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Short communication

Antimicrobial activity and *in vitro* cytotoxicity of selected South African *Helichrysum* species

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Abstract

The antimicrobial activity of 35 indigenous South African *Helichrysum* species was determined against six microorganisms. Seven of the 36 chloroform:methanol (1:1) extracts (leaf and stem extracts for all plants and an additional flower extract for *H. rugulosum*) exhibited minimum inhibitory concentration (MIC) values lower than 0.1 mg/ml against *Bacillus cereus* and/or *Staphylococcus aureus*. The *in vitro* cytotoxicity [against transformed human kidney epithelial (Graham) cells, MCF-7 breast adenocarcinoma and SF-268 glioblastoma cells] of these extracts was also determined at a concentration of 0.1 mg/ml using the sulforhodamine B (SRB) assay. For seven species less than 25% growth was observed for the Graham and MCF-7 cell lines at the test concentration.

Keywords: Antimicrobial activity; Asteraceae; Cytotoxicity; *Helichrysum*

1. Introduction

Helichrysum species are often used to treat conditions associated with infections of the skin and the respiratory and gastro-intestinal tracts. For example, root decoctions of *H. adenocarpum* and *H. ecklonis* are used to treat diarrhoea in children. Different preparations of *H. cymosum*, *H. kraussii* and *H. odoratissimum* are used in the treatment of colds and coughs while the leaves of *H. nudifolium* are applied externally to wounds and used to treat respiratory infections (Hutchings et al., 1996; Lourens et al., 2008 and references therein; Watt and Breyer-Brandwijk, 1962).

Although there are several reports on the antimicrobial activities of *Helichrysum* extracts, comparative toxicity values are often not available (Drewes and van Vuuren, 2008; Drewes et al., 2006; Lourens et al., 2004; Mathekga and Meyer, 1998). In a study by Van Vuuren et al. (2006), the antimicrobial activity of an acetone extract of *H. cymosum* was determined against ten pathogens and toxicity determined against transformed human kidney epithelial cells. The IC_{50} value of 172.01 μg/ml reported in the cytotoxicity assay was lower than the MIC values (313 μg/ml) reported for seven of the screened microorganisms, indicating that cell growth may be inhibited at the concentrations required to inhibit pathogen growth. Heyman and Meyer (2009) investigated the cytotoxicity of 12 *Helichrysum* species and reported IC_{50} values ranging from <3.125 μg/ml to 277 μg/ml, while a dichloromethane extract of *H. nudifolium* roots caused total growth inhibition of three cancer cell lines at concentrations below 34 μg/ml (Fouche et al., 2008). Furthermore, aqueous methanolic extracts from several *Helichrysum* species exhibited mutagenicity (Elgorashi et al., 2008; Reid et al., 2006; Verschaeve and Van Staden, 2008). On the other hand, an aqueous extract of *H. aureonitens* did not exhibit any cytotoxic effects at a concentration as high as 8.44 mg/ml against HF cells (Meyer et al., 1996).

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Table 1
Antimicrobial activity (MIC, mg/ml) and cytotoxicity (average at 0.1 mg/ml ± standard deviation) of *Helichrysum* extracts.

Plant species	Antimicrobial activity[a] Test organisms						Cytotoxicity[a,b]		
	C. neoformans ATCC 90112	*P. aeruginosa* ATCC 9027	*S. aureus* ATCC 12600	*B. cereus* ATCC 11778	*S. epidermidis* ATCC 2223	*K. pneumoniae* NCTC 9633	% T/C of Graham cells	% T/C of SF-268 cells	% T/C of MCF-7 cells
H. acutatum DC. A. Lourens and A. Viljoen 1 Graskop	NS[c]	NS	0.5	1	NS	NS	0.1±0.1	15.8±1.0	6.6±0.1
H. adenocarpum DC. subsp *adenocarpum* S.N. Unknown locality	NS	NS	NS	NS	NS	NS	98.3±0.6	88.1±3.6	37.2±3.2
H. appendiculatum (L.f.) Less A. Lourens and A. Viljoen 28 Graskop	NS	NS	NS	4	NS	NS	91.7±2.1	81.0±2.8	37.5±2.4
H. aureonitens Sch. Bip. J.E. Victor 2439 Amatolas	NS	NS	4	4	4	NS	75.6±0.9	76.0±4.7	37.5±3.7
H. aureum (Houtt.) Merril var. *aureum* J.E. Victor 2428 Amatolas	NS	NS	0.02	0.01	NS	NS	5.0±0.2	35.9±1.9	7.0[d]
H. callicomum Harv. DBH 6565 Transkei	NS	NS	1	0.25	4	NS	27.3±2.9	56.1±2.8	11.2[d]
H. cephaloideum DC. A. Lourens and A. Viljoen 20 Underberg	NS	NS	NS	1	4	NS	93.7±4.6	83.8±2.6	46.1[d]
H. dasyanthum (Willd.) Sweet A. Lourens (ex J. Manning) 33 Cape Agulhas	NS	NS	NS	4	NS	NS	91.3±0.7	81.7±3.6	52.8[d]
H. excisum (Thunb.) Less J. Vlok 2830 Oudtshoorn	NS	NS	0.2	0.03	0.1	NS	63.6±1.1	71.9±3.1	31.4±0.8
H. felinum Less. J. Vlok 2828 Oudtshoorn	NS	NS	2	0.16	2	NS	48.0±1.4	54.1±2.3	23.2±0.2
H. cf. *foetidum* (L.) Moench J. Vlok 2833 George	NS	NS	0.5	0.01	NS	NS	32.7±2.1	57.8±2.1	24.9±0.4
H. herbaceum (Andr.) Sweet A. Lourens and A. Viljoen 17 Northern Drakensberg	0.5	NS	0.5	1	4	NS	50.8±3.0	46.4±1.6	22.9±0.3
H. indicum (L.) Grierson A. Lourens (ex. J. Manning) 34 Cape Agulhas	NS	NS	1	0.5	4	NS	81.1±2.3	70.6±4.2	35.8±0.8
H. kraussii Sch. Bip. A. Lourens and A. Viljoen 4 Lydenburg	NS	NS	0.5	0.004	4	2	28.6±1.4	45.2±4.4	9.0[d]
H. melanacme DC. A. Lourens and A. Viljoen 12 Clarens	NS	NS	0.5	0.25	4	2	18.1±0.4	53.3±2.7	12.2[d]

Table 1 (*continued*).

Plant species	Antimicrobial activity[a] Test organisms						Cytotoxicity[a,b]		
	C. neoformans ATCC 90112	*P. aeruginosa* ATCC 9027	*S. aureus* ATCC 12600	*B. cereus* ATCC 11778	*S. epidermidis* ATCC 2223	*K. pneumoniae* NCTC 9633	% T/C of Graham cells	% T/C of SF-268 cells	% T/C of MCF-7 cells
H. miconiifolium DC. A. Lourens and A.Viljoen 24 Himeville	NS	NS	2	1	4	NS	37.9±6.6	52.4±1.8	20.9[d]
H. montanum DC. F. Van Heerden 1 Murraysburg	NS	NS	1	4	NS	NS	29.4±0.7	46.1±4.0	19.2[d]
H. nudifolium (L.) Less A. Lourens and A. Viljoen 13 Clarens	NS	NS	NS	4	NS	NS	73.1±2.5	83.9±1.8	35.3±0.4
H. odoratissimum (L.) Sweet A. Lourens and A. Viljoen 6 Pilgrims Rest	NS	NS	0.02	0.03	4	2	17.5±0.4	48.2±1.4	7.4±0.7
H. oreophilum Klatt A. Lourens and A. Viljoen 10 Draaikraal	NS	NS	4	4	NS	NS	63.4±2.2	82.9±3.0	34.8±0.1
H. pallidum DC. A. Lourens and A. Viljoen 7 Graskop	NS	NS	NS	NS	NS	NS	88.6±0.9	83.9±9.7	49.0±1.7
H. pandurifolium Schrank A. Lourens (ex J. Manning) 35 Cape Agulhas	NS	NS	2	4	NS	NS	57.1±1.2	71.8±0.8	34.2±0.1
H. patulum (L.) D. Don A. Lourens (ex J. Manning) 36 Cape Agulhas	NS	NS	4	4	NS	NS	63.8±1.3	75.2±2.0	37.8[d]
H. petiolare Hilliard and Burtt J. Vlok 2827 Oudtshoorn	NS	NS	4	2	NS	NS	59.3±3.4	76.6±3.0	33.4[d]
H. platypterum DC. A. Lourens and A. Viljoen 30 Dullstroom	NS	NS	0.05	0.5	NS	NS	0.8±0.3	35.1±1.5	4.6[d]
H. psilolepis Harv. A. Lourens and A. Viljoen 11 Clarens	NS	NS	1	1	NS	NS	25.9±1.9	58.4±7.4	23.1[d]
H. retortum (L.) Willd. A. Lourens (ex J. Manning) 37 Cape Agulhas	ND	NS	4	NS	NS	NS	79.6±3.4	87.3±4.8	ND[e]
H. rosum (Berg.) Less. cf. var. *rosum* J.E. Victor 2436 Amatolas	NS	NS	1	1	4	NS	47.5±1.8	54.8±5.0	22.5±0.7
H. ruderale Hilliard & Burtt. A. Lourens and A. Viljoen 32 Hilton	ND	NS	2	1	NS	NS	45.1±1.2	75.8±3.9	ND

(continued on next page)

Table 1 (*continued*).

Plant species	Antimicrobial activity[a] Test organisms						Cytotoxicity[a,b]		
	C. neoformans ATCC 90112	*P. aeruginosa* ATCC 9027	*S. aureus* ATCC 12600	*B. cereus* ATCC 11778	*S. epidermidis* ATCC 2223	*K. pneumoniae* NCTC 9633	% T/C of Graham cells	% T/C of SF-268 cells	% T/C of MCF-7 cells
H. rugulosum Less. Stems and leaves A. Lourens and A. Viljoen 8 Ventersdorp	NS	NS	0.5	0.25	4	NS	3.0±1.2	44.7±3.6	12.7±2.7
H. rugulosum Less. Flowers A. Lourens and A. Viljoen 8 Ventersdorp	1.0	NS	0.01	0.33	0.01	0.22	ND	ND	ND
H. scitilum Hilliard & Burtt. F. Van Heerden 2 Murraysburg	NS	NS	NS	NS	4	NS	58.6±1.2	85.3±6.8	46.8[d]
H. simillimum DC. A. Lourens and A. Viljoen 23 Himeville	NS	NS	1	4	4	NS	54.6±2.6	66.8±3.9	26.8[d]
H. splendidum (Thunb.) Less A.Lourens and A. Viljoen 2 Graskop	NS	NS	4	NS	NS	NS	82.3±3.2	72.8±3.3	49.2±2.5
H. wilmsii Moeser A. Lourens and A.Viljoen 5 Pilgrims Rest	NS	NS	1	1	4	NS	22.5±2.1	64.1±6.1	15.3±0.5
H. zeyheri Less. F. Van Heerden 3 Koueveld Mountains	NS	NS	2	4	NS	NS	54.4±9.5	58.2±3.1	37.4[d]
Antimicrobial control[f]	2.5×10^{-3}	0.3×10^{-3}	0.3×10^{-3}	1.0×10^{-3}	2.5×10^{-3}	0.7×10^{-3}			
DMSO control[g]	2	4	8	8	16	4			

[a] Experiments at least done in duplicate.

[b] At a concentration of 0.1 mg/ml of 5-flourouracil, more than 80% growth was observed for the SF-268 cells, while an IC_{50} of 1.1 μg/ml was determined for the same drug against the MCF-7 cell line (Kamatou et al., 2008).

[c] Not susceptible, MIC observed equal to that of solvent control.

[d] Experiments only done once.

[e] Not determined.

[f] Ciprofloxacin used as a positive control against bacteria and Amphotericin B used as a positive control against the yeast.

[g] Solvent control.

The above-mentioned results prompted us to investigate the antimicrobial activity and *in vitro* cytotoxicity of chloroform: methanol (1:1) extracts of *Helichrysum* species against three different cell lines at a single concentration of 0.1 mg/ml. The aim was to obtain a general preliminary idea of the cytotoxicity of extracts screened for antimicrobial activity.

2. Materials and methods

2.1. Plant material

The *Helichrysum* species (Asteraceae) were collected in several provinces of South Africa. Voucher specimens were deposited at the University of KwaZulu-Natal herbarium (NU) in Pietermaritzburg, South Africa (voucher numbers, Table 1). Plants were identified at the National Herbarium at the National Biodiversity Institute in Pretoria, South Africa.

2.2. Preparation of extracts

Leaves and stems were air-dried (protected from sunlight), ground and extracted twice with chloroform:methanol (1:1) for 24 h at room temperature. Thereafter the solvent was removed under vacuum.

2.3. Antimicrobial activity

Six microorganisms, including three Gram-positive bacteria (*Bacillus cereus*, ATCC 11778, *Staphylococcus aureus*, ATCC 12600, and *Staphylococcus epidermidis*, ATCC 2223), two Gram-negative bacteria (*Klebsiella pneumoniae*, NCTC 9633 and *Pseudomonas aeruginosa*, ATCC 9027), and a yeast (*Cryptococcus neoformans*, ATCC 90112) were used in the screening of the crude extracts. Selection of test organisms were undertaken according to the traditional use of species of this genus i.e. treatment of respiratory disorders and wounds. The reference stock cultures were obtained from the National Health Laboratory Service (NHLS) (Johannesburg) and were maintained in the microbiology laboratory of the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa.

MIC values were determined by the 96-well microplate method as described by Eloff (1998) and adapted by Magee et al. (2006). Stock solutions were prepared by dissolving samples in dimethyl sulfoxide (DMSO, 64 mg/ml) since dissolution problems occurred with solvents such as acetone. These stock solutions were serially diluted to obtain a final concentration range of 16 to 0.125 mg/ml of extract after addition of the bacterial culture. A fixed bacterial culture of an approximate inoculum size of 1×10^6 colony forming units (CFU)/ml in Tryptone Soya broth was added to all wells. Further dilutions were prepared when activity below these ranges were observed.

The plates were incubated at 37 °C for 24 h for bacteria and 48 h for the yeast. MIC values were determined at least in duplicate. Positive controls included ciprofloxacin for bacteria and amphotericin B for the yeast. A solvent control was included with the assay since DMSO has antimicrobial activity and samples with MIC values equal to that found for DMSO were considered not susceptible (NS, Table 1). After the incubation period, 50 µl of a 0.4 mg/ml *p*-iodonitrotetrazolium violet (INT) solution was added to all wells and left for 6 h (except *C. neoformans* which was left for 12 h) at room temperature before reading the MIC's.

2.4. Cytotoxicity assay

Cytotoxicity was determined with the sulforhodamine B (SRB) assay (Kamatou et al., 2008; Monks et al., 1991). Transformed human kidney epithelial cells (Graham cells, obtained from Dr. R Van Zyl, University of the Witwatersrand), MCF-7 breast adenocarcinoma and SF-268 glioblastoma cells (obtained from the National Cancer Institute (NCI), USA) were used to determine cytotoxicity. The Graham cells were included as a representative of a non-cancerous cell line.

The Graham cells were cultured in Ham's F10 media containing 5% (v/v) heat inactivated fetal calf serum (FCS) and 0.1% gentamicin. The SF-268 and MCF-7 cells were maintained in RPMI-1640 media containing 5% FCS and 0.01% L-glutamine, while media used in the assay included 0.1% gentamicin.

The Graham cells were seeded at 25 000 cells/well, while the MCF-7 and SF-268 cells were seeded at 15 000 cells/well. Cells were incubated for 24 h at 37 °C in 5% CO_2 at 100% relative humidity to allow for attachment to the 96 well plates.

Crude extracts were weighed and dissolved in DMSO to prepare stock solutions of ca. 10 mg/ml. These were further diluted with appropriate culture media, before addition to the microtitre plate (after the 24 h incubation period) to obtain concentrations of 0.1 mg/ml extract per well. Samples were plated out in triplicate or quadruplicate.

After a 48 h incubation period, cells were fixed with 50% trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. Fixed cells were stained with a 0.4% (w/v) SRB solution for 10 min at room temperature after drying. The bound dye was solubilised through the addition of 200 µl Tris base. The absorbance was read at 492 nm (Labsystems iEMS reader MF), connected to Ascent (version 2.4) software. Cytotoxicity results were expressed as average ± standard deviation of duplicate experiments.

The percentage treated cell growth in reference to control growth was calculated as follows:

$$\%T / C = \frac{(\text{mean abs test sample} - \text{mean abs background}) * 100}{(\text{mean abs control} - \text{mean abs blank})}$$

where "mean abs test sample" refers to the mean absorbance at 492 nm obtained for the three/four similar sample wells.

"mean abs background" refers to the mean absorbance at 492 nm of the wells containing only sample and media.

"mean abs control" refers to absorbance of wells containing only cell suspension and no sample.

"mean abs blank" refers to wells containing only media.

3. Results and discussion

The antimicrobial activities and cytotoxicity are reported in Table 1. Of the 36 extracts (35 leaf/stem extracts, one flower extract) tested, seven exhibited MIC's of 0.1 mg/ml or lower against one or more microorganisms. There are reports on the traditional use of five of the seven most active species relating to antimicrobial use. For example, *H. foetidum* is used to treat festering sores while *H. nudifolium* is often used as wound dressing (Lourens et al., 2008 and references therein).

Antimicrobial activity was mainly observed against the two Gram-positive microorganisms, *S. aureus* and *B. cereus*. *H. kraussii* displayed the highest sensitivity (0.004 mg/ml against *B. cereus*) followed by *H. aureum*, *H.* cf. *foetidum* (0.01 mg/ml against *B. cereus*) and *H. rugulosum* (0.01 mg/ml against the *Staphylococcus* spp.). Furthermore, *H. rugulosum* also exhibited the highest broad spectrum activity having noteworthy activity (0.01–0.33 mg/ml) against four of the six pathogens studied. A few extracts had activity against *S. epidermidis*, the Gram-negative organisms and the yeast. These results are in agreement with those obtained by Mathekga and Meyer (1998) for ten *Helichrysum* species.

The extracts of *H. herbaceum* and *H. rugulosum* flowers were the only extracts active against the yeast (*C. neoformans*) and exhibited MIC's of 0.5 and 1.0 mg/ml, respectively. The flower extract of *H. rugulosum* showed much better activity against *S. aureus*, *S. epidermidis* and *K. pneumoniae* than the extract of the leaves and stems of the same plant, indicating that the flower extracts of other species may yield interesting results. This is confirmed by the studies on the flowers of *H. gymnocomum* (Drewes and Van Vuuren, 2008).

Although the MIC values observed for some species seem promising, in several cases cytotoxicity was also observed at 0.1 mg/ml. The MIC's for *H. aureum* extract, for example, are 0.02 and 0.01 mg/ml against *S. aureus* and *B. cereus*, respectively, while only 5% Graham cell growth was observed at 0.1 mg/ml.

Species with potential toxicity include *H. acutatum*, *H. aureum* var *aureum*, *H. platypterum* and *H. rugulosum*, since less than 10% Graham cell growth was observed at the test concentration. Cell growth of the two cancer cell lines were also inhibited to a large extent for these extracts, indicating non-specific cytotoxicity. With extracts of *H. adenocarpum*, *H. appendiculatum*, *H. cephaloideum* and *H. indicum*, more than 80% growth was observed for the 'normal' Graham cells, while the growth of the MCF-7 breast cancer cells were less than half of that at the same concentration of extract, indicating a degree of selectivity. In general, the MCF-7 cells were more sensitive towards the extracts than either the Graham or SF-268 cells.

According to Van Wyk et al. (2000) the best known and most commonly used *Helichrysum* species are *H. cymosum*, *H. odoratissimum*, *H. petiolare* and *H. nudifolium*. In the cytotoxicity assays, the organic solvent extracts from both *H. odoratissium* and *H. cymosum* (Van Vuuren et al., 2006) appears quite toxic against Graham cells. Even for *H. petiolare* and *H. nudifolium*, some growth inhibition was observed at 0.1 mg/ml.

Traditionally, decoctions and infusions (using water) are prepared from these species, smoke or vapours are inhaled or leaves applied externally to wounds. Although *H. argyrosphaerum* and *H. cephaloideum* (Van Wyk et al., 2002) are known to cause poisonings in livestock, *Helichrysum* species in general are not known to cause human poisoning (Van Wyk et al., 2002). It would therefore seem that the cytotoxic components are extracted or are present in higher concentrations in organic solvent extracts and are not necessarily a problem when these plants are used in traditional preparations.

4. Conclusion

Although these cytotoxicity assays were performed only at a single concentration, the fact that cell growth was inhibited to such an extent at concentrations of a similar order to that inhibiting microbial growth is quite disturbing. These preliminary results indicate that toxicity studies are advisable when *Helichrysum* extracts obtained with organic solvents (such as dichloromethane and methanol) is used in antimicrobial screening. The relevance of these results to the traditional use of these plant species need to be investigated further.

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