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Full Length Research Paper

In vitro antibacterial activities of crude extracts of the leaves of *Helichrysum longifolium* in combination with selected antibiotics

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This study has been done to evaluate the interactions between acetone, chloroform, ethyl acetate and methanol extracts of *Helichrysum longifolium* in combination with six first-line antibiotics comprising of Penicillin G sodium, Amoxicillin, Chloramphenicol, Oxytetracycline, Erythromycin and Ciprofloxacin using both the time-kill and the chequerboard methods and against a panel of bacterial isolates comprised of referenced, clinical and environmental strains. The time-kill method revealed the highest bactericidal activity exemplified by a 6.7 Log $_{10}$ reduction in cell density against *Salmonella* spp. when the extract and Penicillin G are combined at ½ × MIC. Synergistic response constituted about 65%, while indifference and antagonism constituted about 28.33% and 6.67% in the time kill assay, respectively. The chequerboard method also revealed that the extracts improved bactericidal effects of the antibiotics. About 61.67% of all the interactions were synergistic, while indifference interactions constituted about 26.67% and antagonistic interactions was observed in approximately 11.66%. These suggest that the crude extracts of the leaves of *H. longifolium* could be potential source of broad spectrum antibiotics resistance modifying compounds.

Key words: Synergism, antibiotics, extracts, chequerboard, time-kill.

INTRODUCTION

Microbial infections represent the word's leading cause of pre-mature death and our well being depends on the pro-duction of new clinically useful antibiotics to curtail and/or eradicate pathogens in our communities (Hugo and Rus-sell, 2003). For over a decade, the pace of development of new antimicrobial agents has slowed down while the prevalence of resistance has grown at an astronomical rate. The rate of emergence of antibiotic resistant bacte-ria is not matched by the rate of development of new antibiotics to combat them (Prescott and Klein, 2002). Today, multiple antibiotic resistance among bacterial pa-thogens is a major public health problem worldwide (Deguchi et al., 1998). It is making a growing number of infections difficult to treat and nosocomial infections more rampant and deadly (Tzouvelekis et al., 1998). Usually, infections resulting from strains that are resistant to main groups of antibiotics like the -lactams and aminoglyco-sides are treatable with vancomycin, chloramphenicol or other antibiotics (Hugo and Russell, 2003). But resistance has been developed to these drugs over the years. In particular infections due to *Staphylococcus aureus* have continued to be a major source of morbidity and mortality in hospitals and these organisms are now exhibiting multi-drug resistance to commonly used antibiotics, hence a significant cause of concern among physicians (Luck et al., 1998; Tzouvelekis et al., 1998). The pre-sence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute signi-ficantly to the intrinsic and acquired resistance in these pathogens (Oluwatuyi et al., 2004). The discovery and development of new compounds that either block or cir-cumvent resistance mechanisms could improve the contain-ment, treatment, and eradication of these strains (Oluwa-tuyi et al., 2004; Sibanda and Okoh, 2008). Com-bination therapy can be used to expand the antimicrobial spectrum, to prevent the emergence of resistant mutants, to minimize toxicity, and to obtain synergistic antimicrobial activity (Pankey et al., 2005).

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There are indications that some herbal materials can act as antibiotic resistant inhibitors (Gibbons et al., 2003; Sibanda and Okoh, 2008; Yam et al., 1998). Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics (Sato et al., 2004). A few studies such as Braga et al. (2005), Dickson et al. (2006) and Gibbons et al. (2003) have reported that plant extracts can enhance the *in vitro* activity of certain antibiotics against strains of MDR *Staphylococcus aureus* and other pathogens.

A number of compounds with an *in vitro* activity of reducing the MICs of antibiotics against resistant organisms have also been isolated from plants. Polyphenols (epicatechin gallate and catechin gallate) have been reported to reverse -lactam resistance in Methicillin Resistant *S. aureus* (MRSA) (Stapleton et al., 2004). Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus* (Marquez et al., 2005; Oluwatuyi et al., 2004; Shibata et al., 2005 and Smith et al., 2007). *Helichrysum longifolium* is a plant that has shown potential as a source of chemotherapeutic compounds (Dilika et al., 1997; Mathekga, 2001) . The leaves are heated over very hot ash before being used to bandage circumcision wounds (Dilika et al., 1997; Mathekga, 2001). Phytochemical studies have revealed that the leaves is rich in flavonoids and other water soluble polyphenolic compounds (Lourens et al., 2008).While the antibacterial potentials of *H. longifolium* extracts have previously been studied, the interactions between the extracts of this plant and antibiotics have not been documented, especially with regards to its potential as a source of resistance modulating compounds. In this paper, we report the effect of combinations between the extracts of *H. longifolium* and some antibiotics on their antibacterial efficacies.

MATERIALS AND METHODS

Plant material

Leaves of *H. longifolium* were collected in December 2007 from a farm at Kidd's Beach Eastern Cape Province of South Africa. The plant materials were compared with the voucher specimen earlier collected from the same spot and deposited at the Griffin's Herbarium of the plant science building of the University of Fort Hare in Alice. The plant materials were later confirmed by the curator of the Herbarium to be *H. longifolium*. The leaves were rinsed with water, air-dried, pulverized in a mill (CHRISTY LABMILL, Christy and Norris Ltd; Process Engineers, Chelmsford, England) and stored in an air-tight container for further use.

Preparation of extract

A 135 g weight of the pulverized leaves of the plant were cold extracted in five different flasks using acetone, chloroform, ethyl acetate, methanol and water respectively, with occasional shaking (Okeke et al., 2001). Each was then filtered (using WHATMANN'S no 1 filter paper) and the filtrates were concentrated to dryness *in vacuo* at 40°C using a rotary evaporator (LABOROTA 4000-EFFICIENT, Heldolph, Germany), while the aqueous extract was freeze-dried (SAVANT REFRIGERATED VAPOR TRAP, RVT4104, USA).

Test bacterial strains

The bacterial isolates used in this study included reference strains obtained from the South African Bureau of Standard (SABS) *Pseudomonas aeruginosa* ATCC 19582, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Proteus vulgaris* ATCC 6830, *Acinetobacter calcaoceticus anitratus* CSIR; clinical isolates obtained from wound sepsis *Staphylococcus aureus* OKOH1; and environmental *strains Shigella flexineri*, *Salmonella* spp., *Micrococcus kristinae*. The inocula of the test organisms were prepared using the colony suspension method (EUCAST, 2000). Colonies picked from 24 h old cultures grown on nutrient agar were used to make suspension of the test organisms in saline solution to give an optical density of approximately 0.1 at 600_{nm}. The suspension was then diluted 1:100 by transfer of 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth to give approximately 1×10^5 cfu/mL bacteria.

Antibiotics used in this study

The following antibiotics were used in this study: Penicillin G sodium, Amoxicillin, Chloramphenicol, Oxytetracycline, Erythromycin all Duchefa product and Ciprofloxacin (Fluka).

Sensitivity testing of the crude plant extract

The sensitivity testing of the crude extracts of the plant was determined using agar-well diffusion method as described by Irobi et al. (1994) with modifications. The bacterial isolates were first grown in nutrient broth for 24 h to prepare bacterial suspension as described above. The bacterial suspension (0.1 mL) was inoculated into molten Mueller-Hinton agar medium at 50°C and then poured into sterile Petri dish, the plate was allowed to set and wells were then bored into the agar medium using a sterile 6 mm cork borer. The wells were later filled up with the extract (0.2 mL) at a concentration of 5 mg/mL taking care to prevent spillage onto the surface of the agar medium. The plates were allowed to stand on the laboratory bench for 1 h to allow proper diffusion of the extract into the medium and then incubated at 37°C for 24 h after which they were observed for zones of inhibition. Tetracycline and ampicilin at concentrations of 1 mg/mL and 10 µg/mL respectively were used as controls.

Determination of the minimum inhibitory concentrations (MICs)

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). Dilutions of the antibiotics, ranging from 0.00-0.824 mg/mL in nutrient agar (Biolab) were prepared by incorporating the antibiotic stock solution into molten agar at 50°C. Dilutions of the extract ranging from 0.05 - 5 mg/mL were also prepared and incorporated into molten nutrient agar (Biolab) at 50°C and poured into sterile plates. The plates were allowed to set and then streaked with standardized inocula of the test bacteria. Plates were incubated at 37°C for 24 h under aerobic conditions. 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 experiment

The time-kill method**:** The effect of combinations of the crude extracts and antibiotics was evaluated using time-kill assay method (Pankey et al., 2005). Controls consisting of nutrient broth

Table 1. Antibacterial activity profile of crude extracts of the leaves of *H. longifolium*.

Bacterial isolates	Zones of inhibition (mm)**						
	Acetone Extract (5 mg/mL)	Aqueous Extract (5 mg/mL)	Chloroform Extract (5 mg/mL)	Ethyl-cetate Extract (5 mg/mL)	Methanol Extract(5 mg/mL)	Tetracycline (1 mg/mL)	Ampicilin (10 µg/mL)
Pseudomonas aeruginosa ATCC 19582	0 ± 0.0	0 ± 0.0	18 ± 2.0	0 ± 0.0	22 ± 0.0	23 ± 1.3	14 ± 0.0
Staphylococcus aureus ATCC 6538	23 ± 0.2	0 ± 0.0	17 ± 0.4	0 ± 0.0	25 ± 0.1	20 ± 1.0	24 ± 0.3
Bacillus cereus ATCC 10702	29 ± 0.5	0 ± 0.0	17 ± 0.7	20 ± 1.2	22 ± 0.1	15 ± 0.9	13 ± 1.0
Bacillus pumilus ATCC 14884	22 ± 0.0	0 ± 0.0	30 ± 0.0	18 ± 1.4	29 ± 0.5	28 ± 0.0	23 ± 1.6
Proteus vulgaris ATCC 6830	0 ± 0.0	0 ± 0.0	22 ± 0.8	0 ± 0.0	22 ± 0.0	20 ± 1.0	24 ± 0.3
Acinetobacter calcaoceticus anitratus CSIR	18 ± 1.6	0 ± 0.0	0 ± 0.0	0 ± 0.0	27 ± 0.9	19 ± 0.4	17 ± 0.4
Staphylococcus aureus OKOH1	20 ± 0.5	0 ± 0.0	0 ± 0.0	0 ± 0.0	20 ± 1.4	28 ± 0.0	23 ± 1.6
Shigella flexineri§	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	22 ± 0.3	30 ± 0.9	28 ± 0.0
Salmonella spp§	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	27 ± 1.4	22 ± 0.6	30 ± 0.9
Micrococcus kristinae§	25 ± 0.2	0 ± 0.0	16 ± 0.4	0 ± 0.0	22 ± 0.5	17 ± 1.2	13 ± 1.6

§ = Environmental strain; = Clinical isolates; ** = Mean of three replicates.

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 standardized organism to a final inoculum density of approximately 10^5 cfu/mL. Immediately after inoculation, aliquots (100 L) 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°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°C for 24 h; numbers of colonies were enumerated and expressed as $\log_{10}$. The chequerboard method: The assay was done as described by (Mandal et al., 2004). Plates were inoculated with standardized cultures by streaking in duplicates and incubated for 24 h at 37°C after which the MICs 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 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 = FIC = FIC (antibiotic) + FIC (plant extract)

DATA ANALYSIS

The means of the two methods (chequerboard and time-kill) were compared using independent t test of significance ($P < 0.05$).

RESULTS

The extraction gave 6, 6.2, 4, 8.2 and 10 g for the acetone, chloroform, ethyl acetate, methanol and aqueous crude extracts, respectively. The results of these experiments revealed that crude extracts of the leaves of *Helichrysum longifolium* exhibited antibacterial activities against the test bacterial isolates comprising of both Gram-negative and Gram-positive bacteria made up of clinical, environmental and standard strains, at a screening concentration of 5 mg/mL (Table 1). Zones of inhibition ranged from 18 - 29 mm for the acetone extract, 16 - 30 mm for chloroform extract, 18 - 20 mm for ethylacetate extract and from 20 - 29 mm for the methanol extract. The aqueous extract exhibited no antibacterial activity against all the test isolates. Chloroform extract gave least activity with an inhibition zone diameter (IZD) of 16 mm against *Micrococcus kristinae*. Interestingly however, the same chloroform extract gave the highest antibacterial activity against *Bacillus pumilus* (ATCC 14884) with zones of inhibition of 30 mm diameter. The antibiotics, tetracycline and ampicilin yielded zones of inhibition of 15 - 30 mm and 13 - 30 mm respectively.

The MICs of the crude extracts and the antibiotics varied between 1 µg/mL and 5.0 mg/mL (Table 2). Specifically, the MICs ranged from 0.5 - 5.0 mg/mL for the acetone extract; 0.1 to 1.0 mg/mL for chloroform extract; 0.5 - 5.0 for methanol extract and it was 5.0 mg/mL for the ethyl acetate extract on the two isolates that were susceptible. For the standard antibiotics, the

Table 2. The minimum inhibitory concentrations (MICs) of the extracts and the antibiotics.

Test isolates	Minimum inhibitory concentrations (mg/mL)**									
	Acetone Extract	Chloroform Extract	Ethyl-cetate Extract	Methanol Extract	PEN G	ERY	AMX	CIP	CHL	OXT
Pseudomonas aeruginosa ATCC 19582	ND	0.1	ND	5.0	0.512	0.256	0.512	0.001	0.128	0.008
Staphylococcus aureus ATCC 6538	1.0	1.0	ND	5.0	0.001	0.008	0.002	0.004	0.002	0.004
Bacillus cereus ATCC 10702	0.5	1.0	5.0	5.0	0.002	0.001	0.004	0.001	0.002	0.001
Bacillus pumilus ATCC 14884	1.0	1.0	5.0	5.0	0.001	0.002	0.001	0.002	0.004	0.002
Proteus vulgaris ATCC 6830	ND	0.5	ND	0.5	0.001	0.512	0.002	0.001	0.008	0.016
Acinetobacter calcaoceticus anitratus CSIR	5.0	ND	ND	1.0	0.512	0.032	0.256	0.002	0.064	0.256
Staphylococcus aureus OKOH1	5.0	ND	ND	1.0	0.001	0.001	0.001	0.001	0.004	0.001
Shigella flexineri§	ND	ND	ND	5.0	0.008	0.064	0.002	0.001	0.004	0.001
Salmonella spp§	ND	ND	ND	5.0	0.004	0.128	0.001	0.001	0.004	0.002
Micrococcus kristinae§	5.0	1.0	ND	0.5	0.001	0.032	0.001	0.002	0.001	0.001

§ = Environmental strain; = Clinical isolates; ** = Mean of three replicates; ND= Not determined; PEN G= Penicillin G sodium; ERY= Erythromycin; AMX= Amoxycillin; CIP= Ciprofloxacin; CHL= Chloramphenicol; OXT= Oxytetracycline.

ranges were 0.001 - 0.512 mg/mL for penicillin G, erythromycin and amoxycillin; 0.001 to 0.004 mg/mL for ciprofloxacin; 0.001 - 0.128 mg/mL for chloramphenicol; 0.001 - 0.256 mg/mL for oxytetracycline. The lowest MIC value for the extracts was exhibited by the chloroform extract against *Pseudomonas aeruginosa* ATCC 19582 (0.1 mg/mL) (Table 2).

The time-kill data on the effects and interactions of the extracts and the antibiotics singly and in combinations are shown in Table 3. 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 extracts showed ability to improve the bactericidal effect of the antibiotics on both Gram positive and Gram negative organisms. The highest bactericidal activity with a 6.7 Log_{10} reduction in cell density was produced by the combination of plant extract and Penicillin G against *Salmonella* spp. (environmental strain).

Synergy rate of 90% (Extract + Amoxycillin), 80% (Extract + Penicillin G; Extract+Chloramphenicol), 50% (Extract + Ciprofloxacin), 60% (Extract + Erythromycin) and 30% (Extract + Oxytetracycline) were observed on all the test isolates. Overall, synergistic response constituted about 65%, while indifference and antagonism constituted about 28.33% and 6.67% respectively of all manner of combinations of extract and antibiotics against all test organisms using the time kill method.

Table 4 shows the interactions of the extract-antibiotic combinations using the chequerboard method, 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 MICs value of the extract or antibiotic, the FIC value was expressed as the average of the individual FIC values (Pankey, et al., 2005). About 61.67% of all the interactions were synergistic, while indifference interactions

Table 3. *In vitro* antibacterial activity of extracts-antibiotic combinations by Time-Kill method.

Isolates	EXT + PEN G	EXT + ERY	EXT + AMX	EXT + CIP	EX + CHL	EXT + OXT
Pseudomonas aeruginosa ATCC 19582	-3.19(S)[2]	-3(S)[2]	-3.19(S)[2]	-3.19(S)[2]	-3.19(S)[2]	-0.91(I)[2]
Staphylococcus aureus ATCC 6538	-3.7(S)[1]	0(I)[1]	-3.5(S)[1]	0.26(I)[1]	2.85(A)[1]	0.58(I)[1]
Bacillus cereus ATCC 10702	-3(S)[3]	-3.2(S)[3]	-3(S)[3]	-3.87(S)[3]	-4(S)[3]	-3.92(S)[3]
Bacillus pumilus ATCC 14884	-6(S)[3]	-5.32(S)[3]	-5(S)[3]	-3(S)[3]	-3(S)[3]	0(I)[3]
Proteus vulgaris ATCC 6830	-3(S)[2]	-5.84(S)[2]	-3(S)[2]	-4.18(S)[2]	-5.12(S)[2]	-1.9(I)[2]
Acinetobacter calcaoceticus anitratus CSIR	0(I)[4]	-4(S)[4]	-3.21(S)[4]	0(I)[4]	-3(S)[4]	0(I)[4]
Staphylococcus aureus OKOH1	0(I)[1]	-2.3(I)[1]	2.95(A)[1]	-1.4(I)[1]	-3.9(S)[1]	-4.6(S)[1]
Shigella flexineri§	-3(S)[2]	2.41(A)[2]	-3(S)[2]	-3.87(S)[2]	-3.47(S)[2]	-3.51(S)[2]
Salmonella spp§	-6.7(S)[2]	0(I)[2]	-5.23(S)[2]	0(I)[2]	1.85(I)[2]	0(I)[2]
Micrococcus kristinae§	-3.3(S)[4]	-4(S)[4]	-3(S)[4]	-0.66(I)[4]	-4(S)[4]	-0.82(I)[4]

1= Acetone extract; 2= Methanol extract; 3= Ethyl acetate extract; 4= Chloroform extract; § = Environmental strain; = Clinical isolates; PEN G= Penicillin G sodium; ERY= Erythromycin; AMX= Amoxycillin; CIP= Ciprofloxacin; CHL= Chloramphenicol; OXT= Oxytetracycline;A= Antagonism; S= Synergism; I= Indifference.

constituted about 26.67% and antagonistic interactions was observed in approximately 11.66%. A comparison of the data for the time kill and chequerboard methods (Table 5) revealed that the degree of agreements between the two methods ranges from 50% to absolute agreement (100%).

DISCUSSION

From this study, *H. longifolium* extracts inhibited the growth of the pathogens tested and these include *P. aeruginosa* known to cause burn wound infections and urinary tract infections, as well as *S. aureus* the causative agent of most skin infections and septicaemia. This findings support the use of *H. longifolium* in the treatment of diseases caused by these pathogens among other pathogens that are susceptible to the antibacterial activity of this plant. The extracts showed broad spectrum activity against both Gram-positive and Gram -negative bacterial strains. The aqueous extract used in our investigations did not show activity against any of the test organisms suggesting that the active constituents of the *H. longifolium* leaves are not water soluble. The time kill assay was used to assess the effect of combinations of the extracts of *H. longifolium* leaves and antibiotics. 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, *Pseudomonas aeruginosa* ATCC 19582 and *Acinetobacter calcaoceticus anitratus* CSIR is noteworthy as these bacteria were resistant to penicillin G, 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. In order to confirm a result of synergy by the time-kill method, chequerboard kinetic studies were as well performed with the crude extracts in combinations against the test bacteria. The method also revealed the ability of the extract to improve the bactericidal effects of the antibiotics on both Gram negative and Gram positive bacteria. The results corroborate the time-kill data; however there are some decree of negligible discrepancies, which indicated overall agreement of between 50 and 100%.

Antimicrobial combinations are used frequently in the clinic to provide broad-spectrum coverage until the causative pathogens are isolated and identified (Rybak and McGrath, 1996). In the clinical setting, combination therapy is most often given empirically without the use of *in vitro* synergy data, as there is a lack of clinical data to correlate the results with patient outcome (Rybak and McGrath, 1996). Although a number of methods are available for

Table 4. *In vitro* antibacterial activity of extracts-antibiotic combinations by Chequerboard method.

Isolates	EXT + PEN G	EXT + ERY	EXT + AMX	EXT + CIP	EXT + CHL	EXT + OXT
Pseudomonas aeruginosa ATCC 19582	-3(S)[2]	-3(S)[2]	-1.19(I)[2]	-3(S)[2]	-3.19(S)[2]	3.1(A)[2]
Staphylococcus aureus ATCC 6538	-3.3(S)[1]	0(I)[1]	-4.5(S)[1]	3.26(A)[1]	3(A)[1]	0(I)[1]
Bacillus cereus ATCC 10702	-3(S)[3]	-3(S)[3]	-3(S)[3]	-3(S)[3]	-3(S)[3]	-3(S)[3]
Bacillus pumilus ATCC 14884	-3(S)[3]	-3.2(S)[3]	-4.1(S)[3]	-3.3(S)[3]	0(I)[3]	3.3(A)[3]
Proteus vulgaris ATCC 6830	-3(S)[2]	-5(S)[2]	-3(S)[2]	-3.41(S)[2]	-3(S)[2]	0(I)[2]
Acinetobacter calcaoceticus anitratus CSIR	0(I)[4]	-6(S)[4]	-3(S)[4]	0(I)[4]	-3(S)[4]	0(I)[4]
Staphylococcus aureus OKOH1	0(I)[1]	-0.91(I)[1]	4(A)[1]	-0(I)[1]	-3(S)[1]	-6(S)[1]
Shigella flexineri§	-3(S)[2]	4(A)[2]	-3(S)[2]	-3(S)[2]	-3(S)[2]	-3(S)[2]
Salmonella spp§	-7(S)[2]	0(I)[2]	-3(S)[2]	0.96(I)[2]	3(A)[2]	0(I)[2]
Micrococcus kristinae§	-5.3(S)[4]	-3(S)[4]	-3(S)[4]	-0.71(I)[4]	-3(S)[4]	0(I)[4]

1= Acetone extract; 2= Methanol extract; 3= Ethyl acetate extract; 4= Chloroform extract; § = Environmental strain; = Clinical isolates; PEN G= Penicillin G sodium; ERY= Erythromycin; AMX= Amoxycillin; CIP= Ciprofloxacin; CHL= Chloramphenicol; OXT= Oxytetracycline;A= Antagonism; S= Synergism; I= Indifference

Table 5. Comparison of results by time kill and chequerboard methods.

Activity	No of test strains out of total of ten											
	EXT + PEN G		EXT + ERY		EXT + AMX		EXT + CIP		EXT + CHL		EXT + OXT	
	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB	TK	CB
Synergy	8	8	6	6	9	8	5	5	8	7	3	3
Antagonism	0	0	1	1	1	1	0	1	1	2	0	2
Indifference	2	2	3	3	0	1	5	4	1	1	7	5
*T-value	-3.19	-3.06	-2.53	-2.01	-2.92	-2.38	-1.99	-1.22	-2.49	-1.52	-1.44	0.04

Legend: EXT = extract; PEN = penicillin G; ERY = erythromycin; AMX = amoxicillin; CIP = ciprofloxacin; CHL = chloramphenicol; OXT = oxytetracycline; TK = time kill; CB = chequerboard; *T- values for data comparisons for Tables 3 & 4 was significant (P<0.05).

evaluating the antimicrobial effect of antibiotics when they are used in combination, the time-kill assay and the agar dilution chequerboard are preferred methods espe-cially in combinations involving crude plant extracts as
they provides detailed information on the bactericidal activity of the antibiotic combination (Darwish et al., 2002), correlates well with cure in animal models (Chadwick et al., 1986) and are better able to predict the outcome of antibiotic treatment (Johnson, 1999).

The use of plants to heal diseases, including infectious ones has been extensively applied by people. Data from the literature as well as our results revealed the great potential of plants for therapeutic treatment, in spite of the fact that they have not been completely investigated. Therefore, more studies need to be conducted to search for new compounds. Once extracted, and before being used in new therapeutic treatments, they should have their toxicity tested *in vivo*. Bioassays (Carvalho et al., 1988; Nascimento et al., 1990) have demonstrated the toxicity of extracts from different plants.

The antimicrobial and resistance modulating potentials of naturally occurring flavonoids and polyphenolic compounds have been reported in other studies such as Cushnie and Lamb, (2005) and Sato et al. (2004) . Some of these compounds like polyphenols have been shown to exert their antibacterial action through membrane perturbations. This perturbation of the cell membrane coupled with the action of -lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone et al., 2006). 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 (that is, peptidoglycan) in the cell wall (Zhao et al., 2001).

Our study revealed the importance of plant extracts when associated with antibiotics to control resistant bacteria, which are becoming a threat to human health. Furthermore, in a few cases, these plant extracts and antibiotics in combinations were active against antibiotic resistant bacteria under very low concentration, thus minimizing the possible toxic effects.

The detection of synergy in this experiment demonstrates the ability of this plant as a potential source of antibiotic resistance modifying compounds. Hence, bioassay guided fractionation of this extracts needs to be done, in a bid to isolate and identify the 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* studies to determine the therapeutic applicability of such compounds in combination therapy, which are subject of ongoing investigation in our group.

Conclusion

Plant extracts have great potential as antimicrobial compounds. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. Our study has shown that crude extracts of the leaves of *H. longifolium* exhibits potentials of synergy in combination with some antibiotics against pathogenic bacte- ria often presenting with problems of drug resistance. This synergistic attributes of crude extracts of *H. longifolium* leaves and antibiotics demonstrates the potential of this plant as a candidate of antibiotic resistance modifying compounds.

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