The Potency of Moringa Oleifera on the Biofilm Formation, Adhesion, and Growth of *Streptococcus Mutants* Based on Incubation Times

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Abstract

Evaluating the antibacterial potential of Moringa leaves in preventing biofilm formation, adhesion, and inhibiting the growth of S. mutans.

This study used Moringa leaves as the test material, and S. mutans isolate ATCC 25175 as the subject of analysis. Evaluation of inhibition, biofilm formation, and adhesion using spectrophotometry and changes in S. mutans cells using microscopy.

All concentrations of M oleifera for 24 and 48 hours had the most practical effect on reducing the growth of S. mutans (OD <0.1), comparable to <300 CFU/mL. M oleifera had the adhesion inhibition ability of S. mutans at 24 hours. At 48 hours, 12.5% and 6.25% had potent adhesion inhibition for S. mutans. At 72 hours, all concentrations had strong S. mutans adhesion inhibition categories. At 48 hours of incubation, the M. oleifera strongly inhibited S. mutans biofilm formation, while at 24 hours, only concentrations of 3.125% and at 72 hours, 12.5% and 6.25%.

M. oleifera has antibacterial properties that can reduce growth, inhibit adhesion, and reduce the formation of S. mutans biofilm. The inhibition was influenced by the incubation time and concentration of M. oleifera.

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Introduction

Dental caries, also known as tooth decay or cavities, is a common dental problem that results from the demineralization of tooth enamel by acids produced by bacteria in the mouth. Streptococcus mutans is one of the bacteria commonly associated with dental caries. S. mutans is a type of bacteria that lives in the mouth and produces acid as a byproduct of its metabolism. This acid can erode the tooth enamel and lead to the formation of cavities. *S. mutans* is part of the normal oral microbiota. These bacteria initiate plaque growth on the tooth surface by producing polysaccharide-based dextran mediated by the enzyme dextransucrase. The *S. mutans* use dextran breakdown to maintain growth and adhesion and form biofilms to increase colonization of the tooth enamel surface and structure, thereby increasing dental caries. [1]

Streptococcus mutans produces a glucan-binding protein (Gbp), which is an intermediate for adhesion to host cells. Together with Glucosyl transferase (gtf), GBP cooperates in biofilm formation through specific interactions. This process aims to increase the accumulation of bacteria and form an insoluble polymer matrix known as exopolysaccharides (EPS). Bacteria attached to the biofilm can help promote growth and spread themselves to other areas of infection. [2] This ability occurs because S. mutans can decompose and metabolize carbohydrates into organic acids such as lactic acid and acetic and propionic acid. Organic acids produced as byproducts of metabolism can cause the pH to drop. [3] When the pH drops below a critical pH (4.5-5.5), it can cause a mineral loss on the enamel surface, called demineralization. Demineralization that occurs continuously results in triggering dental caries.[4]

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Chlorhexidine (CHX) in the form of mouthwash is reported as one of the oral antibacterials. Long-term use, besides irritating the oral mucosa, also increases resistance to *S. mutans* cells. This resistance causes an increase in caries infection and harms the host.[5] Using alternative materials such as *M. oleifera* has antibacterial properties and improves the mucosal defense system. This potential is related to the content of the antibacterial compound *M. oleifera*.[6] Belay (2014) reported that M. oleifera contains active compounds in secondary metabolites such as tannins, saponins, terpenoids, phenols, alkaloids, and flavonoids. This compound has antibacterial, anti-inflammatory, and antioxidant properties.[7] In addition, the fatty acids present in *M. oleifera* are reported to act as solid antimicrobials.[8]

Our previous research has found that the ethanol extract of *M. oleifera* has good antibacterial and cytotoxic properties against *E. faecalis* bacterial cells and can inhibit biofilm formation.[9] This study reports the antibacterial potential of *M. oleifera*, which can reduce growth and inhibit adhesion and *S. mutans* biofilm formation based on incubation time and concentration variables.

Materials and methods

This study used *M. oleifera* material as a test material against *S. mutans* isolates ATCC 25175. The antibacterial properties of M. oleifera were examined based on growth inhibition, adhesion, and biofilm formation. This study used incubation time and concentration variables to measure the effectiveness of *M. oleifera* as an antibacterial at the lowest concentration, which still showed good antibacterial properties.

Plant Material

The plant was collected from Aceh, Indonesia (5.603444, 95.405863). The *M. oleifera was* extracted and assembled in the Chemical Laboratory, education Faculty, Universitas Syiah Kuala, Darussalam Banda Aceh, Indonesia, voucher Co2021.

Preparation of Extract of *Moringa oleifera*

Moringa leaves (*Moringa oleifera*) separated from the stalks was collected as much as 1 kg and then washed with water. Drying is carried out for two days up to. Then *M. oleifera*, powder was placed in a flat-bottomed glass container and soaked with 100 mL of 96% ethanol. The residues and filtrates were separated for three days and interspersed with replacing the same solvent. The filtrate was collected and concentrated using a rotary vacuum evaporator at a temperature of 50°C and a pressure of 75 mmHg to obtain the extract. Making Moringa leaf extract gel preparations begins with weighing all the ingredients needed according to the calculations. The next step is to develop the gelling agent, then add preservatives to increase penetration into the gelling agent. Then the *M. oleifera* extract was added to the gel preparation.

Culture and Growth Assay

Bacteria *S. mutans* ATCC 25175 taken from 50% glycerol stock, then refreshed by re-cultured in Tryptic soy broth (TSB) media (Merck KGaA, Darmstadt, Germany) and then equated with Mc. Farlan 0.5 ($1.5x10^8$). Moringa leaf extract with various concentrations was put into 96 well plates with as much as 100 µL, then incubated at room temperature for 30 min. Then, 10 µL of S. mutans bacterial solution equivalent to McFarlan 0.5 was added and homogenized for 15 min at 200 rpm. They were then incubated for 24 h, 48 h, and 72 h. The antibacterial activity of *S. mutans* moringa leaves was measured by spectrophotometry. The growing quantity of S. mutans was read based on narrowness with a spectrophotometry-Elisa Reader (Bio-Rad, USA) with an Optical density (OD) of 620 nm. OD 0.08-0.1 nm, same as Mc. Farland 0.5 ($1.5x10^8$) or equivalent to <300 CFU. [10]

Quantity of Biofilm formation Assay

A total of 25 µL of saliva was coated on 96 well plates for 15 min. Then removed and continued with the addition of 30 µL of *S. mutans* to the bottom of each well plate, which had been coated with saliva and allowed to stand for 15 minutes. Then adding *M. oleifera* extract gel to each well was 100 µL. Then it was adapted to room temperature for 10 min at 300 rpm. The interaction ability of *S. mutant* biofilm formation was assessed after incubation for 24 hours, 48 hours, and 72 h at 37 ^{0}C. Assessment of the inhibition of biofilm formation begins by removing the mixed solution of S. mutans and the leaves of the test material. Than 1% 100 µL SDS solution was added for 15 min and discarded and washed once with PBS. Then 150 µL of 1% glycerol was added to each well plate, washed once with 200

µL PBS. Visualization of the biofilm mass by adding 1% 150 µL crystal violet for 10 minutes, then washing with 76% ethanol (150 µL) and PBS once. Quantitative data reading with ELISA reader at 520 nm.[11]

Adhesion Assay

The interaction activity (adhesion) was analyzed based on the working principle of Gram staining. Meanwhile, the anti-adhesion value was examined using spectrophotometry. The direction of interaction-adhesion is based on incubation time (24 h, 48 h, and 72 hs) on 96-well microplates using crystal violet and safranin staining. Serial triplo 96-well microplates were coated with 50 µL MHB for 15 min and aspirated. Then 50 µL of bacteria was added and incubated for 15 minutes at room temperature, then 100 µl of test material (extract) was added and incubated for 24 hours, 48 h, and 72 h.

Furthermore, all remaining test material in the microplate was aspirated, then left for 10 minutes at room temperature, and 50 µL of 2% crystal violet was added to each, then allowed to stand for 5 minutes and washed with PBS (Phosphate Buffer Saline) 2 times. Then given Lugol's solution for 1 min and washed with PBS. The rest of cell metabolism that is not bacterial cells is dissolved in 96% alcohol for 20 sec until the dye disappears. Then, 50 µL of safranin solution was given, left for 2 min, and washed again with PBS. The anti-adhesion activity of the ethanol extract of Moringa goes against bacteria based on microplates was assessed by the Elisa Spectrophotometry reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 620 nm.[12]

Statistical Analyses

Data on growth, biofilm inhibition, and adhesin were statistically analyzed using One way ANOVA with a significance limit of p<0.005.

Results

Table 1 shows that all gel concentrations of the ethanol extract of Moringa leaves reduced the growth of S. mutans. The incubation time of 24 and 48 hours had the most practical effect on reducing the growth of *S. mutans* which was affected by all gel concentrations of the ethanol extract of Moringa leaves (OD <0.1: <300 CFU/mL). While for 72 hours, only concentrations of 6.25% and 3.125% had a better effect than other concentrations. The positive control (CHX) gave a stable impact at 24 and 48 hours of incubation. Based on the Kruskall-Wallis analysis showed that the growth of *S. mutans* under the influence of Moringa leaf ethanol extract gel had a significant difference (p<0.05; 0.050) based on the incubation time with a moderate correlation (r=0.653), but based on the concentration of each incubation time it did not show a significant difference. Based on the Mc Farland standard, it can be classified as <300 CFU or Mc. Farland 0.5 (1.5x108). Optical Density 0.05 nm (<150 CFU/mL), 0.08-0.1 nm (Mc Farlan 0.5; <300 CFU), OD 0.11-0.29 nm (Mc Farland 1; 300-600 CFU); OD 0.3-0.49 nm (Mc Farland 2; 600-1200 CFU). These scales were adopted by McFarlad Standard for in vitro use only, Catalog No. TM50-TM60, Scott Sutton, Measurement of Microbial Cells by Optical Density, 2011)[10].

Moringa oleifera	*S. mutans* Growth (Turbinity OD 600 nm)											
	24 h (n=18)				48 h (n=18)				72 h (n=18)			
	Mean	SDV	(CFU/mL)	Freq	Mean	SDV	(CFU/mL)	Freq	Mean	SDV	(CFU/mL)	Freq
Cons 25%	0,09	0,091	<300	5%	0,11	0,012	<300	10%	0,15	0,044	300-600	5%
Cons 12.5%	0,11	0,074	<300	6%	0,11	0,009	<300	10%	0,14	0,026	300-600	5%
Cons 6.25%	0,10	0,006	<300	6%	0,12	0,006	<300	11%	0,10	0,005	<300	4%
Cons 3.125%	0,11	0,031	<300	6%	0,11	0,015	<300	10%	0,11	0,006	<300	4%
CHX	0,10	0,053	<300	6%	0,10	0,007	<300	10%	0,14	0,007	300-600	5%
S. mutans	1,21	0.002	≥600	71%	0,51	0.002	600-1200	48%	2,21	0,003	>1200	78%
*P-value	0,0611				0,105				0,0724			
	0,045											

* Kruskal Wallis Test; OD (Optical Density); SDV (Standard Deviation); Freq (Frequency)

Table 1. Distribution and growth frequency of S. mutans after being affected by Moringa leaf ethanol extract gel based on growth time.

Table 2 shows that *M. oleifera* can inhibit *S. mutans* adhesion for 24 hours, 48 hours, and 72 hours. At 24 hours, all concentrations had weak adhesion inhibition ability of *S. mutans*, except positive control (CHX), which was moderate. There were significant differences between the groups (p<0.05:0.041). At 48 hours, 12.5% and 6.25% concentration groups had strong adhesion inhibition of *S. mutans*. Meanwhile, the concentration of 25% and the positive control were moderate. Significant differences existed between the groups (p<0.05;0.047). At 72 hours, all concentrations had potent *S. mutans* adhesion inhibition, including the positive control, where there was no difference in the five groups (p>0.05;0.219). In addition, there was a significant difference in the adhesion inhibition of *S. mutans* between 24 hours, 48 hours, and 72 hours (p<0.05;0.001). The assessment of the degree of anti-adhesion refers to the OD values, namely, Weak (<0.1), Modedare (> 0.1-0.5), and Strong (>0.5).

Moringa oleifera	N	Adhesion of *S. mutans* (OD 550 nm)											
		24 h (n=15)				48 h (n=15)				72 h (n=15)			
		Mean	SD	Freq	Scale	Mean	SD	Freq	Scale	Mean	SD	Freq	Scale
Cons 25%	3	0.098	0.001	19%	Weak	0.107	0.022	14%	Moderate	0.22	0.101	17%	Strong
Cons 12.5%	3	0.096	0.110	19%	Weak	0.199	0.111	27%	Strong	0.29	0.021	23%	Strong
Cons 6.25%	3	0.086	0.101	17%	Weak	0.19	0.001	26%	Strong	0.29	0.001	23%	Strong
Cons 3.125%	3	0.075	0.021	15%	Weak	0.071	0.211	10%	Weak	0.21	0.001	17%	Strong
CHX	3	0.148	0.012	29%	Moderate	0.175	0.011	24%	Moderate	0.26	0.001	20%	Strong
*p-value		0.041				0.047				0.219			
		0.001											

* One Way ANOVA

Table 2. Adesi inhibition of *S. mutans* by *M. oleifera.*

Figure 1 shows S. mutans cells visually using a 200x magnification microscope. *M. oleifera* decreased the growth of *S. mutans* which was marked by changes in the morphology of *S. mutans*. This change was visually shown by several *S. mutans* cells undergoing lysis and cells experiencing coagulation, and *S. mutans* cells experiencing death. The information from this figure quantitatively indicates the role of *M. oleifera* in inhibiting the growth of *S. mutans* (Table 1) and the ability to inhibit *S. mutans* adhesion to the well plate surface (in-vitro) presented in Table 2.

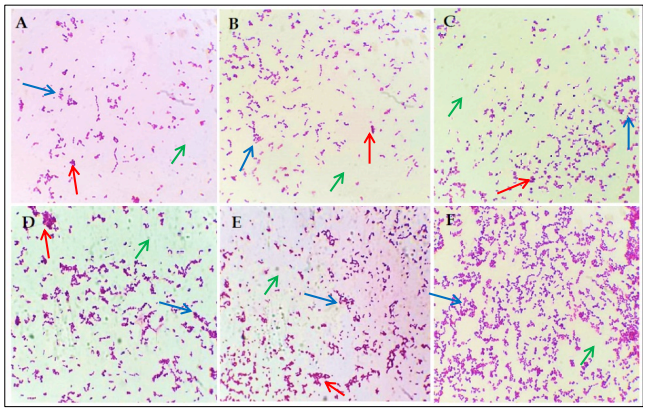

Figure 1. Cell profile of *S. mutans* under the influence of *M. oleifera*. (A) 25%, (B) 12.5%, (C) 6.25%, (D) 3.125%, (E) CHX, and (F) *S. mutans* in BHI medium. Blue arrows (live *S. mutans* cells), red arrows (lyzed S. mutans cells), and green arrows (dead S. mutans cells). Changes in *S. mutans* decreased compared to S. mutans which grew without *M. olaifera* or CHX. Gram stain at 200x magnification.

Figure 2 reported that, in general, the inhibition of *S. mutans* biofilms was in line with the increase in incubation time. The inhibition of *S. mutans* biofilms aligns with the anti-adhesion properties of *M. oleifera* against *S. mutans* (Table 2). Table 3 reports the distribution and frequency of inhibition of *S. mutans* biofilm formation by *M. oleifera*. At 48 hours of incubation, all concentrations had the same ability (strong) to inhibit biofilm formation. This property indicates that *M. oleifera* has the opportunity to prevent the spread of *S. mutans* biofilm mass and can degrade the biofilm mass before maturation occurs. The inhibition rating indicator refers to the Optical Density (OD) value. Weak (OD <0.1), Moderate (01-0.19), Strong (>0.2).

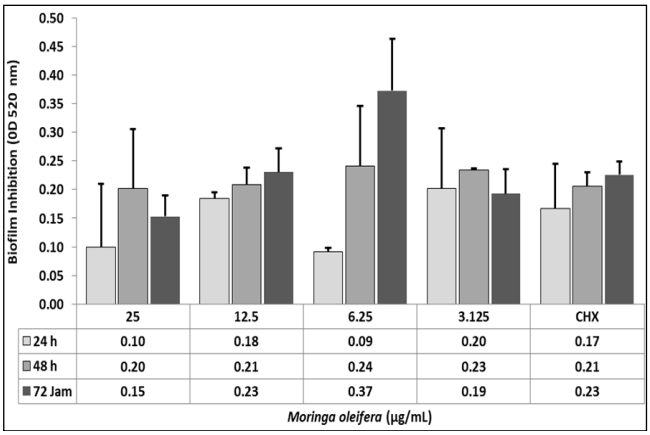

Figure 2. Inhibition of *S. mutans* biofilm. *M. oleifera* can form *S. mutans* biofilms, especially if the incubation time of 48 hours is better than 12 and 48 hours. Bar (inhibition of *S. mutans* biofilm) Bar error (standard deviation).

M. oleifera	N	Biofilm Inhibition of *S. mutans*											
		24 h (n=18)				48 h (n=18)				72 h (n=18)			
		OD	SD	Frek	Skala	OD	SD	Frek	Skala	OD	SD	Frek	Skala
Cons 25%	3	0.10	0.001	13%	Moderate	0.20	0.002	18%	Strong	0.15	0.091	13%	Moderate
Cons 12.5%	3	0.18	0.21	25%	Moderate	0.21	0.01	19%	Strong	0.23	0.011	20%	Strong
Cons 6.25%	3	0.09	0.111	12%	Weak	0.24	0.001	22%	Strong	0.37	0.021	32%	Strong
Cons 3.125%	3	0.20	0.101	27%	Strong	0.23	0.023	21%	Strong	0.19	0.011	16%	Moderate
CHX	3	0.17	0.211	22%	Moderate	0.21	0.011	19%	Strong	0.23	0.01	19%	Strong
*p-value		0.05				0.401				0.021			
		0.001											

* One Way ANOVA

Table 3. Distribution and frequency of inhibition of *S. mutans* biofilms by *M. oleifera*.

Discussion

Moringa oleifera is a plant with several health-promoting properties, including antimicrobial activity. Studies have shown that *M. oleifera* extracts can inhibit the growth of several bacterial species, including Streptococcus mutans, a bacterium associated with developing dental caries. The antimicrobial activity of *M. oleifera* is attributed to several bioactive compounds, such as phenolic acids, flavonoids, and glucosinolates[13]. These compounds have

been shown to have antibacterial properties by disrupting the cell membrane, inhibiting enzymes, and interfering with bacterial metabolism [14]. Raygaert (2018) reported that antimicrobial agents could be divided into several groups based on the mechanism of antimicrobial activity. The main categories have the properties of inhibiting cell wall synthesis, depolarizing cell membranes, inhibiting protein synthesis, inhibiting nucleic acid synthesis, and inhibiting metabolic pathways in bacteria.[15] Based on information from Table 1, Table 2, Table 3, Figure 1, and Figure 2, *M.oleifera* can inhibit growth and biofilm formation and prevent *S. mutans* adhesion.

Table 1 reports that all concentrations of *M. oleifera* tested in this study gave excellent bacteriostatic effects for 24 hours, 48 hours, and 72 hours. This phenomenon indicates that *M. oleifera* can degrade *S. mutans* cell function during growth adaptation. This ability is related to several antibacterial compounds owned by *M. oleifera*. Soraya (2022) reported that *M. oleifera* has antibacterial compounds toxic to *E. faecalis* cells. His research showed that quinic acid was declared the main compound that had a perfect role in causing toxicity to *E. faecalis* cells.[9] Studies have shown that the antibacterial activity of *M. oleifera* extracts against *S. mutans* increases with increasing incubation time [12]. The study found that the antibacterial activity of *M. oleifera* seed extracts against *S. mutans* increased with increasing incubation time, up to 24 hours. Similarly, the incubation time can influence the effect of *M. oleifera* extracts on the virulence of *S. mutans* [16]. One study found that *M. oleifera* leaf extract significantly inhibited the production of extracellular polysaccharides by S. mutans after a 24-hour incubation period. Another study found that *M. oleifera* seed extract significantly reduced the expression of genes associated with virulence factors of *S. mutans* after a 48-hour incubation period [16, 17].

Soraya's research in 2022 is in line with the findings of this study, as presented in Figure 1. M. oleifera can increase damage to *S. mutans* cells, namely, causing severance of septum between cell chains, cell agglutination, failure of division, and cause nucleo-toxicity. This phenomenon shows that *M. oleifera* can act on changes in cell membranes and intra-cell S. mutans. Vazquez-Laslop (2018) reported that the principle of antibacterial action is that it damages the wall or coats the bacteria around it, interfering with the reproduction of bacteria. Blocking the production of proteins in bacteria, such as the release of the GBP protein, plays a role in designating the surface of host cells.[18] Based on this concept, *M. oleifera* qualifies as an antibacterial because it can degrade bacterial cells and work as an antibacterial by reducing growth by damaging the structure/morphological changes of *S. mutans* cells.

One of the virulence properties of *S. mutans* is its ability to adsorb and biofilm formation, which is facilitated by virulence proteins such as Glucan binding protein. This protein facilitates cell attachment to sucrose-dependent host cells and tissues, where this protein tends to attach to dextran/glucan as a product of carbohydrate glycolysis.[19] Table 2 shows that *M. oleifera* can inhibit *S. mutans* adhesion for 24 hours, 48 hours, and 72 hours. At 24 hours, all concentrations had weak adhesion inhibition ability of S. mutans. At 48 hours, the 12.5% and 6.25% concentration groups had strong *S. mutans* adhesion inhibition, and at 72 hours, all concentrations had strong *S. mutans* adhesion inhibition. This adhesion inhibition ability is related to the increased hydrophilic properties of the active compound *M. oleifera*, so several surface proteins from S. mutans cells could not adapt to the surface of the tooth pellicle in in-vivo studies. [20]. In addition, inhibition of bacterial adhesion to tooth surfaces can prevent biofilm formation by S*. mutans* (Table 3). It can be achieved by using agents that can interfere with the ability of the bacteria to attach to tooth surfaces, such as by blocking specific adhesins or surface proteins involved in attachment.

Several studies have investigated the effect of *M. oleifera* extracts on the growth of *S. mutans*. For example, a study published in the Journal of Applied Microbiology in 2016 found that an ethanolic extract of *M. oleifera* leaves effectively inhibited the growth of *S. mutans* [21]. The study showed that the extract had a bacteriostatic effect, which means that it inhibited the development of the bacteria without killing them [22]. Another study published in the Journal of Oral Science in 2017 found that a methanolic extract of *M. oleifera* seeds had an inhibitory effect on the growth and acid production of *S. m*utans. The study showed that the extract had a bactericidal effect, killing the bacteria [23]. Studies

have investigated the cellular response of *M. oleifera* on the virulence of *S. mutans*, a primary cariogenic bacterium in the oral cavity. The findings suggest that *M. oleifera* extracts can modulate the virulence of *S. mutans* by affecting their cellular response [24].

Figure 2 and Table 3 reported that *M. oleifera* could inhibit *S. mutans* biofilm formation, which aligns with the increased incubation time and concentration. At 48 hours of incubation, all concentrations had the same (strong) ability to inhibit biofilm formation. This property indicates that *M. oleifera* has the opportunity to prevent the spread of *S. mutans* biofilm mass and can degrade the biofilm mass before maturation occurs. One study demonstrated that *M. oleifera* leaf extract could inhibit the growth and biofilm formation of *S. mutans*. The extract was found to disrupt the cell membrane integrity of the bacteria, resulting in the leakage of intracellular materials and decreased viability [25]. Another study investigated the effect *of M. oleifera* seed extract on the virulence factors of *S. mutans*. The extract significantly reduced the production of extracellular polysaccharides and adherence of *S. mutans* to the tooth surface. It was also found to inhibit the expression of genes associated with producing virulence factors such as glucosyltransferases and fucosyltransferase [26]. There are several mechanisms by which biofilm formation by *S. mutans* can be inhibited. One mechanism is the disruption of bacterial communication or quorum sensing, which is essential for forming and maintaining biofilms. Quorum sensing involves the production and detection of signaling molecules by bacteria, which enable them to coordinate their behavior in a group. Inhibition of quorum sensing can prevent the formation of biofilms by *S. mutans* [27].

Several research results have described the potential of Moringa oliefera leaves to inhibit the growth, adhesion, and formation of *S. mutans* biofilms. This ability can be a reference for the possibility of *M. oleifera* being used as an active ingredient for mouthwash or irrigation solutions to prevent dental caries and root canal treatment. It requires other strategic research to obtain a good formula for using dental caries reactions triggered by *S. mutans* bacteria.

Conclusions

M. oleifera can be good bacteriostatic at all concentrations at 24 and 48 hours of incubation. *M. oleifera* had the best adhesion inhibition ability of *S. mutans* at 48 hours of incubation at 12.5% and 6.25% concentrations. Meanwhile, at 72 hours, all concentrations had *S. mutans* adhesion inhibition in the strong category. At 48 hours, all concentrations of *M. oleifera* had a strong inhibition of *S. mutans* biofilm formation, while at 24 hours, only 3.125% and at 72 hours, only 12.5% and 6.25%

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Declaration of Interest

The authors report no conflict of interest.

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