Folia Microbiol. **55** (2), 176–180 (2010) **http://www.biomed.cas.cz/mbu/folia/**

Interactions of Antibiotics and Extracts of *Helichrysum pedunculatum* against Bacteria Implicated in Wound Infections

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Received 9 March 2009
Revised version 13 October 2009

ABSTRACT. The effect of combinations of the crude acetone and aqueous extracts of *Helichrysum pedunculatum* leaves and eight antibiotics was determined by means of checkerboard and time-kill methods. In the checkerboard method, synergy of 45.8 % was observed, being independent of Gram reaction, with combinations in the aqueous extract yielding largely (18.8 %) antagonistic interactions. The time-kill assay detected synergy (45.8 %) that was also independent of Gram reaction with a potentiation of more than 3 orders of the bactericidal activity of the test antibiotics. The crude leaf extracts of *H. pedunculatum* could thus be considered to be potential source of a broad-spectrum antibiotic-resistance-modifying compounds.

Abbreviations

FIC fractional inhibitory concentration MDR multi drug resistance MIC minimum inhibitory concentration

Bacteria have the genetic ability to transmit and acquire resistance to drugs used as therapeutic agents (Nascimento *et al.* 2000). An individual may succumb to 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 baumannii* and *Pseudomonas aeruginosa* (Walsh and Amyes 2004).

In developing countries, MDR enteric disease 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 plants are locked-up and due to some chemical components that affect physiological response in man (Edeoga *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 *et al.* 1996).

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

Here we 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.

MATERIALS AND METHODS

Plant extract 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, which were air-dried, pulverized in a mill (Christy *Lab Mill*, England) and the powder stored in a sterile air-tight container at 4 °C for further use.

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The extracts of the leaves were prepared according to Basri and Fan (2005) and filtered through a *Whatman* no.1 filter paper. The acetone filtrates were concentrated under reduced pressure (rotary evaporator *Laborota* 4000-efficient; Germany), while the aqueous extract was freeze-dried at –50 °C under vacuum (*Savant* Refrigerated Vapor Trap RVT4104, USA). The collected crude extracts were allowed to dry at room temperature to a constant mass of 6 g (4 %) and 13 g (8.7 %) for acetone and aqueous crude extracts, respectively.

Preparation of bacterial inocula. Bacteria used included *Bacillus cereus* ATCC 10702 and *Proteus vulgaris* ATCC 6830, and environmental strains of *Micrococcus kristinae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella* spp. 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).

Antibiotics used were: penicillin G sodium salt, amoxicillin, chloramphenicol, oxytetracycline, tetracycline hydrochloride, erythromycin (all *Duchefa*), ampicillin sodium salt (*Calbiochem*) and ciprofloxacin (*Fluka*).

The *minimum inhibitory concentrations* (MICs) 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.

Antibiotic-extract combination experiments. 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 supplied with the extract and the respective antibiotic without the test organism were included in each experiment. The test and control flasks were inoculated with each test organism to a final inoculum concentration of $\approx 10^5$ CFU/mL. Immediately after inoculation, aliquots (100 μL) of the negative control flasks were taken, serially diluted in sterile saline and plated on nutrient agar in order to determine the starting counts. The test flasks were incubated with shaking (120 rpm) at 37 °C. After a 24-h incubation, samples were taken from control and each test flask. 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 saline and plated on nutrient agar in duplicates. The plates were incubated for 24 h at 37 °C, after which numbers of colonies were enumerated and expressed as $\log_{10}$. The 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 *et al.* 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 of the most active single agent alone (Lee *et al.* 2006).

The checkerboard method (Mandal *et al.* (2004). Plates were inoculated with standardized cultures in duplicate for the sake of reproducibility done 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.

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:

$$\text{FIC index} = \Sigma_{FIC} = \text{FIC (antibiotic)} + \text{FIC (extract)}$$

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 *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 *et al.* 2005).

RESULTS

MICs ranged from 500 to 35 000 mg/L for the extract and from 1 to 412 mg/L for the antibiotics (Table I). The extracts were active against the test isolates, although at relatively higher concentrations when compared with MICs of the antibiotics used.

Table I. The minimum inhibitory concentrations (MIC, mg/L) of the extracts (Ext) and the antibiotics

Isolate	Ext	TET	PEN	ERY	AMX	CIP	CHL	OXT	AMP
Bacillus cereus ATCC 10702	5000[a]	1	1	2	4	1	2	1	4
Proteus vulgaris ATCC 6830	30 000[b]	8	1	412	2	1	8	16	4
Pseudomonas aeruginosa[c]	20 000[b]	16	412	412	412	1	64	16	4
Micrococcus kristinae[c]	500[a]	4	1	32	1	2	1	4	1
Salmonella sp.[c]	25 000[b]	32	8	412	2	1	64	16	2
Staphylococcus aureus[c]	35 000[b]	1	1	1	1	1	8	1	1

[a]Acetone extract. [b]Aqueous extract. [c]Environmental strains.

AMP	ampicillin	CHL	chloramphenicol	ERY	erythromycin	PEN	penicillin G
AMX	amoxicillin	CIP	ciprofloxacin	OXT	oxytetracycline	TET	tetracycline

The acetone extract showed ability to improve the bactericidal effect of the antibiotics on both Gram-positive and Gram-negative organisms using the time-kill method (Table II). The aqueous extract and the antibiotics combinations yielded some antagonistic interaction. Synergy, indifference and antagonism of 45.8, 35.4 and 18.8 %, respectively, were observed when the extracts and the antibiotics were combined using this methodology.

Table II. *In vitro* activity of extract–antibiotic combinations by time-kill and checkerboard methods*,‡

Isolate	+TET	+PEN	+ERY	+AMX	+CIP	+CHL	+OXT	+AMP
Time-kill method								
Bacillus cereus ATCC 10702	–3(S)[a]	0.6(I)[a]	–3.0(S)[a]	0.5(I)[a]	–2.3(S)[a]	3.0(A)[a]	–3.0(S)[a]	1.1(A)[a]
Proteus vulgaris ATCC 6830	–6(S)[b]	–3.9(S)[b]	–6.0(S)[b]	–3.7(S)[b]	–0.3(I)[b]	–2.7(S)[b]	–3.9(S)[b]	–4.0(S)[b]
Pseudomonas aeruginosa[c]	–2(S)[b]	0(I)[b]	–2.9(S)[b]	0(I)[b]	0(I)[b]	0(I)[b]	–1.9(I)[b]	0(I)[b]
Micrococcus kristinae[c]	–3(S)[a]	–3.5(S)[a]	0.5(I)[a]	–2.5(S)[a]	1.0(A)[a]	1.7(A)[a]	–3.2(S)[a]	–3.0(S)[a]
Salmonella sp.[c]	–1.3(I)[b]	–2.3(S)[b]	–5.3(S)[b]	0.5(I)[b]	–2.6(S)[b]	–4.1(S)[b]	–1.0(I)[b]	–0.2(I)[b]
Staphylococcus aureus[c]	8(A)[b]	3.3(A)[b]	–0.3(I)[b]	6.2(A)[b]	0.9(I)[b]	–0.4(I)[b]	9.3(A)[b]	6.3(A)[b]
Checkerboard method								
Bacillus cereus ATCC 10702	0.3(S)[a]	1.5(I)[a]	0.31(S)[a]	0.5(I)[a]	0.44(S)[a]	2.5(A)[a]	0.3(S)[a]	3.1(A)[a]
Proteus vulgaris ATCC 6830	0.3(S)[b]	0.6(S)[b]	0.8(S)[b]	6.1(A)[b]	5.3(A)[b]	0.6(S)[b]	3.5(A)[b]	0.4(S)[b]
Pseudomonas aeruginosa[c]	0.2(S)[b]	1.8(I)[b]	0.5(S)[b]	0.2(S)[b]	1.5(I)[b]	2(I)[b]	1.9(I)[b]	3(A)[b]
Micrococcus kristinae[c]	0.5(S)[a]	0.5(S)[a]	0.8(S)[a]	0.6(S)[a]	1.6(I)[a]	2(I)[a]	0.3(S)[a]	0.1(S)[a]
Salmonella spp.[c]	0.6(A)[b]	0.3(S)[b]	0.5(S)[b]	1.5(I)[b]	0.6(S)[b]	0.3(S)[b]	1.6(I)[b]	1.2(I)[b]
Staphylococcus aureus[c]	2.5(A)[b]	2.3(A)[b]	1.6(I)[b]	5.2(A)[b]	0.9(I)[b]	1.7(I)[b]	3.3(A)[b]	2.3(A)[b]

*A – antagonism, S – synergism, I – indifference. ‡For further details and abbreviations *see* footnote at Table I.

Using the checkerboard method (Table II), the extracts again showed ability to improve the bactericidal effects of the antibiotics on both Gram-negative and Gram-positive bacteria. About 45.8 % of all the interactions were synergistic while indifference interactions constituted ≈29.2 %; antagonistic interactions were observed in ≈25%.

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

DISCUSSION

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

Table III. Comparison of results obtained by time-kill and checkerboard methods (number of strains out of total of six tested)*,‡

Activity	+TET		+PEN		+ERY		+AMX		+CIP		+CHL		+OXT		+AMP	
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

**First columns* – time-kill method, s*econd columns* – checkerboard method. ‡For abbreviations *see* footnote at Table I.

The time-kill assay (based on a comparison of the killing rate of the combination to that of the individual agents) detected synergy 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 *P. 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 and did not 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 can be supposed to be much more pronounced if pure compounds are used.

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

The synergy detected was again 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, *e.g.*, Cushnie and Lamb (2005). Some of these compounds were shown to exert their antibacterial action through membrane perturbations. It was also 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 *et al*. 2001). While the above explanations may account for the synergy between the extracts and β-lactam antibiotics, it cannot be applied 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 enhance the activity of antimicrobial compounds by inhibiting MDR efflux systems in bacteria (Tegos *et al.* 2002). The crude leaf extracts of *H. pedunculatum* could contain potential efflux-pump inhibitors. Such compounds are probably 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 *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 *S. 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 especially in combinations involving crude plant extracts as they provide detailed information on the bactericidal activity of the antibiotic combination (Dawis *et al.* 2007), correlate well with cure in animal models (Chadwick *et al.* 1986) and are able to better predict the outcome of antibiotic treatment (Johnson 1999).

The detection of synergy in this experiment demonstrates the ability of the plant as a potential source of antibiotic resistance modifying compounds. Meyer and Dilika (1996) and Dilika *et al.* (2000) reported the antibacterial activity and synergistic potentials of linoleic and oleic acids isolated from *H. pedunculatum*,

therefore, the synergy observed in the present study could be linked with such compounds isolated from the plant. Hence, bioassay-guided fractionation of the extracts should be done, in a bid to isolate and identify compound(s) responsible for the synergism and, after elucidating the mechanisms of action, toxicity assays and *in vivo* tests, the prospective use of such compounds can be suggested in combination therapy.

We are grateful to the *National Research Foundation of South Africa* for supporting this research (ref. TTK2006061400023).

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