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Susceptibility Pattern of a Clinical Isolate of *Staphylococcus aureus* to the Combined Activity of a Herbal Preparation of *Azadirachta indica* and Some Antibiotics

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Abstract

Purpose: To investigated the *in vitro* antimicrobial interaction between the crude water extract of *Azadirachta indica* and some standard antibiotics; doxycycline, gentamicin, streptomycin, erythromycin, ciprofloxacin, and norfloxacin.
Methods: The *in vitro* interactions were evaluated using a combination of agar diffusion and Checkerboard techniques against *Staphylococcus aureus* as test microorganism.
Results: There were synergistic interactions between the crude extract (0.1 mg/ml or 0.25mg/ml extract) and all the antibiotics used except tetracycline in the agar diffusion technique ($p < 0.05$). In the Checkerboard technique, fractional inhibitory concentration (FIC) index reveal that the crude extract were synergistic with either tetracycline (1:9), streptomycin (1:9, 9:1), ciprofloxacin (2:8, 5:5) or norfloxacin (2:8, 6:4).
Conclusion: There is a possible beneficial clinical application of the co-administration of doxycycline, gentamicin, streptomycin, erythromycin, ciprofloxacin or norfloxacin and the crude extract of *A. indica* in the treatment of infections caused by *S. aureus*. However, unguided concomitant usage may result in therapeutic failure.

Keywords: *Staphylococcus aureus*, interaction, antibiotics, FIC index

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Introduction

Several antibiotics are used to treat a variety of infectious human diseases. Many of them have, however, a limited antimicrobial spectrum due to the emergence of multi drug-resistant (MDR) bacterial strains [1-7]. This development has contributed to the use of two or more antimicrobial agents as a combination treatment [8]. The use of antimicrobial combination treatment in microbial infections is one important means of preventing or delaying the emergence of resistant microbial strains. A second important reason is that antibiotic combinations may in certain cases produce a synergistic effect which proves useful in the treatment of bacterial infections especially in otherwise resistant-bacteria cases [9].

The potential usefulness of some plant or herbal extracts in the effective control of several medically important bacterial infections has been reported [10-12]. Concomitant administration of herbs/herbal extracts has equally been reported in patients on antibiotics therapy [13-15] and this portends a potential herb-drug interaction, which could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome). It is therefore important that proper evaluation of any possible drug-herb interaction between commonly administered antibiotics and herbs be carried out in order to report any likely dangerous outcome, or validate any potential synergistic utility of the drug-herb combinations in the management of microbial infections especially those involving MDR in clinically oriented setting.

Azadirachta indica (Meliaceae), popularly known as Neem, is a plant native to India and Burma, growing in tropical and semi-tropical regions [16]. It is also widely available in Nigeria. Different parts of this plant have been reported to have antiseptic, wound healing and skin disease curing activities [17, 18]. Neem leaf extract in the form of tea are used by the people of Nigeria, India and Haiti in the treatment of malaria, and the oil employed in the management of bacteria infections caused by *Staphylococcus aureus* and *Salmonella spp* [19-21]. Clinical studies with the dried neem leaf extract indicated its effectiveness to cure ringworm, eczema and scabies [22, 23].

The aim of this present study was to evaluate the possible clinical suitability of the use of combinations of some antibiotics with neem water extract against some infections of *Staphylococcus aureus.* Such studies as this can furnish information on the use of these combinations which can demonstrate therapeutic and safety relevance when the outcome of combinations allow the proportion of plant extract and drug to be reduced. Moreover, the control of, slow down of, and prevention of the emergence of resistant bacterial strains associated with the use of such combinations could also turn out to be a useful outcome of this probe. Finally, such studies can be useful predictors of a possible beneficial or deleterious outcome when this herb is consumed concomitantly by patients on antibiotics therapy.

Materials and Methods

Plant Materials and Extraction

The leaves of *Azadirachta indica* (Meliaceae) were collected from Nsukka, Enugu State, Nigeria in August 2006 and were authenticated by a Botanist in the Department of Botany, University of Nigeria Nsukka, Nigeria. The leaves were cut into smaller pieces, air-dried and then pulverized. About 200 g of pulverized material was extracted with water and the resulting extract 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 (Bio-chemika, India). antibiotics disc containing 10µg each of doxycycline, gentamicin,

streptomycin, erythromycin, ciprofloxacin, and norfloxacin were used.

Test Microorganism

The test organism used for these experiments is a clinical isolate of *Staphylococcus aureus* obtained from the Pharmaceutical Microbiology Laboratory, University of Nigeria. Identification of the bacterial isolate was performed according to standard bacteriological techniques previously established [24, 25]. A 24 h 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 Neem and the various antibiotics using the overlay-inoculum susceptibility method

A 1 ml each of 2 mg/ml and 5 mg/ml neem water extract was seeded into 19 ml molten nutrient agar respectively and allowed to solidify. Thereafter the test organism was layered on the solidified agar and allowed to dry. Antibiotic discs were then aseptically placed on the solidified agar plate using sterile forceps and the plates were then incubated at 37 ^{o}C for 24 h. The experiment was carried out in triplicates using controls.

Evaluation of the interaction between the various antibiotics using agar diffusion and checkerboard methods

The continuous variation checkerboard method [26] was employed. Briefly, varying proportions ranging from 1:9 to 9:1 of neem extract and either ciprofloxacin or norfloxacin or streptomycin or tetracycline were prepared. Each proportion of the herb-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 *S. aureus* strain was then streaked on the dried plated and incubated at 37 ^{o}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

The MICs for neem extract, tetracycline, norfloxacin, ciprofloxacin and streptomycin were 20 mg/g, 62.5 µg/ml, 2.5 µg/ml, 0.625 µg/ml and 0.625 µg/ml, respectively. MICs of the combined activity of the antibiotics with neem extract using the Overlay-inoculum Susceptibility method is shown in Table 1.

Table 1: Interaction studies between *A. indica* (neem) extract and some antibiotics against *S. aureus* using overlay-inoculum susceptibility method

Antibiotic disc	Neem extract and antibiotics combination	
	2 mg/ml	5 mg/ml
Tetracycline	Antagonism	Antagonism
Doxycycline	Synergism	Synergism
Streptomycin	Synergism	Antagonism
Ciprofloxacin	Synergism	Synergism
Norfloxacin	Synergism	Synergism
Gentamicin	Synergism	Synergism
Erythromycin	Indifference	Synergism

Combinations of the antibiotics with neem extract (2 mg/ml) showed synergism for doxycycline, streptomycin, ciprofloxacin, norfloxacin and gentamycin but antagonism for tetracycline. Similarly, the same pattern was recorded for neem at 5 mg/ml with antibiotics except for slight differences

involving streptomycin (antagonism) and erythromycin (synergism). Evaluation of interaction employing the Checkerboard method showed varying activities/outcomes (Table 2). Neem/norfloxacin combination recorded synergism at ratios of 2:8 and 6:4 while ratios of 2:8 and 5:5 gave synergistic relationship for neem/ciprofloxacin combinations. Conversely, neem/tetracycline combinations showed synergism only at a ratio of 1:9 while 1:9 and 9:1 ratios showed synergism for neem/streptomycin combinations. Indifferent and antagonistic relationships were recorded by other varying ratio combinations.

Discussion

The norfloxacin, ciprofloxacin, streptomycin and tetracycline are antibiotics known to demonstrate useful antibacterial activities in some instances against *Staphylococcus aureus* strains in experimental and clinical conditions [27]. The sensitivity of the *S. aureus* strain used in the study to these antibiotics was ascertained by determining the MIC of the antibiotics against the organism. Results of the MIC determination for norfloxacin, ciprofloxacin, streptomycin and tetracycline, when used singly, against the test organism show that *S. aureus* was susceptible to all the antibiotics utilized in this study with the highest MIC of 62.5 µg/ml recorded for tetracycline which is known to have bacteriostatic effect. It is on the other hand obvious that for the neem aqueous extract, the MIC of 20 mg/g recorded is a clear reflection of the poor antibacterial activity of the crude plant extract. This observed outcome amongst other considerations further necessitated the evaluation of the combination of neem extract with some commonly administered antibiotics used in this study for a possible useful interaction outcome that would present possible clinical applicability.

The outcome of combined activities involving neem extract and antibiotics monitored using the overlay-inoculum susceptibility method (Table 1) show that synergistic interactions were observed for the combinations of 2 mg/ml neem extract with doxycycline, streptomycin, ciprofloxacin, norfloxacin and gentamicin while antagonistic and indifference interactions were observed for tetracycline and erythromycin respectively. In this method of assessment of combined activities, changes is observed between the inhibition zone diameter (IZD) generated for the control (pure antibiotic) and that of the test (neem-antibiotic combination). A positive change in IZD denotes synergism while negative and zero changes denote antagonism and indifference respectively. When the concentration of neem extract was increased to 5 mg/ml, only a slightly different pattern was observed, such that the initial (Table 1) indifferent interaction recorded for neem-erythromycin was now transformed to synergistic interaction, while the initial synergistic interaction of neem-Streptomycin became antagonistic. These observed dose-dependent interaction outcome have equally been reported in other related studies [28]. This behaviour is thought to occur as result of quite unexplainable concentration-dependent changing dynamics within the crude extract. Crude extract are complex mixtures of phytoconstituents of differing activities and inactivities, and so it is relatively difficult to predict with accurate precision the overall pattern of activity of the crude extract at slightly different dose ranges [29]. This explanation could also hold for the various neem-antibiotics combinations. A generalized assessment of the observed results so far shows notable synergism between the neem extract and these antibiotics when used against the test microorganism.

Checkerboard evaluations as a means of monitoring the combined activities of antimicrobial agents is based on the general outcome that FIC index value below unity signifies synergism, ≥ 1 ; indifference, and ≥ 2; antagonism. Therefore, from the study interactions between the neem extract and norfloxacin reveal synergism at ratios 2:8

Table 2: The combined effect of *A. indica (n*eem) extract and antibiotics against *S. aureus* using checkerboard method

Combination ratio (Neem:Drug)		1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1
Neem: norflox-acin	MIC (mg/ml)	0.1: 0.0045	0.05: 0.001	NA	NA	NA	0.15: 0.0005	NA	NA	NA
	FIC (mg/ml)	0.005: 1.8	0.0025: 0.4	NA	NA	NA	0.0075: 0.2	NA	NA	NA
	FIC Index	1.805	0.4025	NA	NA	NA	0.2075	NA	NA	NA
	Effect	Indifferent	Synergism	NA	NA	NA	Synergism	NA	NA	NA
Neem: ciproflo-xacin	MIC (mg/ml)	0.05: 0.00225	0.025: 0.0005	0.075: 0.000875	0.2: 0.0015	0.0625: 0.0003125	0.3: 0.001	0.7: 0.0015	0.8: 0.001	NA
	FIC (mg/ml)	0.0025: 3.6	0.00125: 0.8	0.00375: 1.4	0.01: 2.4	0.00313: 0.5	0.015: 1.6	0.035: 2.4	0.04: 1.6	NA
	FIC Index	3.603	0.801	1.404	2.41	0.503	1.615	2.435	1.64	NA
	Effect	Antagonism	Synergism	Indifference	Antagonism	Synergism	Indifference	Antagonism	Indifference	NA
Neem: Tetracy-cline	MIC (mg/ml)	0.0125: 0.05625	0.2: 0.4	0.00375: 1.4	0.075: 0.0875	0.25: 0.125	NA	NA	0.8: 0.1	NA
	FIC (mg/ml)	0.000625: 0.5	0.01:6.4	0.00375: 1.4	0.01: 2.4	0.0125: 2	NA	NA	0.04: 1.6	NA
	FIC Index	0.5006	6.41	1.404	2.41	2.0125	NA	NA	1.64	NA
	Effect	Synergism	Antagonism	Indifference	Antagonism	Antagonism	NA	NA	Indifference	NA
Neem: Strepto-mycin	MIC (mg/ml)	0.00625: 0.028125	NA	NA	NA	NA	NA	NA	NA	0.05625: 0.003125
	FIC (mg/ml)	0.000313: 0.161	NA	NA	NA	NA	NA	NA	NA	0.00281
	FIC Index	0.161	NA	NA	NA	NA	NA	NA	NA	0.161
	Effect	Synergism	NA	NA	NA	NA	NA	NA	NA	Synergism

FIC = Fractional Inhibitory Concentration; MIC = Minimum Inhibitory Concentration; NA = Not Applicable (ratio combinations where MIC determinations could not be carried out)

and 6:4 (Table 2), and between neem extract and ciprofloxacin reveal showed synergism at ratios 2:8 and 5:5 (Table 2). These two combinatorial outcomes demonstrated quite close similarities in the pattern of interaction, and further suggest that careful selection and predetermined combinations of neem with norfloxacin and ciprofloxacin, respectively, within the range of these established dose ranges can confer synergy in clinically oriented settings. An apparent relatively poorer relationship is shown for neem/tetracycline (Table 2) where synergy is recorded at only ratio 1:9 with antagonism and indifference at different dose ratios. It appears that there may be a more established synergy profile for neem/ streptomycin combinations since the other dose ratios utilized in this study as all the broth tubes dilutions lying between ratios 2:8 to 8:2 actually inhibited the growth of the microorganism thereby giving no clear MIC cutoff points. This major trend (absence of MICs) may further suggest that the fractional MICs of the neem/streptomycin are very low and beyond the range of the tested dilutions in this study. If this is so, then the use of neem/streptomycin "free" combinations may hold great promise in clinical conditions involving *S. aureus* infections. Moreover, from the foregoing the various combinations of neem and the assessed antibiotics have displayed desirable synergy thereby being an improvement on the poor antibacterial activity of the crude neem extract (having MIC = 20mg/ml) when used alone against *S. aureus*. The improvement on the antibacterial activity of the neem aqueous extract (only when fixed combinations are used) should be considered important outcome in spite of the earlier reported usefulness of neem plant (not necessarily the aqueous extract) in certain bacterial infections since none of them however represented an *in vitro* study or evaluation of the aqueous extract. Moreover, *in vivo* may usually introduce the influence of immunological parameters on the overall outcome of infections in the human or animal hosts. This influence is clearly lost *in vitro*, thus better portraying the basal benefits of the drug combinations. This is quite important especially when considerations are made for the immunocompromised. Moreover, such combinations since they allow the use of lower doses of both agents could help minimize any occurrence of possible adverse effects or toxicity associated with either or both agents. In addition to this is the control or prevention of emergent resistant bacterial strains which are a subject of serious concern in clinical practice. On the other hand, the occurrence of antagonism recorded in some cases at certain fixed combinations raises the concern of a possible of treatment failure where patients who are being treated with these antibiotics for *S. aureus*-based infections inadvertently consume herbal preparations containing neem extract. Such kind of "blind" practices by patients can clearly impose a costly compromise on chemotherapy aside the possibility of toxic reaction that could occur with such untoward usage. Patients therefore should by properly instructed on the inherent danger associated with such behaviours, and consequently avoid the consumption of neem extract when on antibiotics therapy.

Staphylococcus aureus is an important cause of serious infections in both hospitals and the community [30]. It has been found to be the most frequently isolated pathogen causing bloodstream infections, skin and soft tissue infections, as well as pneumonia [31-33]. Unfortunately this pathogen has been proven to demonstrate high level of resistance to common antibiotics. Therefore, the synergistic interaction observed between neem aqueous extract and the evaluated antibiotics can be translated into useful clinical applications in *S. aureus*-based infections especially when the constituents of the neem extract are further isolated and characterized.

Conclusion

There is possible potentiation of antibacterial effects of some antibiotics against *S. aureus* infection when co-administrated with neem

water extract. However, the consumption of neem extract while undergoing antibiotics therapy may result in treatment failure. However, the careful use of controlled predetermined combinations of neem water extract and these antibiotics could find clinical applications in the treatment of bacteria infections caused by the susceptible microorganism, and in the prevention of emergent resistant strains of *S. aureus*.

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