



Antibacterial activity of silver nanoparticles-*Clinacanthus nutans* (AgNP-CN) against *Streptococcus mutans*

Siti Aisyah Abd Ghafar¹, Nor Baitie Adura Mohd Fudzi¹, Wan Nur Farhanah Wan Sulaiman¹, Lim Vuanghao², Rohazila Mohamad Hanafiah*¹

¹Department of Oral Biology and Basic Sciences, Universiti Sains Islam Malaysia, Pandan Indah, Kuala Lumpur, Malaysia

²Intergrative Medicine Cluster, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam-13200, Kepala Batas, Penang, Malaysia

Article History:

Received on: 24 Jun 2020
Revised on: 27 Jul 2020
Accepted on: 07 Aug 2020

Keywords:

Antibacterial,
Clinacanthus nutans,
Silver nanoparticles,
Streptococcus mutans

ABSTRACT

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. *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 *Clinacanthus nutans* (AgNP-CN) against *Streptococcus mutans* reported to date. Therefore, objective of this study is to investigate antibacterial properties of silver nanoparticles *Clinacanthus nutans* against *Streptococcus mutans*. *Streptococcus mutans* was subcultured in brain heart infusion (BHI) broth and agar. AgNP-CN with different concentrations was tested against *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 *Streptococcus mutans* growth. AgNP-CN shows the greatest inhibition properties (15.3±1.53 mm) in comparison to *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.



*Corresponding Author

Name: Rohazila Mohamad Hanafiah

Phone: +6010-4081901

Email: rohazila@usim.edu.my

ISSN: 0975-7538

DOI: <https://doi.org/10.26452/ijrps.v11iSPL4.4238>

Production and Hosted by

Pharmascope.org

© 2020 | All rights reserved.

INTRODUCTION

World Health Organization (WHO) estimated almost half of the world's population is affected by dental caries (WHO, 2017). Dental caries is a multifactorial disease that results from four essential factors which are tooth surfaces, substrate, microorganism on biofilm and time (Kumar et al., 2015). Microorganisms are one of the main agents responsible to cause dental caries. Countless facultative as well as obligate anaerobic bacteria overshadow the microbiological population of dental caries. How-

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 (Ramajo 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-*Clinacanthus nutans* (AgNP-CN) possess anticancer properties against oral carcinoma cell lines (HSC-4) (Yakop et al., 2018).

Clinacanthus 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). *Clinacanthus 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-

lic extracts possessed antibacterial activity against anaerobic periodontal bacteria which were *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Hanafiah et al., 2019). Considering vast potentiality of plants as reductants and capping agents in synthesis of silver nanoparticle and medicinal values of *C. nutans*, this study will be focused on antibacterial activity of AgNP-CN against *S. mutans*.

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 1×10^8 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)

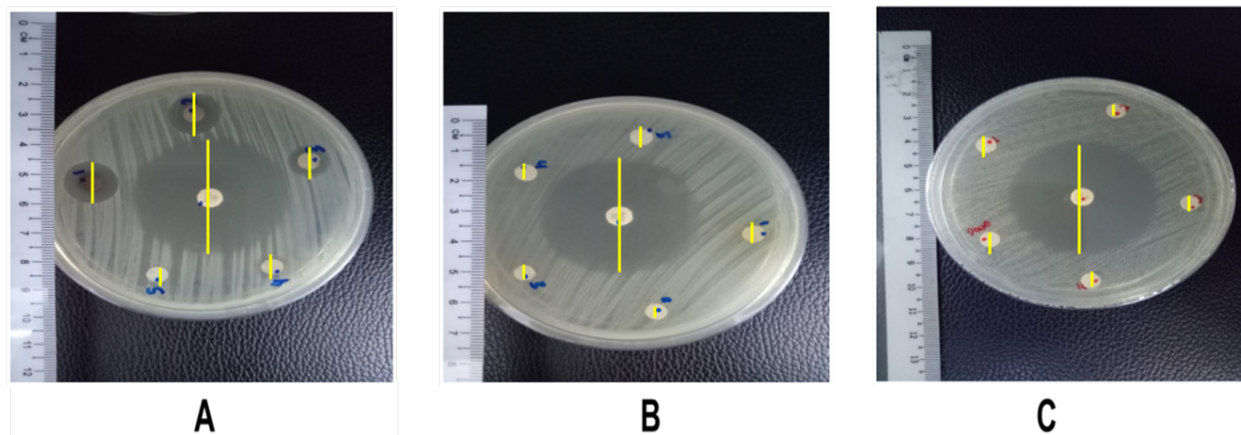


Figure 1: Inhibition zone from disc diffusion assay. (A) AgNP-CN Vs *S.mutans*, (B) CN Vs *S.mutans*, (C) AgNP Vs *S.mutans* on different concentration; (1) 40 mg/mL, (2) 20 mg/mL, (3) 10 mg/mL, (4) 5mg/mL, (5) DMSO, (Center) Amoxicillin 30U.

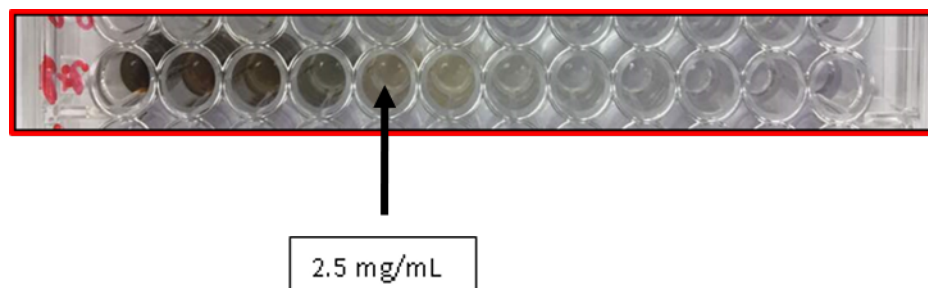


Figure 2: MIC wells. Lowest concentration with no bacterial activity where there is no colour changing is 2.5 mg/mL.

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 ^d	13.0±1.0 ^{c,d}	11.3±1.5 ^{b,c}	9.0±1.0 ^{a,b}	6.0±0.01 ^a	43.0±2.7
CN (leaves)	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	43.0±2.7
AgNP blank	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	6.0±0.01 ^a	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 (Hanafiah et al., 2019). MIC was completed in a sterile 96-well plate. *S. mutans* ($100 \mu\text{L}$) at 10^8 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 $200 \mu\text{L}$ /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 $5 \mu\text{L}$ 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.

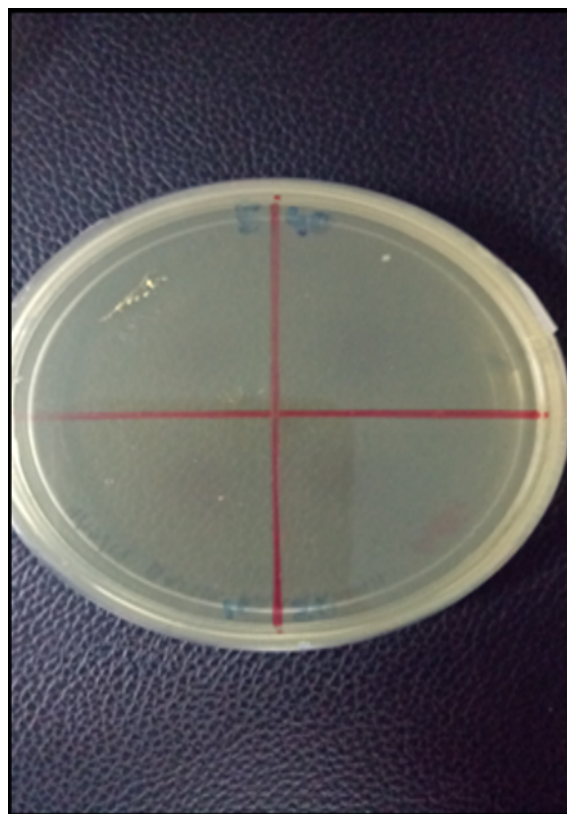


Figure 3: MBC plate inoculate from MIC well (2.5 mg/mL). No growth of bacteria noted. Thus, MBC= 2.5mg/mL

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 (Hanafiah 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.

Funding Support

This study was financially supported by Ministry of High Education (MOHE) Malaysia (USIM/FRGS/FPG/055002/51717) and Universiti Sains Islam Malaysia (P1-16-17019-UNI-USIM-FPG).

REFERENCES

- Ahmad, A., Wei, Y., Syed, F., Tahir, K., Rehman, A. U., Khan, A., Ullah, S., Yuan, Q. 2017. The effects of bacteria-nanoparticles interface on the antibacterial activity of green synthesized silver nanoparticles. *Microbial Pathogenesis*, 102:133–142.
- Ahmed, S., Ahmad, M., Swami, B. L., Ikram, S. 2016. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: A green expertise. *Journal of Advanced Research*, 7(1):17–28.
- Alam, A., Ferdosh, S., Ghafoor, K., Hakim, A., Juraimi, A. S., Khatib, A., Sarker, Z. I. 2016. Clinacanthus nutans : A review of the medicinal uses, pharmacology and phytochemistry. *Asian Pacific Journal of Tropical Medicine*, 9(4):402–409.
- Arullappan, S., Rajamanickam, P., Thevar, N., Kodimani, C. 2014. In Vitro Screening of Cytotoxic, Antimicrobial and Antioxidant Activities of Clinacanthus nutans (Acanthaceae) leaf extracts. *Tropical Journal of Pharmaceutical Research*, 13(9):1455–1455.
- Awwad, A. M., Salem, N. M., Aqarbeh, M. M., Abdulaziz, F. M. 2020. Green synthesis, characterization of silver sulfide nanoparticles and antibacterial activity evaluation. *Chemistry International*, 6(1):42–48.
- CLSI 2019. Performance standards for antimicrobial susceptibility testing 29th ed. Wayne, Pennsylvania, M100. *Clinical and laboratory standard institute*, 100:1–281.
- Hanafiah, R. M., Aqma, W. S., Yaacob, W. A., Said, Z., Ibrahim, N. 2015. Antibacterial and biofilm inhibition activities of Melastoma malabathricum stem bark extract against Streptococcus mutants. *Malaysian Journal of Microbiology*, 11(Specialissue2):199–206.
- Hanafiah, R. M., Kamaruddin, K. A., Saikin, N. A., Abdul, W. N., Yakop, M. F., Lim, V., Ghafar, S. A. A., Azizan, N., Said, S. M. 2019. Antibacterial Properties of Clinacanthus nutans Extracts Against Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans: An In-Vitro Study. *Journal of International Dental and Medical Research*, 12(2):401–404.
- Köhler, B., Andréen, I. 2012. Mutans Streptococci and Caries Prevalence in Children after Early Maternal Caries Prevention: A Follow-Up at 19 Years of Age. *Caries Research*, 46(5):474–480.
- Kumar, A., Puranik, M. P., Sowmya, K. R. 2015. Relationship between untreated dental caries and dental neglect among mentally handicapped children: A cross-sectional study. *Journal of Indian Association of Public Health Dentistry*, 13(2):126–126.
- Lemos, J. A., Quivey, R. G., Koo, H., Abranches, J. 2013. Streptococcus mutans: a new Gram-positive paradigm?
- Rai, M. K., Deshmukh, S. D., Ingle, A. P., Gade, A. K. 2012. Silver nanoparticles: the powerful nanoweapon against multidrug-resistant bacteria. *Journal of Applied Microbiology*, 112(5):841–852.
- Ramajo, L., Parra, R., Reboredo, M., Castro, M. 2009. Preparation of amine coated silver nanoparticles using triethylenetetramine. *Journal of Chemical Sciences*, 121(1):83–87.
- Ready, D., Lancaster, H., Qureshi, F., Bedi, R., Mullany, P., Wilson, M. 2004. Effect of Amoxicillin Use on Oral Microbiota in Young Children. *Antimicrobial Agents and Chemotherapy*, 48(8):2883–2887.
- Roeslan, M. O., Ayudhya, T. D. N., ek Yingyong-

- narongkul, B., Koontongkaew, S. 2019. Anti-biofilm, nitric oxide inhibition and wound healing potential of purpurin-18 phytol ester isolated from *Clinacanthus nutans* leaves. *Biomedicine & Pharmacotherapy*, 113:108724–108724.
- Rouabhia, M. 2012. Diseases Associated with Oral Polymicrobial Biofilms. *The Open Mycology Journal*, 6(1):27–32.
- Sandle, T. 2016. Antibiotics and preservatives. *Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control*, pages 171–183.
- Srikar, S. K., Giri, D. D., Pal, D. B., Mishra, P. K., Upadhyay, S. N. 2016. Green Synthesis of Silver Nanoparticles: A Review.
- Tanase, C., Berta, L., Mare, A., Man, A., Talmaciu, A. I., Roşca, I., Mircia, E., Volf, I., Popa, V. I. 2020. Biosynthesis of silver nanoparticles using aqueous bark extract of *Picea abies* L. and their antibacterial activity. *European Journal of Wood and Wood Products*, 78(2):281–291.
- Veerasamy, R., Xin, T. Z., Gunasagaran, S., Xiang, T. F. W., Yang, E. F. C., Jeyakumar, N., Dhanaraj, S. A. 2011. Biosynthesis of silver nanoparticles using mangosteen leaf extract and evaluation of their antimicrobial activities. *Journal of Saudi Chemical Society*, 15(2):113–120.
- WHO 2017. Sugars and dental caries. Geneva: WHO. [accessed 17 February 2020]. *World Health Organization*, pages 17–17.
- Yakop, F., Ghafar, S. A. A., Yong, Y. K., Yazan, L. S., Hanafiah, R. M., Lim, V., Eshak, Z. 2018. Silver nanoparticles *Clinacanthus Nutans* leaves extract induced apoptosis towards oral squamous cell carcinoma cell lines. *Artificial Cells, Nanomedicine, and Biotechnology*, 46(sup2):131–139.
- Yang, H. S., Peng, T. W., Madhavan, P., Shukkoor, M. A., Akowuah, G. A. 2013. Phytochemical analysis and antibacterial activity of methanolic extract of *Clinacanthus nutans* leaf. *International Journal Drug Development Research*, 5(3):349–355.
- Zulkipli, I. N., Rajabalaya, R., Idris, A., Sulaiman, N. A., David, S. R. 2017. *Clinacanthus nutans*: a review on ethnomedicinal uses, chemical constituents and pharmacological properties. *Pharmaceutical Biology*, 55(1):1093–1113.