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A comparison of the Effects of Lemon Peel -Silver Nanoparticles Versus Brand Toothpastes and Mouthwashes on *Staphylococcus* Spp. Isolated From Teeth Caries

Mais E. Ahmed, Khadija Salama*

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

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Abstract

Thirty one samples of gum swabs were collected from patients with tooth caries (5-30 years old) from the College of Science (Biology department) - University of Baghdad- Iraq for the period from October 2018 to December 2018. , The samples were transported, after inoculation in a transport media (nutrient broth), to the laboratory of the College of Science and then cultured on mannitol salt agar and blood agar). The isolates belonging to *Staphylococcus* spp. were identified by biochemical tests and Vitek 2 compact system, while the more antibiotic resistant isolates were identified by using Polymerase Chain Reaction (PCR) and sequencing of 16SrRNA . The results showed sharp UV absorption peaks at 330 - 340nm and AFM at 56 nm. Transmission Electron Microscope (TEM) results showed that lemon silver nanoparticles (NPs) are spherical in shape. The results also demonstrated that the silver NPs have a higher antibacterial activity with a diameter of inhibition zone that reached 16mm as compared with the common three mouthwash and toothpaste brands (Colgate, Crest and Close up) that had inhibition zone values of 5-8mm.

Keywords: Tooth paste brands, Close up, Mouthwash, PCR

تحديد تأثير جزيئات الفضة النانوية مع قشور الليمون بالمقارنة مع أنواع من المعاجين الأسنان وغسولات الفم ضد البكتريا الكروية العنقودية المعزولة من تسوس الأسنان

ميس عماد احمد، خديجة سلامة*

قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

تم أخذ 31 عينة من مسحات اللثة المعزولة من كلية العلوم (قسم علوم الحياة - جامعة بغداد - العراق) خلال الفترة من أكتوبر (2018) إلى ديسمبر (2018) مع متوسط عمر (5-30) سنوات ، حيث تم نقلها في وسائط النقل (مرق المغذيات) إلى مختبر كلية العلوم ومن ثم زرعها على (وسطان المانتول والدم) . تم تحديد العزلات التي تنتمي إلى *Staphylococcus spp* بواسطة الاختبارات الكيميائية الحيوية ونظام Vitek 2 بينما تم تحديد مزيد من عزلات المضادات الحيوية المقاومة باستخدام تقنيات تفاعل البلمرة المتسلسل

*Email: khadijath.salama@gmail.com

16SrRNA. أظهرت النتائج الموضحة للأشعة فوق البنفسجية ذروة الامتصاص الحادة عند 330 - 340 نانومتر و 56 (AFM) نانومتر نتائج TEM تُظهر أن NPS من الفضة الليمونية لها شكل كروي على التوالي أظهرت النتائج أنه تم تقييم ثلاث غسولات للغم ومعاجين الأسنان (كولجيت ، كريست ، و كلوز اب) على بعض الكائنات الحية الدقيقة في الفم المسببة للأمراض ، والنشاط المضاد للبكتيريا اعتمادا على مكونات نشاطهم الرئيسي مقارنة مع الجسيمات النانوية الفضية و كان قطر منطقة التثبيت (16mm) مقارنة باستخدام أنواع مختلفة من معجون الأسنان ووصلت منطقة الفم الأقل تثبيط (5-8) ملم

Introduction

Dental caries and periodontal diseases are among the most important preventable global infectious diseases. Oral health influences the general quality of life and is linked to chronic conditions and systemic diseases. The oral cavity hosts a group of bacterial flora, some of which are known to cause diseases in humans. Toothpastes are applied to remove stain and drive away bad breath. Their active ingredients play an important role in preventing periodontal diseases and other oral infections [1] Bacteria in the oral cavity include Streptococci, Staphylococci, Lactobacilli and Corynebacteria, with a large proportion of anaerobes. At birth, the oral cavity is sterile but then becomes rapidly colonized by bacteria , especially from the mother during the first feeding [2]. Dental caries is amongst the commonest diseases around the world, influencing 60 – 90% of school youngsters and the vast majority of adults [3]. It is known worldwide that some opportunistic microorganisms, such as *Staphylococcus* spp. and *Candida* spp., must be considered as plausible pathogens, where Staphylococci are thought to be temporarily occupant in the oral cavity [4].

Pathogenic bacteria has increased resistance to antibiotics because of their genetic ability to acquire and transmit this resistance. This problem caused therefore an increase in the last decade of the studies aiming to develop alternative drugs, either natural or synthetic. Plant extracts has been a valuable source of natural products to maintaining health of humans, including in Iraq [5]. The resistance of *Staphylococcus aureus* to ciprofloxacin has complicated the problem of treating staphylococcal-associated infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the causative agent since ciprofloxacin was the drug of choice to treat such infections[6]

The biological synthesis of AgNPS is an eco-friendly and exciting approach in the nanotechnology field which has shown bactericidal effects. Resistance to antibacterial agents derived from fruits and vegetable by pathogenic bacteria has emerged in the last years [7]. Biosynthesis of silver nanoparticles using citrus fruits is an essential approach where a reduction of silver ions is being involved. The peel parts of *Citrus sinensis* and *Citrus limetta* both are rich sources of flavones and poly methoxylated flavones, which are very rare in plant extracts. Citrus fruit extract itself acts as a reducing agent during the synthesis of nanoparticles. Thus, this method of synthesis is considered as ecofriendly, safe and nontoxic [8]. The aims of the present study include the isolation and identification of bacteria causing dental and periodontal decay as well as the investigation of their susceptibility to antimicrobial agents. The study also aimed to compare toothpastes and mouthwashes to silver nanoparticles with lemon peel extract in terms of their impacts against bacteria that cause tooth decay, including *Staphylococcus* spp.

Materials and Method

Ethical Approval

An Ethical Committee Approval for human and animal researches was obtained, while an informed consent was signed by the research participants.

Antibiotics Disk

Table 1-Antibiotic discs for the antibiotic sensitivity test of *Gve* + bacteria.

Antibiotics ($\mu\text{g}/\text{disc}$)	Company (Country)
Clindamycin , (DA) 10	Bioanalyse (USA)
Levofloxacin, (LEV) 5	Bioanalyse (USA)
Amoxicillin, (AX) 25	Bioanalyse (USA)
Erythromycin, (E) 10	Bioanalyse (USA)
Azithromycin ,(AZM) 15	Bioanalyse (USA)

Samples Collection

All the 31 samples were collected from participants (5-30 years of age) from the college of Science (Biology department)- University of Baghdad- Iraq during the period from October 2018 to December 2018. Sterilized swabs and woody sticks were used to collect nine samples from the oral cavity.

All specimens were streaked on mannitol salt agar plates, then incubated at 37°C under aerobic conditions for 18-24 hrs. After incubation, the pure colonies were selected to be diagnosed.

Biochemical Analysis

The physical and biochemical tests were conducted according to the methods described by Bergey's manual of determinative bacteriology.

Antimicrobial susceptibility testing

Kirby-Bauer's disc diffusion method for testing antibiotic sensitivity was performed for each isolate. The test was performed on Mueller–Hinton agar with the antibiotic discs shown in Table- 1. After incubation (24 hrs at 35°C), sensitivity was read. The bacteria isolates were regarded as sensitive or resistant according to CLSI criteria [9].

Molecular assay

DNA extraction

Genomic DNA was extracted from the detected bacterial isolate no. according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Quantus Fluorometer was used to detect the concentration of the extracted DNA [10].

Primers Selection

The set of primers 27F (AGAGTTTGATCTTGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT) was used for the amplification of the 16s rRNA of the identified bacteria [11].

Toothpastes

Three types of the toothpastes (Colgate, Crest, and Close up) were used in this study (Table1), as listed on the (package and the manufacturer's name). Toothpastes without antimicrobial agents and water were used as controls [12].

Mouthwashes

Solutions of three types of mouthwashes were bought from a pharmacy in Baghdad [13], namely Listerine -fresh burst (Italy), Herbis Miswak (Brazil), and Medox (Egypt).

Preparation of Bio Extract

Fresh peels of lemon (20 gm) were washed with tap water and air dried for a short time. They were finely cut and soaked in 100 ml D.W for 10 min and filtered through a Whatman filter paper (no.0.5). This bio extract was always used as fresh.

Preparation of 1mM Silver Nitrate

Silver nitrate was brought from Lobachemie. A weigh of 0.0169 gm was dissolved in 100mL of distilled water in an amber colored bottle.

Synthesis of Silver Nanoparticles

Three milliliters of the prepared peel extract was added to 40mL of silver nitrate solution in a 100mL conical flask and incubated at room temperature for 2- 3 hours. The control sample contained only 40 mL of silver nitrate solution.

Well Diffusion Method

(WDA) Well diffusion method then of the suspension was spread on the test plate. 6 mm diameter were impregnated with 10 µL of the citrus juices and placed on the surface of the test plate was used. The plates were incubated at 37°C for 24 hrs and the inhibition zone was measured [14].

Physio-chemical characterization of silver nanoparticles

Force Field Microscopy (AFM)

UV-Vis spectrophotometer was used at a resolution of 1 nm and a range from 250 nm to 800 nm. The size of nanoparticles was performed with Atomic Force Field Microscopy (AFM)

Scanning Electron Microscope (SEM) Analysis

The morphological features of the synthesized silver nanoparticles prepared with the plant extract were studied by Scanning Electron Microscope (JSM-6480 LV).

Characterization of Silver Nanoparticles

Then synthesis of silver nanoparticles was analyzed using UV-Visible spectroscopy at the wavelength of 300-700nm.

Results and Discussion

Isolation and identification of bacteria

Isolation and identification of Gram positive bacteria (*Staphylococcus. epidermidis* and *Staphylococcus. aureus*)

The bacterial cultures were examined and showed that gram positive cocci appeared as single cells, pairs, tetrads and chains. The macroscopic examination of the isolates on mannitol salt agar demonstrated the ability to ferment mannitol and turn the color of the medium from red to yellow. The bacteria were classified as presumptive *S. aureus* and *S. epidermidis* (Figure- 1).



Figure 1-A presumptive identification of A) *S. aureus* and B) *S. epidermidis* on mannitol salt agar.

The isolates on blood agar showed yellow-gray colonies of 3-4 mm diameter on the zones of β -hemolysis (Figure-2) . The identification was based on previously described features [15]



Figure 2-A presumptive identification of A) *S. aureus* on blood agar B) *S. aureus* on milk agar.

The thirty one samples were taken from patient from both sex groups with an age range of 5-30 years. All the swab samples were positive for microorganisms (Table-2).

Table 2- Distribution of *S.epidermidis* and *S. aureus* infections according to sex.

Bacterial Isolates	Gender		Chi-Square (P-value)
	Male Positive No (%)	Female Positive No (%)	
<i>Staphylococcus epidermidis</i>	5(% 16)	12(% 39)	8.026 ** (0.0062)
<i>Staphylococcus aureus</i>	6(% 19)	8(% 26)	2.881 NS (0.0715)
** (P<0.01).			

The results agree with those previously reported [16]. These bacteria were characterized by using biochemical tests as described. All these isolates were obtained from amalgam tooth filling We show that the percentage of the bacterial isolates was higher in males (24%) than in females (16%), as demonstrated in Table- 2. The percentage increased with the increase in the age of the donors, which

may be due to the decrease in immunity and resistance to infection by bacteria. . The result agrees with those reported by other authors [17].

Antibiotic susceptibility test (AST)

The antibiotic resistance patterns of the isolated gram positive bacteria included resistance to Erythromycin and Azithromycin as well as sensitivity to Clindamycin , Levofloxacin, and Amoxicillin. These results are similar to those obtained by a previous investigation [18] which showed that most of the isolates of *Staphylococcus aureus* were multi-resistant for antibiotics, with a high level of resistance against Methicillin, Penicillin G, Methicillin, Chloramphenicol, whereas they demonstrated sensitivity to Vancomycin.

In this study, the DNA of the bacterial isolates was successfully extracted and showed an appropriate quality to perform PCR (10 ng/ μ l). 16S rDNA was amplified by PCR using specific primers that give a distinct with a size of 1500 bp when analyzed with gel electrophoresis (Figure-3) [19].

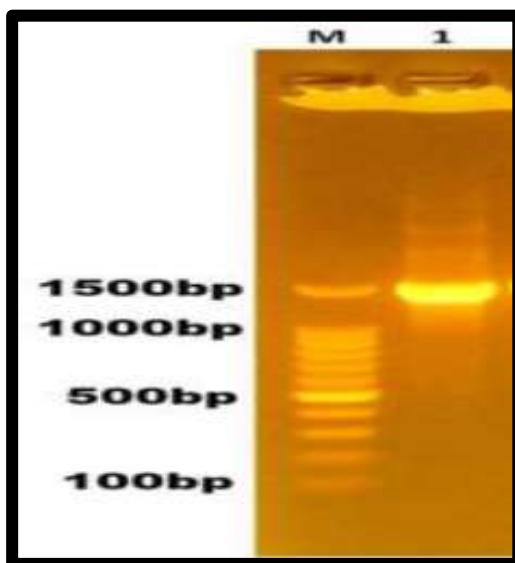


Figure 3-PCR product with a band size of 1250 bp. The product was electrophorised on 2% agarose at 5 volt/cm² and 1x TBE buffer for 1:30 hours. N: DNA ladder (100). *Staphylococcus aureus*

Silver nanoparticles characterization

Spectral Properties of the Silver Nanoparticles

Figure-4 shows a strong surface plasmon centered around 400 nm, which indicates the formation of silver nanoparticles which are not possible to detect at 360 nm.

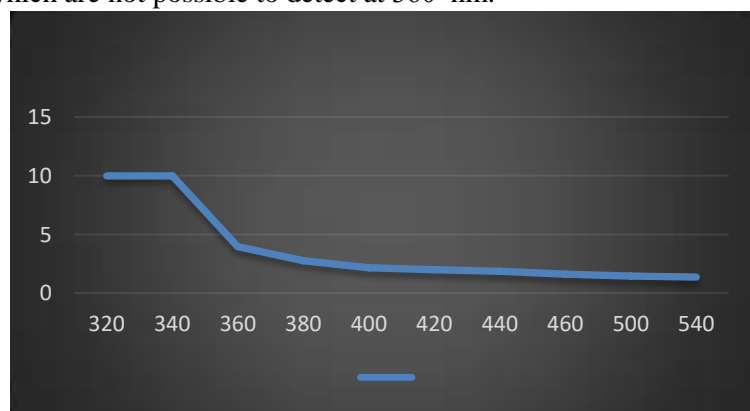


Figure 4-Absorption spectra of silver nanoparticles.

Atomic Force Electron Microscopy (AFM)

The AFM micrograph obtained for the -lemon-silver nanoparticles (Figure- 5) indicates the surface roughness changes values as identified by root mean square (Rp). The sample’s roughness value was 56 nm, while the section analysis of the sample’s grain size showed a value of 34 nm.

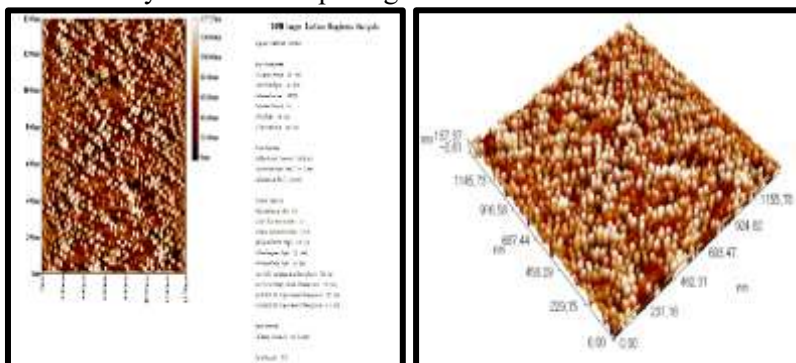


Figure 5-AFM for lemon-silver nanoparticles.

Transmission Electron Microscopy (TEM) analysis of silver nanoparticles

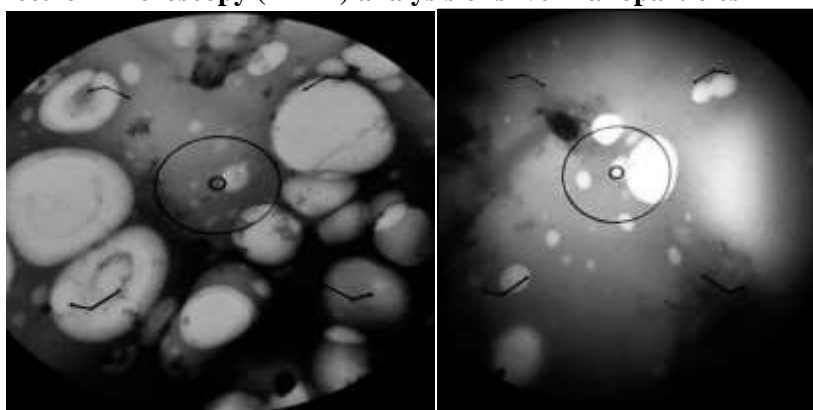


Figure 6-TEM of A) *Staphylococcus aureus* before treatment with lemon-silver nanoparticles B) *Staphylococcus aureus* after treatment with lemon -silver nanoparticles.

We then compared the antimicrobial activities of silver NPs versus those of toothpastes. The NPs synthesized using the natural plant extracts represented the only formulation that had activity against Gram positive bacteria. The diffusion method was used as a preliminary test for detecting the antimicrobial activity. The results showed that the NPs synthesized using lemon had a higher activity with a larger diameter of inhibition zone as compared to the different brands of toothpastes and mouth washes (Table -2).

Table 2-*In vivo* effects of toothpastes, mouth washes and lemon-silver NPs on the growth inhibition zone (mm) of the tested microorganisms.

Microorganism	Toothpastes			Mouth washes			Lemon-silver NPs	LSD (P-value)
	Close up	Crest	Colgate	Liste	Herbis Miswak	Medox		
<i>S. aureus</i>	8 ± 0.36 b	7 ± 0.20 bc	6 ± 0.11 bc	6 ± 0.11 bc	6.5 ± 0.31 bc	5 ± 0.10 c	15 ± 0.48 a	2.833 ** (0.0001)
<i>S. epidermidis</i>	6 ± 0.14 b	5 ± 0.09 b	5.5 ± 0.13 b	7 ± 0.18 b	6 ± 0.23 b	5 ± 0.13 b	16 ± 0.58 a	2.619 ** (0.0001)

** (P<0.01).
Means with the different letters in the same row differed significantly.

The result agree with those of an earlier study [20] which showed that the mouthwash Colgate was the most active solution which caused an inhibitory zone of about 4 mm in diameter, followed by ZAK (3.7 mm.) and Listerine (0.5mm.).

The conventional antimicrobial toothpastes increase the effectiveness in the control involved in a wide variety of the primary etiological agents of dental caries the antimicrobial activity of commercial toothpaste [20]

The inhibitory effects of a 10 % concentration of six different mouthwashes against six oral bacteria was previously measured. The inhibition effect of the six mouthwashes were tested at different dilutions 25%, 50% and 75% using agar well diffusion method against *Streptococcus mutants*, *Streptococcus sanguinis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Haemophilus influenza* and *Streptococcus pyogenes* [21].

Conclusions

Because of the increased bacterial resistance to antibiotics, nanoparticle silver particles were produced with lemon peel and their effectiveness against bacteria isolated from the teeth caries was compared to that of toothpastes and mouthwashes. A high efficacy was observed for the nanoparticles as compared to the toothpastes and mouthwashes.

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