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THE COMBINATORIAL EFFECTS OF *Azadirachta indica* LEAF EXTRACTS WITH AMIKACIN AND TETRACYCLINE AGAINST CLINICALLY IMPORTANT BACTERIA

L.A. Sivasamugham*, K. Tze Sin, K. Thrumaran, G. Subramaniam

Faculty of Health and Life Sciences, INTI International University, Persiaran Perdana BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia

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Neem leaf extract

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ABSTRACT

Antibiotic-resistance is a major threat in the treatment of diseases caused by resistant bacteria. Combination of plant extracts with antibiotics can serve as an alternative to antibiotics. *Azadirachta indica* (neem plant) has many antimicrobial properties due to the presence of secondary metabolites such as alkaloids and flavonoids. In this study, the combinatorial effects of neem leaf extracts with amikacin and tetracycline against eight clinically important gram-positive and gram-negative pathogens were investigated using the agar well diffusion assay. Synergistic effect of neem leaf extract and tetracycline was observed against *Propionibacterium acnes*, *Bacillus subtilis*, and *S. pneumoniae* with a significant enlargement ($p < 0.05$) in the diameter of the zone of inhibition. However, the same combination showed insignificant inhibition against *S. faecalis*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Staphylococcus aureus*. The neem leaf extract-amikacin combination showed insignificant antibacterial activity against *Staphylococcus aureus*, *S. epidermidis*, *S. pneumoniae*, and *Pseudomonas aeruginosa*. An antagonistic effect was observed when *Bacillus subtilis* was exposed to the same combination as there was a significant reduction ($p < 0.05$) in the zone of inhibition. This study suggests the potential development of the neem leaf extract-tetracycline combination as an antibacterial agent against *P. acnes*, *B. subtilis*, and *S. pneumoniae*. However, this preliminary data requires further investigation and test on a wider range of clinical isolates to make a more decisive conclusion. The antagonistic effect of the neem leaf extract and amikacin suggests that the individual agents are potent as antibacterial agents than the combination.

* Corresponding author

E-mail: lalita.sivasamugham@newinti.edu.my (Lalita Ambigai Sivasamugham)

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1 Introduction

Antibiotic resistance is an emerging global health threat (WHO, 2020). The increase of antibiotic resistance urges the need to discover and develop alternative antimicrobial agents from other potential sources including plants (Othman et al., 2019). The use of medicinal plants in treatment has been recorded since ancient history (Petrovska, 2012). Studies show that about 80% of the global population use plants for their primary health care (Ekor, 2014). The medicinal plants such as *Allium sativum* (garlic) and *Prosopis juliflora* (mesquite) contain phytoconstituents such as alkenyl phenols, alkaloids, and flavonoids that have antimicrobial properties (Emad, 2011). Plant extracts also generally show no or very low toxic effects (Cowan, 1999). Also, the cost of production is low (Teka et al., 2015; Cheesman et al., 2017).

Azadirachta indica is widely used as a traditional medicine due to its antimicrobial and other medicinal properties. Studies show antimicrobial activities of the different parts of the neem plant (Raja Ratna Reddy et al., 2013). A combination of neem leaves extracts with other medicinal plants exhibited synergistic antibacterial effects against *Pseudomonas aeruginosa*, *S. mutans*, *S. gordonii*, and *Candida albicans* (Bhuva & Dixit, 2015; Saini et al., 2020). Thus, this study was aimed to investigate the combinatorial effects of *A. indica* leaf extract with amikacin and tetracycline against eight clinically important bacteria using the agar well diffusion assay apart from the qualitative screening of the phytochemicals in the leaf extract.

2 Materials and Methods

2.1 Pure Culture Isolation

The bacterial cultures used in this study were *Streptococcus faecalis*, *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*, *Propionibacterium acnes*, *Bacillus subtilis*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. These cultures were in glycerol stock and were obtained from the culture collections of INTI International University. All bacterial cultures were inoculated onto nutrient agar and incubated at 37°C for 24 h to obtain homogenous colonies.

2.2 Confirmatory Tests

The pure cultures were Gram-stained using the method described by Moyes et al. (2009). The Gram-positive bacteria were subjected to catalase test as described by Iwase et al. (2013) and grown on mannitol salt agar. The IMVIC (Indole, methyl red, Voges-Proskauer, and Citrate) test, oxidase test, and the triple sugar iron test was used to confirm the identity of the Gram-negative bacteria (Holt, 1994; Cappuccino & Sherman, 2005).

2.3 Ethanolic *A. indica* Extract Preparation

Fresh neem leaves were obtained from a residential garden in Seremban, Malaysia. The leaves were rinsed in the following order; water, 80% (v/v) ethanol, and sterile deionized water (Noor et al., 2011). The dust-free neem leaves were sun-dried for 24–48 h, ground using a micro-fine multipurpose powder dry medicine blender (Himitzu), and sieved with a 40-mesh sieve. The neem leaf powder (35 g) was suspended into 70 mL of 80% (v/v) ethanol to a ratio of 1:2. The mixture was incubated in an orbital shaker at room temperature for 24 h and centrifuged at 10,000 rpm for 15 min at room temperature (Itelima et al., 2016). The supernatant was filter-sterilized (0.2 µm) and analyzed for its phytoconstituents before subjecting to the agar well diffusion assay (Echegoyen et al., 2015).

2.4 Agar Well Diffusion Assay

The antibacterial activities of the leaf extracts were detected using the agar well diffusion assay (Perez et al., 1990). The overnight bacterial cultures which were adjusted to 0.5 McFarland standard was lawned onto MH agar using a sterile wooden applicator. Four wells were made onto the agar by puncturing the agar using a sterile micropipette tip. Wells in quadrants 1 and 2 were filled with 200 µL of neem extracts (0.5 g/mL) and 200 µL of antibiotics respectively (Clinical Laboratory Standard Institute, 2017). Quadrant III was filled with 200 µL of 80% (v/v) ethanol whereas; Quadrant IV had 200 µL of neem extract (0.5 g/mL) and antibiotic combination. The agar was incubated at 37°C for 24 hours. This assay was done in triplicates. The effects of the neem leaf extract-antibiotic combinations were analyzed by measuring the diameter of the zone of inhibition (in mm) using a metric ruler.

2.5 Qualitative Phytochemical Screening

The ethanolic neem leaf extracts were screened for phytochemicals using previously described methods to Shu'aibu et al. (2015).

2.6 Statistical Analysis

ANOVA in IBM SPSS Statistics Version 22 was used to determine the significant differences between the neem leaf extracts, antibiotics, and the neem leaf-antibiotic combination when tested against different bacterial strains with a significant value of $p < 0.05$.

3 Results and Discussion

Amikacin is a semi-synthetic aminoglycoside used against a wide spectrum of Gram-negative bacteria, such as *Serratia* spp. and *P. aeruginosa* as well as Gram-positive bacteria, such as *Staphylococcus* (eMC, 2020). Tetracycline is a polyketide

antibiotic that is effective against both Gram-positive and Gram-negative bacteria (Eliopoulos & Roberts, 2003).

The combinatorial effect of neem leaf extract with tetracycline showed a significantly larger zone of inhibition ($p < 0.05$) compared to the individual applications of neem leaf extract and tetracycline against *P. acnes*, *B. subtilis*, and *S. pneumoniae* (Figure 1). This indicates the synergistic effect of the neem leaf extract-tetracycline combination against these bacteria. This finding agrees with the study by Rasha et al. (2015) that showed the synergistic effect of *V. nilotica* and *S. alexandrina* leaf extract-tetracycline combination against *B. subtilis* with a significant increase in the diameter of the zone of inhibition. Kusum & Sharmita (2015) also reported the synergistic effect of neem leaf extract against *E. coli* when combined with tetracycline. The synergistic activity of the neem leaves extract-tetracycline observed in this study could have been

attributed to the bioactive phytoconstituents of the neem leaf extract that enhanced the antibacterial activity of tetracycline (Aslam et al., 2009). Sibanda & Okoh (2007) suggested that bioactive phytoconstituents could inhibit the activity of the efflux pumps in bacteria. Efflux pump is a transport protein found in Gram-positive and Gram-negative bacteria that extrudes toxic compounds such as antibiotics out of the bacterial cells, making the cells to resist the action of antibiotics (Webber & Piddock, 2003). The inhibition of the efflux pump activity increases the entry of antibiotics into bacterial cells enabling them to reach the target site and destroy the cells (Lomovskaya & Bostian, 2006). Efflux pump inhibitors such as ferruginol isolated from *Chamaecyparis lawsoniana* inhibited the tetracycline-resistant efflux pump, quinolone-resistant efflux pump, and erythromycin-resistant efflux pump in *S. aureus* which improved the antimicrobial effect of tetracycline, quinolone, and erythromycin respectively (Aiyegoro et al., 2011).

Figure 1 The mean diameter of zone of inhibition (mm) produced by neem leaf extract, tetracycline and combination of neem leaf extract with tetracycline against the tested bacteria

Qualitative phytochemical screening of the neem leaf extract showed the presence of glycosides, alkaloids, phytosterols, and flavonoids (Table 1) and this was consensus with the findings by Pandey et al. (2014) and Al-Hashemi & Hossain (2016). These phytochemicals could have exhibited a concerted effect eventually causing *P. acnes*, *B. subtilis* and *S. pneumoniae* to be more permeable to the action of tetracycline.

Table 1. Qualitative Phytochemical screening of neem leaf extract

Phytochemicals	Ethanollic Extract
Alkaloids	X
Flavonoids	X
Glycosides	X
Phytosterols	X
Saponins	O

Here X indicates presence and O indicates the absence of the specified phytochemicals in the leaf extract

Flavonoids are suggested to inhibit bacterial growth by inhibiting nucleic acid synthesis, plasma membrane function apart from inhibiting the activity of porin on the bacterial cell membrane. Flavonoids could have also altered the bacterial membrane permeability causing the cells to be more susceptible to the action of tetracycline (Xie et al., 2015). Alkaloids present in the neem leaf extract could have inhibited the efflux pump actions in the tested bacteria as reported by Mabhiza et al. (2016).

Another possible reason for the synergistic effect is the bacterial protein synthesis inhibition that resulted in a potent tetracycline activity (Aiyegoro et al., 2011). In this study, the qualitative preliminary phytochemical screening showed the presence of glycosides, alkaloids, phytosterols, and flavonoids but a quantitative analysis will identify the exact components that exhibited the synergistic activity with tetracycline. The findings in this study strongly suggest the potential use of the neem leaf extract-tetracycline combination as an effective antibacterial agent against *P. acnes*, *B. subtilis*, and *S. pneumoniae*. The combination might reduce the development of tetracycline-resistant bacteria and reduce the dose of tetracycline required to treat infections caused by these bacteria. However, further investigation and tests on different strains of *P. acnes*, *B. subtilis*, and *S. pneumoniae* are required before a more decisive conclusion can be made.

The combination of neem leaf extract and tetracycline showed no difference in the inhibition against *S. aureus*, *S. epidermidis*, *S. faecalis*, and *E. faecalis* (Figure 1). This might be due to the MIC of the combination of neem leaf extract and tetracycline that was not potent enough to inhibit the bacterial activity (Aiyegoro et al., 2011). Apart from that, the genetic variation in neem plant and bacteria might reduce the effectiveness of antimicrobial activity of neem leaf due to the expression of a certain gene in bacteria that could alter or destroy the antimicrobial molecules found in the extract (Muniata & Arias, 2016). However, the mode of action and mechanisms of active phytoconstituents of the neem leaf extract has to be studied to have a greater insight on this.

The neem leaf extract-amikacin combination showed a significant reduction ($p < 0.05$) in the diameter of the zone of inhibition against *B. subtilis* indicating an antagonistic effect (Figure 2).

Figure 2 The mean diameter of zone of inhibition (mm) produced by neem leaf extract, amikacin, and combination of neem leaf extract with amikacin against the tested bacteria

This antagonistic effect might be due to the presence of certain phytoconstituents in the extract that had competitive binding with amikacin to the bacterial membrane hence reducing its antibacterial activity (Passat, 2012; Eze et al., 2013). The antagonistic effect between neem leaf extract and amikacin indicates that amikacin should be used individually as the combination showed reduced activity and possibly an adverse effect.

The combinatorial effect of neem leaf extract and amikacin against *S. aureus*, *S. epidermidis*, *S. pneumoniae*, and *P. aeruginosa* showed no significant difference in the diameter of the zone of inhibition indicating that the combination had no synergistic or antagonistic effect against these bacteria. Similar findings were reported by El-Zawahry et al. (2013) where the combinatorial effect of tamarind extract with amikacin showed a similar diameter of zone of inhibition with amikacin. This might be due to the absence of interaction between phytoconstituents of the neem leaf extract and amikacin (Bhuva & Dixit, 2015). This indicates that it is not crucial to combine neem leaf extract and amikacin for therapeutic purposes. However, to date, no study had been done on the combinatorial effect of neem leaf extract and amikacin against the bacteria used in this study. Hence, further investigations must be done to make a more decisive conclusion.

Conclusion

The combination of neem leaf extract with tetracycline showed synergism against *P. acnes*, *B. subtilis*, and *S. pneumoniae* based on the zone of inhibition suggesting the potential combination to treat related infections. Antagonistic effect exerted by amikacin and neem leaves extracts combination against *B. subtilis* suggests that they should not be combined. However, these are preliminary findings and thus require further investigation and test on a wider range of clinical isolates to make a more decisive conclusion on the potential use of neem leaf extract with tetracycline and amikacin.

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Conflict of interest

The authors declare that they have no conflict of interest.

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