CHAPTER 6

SYNERGISTIC ANTIBACTERIAL EFFECT OF CAESPITATE AND CAESPITIN, TWO PHLOROGLUCINOLS ISOLATED FROM *HELICHRYSUM CAESPITITIUM*

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6.1 Introduction

After a brief review of the present status of the field of antibiotics in general and the isolated compounds in particular, this chapter focuses upon the synergistic effects of the compounds isolated from *Helichrysum caespititium* (DC.) Harv. Antimicrobial tests demonstrated that caespitin (Dekker *et al*., 1983) and caespitate (Mathekga *et al.*, 2000) exhibit significant potency against human bacterial and fungal pathogens (Chapter 4). Caespitin and caespitate are readily obtainable by bioassay-directed isolation techniques. Their antimicrobial spectra are comparatively narrow and their potency is reasonable. Novel lead structures and the possibility of finding additional agents for human or agricultural use based upon these agents, is possible.

It is interesting to note that *H. caespititium* contains more than one antimicrobial agent. Caespitin and caespitate were both active against Gram-positive bacteria only at similar concentrations. In this study we investigated the synergistic effect of caespitate and caespitin on Gram-positive and Gram-negative bacteria.

6.2 Materials and Methods

6.2.1. Plant material.

Shoots of *H. caespititium* were collected from the Drakensberg in the Mont-aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was

deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria.

6.2.2 Preparation of extract

Air dried (80 g) plant material was immersed in acetone and shaken on a rotary shaker for 5 minutes without homogenizing it. The extract was filtered and concentrated to dryness under reduced pressure at 40 ^{0}C with a rotary evaporator. After determining the yield (8.4 g (w/w)), the extract was stored at 4 ^{0}C until antibacterial assays commenced.

6.3 Preparation of caespitate

6.3.1 Isolation and identification of caespitate .

The crude acetone extract of *H. caespititium* was initially subjected to preparative TLC in $CHCl_3$-EtOAc (1:1). The targeted band was recovered and rechromatographed by column chromatography with 100% chloroform on silica gel 60. Direct antibacterial bioassays on TLC of the fractions collected indicated the presence of several antibacterial compounds in the extract. The fraction with the highest antibacterial activity was finally isolated in a pure form by HPLC in H_2O-EtOH (1:1) on a reverse phase Phenomenex column (250 x 4.60 mm; 5 µm). NMR analysis (DEPT, COSY and HETCOR spectra) were obtained using standard pulse sequences on a Varian 200 MHz spectrometer. Mass spectra were recorded on a Hewlett-Packard 5988 GC/MS instrument. High resolution mass spectra were obtained from a Kratos MS 80 RF double-focussing magnetic sector instrument.

6.3.2 Preparation of caespitate and caespitin solutions

Caespitin (obtained from Noristan Laboratories, South Africa) was previously isolated from *H. caespititium* (Dekker *et al.*, 1983) and shown to have antibacterial properties. Equal aliquots of caespitin and caespitate were well mixed and then serially diluted to give a concentration range of 0.1 to 0.001µg/ml in 2% acetone.

6.3.3 Antibacterial activity of caespitate and caespitin

The combined test solutions (sterilised by filtering through a 0.22 µm filter) were added to 5ml of sterilised nutrient agar in Petri dishes and swirled carefully before congealing. The organisms were streaked in radial patterns on agar plates (Mathekga and Meyer, 1998).

Plates were incubated at 37 ^{0}C in the dark and examined after 24 and 48 hours. Complete inhibition of growth was required for the extract to be declared bioactive. The controls consisted of Petri dishes containing only nutrient agar and others containing nutrient agar in 2% acetone. Each treatment was analyzed in triplicate.

6.4 Results

6.4.1 Antibacterial activity

Caespitate exhibited antimicrobial activity at a range of 5.0 to 0.5 mg/ml (Table 6.1) against Gram-positive bacteria only. The bioassay demonstrated that the combination of caespitate and caespitin not only maintained their original broad spectrum antibacterial activity against Gram-positive bacteria but that their synergistic effect enhanced activity down to a range of 0.1 to 0.05 mg/ ml. In addition, the growth of four Gram-negative bacteria, *E. cloacae, E. coli, K. pneumoniae* and *P. aeruginosa* were significantly inhibited. *Serratia marcescens* was not susceptible to the synergistic effect of caespitate and caespitin.

Table 6.1 Synergistic effect of the antibacterial activity of caespitin and caespitate isolated from *H. caespititium.*

		MIC [a]		
	Gram	caespitin	caespitate	caespitate and caespitin
Bacterial species	+/-	(µg/ml)	(µg/ml)	(µg/ml)
Bacillus cereus	+	1.0	0.5	0.05
B. pumilus	+	1.0	0.5	0.05
B. subtilis	+	1.0	0.5	0.05
Micrococcus kristinae	+	1.0	0.5	0.05
Staphylococcus aureus	+	1.0	0.5	0.05
Enterobacter cloacae	-	na[b]	na	0.10
Escherichia coli	-	na	na	0.10
Klebsiella pneumoniae	-	na	na	0.05
Pseudomonas aeruginosa	-	na	na	0.05
Serratia marcescens	-	na	na	na

a Minimum inhibitory concentration

b Not active

6. 5 Discussion.

The rationale for this enhanced effect may be based probably, on an increase in the permeability of the antibiotic through the plasma membrane by the combination of caespitate and caespitin (Kato *et al*., 1990). Hydrophobicity is not the sole determinant for the active stability of a membrane structure. It seems very likely, therefore, that many other molecular elements other than hydrophobicity are involved such as protein flexibility

(Kato *et al.*, 1985), surface charge (Adam *et al.*, 1971), conformational stability (Kato *et al.*, 1990, Song and Damodaran, 1987), solubility (Haling, 1981, Chobert *et al.*, 1988), and molecular size (Kato *et al*., 1990). Therefore, to enhance the antibacterial activity of caespitate, it was combined with caespitin. Here, the strategy is designing an effective anti Gram-negative agent to enable it to fuse into the outer membrane, an amphitropic compound approach. Amphitropic compounds are lipid-binding compounds, such as a-actinin and vinculin, that can traverse through the cell membrane reversibly (Burn, 1988). The combination treatment of caespitate and caespitin provides a simple functional molecule conjugate (Norden, *et al.*, 1979; Nakamura, *et al*., 1990, Masaki, *et al*., 1992), not necessarily target-specific, which can access the outer membrane lipopolysaccharide layer by linking the active molecule to a hydrophobic ligand to facilitate its delivery to the site of action (cytoplasmic membrane) to perform its task (Ibrahim *et al.*, 1991) .

Of the many different types of proteins found capable of passing through the outer membrane of *E. coli*, for example, all appear to possess a hydrophobic sequence (e.g. hemolysin) or contain a covalently bound fatty acid (amphitropic proteins such as a-actinin and vinculin) (Kato *et al.*, 1990). It appears, therefore, that when the synergised molecules are fused into the cell membrane, the positively charged molecules caespitate and caespitin come into contact with or approached the negatively charged phospholipid bilayer of the inner membrane, most probably via zones of adhesion between the outer and inner membranes (Adam *et al.*, 1971). As a result there may be an electrostatic interaction between the positively charged groups of the moderately modified caespitate and caespitin and negatively charged head groups of phospholipids, thus leading to localization of caespitate and caespitin in the vicinity of the site of action (Kato *et al.*, 1990). Therefore, it is rational to expect the results obtained in this study as they are consistent with similar findings by other researchers on synergistic effects.

6. 6 Conclusion

The high activity of crude extracts of the shoots of *H. caespititium* (Chapters 2 and 3) is attributable to the presence of at least two phenolic components, caespitate and caespitin . They are individually less potent than their combined synergistic effect. Combining caespitin and caespitate enhanced the antibacterial activity. This approach heralds fascinating opportunities for engineering potentially active compounds such as caespitate and caespitin that are lethal to Gram-negative and Gram-positive bacteria.

The antimicrobial enhancement (synergistic effect) and non-toxicity of *H. caespititium* as detected in this *in vitro* study may partly explain the popularity of this plant in folk medicine as a remedy for many diseases and skin infections.

Traditional medicine is a potential source of new drugs, a source of cheap starting products for the synthesis of known drugs, as has recently been shown with drugs such as reserpine from *Rauwolfia* species, vinblastine from *Catharanthus reus* and the discovery of a contraceptive in the Zoapatle (*Montanoa tomentosa* (Hahn *et al.*, 1981)).

The traditional practitioner’s potions often come in multi-component preparations (similar to multi-drug therapy in TB treatment) aimed at healing several ailments simultaneously, probably simulating the results obtained by the synergistic effect of caespitin and caespitate and a possible explanation of the efficacy of the traditional practitioner's potion. However, further study is needed to elucidate the mechanism underlying the behaviour of caespitin and caespitate. The combinations made by traditional healers pose an additional scientific investigation challenge in the field of drug discovery.

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