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IV VITRO SYNERGISTIC ANTIMICROBIAL INTERACTIONS BETWEEN THE EXTRACT OF *AZADIRACHTA INDICA* AND ANTIBIOTICS AGAINST *BACILLUS SUBTILIS*

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ABSTRACT

This study aims to investigate the in vitro antimicrobial interactions between crude aqueous extract of Azadirachta indica and standard antibiotics, including ciprofloxacin, and norfloxacin. Using checkerboard techniques with Bacillus subtilis as the test microorganism, the synergistic effect of Azadirachta indica and antibiotics (ciprofloxacin and norfloxacin), was determined. The checkerboard assay presented FIC indices indicative of synergy, particularly notable with the combination of A. indica extract and the antibiotics. Specifically, with the 4:6, 8:2, and 9:1 ratios of ciprofloxacin and 1.9 to 9:1 of norfloxacin. These results suggest a potentiation of antibacterial effects when the plant extract is used in conjunction with conventional antibiotics. The synergistic interaction could potentially reduce the required doses of antibiotics, thereby minimizing side effects and the risk of antibiotic resistance.

Keywords: Norfloxacin, Ciprofloxacin, Checkerboard, *Bacillus subtilis*, FIC index

INTRODUCTION

The escalating challenge of antibiotic resistance poses a significant threat to public health worldwide, undermining the efficacy of conventional antibiotic therapies (Smith and Jones, 2018; WHO, 2017, CDC, 2019). The overuse and misuse of antibiotics have led to the emergence of multidrug-resistant bacterial strains, rendering many standard treatments ineffective (Ventola, 2015). The urgent need for novel antimicrobial strategies has driven research towards alternative and complementary therapies, including the use of plant-derived compounds (Tacconelli*et al*., 2018). Therefore, rigorous investigation into the compatibility and therapeutic implications of combining commonly used antibiotics with herbal remedies is urgently needed. (Nagaraj and Kwang-Hyun, 2022). Among these resistant pathogens, *B. subtilis* stands out due to its prevalence and the severe infections it can cause, ranging from, Endocarditis, Pneumonia, Wound Infections, and Foodborne Illness to life-threatening Bacteremia (CDC 2019, Tanaka *et al*., 2022 and Li *et al*., 2022).

B. subtilis is a Gram-positive, rod-shaped, spore-forming bacterium that is temporarily present in the human gastrointestinal tract

(García-Arribas et al., 1986). In Japan, a case of bacteremia caused by *B. subtilis* variant natto was identified in a 51-year-old man with no significant previous medical history. The patient presented with a generalized tonic-clonic grand mal seizure. A brain magnetic resonance imaging (MRI) scan with intravenous Gadolinium contrast revealed a 3.3 cm × 2.7 cm lesion in the right parietal lobe, surrounded by mild vasogenicoedema, including the posterior central gyrus. This lesion was associated with the presence of *B. subtilis* var. natto (Tanaka et al., 2022).A 67-year-old woman with a 2-day history of a fever, headache and disturbed consciousness was admitted to a hospital, with *B. subtilis* isolated from both the cerebrospinal fluid and blood (Mieko et al., 2023). *B. subtilis*-597 induces changes in lung pathology and inflammation during influenza A virus infection in pigs (Katrineet al., 2024).

Azadirachta indica commonly known as neem, has been traditionally utilized in various medicinal practices for its broad spectrum of therapeutic properties, including antimicrobial, anti-inflammatory, and antioxidant activities (Singh et al., 2020). Indigenous to India and Burma, Neem is widely available in tropical regions, including Nigeria, where it has been utilized in various forms to treat infectious diseases (Ndunguet al., 2020). Recent studies have focused on the potential of neem extracts to enhance the efficacy of conventional antibiotics, offering a promising approach to combat antibiotic resistance (Khan et al., 2019). This synergy between plant extracts and antibiotics could potentiate the antimicrobial effects, thereby reducing the required antibiotic dosage and minimizing adverse effects. Research has shown that plant extracts, such as those from *A. indica*, contain bioactive compounds that can disrupt bacterial cell walls, interfere with essential enzymes, and inhibit biofilm formation, which enhances the effectiveness of antibiotics (Plumet et al., 2022). Specifically, neem extracts have demonstrated significant antimicrobial activity against various pathogens, including multidrug-resistant strains of *E. coli* (Mahapatraet al., 2018). The combination of these extracts with antibiotics could, therefore, provide a synergistic effect, improving treatment outcomes for bacterial infections. Notably also, Neem extracts have demonstrated efficacy against pathogens such as *Staphylococcus aureus*, *E. coli*, and *B. subtilis* highlighting their potential as adjunctive therapies in combating bacterial infections (Ndungu*et al*., 2020).

Despite the documented antimicrobial properties of *A. indica*, there is limited research on its interactions with standard antibiotics against *B. subtilis* Understanding these interactions is crucial for developing effective combination therapies. Thus, the study is aimed at assessing the synergistic antimicrobial interactions between *A. indica* extract and selected antibiotics (ciprofloxacin and norfloxacin) against *B. subtilis*.

MATERIALS AND METHODS

Plant Materials and Extraction

The leaves of *Azadirachta indica* (Meliaceae) were collected from Nsukka, Enugu State, Nigeria and were authenticated by a certified taxonomist, in the Department of Botany, University of Nigeria Nsukka, Nigeria. The leaves were cut into smaller pieces, air-dried, and pulverized. About 200 g of the pulverized material was extracted with water and the resulting extract was stored in the refrigerator after determining the weight of the dry extract per 1 ml of the water extract.

Reagents and Chemicals

Nutrient agar, MacConkey agar (Biochemika, India), and antibiotics discs (OXOID, UK) containing 10 µg each of ciprofloxacin and norfloxacin were used.

Test Microorganism

The test organism used for these experiments is a clinical isolate of *B. subtilis* obtained from the Pharmaceutical Microbiology Laboratory of the Department of Pharmaceutical Microbiology and Biotechnology, University

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of Nigeria, Nsukka. Identification of the bacterial isolate was performed according to standard bacteriological techniques previously established (Cowan and Steel, 1993; Baron and Finegold, 1990). A 24-hour-old culture of the purified test microorganism was harvested and carefully diluted to get a microbial population of 10^5 CFU/ml by comparing with Mcfarland 0.5 standard.

Evaluation of the interaction between the plant (neem) extract and the various antibiotics using the overlay-inoculum susceptibility method

The evaluation of the interaction between the antibiotics using the checkerboard method was employed (Okore, 2005, Esimone et al. 1999). Briefly, proportions ranging from 1:9 to 9:1 of neem extract and either ciprofloxacin or norfloxacin were prepared. Each proportion of the plant extract-drug combination was 2-fold serially diluted. A 1 ml quantity of the sixth dilution was thoroughly mixed with 19 ml molten nutrient agar and allowed to solidify. The *B. subtilis* strain was then streaked on the dried plate and incubated at 37 °C for 24 h. Duplicate determinations and control studies were done. The plates were assessed for growth after incubation and the interaction was accessed by determination of the minimum inhibitory concentration (MIC) of various combinations and their fractional inhibitory concentrations (FIC). This method was employed using the relationship below:

FIC Index= $FIC_A + FIC_B$, Where: (A= Neem extract, B= antibiotic)

FIC_A= Ratio of MIC of A in the presence of B to the MIC of A alone (MIC A'/MIC A)

FIC_B = Ratio of MIC of B in the presence of A to the MIC of B alone (MIC B'/MIC B)

RESULTS AND DISCUSSION

The evaluation of interaction employing the Checkerboard method using *B. subtilis* showed varying activities/outcomes (Table 1). The generalized assessment indicates notable synergism between neem extract and the tested antibiotics against *B. subtilis* revealed synergism between neem extract and the antibiotics at all the ratios 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Norfloxacin and ciprofloxacin are antibiotics known for their antibacterial activities against many bacterial strains. Evaluating the combination of the neem extract with the antibiotics to explore potential synergistic interactions for clinical applicability necessitated this study. Synergism was indicated by a positive change in inhibition zone diameter (IZD) compared to pure antibiotic control, whereas negative or zero changes denoted antagonism or indifference, respectively.

These findings suggest that carefully selected combinations of neem and norfloxacin or ciprofloxacin can enhance antibacterial efficacy in clinical settings. This synergistic effect is significant as it improves the poor antibacterial activity of neem extract alone against *B. subtilis*. Moreover, using these combinations can lower the doses of both agents, potentially reducing adverse effects and toxicity while preventing the emergence of resistant bacterial strains. However, the observed antagonism at certain combinations raises concerns about treatment failure if patients inadvertently consume neem extract while on antibiotics. Such practices could compromise chemotherapy and pose toxicity risks. Therefore, patients should be properly instructed to avoid neem extract during antibiotic therapy to prevent these potential issues. Therefore, the synergistic interaction observed between neem aqueous extract and the evaluated antibiotics can be translated into useful clinical applications in *P. vulgaris and B. subtilis*-based infections especially when the constituents of the neem extract are further isolated and characterized.

Table 1: The combined effect of *A. indica* (neem) extract and antibiotics against *B. subtilis* using checkerboard method

Combinations	Parameters	Combination ratios								
		1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1
Neem:	MIC	0.025:	0.05:	0.15:	0.2:	0.125:	0.15:	0.175:	0.8:	0.9:
Norfloxacin	mg/ml	0.001125	0.001	0.00175	0.0015	0.000625	0.0005	0.000375	0.001	0.0005
	FIC	0.00125:	0.0025:	0.0075:	0.01:	0.00625:	0.075:	0.00875:	0.04:	0.045:
	mg/ml	1.8	1.6	2.8	2.4	1	0.8	0.6	1.6	0.8
	FIC index	1.801	1.6025	2.8075	2.41	1.00625	0.8075	0.60875	1.64	0.845
	Effect	IND	IND	ANT	ANT	IND	SYN	SYN	IND	SYN
	MIC	0,0003125:	0.00625:	0.009375:	0.025:	0.03125:	0.01875:	0.021875:	0.1:	0.225:
Neem:	mg/ml	0.000140	0.000125	0,000109	0.0001875	0.000156:	0.0000625:	0.0000468	.000125:	0.000125
Ciprofloxacin	FIC	0.000156:	0.000313:	0.000461:	0.00125:	0.00156:	0.000938:	0.00109:	0.005:	0.01125:
	mg/ml	0.000014	0.000013	0.000011	0.000019	0.0000156	0.00000625	0.00000468	0.0000125	0.0000125
	FIC index	0.00017	0.00033	0.000472	0.00127	0.00158	0.000944	0.00109	0.00501	0.01126
	Effect	SYN	SYN	SYN	SYN	SYN	SYN	SYN	SYN	SYN

Key: SYN: Synergism, IND: Indifference, ANT: Antagonism.

CONCLUSION

There is possible potentiation of antibacterial effects of antibiotics against *B. subtilis* infection when co-administrated with neem-water extract. The careful use of controlled predetermined combinations of neem water extract and antibiotics could find clinical applications in the treatment of bacterial infections caused by susceptible microorganisms and in the prevention of emergent resistant strains of *B. subtilis*. This study underscores the ultimate need for alternative therapies to conventional antibiotics, as a way of alleviating the antibiotics use associated side effects.

REFERENCES

Baron E.J., Finegold SM. (1990) (eds) Bailey and Scott's Diagnostic Microbiology. C. Mobby. Missouri.

Centers for Disease Control and Prevention (CDC). (2019). Antibiotic resistance threats in the United States, 2019. United States Department of Health and Human Services. Retrieved from https://www.cdc.gov/drug resistance/pdf/threats-report/2019-ar-threats-report-508.pdf

Cowan, S.I., Steel, K.J. (1993) Cowan and Steel's Manual for the identification of medical bacteria. Barrow GI and Feltman RKA (eds) Univ. Press, Cambridge.

Esimone, C.O., Adikwu, M.U., Uzuegbu, D.B., Udeogaranya, P.O. (1999) The effect of ethylenediaminetetraacetic acid on the antimicrobial properties of Benzoic acid and cetrimide. *Journal of Pharmaceutical Research Drug Development* 4: 1-8.

García-Arribas, M.L., de la Rosa, M.C., Mosso, M.A. 1986 Characterization of the strains of *Bacillus* isolated from orally administered solid drugs. *Pharm ActaHelv* 61:303–7.

Katrine, W., Charlotte, K., Betina, L. H., Lea, H. B. H., Pia, R.H., Gisle, V., Kerstin, S., Dorthe, S., Erik, J B., Andrew, R.W., Lars, E.L. (2024) *Bacillus subtilis*-597 induces changes in lung pathology and inflammation during influenza A virus infection in pigs. *Veterinary Microbiology* 291 https://doi.org/10.1016/j.vetmic.2024.110032.

Khan, M., Malik, A., Ahmad, I., & Khan, M. S. (2019). Antibacterial efficacy of neem

Scientia Africana, Vol. 23 (No. 3), August, 2024. Pp 215-220
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(*Azadirachta indica*) leaves extract against antibiotic-resistant pathogenic bacteria. *Asian Pacific Journal of Tropical Biomedicine*, 9(6), 285-289. doi:10.4103/2221-1691.260396.

Li, C., Jia, WW., Yang, Jl. *et al.* (2022) multi-compound and drug-combination pharmacokinetic research on Chinese herbal medicines. *Acta Pharmacol Sin* 43:3080–3095. https://doi.org/10.1038/s41401-022-00983-7).

Mahapatra, A., Koley, H., Pal, C., &Maurya, R. (2018). *In vitro* antibacterial activity of Neem (*Azadirachtaindica*) and Tulsi (*Ocimum sanctum*) extracts against clinical strains of *Escherichia coli* and *Salmonella* ser. *typhi*. *Ancient Science of Life*, 37(1), 20-24. https://doi.org/10.4103/asl.ASL_223_16

Mieko T., Norihito, T., Kazuo, I., Jun S., Takuya M., Toru K., Kazuhide S., Kazushi T., Toshimasa Y., Shigefumi M. (2023) Bacterial Meningitis Caused by *Bacillus subtilis* var. *natto Internal Medicine* 1; 62(13): 1989–1993. https://doi.org/10.2169/internalmedicine.0768-22

Nagaraj, B., Kwang-Hyun B. (2022) Combination Strategies of Different Antimicrobials: An Efficient and Alternative Tool for Pathogen Inactivation. *Biomedicines.* 10 (9):2219. https://doi.org/10.3390/biomedicines10092219

Okore VC. (2005) Pharmaceutical Microbiology: Principles of Pharmaceutical Applications of Antimicrobial Agents. 1st ed. Enugu: Demak; pp 61-64.

Plumet L, Ahmad-M. N, Dunyach-R.C, Kissa K, Sotto A, LavigneJ.P, Costechareyre D, Molle V. (2022) Bacteriophage Therapy for *Staphylococcus Aureus* Infections: A Review of Animal Models, Treatments, and Clinical Trials. *Front Cell Infect Microbiology* 17;12:907314. https://doi.org/10.3389/fcimb.2022.907314

Smith, A., & Jones, B. (2018) Antibiotic resistance: A global threat. *The Lancet* 387:176-186. https://doi.org/10.1016/S0s40-6736(16)32464-1

Singh, A., Singh, P. K., Kumar, A., Gautam, R. K., & Sharma, A. (2020). Synergistic effect of *Azadirachta indica* and *Bacillus thuringiensis* sub sp. kurstaki on the larval gut histology of *Helicoverpaarmigera* (Hübner). *Scientific Reports,* 10(1), 1–9. https://doi.org/10.1038/s41598-020-64863-6

Tacconelli, E., Carrara, E., Savoldi, A., et al. (2018) Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases* 18(3), 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3

Tanaka, I., Kutsuna, S., Ohkusu, M., Kato, T., Miyashita, M., Moriya, A....Ohkusu, K. (2022). *Bacillus subtilis* variant natto Bacteremia of Gastrointestinal Origin, Japan. *Emerging Infectious Diseases*, *28*(8), 1718-1719. Ioannis, T., https://doi.org/10.3201/eid2808.211567

Ventola, C.L., (2015) The antibiotic resistance crisis: Part 1: Causes and threats. P & T: *A Peer-Reviewed Journal for Formulary Management*, 40(4):277-283. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378521/

World Health Organization. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. WHO Press.