GROWTH INHIBITORY EFFECT OF CHLORHEXIDINE AND HEXETIDINE CONTAINING-MOUTHWASHES AGAINST ORAL BACTERIAL ISOLATES

Ibtesam O. Amer[1*], Raja M. Moman[2], Mustafa M. Gebreil[3], and Mohamed R. Alsagher[4]

[1] Department of Biotechnology & Genetic Engineering, Unit of Microbial Biotechnology, Faculty of Medical Technology-Surman, Sabrata University, Libya.
[2,4] Faculty of Pharmacy, Department of Microbiology & Immunology, University of Tripoli, Libya.
[3] Faculty of Pharmacy, Department of Biochemistry, University of Tripoli, Libya.
[*]ibtisam.amer@sabu.edu.ly

Abstract

Background

Frequent use of mouthwash (MW) is one of the most effective methods used to prevent oral bacterial infections and to assist individuals in their efforts to achieve and maintain better oral health. Using a MW containing antibacterial agents would be a simple way to prevent growth and multiplication of pathogenic organisms in oral cavity causing dental caries and other mouth diseases. Chlorhexidine (CHX) and Hexetidine (HX) have been proposed as potent biocides against oral bacteria.

Objective

The present study was performed to investigate oral bacteria growth inhibition when using any of four mouthwashes that are commercially available in the Libyan market and contain either CHX, (*Zordy l*and *Oraxin*), or HX, (*Hextril* and *Givalex*), and to clarify whether CHX and HX were suitable and safe biocides that can be included in mouthwash products.

Materials and Methods

Sixty adult (45 females and 15 males) volunteers had been chosen and divided into four groups and their saliva samples were assessed for microbial count at the beginning and the end of two weeks of treatment, during which they rinsed with 15ml of mouthwashes for 30 seconds twice a day (morning and evening) in addition to their usual oral hygiene procedures. The antibacterial activity of mouthwashes was assayed by cell viable count technique and cell diffusibility measurement.

Results

The results showed wide variations in the effectiveness of mouthwashes; those containing CHX were more effective ($P \leq 0.05$) than formulations containing HX on oral microbial count. The main findings of the present study were that *Zordyl*, *Oraxin*and *Hextril* exerted high effects on the salivary microbiota, causing 90%, 60% and 34% reduction in salivary bacterial counts respectively. 25% reduction was observed for *Givalex*. On the other hand, the zone of inhibition test showed that *Zordyl* and *Oraxin* had large zone inhibitory effects, while *Hextril* and *Givalex*, were less effective on some bacterial species.

Conclusion

It can be concluded that twice daily use of CHX mouthwash (CHX-MW) or HX mouthwash (HX-MW) reduces oral bacterial load counts in healthy subjects when used as an adjunct to their normal oral hygiene procedures. This also suggests that inhibitory power of mouth washes containing CHX is greater on oral bacteria than mouthwashes containing HX.

Keywords: Mouthwashes; Oral cavity hygiene; Antibacterial of mouthwashes; Antibacterial of biocides.

Introduction

The person's general good health and well-being is strongly related to oral health and hygiene. Fact sheet of World Health organization revealed that 60 to 90% of dental cavities was found in school children and nearly 100% in adults worldwide (Chocolatewala N, *et al.*, 2010; Hong CH, *et al*., 2010). The accumulation of microorganisms in dental plaque is related to the aetiology of oral diseases, with a high prevalence globally. The occurrence of various diseases such as dental caries, periodontal diseases, and even oral cancers are usually due to hundreds of bacterial species presented in the oral cavity (Chocolatewala N, *et al.*, 2010).

A bad habit of brushing practiced by majority of people leads to nearly 50% of teeth's surfaces left unclean, thus an additional oral health care can be obtained by the use of a mouthwash (Chen-Ying Su, *et al.*,2019). Usually choosing an appropriate mouthwash relies on user's needs. Therefore, a lot of debate exists about whether mouthwashes are helpful for oral care because mouthwashes include chemicals in their formulations that can cause side effects (Sanz M,2013, Slots J, 2002 &Tehrani MH, *et al.*,2011). Although regular brushing is highly recommended, patients who are facing difficulties in brushing their teeth still benefit from using mouthwashes. Patients who receive radiation for head and neck treatment, their salivary glands could be damaged by this radiation, thus they will be at high risk for the development of dental caries (Hong CH, *et al.*,2010). In addition, cancer patients may develop oral mucositis as another adverse side effect of getting treated with chemotherapy or radiotherapy (Sarvizadeh M, *et al.*,2015). A quick and efficient method to prevent oral bacterial infections

and to assist individuals in their efforts to obtain better oral health can be achieved by using mouthwashes containing antibacterial agents to inhibit the growth and multiplication of pathogenic organisms causing dental caries and other mouth diseases (Joyston-BechalS , *et al.*,1992m Akande, O. O., 2004 &Jong, M. H., , *et al*, 1984). Despite irritation caused by alcohol to many users, still most of commercial mouthwashes contain this biocide in their formulations to inhibit bacterial growth; therefore, it is recommended that the newly prepared mouthwashes must include other biocides that can replace alcohol as antibacterial agent such as Poly-gamma-glutamic acid (g-PGA) which is derived from *Bacillus anthracis* (Shih IL *et al.*,2001). Today, a variety of chemical substances which are usually added to mouthwashes to inhibit microbial growth have also been subjected to numerous investigations against different types of bacteria. Out of all these chemical biocides, CHX and HX are the most potent antimicrobial agents included in a wide range of mouthwash solutions; therefore, they are subjected to extensive research studies (Roland Pianoyyi and Garypitts,1977). The interaction and uptake of CHX by bacteria was shown to be extremely rapid, with a maximum effect occurring within 20 seconds (Fitzgerald, K. A, *et al.*,1989). CHX molecules are positively charged whereas bacterial surface and most surface structures in the oral cavity, including the surfaces of teeth and mucous membranes, are negatively charged. In accordance with the principle of that opposite charges attract each other, CHX binds strongly to all these surface structures (Fitzgerald, K. A, *et al.,* 1989). In this study, we compared the use of CHX and HX as chemical biocides included in some mouthwashes, anticipating that these biocides can exert good inhibitory effect on bacterial strains isolated from oral cavity.

Materials and Methods

Microorganisms

The following bacterial strains were used in this study: Streptococcus mutans, Staphylococcus aureus, Staphylococcus albus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, and Serratia marcescens.

Maintenance and Growth of Organisms

One ml of bacterial culture was taken and inoculated into 10 ml of sterile Mueller-Hinton (MH) broth and incubated for 18 to 24 h at 37^{o}C. From this overnight culture, loopfuls were streaked onto 15 ml MH agar slopes and incubated at 37^{o}C for 18 h. The slopes were kept at 4^{o}C before being used. At the beginning of each week a fresh agar slopes were taken and sub-cultured daily in 5 ml MH broth after incubated at 37^{o}C for further 18 h. A Regular check for organism's identity was maintained throughout this work and each time a new bacterial culture was used. The identification of the organisms was usually carried out either by biochemical tests or microbiological examination. The latter involved the use of gram stain technique whereas the biochemical characteristics of organism were determined by using AulatkaProfalIndaks strips (API system S.A., Montalieu-Vercieu, France).

Mouthwash Rinses Procedure (in-Vivo Test)

The in-vivo study was conducted using volunteer's saliva which then was used to evaluate the effect of different mouthwashes on the bacteria most frequently found in the oral cavity and commonly associated with mouth infections. Sixty adult (45 females and 15 males) volunteers aged between 18-55 years were studied, in 14 days-self-controlled studies, mostly have no history of periodontal diseases, and had not taken antibiotics for the last three months prior study. All volunteers were asked to rinse their mouths for 30 seconds twice a day in the morning (after breakfast) and in the evening (after dinner) with 15 ml of the provided mouthwash. In addition, they were asked not to eat, drink or rinse with water for 30 minutes of their mouthwash use. Each volunteer was provided with a 15 ml volume dispenser to be used in each rinse otherwise rising procedure and volume will not be identical in all groups.

The Measurement of Antibacterial Activity (in-Vitro Test)

Monitoring bacterial growth by measuring the turbidity in the form of viability or diffusibility of bacterial cultures is a quick method, and it has been applied for testing antibacterial activity of materials (Wahab R, et al., 2016 & Wahab R,2013).

Cell Viability Assay

In this assay, viable counting method was used. Triple 20 µl aliquots of 10-fold dilutions (10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) of the bacterial suspension were dispensed onto supper-dried agar plates, and colonies were counted as colony-forming unit per ml (cfu/ml) after overnight incubation at the appropriate temperature.

Cell diffusibility Assay

In this method, 200µl of bacterial suspension of 0.5 McFarland standard that contains approximately 10^8 to 10^9 CFU/ml was swabbed onto the surface of the nutrient agar plate and left to dry. Cups were then bored in the agar plate, and each cup was filled with a specific amount of mouthwash. The plate was incubated at 37ºC for 24 hours. The antibacterial activity was determined by measuring the diameter of the zone of inhibition.

Statistical Analysis

Statistical analysis was carried out by using SPSS for windows version 14 program. The significance level (0.05 parametric) was used to indicate statistical significance. The one-way ANOVA was used to define the effect of mouthwash on all groups.

Results

Saliva samples which serve as control were collected on the first day before the subjects use the mouthwashes; post treatment samples were collected after two weeks. Specimens were then subjected to serial dilution and plating on a variety of selective agar tops to isolate the naturally occurring bacteria which later on were exposed to in-use concentration of CHX-MW or HX-MW to evaluate both viable cell count and zone of inhibition developed. CHX-MW rinses had a substantial effect on the count of bacteria after use. At the end of the study the levels of total oral bacterial count in the saliva samples were significantly lower in groups who used CHX-MW products. Zordyl and Oraxin were the most effective products achieving high reduction in bacterial viability. Zordyl and Oraxin rinses exhibit appreciable antibacterial activity of 90% and 60% respectively Figure (1). While HX-MW products; Hextriland Givalex, which were less effective, exerted 34% and 25% reduction in the bacterial cell count respectively Figure (1).

Table (1): Killing Times for Different Mouthwashes Against Isolated Bacteria from Volunteer's Saliva

Mouthwashes		Killing Times (min) at which Mouthwashes Exhibit Bactericidal Effect on Bacterial Isolates						
		S. Albus	E. Coli	S. Mutans	K. Pneumonia	S. Aureus	S. Marcescens	Ps. Aeruginosa
CHX-MW	Zordyl	4	4	4	4	8	8	8
	Oraxin	4	4	8	8	8	15	15
HX-MW	Hextril	15	15	15	30	15	15	15
	Givalex	15	15	15	30	30	30	30

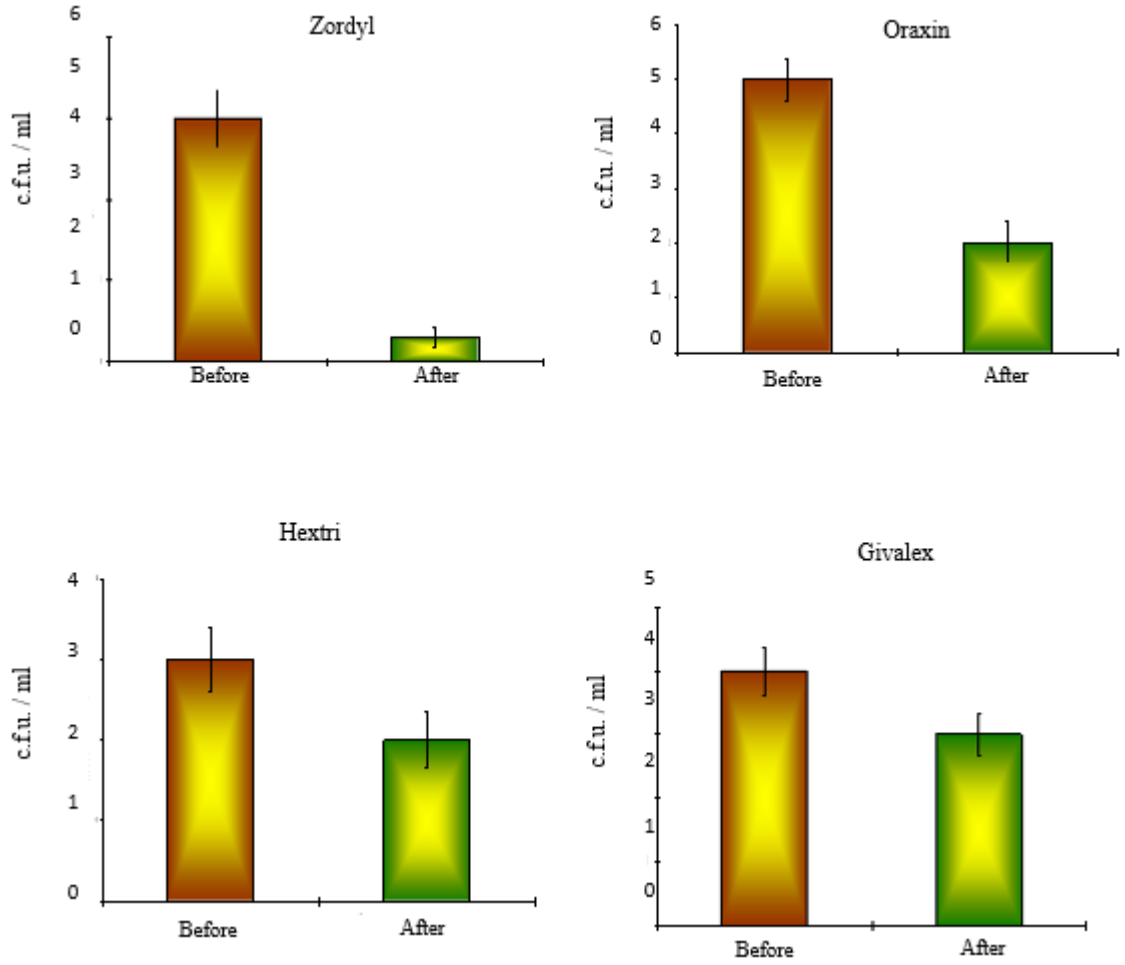


Figure (1): Salivary Bacterial Count of Volunteers before and after Rinsing with Four Mouthwashes.

In order to determine the growth inhibitory activity of the four mouthwashes, suspensions of oral isolated bacteria at final cell density of (10^7 cfu.ml^{-1}) were challenged with the in-use concentrations of mouthwashes solutions and viability was determined by colony counting after 2, 4, 8, 15, 30 and 60 minutes of incubation time as illustrated in figures (2). Figure (2) shows the inhibitory activities of CHX-MW (Zordyl and Oraxin) and HX-MW (Hextriland Givalex) rinses against oral bacterial isolates. This was determined by colony counting over a period of 60 minutes incubation time.

When mouthwashes were tested in volunteers, an immediate significant fall in salivary bacterial counts was produced mainly by Zordyl and Oraxin mouthwashes. The bacterial count reached to bactericidal levels after 4 minutes for Zordyl and Oraxin against S. albus, K. pneumonia and S. marcescens and for Oraxin against E. coli; after 8 minutes for Zordyl and Oraxin against S. aureus, S. albus, S. mutans, E. coli, Ps. aeruginosa, K. pneumonia and S.

marcescens and for GivalexagainstS. mutans; after 15 minutes for Hextril against S. aureus; and Oraxin against S. albus; Givalex against E. coli; for Zordyl against Ps. aeruginosa; and for HextrilandGivalex against S. marcescens; after 30 minutes GivalexandHextril against S. aureus; Ps. aeruginosa and K. pneumonia. On the other hand, zone of inhibition technique was used to assess the antibacterial effects of the four mouthwash products on the bacterial isolates of volunteer's saliva, as shown in table (2) and plates in figure (3). The antimicrobial effect of these mouthwashes was compared with phenol solution (0.5%) as a control that has an effect on the bacterial growth with a zone of inhibition ranging from 10-20 mm. Individual assessment of antibacterial activity of these agents revealed that CHX-MW rinses, Zordyl and Oraxin, were the most effective causing inhibition on the growth of all isolated bacteria. The zone of inhibition was ranging from 17 to 40 mm. By contrast, HX-MW rinses were less effective i. e. Hextril exerts low antibacterial activity giving inhibition zone ranging between 9 to 20 mm. Furthermore, Givalex had low activity against gram positive bacteria and had no effect against gram negative bacteria.

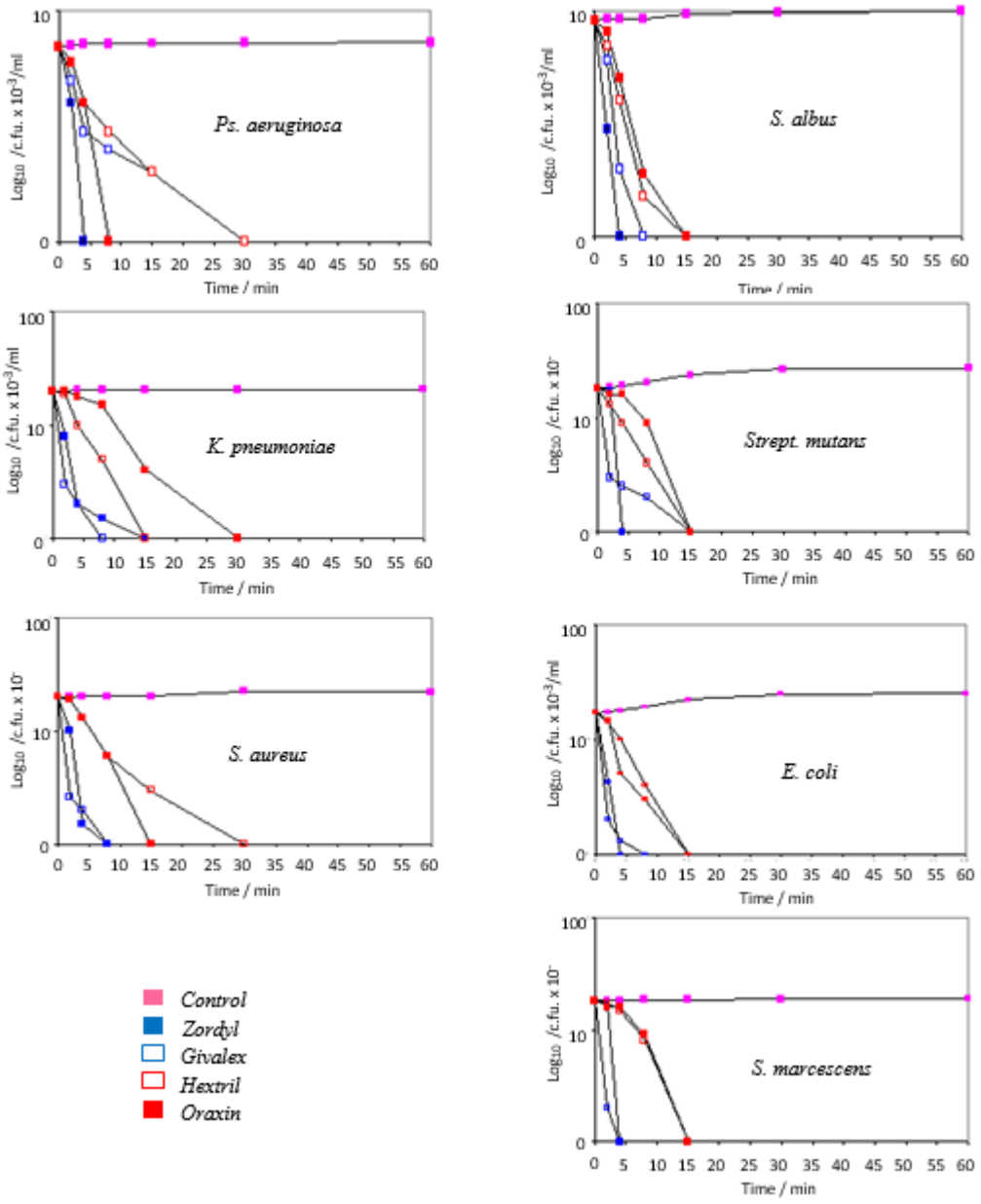


Figure (2): Growth Inhibitory Effect of Different Mouthwashesagainst Bacteria Isolated from Volunteer's Saliva.

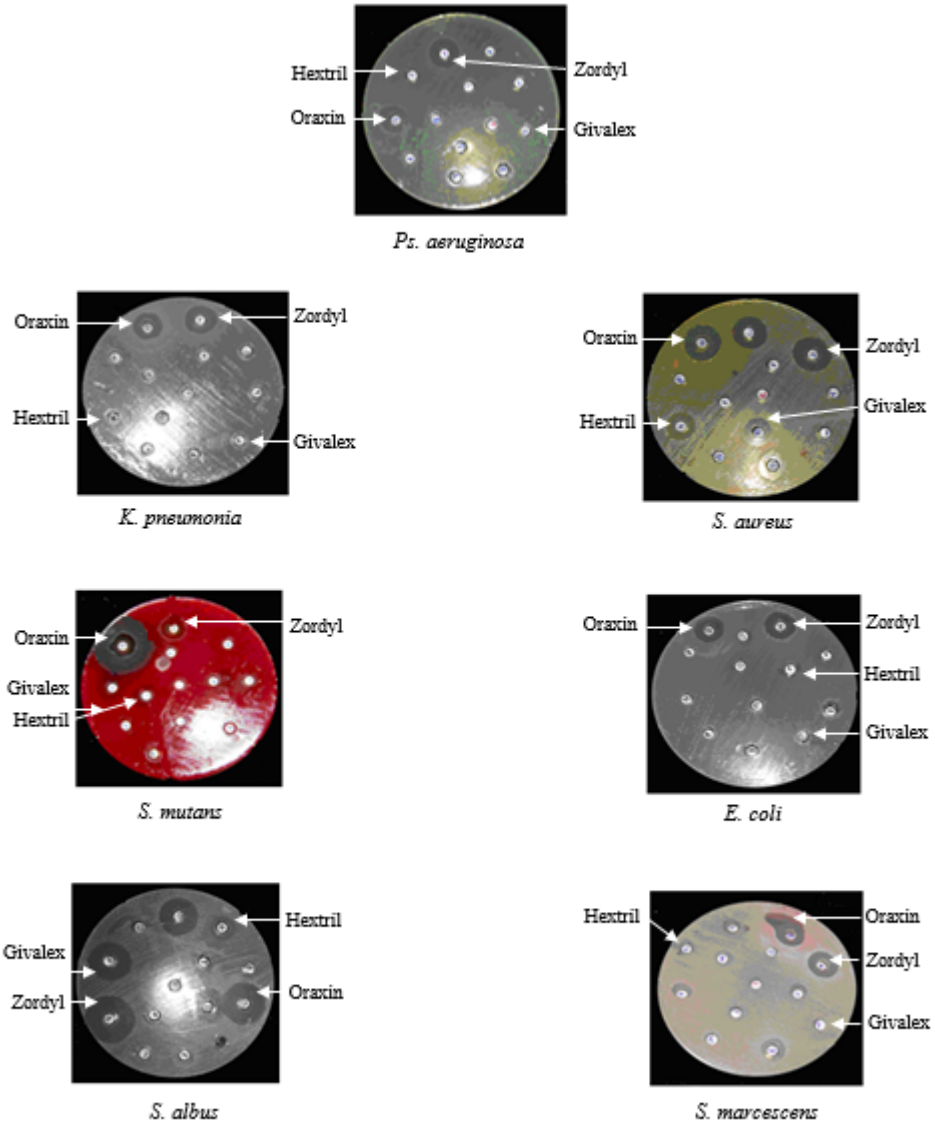


Figure (3): Zone of Inhibition of Different Mouthwashes Against Different Bacteria.

Table (2): Antibacterial Activity of Mouthwashes on Oral Bacterial Isolates Using Cup-Cut Agar Diffusion Method

Mouthwashes		Inhibition Zones (mm) of Mouthwashes						
		S. albus	E. coli	S. mutans	K. pneumonia	S. aureus	S. marcescens	Ps. aeruginosa
CONTROL	Phenol5%	18	20	14	16	10	16	18
CHX-MW	Zordyl	40	25	17	20	30	20	20
	Oraxin	30	20	20	20	30	20	17
HX-MW	Hextril	13	6	9	10	20	16	0
	Givalex	25	0	10	0	20	0	0

Discussion

A total of 60 volunteers (45 males and 15 females) were involved in this study. Subjects were divided into 4 groups and their saliva samples were assessed for microbial counts at the beginning and the end of two weeks of MW use.

Saliva specimens were subjected to serial dilution and plating on a variety of selective and non-selective agar tops to isolate the naturally occurring bacteria which were then exposed to a concentration of CHX-MW and HX-MW to evaluate both viable counting and zone of inhibition developed. CHX-MW showed a better effect on reducing oral bacterial count than HX-MW. It was also observed that in subjects who rinsed with HX-MW, total viable count of salivary bacteria was reduced in much less degree than CHX-MW. It was not surprising that CHX could inhibit bacterial growth since CHX mouth rinses reduced the adhesion and proliferation of human gingival fibroblasts and keratinocytes (Balloni S et al., 2013). However, the safety of CHX-MW has been proven for short-term use or usage at lower concentrations (NajafiMHm et al., 2012 & Quirynen M, et al., 2001). Moreover, the antibacterial activities of these mouthwashes were evaluated using agar cup-cut diffusion assay to determine their zone of inhibition against some of the salivary isolated bacteria which showed that CHX-MW; Zordyl and Oraxin had good inhibitory effect against the tested bacterial strains, namely Ps. aeruginosa,K. pneumonia,S. mutans, S. aureus,S. albus, E. coli and S. marcescens, with zones of inhibition ranging from 17 to 40 mm in diameter for Zordyl and 17 to 30 mm in diameter for Oraxin (Table (1)). In contrast, HX mouthwashes were unable to produce zone of inhibition against the tested bacterial species. Therefore, Zordyl and Oraxinmight are promising mouthwashes for the development of antibacterial effect against human oral pathogens. Furthermore, CHX was proved to be an excellent biocide that can be

applied and used in MW preparations because it could kill all the bacteria in the salivary bacterial isolates in a very short time: 4 min (Table (2)). By contrast, the antibacterial effect HX-MW; Hextril and Givalex took more time to the isolated oral bacteria; 15-30 minutes (in which the time profile is very important in the use of mouthwash, because of their way of application) the shorter the time the better the effect.

Conclusion

From this study, it can be concluded that bacterial growth could be inhibited by using CHX-MW and HX-MW. However, inhibition of bacterial growth was raised in the presence of CHX. Therefore, the use of CHX-MW and HX-MW in their in-use concentrations is an adequate and safe way to maintain oral hygiene that can reduce the bacterial load of oral cavity.

References

- Akande, O. O., A. R. Alada, G. A. Aderinokun, and A. O. Ige. (2004). Efficacy of different brands of mouthrinses on oral bacterial load count in healthy adults. African Journal Biomed. Res.; 125: 125─128.

- Balloni S, Locci P, Lumare A, Marinucci L. (2016). Cytotoxicity of three commercial mouthrinses on extracellular matrix metabolism and human gingival cell behaviour. Toxicol Vitro; 34: 88-96.

- Chen-Ying Su, Chia-Chun Chen, Hsuan-Yu Chen, Chun-Pin Lin, Feng-Huei Lin, Hsu-Wei Fang. (2019). Characteristics of an alternative antibacterial biomaterial for mouthwash in the absence of alcohol. J. Dent. Sci.; 14: 192-197.

- Chocolatewala N, Chaturvedi P, Desale R. (2010). The role of bacteria in oral cancer. Indian J Med PaediatrOncol; 31: 126-31.

- Fitzgerald, K. A., A. Davies, and Russell. (1989). Uptake of ^{14}C- chlorhexidine diacetate to Escherichia coli and Pseudomonas aeruginosa and its release by azolectin. FEMS Microbiology Letters; 60: 327–332.

- Harper, D. S., and Loesche, W. J. (1984). Growth and acid tolerance of human dental plaque bacteria, Arch. Oral Biol; 29: 843─848.

- Hong CH, Napenas JJ, Hodgson BD. (2010). A systematic review of dental disease in patients undergoing cancer therapy. Support Care Canc; 18: 1007-21.

- Jong, M. H., Van Der Hoeven, J. S., van Os, J. H. andOlijve, J. H. (1984). Growth of oral Streptococcus species and Actinomycesviscosus in human saliva. Environ. Microbiol.; 47: 901─904.

- Joyston-Bechal S, Hayes K, Davenport ES, Hardie JM. (1992). Caries incidence, mutans streptococci and lactobacilli in irradiated patients during a 12-month preventive programme using chlorhexidine and fluoride. Caries Res; 26: 384-90.

- Najafi MH, Taheri M, Mokhtari MR, et al. (2012). Comparative study of 0.2% and 0.12% digluconate chlorhexidine mouth rinses on the level of dental staining and gingival indices. Dent Res J; 9: 305-8.

- Quirynen M, Avontroodt P, Peeters W, Pauwels M, Coucke W, van Steenberghe D. (2001). Effect of different chlorhexidine formulations in mouthrinses on de novo plaque formation. J ClinPeriodontol; 28: 1127-36.

- Roland Pianoyyi and Garypitts. (1977). Effects of an Antiseptic Mouthwash on Odorigenic Microbes in the Human Gingival Crevice Group, Personal Products Division, Department of Chemical and Biological Research, Warner-Lambert Company, Morris Plains, New Jersey 07950, USA.

- Sanz M, Serrano J, Iniesta M, Santa Cruz I, Herrera D. (2013). Antiplaque and antigingivitis toothpastes. Monogr Oral Sci; 23: 27-44.

- Sarvizadeh M, Hemati S, Meidani M, Ashouri M, Roayaei M, Shahsanai A. (2015). Morphine mouthwash for the management of oral mucositis in patients with head and neck cancer. Adv Biomed Res; 4: 44.

- Shih IL, Van YT. (2001). The production of poly- (gamma-glutamic acid) from microorganisms and its various applications. BioresourTechnol; 79: 207-25.

- Slots J. (2002). Selection of antimicrobial agents in periodontal therapy. J Periodontal Res; 37: 389e98.

- Tehrani MH, Asghari G, Hajiahmadi M. (2011). Comparing Streptococcus mutans and Lactobacillus colony count changes following green tea mouth rinse or sodium fluoride mouth rinse use in children (Randomized double-blind controlled clinical trial). Dent Res J; 8: S58e63.

- Wahab R, Khan F, Mishra YK, Musarrat J, Al-Khedhairy AA. (2016). Antibacterial studies and statistical design set data of quasi zinc oxide nanostructures. RSC Adv; 6: 32328-9.

- Wahab R, Khan ST, Dwivedi S, Ahamed M, Musarrat J, AlKhedhairy AA. (2013). Effective inhibition of bacterial respiration and growth by CuO microspheres composed of thin nanosheets. Colloids Surfaces B Biointerfaces; 111: 211-7.