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Full Length Research Paper

Antimicrobial evaluation of the interaction between methanol extract of the lichen, *Ramalina Farinacea (Ramalinacea)* and Ampicilin against clinical isolates of *Staphylococcus Aureus*

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Ampicillin is a member of the group of antibiotics called penicillin otherwise known as B-lactam drugs; it is a selective inhibitor of bacterial cell wall synthesis and therefore is active against growing bacteria. Ampicillin is one of the most widely prescribed antibiotics. It is considered as penicillin and is a close relative of amoxicillin. 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 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*. Antimicrobial interaction screening of the methanol extract with ampicillin, reveled that most of the combination ratio of methanol extract and ampicillin shows synergism while few were additive and no indifference and antagonism. This shows that in the treatment of infections of *S. 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 cases of infection by *S. aureus*.

Key words: Antimicrobial interactions, Lichen, *Ramalina farinacea*, ampicillin, Methanol extract, *Staphylococcus aureus*.

INTRODUCTION

Some of the possible reasons for employing two or more antimicrobials simultaneously instead of a single drug are as follows:

(1)To give prompt treatment in desperately ill patients suspected of having a serious microbial infection (Geo et al., 1995). (2) To delay the emergence of microbial mutants resistant to one drug in chronic infections by the use of a second or third non-cross-resistant drug. The most prominent example is active tuberculosis of an organ, with large microbial populations (Abercrombie et al., 1992). (3) To treat mixed infections, particularly those following massive trauma or those involving vascular structures (Geo et al., 1995; Abercrombie et al., 1992). (4) To achieve bactericidal synergism or to provide bactericidal action in a few infections, e.g. enterococcal sepsis (Geo et al., 1995; Lewis et al., 2002).

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 (Geo et al., 1995). Indifference: The combined action is not different from the more effective agent when used alone

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(Geo et al., 1995; Vincent, 2005). Additive, the combined action is equivalent to the sum of the actions of each drug when used alone (Lewis et al., 2002). Synergism, this is when combined action is significantly greater than the sum of the both effects (Geo et al., 1995; Eugene et al., 2004).

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 (Vincent, 2005). 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 (Lewis et al., 2002; Vincent, 2005).

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

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 (Lewis et al., 2002; Vincent, 2005).

The genus *staphylococcus* has at least 30 species. The three main species of clinical importance are *S. aureus, Staphylococcus epidermidis* and *Staphylococcus albus*. *S. aureus* is coagulase-positive, which differentiates it from the other species. *S. aureus* is a major pathogen for humans. Almost every person will have some type of *S. aureus* infection during a lifetime, ranging in severity from food poisoning or minor skin infections to severe life - threatening infections (Geo et al., 1995).

Ampicillin is a member of the group of antibiotics called penicillin otherwise known as B-lactam drugs (Geo et al., 1995). Ampicillin is selective inhibitors of bacterial cell wall synthesis and therefore is active against growing bacteria (Eugene et al., 2004).

Screening tests with lichens have indicated the frequent occurrence of antimicrobial substances (Isaac, 1992; Werner, 1992; Ingolfsdottir, 1985)

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* (Lewis et al., 2002; Eugene et al., 2004). The mechanism of the antimicrobial action of lichen substances have been variously described (Eugene et al., 2004; Isaac, 1992; Werner, 1992; Ingolfsdottir, 1985).

The aim of this study is to investigate *in vitro* antimicrobial interaction between methanol extract of the lichen, *Ramalina farinacea* (L) Ach (FAM*: Ramalinacea,* fruticose lichen that grows abundantly in Oba forest, Nsukka and Ampicillin.

MATERIALS AND METHODS

Test organism

Clinical isolates A and B of *S. aureus* were collected from Bishop Shanahan Hospital, Nsukka and University of Nigeria Teaching Hospital, Enugu.

Culture media

The culture media used include nutrient agar (Becton and Dickson Co, USA), 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 lichen, *R. farinacea* (L) Ach. was collected in October 2006 from palm and dead tree trunks in Oba, Nsukka. It was identified by plant taxonomist, Mr. J. M. C. Ekwere of the Botanical Garden, University of Nigeria, Nsukka and voucher specimen was deposited in the same department.

Maintenance and standardization of stock cultures

A stock culture of each clinical isolate of *S. 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 h) cultures were standardized by diluting with Normal saline 1:1000 to obtain population density of approximately 10^6 cfu/ml before use (Vincent, 2005).

Extraction of lichen and preparation of drug stock solution

A 200 g 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, Heraeus) at 170°C for 1 h. at each occasion. Solution of the extract and culture media were autoclaved at 121°C for 15 min.

Antimicrobial screening tests of the lichen extract

Preliminary sensitivity tests of S. aureus against methanol extract

The sensitivity of *S. aureus to* methanol extract of the lichen was evaluated by the cup-plate agar diffusion (Vincent, 2005). A small portion of the extract were dissolved in 2 ml DMSO and the resulting solution diluted to a concentration of 800 µg /ml stock solution of the extracts using sterile distilled water.

Molten nutrient agar in a plate (Petri dish) were seeded with

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

Drug ratio Li: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	-	-	-	-	-

The MIC of Lichen alone against *S. aureus* strain A is 400 µg/ml and ampicilin alone is 25 µg/ml. At combination ratios (9:1, 8:2, 6:4, 5:5, 4:6 and 3:7) the interactions were synergistic and at (7:3, 2:8 and 1:9) interactions were additive.

0.1 ml of standardized broth culture of bacteria and allowed to set. A total of 4 wells, 8 mm in diameter, were made in the agar using a sterile cork borer. Two drops (32 µg/0.02 ml) of each of the extracts were carefully placed into each well as control. The plates were left for 1 h at room temperature for diffusion, after which they were incubated at 37°C for 24 h.

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 *S. aureus*.

***In vitro* interactions of methanol extract with ampicilin against *S. aureus* strains A and B using checkerboard method.**

A 200 g 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, 800 g/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 (*S. aureus*) and then incubated for 24 h at 37°C. Two (2) strains of clinical isolates of *S. 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.

RESULTS

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 *S. aureus* is 1.05µg/ml. Showing that the methanol extract of the Lichen is very active at a very low concentration against a sample strain of *S. aureus.*

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. 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 *S. aureus* strains A and B are as presented in Tables 2 and 3.

In Table 1 and Figure 1, the preliminary sensitivity test revealed that methanol extract is active against *S. aureus* strain at a very low inhibitory concentration of MIC 1.05 µg/ml. This enhances the combination result of the methanol extract of the lichen with ampicilin.

Table 2 shows the interaction of the methanol extract and ampicillin against *S. aureus* strain A. At ratios 9:1, 8:2, 6:4, 5:5, 4:6, 3:7 the interaction was synergistic while at ratios 7:3, 2:8 and 1:9 was additivity.

Table 3 shows the interaction of methanol extract and ampicilin against *S. aureus* strain B. At all the combination ratios there were inhibition and the combination interactions were synergistic.

These are encouraging combination results that needs to be employed in clinical treatment of infectious disease caused by *S. aureus* that are resistant to ampicilin alone

Conclusion

The best synergistic interactions against *S. aureus* strains considered for this research were obtained with combination of methanol extracts and ampicillin against

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

Drug ratio Li: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	-	-	-	-	-

The MIC of Lichen alone against *S. aureus* strain B is 400 µg/ml and ampicilin alone is 12.5 µg/ml. While at all combination ratios their interactions were synergistic. Key: MIC= Minimum inhibitory concentration; Amp = Ampicillin; Li = Methanol extract of Lichen; FIC = Fractional inhibitory concentration; ADD = Additive; SYN = Synergism.

Table 1. Preliminary sensitivity tests of a sample *S. aureus* strain against methanol extract of Lichen.

(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

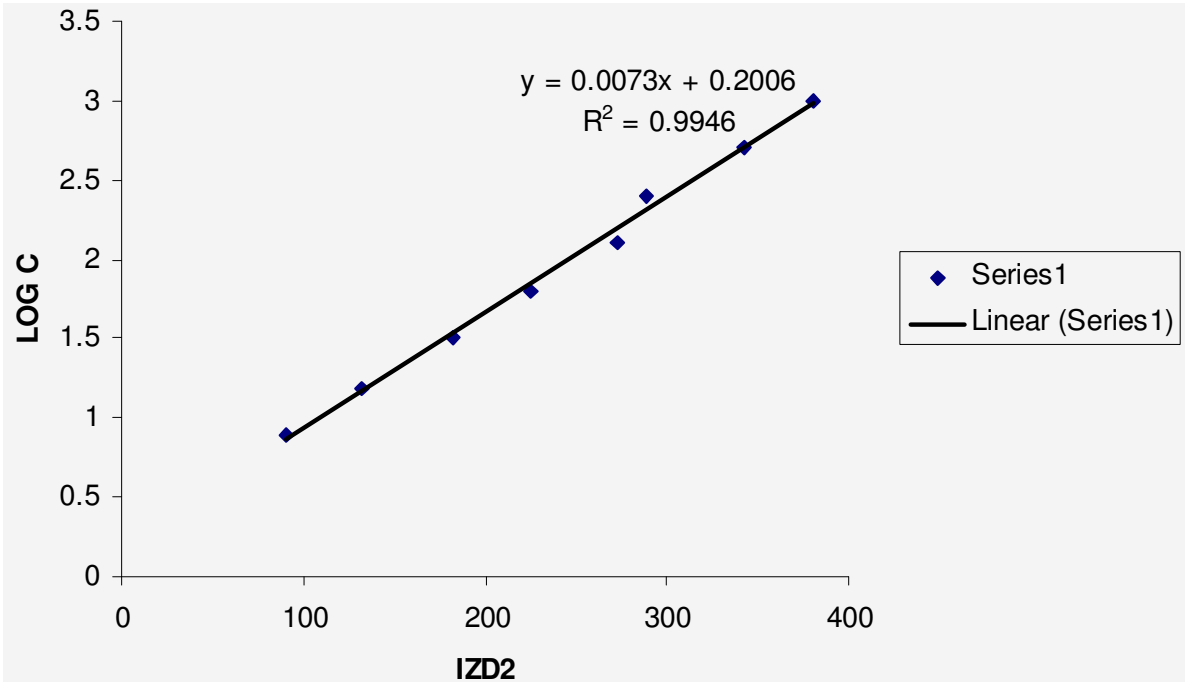

Figure 1. Log conc. against the squares of the inhibition zone diameter IZD^2.

S. 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 *S. aureus* strain B gave 70% synergy and 30% additive.

This study shows that combination therapy with this 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 *S. aureus* infections.

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