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In vitro evaluation of the combined antibacterial activity of the leaf extracts of *Dissotis theifolia* with some disc antibiotics

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The combined antibacterial effect of the leaf extracts of *Dissotis theifolia* with some disc antibiotics was investigated. Extraction of the dried pulverized leaves of the plant was carried out using maceration method and some portion of the extract further fractionated in a chromatographic column. The antibiogram of the test microorganisms was determined using the agar-diffusion method while the *in vitro* activity of the combination of sub-bacteriostatic concentrations of the extract and column fraction F_2 with some disc antibiotics was evaluated using the overlay inoculum susceptibility disc method. Fractionation of the extract yielded three column fractions (F_1–F_3) with only F_2 showing remarkable antibacterial activity. Results of the antibiograms clearly demonstrated the resistance and susceptibility pattern of the test isolates against the disc antibiotics. Combinations of most of the disc antibiotics with the extract and column fraction F_2 of *D. theifolia* produced antagonistic ($P \leq 0.05$) and indifferent effects ($P \leq 0.05$), respectively.

Antibacterial agents are sometimes used in combination to achieve certain therapeutic effects. Among the methods adopted in evaluating the combined activity of chemotherapeutic agents are the checkerboard method[1], the time-kill curve[2] and the overlay inoculum susceptibility disc method[3]. In this last method, synergism is established when combinations of two antimicrobial agents produce 19 % increment or more over the inhibition zone diameter of either of the single agents. Increments of less than 19 % correspond to additivity while no difference in the inhibition diameters corresponds to indifference[3].

In interactions, the presence of one drug alters the pharmacological effect or modifies the pharmacokinetics of another drug. Cases abound where a drug enhances the effectiveness of an antimicrobial agent. Interactions resulting from the use of two drugs that have opposing pharmacological effects give rise to antagonism. Drugs that have different receptors on microorganisms and induce opposite effects or contribute in an opposite way to a specific response produce antagonistic effects. The interaction between a tetracycline derivative and certain metal ions may form complexes that are poorly absorbed. Many drug interactions also result from the ability of one drug to stimulate the metabolism of another, most often by increasing the activity of the enzymes involved in the metabolism of numerous therapeutic agents. There are also cases where a drug inhibits the metabolism of a second agent, resulting in a prolonged and intensified activity of the first drug. An interaction where a drug displaces another from its protein binding sites occurs when two drugs that are capable of binding to proteins are administered concurrently. Drug interactions may result in synergistic, antagonistic, indifferent or additive effects.

Concurrent use of orthodox and herbal medicines is common in many urban and rural communities in Africa and Asia. It is probable that varying levels of interaction may be occurring in individuals who have this habit of simultaneous use of orthodox medicine with herbal prepara-

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TABLE 1: ANTIBIOGRAM OF ISOLATES OF *P. AERUGINOSA* AND *E. COLI*.

Organisms	Antibiotic disc (Mean Inhibition zone diameter in mm)								
	OFX_{10}	CN_{10}	N_{200}	CRO_{30}	SCT_{25}	CT_{30}	T_{25}	CXM_{30}	C_{25}
P. aeruginosa 1	20	25	0	18	12	16	0	0	0
P. aeruginosa 2	0	26	0	16	0	15	0	0	0
P. aeruginosa (ATCC 10145)	20	22	0	20	0	20	0	0	0
E. coli 1	16	16	10	22	22	20	0	17	0
E. coli 2	14	14	12	18	30	20	24	18	12
E. coli (ATCC 11775)	20	20	21	20	30	20	16	14	20

OFX_{10} stands for 10 μg ofloxacin, CN_{10} denotes 10 μg gentamicin, N_{200} is 200 μg nitrofurantoin, CRO_{30} represents 30 μg ceftriaxone, SXT_{25} is 25 μg cotrimoxazole, CT_{30} denotes 30 μg cefotaxime, T_{25} stands for 25 μg tetracycline, CXM_{30} is 30 μg cefuroxime, C_{25} means 25 μg chloramphenicol and 0 indicates that there is no zone of inhibition.

tions. Preliminary antimicrobial screening revealed the leaf extract and column fraction, F_2 of *D. theifolia* to have broad-spectrum antibacterial activity. The present study aims at evaluating the overall *in vitro* antibacterial effect achieved when some disc antibiotics are used in combination with the extract of *D. theifolia*.

MATERIALS AND METHODS

A total of seven bacterial samples were used in this investigation. These include two clinical isolates of *Escherichia coli* collected from two different diarrhoeal patients at the University of Nigeria Teaching Hospital, Enugu (designated as *E. coli* 1 and *E. coli* 2); two clinical isolates of *Pseudomonas aeruginosa* from urine samples (*P. aeruginosa* 1 and *P. aeruginosa* 2); Typed cultures of *E. coli* (ATCC 11775), *S. aureus* (ATCC 12600) and *P. aeruginosa* (ATCC 10145) collected from Bioresources Development and Conservation Program (BDCP) Centre, Nsukka. Comdisc® an antibiotic multidisc manufactured by Jireh Laboratory (Nigeria) was used in the study. The product contains the following antibiotics, tetracycline (T, 25 μg), chloramphenicol (C, 25 μg), ceftriaxone (CRO, 30 μg), nitrofurantoin (N, 200 μg), gentamicin (CN, 10 μg), cotrimoxazole (SXT, 25 μg) and ofloxacin (OFX, 10 μg).

Collection of plant materials:

The plant was collected in May 1999 from Nsukka in Enugu State, Nigeria. Botanical identification was made in the Department of Botany, University of Nigeria, Nsukka and a voucher specimen has been deposited at the University Herbarium. The leaves were dried in an oven at 45–50° for 24 h. The dried leaves were pulverized and stored at room temperature (30°) until use.

Extraction and fractionation:

Extraction of the dried pulverized leaves of the plant was carried out in accordance with methods used by earlier workers[4,5]. For the column chromatography, 15 g of the extract was used as the starting material. This was mixed thoroughly with adequate quantity of silica gel (particle size 0.063–0.200 mm, i.e. 70–230 mesh, ASTM) to produce free-flowing powder. The powder was placed on top of the dry-packed column (width 4 cm, length 80 cm) and eluted first with acetone: concentrated ammonia (AA, 1:1) until the eluents, collected at 15 ml aliquots became clear. This occurred at the 50th collection. After allowing the AA solvent system to drip-dry overnight, methanol:ethylamine (40:0.7) was used to further elute the constituents. The first part of the eluents from methanol: ethylamine solvent system (51–72 samples) appeared to be the remnant of the part that was being eluted by AA solvent system; after which the methanol: ethylamine solvent system eluents started to be eluted (coloured greenish brown). This was collected in 8 ml aliquots until sample number 200 was collected. This was when the elution was deemed to be complete. The R_f values of the sample were subsequently determined via

TABLE 2: EFFECTS OF COMBINATIONS OF SOME DISC ANTIBIOTICS WITH SUB-BACTERIOSTATIC CONCENTRATION OF AQUEOUS EXTRACT OF *D. THEIFOLIA*

Test organisms	Disc Antibiotic	IZD of disc antibiotic	IZD disc/extract	% variation	Effect
P. aeruginosa 1	N	0	0	0	Indifference
	SXT	15	17	13.3	Additivity
	CXM	0	0	0	Indifference
	T	0	0	0	Indifference
	CN	25	10	-60.0	Antagonism
	C	0	0	0	Indifference
E. coli	N	0	0	0	Indifference
(ATCC 11775)	SXT	30	24	-20.0	Antagonism
	CXM	14	11	-21.4	Antagonism
	T	16	15	-6.3	Antagonism
	CN	20	18	-10.0	Antagonism
	C	20	23	15	Additivity
E. coli 1	N	22	24	18.2	Additivity
	SXT	19	17	-10.5	Antagonism
	CXM	20	17	-15.0	Antagonism
	T	0	0	0	Indifference
	CN	16	12	-25.0	Antagonism
	C	0	0	0	Indifference
E. coli 2	N	16	19	18.8	Additivity
	SXT	17	19	11.8	Additivity
	CXM	18	18	0	Indifference
	T	19	20	5.3	Additivity
	CN	14	12	-14.3	Antagonism
	C	24	29	20.8	Synergism

*Effects were found to be significant using the Student's t-test at 5 % level of significance.

thin layer chromatography, using ultraviolet lamp of long and short wavelength ranges as a detector. Characterization of the spots after the TLC procedure yielded three column fractions F_1, F_2 and F_3 of yield 11.2, 8.4 and 3.3 % respectively. Different concentrations of the plant extract and the column fraction, F_2 were made using the serial dilution technique[6] and using distilled water as the diluent.

Maintenance, activation and standardization of stock microbial cultures:

The stock cultures were maintained on nutrient agar slants at 4°. In order to activate these cultures, subcultures were freshly prepared and incubated at 37° for 18–24 h before use[7]. Standard suspensions of each test microorganism were made by transferring a colony from the sub-

TABLE 3: EFFECTS OF COMBINATIONS OF SUB-BACTERIOSTATIC CONCENTRATION OF COLUMN FRACTION F WITH SOME DISC ANTIBIOTICS

Test microorganisms	Disc Antibiotic	IZD of disc antibiotic	IZD disc/extract	% variation	Effect
E. coli (ATCC 11775)	N	22	28	27.2	Synergism
	CN	23	24	4.4	Additivity
	C	0	0	0	Synergism
	SXT	30	30	0	Indifference
	CXM	14	14	0	Indifference
	T	18	22	22.2	Synergism
P. aeruginosa (ATCC 10145)	N	0	0	0	Indifference
	CN	20	28	40.0	Synergism
	C	0	0	0	Indifference
	SXT	0	0	0	Indifference
	CXM	0	0	0	Indifference
	T	0	0	0	Indifference
P. aeruginosa 1	N	0	0	0	Indifference
	CN	12	16	33.0	Synergism
	C	0	0	0	Indifference
	SXT	0	0	0	Indifference
	CXM	0	0	0	Indifference
	T	0	0	0	Indifference
P. aeruginosa 2	N	20	24	20.0	Synergism
	CN	10	10	0	Indifference
	C	0	0	0	Indifference
	SXT	0	0	0	Indifference
	CXM	0	0	0	Indifference
	T	0	0	0	Indifference

*Effects were found to be significant using the Student's t-test at 5 % level of significance.

culture into 5 ml of sterile distilled water, and adjusting the volume to obtain a cell population of approximately $1x10^6$ CFU/ml. A volume of 0.1 ml of such suspensions was used as inoculum in all the tests.

Determination of the antibiogram against the test microorganisms:

Disc-agar diffusion method was used to determine the antibiogram of the test microorganisms[8-10]. Mueller-Hinton agar plates were individually seeded with 0.1 ml (approximately $1x10^6$ CFU/ml) each of the standardized test microorganism suspension. The plates were allowed to solidify and the disc antibiotics aseptically placed on the surface of the plates. Two replicate tests were performed and the plates were incubated at 37° for 18–24 h. The diameters of zones of inhibition were measured and the mean values calculated.

Evaluation of the combined antimicrobial activity of the plant extract with disc antibiotics:

An overlay inoculum susceptibility disc method as described by earlier workers[3,11] was adapted to determine the

in vitro activity of the combination of sub-bacteriostatic concentrations of the aqueous extract and column fraction, F_2 of *D. theifolia* with some disc antibiotics. The extract and column fraction, F_2 were separately used to prepare seeded Mueller-Hinton agar plates. A control experiment was set up with seeded Mueller-Hinton agar plates without the extract or its column fraction. Disc antibiotics were aseptically placed on the solidified agar plates. All the plates were incubated at 37° for 24 h before taking measurements of the inhibition zone diameters. The combined antimicrobial activity was determined by comparing the percentage changes in the inhibition zone diameters of the test experiment with the control experiment.

RESULTS

Fractionation of the crude extract of *D. theifolia* yielded three column fractions (F_1–F_3). From preliminary antimicrobial screening, only F_2 showed remarkable antibacterial activity, hence, further experiments were conducted using the aqueous extract of the plant and the column fraction F_2. F_2 was completely soluble in water (pH 6.8) and found to have activity against *E. coli, B. subtilis* and *P. aeruginosa* (all isolates) while the aqueous extract was found to have activity against *P. aeruginosa* (all isolates), *E. coli* (all isolates), *B. subtilis* and *S. aureus*. The antibiograms of the different isolates of *P. aeruginosa* and *E. coli* are presented in Table 1 and they demonstrate the resistance and susceptibility pattern of the test microorganisms against the disc antibiotics. Inhibition zone diameters which are equal to or less than 14 mm signify resistance by the microorganisms while inhibition zone diameters equal to or more than 19 mm signify susceptibility[12]. The combined effects of the plant extract and the column fraction, F_2 with some disc antibiotics are clearly shown in Tables 2 and 3. The results from this combination study using the overlay inoculum susceptibility disc method show that combinations of the aqueous extract of *D. theifolia* with most of the disc antibiotics produced antagonistic effect (Table 2) while combinations of F_2 with the disc antibiotics produced mainly indifferent effect (Table 3).

DISCUSSION

From the results of the antibiograms (Table 1), it is evident that some of the commonly used antibiotics such as tetracycline, cefuroxime, nitrofurantoin and chloramphenicol showed no zone of inhibition against *P. aeruginosa*. It is deducible from these results that although tetracycline and chloramphenicol are used in the treatment of infections caused by Gram-negative microorganisms, organisms such as *P. aeruginosa* may sometimes not be susceptible to these agents. This may be attributed to poor penetration of the antibiotics into the cells or the rapid efflux of the antibiotic due to the production of inactivating enzymes by the microorganism, which rendered the antibiotics less active[2]. The potency of the disc antibiotics might also influence the formation of inhibition zones[13]. This means that at the antibiotic load of 25 µg per disc, tetracycline does not inhibit the growth of *P. aeruginosa*. The low concentration of the disc tetracycline or the indiscriminate use of the drug for trivial illnesses, ill-defined prophylactic purposes and in animal feed stuff may contribute to the observed absence of activity[14–17]. All the isolates of *E. coli* showed marked susceptibility to gentamicin, cotrimoxazole, ceftriaxone and cefotaxime while only the Typed *E. coli* (ATCC 11775) was susceptible to nitrofurantoin.

The result of the combined antibacterial activity of the extract of *D. theifolia* with the disc antibiotics shows that there may be some antagonistic interaction between the plant extract and the antibiotic discs (Table 2). This observation may be due to a situation where the extract, which may have affinity to and also an intrinsic activity on particular receptors, competes for these receptors with the antibiotics which may have only an affinity to the receptors but no intrinsic activity on them. The antagonistic effect may also be attributed to the probability that both the extract and the antibiotic disc in combination, act on different receptors and they induce opposite effects or contribute in an opposite way thereby could not produce zone of inhibition against the test microorganisms.

Most of the combinations of fraction F_2 with the disc antibiotics produced mostly indifferent effects (Table 3). It is discernible from this result that the activity of the fractions was unaffected by the presence of the disc antibiotics. Overall, it is being recommended, based on the results from this study that herbal preparations containing crude aqueous extracts of *D. theifolia* should not be taken simultaneously with almost all these commonly used antibiotics. The column fraction F_2, when finally isolated and purified may be used concurrently with these antibiotics to achieve a synergistic or additive affect or to widen the spectrum of antibacterial effect in chemotherapy. It may be necessary for health care providers especially pharmacy practitioners to always establish the type of interaction occurring between herbal extracts and antibiotics in common use before advising patients on the possible combination of such extracts with antibiotics.

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