$0.96 \pm 0.01$	$0.81 \pm 0.31^d$

	Dichloromethane (3.12 mg/mL)	0.50 ± 0.06	0.33 ± 0.01	0.41 ± 0.00	0.41 ± 0.26 <sup>c</sup>
	1% DMSO + Milli-Q H <sub>2</sub> O (1:1)	1.09 ± 0.02	1.10 ± 0.00	1.23 ± 0.00	1.14 ± 0.34 <sup>f</sup>
Control	Chlorhexidine (0.12%)	0.07 ± 0.00	0.09 ± 0.00	0.04 ± 0.00	0.07 ± 0.53 <sup>a</sup>
	Without extract	0.89 ± 0.00	1.04 ± 0.00	1.36 ± 0.00	1.09 ± 0.51 <sup>e</sup>

#ANOVA (F=4026.24, p=0.000) and Tukey test (a, b, c, d, e, and f).

Table 4 outlines the multivariate repeated measures analysis of the effects of guava fractions on biofilm adhesion. All statistics reveal that adhesion varied over time (p=0.000) and that the effects of one guava fraction can differ from those of other fractions over time (p=0.000). In addition, Mauchly's sphericity test (p=0.425) indicated the presence of sphericity, which indicates that the repeated measures analysis is appropriate.

**Table 4. Effects of guava on biofilm adhesion by repeated measures analysis#.**

Effect	Multivariate Statistics	Value	F	p-value
Time	Pillai's Trace	0.968	168.699	0.000
	Wilks' Lambda	0.032	168.699	0.000
	Hotelling's Trace	30.673	168.699	0.000
	Roy's Largest Root	30.673	168.699	0.000
Time * Fraction	Pillai's Trace	1.854	30.484	0.000
	Wilks' Lambda	0.002	52.019	0.000
	Hotelling's Trace	86.657	86.657	0.000
	Roy's Largest Root	80.175	192.42	0.000

#Mauchly's sphericity test (W=0.056, p=0.425).

## Discussion

Considering that periodontal disease results from an imbalance in the oral ecosystem that affects periodontal tissues [22,23], this disease must be prevented and/or treated because it is closely linked to systemic problems [24]. Therefore, effective plaque control strategies have become the guiding principle for prevention of plaque-related diseases such as periodontitis [25].

Few studies have examined the control of microorganisms associated with periodontal disease based on natural products. Therefore, this study was proposed to evaluate the antibacterial and antiadhesion activity of *P. guajava* fractions extracted from its chloroform residue.

In a previous study, Millones-Gómez et al. [17] found that both the crude extract and the chloroform portion of guava showed antimicrobial efficacy against *S. gordonii*, with a mean zone of inhibition diameters of 10.4 mm and 9.12 mm at a concentration of 50 mg/mL, respectively. In the present study, of the three *P. guajava* fractions analyzed, the methanol fraction showed antimicrobial action against *S. gordonii* and *P. gingivalis*. The dichloromethane fraction also showed antimicrobial activity against *S. gordonii* and *Fusobacterium*. Furthermore, Shetty et al. [18] demonstrated that the ethanol extract of *P. guajava* has greater antimicrobial activity against *P. gingivalis* than the methanol fraction. Similar results have been reported for Indian *P. guajava* [26,27]. Some solvents can attract components as a function of their polarity, which is reflected in their biological properties [28].

Biofilm adhesion inhibition was shown by a decrease in the mean OD. *P. guajava* leaf extracts contain chemical compounds with antiadhesion properties derived from flavonoids and polyphenols. Flavonoids consist of active substances, such as flavone and naringenin, and flavone has been shown to inhibit biofilm formation. These compounds can interfere with the quorum signaling pathway by disrupting the interaction between acyl-homoserine lactone (AHL) and its receptor. AHL is an autoinducer or signaling molecule of Gram-negative bacteria used in the quorum sensing process [29]. Naringenin also plays a role in inhibiting biofilm












formation through its activities as a quorum sensing inhibitor. The action of naringenin in inhibiting the quorum sensing system is likely caused by the combination and reduction of AHL molecules and by the transcription factor Lux-R, followed by a decrease in the expression of the quorum sensing-related gene [30]. Quorum sensing is one of the regulatory mechanisms of extracellular polymeric substances (EPSs), commonly known as polysaccharides, and plays a role in bacterial biofilm formation. Therefore, if the quorum sensing pathway is inhibited, EPS formation will also be inhibited, thereby inhibiting bacterial biofilm formation [31].

Based on these results, the mean OD of the samples treated with the methanol fraction of *P. guajava* showed a moderate decrease in adhesion to the biofilm, apparently representative of the antibacterial effect shown on *S. gordonii*, the microorganism responsible for biofilm adherence in periodontal biofilm models [32,33]. Despite the promising results, it should be emphasized that this experimental trial does not reflect the complex polymicrobial and environmental interactions present in the oral cavity. Environmental interactions are present in the oral cavity. On the other hand, complementing the results of this study with the separation and identification of the components, understanding the therapeutic targets, as well as the mechanisms of action of these propolis would help to the mechanisms of action of these propolis would help to evaluate new molecules for the formulation of new pharmacological supplies that could be valuable in the field of dentistry.

## Conclusion

The methanol fraction of *P. guajava* had an antibacterial effect on *S. gordonii* and *P. gingivalis*, and the 1.56 mg/mL methanol fraction decreased biofilm adhesion.

## Authors' Contributions

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LAPC 	<a href="https://orcid.org/0000-0002-5523-8134">https://orcid.org/0000-0002-5523-8134</a>	Writing - Review and Editing and Supervision.
All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.		

## Financial Support

None.

## Conflict of Interest

The authors declare no conflicts of interest.

## Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

## References

- [1] Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RLJ. Microbial complexes in subgingival plaque. J Clin Periodontol 1998; 25(2): 134-44. <https://doi.org/10.1111/j.1600-051X.1998.tb02419.x>
- [2] Kolenbrander PE, Palmer RJJ, Periasamy S, Jakobovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol 2010; 8(7):471-80. <https://doi.org/10.1038/nrmicro2381>
- [3] Hajishengallis G, Lamont RJ. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. Mol Oral Microbiol 2012; 27(6):409-19. <https://doi.org/10.1111/j.2041-1014.2012.00663.x>

- [4] Millones-Gómez P, Aguilar A. Eficacia de la azitromicina asociada al RAR en periodontitis crónica: ensayo clínico, aleatorizado, controlado y triple ciego en grupos en paralelo. *Rev Esp Cirug Oral Maxilofac* 2018; 40(3):129-34. <https://doi.org/10.1016/j.maxilo.2017.08.001>
- [5] Pensantes-Sangay SJ, Calla-Poma RD, Requena-Mendizabal MF, Alvino-Vales MI, Millones-Gómez PA. Chemical composition and antibacterial effect of plantago major extract on periodontal pathogens. *Pesqui Bras Odontopediatria Clín Integr* 2020; 20:e0012. <https://doi.org/10.1590/pboci.2020.100>
- [6] Rodríguez JAL, Casana STV, Gómez PAM. Effectiveness of chlorhexidine and essential oils associated with scaling and root planing in the treatment of chronic periodontitis. *Rev Cienc Salud* 2020; 18(3):1-11. <https://doi.org/10.12804/revistas.urosario.edu.co/revsalud/a.9795>
- [7] Figueredo CM, Lira-Junior R, Love RM. T and B cells in periodontal disease: new functions in a complex scenario. *Int J Mol Sci* 2019; 20(16):3949. <https://doi.org/10.3390/ijms20163949>
- [8] Millones-Gómez PA, Amaranto REB, Torres DJM, Calla-Poma RD, Requena-Mendizabal MF, Alvino-Vales MI, et al. Identification of proteins associated with the formation of oral biofilms. *Pesqui Bras Odontopediatria Clín Integr* 2021; 21:e0128. <https://doi.org/10.1590/pboci.2021.084>
- [9] Wang RP, Ho YS, Leung WK, Goto T, Chang RC. Systemic inflammation linking chronic periodontitis to cognitive decline. *Brain Behav Immun* 2019; 81:63-73. <https://doi.org/10.1016/j.bbi.2019.07.002>
- [10] Abiko Y, Sato T, Mayanagi G, Takahashi N. Profiling of subgingival plaque biofilm microflora from periodontally healthy subjects and from subjects with periodontitis using quantitative real-time PCR. *J Periodontol Res* 2010; 45(3):389-95. <https://doi.org/10.1111/j.1600-0765.2009.01250.x>
- [11] Belstrøm D, Grande MA, Sembler-Møller ML, Kirkby N, Cotton SL, Paster BJ, et al. Influence of periodontal treatment on subgingival and salivary microbiotas. *J Periodontol* 2018; 89(5):531-9. <https://doi.org/10.1002/jper.17-0377>
- [12] Demoliner F, Policarpi PDB, Vasconcelos LFL, Vitali L, Micke GA, Block JM. Sapucaia nut (*Lecythis pisonis* Cambess) and its by-products: a promising and underutilized source of bioactive compounds. Part II: phenolic compounds profile. *Food Res Int* 2018; 112:434-42. <https://doi.org/10.1016/j.foodres.2018.06.050>
- [13] Nhu TQ, Dam NP, Bich Hang BT, Bach LT, Thanh Huong DT, Buu Hue BT, et al. Immunomodulatory potential of extracts, fractions and pure compounds from *Phyllanthus amarus* and *Psidium guajava* on striped catfish (*Pangasianodon hypophthalmus*) head kidney leukocytes. *Fish Shellfish Immunol* 2020; 104:289-303. <https://doi.org/10.1016/j.fsi.2020.05.051>
- [14] Qin XJ, Yu Q, Yan H, Khan A, Feng MY, Li PP, et al. Meroterpenoids with Antitumor Activities from Guava (*Psidium guajava*). *J Agric Food Chem* 2017; 65(24):4993-9. <https://doi.org/10.1021/acs.jafc.7b01762>
- [15] Lin CY, Yin MC. Renal protective effects of extracts from guava fruit (*Psidium guajava* L.) in diabetic mice. *Plant Foods Hum Nutr* 2012; 67(3):303-8. <https://doi.org/10.1007/s11130-012-0294-0>
- [16] Hassan EM, El Gendy AEG, Abd-ElGawad AM, Elshamy AI, Farag MA, Alamery SF, et al. Comparative chemical profiles of the essential oils from different varieties of *Psidium guajava* L. *Molecules* 2020; 26(1):119. <https://doi.org/10.3390/molecules26010119>
- [17] Millones-Gómez PA, Maurtua-Torres D, Bacilio-Amaranto R, Calla-Poma RD, Requena-Mendizabal MF, Valderrama-Negron AC, et al. Antimicrobial activity and antiadherent effect of peruvian *Psidium guajava* (Guava) leaves on a cariogenic biofilm model. *J Contemp Dent Pract* 2020; 21(7):733-40. <https://doi.org/10.5005/jp-journals-10024-2893>
- [18] Shetty YS, Shankarapillai R, Vivekanandan G, Shetty RM, Reddy CS, Reddy H, et al. Evaluation of the efficacy of guava extract as an antimicrobial agent on periodontal pathogens. *J Contemp Dent Pract* 2018; 19(6):690-7. <https://doi.org/10.5005/jp-journals-10024-2321>
- [19] Adiguzel A, Ozer H, Sokmen M, Gulluce M, Sokmen A, Kilic H, et al. Antimicrobial and antioxidant activity of the essential oil and methanol extract of *Nepeta cataria*. *Pol J Microbiol* 2009; 58(1):69-76.
- [20] Ebersole JL, Peyyala R, Gonzalez OA. Biofilm-induced profiles of immune response gene expression by oral epithelial cells. *Mol Oral Microbiol* 2019; 34(1):10.1111/omi.12251. <https://doi.org/10.1111/omi.12251>
- [21] Montgomery D. Design and Analysis of Experiments. Hoboken, NJ: John Wiley & Sons, Inc.; 2000. pp: 105-110.
- [22] Bermúdez-Vásquez MJ, Granados-Chinchilla F, Molina A. Composición química y actividad antimicrobiana del aceite esencial de *Psidium guajava* y *Cymbopogon citratus*. *Agron Mesoam* 2019; 30(1):147-63. <https://doi.org/10.15517/am.v30i1.33758>
- [23] Millones Gómez PA. Mouthwashes in Covid 19: benefit or harm to the oral microbiome? *Oral Dis* 2021; 10.1111/odi.13975. <https://doi.org/10.1111/odi.13975>.
- [24] Bacilio R, Millones P. Efectividad analgésica del clonixinato de lisina asociado con el paracetamol en el tratamiento posoperatorio de exodoncias. *Rev Cienc Salud* 2019; 17(2):321-33. <https://doi.org/10.12804/revistas.urosario.edu.co/revsalud/a.7943>
- [25] Ravi K, Divyashree P. *Psidium guajava*: a review on its potential as an adjunct in treating periodontal disease. *Pharmacogn Rev* 2014; 8(16):96-100. <https://doi.org/10.4103/0973-7847.134233>

- [26] Shekar C, Nagarajappa R, Singh R, Thakur R. Antimicrobial efficacy of *Acacia nilotica*, *Murraya koenigii* L. Sprengel, *Eucalyptus hybrid*, and *Psidium guajava* on primary plaque colonizers: an in vitro comparison between hot and cold extraction process. *J Indian Soc Periodontol* 2015; 19(2):174-9. <https://doi.org/10.4103/0972-124x.145814>
- [27] Blanco-Olano J, Millones-Gómez P. Cicatrizing effect of Aloe vera gel with *Erythroxyllum coca* in animal model. *Med Nat* 2020; 14(1):65-74.
- [28] Esonye C, Onukwuli OD, Anadebe VC, Ezeugo JNO, Ogbodo NJ. Application of soft-computing techniques for statistical modeling and optimization of *Dyacrodes edulis* seed oil extraction using polar and non-polar solvents. *Heliyon* 2021; 7(3):e06342. <https://doi.org/10.1016/j.heliyon.2021.e06342>
- [29] Manner S, Skogman M, Goeres D, Vuorela P, Fallarero A. Systematic exploration of natural and synthetic flavonoids for the inhibition of *Staphylococcus aureus* biofilms. *Int J Mol Sci* 2013; 14(10):19434-51. <https://doi.org/10.3390/ijms141019434>
- [30] Vandeputte OM, Kiendrebeogo M, Rasamiravaka T, Stévigny C, Duez P, Rajaonson S, et al. The flavanone naringenin reduces the production of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Microbiology (Reading)* 2011; 157(Pt 7):2120-32. <https://doi.org/10.1099/mic.0.049338-0>
- [31] Vu B, Chen M, Crawford RJ, Ivanova EP. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* 2009; 14(7):2535-54. <https://doi.org/10.3390/molecules14072535>
- [32] Sánchez MC, Alonso-Español A, Ribeiro-Vidal H, Alonso B, Herrera D, Sanz M. Relevance of biofilm models in periodontal research: from static to dynamic systems. *Microorganisms* 2021; 9(2):428. <https://doi.org/10.3390/microorganisms9020428>
- [33] Marchesan JT, Moss K, Morelli T, Teles FR, Divaris K, Styner M, Ribeiro AA, Webster-Cyriaque J, Beck J. Distinct microbial signatures between periodontal profile classes. *J Dent Res* 2021; 100(12):1405-13. <https://doi.org/10.1177/00220345211009767>