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ANTIMICROBIAL AND SYNERGISTIC EFFECTS OF MISWAK, NANO-SILVER DRUG, AND CHLORHEXIDINE ALONE AND THEIR COMBINATIONS UPON CERTAIN ORAL MICROBIOTA

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The current study target to compare the antimicrobial efficacy of miswak, silver nanoparticles (AgNPs), and chlorhexidine with different concentrations alone and in combinations with each other against two bacteria Streptococcus mutans (S. mutans), Staphylococcus aureus (S. aureus), and one fungus Candida albicans (C. albicans). Manually prepared miswak extract, chlorhexidine gluconate (0.2% and 0.12% concentrations), and AgNPs (20 to 50 nm) were used. The following combinations were prepared and their synergistic effect were evaluated: AgNPs with chlorhexidine, miswak with chlorhexidine, and miswak with AgNPs. The antimicrobial efficiencies were defined using agar well diffusion method. The mean difference was tested using ANOVA. When tested materials were used alone, the mean diameter of inhibition zone (DIZ) of AgNPs, chlorhexidine and miswak against S. aureus and S. mutans were significantly higher than C. albicans. The AgNPs had greatest effect than the other two tested materials (AgNPs> chlorhexidine> miswak) upon tested bacteria. When tested combinations are used, the mean DIZ of AgNPs with miswak extract combination was significantly highest more than miswak with chlorhexidine 0.2% or 0.12% combinations at all the tested microbe levels. Mean DIZ of AgNPs with 0.2% chlorhexidine was significantly highest more miswak with 0.2% or 0.12% chlorhexidine combinations at all tested microbe levels. Finally, the combination of AgNPs with miswak extract has superior antimicrobial efficacy when compared to the other tested combinations. Therefore, AgNPs, and miswak extract can be used as a promising antimicrobial biomaterial in dental applications.

Keywords: *AgNPs; miswak extract; chlorhexidine; combinations; antimicrobial efficacy.*

INTRODUCTION

The dental biofilm is formed of microorganisms through an ordered sequence of events, resulting in a rich, well-ordered and functionally organized microbial community in the teeth. Nowadays, Oral biofilm is a dense environment for a large number of microbial species, which causes periodontal infections and dental caries[1]. Dental biofilms cause dental caries which is dominated by carbohydrate-fermenting bacteria. Dental caries considers as

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multifactorial disease with high sugar diet and *S. mutans* as main etiological factors[2]. Along with *S. mutans,* studies the highlighted role of *C. albicans* in increase of dental caries[3]. Oral microorganism like *S. aureus, S. mutans* and *C. albicans* with other microorganisms forms dental plaque[1]. The *S. aureus, S. mutans* and *C. albicans* strains are resistant to the currently used antimicrobials[4], and shifted the focus of research to other alternatives, offering therapeutic benefits and more inexpensive treatment[5]. The AgNPs are metallic nanoparticles that serve as an antimicrobial agent with long term antibacterial activity[6], low bacterial resistance, high surface to volume ratio, and low volatility[7&8]. Nano-sized silver particles penetrate into the cell membranes/cell wall by thiol groups or sulfur-containing proteins, ultimately resulting in the microbial DNA damage and cell death[9&10]. Antimicrobial efficacy of miswak is related to its b-sitosterol, m-anisi acid, and chloride content[11&12] and commonly used tooth cleaning tools[13]. Chlorhexidine is commonly used mouth wash because of its efficacy against common oral pathogens[14&15]. However, continuous use of high concentration of chlorhexidine is associated with dental complications like dryness of mouth, dental stains, changes in taste, and gingival irritation[16]. Previously, limited research was conducted to check the antimicrobial efficiency of AgNPs, miswak, and chlorhexidine combinations against common oral microbes and researches highlighted the need for further studies in this regard[17&18]. The current study was carried for the comparison of the antimicrobial efficiency of miswak, AgNPs, and chlorhexidine with different concentrations alone and their combinations against *S. aureus, S. mutans,* and *C. albicans*

MATERIAL AND METHODS

Study design, ethical approval, microbial strains used

An *in-vitro* study was conducted at University Dental Hospital and Department of Microbiology, Taif University, KSA. Ethical clearance was obtained from Taif University, Institutional review board (Ethical clearance number- 41-1107-00152). Two bacterial biofilm producing strains,*S. aureus, S.mutans* [19&20] and one strain of *C. albican* were used in this study to evaluate the antimicrobial activity. The microbes were isolated from patients visiting University Dental Hospital, Taif, Saudi Arabia. The isolated microbes were confirmed using standard microbiological methods[21]. This study was a continuation of previous studies, so we relied on the same previous strains.[19&20]

Preparation of test solutions

Miswak extract

The roots of miswak were brought from the local markets, Taif City, KSA and ground into powder. The miswak was placed into a thimble, a thick filter paper tube with Dx l= 35 x 150 mm and grade 603 (Carl Schleicher und Schull, Dassel, Germany), for extraction with methanol which was removed after extraction leaving a brownish oil. A methanol was added to dissolve the oil and to precipitate it again by adding diethyl ether and the precipitate with ether-methanol were separated by decantation. This step was repeated and finally, the miswak oily extract was kept in a refrigerator at 4ºC. Then, 50 mg/ml miswak extract was prepared[22].

Miswak extract characterization

Miswak extract was characterized using Fourier transform infrared analysis (FT-IR)[19].

Silver nanoparticles

Chemically derived, commercially available AgNPs powder of size 20 to 50 nm (Alibaba Company, Shanghai Xinglu Chemical Technology Co., LTD, China) was purchased. The AgNPs (100 μg/ml) was prepared by adding 1 mg of AgNPs powder to 10 ml of normal saline.

Chlorhexidine solution

Commercially available chlorhexidine gluconate with two different concentrations (0.2% and 0.12%) were purchased (Perioshield mouthwash, Sunstar GUM-Butler, USA).

Preparation of solution combinations

Miswak and AgNPs combinations

Equal quantity of 50 mg/ml miswak extract and 100 μg/ml AgNPs were stirred together.

Chlorhexidine and AgNPs combinations

Equal quantity of chlorhexidine with 0.2% and 0.12% concentration individually and 100 µg/ml AgNPs were stirred together.

Miswak and chlorhexidine combinations

Equal quantity of chlorhexidine with 0.2% and 0.12% concentration, individually and 50 mg/ml miswak extract were stirred together using sterile glass rod to get uniformly.

Determination of antimicrobial activity

The agar-well diffusion method was used to determine antimicrobial efficiency of the tested solutions [AgNPs, miswak, and chlorhexidine with 0.2% and 0.12%].[23&24]. The inoculum (100 µl) of The bacterial strains or Candida (~10^6 CFU/ml) was put onto the surface of the Mueller-Hinton agar and Sabouraud dextrose agar, respectively. Wells (7 mm in diameter) were cut from the agar with a sterile borer and different volume (50, 100, and 200 µl) of each solution (AgNPs, miswak, and chlorhexidine) were delivered into them in addition to phosphate-buffered saline as negative controls. The inoculated plates of the tested microbes were incubated for 24 h at 37°C. The diameter of the inhibition zone (DIZ) of the tested microbes were obtained to determine antimicrobial efficiency of AgNPs, miswak, and chlorhexidine. Minimum Inhibitory Concentration (MIC) of AgNPs, miswak, and chlorhexidine was determined by broth micro-dilution method[25&26]. each test was occurred in triplicates.

Statistical analysis

Mean difference was tested using One-way Analysis of variance (ANOVA) followed by Tukey Post Hoc using the Statistical Package for Social Science version 17 (SPSS Inc Chicago link). All statistical tests were two-sided, and the significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Results

Determination of minimum inhibitory concentration

The MIC was the lowest for AgNPs (25 µg/ml) and chlorhexidine 0.05% against *S. mutans* (Table 1). The mean diameter of inhibition zone (DIZ) of AgNPs against *S. aureus* (23.4 ± 0.5) was significantly (p= 0.04) higher than *Candida albicans* (19.8 ± 0.4) (Table 2 and Figure 1). The mean DIZ of miswak against *S. aureus* and *S. mutans* was significantly (p= 0.04) higher than *C. albicans* at 100 µl and 200 µl solutions (Table 2 and Figure 1).

Table 1: MIC of tested solutions against tested microbes

Material name and concentration	Bacteria	Concentration and bacterial growth				MIC
		1/2	1/4	1/8	1/16	
Miswak (50 mg/mL)	*S. aureus*	-	-	-	+	6.25 mg/mL
	S. mutans	-	-	-	+	6.25 mg/mL
	C. albicans	-	-	+	+	12.50 mg/mL
AgNPs (100 µg/mL)	*S. aureus*	-	-	+	+	25 µg/mL
	S. mutans	-	-	+	+	25 µg/mL
	C. albicans	-	+	+	+	50 µg/mL
0.2% CHX	*S. aureus*	-	-	+	+	0.05%
	S. mutans	-	-	+	+	0.05%
	C. albicans	-	+	+	+	0.10%

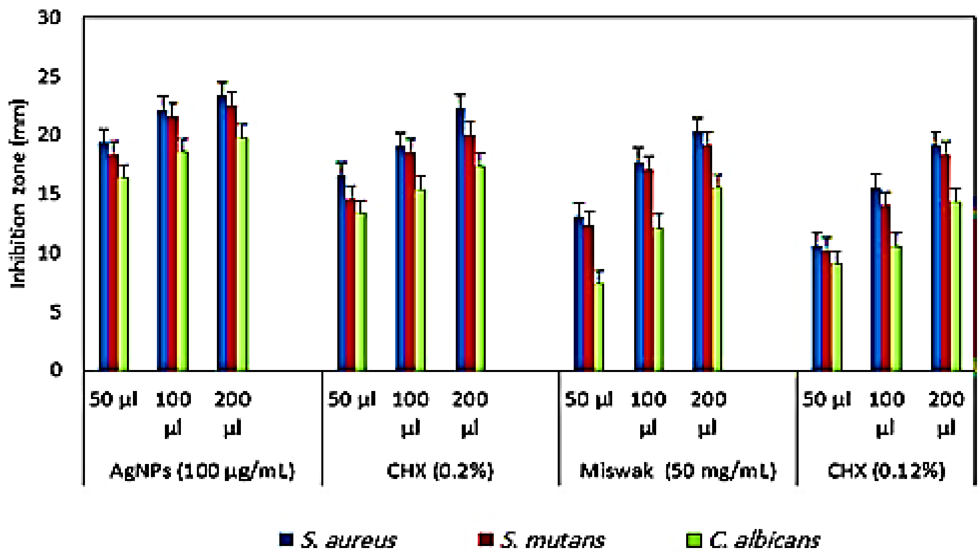

Fig. 1: Mean diameter of inhibition zone (DIZ) of AgNPs (100 µg/mL), CHX (100 µg/mL) and miswak (50 mg/mL) against different oral microbes

Table 2: Mean diameter of inhibition zone (DIZ) of AgNPs (100 µg/mL), Chlorhexidine 0.2% and miswak (100 µg/mL) against different oral microbes.

Microbes	AgNPs, Mean (SD)			Chlorhexidine 0.2%, Mean (SD)			Chlorhexidine 0.12%, Mean (SD)			Miswak, Mean (SD)		
	50 µl	100 µl	200 µl	50 µl	100 µl	200 µl	50 µl	100 µl	200 µl	50 µl	100 µl	200 µl
a- S. aureus	19.4 (0.5)	22.2 (0.4)	23.4 (0.5)	16.6 (0.5)	19.1 (0.1)	21.4 (0.5)	10.6 (0.4)	15.6 (0.5)	19.2 (0.8)	13.1 (0.1)	17.8 (0.4)	20.4 (0.5)
b- S. mutans	18.4 (0.5)	21.6 (0.5)	22.6 (0.5)	14.6 (0.5)	18.6 (0.5)	20.1 (0.1)	10.2 (0.5)	14.1 (0.1)	18.4 (0.5)	12.4 (0.5)	17.1 (0.1)	19.2 (0.5)
c- C. albicans	16.4 (0.5)	18.6 (0.5)	19.8 (0.4)	13.4 (0.5)	15.4 (0.5)	16.6 (0.5)	9.1 (0.5)	10.6 (0.5)	14.4 (0.5)	7.4 (0.5)	12.2 (0.3)	15.6 (0.5)
ANOVA F value	2.17	2.28	5.63	2.16	2.35	2.27	1.12	5.32	5.13	2.19	5.78	5.78
P value	0.07	0.06	0.04	0.11	0.06	0.09	0.11	0.04	0.04	0.12	0.04	0.04
Tukey post hoc	NA	NA	c < a and b	NA	NA	NA	NA	c < a and b	c < a and b	NA	c < a and b	c < a and b
SD – standard deviation, NA – not applicable.												

FT-IR analysis of miswak

Alkaloids (salvadorine), benzyl isothiocyanate, benzyl cyanates, and sulfur (Table 3 and Figure 2) are more considerable in the extract of miswak, that are all responsible for the growth inhibition of bacterial and fungal strains (figure 3).

Antimicrobial activity of test solutions

The mean DIZ of chlorhexidine 0.12% against *S. mutans* was significantly (p= 0.04) higher than *C. albicans* at 100 µl and 200 µl solutions (Table 2).

Table 4 and Figure 4 shows the mean DIZ of tested solutions against different oral microbes at 200 µl solutions. The mean DIZ of AgNPs against *S. aureus* was 23.4 ± 0.5 and it was significantly higher than chlorhexidine 0.12% (19.2 ± 0.8, p= 0.03). The mean DIZ of AgNPs against *S. mutans* was 22.6 ± 0.5 and it was significantly higher than chlorhexidine 0.12% (18.4 ± 0.5, p = 0.04). The mean DIZ of AgNPs against *C. albicans* was 19.8 ± 0.4 and it was significantly higher than chlorhexidine 0.12% (14.4 ± 0.5, p= 0.04).

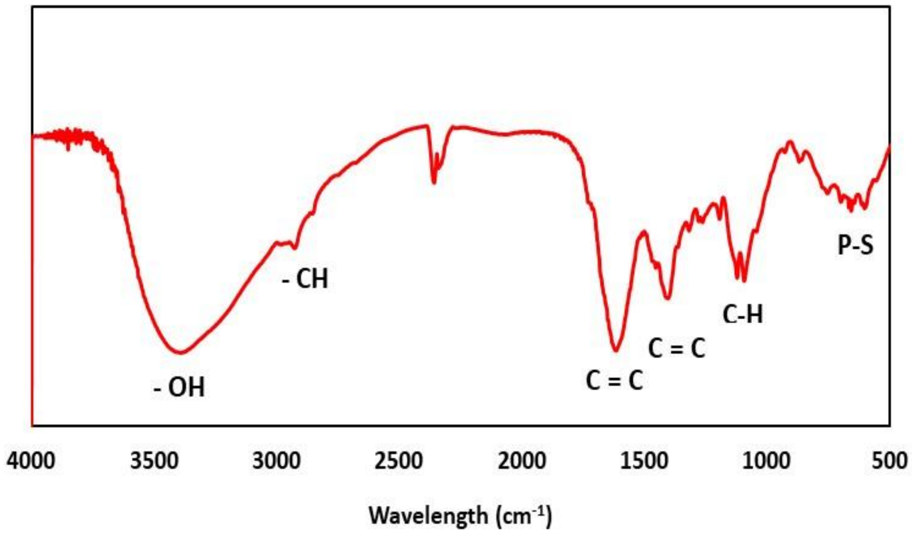

Fig. 2: FTIR of miswak extract

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Table 3: Peaks were obtained by miswak FTIR analysis and corresponding functional groups.

Peak (cm^{-1})	Functional group	Compounds
3381.73	• O–H stretch • stretch vibration of N–H	• Phenols • benzyl amides and three methylamine
2925.67	Aldehyde –CH	aliphatic compounds
2357.58	N=C	Isothiocyanate and isocyanate
1616.08	Alkene C = C	Aliphatic compounds
1403.98	C–H vibration in benzene ring skeleton	lignans, salvadorine, benzyl amides, benzyl cyanates
1093.42	C-H bending	Aromatic compounds
657.61	P = S	Compounds containing phosphorus and sulfur

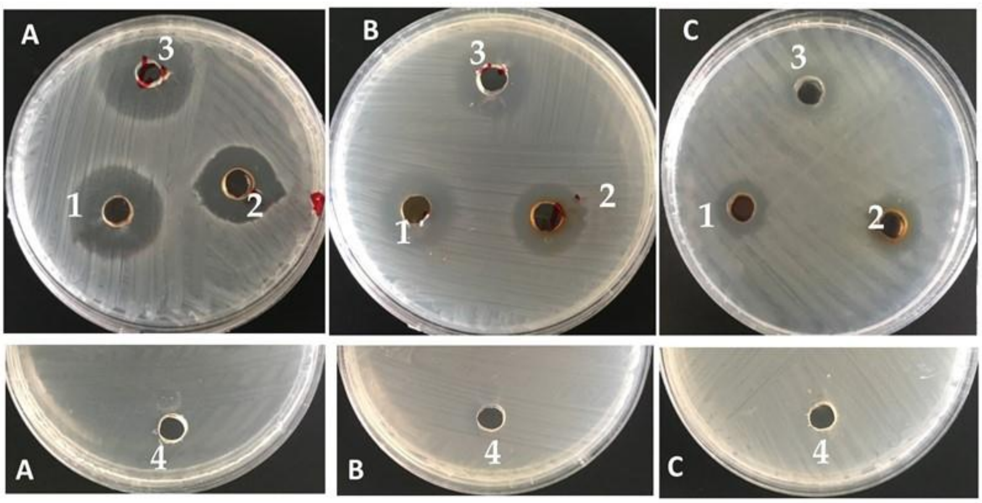


Fig. 3: Inhibition zone of 1-AgNPs with miswak, 2- AgNPs with 0.12% CHX, 3- 0.12% CHX with miswak 4- Negative control against A- *S. aureus*, B- *S. mutans* and C-*C. albicans*.

Table 4: Mean diameter of inhibition zone (DIZ) of tested solutions against different oral microbes at 200 µl solutions.

Microbes	AgNPs (100 µg/ml)	Miswak (50 mg/ml)	0.2% CHX	0.12%CHX	ANOVA F value	ANOVA p value	Tukey post Hoc
	200 µl	200 µl	200 µl	200 µl			
S. aureus	23.4 (0.5)	20.4 (0.5)	21.4 (0.5)	19.2 (0.8)	7.12	0.03	AgNPs > 0.2% CHX, Miswak > 0.12% CHX
S. mutans	22.6 (0.5)	19.2 (0.5)	20.1 (0.1)	18.4 (0.5)	6.13	0.04	AgNPs > 0.2% CHX, Miswak > 0.12% CHX
C.albicans	19.8 (0.4)	15.6 (0.5)	16.6 (0.5)	14.4 (0.5)	6.19	0.04	AgNPs > 0.2% CHX, Miswak > 0.12% CHX

SD – Standard deviation, CHX - Chlorhexidine.

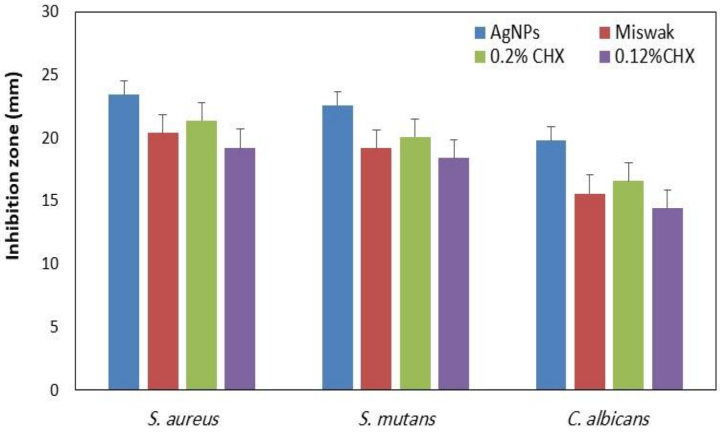


Fig. 4: Mean diameter of inhibition zone (DIZ) of tested solutions against different oral microbes at 200 µl solutions.

Antimicrobial activity of combination solutions

Table 5 and Figure 3 and 5 show the mean DIZ of the tested combination of solutions against different oral microbes at 200 µl solutions. Mean DIZ of AgNPs with miswak extract combination was significantly higher than miswak extract with chlorhexidine 0.2% or 0.12% combinations at all the tested microbe levels. The mean DIZ of AgNPs with 0.2% chlorhexidine was significantly higher than miswak with 0.2% or 0.12% chlorhexidine combinations at all tested microbe levels.

Table 5: Mean diameter of inhibition zone (DIZ) of tested solution combinations against different oral microbes at 200 µl solutions.

Microbes	AgNPs + miswak (a)	AgNPs + 0.2% CHX (b)	AgNPs + 0.12% HX (c)	Miswak + 0.2% CHX (d)	Miswak + 0.12%CHX (e)	ANOVA F value	ANOVA p value	Tukey post Hoc
	200 µl	200 µl	200 µl	200 µl	200 µl			
S. aureus	29.6 (0.3)	30.4 (0.4)	28.4 (0.5)	24.3 (0.5)	23.2 (0.3)	7.16	0.03	b > d, e a >d, e c > d, e
S. mutans	25.3 (0.5)	25.5 (0.4)	23.2 (0.3)	23.4 (0.4)	22.1 (0.2)	6.19	0.03	a > c, d b > d, e
C. albicans	24.8 (0.4)	25.4 (0.5)	22.2 (0.1)	19.4 (0.5)	18.4 (0.3)	5.23	0.04	b > d, e a > d, e c > d, e

SD – Standard deviation, CHX - Chlorhexidine.

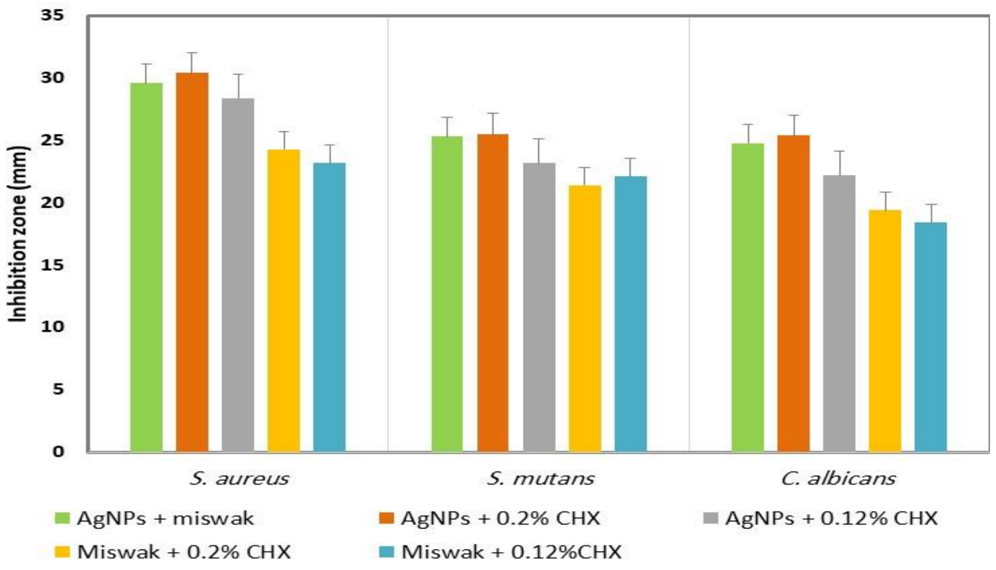


Fig. 5: Mean diameter of inhibition zone (DIZ) of tested solution combinations against different oral microbes at 200 µl solutions.

Discussion

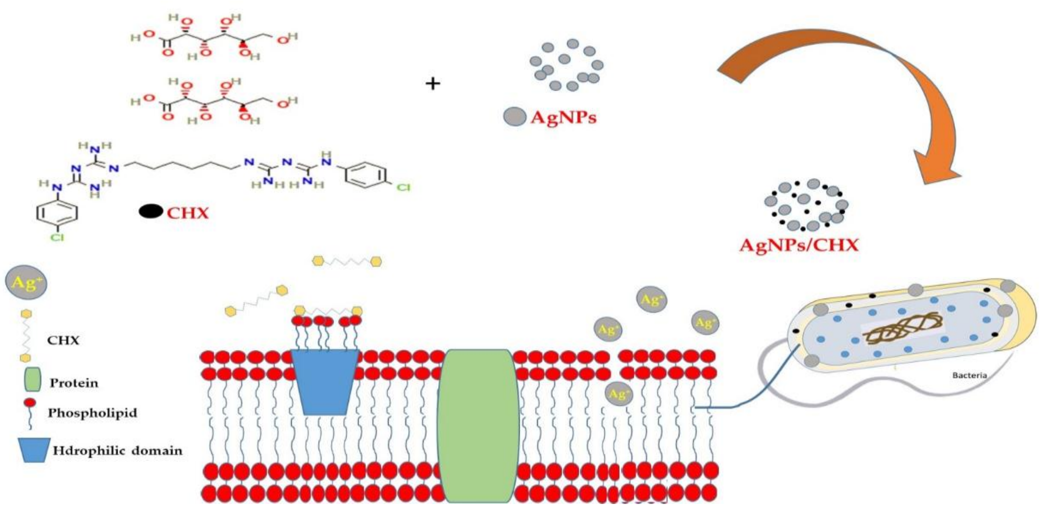
Development of antimicrobial resistance to synthetic alternatives shifted the research focus towards natural medicines and inorganic antimicrobials like AgNPs[5&15]. The present *in-vitro* study was the first to assess the antimicrobial efficacy of AgNPs, miswak extract, chlorhexidine gluconate alone, and their combinations against oral microbes.

Our study showed superior antibacterial efficiency of miswak against tested *S. aureus* and *S. mutans* strains than antifungal efficiency against *C. albicans* because of the presence of Alkaloids (salvadorine), benzyl isothiocyanate, benzyl cyanates and sulfur. These data are agreeing with Abhary and Al-Hazmi[11]. These results were in agreement with previous studies which reported different zone of growth inhibition against different oral microbes because of discrepancy in the membrane permeability of the studied microorganism[27]. Also, Constituents of miswak such as cyanides, chlorides, sulfur, and fluorides, possess an antimicrobial efficacy[28] by inhibiting oxygen uptake and disrupting the transport system, and disrupting the bacterial cell wall[11]. A recent systematic review showed that chlorhexidine was more effective against oral microbes than miswak extract with a mean difference of 0.19 ($P = 0.04$, 95% CI: 0.01 to 0.37)[29]. In contrast to this, the present study showed no significant difference in antibacterial efficacy of miswak extract and chlorhexidine. However, antifungal activity against *C. albicans* was significantly higher for chlorhexidine gluconate at a concentration of 0.2% than miswak extract. In the current study, no significant difference was observed between antimicrobial efficacy of different chlorhexidine concentrations used, prompting the use of a lower concentration of chlorhexidine (0.12%) to avoid the adverse effect of high concentration (0.2%). A combination of miswak extract and chlorhexidine with 0.2% and 0.12% concentrations showed significantly higher antimicrobial efficiency than when the solutions are used alone. This indicates that the combination of two solutions synergized the antimicrobial efficiency of solutions. Ashour et al.[30] reported that each solution of miswak extract and chlorhexidine (0.2%) with glass ionomer cement showed antibacterial activity.

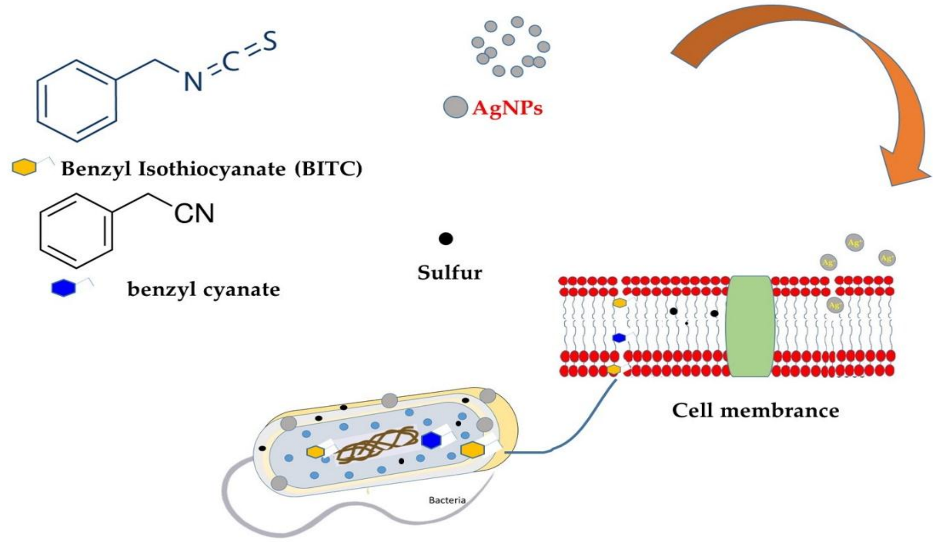
Previous *in-vitro* research showed that nano-sized silver particles penetrate the microbial cell membranes/cell wall by thiol groups or sulfur-containing proteins, ultimately by damaging the microbial DNA and leading to cell death[8]. In agreement with the recently conducted study by Panpaliya et al.[31], present result showed that AgNPs has significantly higher antibacterial and antifungal efficacy in comparison to chlorhexidine tested concentrations. This may be attributed to the nano-size of silver particles which can penetrate a deeper layer of microbes leading to its destruction[9]. Though, chlorhexidine has good antimicrobial properties, because of its size, it cannot penetrate deeper layers of microbes[31]. When AgNPs were combined with chlorhexidine, the antimicrobial efficacy was enhanced (scheme 1) in comparison to using the solutions alone. Chlorhexidine interacts with the negatively charged cytoplasmic membrane. Nano-sized silver particles penetrate the microbial cell membranes and/or cell wall. The current result is agreeing with a recently conducted study by Ashour et al.[30] showed the incorporation of chlorhexidine and AgNPs enhanced its antimicrobial efficiency against oral microorganisms compare to each solution of chlorhexidine and AgNPs individually.

Our study results exhibited that the combination of AgNPs and miswak extract components significantly enhanced their antimicrobial efficiency (scheme 2) due to synergized effect of solutions. AgNPs, and miswak extract can be used as a promising antimicrobial agent in dental applications instead of AgNPs and chlorohexidine because of using chlorohexidine cause dryness of mouth, dental stains, changes in taste, and gingival irritation[32&33].

The limitation of the current study is its *in-vitro* nature; it was not possible to mimic all the oral conditions in the lab environment.



Scheme 1: CHX (as a cationic agent) interacts with the negatively charged cytoplasmic membrane. Nano-sized silver particles penetrate the microbial cell membranes/cell wall.



Scheme 2: Benzyl isothiocyanate, sulfur, and benzyl cyanates interact and penetrate cytoplasmic membrane. Nano-sized silver particles penetrate the microbial cell membranes/cell wall

Conclusion

To conclude, the present result exhibited the combination of miswak, and AgNPs with chlorhexidine increases the antimicrobial efficiency of combined solutions against common oral microbes. AgNPs, and miswak displayed insignificant antimicrobial activity in comparison to AgNPs, and chlorohexidine so we advise to use AgNPs, and miswak as a promising antimicrobial agent in dental applications.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Taif University (protocol code-41-1107-00152 and 1/12/2019).

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التأثيرات المضادة للميكروبات والتأثير التآزرية للمسواك والفضة النانوية، والكلورهيكسيدين بمفرده وتوليفاتهم على بعض الميكروبات الفموية

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تهدف الدراسة الحالية إلى مقارنة الفعالية المضادة للميكروبات لكل من المسواك و جسيمات الفضة النانوية والكلورهيكسيدين بتركيزات مختلفة بمفردها وبالاقتران مع بعضها البعض ضد نوعين من البكتيريا و هي العقدية الطافرة و المكورات العنقودية الذهبية وفطر واحد هو كانديدا البيكانز. تم استخدام مستخلص السواك المحضر يدويا، وجلوكونات الكلورهيكسيدين بتركيزات (٠.٢ ٪ و٠.١٢٪)، وجسيمات الفضة النانوية (٢٠-٥٠ نانومتر). تم تحضير التركيبات التالية: جسيمات الفضة النانوية مع الكلورهيكسيدين، المسواك مع الكلورهيكسيدين، المسواك مع جسيمات الفضة النانوية. تم تقييم تأثيرها التآزري وتم تحديد كفاءاتها المضادات للميكروبات باستخدام طريقة الانتشار في حفر الاجار. تم تحليل متوسط الفروق باستخدام انوفا. عندما تم استخدام المحاليل كل على حدا، كان متوسط قطر منطقة التثبيط لجسيمات الفضة النانوية والكلورهيكسيدين والمسواك ضد البكتيريا العقدية الطافرة والمكورات العنقودية الذهبية أعلى بشكل ملحوظ من كانديدا البيكانز وكان لـ جسيمات الفضة النانوية تأثير أكبر من المادتين الأخريين المختبرتين (جسيمات الفضة النانوية > الكلورهيكسيدين> المسواك) على البكتيريا المختبرة. كان متوسط قطر منطقة التثبيط لتركيبة جسيمات الفضة النانوية مع مستخلص المسواك أعلى بكثير من تركيبات السواك مع الكلورهيكسيدين ٠.٢٪ أو ٠.١٢٪ على جميع مستويات

الميكروبات المختبرة. كان متوسط قطر منطقة التثبيط لتركيبة جسيمات الفضة النانويـــة مـــع ٠.٢ ٪ كلورهيكسيدين أعلى بكثير من تركيبات المسواك مع الكلورهيكسيدين ٠.٢ ٪ أو ٠.١٢ ٪ وذلك علـــى جميع مستويات الميكروبات المختبرة. أخيرًا، فإن الجمع بين جسيمات الفضة النانوية مـــع مســـتخلص المسواك له فعالية فائقة كمضادات للميكروبات عند مقارنته بالتركيبات الأخرى المختبرة. لذلك، يمكـــن استخدام جسيمات الفضة النانوية ومستخلص المسواك كمواد حيوية واعدة مضـــادة للميكروبـــات فـــي تطبيقات طب الأسنان.