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IN VITRO COMPARISON OF THE ANTIBACTERIAL EFFECT BETWEEN MISWAK AND SOME TOOTHPASTES ON ORAL BIOFILM FORMING BACTERIA.

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ABSTRACT

The dental biofilms are the most prevalent cause of the oral diseases that colonize in the gingival and sub-gingival regions of the mouth. This study aimed to isolate and characterize the oral biofilm forming bacteria and also to estimate the antibacterial effect of Miswak and some toothpastes which mainly contain sodium fluoride. Fifty Samples were collected from the mouth by swabbing across the gingival and the buccal cavity. Thirty three of them were isolated and characterized by the analytic profile index and some important routine laboratory tests. Eighteen isolates were diagnosed as *Staphylococcus aureus* and fifteen isolates were diagnosed as *Streptococcus oralis*. Biofilm formation was examined by tube staining assay. Some isolates formed biofilm after 24 hours incubaion. Others has formed after 48 hours. Interesting thing that biofilm formation increased after 72 hours. Obviously, it was observed that cells adherence reach the highest peak by escalating the time of incubation. Miswak is a natural toothbrush which has been documented as potent antibacterial effect. Also toothpaste is used to promote oral hygiene. In this study, it has been compared the antibacterial effect on Miswak and some toothpastes by using agar - wells diffusion method. The zone diameters of inhibition for Miswak were (4mm) on the *Staphylococcus aureus,* and (3.5mm) on the *Streptococcus oralis as* the average of zone diameters of inhibition for some toothpastes were (3mm) on the *Staphylococcus aureus,* (2.5mm) on the *Streptococcus oralis*. The obtained results for this study were observed that Miswak extract has a very significant effect on the isolated biofilm forming bacteria in comparison to some toothpastes and this what was indicated by our prophet Muhamed (PBUH) in his honorable talks about Miswak.

KEYWORDS: *Staphylococcus aureus,* Miswak, *Streptococcus oralis.*

1. INTRODUCTION

The toothbrush tree, *Salvadora persica*, L., locally called Miswak, its a member of the Salvadoraceae family which has been used by many Islamic communities as toothbrushes and has been scientifically proven to be very useful in the prevention of tooth decay, even when used without any other tooth cleaning methods (Salehi and Momeni Danaie, 2006). Chewing sticks that are made from the roots, twigs, or stems of *S. persica* are commonly used in the Middle East as a means of maintaining oral hygiene. Studies indicate that *S. persica* extract is somewhat comparable to other oral disinfectants and anti-plaque agents, such as triclosan and chlorhexidine gluconate, if used at a very high concentration (Almas, *etal*., 2005).

It has been reported that extracts of Miswak posses various biological properties, including significant antibacterial, antifungal (Al-Lafi and Ababneh, 1995).

Salvadora persica and other related plants are reported to be effective against bacteria which are important for the development of dental plaque. Despite the wide use of Miswak, the plant in Iraq has not received much attention and has not been fully studied. Therefore, the aim of the present study is to evaluate the antimicrobial activity of Iraqi Miswak by using agar-well diffusion method.

A biofilm is a complex aggregation of micro−organisms growing on a solid substrate. Biofilms occur in a range of everyday situations, it grow in a three stage process. The initial stage includes the attachment of bacteria into the substratum. Bacterial growth and division then leads to the colonization of the surrounding area and the formation of the biofilm. Bacteria do not act individually to form biofilms, but it congregates into long chains to help initiate the early stages of biofilm formation.

The major features that distinguish biofilm forming bacteria from their planktonic counterparts are their surface attachment ability, high population density, extracellular polymeric substances slime (EPS) and a wide range of physical, metabolic and chemical heterogeneities (Beer and Stoodley, 2006). It is now recognized that biofilm formation is an important aspect of many diseases, including native valve endocarditic, osteomyelitis, dental caries, middle ear infections, medical device-related infections, ocular implant infections and chronic lung infections in cystic fibrosis patients (Donlan and Costerton, 2002). Biofilms can tolerate antimicrobial agents at concentrations of 10–1000 times than that needed to kill

genetically equivalent planktonic bacteria, making biofilms extremely difficult to eradicate from living hosts (Lewis, 2001).

Oral bacterial species mostly lack an environmental niche and are found almost exclusively within the mouth (Rachid et al., 2000). For these bacteria, planktonic growth would cause them to be quickly washed away by saliva, swallowed and destroyed within the acidic juices of the stomach. These bacteria likely spend the majority of their natural existence growing as a biofilm. Therefore, the present study aimed to fulfill the following objectives:

1. Isolation of the oral biofilm forming bacteria.
2. Biochemical identification of bacterial strains.
3. In vitro screening for biofilm formation.
4. Comparison of the antibacterial effect between Miswak and Some toothpastes on oral Biofilm forming bacteria.

2. MATERIALS AND METHODS

2.1 Isolation of oral bacteria

2.1.1 Sample Collection

Sample was collected from mouth by swabbing across the gingival and sub-gingival region as well as from the roof and floor of the buccal cavity. The samples were collected from the fifty persons and were inoculated in Nutrient broth (HiMedia, India) until reachs to the laboratory.

2.1.2 Isolation and screening of biofilm forming bacteria

Samples directly streaking on Blood agar plates and incubated at 37ºC for 24 hours. After incubation the isolated colonies were then restreaked onto the mannitol salts agar and nutrient agar plates to obtain pure cultures and those found to be viable were screened for biofilm formation. Primary biofilm screening was done using tube staining assay (Christensen *et al*., 1982).

2.1.3 Biochemical identification of bacterial strains

Biochemical identification of the selected strains was performed by a physical and biochemical characterization.

2.1.4 Physical characterization

1- Gram staining

The diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute. The slide were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off solution. The slides were washed with water and counter stained with safranin stain for about one minute and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

2- capsule stain: using sterile technique, add a loop-full of bacterial culture to slide, smearing it in the dye (India ink), allow 5-7 minutes to air dry (do not heat fix), flood the smear with crystal violate by tilting the slide at 45 degree angle and lrt stain run off until it air dries, then examine the smear microscopically (100X) for the presence of encapsulated cells as indicated by clear zone surrounding the cells.

3- Colony morphology

Shape, size, color, elevation and margin of colony and appearance are observed in overnight plate culture on enriched and different selective media.

4- Cell morphology

The gram stained cells were viewed under light microscope under 100x oil immersion to determine the morphological characteristics of the cells.

2.1.5 Biochemical identification

API 20 Strep C (bioMerieux, Inc France) was used as a confirmatory identification method for all isolates. The procedures were done according to the manufacturer's instructions. The reactions are read according to the reading table and the identification is obtained by using the identification software.

2.1.6 Optochin and Bacitracin Test

Inoculate Muller -Hinton agar plate with suspected alpha-hemolytic isolates, then apply commercially available optochin and Bacitracin discs on the streaked Muler - Hinton agar

plates, finally Incubated plates at 37°C with 5-10% CO2 for 18-24 hours. After that observe the zone of growth inhibition around the disc, alpha-haemolytic colonies with zone of inhibition between 9 and 13 mm should be tested for bile solubility.

2.1.7 Bile Solubility test

1. Grow the isolates to be tested for 18-24 hours on a Blood agar plate at 37°C with ~5% CO2 (or in a candle-jar).
2. Add bacterial growth from the overnight Blood agar plate to 1.0 ml of 0.85% saline to achieve turbidity in the range of a 0.5-1.0 McFarland standard.
3. Divide the cell suspension equally into 2 tubes (0.5 ml per tube).
4. Add 0.5 ml of 2% sodium deoxycholate (bile salts) to one tube. Add 0.5 ml of 0.85% saline to the other tube. Mix each tube well.
5. Incubate the tubes at 37°C in CO2.
6. Vortex the tubes, then observe the tubes for any clearing of turbidity after 10 minutes. Continue to incubate the tubes for up to 2 hours at 37°C in CO2, if negative after 10 minutes. Observe again for clearing.

2.2 The Tube staining assay

The qualitative assessment of biofilm formation was determined by the tube staining assay following Christensen *et al*.; nutrient broth (1mL) was inoculated with 100µl of overnight culture broth and incubated for 24, 48 and 72 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.3) and dried. Staining of dried tubes was done with 0.1% crystal violet. Excess stain was removed by washing the tubes with deionized water. Biofilm formation in tubes was then observed. Formation of biofilm was confirmed with the presence of attachment (visible film) on the wall and bottom of the tube. However, the liquid interface did not indicate biofilm formation (Mathur et al., 2006).

2.3 Preparation of the aqueous extraction

2.3.1 Preparation of Miswak Extract

A sample of the most commonly used chewing sticks from Miswak trees was collected from local market. The fresh Miswak was cut into small pieces and allowed to dry at room temperature for couple of days. Then it was ground to powder. Successive 10 g quantity was put into sterile screw-capped bottle to which 100 ml of sterile de-ionized distilled water was added. The extract was allowed to soak for 48 hours at 4°C and then centrifuged at 2000 rpm

for 15 minutes. The supernatant was passed through filter paper (0.45 µm pore size). The extract was stored at 4°C and used within one week (Al-lafi and Ababneh, 1995).

2.3.2 Preparation of toothpaste Extract

Aqueous extraction about 1gm of toothpaste and10 ml of coldwater was added to them in sterile test tubes kept for 1 week at room temperature until use (Rehab, 2015).

2.4 Preparation of inoculums

The *Staphylococcus aureus* (*S.aureus*) and *streptococcus species* microorganisms were isolated from mouth by oral swab and were cultured on both mannitol salt agar and blood agar for overnight at 37°C then the growing microorganisms were examined microscopically using Gram stain and biochemical tests were made for identification.

2.5 Antimicrobial screening

The antibacterial activity of the Miswak (*S. persica*) extract and toothpaste was evaluated by using the disc diffusion test. Oral swab streaked on muller hinton agar plates then a sterile 4 mm cork borer was used to cut three wells at equidistance in each of the plates. 0.2 ml of each extract was introduced into each of the three wells. The plates were incubated at 37°C for 24 h The antimicrobial activity was evaluated by measuring the diameter of zones of inhibition (In mm) (Christofilogiannisp, 2001).

3. RESULTS

3.1. Sample collection and Characterization of Bacterial Strains

Thirty three bacterial strains with observable difference in colony morphology were randomly selected from initial spread plate and re-streaked on Blood agar and mannitol salts agar (Fig.1, 2).

Fig. 1: *Staphylococcus aureus* show Beta- hemolysis on Blood agar and Golden yellow on mannitol salts agar.

Fig. 2: *Streptococcus* sp. show alpha- hemolysis on Blood agar.

The isolated bacterial species were morphologically characterized by Gram staining; eighteen isolates were found to be gram positive *Staphylococcus* while fifteen isolates were found to be gram positive *Streptococcus*.

3.2 Biochemical identification

Staphylococcus aureus was biochemically identified using analytic profile index kit (Api 20 E system) (Fig-3).

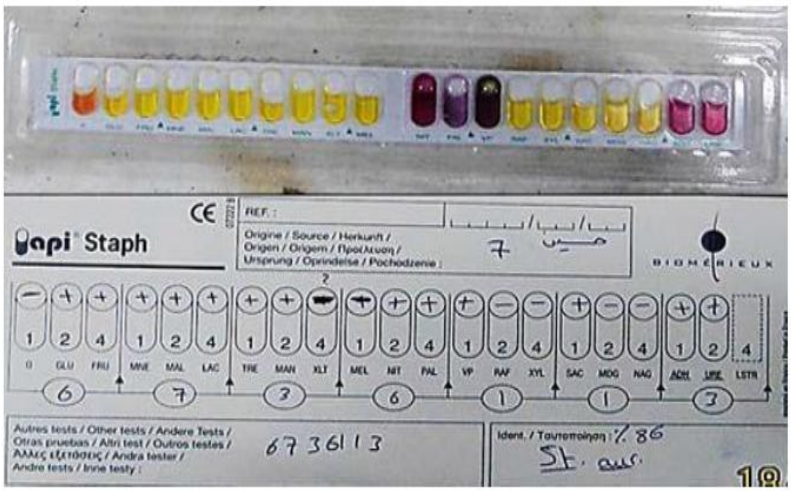

Fig. 3: Api 20 E system for *Staphylococcus aureus*

Laboratory diagnosis of *Streptococcus* as *Streptococcus mutans* was based on characteristic colony morphology (Ryan and Ray, 2004) which were small, round on blood agar plate, transluscent or mucoid with alpha-hemolysis (A green discolouration of the agar around the colonies), also diagnosis depend on susceptibility to certain diagnostic discs such as optochin

and Bacitracin discs (Fig- 4) in addition to the bile susceptibility test (Fig.5); results were shown in (table-1).

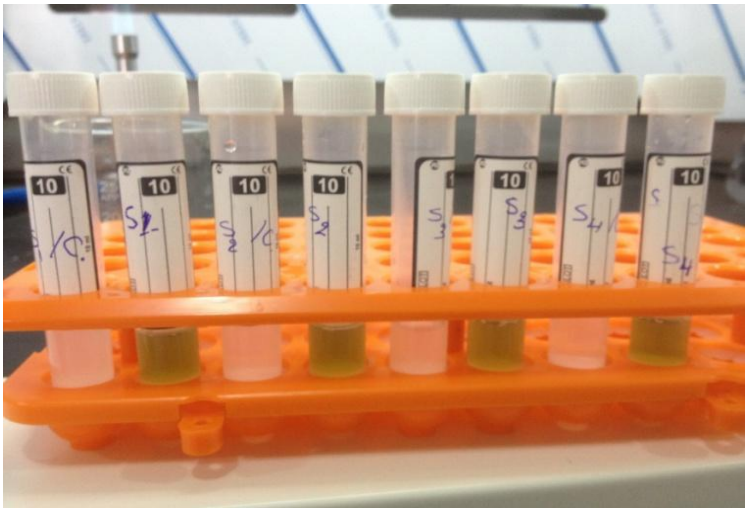

Fig.4: Bile susceptibility test for *Streptococcus oralis*

Fig.4: Sensitivity test to the optochin and Bacitracin discs for *Streptococcus oralis*

Table-1: Laboratory diagnostic methods for *Streptococcus sp.*

Tests	***Streptococcus oralis***
Optochin test	Sensitive (2.5mm)
Bacitracin test	Sensitive(3mm)
Bile soluble test	Bile soluble
Capsule stain	Capsulated

3.3. Tube assay

Twenty seven strains were found positive for biofilm formation. The tubes when stained with crystal violet showed biofilm attachment. (Fig-4,5,6), It was observed that cell attachment

was maximum with increasing the time of incubation and this results agreements with the study of Nisha and Thangave (2014) in which exopolysacharide (EPS) were estimated at different incubation period (24hr, 48hr, 72hr and 96hr) and temperature (27°C, 37°C) and at pH 6 - 8. From the quantification results of Crude EPS, higher carbohydrate content was at 96 hrs, at pH 8 and at 27°C of incubation time. Another study reported that glass and stainless steel are surfaces that provide a greater bacterial adherence (Djordjevic, *etal*., 2002). The process of biofilm formation is influenced by various factors including nutrients level, pH, and temperature.

Study of (Kaustubh, *etal*., 2015) also deals with the isolation and characterization of biofilm forming bacteria from oral microflora which demonstrated that the *Streptococcus* species is most dominating out of the total microflora.

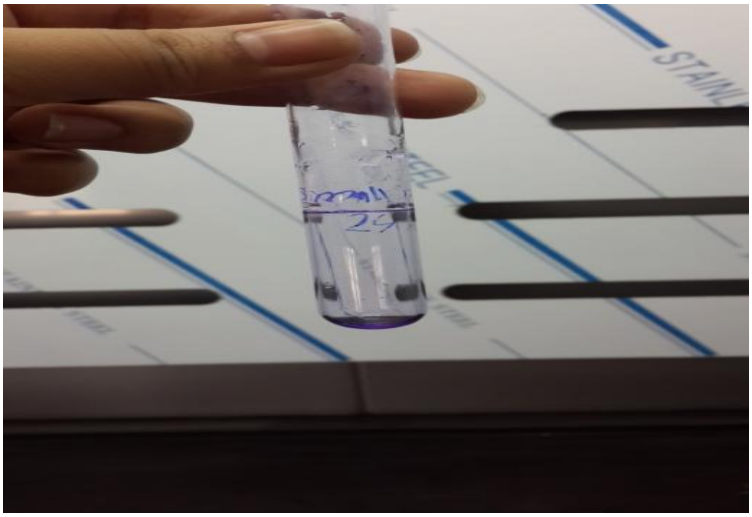

Fig. 4: Tubes showing biofilm formation after 24 hrs.

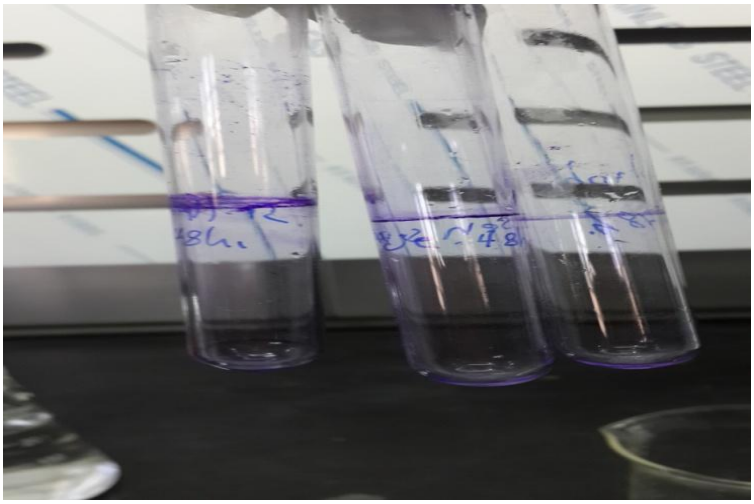

Fig. 5: Tubes showing biofilm formation after 24 hrs. after 48 hrs.

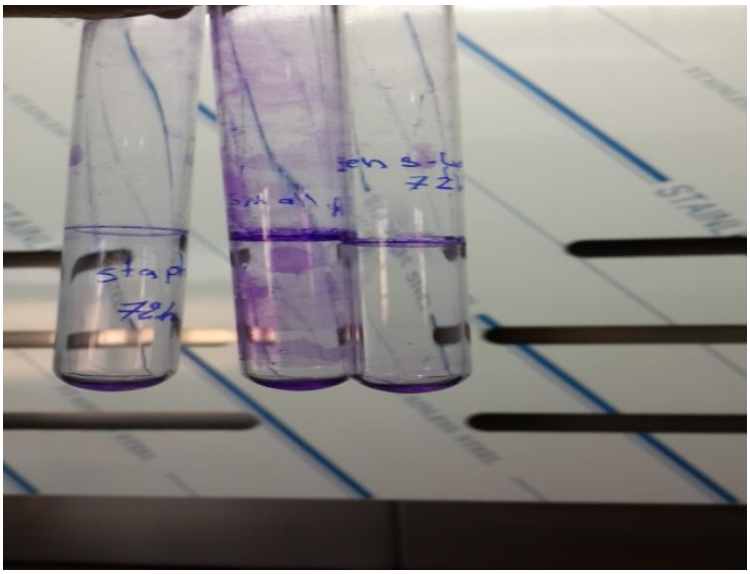

Fig. 6: Tubes showing biofilm formation after 72 hrs.

3.4 Antibacterial activity test

Based on the diameter of the zone of microbial inhibition produced by the Miswak and toothpaste in agar well diffusion method against the tested oral pathogen, the hot crude extract of Miswak was more effective and has antibacterial activity against *Staphylococcus aureus* and *Streptococcus mutans.* (Fig.7) than the toothpastes(Fig.8). Most of the extracts exerted their antimicrobial activity only at the highest concentrations used.

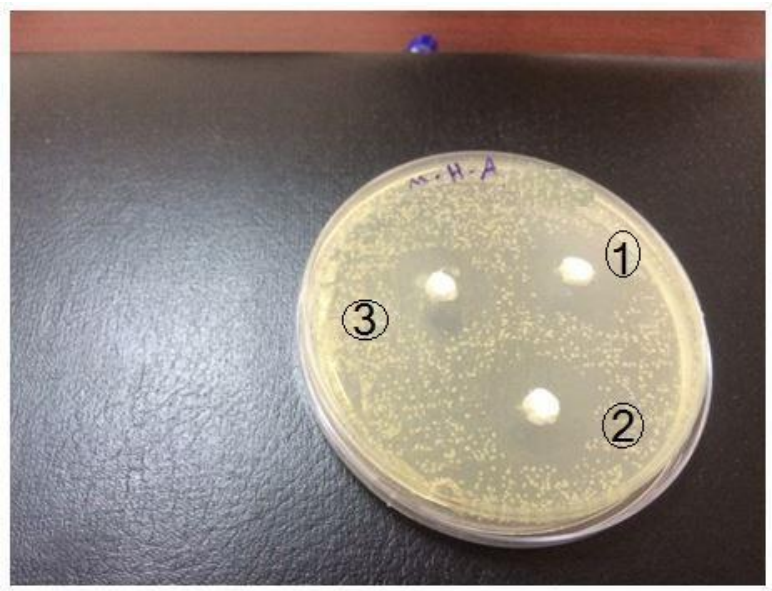

Fig. 7 Antibacterial effect of Miswak(1,2) and toothpaste (3) extract on *Staphylococcus aureus*

Fig. Antibacterial effect of Miswak(1,2) and toothpaste (3) extract on *Streptococcus mutans.*

The inhibition zone of the various concentrations, which were recorded after 24 hour incubation at 37°C were shown in (Table-2).

Table 2: The diameter (mm) of zone of inhibition produced by Miswak and toothpaste.

Bacteria isolates	Diameter of inhibition zone of the Miswak	Diameter of inhibition zone of the toothpaste
Staphylococcus aureus	4 mm	3 mm
Streptococcus Oralis	3.5 mm	2.5 mm

The observed antibacterial activities can be whether because of the pH of the crude extracts or due to biological active compounds present in Miswak like trimethylamine, salvadorine chlorides, fluoride, silica, sulphur, vitamin C and small quantities of tannins, saponins flavenoids and sterols (Akhtar and Ajmal ,1981). More studies performed by independent researchers using other techniques published antibacterial results of *S. persica* extracts obtained with the agar diffusion test.

These results harmony with the study of (Vahabi, *etal*., 2011), who improved that the *S. persica* is a medical plant whose roots, twigs or stems have been used over centuries as oral hygiene tools in many parts of the world, also its accord with the study of (El-Desoukey, 2015) who demonstrated that Miswak is more efficient antimicrobial agent against *Staphylococcus aureus.*

The high amount of NaCl, KCl, trimethylamine and sulphur-containing organic substances (salvadourea and salvadorine) in Miswak might be in somehow responsible for the observed antibacterial and gum-stimulating effects (Cushnieand. Lamb, 2005 and Monks, *etal*., 1990) Results of this study on Miswak and toothpaste approved that Miswak was more effective based on the diameter of the zone of inhibition produced by the Miswak in agar well diffusion method against the tested oral pathogen and it was safe antibacterial toothbrush than toothpaste, especially that contain fluoride.

Interestingly, the children who under age 2 years can swallow most of the toothpaste during brushing and that gives an enamel fluorosis for the front permanent incisors. Enamel fluorosis is a condition which can vary from minor white spots to unsightly yellow or brown of the enamel due to excessive intake of fluoride (Van Loveran, etal., 2004).

Aqueous and methanol extracts of *Salvadora persica* were investigated by Firas *et al.* (2008) for its antimicrobial activities against seven isolated oral pathogens. The aqueous extract hindered all isolated microorganisms, especially the *Streptococcus mutans* and was more efficient than the methanol extract. Study of Aires *et al.* (2009) and Beevi *et al*. (2009) were confirmed more effect of cold aqueous extract of Miswak. on the contrary of that, in my study which the hot aqueous extract was used. So its strongly recommended to use fresh soaked Miswak in a cold water instead of hot water, because that leads to some active ingredients loss.

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