RESEARCH ARTICLE

Screening for Antibacterial and Antibiofilm Activity of Some Plant Extract and Chlorhexidine against *Streptococcus mutans*

Hala Hussein[1]*, Abdalnabi J. Abid[2], Zainab H. Alsaadi[1]

[1]*Department of Microbiology, College of Medicine, University of Babylon, Babylon, Iraq.*
[2]*Department of Biology, College of Science for Women, University of Babylon, Babylon, Iraq*

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ABSTRACT

Objectives: Dental caries is an oral illness caused by *Streptococcus mutans* formation biofilms, which playing an important role in the formation of virulent cariogenic biofilms. As a result, the most important biological aim in avoiding dental caries is to reduce the quantity of bacteria in the mouth. Controlling the proliferation of *S. mutans* in the oral cavity is critical for excellent oral hygiene. Additionally, antibiotic abuse and misuse in dentistry concludes in drug resistance among commensal and pathogenic bacteria of the oral cavity, including *S. mutans*. As a result, alternatives to traditional antimicrobials are urgently needed. Plant-based materials are one of the alternate antimicrobials.

Aim: This study test the inhibitory efficacy of certain herbal plant extracts and chlorohexidine on *S. mutans* bacterial growth and biofilm forming abilities.

Materials and Methods: A total of 150 samples were considered from dental caries patients. *S. mutans* was found in 41 of the isolates. These bacterial isolates were identified using normal laboratory procedures, followed by molecular detection utilizing particular primers based on the 16srRNA gene and the 16S-23S ribosomal RNA intergenic spacer gene as a genetic marker for *S. mutans* separation by polymerase chain reaction (PCR). There are only 35 isolates that test positive for these genes.

The antibacterial activity of plant extract and chlorhexidine against *S. mutans* was determined using the agar diffusion method. Tissue culture plate technique (TCP) experiment was used to investigate their activity on biofilm development.

Results: Between150 samples were collected from patients with dental caries 41 isolates were diagnosed as *S. mutans*. Only 35 of these isolates gives positive results for *16srRNA* gene and 16S-23S ribosomal RNA intergenic spacer gene (*MUT* gene). all *S. mutans* isolates were biofilm former, high and moderate biofilm formation mode were account for (65.7%) and (34.2%) respectively while there is no isolates that express non biofilm formation. Regarding to effect of chlorhexidine (CHX) gluconate (0.12%) on biofilm formation, the result of this study demonstrate CHX effectively reduces the biofilm formation to 22% moderate ability, 77% weak and no strong. While ginger and clove aqueous extract at different concentrations also prevents the biofilm formation by *S. mutans* except 3% of both plant extract was no effect on biofilm. Regarding to antibacterial activity, the results found that the CHX (0.12 %) produced the highest inhibition activity against *S. mutans* with inhibition zone range 25 to 40 mm. Regarded aqueous plant extracts the maximum inhibition zone was observed in 50% concentration Clove (28 mm) and Ginger (25 mm).

Conclusion: Our study results showed the molecular technique by using specific primers based on 16srRNA gene and 16S-23S ribosomal RNA intergenic spacer gene as a genetic marker give accurate identification for *S. mutans*. chlorhexidine, Clove and ginger have an effect on *S. mutans* growth and biofilm formation.

Keywords: Chlorhexidine (CHX), MUT gene, polymerase chain reaction (PCR), Tissue culture plate (TCP).

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INTRODUCTION

The mouth cavity is habitat to the greatest amount of biodiversity. Oral mucosa, saliva, denture surfaces, and dental plaque have yielded over 70 species.[1]

Oral streptococci, which account for more than 80% of the mouth microflora, can produce glucosyltransferases, which are enzymes involved in the formation of glucans. Glucans have a function in the creation of a dental plaque

**Author for Correspondence: halahussein430@gmail.com*

biofilm, which plays a key role in the pathogenesis of dental caries.[2]

The structure of the biofilm and the physiological properties of biofilm organisms impart an inherent resistance to antimicrobial agents distinguish biofilm and planktonic cells significantly.[3] Biofilms, particularly the cells that compose the deeper layers of a dense biofilm, are difficult or impossible to eliminate.[4] Several antibiotics, such as penicillins and cephalosporins, as well as erythromycin and metronidazole, are commercially available to treat oral infections.[5] These substances have the potential to change the microbiota in the mouth and cause negative side effects.[6] Other antibacterial drugs, such as chlorhexidine, that are used in the prevention and treatment of oral illnesses, have been employed as methods to prevent and minimize carious lesions.[7] Chlorhexidine works by destroying prokaryotic cell membranes and altering cytoplasmic components. The fast buildup of metal ions inside the cells as they become more permeable causes cell death.[8] Antimicrobial action is also conferred by medicinal herbs against oral microorganisms. Plant extracts can be used to prevent and cure dental caries by inhibiting the formation of oral pathogens, reducing the rate of biofilms and dental plaque, and influencing the adherence of bacteria to surfaces.[9]

Clove (Sygium aromaticum) is a dried, unopened clove tree inflorescence containing 20% essential oil. Clove has been proven to be effective against a wide range of germs, including those related with tooth caries and peri-odontal illness. Syzygium aromaticum also has antifungal, anticarcinogenic, antiallergic, and antimutagenic properties.[10]

The Zingiberaceae family includes ginger (*Zingiber officinale*). Rhizome is the plant portion that is utilized. The gingerols were discovered to be the most active components in fresh ginger rhizomes.[11]

MATERIALS AND METHODS

Diagnosis Bacterial Isolates

One hundred fifty samples were collected from dental caries patients who admitted to Center of Dentistry in Hilla city, during the period from April to October 2017. *S. mutans* were identified as per the standard microbiological and biochemical protocols.[12] Further identification for *S. mutans* was done by molecular detection method using specific primers based on 16srRNA gene as a genetic marker and 16S-23S ribosomal RNA intergenic spacer gene for confirmed isolation *S. mutans* by PCR. From these samples 35 isolates were diagnosed as *S. mutans*.

Molecular Detection of *S. mutans*

Genes amplifications were performed in a thermocycler (Applied Biosystems, USA) and the primers sequence and PCR amplification condition are illustrated in the (Table 1).

S. mutans samples were further diagnosed by using these specific primers.

Preparations of Hot Water Extract (HWE)

Using the basic decoction procedure, hot water extract was made. A 30 gram powdered plant components were placed in a beaker with 300 mL sterile distilled water for this experiment. This was cooked in a water bath until the menstrual fluid was reduced to less than a quarter of its original volume (approx. to 75 mL). To get a dried form of extract, the water content in the extract was entirely evaporated, and the remaining liquid was filtered using Whatman's filter paper no.1.[15]

Antibacterial Assay of Plant Extracts by Agar Well Diffusion Method

The hot water extracts were analyzed for antibacterial activity using agar diffusion on Mueller Hinton Agar. Using a sterile swabs lawn of the test organism was spread onto the Mueller Hinton agar plates. The wells were punctured in the centre by using a sterile cork borer and filled with extract. The plates were incubated at 37ºC for 24 hours. Further the plates were observed for the zone of inhibition. The zones were measured using zone measuring scale.[16]

Detection of Biofilm Formation

Tissue culture plate method (TCP) assay (also called semi quantitative microtiter plate test (biofilm assay) described by Christensen[17] was considered as standard test for detection of biofilm formation.

The Biofilm Inhibition Activity

Tissue culture plate method was done with modification The plant extracts and CHX was added into each well. Then the plate was incubated at 37ºC for 18 hours. Further to that, content of each well was removed by tapping the plates and washed 4 times with phosphate buffer saline (PBS pH 7.2) to

Table 1: Detection primers sequences with their amplicon size (bp) and their PCR condition

Genes	*Primer sequence (5'-3')*	*PCR condition*	*Size (bp)*	*Reference*
16S rRNA	F- TTCGAAGCAACGCGAAGAAC R- AACCCAACATCTCACGACAC	95ºC, 2 min 1x 95ºC, 30 sec 57ºC, 30 sec 30x 72ºC, 20 sec 72ºC, 5 min 1x	132bp	[13]
MUT gene	F-CTCCTTTCTAAGGAAAAACGCA R-TGAACTCCAGACTGACTTATTAGAAAA	94ºC, 5 min 1x 94ºC, 30 sec 58ºC, 40 sec 35x 72ºC, 30 sec 72ºC, 5 min	225bp	[14]

remove free-floating 'planktonic' bacteria. Biofilms formed by adherence in plate were fixed by placing in oven at 37°C for 30 minutes. All wells were stained with crystal violet (0.1% w/v). Excess stain was rinsed off and plates were kept were dried. The optical density (O.D.) at 630 nm.[18]

RESULTS AND DISCUSSION

Forty one isolates identified biochemically were subjected to further molecular detection method using specific primers based on *16srRNA* gene as a genetic marker for confirmed identification of *S. mutans* by PCR. The results revealed that only 35 isolates were positive for PCR as shown in (Figures 1 and 2).

The findings of this study were confirmed by Salman *et al*.,[19] who found that 36 isolates were *S. mutans* based on 16S rDNA and explained that morphotyping was found to be unreliable for species identification, whereas 16S rDNA was found to be highly sensitive for differentiation between *S. mutans* and other Streptococcus when compared to conventional methods.

In general, the 16SrRNA gene was employed for bacterial identification, taxonomy, and phylogeny since it is one of the most widely used housekeeping genetic markers, and for a variety of reasons, including: Their function has remained constant across time, implying that random sequence alterations are a more accurate indicator. operon or multigene family.[20]

To confirm the previous results other specific primers were used, 16S-23S ribosomal RNA intergenic spacer gene (*MUT* gene). This result showed also 35/150 (23%), produced the specific 225bp DNA fragment when compared with allelic ladder; as shown in Figure 2.

The results of this study was accepted with [21] who demonstrated that ribosomal 16S-23S Intergenic Spacer (ITS) region is agood candidate for bacterial identification.

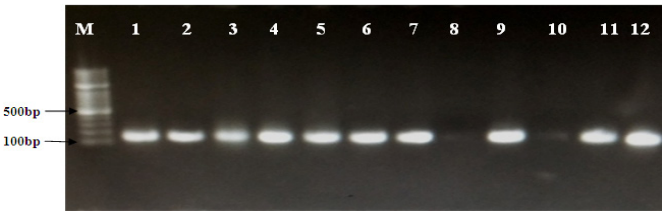


Figure 1: 1%Agarose gel electrophoresis at70 volt for 50 min for *16sRNA*PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane (1,2,3,4,5,6,7,9,11,12) were positive for this gene, the size of product is 132 bp.

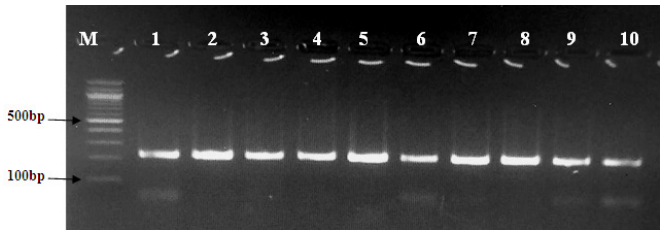


Figure 2:1%Agarose gel electrophoresis at70 volt for 50 min for *MUT gene* PCR products visualized under U.V light at 280 nm after staining with ethidium bromide. L: 1500 bp ladder; lane(1,2,3,4,5,6,7,9,11,12) were positive for this gene, the size of product is 225bp.

Khan *et al,*[22] showed 16S and 23S ribosomal genes may be a better target for accurate identification of bacterai at the species level.

Antibacterial Activity of Chlorhexidine (Mouth Wash) against *S. mutans*

The screening of antimicrobial activity of Chlorhexidine (CHX) gluconate (0.12%) was carried out using the well-agar diffusion test and the results were shown in Figure 3.

Our results showed that the 0.12 % CHX produced high inhibition activity against *S. mutans* with inhibition zone range 25 to 40 mm as show in Figure 3.

The obtained results were in agreement with Neeraj *et al.*[23] who illustrated that CHX) showed significantly largest inhibition zone against *Streptococcus mutans*.

But these results were disagreement with the results obtained[24] who explained 0.12% CHX did not show antibacterial activity.

This high activity related to CHX is a positively charged hydrophobic and lipophilic molecule that interacts with phospholipids and lipopolysaccharides on the bacteria's cell membrane. Further, it enters the cell through some active or passive transport mechanism.[25]

Antibacterial Activity of Aqueous Clove and Ginger Plant Extract Against *S. mutans*

Determination of antimicrobial activity by the agar well diffusion assay is most used to determine antimicrobial susceptibility. Five concentrations of pant extract were used for detection of their inhibitory activity on *S. mutans* isolates. All tested isolates were inhibited by aqueous extracts at 50, 25 and 12.5% concentration. The maximum inhibition zone was observed in 50% concentration clove (28 mm) and ginger (25 mm) aqueous extracts as show in Figure 4, the antibacterial actions of 25% concentration of clove gave lower inhibition zone than 50% concentration of that extract (22 mm) and the minimum inhibition was shown in ginger (20 mm) (Figure 5). While 12.5% gave lower inhibition zone than other concentration 18 mm in clove and 16 mm in ginger. No

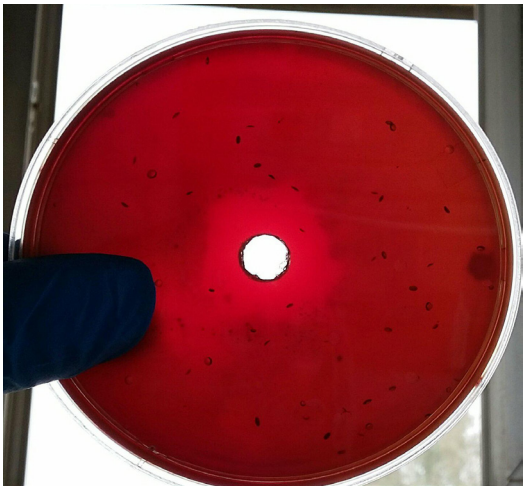


Figure 3: Inhibition zone of *S. mutans* with 0.12% concentrations of CHX

inhibition zone in 6% and 3% concentrations for both plant extract.

The results of this study agreed with a study done.[26] who showed a zone of inhibition (20 ± 0.7 mm). Also, aqueous ginger extract had a significant antibacterial activity against *S. mutans* and produced inhibition zones larger than those produced by most potent antibiotics,

While the present results was disagreement with the results obtained[27] who showed the aqueous extract of ginger gave moderate antibacterial activity against *S. mutans* and no inhibition zone at 50% concentration or less

Z. officinale which is a medical plant used worldwide. The major reason of using ginger is its proven direct anti-microbial activity, anti-inflammatory and antioxidative, that suggested its use in treatment of different bacterial infections.[28]

Regarded clove extract the results in this study was accepted with the results obtained by Abdullah *et al.*[29] who showed the aqueous clove extract has antibacterial activity against *S. mutans* isolates and the inhibition zones were ranged between 23 to 28 mm.

Eugenol, a chemical molecule found in cloves, is known to inhibit bacterial growth and is a natural antibiotic with wide antibacterial properties against gram-positive and gram-negative bacteria. Eugenol is a chemical compound found in cloves and is known to inhibit bacterial growth.[30]

Biofilm Formation of *S. mutans*

The results revealed that all *S. mutans* isolates were biofilm former, high and moderate biofilm formation mode were account for 65.7% and 34.2% respectively while there is no isolates that express non biofilm formation, as shown in Table 2.

Effect of Clove, Ginger Extract and Chlorhexidine on Biofilm Formation

Multiple factors were used in this study to reduce biofilm production, regarding to effect of CHX gluconate (0.12%) on biofilm formation, the result of this study demonstrated CHX effectively reduces the viability of biofilm-forming by *S. mutans*. It reduced the biofilm formation to 22% moderate ability, 77%weak and no strong, as shown in Table 3.

These results accepted with Marsh[31] who showed that the using of high concentrations (0.12% or more) of CHX is bactericidal, causing a lethal damage to the bacterial membrane, being active on both gram-positive and gram-negative bacteria.

Table 2: Production of biofilm in *S. mutans*

	Biofilm formation			
Bacterial isolate No.	*Strong No %*	*Moderate No %*	*Weak No %*	*Total No %*
S. mutans (35)	23(65.7%)	12(34.2%)	0	35(100 %)

Table 3: Effect of CHX (0.12 %) on biofilm formation

	Biofilm formation after adding CHX (0.12 %)			
Bacterial isolate No.	*Strong No %*	*Moderate No %*	*Weak No %*	*Total No %*
S. mutans (35)	0	8 (22.8%)	27(77.1%)	35(100 %)

The treatment with 0.12% CHX prevented the growth of biofilm mass and eliminated a considerable proportion of the biofilm's viable bacteria. It works by causing direct damage to the interior cytoplasmatic membrane and is bacteriostatic at low doses and bactericidal at high doses.[32]

The results showed that using of these extracts at different concentrations have different effect on biofilm formation. From present results in Figures 6 and 7, the biofilm formation affected by using Clove and Ginger at (50, 25, 12.5, 6 and 3%) concentrations, respectively as show in Figures 4 and 5.

For both plant extract in 3% concentration the biofilm formation was not affected.

The results of this study was agreement with with Hossam[33] who find the clove extract showed 78.7 % of biofilim inhibition compared with *S. mutans* with no additions.

Figure 4: Inhibition zone of *S. mutans* at 50% concentrations of aqueous Ginger extract concentration

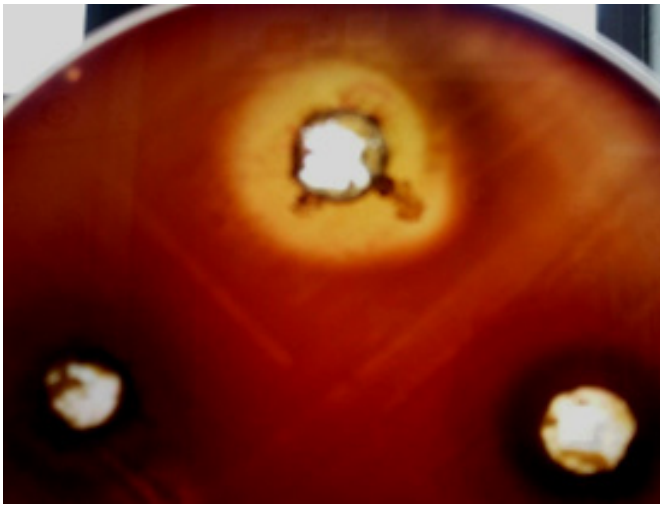
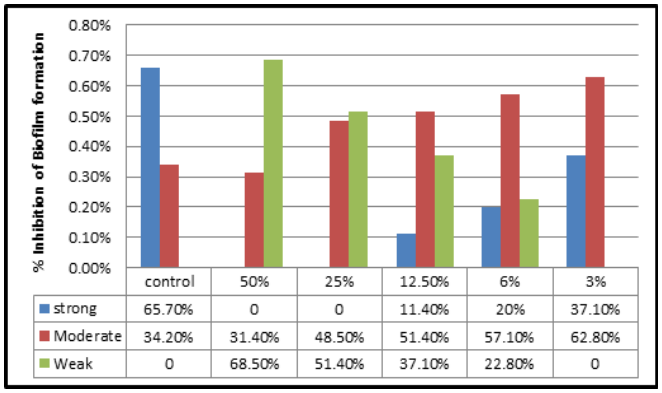
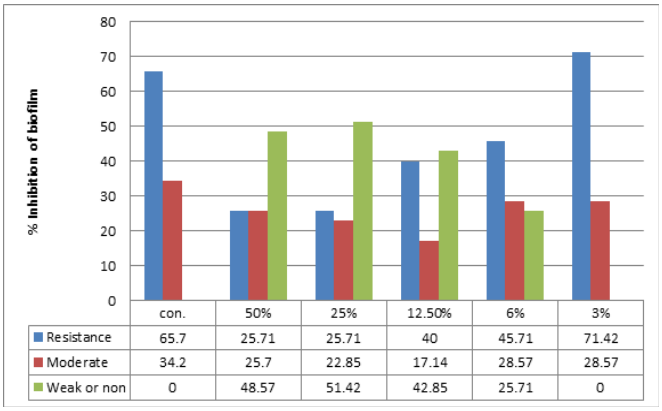


Figure 5: Inhibition zone of *S. mutans* at 25% of aqueous Ginger extract, No inhibition zone at 6% and 3% concentrations



	control	50%	25%	12.50%	6%	3%
strong	65.70%	0	0	11.40%	20%	37.10%
Moderate	34.20%	31.40%	48.50%	51.40%	57.10%	62.80%
Weak	0	68.50%	51.40%	37.10%	22.80%	0

Figure 6: Antibiofilm activity of clove (50, 25, 12.5, 6 and 3%) concentrations



	con.	50%	25%	12.50%	6%	3%
Resistance	65.7	25.71	25.71	40	45.71	71.42
Moderate	34.2	25.7	22.85	17.14	28.57	28.57
Weak or non	0	48.57	51.42	42.85	25.71	0

Figure 7: Antibiofilm activity of ginger (50, 25, 12.5, 6 and 3%) concentrations

Regarded the antibiofilm activity of Ginger the obtained results are accepted with Arash *et al.*[34] how showed that *Z. officinale* exhibit good antibacterial activity against *S. mutants*.

These results agreed with Kothiwale *et al.*[35] who showed that the uses of different type of extract as newly formulated mouth rinse containing clove demonstrates antiplaque properties, possibly useful as an adjunctive to mechanical therapy in the treatment and prevention of oral diseases.

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ETHICAL APPROVAL

This work was approved by the Ethical Committees of the hospital following the recommendations of the Ethical Committee.

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