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Full Length Research Paper

In vitro antibacterial time kill studies of leaves extracts of *Helichrysum longifolium*

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In vitro **antibacterial time kill studies of extracts of *Helichrysum longifolium* was assessed using twenty-three bacteria species made up of eleven Gram positive and twelve Gram negative strains. All test bacteria were susceptible to the methanol extract, while none was susceptible to the aqueous extract. Two of the test bacteria were susceptible to the ethyl acetate extract, while ten and seven were susceptible to the acetone and chloroform extracts respectively at the test concentration of 5 mg/ml. The minimum inhibitory concentrations (MICs) ranged between 0.5 and 5.0 mg/ml, for acetone and methanol extracts; 0.1-5.0 mg/ml for chloroform extract and 5.0 mg/ml for the ethyl acetate extract; while minimum bactericidal concentrations (MBCs) ranged between 1.0 and >5 mg/ml for all the extracts. Average log reductions in viable cell counts for all the extracts ranged between 0.1 Log_{10} and 7.5 Log_{10} cfu/ml after 12 h interaction at 1 × MIC and 2 × MIC. Most of the extracts were rapidly bactericidal at 2 × MIC achieving a complete elimination of most of the test organisms within 12 h exposure time.**

Key words: *Helichrysum longifolium,* crude extract, MIC, time-kill.

INTRODUCTION

Medicinal plants are sources of enormous quantities of chemical substances which are able to initiate different biological activities including those useful in the treatment of human diseases. Scientific investigations of medicinal plants used in folklore remedies have attracted increased attention in the "medical world," especially in a bid to finding solutions to the problems of multiple resistance to the existing synthetic antimicrobials. Most of the synthetic antibiotics now available in market have major setbacks due to the accompanying side effects on patients and the multiple resistances developed by pathogenic microorganisms to them. Hence, there is a justifiable need to explore for new and more potent antimicrobial compounds of natural origin to combat these pathogens.

The genus *Helichrysum* belongs to the Asteraceae family and consists of an estimated 600 species. The name is derived from the Greek words *helisso* (to turn around) and *chrysos* (gold). Common name include "strawflower". It occurs in Africa (with 244 species in South Africa), Madagascar, Australasia and Eurasia. The plants may be annuals, herbaceous perennials or shrubs, growing to a height of 90 cm (http://en.wikipedia.org/wiki/Strawflower).

Helichrysum species are used extensively for stress-related ailments and as dressings for wounds normally encountered in circumcision rites, bruises, cuts and sores (Dilika et al., 1997; Mathekga, 2001; Grierson and Afolayan, 1999; Lourens et al., 2004). The antimicrobial activeties of extracts from *Helichrysum* species have been widely reported (Bougatsos et al., 2004; Eloff, 1999; Grierson and Afolayan, 1999; Mathekga et al., 2000; Aiyegoro et al., 2008). Different compounds like phenolics e.g. flavonoids and chalcones, phthalides, α-pyron derivatives, terpenoids, essential oils, volatiles and fatty acids have been found in the genus (Czinner et al., 2000). However the compounds responsible for these activities have been identified in only a few cases.

Helichrysum longifolium leaves are used by the "Pon-

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dos" to treat circumcision wounds. The leaves are heated over very hot ash before being used as a bandage for the treatment of wounds after circumcision (Dilika et al., 1997; Mathekga 2001). Information on *H. longifolium* is scanty thus suggesting that not much work has been done on the antimicrobial potentials of this specie especially with regards to its *in vitro* time kill characteristics. In addition, while previous researchers have used MICs and MBCs as prediction tools for antimicrobial action of plant crude extracts, there are limitations to the use of such data since it does not consider time-related antimicrobial effects (Kiem and Schentag, 2006), such as killing rate. The bactericidal potencies of the extracts of the plant in terms of the kinetics of bacterial death have not been reported. In this paper, we report the antibacterial and time kill regimes of crude extract of the leaves of *H. longifolium* as part of our exploration for new and novel bioactive compounds from indigenous plants.

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 picked and washed with water to remove all unwanted plant materials and sand, air-dried, pulverized in a mill (CHRISTY LAB MILL, Christy and Norris Ltd; Process Engineers, Chelmsford, England) and stored in an air-tight container for further use.

Preparation of extract

Exactly 135 g of the pulverized leaves of the plant was cold extracted in five different flasks using acetone, chloroform, ethyl acetate, methanol and water with occasional shaking (Okeke et al., 2001). Each extract was then filtered separately (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 using (SAVANT REFRIGERATED VAPOR TRAP, RVT4104, USA). The extraction gave a yield of about 6, 6.2, 4, 8.2 and 10 g for the acetone, chloroform, ethyl acetate, methanol and aqueous crude extracts respectively.

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 7700, *Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 10702, *Bacillus pumilus* ATCC 14884, *Proteus vulgaris* ATCC 6830, *P. vulgaris* CSIR 0030, *Serratia marsecens* ATCC 9986, *Acinetobacter calcaoceticus* UP, *A. calcaoceticus anitratus* CSIR; clinical isolates obtained from wound sepsis (*S. aureus* OKOH1, *S. aureus* OKOH2A, *S. aureus* OKOH2B, *S. aureus* OKOH3); and environmental strains (*Klebsiella pneumoniae, Shigella flexineri, Salmonella* spp*., P. aeruginosa, P. vulgaris, Escherichia coli, S. aureus, Micrococcus luteus, Micrococcus kristinae*). The organisms were sub-cultured in nutrient broth and nutrient agar (BIOLAB) while Mueller Hinton II Agar (BIOLAB) was used for susceptibility, minimum inhibitory concentration (MIC) and time-kill tests.

Antibacterial susceptibility test

Screening of the crude extracts of the plant for antibacterial activity was done in accordance with the method of Afolayan and Meyer (1997). Stock solutions of the extracts were prepared by reconstituting the dried extracts in the extracting solvents and used to prepare dilutions of the extracts in molten Mueller Hinton agar maintained in a water bath at 50°C to attain a concentration of 5 mg/ml and final methanol concentration of 5%. The inoculum size of each test strain was standardized at 5×10^5 cfu/ml using McFarland Nephelometer standard according to the National Committee for Clinical Laboratory Standards (NCCLS, 1993) (now Clinical and Laboratory Standards Institute (CLSI)). The inocula were streaked in radial patterns on the agar plates (Afolayan and Meyer, 1997) and the plates were incubated under aerobic conditions at 37°C for 24 h. Two blank plates containing nutrient agar and 5% methanol without the extract, served as controls. Each test was done in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was determined using the agar dilution method (EUCAST, 2000). The extracts were diluted such that the highest concentration of the solvent in agar was 5% (Predetermined to have no inhibitory effect on the test organism). Plates were inoculated with overnight broth cultures of the test organisms diluted 1:100 with fresh sterile nutrient broth and incubated for 18 h at 37°C. The MIC was taken as the least concentration of extract showing no visible growth of the test organism. The MBC of the fractions were determined as described by (Olorundare et al., 1992) with little modifycations. Samples were taken from plates with no visible growth in the MIC assay and subcultured onto freshly prepared nutrient agar plates and later incubated at 37°C for 48 h. The MBC was taken as the lowest concentration of the extract that did not allow any bacterial growth on the surface of the agar plates.

Time-kill assay

Determination of the rate of kill of the crude extract was done following the procedure described by Okoli and Iroegbu (2005). Inocula were prepared following the described guidelines of EUCAST (2003). The resultant suspension was diluted 1:100 with fresh sterile broth and used to inoculate 50 ml volumes of Mueller Hinton broth incorporated with extract at MIC and 2 × MIC to a final cell density of approximately 5×10^5 cfu/ml. The flasks were incubated at 37°C on an orbital shaker at 120 rpm. A 500 µl sample was removed from cultures at 0, 6 and 12 h, diluted serially and 100 µl of the diluted samples were plated on Mueller Hinton agar plates and incubated at 37°C for 24 h. Controls included extract free Mueller Hinton broth seeded with the test inoculum.

RESULTS AND DISCUSSION

The antibacterial activities of *H. longifolium* leaves extract was investigated against a panel of test bacteria including

Table 1. Determination of the MIC & MBC of the susceptible organisms on *Helichrysum longifolium* in Different Crude Extracts.

Test Organisms	Gram Reaction	MIC/MBC (mg/ml)			
		Methanol Extract	Acetone Extract	Chloroform Extract	Ethyl acetate Extract
Pseudomonas aeruginosa ATCC 19582	-	5.0 / >5	N/A	0.1 / 1.0	N/A
Staphylococcus aureus ATCC 6538	+	5.0 / 5.0	1.0 / 5.0	1.0 / 5.0	N/A
Streptococcus faecalis ATCC 29212	+	5.0 / 5.0	N/A	1.0 / 5.0	N/A
Bacillus cereus ATCC 10702	+	5.0 / >5	0.5 / 5.0	1.0 / 5.0	5.0 / 5.0
Bacillus pumilus ATCC 14884	+	5.0 / >5	1.0 / 5.0	1.0 / 5.0	5.0/ 5.0
Pseudomonas aeruginosa ATCC 7700	-	0.5 / 5.0	N/A	0.5 / 1.0	N/A
Proteus vulgaris CSIR 0030	-	5.0 / >5	N/A	N/A	N/A
Serratia marsecens ATCC 9986	-	5.0 / 5.0	N/A	N/A	N/A
Acinetobacter calcaoceticus UP	-	5.0 / 5.0	N/A	N/A	N/A
Acinetobacter calcaoceticus anitratus CSIR	-	1.0/ 5.0	5.0 / >5	N/A	N/A
Klebsiella pneumoniae♣	-	1.0 / 5.0	N/A	N/A	N/A
Shigella flexineri♣	-	5.0 / 5.0	N/A	N/A	N/A
Salmonella spp.♣	-	5.0 / 5.0	N/A	N/A	N/A
Pseudomonas aeruginosa♣	-	5.0 / 5.0	N/A	N/A	N/A
Proteus vulgaris♣	-	0.5 / 5.0	N/A	N/A	N/A
Escherichia coli♣	-	0.5 / 1.0	N/A	N/A	N/A
Staphylococcus aureus♣	+	1.0 / 5.0	5.0 / 5.0	N/A	N/A
Staphylococcus aureus OKOH 1♪	+	1.0 / 5.0	5.0 / >5	N/A	N/A
Staphylococcus aureus OKOH 2A♪	+	1.0 / 5.0	5.0 / 5.0	N/A	N/A
Staphylococcus aureus OKOH 2B♪	+	1.0 / 5.0	N/A	N/A	N/A
Staphylococcus aureus OKOH 3♪	+	1.0 / 5.0	5.0 / >5	N/A	N/A
Micrococcus kristinae♣	+	0.5 / 5.0	5.0 / 5.0	1.0 / 5.0	N/A
Micrococcus luteus♣	+	1.0 / 1.0	5.0 / 5.0	N/A	N/A

N/A = Not Applicable (because they lack antibacterial activity); ♣= Environmental Strains; ♪= Clinical Strains.

those implicated in wound and wound infections and found to possess bioactivity against the test organisms. Twenty-three bacteria species made up of eleven Gram positive and twelve Gram negative bacteria were screened for susceptibility to the crude extracts (Table 1). Four of the test organisms were clinical isolates; nine were environmental strains and ten were reference strains. All test bacteria were susceptible to the crude methanol extract, while none was susceptible to the aqueous extract at the test concentration (5 mg/ml). Two species (*B. cereus* ATCC 10702 and *B. pumilus* ATCC 14884) were susceptible to the ethyl acetate extract, while ten of the test bacteria were susceptible to the acetone extract of which only one of them is a Gram negative reference strain (*A. calcaoceticus anitratus* CSIR). The remaining nine test bacteria were Gram posi-tive and they include clinical, environmental and refe-rence strains. Seven of the test bacterial strains were susceptible to chloroform extract, out of which six were reference strains (4 Gram positive and 2 Gram negative) and the remaining one, *M. kristinae* a Gram positive environmental strain (Table 1).

Most of the bacteria species used in this study such as *Escherichia coli, S. aureus, K. pneumoniae* all have been implicated in wound infections. Others such as *Shigella* is associated with diarrhoea and food infections; and *K. pneumoniae* is the causative agent of pneumonia (Pelczar et al., 2006). All these pathogens were suscep-tible to the plant extract used in this study, thus supporting the use of *H. longifolium* in folklore remedies in the treatment of diseases caused by these microorganisms. The extract was observed to inhibit the growth of both Gram positive and Gram negative organisms and thus suggest a broad spectrum activity.

The minimum inhibitory concentrations (MICs) of the crude extracts was observed to range between 0.5-5.0 mg/ml, for acetone and methanol extracts; between 0.1-5.0 mg/ml for chloroform extract; and 5.0 mg/ml for the ethyl acetate extract, while the minimum bactericidal concentrations (MBC) ranged between 1.0->5 mg/ml for all the crude extracts (Table 1). Considering that the extract is in the crude form, this observation suggests this plant as a promising source of active pure antimicrobial compounds.

Table 2. Nature of inhibition of crude extracts of *Helichrysum longifolium* leaves against bacterial isolates.

Organism	Acetone Extract				Chloroform Extract				Ethyl acetate Extract				Methanol Extract			
	Log_{10}Kill (MIC)		Log_{10}Kill (2MIC)		Log_{10}Kill (MIC)		Log_{10}Kill (2MIC)		Log_{10}Kill (MIC)		Log_{10}Kill (2MIC)		Log_{10}Kill (MIC)		Log_{10}Kill (2MIC)	
	6 h	12 h	6 h	12h	6 h	12 h	6 h	12h	6 h	12h	6 h	12h	6 h	12 h	6 h	12h
Pseudomonas aeruginosa ATCC 19582	N/A	N/A	N/A	N/A	6.2*	6.2*	6.2*	6.2*	N/A	N/A	N/A	N/A	1.5	1.1	1.3	0.7
Staphylococcus aureus ATCC 6538	3.4*	4*	3.5*	4*	3*	5.9*	3.9*	5.9*	N/A	N/A	N/A	N/A	4.1*	4.1*	4.1*	4.1*
Streptococcus faecalis ATCC 29212	N/A	N/A	N/A	N/A	6*	6*	6*	6*	N/A	N/A	N/A	N/A	2	2	1	2.2
Bacillus cereus ATCC 10702	3.7*	6*	3.7*	6*	2.9	3.5*	3.2*	4*	1.8	2.1	2.5	3.1*	4.1*	4.1*	4.1*	4.1*
Bacillus pumilus ATCC 14884	2.8	3.1*	3.1*	3.6*	2.4	2.9	2.4	3*	2.3	2.9	2.9	3.9*	0.1	4.1*	0.1	4.1*
Pseudomonas aeruginosa ATCC 7700	N/A	N/A	N/A	N/A	6*	6*	6*	6*	N/A	N/A	N/A	N/A	4.1*	4.1*	4.1*	4.1*
Proteus vulgaris CSIR 0030	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4.1*	4.1*	4.1*	4.1*
Serratia marsecens ATCC 9986	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4.1*	4.1*	4.1*	4.1*
Acinetobacter calcaoceticus UP	3.7*	4*	4*	6*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.4	1.4	4.1*	4.1*
Acinetobacter CSIR *calcaoceticus anitratus*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.1	2.2	4.2*	4.2*
Klebsiella pneumoniae♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.5	0.1	4*	4*
Shigella flexineri♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	4.3*	4.3*	4.3*	4.3*
Salmonella spp.♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.5*	7.5*	7.5*	7.5*
Pseudomonas aeruginosa♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5.6*	5.6*	5.6*	5.6*
Proteus vulgaris♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.3	6.3*	6.3*	6.3*
Escherichia coli♣	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7.5*	7.5*	7.5*	7.5*
Staphylococcus aureus♣	6*	6*	6*	6*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3.7*	6*	6*	6*
Staphylococcus aureus OKOH 1♪	4.2*	4.4*	4.2*	6.7*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3.7*	4*	3.7*	4*
Staphylococcus aureus OKOH 2A♪	3.1*	3.1*	3.4*	6*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2	2.8	6*	6*
Staphylococcus aureus OKOH 2B♪	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.4	2.6	4*	6*
Staphylococcus aureus OKOH 3♪	4.5*	6.8*	6.8*	6.8*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3.8*	5.8*	3.8*	5.8*
Micrococcus kristinae♣	3*	0	3.3*	0	6*	6*	6*	6*	N/A	N/A	N/A	N/A	3.1*	7.4*	7.4*	7.4*
Micrococcus luteus♣	3.8*	6.6*	4.6*	6.6*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.7	5*	5*	5*

N/A = Not Applicable (because they lack antibacterial activity); ♣= Environmental Strains; ♪= Clinical Strains; *represents bactericidal effect.

The bactericidal activity of *H. longifolium* crude leaves extracts against the test isolates was determined using a time–kill assay, as this method, unlike an MIC/MBC assay, allows determination of the speed of cidal activity of the extract. A significant decrease in mean viable count of isolates was seen at each time interval. The results of time-kill studies are presented in Table 2. Data are presented in terms of the log_{10} cfu/ml change and are based on the conventional bactericidal activity standard, that is, a $3Log_{10}$ cfu/ml or greater reduction in the viable colony number (Pankey and Sabath, 2004). Average log reduction in viable cell count for the acetone extract ranged between 2.8 Log_{10} and 6.8 Log_{10} cfu/ml after 6 h of interaction, and between 3.1 Log_{10} and 6.8 Log_{10} cfu/ml after 12 h interaction in 1 × MIC and 2 × MIC. For the chloroform extract log reduction in viable cell count vary from 2.4

Log_{10} and 6.2 Log_{10} cfu/ml after 12 h interaction at 1 × MIC and 2 × MIC; while for ethyl acetate and methanol extracts the log reduction in viable cell density ranged between 1.8 Log_{10} and 3.9 Log_{10} and between 0.1 Log_{10} and 7.5 Log_{10} cfu/ml respectively. The greatest reductions in cell densities were achieved for *S. aureus* OKOH 3 (a clinical isolate) with the average reduction in viable cell counts of 6.8 Log_{10} cfu/ml, *P. aeruginosa* ATCC 19582 with the average reduction in viable cell counts of 6.2 Log_{10} cfu/ml, and *B. pumilus* ATCC 14884 and *E. coli* (environmental strain) with the average reduction in viable cell counts of 7.5 Log_{10} cfu/ml for acetone, chloroform, ethyl acetate and methanol extracts respectively.

The crude acetone extract was bactericidal against 10 of the test bacteria (9 Gram positive and 1 Gram negative) at 1 × MIC and 2 × MIC after a 12 h interaction period. At both MIC levels, the extract was bactericidal against the reference strain *B. pumilus* ATCC 14884 after 12 h and bacteriostatic during the first 6 h of interaction at 1 × MIC. Also the chloroform extract was bactericidal against seven of the test bacterial strains (5 Gram positive and 2 Gram negative) at both MIC levels after 12 h of interaction. On the other hand, the ethyl acetate extract exhibited bactericidal effect on 2 test bacteria, (*B. cereus* ATCC 10702 and *B. pumilus* ATCC 14884), after 12 h interaction at 2 × MIC. Its effect was only bacteriostatic at 1 × MIC and 2 × MIC for the first 6 hours, but was able to kill off the two organisms after 12 h exposure time. Lastly, the methanol extract was also bactericidal against 21 of the test bacterial made up of 10 Gram positive and 11 Gram negative including clinical, environmental and reference strains at 1 × MIC and 2 × MIC after a 12 h interaction period. The methanol extract was only bacteriostatic against *P. aeruginosa* ATCC 19582 and *S. faecalis* ATCC 29212. The bactericidal activities of the acetone, chloroform and methanol extracts on the clinical *Staphylococcus* isolates used in this study is worth noting, because these organisms were isolated from septic wound abrasions. Inhibitory levels of crude extracts of *H. longifolium* could therefore be bacteriostatic or bactericidal independent of Gram's staining characteristic. Also the rate of killing of the extracts appears to be both concentration and time dependent. Our study revealed that the extracts were rapidly bactericidal at 2 × MIC achieving a complete elimination of most of the test organisms after 12 h exposure time (Table 2).

The result obtained from this study corroborates our previous studies on *Helichrysum pedunculatum* (Aiyegoro et al., 2008). From the study, the trend of cidal activities of *H. pedunculatum* is also time and concentration dependent. At higher concentration (2×MIC) and longer duration of interaction (12 h), more bacteria were killed. Overall, the *in vitro* data from this study corroborates the reported efficacies of the several different crude extracts of *Helichrysum* species on a wide range of microorganisms and this support the folkloric uses of these plants in treatment of different topical ailments among the traditional people.

Conclusion

In this study, the acetone, chloroform, ethyl acetate and methanol extracts of the test plant exhibited a broad spectrum antibacterial activity at the test concentration of 5 mg/ml and the rate of killing of the extract appear to be time and concentration dependent. The antibacterial activities exhibited by this plant suggest it as a potential candidate in bioprospecting for antimicrobial drugs and the isolation and the identification of the active principles of the plant is currently the subject of on going investigation in our group.

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