African Journal of Biotechnology Vol. 10(12), pp. 2314-2318, 21 March, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1811
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vitro evaluation of the interaction between methanol extract of lichen (*Ramalina farinacea*) and tetracycline against clinical isolates of *Staphylococcus aureus*

Akeem Agboke[1], Clement Jackson[1]*, Musiliu Adedokun[2] and M. A. Momoh[3]

[1]Faculty of Pharmacy, University of Uyo, Akwa Ibom State, Nigeria.
[2]Faculty of Pharmacy, Olabisi Onabanjo University, Ogun State, Nigeria.
[3]Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

Accepted 21 January, 2011

Antimicrobial interaction studies between methanol extract of lichen (*Ramalina farinacea* (l) ach. (Fam: *Ramalinacea*) and tetracycline, against clinical isolates of *Staphylococcus aureus* was evaluated. Preliminary antimicrobial properties of the extract were evaluated. Antimicrobial interaction studies between the methanol extract of lichen in combination with tetracycline against strains of *S. aureus* I, J and K was carried out using checkerboard method. The preliminary antimicrobial screening revealed that the extract was effective against *S. aureus* J and K. The interaction of lichen methanol extract with tetracycline against *S. aureus* strain I at ratios 8:2, 7:3, 6:4 and 4:6 was not significantly different, while ratios 9:1 and 3:7 showed antagonism. However, the combination ratios of methanol extract and the antibiotic against *S. aureus* J shows synergism (8:2, 7:3, 6:4, 5:5, 4:6 and 1:9), while ratios 9:1, 3:7 and 2:8 exhibit indifference. Interaction of the methanol extract and tetracycline against strain K at ratios 8:2, 6:4, 4:6 and 1:9 showed synergism, while ratios 9:1, 7:3, 5:5, 3:7 and 1:9 were not significantly different in their interactions. This shows that, in the treatment of infections caused by strains I, J and K of *S. aureus*, the combination of the methanol extract of lichen (*R. farinacea*) and tetracycline can be used.

Key words: Antimicrobial, checkerboard, combination ratio, minimum inhibitory concentration, strains.

INTRODUCTION

Lichen is a symbiotic association of an algae and a fungal partner (Cheji, 1988). The New Penguin Dictionary of Biology (Berdy, 1982) defines lichens symbiotic associations between a fungus and an alga, as the one that develops into a unique morphological form, distinct from either partner. This composite form generally has separate chemical and physiological properties as well (Ebirim and Ofoefule, 1997). It is not every association between fungi and algae that is regarded as lichenized. Frequently, fungi and algae occupy the same natural substrates and may form fairly permanent associations which involves physiological and physical interactions that are similar to those which occur in lichens (Keele et al., 2001; Prea et al., 2002; Lewis and Klepser, 1999) but nevertheless, could not be described as such because they have not formed a "new morphological entity" distinct from their separate components. At best, such associations could be described as lichen-like associations.

The antimicrobial actions of lichen substances are well known (Berdy, 1982; Keele et al., 2001; Lewis and Klepser, 1999). It is estimated that more than half of the lichen species have antibiotic properties (Prea et al., 2002). They are active against bacteria, mostly gram-positive forms, free-living moulds, yeast and viruses (Lewis et al., 2002; Odis et al., 2001).

Lichens have been in medical use for a long time (Odds et al., 2001). Lichens and lichen extracts "helped" in fever, jaundice, epilepsy, convulsions etc., as described in several folklores (Rex et al., 2001). Iceland moss, *Cetraria isalandica* which contains a very bitter despidone (cetraric acid) is used for disguising the taste of nauseous medicines (Rex et al., 2001).

*Corresponding author. E-mail: clementjackson1@yahoo.com.

Usnic acid, the most widely distributed and the best known lichen antibiotic, has been used in several countries (Germany: Evosin®; Austria: Usuikin®; U.S.S.R: Vinan®; Finland: USNO® as a topical antibacterial agent for human skin diseases (Odds et al., 2001). Tetracycline was the first therapeutically superior drug to be made by chemical alteration of an antibiotic produced by microbial metabolism. It sparked a wide-scale search for superior structurally modified antibiotics which has provided most of the important antibiotic discoveries made since then (Cheij, 1988).

Tetracycline is prescribed for many different infections particularly, respiratory tract infections due to *Haemophilus influenza, Streptococcus pneumonia* or *Mycoplasma pneumonia.* It is also used for Rocky Mountain spotted fever, typhus, chancroid, cholera, brucellosis, anthrax and syphilis acne (Cheij, 1988; Lewis and Klepser, 1999). Tetracycline bind to the 30s subunit of microbial ribosomes. They inhibit protein synthesis by blocking the attachment of charged aminoacyl-tRNA. Thus, they prevent introduction of new amino acids to the nascent peptide chain. The action is usually inhibitory and reversible upon withdrawal of the drug. Resistances to tetracycline result from changes in permeability of the microbial cell envelope.

The aim of this study was to investigate *in vitro* antimicrobial interaction between methanol extract of lichen (*Ramalina farinacea* (L) Ach (fam: *Ramalinacea,* a fruticose lichen) and tetracycline. Checkerboard method of evaluation of the *in vitro* antimicrobial interactions was employed in this study; it involves the determination of percent 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 (Prea et al., 2002). 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 (Keele et al., 2001; Lewis and Klepser, 1999):

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

Where MIC_A and MIC_B are the minimum inhibitory concentrations 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 (Chinwuba et al., 1994; Esimone et al., 1999).

MATERIALS AND METHODS

Test organism

Clinical isolates I, J and K of *Staphylococcus aureus*, were collected from Bishop Shanahan Hospital, Nsukka and 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 and selenite F broth (Oxoid). All media were prepared according to manufacturer's instructions.

Reagents

The following reagents were used: Tetracycline HCI, Helm Pharmaceuticals GmbH Hamburg, Germany, methanol (Janssen) and dimethylsulphoxide (DMSO, BDH, England). All solvents and chemicals were of analytical grade.

Collection and identification of lichens

The lichens, *R. 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 and was kept sample in the herbarium of the University of Nigeria, Nsukka.

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 (Odds et al., 2001).

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, 800 µg/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°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 (Okore, 2005). A small portion of the extract was dissolved in 2 ml DMSO and the resulting

Table 1. The effect of methanol extracts of *R. ferinacea* against *S. aureus* strain I.

Concentration (µg/ml)	Log Conc.	IZD1	IZD2	IZD AVERAGE	$1ZD^2$
1000	3.00	18	20	19	361.00
500	2.70	17	18	17	306.25
250	2.40	16	16	16	256.00
125	2.10	15	15	15	225.00
625	1.80	13	14	13.5	182.25
31.25	1.50	12	13	12.5	156.25

Conc., Concentration; IZDs, inhibition zone diameters.

Table 2. Interaction of methanol extract of *R. farinacea* – 800 µg/ml and tetracycline – 50 µg/ml against *S. aureus* strain I.

Drug ratio (Lichen : TCN)	MIC (µg/ml) of extract	MIC (µg/ml) TCN	FIC of extract	FIC of Amp	FIC index	Activity index	Inference
10:0	50	-	-	-	-	-	-
9:1	180	1.25	3.6	0.005	3.60	0.56	ANT
8:2	80	1.25	1.6	0.005	1.60	0.20	IND
7:3	70	1.88	1.4	0.0075	1.40	0.15	IND
6:4	60	2.5	1.2	0.01	1.20	0.07	IND
5:5	50	3.13	1.0	0.013	1.0	-	-
4:6	80	7.5	1.6	0.03	1.60	0.20	IND
3:7	120	17.5	2.4	0.07	2.5	0.39	ANT
2:8	No inhibition	-	-	-	-	-	-
1:9	No inhibition	-	-	-	-	-	-
0:10	-	25	-	-	-	-	-

MIC = Minimum inhibitory concentration; Amp = ampicillin; Lichen = methanol extract of lichen; FIC = fractional inhibitory concentration; ADD = additive; SYN = synergism.

solution was diluted to a concentration of 800 ug/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, 8 mm in diameter, were made in the agar using a sterile cork borer. Two drops (32 ug / 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 minimum inhibitory concentration (MIC) was obtained from the intercepts on the log concentration 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 interaction of methanol extract with tetracycline against *S. aureus* strains I, J and K

A 200 g of the sun-dried lichen was extracted with methanol as earlier stated and 800 µg/ml was prepared as the stock solution of the lichen extracts in dymethylsulphoxide (DMSO). Stock solution of antibiotics 50 µg/ml was also prepared in sterile distilled water. Thereafter, varying proportions of tetracycline 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. Three (3) 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 as discussed earlier.

RESULTS AND DISCUSSION

The combination of methanol extract of lichen *R. farinacea* and tetracycline is hoped to achieve a desirable synergistic effect in order to increase the antibiotic spectrum of tetracycline. Combined drug use is occasionally recommended to prevent resistance emerging during treatment and to achieve higher efficacy in the treatment of infections diseases.

The results of the interaction studies carried out on the methanol extract and tetracycline against *S. aureus* strains I, J and K are presented in Tables 2 to 4. From Table 1, the preliminary sensitivity test revealed that methanol extract is effective against *S. aureus* strain I at a very low inhibitory concentration (MIC, µg/ml). Table 2 shows the interaction of methanol with tetracycline against *S. aureus* strain I. At ratios 8:2, 7:3, 6:4 and 4:6, the interaction was similar, while at 9:1 and 3:7 there was antagonism. From Table 3, interaction of methanol

Table 3. Interaction of methanol extract of *R. farinacea* 800 µg/ml and tetracycline – 50 µg/ml against *S. aureus* strain J.

Drug ratio (Lichen : TCN)	MIC (µg/ml) of extract	MIC (µg/ml) TCN	FIC of extract	FIC of TCN	FIC index	Activity index	Inference
10:0	50	-	-	-	-	-	-
9:1	90	10	1.8	0.005	1.80	0.25	IND
8:2	40	10	0.8	0.03	0.83	-0.08	SYN
7:3	35	15	0.7	0.04	0.74	-0.13	SYN
6:4	30	20	0.6	0.05	0.65	-0.18	SYN
5:5	25	25	0.5	0.06	0.56	-0.25	SYN
4:6	40	60	0.8	0.15	0.95	-0.02	SYN
3:7	60	140	1.2	0.35	1.55	0.19	IND
2:8	40	160	0.8	0.40	1.20	0.07	IND
1:9	20	180	0.4	0.45	0.85	-0.07	SYN
0:10	-	1.56	-	-	-	-	-

MIC = Minimum inhibitory concentration; Lichen = methanol extract of lichen; FIC = fractional inhibitory concentration; ADD = additive; SYN = synergism.

Table 4. Interaction of methanol extract of *R. farinacea* – 800 µg/ml and tetracycline – 50 µg/ml against *S. aureus* strain K.

Drug ratio (Lichen : TCN)	MIC (µg/ml) of extract	MIC (µg/ml) of TCN	FIC of extract	FIC of TCN	FIC index	Activity index	Inference
10:0	50	-	-	-	-	-	-
9:1	90	0.63	1.8	0.02	1.80	0.25	IND
8:2	40	0.63	0.8	0.02	0.82	-0.08	SYN
7:3	70	0.94	1.4	0.03	1.43	0.15	IND
6:4	30	2.5	0.6	0.1	0.7	-0.15	SYN
5:5	50	3.13	1.0	0.12	1.12	0.04	IND
4:6	40	3.80	0.8	0.15	0.95	-0.02	SYN
3:7	60	8.75	1.2	0.35	1.55	0.19	IND
2:8	40	10	0.8	0.40	1.20	0.07	IND
1:9	20	11.30	0.4	0.45	0.85	-0.07	SYN
0:10	-	25	-	-	-	-	-

MIC = Minimum inhibitory concentration; Amp = ampicillin; Lichen = methanol extract of lichen; FIC = fractional inhibitory concentration; ADD = additive; SYN = synergism.

extract of *R. farinacea* and tetracycline exhibited synergism against *S. aureus* strain J at ratios of 8:2, 7:3, 6:4, 5:5, 4:6 and 1:9, while ratios 9:1, 3:7 and 2:8 exhibit similar interactions, no antagonism. Table 4 shows interaction of the methanol extract and tetracycline against strain K. At ratios 8:2, 6:4, 4:6 and 1:9, there was synergism, while ratios 9:1, 7:3, 5:5, 3:7 and 1:9, were alike.

The best synergistic interactions against *S. aureus* strains considered for this research were obtained with combination of methanol extracts and tetracycline against *S. aureus* strain J, the interaction was synergistic at six combination ratios giving 66.6 and 33% similarity. However, the combination interaction of the extract and tetracycline against *S. aureus* strain K gave 44.4% synergy and 55.6% similarity.

This study go a long way to show that combination therapy with these commonly used antibiotic (tetracycline) 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.

REFERENCES

Berdy J (1982). Handbook of Antibiotic Compounds. Vol. VIII Part 1: Antibiotics from Higher forms of life: Higher Plants. CRC Press, Boca Raton.

Cheij R (1988). The McDonald Encyclopedia of Medicinal Plants. Mcdonald & Co. Ltd., London. pp. 54-59.

Chinwuba GN, Chiori GO, Ghobashy AA, Okoro VC (1994). Determination of synergy of Antibiotic combination by overlay inoculum susceptibility disc method, Arzneimittel for schung\drug research, 41: 148-150.

Ebirim GC, Ofoefule SI (1997). Investigations into the Folkloric

antimicrobial activities of Landolphia, Owrrience. Plytother. Res. II: 149-151.
Esimone CO, Adikwu MU, Uzuegbu DB, Udeogaranya PO (1999). The effect of ethylenediaminetetraacetic acid on the antimicrobial properties of Benzoic acid and cetrimide J. Pharm. Res. Dev. 4(1): 1-8.
Keele J, DeLallo VC, Lewis RE, Ernest EJ, Klepser ME (2001). Evaluation of time kill methodology. Amphotericin B and flucytosine in combination against *Candida albicans* and *Cryptococcus neoformans* using Diagn. Microbiol. Infect. Dis. 41: 121-126.
Lewis RE, Klepser ME (1999). The changing face of nosocomial candidemia: epidemiology, resistance, and drug therapy. Am. J. Health Syst. Pharm. 56: 525-533.
Odds FC, Rinaldi MG, Sheehan DJ, Warnock DW (2001). Antifungal susceptibility testing: practical aspects and current challenges. Clin. Microbiol. Rev. 14: 643-658.
Prea S, Gonzalez G, Fothergill AW, Sutton DA, Rinaldi MG (2002). *In vitro*, against clinical isolates of *Candida glabrata* with decreased susceptibility to azoles. J. Clin. Microbiol. 40:1831-1833.
Rex JH, Pfaller MA, Walsh TJ, Chaturvedi V, Espinel-Ingroff A, Ghannoum MA, Gosey LL, Odds FC, Rinaldi MG, Sheehan DJ, Warnock DW (2001). Antifungal susceptibility testing practical aspects and current challenges. Clin. Microbiol. Rev. 14: 643-658.