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Abstract.

The *in vitro* antibacterial activities and time kill regimes of crude methanol extract of *H. pedunculatum* was assessed using standard microbiological procedures. The experiment was conducted against a panel of bacterial species made up of clinical, environmental and reference strains. The extract was active against eleven of the twenty-one bacteria tested at a concentration of 10 mg/ml. Minimum Inhibitory Concentration (MIC) values for all the susceptible bacteria ranged between 0.1 – 5.0 mg/ml. The average log reduction in viable cell count in time kill assay ranged between 0.17 Log_{10} to 6.37 Log_{10} cfu/ml after 6 hours of interaction, and between 0.14Log_{10} and 6.99 Log_{10} cfu/ml after 12 hours interaction in 1 × MIC and 2 × MIC of the extract. The extract was bactericidal against 8 of the test bacteria at 1 × MIC and against 9 of the test bacteria at 2 × MIC from 12 hour interaction period. At both MIC levels, the extract was bactericidal to all the reference strains after 12 hours and generally bacteriostatic during the first 6 hours of interaction. Also the extract was bactericidal to four of the six environmental strains at both MIC levels after 12 hours of interaction and bacteriostatic during the first 6 hours of interaction. Inhibitory levels of crude methanol extract of *H. pedunculatum* could be bacteriostatic or bactericidal independent of Gram's characteristic.

Key words: *Helichrysum pedunculatum*, methanol extract, MIC, time-kill.

3.1 Introduction

The Genus *Helichrysum,* from the family Asteraceae, is large, comprising of about 500 species with 246 growing in South Africa (Afolayan and Meyer, 1997). Some members of the family are: *H. pedunculatum, H. longifolium, H. stoechas*, *H. apendunculatum, H. kraussii* and *H. nudifolium*. Members of the genus are usually aromatic, perennial shrubs, having dense-woolly leaves with hardy inflorescence parts that are usually brightly coloured (Mathekga, ADM et *al*., 2000).

Extracts of various species have been used to treat topical infections, respiratory ailments and as dressing of circumcision wounds (Stafford, GI *et al*., 2005). The antimicrobial activities of extracts from *Helichrysum* species have been widely reported (Bougatsos, C *et al*., 2004; Eloff, 1999; Grierson and Afolayan, 1999; Mathekga, ADM *et al*., 2000), and they are used in East Africa, for menstrual and abdominal pains (Stafford, GI *et al*., 2005). While the first therapeutic uses of the plant were based on folk medicine, i*n vivo* and *in vitro* studies have also proved its choleric and hepatoprotective properties (Czinner, E *et al*., 2001). In Europe, infusions are prepared from inflorescences of *Helichrysum* because of their bile regulatory and diuretic effects (Cosar and Cubucku, 1990). In South Africa, *Helichrysum* species are used extensively for stress-related ailments and as dressings for wounds normally encountered in circumcision rites, bruises, cuts and sores (Grierson and Afolayan, 1999; Lourens, ACU *et al*., 2004).

H. pedunculatum (Hilliard and Burtt) is a perennial herb with a wide distribution, it is found within the boarder range of Southern Lesotho to the Eastern Cape Province of South Africa

(Meyer and Dilika, 1996). The Xhosas of the Eastern Cape Province of South Africa commonly use the plant to dress wound acquired after circumcision rite. However, there is paucity of information on the nature of bacterial inhibition of the plant. Hence, in this paper, we report the antibacterial activity of the methanol extract of *H. pedunculatum* and the nature of their inhibition by *in vitro* time-kill assay.

3.2. Materials and methods

3.2.1 Plant material

Leaves of *H. pedunculatum* were collected from the vicinity of the Research Farm of the University of Fort Hare, Alice, Eastern Cape Province of South Africa, during September 2007. 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. pedunculatum.* The leaves were picked and washed with water to remove all unwanted plant materials, 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.

3.2.2 Preparation of Extract

Exactly 135 g of the pulverized leaf of the plant was cold extracted using methanol with occasional shaking (Okeke, MI *et al.*, 2001). The mixture was then filtered (using Whatmann's

no 1 filter paper); the filtrate was concentrated to dryness *in vacuo* using a rotary evaporator. This gave a yield of about 12 g of the crude extract.

3.2.3 Test bacterial strains

Bacterial isolates used in this study included reference strains obtained from the South African Bureau of Standard (SABS) (*Acinetobacter calcaoceticus anitratus* CSIR, *Serratia marsecens* ATCC 9986, *Proteus vulgaris* CSIR 0030, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 7700, *Bacillus pumilus* ATCC 14884, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 19582, *Escherichia coli* ATCC 25922); clinical isolates obtained from wound sepsis (*Staphylococcus aureus* OKOH 2B, *Staphylococcus aureus* OKOH 3); and environmental strains, (*Micrococcus luteus, Micrococcus kristinae, Escherichia coli, Enterococcus faecalis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella* sp., *Shigella flexineri, Bacillus subtilis, Klebsiella pneumoniae*). 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.

3.2.4 Antibacterial susceptibility test

Screening of the methanol extract of the plant for antibacterial activity was done in accordance with the method of Afolayan and Meyer (1997). Stock solution of the extract was prepared by reconstituting the dried extract in the extracting solvent. This was used to prepare dilution of the extract in molten Mueller Hinton agar maintained in a water bath at 50^{o}C to attain a

concentration of 10 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). Plates were incubated under aerobic conditions at 37^{o}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.

3.2.5 Determination of minimum inhibitory concentration (MIC)

The MIC was determined using the agar dilution method (EUCAST, 2000). The extract was diluted in 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^{o}C. The MIC was taken as the lowest dilution (least concentration) of extract showing no visible growth of the test organism.

3.2.6 Time-Kill assay

Determination of the kill rate of the crude extract was done following the procedure as 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^{o}C on an orbital shaker at 120 rpm. A 500 µl sample was removed from cultures at 0, 6 and 12h, diluted serially and 100 µl of the diluted samples were plated on Mueller Hinton agar plates and incubated at 37^{o}C for 24 h. Controls included extract-free Mueller Hinton broth seeded with the test inoculum.

3.3 Results and Discussion

Twenty-one bacterial species made up of 7 Gram-positive and 14 Gram-negative bacteria were screened for susceptibility to crude methanol extract of *H. pedunculatum* (Table 3.1). Two of test organisms were clinical isolates; 10 were environmental strains and 9 were reference strains. Eleven of the test bacteria were susceptible at the test concentration (10 mg/ml) (Table 3.1), of which 5 were reference strains and the remaining six were environmental strains. The 2 clinical isolates *Staphylococcus aureus* OKOH 2B and *Staphylococcus aureus* OKOH 3 were not susceptible to the extract.

The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.1-5.0 mg/ml. Specifically, MICs for the reference and environmental strains ranged between 0.5 - 5.0 mg/ml and 0.1 - 5.0 mg/ml respectively (Table 3. 1).

The results of time-kill studies are presented in Table 3.2. 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. Average log reduction in viable

cell count in time kill assay ranged between 0.17 Log_{10} to 6.37 Log_{10} cfu/ml after 6 hours of interaction, and between 0.14 Log_{10} and 6.99 Log_{10} cfu/ml after 12 hours interaction in 1 × MIC and 2 × MIC of the extract. The extract produced a range of $0.41\log_{10} - 1.19\log_{10}$ and $0.54\log_{10} - 6.37\log_{10}$ reduction after 6 hour at concentration equal to 1 × MIC and 2 × MIC respectively and a range of $5.98\log_{10} - 6.99\log_{10}$ reduction after 12 hour at both 1 × MIC and 2 × MIC respectively on the reference strains. On the other hand, the range for environmental strains is between $0.17\log_{10} - 5.44\log_{10}$ and $0.65\log_{10} - 5.44\log_{10}$ reduction after 6 hour at concentration equal to 1 × MIC and 2 × respectively and a range between $0.99\log_{10} - 6.44\log_{10}$ after 12 hours at 1 × MIC and 2 × MIC respectively. Log reduction on *Bacillus subtilis* (environmental strain) was $5.44\log_{10}$ cfu/ml after 6 hour of interaction at 1 × MIC, the log reduction was constant until after 12 hours even at 2 × MIC, thus suggesting that the total population of the bacteria have been wiped off by the sixth hour at both 1 × MIC and 2 × MIC. There is an increase in inoculum size for *Micrococcus kristinae* (environmental strain), the increase in log range from $0.05\log_{10} - 0.44\log_{10}$ after 12 hour at both MIC levels and this indicate resistance to the extract. The greatest reductions achieved within the reference strains are in *Pseudomonas aeruginosa* ATCC 19582 and *Pseudomonas aeruginosa* ATCC 7700, the value is on the average of $6.99\log_{10,}$ after 12 hour at 2 × MIC, while the greatest reduction with the environmental strains is achieved with *Micrococcus luteus* which is $6.44\log_{10}$ reduction after 12 hour at 2 × MIC. Generally the reference strains were more susceptible to the extract than the environmental strains.

The extract was bactericidal against 8 of the test bacteria at 1 × MIC and against 9 of the test bacteria at 2 × MIC from 12 hour interaction period. At both MIC levels, the extract was bactericidal to all the reference strains after 12 hours and generally bacteriostatic during the first

6 hours of interaction. Also the extract was bactericidal to four of the six environmental strains at both MIC levels after 12 hours of interaction and bacteriostatic during the first 6 hours of interaction. The extract exhibited bacteriostatic effect on *Klebsiella pneumoniae* (environmental strain) after 12 hour interaction at both the 1 × MIC and 2 × MIC, but the effect was more pronounced at 2 × MIC. It was not able to inhibit the growth of *Micrococcus kristinae* (environmental strain) at both 1 × MIC and 2 × MIC, but after 12 hour of interaction at 2 × MIC, it only showed about $0.14Log_{10}$ cfu/ml reduction in inoculum size (a weak activity). Inhibitory levels of crude methanol extract of *H. pedunculatum* could therefore be bacteriostatic or bactericidal independent of Gram's staining characteristic of the organisms.

Results obtained from this study suggest the possible reason why *H. pedunculatum* is being used in folkloric medicines for the treatment of various human topical infections. The effectiveness of an antibacterial agent is measured by its ability to inhibit and kill bacteria (Nostro, A *et al.*, 2001). *In vitro* time-kill assays are expressed as the rate of killing by a fixed concentration of an antimicrobial agent and are one of the most reliable methods for determining tolerance (Nostro, A *et al*., 2001). Generally, the effect of the crude methanol extract of *H. pedunculatum* on the test bacteria in this experiment is time and concentration dependent, as it is evident from the data presented. At higher concentration (2 × MIC) and longer duration of interaction (12 hour), more bacteria were killed. The *in vitro* data corroborates well with the reported efficacies of the several different crude extracts of *H. pedunculatum* on a wide range of microorganisms and this support the folkloric uses of this plant in treatment of different topical ailments among the traditional people (Nostro, A *et al*., 2001).

3.4 Conclusion

Crude methanol extract of *H. pedunculatum* has a broad spectrum antibacterial activity against many bacterial species. The nature of bacterial inhibition of extract could either be bacteriostatic or bactericidal depending on the concentration of the extract and the exposure time irrespective of Gram's staining characteristic of the test organism. The resistance of the clinical isolates to the extract is worrisome and this could be one reason why, there is an incidence of high death rate resulting from circumcision wounds infection even after treating such wounds with *H. pedunculatum* leaf. Perhaps the plant could be of more relevance in combination therapy and a source of resistance modifying principles which is the subject of on going studies in our group.

Table 3.1. Antibacterial activities of crude methanol extract of *H. pedunculatum* leaves on bacterial isolates.

Isolate Identity	**Gram's Reaction**	**Antibacterial activity (10 mg/ml)**	**MIC (mg/ml)**
Escherichia coli ATCC 25922	-	-	ND
Pseudomonas aeruginosa ATCC 19582	-	+	0.5
Staphylococcus aureus ATCC 6538	+	+	5.0
Bacillus pumilus ATCC 14884	+	+	5.0
Pseudomonas aeruginosa ATCC 7700	-	+	0.5
Klebsiella pneumoniae ATCC 4352	-	-	ND
Proteus vulgaris CSIR 0030	-	+	5.0
Serratia marsecens ATCC 9986	-	-	ND
Acinetobacter calcaoceticus anitratus CSIR	-	-	ND
Klebsiella pneumoniae[§]	-	+	5.0
Bacillus subtilis[§]	+	+	1.0
Shigella flexneri[§]	-	-	ND
Salmonella sp.[§]	-	-	ND
Pseudomonas aeruginosa[§]	-	+	0.5
Proteus vulgaris[§]	-	+	0.5
Enterococcus faecalis[§]	-	-	ND
Escherichia coli[§]	-	-	ND
Micrococcus kristinae[§]	+	+	0.1
Micrococcus luteus[§]	+	+	0.5
Staphylococcus aureus OKOH 2B[Ω]	+	-	ND
Staphylococcus aureus OKOH 3[Ω]	+	-	ND

Key: [Ω] are clinical isolates; [§]are environmental strains; - represents no antibacterial activity; + represent presence of antibacterial activity; MIC represents minimum inhibitory concentration; ND represents not determined.

Table 3.2**.** Nature of inhibition of crude methanol extracts of *H. pedunculatum* leaves against bacterial isolates.

Susceptible isolates	MIC (mg/ml)	Log_{10} Kill (MIC) 6hr	Log_{10} Kill (MIC) 12hr	Log_{10} Kill (2×MIC) 6hr	Log_{10} Kill (2×MIC) 12hr
Pseudomonas aeruginosa ATCC 19582	0.5	0.47	6.99*	0.63	6.99*
Staphylococcus aureus ATCC 6538	5.0	1.19	6.37*	6.37*	6.37*
Bacillus pumilus ATCC 14884	5.0	0.79	6.73*	1.51	6.73*
Pseudomonas aeruginosa ATCC 7700	0.5	0.41	6.99*	0.54	6.99*
Proteus vulgaris CSIR 0030	5.0	1.02	5.98*	1.58	5.98*
Klebsiella pneumoniae§	5.0	0.17	0.99	0.65	1.47
Bacillus subtilis§	1.0	5.44*	5.44*	5.44*	5.44*
Pseudomonas aeruginosa§	0.5	0.71	6.28*	0.83	6.28*
Proteus vulgaris§	0.5	0.72	6.20*	0.92	6.20*
Micrococcus kristinae§	0.1	+0.44	+0.05	+0.36	0.14
Micrococcus luteus§	0.5	0.91	3.40*	1.07	6.44*

Key: MIC represents minimum inhibitory concentration; §are environmental strains; growth relative to the starting inoculum is indicated by plus (+) sign; * represents bactericidal effect.

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