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Article

The effect of neem on common nosocomial infection-causing organisms

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SUMMARY

Nosocomial infections are a major source of morbidity and mortality, affecting more than 2 million patients annually in the United States. Furthermore, the hospital environment supporting the acquisition of resistance to antibiotic agents by pathogens, complicates the treatment of infections due to drug-resistance of these pathogens. Ethnopharmacological reports support the use of neem (*Azadirachta indica*) against bacterial and fungal infections such as typhoid, yeast infection, and periodontitis. However, there is a lack of research about the effect of neem specifically on nosocomial organisms. We conducted this study to evaluate the effect of aqueous and ethanolic extracts from neem leaves and neem oil on the growth of several human pathogens which are known to cause hospital-acquired, or nosocomial, infections, including *Saccharomyces cerevisiae*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Neem extract in distilled water showed the strongest average inhibition across all microorganisms except *S. aureus*. Activity of neem extract in 95% ethanol was comparable to that of 10% bleach. Under the conditions of this study, we concluded that neem leaf extract has a significant antimicrobial effect against nosocomial organisms, supporting its use as an alternative or combination treatment for hospital-acquired infections.

INTRODUCTION

Bioactive compounds obtained from certain plants have been recognized worldwide for their medicinal uses (1). For the past few years, there has been increasing interest in using these properties in therapeutic fields to fight against harmful pathogenic microorganisms (1). The resilience of these microorganisms to widely used drugs has furthered this focus. One such plant that has been researched to solve this problem is neem. Neem, or *Azadirachta indica*, is a member of the *Meliaceae* family, subfamily *Meloidae*, and tribe *Melieae* mainly cultivated on the Indian subcontinent (1). The versatile multifarious tropical tree has been widely renowned for its various biomedical properties due to its many bioactive components. As a result, neem has been indigenously cultivated for at least 4,000 years. (2). Neem's properties include being antiallergenic, antidermatitic, antiviral, antifungal, anti-inflammatory, anti-pyorrhoeic, insecticidal, larvicidal, and nematicidal (2).

Neem is effective against many bacteria, protozoa, fungi, and viruses, and is traditionally consumed through dietary sources after being grown naturally (1, 2). However, while there are extensive studies on the effectiveness of neem on these microorganisms, there is a gap in knowledge of neem's potential effectiveness against nosocomial infections, defined as hospital-acquired infections of microorganism origin. The aim of this work is to study the effectiveness of neem against common nosocomial organisms.

Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Saccharomyces cerevisiae, and *Micrococcus luteus* are the primary microorganisms that cause nosocomial infections (3). These microorganisms colonize a patient's skin, mucous membrane, anterior urethra, or other soft tissues (3). These microorganisms mainly cause nosocomial infections by forming biofilms on colonized surfaces, which are difficult to eliminate and are highly correlated with nosocomial infections (3). *E. coli* is known to cause diarrhea, urinary tract infections, respiratory illness, and pneumonia (4). Most *E. coli* are harmless and serve an important role in a healthy human intestinal tract (4). However, some *E. coli* are pathogenic, meaning they can cause illness outside of the intestinal tract (4). *S. aureus* is a bacteria that causes infections such as bacteremia, pneumonia, endocarditis, and osteomyelitis (5). Strains of bacteria found widely in the environment cause Pseudomonas infections, of which the most common type causing infections in humans is called *Pseudomonas aeruginosa* (6). *S. cerevisiae*, or brewer's yeast, is a one-celled fungus which is rich in minerals and nutrients and causes fungemia, endocarditis, pneumonia, and skin infections in people with weakened immune systems (7). *M. luteus* is a common gram-positive bacteria that causes endocarditis after surgery of patients, as it colonizes the surface of heart valves (8).

Neem most likely inhibits the microorganisms listed above because of its strong antibacterial and antifungal properties. Neem is composed mainly of quercetin and a number of limonoids, the main substances in neem that are active against bacteria and fungi (9). Quercetin, a polyphenolic flavonoid, accounts for many antifungal and antibacterial properties in neem (9). Limonoids found in neem oil are oxygenated modified triterpenes that have many antifungal properties (9). It can be assumed that neem has these properties because of this distinctive chemical structure such as hydroxyl groups at sites on the aromatic rings. This results in inhibition of

energy metabolism, inhibition of the attachment and biofilm formation, alteration of the membrane permeability, and attenuation of the pathogenicity.

Previous studies have found neem to be an effective antibacterial and antifungal against organisms such as *S. aureus* and *P. aeruginosa* (2, 11, 12). For example, Mahmoud et al. showed that all concentrations of the aqueous extract effectively suppressed the mycelial growth of *Aspergillus* species (*A. niger, A. flavus*, *A. terreus* and *A. fumigatus*), *Microsporum gypseum* and *Candida albicans* and that this effect was found to increase with concentration where a maximum activity was reached using the last one (20%) (12). The authors also reported that complete inhibition in the growth of *A. niger* was obtained in the assay with 20% concentration of aqueous leaf extract of neem (12). It was concluded that all concentrations of organic extracts effectively suppressed the mycelial growth and the recorded values were increasing gradually with concentration, reaching the highest values with 20% (12). Similarly, Sultana et al. showed that the minimum inhibitory concentration of neem was 1.4 g/mL to kill *S. aureus* and *P. aeruginosa* (11). Mondali *et al.* studied the efficacy of different extracts of neem leaf on *Aspergillus*, and *Rhizopus* (2). The growth of both fungal species was inhibited significantly and controlled with both alcoholic and water extracts of all tested concentrations (2). The alcoholic extracts of neem leaf were most effective in comparison to aqueous extract for retarding the growth of these species (2).

Nosocomial infections are a large, growing problem because as multiple bacteria and fungi are becoming resistant to drugs, the number of people fatally succumbing to these infections is growing at an alarming pace (3). The purpose of our experiment is to analyze the effectiveness of neem against these common nosocomial organisms so that hospitals will be able to properly combat these infections. In this experiment, the process of finding the zone of inhibition and the minimum inhibitory concentration will be replicated with five different microorganisms. We propose that neem extract, if applied to the common nosocomial organisms *E. coli, S. aureus, P. aeruginosa, S. cerevisiae,* and *M. luteus*, will inhibit microorganism growth. This experiment fits into the existing body because it continues to compare and study neem's effectiveness against certain infections. However, our approach is unique because we investigate neem's effectiveness purely on nosocomial infection-causing organisms and analyze how neem plays a role in growth inhibition. Previous studies have only looked at the infections themselves, but not clearly at the microorganisms causing the infections; this experiment aims to address that gap.

RESULTS

To investigate the effectiveness of neem we cultured each of the five microorganisms on agarose with varying concentrations of neem. Evaluations of zone of inhibitions showed that neem oil had the clearest agar surrounding it in contrast to the bacterial and fungal growth on the plate and produced the least erosion of the agar-drilled wells it was in (**Figure 1**). In this context, the zone of inhibition is the radius surrounding a well filled with solution in which bacterial colonies do not grow. As a control, 10% bleach had the most erosion of the agar-drilled wells in comparison to the other wells. Neem powder resuspended in 95% ethanol had the most opaque agar, indicating a smaller zone of inhibition surrounding it compared to the other solutions.

The antibacterial effectiveness of negative control (distilled water), positive controls (95% ethanol and 10% bleach) and test materials (neem powder in distilled water, neem powder in 95% ethanol, and neem oil) against *E. coli, M. luteus, P. aeruginosa,* and *S. aureus* are shown in **Figure 1**. *S. cerevisiae* was also studied, however, due to contamination the data were eliminated.

P. aeruginosa was found to be the most susceptible to neem extract in distilled water, followed by *E. coli*. Neem extract in distilled water showed significantly higher inhibitory activity than positive control 95% ethanol for *E. coli* and *M. luteus*. Neem extract in 95% ethanol produced comparable zones of inhibition for all organisms, with significantly higher inhibition than 95% ethanol alone for all organisms except *P. aeruginosa*. The test solution neem oil was most effective against *P. aeruginosa* with a 2.1 mm zone of inhibition, followed by a 1.4 mm zone of inhibition for *S. aureus*. However, neem oil was not comparable in effectiveness to either positive control and was shown to be least effective of all test solutions. The positive controls demonstrated a similar pattern to neem oil with both being most effective against *P. aeruginosa*, followed by *S. aureus*. *P. aeruginosa* cleared a 4.5 mm zone of inhibition for the 10% bleach solution and a 4.3 mm zone of inhibition with 95% ethanol, while *S. aureus* produced a 2.9 mm zone of inhibition for the 10% bleach solution and a 1.1 mm zone of inhibition for 95% ethanol. *M. luteus* was least susceptible, with the smallest zones of inhibition in all tested solutions except neem powder in distilled water. *S. aureus* was least affected by neem powder in distilled water.

Figure 1 shows a graphical representation of the mean zone of inhibition of all solutions. **Figure 1** demonstrates the differences in the zones of inhibition between the experimental solutions, neem in distilled water, neem in 95% ethanol, and neem oil, and the control solutions, distilled water, 95% ethanol, and 10% bleach. The high variance is due to several outliers in the data, including a 2 mm zone of inhibition in 10% bleach for *S. aureus*, a 2 mm zone of inhibition in neem in distilled water for *E. coli*, a 1.5 mm zone of inhibition in 95% ethanol for *P. aeruginosa*, and a 1.5 mm zone of inhibition in neem powder in distilled water for *P. aeruginosa*. It is interesting to note that the zones of inhibitions measured for neem powder in 95% ethanol and neem oil have smaller standard deviations in comparison to the other solutions. It is also interesting to note that 95% ethanol and 10% bleach inhibited *P. aeruginosa*.to similar levels.

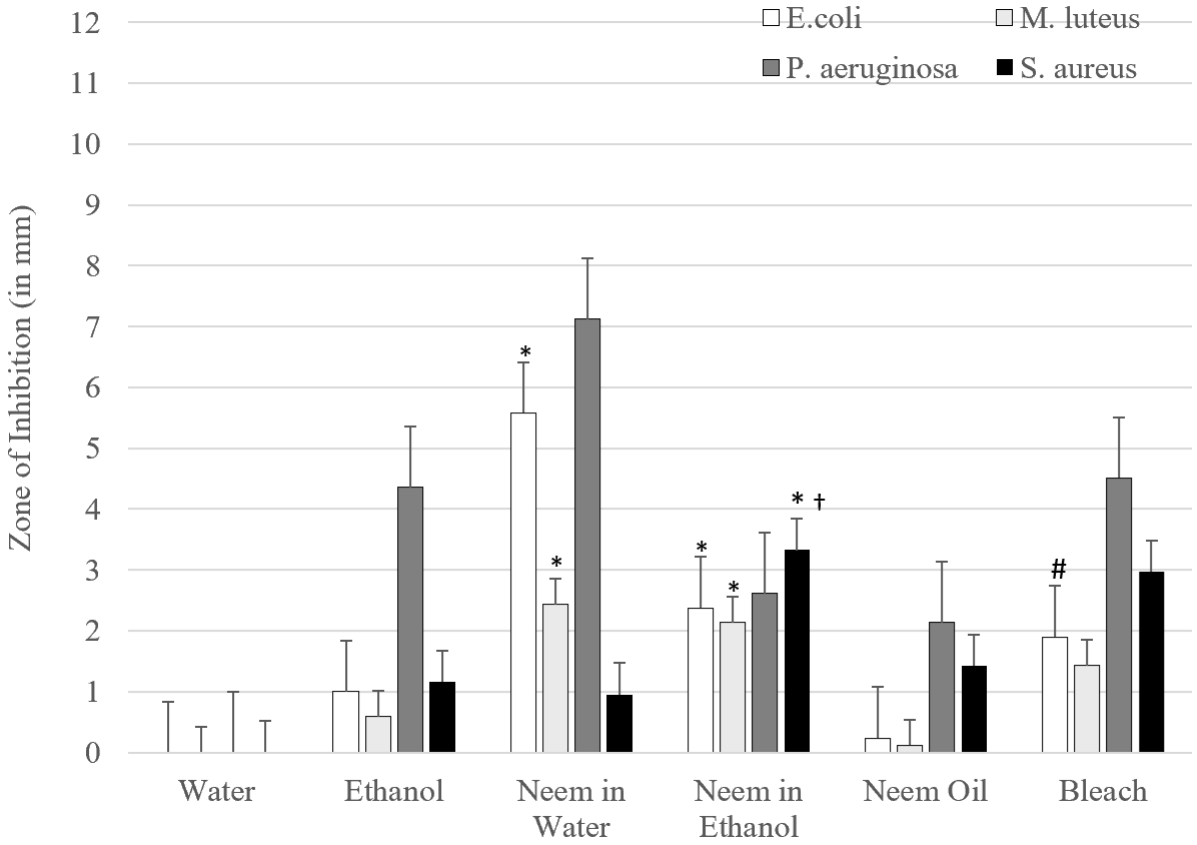

Figure 1. **Inhibition of bacterial growth by Neem.** Bars represent average radius for zone of inhibition for neem extracts, neem oil, and controls against select nosocomial organisms (n=8). * represents statistically significant differences ($p < 0.05$) between the zone of inhibition from the species highlighted compared to the zone of inhibition of the same species in 95% ethanol. † represents significant differences ($p < 0.05$) between the zone of inhibition from the species highlighted compared to the zone of inhibition of the same species in 10% bleach. #represents statistically significant differences ($p < 0.05$) that show that the highlighted control outperformed neem oil.

We visually observed via the opacity of solutions and reported the minimum inhibitory concentration (MIC) in **Table 1**. In general, neem powder in distilled water tended to have the most opacity and have the darkest, murkiest color for all test tubes because of the growth of the bacteria. This was determined based on visual comparison of the solutions before and after growth. *E. coli* and *M. luteus* were the least opaque of each test tube out of neem powder in distilled water and neem oil, respectively, while *P. aeruginosa* was the most opaque of each test tube overall.

Antimicrobial agents with low activity against an organism usually give a high MIC, while those that are highly effective give low MIC values. **Table 1** summarizes the MICs of neem powder in distilled water and neem oil for *E. coli*, *M. luteus*, *P. aeruginosa*, *S. aureus*, and *S. cerevisiae.* Data show that *E. coli* were the least susceptible bacteria. *S. aureus* and *S.* cerevisiae were the most susceptible, and neem oil produced the lowest MIC.

MIC values for *P. aeruginosa, S. aureus,* and *S. cerevisiae* were 0.1 g/mL for neem extract in water. *M. luteus* had a higher MIC of 0.6 g/mL, while no MIC was determined for *E. coli* due to limited resources. Neem oil produced similar MIC values for *M. luteus*, *S. aureus,* and *S. cerevisiae*, all at 0.1 g/mL. Neem oil also produced MICs of 0.4 g/mL for *E. coli* and *P. aeruginosa*.

DISCUSSION

Our experiments indicate that neem powder in distilled water, neem powder in 95% ethanol, and neem oil were effective in killing *S. aureus*, *E. coli*, *P. aeruginosa*, and *M. luteus*. We also concluded that neem powder in distilled water was more effective in inhibiting the microorganisms listed above than 95% ethanol, distilled water, 10% bleach, and neem oil. Lastly, we concluded from the minimum inhibitory concentration experiment that neem oil produced a lower inhibitory concentration compared to neem powder in distilled water. Collectively, these conclusions support the hypothesis that various neem extracts, when applied to the common nosocomial organisms *E. coli, S. aureus, P. aeruginosa, S. cerevisiae,* and *M. luteus*, would inhibit the growth of these microorganisms.

We speculate that neem powder in distilled water showed higher inhibitory activity because the antibacterial and antifungal components were readily soluble in the aqueous

Microorganism	MIC of neem powder in distilled water (n=4)	MIC of neem oil (n=4)
E. coli	Exceeded 0.6 g/mL	0.4 g/mL
M. luteus	0.6 g/mL	0.1 g/mL
P. aeruginosa	0.1 g/mL	0.4 g/mL
S. aureus	0.1 g/mL	0.1 g/mL
S. cerevisiae	0.1 g/mL	0.1 g/mL

Table 1. Minimum Inhibitory Concentrations (MICs) of neem powder in distilled water and neem oil against select nosocomial organisms.

solution. In addition, the solution could diffuse easily through the solid agar plate to produce the larger zones of inhibition. On the other hand, neem oil, being highly lipophilic, had limited diffusivity in solid agar plates and hence produced relatively smaller zones of inhibition. This finding also explains neem oil's effectiveness in the minimum inhibitory concentration experiments, where neem oil was mixed well in liquid nutrient broth. This helped produce comparable MICs to those of neem powder in distilled water.

Because of limited resources, MIC for *E. coli* in neem powder in distilled water was not determined was due to limited resources, as higher concentrations could not be tested. Other limitations of this experiment include contamination of *S. cerevisiae* during the agar-well diffusion experiment, not having enough test tubes and nutrient broth to measure the minimum inhibitory concentration for *E. coli*, inability to accurately measure zone of inhibition due to the streaking method, and inability to accurately measure turbidity for minimum inhibitory concentration. These problems, if addressed, could statistically change the outcome of the experiment. Because the *S. cerevisiae* culture had experienced contamination, the results were erroneous and have not been reported. The addition of this data would have strengthened the support for the conclusions. Lack of a sufficient number of test tubes and nutrient broth to test higher concentrations of *E. coli* in the minimum inhibitory concentration experiment did not allow for the obtaining of the exact MIC value and of the data to support the hypothesis for this organism. Accuracy in the measurements of streaking of zone of inhibition and the turbidity for minimum inhibitory concentration are crucial to substantiate the findings and reinforce the conclusions. The streaking method is slightly weaker in comparison to the pouring technique because microorganisms do not grow uniformly and do not distribute in the plate evenly. Visual assessment of turbidity is a subjective method in comparison to an objective method of measuring turbidity using techniques such as spectroscopy. These problems can be fixed by creating more sterile conditions to stop contamination, using more test tubes to measure the minimum inhibitory concentration, using a pouring technique for the zone of inhibition for uniform distribution of the microorganisms, and using spectroscopy to determine the minimum inhibitory concentration. Specifically, more sterile conditions could be achieved by cleaning the workplace before working, sterilizing the inoculating loop and straws for longer periods of time, and working closer to a lit Bunsen burner.

The nosocomial bacteria and fungi are known to survive and remain infectious under varieties of environmental conditions, including pH and temperature fluctuations (4, 5, 6, 7, 8). Hence, future experiments should include evaluating the effect of temperature on neem extract activity against the microorganisms, the effect of pH on neem extract activity, the inclusion of other nosocomial organisms that would be extracted from hospitals, and the addition of an antibiotic as a positive control in the experiment. By studying more variables in the experiment, such as temperature and pH, the best use of neem extract to inhibit certain nosocomial organisms will be narrowed down and scientific knowledge about the therapeutic benefits of neem will be enhanced. By using more species of bacteria and fungi, effectiveness of neem can be studied more accurately, and the data can be used to determine better treatment options, specifically for hospital-like conditions. Also, adding a commonly-used antibiotic by hospitals that kill nosocomial organisms to the experiment would allow for better comparison of results against neem and the antibiotic.

MATERIALS AND METHODS

All experimental work was performed in a biosafety level 2 microbiology laboratory at Kennesaw State University. Neem oil and neem powder, manufactured respectively by Mary Tylor Naturals LLC and Metiista LLC, were purchased and stored in plastic bags in shade to prevent contamination and degradation. The following reagents were prepared: 1) A 10% solution of neem in distilled water was made with 10 grams of neem powder dissolved in 100 mL of distilled water, and, 2) A 10% solution of neem in 95% ethanol was made with 10 grams of neem powder dissolved in 100 mL of 95% ethanol. Distilled water was used as a negative control, expected to have no effect on organisms, and a 10% bleach solution and 95% ethanol served as positive controls, expected to inhibit growth of microorganisms.

The following pathogen strains obtained from the American Type Culture Collection and were used in this study: *Saccharomyces cerevisiae* (S288C), *Micrococcus luteus* (NCTC2665), *Staphylococcus aureus* (MRSA), *Escherichia coli* K12, and *Pseudomonas aeruginosa* (PA01). Samples were refrigerated at 4 degrees Celsius. Under sterile conditions, one colony of each microorganism was scooped using a loop and inoculated into 40 mL of nutrient broth (Standard I Nutrient Broth obtained from HiMedia Labs) (13). Eight inoculations were done for each microorganism, from which sterile inoculation loops were used to transfer small

amounts of the cultures into test tubes containing nutrient broth for each microorganism. The microorganisms were taken from the parental microorganism strain's petri plate. Similarly, petri plates were prepared and streaked to create a lawn with uniform growth (14). The test tubes placed in an orbital shaker and petri plates were incubated for 24 hours at 37°C.

To analyze antibacterial activity, an agar well diffusion method was followed. After incubation and growth of the microorganisms, 6 holes (4 mm in diameter) were punched aseptically on each petri plate using a sterile plastic straw. On each plate, 100 µL of each test material or control was pipetted into a well, which was labeled with different colored tape on the bottom of the plate for identification. The petri plates were left on a flat bench to dry for one hour and were then incubated for 18 hours at 37°C. Thereafter, the petri plates were analyzed for the radius of the zone of inhibition around each well of each solution using a ruler. There were 8 replicates for each strain.

To calculate the minimum inhibitory concentration (MIC), 1, 2, 4, and 6 mL of 10% neem powder in water and neem oil were pipetted into a test tube for each microorganism. The test tubes were then incubated at 37°C and inserted back into the orbital shaker for another 18 hours. The test tubes were analyzed for MIC by observing for absence of visible microbial growth against natural light and noting that concentration of the solution. The MIC for each set of 4 test tubes for each microorganism was recorded in a data table.

Statistical analyses were performed using Microsoft Excel version 16.16.7. For antimicrobial effectiveness, the mean and standard error of 8 data points for each organism was calculated and the zone of inhibition data were analyzed using the Student's t-test, defining statistical significance at a p-value of < 0.05. All comparisons were made against 10% bleach and 95% ethanol, the positive controls. Statistical analysis ($p \leq 0.05$) was performed to show differences between the sizes of the zone of inhibition in positive control - 95% ethanol and neem powder in distilled water, neem powder in 95% ethanol, and neem oil, which are indicated with an * in **Figure 1**. In addition, the same analysis was also conducted between positive control - 10% bleach and neem powder in distilled water, neem powder in 95% ethanol, and neem oil, which are indicated with a † **in Figure 1**. # indicates that the control outperformed neem oil. Apart from the significant data listed in the **Table 1**, all other comparisons showed no differences in statistical significance.

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