AFOLAYAN, ANTHONY JIDE

THE MEDICINAL POTENTIAL OF HELICHRYSUM AUREONITENS

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THE MEDICINAL POTENTIAL OF *HELICHRYSUM AUREONITENS*

by

ANTHONY JIDE AFOLAYAN

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Chapter 1

Introduction

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Background

Plants have been used successfully for centuries by herbalists all over the world and especially in Africa, Asia and Latin America for the treatment of various human diseases. It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs (Farnsworth 1990; Nwosu and Okafor 1995). Higher plants are known to be the main source of drug therapy in traditional medicine.

Out of the 250 000 species of higher plants known to exist on earth, only a relative handful have been thoroughly investigated for their therapeutic values. Yet, in terms of the quantity consumed, the plant kingdom has yielded more than 25% of the drugs used in prescriptive medicines today (Farnsworth 1988; Balick 1990). On the basis of global survey data it has been discovered that about 119 plant-derived chemical compounds of known structure are currently used as drugs or as biodynamic agents that affect human health. Less than a dozen of these are produced commercially by synthesis or by simple chemical modification of the extracted active compounds; the remainder are extracted and purified directly from plants. These 119 useful drugs are obtained from only about 90 species of plants (Farnsworth *et al.* 1985; Farnsworth 1990).

Considering the current rate of deforestation and concurrent loss of biodiversity throughout the world, there is an urgent need to examine the rest of the plant kingdom for new pharmaceutical compounds.

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In South Africa alone, between 12 and 15 million people are believed to be dependent on traditional remedies from as many as 700 indigenous plant species (Meyer and Afolayan 1995). The genus *Helichrysum* with 245 species in South Africa (Hilliard 1983) constitutes a major group of angiosperms exploited for their medicinal value by the indigenous South Africans (Phillips 1917).

Species of the genus *Helichrysum* have been widely reported for their antimicrobial activities (Tomas-Barberan *et al.* 1990). Ethanol extracts from *H. armenium, H. graveolens* and *H. plicatum* have been reported to be active against *Staphylococcus albus* and *S. aureus* (Cosar and Cubukcu 1990). The dichloromethane extract of *H. stoechas* was found to be active only on gram postive bacteria (Rios *et al.* 1991). Antifungal activity of *H. decumbens* and *H. nitens* has been respectively reported by Tomas-Barberan *et al.* (1988) and Tomas-Lorente *et al.* (1989). However, at the commencement of this research project, no such information was available on *H. aureonitens* Sch. Bip. Information from folklore, revealed that the Zulu people of the KwaZulu-Natal province of South Africa have used extracts from this plant topically for centuries against infections. There was therefore, the need to examine this herb scientifically for its antimicrobial properties.

H. aureonitens is a tufted perennial herb which often forms extensive colonies visible from afar as grey patches in grasslands (Figure 1). Its stems are slender, about 30 cm tall, simple or sparingly branched from a creeping stock and leafy throughout. Leaves are up to 20 x 3 mm becoming slightly smaller upwards. In shape, they are oblong or oblong-spatulate with subacute or obtuse apex and subrevolute margins. The inflorescence is heterogamous, campanulate, about 4 x 3 mm and up to 30 in compact corymbose clusters at the tips of

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Figure 1: *Helichrysum aureonitens* in its natural habitat near Melmoth, Kwazulu-Natal.

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the branches. Involucral bracts are in three series, imbricate, mostly obtuse, and of the same number with the flowers appearing yellow or pale brown. The receptacle is with fimbrils about the same number as the ovaries. There are usually 37-57 flowers consisting of 13-23 males and 21-37 females. Achenes are less than 0.5 mm in length and glabrous. Pappus bristles are many and are of the same number as the corolla. *H. aureonitens* flowers between September and February (Hilliard 1983).

In distribution, *H. aureonitens* is widespread in southern Africa (Figure 2). It grows from Huila in Angola to the Highveld and eastern highlands of South Africa, Swaziland, western Lesotho, Free State, KwaZulu-Natal and the Eastern Cape about as far south as King William's Town and the Amatola mountains. It was also recorded from southmost Mozambique (Hilliard 1983).

The choice of *Helichrysum aureonitens*

Four basic methods are usually employed when selecting a plant for screening to seek antimicrobial leads. These are: (1) random choice of plant species; (2) choice based on ethnomedical uses; (3) follow-up of existing literature on the use of the species and (4) chemotaxonomic approaches (Suffness and Douros 1979). Comparison of the four methods showed that the choice based on folklore has given about 25% more positive leads than other methods (Vlietinck and Vanden Berghe 1991). *H. aureonitens* was chosen for this research project on the basis of the verbal information collected from the indigenous people of the KwaZulu-Natal province of South Africa which was corroborated by five medical practioners in the province. According to these people, aqueous extracts from this plant have been used topically for ages against infections.

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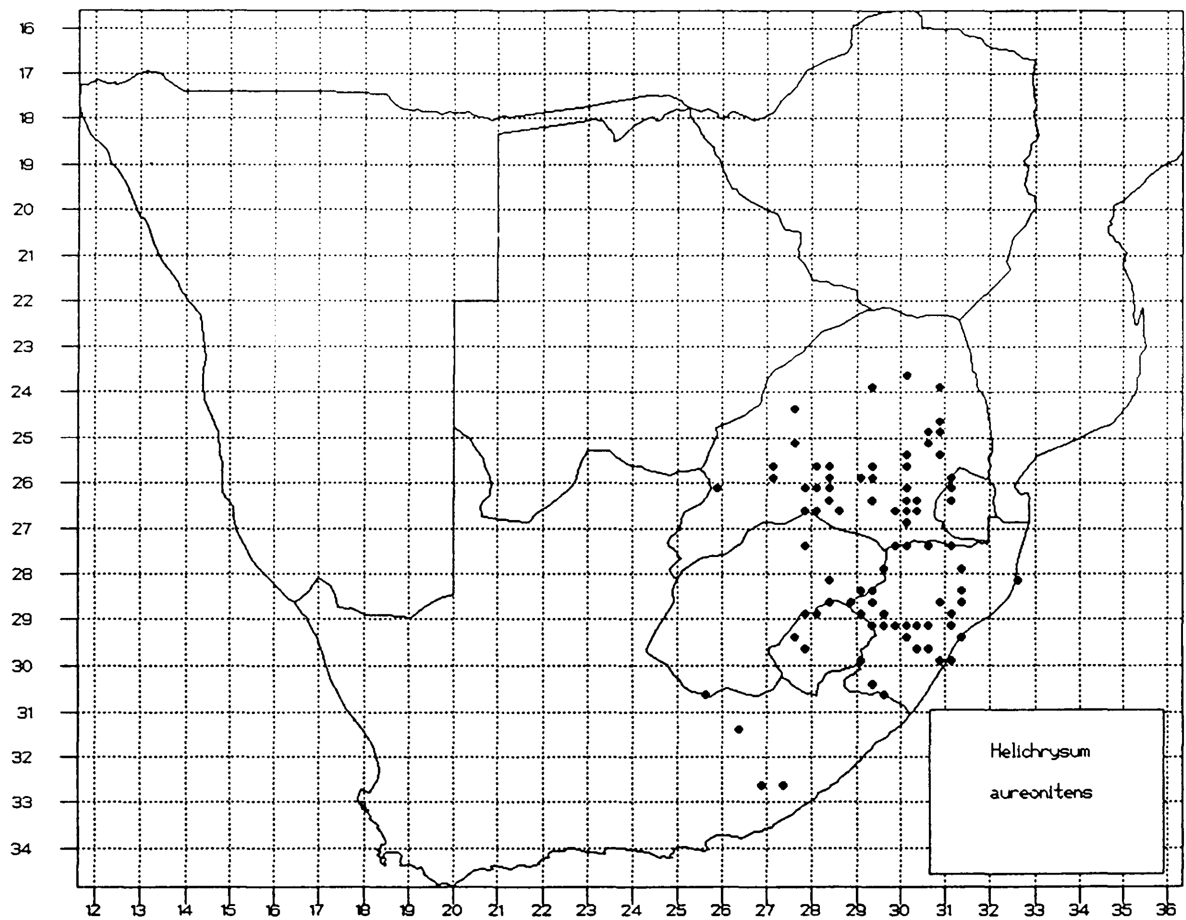

Figure 2: Distribution of *Helichrysum aureonitens* in southern Africa (Hilliard 1983).

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Scope of the thesis

Morphology and ultrastructure of the trichomes:

The stems and leaves of *H. aureonitens* are covered by densely arranged whitish epidermal hairs (trichomes) which render the plant visible from afar as grey patches in the grasslands. No information on the morphology and ultrastructure of these trichomes was available. Foliar trichomes are a common feature of plant surfaces. In some plants they are so dense that they form a purely physical obstruction to foraging insects. In addition, some trichomes are glandular and produce volatile substances which in some plant species inhibit insect feeding as well as producing a chemical barrier against propagules of pathogenic fungi (Franceschi and Giaquinta 1983).

Production of epicuticular compounds with antimicrobial properties has been reported in many plant species including some species of *Helichrysum*. In most cases, the sources of these compounds have been attributed to the trichomes (Wollenweber 1984). The eight antifungal flavones isolated from *H. nitens* were observed to be externally deposited on the leaves and stems of the plant (Tomas-Barberan *et al.* 1988) and various antimicrobial compounds from *H. decumbens, H. graveolens, H. italicum* and *H. stoechas* were isolated from the rinses of the leaves and stems of these plants for two minutes in dichloromethane or chloroform, an indication of the external deposition of the compounds (Tomas-Lorente *et al.* 1989; Tomas-Barberan *et al.* 1990). The glandular trichomes were reported to be the sites of accumulation of the antimalarial drug, artemisinin, produced by *Artemisia annua* (Klayman 1985; Duke and Paul 1993)

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Developmental and structural studies of trichomes, according to Franceschi and Giaquinta (1983), can shed light on the nature of the secreted material and the functional significance of the glands. It is possible that the medicinal compounds produced by *H. aureonitens* are among the exudates produced on its leaves. One of the objectives of this study was to describe the morphology and ultrastructure of the foliar trichomes of this plant and to relate these observations to their possible functional role in the production of medicinal compounds.

Antibacterial activities of the extracts:

In the Spanish Mediterranean area (Andalucia, Cataluna, Valencia), antimicrobial herbs are popular among the inhabitants of the little towns and rural villages because of their spectacular efficacy especially in cases where conventional chemotherapy has failed (Rios *et al.* 1987). *H. italicum* and *H. stoechas* are among these herbs which are used to treat general infectious diseases. Extracts from both species have been reported to be active against *Staphylococcus aureus, Candida albicans* and *Mycobacterium phlei* (Rios *et al.* 1987). Similarly, the indigenous people of the KwaZulu-Natal province of South Africa have used *H. aureonitens* for the treatment of infections for ages. Another objective of this project was to test dichloromethane, methanol and water extracts from this plant against some bacteria species. For this purpose, 10 bacteria species were chosen at random consisting of five gram positive and five gram negative. The gram positive ones were *Bacillus cereus, B. pumilus, B. subtilis, Micrococcus kristinae* and *Staphylococcus aureus* while the gram negative species were *Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marsescens*.

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Antiherpes activity of aqueous extract:

The identification of a retrovirus, human immunodeficiency virus (HIV) as the causative agent of AIDS (acquired immunedeficiency syndrome), the steadily increasing incidence of various viral infections caused by viruses such as herpes simplex virus (HSV), varicella-zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) in immunodeficient patients and the socio-economic impacts of viral infections of the respiratory tract (influenza, adeno, corona and rhinoviruses) and gastrointestinal tract (rotaviruses) have all been important factors in boosting the search for new antiviral agents (Vlietinck and Vanden Berghe 1991). Although many compounds having potent antiviral activity in cell cultures and in experimental animals have been detected, at present, only a few have been approved for therapy of viral infections in humans. Unfortunately, none of these drugs is without toxicity. There is, therefore, a strong need not only to improve the actual antiviral armamentarium, but also to find an effective therapy of viral infections for which at present, no clinically useful drugs or vaccines are available.

Verbal ethnomedical information from some indigenous people has revealed that extracts from *H. aureonitens* have been used topically by Zulus against skin diseases for many years. Notably among these are herpes simplex (cold sore) and herpes zoster (chicken pox). One of the objectives of this project was to test extracts from *H. aureonitens* against herpes simplex virus type 1 (HSV-1). HSV-1 is an enveloped double stranded DNA virus which is known for causing a wide variety of clinical syndromes (Timbury 1986). It is commonly used *in vitro* for the preliminary screening for antiviral activities of drugs. This work was done in collaboration with the Department of Virology of the University of Pretoria.

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Isolation, purification and identification of the active compounds:

Many *Helichrysum* species have been examined for their chemical components. These include 38 species from South Africa (Jakupovic *et al.* 1986, 1989a), eight from Madagascar (Randriaminahy *et al.* 1992), several from Spain (Tomas-Barberan *et al.* 1988, 1990) and many speices from Australia (Jakupovic *et al.* 1989b). However, none of the studies included *H. aureonitens*.

After it was established that the *H. aureonitens* extracts had antibacterial and antiherpes activities, it became essential to isolate and identify the active compound(s) in these extracts. Through the activity-guided fractionation by bioautography of the acetone extract, two closely related bioactive compounds were isolated and identified. Another objective of this study was to confirm and determine the minimum inhibitory concentration (MIC) of the main bioactive compound (galangin) against each of the bacteria used in the previous tests and further screen the compound on some selected fungal species. In addition, galangin was once again tested against HSV-1 and also on reovirus, adenovirus and coxsackievirus. While HSV-1 and adenovirus are double stranded DNA viruses, the genetic material of reovirus and coxsackie virus consists only of RNA.

The cultivation of many species of Asteraceae has been limited by poor germination (Sharman 1993; Bunker 1994). Efforts to improve seed germination in the genus *Helichrysum* have received attention from a few workers (Mott 1972; Willis and Groves 1991; Brown 1993). Self propagation in *H. aureonitens* is mainly by resprouting from roots. In an effort to improve seed germination in this herb, the effects of temperature, light, gibberellic acid,

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scarification and smoke extract on the germination of its seeds were investigated in the light and dark. The viability as well as the average mass of seeds were also determined.

Structure of the thesis

This thesis consists of contributions in the form of reprints of published articles (Chapters 2, 3 and 4) and three manuscripts submitted for publication (Chapters 5, 6 and 7).

The morphology and ultrastructure of secreting and nonsecreting foliar trichomes of this plant are described in Chapter 2. Efforts to link the glandular trichomes on the leaves and stems to the sites of accumulation of the antimicrobial compounds found in the epicuticular extracts from the plant is reflected in the discussion of the chapter. The antibacterial activities of the dichloromethane and methanol extracts obtained by shaking the aerial parts of the plant in these solvents are reported in Chapter 3, while Chapter 4 reports on the antiherpes activity of the aqueous extract. Chapter 5 describes the isolation, purification and eventual identification of the main active compound in the acetone extract from the shoots of *H. aureonitens*. The MIC of the active compound on 10 species of bacteria and its activity on 6 selected fungi species are also reported in this chapter. Chapter 6 is concerned with the results of the bioassay of the active compound, galangin, on the four selected viruses. In Chapter 7, the germination response of the seeds of the plant under some chemical and environmental conditions is described.

In the General Discussion in Chapter 8, an attempt is made to distil out from all the chapters, a more coherent picture of the results of this study. In this Chapter the importance of

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secondary plant metabolites as possible future therapeutic remedies and the medicinal potential of *H. aureonitens* are emphasized.

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Chapter 2

Morphology and ultrastructure of secreting and nonsecreting foliar trichomes of *Helichrysum aureonitens* (Asteraceae)

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CHAPTER 2

MORPHOLOGY AND ULTRALSTRUCTURE OF SECRETING AND NONSECRETING FOLIAR TRICHOMES OF *HELICHRYSUM AUREONITENS* (ASTERACEAE)

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MORPHOLOGY AND ULTRASTRUCTURE OF SECRETING AND NONSECRETING FOLIAR TRICHOMES OF HELICHRYSUM AUREONITENS (ASTERACEAE)

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Electron microscopical examination of foliar appendages of *Helichrysum aureonitens* Sch. Bip. has revealed two types of trichomes. The nonsecreting type consisting of four cells form a dense mass of long, fibrous hairs, covering the entire surface of the shoot. The glandular secreting type are few in number, club shaped, and consist of several cells. Glandular trichomes consist of secretory cells with dense cytoplasm, free ribosomes, numerous cisternae of endoplasmic reticulum, and scattered mitochondria. Secretions accumulate in a cavity beneath a sheath derived from separation of the cuticularized outer wall surface of the top tier of secretory cells and are released to the outside when the cavity ruptures. Only the apical pair of cells participate in secretion. Cells of both types have plastids without thylakoids. We hypothesize that the active therapeutic compounds (probably flavonoids) secreted by *H. aureonitens* are produced by the endoplasmic reticulum in the glandular trichomes.

Introduction

Helichrysum aureonitens Sch. Bip. (Asteraceae) is a tufted perennial herb that is widespread in southern Africa (Hilliard 1983) and used in herbal medicine (Phillips 1917). Exudate of this herb is believed to be effective against *Herpes zoster* skin disease and *Herpes simplex* virus Type I in human lung-tissue cultures (J. J. M. Meyer et al., unpublished). Its stems and leaves are covered by densely arranged whitish epidermal trichomes that render the plant visible from afar as grey patches in grasslands. The morphology and ultrastructure of these hairs (trichomes) have not been reported.

Production of epicuticular flavonoids with antimicrobial properties have been reported in many species of *Helichrysum* (Tomas-Barberan et al. 1988, 1990; Tomas-Lorente et al. 1989; Cosar 1990). In most species, the source of these flavonoid exudates has been attributed to the trichomes (Wollenweber 1984).

Developmental and structural studies of trichomes, according to Franceschi and Giaquinta (1983), can shed light on the nature of the secreted material and the functional significance of the glands. It is possible that the antiviral compounds produced by *H. aureonitens* are among the flavonoid exudates produced on its leaves.

In this article, we examine the morphology and ultrastructure of foliar trichomes of *H. aureonitens* with scanning (SEMs) and transmission electron microscopes (TEMs). The objective of the research is to describe the morphology and ultrastructure of these trichomes and to relate our observations to their possible functional role in the production of antiviral compounds.

[1]Author for correspondence and reprints.

Manuscript received July 1994; revised manuscript received January 1995.

Material and methods

Transmission electron microscopy

Plants used in this study were grown in a phytotron at 25°C, with a light/dark period of 14/10 h and light level of 200 μmol m^{-2} s^{-1}. Leaves 6–8 mm in length were removed from the upper part of *Helichrysum aureonitens.* Slices of these were fixed in 2.5% glutaraldehyde for 1 h and postfixed for 2 h in 0.25% aqueous osmium tetroxide, both buffered with 0.075 M phosphate (pH 7.4) at room temperature. After rinsing with water, the tissue was dehydrated in a graded acetone series, gradually infiltrated with graded quetol in acetone and polymerized at 65°C for 36 h. Thin sections were cut on a Reichert-Jung Ultracut E microtome, stained with 4% aqueous uranyl acetate and Reynold's (1963) lead citrate, and examined with a Philips EM301 TEM at 60 kV.

Scanning electron microscopy

Leaf slices dehydrated in a graded acetone series were dried to critical point in liquid CO_2 and sputter-coated with gold. The specimens were examined with JEOL 840 SEM at 8 kV.

Results and discussion

The leaves of *Helichrysum aureonitens* have two types of epidermal appendages: glandular and nonglandular trichomes. The nonglandular hairs are long and abundant, forming a dense covering that completely obscures the epidermal surfaces (fig. 1*A*). A mature nonglandular trichome consists of four cells. The three bottom cells remain alive with dense cytoplasmic inclusions and relatively small vacuoles, while the apical cell, though highly vacuolated and many times elongated, has collapsed and is dead, thereby giving the trichome a more or less fibrous appearance (figs. 1*B–D,* 2*A*). Their abundance, distribution over the entire surface of the leaves, and fibrous

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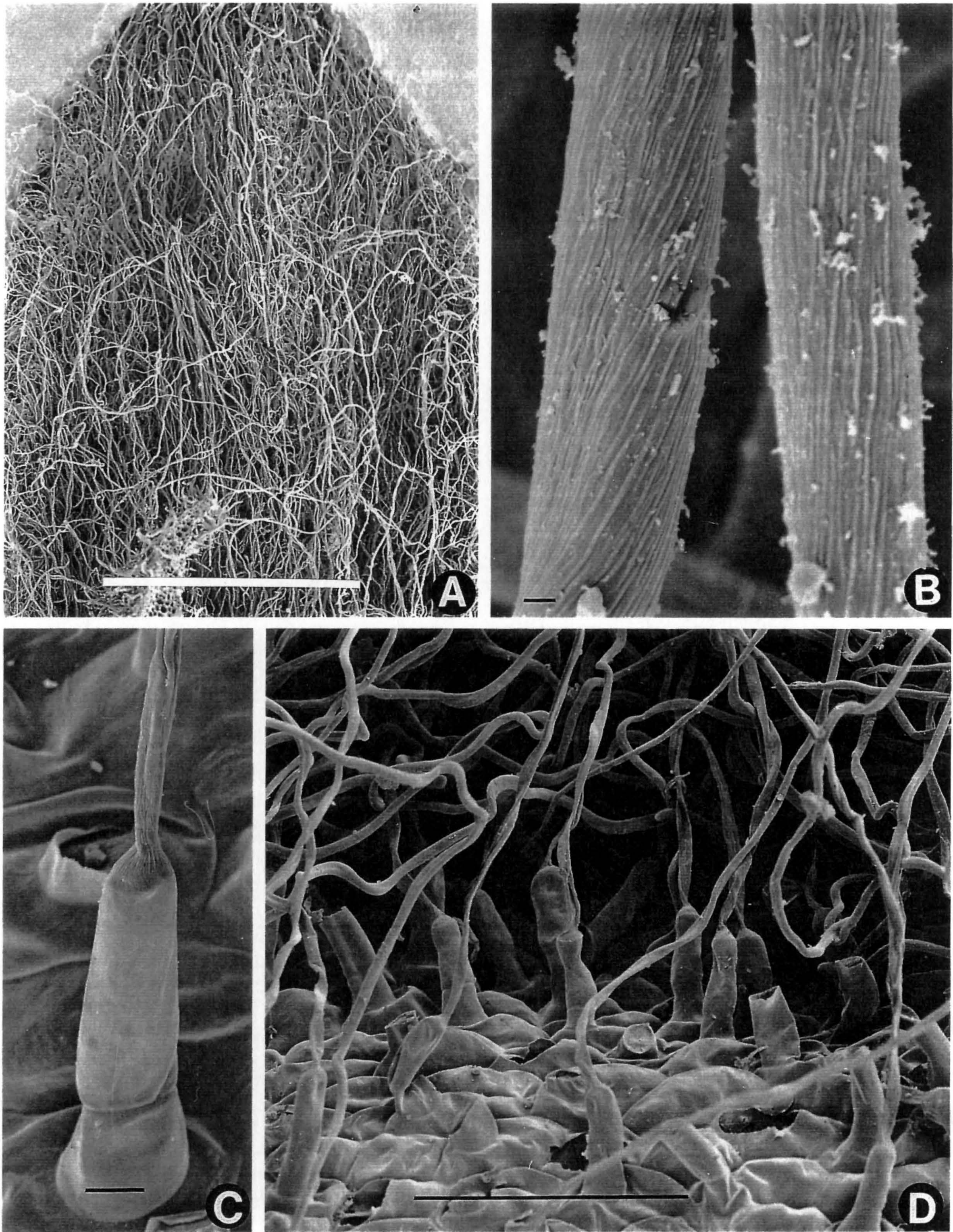

Fig. 1 SEM of nonsecreting foliar trichomes of *Helichrysum aureonitens*. *A,* Abaxial surface of leaf with dense mass of nonglandular trichomes, which completely obscure the epidermis. Scale bar = 1 mm. *B,* High magnification of the fibrous part of trichome. Scale bar = 1 μm. *C,* A nonsecreting trichome; note two of three living and one dead (fibrous) cells. Scale bar = 10 μm. *D,* High magnification of abaxial surface revealing the morphology of the trichomes. Scale bar = 100 μm.

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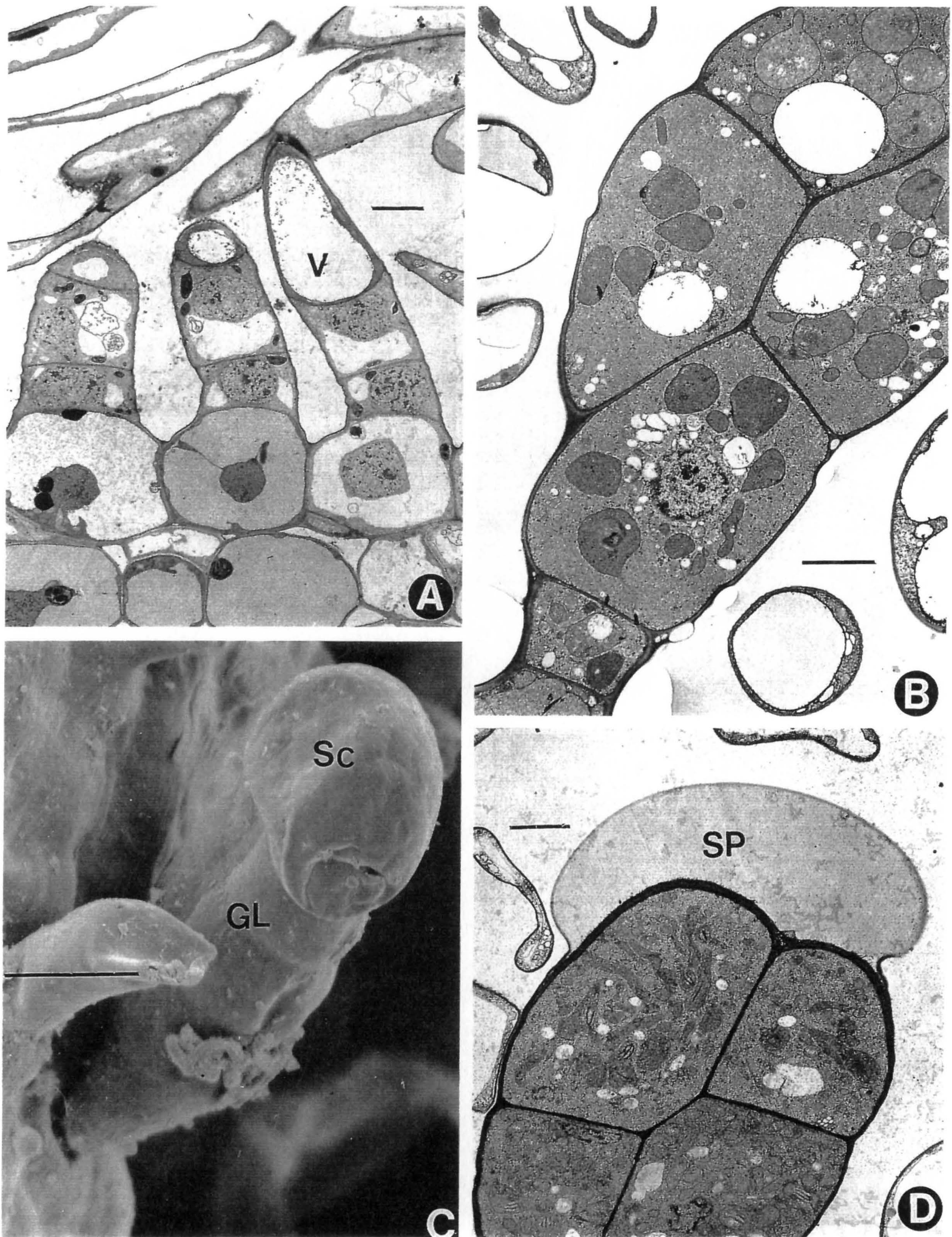

Fig. 2 EM of trichomes of *Helichrysum aureonitens. A,* Developing four-celled nonglandular trichomes. Note the high vacuolation of the apical cell (*V*) and the beginning of elongation. Scale bar = 2.5 μm. *B,* Club-shaped glandular trichome with two stalk cells prior to secretion. In lateral view, the biseriate nature of the trichome is obscured. Scale bar = 2.5 μm. *C,* SEM of mature secreting trichome. Note the biseriate arrangement of the gland cells (*GL*) and fully expanded secretory cavity (*Sc*) before rupturing. Scale bar = 10 μm. *D,* Face view of the secreting trichome. Note the biseriate arrangement of the head cells. The secretory product (*SP*) is noticeable within the subcuticular space formed over the apical pair of cells of the trichome. Scale bar = 2.5 μm.

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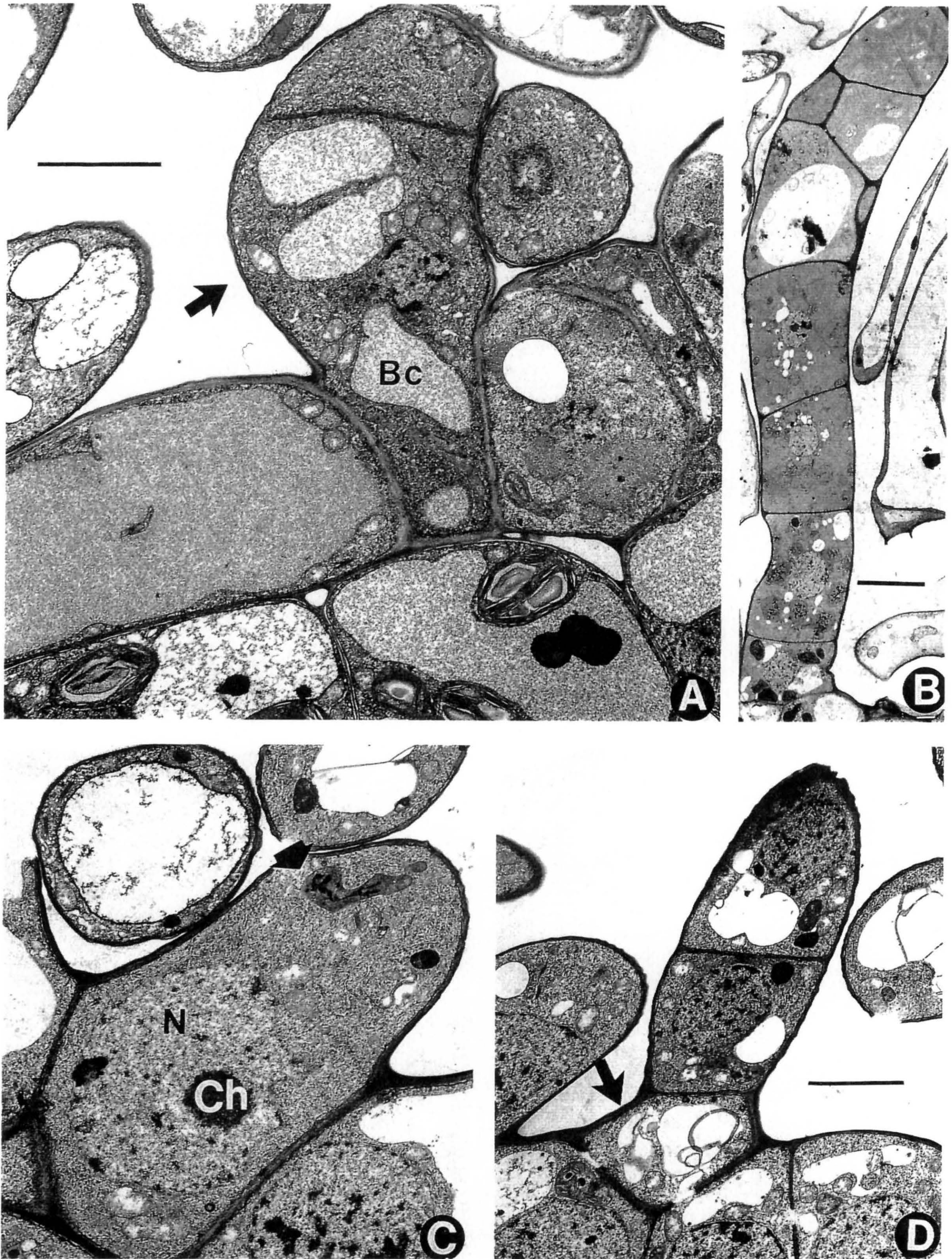

Fig. 3 TEM of early stages of development of glandular trichome of *Helichrysum aureonitens*. *A*, Young trichome after the first periclinal division (arrow). The basal cell (*BC*) grows out of the epidermal layer before division. Scale bar = 2.5 μm. *B*, Mostly uniseriate arrangement of cells are visible in the glandular trichomes. Scale bar = 5 μm. *C*, Elongating trichome initial (arrow) at one-celled stage prior to division. Note the large nucleus (*N*) and the condensed chromatin (*Ch*). Scale bar = 2.5 μm. *D*, The parent basal cell (arrow) grows out of the epidermal layer thereby exposing two-celled base for the glandular trichome. Scale bar = 2.5 μm.

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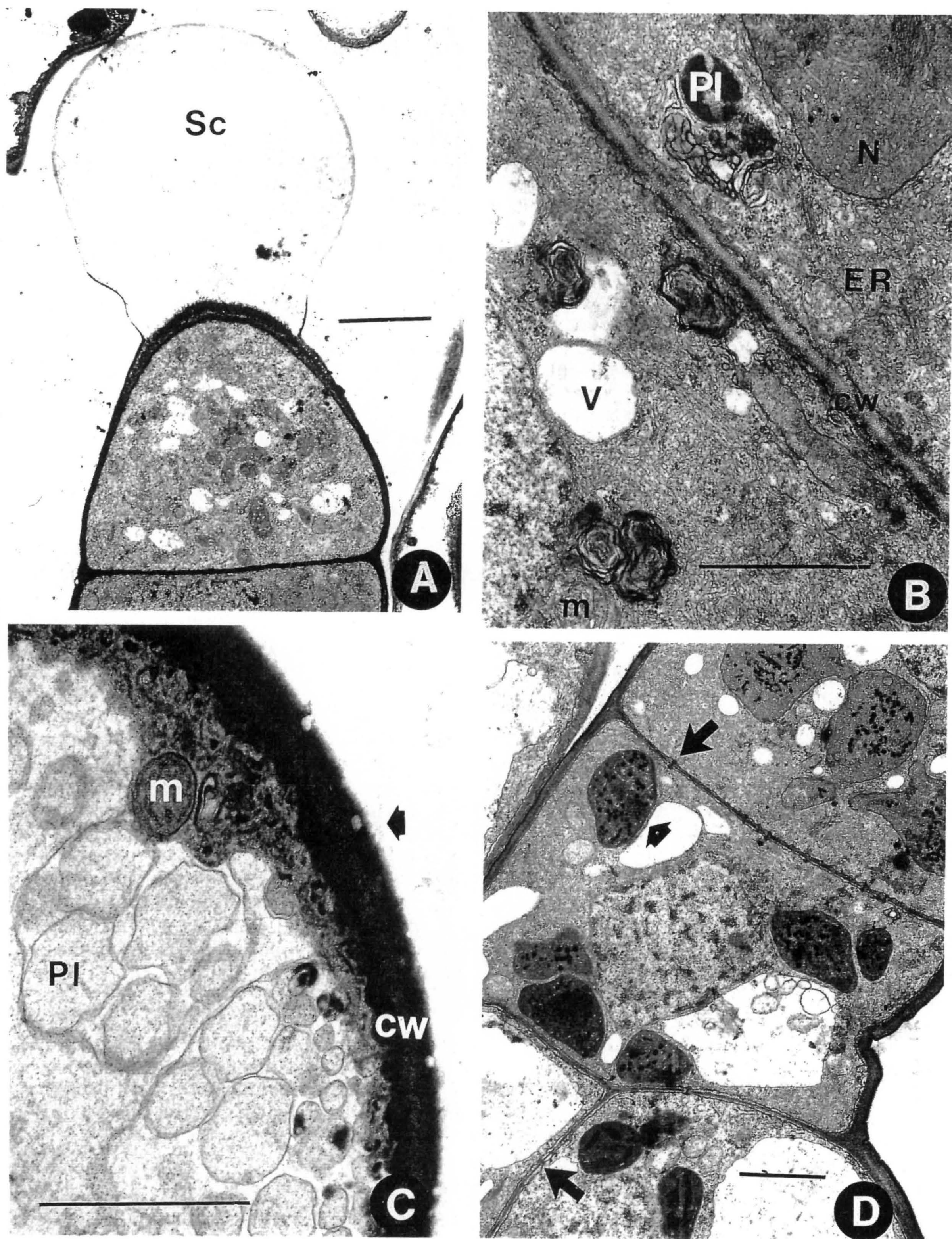

Fig. 4 Ultrastructure of secreting trichomes of *Helichrysum aureonitens*. *A*, Lateral view of secreting trichome with the bulbous secretory cavity (*Sc*). Scale bar = 2.5 µm. *B*, Enlarged portions of top tier of cells. *cw* = cell wall, *m* = mitochondria, *N* = part of a large nucleus, *ER* = endoplasmic reticulum, *V* = vacuole, and *Pl* = plastid. Scale bar = 1 µm. *C*, High magnification of part of the apical cell prior to secretion. Note an electron dense cuticle (arrow head) along the cell wall, and also the reticulate ingrowths formed by the cell wall (*cw*), which probably serve to increase the surface area of the inner wall. *m* = mitochondria, *Pl* = degenerated plastids. Scale bar = 1 µm. *D*, Bottom and stalk cells of a glandular trichome. Note the chloroplasts of bottom cells, the plastids (arrow head) without thylakoids of trichome cell and the plasmodesmata (arrows). Scale bar = 1 µm.

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nature indicate a role in protection of the aerial shoot of the plant, possibly against foraging insects and airborne propagules of fungi. A positive correlation between trichome density and insect resistance has been demonstrated in various plant species (Levin 1973).

Glandular trichomes are club shaped and are relatively short with two stalk cells and several gland cells (fig. 2*B–D*). In some trichomes, the gland cells are arranged biseriately (fig. 2*D*).

Both trichome types are initiated by an outgrowth of a single epidermal cell that divides periclinally to establish a uniseriate arrangement (fig. 3*A–D*). At very early stages in development, it may be difficult to distinguish between the two trichome types; however, as development continues, two basal cells become apparent in the young glandular trichome (fig. 3*D*), while the nonglandular trichome retains a one-celled base (fig. 2*A*). In addition, the apical cell of a nonglandular trichome is exceptionally long, dead, and fibrous with a large vacuole (fig. 2*A*).

The most striking features of secretory trichome cells are dense cytoplasm and a large nucleus with scattered chromatin and a prominent nucleolus. Other conspicuous features are numerous free ribosomes, scattered mitochondria, small plastids without thylakoids, and dictyosomes with Golgi vesicles (fig. 4*A–D*). Polyribosomes, numerous cisternae of ER and mitochondria with long cristae are evident at higher magnification (fig. 4*B*).

A distinct electron dense cuticle (fig. 4*A, C, D*) is continuous from the epidermis along the walls of the trichomes. Secretory products accumulate in the subcuticular space/secretory cavity above the upper tier of head cells (figs. 2*D*, 4*A*). We think only the apical pair of cells are secretory because cuticular separation only occurs in the region bordering the upper part (fig. 2*D*) of these cells. Formation of a subcuticular space inside of which secretory substances accumulate is common during secretion in many types of exotropic glands. The secretory products are usually released through pores in the cuticle (Schnepf 1974) or through breaks in the cuticle caused by external pressure (Hammond and Mahlberg 1977). Absence of pores in the cuticle of *H. aureonitens* signified that secretory products are probably released when the secretory cavity ruptures.

The cell walls of secretory cells are electron dense with reticulate ingrowths (fig. 4*C*). Ingrowth formation is characteristic of some secretory structures (Gunning 1977). This property may serve to increase surface area, possibly to facilitate export from the cytoplasm to the subcuticular space (Vermeer and Peterson 1979).

Plasmodesmata are observed in crosswalls between the bottom cells and adjacent leaf epidermal cells (fig. 4*D*). These intercellular connections, which permit free exchange of cytoplasmic materials, were not observable between gland cells of the trichomes (fig. 4*A*). However, the obscurity of plasmodesmata could possibly result from high electron density of the secretory materials that might be present in high concentrations at both sides of the cell wall. This same observation was reported in secretory trichomes of *Origanum dictamus* L. (Bosabalidis and Tsekos 1982).

Unlike the subepidermal cells that contain chloroplasts with well-developed stroma and granal stackings (figs. 3*A*, 4*D*), trichome cells of *H. aureonitens* contains numerous plastids without thylakoids. They are mostly present as organelles of increased electron density within the cytoplasm (fig. 2*B*). Werker and Fahn (1982) speculated that plastids function in the production or accumulation of lipophilic substances.

The mature gland cells at the secretory stage contain numerous dictyosomes (fig. 4*B*) that appear to be active in vesicle formation. The glands also contain an extensive network of ER and numerous mitochondria. ER, especially tubular ER, has been implicated in flavonoid secretion (Werker and Fahn 1982). Although ultrastructural studies alone do not provide the information required to establish sites of synthesis in cells, it is plausible to assume that the active therapeutic flavonoids are produced by the ER in the glandular trichomes.

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Chapter 3

Antibacterial activity of *Helichrysum aureonitens* (Asteraceae)

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CHAPTER 3

ANTIBACTERIAL ACTIVITY OF *HELICHRYSUM AUREONITENS* (ASTERACEAE)

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Short communication

Antibacterial activity of *Helichrysum aureonitens* (Asteraceae)

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Received 29 December 1994; revision received 16 March 1995; accepted 1 April 1995

Abstract

The antibacterial activity of extracts from *Helichrysum aureonitens* was investigated. The dichloromethane extract was active against all five gram positive bacteria tested and the methanol extract was active only against *Bacillus cereus*, *B. pumilus* and *Micrococcus kristinae*, while the water extract had no activity against any of the organisms. None of the extracts inhibited the growth of the five gram negative bacteria tested.

Keywords: *Helichrysum aureonitens*; Antibacterial; Medicinal

1. Introduction

Species of the genus *Helichrysum* have been widely reported for their antimicrobial activities (Tomas-Barberan et al., 1990). Ethanol extracts from *Helichrysum armenium*, *Helichrysum graveolens* and *Helichrysum plicatum* have been reported to be active against *Staphylococcus albus* and *Staphylococcus aureus* (Cosar and Cubukcu, 1990). The dichloromethane extract of *Helichrysum stoechas* was found to be active only on gram positive bacteria (Rios et al., 1991). Antifungal activity of *Helichrysum decumbens* and *Helichrysum nitens* has been respectively reported by Tomas-Barberan et al., (1988) and Tomas-Lorente et al., (1989). However, no such information is available on *Helichrysum aureonitens*. Information from folklore, revealed that the Zulu people of the Kwazulu-Natal province of South Africa have used this plant for centuries against infection.

Helichrysum aureonitens Sch. Bip. (Asteraceae), is a perennial herb which is widespread in southern Africa (Hilliard, 1983) and is widely used in folk medicine (Phillips, 1917). It is estimated that between 12 and 15 million South Africans use traditional remedies from as many as 700 indigenous plant species.

In this study, we examined the antibacterial effects of extracts of *Helichrysum aureonitens* on ten selected bacteria species.

2. Materials and methods

2.1. Plant material

Shoots of *Helichrysum aureonitens* (excluding

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flowers) were collected from the Kwazulu-Natal province of South Africa in September 1994. A voucher specimen (Afol. 2001) of the herb was prepared and deposited at the National Botanical Institute of South Africa, Pretoria.

2.2. Preparation of extracts

Portions of 150 g each of air dried plant material were shaken separately for 5 min in dichloromethane (CH_2Cl_2), methanol (MeOH) and water (H_2O). The three extracts were filtered and concentrated to dryness under reduced pressure.

Plant extracts were introduced aseptically into sterile Petri dishes by filtering through 0.22 μm syringe fitted filters. To test at 1 mg/ml, 10 mg of extract was dissolved in 0.2 ml of solvent and added to 9.8 ml of nutrient medium. MeOH was chosen as solvent because, in addition to dissolving the extracts completely, it showed no inhibition of the cultures at this level (2% final concentration). Nutrient agar (Biolab) was prepared in the usual fashion by autoclaving before the addition of the extracts. Before congealing, 10 ml of agar medium containing the plant extract was added to each Petri dish and swirled carefully until the agar began to set.

2.3. Bacteria

Ten selected bacteria species were collected from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on nutrient agar slant (Biolab) and was recovered for testing by growth in nutrient broth no. 2 (Biolab) for 24 h. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth.

2.4. Antibacterial testing

The organisms were streaked in radial patterns on the agar plates (Mitscher et al., 1972). Plates were incubated at 37°C and examined after 24 and 48 h. Complete suppression of growth was required for an extract to be declared active. The three extracts were tested at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml. Two blank plates containing only nutrient agar and two containing nutrient agar and 2% MeOH without the extracts, served as controls. Each test was replicated 3 times.

3. Results and discussion

CH_2Cl_2, MeOH and H_2O extracts from *Helichrysum aureonitens* showed no activity against gram negative bacteria. However, CH_2Cl_2 and MeOH extracts indicated antibacterial activity with significant inhibition against gram positive bacteria (Table 1). Both extracts inhibited the growth of *Bacillus cereus, Bacillus pumilus*, and *Micrococcus kristinae*. In addition, the CH_2Cl_2 extract was active against *Bacillus subtilis* and

Table 1
Antibacterial activity of the methanol, dichloromethane and water extracts from shoots of *Helichrysum aureonitens*

Bacteria species	Gram +/−	MIC (mg/ml)[a]		
		MeOH	CH_2Cl_2	H_2O
Bacillus cereus	+	1.0	0.5[b]	na[c]
Bacillus pumilus	+	2.5	1.0	na
Bacillus subtilis	+	na	2.0	na
Micrococcus kristinae	+	0.5	0.5	na
Staphylococcus aureus	+	na	1.5	na
Enterobacter cloacae	−	na	na	na
Escherichia coli	−	na	na	na
Klebsiella pneumoniae	−	na	na	na
Pseudomonas aeruginosa	−	na	na	na
Serratia marcescens	−	na	na	na

[a]Minimum inhibitory concentration.
[b]Lowest concentration of extracts tested.
[c]Not active.

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Staphylococcus aureus. The minimum inhibitory concentrations (MIC) for *Bacillus cereus* and *Bacillus pumilus*, of the CH_2Cl_2 extract were lower than those of the MeOH extract. The two extracts, however, inhibited the growth of *Micrococcus kristinae* at 0.5 mg/ml which was the highest dilution used in this study. The water extract had no activity on any of the organisms tested. Inhibition of only gram positive bacteria was similarly reported on the extracts of *Helichrysum stoechas* (Rios et al., 1991).

Different *Helichrysum* species produce different secondary metabolites (phloroglucinols, acetophenones, flavonoids) as a biochemical defence mechanism (chemical barriers) against microorganisms (Tomas-Barberan et al., 1990). This indicates the use of different metabolic pathways to produce chemical barriers which has a single ecological role of defence against bacteria and fungi.

Work is in progress on the isolation and identification of the antibacterial compound from *Helichrysum aureonitens*.

Acknowledgement

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CHAPTER 4

INHIBITION OF HERPES SIMPLEX VIRUS TYPE 1 BY AQUEOUS EXTRACTS FROM SHOOTS OF *HELICHRYSUM AUREONITENS* (ASTERACEAE)

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Inhibition of herpes simplex virus type 1 by aqueous extracts from shoots of *Helichrysum aureonitens* (Asteraceae)

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Abstract

Helichrysum aureonitens, a southern African medicinal plant reported to have antibacterial properties, was evaluated for antiviral activity against herpes simplex virus type 1 (HSV-1) in vitro. The crude aqueous extract from shoots of *H. aureonitens* at a concentration of 1.35 mg/ml (w/v) showed significant antiviral activity on HSV-1 in human lung fibroblasts as demonstrated by the absence of a cytopathic effect.

Keywords: Anti-herpes; Extract; HSV-1; *Helichrysum aureonitens*; Medicinal

1. Introduction

Antimicrobial properties of the genus *Helichrysum* have been widely reported in the literature (Tomas-Barberan et al., 1988, 1990; Tomas-Lorente et al., 1989; Cosar and Cubukcu, 1990; Rios, 1991; Meyer and Afolayan, 1995). These reports have focused on the antifungal and antibacterial properties of the members of the genus, while no information is available on the possible antiviral activity of these plants.

Helichrysum aureonitens Sch. Bip. (Asteraceae) is a tufted perennial herb which grows predominantly in southern Africa (Hilliard, 1983) and is widely used in herbal medicine (Phillips, 1917). According to traditional healers, the people of the Kwazulu-Natal province have been using extracts from this plant topically for many generations against skin infections. Ethnomedical information from the region revealed that exudate from *H. aureonitens* has been used topically against herpes zoster. It is estimated that between 12 and 15 million South Africans still depend on traditional herbal medicine from as many as 700 indigenous plant species.

In this study, we examined the antiviral effect of the aqueous extracts from *H. aureonitens* on herpes simplex virus type 1 (HSV-1) in vitro.

2. Materials and methods

2.1. Plant collection and preparation of extract

Shoots of *H. aureonitens* (excluding flowers) were collected from the Kwazulu-Natal province

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of South Africa in September, 1994. A voucher specimen (Afol. 2001) of the herb was prepared and deposited at the National Botanical Institute of South Africa, Pretoria.

Non-homogenised plant material (100 g) was boiled in 1750 ml of distilled water for 30 min under reflux. The extract was filtered and concentrated to dryness at reduced pressure. The dry extract was later dissolved in water to a final concentration of 270 mg/ml.

2.2. Cell culture

Standard cell culture techniques, as outlined by Grist et al. (1979), were used for all procedures utilizing cell cultures. Monolayers of secondary human lung fibroblast (HF) cells were prepared by seeding 25 cm^3 flasks or 96-well microtitre trays with 10^5 cells/ml. Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) Ltd, Kelvin, South Africa), supplemented with 10% heat inactivated (56°C for 30 min) foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) and containing 100 U/ml penicillin and 100 μg/ml streptomycin was used for the propagation of the cells. Cell cultures were incubated in a humidified CO_2 atmosphere (4% CO_2/96% filtered air) at 37°C. Maintenance medium (MM) was essentially the same as the propagation medium except that it contained only 2% FCS.

2.3. Virus stock

A stock suspension of virus, with a titre of $10^{6.5}$ $TCID_{50}$/ml, was prepared from a clinical isolate of HSV-1. The virus suspension was diluted in MM and used at a final concentration of 100 $TCID_{50}$/ml.

2.4. Cytotoxicity assay

The plant extract was tested for cytotoxicity by exposing monolayers of HF cells to dilutions of the filtered sterilised extract at 37°C. Doubling dilutions of the extract in MM, from a concentration of 8.44 mg/ml to 0.53 mg/ml, were used and the cells monitored over a period of six days for cytotoxic effects. Monolayers of cells exposed to MM alone were used as a control.

2.5. Antiviral assay

Dilutions of the plant extract were tested for antiviral activity at the final concentrations of 8.44, 4.22, 2.70, 2.11, 1.80, 1.35, 1.17, 1.05 and 0.53 mg/ml. The final concentration of the virus in the assay was 100 $TCID_{50}$/ml. 2 ml of plant extract and 2 ml of the viral suspension, both diluted in MM, were added simultaneously to monolayers of HF cells in 25 cm^3 flasks. Prior to incubation at 37°C, the inoculated flasks were gently swirled to ensure the even distribution of virus and extract over the cell monolayer. As a positive control, cells were infected with virus without added extract, and as a cell control only MM was added to the cells. Cells were examined daily, by light microscopy, for the appearance of a cytopathic effect (CPE). The absence of CPE at a specific concentration of extract was considered to be indicative of antiviral activity.

3. Results and discussion

After one week, even at the high concentration of 8.44 mg/ml, HF cells treated with aqueous extract of *H. aureonitens* alone did not exhibit altered morphology or growth characteristics indicative of cytotoxic effects.

In the assay to assess the possible antiviral properties of the extract, the virus control showed extensive CPE 36 h after infection, whereas the HSV-1 infected cells treated with the extract showed no CPE after one week. The plant extract exhibited significant antiviral activity at a concentration of 1.35 mg/ml (w/v).

Extracts from *Cedrela tubiflora* and *Pongamia pinnata* have been reported to be active against HSV-1 at concentrations of 1.0 mg/ml and 0.108 mg/ml, respectively (Cordoba et al., 1991; Elancheshiyan et al., 1993). Although the minimum inhibitory concentration (MIC) of extract from *H. aureonitens* on HSV-1 is higher than the 7.2 μg/ml of pure aloe emodin from *Aloe barbadensis* leaves (Sydiskis et al., 1991), further purification of the extract will permit the isolation of the active compound and decrease the level of its actual effective dose. We have embarked on the purification and chemical characterization of the active principles and analysis of their antiviral properties.

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Chapter 5

The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*

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CHAPTER 5

THE ANTIMICROBIAL ACTIVITY OF 3,5,7-TRIHYDROXYFLAVONE ISOLATED FROM THE SHOOTS OF *HELICHRYSUM AUREONITENS*

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[a]The antimicrobial activity of 3, 5, 7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*

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Abstract

Extracts from *Helichrysum aureonitens* are used topically by the indigenous people of South Africa against infections. The antimicrobial activity-guided fractionation by bioautography of the acetone extract from the aerial parts of *H. aureonitens* led to the isolation of 3,5,7-trihydroxyflavone (galangin). Evaluation of the antibacterial activity of the compound against 10 randomly selected bacteria indicated significant activity against all the gram positive bacteria tested with the MIC ranging from 0.1 to 0.5 mg/ml. The compound was not active on gram negative bacteria except for *Enterobacter cloacae* which was significantly inhibited at an MIC of 0.1 mg/ml. Galangin indicated considerable activity against the fungi tested with the exception of *Cladosporium herbarum*. *Penicillium digitatum* and *P. italicum* appeared to be particularly susceptible at a concentration of 0.01 mg/ml.

Keywords: 3,5,7-trihydroxyflavone, antimicrobial, galangin, *Helichrysum aureonitens*, medicinal.

[a]Written in the format and submitted to the Journal of Ethnopharmacology.

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Introduction

Helichrysum aureonitens Sch. Bip. (Asteraceae) is a hairy perennial herb which grows mostly in the KwaZulu-Natal province of South Africa. It belongs to a large genus of about 500 species with 246 growing in South Africa. Extracts from *Helichrysum aureonitens* are used topically by the indigenous people of South Africa against infections.

The antimicrobial activities of extracts from *Helichrysum* species have been widely reported (Tomas-Barberan et al., 1988; Tomas-Lorente et al., 1989; Cosar and Cubukcu, 1990; Tomas-Barberan et al., 1990; Rios, et al. 1991). However, the compounds responsible for these activities have been identified in only a few cases. The antibacterial and antiherpes activities of extracts from *H. aureonitens* were recently reported from our laboratory (Meyer and Afolayan, 1995; Meyer et al., 1996).

In this paper we report on the isolation and structural elucidation of the main antimicrobial compound found in the epicuticular acetone extract of this herb. We also report our observations on the inhibitory properties of this compound on some bacteria and fungi.

Materials and methods.

Plant material

Aerial parts of *H. aureonitens* (excluding flowers) were collected from the KwaZulu-Natal province of South Africa and a voucher specimen (*Afol. 2001*) of the herb was prepared and deposited at the National Botanical Institute of South Africa, Pretoria.

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Extraction and isolation of the antimicrobial compound.

The air-dried aerial parts (200 g) were shaken in acetone for 5 minutes. The resultant extract was concentrated to dryness and gave 2.362 g of dry matter. This was dissolved in acetone and subjected to column chromatography on Sephadex LH-20 (Pharmacia) and eluted with absolute ethanol. The different fractions obtained were tested for antimicrobial activity by direct bioautography on TLC plates using *Bacillus subtilis* (Mitscher et al., 1972). The main active fraction was further purified by preparative TLC on silica gel 60 F_{254} (MERCK) with acetone:chloroform (2:25) and by HPLC on a reverse-phase column (Phenomenex C-18, 5 μm, 10 x 250 mm) eluted with chloroform:aceteone (40:60) at a flow rate of 1.5 cm^3/min. The eluant was monitored with a variable wavelength UV-VIS detector at 366 nm. Finally, 122.6 mg of the active compound was isolated.

Addition of the bioactive compound (galangin) to the nutrient medium.

Nutrient agar (NA) for the bacteria and potato dextrose agar (PDA) for the fungi (both Biolab) were prepared in the usual fashion by autoclaving before the addition of the bioactive compound. Galangin was introduced aseptically into sterile Petri dishes by filtering through sterile 0.22μm syringe fitted filters. To test at 0.5 mg/ml, 5 mg of the compound was dissolved in 0.1 ml of solvent and added to 9.9 ml of nutrient medium. Before congealing, 10 ml of agar medium containing galangin was added to each Petri dish, swirled carefully until the agar began to set and left overnight for acetone to evaporate. Acetone was chosen as solvent because in addition to dissolving the compound completely, it showed no inhibition of the microbes at this level (1% final concentration).

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Antibacterial testing

Ten selected bacteria species were obtained as previously described (Meyer and Afolayan, 1995). Each organism was maintained on NA slant (Biolab) and was recovered for testing by growth in nutrient broth No 2 (Biolab) for 24 h. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth.

The organisms were streaked in radial patterns on the agar plates (Mitscher et al., 1972). Plates were incubated at 30°C and examined after 24 and 48 hr. Complete suppression of growth by a specific concentration of galangin was required for it to be declared active. The compound was tested at 0.5, 0.1, 0.05 and 0.01 mg/ml (three replicates). Blank plates containing only nutrient agar and others containing nutrient agar and 1% acetone without galangin served as controls.

Antifungal testing

Six fungal species were obtained fom the Institute of Plant Protection, Pretoria. Each culture was maintained on PDA and was recovered for testing by subculturing in fresh PDA for 3 days. The prepared plates containing galangin at concentrations of 0.5, 0.1, 0.05, and 0.01 mg/ml respectively, were innoculated with plugs obtained from the actively growing margin of the fungi plates and incubated at 25°C for 3 days. Fungal growth was measured and expressed as means of percentage growth inhibition of three replicates. Significance differences within the means of the treatments and the control were calculated using the LSD statistical test.

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Results

The antimicrobial activity-guided fractionation by bioautography of acetone extract from *H. aureonitens* led to the isolation of the bioactive galangin (3,5,7-trihydroxyflavone). Spectra data were compared with those found in the literature (Markham et al., 1982; Markham and Geiger, 1993). ^{1}H-NMR (in acetone- d_6): 6.28 (1H, d J=2Hz, H-6), 6.55 (1H, d J=2Hz, H-8), 7.49-7.57 (3H, m, H-3', H-4', H-5'), 8.25 (2H, dd J=1.5Hz and 8.0Hz, H-2', H-6'). ^{13}C-NMR (in acetone- d_6): 94.5 (C-8), 99.3 (C-6), 104.4 (C-10), 128.4 (C-2', C-6'), 129.3 (C3', C-5'), 130.7 (C-1'), 137.9 (C-3), 147.0 (C-2), 158.0 (C-9), 162.3 (C-5), 165.3 (C-7), 176.9 (C-4). MS *m/z*: 270 (M^+)

Evaluation of the antibacterial activity of the compound against 10 randomly selected bacteria indicated significant activity against all the gram positive bacteria tested (Table 1). However, the minimum inhibitory concentration (MIC) was higher on *Bacillus pumilus, B. subtilis* and *Staphylococcus aureus* than on *Bacillus cereus* and *Micrcoccus kristinae*. The compound was not active on gram negative bacteria except for *Enterobacter cloacae* which was significantly inhibited at an MIC of 0.1 mg/ml.

Although not fungicidal, galangin caused appreciable inhibition of growth against the fungi tested with the exception of *Cladosporium herbarum*. *Penicillium digitatum* and *P. italicum* appeared to be particularly susceptible at 0.01 mg/ml (Table 2).

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Table 1. Antibacterial activity of 3,5,7-trihydroxyflavone isolated from shoots of *H. aureonitens*.

Bacteria species	Gram +/-	MIC (mg/ml)[a]
Bacillus cereus	+	0.1
B. pumilus	+	0.5[b]
B. subtilis	+	0.5
Micrococcus kristinae	+	0.1
Staphylococcus aureus	+	0.5
Enterobacter cloacae	-	0.1
Escherichia coli	-	na[c]
Klebsiella pneumoniae	-	na
Pseudomonas aeruginosa	-	na
Serratia marcescens	-	na

[a]Minimum inhibitory concentration

[b]Maximum concentration of compound tested.

[c]Not active

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Table 2. Antifungal activity of 3,5,7-trihydroxyflavone isolated from shoots of *H. aureonitens*

Values are means of percentage growth inhibition of three replicates; values within a column followed by the same superscript are not significantly different at $P < 0.05$ according to the LSD test.

Treatment	Growth inhibition (%)					
	AF	CS	AT	PD	CH	PI
0.01mg/ml	8.8^{c}	16.7^{d}	22.2^{b}	29.9^{b}	0.0^{c}	29.4^{a}
0.05mg/ml	14.3^{b}	25.4^{c}	8.3^{c}	29.9^{b}	7.0^{ab}	20.6^{ab}
0.1mg/ml	16.5^{b}	34.2^{b}	18.5^{b}	29.9^{b}	9.3^{a}	17.6^{ab}
0.5mg/ml	30.8^{a}	46.5^{a}	35.2^{a}	39.1^{a}	14.0^{a}	29.4^{a}
Acetone	3.3^{d}	4.4^{e}	-5.6^{d}	9.2^{c}	-2.4^{c}	8.8^{bc}
Control	0.0^{d}	0.0^{e}	0.0^{cd}	0.0^{d}	0.0^{bc}	0.0^{c}

AF, *Aspergillus flavus*; CS, *Cladosporium sphaerospermum*; AT, *Aspergillus tamarii*; PD, *Penicillium digitatum*; CH, *Cladosporium herbarum*; PI, *Penicillium italicum*.

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Discussion

Galangin, was first isolated in 1881 from the root of galangal, *Alpinia officinarum* (Wollenweber, 1988) and synthesised by Heap and Robinson (1926). Since then, it has been found in extracts of many angiosperms (Ferraro et al., 1981; Wollenweber et al., 1985; McDowell et al., 1988; Sabatier et al., 1992). Galangin together with chrysin, tectochrysin, pinobanksin and pinocembrin are characteristic flavonoids from propolis (bee glue) and bee wax (Tomas-Barberan et al., 1993; Ferreres et al., 1994; Johnson et al., 1994; Soler et at., 1995). It is a major constituent of fruits and vegetables which lowers cancer risk in humans with higher dietary intake of fruits and vegetables (Phang et al., 1993). It was, however, implicated together with quercetin and kaempferol in the increase in the frequencies of chromosomal aberrations and mutations in Chinese hamster ovary cells (Middleton and Kandaswami, 1993). Information on the antimicrobial property of galangin is scanty. Nishino et al. (1987) reported galangin as the most active compound of the 26 flavonoids tested on *Staphylococcus epidermidis*, Chabot et al. (1992) observed a reduction in the hyphal growth of *Gigaspora margarita* by galangin while Kanazawa et al. (1995) reported on the desmutagenic activity of galangin and quercetin on *Salmonella typhimurium*.

We have earlier reported the antibacterial activity of the dichloromethane and methanol extracts from this plant against gram positive bacteria (Meyer and Afolayan, 1995) with no visible activity on the gram negative bacteria including *E. cloacae*. This was probably due to the relatively low concentration of galangin in the crude methanol and dichloromethane extracts. The antimicrobial property of galangin might be associated with its lack of hydroxyl groups on the B ring. Chabot et al. (1992) observed that flavonoids lacking hydroxyl groups on the B ring are known for their antimicrobial activities. Six flavones that are externally

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deposited on the leaf and stem surfaces of *H. nitens* from Malawi showed antifungal activity against *Cladosporium cucumerinum*. The antifungal activity decreases dramatically when the methyl group at position 5 is removed (Tomas-Barberan et al. 1988).

In this study, we observed galangin to be a major constituent of the epicuticular extract from *H. aureonitens* (5.17%). The ability of galangin to inhibit the growth of all the gram positive bacteria tested, *Enterobacter cloacae*, a gram negative bacterium and a number of fungal species is an indication of the compound as a broad spectrum antimicrobial agent. This probably explains the use of extracts from this plant by the indigenous people of South Africa against a number of infections for generations.

Acknowledgement

This study was financed by a grant from the Foundation for Research Development of South Africa.

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Chapter 6

Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*

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CHAPTER 6

ANTIVIRAL ACTIVITY OF GALANGIN ISOLATED FROM THE AERIAL PARTS OF *HELICHRYSUM AUREONITENS*

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[a]Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*.

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Abstract

The *in vitro* antiviral activity of galangin (3,5,7-trihydroxyflavone), the major antimicrobial compound isolated from the shoots of *Helichrysum aureonitens*, was investigated against herpes simplex virus type 1 (HSV-1), coxsackie B virus type 1 (Cox B1), adenovirus type 31 (Ad31) and reovirus. At concentrations ranging from 12-47 μg/ml galangin showed significant antiviral activity against HSV-1 and CoxB1 , limited activity against reovirus, and no antiviral activity against Ad31.

Keywords: 3,5,7-trihydroxylflavone, antiviral, galangin, *Helichrysum aureonitens*, medicinal.

[a]Written in the format and submitted to the Journal of Ethnopharmacology.

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1. Introduction

The importance of ethnopharmacological investigations in the discovery of new therapeutic agents from plants has been discussed extensively (Malone, 1983; Vlietinck and Vanden Berghe, 1991). Acute, recurrent and chronic viral infections occur worldwide and several antiviral compounds have been introduced into therapeutic use during the past decades (Meyers et al., 1982; Merigan, 1995). The variability in efficacy of these antiviral compounds in recurrent and chronic infections, and in immunodeficient patients, as well as the problem of prohibitive costs in developing countries have necessitated the search for alternative drugs (Elanchezhiyan et al., 1993).

The people of the KwaZulu-Natal province of South Africa have been using extracts from *Helichrysum aureonitens* topically for many generations against skin infections, especially herpes zoster and infections associated with herpes simplex virus (HSV)(Meyer et al., 1996). The antibacterial activity of the crude dichloromethane and methanol extracts of *Helichrysum aureonitens* shoots, and the *in vitro* antiviral activity of the aqueous extract against HSV type 1 (HSV-1) at a concentration of 1.35 mg/ml have been reported previously (Meyer and Afolayan, 1995; Meyer et al., 1996). The bioactive-guided fractionation of the epicuticular acetone extract of this plant using *Bacillus subtilis* has revealed 3,5,7-trihydroxyflavone (galangin) as the major antimicrobial compound present in the extract (Afolayan and Meyer, submitted for publication).

In this study we examined the effect of galangin *in vitro*, on HSV-1, coxsackie B virus type 1 (Cox B1), adenovirus type 31 (Ad31) and reovirus.

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2. **Materials and methods**

2.1. *Stock solution of galangin*

A stock solution (60 mg/ml) of galangin (Aldrich, Germany) was prepared in cell culture tested dimethyl sulfoxide (DMSO)(Sigma, St.Louis, USA) from which aliquots were diluted with Eagle's minimum essential medium (MEM) to obtain the desired concentrations.

2.2. *Cell culture*

Two types of cell cultures were used: primary vervet monkey kidney (VK) cells and the human hepatoma cell line, PLC/PRF/5 (ATCC CRL 8024). Standard cell culture techniques, as outlined by Grist *et al.* (1979), were used for all procedures utilizing cell cultures. Monolayers of VK and PLC/PRF/5 were prepared separately by seeding 25 cm^3 flasks or 96-well microtitre trays with 10^5 cells/ml. Eagle's MEM (Highveld Biological (Pty) Ltd, Kelvin, South Africa), supplemented with 10% heat inactivated (56°C for 30 minutes) foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) and containing 100 U/ml penicillin and 100 μg/ml streptomycin was used for the propagation of the cells. Cell cultures were incubated in a humidified CO_2 atmosphere (4% CO_2/96% filtered air) at 37°C. Maintenance medium (MM) was essentially the same as the propagation medium except that it contained only 1-2% FCS.

2.3. *Virus stock*

Stock suspensions of clinical isolates of HSV-1 and Cox B1, and environmental isolates of Ad31 and reovirus, were prepared in the appropriate cell cultures. The viral titre of each suspension was determined before dilution in MM for use at a final concentration of 100 $TCID_{50}$/ml.

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2.4. *Cytotoxicity assay*

Monolayers of VK and PLC/PRF/5 cells were prepared separately in 96-well microtitre trays by seeding each with 200 μl 10^5 cells/ml in 10% MEM. Doubling dilutions of galangin from a concentration of 1.5 mg/ml to 0.0002 mg/ml, were prepared in MM. The galangin was tested for cytotoxicity by exposing the monolayers of the cell cultures to dilutions of the compound in MM at 37°C. The cells were monitored over a period of six days for cytotoxic effects. Monolayers of cells exposed to MM, without the addition of galangin were used as controls.

2.5. *Antiviral assay*

HSV-1, Cox B1 and reovirus were propogated and assayed in VK cells while the PLC/PRF/5 cells were used for Ad31. The galangin was tested for antiviral activity at the final concentrations of 47, 23, 12 and 6 μg/ml. The final concentration of each virus in the assay was 100 $TCID_{50}$/ml. 2 ml of galangin solution and 2 ml of the viral suspension, both diluted in MM, were added simultaneously to monolayers of each cell culture in 25cm^3 flasks. Prior to incubation at 37°C the inoculated flasks were gently swirled to ensure the even distribution of virus and the compound over the cell monolayer. As positive controls, cells were infected with the same concentration of virus but without the addition of galangin, and as a negative or cell control, only MM was added to the cells. Cells were examined daily by light microscopy for the appearance of a cytopathic effect (CPE). The absence of CPE at a specific concentration of compound at the same time that the corresponding positive control showed CPE, was considered to be indicative of antiviral activity.

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3. Results and discussion

The maximum non-toxic concentration of galangin on the VK and PLC/PRF/5 cell cultures was 47 μg/ml. At this concentration, the cells did not exhibit altered morphology or growth characteristics indicative of cytotoxic effects.

As is evident from Table 1 galangin showed no antiviral activity against Ad31, a non-enveloped double-stranded DNA virus, with the same level of CPE being visible in both the positive controls and galangin treated infected cell cultures at the same time intervals. The limited antiviral effect of galangin on reovirus is shown in Table 2, with the infected cells showing 25% CPE 96 h after infection at 47 μg/ml of galangin. The compound showed significant antiviral activity against HSV-1 (an enveloped double-stranded DNA virus) and Cox B1 (an unenveloped single-stranded RNA virus) at concentrations varying from 12-47 μg/ml (Tables 3 & 4). HSV-1 was significantly inhibited at a low concentration of 12 μg/ml and cells infected with Cox B1 showed less than 1% CPE 96 h after infection at a galangin concentration of 47 μg/ml.

In a comparative assay of the antiviral activity of galangin, kaempferol and quercetin on HSV-1 in Vero cells, galangin was reported to be the most active, being able to reduce the viral titre by $2.3\log_{10}$ at 10.8 μg/ml (Amoros et al., 1992). Xylogalactans isolated from the red seaweed, *Nothogenia fastigiata*, also achieved a dose-dependent inhibition of the replication of HSV-1 in Vero cells with 50% effective doses in the range of 15.0-32.6 μg/ml (Damonte et al., 1996). Comparing the effects of two synthetic nucleoside analogues (penciclovir and acyclovir) on HSV-1, Bacon et al., (1996) reported similar activities in the plaque reduction assay with 50% effective concentrations of 0.8 and 0.6 μg/ml respectively.

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Table 1

Dose response pattern of Adenovirus type 31 on PLC/PRF/5 cells to galangin.

Concentration of galangin (μg/ml)	Percentage cytopathic effect			
	24 h	48 h	72 h	96 h
	post infection			
47	25-50	95	100	100
23	25-50	95	100	100
12	25-50	100	100	100
6	25-50	100	100	100
Positive control	25-50	100	100	100
Negative control	0	0	0	0

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Table 2

Dose response pattern of Reovirus on VK cells to galangin.

Concentration of galangin (μg/ml)	Percentage cytopathic effect 24 h post infection	48 h	72 h	96 h
47	0	5-10	5-10	25
23	0	5-20	50	50
12	0	10-25	50	75
6	0	10-25	50	75
Positive control	0-10	25	50	75
Negative control	0	0	0	0

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Table 3

Dose response pattern of Coxsackie B virus type 1 on VK cells to galangin.

Concentration of galangin (μg/ml)	Percentage cytopathic effect			
	24 h	48 h	72 h	96 h
	post infection			
47	1	1	1	1
23	5	5-25	25	50
12	5	25-50	95-100	100
6	5	90-100	100	100
Positive control	5	100	100	100
Negative control	0	0	0	0

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Table 4

Dose response pattern of Herpes simplex virus type 1 on VK cells to galangin.

Concentration of galangin (μg/ml)	Percentage cytopathic effect			
	24 h	48 h	72 h	96 h
	post infection			
47	0	0	0	0
23	0	1	1	1
12	5	5	5	5
6	5	5-25	25-50	50-75
Positive control	5	5-25	50	100
Negative control	0	0	0	0

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The natural occurrence of galangin in many plants (Ferraro et al., 1981; Wollenweber, 1988; Sabatier et al., 1992) and its significant antiviral activity against HSV-1 at low concentrations are indications of the potential of the compound as an alternative antiherpes agent.

Acknowledgement

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Chapter 7

Germination in *Helichrysum aureonitens* (Asteraceae): Effects of temperature, light, gibberellic acid, scarification and smoke extract

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CHAPTER 7

GERMINATION IN *HELICHRYSUM AUREONITENS* (ASTERACEAE): EFFECTS OF TEMPERATURE, LIGHT, GIBBERELLIC ACID, SCARIFICATION AND SMOKE EXTRACT

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Germination in *Helichrysum aureonitens* (Asteraceae): Effects of temperature, light, gibberellic acid, scarification and smoke extract.

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Abstract

Self propagation in *Helichrysum aureonitens* Sch. Bip. (Asteraceae) is mainly by resprouting from roots. In an effort to improve seed germination of this herb, the effects of temperature, light, gibberellic acid (GA_3), scarification and smoke extract on the germination of its seeds in the light and dark were investigated. Seed germination in this species was temperature- and light-dependent and the optimum temperature range appeared to be 25-30°C. Although seed viability was 82% as determined by tetrazolium staining, maximum germination of the control was only 34%. Germination was significantly improved by treatment with GA_3 in continuous illumination of 24 μmol m^{-2} s^{-1}. Only 12.0% of the scarified seeds and 10.0% of the seeds treated with smoke extract germinated. Germination in the dark and of controls ranged from 0-9%. Unlike other fynbos species, smoke extract inhibited germination rather than enhanced it.

Keywords: Germination, *Helichrysum aureonitens*

[a]Written in the format and submitted to the South African Journal of Botany.

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Introduction

The cultivation of many species of the family Asteraceae has been limited by poor germination (Sharman 1993; Bunker 1994). The genus *Helichrysum* is a large group in this family with more than 500 species worldwide of which about 245 are widely distributed in South Africa (Hilliard 1983). Efforts to improve seed germination in this genus have received attention from a few workers. Mott (1972) found that the after-ripening period of *H. cassinianum* was shortened by dry heat storage and Willis and Groves (1991) reported that seeds of *H. apiculatum* require a short period of exposure to high temperature to overcome innate dormancy. Brown (1993a) succeeded in breaking dormancy in *H. vestitum* only after exposing its seeds to smoke extracts, whereas gibberellic acid was reported to promote germination of *H. apiculatum* in the dark (Bunker 1994).

Helichrysum aureonitens Sch. Bip. is a perennial herb commonly found in southern Africa (Hilliard 1983). Its distribution is limited mostly to the fire-prone areas of the KwaZulu-Natal province in South Africa and it has been widely used for centuries by the Zulu people as a remedy for a number of infections (Phillips 1917; Meyer and Afolayan 1995). Self propagation of the plant is by resprouting from underground roots. Most resprouters rarely set viable seed and seedlings are uncommon (Bell *et al.* 1993).

This paper reports on the effects of temperature, light and gibberellic acid (GA_3) on the germination of seeds of *H. aureonitens* carried out under light and dark conditions. We also present our observations on the effect of scarification and treatment with smoke extract. Viability as well as average mass of seeds was also determined.

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Material and Methods

SEED COLLECTION

Propagules of *H. aureonitens* are one-seeded fruits (achenes); hereafter, they will be refered to as 'seeds'. Seeds were collected from a single population growing in the wild in the KwaZulu-Natal province of South Africa (28° 35'S; 31°23'E). They were stored in paper bags at room temperature (*ca*. 15-25°C) until the various trials were conducted, which was within 20 weeks of collection. Only dark-grey and plump, mature seeds were used in the experiments.

SEED MASS DETERMINATION

Average seed mass was determined by weighing four replicates of 100 seeds each and the mean mass of one seed was calculated.

VIABILITY TEST

Viability percentage was determined using the tetrazolium technique (Grabe 1970). Seeds were imbibed for 24 h in water, pierced with a needle under a dissecting microscope without damaging the embryo and soaked in colourless 2,3,5-tetraphenyltetrazolium chloride (TTC) solution for 16 h at 25°C in the dark. Excess TTC solution was siphoned from the seeds using filter paper. Due to the small size of the seeds, the testa from each seed was clarified with a solution of lactophenol to permit direct observation of the embryo (Grabe 1970). This was achieved by soaking the seeds in a solution of 20% lactic acid, 20% phenol, 40% glycerine and 20% water for 40 minutes, whereafter the embryos of viable seeds appeared reddish in colour.

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GERMINATION TESTS

Germination trials were conducted in 9 cm sterile Petri dishes lined with two Whatman No. 1 filter papers moistened with sterile, distilled water. Four replicates of 25 seeds each were used for each treatment. Each trial was a completely randomised design with two light levels (light and dark). In the dark treatment, the Petri dishes were covered with aluminium foil and observations carried out daily under a green safe light. Light treatments were exposed to continuous illumination produced by white fluorescent tubes with a mean photon flux density of 24 μmol m^{-2} s^{-1}. Germinated seeds were counted daily for 30 days. At the end of the trials, ANOVAs were performed and means compared using Tukey's test (Steel and Torrie 1960).

TEMPERATURE

The effect of temperature on germination was determined in incubators set at 15, 25, 30 and 35°C . Seeds were surface sterilised in 0.1% mercuric chloride solution for one minute to prevent fungal attack and rinsed in several changes of sterile water. Subsequent trials were conducted at the optimum temperature (30°C).

GIBBERELLIC ACID (GA_3)

Effect of GA_3 on germination in *H. aureonitens* was investigated by moistening the incubation medium with a 500 mg l^{-1} solution of GA_3. The seeds for the control trial were moistened in water only.

EFFECT OF AQUEOUS SMOKE EXTRACT ON SEED GERMINATION

Dehydrated fynbos-smoke-saturated paper was purchased from the Conservation Biology Research Unit, Kirstenbosch, South Africa. The smoke extract was prepared by shaking the

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paper in 50 ml of distlled water for five minutes after which the seeds were soaked in the aqueous smoke extracts for 24 h (Brown 1993*a*; Brown 1994). Seeds for the control were soaked in water.

SEED SCARIFICATION

The effect of seed scarification on germination was determined by piercing the testa of water-imbibed seeds with a needle under a dissecting microscope without damaging the embryos. Seeds for the control trial were left intact.

Results

SEED MASS AND VIABILITY

The average mass of one seed was 4.93±0.14 mg, and the viability of the seed lot as determined by the tetrazolium test was 82.0±4.1%.

EFFECT OF TEMPERATURE AND LIGHT

Seeds of *H. aureonitens* germinated in all the temperature treatments except at 35°C in the dark. Significant differences were obtained between germination percentages at the optimum temperature range and both of the extremes. The optimum temperature for germination was 25-30°C under continuous illumination (Table 1). Light also significantly increased germination of the seeds.

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Table 1. Germination of *H. aureonitens* seeds at four different temperature regimes and light and dark conditions after 30 days

Temperature (°C)	Germination (%)	
	Light	Dark
15	11(1.00)[a]	3(0.90)[a]
25	30(1.00)[b]	3(1.00)[a]
30	34(3.06)[b]	9(0.05)[b]
35	1(0.50)[a]	0[c]

Note. Each value represents the mean percentage germination of four replicates of 25 seeds with standard deviations in parenthesis; means within a column followed by the same superscript do not differ significantly ($P > 0.05$) according to Tukey's significance tests.

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Table 2. The influence of four dormancy-breaking treatments on the germination of *H. aureonitens* seeds after 30 days

Treatment	Germination (%)	
	Light	Dark
Aqueous smoke extract	10.0(1.00)[a]	0.0[a]
Gibberellic acid	45.2(2.80)[b]	6.0(0.00)[b]
Scarified seeds	12.0(1.73)[a]	1.0(0.50)[a]
Water (control)	29.3(1.53)[c]	0.0[a]

Note. Values are means of percentage germination of four replicates of 25 seeds with standard deviations in parenthesis; values within a column followed by the same superscript are not significantly different ($P > 0.05$) according to Tukey's significance tests.

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EFFECT OF GIBBERELLIC ACID

Treatment of seeds of *H. aureonitens* with GA_3 significantly ($P < 0.05$) increased germination both in the light and dark when compared to the smoke treatment and control (Table 2).

EFFECT OF SEED SCARIFICATION AND SMOKE EXTRACT

Scarification and treatment with smoke extract did not improve germination when compared with the control (Table 2). Both treatments significantly inhibited germination.

Discussion

Poor germination has always been a problem limiting the evaluation and commercialisation of many members of the Asteraceae family (Bunker 1994). This study showed a maximum germination of seeds of *H. aureonitens* (in the control) to be only 34% in the light at 30°C. This value is low considering that the TTC test indicated that 82% of the seeds were viable. According to Delfs *et al.* (1987), long-lived shrub species which resprout after fire regenerate only rarely from seeds.

Germination of seeds at 25 and 30°C was significantly higher than at 15 or 35°C. The optimum temperature range for germination (25-30°C) in *H. aureonitens* is high in comparison to other temperature-sensitive species of *Helichrysum*. Mott (1972) and Willis and Groves (1991) reported that seeds of *H. cassinianum* and *H. apiculatum* germinated best at a constant temperature of 20°C and at an alternating temperature of 20/10°C respectively. Bell *et al.* (1993) reported that species growing in microhabitats where moisture is likely to be available in warmer seasons tend to have germination maxima at higher temperatures. *H.*

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aureonitens is mostly found in the KwaZulu-Natal province of South Africa where most of the rain falls in the warmer summer periods. Therefore, the temperature optimum for germination in this herb matches the summer rainy period of its natural environment.

H. aureonitens seeds required light over the entire range of temperatures and treatments applied. Willis and Groves (1991) similarly reported a significant increase in seed germination percentages of *H. apiculatum* exposed to light in comparison to those germinated in the dark. The light stimulus of this small-seeded species is ecologically effective because germination at soil depth would result in insufficient endosperm reserves for emergence and seedling survival.

Treatment of *H. aureonitnes* seeds with GA_3 significantly increased germination in the presence of light. The positive germination response to GA_3 coupled with the fact that maximum germination in the control was only 34% could have suggested a need for an after-ripening period for maximum germination. However, the maximum after-ripening periods of *Helichrysum* species are between four and five months. Mott (1972) observed a decrease in seed germination in *H. cassinianum* after five months and Willis and Groves (1991) also reported a decline in germination percentages in *H. albicans* and *H. apiculatum* after 12 weeks. Karssen and Lacka (1986) suggested that light may affect germination by changing the sensitivity of seeds to GA_3. Our results support this suggestion since germination of this plant was higher after treatment with GA_3 in the light than in the dark. It further suggests that seed sensitivity to GA_3 increased in the presence of light. Promotion of germination was also reported for seeds of *H. apiculatum* treated with GA_3 (Bunker 1994). GA_3 appears to be essential for seed germination and almost universally stimulates germination, being

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frequently associated with mobilization of endosperm reserves and growth of embryonic tissues (Bell *et al.* 1993).

Mechanical scarification of the seed coats is a common method of improving seed germination. Atwater (1980) proposed that the principal block to germination in the Asteraceae was the testa surrounding the embryo. Physical or chemical attrition of both the pericarp and testa is, therefore, often necessary for germination to take place. A single puncture to the pericarp and testa is sufficient to bring about germination in many daisies (Atwater 1980; Mullett 1981), although complete excision of the embryo has been recommended for some species. Puncturing or scarifying the seed coats of *H. aureonitens* did not improve germination of dormant seeds. Heydecker and Coolbear (1977) observed that different species may require different proportions of the embryo to be exposed to promote germination. In our study, only a small portion of the embryo was exposed in the scarification treatment which may have been insufficient to remove any germination inhibitors that were present. Complete removal of the seed coats in *H. cassinianum* had no effect on germination (Mott 1972).

In a study of seed germination of 37 taxa from 10 fynbos families, Brown (1993*b*) found that 18 taxa from Asteraceae showed a statistically significant improvement in germination following treatment with smoke and/or smoke extracts. Brown (1993*a*) reported an increase from $8 \pm 3\%$ to $81 \pm 7\%$ in germination of seeds of *Syncarpha vestita* (syn. *H. vestitum*). Although fire is a major ecological factor affecting the vegetation of KwaZulu-Natal province (Tainton 1981), smoke extract significantly inhibited germination of seeds *H. aureonitens*. The contrasting results may be attributed to differences in the concentration of the active smoke

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extract in the aqueous medium. Examination of water saturated with smoke showed the presence of ethylene as well as octanoic acid, both of which are capable of stimulating germination (Sutcliffe and Whitehead 1995). It is possible that the presence of octanoic acid in the smoke extract resulted in the inhibition of germination depending on the period of exposure of the seeds to the acid. The inhibition of germination by the continuous presence of octanoic acid could be the result of its incorporation into the cell membranes in such a way that the integrity of the membrane is affected (Stewart and Berrie 1979).

Generally, this study has shown that germination in *H. aureonitens* is improved by GA_3 treatment in light. The fact that percentage germination of GA_3-treated seeds was low but viability was high indicates that more than one mechanism may be required to break dormancy in the seeds of this plant.

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Chapter 8

General discussion and conclusion

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

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GENERAL DISCUSSION AND CONCLUSION

Motivation for the study

The importance of continuous investigation of the abundant flora of the world for the presence of new drugs that will benefit humans cannot be over emphasized. Natural plant products are a major component of the "natural" pharmaceutical industry that is growing rapidly in Europe and North America, as well as in traditional herbal medicine programmes being incorporated into the primary health care system of the developing countries (Cox 1990). The importance of plant-derived pharmaceuticals can be deduced from their prominence in the market. According to Farnsworth (1984), 25% of all prescriptions issued in the United States and Canada from 1959 to 1973 contained an active component derived or originally isolated from higher plants. Considering the rapid rate of deforestation and the concurrent loss of biodiversity throughout the world, the need to examine more plants for new drugs is even more urgent.

Selection of plants for screening based on ethnomedical information is not only much more productive in drug discovery but has a far higher chance of success than random screens (Cox 1990; Farnsworth 1990). The choice of *H. aureonitens* for this study was based on the ethnomedical information from the indigenous people of South Africa who have been using the herb for ages against infections including herpes simplex and herpes zoster skin diseases.

Foliar appendages and secretion in *H. aureonitens*

Production of epicuticular compounds with antimicrobial properties has been well documented in *Helichrysum* species (Tomas-Barberan *et al.* 1988; Tomas-Lorente *et al.* 1989;

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Cosar and Cubukcu 1990). In most species, the source of these compounds has been attributed to the foliar trichomes (Wollenweber 1984). Examination of the morphology and ultrastructure of the foliar appendages of *H. aureonitens* revealed two types of trichomes: secretory and nonsecretory types. The nonsecretory trichomes are long and abundant, forming a dense covering that completely obscures the epidermal surfaces of the leaves. The secretory trichomes are few, club shaped and are relatively short with two stalk cells and several gland cells. Each secretory trichome is characterised by a terminal secretory cavity above the upper tier of head cells inside of which the secretory products accumulate. Formation of a subcuticular space inside of which secretory substances accumulate, is common during secretion in many types of exotropic glands. The secretory products are usually released through pores in the cuticle (Schnepf 1974), or through breaks in the cuticle caused by external pressure (Hammond and Mahlberg 1977). Absence of pores in the cuticle of *H. aureonitens* signified that secretory products are probably released when the secretory cavity ruptures. The mature gland cells at the secretory stage contains numerous dictyosomes that appear to be active in vesicle formation. The glands also contain an extensive network of endoplasmic reticulum (ER) and numerous mitochondria. Endoplasmic reticulum, especially tubular ER has been implicated in flavonoid secretion (Werker and Fahn 1982). Although ultrastructural studies alone do not provide the information required to establish sites of synthesis in cells, it is plausible to assume that the active therapeutic compounds are produced by the ER in the glandular trichomes of *H. aureonitens*.

Antimicrobial activties of extracts from *H. aureonitens*

The antibacterial activity of extracts from *H. aureonitens* was investigated. CH_2Cl_2, MeOH and H_2O extracts showed no activity against gram negative bacteria. However, CH_2Cl_2 and

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MeOH extracts indicated antibacterial activity with significant inhibition against gram positive bacteria. Both extracts inhibited the growth of *Bacillus cereus, B. pumilus,* and *Micrococcus kristinae*. In addition, the CH_2Cl_2 extract was active against *Bacillus subtilis* and *Staphylococcus aureus*. The MIC for *B. cereus* and *B. pumilus*, of the CH_2Cl_2 extract were lower than those of the MeOH extract. The two extracts, however, inhibited the growth of *M. kristinae* at 0.5 mg/ml which was the highest dilution used in the study. The water extract had no activity on any of the organisms tested.

Aqueous extract of this herb was evaluated for antiviral activity against herpes simplex virus type 1 (HSV-1) *in vitro*. The crude extract from its shoots showed significant antiviral activity on HSV-1 in human lung fibroblasts as demonstrated by the absence of a cytopathic effect at a concentration of 1.35 mg/ml.

The discovery of the antibacterial and antiherpes activity of crude extracts from *H. aureonitens* has substantiated the use of extracts from this plant by the indigenous people of South Africa against a number of infections for generations.

Isolation and identification of the active compounds

Two closely related bioactive compounds (pinobanksin and galangin) were isolated from this plant. Spectral data were compared with those found in the literature (Kuroyanagi *et al.* 1982; Markham *et al.* 1982; Markham and Geiger, 1993). Pinobanksin (the *cis* isomer of 3,5,7-trihydroxyflavanone) was isolated in a very small quantity and is highly unstable. Flavonoids with 2,3-*cis* configuration are known to be relatively unstable as compared to their 2,3-*trans* isomers (Foo 1987). Galangin (3,5,7-trihydroxyflavone) constituted the major

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bioactive compound in the extract and is more stable than pinobanksin. It was first isolated in 1881 from galangal root, *Alpinia officinarum* (Wollenweber 1988) and first synthesised by Heap and Robinson (1926). Since then, it has been found in extracts of many angiosperms (Ferraro *et al.* 1981; Wollenweber *et al.* 1985; McDowell *et al.* 1988; Sabatier *et al.* 1992). Information on the antimicrobial property of galangin is however scanty.

Antimicrobial properties of galangin

Evaluation of the antibacterial activity of the compound against ten randomly selected bacteria indicated significant activity against all the gram positive bacteria tested. However, the MIC was higher on *Bacillus pumilus, B. subtilis* and *Staphylococcus aureus* than on *Bacillus cereus* and *Micrococcus kristinae*. The compound was not active on gram negative bacteria except for *Enterobacter cloacae* which was significantly inhibited at an MIC of 0.1 mg/ml. Galangin is more active on gram positive bacteria than on the gram negative species. This might be due to the possession of an outer membrane composed mainly of lipopolysaccharides by the gram negative bacteria, which is impermeable to hydrophobic molecules like galangin. Galangin does not dissolve in water.

We have earlier reported the antibacterial activity of the dichloromethane and methanol extracts from this plant against gram positive bacteria (Meyer and Afolayan, 1995) with no visible activity on the gram negative bacteria including *E. cloacae*. This was probably due to the relatively low concentration of galangin in the crude methanol and dichloromethane extracts. The antimicrobial property of galangin might be associated with its lack of hydroxyl groups on the B ring. Chabot et al. (1992) observed that flavonoids lacking hydroxyl groups on the B ring are known for their antimicrobial activities.

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The *in vitro* activity of galangin was investigated against herpes simplex virus type 1 (HSV-1), coxsackie B type 1 (CoxB1), adenovirus type 31 (Ad31) and reovirus. Galangin showed significant activities against HSV-1 and CoxB1 at concentrations varying from 12-47 μg/ml. The compound showed no antiviral activity on Ad31 and indicated limited activity against reovirus.

Galangin indicated considerable activity against the fungi tested with the exception of *Cladosporium herbarum*. *Penicillium digitatum* and *P. italicum* appeared to be particularly susceptible at 0.01 mg/ml.

Germination in *H. aureonitens*

Self propagation in *H. aureonitens* is mainly by resprouting from roots. The effects of temperature, light, gibberellic acid (GA_3), scarification and smoke extract on the germination of its seeds in the light and dark were investigated. Seed germination in this species was temperature- and light-dependent and the optimum temperature range was 25-30°C. Although seed viability was 82% as determined by tetrazolium staining, maximum germination of the control was only 34%. Germination was significantly improved by treatment with GA_3 in continuous illumination of 24 μmol m^{-2} s^{-1}. Unlike other fynbos species, smoke extract inhibited germination rather than enhanced it in *H. aureonitens*.

Conclusion

In this study, we observed galangin to be a major constituent of the epicuticular extract from *H. aureonitens* (5.17% of the dry weight of crude extract). The antiherpes properties of galangin and its ability to inhibit the growth of all the gram positive bacteria tested,

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Enterobacter cloacae, a gram negative bacterium and a number of fungal species is an indication of the compound as a broad spectrum antimicrobial agent. This probably explains the successful use of extracts from this plant by the indigenous people of South Africa against a number of infections for generations.

Ethnomedically directed research such as this, is necessary in order to optimize the search for novel pharmaceuticals. About three quarters of the biologically active plant-derived compounds presently in use worldwide were discovered through follow-up research to verify the authenticity of data from folk and ethnomedical uses (Farnsworth *et al.* 1985; Soejarto and Farnsworth 1989; Lewis and Elvin-Lewis 1995). The indigenous people of KwaZulu-Natal are relatively, a culturally intact tribe still using plants today as they have for generations for health care on daily basis. The survival of the culture of this people and the plants that nurture them depends not only on the current global race against ecosystem destruction but also on the acceleration of research activities on the plant species which they use for their health care delivery.

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Chapter 9

Summary

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CHAPTER 9

SUMMARY

The Medicinal Potential of *Helichrysum aureonitens*

by

Anthony Jide Afolayan

Supervisor: Prof J.J.M. Meyer

Department of Botany

Doctor of Philosophy

Helichrysum aureonitens Sch. Bip. (Asteraceae) is a perennial herb which grows mostly in the KwaZulu-Natal province of South Africa. The Zulu people have been using extracts from this plant for ages against infections.

Electron microscopical examination of foliar appendages of *H. aureonitens* revealed two types of trichomes: secreting (glandular) and nonsecreting (fibrous) hairs. Secretions accumulate in a cavity found on the top tier of secretory cells, and are released to the outside when the cavity ruptures.

The antibacterial activity of extracts from this plant was investigated. Dichloromethane extract was active against all five gram positive bacteria tested and the methanol extract was active only against *Bacillus cereus, B. pumilus* and *Micrococcus kristinae*, while the water extract had no activity against any of the organisms. None of the extracts inhibited the growth of the five gram negative bacteria tested.

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Aqueous extract from shoots of *H. aureonitens* was evaluated for antiviral activity against herpes simplex virus type 1 (HSV-1) *in vitro*. The crude extract showed significant antiviral activity at a concentration of 1.35 mg/ml.

The main antimicrobial compound from the aerial parts of this herb was isolated and identified as 3,5,7-trihydroxyflavone (galangin). Evaluation of the antibacterial activity of the compound indicated significant activity against all the gram positive bacteria tested at concentrations ranging from 0.1 to 0.5 mg/ml. The compound was not active on gram negative bacteria except for *Enterobacter cloacae* which was inhibited at 0.1 mg/ml. Galangin indicated considerable activity against the fungi tested with the exception of *Cladosporium herbarum*. It showed activities against HSV-1 and CoxB1 at concentrations varying from 12-47 μg/ml.

Self propagation in *H. aureonitens* is mainly by resprouting from roots. The effects of temperature, light, gibberellic acid (GA_3), scarification and smoke extract on the germination of its seeds in the light and dark were investigated. Seed germination in this species was temperature- and light-dependent and the optimum temperature range was 25-30°C. Although seed viability was 82% as determined by tetrazolium staining, maximum germination of the control was only 34%. Germination was significantly improved by treatment with GA_3 in continuous illumination of 24 $\mu mol\ m^{-2}\ s^{-1}$. Unlike other fynbos species, smoke extract inhibited germination rather than enhanced it in *H. aureonitens*.

In this study, we observed galangin to be a major constituent of the epicuticular extract from *H. aureonitens* (5.17% of the dry weight of crude extract). The antiherpes property of

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galangin and its ability to inhibit the growth of all the gram positive bacteria tested, *Enterobacter cloacae*, a gram negative bacterium and a number of fungal species is an indication that the compound is a broad spectrum antimicrobial agent. This study has substantiated the use of extracts from this plant by the indigenous people of South Africa against a number of infections for generations.

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Chapter 10

Acknowledgements

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CHAPTER 10

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