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Abstract

The effect of combinations of the crude acetone and aqueous extracts of *Helichrysum pedunculatum* leaves and eight antibiotics was investigated by means of checkerboard and time-kill methods. In the checkerboard method, synergy of 45.83% were observed and this is independent of Gram's reaction, with combinations in the aqueous extract yielding largely (18.75%) antagonistic interactions. The time kill assay detected synergy (45.83%) that is also independent of Gram's reaction with a $\geq 3Log_{10}$ potentiation of the bactericidal activity of the test antibiotics. I conclude that the crude leaf extracts of *H. pedunculatum* could be potential source of broad spectrum antibiotics resistance modifying compounds.

Keyword: MIC: Minimum Inhibitory Concentration, MDR: Multi drug resistance, FIC: Fractional Inhibitory Concentration

7.1 Introduction

Bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents (Nascimento, GGF *et al.* 2000). Individual may succumb to multi drug resistance (MDR) infections because all available drugs have failed (Levy 2002). Notable global examples include hospital and community MDR strains of *Mycobacterium tuberculosis, Enterococcus faecium, Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter baumanni* and *Pseudomonas aeruginosa* (Walsh and Amyes 2004).

In developing countries, MDR enteric diseases agents such as *Salmonella enteritidis, Shigella flexneri* and *Vibrio cholerae* threaten and circumvent public health measures (Stuart and Bonnie 2005). *M. tuberculosis,* particularly in some endemic areas, bears resistance to as many as eight drugs, making some individuals with tuberculosis incurable (Bloom and Murray 1992).

The frequency of drug resistance in the community has extended the resistance problem beyond the confines of the hospital. Resistant strains can be traced from the community to the hospital and vice versa, indicating that drug resistance is no longer localized (Stuart and Bonnie 2005).

Plants are of great medical importance to man. The curative potentials of these plants are locked-up and embedded in some chemical components that effects physiological response in man (Edeoga, HO *et al.* 2005). They are effective with minimal or no side effects in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic drugs (Robbers, J *et al.* 1996).

A number of compounds with an *in vitro* activity of reducing the minimum inhibitory concentrations (MICs) of antibiotics against resistant organisms have been isolated from plants.

In this study, I investigated the inhibitory activity of crude aqueous and acetone extracts of *H. pedunculatum* leaves against bacterial pathogens that are implicated in wound infections. Also the effects of combinations of the extracts and some antibiotics on their resistance modifying potencies were evaluated.

7.2 MATERIALS AND METHODS

7.2.1 Plant extracts preparation

H. pedunculatum leaves were collected from the vicinity of the Research Farm of the University of Fort Hare, Alice, Eastern Cape Province of South Africa and the leaves were compared with the voucher specimen at the Griffin's Herbarium, University of Fort Hare. Unwanted materials were removed from the leaves, air-dried, pulverized in a mill (Christy Lab Mill, England) and the powder stored in a sterile air-tight container at 4^0C for further use.

The extracts of the leaves were prepared in accordance to the description of (Basri and Fan 2005). The resultant extracts were filtered through a Whatman No.1 filter paper, the acetone filtrates were concentrated under reduced pressure using a rotary evaporator (Laborota 4000-efficient, Heldolph, Germany), while the aqueous extract was freeze-dried at -50^{o}C under vacuum (Savant Refrigerated Vapor Trap, RVT4104, USA). The crude extracts collected

were allowed to dry at room temperature to a constant weight of 6 g (4%) and 13 g (8.7%) for acetone and aqueous crude extracts respectively.

7.2.2 Preparation of bacterial inocula

Bacterial isolates used in this study included *Bacillus cereus* (ATCC 10702) and *Proteus vulgaris* (ATCC 6830), and environmental strains of *Micrococcus kristinae, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella* sp. which were obtained from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The inocula of the test organisms were prepared using the colony suspension method (EUCAST 2000).

7.2.3 Antibiotics used in this study

The following antibiotics were used in this study: penicillin G sodium (Duchefa), amoxicillin (Duchefa), chloramphenicol (Duchefa), oxytetracycline (Duchefa), ampicillin sodium salt (Calbiochem), tetracycline hydrochloride (Duchefa), erythromycin (Duchefa) and ciprofloxacin (Fluka).

7.2.4 Determination of the minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MIC) of the antibiotics and plant extracts were determined using the standard method of the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2000) with little modifications. The MIC was defined as the

lowest concentration of the antibiotic or extracts that completely inhibited visible growth of the test organism.

7.2.5 Antibiotic-extract combination experiment

7.2.5.1 The time-kill method

The effect of combinations of the crude extracts and antibiotics was evaluated using time-kill assay method (Pankey and Ashcraft, 2005). Controls consisting of nutrient broth incorporated with the extract and the respective antibiotic without the test organism at the test concentrations were included in each experiment. The test and control flasks were inoculated with each test organism to a final inoculum density of approximately 10^5 cfu/ml. Immediately after inoculation, aliquots (100 µl) of the negative control flasks were taken, serially diluted in sterile physiological saline and plated on nutrient agar in order to determine the zero hour counts. The test flasks were incubated at 37^{o}C with shaking at 120 rpm. After 24 h of incubation, samples were taken from control and each test flasks. The samples from the test flask were transferred to a recovery medium containing 3% "Tween-80" to neutralize the effects of the crude extracts and antibiotics carry-overs from the test suspensions. Both samples from the recovery medium and the control flasks were then serially diluted in sterile physiological saline and plated on nutrient agar in duplicates. The plates were incubated at 37^{o}C for 24 h, after which numbers of colonies were enumerated and expressed as $\log_{10}$. This experiment was carried out in duplicate.

The interactions were considered synergistic if there was a decrease of ≥ 2 $\log_{10}$ cfu/ml in colony counts after 24 h by the combination compared to the most active single agent

(Pankey and Ashcraft, 2005). Additivity or indifference was described as a < 2 $\log_{10}$ cfu/ml change in the average viable counts after 24 h for the combination, in comparison with the most active single drug. Antagonism was defined as a ≥ 2 $\log_{10}$ cfu/ml increase in colony counts after 24 h by the combination compared with that by the most active single agent alone (Lee, JY *et al.* 2006).

7.2.5.2 The checkerboard method

The method was done as described by (Mandal, S *et al.* (2004). Plates were inoculated with standardized cultures and for reproducibility done in duplicate by streaking and incubated for 24 h at 37°C after which the MIC values were estimated.

The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic and extract combination permitting no visible growth of the test organisms on the plates (Mandal, S *et al.* 2004).

The FIC value for each agent was calculated using the formula:

FIC (antibiotic) = MIC of antibiotic in combination / MIC of antibiotic alone

FIC (extract) = MIC of extract in combination / MIC of extract alone

The interactions between the antibiotics and the extracts were assessed in terms of the FIC indices calculated using the formula:

FIC Index = ΣFIC = FIC (antibiotic) + FIC (plant extract)

Combinations were classified as synergistic, if the FIC indices were < 1, additive if the FIC indices were = 1, indifferent if the FIC indices were between 1 and 2 and antagonistic if the FIC indices were >2 (Kamatou, GPP *et al.* 2006). Where more than one combination resulted in a change in the MIC value of the extract or antibiotic, the FIC value was expressed as the average of the individual FIC values (Pankey, G *et al.* 2005).

7.3 RESULTS

The MIC values in this study are shown in Table 7.1. The MIC ranged from 0.5 to 35 mg/ml for the extract and from 0.001 to 0.412 mg/ml for the antibiotics. The extracts were active against the test isolates, although at relatively higher concentration when compared with MICs of the antibiotics used.

The acetone extract showed ability to improve the bactericidal effect of the antibiotics on both Gram positive and Gram negative organisms using time-kill method (Table 7.2). The aqueous extract and the antibiotics combinations yielded some antagonistic interaction. Synergy, indifference and antagonism of 45.83%, 35.42% and 18.75% respectively were observed when the extracts and the antibiotics were combined using time-kill methodology (Table 7.2).

Using the checkerboard methodology (Table 7.3), the extracts showed ability to improve the bactericidal effects of the antibiotics on both Gram-negative and Gram-positive bacteria.

About 45.83% of all the interactions were synergistic, while indifference interactions constituted about 29.17% and antagonistic interactions was observed in approximately 25%.

A comparison of the data for the time kill and checkerboard methods (Table 7.4) revealed that the degree of agreements between the two methods ranges from 50% to absolute agreement (100%).

7.4 DISCUSSION

The presence of elevated MIC values of some of the organisms used in this study (Table 7.1) against common first-line antibiotics reflects the common presence of resistance mechanisms universally present in bacteria, and justifies the need to seek strategies to inhibit such mechanisms.

The time kill assay was used to assess the effect of combinations of the extracts of *H. pedunculatum* leaves and antibiotics. This method was based on a comparison of the killing rate of the combination to that of the individual agents. Synergy was detected for combinations involving all the antibiotics. Since synergy was not specific to any class of antibiotics in this experiment, it is likely that the target for this interaction was genetic, hence there is need to establish the molecular basis of this interaction.

The synergy against *Proteus vulgaris* ATCC 6830 and *Salmonella* sp. is noteworthy as these bacteria were resistant to penicillin G, tetracycline, chloramphenicol, amoxycillin, oxytetracycline, ciprofloxacin and erythromycin with MIC values much higher than their predicted breakpoints. Although the level of antibiotic potentiation was low as not to lead to a restoration of susceptibility (lowering the MIC values to below the breakpoint values) the results seem promising considering that crude extracts were used. The potentiation is likely to have been much more pronounced if pure compounds were used.

As an alternative method, checkerboard method is based on the increased susceptibility of the test organism to the presence of both antimicrobial agents which is reflected by changes in the MIC values (Odds 2003). In agreement to the time kill assay the checkerboard method, also detected synergy against both Gram positive and Gram negative bacteria (Table 7.3).

The synergy detected in this study was not specific to any group of organisms or class of antibiotics. This suggests that crude extracts of this plant could contain a mixture of compounds that can enhance the activity of different antibiotics. The antimicrobial and resistance modifying potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies such as (Cushnie and Lamb 2005). Some of these compounds have been shown to exert their antibacterial action through membrane perturbations. It has also been shown that some plant derived compounds can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site (i.e. peptidoglycan) in the cell wall (Zhao, WH *et al*. 2001) While the above explanations may account for the synergy between the extracts and beta-lactam antibiotics, it might not apply in the case of the observed synergy with other classes of antibiotics with different targets such as tetracyclines, erythromycin, ciprofloxacin and chloramphenicol. Bacterial efflux pumps are responsible for a significant level of resistance to antibiotics in pathogenic bacteria (Kumar and Schweizer 2005). Some plant derived compounds have been observed to enhance the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria (Tegos, G *et al.* 2002). It is likely that the crude leaf extracts of *H. pedunculatum* could be containing potential efflux pump inhibitors. Such compounds are likely to be broad spectrum efflux inhibitors considering that the synergistic effect of the extract was observed on both Gram positive and Gram negative organisms as well as in combination with, cell wall inhibiting and protein synthesis inhibiting antibiotics. Smith, ECJ *et al.* 2007 reported one

efflux inhibitor (ferruginol) from the cones of *Chamaecyparis lawsoniana* that inhibited the activity of the quinolone resistance pump (NorA), the tetracycline resistance pump, (TetK) and the erythromycin resistance pump, (MsrA) in *Staphylococcus aureus*.

Although a number of methods are available for evaluating the antimicrobial effect of antibiotics when they are used in combination, the time-kill assay and the agar dilution checkerboard are preferred methods especially in combinations involving crude plant extracts as they provides detailed information on the bactericidal activity of the antibiotic combination (Dawis, MA *et al.* 2005), correlates well with cure in animal models (Chadwick, EG *et al.* 1986) and are better able to predict the outcome of antibiotic treatment (Johnson 1996).

The detection of synergy in this experiment demonstrates the ability of this plant as a potential source of antibiotic resistance modifying compounds. Meyer and Dilika (1996) as well as Dilika, F *et al.* (2000) have reported the antibacterial activity and synergistic potentials of linoleic and oleic acids isolated from *H. pedunculatum*, therefore, the synergy observed in this present study could be linked with such compounds isolated from the plant. Hence, bioassay guided fractionation of this extracts should be done, in a bid to isolate and identify more compound(s) responsible for the synergism. Finally, an elucidation of the mechanisms of action of the compounds must be followed by toxicity and *in vivo* test to determine the therapeutic applicability of such compounds in combination therapy.

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Table 7.1 The minimum inhibitory concentrations (MIC) of the extracts and the antibiotics

	MIC (mg/ml)								
Isolate	EXT	TET	PEN	ERY	AMX	CIP	CHL	OXT	AMP
Bacillus cereus ATCC 10702	5.0♪	0.001	0.001	0.002	0.004	0.001	0.002	0.001	0.004
Proteus vulgaris ATCC 6830	30.0Ω	0.008	0.001	0.412	0.002	0.001	0.008	0.016	0.004
Pseudomonas aeruginosa §	20.0 Ω	0.016	0.412	0.412	0.412	0.001	0.064	0.016	0.004
Micrococcus kristinae§	0.5♪	0.004	0.001	0.032	0.001	0.002	0.001	0.004	0.001
Salmonella sp.§	25.0 Ω	0.032	0.008	0.412	0.002	0.001	0.064	0.016	0.002
Staphylococcus aureus§	35.0 Ω	0.001	0.001	0.001	0.001	0.001	0.008	0.001	0.001

Ω = aqueous extract; ♪ = acetone extract;

§ = environmental strains; EXT = extract;

TET = tetracycline; PEN = penicillin G;

ERY = erythromycin; AMX = amoxicillin;

CIP = ciprofloxacin; CHL = chloramphenicol;

OXT = oxytetracycline; AMP = ampicillin

Table 7.2 *In vitro* activity of extract-antibiotic combinations by Time-Kill method

Isolate	EXT+ TET	EXT+ PEN	EXT+ ERY	EXT+ AMX	EXT+ CIP	EXT+ CHL	EXT + OXT	EXT+ AMP
Bacillus cereus ATCC 10702	-3(S)♪	0.6(I)♪	-3.0(S)♪	0.5(I)♪	-2.3(S)♪	3.0(A)♪	-3.0(S)♪	1.1(A)♪
Proteus vulgaris ATCC 6830	-6(S)Ω	-3.9(S)Ω	-6.0(S)Ω	-3.7(S)Ω	-0.3(I)Ω	-2.7(S)Ω	-3.9(S)Ω	-4.0(S)Ω
Pseudomonas aeruginosa §	-2(S)Ω	0(I)Ω	-2.9(S)Ω	0(I)Ω	0(I)Ω	0(I)Ω	-1.9(I)Ω	0(I)Ω
Micrococcus kristinae§	-3(S)♪	-3.5(S)♪	0.5(I)♪	-2.5(S)♪	1.0(A)♪	1.7(A)♪	-3.2(S)♪	-3.0(S)♪
Salmonella sp.§	-1.3(I)Ω	-2.3(S)Ω	-5.3(S)Ω	0.5(I)Ω	-2.6(S)Ω	-4.1(S)Ω	-1.0(I)Ω	-0.2(I)Ω
Staphylococcus aureus§	8(A)Ω	3.3(A)Ω	-0.3(I)Ω	6.2(A)Ω	0.9(I)Ω	-0.4(I)Ω	9.3(A)Ω	6.3(A)Ω

§ = environmental strain; A = antagonism; S = synergism;

I = indifference; EXT = extract; TET = tetracycline;

PEN = penicillin G; ERY = erythromycin; AMX = amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol;

OXT = oxytetracycline; AMP = ampicillin; Ω = aqueous extract;

♪ = acetone extract

Table 7.3 *In vitro* activity of extract-antibiotic combinations by Checkerboard method

Isolate	EXT + TET	EXT+ PEN	EXT+ ERY	EXT+ AMX	EXT+ CIP	EXT+ CHL	EXT+ OXT	EXT+ AMP
Bacillus cereus ATCC 10702	0.3(S)♪	1.5(I)♪	0.31(S)♪	0.5(I)♪	0.44(S)♪	2.5(A)♪	0.3(S)♪	3.1(A)♪
Proteus vulgaris ATCC 6830	0.3(S)Ω	0.6(S)Ω	0.8(S)Ω	6.1(A)Ω	5.3(A)Ω	0.6(S)Ω	3.5(A)Ω	0.4(S)Ω
Pseudomonas aeruginosa §	0.2(S)Ω	1.8(I)Ω	0.5(S)Ω	0.2(S)Ω	1.5(I)Ω	2(I)Ω	1.9(I)Ω	3(A)Ω
Micrococcus kristinae§	0.5(S)♪	0.5(S)♪	0.8(S)♪	0.6(S)♪	1.6(I)♪	2(I)♪	0.3(S)♪	0.1(S)♪
Salmonella sp.§	0.6(A)Ω	0.3(S)Ω	0.5(S)Ω	I.5(I)Ω	0.6(S)Ω	0.3(S)Ω	1.6(I)Ω	1.2(I)Ω
Staphylococcus aureus§	2.5(A)Ω	2.3(A)Ω	1.6(I)Ω	5.2(A)Ω	0.9(I)Ω	1.7(I)Ω	3.3(A)Ω	2.3(A)Ω

§ = environmental strain; A = antagonism; S = synergism;

I = indifference; EXT = extract; TET = tetracycline;

PEN = penicillin G; ERY = erythromycin; AMX = amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol;

OXT = oxytetracycline; AMP = ampicillin; Ω = aqueous extract;

♪ = acetone extract

Table 7.4 Comparison of results by time kill and checkerboard methods

Activity	No of test strains out of total of six															
	EXT+ TET		EXT+ PEN		EXT+ ERY		EXT+ AMX		EXT+ CIP		EXT+ CHL		EXT+ OXT		EXT+ AMP	
	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB
Synergy	4	4	3	3	4	5	2	2	2	2	2	2	3	2	2	2
Indifference	1	0	2	2	1	1	3	2	3	3	2	3	2	2	2	1
Antagonism	1	2	1	1	1	0	1	2	1	1	2	1	1	2	2	3

EXT = extract; TET = tetracycline;

PEN = penicillin G; ERY = erythromycin;

AMX = amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol;

OXT = oxytetracycline; AMP = ampicillin

TK = time kill; CB = checkerboard