ORIGINAL ARTICLE

Chitosan Antibacterial Activity Against *Streptococcus viridans*

Dian Agustin Wahjuningrum[1], Halida Dwi Pramesti[2], Manuel Raynaldy Sihombing[2], Zabrina Luthfiana Devi[2], M. Roelianto[1] and Setyabudi[1]

[1] Lecturer in Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga-Surabaya, Indonesia
[2] Undergraduate Student, Faculty of Dental Medicine, Universitas Airlangga-Surabaya, Indonesia

ABSTRACT

Introduction: *Streptococcus viridans* is the most common facultative anaerobic bacteria which can cause pulp and root canal infections in oral cavity. This Gram-positive bacterium can cause pulp necrosis, an indication for root canal treatment. One of the important steps in root canal treatment is root canal irrigation and a good root canal irrigant has a maximum antibacterial effect with minimum toxicity effect. Chitosan is a natural ingredient from shrimp shells. Chitosan has high antibacterial activity against many pathogenic microorganisms and has been used as an antibacterial agent to eliminate Streptococcus viridans. **Methods:** *Streptococcus viridans* was grown in a tube which contained BHIB medium and 2% chitosan suspension in each sample group with the concentration of 0.0156%; 0.03125%; .0625%; 0.125%; 0.25%; 0.5%; 1%; 2%. Positive control (BHIB medium and *Streptococcus viridans* bacteria), 2% acetic acid solution control (2% acetic acid solution and *Streptococcus viridans*), and negative control (BHIB medium) were made to ensure that there was not any antibacterial effect on the medium. Each tube was incubated at 37°C for 24 hours. The number of living colonies were calculated and compared with the positive control to determine the antibacterial activity, MIC, and MBC. **Results:** In this study, the percentage of the living colonies in the sample groups with chitosan concentration of 0.125%; 0.0625%; 0.03125% were 0%; 7.2%; 16.4%, respectively. **Conclusion:** Chitosan has an antibacterial activity with 0.0625% MIC and 0.0125% MBC against *Streptococcus viridans*.

Keywords: Antibacterial Activity, Minimum Inhibitory Concentration-Minimum Bactericidal Concentration, Chitosan, *Streptococcus viridans*, Infectious Disease

Corresponding Author:
Setyabudi,
Email: setyabudi@fkg.unair.ac.id
Tel: +62315030255

INTRODUCTION

Based on Indonesia's National Basic Health Research (RISKESDAS), the national prevalence of oral and dental problems in 2007 was 23.4% and increased in 2013 to 25.9% (1). Based on these data, it can be concluded that the caries prevalence in Indonesia is still high and can increase the risk of pulp disease. The prevalence of pulp disease in Indonesia can be categorized as high. The 2011 Indonesia Health Profile recorded that pulp and periapical disease were ranked 7th for outpatient diseases in 2010 (2). Pulp necrosis or dead pulp can be caused by bacteria, trauma, and chemical irritants. Infection of the pulp and root canal is not only caused by one type of bacteria; however, several types of bacteria (3).

The facultative anaerobes are the most common type of bacteria found in pulp and root canal infections (4), because in necrotic root canal was found a number of 57.14% facultative anaerobic bacteria, while it was 42.86% for aerobic bacteria (5). *Streptococcus viridans* is 63% of the facultative anaerobic bacteria inhabiting the root canals (6) and the most dominant and pathogenic bacteria in root canal infections in the oral cavity (7). Streptococcus viridans, a gram-positive bacterium, can cause oral infectious diseases such as caries, periodontitis, and dental abscess which can then leads to bacteremia and endocarditis (8). Condition of a bacterial infection that can cause pulp necrosis is an indication for root canal treatment.

Root canal treatment is a treatment performed by removing non-vital or necrotic pulp from the root canal and then replacing it with a filling material (3). Preparation, sterilization, and filling are the important steps in root canal treatment on the infected tooth.

The success of a root canal treatment depends on the elimination of bacteria and their organic substrates and it can be achieved by using an antibacterial root canal filling material (9). At the preparation step, a root canal irrigant is used to remove necrotic tissue and pile of dentin debris, also to wet the root canal of the tooth which makes the preparation easier. In addition, the number of microorganisms in the root canal are reduced, thus the remaining bacteria can be killed with drugs (3,9).

Root canal irrigation is an important step in supporting successful root canal treatment (10). A good root canal irrigant has a maximum antibacterial effect with minimum toxicity effect (9). Moreover, an irrigant should be able to disinfect and penetrate dentin tubules, lubricate endodontic devices, offer long-term antibacterial effect, remove smear layer, dissolve necrotic tissue, be relatively inexpensive, non-toxic, and non-carcinogenic (3,11). The existing irrigants nowadays still have several weaknesses, for example, sodium hypochlorite (NaOCl), a synthetic irrigant, is toxic when used at a high concentration can cause periapical inflammation, while at a low concentration, the irrigants is neither ineffective to kill all bacteria nor can remove the smear layers completely (11). Therefore, it is necessary to use natural ingredients that have a maximum antibacterial effect with minimum toxicity as an alternative for root canal irrigants.

One of the natural ingredients that can be used is chitosan. Chitosan is a chitin derivative from the deacetylation process (12,13,14), which can be obtained from shrimp shell waste or other crustaceans (15,16). Chitosan is a natural biopolymer that has biocompatible properties, is degradable, has high bioactivity, amino group reactivity, selective permeability, antibacterial, non-toxic, non-allergenic, and non-carcinogenic (13). Chitosan has high antibac-terial properties against a wide variety of pathogenic microorganisms, including fungi, as well as Gram-positive and Gram-negative bacteria (14). Therefore, chitosan can be used as an option to be developed into root canal irrigants.

This research was conducted at the Dental Research Center of Faculty of Dental Medicine, Universitas Airlangga, Indonesia. The material that was used is chitosan from white leg shrimp (Litopenaeus vannamei), because of the chitin content in shrimp shells that can reach 40-60% (15). The crustacean processing industry produces a large amount of solid waste in the form of shells which has an impact on environmental pollution thus it has the potential to be used as the raw material for chitosan (16).

MATERIALS AND METHODS

Chitosan Suspension Preparation

The chitosan powder (Batan, Indonesia) was made into a suspension by dissolving 2 grams of chitosan powder in 50 ml of 2% (v/v) acetic acid solution and 50 ml of distilled water in order to obtain 2% (w/v) chitosan suspension. This suspension was further diluted to prepare 2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, 0.0156% chitosan suspension. The dilution method was based on the formulation: C1 x V1 = C2 x V2 (C1 and V1 were the initial concentration and volume, respectively, before the dilution, and C2 and V2 were the concentration and volume, respectively, after the dilution).

Streptococcus viridans Preparation

The *Streptococcus viridans* cultures (Dental Research Center of Faculty of Dental Medicine, Universitas Airlangga, Indonesia) to be used were taken and planted on Brain Heart Infusion Broth (BHIB) medium. Then, it was incubated at 37 C for 24 hours. The culture was adjusted with 0.5 McFarland standard that is equivalent to 1.5 x 108 CFU/ml. The turbidity was matched by holding both the culture and the standard side by side against a white background with black horizontal lines.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was done by using the dilution method. Eleven sterile test tubes were prepared. Eight test tubes marked with a number from 1 to 8 for treatment groups which contained 2% chitosan suspension with various concentrations, and three test tubes for control with two test tubes marked with (+) and ((+) Acetic acid) for positive control and one test tube marked with a (-) for negative control. Tube 2 to 7 from those eight tubes contained 2.5 ml of BHIB medium. Tube 1 was filled with 10 ml of 2% chitosan suspension, then 5 ml from tube 1 was taken and filled into tube 2 until it reached 10 ml, followed by the tube 2 to tube 3, and continued until tube 8. Thus, those eight test tubes contained a chitosan concentration of 0.0156%; 0.03125%; .0625%; 0.125%; 0.25%; 0.5%; 1%; 2% each. A total of 0.05 ml of bacterial suspension that has been standardized with 0.5 McFarland (1,5 x 108 CFU/ml) was grown in those eight test tubes. Positive control test tube (+) which contained BHIB medium and 0.05 ml bacterial suspension as well as a positive control test tube of 2% acetic acid solution ((+) Acetic acid) which contained 2% acetic acid solution and 0.05 ml bacterial

suspension were made to ensure that there was not any antibacterial effect on the medium. Moreover, negative control test tube (-) which contained BHIB medium without *Streptococcus viridans* nor 2% chitosan suspension were also made to ensure that there was not any bacteria contamination on the medium. Each tube was incubated anaerobically in an incubator at 37 C for 24 hours. The reading of the results was done by observing the presence or absence of bacterial growth marked by turbidity and precipitation with the naked eye, then the colony was calculated.

Bacteria subculture was done by taking 0.1 ml of bacteria from each test tube as well as positive control, positive control of 2% acetic acid solution, and negative control tube, then grown it on nutrient agar medium with spread plate method, and incubated anaerobically at 37 C for 24 hours. Results of bacteria subculture were observed, then replication was only done for groups which medium shown presence of bacterial growth and all control tests, because the medium with the lowest concentration that shown absence of bacterial growth was determined as the concentration value that has antibacterial effect. Replication was done four times on a nutrient agar medium with the spread plate method, then the medium was incubated anaerobically at 37 C for 24 hours to obtain a significant difference in the result of the bacterial growth.

MIC and MBC were determined by calculating the number of colony growth on nutrient agar medium with Quebec Colony Counter which was compared with the number of colony growth in positive control and expressed as CFU/ml. The results of the concentration that showed (>90%) inhibition of the *Streptococcus viridans* bacteria which compared to the positive control were considered as MIC22. Meanwhile, the concentration that showed (>99.9%) inhibition of the *Streptococcus viridans* bacteria which compared to the positive control were considered as MBC18.

Data Analysis

Data of the research results in the form of the number of colonies was processed in a statistical test. One-Way ANOVA test was done to find out the significant differences from each sample group and LSD Post Hoc test was done to find out which particular differences between groups are significant.

RESULTS

Chitosan Chemical Analysis

Before determining the MIC and MBC, the result of chemical analysis was obtained from Badan Penelitian dan Konsultasi Industri (BPKI) to determine content of compounds and chemical characteristics of the chitosan which are in the following table (Table I).

Table I : Results of Chitosan Chemical Analysis (BPKI).

Compound/Characteristic	Concentration Level
Chitosan	81.80%
Degree of Acetylation	2.81%
Degree of Deacetylation	96.82%
Water	6.88%
Molecular Weight	3568 g

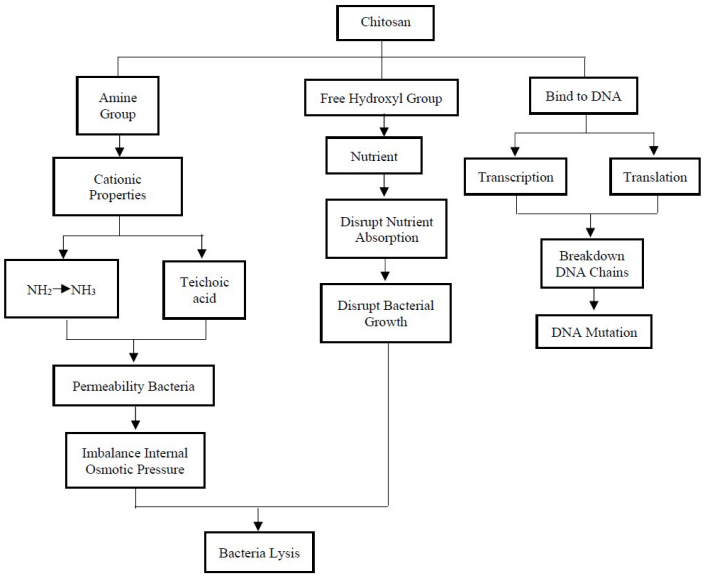

Figure 1. Graphical abstract.

Figure 1 : Graphical Abstract of mapping concept.

Table I shows the results of chemical analysis of the chitosan. The powder used in this study contained 81.80% chitosan with 2.81% degree of acetylation (DA) and of 96.82% degree of deacetylation (DDA). In addition, the powder contained 6.88% of water found with a molecular weight of 3568 g.

Research Results

Bacterial colony growth on nutrient agar which had been grown from serial dilution on all sample groups using the spread plate method was observed to determine the MIC and MBC.

Figure 2 explains about the results of *Streptococcus viridans* growth on nutrient agar medium. It could be seen that the zone of sample group 5 with a concentration of 0.125% was the zone with the smallest concentration that did not show *Streptococcus viridans* growth. Thereafter, few sample groups which

showed *Streptococcus viridans* growth were regrown on nutrient agar medium to determine MIC and MBC. The sample groups that were regrown were 7 groups, group 7 (concentration of 0.03125%), group 6 (concentration of 0.0625%), group 5 (concentration of 0.125%), group 4 (concentration of 0.25%), positive control group, 2% acetic acid solution control group, and negative control group. Regrowth in group 1, 2, 3, and 8 was not done, because the bacterial growth was far from the MIC and MBC concentrations based on the growth with the spread plate method. Then, the *Streptococcus viridans* bacterial colonies were calculated using the Quebec Colony Counter.

Figure 3 shows about the results of sample groups growth on Nutrient Agar Medium. Observations were made in the negative control group and no bacterial colonies were found, thus there was certainly no contamination in this research. In the positive control group, the mean bacterial colony of the sample group was 179.2 CFU/ml assuming the number of living colonies was 100%, and in the 2% acetic acid solution control group, the mean bacterial colony of the sample group was 176.5 CFU/ml assuming the number of living colonies was 100%, thus it is confirmed that the two control groups did not have an antibacterial effect.

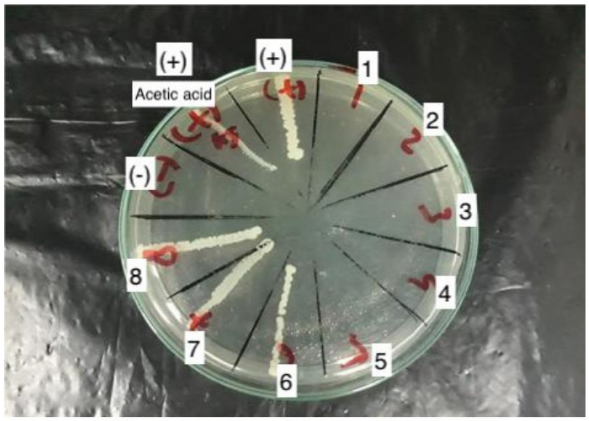

Figure 2 : Results of *Streptococcus viridans* Growth on Nutrient Agar Medium using Spread Plate Method.

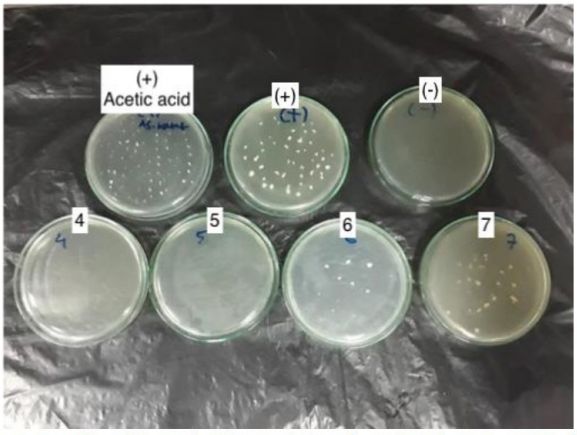

Figure 3 : Results of Sample Groups Growth on Nutrient Agar Medium. .

Table II : The Mean and Standard Deviation of *Streptococcus viridans* Colonies.

		N	x (CFU/ml)	SD
(-)	Negative control	7	0	0
(+)	Positive control	7	179.25	1.25 0.45
(+) Acetic acid	2% Acetic Acid Solution Control	7	176.5	2.16 0.72
7	0.03125% Concentration	7	29.5	2.16 0.72
6	0.0625% Concentration	7	13	1.055 0.355
5	0.125% Concentration	7	0	0
4	0.25% Concentration	7	0	0

Table III : One-Way ANOVA Test Results to Determine Differences in All Sample Groups.

ANOVA
strep.viridans

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	98685.188	3	32895.063	6096.382	.000
Within Groups	64.750	12	5.396		
Total	98749.938	15			

The percentage of living bacterial colonies in group 4 (concentration of 0.25%) was 0%, with the conclusion of 100% dead bacterial colonies, and this additionally occurred in group 5 (concentration of 0.125%). However, in group 6 (concentration of 0.0625%), it was found that the number of living *Streptococcus viridans* colonies was 13 CFU/ml, that was 7,2%, with the conclusion that it could kill 92.8% of *Streptococcus viridans* colonies. Lastly, in group 7 (concentration of 0,03125%), it was found that the number of living Streptococcus viridans colonies was 29.5 CFU/ml, that was 16%, with the conclusion that it could kill 84% of *Streptococcus viridans* colonies.

The concentration of 0.0625% succeeded in killing 92.8% of *Streptococcus viridans* bacterial colonies, and that means it is the MIC of chitosan against *Streptococcus viridans* bacteria. Meanwhile, the concentration of 0.125% succeeded in killing 99.9% of the *Streptococcus viridans* bacterial colonies, and that means it is the MBC of chitosan against *Streptococcus viridans* bacteria.

One-Way ANOVA test was done to find out significant differences in all sample groups. The results of the test can be seen in the table below (Table III).

From Table III, it can be seen that the value $p = 0.000$ ($p < 0.05$) indicates that there was a significant difference in all sample groups

DISCUSSION

This research was conducted to prove the ability of chitosan to inhibit and kill *Streptococcus viridans* bacteria (as described in figure 3). Chitosan is able to inhibit bacterial growth because it has cationic properties in the presence of amine groups that protonated from NH_2 to NH_3+ and interacts with the teichoic acid in *Streptococcus viridans* bacteria which is negatively charged. This interaction results in changes in the permeability of the bacterial cell wall which results in the material passing through the cell membrane and the formation of peptidoglycan to be disrupted, causing an imbalance in internal osmotic pressure that triggers bacterial cell lysis. Lysed bacteria cause protein fluid and intracellular electrolytes leakage such as potassium ions and molecules such as proteins, as well as nucleic acids to leave the bacteria (24).

In addition, chitosan is able to kill bacteria because it has a reactive free hydroxyl group that can interact with the nutrients needed by bacteria thus can disrupt nutrient absorption and bacterial growth20. Chitosan additionally has the ability to bind to DNA which can inhibit the transcription and translation of *Streptococcus viridans* DNA, and the DNA that is inhibited will undergo mutations caused by the breakdown of DNA chains (17,26).

In this study, 2% (w/v) chitosan suspension was used, because it is the optimal concentration and has the best antibacterial ability. In addition, chitosan is a weak base that is insoluble in water, organic solvents, and neutral or alkaline solution with a pH of more than 6.5 thus the best solubility of chitosan is in 2% acetic acid solution (27). Chitosan solubility in acetic acid is one of the main parameters in the quality assessment standard of chitosan. In accordance with the theory, the MIC value is obtained when the inhibited bacteria are as much as 90% and the MBC value is obtained when the killed bacteria are as much as 99.9%. It can be concluded that chitosan has an antibacterial activity with an MIC of 0.0625% and an MBC of 0.125% against *Streptococcus viridans* bacteria

CONCLUSION

Chitosan has an antibacterial activity with 0.0625% MIC and 0.0125% MBC against *Streptococcus viridans* bacteria.

ACKNOWLEDGEMENT

This research is supported by Ministry of Research and Technology Indonesia and Dental Research Center of Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

REFERENCES

1. Kementrian Kesehatan RI. 2013. Riset Kesehatan Dasar. Jakarta: Kementrian Kesehatan RI, 113.
2. RI DK. (2012). Profil Data Kesehatan Indonesia 2011. Jakarta: Kementrian Kesehatan RI, Lampiran 3.4.
3. Torabinejad, M. & Walton, R. Endodontics : Principles and Practice 4th Ed. Elsevier Health Sciences, 2009
4. Sakko M, TjΔdeRhane L, Richardson R. Microbiology of Root Canal Infections. Primary Dental Journal. 2016, 5(2): 84-9.
5. Yamin I, Natsir N. Bakteri Dominan di Dalam Saluran Akar Gigi Nekrosis. Dentofasial. 2014, 13(2): 115.
6. Guimarres N, Otoch H, Andrade L, Ferreira C, Rocha M, Gomes F. Microbiological Evaluation of Infected Root Canals and Their Correlation with Pain. RSBO. 2012; 9(1); 31 - 3.
7. Pujiastuti P, Lestari S. Perbedaan Efektifitas Antibakteri Ekstrak Daun Sirih Merah (Piper crocatum) pada Porphyromonas gingivalis dan

Streptococcus viridans. Stomatognatic (Jurnal Kedokteran Gigi Universitas Jember). 2015; 12(1): 1 – 4.
8. Samaranayake LP. Essential Microbiology for Dentistry 4th Ed. Churchill Livingstone, London, 2012: 123-4.
9. Trilaksana AC, Saraswati A. Efficacy of Green Tea Leaf Extract (Camellia sinensis) with NaOCl 2,5% Againts Enterococcus Faecalis as an Alternative Solution for Root Canal Irigation. Journal of Dentomaxillofacial Science. 2016 April, 1(1): 58-62.
10. Kandaswamy D, Venkateshbabu N. Root Canal Irrigations. Journal of Conservative Dentistry. 2010, 13(4): 256-62.
11. American Association of Endodontists. Root Canal Irrigants and Disinfectants. Chicago: American Association of Endodontists 2011: 3.
12. Mooduto L, Wahjuningrum, DA, Agatha PA, Lunardhi CGJ, Antibacterial effect of chitosan from squid pens against Porphyromonas gingivalis bacteria. Iran Journal of Microbiology. 2019, 11(2), 177-80.
13. Hasib A, Wahjuningrum DA, Ibrahim MHR, Kurniawan HJ, Ernawati R, Hadinoto MEK, et al. ALP (Alkaline Phosphatase) Expression in Simple Fracture Incident in Rat (Rattus norvegicus) Femur Bone Supplemented by Apis Mellifera Honey. Journal of International Dental and Medical Research. 2020; 13(3): 887-91
14. Basri H, Prakoso AT, Sulong MA, Saad APM, Ramlee MH, Wahjuningrum DA, et al. Mechanical degradation model of porous magnesium scaffolds under dynamic immersion. Journal of Materials: Design and Application. 2019; 0(0), 1-11. doi: 10.1177/1464420719881736.
15. Wahjuningrum DA, Subijanto A, Taqiya AK, Anggini FD, Hasib A, Mooduto L, et al. Cytotoxicity of Chitosan Derived from Shrimp for Bone Scaffold on Adipose Tissue-Derived Mesenchymal Stem Cells. Asia Pacific Journal of Medical Toxicology. 2019; 8(4), 115-7.
16. Saad AMP, Prakoso AT, Sulong MA, Basri H, Wahjuningrum DA, Syahrom A. Impacts of dynamic degradation on the morphological and mechanical characterization of porous magnesium scaffold. Biomechanics and Modeling in Mechanobiology. 2019; doi: 10.1007/s10237-018-01115-z.
17. Hajji S, Younes I, Rinaudo M, Jellouli K, Nasri M. Characterization and In Vitro Evaluation of Cytotoxicity, Antimicrobial and Antioxidant Activities of Chitosans Extracted from Three Different Marine Sources. Application of Biochemistry Biotechnology. 2015; 177, 18-35.
18. Wardani G, Mahmiah, Sudjarwo SA. In vitro Antibacterial Activity of Chitosan Nanoparticles Against Mycobacterium tuberculosis. Pharmacognosy Journal. 2018; 10(1), 162-166.
19. Rocassa IN, Siqueira JF. Root Canal Microbiota of Teeth with Chronic Apical Periodontitis. Journal of Clinical Microbiology. 2018; 46: 3599-606.
20. Kaimudin M, Leounupun MF. Characterization Chitosan from Waste Leather with Shrimp Process using Different Bleaching and Deacetylation. Journal BIAM. 2016; 12(01), 1-7.
21. Afriani Y, Fadli A, Maulana S, Karina I. Sintesis, Kinetika Reaksi dan Aplikasi Kitin dari Cangkang Udang. Program Insentrif Riset Terapan. Badan Penelitian Pengembangan Kelautan dan Perikanan. Jakarta; 2016
22. Capoor MD, Nair D, Posti J, Singhal S, Deb M, Aggarwal P, et al. Minimum Inhibitory Concentration of Carbapenems and Tigecycline Against Salmonella spp. Journal of Medical Microbiology. 2009; 58(3): 337-41.
23. Akram F, El-Tayeb T, Abou-Aisha K, El-Azizi M. A Combination of Silver Nanoparticles and Visible Blue Light Enhances the Antibacterial Efficacy of Ineffective Antibiotics Against Methicillin-Resistant Staphylococcus aureus (MRSA). Annals of Clinical Microbiology and Antimicrobials. 2016; 15(1), 3.
24. Goy RC, Morais STB, Assis OBG. Evaluation of the Antimicrobial Activity of Chitosan and Its Quaternized Derivative on E. coli and S. aureus Growth. Revista Brasileira de Farmacognosia. 2015; 26(11): 122-7.
25. Kara F, Aksoy EA, Yuksekdag Z, Hasirci N, Aksoya S. Synthesis and Surface Modification of Polyurethanes with Chitosan for Antibacterial Properties. 2014; 112(5), 39-47.
26. Martinez LR, Mihu MR, Cordero RJB, Han G, Friedman AJ, Friedman JM, et al. Demonstration of Antibiofilm and Antifungal Efficacy of Chitosan Againts Candidal Biofilms, Using an In Vitro Central Venous Catheter Model. Journal of Infectious Disease. 2010; 201(9): 1436-40.
27. Dompeipen EJ, Kaimudin M, Dewa RP. Isolation of Chitin and Chitosan from Waste of Skin Shrimp. Majalah BIAM. 2016; 12(01), 32-8.