The Study of Antimicrobial Activity on *Aggregatibacter actinomycetemcomitans* of AgNPs Capping with Roselle

Sirorat wacharanad[1]*, Nattaporn sukprasert[2], Wichaya choonium[2], Thitirat Taya[2], Nuttaya Phrai-in[2]

1. Department of preventive, Faculty of Dentistry, Naresuan university, Thailand.
2. Faculty of Dentistry, Naresuan university, Thailand.

Abstract

The aim of the experiment is to study the antimicrobial effect of silver nanoparticles capping with Roselle (SNP-Ro) against *Aggregatibacter actinomycetemcomitans (Aa)*.

Silver nanoparticles were prepared from silver nitrate solution and Roselle extract using microwave-assisted synthesis method. The concentration of Roselle used in the synthesis step was varied from 0.1 mM to 2.5 mM. The SNP-Ro were analyzed by UV-Vis spectroscopy, exhibited yellow color and showed maximum absorbance at 400 nm which is the specific value of silver nanoparticles. The particles under the microscope were relatively spherical and the average size of SNP-Ro from 1:0.5 and 1:2.5 ratios were 14.43 ± 3.71 nm and 9.06 ± 3.32 nm in diameter respectively. Their antimicrobial activity against *Aa* was 99.94 percent reduction.

The statistical analysis of percent reduction showed no significant difference between SNP-Ro and 0.12% chlorhexidine gluconate which is positive control group ($p>0.05$) and the comparison of antimicrobial effect between each concentration of SNP-Ro had no significant difference ($p>0.05$).

Experimental article (J Int Dent Med Res 2019; 12(3): 912-916)

Keywords: Silver nanoparticles, Roselle, *Aggregatibacter actinomycetemcomitans*.

Received date: 27 November 2018 **Accept date:** 28 January 2019

Introduction

During the 21st Century, Silver nanoparticles is the topic that has been researched widely as its benefits can be applied to many fields. In the past, according to the knowledge, silver was regarded as the natural antibiotic preventing infection. Generally, the use of antibiotic makes drug-resistance to the bacteria. On the other hand, this is not happening with silver nanoparticles. For this reason, it is interesting to use it with other material to resist bacteria.[1,2]

The synthesis process of silver nanoparticles mostly uses chemical substance which produces biological toxins. Thus, there is more interest in studying the synthesis process of silver nanoparticles using natural extract to minimize the biological toxins. One step of the process helps to reduce the toxins is the use of coating from plant extracts. As the coating is anionic that can hold with cation of silver particles and prevent its precipitation. Furthermore, plant extracts function as the reducing agent that synthesize the silver nanoparticles.[3,4,5] Thus, Roselle is one of the interesting alternatives as it is herb that is generally found and can be used in various purpose such as in cooking or for medicine as the diuretic to treat the malfunction of digestive system and hyperlipidemia, resist atherosclerosis, perspire, reduce blood pressure, prevent and heal infection, including to function as the antioxidant.[6] The study on antibacterial activity of Roselle stem extract to *Escherichia coli* and *Staphylococcus aureus* in culture medium and milk that had different fat concentration showed that the sterilized Roselle stem extract had antibacterial activity to *Escherichia coli*. Likewise, the study on the antibacterial activity to *Staphyloccocus aureus* indicated that sterilized Roselle stem extract had antibacterial activity to *Staphyloccocus aureus* after 24 hours.[7] Thus, Roselle is the herb containing antibacterial activity. For this reason, the researcher is interested to use it in silver nanoparticles synthesis to minimize toxicity.

In dentistry field, silver nanoparticle is diversely used; it is mostly compounded with

Corresponding author:
Sirorat Wacharanad
Department of Preventive,
Faculty of Dentistry, Naresuan University,
Thailand.
E-mail: sirorat.w@gmail.com

other material to disinfect bacteria in the mouth where numerous microbes adhere in form of plaque that is the cause of many oral diseases such as periodontal disease leading to the loss of tooth.[8,9,10] Therefore, the researcher was interested in silver nanoparticles synthesis using Roselle extract as the coating and reducing agent. Moreover, the heat waves from microwave was used to activate the completion of synthesis process. It was aimed to produce the non-toxic silver nanoparticles to human and environment. The synthesized silver nanoparticle was used to study the antibacterial activity *Aggregatibacter actinomycetemcomitans(Aa),* which was the cause of periodontal disease[11], which would be the primary information for the further research for drug and oral care products development.

Materials and methods

Silver nanoparticles preparation

Microwave-assisted synthesis method was used to prepare SNP capped with Roselle (SNP-Ro). Stock concentration of silver nitrate and Roselle was prepared at 10 mM. The ratio of silver nitrate Roselle and distilled water was showed in Table 1. After mix solution as Table 1, the solution was stirred with magnetic stirrer 2 minutes. All solution was taken to microwave by adjusted power at medium for 2 minutes. The solution was change into yellow color. They were left 48 hours in room temperature. After that they were analyzed via UV-Vis spectroscopy and Transmission electron microscopy.

ratio	$AgNO_3$ (ml)	Roselle (ml)	Distilled water (ml)
1: 0.1	5	0.5	44.5
1: 0.5	5	2.5	42.5
1: 1	5	5	40
1: 1.5	5	7.5	37.5
1: 2	5	10	35
1: 2.5	5	12.5	32.5

Table 1. This table showed the proportion of silver nitrate and Roselle which use to synthesize silver nanoparticles.

Antibacterial property against *Aa*

The antibacterial activity against Aa was tested using the standard method. The SNP films were exposed to 20 µl of Aa in brain heart infusion broth (2 ml). After 24 h incubation at 37 ◦C, 5% CO_2 condition, the bacteria/broth mixture was diluted five times. Then 50 µl of diluted bacteria was placed onto brain heart infusion agar using the spread plate method. After 24 h incubation the bacteria were counted. The result was corrected by the dilution factor to give the number of colonies forming units (CFU) per milliliter. The percentage of bacterial reduction was then calculated and compared to blank condition[12]. After the experiment, the counting pathogen from each concentration level was calculated for the percentage of pathogen reduction with the following formula:

$$R = \frac{A - B}{A} x\ 100$$

From the formula:
R was the percentage of pathogen reduction
A was the number of pathogen before experiment (CFU/ml)
B was the number of pathogen after experiment (CFU/ml)

Results

Silver nanoparticles synthesis with Microwave assisted Synthesis method using Roselle extract to $AgNO_3$ at the different ratio. The concentration of $AgNO_3$ was stable at 1 mM. and the physical properties was tested by measuring the absorbance using Spectrophotometer. From the experiment, it was found that the silver nanoparticles synthesizing at different ratio changed the color of solution, from no color (before heating) to light yellow to dark yellow (after heating) depending on the ratio of the Roselle, as shown in Figure 1(B). Then, tested the absorbance with the spectrophotometer and it showed that the result was in the range of 350-450 nm., as shown in Figure 1(A). This was to say the silver nanoparticles emerged. After using Transmission Electron Microscope (TEM) to examine SNP-Ro at the ratio 1:0.5 and 1:2.5, it was found that the morphology of SNP-Ro was in round-shaped, both single circle spreading around and the cluster of circle at the size of 14.43 ± 3.71 nm. and 9.06 ± 3.32 nm. respectively, as shown in Figure 2.

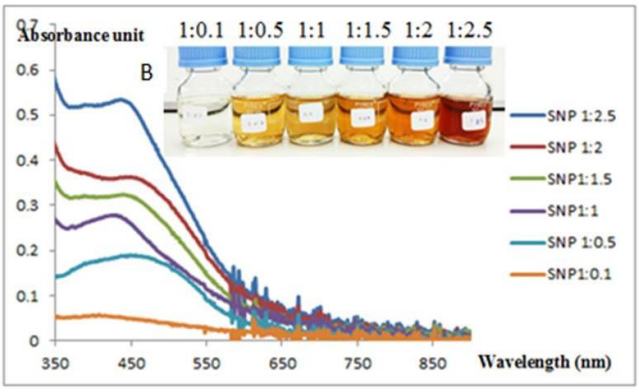

Figure 1. Graph of absorbance of silver nanoparticles coating with Roselle at different ratio (A). Silver nanoparticles coating with Roselle at different ration leaving at room temperature for 48 hours after the synthesis (B).

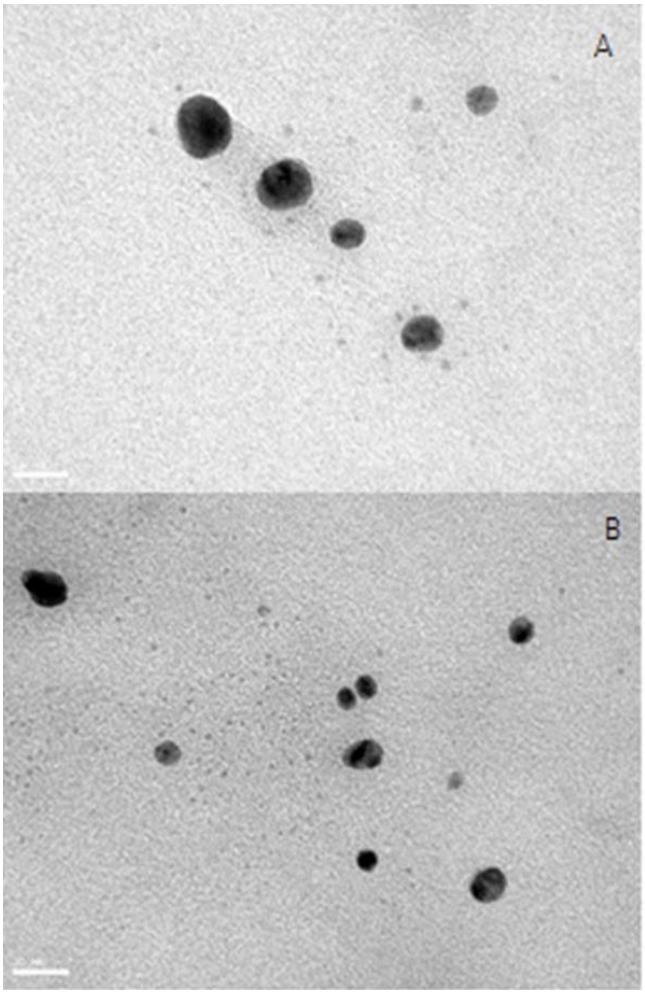

Figure 2. Morphology of (A) SNP-Ro1:0.5 and (B) SNP-Ro1:2.5 from Transmission Electron Microscope (TEM) at magnificationที่ 310Kx. (scale bar 20 nm).

The hypothesis test on the antibacterial to *Aa* of the silver nanoparticles coating with Roselle(SNP-Ro) at the ratio of $AgNO_3$ concentration to the different concentration of Roselle extract at 1:0.1, 1:0.5, 1:1, 1:1.5, 1:2 and 1:2.5 applying the drop plate technique and find the percentage of reduction. The experiment indicated that SNP-Ro at all ratios had the average reduction at 99.70 - 99.99%, as shown in Table 2. Therefore, it was assumed that silver nanoparticles with Roselle coating could restrain the growth of *Aa*.

The positive control group was CHX with 0.12 concentration. The experiment result showed that there was no growth of the pathogen, accounted for 100% of reduction. For the negative control group, Brain Heart Infusion and the pathogen was used. Result illustrated that there were more pathogens than before the experiment, as same as with Roselle extract, as shown in Table 2. The study on the antibacterial activity of SNP-Ro to *Aa* indicated that it could resist *Aa* and at each concentration, there was no difference with statistical significance ($p \geq 0.05$). When comparing to the positive control group, CHX at 0.12 concentration, it was found that the antibacterial activity was not different with statistical significance ($p \geq 0.05$). On the other hand, there was obvious difference in the negative control group, between Brain-Heart infusion and Roselle extract.

samples	Percent of reduction (mean ± SD)
SNP-RO1:0.1	99.99±0.01
SNP-RO1:0.5	99.98±0.03
SNP-RO1:1	99.99±0.00
SNP-RO1:1.5	99.99±0.01
SNP-RO1:2	99.99±0.03
SNP-RO1:2.5	99.70±0.88
0.12%CHX	100±0.00
BHI	-
Roselle	-

Table 2. Resistance results of SNP-Ro to *Aa* at the different ratio by percentage of reduction.

Discussion

Nowadays, the medical field is interested in using silver nanoparticle as a compound to resist bacteria. However, the synthesis process that uses the chemical substance as the reducing

agent produce physical toxins. Therefore, the use of natural extract is the approach to minimize such problem. For this reason, the researcher chose Roselle, the natural plant extract, to use in synthesis process. Roselle is not only the non-toxic herb but also contains antibacterial activity to *Acinetobacter baumannii*, which is mostly found in the hospital, according to the study of Emad Mohamed Abdallah in 2016[13] Moreover, the research of Higginbothamin 2013 revealed that Roselle stem extract was able to resist *Escherichia coli*. The study on antibacterial activity to *Staphyloccocus aureus* showed that the sterilized Roselle stem extract restrain *Staphyloccocus aureus* after 24 hours[7]. Roselle that is the natural substance was used as the reducing agent and coating in this experiment to prevent precipitation. It was in line with the study of Begum NA in 2009 that black tea leaves extracting with Ethyl Acetate was used in silver nanoparticles synthesis in order to maintain the form and prevent precipitation[14]. Furthermore, the study of Song JY in 2009 extracted the fern leaves, persimmon leaves, gingko leaves, and magnolia leaves to use as the reducing agent in the synthesis process[15]. This research synthesized silver nanoparticles with the Roselle coating with Microwave-assisted synthesis. Results showed that Roselle extract functioned as the reducing agent that helped to synthesize and coat to prevent precipitation of silver nanoparticles. It was found that if the solution was left at the room temperature for 48 hours after the synthesis, its color changed to yellow and would be darker when the time passed. It was consistent with the study of DarshanSingh et al. in 2015 who examined the silver nanoparticles synthesis using *Origanummajorana* extract and *Citrus sinensis* as the coating and reducing agent. Likewise, the solution's color changed to yellow[16].

For measuring the absorbance of SNP-Ro using Spectrophotometer with UV-Vis spectroscopy technique, it was found that the solution with six concentration levels had the absorbance result at 390-450 nm. This result was in line with the study result of Darshan Singh et al. in 2015 who synthesized silver nanoparticles with marjoram and used *Origanummajorana* leaves extract as the coating. The absorbance was 340-410 nm[16]. Further, the study of of Krishnara et al. in 2010 used leaf extract of Acalypha indicain the synthesis and the absorbance were 450 nm[17]. Lastly, the study of Preetha Devaraj et al. in 2013 applied Cannonball leaves extract in the experiment and the absorbance was 434 nm[18]. Anyway, the absorbance value was slightly different, because of the use of different extract, comparing to other researches, however, they were still in the same range. When analyzing SNP-Ro synthesizing with the TEM, it was found that silver nanoparticles were in round-shaped at the size 10-15 nm. This was similar to the study of Darshan Singh et al. in 2015 who had the particle size less than 70 nm.[16] Furthermore, the research of Krishnara et al. in 2010 had 20-30 nm. size in the round shaped as well[17]. Report from PreethaDevaraj et al. in 2013 show that the particles were likely in round-shaped at 13-61 nm.[18]

The study of the antibacterial activity on SNP-RO to *Aa* found that it could resist *Aa.* This was consistent with the previous study on the antibacterial activity of silver nanoparticles using plant in the synthesis process which found out that it contained the antibacterial activity as well. The study that used aloe vera extract to examine the antibacterial activity indicated that it was able to resist *S. epidermidis* and *P. aeruginosa*[19] Moreover, there was the research that used *Bidens biternata (Lour.) Merr. & Scherff.* extract to observe the antibacterial activity of silver nanoparticles. Results showed that it resisted both Gram-positive bacteria and Gram-negative bacteria, which were *S. aureus*, *Bacillus subtilis*, *Salmonella typhi,* and *Escherichia coli.* [20]

Silver nanoparticle has various mechanisms for antibacterial activity. There was the assumption that silver nanoparticles adhered and got through to the microbes' cell wall which caused the change of plasma membrane structure. Consequently, it could not control the movement of cytoplasmic membrane so many things got through the membrane. Finally, the cells died.[21] Besides, the report about the mechanism for disinfection of silver nanoparticles that it derived from the free radicals which destroyed the membrane. Therefore, silver nanoparticle got through the cells and they died eventually.[22] Another mechanism was the reaction between silver nanoparticles and DNA compound which did not allow the fission and DNA creation. Finally, the cells died.[23,24] In the meantime, the research of Shrivastava et al.[25] explained that silver nanoparticles minimized

phosphotyrosine in bacteria's peptide which influenced the cell signal to obstruct the growth of cells. As a result, the cells died.[26]

Conclusions

SNP-Ro solution containing the different ratio of Roselle could be reduced with Microwave-assisted synthesis to obtain the yellow solution in small size, about 10-15 nm. Making the silver particle became smaller would increase the special properties such as more efficiency in antibacterial or the easy access to the cells. It could be seen from SNP-Ro that all ratios were able to resist bacteria at 100%. The experiment results could be concluded that silver nanoparticles synthesis could be processed with natural extract to reduce toxicity and utilize in medical area efficiently. Furthermore, this knowledge might be applied to dental supplies development to resist bacteria and decrease the risk of disease.

Declaration of Interest

The authors report no conflict of interest and this work was funded by Faculty of dentistry, Naresuan university, Thailand and supported by Mrs. Suttimas Yuakyong, Faculty of Dentistry, Naresuan University

References

1. Rai MK, Deshmukh SD, Ingle AP, Gade AK. Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. J Appl Microbiol 2012;112(5):841-52.
2. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 2009;27(1):76-83.
3. Nanomaterials in REACH Nanosilver as a case study. Available at: "http://www.rivm.nl/bibliotheek/rapporten/601780003.pdf". Accessed November 17, 2009.
4. Jancy ME, Inbathamizh L. Green synthesis and characterization of nano silver using leaf extract of morinda pubescens. Asian J Pharm Clin Res 2012;5(1):159-62.
5. Vasquez RD, Apostol JG, De Leon JD, et al. Polysaccharide-mediated green synthesis of silver nanoparticles from Sargassum siliquosum J.G. Agardh: Assessment of toxicity and hepatoprotective activity. OpenNano 2016;1:16–24.
6. Tounkara F, Sodio B, Chamba MVM, Le GW, Shi YH. Nutritional and functional properties of Roselle (Roselle L.) seed protein hydrolysates. Emir. J. Food Agric 2014;26(5):409-17.
7. Higginbotham KL, Burris KP, Zivanovic S, Davidson PM, Stewart CN. Antimicrobial Activity of Roselle Aqueous Extracts against Escherichia coli O157:H7 and Staphylococcus aureus in a Microbiological Medium and Milk of Various Fat Concentrations. J Food Prot 2014;77(2):262–8.
8. Haghgoo R, Saderi H, Eskandari M, Haghshenas H, Rezvani MB. Evaluation of the Antimicrobial Effect of Conventional and Nanosilver Containing Varnishes on Oral Streptococci. J Dent Shiraz Univ Med Sci 2014;15(2):57-62.
9. Hamouda IM. Current perspectives of nanoparticles in medical and dental biomaterials. Journal of Biomedical Research J *Biomed. Res* 2012;26(3):143-51.
10. Melo MAS, Guedes SFF, Xu HHK, and Rodrigues LKA. Nanotechnology-based restorative materials for dental caries management. Trends Biotechnol 2013;31(8):1-18.
11. Ranganathan AT, Sarathy S, Chandran CR, and Iyan K. Subgingival prevalence rate of enteric rods in subjects with periodontal health and disease. J Indian Soc Periodontol 2017;21(3):224–8.
12. Wacharanad S , Sasimomthon W, Wongyai P, Vudhivanich A, Tippawan K. Activity of Chlorhexidine Gluconate Loaded at Varying Polyelectrolyte Multilayers against Aggregatibacter Actinomycetemcomitans. MATEC Web of Conferences 2016;77:11003.
13. Abdallah EM. Antibacterial efficiency of the Sudanese Roselle (Roselle L.),a famous beverage from Sudanese folk medicine. J Intercult Ethnopharmacol 2016;5(2)):186–190.
14. Begum NA, Mondal S, Basu S, Laskar RA, Mandal D. Biogenic synthesis of Au and Ag nanoparticles using aqueous solutions of Black Tea leaf extracts. Colloids Surf B Biointerfaces 2009;71(1):113-8.
15. Song JY, Kim B. Rapid biological synthesis of silver nanoparticles using plant leaf extracts. Bioprocess Biosyst Eng 2009;32(1):79-84**.**
16. Singh D, Rawat D, Isha. Microwave-assisted synthesis of silver nanoparticles from Origanummajorana and Citrus sinensis leaf and their antibacterial activity: a green chemistry approach. Bioresour. Bioprocess 2016;3(1):1-7.
17. Krishnaraj C, Jagan EG, Rajasekar S, Selvakumar P, Kalaichelvan PT, Mohan N. Synthesis of silver nanoparticles using Acalypha indica leaf extracts and its antibacterial activity against water borne pathogens. Colloids Surf B Biointerfaces 2010;76:50–6.
18. Preetha D, Prachi K, Chirom A and Arun R. Synthesis and Characterization of Silver Nanoparticles Using Cannonball Leaves and Their Cytotoxic Activity against MCF-7 Cell Line. J Nanotechnol 2013;1-5
19. Zhang Y, Yang D, Kong Y, Wang X, Pandoli O, Gao G. Synergetic antibacterial effects of silver nanoparticles Aloe Vera prepared via a green method. Nano Biomed Eng 2010;2(4):252–7.
20. Sadeghi B, Gholamhoseinpoor F. A study on the stability and green synthesis of silver nanoparticles using Ziziphoratenuior (Zt) extract at room temperature. SpectrochimActa Part A:MolBiomolSpectrosc 2015;134:310–5.
21. Nanomaterial Case Study: Nanoscale Silver in Disinfectant Spray. Available at: "file:///C:/Users/User/Downloads/NANOMATERIALS_CASESTUDY_SILVER_FINAL.PDF". Accessed April 20,2016.
22. Sharma H, Hussain S, Schlager J, Ali S, Sharma A, Uppsala universitet M. Influence of Nanoparticles on Blood-Brain Barrier Permeability and Brain Edema Formation in Rats. Acta Neurochir Suppl 2010;106:359-64.
23. Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, Lara HH, et al. Interaction of silver nanoparticles with HIV-1. J Nanobiotechnology 2005;3(6):1-10.
24. Yang W, Shen C, Ji Q, An H, Wang J, Zhang Z, et al. Food storage material silver nanoparticles interfere with DNA replication fidelity and bind with DNA. Nanotechnology 2009;20(8):1-7.
25. Leu L, Sun RW, Chen R, Hui CK, Ho CM, Luk JM, et al. Silver nanoparticles inhibit hepatitis B virus replication. AntivirTher 2008;13(2):253–62.
26. Stevens K, Crespo-Biel O, van den Bosch E, et al. The relationship between the antimicrobial effect of catheter coatings containing silver nanoparticles and the coagulation of contacting blood. Biomaterials 2009;30:3682-90.