AN IN-VITRO STUDY ON THE ANTIBACTERIAL EFFECT OF NEEM (*AZADIRACHTA INDICA*) LEAF EXTRACT ON METHICILLIN-SENSITIVE AND METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

AUTHORS: Wendy C. Sarmiento, MD*, Cecilia C. Maramba, MD*, Ma. Liza M. Gonzales, MD*
University of the Philippines College of Medicine- Philippine General Hospital
CORRESPONDENCE:
Wendy C. Sarmiento
Email: purplefizz@yahoo.com
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ABSTRACT
The most common cause of pyogenic infection of the skin and soft tissues in children is *Staphylococcus aureus,* a fast-emerging problem due to its accompanying significant cost and morbidity. The popularity of herbal medications has increased due to the search for cheaper and more accessible alternatives. However, data is still lacking to back up these claims. Although a few in vitro studies have tested Neem leaf extract on S. aureus, there are none done on Methicillin-resistant *Staphylococcus aureus* (MRSA) despite the fact that it is being marketed for such purposes.
Objectives: This study aims to determine if Neem leaf extract (Azadirachta indica) has antibacterial properties against Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus* and to compare the anti-staphylococcal properties of Neem leaf extract with oxacillin, vancomycin, mupirocin, and povidone iodine.
Methods: An in vitro experimental study was performed using Neem leaf, properly identified and verified, was subjected to ethanol extraction of its active ingredients then diluted to produce 25%, 50%, 75%, and 100% concentrations. Standard strains of *Staphylococcus aureus* and clinical isolates of MRSA where inoculated on blood agar plates and subjected to the standardized disc susceptibility testing method. Zones of inhibition were measured for each test extract and compared to currently used medications, namely oxacillin, vancomycin, mupirocin, and povidone iodine with the pure diluent as negative control. The data was analyzed using difference of means hypothesis testing; it utilized the student's *t*-test to determine significance.
Results: A trend of increasing antibacterial activity was noted with increasing concentration of the extract. Zones of inhibition started to appear at 50% concentration for S. aureus and 75% for MRSA. The antibiotics were able to produce greater zones of inhibition than the Neem extracts.
Conclusion: Data from this study strongly suggest that the ethanol extract from Neem leaves exhibits in vitro antibacterial activity against both *Staphylococcus aureus* and MRSA with greatest zones of inhibition noted at 100% concentration.

INTRODUCTION

Worldwide, the most common cause of pyogenic infection of the skin and soft tissues in children remains to be *Staphylococcus aureus*. Review of wound isolates in the Philippine General Hospital in the year 2000 revealed similar results, with *Staphylococcus aureus* infecting the vast majority at 42% and Group A beta-hemolytic Streptococcus coming in second at 15%.[1] *Staphylococcus aureus* causes impetigo, furuncles, cellulitis, abscess, and wound infections among other diseases. Pathology results not only from tissue destruction and toxin production but also from its propensity to disseminate. This produces significant morbidities such as osteomyelitis, suppurative arthritis, pneumonia, deep abscesses, endocarditis and other disseminated infections.[2]

Methicillin-resistant *Staphylococcus aureus* (MRSA) is fast becoming a worrisome threat. Initially considered a purely nosocomial infection, it has now become a common, community-acquired infection in the United States.

Treatment of such diseases entails considerable cost. Currently accepted standards for treatment include oral and intravenous oxacillin for susceptible *Staphylococcus aureus,* and vancomycin for MRSA. Topical mupirocin and povidone iodine are also some of the most commonly used medications to treat localized skin infections. Continued search for cheaper and accessible drugs is of utmost importance. Thus, it is only reasonable that we turn to our own natural resources. Since approval of the Traditional/ Alternative Medicine Act of 1997, more focus has been placed on using locally found medicinal plants as therapeutic agents. Since then, we have been seeing an upsurge in the use of herbal medications and supplements, which are now being commercialized. With some products claiming to be a cure-all, it is becoming increasingly important to differentiate fact from fiction.

The Neem tree has been used for centuries in Ayurveda medicine and is sometimes referred to as the "village pharmacy". Initially, it was native to the lands of India, Pakistan, Nepal and Sri Lanka, but eventually it has found its way to and thrived in Philippine soil. There have been claims that Neem has antibacterial, anti-fungal, anti-parasitic, anti-viral, and insecticidal properties.[1] It has purported uses such as preventing hepatitis, male contraception, and controlling diabetes. It is currently marketed as oils, pills, powders, tea, and various lotions and creams. However, at present, there is still a lack of scientific data to back up these claims. This study aims to elucidate on the possible anti-staphylococcal properties of the Neem leaf.

Despite discovering that some websites advertise Neem capsules for MRSA infections, literature search revealed that no clinical trials have yet been done on the efficacy of such claims. On the other hand, a few in vitro studies were identified which tested the activity of Neem leaf extract on *Staphylococcus aureus.* In the Philippines, 2 studies have been conducted—1 published and 1 unpublished, both with promising results—which showed inhibition of *Staphylococcus aureus* with the ethanol extract of Neem.[1,2] However, no comparison to an antibiotic was made. Internationally, other in vitro studies were found which compared the effects of Neem extract with gentamicin, tetracycline,[1] and cefepime.[1] Common to all these trials was the finding that ethanol or methanol extracts were most effective, with aqueous extracts showing no anti-staphylococcal activity. Data from these studies show zone of inhibition to be 18 to 19 mm. Other data assert that the minimum inhibitory concentration was 5% solution and

that the most effective concentration was 90% followed by 45%.

OBJECTIVES

This aim of this study was to determine if Neem leaf extract (*Azadirachta indica*) has antibacterial properties against Methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus.*

MATERIALS AND METHODS

Study Design

The study utilized an in vitro experimental study.

Test Organisms

Bacterial colonies derived from standard strains of *Staphylococcus aureus* ATCC 25923 and clinical isolates of Methicillin resistant *Staphylococcus aureus.*

Data Collection:

Identification and sourcing of Neem leaf

Leaves from the mature Neem tree obtained from Siniloan, Laguna and verified to be *Azadirachta indica* by the College of Botany of the Laguna Polytechnic University were used.

Extracting Neem leaf concentrate

Neem leaves were manually washed with running water to remove impurities. Ten grams of leaves, not including stems, were then air-dried, cut, and ground to small pieces. They were subjected to standard procedures of percolation to obtain the ethanol extract.[1, 2, 3] The final extract of 1 g/ml was diluted accordingly with ethanol to produce concentrations of 25%, 50%, 75%, 100% and pure diluent for 0%. The extracts were stored in sterile vials.

Obtaining and inoculating bacterial colonies

To comply with international standards for disc susceptibility testing, the *Staphylococcus aureus* susceptible strain ATCC 25923 was used.[1] For MRSA, clinical isolates obtained from the Research Institute for Tropical Medicine, which were proven to be methicillin resistant, were used. They were then inoculated on Mueller Hinton agar with 2% NaCl. Sufficient molten agar was poured into sterile Petri dishes to give a depth of 4 +/- 0.5mm (25 mL in a 90 mm Petri dish).

Application of antibiotic extract and controls

After drying the plate the discs impregnated with the drugs and test extracts were applied to the surface within 15 min of inoculation. Plates were incubated 15 minutes after application of discs. Following standard procedure, staphylococci using oxacillin discs were incubated at 30°C for 24 hours, while Staphylococci using vancomycin discs were incubated at 35 to 37°C for 18 to 20 hours. All tests were done in triplicate.

Measuring zones of inhibition

The zones of inhibition were measured by an experienced laboratory technician and verified by a separate second observer. Because antibiotic discs used were standard and had their name printed on them, the observers were not blinded as to the substances used. Zones of inhibition for each test drug were measured to the nearest millimeter using a caliper.

Outcomes measured: Test drugs used were 25%, 50%, 75% and 100% concentration of the Neem leaf extract. The zones of inhibition produced by the different concentrations were compared with the control which was pure diluent, otherwise denoted as 0%, as well as, to povidone iodine 10%, mupirocin, oxacillin 1mcg/disc, and vancomycin 30 mcg/disc.

Data analysis: Raw data in the form of millimeters representing the measured zones of inhibition was tabulated, after which standard deviation and mean values were computed. Qualitative analysis was done and then confirmed by computing for t-values to determine significance (p-value). Graphs to demonstrate Neem concentration versus zone of inhibition were plotted.

RESULTS

The zones of inhibition measured by the two independent lab technicians were noted to have no significant differences. The computed means are shown in Table 1.

Table 1. Mean Zones of Inhibition of Different Concentrations of Neem Leaf Extract against S. Aureus.

Extract/Antibiotic tested	Mean zone of inhibition (mm)	
	MSSA	MRSA
Control (0%)	6.00	6.00
25 %	6.00	6.00
50 %	8.33	6.00
75%	12.00	7.20
100%	14.33	7.67
Oxacillin	22.50	6.00
Vancomycin	21.00	19.80
Povidone Iodine	17.67	13.53
Mupirocin	35.17	38.00

Since the diameter of the blank disc is 6mm, this value in the chart means that no zone of inhibition was produced and that the bacterial colonies remained undisturbed. It is pertinent to note that the pure diluent (95% ethyl alcohol) did not produce any effect on the bacterial colonies. Analyzing Table 1, it is evident that a significant zone of inhibition started appearing around bacterial colonies (with zones of >6mm) at a Neem leaf concentration of 50% on S. aureus and 75% on MRSA ($p < 0.05$).

Figure 1. Mean Zones of Inhibition of Different Concentrations of Neem Leaf Extract against MSSA

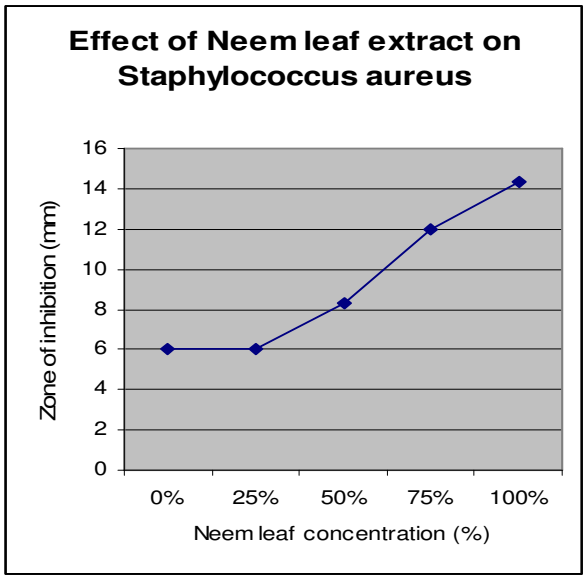
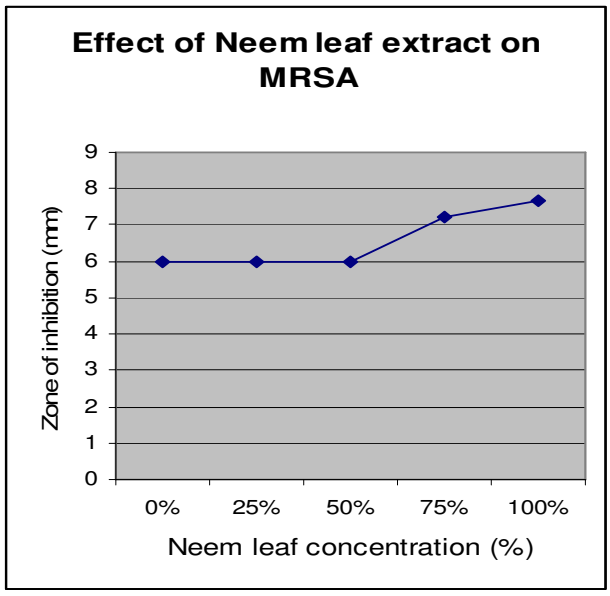


Figure 2. Mean Zones of Inhibition of Different Concentrations of Neem Leaf Extract against S. Aureus.



In general, the antibiotic controls produced greater zones of inhibition except for povidone iodine 10% which antibacterial activity was comparable to the 100% extract on *S. aureus*.

Figures 1 and 2 graphically demonstrate how increasing concentrations of Neem affect the zone of inhibition they were able to produce on *Staphylococcus aureus* and MRSA colonies, respectively. It appears that a positive trend is noted with higher concentrations.

DISCUSSION

Data from this study validated previous studies which suggested that the alcohol extract of Neem leaf has anti-staphylococcal properties on methicillin sensitive strains. However, the international studies were able to produce larger zones of inhibition (18-19mm) with much lower concentrations. This may be due to the known botanical fact that trees from different locations may demonstrate different properties since they are grown on different soils and in varying climates and are exposed to different chemical and biologic flora. However, after noting that different countries produced the same zones, the disparity may be attributed to their superior equipment and extraction methods wherein the active component of the

leaf was better isolated. Jahan et al. from Bangladesh documented this zone of inhibition at 1:32 dilution extracting Neem leaf with petroleum ether at 60 to 80°C in soxhlet apparatus, then filtered and evaporated under reduced pressure using rotary vacuum evaporator. Yagoub et al. from Sudan using the same extraction method were able to demonstrate the same zone of inhibition. Despite the crude extraction methods used in this study, antibacterial effects were already demonstrated even for MRSA, which indicate some potential for these drugs.

It is useful to note however that zones of inhibition for methicillin sensitive strains were twice as large as those for MRSA. The MRSA strains appeared to be more sensitive to Neem than oxacillin so it appeared that these organisms had not yet developed full resistance to the Neem leaf active ingredient.

Our study differs from the unpublished study of Galang et al., wherein a non-linear trend was noted for increasing concentrations, in which the most effective solution was 90% followed by 45%, with the least effective being 75%. A limitation in this study, which used disc diffusion, is the inability to conclude the minimum inhibitory concentration which was reported by one study from the Philippines by Pascua et al. to be equal to 5%. All other studies failed to compare the extract to oxacillin and vancomycin, which are considered the antibiotic standards for treating S. aureus. No other studies attempted to test Neem for MRSA. Another difference is that the other studies utilized distilled water as their control (or 0%) which did not make it clear whether it was the Neem extract or ethyl alcohol which exhibited antibacterial properties.

Clinically, there is potential for the usage of the 100% extract on methicillin sensitive staphylococcus aureus instead of povidone iodine but studies on toxicity and safety still need to be performed. It is difficult to infer based on this study whether Neem leaf can be used as home brews because what are generally used are water decoction methods and not ethanol extracts and studies have shown aqueous extracts to have no anti-staphylococcal activity.

CONCLUSIONS

Data from this study strongly suggest that the ethanol extract of Neem leaves exhibits in vitro antibacterial activity against both *Staphylococcus aureus* starting at 50% concentration and MRSA starting at 75% concentration. It appears that the antibacterial activity follows a dose-dependent pattern with the greatest zones of inhibition noted at 100% concentration. There is much potential for the development of these drugs especially for use against methicillin sensitive *Staphylococcus aureus* and with ideal extraction methods. Antibiotics produced greater zones of inhibition compared to the Neem leaf extracts. Povidone iodine 10% was comparable to the 100% Neem extract on S. aureus.

RECOMMENDATIONS

Provided with more funding and equipment, it is recommended that Neem leaf from the same area be subjected to better extraction methods as acknowledged in other studies which used petroleum ether at 60-80°C in soxhlet apparatus then filtered and evaporated under reduced pressure using rotary vacuum evaporator. Thus, before in vivo studies can be done, it is our recommendation that future studies should explore the best method for extracting the active ingredient of the Neem leaf.

Future studies can include a greater range of organisms. Only methicillin sensitive and resistant *Staphylococcus aureus* were tested in this study and they were limited to what is currently available at the mentioned institutions. While *S. aureus* had a standard ATCC strain which is recognized internationally, MRSA standard strain was not available, which may make tests difficult to interpret if the species are different from institution to institution. Since this is the first study to test Neem leaf against MRSA, future studies are

recommended to explore this area, especially with superior extracts.

Because it is not well documented whether the ethanol extract of the Neem leaf is suitable for intravenous, oral or topical administration this should be investigated further.

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