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# The Antibacterial Activity of Nanosilver Coupled Edible Plant Extracts against *Streptococcus mutans*, the Cause of Dental Caries

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

*Streptococcus mutans* strains were found to have a substantial role in the development of caries. The objective of this study is to evaluate the potential antibacterial activity of some edible plant extracts against *Streptococcus mutans* isolates. Fifty oral dental plaque specimens were obtained from various dental clinics in Jeddah city from 23 males and 27 females with median ages ranging between 19.9 to 51.25 years. All isolates were subjected to microbiological, biochemical, and molecular identification. The results showed that 36.3% of the bacterial isolates were identified as *Streptococcus mutans*, 4.5% as *Streptococcus gallolyticus*, and 4.5% as *Staphylococcus aureus* followed by two Yeast species *Candida albicans* (45.4%) and *Candida glabrata* (9.0%). The PCR products of the size of 600 and 517 bp DNA fragments were successfully amplified from 16S rRNA and glucosyltransferase gene (*gtfb*) from the *S. mutans* isolates and reference strain ATCC25175. The *gtfb* sequences were submitted to NCBI and assigned accessions MW491783, MW491784, MW491785, and MW491786. Six plant extracts (Guava leaves, Olive Leaves, Clove,

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Garlic, Green Ginger, and Cinnamon), Nanosilver 25 nm, and 15% of two commercially available toothpastes (A and B) were evaluated against the *S. mutans* isolates by agar well diffusion test. Most plant extracts tested exhibited considerable inhibitory effects when coupled with Nanosilver 25 nm (1:1). Clove/SNP; 106.8±4.95, and Garlic/SNP; 105.3±3.2) had the greatest mean values of MIC, whereas (Olive/SNP) exhibited the lowest (56.5±3.89). In conclusion, although there are lot of studies on edible plant extracts, incorporating nanoparticles can synergistically increase its antibacterial effectiveness.

*Keywords: Streptococcus mutans; gtfb gene; dental carries; PCR; DNA sequencing; MIC; olive; clove; garlic extracts; nanosilver; and commercialy available toothpastes.*

## 1. INTRODUCTION

Tooth decay is one of the most common oral illnesses, caused by oral bacteria that survive in the habitation of teeth and coexist peacefully with the immune system (Reyes et al., 2012). Dental caries is a bacterial infection of the calcified tissues of the teeth that are characterized by demineralization of the tooth's inorganic component and destruction of the tooth's organic material, with the first permanent molar being the first casualty due to its early involvement. Caries have long been recognized as a disease caused by bacteria fermenting food, generating acids, and destroying tooth material [1].

The human oral cavity houses a rich and diverse microbial community that has been the focus of recent intense research. Ribosomal RNA metabarcoding, whole metagenome shotgun sequencing, and meta-transcriptomics are newly developed sequencing-based approaches. The term “human microbiome” is used to describe the combined genomes of the commensal microorganisms which reside in or on the human body [2,3].

Even if unculturable, oral microbiomes have great diversity and roles. A recent sequencing-based study has charted oral ecosystems and how they change due to lifestyle or disease conditions. As studies progress, the relationship between the oral microbiome and health conditions other than oral diseases is becoming more evident.

New horizons are opened up for microbiome-based diagnostics and therapeutics, which benefit from the use of the oral cavity for microbiome monitoring and manipulation [4]. Multiple diverse microbial phylotypes are found in the oral cavity. The humid, sticky nature of the mouth provides optimal environmental conditions and a reasonable degree of openness for microorganisms. If there is low oxygen and low pH, only organisms that can live in the human mouth can survive. However, only a small number of these organisms engage in oral diseases such as cavities or tooth decay. *Streptococcus mutans*, *Streptococcus gordonii*, and *Streptococcus sanguinis* are the primary bacteria responsible for dental decay, although there are also certain Lactobacillus bacteria (Okada et al., 2005).

*Streptococcus mutans* is important to understand, not only because it is globally distributed, but also because it exhibits a variety of symptoms that influence our daily life. As bacteria grow in the mouth, they can cause tooth decay, delayed speech, chewing trouble, numerous infections, behavioral issues socialization, and concentration difficulties, and so on (Philip et al., 2009). Saudi Arabians suffer from the same oral problems as other populations, although to a lower extent and severity. While the prevalence of caries is minor, it appears to be growing. Periodontal disease and fluorosis of the teeth are prevalent in the general population. Malocclusion is nearly as prevalent as in Western countries. Oral cancer statistics in Saudi Arabia are quite scarce [5].

As a primary etiology of dental caries, *Streptococcus mutans* poses a strong adhesive ability to attach to the tooth surface, which is the decisive initial step in colonization, biofilm formation, and caries development [6,7].

*Streptococcus mutans* bind to the teeth surface by hydrophobic attachments and ferment dietary carbohydrates like sucrose.

Sucrose metabolism results in increased adherence to bacteria to tooth enamel, and acidic compounds produced are crucial in the production of dental caries, periodontal diseases, and gum recession. In addition to the involvement of bacterial communities associated with fermentative metabolism, an increase in

dietary carbohydrates, particularly sucrose (Figueroa et al., 2009), resulting in more acid production that may exceed both the capacity of the saliva to extract acid end-products and the neutralizing power of the salivary/plaque buffer system, causing additional acidification of the biofilm [8].

The role of *Streptococcus mutans* in the initiation of caries was because it is acidogenic and aciduric [9]. *S. mutans* also produce an extracellular layer of polysaccharides in dental biofilms which are the glucosyltransferases products synthesized by *gtfb* and *gtfc* genes [10] (Koo et al., 2010).

Since, the occurrence of *S. mutans* can thrive and produce lactic acid, promoting the formation of dental caries, the study aimed to (1) identify these bacteria and to examine the presence of virulence gene *gtfb* by PCR. Phenotypic, biochemical, and molecular biological methods were used to identify Streptococcus species causing dental caries among adults; and (2) evaluate the antibacterial activity of the edible plant extracts available in Saudi Arabia against *S. mutans* isolates.

## 2. MATERIALS AND METHODS

### 2.1 Clinical Oral Samples Collections

Fifty oral dental plaque (DP) samples were collected with sterile cotton rolls and pure cotton buds from different age and sex groups of patients with dental caries (DC) by the dentists from dental clinics and hospitals in Jeddah city, Saudi Arabia. In each group, samples collected from molar or incisor, upper or lower teeth were not separated. The Control group with free caries (FC) was compared to the outcomes with those subjects noted as (DC). Therefore, the obtained plaques were pooled to give a single sample for each subject and aseptically transferred into a microcentrifuge tube containing 1 ml PBS (pH 7.4). Plaque samples were immediately cold-transported to the laboratory then stored at -80°C until use.

Tryptone Yeast Extract Cystine w/Sucrose (TYCSB) agar media containing bacitracin antibiotic, Blood agar (BA), and Sabouraud dextrose agar (SDA) were inoculated with toothpicks from dental carries samples and incubated under aerobic and anaerobic conditions at CO2 incubator at 37°C for 48 hours. Further, a single bacterial colony from each plate was streaked on Blood Agar (BA) media and Bile esculin slant agar then incubated at 37°C for 48 hours under anaerobic conditions. All the patients did not have any chronic diseases. Patients who had taken antibiotic therapy for the last two weeks were excluded from the study.

### 2.2 Biochemical Analysis of Bacterial Isolates

Vitek®2 (bioMérieux, La Balme Les Grottes, France) was performed to permits both species identification and antibiotic susceptibility testing. It contains the VITEK 2 GP ID and AST Cards that allow species identification and antibiotic susceptibility testing by comparison of the biochemical profile with an extensive database. Strain identification was performed following the manufacturer's instructions. This system is based on kinetic analysis detecting metabolic changes and by additional continuous monitoring of reactions, provides much faster species identifications [11]. A pure single colony was inoculated on TYCSB and incubated anaerobically at 37°C for 24h. Each isolate was picked and suspended in 3ml of sterile saline (0.45% to 0.50% NaCl, pH 4.5 to 7.0) using a sterile polystyrene tube to achieve turbidity equivalent to a 1.8 - 2.2 McFarl and standard according to the manufacturer's recommendations using VITEK2 ID/AST testing instrument. The inoculum was placed into the VITEK®2 Cassette at the SMART CARRIER STATION™, where the VITEK® 2 Card and sample are linked virtually. Once the Cassette is loaded, the incubation and reading of each card are managed by the system without any intervention. All cards used were automatically discarded into a waste container. Based on these readings, the final profile results were compared with the database and the identification of the unknown organism was described according to a specific algorithm.

### 2.3 Antibiotic Susceptibility Test (AST) with Vitek 2

The antibiotic susceptibility test using the AST-ST01 card VITEK 2 system (Vitek® 2 (bioMérieux, La Balme Les Grottes, France) to determine the antibiotic susceptibility of four isolates of *S. mutans* (n = 4) compared with the Clinical and Laboratory Standards Institute (CLSI M100-S25, 2015) reference standards within 24 hours. The 0.5 McFarland bacterial suspension was diluted to 1.5 × 107 CFU/ml in 0.45% saline

(0.45% to 0.50% NaCl, pH 4.5 to 7.0) and loaded into the VITEK 2 instrument for incubation and reading. the AST-ST01 card used for Streptococcus species contained Cefotaxime (CTX), Ceftriaxone (CRO), Cefepime (FEP), Vancomycin (VAN), Levofloxacin (LVX), Ampicillin (AMP), and Penicillin (PCN).

## 2.4 Genomic DNA Isolation and PCR Amplification from Bacterial Isolates

Bacterial genomic DNAs were isolated using QIAamp DNA Mini Kit according to QIAGEN kit handbook. The DNA concentration and purity were measured at 260 and 280 nm using a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific, Massachusetts, US). The absorbance ratio (A260/280) of 1.8 − 2.2 was used for PCR analysis and stored at −20°C until required [12]. The eluted DNAs were kept at -20°C until use. The PCR assay was performed using the thermal cycler (Veriti Thermal Cycler, Applied Biosystems, USA) for 16S ribosomal RNA (rRNA). The forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3') were used.

For gtfb gene amplification, a specific primer set GTFB-F 5'- ACTACACTTTCGGGTGGCTTGG-3' and GTFB-R 5'-CAGTATAAGCGCCAGTTTCATC-3' (Oho et al., 2000) were used. The PCR mixture (10 µl) consisted of 0.2 mM each deoxyribonucleoside triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 2 mM MgCl2, 1 U of Ex Taq DNA polymerase (Qiagen®Taq PCR), a 0.5 µM concentration of each primer, and 10 ng of template DNA. After denaturation at 96°C for 2 min, a total of 25 PCR cycles were performed; each cycle consisted of 15 s of denaturation at 96°C, 30 s of annealing at 61°C, and 1 min of extension at 72°C, followed by final extension cycle at 72°C for 10 min, and a 10°C soak. An aliquot of 2 µL of each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and visualized on a UV transilluminator to confirm the presence of a 517 bp band. Then the PCR product solutions were sent to Macrogen Company, South Korea for purification and sequencing.

## 2.5 DNA Sequencing

The PCR products were purified using a Multiscreen filter plate (Millipore Corp.) The purified PCR products of *gtfb* gene were sequenced using an automated DNA sequencer ABI PRISM 3730XL Analyzer (96 capillaries) using BigDye (R) Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's instructions. Sequences were submitted to GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov). Sequences obtained in this study were compared with the GenBank database using the BLAST software on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). *gtfb* gene sequences were aligned using BLAST program and Phylogenetic trees were constructed after multiple sequence alignments using CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics) to construct a neighbor-joining tree using Jukes-Cantor model [13].

## 2.6 Evaluation of the Antibacterial Activity of Plant Extracts and Commercial Toothpastes

### 2.6.1 Preparation of aqueous plant extracts

Six plants (Guava; leaves) (*Psidium guajava*), (Olive; Leaves) *Olea europaea*, (Clove; fruits) *Syzygium aromaticum*, (Garlic; bulb) *Allium sativum*, (Green Ginger; rhizome) *Zingiber officinale*, (Cinnamon; bark) *Cinnamomum zeylanicum* were purchased from local stores in Jeddah, KSA. The collected plants were re-identified, and the nomenclature was rechecked and confirmed with the help of a plant taxonomist. All raw substances had been washed with clean tap water, then through sterilized distilled water and air-dried. An exception for Ginger rhizomes which had dried in a vacuum oven at 80°C for two days. Five grams of each plant powder were dissolved in 100 mL distilled water in a glass beaker for maceration and then incubated on a rotary shaker for 72 h [14]. Each extract was filtered through filter paper (Whatman No.1) then concentrated by complete evaporation with a rotary evaporator under reduced pressure. The resulting dry extracts were re-weighed, and the percentage of the resultants was calculated from the quantity of the initial plant material (25 g). Crude extracts stock solutions were prepared by mixing dried extracts with an appropriate amount of DMSO (100%) and stored at 4°C in an airtight sterilized dark bottle till use [15]. The filtrated aqueous extracts were evaporated and dried at room temperature. The extracts were stored in air-tight containers at -20˚C until future use. Crude extracts stock solutions were prepared by mixing dried extracts with an appropriate amount of DMSO (100%)

and stored at 4°C in an airtight sterilized dark bottle till use. Two commercially available toothpastes (A and B) were prepared with concentration 15% according to the procedure adopted by Sekar and Zulhilmi Abdullah [16] at four different dilutions, namely 1:4 (25%), 1:1 (50%), 3:4 (75%), and (100%), with sterile distilled water. The two commercial toothpastes were evaluated using the agar well diffusion assay against four *S. mutans* isolates.

### 2.6.2 Agar well diffusion assay

The antibacterial activity of tested plant extracts was evaluated *in vitro* by using agar well diffusion assay using Blood Agar (BA) as selective media. The agar well diffusion assay was accomplished as illustrated by [17]. The inhibition zones were measured in millimeters [18]. The *S. mutans* isolates as well as the reference ATCC 25175 strain was subcultured, and then diluted in a sterile normal saline solution (0.9%) to obtain a colony-forming unit of 1x108 CFU/ml adjusted with the turbidity of 0.5 McFarland. Blood agar plates were applied with 50 µL of each plant extracts and 15% of two commercially available toothpastes (A and B) and Amoxicillin (10 µg). Fifty microliters of six plant extracts (Guava; leaves) (*Psidium guajava*), (Olive; Leaves) *Olea europaea*, (Clove; fruits) *Syzygium aromaticum*, (Garlic; bulb) *Allium sativum*, (Green Ginger; rhizome) *Zingiber officinale*, (Cinnamon; bark) *Cinnamomum zeylanicum* and Nano Silver, were diluted in Dimethyl sulfoxide (100% DMSO) at concentrations 12.5, 25, 50 and 100 µg/ml separately and applied into the wells provided in the agar medium. Then the plates were incubated in CO2 incubator at 37°C for 24, 48, and 72 h. The antibacterial activity was assessed by measuring the diameter of the bacterial inhibition zone in comparison with 10 µg/mL Amoxicillin antibiotic [19]. The inhibition zones were measured as described by Fayaz et al., [20] and compared with those obtained from amoxicillin antibiotics. Statistical analysis was performed using all the assays were performed in triplicate and the results are presented as means ± SD.

### 2.6.3 Broth dilution method

The plant extracts that showed effective antimicrobial activity were combined in 1:1 Nanosilver 25 nm and subjected to demonstrate their minimum inhibitory concentrations (MICs) and Minimum Bactericidal Concentrations (MBC) according to the Clinical and Laboratory Standard Institution strategies by serial two-fold micro broth dilution technique [21]. Mueller Hinton broth (MHB) was prepared and poured into sterile test tubes. One colony of each tested bacterial isolates (*Streptococcus mutans* RHA1, RHA2, RHA3, RHA4, and ATCC 25175 strain were inoculated separately in 2.5 ml MHB and incubated overnight at 37°C on a shaker (250 rpm). 0.5 ml of each overnight culture were inoculated into 5 ml pre-warmed MHB then incubated at 37°C on a shaking incubator for about 18 h to a final optical density (OD600) of 1. A serial dilution was done to different plant extracts in MHB medium to reach concentrations ranging from 100 to 1.563 mg/ml. 100 µl of each prepared bacterial strain was inoculated to the tubes with different concentrations of plant extracts. A tube of MHB supplemented with different plant extracts was left uninoculated and used as a negative control for each dilution. For positive control, 100 µl of both bacterial strains were inoculated to MHB tubes without plant extract. Tubes were incubated at 37°C overnight and the lowest concentration that inhibits the bacterial growth was considered as the MIC value for each of the tested bacteria strains. To determine MBC value, sterilized Muller–Hinton agar (MHA) was poured into a Petri dish and was let to solidify. Samples that showed no obvious bacterial growth were streaked on the surface of the agar separately then incubated for twenty-four hours at 37°C. The lowest concentration which displayed no growth on the MHA plates was recorded as the MBC.

## 2.7 Data Analysis

Statistical analysis was performed in a statistical package for social sciences (SPSS) software (version 20.0). Each sample had three replicates. The mean diameters of the inhibition zones were calculated. A *p*-value less than 0.05 was considered statistically significant.

# 3. RESULTS

## 3.1 Colony Identification of Potential Species Associated with Dental Plaque (DP)

Oral microorganisms were detected within 22 (44%) dental plaque samples and 28 (56%) did not show any microbial growth from both male and female groups. The microbial analyses

revealed that there was (1 - 3 species) isolated from each sample that was available for testing. Two yeast species in 12 (24%), two bacterial species in 5 (8%), and one bacterial species in two (4%) were isolated from 22 DP samples. The control group with free caries (FC) showed no microbial growth. Four bacterial isolates (36.3%) were identified as *Streptococcus mutans*, one was identified as *Streptococcus gallolyticus* (4.5%), and one species was identified as *Staphylococcus aureus* (4.5%) followed by two Yeast species *Candida albicans* (45.4%) and *Candida glabrata* (9.0%) were identified, which *Candida albicans* was the predominant species to be isolated as shown in Table 1 and Fig. 1.

## 3.2 API-20E Test

In the current study, four isolates of *S. mutans* were subjected to biochemical analysis for identification as shown in Table 2. Each colony from all plates was tested by API-20E Test (bioMérieux Inc., Durham, NC). *S. mutans* isolates were positive for ONPG: β-galactosidase, ADH: arginine-dihydrolase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, GLU: glucose, MAN: mannitol, INO: inositol, SOR: sorbitol, RHA: rhamnose, SAC: sucrose, and MEL: melibiose, however it is negative for CIT: citrate utilization, Production of $H_2S$, URE: Urease, TDA: tryptophan deaminase, IND: indole, VP: acetoin, GEL: gelatinase, AMY: amygdalin and ARA: arabinose. It produces glucan from the sucrose *in vitro* which is a major cause of caries.

## 3.3 Antibiotic Susceptibility Test

The bacterial isolates were subjected to antibiotic susceptibility test (AST) by AST-ST01 card (bioMérieux) VITEK 2 using eight different common antibiotic agents including Cefotaxime (CTX), Ceftriaxone (CRO), Cefepime (FEP), Vancomycin (VAN), Levofloxacin (LVX), Ampicillin (AMP), Penicillin (PCN) and Benzylpenicillin (Penicillin G) as shown in Table 3. RHA1, RHA2, and RHA3 *Streptococcus mutans* isolates were sensitive to Cefotaxime, Ceftriaxone, Cefepime, Vancomycin, Levofloxacin and resistant to Ampicillin and Penicillin. While RHA4 isolate was sensitive to Benzylpenicillin, Cefotaxime, Ceftriaxone, Cefepime, Vancomycin, Levofloxacin, and intermediate to Ampicillin.

## 3.4 Molecular Identification of *S. mutans* by PCR Amplification

*S. mutans* isolates were identified using PCR amplification of 16S rRNA region and confirmed by successful amplification of glucosyltransferase gene *(gtfb)* virulence gene. The PCR amplification of DNA extracted from the bacterial isolates was done with the universal 16S rRNA primers & species-specific primers for gtfb gene. The PCR products appeared as intense sharp bands on agarose gel with a molecular weight size of ~600 and 517 bp DNA fragments from all *S. mutans* bacterial isolates (Fig. 2).

## 3.5 DNA Sequencing of *gtfb* Gene

Partial nucleotide sequence analysis of the identified bacterial isolates accessions MW491783, MW491784, MW491785, and MW491786 aligned with other *S. mutans* strains and their sources from NCBI showed 98 to 99% similarities are given in (Table 4). The gtfb partial gene sequence analysis was performed with CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics) to construct a neighbour-joining tree using Jukes-Cantor model (Fig. 3).

**Table 1. The total number of potentially pathogenic microorganisms associated with dental plaque (DP) samples**

| Sample number | Microbial species | Frequency | Percentages (%) |
|---|---|---|---|
| 1 | *Streptococcus mutans* | 8 | 36.3 % |
| 2 | *Streptococcus gallolyticus* | 1 | 4.5 % |
| 3 | *Candida albicans* | 10 | 45.4% |
| 4 | *Candida glabrata* | 2 | 9.0 % |
| 5 | *Staphylococcus aureus* | 1 | 4.5 % |
| Total | | 22 | 100% |

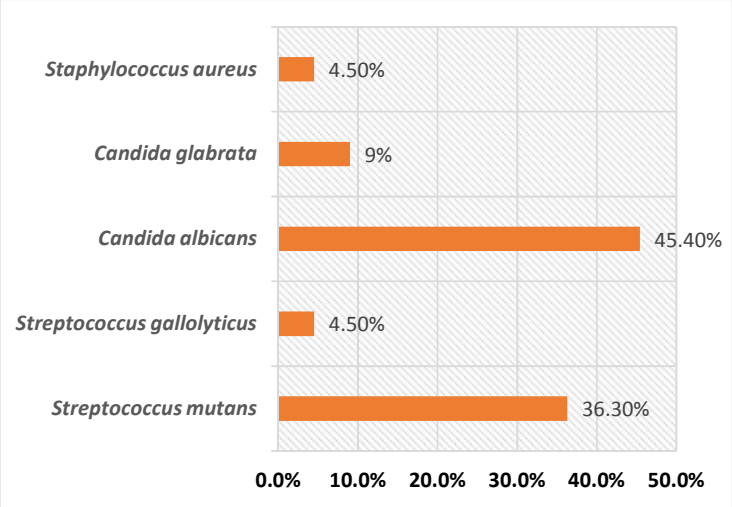


**Fig. 1. The percentage of potentially pathogenic microorganisms associated with dental plaque (DP) samples. The graph shows that the predominant species was *C. albicans* (45.4 %), followed by *S. aureus* (36.30%), *C. glabrata* (9.0 %). The other microbial species were *Streptococcus gallolyticus* and *Staph aureus* (4.5%)**

**Table 2. The biochemical analysis of *S. mutans* by API-20 STREP system**

| Test | Substrate | Reaction |
|---|---|---|
| ONPG | O-nitrophenyl-D-galactopyranoside | +ve |
| ADH | L-Arginine | +ve |
| LDC | L-Lysine | +ve |
| ODC | L-Ornithine | +ve |
| CIT | Trisodium Citrate | -ve |
| $H_2S$ | Sodium thiosulphate | -ve |
| URE | Urea | -ve |
| TDA | L-Tryptophane | -ve |
| IND | L-Tryptophane | -ve |
| VP | Sodium pyruvate | -ve |
| GEL | Gelatin - | ve |
| GLU | D-Glucose | +ve |
| MAN | D-Mannitol | +ve |
| INO | Inositol | +ve |
| SOR | D-Sorbitol | +ve |
| RHA | L-Rhamnose | +ve |
| SAC | D-Sucrose | +ve |
| MEL | D-Melibiose | +ve |
| AMY | Amygdalin | -ve |
| ARA | L-Arabinose | -ve |

**Table 3. Antibiotic susceptibilities of *S. mutans* isolates as determined by the VITEK 2 system**

| Bacterial isolate | CTX | CRO | FEP | VAN | LVX | AMP | PCN | Benzylpenicillin (Penicillin G) |
|---|---|---|---|---|---|---|---|---|
| RHA1 | S | S | S | S | S | R | R | S |
| RHA2 | S | S | S | S | S | R | R | S |
| RHA3 | S | S | S | S | S | R | R | S |
| RHA4 | S | S | S | S | S | I | R | S |

*(S) Susceptible; (R) Resistant; (I) Intermediate*

**Table 4. Information and identification for *gtfb* gene sequences of *S. mutans* RHA1(MW491783.1), RHA2 (MW491784.1), RHA3 (MW491785.1) and RHA4 (MW491786.1)**

| No. | Strain | *gtfb* gene sequences | | | | | |
|---|---|---|---|---|---|---|---|
| | | Identification | Identity (%) | | | | Reference accession Number |
| | | | RHA(MW491783.1) | RHA2(MW491784.1) | RHA3(MW491785.1) | RHA4(MW491786.1) | |
| 1 | UA140 | | 98%<br>436/447 | 99%<br>453/459 | 98%<br>455/464 | 98%<br>443/452 | JX072983.1 |
| 2 | T8 | *S. mutans* | 98 %<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | CP044492.1 |
| 3 | NCH105 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | CP044221.1 |
| 4 | S1 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | CP050271.1 |
| 5 | FDAARGOS_685 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | CP050962.1 |
| 6 | UA96 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | JX072987.1 |
| 7 | NG8 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | CP013237.1 |
| 8 | BM31 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | MG766902.1 |
| 9 | BM35 | *S. mutans* | 98%<br>443/452 | 99%<br>457/459 | 99%<br>459/464 | 98%<br>450/457 | MG766903.1 |

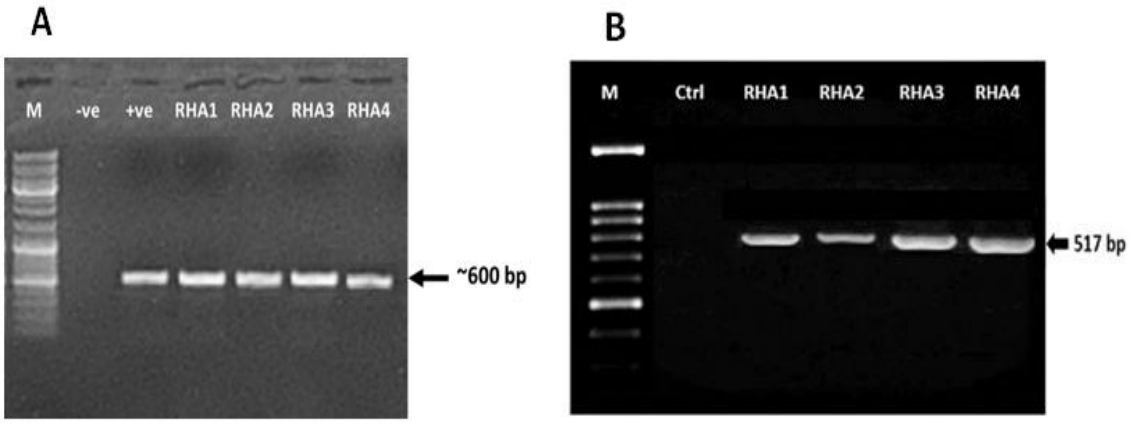


**Fig. 2. (A) 1% agarose gel electrophoresis showing the amplified PCR products of 16S rDNA from S. mutans isolates (RHA1, RHA2, RHA3 and RHA4). M: Thermo Scientific GeneRuler DNA Ladder Mix. Positive control (+ve): ATCC 25175 and Negative control (-ve) are included. (B) PCR amplification of gtfb virulence gene. M: Molecular weight DNA marker. Lane Ctrl: negative control. The size of the PCR products is indicated by arrow**

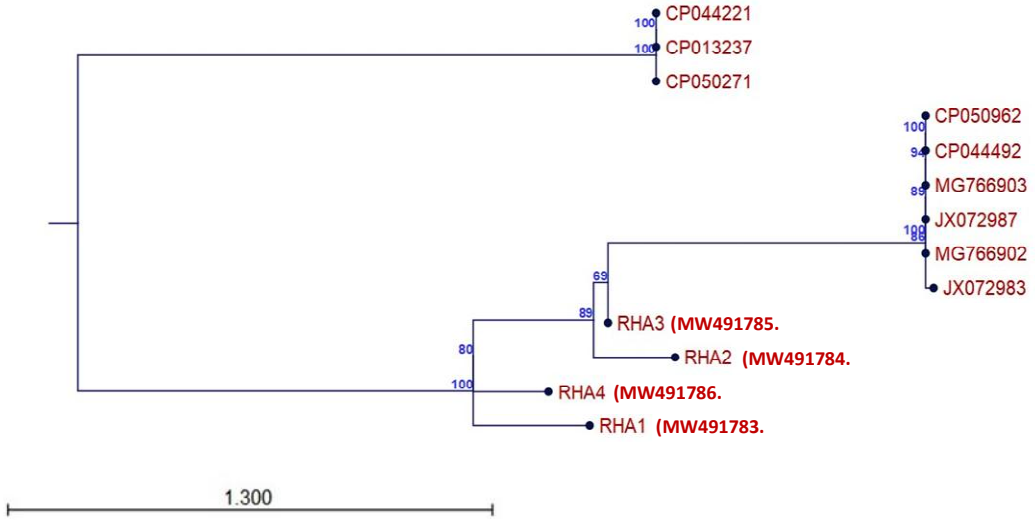


**Fig. 3. Phylogenetic relationships based on the *gtfb* gene sequence alignment of *S. mutans* isolates RHA1 (MW491783.1), RHA2 (MW491784.1), RHA3 (MW491785.1), and RHA4 (MW491786.1) with other *S. mutans* strains. The dendrograms were obtained by neighbour-joining (NJ) using CLC Main Workbench V8.1.3 (Qiagen, Bioinformatics)**

### 3.6 Determination of the inhibition zones of plant extracts combined with NanoSilver

The antibacterial activity of tested plant extracts Guava/SNP, Olive/SNP, Clove/SNP, Garlic/SNP, Green Ginger/SNP, and Cinnamon/SNP extracts combined with NanoSilver (25 nm) (1:1) was examined against the four *S. mutans* isolates and the mean values of inhibition zones was recorded (Figs. 4 and 5 and Table 5). When silver nanoparticles were combined with plant extracts, a significant synergistic effect against dental caries bacteria was observed. The order of highest mean value of inhibition zones of tested plant extracts combined with NanoSilver (25 nm) (1:1) was Clove 27.1±0.1 to 35.2±0.1, followed by Olive 27.3±0.1 to 34.3±0.1, Cinnamon 27.1±0.1 to 31.2±0.08, Green Ginger 26.0±0.1 to 33.1±0.08, and finally, Garlic 25.1±0.04 to 35.0±0.04.

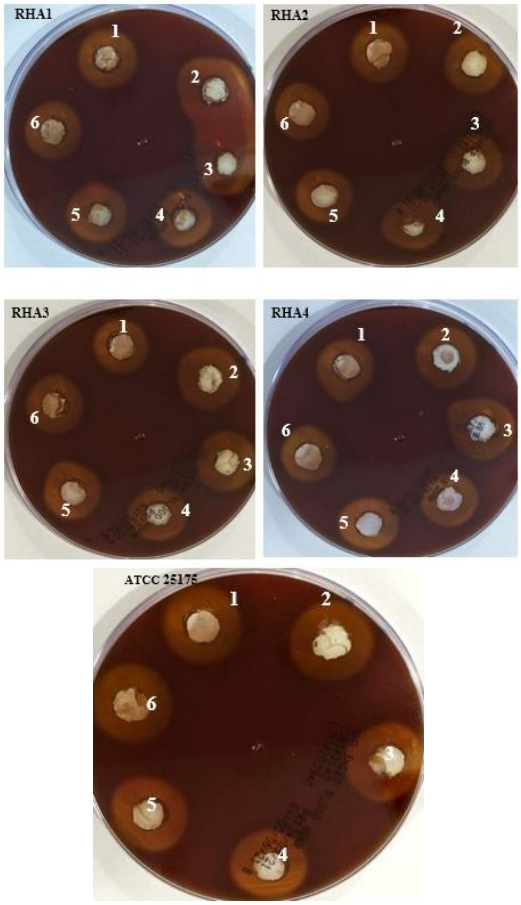


**Fig. 4. Assessment of antibacterial activity by agar well diffusion test against four *S. mutans* isolates RHA1 (MW491783.1), RHA2 (MW491784.1), RHA3 (MW491785.1), and RHA4 (MW491786.1) and the reference strain ATTC 25175. 100 µg/ml of combined plant extract with Nanosilver (25 nm) 1:1 against *S. mutans* isolates and reference control ATCC 25175 at concentration 100 µg/ml. (1) Guava/SNP, (2) Olive/SNP, (3) Clove/SNP, (4) Garlic/SNP, (5) Green Ginger/SNP, and (6) Cinnamon/SNP**

**Table 5. The diameter of zones of inhibition (mm) of combined plant extract with Nanosilver 25 nm 1:1 against *S. mutans* isolates and reference control ATCC 25175 at concentration 100 µg/ml**

| | Plant extract | RHA1 | RHA2 | RHA3 | RHA4 | ATCC 25175 |
|---|---|---|---|---|---|---|
| 1 | Guava/ SNP (1:1) | 28.6±0.4 | 25.4±0.2 | 27.4±0.2 | 25.3±0.1 | 33.3±0.1 |
| 2 | Olive/ SNP (1:1) | 30.3±0.1 | 27.3±0.1 | 30.3±0.1 | 29.5±0.08 | 34.3±0.1 |
| 3 | Clove/ SNP (1:1) | 31.3±0.0 | 27.1±0.1 | 32.2±0.1 | 31.3±0.04 | 35.2±0.1 |
| 4 | Garlic/SNP (1:1) | 29.3±0.1 | 27.1±0.09 | 25.1±0.04 | 28.0±0.04 | 35.0±0.04 |
| 5 | Green Ginger/ SNP (1:1) | 28.2±0.08 | 26.0±0.04 | 26.0±0.1 | 27.1±0.08 | 33.1±0.08 |
| 6 | Cinnamon/ SNP (1:1) | 28.0±0.04 | 27±0.04 | 28±0.04 | 27.1±0.1 | 31.2±0.08 |

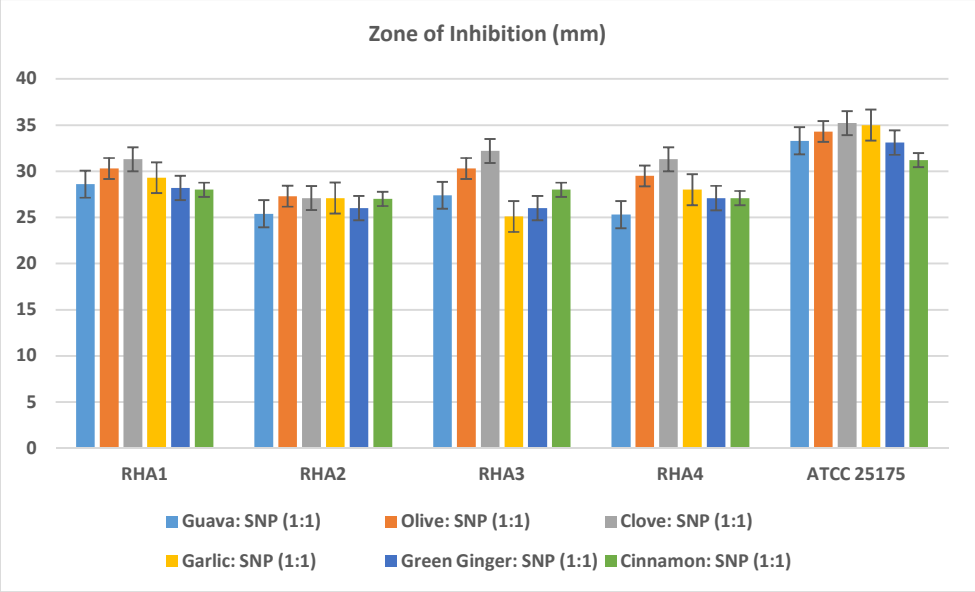


**Fig. 5. Bar chart representing comparison in the mean value of inhibition zones with the different plant extracts combined with Nanosilver 25 nm against *Streptococcus mutans* isolates**

### 3.7 Determination of the Minimum Inhibitory Concentrations (MICs) of Plant Extracts Combined with Nanosilver

MIC was carried to analyze the antimicrobial activity of olive, clove, and garlic extracts combined with Nanosilver 25 nm (1:1) against the four isolates of *Streptococcus mutans* by broth microdilution method after confirmation of their antimicrobial activity through agar well diffusion assay. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the growth of a microorganism. After 24 h of incubation, no growth of any of the tested bacterial isolates was seen in the microtitre plate supplemented with different concentrations ranged between 0.032 to 128 mg/ml. The MIC of the most effective plant extract combined with silver nanoparticles against each bacterial isolate was shown in (Table 6). No statistically significant difference was found between Clove, Garlic, and Olive in MIC with *Streptococcus mutans* (RHA1) where ($p$ = 0.97) and ATCC 25175 strain where ($p$ =0.5636). However, a statistically significant difference ($p < 0.001$) was found in the MIC values for *S. mutans* isolates RHA2, RHA3, and RHA4 where $p$ values were 0.0359, 0.0458, and 0.0046 respectively. Where the highest mean value of MIC for positive inhibitory effects against *Streptococcus mutans* isolates for Clove/SNP was ranged between (50.53±4.22 to 92.4±0.41); Garlic/SNP; 52.4±7.72 to 103.5±5.90) while Olive/SNP showed the lowest mean values (55.80±6.35 to 106.8 ±4.95) (Fig. 6). The MIC results suggest that Nanosilver in combination with each plant extract has a very high antibacterial activity against *S. mutans* isolates and the reference ATCC 25175 strain.

**Table 6. MIC results of Clove, Garlic and Olive extracts combined with Nanosilver (25 nm)**

| Variables | MIC (mg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RHA1 | | RHA2 | | RHA3 | | RHA4 | | ATCC 25175 | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Clove/SNP | 56.5 | 3.89 | 83.63 | 4.0 | 90.2 | 7.70 | 92.4 | 0.41 | 50.53 | 4.22 |
| Garlic/SNP | 52.4 | 7.72 | 91.99 | 3.47 | 103.5 | 5.90 | 95.4 | 4.6 | 53.7 | 6.48 |
| Olive/SNP | 57.2 | 3.80 | 95.44 | 5.10 | 106.8 | 4.95 | 105.3 | 3.2 | 55.80 | 6.35 |
| *P*-value | $p$=0.97 ns | | $p$= 0.0359* | | $p$= 0.0458* | | $p$=0.0046* | | $p$= 0.5636 ns | |

**Significant (P≤0.05) ns; nonsignificant (P>0.05).*

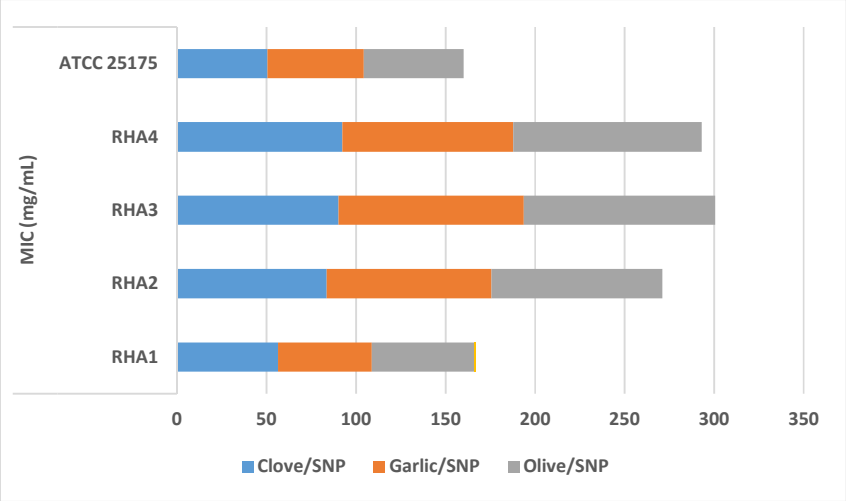


**Fig. 6. A stacked bar chart showing the means of the MIC values for Clove/SNP, Garlic/SNP, and Olive/SNP against four S. mutans isolates (RHA1, RHA2, RHA3, and RHA4) and the reference strain ATCC 25175. The lower the mean value of the MIC, the higher the antibacterial activity. Clove/SNP, Garlic/SNP, and Olive/SNP showed the highest antibacterial activity against S. mutans RHA1 isolate and ATCC 25175 strain**

## 4. DISCUSSION

Oral microbiota taxonomic composition and metabolic activity vary substantially across individuals and may also vary considerably between different sites in the mouth. Not only does the microbiota's composition vary, but environmental factors such as pH, salivary flow, and food intake are also difficult to control [22]. Dental caries is one of the major causes of the destruction of mineralized tissue of the teeth. *Streptococcus mutans*, Lactobacillus acidophilus, and Candida albicans are dominant microorganisms in the lesion of advanced caries. *S. mutans* is the organism causing the initiation of caries, whereas L. acidophilus causes the progression of dental caries [23,24,25]. Thus, the existence of S. mutans in dental structure is a signal of a cariogenic biofilm, and any chemical substance which can use to decrease these bacterial levels can offer additional means of stopping dental caries [26].

Like many other aspects of human health, oral health differs between men and women, especially at specific life stages. Since many stages of the female life are characterized by vast changes in reproductive hormones, there are corresponding changes that occur throughout the body, including the oral cavity. The sex and age-specific changes and risk factors associated with oral health are often overlooked by health care professionals and the general population [27]. Although many studies have demonstrated that caries rates are higher in women than in men [28], our investigations found that the mean age between males and females was statistically non-significant where $p>0.05$. The increased prevalence of caries in females versus males can be explained due to the sex chromosomes, exhibiting sex-linked modes of inheritance. Genes present on the X or Y chromosome can contribute to the development of caries. Variations in sex-linked genes would alter the host's oral environment and the host's response to the initiation of caries [29]. Another study was consistent with our finding, which reported that there was no significant difference between males and females in periodontal problems except the calculus scores [30].

The purpose of this study was to study the association between the *S. mutans* isolated from fifty dental plaque samples and dental caries (DC), as well as the efficacy of six plant extracts against cariogenic bacteria and their beneficial role in dental caries prevention.

In the present study, three genera (Streptococcus, Staphylococcus, and Candida); and 5 microbial species were identified from 22 DP specimens. The microbial isolates were present in 44% of DP while there were 56% of DP did not show any microbial growth.

We concluded that the presence of gram-positive facultative anaerobic bacteria *Streptococcus mutans*, *Streptococcus gallolyticus*; and *Staphylococcus aureus*, and two Candida species: *Candida albicans*, and *Candida glabrata* isolated from dental plaques (DP) samples did not show any significant difference in terms of numbers within males and females patient groups ($p$>0.05) and were more likely to be significantly associated with dental caries. Additionally, the composition of all bacterial isolates obtained from DP samples was nearly similar. These results support observations reported in a previous study where the microbial complexes found in dental plaques were similar [31,32]. Several studies on oral microbial diversity have been identified in the plaque biofilm of infants and their mothers [33]. Pathogenic microorganisms, not limited to *Streptococcus mutans*, have long been identified as an important and indispensable role in the development of dental caries [34]. High-throughput sequencing studies on early childhood caries (ECC) comparing microbial diversity at caries and caries-free sites have resulted in the proposal of several microorganisms related to ECC [33]. Ling et al., [35] indicated that Streptococcus, Veillonella, Actinomyces, Granulicatella, Leptotrichia, and Thiomona were all related to ECC. Kanasi et al., [36] suggested that 139 types of different microorganisms were associated with ECC. Jiang et al., (2013) believed that three genera, Streptococcus, Granulicatella, and Actinomyces, were related to ECC. Previous studies on caries microorganisms using sequencing technology tended to conclude that some microorganisms may be related to the occurrence of dental caries (Asa et al., 2008), or that microbial diversity at caries sites is less than that at healthy sites Jiang et al., (2013).

Only four isolates represented a percentage of about (20.0%) out of 22 DP samples were identified as *S. mutans* serotype (e) based on biochemical and molecular identification. *Streptococcus mutans* was first isolated using selective enrichment technique including culturing of cells on TYCSB agar, Blood Agar (BA), and Bile esculin slant agar, which promotes the growth of *Streptococcus mutans* and suppress other bacterial species Gold et al., [37]; Hamada et al., [38]; Momeni et al., [39].

For further identifications of the *S. mutans* isolates for acid production since acidogenesis is the most important virulence factor for dental caries. The results showed that the pH value varied between 5.5 and 6.0 which indicates that the bacterial isolates could grow on an acidic medium. These results were in agreement with that obtained by Lemos et al., [40].

In order to confirm the biochemical tests and their results that had been done and recorded for bacterial isolates., identification was made finally by API-20 STREP system according to the method that was described by the manufacturing company (API system S. A., Montalieu-Vercien, France). Our biochemical results were in agreement with other studies reported by Teng et al., [41] who identified 71 strains of viridans streptococci isolated from blood by the API 20 STREP system. All strains of *S. mitis*, *S. mutans*, *S. salivarius* and *S. anginosus-constellatus* were correctly identified.

The *streptococcus mutans* isolates identified as RHA1, RHA2, RHA3, and RHA4 were subjected to an antibiotic susceptibility test (AST) by VITEK 2 using eight different common antibiotics. The results revealed that three isolates (RHA1, RHA2, and RHA3) were sensitive to Cefotaxime, Ceftriaxone, Cefepime, Vancomycin, Levofloxacin and resistant to Ampicillin and Penicillin. While only one isolate (RHA4) was sensitive to Benzylpenicillin, Cefotaxime, Ceftriaxone, Cefepime, Vancomycin, Levofloxacin, and intermediate to Ampicillin. The accuracy of AST was excellent with S. mutans isolates, and the antimicrobial agreement rate was comparable or superior to that of other studies [42,43,44].

Although Candida's role in dental caries has been studied extensively, limited homogenous studies have been conducted and none have been found, that associate Candida with dental caries [45]. A recent study reported that Candida albicans promotes tooth decay by inducing oral microbial dysbiosis [46]. Our results showed that two Yeast species *Candida albicans* (45.4%) and *Candida glabrata* (9.0%) were identified in the DC samples collected in the current study, which Candida albicans was the predominant species. In vitro studies showed that *C. albicans* colonization increased the cariogenicity of oral biofilm by altering its microbial ecology, leading to a polymicrobial biofilm with enhanced acidogenicity, and consequently exacerbated tooth demineralization and carious lesion severity [46]. As stated, the origin of dental caries is multifactorial with acidogenic bacterial biofilm an absolute requirement for bacterial acid

generation. Many investigators have shown the association between Candida species and dental caries [47-52]. In addition, it has been reported that the level of oral Candida species could be useful as an indicator of microbial risk in dental caries [53,54].

To ensure suitable differentiation and confirmation of the isolates to the species level, we used the advanced molecular technique based on amplifying the 16S rRNA segment and DNA sequencing of Glucosyltransferases (*gtfb*) gene.

The use of 16S rDNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. It can be used for reproducible and reliable bacterial identification, and also to define the species or at least provide a medically useful distinction between the bacterial strains. Moreover, 16S rDNA is the target of multiple antimicrobials. Several researchers reported 16S rRNA ribotyping of *Streptococcus mutans* from dental caries patients based on this technique to confirm species identification [55,56].

The molecular identification for the *S. mutans* isolates revealed that the identification of microbial isolates using the morphological features and the biochemical tests were correct, thus yielding the correct identification for all isolates tested. For instance, consistent with our data which reported a molecular size of the 16S rRNA region nearly 600 bp for all bacterial isolates were mentioned by [57,58].

Glucosyltransferases (*Gtfs*) genes found in *S. mutans* are the main enzymes involved in glucan synthesis, which cause biofilm formation. These are encoded by *GtfB*, *GtfC*, and *GtfD* genes, respectively. *GtfB* tends to synthesize a majority of the water-insoluble glucans (WIG), *GtfC* synthesizes both soluble and insoluble glucans, and *GtfD* is primarily for soluble glucans [59,60].

In the current study, DNA-PCR has successfully amplified the *GtfB* virulence gene from the isolated *S. mutans* (RHA1, RHA2, RHA3, and RHA4). The PCR findings of the four *Streptococcus mutans* isolates supported the detection of the serotype (e) and were compatible with the results obtained by [61,62]. The nucleotide sequencing of the four isolates was deposited in the GenBank, NCBI under the accession numbers (MW491783.1; RHA1), (MW491784.1; RHA2), (MW491785.1; RHA3) and (MW491786.1; RHA4).

*S. mutans* strains isolated from DP samples harbour two genes encoding enzymes capable of synthesizing water-insoluble glucans (WIG), *GtfB*, and *GtfC*. There is a strong homology between these two genes and they might be tandemly arranged in the bacterial chromosome, which indicates that they might originate from gene duplication. A chimeric *GtfB* gene thought to have arisen from recombination between *GtfB* and *Gtf*C genes, which has been detected in UA101strain [63,64].

In the current study, the partial nucleotide sequencing analysis indicated that only one open reading frame (ORF) be identified in the *GtfB* gene and sharing homology 98 to 99% with the *Gtfb* genes of other *S. mutans* in GenBank, NCBI.

The phylogenetic tree would suggest that the *GtfB* gene showed high similarity with other accession numbers in NCBI. The phylogenetic tree was constructed using neighbour-joining (NJ) using CLC Main Workbench V8.1.3 method.

In recent years, researchers gave attention to the use of plant extracts against cariogenic bacteria regarding their effect on growth. For this reason, the present study selected six plants (Guava, Olive, Clove, Garlic, Green Ginger, and Cinnamon) [65,66], which are known for their medical applications to evaluate their effect on S. mutans isolates.

Sensitivities of *S. mutans* to these aqueous plant extracts in comparison with 15% of two commercially available toothpastes (A and B), and Nanosilver (25 nm) were tested using agar well diffusion technique. The findings of our investigation indicate that three extracts, Clove Garlic and Olive, have a high level of antibacterial activity against *S. mutans* isolates. While none of the other plant extracts shown substantial antibacterial action when administered alone. When Nanosilver (25 nm) was coupled with each plant extracts, extremely potent antibacterial activity was observed. Similar results were obtained by Cinthura and Rajasekar, [67]; Ipe Deepak et al., [68] who stated that Cinnamon-Clove mediated Silver nanoparticles have good antibacterial activity (10 mm-zone of inhibition) in their highest volume and synergistically increases bacterial susceptibility to antibiotics, this property was

higher than a routinely used antibiotic (Amoxicillin-8 mm).

According to literature data on the effectiveness of plant extracts the results are inconsistent probably because of differences in extract preparation methods [69], therefore we assumed that the solvent used for the experiment could influence the result.

In this study, we used water extract for each tested plant as it could allow releasing of active ingredients from tested plant extracts causing their antibacterial efficiency against S. mutans. The previous investigation could be supported by Jain *et* al., [70] who evaluated the antibacterial activity of six Indian plant extracts against *Streptococcus mutans*. Antimicrobial activity of the aqueous, organic solvent, and crude extracts was determined by measuring the mean zones of inhibition (mm) produced against the bacterial isolates. The results showed that aqueous extract of amla and organic solvent-based extract of ginger showed the maximum antibacterial activity against *S. mutans*.

The current study suggests that aqueous plant extracts of clove, olive, and garlic combined with 25 nm NanoSilver (1:1) have potential for the control of *S. mutans*. These extracts can be used as an alternative remedy for dental caries prevention or in the form of mouthwash, which is safe and economical.

According, to our study results, *S. mutans* isolates were found to be sensitive to Clove/SNP, with MIC ranged between (50.53±4.22 to 92.4±0.41 mg/ml) followed by Garlic/SNP (52.4±7.72 to 103.5±5.90 mg/ml), and finally, Olive/SNP (55.80±6.35 to 106.8 ±4.95 mg/ml) where ($p$>0.05). While the same extracts showed highly variable antibacterial activity against the other S. mutans isolates (RHA2, RHA3, and RHA4) where p values were 0.0359, 0.0458, and 0.0046 respectively.

The high antibacterial activity of the Clove aqueous extract is mainly attributed to its secondary metabolites. Shoji et al., [71] presented that Clove's antimicrobial properties are mainly related to its flavonoids and saponins.

In our experiment, we noticed that the 1:1 ratio of silver nanoparticles (25 nm) to clove was the most active ratio as compared to the other ratios (1:2) and (2:1). This latter result appeared to reflect an increase in the antibacterial activity of the Clove extract when the same amount of silver nanoparticles (SNP) was added, which could be explained by the synergistic effect of the silver nanoparticles and the presence of active phytochemical components such as sugars, terpenoids, polyphenols, alkaloids, phenolic acids, and proteins, which mostly play a critical role in the reduction of silver ions into nanoparticles and in their subsequent stability [72]. Furthermore, a phytochemical coating of silver nanoparticles could make the nanoparticles extra stable to preserve particles at the nano-scale range [73].

Anywise, the synergistic effect between SNP and Clove extract enhanced the antimicrobial activity of Clove extract, and this could be explained by the low MIC mean value (50.53±4.22). Although both commercial toothpastes displayed significant antibacterial activity, the findings of this investigation suggested that incorporating clove extract in combination with SNP (25 nm) resulted in increased antibacterial activity.

In the current study, garlic/SNP was used to evaluate its antibacterial activity against *Streptococcus mutans* isolates. The results showed that the synergism of silver nanoparticles and garlic extract on *S. mutans* isolates (RHA1, RHA2, RHA3, and RHA4) yielded larger-sized inhibition zones than garlic extract alone ($P < 0.01$) and the MIC ranged between 52.4±7.72 to 103.5±5.90 mg/ml. Garlic has been used for centuries worldwide by various societies to combat infectious diseases; it is also effective against antibiotic-resistant organisms. Garlic extract has antimicrobial activity against oral bacterial species, particularly gram-negative species. Allicin (allyl 2-propenethiosulfinate or diallyl thiosulfinate) is the principal bioactive compound present in the aqueous extract of garlic or raw garlic homogenate. When garlic is chopped or crushed, allinase enzyme is activated and produces allicin from alliin (present in intact garlic). The antibacterial activity of garlic is widely attributed to allicin. It is known that allicin has sulfhydryl modifying activity and is capable of inhibiting sulfhydryl enzymes. it is thought that its main mechanism of antimicrobial action is through interaction with thiol-containing enzymes, including cysteine proteases and alcohol dehydrogenases. Because these enzymes tend to be essential for bacterial nutrition and metabolism, it has been suggested that the development of resistance to allicin arises 1000-fold less easily than it does to certain

antibiotics. Garlic juice was more effective against oral pathogens when compared with Chlorhexidine mouthwash and can be recommended as a new type of mouthwash. This is following the study conducted by Mansour et al., [74] who reported the efficacy of garlic juice was higher than Chlorhexidine against tested organisms. Fatemeh et al., [75] compared alcoholic extracts of eucalyptus and garlic against cariogenic pathogens i.e., *Streptococcus mutans* and Lactobacillus acidophilus, and showed that both bacteria, were resistant to eucalyptus extract but sensitive to garlic extract and the inhibition zone diameter increased as the concentration of garlic extract increased.

Due to the antibacterial property of olive leaf extracts (*Olea europaea*), we evaluated the antibacterial effects of olive leaf (aqueous extracts) on *Streptococcus mutans* isolates. The MICs of olive leaf extracts on *S. mutans* isolates was ranged between (55.80±6.35 to 106.8 ±4.95 mg/ml). In addition, the inhibition zone of *S. mutans* (30.3±0.1; 27.3±0.1; 30.3±0.1; 34.3±0.1; 29.5±0.08 and 34.3±0.1) significantly increased when combined to silver nanoparticles (1:1). In general, olive leaf extracts induce growth inhibition of S. mutans. Consequently, it can be used as chewing gum, chocolate, and toothpastes to prevent dental caries. Golestannejad et al., [76] observed that alcoholic extraction of *Olea europaea* inhibited the growth of *S. mutans*, so gradually prevents damage to tooth enamel and dental caries [77,78,79]. The polyphenolic compounds of olive leaf extracts can interact with proteins, enzymes, and membranes of microbes, and thus changes in cell permeability, and loss of protons, ions, and macromolecules, which can lead to inhibition of bacterial adhesion [80].

## 5. CONCLUSIONS

In conclusion, the results suggest that the identification methods employed here are useful for the identification of *Streptococcus mutans* isolated from decayed teeth. And also indicate that PCR analysis is suitable for simple, rapid and reliable identification of *S. mutans* using species-specific primers. *GtfB* DNA sequencing was highly sensitive for the identification of *S. mutans* compared to the conventional methods. Serotype "e" was found to be the predominant among the study subjects. Regarding the antibacterial activity of tested plant extracts, it was suggested in the accompanying conclusions that SNP coupled with Clove (*Syzygium aromaticum*), Garlic (*Allium sativum*), and Olive (*Olea europaea L.*) aqueous extracts would be valuable compounds for the manufacture of antibacterial agents against *S. mutans*, and this property was proven to be similar to an antibiotic. Although they were almost as effective as commercial toothpastes, they may have a significant role in dental toothpastes for the prevention of dental caries. Thus, the study results may establish a scientific basis for the traditional use of clove, garlic, and olive against dental caries, contribute to the improvement of oral health, and reduce the adverse effects and costs associated with allopathic medical treatment. Additional clinical trials, however, appear essential to determine their safety and effectiveness.

## CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline patients consent and ethical approval has been collected and preserved by the authors.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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