Original Article

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Effect of different molecular weight of chitosan mouthwash formulations against *Streptococcus mutans*

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Objectives: This study aimed to examine the antibacterial and antibiofilm properties of chitosan mouthwash formulations against *Streptococcus mutans*, using chitosan polymers of different molecular weight.

Materials and methods: Chitosan mouthwashes at pH 6.0 were prepared from different molecular weights of shrimp chitosan (30 kDa, 890 kDa) at various concentrations (2.5, 5.0 mg/mL). Chlorhexidine mouthwash was used as a positive control. All formulations were evaluated for their antibacterial and antibiofilm properties against *Streptococcus mutans* ATCC 25175. Antibacterial property was determined by using the time-dependent killing effect at 0 minute, 30 minutes, and 2 hours. Reductive and preventive properties on biofilm were assessed by adapting the microtiter plate test for quantification of biofilm formation. Statistical analyses were performed using SPSS program version 17.0, with the significance level at $p<0.05$.

Results: All chitosan mouthwashes, of different molecular weight and concentration, could reduce the planktonic form of *Streptococcus mutans* within 2 hours. Chitosan mouthwashes reduced established *Streptococcus mutans* biofilm by up to 92.27%, depending on the exposure time. The preventive effect of chitosan mouthwash against new *Streptococcus mutans* biofilm formation was improved to 97%.

Conclusion: Both low molecular weight and high molecular weight chitosan polymer could be used for mouthwash formulations with effective antibacterial and antibiofilm properties. Moreover, chitosan mouthwash had a greater antibiofilm property than chlorhexidine mouthwash.

Keywords: Chitosan, mouthwash, Streptococcus mutans

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Introduction

Dental caries is a chronic infectious oral disease, caused by cariogenic bacteria in saliva and oral biofilm [1]. Based on the Thailand's 8th national dental status survey in 2017, dental caries is the major oral infection in Thai children and has been documented among 75.6% and 52.0% at ages five and twelve years old, respectively. *Streptococcus mutans* (*S. mutans*) is a primary colonizing bacteria that can synthesize extracellular polysaccharides from sucrose. The firm attachment of cariogenic bacteria to teeth and rapid fermentation of carbohydrates, leads to acidic conditions and imbalance between demineralization and the remineralization [2]. Currently, caries management is focused on the prevention concept rather than the restoration or surgical approach. Caries prevention can be performed by reducing the number of pathogenic microorganisms and dealing with biofilm [3]. One effective method is treatment with chemotherapeutic antimicrobial agents such as mouthwash [4]. Chlorhexidine mouthwash is effective for preventing dental plaque formation and gingivitis, and has been

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approved for safety by the American Dental Association in 1987 and the Food and Drug Administration (FDA) in 2000. Unfortunately, it has adverse effects, such as staining of teeth, tongue and restorations. It can cause erosion of oral mucosal and disturbs taste perception [5]. Moreover, chlorhexidine mouthwash should be used only for a short period of time or no more than 2 weeks [6] and not suitable for children younger than 6 years old who cannot control swallowing. Alcohol containing mouthwash may not be suitable for children. However, mouthwash may be helpful for children who are unable to clean their teeth properly due to disability of hands and brain function.

Chitosan is a natural polysaccharide, a biopolymer derived by deacetylation of chitin found in crustacean shells, such as shrimp shells [7]. Chitosan has been shown to exhibit antimicrobial activity in terms of bacteriostatic, bactericidal [8, 9], and anti-biofilm formation [10]. Kong *et al*, in 2010, demonstrated that chitosan has very low toxicity, good biocompatibility and biodegradability, without development of any resistance [11]. Different molecular weights of chitosan polymer were reported to show different antibacterial properties and different solubility. Low molecular weight chitosan has greater solubility and easily penetrates into bacterial cells. The high molecular weight chitosan can bind to the negatively charged on the bacterial cell wall and act as a chelating agent [12, 13]. Although there are several reports on the antibacterial and antibiofilm activity of chitosan on S. *mutans*. There are no study reports about the effect on mouthwash formulation. The aims of this study were to compare the effectiveness of chitosan mouthwashes prepared with very low molecular weight, less than 30 kDa, and high molecular weight higher than 890 kDa of chitosan polymer with higher than 90% acetylation to chlorhexidine mouthwash in terms of antimicrobial activity and biofilm formation. This may help in the development of alternative mouthwash which is environmentally friendly for children who are unable to properly clean their teeth in the future.

Materials and methods

Mouthwash preparation

Chitosan mouthwashes were prepared from commercially available food grade chitosan powders (Marine Bio Resources Co., LTD., Thailand) extracted from shrimp shells, having molecular weight of 30 kDa and 890 kDa, degree of deacetylation above 90% and size less than 150 µm. The chitosan mouthwash formulation was modified from Sano, 2003 [14]. Chitosan powder was dissolved in 1% acetic acid and prepared at the concentration of 2.5 and 5 mg/ml in non-alcohol mouthwash. All compositions were mixed at room temperature and adjusted to pH 6.0. A negative control was mouthwash based solution which had similar compositions and pH as chitosan mouthwash, but excluding the chitosan powder. A commercial 0.12% chlorhexidine mouthwash (MDent®, Thailand) was used for a comparison.

Microorganism cultivation

Streptococcus mutans ATCC 25175 purchased from American Type Culture Collection (ATCC) was grown in Brain Heart Infusion agar plates (BHI, Difco™) incubated at 37°C for 48 h. Two to three colonies were selected for inoculation in 5 ml BHI broth and incubated overnight (18 h) in an anaerobic jar at 37°C. Cells in the exponential growth phase were centrifuged, washed and re-suspended in phosphate buffered saline (PBS) to obtain S. mutans cells at about 10^6 CFU/mL. For biofilm formation, S. mutans was re-suspended in BHI with 10% sucrose (BHI-S) to induce biofilm formation.

Time dependent killing effect on planktonic Streptococcus mutans

The antibacterial susceptibility test against *S. mutans* was determined by viable count technique. The 1 ml of re-suspended *S. mutans* in PBS was mixed with 1 ml of each mouthwash formulation, with chlorhexidine mouthwash and mouthwash base as positive and negative control, respectively. The tested samples were collected at time 0 minute, 30 minutes and 2 hours. The solution was diluted with sterile phosphate buffered saline (PBS) by 10, 100, and 1,000-fold and spotted on a bacterial culture plate on BHI agar. Bacterial agar plates were incubated for 48 hours at 37°C, before evaluating the bacterial viability by counting colony-forming units (CFUs/ml). The experiment was performed in triplicate.

Effect on Streptococcus mutans biofilm formation

The biofilm study method was modified from Stepanovic *et al*., 2000 [15]. The 200 µL of *S. mutans* was re-suspended in BHI-S into each well of a flat bottom 96 well plate and incubated at 37°C in an anaerobic jar for 24 h. After formation of biofilm, the medium was gently removed and washed with phosphate buffered saline (PBS) to remove non-adherent cells. Then, 200 µL of various mouthwash formulations were added as test samples, using chlorhexidine and mouthwash base for positive and negative controls. The biofilm plate was incubated at 37°C for 2 and 24 hours. After that, the media was discarded and the plate washed with sterile deionized water, fixed with 200 µL absolute methanol for 15 minutes, and air-dried at room temperature. The biofilm was stained with 200 µL of 0.4% crystal violet solution for 30 minutes and washed with sterile deionized distilled water. The stain was eluted with 200 µL of 95% ethanol for 30 minutes. Adherence of remaining biofilm was compared to completely formed biofilm (negative controls) and quantified by measuring optical density (OD) at wavelength 596 nm using a microplate reader (TECAN, Tecan Group Ltd., Switzerland). All assays were repeated six times. Results were interpreted as percentage of mature biofilm reduction by using equation: Mature biofilms reduction (%) = $[(OD_{control} - OD_{test}) / OD_{control}] \times 100$

Preventive effect on Streptococcus mutans biofilm formation

The biofilm formation assay used in this study is based on Stepanovic *et al*., 2000 [15]. Each well of a flat bottom 96 well plate was filled with 200 µL of a mixture of test solutions and 10% (v/v) of *S. mutans* in BHI-S. The plate was incubated at 37°C for 48 hours. After that the medium was gently decanted and washed with 200 µL PBS. The biofilm was fixed with 200 µL of absolute methanol for 15 minutes and air dried at room temperature. The remaining biofilm was stained with 200 µL of 0.4% crystal violet for 30 minutes and rinsed with sterile deionized distilled water and eluted with 200 µL of 95% ethanol for 20 minutes. Adherence of remaining biofilm was compared to completely formed biofilm (negative controls). The optical density (OD) was measured at wavelength 596 nm. All assays were repeated six times. Results were interpreted as a percentage of inhibition of biofilm formation of *S. mutans* by using the equation: Preventive effect (%) = $[(OD_{control} - OD_{test}) / OD_{control}] \times 100$

Statistical analysis

The normal distribution of means was analyzed by using the Shapiro-Wilk Test. The difference of means was statistically analyzed by ANOVA, along with multiple comparisons by Tukey's HSD post hoc test. Each antimicrobial experiment was performed in triplicate, while the anti-biofilm experiments were performed in six repeats. The statistical analyses were analyzed by SPSS program version 17.0 for IBM (SPSS, Chicago, IL, USA), with the significance level defined as $p < 0.05$.

Results

Time dependent killing effect of mouthwash on planktonic Streptococcus mutans

The killing effect of chitosan mouthwash formulations against planktonic *S. mutans*, with an initial load about 10^6 CFU/mL, showed that all chitosan mouthwash formulations could statistically reduce the number of *S. mutans* immediately or at 0 minute, with continuous reduction over time ($p < 0.05$). Moreover, there is no significant difference ($p > 0.05$) between different molecular weight and different concentration of chitosan mouthwashes in terms of antibacterial activity. All chitosan mouthwash formulations could reduce the number of S. *mutans* from its initial load approximately by 0.70 to 0.85 log CFU/mL at 0 minute, 1.54 to 1.78 log CFU/mL at time 30 minutes, and around 6 log CFU/mL at 2 hours (Figure 1). These log reduction CFU/mL can be compared as a percentage as follows: 80.05% to 85.57% at 0 minute, 97.12% to 98.34% at 30 minutes, and more than 99.999% at 2 hours. While the mouthwash-based solution and PBS could not reduce a number of *S. mutans* at any time point.

Reductive effect on Streptococcus mutans mature biofilm

At 2 hours treatment time, mouthwash containing 30 kDa chitosan caused *S. mutans* biofilm reduction around 88.56% to 89.97%, while 890 kDa caused 80.29% to 80.85%. For 24 hours treatment time, mouthwash containing 30 kDa chitosan caused *S. mutans* biofilm reduction around 90.37% to 92.27%, while 890 kDa caused 90.27% to 91.22% (Figure 2). The results show that mouthwash containing 30 kDa chitosan had a significantly greater reduction of biofilm formed compared to that containing 890 kDa chitosan at 2 hours treatment time ($p < 0.05$). Whereas, there is no significant difference ($p > 0.05$) between different molecular weight and different concentration of chitosan mouthwashes at 24 hours treatment time. In addition, chlorhexidine mouthwash could reduce *S. mutans* mature biofilm by only 38.91% and 27.06% at 2 hours

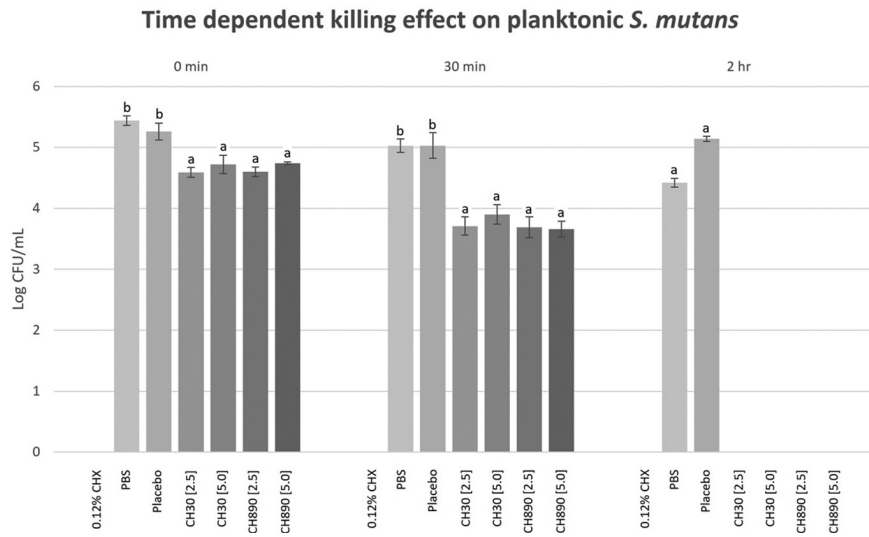

Figure 1 Time dependent killing effect on planktonic *S. mutans* after treatment with 0.12% Chlorhexidine mouthwash (0.12% CHX), phosphate buffered saline (PBS), mouthwash base (placebo), chitosan mouthwash with molecular weights of 30 and 890 kDa (CH30, CH890) and concentrations of 2.5 and 5.0 mg/mL ([2.5], [5.0]). The testing times were 0 minute, 30 minutes, and 2 hours. Different alphabets above the bar (a, b) means significant differences between test solutions in the same period of time.

and 24 hours, respectively. Moreover, plates treated with chitosan mouthwash for a longer time showed greater effect on biofilm reduction. The results show that chitosan mouthwash showed greater percent reduction of biofilm than chlorhexidine mouthwash ($p < 0.05$).

Preventive effect on formation of biofilm by Streptococcus mutans

One notable result was that chitosan mouthwashes showed very good preventive effect on new biofilm formation of up to 97% with statistically significant preventive effect when compared to chlorhexidine mouthwash ($p < 0.05$) (Figure 3).

Discussion

Chitosan polymer shows interesting physicochemical and biological properties, depending on source, preparation procedure, molecular weight and degree of deacetylation [16]. Both low molecular weight (100 kDa) and high molecular weight (600-800 kDa) was reported to have a significant antibacterial effect on S. *mutans* and S. *sobrinus* and inhibit biofilm formation [17]. This study clearly showed that both molecular weight forms of chitosan (30 and 890 kDa) with high percentage of deacetylation (>90%) in mouthwash formulations possess antibacterial activity, mature biofilm inhibition and could be used for prevention of dental biofilm formation. The antibacterial study showed that all chitosan mouthwashes had similar bactericidal effects against S. *mutans* within 2 hours, with no statistical difference in log reduction of number of S. *mutans* at each period of time. Electron microscopic studies [18, 19] have shown that the positively charged chitosan can bind to the negatively charged bacterial cell wall, causing weakening or even breakage, leading to leakage of intracellular substances and eventually cell death.

The results from this study agreed with Abedian Z. 2019 [17] and Costa *et al*. 2012 [20], that chitosan mouthwash could inhibit cariogenic bacteria. It was also reported that the chitosan

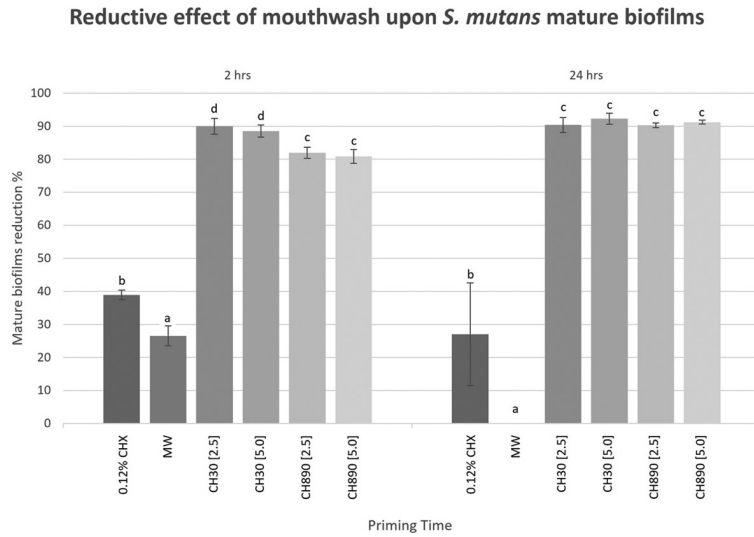

Figure 2 Effect of mouthwash formulations on *S. mutans* mature biofilms at different priming times (2, 24 hours) with Chlorhexidine mouthwash (CHX), mouthwash base (MW), chitosan mouthwash with molecular weights of 30 and 890 kDa (CH30, CH890), and concentrations of 2.5 and 5.0 mg/mL ([2.5], [5.0]). Results are shown as percentage reduction in mature biofilm. Different alphabets on the bar (a, b, c, d) means significant differences.

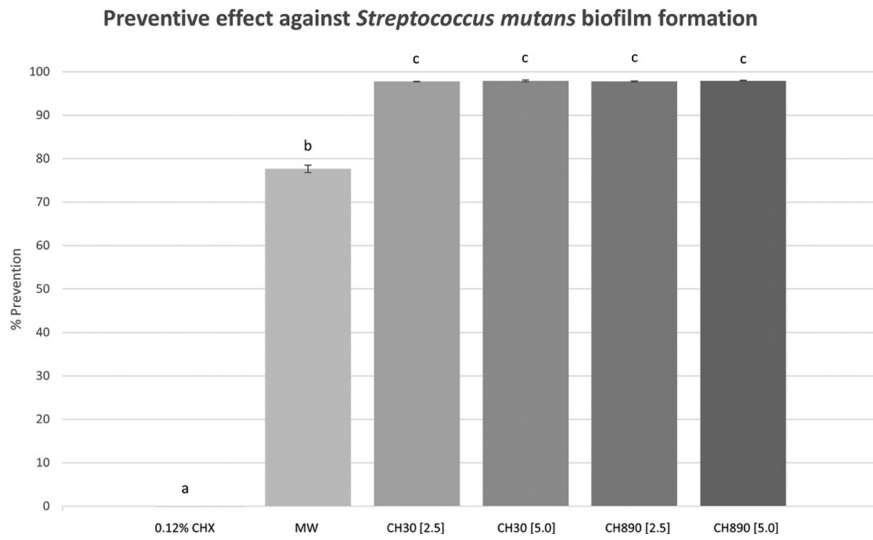

Figure 3 Preventive effect of mouthwash formulations upon *S. mutans* biofilm formation with chlorhexidine mouthwash (CHX), mouthwash base (MW), chitosan mouthwash with molecular weights of 30 and 890 kDa (CH30, CH890), and concentrations of 2.5 and 5.0 mg/mL ([2.5], [5.0]). Results are shown as percent inhibition of biofilm formation. Different alphabets above the bar (a, b, c) means significant differences between test solutions in the same priming time group.

mouthwash possesses lower toxicity and higher antimicrobial activity than chlorhexidine mouthwash and the commercial mouthwash tested. An *in vitro* study of Thongaroon K. *et al*., also reported that the antibacterial property of chitosan mouthwash was time dependent. However, our chitosan mouthwash preparations could inhibit S. *mutans* growth after 2 hours, faster than their study showing reduction in bacteria after 6 hours [21].

Our study showed that chitosan mouthwash had prominent action against mature biofilm compared with chlorhexidine mouthwash. Previous reports showed that chitosan mouthwash could reduce mature *S. mutans* biofilm by up to 94% [22] and had greater activity than commercial chlorhexidine mouthwash [23]. In addition, at 2 hours, our study also showed that low molecular weight chitosan mouthwash (30 kDa) had superior reduction of mature *S. mutans* biofilm than high molecular weight chitosan mouthwash (890 kDa). The low molecular weight chitosan consists of smaller molecules, which are presumably greater mobility attraction and able to interact better with the bacterial cell wall than larger molecules.

Costa EM *et al* found that chitosan mouthwash could be a potent inhibitor of *S. mutans* biofilm formation [21, 22]. Our study showed greater ability of chitosan mouthwashes to inhibit new biofilm formation, compared to chlorhexidine mouthwash. Consistent with Decker EM. *et al,* chitosan could reduce the attachment ability of *Streptococci* by about 90% [24]. As an antiplaque agent, chitosan is naturally occurring, environmentally friendly, and has very low toxicity [25]. In acidic solution, the amine groups (-NH2) of chitosan are protonated to –NH3+, so that the cationic molecules of chitosan can form electrostatic interactions with anionic molecules on the cell surface of bacteria. These binding interactions may intercept the attachment of cariogenic bacteria to tooth surfaces [26]. It was also noticed that the mouthwash base also gave positive results however including chitosan into the formulation gave significant difference. This may be due to the formulation

used in this study being similar to other non- alcoholic commercial mouthwash available in the market.

Although chitosan has been reported to have stronger bactericidal effects at acidic pH, the acidic pH may cause demineralization and damage the enamel surface of teeth. The chitosan mouthwash formulations used in this study have a final pH of 6.0, which is closer to the normal pH of saliva than those of previous studies using formulations at pH 5.0. Moreover, the chitosan powder used in our study has a high degree of deacetylation (>90%), which makes the chitosan easily ionized and readily bind to the negatively charges on bacterial cell surface, resulting in stronger antibacterial and antibiofilm properties.

There have been several attempts to make a high potency mouthwash with low toxicity. The toxicity of our chitosan mouthwash was verified by the MTT cytotoxicity test according to the international standard, ISO 10993-5, 2009 [27]. Thus, our chitosan mouthwash formulations show no cytotoxicity against L929 cells or mouse fibroblasts which allow cell viability over 80% when tested with all formulations (data not shown). The MTT assay from the study of Costa E.M. *et al*, has also shown that chitosan mouthwash was less toxic than the chlorhexidine based commercial mouthwash [20]. Therefore, chitosan mouthwash unlike fluoride mouthwash or chlorhexidine mouthwash is safe and can be an alternative mouthwash for prevention of dental caries especially in children and in disabled patients who are unable to clean their teeth properly. Moreover, this preparation tastes good and has no alcohol content.

In actual daily use of mouthwash, patients are advised to hold mouthwash in the oral cavity for 30 seconds, which may be comparable to our results from an antibacterial property at 0 minute, that showed up to 80.05% to 85.57% S. *mutans* reduction. Alternatively, if patients use mouthwash before bedtime without rinsing or eating anything else, mouthwash may be coated in their mouth longer and may increase its effectiveness in antimicrobial and antibiofilm properties. Therefore, further clinical studies may be required.

Conclusion

Chitosan mouthwash prepared from both low and high molecular weight chitosan with similar degree of deacetylation had similar antibacterial and antibiofilm properties against the S. *mutans*. Although, chlorhexidine mouthwash could significantly reduce the number of planktonic S. *mutans* more than chitosan mouthwash, chitosan mouthwash could significantly reduce mature biofilm formation and inhibit newly biofilm formation better than chlorhexidine mouthwash. Therefore, chitosan mouthwash may be used as an alternative treatment to prevent dental caries with low toxicity and environmental friendliness.

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References

1. Featherstone JD. Caries prevention and reversal based on the caries balance. ***Pediatr Dent*** 2006; 28: 128-32.
2. Forssten SD, Björklund M, Ouwehand AC. Streptococcus mutans, caries and simulation models. ***Nutrients*** 2010; 2: 290-98.
3. Anderson MH, Bales DJ, Omnell K-A. Modem management of dental caries: the cutting edge is not the dental bur. ***J Am Dent Assoc*** 1993;124(6): 36-44.

4. Wolff MS, Larson C. The cariogenic dental biofilm: good, bad or just something to control? ***Braz Oral Res*** 2009; 23: 31-8.
5. Flötra L, Gjermo P, Rölla G, Waerhaug J. Side effects of chlorhexidine mouth washes. ***Eur J Oral Sci*** 1971; 79: 119-25.
6. Emilson C, Lindquist B, Wennerholm K. Recolonization of human tooth surfaces by Streptococcus mutans after suppression by chlorhexidine treatment. ***J Dent Res*** 1987; 66: 1503-8.
7. Chung Y-C, Chen C-Y. Antibacterial characteristics and activity of acid-soluble chitosan. ***Bioresour Technol*** 2008; 99: 2806-14.
8. Chen C-Y, Chung Y-C. Antibacterial effect of water-soluble chitosan on representative dental pathogens Streptococcus mutans and Lactobacilli brevis. ***J Appl Oral Sci*** 2012; 20: 620-27.
9. Fujiwara M, Hayashi Y, Ohara N. Inhibitory effect of water-soluble chitosan on growth of Streptococcus mutans. ***New Microbiol*** 2004; 27: 83-6.
10. Pasquantonio G, Greco C, Prenna M, Ripa C, Vitali L, Petrelli D, *et al.* Antibacterial activity and anti-biofilm effect of chitosan against strains of Streptococcus mutans isolated in dental plaque. ***Int J Immunopathol Pharmacol*** 2008; 21: 993-97.
11. Kong M, Chen XG, Xing K, Park HJ. Antimicrobial properties of chitosan and mode of action: a state of the art review. ***Int J Food Microbiol*** 2010; 144: 51-63.
12. Younes I, Sellimi S, Rinaudo M, Jellouli K, Nasri M. Influence of acetylation degree and molecular weight of homogeneous chitosans on antibacterial and antifungal activities. ***Int J Food Microbiol*** 2014; 185: 57-63.
13. Zheng L-Y, Zhu J-F. Study on antimicrobial activity of chitosan with different molecular weights. ***Carbohydr Polym*** 2003; 54: 527-30.
14. Sano H, Shibasaki KI, Matsukubo T, Takaesu Y. Effect of chitosan rinsing on reduction of dental plaque formation. ***Bull Tokyo Dent Coll*** 2003; 44: 9-16.
15. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. ***J Microbiol Methods*** 2000; 40: 175-79.
16. Naveed M, Phil L, Sohail M, Hasnat M, Baig MMFA, Ihsan AU, et al. Chitosan oligosaccharide (COS): An overview. ***Int J Biol Macromol*** 2019; 129: 827-43.
17. Abedian Z, Jenabian N, Moghadamnia AA, Zabihi E, Tashakorian H, Rajabnia M, *et al.* Antibacterial activity of high-molecular-weight and low-molecular-weight chitosan upon oral pathogens. ***J Conserv Dent.*** 2019; 22:169-74.
18. Costa E, Silva S, Pina C, Tavaria F, Pintado M. Evaluation and insights into chitosan antimicrobial activity against anaerobic oral pathogens. ***Anaerobe*** 2012; 18: 305-9.
19. Yadav A, Bhise S. Chitosan: A potential biomaterial effective against typhoid. ***Curr Sci*** 2004; 87: 1176-78.
20. Costa E, Silva S, Costa M, Pereira M, Campos D, Odila J, *et al.* Chitosan mouthwash: Toxicity and in vivo validation. ***Carbohydr Polym*** 2014; 111: 385-92.
21. Thongaroon K, Phonghanyudh A, Surarit R, Kraivaphan P, Niamsiri N. In vitro antimicrobial activity of chitosan mouthwash against Streptococcus mutans and Lactobacillus casei. ***Walailak Procedia*** 2019; 2019(4):113.
22. Costa E, Silva S, Tavaria F, Pintado M. Study of the effects of chitosan upon Streptococcus mutans adherence and biofilm formation. ***Anaerobe*** 2013; 20: 27-31.
23. Costa E, Silva S, Madureira A, Cardelle-Cobas A, Tavaria F, Pintado M. A comprehensive study into the impact of a chitosan mouthwash upon oral microorganism's biofilm formation in vitro. ***Carbohydr Polym*** 2014; 101: 1081-86.
24. Decker EM, Von Ohle C, Weiger R, Wiech I, Brecx M. A synergistic chlorhexidine/chitosan combination for improved antiplaque strategies. ***J Periodontal Res*** 2005; 40: 373-77.
25. Farias JM, Stamford TCM, Resende AHM, Aguiar JS, Rufino RD, Luna JM, *et al.* Mouthwash containing a biosurfactant and chitosan: An eco-sustainable option for the control of cariogenic microorganisms. ***Int J Biol Macromol*** 2019; 129: 853-60.
26. Chung Y-C, Su Y-P, Chen C-C, Jia G, Wang H-I, Wu JG, *et al.* Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. ***Acta Pharmacol Sin*** 2004; 25: 932-36.
27. International Standard Organization; ISO 10993-5(2009) Biological evaluation of medical devices—part 5: tests for in vitro cytotoxicity. [accessed 2019 Jan 24] Available from: http://www.dent.chula.ac.th/upload/images2/dent-bio-material/cytotoxicitytest/ISO10993_5_2009.pdf.