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Abstract.

Helichrysum pedunculatum is used in folklore remedies to dress wound acquired after circumcision rite. This led to the study of *in vitro* antibacterial activities of this medicinal plant. Using standard microbiological procedures, six bacteria species made up of four Gram positive and two Gram negative bacteria were screened for susceptibility to crude acetone and aqueous extracts of this plant. The minimum inhibitory concentrations (MICs) of the acetone extract against the susceptible bacteria was 5.0 mg/ml while that of the aqueous extract ranged between 0.5 - 35 mg/ml. Average log reduction in viable cell count in time kill assay of the acetone extract ranged between 0.64 Log₁₀ and 5.99 Log₁₀ cfu/ml after 6 hours of interaction, and between 5.99 Log₁₀ and 6.06 Log₁₀ cfu/ml after 12 hours interaction in 1 × MIC and 2 × MIC, and between 0.10 Log₁₀ to 0.33 Log₁₀ cfu/ml after 6 hours of interaction, and 0.23 Log₁₀ and 0.56 Log₁₀ cfu/ml after 12 hours interaction in 1 × MIC and 2 × MIC for the aqueous extract. The effect of the aqueous extract was only bacteriostatic on both reference and environmental strains and the clinical isolates were outrightly resistant to this extract (not reported here), this 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.

Key words: *Helichrysum pedunculatum*, Minimum Inhibitory Concentration (MIC), Log reduction, Time-Kill, Bacteriostatic, Bactericidal, Antibacterial.

4.1 Introduction

The genus *Helichrysum* belongs to the tribe Inuleae in the Asteraceae family and is known for its aromatic and therapeutic properties. It is a large family of about 500 species, with 245 species indigenous to South Africa (Mathekga, ADM *et al.*, 2000). *Helichrysum* species have been used to treat coughs, colds, fever, infections, headache, menstrual pain, and as a wound dressing in areas as far apart as Europe, Egypt, North America, China, Australia and South Africa (Balick, 1990; Rood, 1994; Cosar & Cubucku, 1990; Hutchings, 1996; Bhat & Jacobs, 1995), and are included in many pharmacopoeias. The South African *Helichrysum* species are used extensively for stress-related ailments and as dressings for wounds normally encountered in circumcision rites, bruises, cuts and sores (Grierson & Afolayan, 1999; Lourens, ACU *et al.*, 2004). *In vitro* and *in vivo* studies had proved the cholaretic (Czinner, E *et al.*, 2000) and hepatoprotective (Mathekga, ADM *et al.*, 2000) properties of this genus.

Extracts from different *Helichrysum* species are used topically by the indigenous people of South Africa against infections associated with herpes simplex virus herpes zoster (Mathekga, ADM *et al.*, 2000). Also, Meyer and Afolayan (1995) and Meyer & Dilika (1996) reported antiherpes and antibacterial activities of *H. aureonitens*. *H. pedunculatum* Hilliard and Burt (Asteraceae) is a perennial herb with a wide distribution, it is found within the range of borders of Southern Lesotho to the Eastern Cape Province of South Africa (Meyer & Dilika, 1996). Antibacterial assays of *H. pedunculatum* (a plant used during circumcision rites) showed that dichloromethane extracts are active against all the Gram positive bacteria tested, as well as two Gram negative bacteria, *Enterobacter cloacae* and *Serratia marcescens*. A water extract was effective against *Staphylococcus aureus* and *Micrococcus kristinae* (Meyer & Dilika, 1996). The plant is locally

called *Icziwe* by the Xhosas of the Eastern Cape Province of South Africa, where it is commonly used to dress wound acquired after circumcision rite. The aerial parts of *H. italicum* and *H. stoechas* are employed in the Spanish Mediterranean area in therapy for their antifever, anticold, wound healing and antiinfectious qualities. In addition, *H. italicum* is recognised for its ability to heal skin problems, as well as its diuretic and disinfectant qualities. *H. foetidum* are warmed and applied as a poultice for infected sores, the tea from dried leaves of *H. appendiculatum* and the sap of *H. pedunculatum* are applied to circumcision wounds.

Many cultures, including African, European, Eastern and North American cultures use *Helichrysums* in food and medicine. For Europeans, the *Helichrysum* ranks as one of the most ancient and valuable healing substances - it is said to be more anti-inflammatory than German Chamomile, have more tissue regenerating power than Lavender and more cicatrisant (the formation of scar tissue) than Frankincense [<http://herbalafrica.co.za/HerbsHelichrysum.htm>]. Furthermore, oil of *Helichrysum* has been found by European researchers to generate tissue, reduce tissue pain, helps improve skin conditions, circulatory function, prevents phlebitis, helps regulate cholesterol, stimulates liver cell function, reduces scarring and discoloration. It is anticoagulant, anticatarrhal, expectorant, and antispasmodic (Czinner, E *et al.*, 2000). In Africa, *Helichrysums* are often used for food - the leaves are cooked and eaten. Medicinally the roots, leaves, stems and flowers are used as traditional medicine for chest complaints, colic in children, coughs, colds, internal sores, fever, headaches, and for dressing wounds (Mathekga, ADM *et al.*, 2000; Czinner, E *et al.*, 2000).

Although, several studies have been conducted on this plant, there is still paucity of information on the nature of bacterial inhibition of the crude acetone and aqueous extracts of the plant. In addition to using minimum inhibitory concentrations (MICs) as prediction tools for antibacterial

potencies of these plant extracts, we investigated the time-kill bactericidal patterns of two crude extracts against a panel of bacteria species that are implicated in wound infections. In this paper, we therefore report on the antibacterial activity of the aqueous and acetone extracts of *H. pedunculatum* and the nature of their inhibition using *in vitro* time-kill assay as a predicting tool of their bactericidal efficacy.

4.2 Materials and methods

4.2.1 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 and sand, air-dried (30°C), pulverized in a mill (Christy Lab Mill, Christy and Norris Ltd; Process Engineers, Chelmsford, England) and stored in a sterile air-tight container for further use.

4.2.3 Preparation of Extract

Exactly 135 g of the pulverized leaf of the plant was cold extracted using acetone and water separately with occasional shaking (Stuart Scientific Orbital Shaker, UK) for 48 h (Okeke, MI *et al.*, 2001). The extracts were filtered through Whatman no. 1 filter paper; The acetone extract was evaporated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany), while the water extract was freeze-dried at -50°C under vacuum using Savant Refrigerated Vapor Trap, RVT4104, USA. The acetone filtrate gave a yield of about 6 g (4.44 %) of the crude extract, while, the aqueous filtrate gave a yield of about 13 g (9.63 %) of the crude extract. Extracts were stored at 4°C in air tight sterile containers, until ready for use.

4.2.4 Test bacterial strains

Bacterial isolates used in this study included reference strains obtained from the South African Bureau of Standard (SABS) (*Bacillus cereus* ATCC 10702, *Proteus vulgaris* ATCC 6830) and environmental strains- (*Micrococcus kristinae*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*) from the Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa. The bacteria 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 assay.

4.2.5 Antibacterial susceptibility test

Screening of the crude extracts of the plant for antibacterial activity was done in accordance with the method of Afolayan & Meyer (1997). Stock solution of the extract was prepared by reconstituting the dried extract in the extracting solvent to make 50 mg/ml. This was used to prepare dilutions of the extract in molten Mueller Hinton agar maintained in a water bath at 50°C to attain a concentration of 10 mg/ml and final acetone concentration of 5 % (for the crude acetone extract). 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 & Meyer, 1997). Plates were incubated under aerobic conditions at 37 °C for 24 h. Two blank plates containing nutrient agar and 5 % acetone (this represented the final acetone concentration in the test plates) without the extract, served as positive controls, while another two blank plates containing only nutrient agar, served as negative controls. No visible growth on the test plates compared with the positive control was used to indicate the inhibitory activity of the extracts. Each test was done in triplicate.

4.2.6 Determination of minimum inhibitory concentration (MIC)

The MIC was determined using the agar dilution method (EUCAST, 2000). For the acetone extract, the highest concentration of the solvent in agar was 5 %. At this concentration, acetone had no inhibitory effect on the test organisms. 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 highest dilution (lowest concentration) of extract showing no visible growth of the test organism.

4.2.7 Time-Kill assay

Determination of the kill rate of the crude extract was done following the procedure described by Okoli & 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 $2 \times \text{MIC}$ 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, and transferred to 4.5 ml of Mueller Hinton broth and recovery medium containing 3% "Tween-80" to neutralize the effects of the crude extracts carry-overs from the test suspensions. The suspension was then diluted serially and 100 µl of the diluted samples were plated in triplicate 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.

4.3 Results

Six bacterial species made up of four Gram-positive and two Gram-negative bacteria were screened for susceptibility to crude acetone and aqueous extracts of *H. pedunculatum* (Table 4.1). Four of the test organisms were environmental strains and 2 were reference strains. Two of

the test bacteria were susceptible to the acetone extract at the test concentration (10 mg/ml), while four bacteria were susceptible to the aqueous extract at the test concentration of 35 mg/ml (Table 4.1). The acetone extract was inhibitory only to Gram positive organisms at the test concentration, while the aqueous extract had activity against both Gram-positive and Gram-negative organisms, although at relatively higher test concentration.

The minimum inhibitory concentrations (MICs) of the extract against the susceptible bacteria generally ranged between 0.5 - 35 mg/ml for both extracts. Specifically, MICs for the acetone and aqueous extracts ranged between 0.5 – 5.0 mg/ml and 20 – 35 mg/ml respectively (Table 4.1).

The results of time-kill studies are presented in Table 4.2. Data are presented in terms of the $\log_{10}$ cfu/ml change and are based on the conventional bactericidal activity standard, that is, a $3\log_{10}$ cfu/ml or greater reduction in the viable colony number. For the acetone extract, average log reduction in viable cell count in time kill assay ranged between 0.64 $\log_{10}$ and 6.06 $\log_{10}$ cfu/ml after 12 hours interaction in $1 \times \text{MIC}$ and $2 \times \text{MIC}$. For the aqueous extract, the log reduction ranged between 0.10 $\log_{10}$ and 0.56 $\log_{10}$ cfu/ml after 12 hours interaction in $1 \times \text{MIC}$ and $2 \times \text{MIC}$. The greatest reductions achieved with the acetone extract was on the reference strain *Bacillus cereus* ATCC 10702 with the average value of 6.06 $\log_{10}$ cfu/ml, while the greatest reduction achieved by the aqueous extract was on the environmental strain *Enterococcus faecalis* with the average value of 0.56 $\log_{10}$ cfu/ml.

4.4 Discussion

H. pedunculatum is a plant that represents a promising source of novel chemotherapeutic agents. It has been used comprehensively around the world in traditional medicine practices; therefore, it will be of enormous relevance in solving health-related problems to the poor people of Africa, if its potentials are effectively annexed. The antibacterial activity of the aqueous extract from the leaves of this plant was observed against both Gram-positive and Gram-negative bacteria from this experiment, thus it confirms the broad antibacterial efficacy of this plant and the validation of its traditional use in the treatment of circumcision wounds and wound infections..

The crude acetone extract was bactericidal against two of the test bacteria at $1 \times \text{MIC}$ and $2 \times \text{MIC}$ after a 12 hour interaction period. At both MIC levels, the extract was bactericidal to the reference strain (*Bacillus cereus* ATCC 10702) after 12 hours and bacteriostatic during the first 6 hours of interaction at $1 \times \text{MIC}$. Also the extract was bactericidal to the environmental strain at both MIC levels after 6 hours of interaction. On the other hand, the aqueous extract exhibited bacteriostatic effect on both the environmental and reference strains after 12 hour interaction at both the $1 \times \text{MIC}$ and $2 \times \text{MIC}$, but the effect was more pronounced at $2 \times \text{MIC}$ and after 12 hours of interaction. Inhibitory levels of crude acetone extract of *H. pedunculatum* from this study could therefore be bacteriostatic or bactericidal on Gram positive bacteria, while that of the aqueous extract is bacteriostatic and independent of Gram's staining characteristic.

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). Aqueous extracts showed less activity (35 mg/ml) than acetone extracts (10 mg/ml) possibly because the same active substances present in water extracts, is lower in concentration than that of acetone extract. This might be attributed to the fact that these active substances are more soluble in organic solvents than in water since water is only able to extract hydrophilic compounds as also suggested by de Boer, HJ *et al.* (2005). The antibacterial action of the two crude extracts is more pronounced on Gram-positive than on Gram-negative bacteria, and these findings correlate to the observations of previous screenings (Nair, R *et al.*, 2005; Rabe & van Staden, 1997) of medicinal plants for antibacterial activity. The differences in the susceptibilities of Gram-positive and Gram-negative bacteria to plant extracts have been observed by several researchers (Nostro, A *et al.*, 2000; Suffredini, IB *et al.*, 2006; Parekh and Chanda, 2006). Gram-negative bacteria are inherently more resistant to antimicrobials than Gram-positive organisms and this has been ascribed to the combined exclusion of the antimicrobial compounds by the double membrane barrier and transmembrane efflux present in this group of organisms (Zgurskaya and Nikaido, 2000). The antibacterial activity shown by the water extract in this study could be of interest since traditional healers use water generally as a solvent of preparing remedies from medicinal plants. This study showed that *H. pedunculatum* water extract was active against Gram-negative bacteria such as *Proteus vulgaris* ATCC 6830 and *Enterococcus faecalis*. This is in contrast to the work done by other researchers who have reported water extract to show low activities especially towards Gram-negative bacteria, as compared to organic extracts (Shale, TL *et al.*, 1999; Lall and Meyer, 2000; Matu and Van

Staden, 2003). This seems to confirm the antibacterial potential of *H. pedunculatum* and its use in traditional medical practice. Some researchers have attributed this type of activity to the presence of water soluble tannins which are well known to possess antimicrobial properties (Djipa, CD *et al.*, 2000; Otshudi, AL *et al.*, 2000). However, the relative weak activity of the aqueous extract is worrisome and this could be one reason why, there is incident of high death rate resulting from circumcision wounds infection even after treating such wounds with *H. pedunculatum* leaf.

Generally, the effect of the crude acetone and aqueous extracts 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 ($2 \times \text{MIC}$) 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.

4.5 Conclusion

On the basis of the results obtained, it can be concluded that the crude extracts of *H. pedunculatum* exhibit significant antibacterial activities and properties that support folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents. This probably explains the use of extracts from this plant by the indigenous people of South Africa against a number of

infections for generations. Consequently, we propose a detailed study of the plant in order to determine its pharmacological effects, active compounds as well as their mechanism of action. The effect of the aqueous extract was only bacteriostatic on both reference and environmental strains and the clinical isolates were outrightly resistant to this extract (not reported here), this is worrisome and 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, since the poultice from this leaf that is used during circumcision is normally prepared with water. Work is in progress on the isolation, purification and structural elucidation of the bioactive compounds in this plant in order to further validate the claims for its use in traditional medicine by the people of the Eastern Cape in South Africa. 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.

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Table 4.1. Antibacterial activities of crude Acetone and Aqueous extracts of *H. pedunculatum* leaves on bacterial isolates.

Isolate Identity	Gram's Reaction	Acetone Extract		Aqueous extract	
		Antibacterial activity (10 mg/ml)	MIC (mg/ml)	Antibacterial activity (35 mg/ml)	MIC (mg/ml)
<i>Bacillus cereus</i> ATCC 10702	+	+	5.0	-	ND
<i>Proteus vulgaris</i> ATCC 6830	-	-	ND	+	30
<i>Micrococcus kristinae</i> [§]	+	+	0.5	-	ND
<i>Staphylococcus epidermidis</i> [§]	+	-	ND	+	20
<i>Enterococcus faecalis</i> [§]	-	-	ND	+	25
<i>Staphylococcus aureus</i> [§]	+	-	ND	+	35

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

Table 4.2. Nature of inhibition of crude Acetone and Aqueous extracts of *H. pedunculatum* leaves against bacterial isolates.

Susceptible isolates	Acetone extract					Aqueous extract				
	MIC (mg/ml)	Log ₁₀ Kill(MIC)		Log ₁₀ Kill(2*MIC)		MIC (mg/ml)	Log ₁₀ Kill (MIC)		Log ₁₀ Kill(2*MIC)	
		6hr	12hr	6hr	12hr		6hr	12hr	6hr	12hr
<i>Bacillus cereus</i> ATCC 10702	5.0	0.64	6.06*	0.76	6.06*	NA	NA	NA	NA	NA
<i>Proteus vulgaris</i> ATCC 6830	NA	NA	NA	NA	NA	30	0.10	0.23	0.19	0.34
<i>Micrococcus kristinae</i> [§]	0.5	5.99*	5.99*	5.99*	5.99*	NA	NA	NA	NA	NA
<i>Staphylococcus epidermidis</i> [§]	NA	NA	NA	NA	NA	20	0.12	0.25	0.21	0.38
<i>Enterococcus faecalis</i> [§]	NA	NA	NA	NA	NA	25	0.24	0.42	0.33	0.56
<i>Staphylococcus aureus</i> [§]	NA	NA	NA	NA	NA	35	0.19	0.42	0.32	0.52

Key: MIC represents minimum inhibitory concentration; [§]are environmental strains; * represents bactericidal effect; NA represents no activity.