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IN VITRO EVALUATION OF THE INTERACTION BETWEEN METHANOL EXTRACT OF THE LICHEN, *RAMALINA FARINACEA* AND AMPICILIN AGAINST CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Ampicillin is used mainly to treat infections of the middle ear, sinuses, bladder, kidney, and uncomplicated gonorrhea. It is used intravenously to treat meningitis and other serious infections**.** The activities of this antibiotic were in some cases hindered by the β-lactamase producing resistant strains of *Staphylococcus aureus*. Methanol extract of the lichen gave minimum inhibitory concentration (MIC) of 1.05µg/ml against the *Staph. aureus*, showing that the methanol extract is very potent against the microorganism. Two clinical isolates of *Staph. aureus* were used for this work, Staph. aureus strains A and B. Antimicrobial interaction screening of the methanol extract with ampicillin, reveled that at combination ratios 9:1, 8:2, 6:4, 5:5, 4:6, 3:7 of methanol extract and ampicillin showed synergism against *Staph. aureus* strain A, while at all the 8 combination ratios of Methanol extract and Ampicilin showed synergism against *Staph. aureus* strain B. This shows that in the treatment of infections of *Staph. aureus* the combination of the methanol extract of Lichen, *Ramalina Farinacea* and ampicilin can be used together to enhance potency of the ampicilin in some resistance cases of infection by Staph. aureus.

Keywords: Antimicrobial interactions, Lichen, *Ramalina farinacea*, ampicillin, Methanol extract, *Staphylococcus aureus*, Combination ratio.

INTRODUCTION

Antimicrobial Interactions can be antagonistic, indifference, additive or synergistic. Antagonism, this is when the combined action is less than that of the more effective agent when used alone [1]. It occurred when a bacteriostatic drug (which inhibited protein synthesis in bacteria) such as chloramphenicol or tetracycline was given with a bactericidal drug such as a penicillin or an aminoglycoside. [1, 2].

Indifference: The combined action is not different from the more effective agent when used alone [1, 3]. Additive, the combined action is equivalent to the sum of the actions of each drug when used alone [3]. Synergism, this is when combined action is significantly greater than the sum of the both effects. [1, 4].

Checkerboard method of evaluation of the *in vitro* antimicrobial interactions was employed in this study; it involves the determination of per cent growth inhibition of microbial cells in the presence of different combinations of drugs. The specific merits and limitations of checkerboard testing have been described and summarized in detail [5]. Briefly, the checkerboard method is relatively simple to perform and the results are easily interpreted, making them useful for extensive screening.

Studies investigating the *in vitro* efficacy of antimicrobial agents in combination using checkerboard method interpret results in terms of the Fractional Inhibitory Concentration Index (FICI), which is defined by the following equation [3,5]:M

$$FICI = FIC_A + FIC_B = \frac{MIC_A \text{ in combination}}{MIC_A \text{ tested alone}} + \frac{MIC_B \text{ in combination}}{MIC_B \text{ tested alone}}$$

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Where MIC_A and MIC_B are the MICs of drugs A and B respectively.

FIC_{index} values < 1 were considered as synergy and the degree of synergy increases as the value tends towards zero. FIC_{index} value of 1 indicates additivity, values greater than 1, but less than 2 represent indifference while values greater than 2 shows antagonism[3,5].

The genus *staphylococcus* has at least 30 species. The three main species of clinical importance are *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus albus*. *Staphylococcus aureus* is coagulase-positive, which differentiates it from the other species. *Staphlococcus aureus* is a major pathogen for humans. Almost every person will have some type of *Staphlococcus aureus* infection during a lifetime, ranging in severity from food poisoning or minor skin infections to severe life -threatening infections [1].

Ampicillin is a member of the group of antibiotics called penicillin otherwise known as B-lactam drugs [1]. Ampicillin is selective inhibitors of bacterial cell wall synthesis and therefore is active against growing bacteria [4]. This inhibition is only one of the several different activities of these drugs, but it is the best-understood.

Ampicillin is one of the most widely prescribed antibiotics. Unlike penicillin, ampicillin and amoxicillin can penetrate and prevent the growth of certain types of bacteria, called gram-negative bacteria. Ampicillin is used mainly to treat infections of the middle ear, sinuses, bladder, kidney, and uncomplicated gonorrhea. It is also used intravenously to treat meningitis and other serious infections [1, 5].

The antimicrobial actions of lichen substances are well known [6, 7, 8].

It is estimated that more than half of the lichen species have antibiotic properties [8]. The compounds responsible for this activity are polysaccharides (with host-mediated antitumour activity), depsides, depsidones, coumarones, benzofuran derivatives, xanthones, or fatty acid derivatives (ox*y-oxo-acids. Lactones) [8]. They are active against bacteria, mostly Gram-positive forms, free-living moulds, yeast and viruses [7, 8].Screening tests with lichens have indicated the frequent occurrence of antimicrobial substances [9]. Antibacterial screening of the light petroleum extracts of *Thamnolia subuliformis* showed it is active *in-vitro* against Gram positive organisms as well as against *Escherichia coli* and *Candida albicans* [10].

Harmala *et al* screened extracts from *Cladina mitis*, *Cladina stellaris* and *Certraria islandica* against six pathogenic microorganisms. It was found that extracts from *Cladina mitis* and *Cladina stellaris* were active against *Staph. aureus* and *Bacillus subtilis* but did not affect the other test organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergilius niger*). Pure usnic acid (used as control gives a similar result and it is found that the 2 species of *Cladinas* above contained usnic acid. The mechanisms of the antimicrobial action of lichen substances have been variously described [6, 7].

The aim of this study is to investigate *in vitro* antimicrobial interaction between methanol extract of the lichen, *Ramalina farinacea* (L) Ach (f*am: Ramalinacea,* and ampicilin against 2 clinical isolates of *Staphylococcus aureus* strains A and B for possible considerations in resistance cases.

RESEARCH METHODOLOGY

Materials and Methods

Test organism

Clinical isolates A and B of *Staph. aureus*, were collected from University of Nigeria Teaching Hospital, Enugu.

Culture Media

The culture media used include nutrient agar, McConkey agar, nutrient broth No.2, mannitol salt agar, deoxycholate citrate agar, selenite F broth (Oxoid). All media were prepared according to manufacturer's instructions.

Reagents

The following reagents were used: ampicillin, Beecham Pharmaceuticals Brentfords England, methanol (Janssen), di-methyl-sulphoxide (DMSO, BDH, England). All solvents and chemicals were of analytical grade.

Collection and Identification of Lichens

The lichens, *Ramalina farinacea* (L) Ach. were collected in October 2006 from palm and dead tree trunks in oba, Nsukka. They were identified by plant taxonomist, Mr. J. M. C. Ekwere of the Botanical Garden, University of Nigeria, Nsukka.

Maintenance and Standardization of Stock Cultures

A stock culture of each clinical isolate of *Staph. aureus* was stored in nutrient agar slant. Prior to use, the culture were activated by successive daily sub-culturing into fresh agar slants for a period of 3 days. The Overnight (18 hr) cultures were standardized by diluting with Normal saline 1:1000 to obtain population density of approximately 10^6 cfu/ml before use [5].

Extraction of Lichen and Preparation of drug stock solution

A 200g of the sun-dried lichen was extracted with appropriate quantity of methanol by cold maceration and the solution was allowed to air dry to obtain the extracted quantity of the lichen, 800ug/ml was prepared as the stock solution of the lichen.

Sterilization of materials

The Petri dishes and pipettes packed into metal canisters were appropriately sterilized in the hot air oven (Ov – 335, Hareus) at 170^0c for 1 hr. at each occasion. Solution of the extract and culture media were autoclaved at 121^0c for 15 min.

Determination of minimal inhibitory concentration (MIC) of Methanol extract against *Staph. Aureus*

The sensitivity of *Staph. aureus to* methanol

extract of the lichen was evaluated by the cup-plate agar diffusion method [5]. A small portion of the extract were dissolved in 2 ml DMSO and the resulting solution diluted to a concentration of 800ug/ml stock solution of the extracts using sterile distilled water.

Molten nutrient agar in a plate (Petri dish) were seeded with 0.1 ml of standardized broth culture of bacteria and allowed to set. A total of 4 wells, 8mm in diameter, were made in the agar using a sterile cork borer. Two drops (32ug/0.02ml) of each of the extracts were carefully placed into each well as control. The plates were left for 1hr at room temperature for diffusion, after which they were incubated at 37^{o}C for 24hrs.

The inhibition zone diameters (IZDs) of the different concentrations of the extract were measured and the MIC obtained from the intercepts on the log conc. axis of the graphs of logarithm of concentration (log conc.) against the squares of the inhibition zone diameter (IZD ^{2}) of Staph. *aureus*.

In vitro interactions of methanol extract with ampicilin against *Staph. aureus*
Strains A and B.

A 200g of the sun-dried lichen was extracted with appropriate quantity of methanol using cold maceration method and the solution was allowed to air dry to obtain the extracted quantity of the lichen, 800ug/ml was prepared as the stock solution of the lichen extracts in dymethylsulphoxide (DMSO).

Stock solution of antibiotics 50 ug/ml was also prepared in sterile distilled water. Thereafter, varying proportions of ampicilin and the extract were prepared according to the continuous variation checkerboard method; each proportion of antibiotic combination was serially diluted (2-fold), inoculated with 0.1 ml of the standardized 10^{6} cfu/ml culture of nutrient broth of the test microorganism (*Staph. aureus*) and then incubated for 24h at 37 ^{o} C. Two (2) strains of clinical isolates of *Staphylococcus aureus* were used for the research work. Interaction was assessed algebraically by determining the fractional inhibitory concentration (FIC) indices at the combination ratios obtained using the continuous variation method.

The MIC obtained from the intercepts on the Log conc. axis of the graphs of logarithm of concentration (Log conc.) against the squares of the inhibition zone diameter (IZD ^{2}) of Staph. *aureus*. Given 1.05µg/ml. The methanol extract of the lichen is very potent antibiotic against Staph. aureus strain.

In vitro interactions of methanol extract with ampicilin against *Staph. aureus*
Strains A and B.

From the above table 2, interaction result at the combination ratios of 9:1, 8:2, 6:4, 5:5, 4:6, 3:7 of the extract and ampicilin showed synergism while combination ratios 7:3, 2:8 and 1:9 showed Additive.
From the above table 3, interaction result at all combination ratios of the extract and ampicilin showed synergism.

Key:
MIC = Minimum Inhibitory Concentration
Amp = Ampicillin
Lichen = Methanol extract of Lichen
FIC = Fractional Inhibitory Concentration
ADD = Additive
SYN = Synergism

RESULTS

Figure 1: **Log conc. against the squares of the inhibition zone diameter IZD ^{2}**

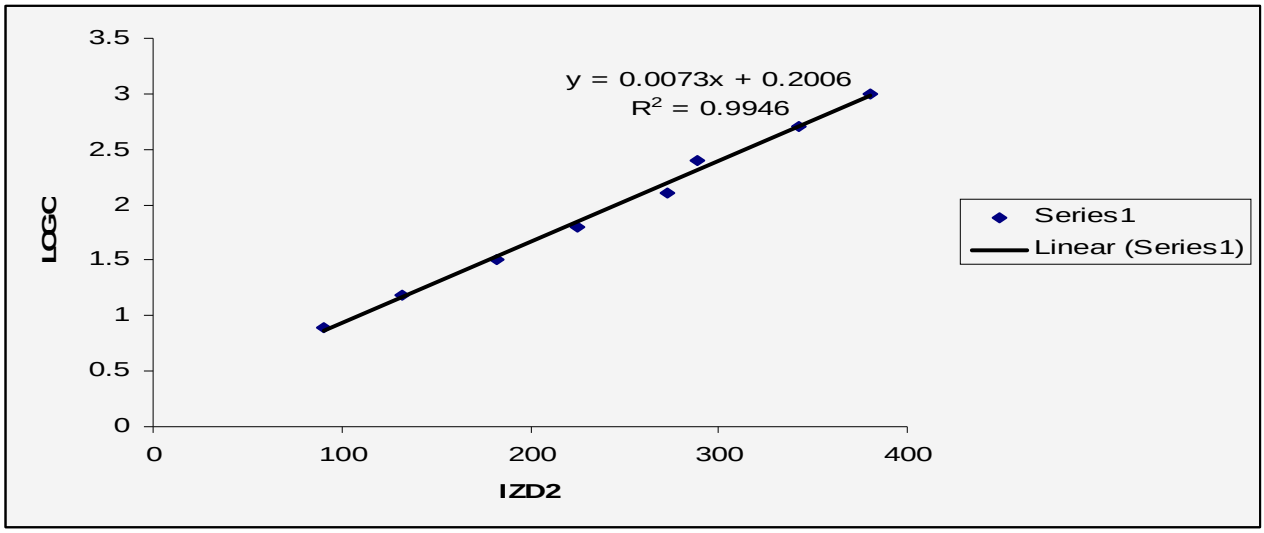

Table 1: Minimal inhibitory concentration (MIC) of Methanol extract against *Staph. Aureus*

(Conc. µg/ml)	Log Conc.	IZD1	IZD2	IZD AVERAGE	$1ZD^2$
1000	3.00	19	20	19.5	380.25
500	2.70	18	19	18.5	342.25
250	2.40	17	17	17	289.00
125	2.10	16	17	16.5	272.25
625	1.80	15	15	15	225.00
31.25	1.50	13	14	13.5	182.25
15.62	1.19	11	12	11.5	132.25
7.81	0.89	9	10	9.5	90.25

Table 2: Interaction of Methanol extract of *Ramalina farinacea* – 800 µg/ml and ampicillin - 50 µg/ml against *Staph. aureus* strain A .

Drug Ratio Lichen: Amp	MIC µg/ml Extract	MIC µg/ml Amp	FIC Extract	FIC Amp	FIC Index	Activity Index	Inference
10:0	400	-	-	-	-	-	-
9:1	180	1.25	0.45	0.05	0.5	-0301	SYN
8:2	160	2.5	0.4	0.10	0.5	-0.301	SYN
7:3	280	7.5	0.7	0.30	1.0	-0.0	ADD
6:4	60	2.5	0.15	0.10	0.25	-0.6020	SYN
5:5	100	6.25	0.25	0.25	0.5	-0.3010	SYN
4:6	80	7.5	0.2	0.30	0.5	-0.3010	SYN
3:7	60	8.75	0.15	0.35	0.5	-0.3010	SYN
2:8	80	20	0.2	0.8	1.0	0.0	ADD
1:9	40	22.5	0.1	0.9	1.0	0.0	ADD
0:10	-	25	-	-	-	-	-

Table 3: Interaction of Methanol extract of *Ramalina farinacea* – 800 µg/ml and ampicillin - 50 µg/ml against *Staph.aureus* strain B.

Drug Ratio Lichen: Amp	MIC µg/ml Extract	MIC µg/ml Amp	FIC Extract	FIC Amp	FIC Index	Activity Index	Inference
10:0	400	-	-	-	-	-	-
9:1	180	1.25	0.45	0.1	0.55	-0259	SYN
8:2	160	2.5	0.4	0.20	0.60	-0.221	SYN
7:3	140	3.75	0.35	0.30	1.65	-0.187	SYN
6:4	60	2.5	0.15	0.20	0.35	-0.455	SYN
:5	100	6.25	0.25	0.50	0.75	-0.124	SYN
4:6	40	3.75	0.1	0.30	0.40	-0.397	SYN
3:7	60	8.75	0.15	0.70	0.85	-0.071	SYN
2:8	20	5	0.05	0.40	0.45	-0.346	SYN
1:9	2.5	1.4	0.00625	0.11	012.	-0.921	SYN
0:10	-	12.5	-	-	-	-	-

DISCUSSION

The combination of methanol extract of lichen *Ramalina farinacea* and ampicillin is hoped to achieve a desirable synergistic effect in order to increase the antibiotic spectrum of ampicillin or given a more potent combination therapy for ampicilin resistant Staph. aureus infection.

Combined drug use is occasionally recommended to prevent resistance emerging during treatment and to achieve higher efficacy in the treatment of infections and diseases.

The results of the interaction studies carried out on the methanol extract and ampicillin against *Staph. aureus* strains A and B as presented in Tables 2 and 3.

Table 2: Shows the interaction of the methanol extract and ampicillin against *Staph. aureus* strain A. At ratios 9:1, 8:2, 6:4, 5:5, 4:6, 3:7 to be synergistic while at ratios 7:3, 2:8 and 1:9 was additive. Table 3: Shows the interaction of methanol extract and ampicilin against *Staph. aureus* strain B to be 100% synergistic.

CONCLUSION

The best synergistic interactions were obtained with combination of methanol extracts and ampicillin against *Staph. aureus* strain B, the interaction was synergistic at all the combination ratios used given us 100% synergy. While the combination interaction of the extract and ampicillin against Staph. aureus strain B gave 70% synergy and 30% additive.This study go a long way to show that combination therapy with these commonly used antibiotic (ampicillin) and lichen methanol extract can possibly improve survival and treatment outcome in some seriously debilitated patients who are afflicted with life threatening *Staph. aureus* infections both in the community and hospital.

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