

Antimicrobial Activity of Methanolic Neem Extract on Wound Infection Bacteria

Syarifah Masyitah Habib Dzulkarnain, and Izham bin Abdul Rahim

Abstract—Wound causes an increasing burden to healthcare systems as the availability of drugs capable of stimulating the process of wound repair is still limited in order to treat the patient. In addition, the increasing prevalence of antibiotic resistance bacteria is widely recognized and poses a continuing burden to healthcare. Therefore, the need of novel antibiotics for effective treatment for wound infection is necessary in order to eliminate the threat of the infections. Objective of this study was to investigate the in vitro antibacterial activity of medicinal plant, *Azadirachta indica* or its local name 'neem', on wound infection bacteria. Methanolic neem extract was used to test on the bacteria at four different concentrations. Antimicrobial susceptibility testing (AST) were used in this study. Zone of inhibition have been shown to *Staphylococcus aureus* and was considered significant because the p-value was less than 0.05 at concentration 150mg/ml and 200mg/ml when compared with positive control. This study showed that methanolic neem extract possess antimicrobial activity and further studies need to be done in order to exploit the bioactive compounds of neem in order to reveal its potential as a new source of antibacterial agent.

Keywords—antibiotic resistance bacteria, antimicrobial susceptibility testing, medicinal plant, wound

I. INTRODUCTION

HERBAL remedies are known to treat many infectious diseases throughout the history of mankind. Plant material continues to play a major role in the primary health care as therapeutic remedies in many developing countries. Thus, the discovery of medicinal plants as antimicrobial agents is useful in expanding the wide varieties of antibiotics available [1]. When it comes to wound healing, no one can go past *Azadirachta indica*, or its local name neem, for its wound healing properties [2]. Various parts of the neem tree have been used as traditional Ayurvedic medicine in India. For instance, the leaves, seed and bark possesses a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms. However, the leaves are in concern for its medicinal properties for wound healing. Neem leaf is effective in the healing of chronic wounds, diabetic foot and gangrene developing conditions [3].

As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, thus the development of modern drugs from neem should be

emphasized for the control of various infections. In fact, time has come to make good use of centuries-old knowledge on neem through modern approaches of drug development since the widespread prescription of antibiotics has promoted the species population to evolve to a condition of multiple drug resistance.

This study is aimed at determining the antimicrobial activity of neem against possible bacteria that can cause wound infections which are *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*.

II. METHODOLOGY

A. Neem extraction

Air dried powder (100 g) of the leaves was mixed with 500 mL of methanol and were kept at room temperature for 36 hours. The mixture was then filtered through Whatman No.1 filter paper and the filtrate was evaporated to dryness by leaving it inside the oven at constant temperature of 60°C for 3 to 4 days. The residues obtained were stored at 4°C until testing. Four different concentrations, 50,100,150 and 200 (mg/ml) in 20% dimethyl sulfoxide (DMSO) were prepared.

B. Bacterial strains

All the bacterial cultures preparations were performed by using the stock culture from ATCC. The bacteria were *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615) and *Pseudomonas aeruginosa* (ATCC 10145). From the stock culture, one colony was taken to be streaked on nutrient agar (NA) and incubated for 18 hours at 37°C. Culture purity of each organism was confirmed through colony morphology identification, Gram stain, coagulase, catalase and biochemical testing where one colony was taken to perform the confirmation. All bacterial cultures were maintained in NA slants stored at 4°C in the Preparation Laboratory of the Health Sciences Faculty and periodically sub-cultured.

C. Antimicrobial susceptibility testing by NCCLS method.

Muller Hinton agar plates were subcultured with standardized inoculums of bacterial strains. The plates were allowed to dry for 3 to 5 minutes. Then, the prepared disk were placed onto MHA plates and were incubated for 18 hours at 37°C. This experiment was carried out in triplicate. For positive control, standard antibiotic were used. On the other hand, 20% DMSO impregnated disks were used as the negative control. After the incubation period, each plate was examined. If there

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is the resulting zone of inhibition, uniformly circular zone of inhibition will be formed. The diameters of complete zone inhibition were measured to the nearest whole millimeter by using a ruler. The results of the measurement were recorded and the pictures of the plates were taken by using Molecular Imager Gel Doc™ System.

III. RESULT

A. Product of Neem Extract

Methanolic extracts as seen in Fig.1 of the 100g dried neem leaves obtained was 4.18g (4.18%). For quality control, the neem extract was cultured on nutrient agar (NA) to determine its purity. After overnight incubation, no growth of any colonies of bacteria has been shown.

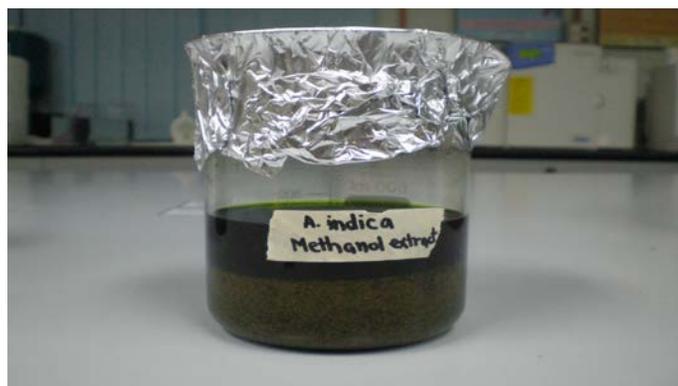


Fig. 1 Methanolic extract of neem

B. Antimicrobial susceptibility testing

TABLE I
ZONE INHIBITION OF BACTERIA

Bacteria	Neem (mg/ml)	50	100	150	200	Positive control	Negative control
<i>S. aureus</i>	Average inhibition zone (mm)	8.7	9.3	10	10	20.7	-
	Standard deviation (SD)	0.577	0.577	0	0	0.577	-
<i>S. pyogenes</i>	Average inhibition zone (mm)	9.3	10.7	11	11.3	21.7	-
	Standard deviation (SD)	0.577	0.577	0	0.577	0.577	-
<i>P. aeruginosa</i>	Average inhibition zone (mm)	-	-	-	-	-	-
	Standard deviation (SD)	-	-	-	-	-	-

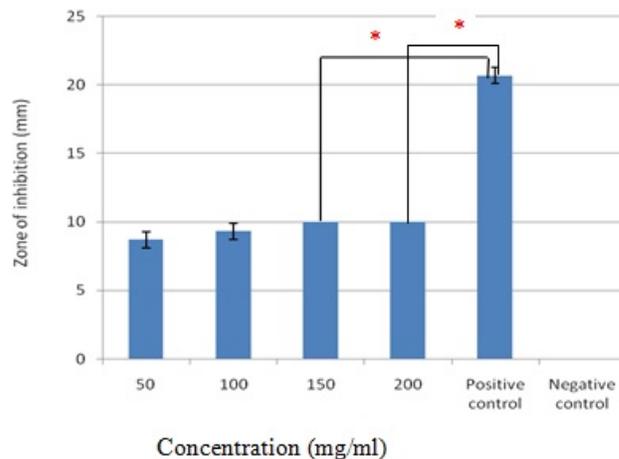


Fig. 2 Bar chart for the average of inhibition zone against the neem extract concentration(mg/ml) for *S. aureus*. ‘*’ indicates p-value<0.05

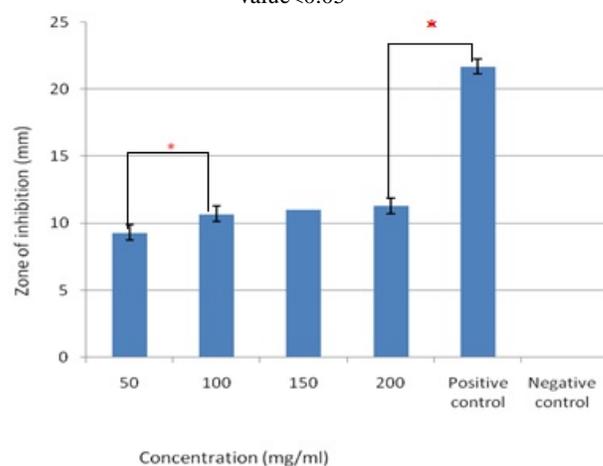


Fig. 3 Bar chart for the average of inhibition zone against the neem extract concentration(mg/ml) for *S. pyogenes*. ‘*’ indicates p-value<0.05

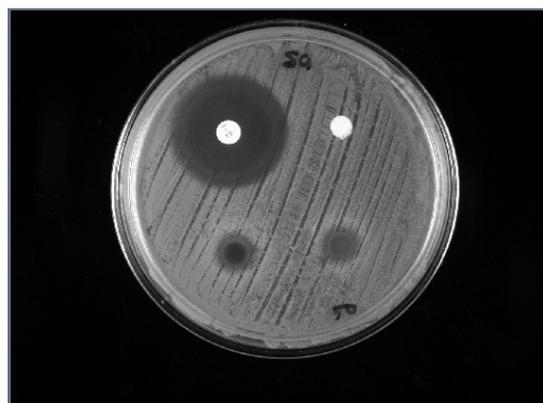


Fig. 4 Zone of inhibition of neem extract on *Staphylococcus aureus*

IV. DISCUSSION

A. Extraction Method

The method of choice that had been used in this study was the solvent extraction. This method was chosen due to the

limitation of equipments available in the laboratory and the simplicity of the method to be done. Dried neem leaves were used by following the method used by the previous study [4]. According to [5], the differences in water content may affect solubility of subsequent separation by liquid-liquid extraction and the secondary metabolic plant components should be relatively stable, especially if it is to be used as an antimicrobial agent. Therefore, the dried neem leaves had been chosen.

A slightly small amount methanolic neem extract which was 4.18g (4.18%) was obtained in this study instead of what was obtained in the previous study [4] which was 4.36%. Variations in extraction methods which are usually found in the length of the extraction period may contribute to this outcome. Reference [5] shows, the longer the contact between solvent and material, thus the more desirable extract will be produced until all possible materials have been extracted. Moreover, the particle size also may give the impact for the extraction period. Grinding the plant material as finer as possible will increase the surface area for the extraction thereby increasing the rate of extraction and shaking the plant material in the solvent mixture will also increase the rate of extraction [5]. In addition, the equipment used in the previous study [4] was Soxhlet apparatus for the extraction method. In soxhlet extraction, the sample is continuously exposed to a fresh solvent thus allowing the ground neem leaves to react with the solvent which is methanol more effectively thus improve the efficiency. Quality control that have been done to determine the purity of the extract showing that there was no growth of any colonies of bacteria thus suggesting that the test done was validated.

A 100% methanol was used as a solvent of choice. The reason is because the organic solvent was found to provide more consistent antimicrobial activity compared with water extraction method [6]. This was proved by the previous study [7] that showing remarkable zone of inhibition by using methanol compared to aqueous (water) results. These observations also can be due to the polarity of the compounds being extracted by the solvent [6] in order to ensure that a wide polarity range of compounds could be extracted. In addition, this organic solvent was also used because most of the antimicrobial active components are not water soluble. Moreover, many of the previous study of neem also used methanol as a solvent for extraction [4]-[7]-[8]-[9].

In addition, dimethyl sulfoxide (DMSO) was used to prepare neem extract concentration. It was used because it has a unique capability of penetrating living tissues without causing significant damage to the resulting neem extract. This unique capability of DMSO most probably related to its relatively polar nature, its capacity to accept hydrogen bonds, and its relatively small and compact structure [10]. The important thing here is the solvent should be non-toxic and must not interfere with assay to be done since the end product will contain traces of residual solvent.

Therefore, the solvent used for extraction and the extraction

technology being performed are the important parameters as these are the basic parameters influencing the quality of an extract[5].

B. Antibiotic Susceptibility Testing (AST)

Methanolic neem extract in this study had shown to possess antibacterial activities against *Staphylococcus aureus* and *Streptococcus pyogenes* but not for *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was not inhibited by the concentration ranging from 50mg/ml to 200mg/ml. However, very poor growth of *Streptococcus pyogenes* on Muller Hinton agar showed that the growth criteria did not met thus the antimicrobial effect of neem extract against *Streptococcus pyogenes* could not be concluded. The reason was, *Streptococcus pyogenes* is a fastidious bacteria that required enriched medium containing blood to grow. Therefore, for the next testing, the agar used should be blood agar or chocolate agar.

Staphylococcus aureus was susceptible to the methanolic neem extract starting at concentration 50mg/ml through 200mg/ml. The result in Table 1 showed that the higher concentration of neem extract, the larger the diameter of inhibition zones, thus, this exhibit the concentration dependant activity of the bacterium. At concentration 150mg/ml and 200mg/ml, diameter of inhibition zone as seen in Fig. 4 were considered significant if compared with positive control with p-value less than 0.05 as seen in Fig. 2. These results suggest that neem extract at concentration 150mg/ml and 200mg/ml has significance antimicrobial effect over positive control against *Staphylococcus aureus*.

In this study, neem extracts had shown to have antibacterial effect on Gram positive bacteria but not on Gram negative bacteria. This was supported by the previous study performed [11] that had found that plant extracts inhibited the Gram-positive microorganisms more than the Gram-negative ones. *Pseudomonas aeruginosa* showed antibiotic resistant because it may be chromosomally or plasmid mediated and may also be affected by changes in the cellular membrane or intracellular environment [12]. This could possibly be explained by the fact that the antibacterial activity of neem had been found to be mainly due to the inhibition of cell membrane synthesis within the bacteria where the "L" forms of *Pseudomonas aeruginosa* was develop thus becoming resistant strains [8]. However, the other study [9] found that methanolic extract of neem leaves at 100mg/ml exhibit 10 to 23 mm diameter of inhibition. This variation may be due to the different method where agar well diffusion method was used instead of the susceptibility testing.

V.CONCLUSION

In this study, the antibacterial activity of neem extracts towards significant microbes that causing wound infection has been investigated. The methanolic extract of neem, is remarkably inhibited the growth of the tested bacteria, they was *Staphylococcus aureus* but not for *Streptococcus pyogenes* and *Pseudomonas aeruginosa*. In this study, *Staphylococcus aureus* exhibit an antimicrobial effect with significant zone of inhibition at concentration of 150mg/ml and at 200mg/ml as compared with positive control since the

p-value of less than 0.05. As this study was a basic approach to investigate the antibacterial activity of neem extracts, the addition of the species of microorganism to be tested and the drug combination activity study were recommended for future study in order to determine the effectiveness of this plant.

ACKNOWLEDGMENT

We would like thank Universiti Teknologi MARA for providing fund to carry out this project. In addition, we also would like to express our gratitude to UiTM's laboratory staffs for their cooperation and technical experts.

REFERENCES

- [1] Zaidan, M.R.S., Noor Rain, A., Badrul, A.R., Adlin, A., Norazah, A., Zakiah, I., (2005). In vitro screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Tropical Biomedicine* 22(2): 165-170.
- [2] Sekar, S. (2008). Pharmaceutical Plant Company herbs. Retrieved February, 2009, from Metabalm: <http://www.ppcherbs.com.au/metabalm.htm>
- [3] Yagoub, S.O., Al Safi, S.E.H., Ahmed, B., El Magbol, A.Z., (2005). Antimicrobial activity of some medicinal plants against some Gram positive, Gram negative and fungi. Retrieved February, 2009 from <http://www.astf.net/sro/sro4/third%20scope%20priorities%20of%20scientific%20research%20and%20specialized%20domains%20in%20science/Specialized%20scientific%20fields/Medicine/accepted/872P.pdf>
- [4] Thakurta, P., Bhowmika, P., Mukherjee, S., Hajra, T.K., Patra, A., Baga, P.K., (2007). Antibacterial, antisecretory and antihemorrhagic activity of *Azadirachta indica* used to treat cholera and diarrhea in India. *Journal of Ethnopharmacology* 111 (2007) 607–612. <http://dx.doi.org/10.1016/j.jep.2007.01.022>
- [5] Ncube, N. S., Afolayan, A. J., Okoh A. I., (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology* Vol. 7 (12), pp. 1797-1806.
- [6] Parekh, J., Jadeja, D., Chanda, S., (2005). Efficacy of Aqueous and Methanol Extracts of Some Medicinal Plants for Potential Antibacterial Activity. *Turk J Biol* 29 (2005) 203-210.
- [7] Sharma, D., Lavania, A.A., Sharma, A., (2009). In vitro Comparative Screening of Antibacterial and Antifungal Activities of Some Common Plants and Weeds Extracts. *Asian J. Exp. Sci.*, Vol. 23, No. 1, 2009; 169-172.
- [8] Okemo, P.O., Mwatha, W.E., Chhabrab, S.C., Fabry, W., (2001). The kill kinetics of *Azadirachta indica* a. Juss. (meliaceae) Extracts on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. *African Journal of Science and Technology (AJST) Science and Engineering Series* Vol. 2, No. 2, pp. 113-118.
- [9] Odunbaku, O.A., Ilusanya O.A., (2008). Antibacterial Activity of the Ethanolic and Methanolic Leaf Extracts of Some Tropical Plants on Some Human Pathogenic Microbes. *Research Journal of Agriculture and Biological Sciences*, 4(5): 373-376.
- [10] Szmant, H.H., (2007). Physical properties of dimethyl sulfoxide and its function in biological systems. Retrieved January, 2009 from <http://www.dmsol.org/articles/information/szmant.html>
- [11] Lin, J., Opokua, A.R., Geheeb-Kellera, M., Hutching, A.D., Terblanche, S.E., Jagerc, A.K., van Staden, J., (1999). Preliminary screening of some traditional zulu medicinal plants for anti-inflammatory and antimicrobial activities. *Journal of Ethnopharmacology* 68 (1999) 267–274. [http://dx.doi.org/10.1016/S0378-8741\(99\)00130-0](http://dx.doi.org/10.1016/S0378-8741(99)00130-0)
- [12] Cunha, B.A., (2002). *Pseudomonas aeruginosa*: Resistance and therapy. *Seminars in Respiratory Infections*. Volume 17, Issue 3 <http://dx.doi.org/10.1053/srin.2002.34689>

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