Antibacterial activity of silver nanoparticles-\textit{Clinacanthus nutans} (AgNP-CN) against \textit{Streptococcus mutans}

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\textbf{ABSTRACT}

\textit{Clinacanthus nutans} was found to possess anti-venom, anti-inflammatory, analgesic, anti-diabetic, anti-rheumatism, antiviral and antioxidant properties. Silver nanoparticles are nanoparticles between 1nm to 100nm in size and play significant role in medicinal fields. Silver nanoparticles exhibit unique properties, such as excellent conductivity, chemical stability, catalytic and antimicrobial activity. \textit{Streptococcus mutans} is usually discovered in human oral cavity and the main aetiological of tooth decay. There is no study on antibacterial effect of silver nanoparticles \textit{Clinacanthus nutans} (AgNP-CN) against \textit{Streptococcus mutans} reported to date. Therefore, objective of this study is to investigate antibacterial properties of silver nanoparticles \textit{Clinacanthus nutans} against \textit{Streptococcus mutans}. \textit{Streptococcus mutans} was subcultured in brain heart infusion (BHI) broth and agar. AgNP-CN with different concentrations was tested against \textit{Streptococcus mutans} via disc diffusion assay, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Amoxicillin was used as positive control while DMSO and blank silver nanoparticles were used as negative control. Disc diffusion assay shows AgNP-CN inhibit \textit{Streptococcus mutans} growth. AgNP-CN shows the greatest inhibition properties (15.3±1.53 mm) in comparison to \textit{Clinacanthus nutans} leaves extract (6.0±0.01 mm) and blank silver nanoparticles (6.0±0.01 mm). MIC value for AgNP-CN is 2.5±0.01 mg/mL while amoxicillin is 0.007±0.01 mg/mL. Whereas MBC values for AgNP-CN is 2.5 mg/mL. Results are concentration dependent, with higher concentration shows better inhibition property.

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ever, the greatest crucial aetiological representative of dental caries is *Streptococcus mutans* (Lemos et al., 2013). Tooth decays occur when a susceptible tooth surface is occupied by cariogenic microbes and nutritional resource of sucrose or processed sugar. Fermentation of sugar will lead to fabrication of lactic acid by the action of bacteria which dissolves the hydroxyapatite crystal formation of the tooth which foundations caries. *Streptococcus mutans* is one of Gram-positive cocci bacteria and the most common microorganism that caused dental caries (Hanafiah et al., 2015). Cavitation produced by *Streptococcus mutans* may provide an ecological niche where the microorganisms form a protected biofilm, and enabling caries progression (Rouabhia, 2012). *Streptococcus mutans* can colonize the oral cavity at any age if the environment is favorable and the amount may increase with age (Köhler and Andréen, 2012).

Silver in colloidal state reveals unique properties such as good conductivity, organic stability, catalytic and antibacterial activity (Ramaio et al., 2009). Silver nanoparticles have tremendous applications in the field of antimicrobials and therapeutics (Rai et al., 2012). Biosynthetic methods to synthesize AgNP utilizing either one of biological microorganisms or plant extracts have become apparent as a straightforward and sustainable substitute to chemical and physical methods (Ahmed et al., 2016). For example, the use of plant extracts such as *Garcinia mangostana* and *Coptis sinensis* in synthesizing AgNP have shown excellent antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Veerasamy et al., 2011; Ahmad et al., 2017). Recent study shows silver nanoparticles-Clínicanthus nutans (AgNP-CN) possess anticancer properties against oral carcinoma cell lines (HSC-4) (Yakop et al., 2018).

*Clinicanchus nutans* (CN) is a small shrub belongs to the family of Acanthaceae, which is well-known to the South East Asian countries primarily Malaysia, Thailand and Indonesia (Zulkipli et al., 2017). *Clinicanchus nutans* was found to have anti venom, anti-inflammatory, analgesic, anti-diabetic, anti-rheumatism, anti-viral and antioxidant properties (Arullappan et al., 2014). The flavonoids and phenolic complexes that are produced generally in therapeutic plants can stimulate antibacterial response owing to the existence of carbonyl group (Alam et al., 2016). Methanolic extract of *C. nutans* leaves possessed antibacterial effect against *Staphylococcus aureus*, *Escherichia coli*, *Propionibacterium acnes*, *Staphylococcus epidermidis* and *Bacillus cereus* (Yang et al., 2013). Recent study showed *C. nutans* chloroform and ethano-

**MATERIALS AND METHODS**

**Preparation of C. nutans water extract**

*C. nutans* leaves were washed, separated and oven-dried at 45°C. The leaves were ground into fine powder and drenched into purified water at room temperature. Water extracts were accumulated in clean glass bottles and filtered using Whatman no. 1 paper then freeze dried (Hanafiah et al., 2019). *C. nutans* water extract was kept at -20°C until further use.

**Green synthesis of silver nanoparticle**

For production of silver nanoparticle, 1 mM of silver nitrate were combined with *C. nutans* leaves water isolate and incubated in room temperature until changes of colour to reddish brown pigment which signified the development of AgNP-CN (Yakop et al., 2018).

**Bacteria Culture**

*Streptococcus mutans* (ATCC 25175 strain) obtained from Microbiology laboratory, Faculty of Dentistry, Universiti Sains Islam Malaysia and subcultured in Brain Heart Infusion (BHI) broth.

**Disk diffusion assay**

Bacteria were cultured on brain heart infusion (BHI) agar and incubated under 37°C at anaerobic environment for 24 h. Approximately 1 x 10⁹ CFU/mL of bacteria intensities equivalent towards 0.5 McFarland turbidity guideline was employed to inoculate *S. mutans* into fresh BHI broth then incubated at 37°C overnight. A sterilized cotton swab was used to disseminate the culture on BHI agar before application of 5 x 6mm paper discs (Whatmann No. 1) infused with 10 μL tested materials (AgNP-CN, AgNP blank (Sigma) and CN) at concentration between 5-40 mg/mL. DMSO 10% was used as negative control and amoxicillin (30U) as positive control. Inhibition zones were measured from the circumference of disks to the circumference of inhibition zone after incubation under 37°C for 24 h at anaerobic state.

**Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**
Figure 1: Inhibition zone from disc diffusion assay. (A) AgNP-CN Vs S. mutans, (B) CN Vs S. mutans, (C) AgNP Vs S. mutans on different concentration; (1) 40 mg/mL, (2) 20 mg/mL, (3) 10 mg/mL, (4) 5 mg/mL, (5) DMSO, (Center) Amoxicillin 30U.

Figure 2: MIC wells. Lowest concentration with no bacterial activity where there is no colour changing is 2.5 mg/mL.

Table 1: Zone of inhibition against S. mutans from disc diffusion assay

| Sample         | Concentration (mg/mL) | DMSO     | AMO 30U |
|----------------|-----------------------|----------|---------|
|                | 40    | 20    | 10    | 5     |          |
| AgNP-CN (leaves) | 15.3±1.5<sup>d</sup> | 13.0±1.0<sup>c,d</sup> | 11.3±1.5<sup>b,c</sup> | 9.0±1.0<sup>a,b</sup> | 6.0±0.01<sup>a</sup> | 43.0±2.7 |
| CN (leaves)     | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 43.0±2.7 |
| AgNP blank      | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 6.0±0.01<sup>a</sup>  | 42.7±2.5 |

Values on the same row with different superscript letter differ significantly (p<0.05).

Table 2: MIC and MBC value of AgNP-CN Vs S. mutans

| Sample       | MIC value          | MBC value          |
|--------------|--------------------|--------------------|
| AgNP-CN      | 2.5±0.01 mg/mL     | 2.5 mg/mL (bactericidal) |
| Amoxicillin  | 0.007±0.01 mg/mL   | 0.020 mg/mL (bactericidal) |

Abbreviation: AgNP-CN: Silver nanoparticle-Clinacanthus nutans
The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was done using two-fold sequential dilution approach as portrayed by (Hanafäiah et al., 2019). MIC was completed in a sterile 96-well plate. S. mutans (100 μL) at 10^6 CFU/mL was added to several concentrations of AgNP-CN (0.08-40 mg/mL) diluted in fresh BHI broth to a finishing volume of 200 μL/well. DMSO 10% (v/v) was used as negative control and amoxicillin (0.008-0.128 mg/mL) was used as positive control (Ready et al., 2004). Following incubation at 37 °C under anaerobic condition for 24 h, MIC value was defined as the lowest concentration that impedes the observable growth of bacteria. Minimum bactericidal concentration (MBC) was established from culturing a 5 μL aliquot from well that demonstrated no microbial growth in MIC wells onto sterile BHI agar and incubated overnight at 37 °C and the MBC was demarcated as the lowest concentration stopping microbial growth.

**Statistical Analysis**

The data was collected and analyzed using two-way ANOVA test where the p value is less than 0.05 (p<0.05).

**RESULTS AND DISCUSSION**

This study was done to evaluate antibacterial activity of silver nanoparticle-Clinacanthus nutans (AgNP-CN) against S. mutans. AgNP-CN was synthesized via green synthesis method whereby Clinacanthus nutans leaves water extract was used as capping and reducing agents (Yakop et al., 2018). Silver nanoparticles have been known as new emerging antimicrobial agents in the field of nanomedicine (Awwad et al., 2020). Green synthesis method provides more economical and environment-friendly end products. Recent study shows AgNP-CN was not cytotoxic towards normal cell lines (3T3-L1 cell lines) (Yakop et al., 2018). With green synthesis method, silver nanoparticles produced were reported to exhibit better antimicrobial activities compared to physical and chemical methods (Srikar et al., 2016). This corresponds with present study whereby AgNP-CN shows more antibacterial activity as compared to AgNP blank and CN water extract. AgNP-CN shows antibacterial activity in concentration dependent manner. Disc diffusion assay is the most common technique used to determine antimicrobial resistance worldwide. It is because this test did not require any special equipment to execute. A vibrant rounded zone of no growth in the immediate part of a disc specifies susceptibility to that antimicrobial (Sandle, 2016). As shown in disc diffusion assay result (Table 1 and Figure 1), with higher concentration, more pronounced inhibition zones were observed.

Determination of MIC is important in research and laboratory diagnostics. It is used to find new alternative drugs and to confirm microbial resistance such as S. mutans from existing antibiotics. As for personalized medicine, clinicians use MIC value to choose which antibiotics with specific dose to administer to patient for certain infections (Hanafäiah et al., 2019). Study done by Roeslan et al., reported that C. nutans from hexane fraction did not show any MIC value but able to reduce biofilm formation of S. mutans (Roeslan et al., 2019). This study showed that C. nutans could reduce biofilm formation but did not kill S. mutans. As proposed by green synthesis method, synthesis of AgNP using plants extract especially C. nutans will enhance its antibacterial activity. This supported present study whereby the MIC value is 2.5 mg/mL (Table 2 and Figure 2). This proved that C. nutans able to enhance AgNP-CN activity against S. mutans. This event could be explained with AgNP is reduced and stabilized by bioactive compounds such as polyphenol, flavonoids, terpenoids, alkaloids in CN water extracts that reported to possess antibacterial activity (Tanase et al., 2020).
MBC always complemented with MIC. If only MIC value is obtained, it cannot conclude what is the nature of inhibition of tested materials such as drugs, bioactive compounds, plants extract or in this case AgNP-CN against certain microorganism. Antibacterial agents were considered as bactericidal if MBC value was not farther than four times of MIC value, the closer the MIC value to MBC the more bactericidal the compounds or extracts (CLSI, 2019).

In this study, AgNP-CN MBC value (2.5 mg/mL) (Figure 3) was less than four times of MIC value (2.5 mg/mL). Meanwhile MIC and MBC values of amoxicillin were 0.007 mg/mL and 0.020 mg/mL, respectively. With the same value of MIC and MBC AgNP-CN is regarded as bactericidal agent against S. mutans.

CONCLUSIONS

AgNP-CN shows potential in inhibiting growth of S. mutans as compared to the CN leaves extract and AgNP blank. The antibacterial effect of AgNP-CN against S. mutans is concentration dependent whereby higher concentration gives larger inhibition zones. AgNP-CN has bactericidal properties against S. mutans.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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