CHAPTER 2

*ANTIBACTERIAL ACTIVITY OF LINOLEIC- AND OLEIC ACID ISOLATED FROM *HELICHRYSUM PEDUNCULATUM*, A PLANT USED DURING CIRCUMCISION RITES

*Antibacterial activity of linoleic- and oleic acid isolated from *Helichrysum pedunculatum*, a plant used during circumcision rites

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2.1. Abstract

Helichrysum pedunculatum (Asteraceae) is a commonly used plant to dress wounds during male circumcision rites by the Xhosas in South Africa. The antibacterial activity-guided fractionation of the dichloromethane extract of leaves of *H. pedunculatum* resulted in the isolation of linoleic- and oleic acids. These two fatty acids were then evaluated for their antibacterial activity on ten selected bacteria. Linoleic acid inhibited the growth of all the Gram-positive bacterial species tested with the minimum inhibitory concentration (MIC) varying between 0.01 and 1.0 mg/ml. Oleic acid was active against three of the five Gram-positive bacteria at a MIC of 1.0 mg/ml. Both compounds were inactive against all the Gram-negative species tested. A synergistic effect between the two fatty acids was observed against *Staphylococcus aureus* and *Micrococcus kristinae.*

*Written in the format of a paper for Fitoterapia.

Keywords: Antibacterial, Circumcision, *Helichrysum pedunculatum,*

Linoleic acid, Oleic acid

2.2. Introduction

Traditional male circumcision is a common practice among the indigenous people of South Africa. Usually, the whole ceremony takes place in a secluded area in the wild and the patients who are mainly teenagers are kept far away from families and friends throughout the period. Circumcision performed in the wild has a high risk of infection. Information obtained from various local communities has revealed a high incidence of complications arising from wound contaminations. *Staphylococcus aureus* is a bacterium which is commonly- implicated in hospitalised circumcised patients. *Helichrysum pedunculatum* Hilliard & Burtt (Asteraceae) is commonly used by the Xhosas of Transkei to bandage circumcision wounds. The folkloric use of this plant was verified by [1] when they showed that extracts of the plant have antibacterial activity. A number of other *Helichrysum* species have been reported to have antimicrobial activity [2-4]. In this study, we report on the isolation and identification of two antibacterial fatty acids from *H. pedunculatum.* Each compound was first tested individually for activity against ten bacterial species followed by an investigation of synergistic enhancement in activity against two of the bacteria.

2.3. Experimental

2.1 Plant material

Leaves of *H. pedunculatum* were collected during December 1996 from the Transkei, a region in the Eastern Cape province of South Africa. A

voucher specimen (Dilika 299) has been deposited at the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria.

2.2 Chemicals

Chromatography material and solvents were purchased from Merck-Johannesburg. Nutrient agar and broth (no 2) were obtained from Biolab. The two fatty acid standards, linoleic- and oleic acid were purchased from Aldrich.

2.3 Isolation and identification of the antibacterial compounds

Air dried *H. pedunculatum* leaves were shaken for 5 min in a orbital shaker in dichloromethane and the resulting extract was then further fractionated by column chromatography and HPLC. Column chromatography was performed on silica gel 60 using two different gradients of either hexane, chloroform, ethyl acetate and ethanol or diethyl ether, petroleum ether and methanol as eluents. Those fractions showing antibacterial activity were then rechromatographed on column chromatography using silica gel 60 (diethyl ether-light petroleum ether-methanol, 15:85:7) to separate the fraction containing oleic acid and Sephadex LH-20 (96% ethanol) for the fraction containing linoleic acid. The oleic acid fraction was further purified by HPLC utilising an analytical Phenomenex C_{18}, 250 x 4.6 mm column (methanol-water 80:20, flow rate 1.0 ml/min, 40 °C, 206nm).

GC-MS analysis (full scan mode was utilised at 280°C) of the two fatty acid fractions was conducted on a VG micromass gas chromatograph

equiped with an SE 30 capillary column (25 x 0.32 mm ID). The two isolated acids as well as their standards were also analysed by nuclear magnetic resonance spectroscopy (^{1}H- and ^{13}C-NMR).

2.4 Bioautography

Bioautography to guide fractionation was conducted on TLC plates. The chromatographic fractions were spotted and developed with chloroform-ethyl acetate (1:2) and diethyl ether-light petroleum ether-methanol (15:85:7). A 24 h old *S. aureus* culture was centrifuged at 3000 rpm for 20 minutes, the pellet resuspended in fresh sterile nutrient broth to an absorbance of 0.84 at 560 nm and sprayed on the TLC plate. After a 24 h incubation at 37 °C in humid conditions, the plates were sprayed with *p*-iodonitrotetrazolium violet (INT) and reincubated at 37 °C for 6 h [1,5].

2.5 Antibacterial testing

Standards of the two fatty acids were dissolved in acetone and mixed with autoclaved nutrient agar to final concentrations of 0.01, 0.05, 0.1 and 1.0 mg/ml. These mixtures containing 1 % acetone were then added to Petri dishes and swirled until set. The plates (including a 1% acetone control) were left overnight for the acetone to evaporate. Three replicates were used per treatment.

The ten bacterial species (Table 1) were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained and recovered as described previously [1]. Bacterial species

were cultured in nutrient broth no 2 for 24 h. Before streaking onto set agar, each culture was diluted 1:100 with fresh nutrient broth. The bacteria were streaked in a radial pattern on the plates that were left overnight and then incubated at 37 °C for 24 h [6]. Complete suppression of growth by linoleic- and oleic acid was required for the acids to be declared active at a specific concentration (Table 1).

Table 1. Antibacterial activity (MIC[a] mg/ml) of linoleic- and oleic acid isolated from *H. pedunculatum*

Bacterial Species	Gram +/-	Linoleic acid	Oleic acid
Bacillus cereus	+	0.01	na[b]
B. pumilus	+	1.0	na
B. subtilis	+	0.01	1.0
Micrococcus kristinae	+	1.0	1.0
Staphylococcus aureus	+	1.0	1.0
Enterobacter cloacae	-	na	Na
Escherichia coli	-	na	Na
Klebsiella pneumoniae	-	na	Na
Pseudomonas aeruginosa	-	na	Na
Serratia marcescens	-	*na*	*na*

[a] minimum inhibitory concentration
[b] not active

2.6 Synergism of the two antibacterial compounds

The antibacterial activity of linoleic- and oleic acids was also analysed in combination to determine if there was a synergistic effect between them. This was also done by the agar dilution method. The final concentration of each compound in the mixture was 0.05, 0.1 and 0.5 mg/ml, and were tested against *M. kristinae* and *S. aureus* bacteria.

2.4. Results

The antibacterial-guided fractionation of the dichloromethane extract led to the isolation of linoleic- and oleic acid. The GC-MS spectra and the ^{1}H and ^{13}C-NMR spectra of the isolated compounds matched those of standards of these fatty acids.

Linoleic acid inhibited the growth of all the Gram-positive bacterial species tested with the MIC varying between 0.01 and 1.0 mg/ml (Table 1). Oleic acid was active against three of the five Gram-positive bacteria at a MIC of 1.0 mg/ml. None of the Gram-negative bacteria was inhibited by these fatty acids.

Both fatty acids were inhibitory to *S. aureus* and *M. kristinae* at 1.0 mg/ml when administered singularly. However, when administered in combination, the growth of both bacteria was inhibited at a concentration of 0.05 mg/ml of each fatty acid, indicating a strong synergistic effect. This is the first report of the synergistic effect of these two fatty acids on bacterial species.

2.5. Discussion

The insensitivity of the Gram-negative bacteria to fatty acids may be due to the prevention of fatty acid penetration by lipids in the cell wall [7-9]. Although there are rare strains sensitive to the inhibitory effect of the acids, most of the fatty acid sensitive strains belong to the genus *Bacillus* [7,9]. This is evident in the findings of [9; 10] on the antibacterial activity against *B. megaterium* and *B. subtilis*. This was also confirmed in this study by the activity of linoleic acid against *B. cereus, B. pumilus* and *B. subtilis,* however oleic acid acid showed no antibacterial activity against *B. cereus* and *B. pumilus.*

Linoleic acid has previously been reported for its antimicrobial properties against bacteria and fungi [11,12]. Although, [13] suggest 0.01mg/ml of the test compound for extracts to be considered active, the compounds recognised so far as constituents of *H. pedunculatum* could rationalise the use of the plant in wound treatment especially against bacterial infection. This activity might be attributed to fatty acids alone or in synergy with other compounds as they are known to have a potent antibacterial action [9].

The antibacterial activity of linoleic- and oleic acids as well as their synergistic effect to inhibit the growth of *S. aureus* might explain the use of this herb by the Xhosas of South Africa during male circumcision rituals. *S. aureus* is the most common implicated bacterial species in hospitalised circumcised patients.

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2.7. References

[1] Meyer JJM, Dilika F. J Ethnopharmacol 1996;53:51.

[2] Tomas-Barberan FA, Iniesta-Sanmartin E, Tomas-Lorente F, Rumbero A. Phytochemistry 1990;29:1093.

[3] Rios MC, Villa AI. J Ethnopharmacol 1991;33:51.

[4] Salie F, Eagles PFK, Leng HMJ. J Ethnopharmacol 1996;52:27.

[5] Lund BM, Lyon GD. J Chromatography 1975;110:193.

[6] Mitscher LA, Leu R, Bathala MS, Wu W, Beal JL. Lloydia 1972;35:157

[7] Galbraith H, Miller TB, Paton AM, Thompson JK. J Appl Bacteriol 1971;34:803.

[8] Kondo E, Konai K. Jap J Med Sci Biol 1976;29:25.

[9] Gonzalez MD, Moreno E, Quevedo-Sarmiento J, Ramos-Cormenzana A. Chemosphere 1990;20:423.

[10] Geissberger P, Sequin U. Acta Tropica 1991;48:251.

[11] Kabara JJ. Symp on the Pharmacol Effects of Lipids 1978:1.

[12] Hattori M, Miyachi K, Hada S, Kakiuchi N, Kiuchi F, Tsuda Y, Namba T. Chem Pharmaceut Bull 1987;35:3507.

[13] Rahalison L, Hamburger M, Manod M, Frenk E, Hostettman K. Planta Med 1994;60:41.