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Identification and characterization of a novel triyne and cinnamate 4-hydroxylase in *Helichrysum aureonitens* Sch. Bip.

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

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DECLARATION

I, Sayed Mahdi Ziaratnia, declare that the thesis submitted herewith, for the degree Philosophiae Doctor, to the University of Pretoria, contains my own independent work and has not previously been submitted by me for a degree to this or any other tertiary institution.

S.M. Ziaratnia

May 2009

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ABSTRACT

For centuries people have used plants as medicine or food additives with varying success to cure and prevent diseases. Written records about medicinal plants date back at least 5 000 years to the Sumerians. According to World Health Organization (WHO) around 80 % of the population in developing countries is dependent on herbal medicine for basic healthcare needs. Even at the dawn of the twenty-first century, 11 % of the 252 drugs considered as basic and essential by WHO were exclusively of flowering plant origin.

The genus *Helichrysum*, belongs to the family Asteraceae and is represented by approximately 600 species in Africa, of which 244 species are indigenous in South Africa. In *Helichrysum aureonitens,* galangin is one of the flavonol compounds with good medicinal properties. *H. aureonitens* was targeted to be enhanced via cell suspension culture to potentially produce valuable natural products. In ethanol extracts of cell suspension cultures, galangin was not detected even though it was present in the leaves of the intact plants. Some other compounds were induced in higher amounts in the cells of *H. aureonitens* suspension cultures when compared to that produced in the intact plants. To find out the reasons for the absence of galangin in the cells of *H. aureonitens* suspension cultures, some of the intermediates of the 4'-OH biosynthetic pathway for production of flavonols were analyzed by GC-MS, including cinnamic acid, *p*-coumaric acid, naringenin and kaempferol. None of these were detected in the *H. aureonitens* cell suspension cultures. The major compound from *H. aureonitens* cell suspension cultures was isolated and identified as a new chlorophenol compound named 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol (a triyne). This triyne has previously been proposed as being an intermediate in the acetylene biosynthetic pathway in *Helichrysum spp.*, however only the methyl ether form had previously been isolated from the roots of *H. coriaceum*. The triyne isolated from the *H. aureonitens* cell suspension cultures in the present study was detected in intact plant extracts, but at very low concentrations. Results of the anti-tuberculosis assay of the cell suspension culture extracts and the triyne showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol extract of cell suspension cultures against *Mycobacterium tuberculosis* H37Rv were found to be

1.0 mg/ml and 2.0 mg/ml respectively and the triyne was not active at 200 µg/ml. The ethanol extract of the cell suspension cultures and the triyne were also evaluated for their cytotoxicity against monkey kidney Vero cells and human prostate epithelial carcinoma (DU145) cell lines. The inhibitory concentrations (IC_{50}) of the crude extract and the triyne was found to be 12.11 and 1.51 µg/ml against the Vero cells respectively. The crude extract and the triyne showed similar activity in the prostate cancer cell lines by exhibiting IC_{50} values of 3.52 and 2.14 µg/ml respectively. The triyne therefore warrants further investigation for its potential as an anticancer drug.

Flavonoids represent the major phenolic compounds which are responsible for the medicinal properties in the *Helichrysum* genus. Some of flavonols, including kaempferol, quercetin, and galangin are also present in *H. aureonitens*. In this study both galangin and kaempferol (containing a 4'-OH group) were detected in leaf samples of *H. aureonitens.* But GC-MS analysis of the leaf samples of *H. aureonitens* did not show the existence of biosynthetic intermediates such as *p*-coumaric acid and naringenin (compounds having a 4'-OH) while cinnamic acid and some other compounds with no OH at the 4' position on the B ring, were detected. The chemical structure analysis of the isolated compounds showed that they are pinocembrin chalcone, pinocembrin, pinobanksin and galangin, all containing no OH group at the 4' position. This indicates that some part of the 4'-OH biosynthetic pathway for 4'-OH flavonoids is not functional in *H. aureonitens*. Since the only (yet identified) enzyme responsible for hydroxylation at the 4' position on the B ring is cinnamate 4-hydroxylase (C4H), it can be postulated that C4H might be able to hydroxylate other substrates in *H. aureonitens* plants.

One copy of C4H was isolated and cloned from *H. aureonitens*. It has 1518-base pairs (including stop codon, TAA) and an open reading frame encoding a 506-amino-acid polypeptide. It showed the highest homologies to *Echinacea angustifolia* (Asteraceae) C4H with 83.6 % identity on the nucleotide level but 93 % identity on the amino acid level. The genomic DNA sequence of the isolated C4H from *H. aureonitens* indicates the presence of three introns with a longer size compared to the *Arabidopsis thaliana* C4H gene structure. The presence of the first intron has not been reported before in the C4H gene from other plants and it is therefore a new finding from the isolated C4H in *H. aureonitens.*

To check the putative isolated C4H, the full length cDNA of C4H was isolated from *H. aureonitens* and for the first time integrated in a secreted expression vector, pPICZαC, and transformed into *Pichia pastoris*. After the 48 hrs induction protein was collected, precipitated by ammonium sulphate and finally column purified. The results of SDS-PAGE electrophoresis and Western blot showed the expression of a protein with a size of 50-60 kDa. The calculated mass of C4H with regarding to a polyhistidine tag is about 60.5 kDa. The secreted expression was found as an effective system for the production of a soluble C4H protein with easy purification.

THESIS COMPOSITION

Chapter 1: Introduces briefly the history of medicinal plants in the world and discusses its benefits for human health. It also reviews the current situation of the medicinal plant product trade in the world focusing on the international trade of these plants. This chapter discusses the opportunities and limitations of the production of products from medicinal plants and also reports on the application of modern techniques for natural products production. In the last part of the chapter, there is an introduction to the genus of *Helichrysum* as well as a description of the medicinal properties of the isolated compounds from *H. aureonitens*.

Chapter 2: Reports on the establishment of *H. aureonitens* cell suspension cultures. This chapter focuses on the chemical analysis of the galangin intermediates based on the 4'-OH biosynthetic pathway of flavonols.

Chapter 3: Deals with the isolation and identification of the major compound from the *H. aureonitens* cell suspension cultures. The chapter also reports on the characteristics of the major compound and crude extracts of the *H. aureonitens* cell suspension cultures including, their toxicity, anti-TB (*Mycobacterium tuberculosis*) as well as anticancer activity.

Chapter 4: Investigates the 4'-OH biosynthetic pathway for flavonol production in *H. aureonitens* by the detection of flavonol intermediates in the leaf samples. It also reports on

the detection of some other intermediates of the non 4'-OH biosynthetic pathway of flavonols which can be involved in the galangin production of *H. aureonitens* plants. Finally it is postulated that the difference between these two biosynthetic pathways is the functionality of cinnamate-4-hydroxylase (C4H).

Chapter 5: Focuses on the full length isolation and characterization of C4H in *H. aureonitens.* It includs the neucleotide, amino acid sequence, gene structure and the phylogenetic relationships between the isolated C4H from *H. aureonitens* and those in other plants.

Chapter 6: Presents the expression of C4H in the yeast, *Pichia pastoris* with the secreted system of expressed protein into the medium. The precipitation and purification of C4H will also be discussed in this chapter.

Chapter 7: Summarizes the results of this study regarding the novel phenolic compound isolated from *H. aureonitens*, and as well as the characterization of the isolated and expressed C4H. Finally this chapter outlines possible further research which includes the functional expression of the isolated C4H from *H. aureonitens* in yeast and also determine the function of this gene in the *H. aureonitens* cells as well as the characterization of C4H from other *Helichrysum* species.

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LIST OF ABBREVIATIONS

^{13}C-NMR: Carbon-nuclear magnetic resonance

^{1}H-NMR: Proton-nuclear magnetic resonance

2D-TLC: Two-dimensional TLC

4CL: 4-Coumarate:CoA ligase

$AgNO_3$: Silver nitrate

AOX: Alcohol oxidase

ATG: Methionine (start codon)

ATP: Adenosine-5'-triphosphate

BA: 6-benzylaminopurine

BMGY: Buffered Glycerol-complex medium

BMMY: Buffered Methanol-complex medium

bp: Base pair

BSA: Bovine serum albumin

C4H: Cinnamate-4-hydroxylase

$CaCl_2$: Calcium Chloride

CAD: Cinnamyl alcohol dehydrogenase

CCoA-3H: Coumaroyl-coenzyme A 3-hydroxylase

CCR: Cinnamoyl-CoA reductase

$CDCl_3$: Deuterated chloroform

cDNA: Complimentary DNA

CHI: Chalcone isomerase

CHS: Chalcone synthase

CODEHOP: Consensus-degenerate hybrid oligonucleotide primers

COMT: Caffeic acid 3-O-methyltransferase

COSY: Correlated spectroscopy

CPR: Cytochrome P450 oxidoreductase

CTAB: Cetyltrimethylammonium bromide

dATP: Deoxyadenosine triphosphate (A)

dCT: Deoxycytidine triphosphate (C)

DDT: Dichlorodiphenyltrichloroethane

dGTP: Deoxyguanosine triphosphate (G)

DMF: Dimethylformamide

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

dNTP: Deoxynucleoside triphosphate

dTTP: Thymidine triphosphate (T)

E. coli: *Escherichia coli*

EDTA: Ethylenediamine tetra acetic acid

EtOH: Ethanol

F3H: Flavonone 3-hydroxylase

F5H: Flavonone 5-hydroxylase

FLS: Flavonol synthase

g: Gram

GC: Gas chromatography

GC-MS: Gas chromatography-mass spectrometry

gDNA: Genomic DNA

h: hours

His-tag: Polyhistidine (6xHis) tag

HIV: Human immunodeficiency virus

HMBC: Heteronuclear multiple bond correlation

HMQC: Heteronuclear multiple quantum correlation

IC_{50}: The half maximal inhibitory concentration

IR: Infrared spectra

KAc: Potassium acetate

KCl: Potassium chloride

KEX2: The preliminary cleavage of the signal sequence by the KEX2 gene product

L: Litre

LB: Luria broth

LC-MS: Liquid chromatography-mass spectrometry

M: Molar

MBC: Minimum bactericidal concentration

MeOH: Methanol

$MgCl_2$: Magnesium chloride

MHz: Megahertz

MIC: Minimum inhibitory concentration

min: Minutes

mL: Millilitres

mM: Millimolar

$MnCl_2$: Manganese chloride

mRNA: Messenger ribonucleic acid

MS medium: Murashige and Skoog medium

MS: Mass spectrometry

Myc-tag: Myc epitope tag

Na_2CO_3: Sodium carbonate

$Na_2S_2O_3$: Sodium thiosulfate

NAA: α-Naphthalene acetic acid

NaAc: Sodium acetate

NaCl : Sodium chloride

NADPH: Nicotinamide adenine dinucleotide phosphate

NaH_2PO_4: Sodium dihydrogen phophate

NaOAc: Sodium acetate

NaOH: Sodium hydroxide

ng: Nanogram

NMR: Nuclear magnetic resonance

OD: Optical density

ORF: Open reading frame

P. pastoris: *Pichia pastoris*

PAL: Phenylalanine ammonia lyase

PCR: Polymerase chain reaction

Pmol: Pico mole

PMSF: Phenyl methyl sulphonyl fluoride

PVDF: Polyvinylidene fluoride

RAPD: Random amplification of polymorphic DNA

RNA: Ribonucleic acid

rpm: Revolutions per minutes

S. cerevisiae: *Saccharomyces cerevisiae*

sd H_2O: Sterile distilled water

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

TAE: Tris-acetate EDTA

TAL: Tyrosine ammonia-lyase

Taq: *Thermus aquaticus*

TB: Tuberculosis

TEMED: N,N,N',N'-tetramethyl-ethylenediamine

TLC: Thin layer chromatography

Tris: 2-Amino-2-(hydromethyl)propane-1.3 diol

tRNA: Transfer RNA

U: Unit

UV: Ultraviolet

WHO: World Health Organization

YNB: Yeast nitrogen base with ammonium sulphate without amino acids

YPDS: Yeast extract peptone dextrose medium

Chapter 1

Introduction

1.1 Medicinal plants past, present

1.1.1 Medicinal plants and human health care

People have used plants for centuries as medicine or food additives with varying success to cure and prevent diseases. Written records about medicinal plants date back at least 5000 years to the Sumerians (Swerdlow, 2000). According to World Health Organization (WHO) around 80 % of the population in developing countries is dependent on herbal medicine for basic health care needs (Vines, 2004). This high demand for natural products is not limited only to the developing countries; the use of herbal medicine in developed countries is also increasing. For instance, 25 % of the population in the UK consumes herbal medicine regularly (Vines, 2004). In the 1970s, 25 % of all drugs dispensed in the USA contained compounds derived from flowering plants. Even at the dawn of the twenty-first century, 11 % of 252 drugs considered as basic and essential by WHO was exclusively of flowering plant origin (Rates, 2001). The recent findings in plant-derived antitumor drugs such as taxol, vinblastine, vincristine and camptothecin has dramatically improved the effectiveness of chemotherapy against some of the deadliest cancers (Raskin et al., 2002). Some of the most important drugs derived directly or as precursors from plants are listed in Table 1.1.

The strong relationship between plants and human health is not limited only to medicine. There is also a historic bond between food and the treatment of diseases such as “An apple a day keeps the doctor away”. Plant-derived products now have a considerable share in the market. These include phytopharmaceuticals, pharmaceuticals, herbal remedies, dietary supplements, homeopathics, medicinal and herbal teas, spirits, aromas and essences, perfumes, cosmetics, colouring agents, varnishes, and also detergents. As a consequence, there is a huge trade on the local, regional, national and international level, both for domestic use and commercial trade and this result in an enormous demand in botanicals.

Table 1. 1. Some of the most important pharmaceuticals (or their precursors) derived from plants (Raskin et al., 2002).

Name	Type	Source	Therapeutic use
Alkaloids:			
Atropine[a], hyoscyamine, scopolamine	Tropane alkaloid	*Solanaceous* spp.	Anticholinergic
Camptothecin[a]	Indol alkaloid	*Camptotheca acuminata* Decne	Antineoplastic
Capsaicin	Phenylalkyl-amine alkaloid	*Capsicum* spp.	Topical analgesic
Codeine, morphine	Opium alkaloid	*Papaver somniferum* L.	Analgesic, antitussive
Cocaine	Cocaine alkaloid	*Erythroxylum coca* Lamarck	Local anaesthetic
Colchicine	Isoquinoline alkaloid	*Colchicum autumnale* L.	Antigout
Emetine	Isoquinoline alkaloid	*Cephaelis ipecacuanha* (Brot.)A. Rich.	Antiamoebic
Galanthamine	Isoquinoline alkaloid	*Leucojum aestivum* L.	Cholinesterase inhibitor
Nicotine	Pyrrolidine alkaloid	*Nicotiana* spp.	Smoking cessation therapy
Physostigmine	Indole alkaloid	*Physostigma venenosum* Balfor	Cholinergic
Pilocarpine	Imidazole alkaloid	*Pilocarpus jaborandi* Holmes	Cholinergic
Quinine	Quinoline alkaloid	*Cinchona* spp.	Antimalarial
Quinidine	Quinoline alkaloid	*Cinchona* spp.	Cardiac depressant
Reserpine	Indole alkaloid	*Rauwolfia serpentina* L.	Antihypertensive, psychotropic
Tubocurarine	Bisbenzyl isoquinoline alkaloid	*Chondodendron tomentosum* Ruiz,	Skeletal muscle relaxant
		Strychnos toxifera Bentham	
Vinblastine, vincristine	Bis-indole alkaloid	*Catharanthus roseus* L.	Antineoplastic
Yohimbine	Indole alkaloid	*Apocynaceae, Rubiaceae* spp.	Aphrodisiac
Terpenes and steroids:			
Artemisinin	Sesquiterpene lactone	*Artemisia annua* L.	Antimalarial
Diosgenin[a], hecogenin[a], stigmastero	Steroids	*Dioscorea* spp.	Oral contraceptives and hormonal drugs
Taxol and other taxoids[a]	Diterpenes	*Taxus brevifolia* Nutt.	Antineoplastic
Glycosides:			
Digoxin, digitoxin	Steroidal glycosides	*Digitalis* spp.	Cardiotonic
Sennosides A and B	Hydroxy-anthracene glycosides	*Cassia angustifolia* Vahl.	Laxative
Others and mixtures:			
Ipecac	Mixture of ipecac alkaloids	*Cephaelis ipecacuanha* (Brot.)	Emetic
	and other components A. Rich.		
Podophyllotoxin[a]	Lignan	*Podophyllum peltatum* L.	Antineoplastic
[a]Most often used as precursors in chemical synthesis of final products.			

1.1.2 Medicinal plants in trade

Traded raw plant material consists mainly of dried roots, leaves, bark, wood, flowers, seeds or sometimes the whole plant (Lange, 1996). In the 1990s, the overall import trade amounted on average of 400 000 tons valued at USD 1 224 million. The international trade is dominated by only a few countries: 82 % of the world-wide imports are channelled to just 12 countries (Table 1.2), and 12 countries are also responsible for 80 % of the overall world exportation. Temperate Asia and Europe dominates the domestic import at 42 % and 33 % respectively. Hong Kong and China are the most important export and import countries (Table 1.2). On the other hand three countries, Hong Kong, USA and Germany are important trade centres for pharmaceutical plants.

Table 1. 2. List of the important countries for trading of medicinal plants (Lange, 1996).

Country of import	Quantity [t]	Value [USD]	Country of export	Quantity [t]	Value [USD]
Hong Kong	67,000	291,200,000	China	147,000	281,800,000
Japan	51,350	136,000,000	Hong Kong	63,150	228,800,000
USA	49,600	135,500,000	India	33,900	56,650,000
Germany	45,350	110,200,000	Germany	15,100	70,050,000
Rep. Korea	32,250	52,300,000	USA	13,500	115,500,000
France	21,350	52,000,000	Mexico	13,000	11,250,000
China	13,650	41,600,000	Egypt	11,750	13,850,000
Italy	11,700	42,850,000	Chile	11,600	28,200,000
Pakistan	11,050	11,150,000	Bulgaria	10,050	14,500,000
Spain	9,100	27,650,000	Singapore	9,600	56,600,000
United Kingdom	7,650	27,000,000	Morocco	8,000	13,300,000
Singapore	6,300	50,600,000	Pakistan	7,800	4,950,000
Total	**326,300**	**978,150,000**	**Total**	**344,400**	**893,400,000**

Fig. 1.1 shows that Japan is by far the most important consumer country, followed by the USA, Germany and the Republic of Korea. In these countries, the raw material is mainly processed as a industry of the country and then sold as finished products either on the domestic market or exported. In addition, China is the most important supplier of raw material to the world's medicinal and aromatic plant markets.

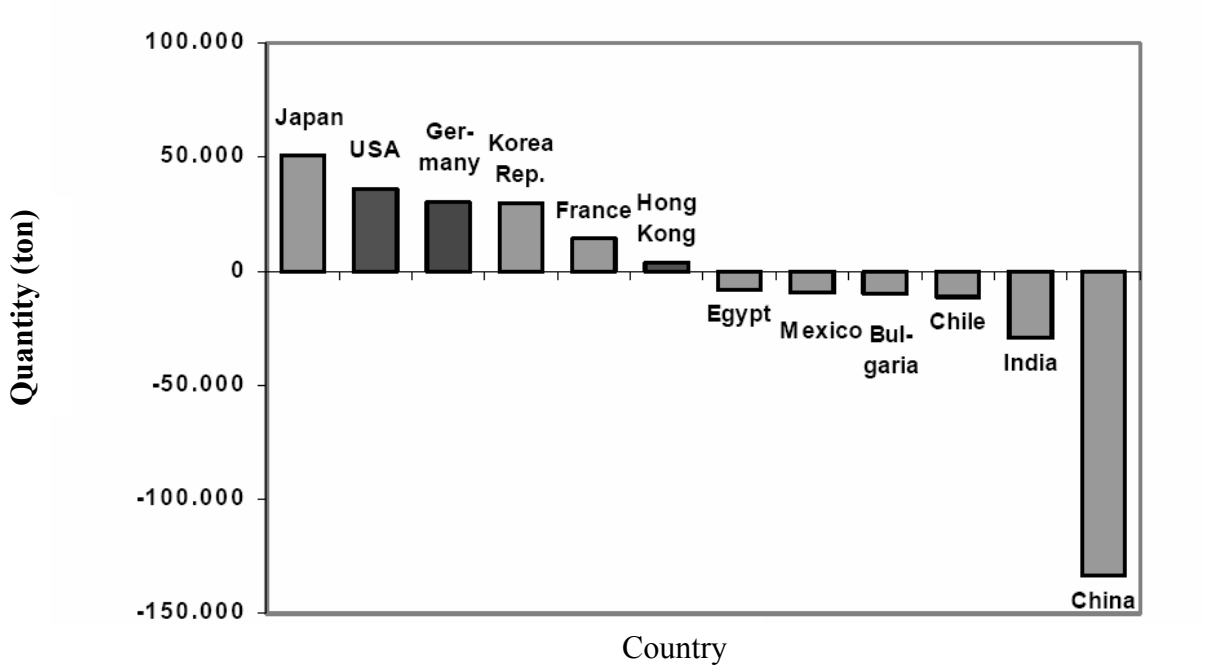


Fig. 1.1. Traded medicinal plants, cultivated or of wild origin (Lange, 1996).

In general, the cultivation of medicinal plant species has declined in the last thousand years. An example of cultivation would be the opium poppy (*Papaver somniferum*) which has been cultivated since 2700 B.C. (Heeger, 1989). Unfortunately, there is no exact information available of medicinal plant material produced under cultivation. On average over 80 000 tons of the annual exports of medicinal plants are wild-harvested. But, the botanicals exported are only a small part

of the annual domestic production which amounted to 700 000 tons in the early 1990s (Xiao, 1991).

In China, 60 % of the quantity in the trade are wild-collected (He and Sheng, 1997). In Nepal, every year more than 15 000 tons of herbs are taken from the wild (Bhattarai, 1997). Dozens of medicinal plant species are collected in large amounts from the wild in Canada, Mexico and the United States (Robbins, 1999; Fuller, 1991). In Europe, around 2 000 medicinal plant species are commercially used, of which 130-140 species are cultivated and the remainder are harvested from the wild (Lange, 1998). About 90 % of the 1 200 - 1 300 European native species are therefore primarily harvested from the wild (Lange, 1998). The quantity of wild-collected botanicals in east and southeast Europe in the late 1990s has been estimated to be at least 30 000 - 45 000 tons of dry plant material (the fresh material weighs two to three times more) (Lange, 2004). Harvesting from wild areas has resulted that some species are now threatened. Within Europe, for instance, at least 150 species are reported to be threatened. This is more than 10 % of the European native medicinal plant species and 1.2 % of the total European flora (Lange, 1998). Well-known medicinal plants that are threatened include *Arnica montana*, *Arctostaphylos uva-ursi* (bear-berry), *Gentiana lutea* (Lange, 1998), *Piper methysticum* (kava) and *Glycyrrhiza glabra* (liquorice) (Vines 2004). Another serious problem caused by wild harvesting is loss of genetic diversity, local extinctions and habitat destruction (Canter et al., 2005). Between 4 000 to 10 000 medicinal species may now be threatened globally (Edwards 2004).

According to the IUCN, WHO and WWF (1993) the cultivation of medicinal plants would be the best way to fulfill market demands. However, according to Lange (1998), cultivation has not proven to be useful for the majority of taxa in the trade due to the following reasons:

1- Many plants are difficult to cultivate due to their low germination potential or specific germination requirements. There is a lack of knowledge of the specific requirements for pollination, seed germination and growth, (Canter et al., 2005).

3- Cultivation of some species takes long time.

4- Many plants are only required in a small quantities.

5- In some cases the quality of wild-harvested material is better.

6- The cost of wild plant material is in general lower than for cultivated material (Lange, 1997).

1.1.3 Medicinal plants: opportunities

During the twentieth century, the emphasis gradually shifted from natural compounds obtained directly from plants to chemically synthesized compounds and their analogues. However, the natural products were widely considered as templates for the structure optimization programs designed to make perfect new drugs (Raskin et al., 2002). Although synthetic chemical drugs have a significant market share, the contribution of plants to disease treatment and prevention is still enormous. This is due to the fact that most herbal medicine is natural and therefore considered be safe for human consumption (Canter et al., 2005). In the twenty-first century the new focus on botanical pharmaceuticals as sources of human health products has became considerable. According to Raskin et al. (2002), this re-attention is based on some of the advantages of medicinal plants and these include:

- Potential of plants to synthesize mixtures of bioactive compounds with multiple therapeutic effects.
- Low-cost and better ability for scaling up the production of proteins and secondary metabolites.

- Changing the attention from chemically synthetic medicine to drug discovery and disease treatment and prevention.
- Cost limitation on the chemical synthesis of complex bioactive molecules.
- Public concern has indicated that plant phytochemicals provide a safer approach to disease treatment and prevention.

In 1999 the global market for herbal supplements exceeded US$15 billion, with a US$7 billion market in Europe, US$2.4 billion in Japan, US$2.7 in the rest of Asia and US$3 billion in North America (Glaser 1999). Many experts believe that the majority of plant-derived natural products remain undiscovered or unexplored for their pharmacological activity (Gentry, 1993; Mendelson and Balick, 1995).

The prospect of natural products to treat complex diseases is currently a good opportunity. In the twentieth century natural products have been used (applied) to treat complex diseases, such as diabetes, heart disease, cancer, and psychiatric disorders. However, there is no 'single golden molecular' solution as most of these diseases are not caused by a single genetic or environmental change, but are a result of interactions between genetics, environmental or behavioral factors (Kibertis, 2002). The traditional medicine systems are always based on the belief that a complex disease can only be effectively treated with a combination of botanical and non-botanical remedies (Raskin et al., 2002). For instance, the root extract of *Tripterygium wilfordii* has been traditionally used as a Chinese medicine to treat rheumatoid arthritis. The main active ingredient of this extract, a triptolide, is toxic unless given as a part of the root extract. This suggests that other unidentified extract components increase its safety and effectiveness (Su, et al., 1990).

1.1.4 Medicinal plants: limitations

There are certain limitations in the usage of medicinal plants. One of the major problems is the lack of reproducibility of the activity. In more than 40 % of plant extracts tested the activities could not be repeated when the extracts were re-sampled and re-extracted (Cordell, 2000). In addition, the biochemical profiles of plants can be changed due to the time and the location of harvesting (Raskin et al., 2002). Moreover, the current popular interest in drug discovery is to search for an active single compound rather than for a mixture. However, these valuable bioactive molecules are often associated with pigments and poly-phenols that interfere with their medicinal properties. A further limitation is the lack of an efficient and rapid strategy with a high-throughput screen to isolate and characterize a natural product from a plant extract. Poorly proven efficacy and safety, lack of standardization and quality standards are also a current limitation (Osowski et al., 2000, Kressmann et al., 2002).

1.1.5 Production of medicinal compounds

1.1.5.1 Traditional cultivation

Domestic cultivation is an alternative to overcome the problems that are inherent when working with medicinal plants. Problems include: misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. The use of controlled environments can overcome cultivation difficulties and could be a means to manipulate phenotypic variation in bioactive compounds and toxins. Improvement of domestic cultivation of medicinal plants requires using traditional and modern techniques such as biotechnological plant-breeding technologies. The techniques can be applied at the genetic level for improving the yield through seed uniformity, modifying the potency and toxicity, which are all agronomical traits of medicinal plants. Extracts of *Ginko biloba* are for example standardized to contain 24-27 %

flavonoid glycosides and 6-7 % terpene lactones while the toxic component, ginkgolic acid, is kept below 5 ppm (Canter et al., 2005). This is done during the characterization of the plant extracts. If these kinds of traits could be successfully identified at early stages, it would translate into a more cost-effective extraction process. The technique can be applied for the selection of desirable genotypes at early stages with molecular markers, and could speed up the selection of desirable plants at an early stage. There are several publications on the use of one particular type of molecular technique (RAPDs) for population analyses (Sangwan et al., 1999; Bai et al., 1997).

Controlled growth systems can also make it feasible to increase potency, reduce toxin levels and increase uniformity and predictability of plant extracts. This is due to the fact that the target compounds in plants frequently serve as adaptations to fluctuating temperature and light conditions (e.g. antioxidants), stress (e.g. proline), infection (e.g. flavanoids) or herbivory (e.g. alkaloids) (Canter et al., 2005). According to McChesney (1999) for example Caucasian-grown *Atropa belladonna* has an alkaloid content of 1.3%, compared to 0.3% in plants grown in Sweden. Shade-grown *Mentha piperata* has a lower essential oil content (1.09% v 1.43%) and lower menthol content within the oil (57.5% v 61.8%) compared to those grown in the light (McChesney, 1999). *Papaver somniferum* (poppy) grown in cool conditions contains more morphine but has a lower alkaloid content than *P. somniferum* grown in warmer conditions (Mc Chesney, 1999). These examples indicate the dependence of secondary metabolite accumulation on environmental conditions such as water availability, exposure to soil microorganisms and variations in soil pH and nutrients (McChesney, 1999).

1.1.5.2 Tissue and cell suspension cultures

Tissue culture experimental approaches used for the propagation of medicinal plants can be divided into three broad categories. The most common approach is the propagation of whole

plants through meristematic tissues such as shoot tips and auxiliary buds. This process is referred to as micropropagation. It can also be used for the conservation of valuable biodiversity (Augustine and D'Souza, 1997; Purohit et al., 1994). These techniques have a potentially significant role to play in establishing breeding material from wild populations (Kintzios and Michaelakis, 1999) and for mass-producing material for selection or engineering (Lakshmanan et al., 2002).

In the second approach, adventitious shoots are initiated from leaf, root or stem segments or from a callus derived from these organs, and this is known as organogenesis.

The third system of propagation involves induction of somatic embryogenesis in cell and callus cultures. Tissue cultures often promote genetic disturbances, which result in somaclonal variation, greatly extending the range of useful variation available to the breeder (Rout et al., 2000; Sevon and Oksman-Caldentey, 2002). Plant cell culture also provides an alternative approach, which is the production of secondary metabolites. Metabolite yield in cell suspension cultures may significantly exceed that observed in the parent plants. By using this technology therefore, the metabolites can be produced under controlled and reproducible conditions, independent of geographical and climatic factors. Table 1.3 shows some plant cell suspension cultures with higher production of secondary metabolites than when compared to their production in the parent plants. Although tissue and root cultures offer genetic stability (Flores and Curtis, 1992), as well as, in some cases, superior metabolic performance over suspension cultures of the same lines, cell suspension culture still has more potential for industrial application than plant tissue or organ cultures (Kieran et al., 1997).

Table 1.3. List of some cell suspension cultures with higher production of secondary metabolites as compared to what is present in intact plant (Ramachandra and Ravishankar, 2002).

Product	Plant species	Yield (%D.W.)	Reference
Rosmarinic acid	*Sa. officinalis*	36	Hippolyte et al. (1992)
Rosmarinic acid	*Col. blumei*	21.4	Ulbrich et al. (1985)
Anthroquinones	*M. citrifolia*	18	Zenk et al. (1975)
Shikonin	*L. erythrorhizon*	12.4	Fujita (1988)
Berberine	*Th. minus*	10.6	Kobayashi et al. (1988)
Jatrorhizine	*Berberis wilsonae*	10	Breuling et al. (1985)
Anthocyanins	*Pe. frutescens*	8.9	Zhong et al. (1994)
Berberine	*C. japonica*	7.5	Matsubara et al. (1989)
Diosgenin	*Diosc. deltoidea*	3.8	Sahai and Knuth (1985)
Sanguinarine	*P. somniferum*	2.5	Park et al. (1992)
Serpentine	*Cath. roseus*	2.2	Zenk et al. (1977)

1.1.5.3 Genetic manipulation of medicinal plants

DNA manipulation to change the gene expression in medicinal plants is now expanding. The modification of a desirable trait, which is related to one or a small number of genes, is now feasible (Canter et al., 2005). Although the primary target for trait manipulation in medicinal plants is the content of the active compounds, the other traits, including basic agronomic characters related to uniformity, stability, growth and development, and resistance to the pests, diseases and stresses (biotic and abiotic), must also be improved (Bajaj, 1998; Bernath, 2002). There are a considerable number of publications about the manipulation of plant biosynthetic pathways to produce drug precursors, food components or pesticides (Stevenson, 2004). In *Mentha spp* (mints), for example the biosynthetic pathway has been engineered to modify the essential oil production in the trichomes and to enhance the resistance of the plant to fungal infection and abiotic stresses (Veronese, et al., 2001). There is a long history of commercial production of high-valued phytochemicals by genetic modification through gene transfer into the

plant genome. An excellent example would be where the modification is carried out by infecting cells with *Agrobacterium rhizogenes*, carrying the gene of interest. The hairy root technique has been successfully applied for transformation of several important medicinal plants, including *Papaver somniferum* (Park and Facchini, 2000), *Artemisia spp*. (wormwoods) (Chen et al., 1999), members of the Solanaceae family (Moyano et al., 1999) and *Taraxacum platycarpum* (Lee et al., 2004).

A second modification technique is based on the transformation of the plants with *Agrobacterium tumefaciens*. This has been used for *Taxus* spp. (yew) (Han et al., 1994), *Echinacea spp.* (Wang and To, 2004), *Scrophularia spp.* (figwort) (Park et al., 2003), *Digitalis spp.* (foxglove) (Sales et al., 2003), *Thalictrum spp.* (meadow rues) (Samanani et al., 2002) and *Artemisia spp.* (Chen et al., 2000). A general problem however is where some important species still remain recalcitrant, notably *Ginko biloba* to regenerating the whole plant from transformed cells (Balz et al., 1999).

1.1.5.4 Pathway engineering in medicinal plants

Recent advances in metabolic engineering of both native and heterologous secondary metabolite producing organisms have allowed higher levels of production, direct synthesis of desired products, and the biosynthesis of novel products (Mijts and Schmidt-Dannert, 2003). Increasing the production of active phytochemical compounds is targeted through genetic manipulation of the metabolic biosynthetic pathways of the active compounds. Metabolic engineering utilizes knowledge of cellular metabolism to alter biosynthetic pathways and has many advantages over traditional methods of strain improvement through extensive screening. Although the metabolic pathways of active compounds are mostly poorly understood, and relatively few key genes have been isolated, there are some successful reports with regards to the engineering of biosynthetic

pathways leading to improved breeding of medicinal plants (Ferreira and Duke, 1997; Charlwood and Pletsch, 2002). An example is the nine-fold enhancement in the production of the sedative compound scopolamine in hairy root cultures of *Hyoscyamus niger* (black henbane). This has been performed by the simultaneous overexpression of two genes encoding the rate-limiting upstream and downstream biosynthetic enzymes (Zhang et al., 2004). A threefold enhancement has also been reported in the production of artemisinin, with anti-malarial and anti-cancer activity, in transgenic *Artemisia annua* plants through the overexpression of farnesyl diphosphate synthase, the enzyme involved in the process in the first biosynthetic step (Chen et al., 1999; Chen et al., 2000). In another example a 78-fold increase in flavonoid levels in the tomato peel was achieved by the overexpression of the *Petonia* chalcone isomerase (CHI) gene, an early enzyme of the flavonoid pathway, which was found to be a key enzyme in the increase of flavonol production (Muir et al., 2001). This technique can also be used for the induction of new compounds in plants. Isoflavones in legumes, for instance act as phytoalexins, that is, the biosynthesis of these antimicrobially active compounds are induced by microbial infection. By overexpression of isoflavone synthase, a cytochrome P450 enzyme, these compounds could be produced in *Arabidopsis*, tobacco plants and maize, which normally lack the ability to synthesize these compounds (Jung et al., 2000; Yu et al., 2000).

Another approach in the engineering pathway is combinatorial engineering by which genes responsible for individual metabolic pathway steps from different source organisms are combined to generate novel branches in the metabolic pathways and biosynthesize products that were previously inaccessible (Mijts and Schmidt-Dannert, 2003). Metabolic and combinatorial engineering of isoprenoid compound pathways includes those synthesizing many important industrial and pharmaceutical products such as terpenes and carotenoids. They provide excellent examples of the utility of this approach in secondary metabolite production. Overexpression of the

dxr (1-deoxy-D-xylulose- 5-phosphate reductoisomerase) and dxs (1-deoxy- xylulose- 5-phosphate synthase) genes (from non-mevalonate pathway), individually and in combination improves the yield of carotenoids when combined with engineered carotenoid biosynthetic pathways in *E. coli* (Matthews and Wurtzel, 2000; Jones et al., 2000).

1.2 Medicinal plants in South Africa

South Africa is rich in plant biodiversity, with almost 10 % (about 25 000 plant species) of all the plants known to humankind and is home to one of the 6 richest plant kingdoms in the world. About 70 % of these species are endemic to South Africa. (Torssel, 1997). It is estimated that there are more than 100 000 practising traditional healers in the country, with a contingent industry worth about R500 million per annum (Mander and Le Breton, 2005). More than 80% of South Africans rely on indigenous medicinal plants for their health care. Mander (1998) estimated that there are 27 million indigenous medicine consumers in South Africa with a large supporting industry. Up to 700 000 tons of plant materials are consumed annually which most of these plant species are collected in the wild for local use (Cunningham, 1988) and international trade (Lange, 1997). The current demand for numerous plant species resulting in intensive harvesting of indigenous plant stocks, and this is in association with the lack of major resource management and plant production, has resulted in the paucity of numerous indigenous medicinal plants. According to the South Africa Trade Directory of Indigenous Natural Products, this situation has already "killed more than 80% of the country's highly-valued medicinal trees". Twenty thousand harvested tons of more than 1 000 plant species are harvested with a street-value of R270 million and traded annually in the informal and commercial sectors for indigenous medicinal use (Technology for sustainable livelihood, Department of Science and Technology, Republic of South Africa.

1.3 The genus *Helichrysum*

The genus *Helichrysum* belongs to the family Asteraceae and is represented by approximately 600 species in Africa with about 244 species indigenous to South Africa (Drewes and van Vuuren, 2008). The name *Helichrysum* is derived from the Greek *helios*, the sun, and *chrysos,* gold, as there are many species with yellow flower heads.

Bioactivities of different species of *Helichrysum* such as antioxidant, antimicrobial, antifungal, antimalarial, antiviral and antidiabetic, have been widely reported in literature (Tomas-Barberan et al., 1988; Tomas-Lorente et al., 1989; Tomas-Barberan et al., 1990; Meyer et al., 1996; Tape et al., 2005; Drewes et al., 2006; van Vuuren et al., 2006; Lall et al., 2006; Aslan et al., 2006). Ethanol extracts of *Helichrysum 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 against Gram positive bacteria (Rios et al., 1991) and extracts of *H. decumbens* and *H. nitens* showed antifungal activity (Tomas-Barberan et al., 1988; Tomas- Lorente et al., 1989). An antimalarial study has shown that the essential oil of *H. cymosum* is active against the chloroquine-resistant *Plasmodium falciparum* FCR-3 strain (Van Vuuren et al., 2006).

1.3.1 Compounds isolated and identified from the genus *Helichrysum*

Many *Helichrysum* species have been studied for their chemical components. These include 38 species from South Africa (Jakupovic et al., 1986; Jakupovic et al., 1989b; Meyer et al., 1997) eight from Madagascar (Randriaminahy et al., 1992), several from Spain (Tomas-Baberan et al., 1988; Tomas-Barberan et al., 1990) and many species from Australia (Jakupovic et al., 1989a) The fact that different *Helichrysum* species produce different secondary metabolites (acetophenones, favonoids, phloroglucinols) as a biochemical defense mechanism against bacteria

and fungi is of great interest, since it indicates the use of different metabolic pathways (Mathekga et al., 2000). Figure 1.2 indicates some of isolated and identified compounds from different parts of *Helichrysum* species.

1.3.2 *Helichrysum aureonitens*

Helichrysum aureonitens Sch. Bip. is a perennial, with many slender, erect, stems about 300 mm high, and arising from a creeping rootstock. The stems and leaves are covered with white woolly hairs. The leaves are narrowly oblong to oblong-spathulate, with margins flat or a little revolute (rolled to the underside of the leaf). Each leaf has a tiny, hard, hairless tip (Ready, 2007). Globose flower heads compact clusters with bracts around each head, and bright yellow, often tinged with brown (Fig. 1.2). Flowering time is from September to February. The fruits are one-seeded and each bears a number of fine bristles. *H. aureonitens* is widespread in the grasslands of Limpopo, North-West, Gauteng, and Mpumalanga in the north, and south through Swaziland, eastern Free State, Kwazulu-Natal and western Lesotho to the Eastern Cape, as far as King William's Town. It is therefore found in the summer rainfall region of South Africa. It grows in full sun in moist to well-drained sandy loam soils (Ready, 2007). Seeds of *H. aureonitens* are usually difficult to grow as they need to be stimulated by fire to germinate (Ready, 2007).

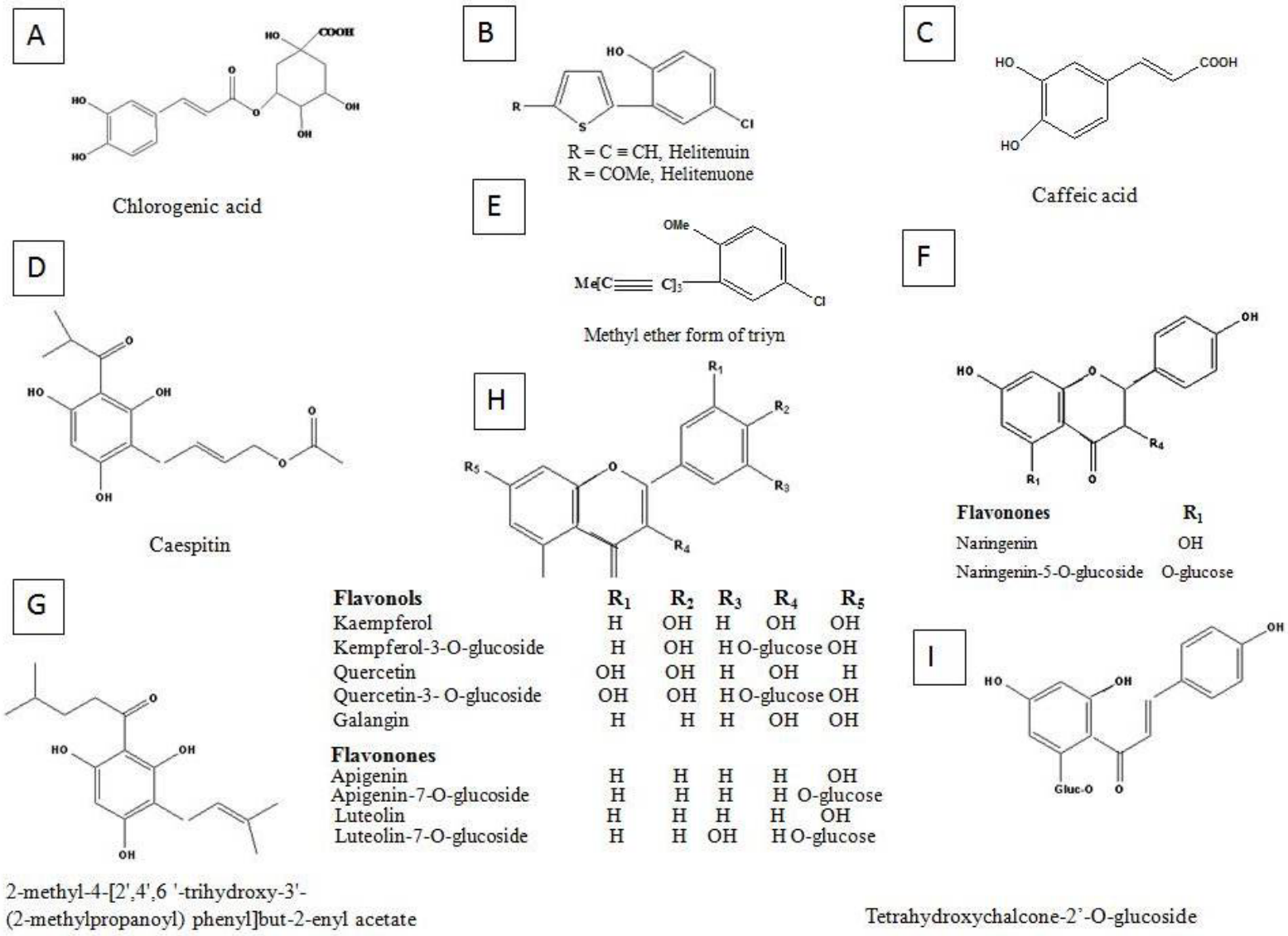


Fig. 1. 2. Isolated compounds from different parts of *Helichrysum* species. **A:** *H. arenarium* (Czinner et al., 2000); **B:** *H. tenuifolium* (Bohlmann et al., 1979); **C:** *H. arenarium* (Czinner et al., 2000); **D:** *H. caespitium* (Mathekga et al., 2000); **E:** *H. coriaceum* (Bohlmann et al., 1984); **F:** *H. arenarium* (Czinner et al., 2000); **G:** *H. caespitium* (Dekker et al., 1983); **H:** *H. arenarium* (Czinner et al., 2000); **I:** *H. stoechas* (Carini et al., 2001).

1.3.3 Medicinal properties of *H. aureonitens*

H. aureonitens is used 'to invoke the goodwill of ancestors, and by diviners to induce trances' (Pooley, 1998). It is also used as a remedy for bed-wetting in children and for treating a wide range of infections (Ready, 2007). In rural areas it is used to kindle fires by friction. It can be used to keep red mites away (Ready, 2007). It is also widely used in folk medicine (Phillips, 1917). Extracts from *H. aureonitens* are used topically by the indigenous people of South Africa against infections such as *Herpes zoster* (Meyer et al., 1996; Afolayan and Meyer, 1997). Antimicrobial and antiviral activity has been also reported (Meyer et al., 1996; Afolayan and Meyer, 1997). The dichloromethane extract has been found to be active against five Gram-positive bacteria and the methanol extract is active against *Bacillus cereus, B. pumilus* and *Micrococcus kristinae* (Meyer and Afolayan, 1995). Essential oils of the aerial parts of *H. aureonitens* has also shown a significant inhibitory effect on Gram-positive and Gram-negative bacteria (Yani et al., 2005).

Fig. 1. 3. Aerial parts of *Helichrysum aureonitens*.(www.plantzafrica.com).

1.4 Aims of the study

Galangin (3,5,7-trihyroxyflavonone) is one of the compounds that has been isolated from the aerial parts of *H. aureonitens*. Galangin has shown inhibitory activity against bacteria, fungi and viruses (Afolayan and Meyer, 1997; Meyer et al., 1997). Based on these results, this study was planned initially to investigate how the production of galangin can be enhanced in *Helichrysum aureonitens*? For that purpose the strategy of elicitation of galangin in different systems including hairy root induction and cell suspension cultures was planned. The intention was to establish a cell suspension culture system as well as the induction of a hairy root culture with *Agrobacterium sp*., and then investigate the elicitation of galangin using biotic and abiotic elicitors. Based on the initial negative results of the hairy root induction experiments it was found that hairy root induction is not feasible and the experiment was terminated.

After the establishment of the cell suspension culture of *H. aureonitens*, it was found that galangin was not detected in these samples, but surprisingly some other compounds which were not observed in intact plants of *H. aureonitens*.

These preliminary results lead to the following hypotheses for this study:

Hypotheses:

1- Cells of *H. aureonitens* cell suspension culture might produce interesting and perhaps novel compounds when growing in the dark, while the PAL enzyme is not activated.

2- Since previous results on *H. aureonitens* in our laboratory have shown the presence of the kaempferol, but none of its 4'-OH precursors, it is hypotesised that kaempferol is probably synthesized via galangin by the C4H enzyme.

1.5 Objectives of this study

According to the defined hypotheses, the objectives of this study were as follows:

1- Investigation on the reasons for the absence of galangin in the cell suspension cultures of *H. aureonitens.*

2- Isolation and identification of the major compound in the cell suspension culture of *H. aureonitens.*

3- The bioactivity of the isolated major compound against *Mycobacterium tubercolusis* and a human cancer cell line, prostate epithelial carcinoma (DU145).

4- Investigation on the biosynthetic pathway for galangin and subsequently kaempferol production in *H. aureonitens* and its relation with the (4'-OH) biosynthetic pathway of flavonols.

Chapter 2

Production of galangin in the cell suspension cultures of *Helichrysum aureonitens*

2.1 Abstract

In *Helichrysum aureonitens* galangin is one of the flavonol compounds with good medicinal properties. *H. aureonitens* was targeted to be enhanced via cell suspension culture as a potential system to produce valuable natural products. In ethanol extracts of the cell suspension cultures, galangin was however not detected, even though it was present in the leaves of intact plants. Other compounds were induced to be synthesised in higher amounts in the cells of the *H. aureonitens* suspension cultures than in the intact plants. According to the known biosynthetic pathway for the production of flavonols, some intermediates were searched for by GC-MS, including cinnamic acid, *p*-coumaric acid and naringenin as well as kaempferol. None of the mentioned intermediates was detected in the *H. aureonitens* cells suspension cultures. It was therefore concluded that the biosynthetic pathway of 4'-OH flavonol compounds is not functioning in the *H. aureonitens* cells suspension cultures.

2.2 Introduction

According to the medicinal properties of *H. aureonitens* described in the previous chapter in general and those of galangin specific, the enhancement of pharmaceutical compounds in cell suspension cultures of *H. aureonitens* seems to be important. *H. aureonitens* is only found in the wild and the negative effect of unsustainable collection from the wild has been mentioned before. Therefore it is important that other strategies should be considered to promote conservation of the plants and to try to increase the quality and quantity of medicinal compounds through different methods. Plant cell culture systems represent a potential renewable source of valuable medicinal compounds such as flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses. Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products (DiCosmo and Misawa, 1995). According to Ramachandra Rao and Ravishankar (2002) the advantages of this technology over the conventional agricultural production are as follows:

1- Independence of geographical and seasonal variations and various environmental factors.
2- Offering a defined production system, that ensures the continuous supply of products uniform in quality and yield.
3- Producing novel compounds that are not normally found in the parent plant.

Optimizing the cultural conditions, such as media components, phytohormones, pH, temperature, aeration, agitation and light affects the production of secondary metabolites (Mulabagal and Tsay, 2004). Through optimization of cultural conditions several products were found to be accumulating in the cultured cells at higher levels than compared to the native plants. Manipulation of the physical aspects mentioned before and the nutritional elements in a

culture is perhaps the most efficient way for the optimization of culture productivity. Good examples are the production of ginsenosides by *Panax ginseng* (Furuya et al., 1984; Furuya, 1988; Choi et al., 1994; Franklin and Dixon, 1994), rosmarinic acid by *Coleus bluemei* (Ulbrich et al., 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), ubiquinone-10 by *Nicotiana tabacum* (Fontanel and Tabata, 1987), berberin by *Coptis japonica* (Matsubara et al., 1989), all which accumulated at higher levels in the cultured cells than in the intact plants. Moreover, secondary pathways are activated in response to stress by induction of secondary metabolites as a defence to the stress condition. This response can be used for increasing the production of secondary metabolites by the application of biotic and abiotic elicitors in the cell suspension culture system (DiCosmo and Tallevi, 1985; Eilert, 1987; Barz et al., 1988).

There is little information with regards to the yield and biological activity of compounds in *Helichrysum* species in cell suspension culture systems. This part of the study was therefore carried out to establish a cell suspension culture system of *H. aureonitens* with the purpose of the enhancement of galangin using elicitors as well as the chemical analysis of some intermediates from the galangin biosynthetic pathway.

2.3 Materials and methods

2.3.1 Induction of plant callus and establishment of cell suspension cultures

Cell suspension cultures were induced from fully expanded leaves, which were harvested from *H. aureonitens* growing in the greenhouse. Leaves were surface sterilized for 30 seconds with 70 % (v/v) ethanol, followed by treatment with 5 % sodium hypochlorite plus 1 drop Tween-20 for 10 minutes and then washed three times with sterile distilled water. Callus induction was carried out by culturing the leaf explants on Murashige and Skoog (1962) (MS) medium supplemented with MS vitamins, 3 % (w/v) sucrose, 5.37 µM α-naphthalenacetic acid (NAA) and 0.85 µM 6-benzylaminopurine (BA). The pH was adjusted to 5.7 with NaOH (1 M) and the cultures were incubated at 25 °C in the dark. Suspension cultures of *H. aureonitens* were established from the leaf-derived callus in the liquid medium with the same medium composition as that used for callus induction medium and incubated at 25 °C in dark conditions on a rotary shaker at 110 rpm. Subcultures were created at 20-day intervals by transferring 200 mg of fresh cells into a 100-ml Erlenmeyer flask containing 20 ml fresh medium and incubated on a rotary shaker at 110 rpm at 25 °C in the dark.

2.3.2 Extraction and thin layer chromatography (TLC)

In a preliminary experiment the fresh leaves from greenhouse grown plants and the cells from the cell suspension cultures of *H. aureonitens* were extracted with different solvents including methanol, ethyl acetate, acetone, ethanol and dichloromethane, but for the rest of study only 100 % ethanol was used for extraction. Detection of compounds, including galangin in the extracts and fractions were initially carried out by using thin layer chromatography (TLC). The samples were spotted on TLC plates, developed in a hexane, dichloromethane, methanol

system (10: 10: 0.5) followed by exposure to short wave UV light to detect the conjugated compounds. The TLC plates were then sprayed with a 0.34 % vanillin in a 3.5 % sulphuric acid in methanol solution to visualize all the compounds on the TLC plates.

2.3.3 GC-MS analysis

GC-MS analysis was carried out under the following conditions:

A mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Technologies), with the following parameters were used, EI (70 eV), source temperature 250 °C, HP-5 column (30 m x 0.25 mm, 0.32 µm film thickness, J&W Scientific), injection temperature 250 °C, column temperature program: 80 °C for 1 min, then raised to 300 °C at a rate of 10 °C min^{-1}, and held on this temperature for 10 min; interface temperature 280 °C, carrier gas He, flow rate 1.2 mL min^{-1}, splitless injection.

The chemical structure of the isolated compound was identified by applying different spectroscopic techniques including NMR (1D and 2D) IR, MS and UV.

2.4 Results

Results showed that galangin is present in all leaf extracts, but was not observed in the extracts of cell suspension cultures (Fig. 2.1). However, two other major compounds were produced in the cell suspension cultures.

In addition to TLC, GC-MS analysis was carried out for the detection of galangin in the cell suspension cultures of *H. aureonitens*. GC-MS results also confirmed that galangin is not produced in the cell suspension cultures, although it is present in leaves of *H. aureonites* (Fig. 2.2). To investigate any blockage of galangin biosynthesis, some intermediates from the known galangin biosynthetic pathway which includes cinnamic acid, *p*-coumaric acid, naringenin as well as kaempferol were GC-MS analyzed in the cell suspension culture sample. Figures 2.3 to 2.6 show that these intermediates were also not detected in the cell suspension of *H. aureonitens*.

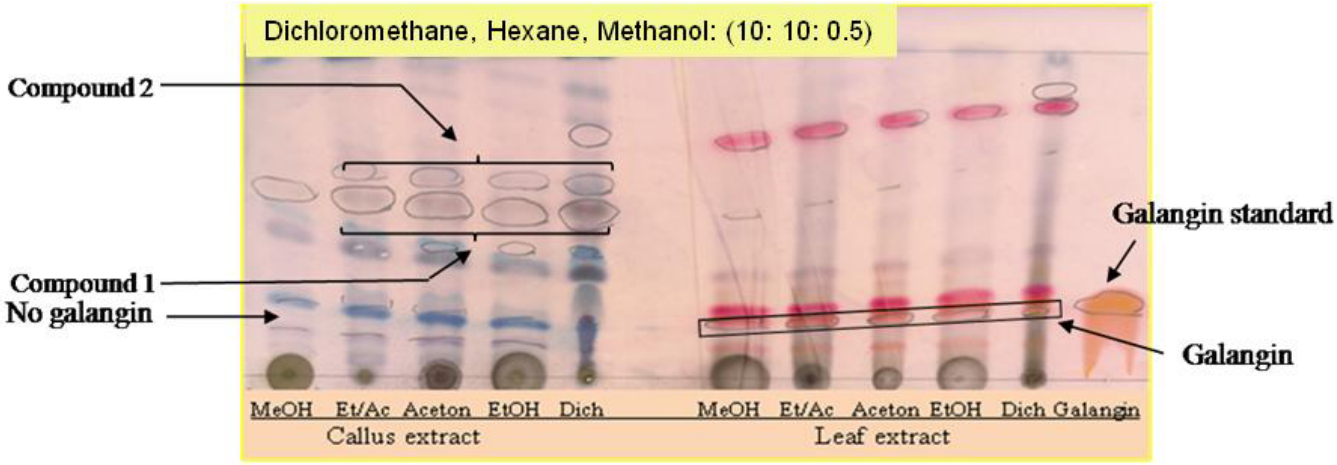


Fig. 2.1: Comparison of the galangin production in the leaf and in the cell suspension cultures of *H. aureonitens* with different solvents. Compounds **1** and **2** are the compounds produced in the cell suspension cultures of *H. aureonitens*, but not observed in its leaves.

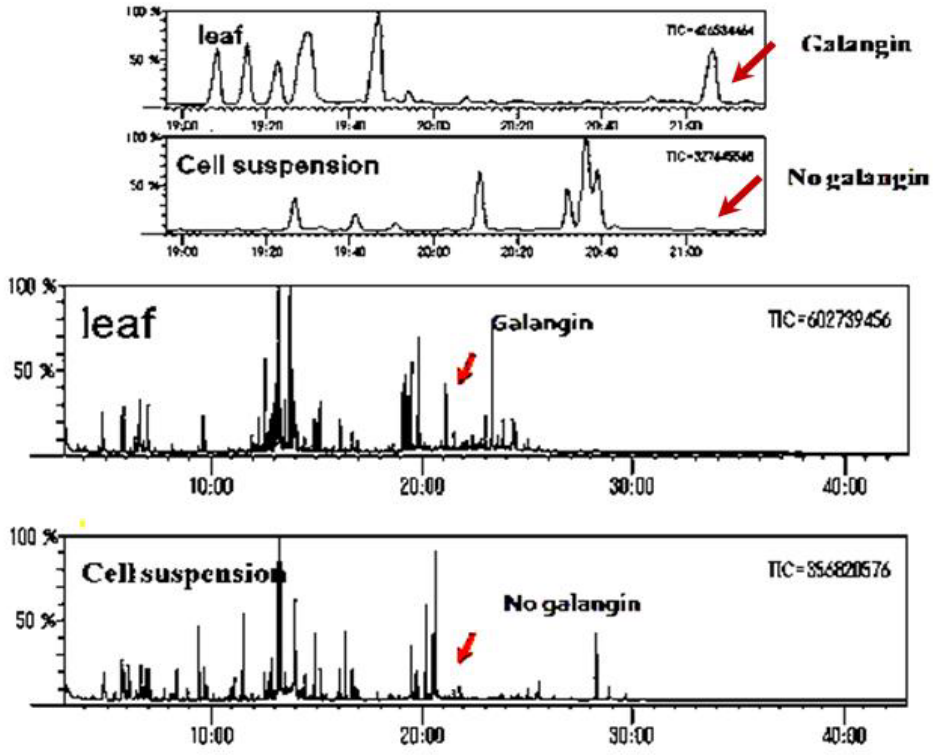


Fig. 2.2: GC-MS analysis comparison of the galangin production in the leaf and in cell suspension cultures of *H. aureonitens.* Arrow show the position of galangin picks.

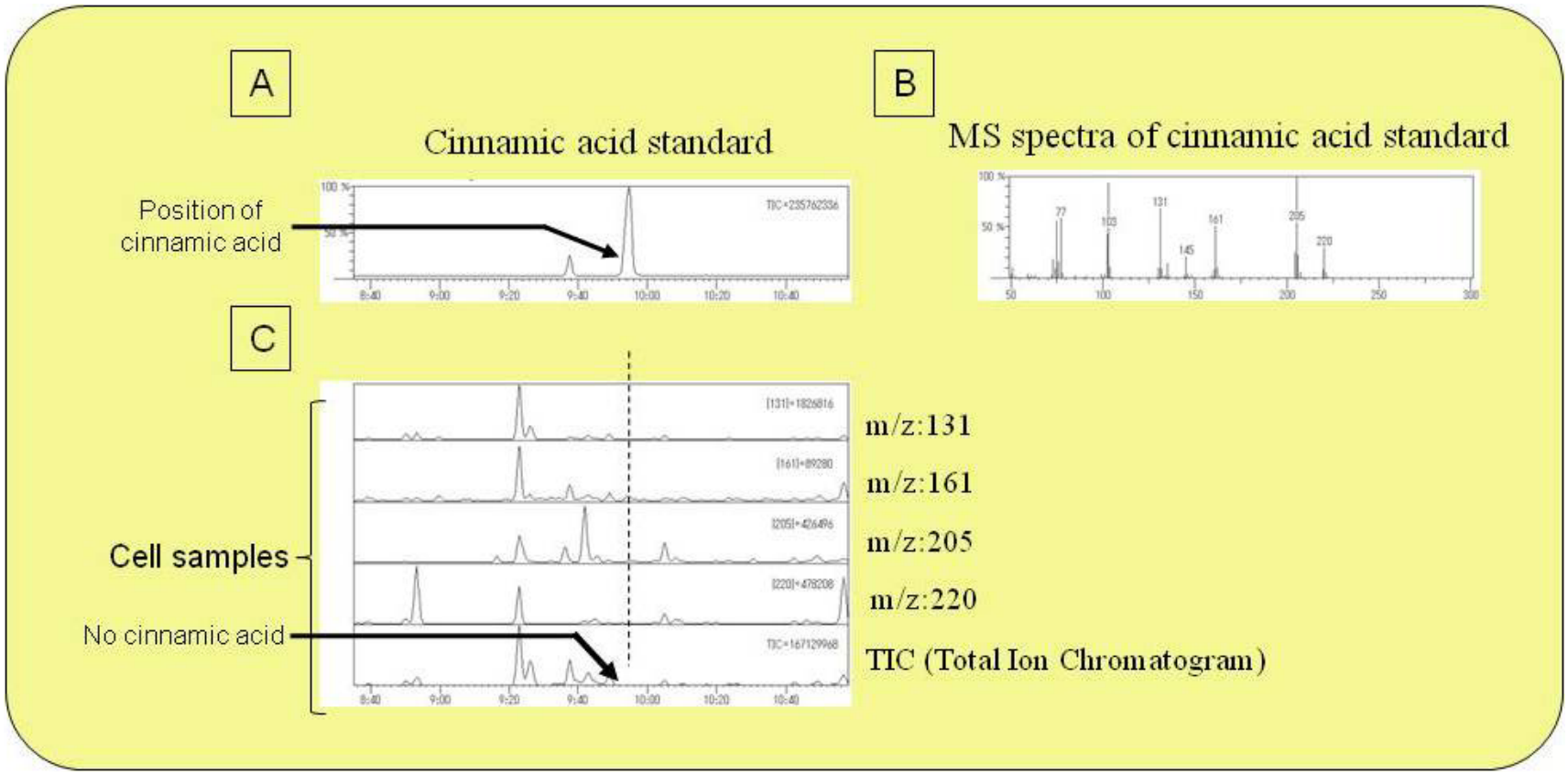


Fig. 2.3: GC-MS analysis of the cell suspension culture extracts for the detection of cinnamic acid. **A:** Cinnamic acid standard chromatograph; **B:** MS chromatograph of the cinnamic acid standard; **C:** Total ion chromatogram.

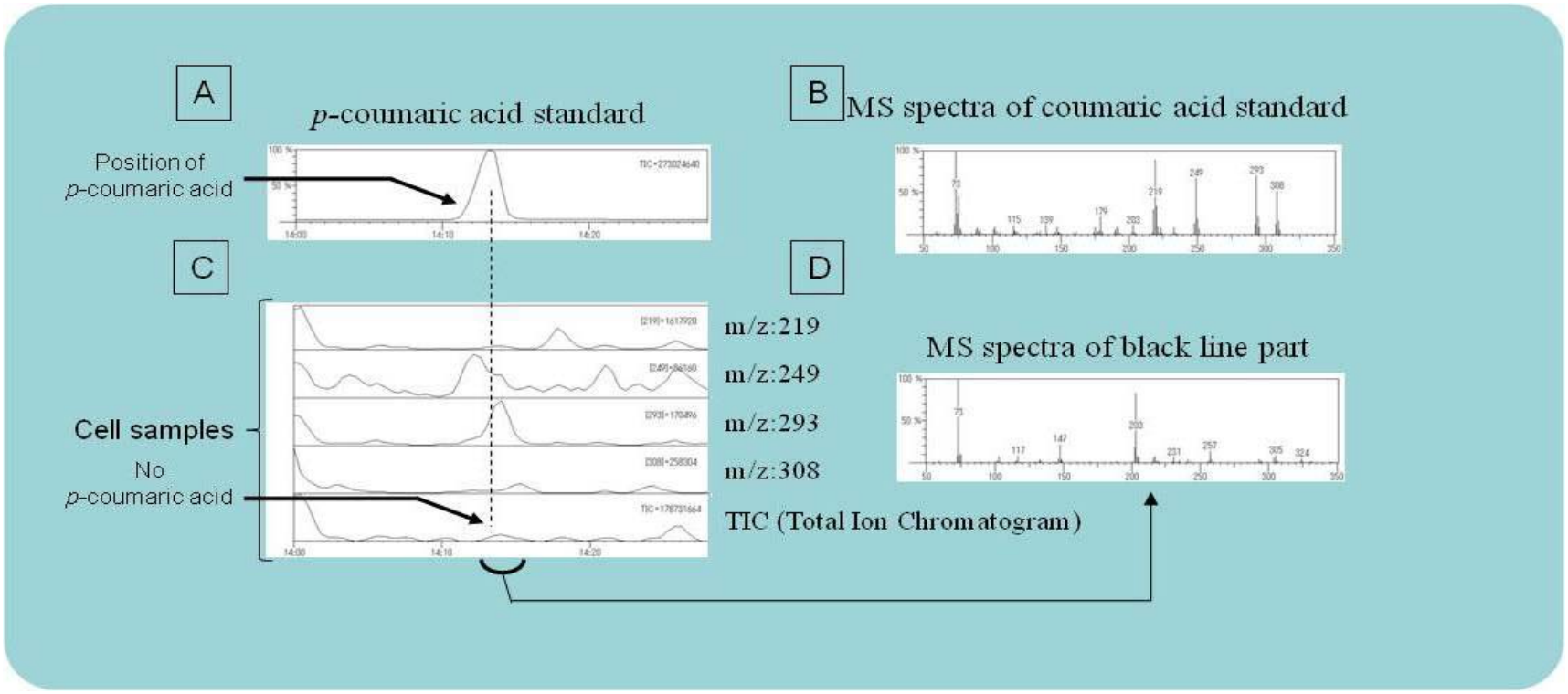


Fig. 2.4: GC-MS analysis of the cell suspension culture extracts for the detection of *p*-coumaric acid. **A:** *p*-coumaric acid standard chromatograph; **B:** MS chromatograph of the *p*-coumaric acid standard; **C:** Total ion chromatogram. **D:** MS chromatograph of the arrowed pick in TIC chromatogram.

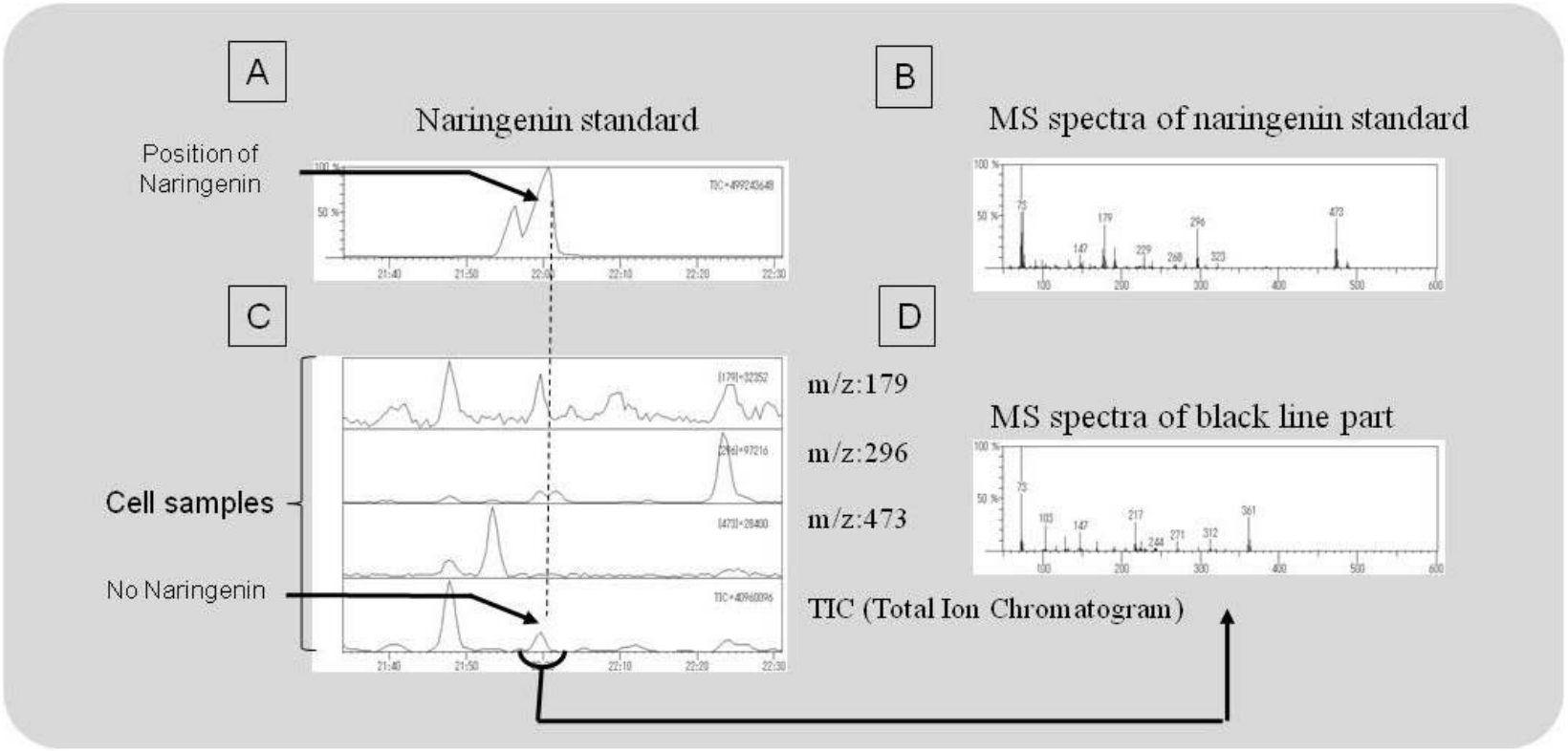


Fig. 2.5: GC-MS analysis of the cell suspension culture extracts for the detection of naringenin. **A:** naringenin standard chromatograph; **B:** MS chromatograph of the naringenin standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in the TIC chromatogram.

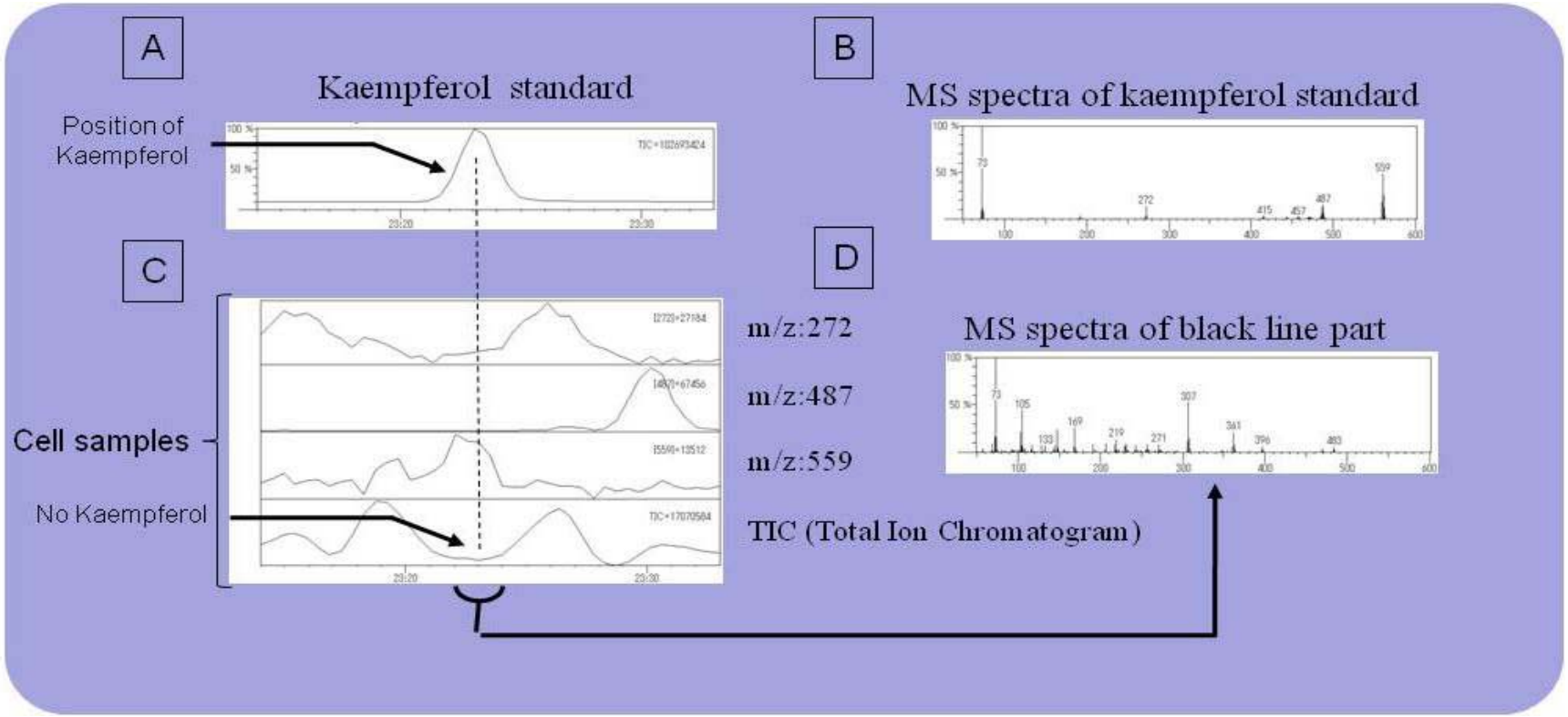


Fig. 2.6: GC-MS analysis of the cell suspension culture extracts for the detection of kaempferol. **A:** Kaempferol standard chromatograph; **B:** MS chromatograph of kaempferol standard; **C:** Total ion chromatogram. **D:** MS chromatograph of the arrowed pick in TIC chromatogram.

2.5 Discussion

The above results demonstrate that galangin is not produced in the cell suspension culture of *H. aureonitens.* There are several other reports that show the absence of certain compounds in the cells suspension cultures while they are present in the intact plants. An example of this is the callus culture of ginseng (*Panax ginseng*), in which polyacetylene, one of the anti-cancer compounds in intact ginseng plants, was not detected in the callus cultures (Yang et al., 1999). The absence of galangin in the cell suspension cultures of *H. aureonitens* could be due to different reasons. It has been stated that many of the natural products of interest are synthesized in organized tissues including leaves, roots and flowers. It is widely accepted that some levels of cellular or tissue differentiation is necessary for the biosynthesis of many compounds (Lindsey and Yeomem, 1983). The absence of peroxidise in carrot cells suspension cultures was attributed to lack of cell or tissue differentiation (Dornenburg and Knorr, 1995). In this experiment undifferentiated cells were used in the cell suspension cultures. This could be a possible reason for the absence of galangin in the cell suspension cultures of *H. aureonitens.* The experiment also showed that the tested flavonoid intermediates as well as kaempferol were not detected in the of *H. aureonitens* cell suspension culture. Accordingly it can be concluded that the known flavonol biosynthetic pathway in the cell suspension culture of *H. aureonitens* is not functioning. Previous experiments demonstrated that in some species of cell suspension cultures of parsley (*Petroselinum crispum*) and a few related species, flavonoids were absent in dark-grown cells and accumulate rapidly upon UV irradiation (Matern et al., 1983). It has also been shown that flavonoids, CHS protein, and CHS, PAL, and 4CL transcripts all accumulate in the same epidermal cells, following UV irradiation (Schmelzer et al., 1988; Wu and Hahlbrock, 1992). When using *Arabidopsis,* UV irradiation induces flavonoids (particularly kaempferol derivatives) and sinapate esters and isoflavonoids and psoralens in other species

(Hahlbrock, 1981; Beggs et al., 1985; Li et al., 1993; Lois, 1994). This phenomenon is attributed to the UV protection role of flavonoids in plants (Jordan, 1996; Reuber, et al., 1996).

It has been also mentioned that some of the phenylpropanoid compounds are induced in response to wounding or to feeding by herbivores. A good example would be kaempferol, another member of the flavonol group of compounds. The accumulation of flavonols such as kaempferol and its glycosides are induced by both wounding and pollination in petunia stigmas and appear to be required for normal pollen development (Mo et al., 1992; van der Meer et al., 1992; Vogt et al., 1994). The absence of galangin in the cells of *H. aureonitens* suspension cultures can be attributed to the dark condition or the absence of any kind of stresses in this experiment.

2.6 Conclusions

The results in this chapter showed that galangin and all the tested intermediates from the known flavonol biosynthetic pathway were not detected in the cell suspension culture samples of *H. aureonitens*. It can thus be concluded that galangin is a flavonoid-group member which is not inducible in this system, although it is produced in the leaf samples. One of the differences between these two samples is the different growing conditions, such as the absence or presence of light. It is therefore recommended that further experiments with *H. aureonitens* suspension cultures should include factors such as light in association with some biotic elicitors.

There are some compounds present in large amounts in the cell suspension cultures of *H. aureonitens,* and these were observed in much lower quantities in the leaf samples (Fig. 2.2). This leads to some questions that will be addressed in further chapters.

1- What is the observed compound in the cell suspension cultures of *H. aureonitens*?

2- Is the galangin biosynthetic pathway blocked and is there any relationship between these compounds and the galangin biosynthetic pathway?

3- Whether the tested intermediates of the galangin biosynthetic pathway in the cell suspension cultures are detected in the intact plant samples as well.

Chapter 3

Isolation, identification and bioactivity of a novel chlorophenol derivative from *Helichrysum aureonitens* cell suspension cultures

3.1 Abstract

A chlorophenol, 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol, was isolated as the major phenolic compound from the *Helichrysum aureonitens* cell suspension culturess. This triyne has been proposed to be an intermediate in the acetylene biosynthetic pathway in *Helichrysum* spp., but only the methyl ether form has previously been isolated from the roots of *H. coriaceum*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol extracts of cell suspension cultures of *H. aureonitens* against *Mycobacterium tuberculosis* H37Rv were found to be 1.0 mg/ml and 2.0 mg/ml respectively and the triyne was not active at 200 µg/ml. The ethanol extract of the cell suspension cultures and the triyne were evaluated for their cytotoxicity against monkey kidney (Vero cells) and human prostate epithelial carcinoma (DU145) cell lines. The inhibitory concentrations (IC_{50}) of the triyne and the crude extract were found to be 1.51 and 12.11 µg/ml against Vero cells respectively. The crude extract and the triyne showed similar activity in the prostate cancer cell lines by exhibiting IC_{50} values of 3.52 and 2.14 µg/ml respectively. The triyne warrants further investigation for its potential as an anticancer drug.

3.2 Introduction

According to the medicinal properties of *Helichrysum aureonitens* described in previous chapters, it can be considered as an important medicinal plant, which could benefit human health. One of the most efficient ways to produce secondary plant products is by a cell suspension culture system. Some plant cell suspension cultures produce the same secondary metabolites as the parent plants, while others produce different profiles in culture systems (Khafagi et al., 2003). This method offers an alternative opportunity to produce secondary metabolites by changing some culturing factors, plant growth regulators and elicitors or nutritional factors *in vitro*. This process can up-regulate or down-regulate the expression of genes involved in different biosynthetic pathways and consequently produce higher quantities of metabolites (Rao and Ravishankar, 2002). Research has shown that the cell suspension culture system can induce new compounds, not previously observed in the intact plants (Monache et al., 1994). A good example would be *Sophora flavescens*. Application of cork tissue and/or methyl jasmonate to cell suspension cultures of *S. flavescens*, not only increased the amount of sophoraflavanone G as a major flavonoid but also produced two precursors of sophoraflavanone G. Both of these precursors could not be detected either in cultured cells or in the original plants (Zhaoa et al., 2003). Elicitation of cell suspension cultures of *H. kraussii* also induced the production of two compounds which were not present in the control cultures (Prinsloo and Meyer, 2006). There are several successful cell suspension culture experiments for producing higher amount of secondary metabolites than in the intact plants. Scragg et al. (1990) reported that quinoline alkaloids were produced in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana*. Significant amounts of

sanguinarine were also produced in cell suspension cultures of *Papaver somniferum* using bioreactors (Park et al., 1990).

Drapeau et al. (1987) estimated that a 40-fold increase in the ajmalicine productivity of *Catharanthus roseus* would be required to justify the production of this compound by cell culture methods. In contrast the cells of ginseng (*Panax ginseng*) callus culture did not produce polyacetylene, an anticancer compound, although it was present in the intact ginseng plants (Yang et al., 1999).

When analysing results in the previous chapter it was found that galangin was not detected in the *H. aureonitens* cell suspension cultures. However, the production of two other compounds was induced to much higher concentrations when compared to the intact plant samples.

This part of the study will focus on the isolation, identification and the structural elucidation of the compound found in the ethanol extract of the *H. aureonitens* callus. The observations on the medicinal properties of the compound of the callus cultures are also reported.

3.3 Materials and methods

3.3.1 Induction of plant callus and the establishment of cell suspension cultures

The cell suspension cultures *H. aureonitens* were induced from fully expanded leaves, which were harvested from *H. aureonitens* grown in the greenhouse as described in chapter 2. Subcultures were created at 20-day intervals by transferring 200 mg fresh cells into a 100-ml Erlenmeyer flask containing 20 ml fresh medium and incubated on a shaker at 25 °C in the dark.

3.3.2 Extraction

Fresh leaves from greenhouse grown plants and cells from cell suspension cultures of *H. aureonitens* were extracted with pure ethanol.

3.3.3 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used as a preliminary step for the detection of compounds in all extracts and fractions (eg. galangin). The samples were spotted on TLC plates, developed in a hexane: dichloromethane: methanol system (10: 10: 0.5) followed by spraying with 0.34 % vanillin in 3.5 % sulphuric acid in methanol.

Two dimensional TLC analysis was carried out to determine the degradation of compounds. Second development was done immediately after the first one finished in a hexane: ethyl acetate system (7: 1). The main and decomposed spots were visualized under UV light. Control TLC plates were developed with a similar procedure, but without UV irradiation. To visualize the spots on control TLC plates, *p*-anisaldehyde was used.

3.3.4 Isolation and purification and identification of the compound from the ethanol extract of the cell suspension cultures

In a preliminary fractionation of compounds in the crude ethanolic extracts of *H. aureonitens* cell suspension cultures, the samples were applied to a Sephadex column (LH-20, Sigma-Alderich, South Africa) and eluted with 95 % ethanol. The eluted fractions were developed on TLC plates and the fractions that contained major compounds were collected and further purified using prep-TLC under dark conditions. The solvent system for prep-TLC developing was hexane: ethyl, acetate (7:1).

The fractions containing the major compounds were pooled together and column chromatograghy was again performed on a Sephadex LH-20 column using 95 % ethanol as eluent. The partially pure compound obtained from the Sephadex column was finally purified using prep-TLC in a hexane: dichloromathane: methanol system (10: 10: 0.5).

The chemical structure of the isolated compound was identified by applying different spectroscopic means including NMR (1D and 2D) IR, MS and UV.

3.3.5 Identification of the isolated compound from the cell suspension cultures

GC-MS analysis was carried out as described in chapter 2. Briefly a mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Tecnologies) with the following parameters, EI (70 eV), source temperature 250 °C, HP-5 column (30 m x 0.25 mm, 0.32 μm film thickness, J&W Scientific), injection temperature 250 °C, column temperature program: 80 °C for 1 min, then raised to 300 °C at a rate of 10 °C min^{-1}, and held on this temperature for 10 min; interface temperature 280 °C, carrier gas He, flow rate 1.2 mL min^{-1}, splitless injection.

3.3.6 Bioassays

3.3.6.1 Antituberculosis activity

The radiometric respiratory technique using the BACTEC system (Becton Dickinson Diagnostic Instrument, Spars, md) was used for testing susceptibility of *Mycobacterium tuberculosis* H37Rv (ATCC 27264) using the method described by Heifets and Good (1994). Solutions of the cell suspension culture and compound **1** were prepared by maceration of a requisite amount of the sample in a known volume of dimethyl sulphoxide (DMSO) to obtain a concentration of 5.0 mg/ml for both the crude extract and compound **1**. The solutions were stored at 4 ˚C until used. Subsequent dilutions were done in DMSO and added to 4.0 ml of BACTEC 12B (7H12 medium) broth to achieve the desired final concentrations of 2.0, 1.0, and 0.5 mg/ml for the crude extract and 200, 100, 50 µg/ml for compound **1**, with PANTA (Becton Diskinson & Company), an antimicrobial supplement. Control experiments showed that the final amount of DMSO (1 %) in the media had no effect on the growth of *M. tuberculosis*.

The radiometric respiratory techniques using the BACTEC 460 system (Becton Dickinson Diagnostic Instrument, Sparks, md) was used for testing susceptibility against *M. tuberculosis* as previously described (Mativandlela et al., 2006). Isoniazid (INH) (Sigma-Aldrich, South Africa) at a final concentration of 0.2 µg/ml served as the drug-control. The MIC was defined as the lowest concentration of the compound that inhibited more than 99 % of the bacterial population. The bactericidal effect (minimum bactericidal concentration, MBC) of the extract was assessed by plating the bacterial suspensions from the BACTEC vials, which exhibited a MIC effect, on 7H11 agar medium at the end of the experiment. The MBC was defined as the minimal bactericidal concentration, which effectively reduced by at least 99 % the viable

counts in the extract or compound containing samples as compared with those in the control vials (extract and compound free vials). The experiment was repeated three times.

3.3.6.2 Cytotoxicity assay

Microtitre well plates with Vero and human prostate epithelial carcinoma (DU145) cells were used for toxicity analysis of the ethanolic crude extract and the triyne according to Zheng et al. (2001). The positive toxicity drug, Zelaraleonone was tested at the final concentrations of 7.52 - 0.26 µg/ml. Cytotoxicity was measured by the XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) method using the cell proliferation kit II (Roche Diagnostics GmbH). Vero and DU145 cells (100 µl) were seeded at 1 x 10^5 into microtitre plates and incubated for 24 h. A dilution series was made of the *H. aureonitens* cell suspension crude extract (400.0 to 3.125 µg/ml) and the triyne (200.0 to 1.563 µg/ml), and was added to the microtitre plates and incubated for 72 hrs. Samples were used in triplicates. The absorbance of the colour complex was quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm. The IC_{50} value was defined as the concentration of the compounds at which absorbance was reduced by 50 %. The 'GraphPad Prism 4', statistical program was used to analyze the 50 % inhibitory concentration (IC_{50}) values (Motulsky, 2003).

3.4 Results

3.4.1 Isolation and purification of compound 1

Figure 3.1 shows the TLC chromatogram of the fractios of *H. aureonitens* cell suspension crude extracts that were eluted from the Sephadex column containing two major compounds. In most of the fractions both compounds **1** and **2** eluted together. But only compound **1** could be isolated and purified after column chromatography.

3.4.2 Identification of compound 1

Compound **1** was isolated as a semi-solid from the non-polar part of the ethanol extract of *H. aureonitens* cell suspension cultures. HRESI (negative mode) gave 213.0181 $(M-1)^+$ corresponding to the molecular formula $C_{13}H_7OCl$. ^1H NMR showed four signals at 7.33 (b s), 7.23 (d, 7.8 Hz), and 6.86 (d, 7.8 Hz), in addition to the methyl singlet signal at 2.05. The ^{13}C NMR showed 13 carbons, six of them were aromatic 157.4, 132.2, 131.6, 125.2, 116.6, 109.2, six carbons of acetylenic bonds at 82.2, 80.1, 70.1, 68.1, 64.6, 57.7 and a signal of methyl carbon at 4.7. HMBC cross-peak showed a correlation of H-3/ C-1, C-2, C-4, C-5, C-7, H-4/ C-3, C-2, C-5, C-6 and H-6/ C-1 C-2, C-5, C-4. The above data indicated the compound to be 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol (Fig. 3.2 and 3.3). The other data which included 2D NMR, HMQC, HMBC and COSY experiments also supported the structure of compound **1** (Fig. 3.4 to 3.8 and Table 3.1).

3.4.3 GC-MS analysis of detection of the triyne in the cells and leaves sample

Figure 3.9 shows the comparison of the GC-MS analyses of the triyne content of the cells of suspension cultures and leaves of *H. aureonitens*. It indicates that the triyne is also present in the leaves sample, but at very low concentrations.

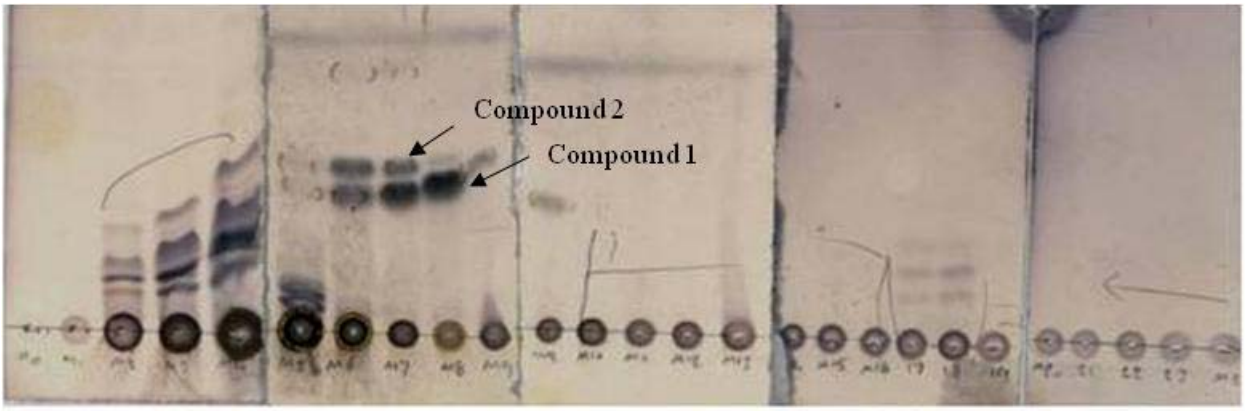


Fig. 3. 1. Results of developed collected fractions from the Sephadex column on TLC plates. Arrows show the presence of two compounds in some of the fractions of the cell suspension culture extracts which were not observed in the leaf samples of *H. aureonitens*.

Table 3. 1: NMR data of the isolated compound ($CDCl_3$, 300 MHz/75 MHz).

C	δ_C	δ_H	HMBC
	1 157.45		
	2 109.22		
	3 132.20	7.330 (d, 2.4)	C-1, -4, -5, -7
	4 125.24		
	5 131.63	7.226 (dd, 5.7, 2.4)	C-1, -2, -3, -4, -6
	6 116.59	6.867 (d, 5.7)	C-1, -2, -4, -5, -7
	7 68.09		
	8 57.76		
	9 64.58		
	10	70.08	
	11	80.09	
	12	82.05	
	13	4.72 2.057 (s)	C-8, -9, -10, -11, -12
-OH		5.739 (s)	

Coupling constants are expressed in Hz.

Carbon assignments from C-8 to 12 were not complete.

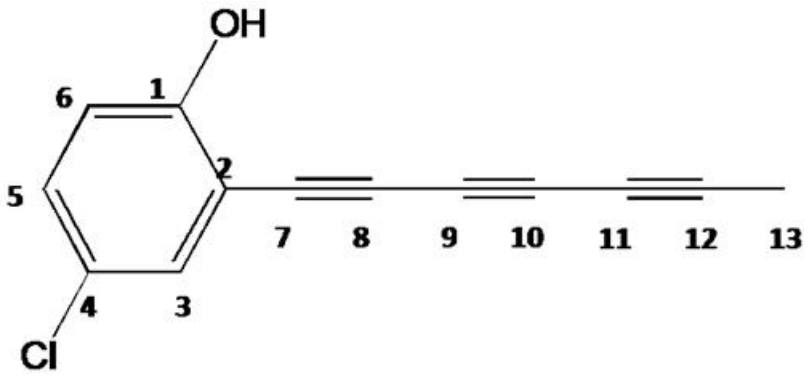


Fig. 3. 2. Chemical structure of the major compound based on the results of NMR (Carbon number) (4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol).

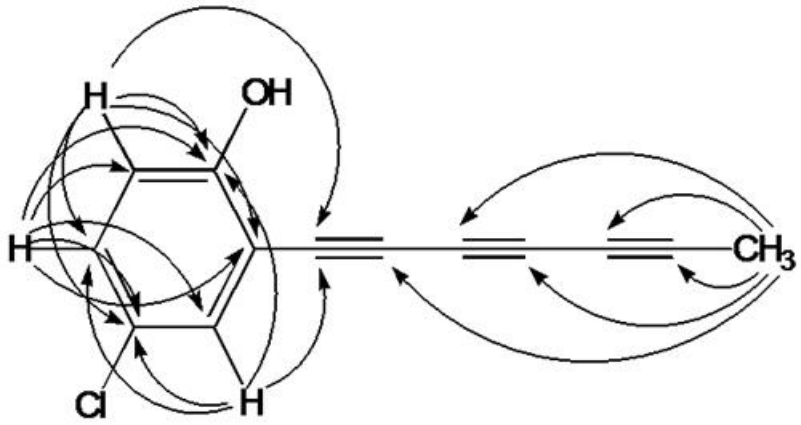


Fig. 3. 3. Chemical structure of the major compound based on the result of NMR (HMBC correlation).

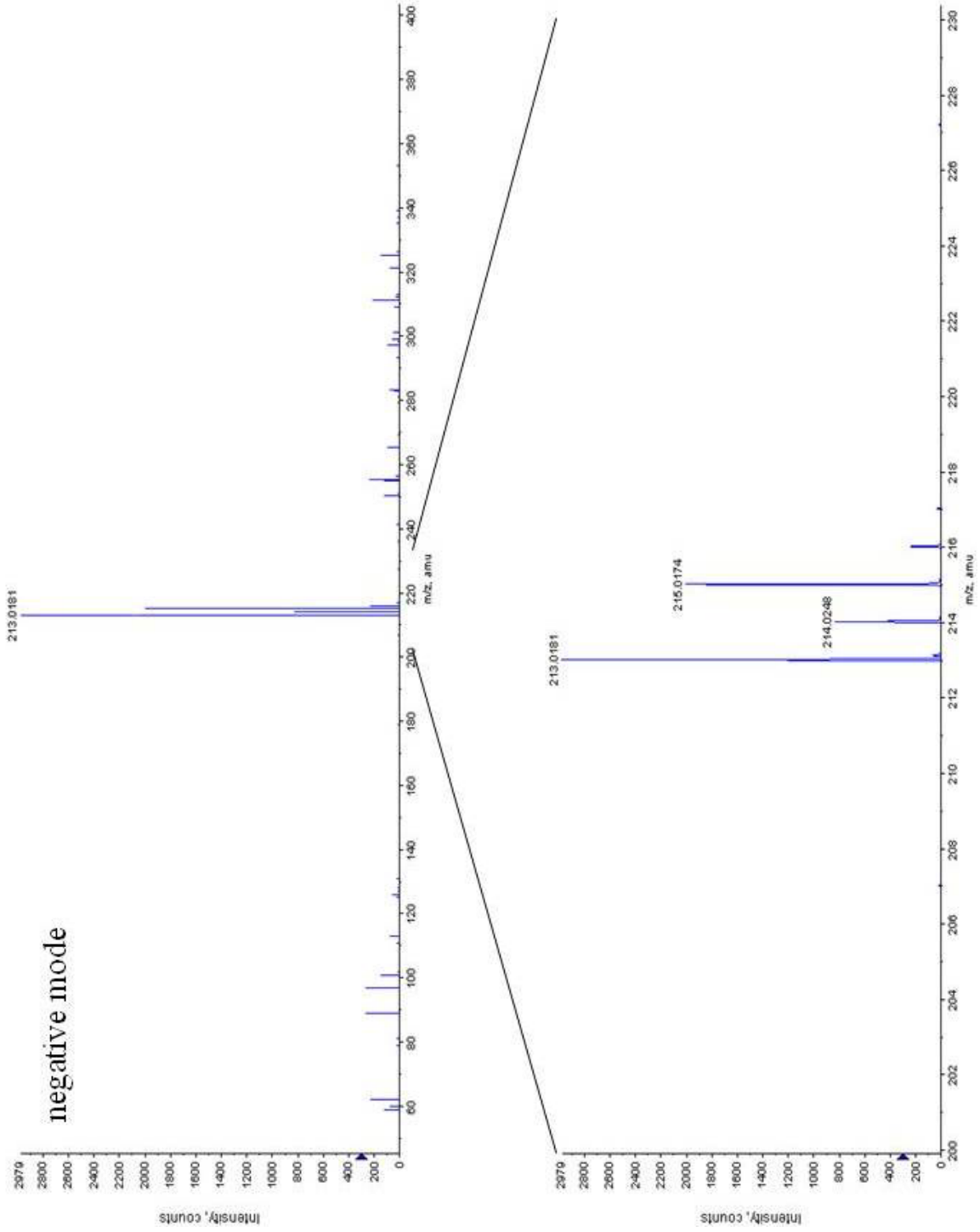


Fig. 3. 4. MS spectrum of the triyne using ESI.

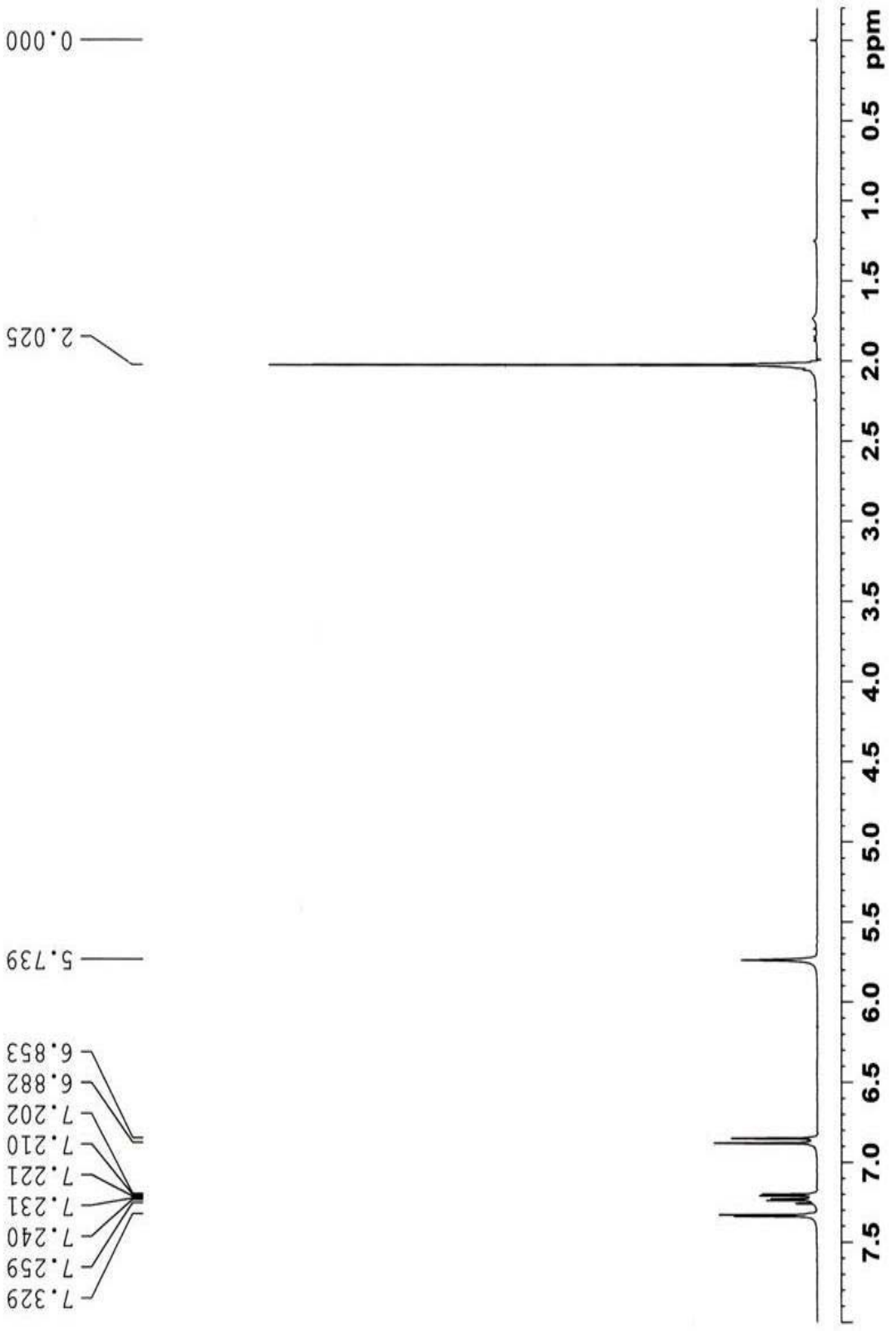


Fig. 3. 5. ^{1}H-NMR of the triyne

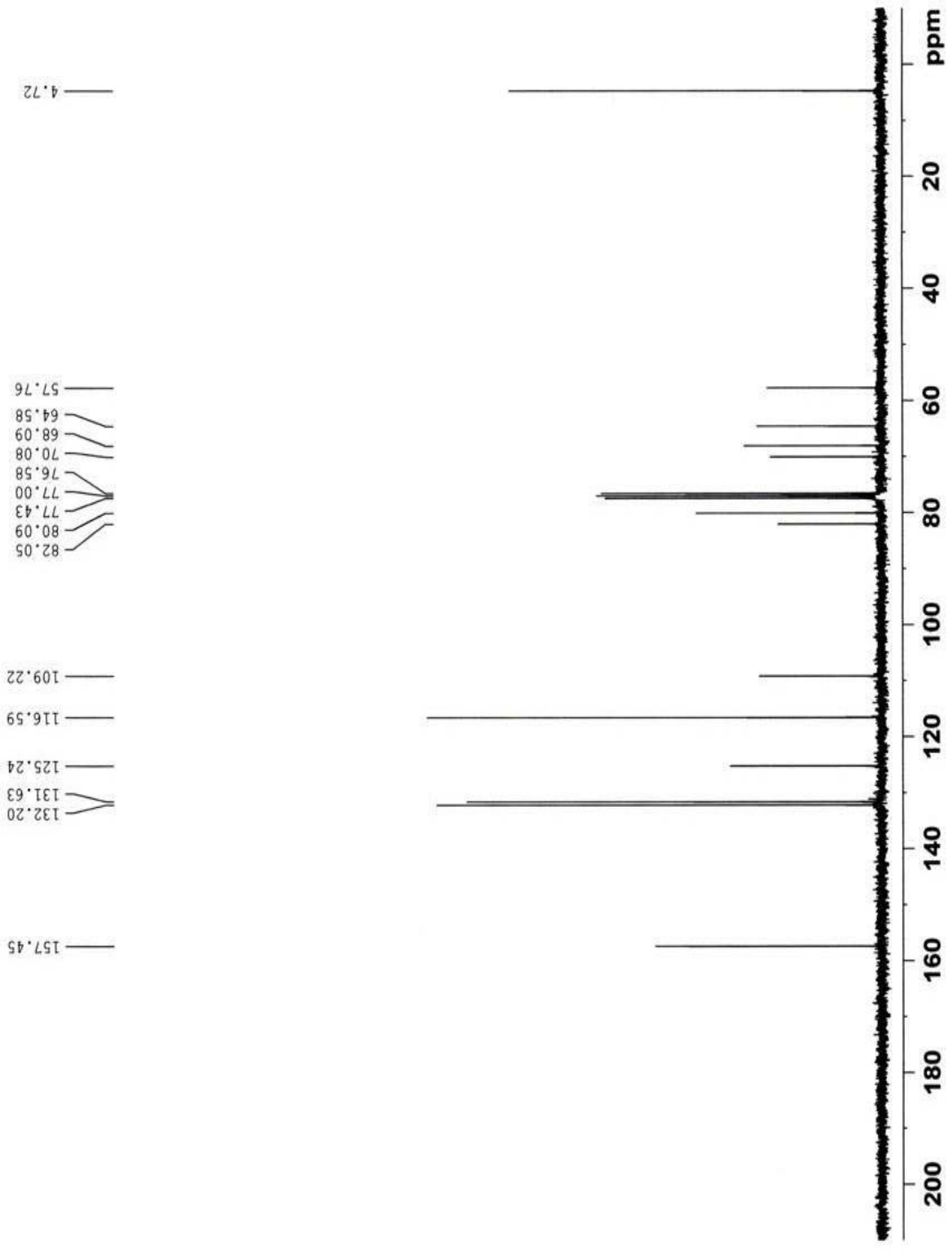


Fig. 3. 6. ^{13}C-NMR of the triyne.

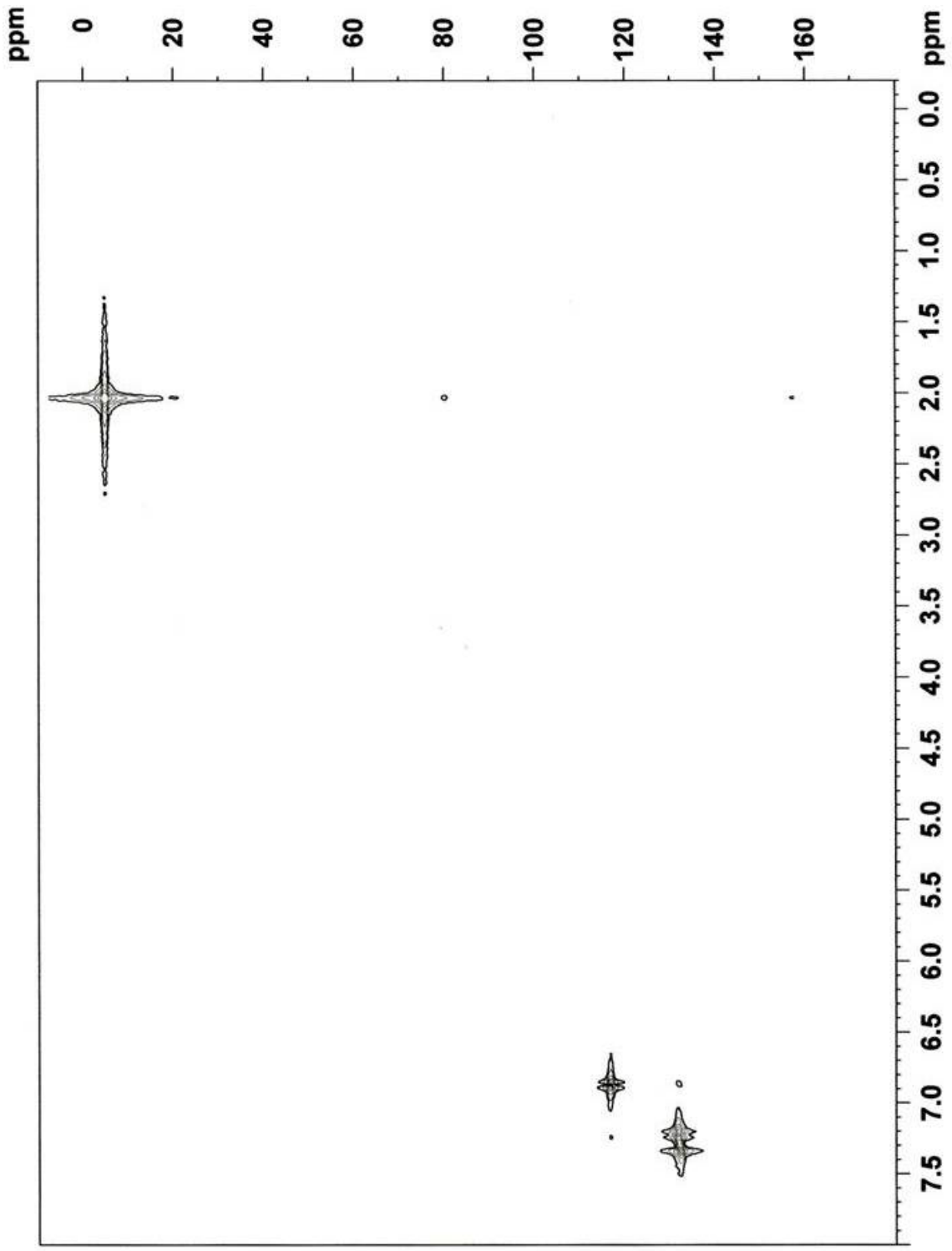


Fig. 3. 7. HMQC of the triyne.

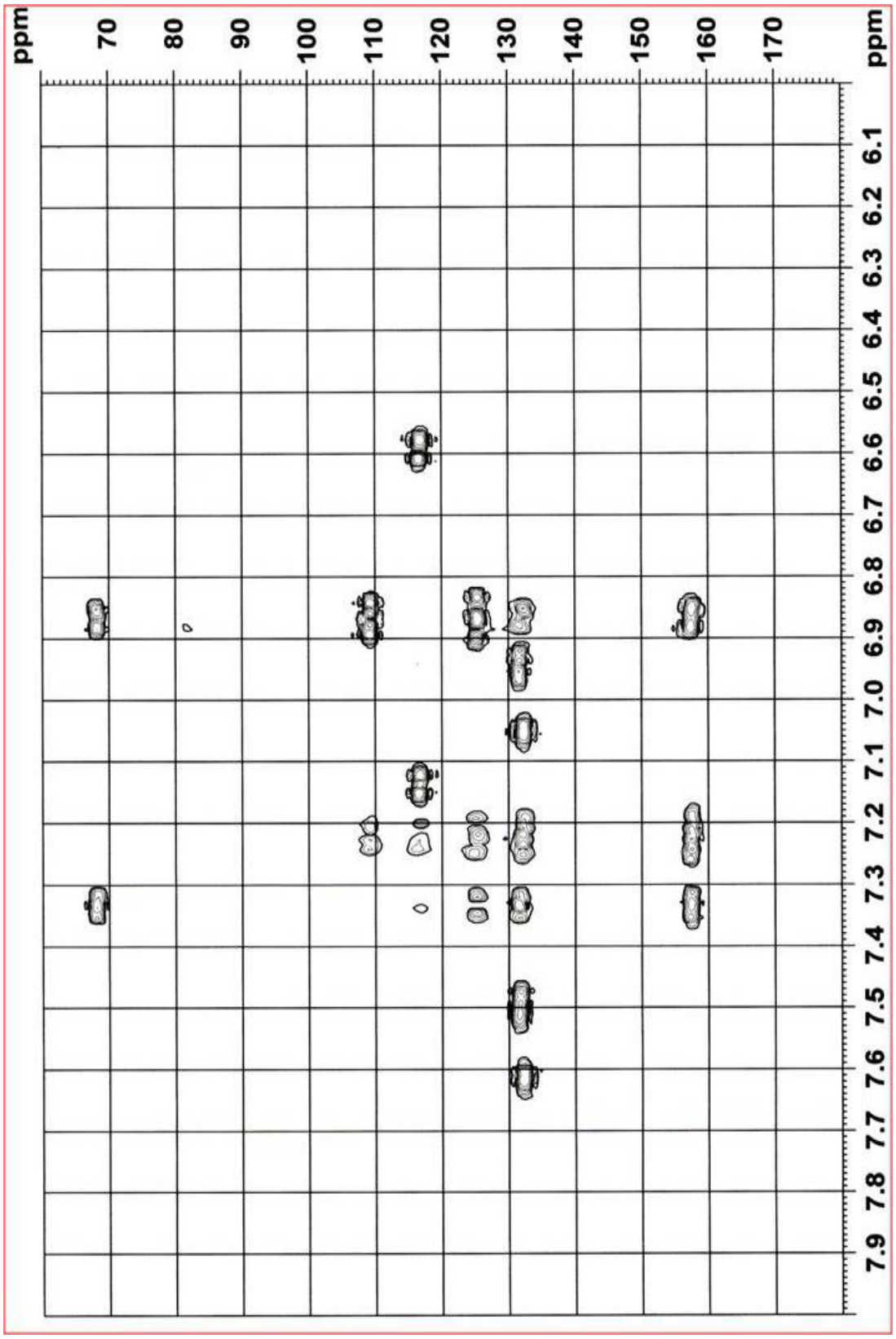


Fig. 3. 8. HMBC of the triyne.

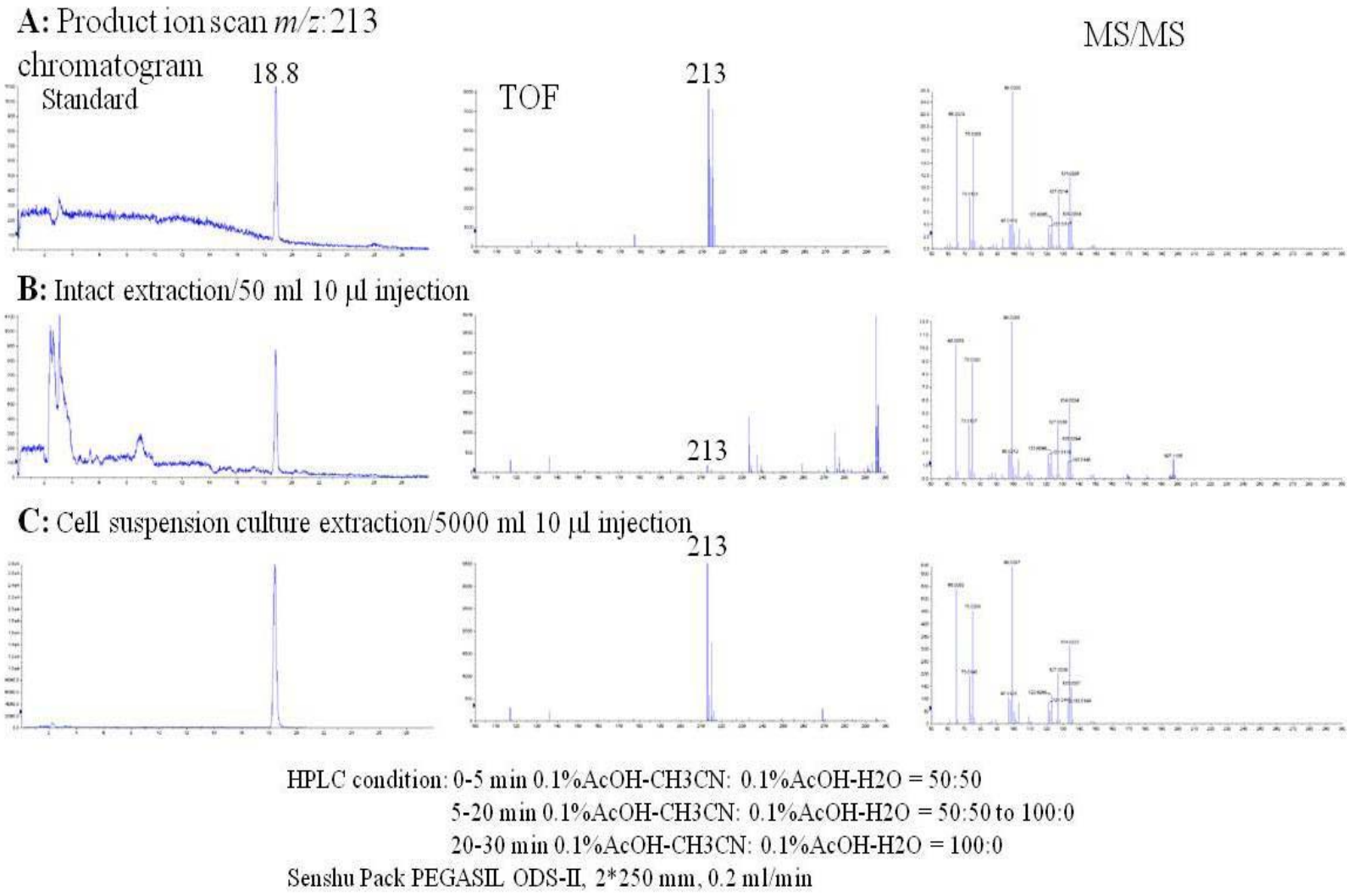


Fig. 3. 9. Detection of the triyne in different ethanolic extracts of *Helichrysum aureonitens*. **A:** Pure triyne as a standard; **B:** Intact plant **C:** Cell suspension cultures.

3.4.4 Instability of the triyne

On TLC analysis, the compounds were always visualized using UV light. After developing compound **1** on TLC plates and irradiating with UV light, a part of compound **1** was coloured to yellow by UV irradiation. Compound **1** was therefore also analyzed with two dimensional development on TLC (2D-TLC) followed by UV irradiation. Control plates were visualized by *p*-anisaldehyde. The result of the 2D-TLC elucidated that compound **1** from the cells of *H. aureonitens* cell suspension was decomposed to a yellow compound at the starting point with UV light (Fig. 3.10).

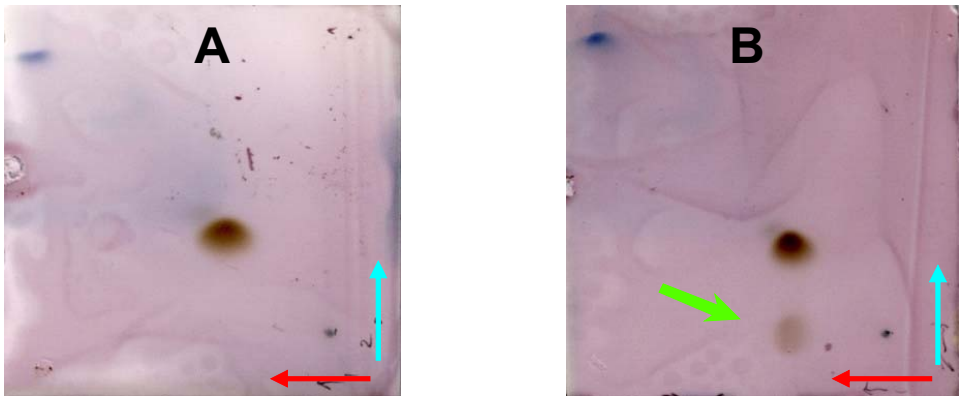


Fig. 3. 10. Two dimension thin layer chromatography (TLC) of the triyne. **A**: Control without UV light and emission with *p*-anisaldehyde; **B**: Degradation of the triyne after irradiation for 30 seconds with UV light after first TLC development. Green arrow shows the decomposed triyne by UV light. Narrow red and blue arrows show the direction of the first and second development respectively.

3.4.5 Bioassays

3.4.5.1 Antituberculosis activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol crude extracts of the cell suspension cultures against *M. tuberculosis* H37Rv were found to be 1.0 mg/ml and 2.0 mg/ml respectively. Compound **1** (the triyne) did not show antituberculosis activity at the highest concentration (200 µg/ml) tested. The MIC for the positive control "isoniazid" was found to be 0.2 µg/ml.

3.4.5.2 Toxicity and anticancer activity

The ethanol crude extracts of the cell suspension cultures and the triyne were evaluated *in vitro* for their cytotoxicity against Vero cells and the human prostate epithelial carcinoma (DU145) cell line. The triyne was about 8 times more toxic (IC_{50} value, 1.51 µg/ml) than the crude ethanol extracts (12.11 µg/ml) on a Vero cell line. The crude extracts and the triyne showed similar toxicity on a prostate cancer cell 4 line with IC_{50} values of 3.52 and 2.14 µg/ml respectively. The IC_{50} values of the positive control "Zeralenone" were found to be 1.63 and 0.53 µg/ml against Vero and DU145 cell lines respectively (Fig. 3.11).

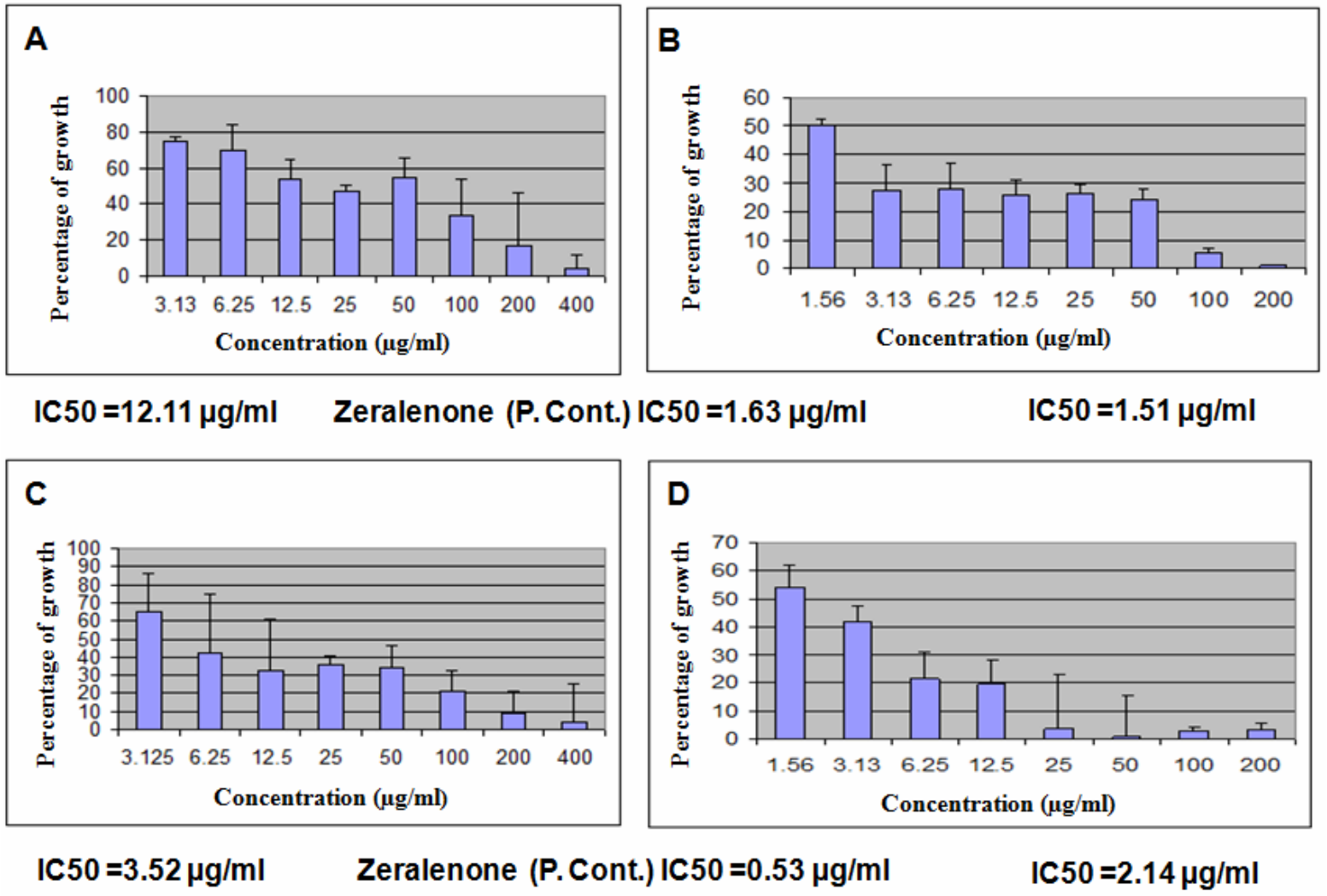


Fig. 3. 11. Effect of the ethanol crude extracts and the isolated the triyne (µg/ml) on the growth of Vero and DU145 cell lines. A: Crude extract (Vero cells); B: Chlorophenol triyne (Vero cells); C: Crude extracts (DU145); D: Chlorophenol triyne (DU145)

3.5 Discussion

The results of this part of the study regarding the production of the triyne in the *H. aureonitens* cell suspension cultures confirm what Bohlmann et al. (1984) found for the biosynthesis of the triyne in the roots of *H. tenuifolium*. They isolated the methyl ether derivative of the triyne and two other unusual chlorophenol acetylenes, helitenuin and helitenuone from the roots of *H. tenuifolium* (Fig. 3.12). They proposed that the isolated triyne could be synthesized from compounds **2** and **3** (Fig. 3.12), and the triyne would be the precursor of the compounds heliteniun and helitenuone **(**Bohlmann and Abraham, 1979). Based on this, it was proposed that the triyne is an intermediate in the acetylene biosynthetic pathway and the substrate for both helitenuin and helitenuone **(**Bohlmann et al., 1984). Although they were not able to isolate the triyne from the roots of *H. coriaceum,* the isolation of the triyne from the cell suspension cultures of *H. aureonitens* confirms their hypothesis.

The triyne was also detected in the aerial parts of the intact plants and the tissue cultured plants of *H. aureonitens* by LC-MS analysis, but at very low concentrations (Fig. 3.9). This indicates the higher induction of the polyacetylenase enzyme under the cell suspension conditions. This response of the cells in the cultures could have been driven by the exogenously applied phytohormones through up-regulating the involved genes in the triyne production biosynthetic pathway. Bohlmann (1984 and 1979) proposed the presence of the triyne in the roots (which normally grow in darkness) of *H. coriaceum* and *H. tenuifolium*. In our experiment the triyne was produced in the *H. aureonitens* cell suspension cultures grown in dark conditions. It might therefore be that dark conditions are necessary for the induction of the triyne, but more experimentation is needed to confirm this. This was confirmed in another

experiment in which the triyne could not be detected after 72 hrs exposure of the cell cultures to UV light (data not shown).

Two biosynthetic pathways are proposed for the production of acetylenic compounds including fatty acids and polyketide (Bohlmann et al., 1984 and Paiva, 2000). In the fatty acids pathway, the acetylenes are formed by the condensation of "activated" acetate units supplied either as malonyl CoA or acetyl CoA or from common fatty acids like linoleic acid. Polyacetate molecules can also be synthesized by specific "polyketide synthases" and modifying enzymes (Paiva, 2000). Originally the fatty acids were proposed as the starting point in the biosynthesis of linear polyacetylenic compounds, in contrast the poliketide biosynthetic pathway has also been proposed for the aromatic polyacetylenic compounds, e.g. the triyne can be biosynthesized from this pathway as well as phloroglucinols. The presence of phloroglucinols in the genus *Helichrysum* supports the contribution of the poliketide enzymatic system in the production of acetylenic compounds in this genus. However, because we could not isolate any intermediate related to the triyne we were not able to indicate which biosynthetic pathway is driving the chlorophenoltriyne production.

Antituberculosis activity has also been reported in other *Helichrysum* species. The MIC for example of the acetone extracts of *H. melanacme* and *H. odorastissimum* were found to be 0.50 mg/ml whereas the acetone extracts of *H. caespititium* exhibited an MIC of 0.10 mg/ml (Lall and Meyer 1999; Meyer et al., 2002). It has been shown that the higher activity of crude extracts when compared to the pure component is due to the interaction between different molecular components, and this is required for the optimal therapeutic effect of the plant extracts (Raskin et al., 2002). In root extracts of *Tripterygium wilfordii*, the main active ingredient identified was a triptolide, a traditional Chinese medicine to treat rheumatoid

arthritis, is shown to be toxic unless given as a part of the root extract, suggesting that other unidentified extract components increase its safety and, possibly, efficacy (Su et al., 1990).

The anticancer activity of some acetylenic compounds has already been reported (Siddiq and Dembitsky, 2008). Polyacetylenes found in ginseng (Matsunaga et al., 1990) and other medicinal plants (Jung et al., 2002) have been reported to exhibit anticancer activity. There are also some reports on the anticancer activities in the *Helichrysum* genus. Extracts from *H. pallassi, H. armenium, H. plicatum* have been reported to have considerable inhibition on DNA topoisomerase I. The inhibitors of DNA topoisomerase I represent a major class of anticancer drugs (Kucukogola et al., 2006).

Results obtained from the TLC analysis of the ethanol extracts showed that there are no observable flavonoid compounds in the *H. aureonitens* cell suspension cultures. This result was confirmed by GC-MS and LC-MS analysis. Some flavonoids including quercetin and galangin were detected in the ethanolic extracts of intact plants. This result was confirmed by LC-MS analysis.

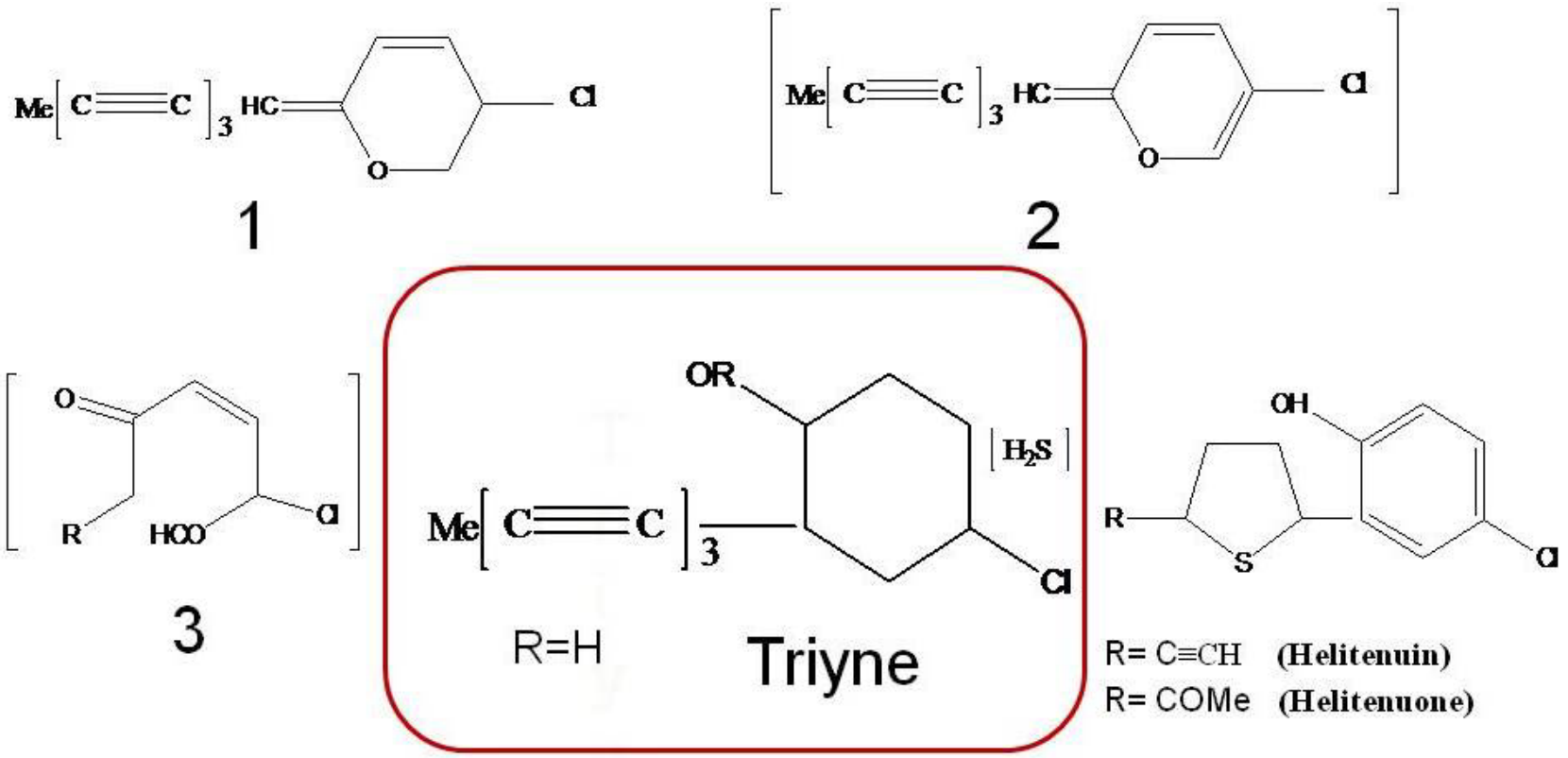


Fig. 3. 12. Proposed biosynthetic pathway for the chlorophenol compounds in *Helichrysum coriaceum***.** The boxed compound shows the position of the proposed the triyne in the biosynthetic pathway of the chlorophenol compounds. Bohlmann et al., 1984.

3.6 Conclusions

As mentioned before, one of the initial objectives for this study was the enhancement of galangin in cell suspension cultures of *H. aureonitens*. The results of this project showed that galangin is not produced in the cell suspension culture system. In fact none of the detected flavonoids in the intact plants were present in the cell suspension cultures of *H. aureonitens.* Instead there were two other compounds detected in a high concentration of which only compound **1** was isolated, purified and identified as a new chlorophenol compound. The new compound was identified as 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol. According to the chemical structure and the biosynthesis of the triyne the possibility of a direct relationship between it and the galangin pathway was rejected. Flavonol compounds e.g. galangin are light dependent compounds, while the triyne was detected in roots or in cell suspension cultures, which normally grow in dark conditions.

According to the results of this chapter, it could also be concluded that the triyne is a novel chlorophenol compound in the genus *Helichrysum* isolated for the first time from *H. aureonitens* cell suspension cultures. The triyne is perhaps the missing link in the chlorophenol compounds' biosynthetic pathway in *Helichrysum spp,* which was proposed by Bohlmann et al. (1984). Another interesting characteristic of the triyne is the presence of a chlorine molecule in the chemical structure. The chlorinated phenolic compounds are hardly found in nature. Results obtained at tested concentrations of the triyne against *M. tuberculosis*, showed no activity, but its toxicity against Vero cells was considerable when compared to the crude extracts. This indicates that the potential benefits of the triyne might be in anticancer activity.

Chapter 4

Biosynthetic pathway for flavonoids in cell suspension cultures of *Helichrysum aureonitens*

4.1 Abstract

Most phenolic compounds are derived from phenylalanine via the core phenylpropanoid pathway leading to formation of the flavonoids and other phenolic compounds. Flavonoids, which represent most of the medicinal properties, are the major phenolic compounds in the genus *Helichrysum*. Flavonols are present in *H. aureonitens*, and are represented by three compounds, kaempferol, quercetin, and galangin. Chemically the difference between galangin and kaempferol is the presence of an OH at the 4' position on the B ring of kaempferol. The only enzyme catalyzing the hydroxylation at the 4' position is cinnamate 4 hydroxylase (C4H) (Boudet, 2003). The chemical analysis of some of the intermediates by GC-MS showed that, except for cinnamic acid and kaempferol, the 4'-OH other intermediates including *p*-coumaric acid and naringenin, were not present in the leaf samples of *H. aureonitens,* while other intermediate compounds without OH at the 4' position on the B ring were detected. It was therefore proposed that for the production of galangin, an alternative pathway (non 4'-OH pathway) might be present in the *H. aureonitens* leaf extracts, which starts with phenylalanine and then moves to cinnamic acid. To produce kaempferol in the leaves of *H. aureonitens* plants, C4H, the only enzyme that can specifically catalyze hydroxylation at the 4' position, is necessary. Although C4H should be active in the leaves of *H. aureonitens*, it is proposed to be able to use galangin also as a substrate.

4.2 Introduction

Flavonoids represent an important class of natural products in plants. This category of secondary metabolites has important functions in the biochemistry and ecology of plants (Forkman and Heller, 1999). Flavonoids consist of several subgroups of compounds which include, anthocyanins, flavonones and flavonols. The phenylpropanoid pathway provides a variety of biologically important secondary metabolites through several important branches (Chen et al., 2007). The major one synthesizes lignins, which play a fundamental role in mechanical support, solute conductance and disease resistance in higher plants (Harakava, 2005; Chen et al., 2007). Another important branch of the pathway synthesizes various flavonoid compounds, which are involved in the attraction of pollinators and for protecting plants from UV irradiation and attacks by fungi and animals (Betz et al., 2001; Chen et al., 2007).

Flavonoids represent the major phenolic compounds in the genus *Helichrysum* (Czinner et al., 2001) and mainly confer the medicinal properties to this genus (Dombrowicz et al., 1994). Flavonoids are an important class of low molecular weight plant secondary metabolites which are widespread throughout the plant kingdom ranging from mosses to angiosperms (Koess et al., 1994). They are most commonly known for their antioxidant activity, as most of the polyphenols are ideal for free radical scavenging. *In vitro* experiments have showed that the majority of flavonoids are more effective in antioxidant activity than vitamin E and C (Rice-Evans et al., 1996; Rice-Evans et al., 1997).

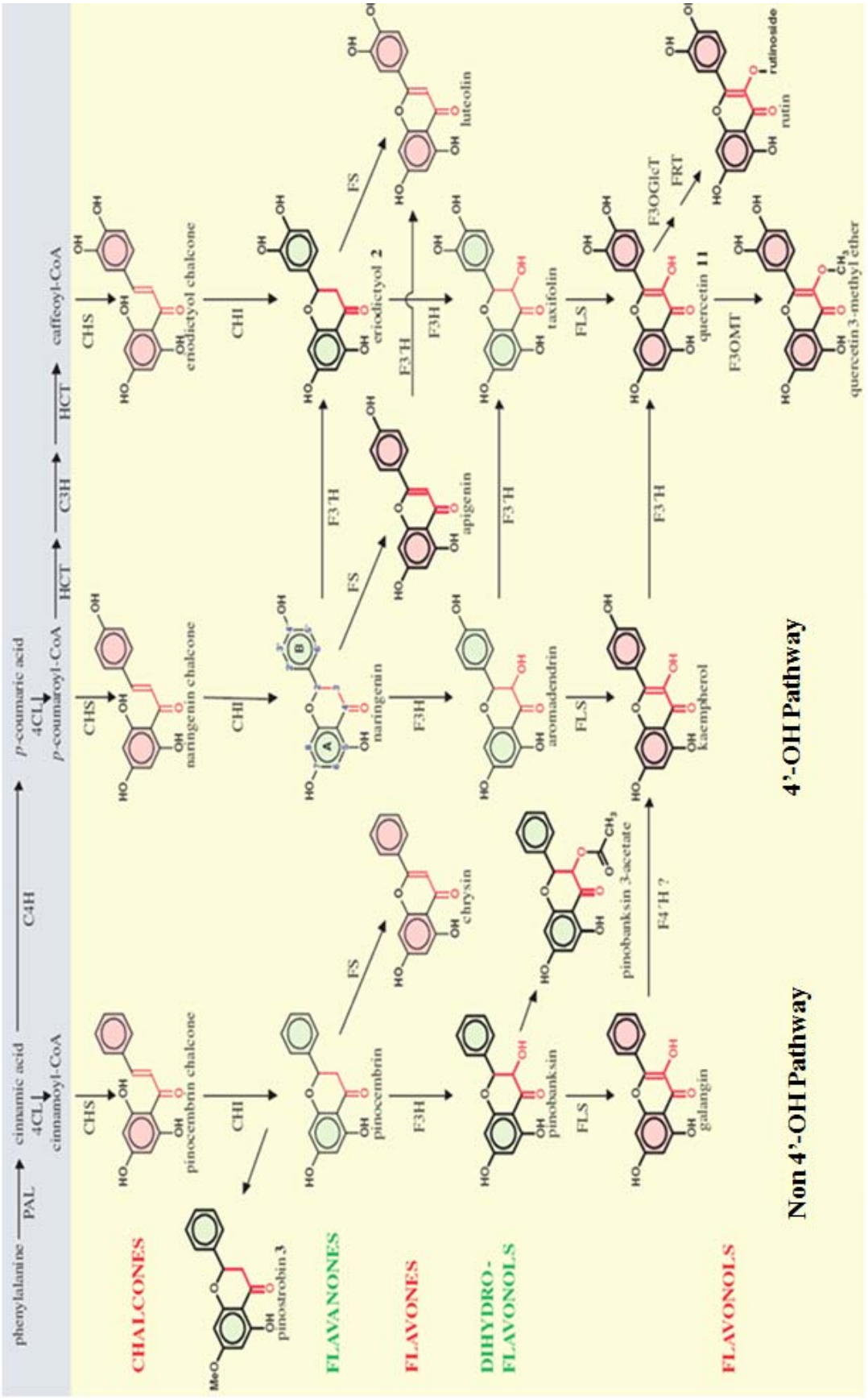
After the discovery of phenylalanine ammonia-lyase by Koukol and Conn (1961), rapid and substantial progress has been made to elucidate the metabolic pathways of flavonoids. Although the main reactions are known, there are still a few gaps.

Flavonoids are synthesized by the phenylpropanoid metabolic pathway in which phenylalanine is used as a substrate to produce 4-coumaroyl-CoA. This then reacts with

malonyl-CoA to synthesise the backbone of flavonoids. They all share a basic skeleton that contains two aromatic rings (A and B) and a heterocyclic ring (C) that contains one oxygen atom (Fig. 4.1). Based on the modification of the C-ring, they can be classified into several groups such as, flavonones, isoflavones, flavones, flavonols, flavanols and anthocyanines (Schijlen et al., 2004). Along this pathway, many products can be formed, including the flavonols, flavan-3-ols, proanthocyanidins (tannins) and a host of other polyphenolics (Scheme 4.1).

The flavonol group contains galangin, kaempferol, quercetin and myrecetin (Fig. 4.2). Most phenolic compounds are derived from phenylalanine via the core phenylpropanoid pathway. Chemically the difference among flavonoids is mainly the number and the position of the hydroxyl groups on the B ring. The main difference between galangin and most other members of the flavonols is the absence of the 4'-OH of ring B in galangin whereas most other flavonols have been hydroxylated. Although there is no clear evidence on the whole pathway of galangin biosynthesis in plants, it is logical to propose that there is an alternative pathway (non 4'-OH pathway) for the production of galangin. This pathway has already been tested in an engineered combinatorial biosynthetic pathway in *E. coli* in which phenylalanine was converted to galangin (Miyahisa et al., 2006).

It probably starts with phenylalanine and then goes to cinnamic acid. Since the only enzyme catalyzing the hydroxylation at the 4' position is cinnamate-4-hydroxylase (C4H) and galangin does not have an OH^- at this position, it can be concluded that C4H is not involved in this pathway. This means that cinnamic acid reacts directly with malonyl-CoA to synthesisze cinnamoyal-CoA and is then converted to pinocemberin chalcone. This pathway also produce pinocembrin which itself will be used as a substrate to synthesize pinobanksin, which is then probably converted to galangin. All the enzymes involved in this pathway would be similar to the 4'-OH pathway except for the C4H. The presence of the alternative



Scheme. 4. 1. Biosynthetic pathway of flavonoids. (Morreel *et al.*, 2006).

biosynthetic pathway is logical, but to date, some of its intermediates have not been tested for the production of the relevant products. It has not been shown by experimentation that pinobanksin is the substrate used to synthesize galangin and that this reaction is catalyzed by flavonol synthase (FLS) (Forkman and Heller, 1999).

In some plants the intermediates and products of both types of pathways have been identified in one plant species. For instance in the extract of Mexican oregano (*Lippia graveolens*), quercetin, naringenin, pinocembrin and galangin was identified by direct comparison with standards (Lin et al., 2007). Identification of some flavonoids such as chrysin, galangin and pinocembrin as major components has been reported in bud coats of *Populus* (Wollenweber, 1975). This information raises some questions. Is there an alternative pathway to synthesize galangin and its intermediates? If yes, are there links between the galangin pathway and the normal biosynthetic pathway for flavonols?

Since galangin has already been isolated and identified from *H. aureonitens* (Meyer et al., 1997), it represented a good opportunity to study the relationship between the galangin production pathway and the normal pathway for flavonols in this plant. In this chapter, a study on the biosynthetic pathway of flavonols in *H. aureonitens,* by chemical analysis of some intermediates based on the 4'-OH flavonoids biosynthetic pathway was done.

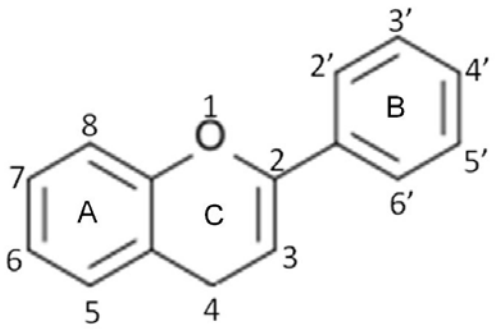
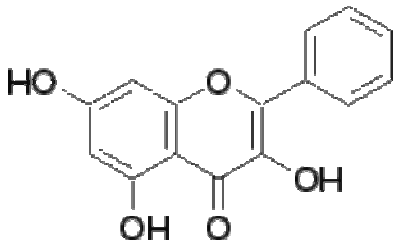
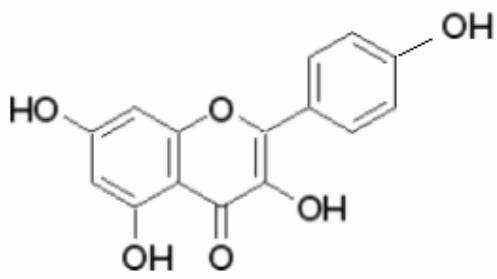


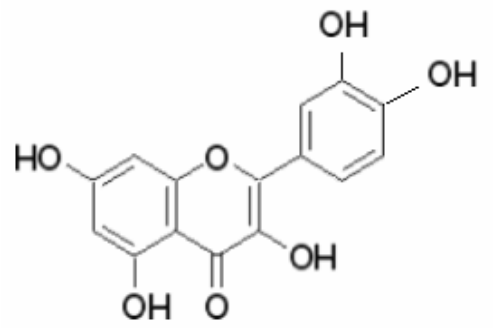
Fig. 4. 1. Molecular structure of the flavonoid backbone.



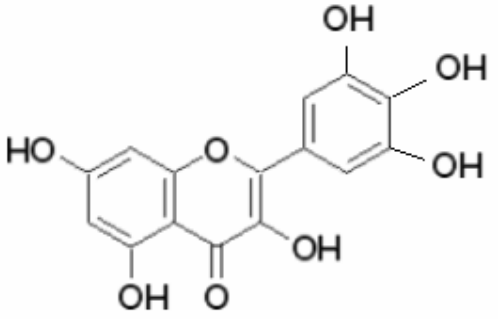
Galangin



Kaempferol



Quercetin



Myrecetin

Fig. 4. 2. Chemical structures of some members of the flavonols. The differences between compounds are based on the presence of OH-groups at the 4' position and the number of OH-groups on the B aromatic ring.

4.3 Materials and methods

4.3.1 Plant materials

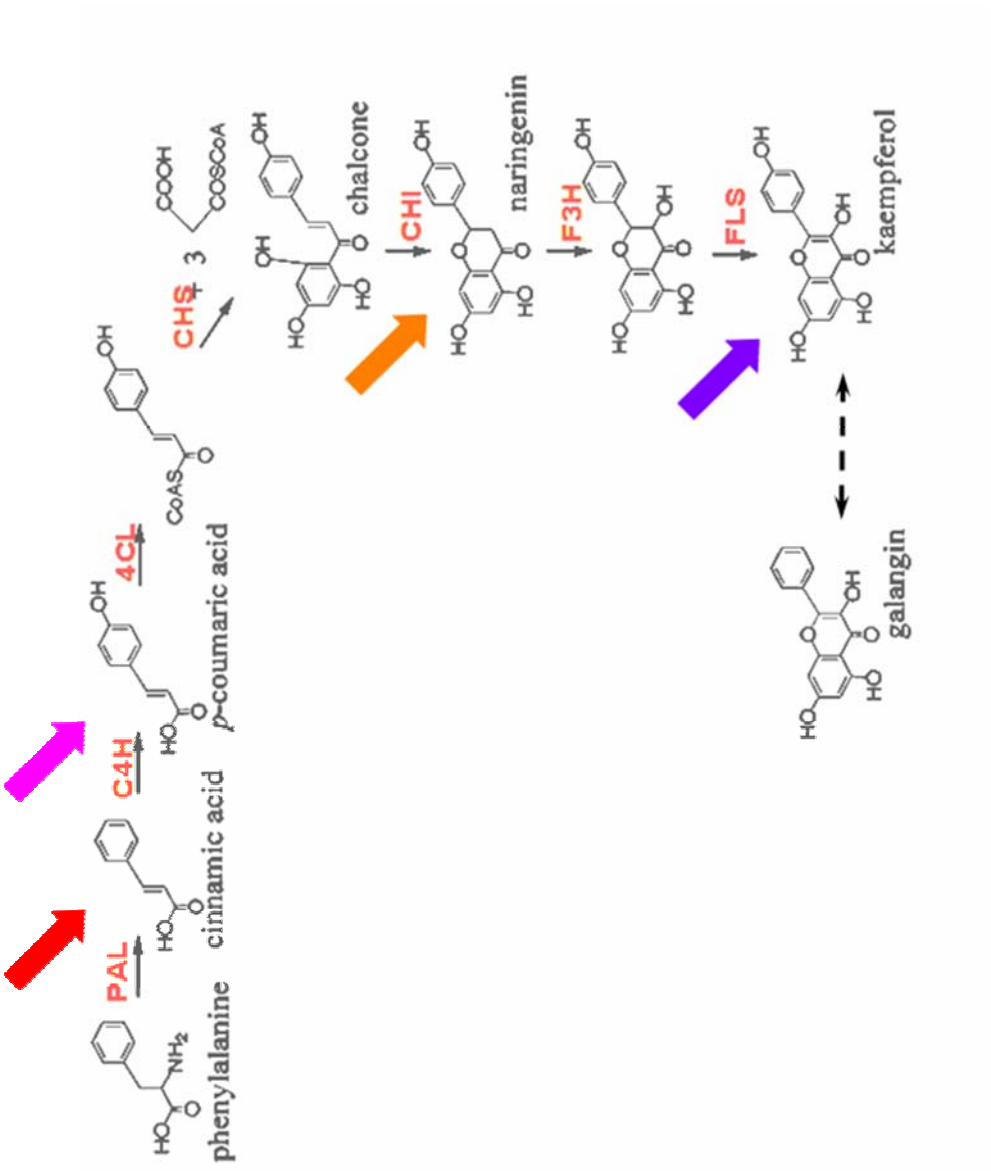
Helichrysum aureonitens plants were collected from the Kwazulu-Natal province of South Africa. For the chemical analysis the air dried leaves were extracted with ethanol. The combined extracts were concentrated under reduced pressure to produce a brown, oily residue. A voucher specimen (Afol. 2001) of the herb was deposited at the South African National Biodiversity Institute, Pretoria.

4.3.2 GC-MS analysis

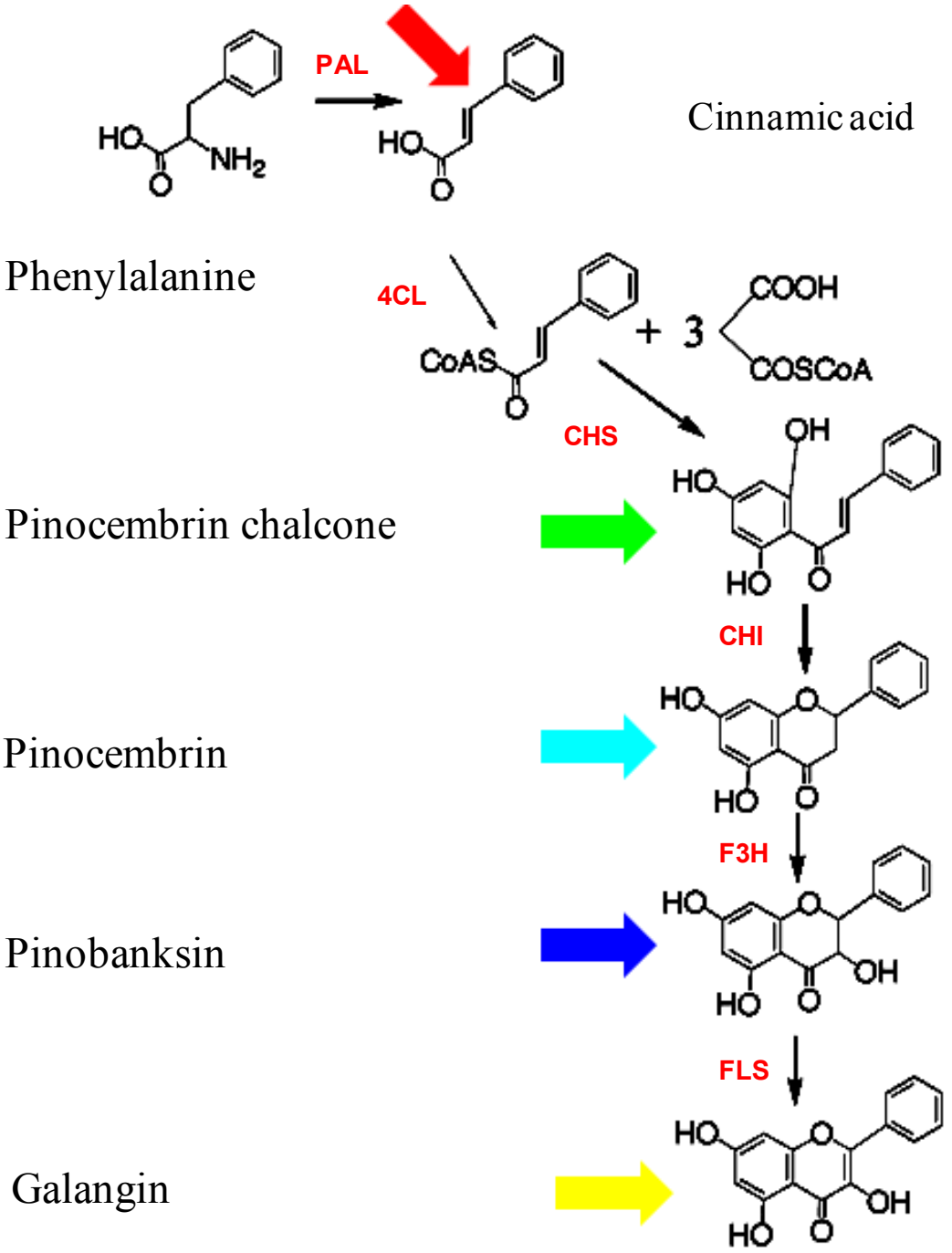
According to the 4'-OH biosynthetic pathway of flavonoids (Scheme 4.1), the presence of some intermediates including cinnamic acid, *p*-coumaric acid, naringenin and kaempferol (as a final product in flavonols biosynthetic pathway) were chemically analyzed by GC-MS. The standard chemicals for each of the intermediates were obtained from Wako, Japan. A part of the residue of the samples were trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, purchased from SIGMA) at 80°C for 30 min followed by GC-MS analysis. GC-MS analyses were carried out under the following conditions. A mass spectrometer (JMS-AM SUN200, JEOL) was connected to a gas chromatograph (6890A, Agilent Technologies), EI (70 eV), source temperature 250°C, HP-5 (30 m × 0.32 mm, 0.25-µM film thickness, J&W), injection temperature 250°C, column temperature program: 80°C for 1 min, then raised to 280°C at a rate of 20°C min^{-1}, and held at this temperature for 8 min; interface temperature 300°C, carrier gas He, flow rate 1.2 mL min^{-1}, splitless injection.

4.4 Results

To assess the pathway for galangin production in *H. aureonitens,* the presence of some intermediates such as cinnamic acid, *p*-coumaric acid, naringenin, galangin and kaempferol were analyzed by GC-MS (Scheme 4.2). The GC-MS analysis of methylated ethanolic leaf extracts of *H. aureonitens* showed the presence of identical peaks with the methylated derivatives of cinnamic acid (identified peaks 220, 205, 161, 131), galangin (471, 399, 327, 105 and 73) and kaempferol (574, 559, 415, 272) (Fig. 4.3 to 4.7). This means that three of the tested intermediates, cinnamic acid, galangin and kaempferol were detected, but *p*-coumaric acid and naringenin, were not present in the ethanolic leaf extracts of *H. aureonitens*. There were however some other peaks which illustrated the production of several compounds in association with galangin in the ethanolic leaf extracts of *H. aureonitens* (Fig. 4.7-A). The GC-MS analysis of the compounds revealed that these compounds are pinocembrin chalcone, pinocembrin and pinobanksin (Fig. 4.8). The difference of these intermediates with ones in the 4'-OH biosynthetic pathway is only in the OH group at the 4' position on the B ring (Scheme 4.1).



Scheme 4. 2. 4'-OH biosynthetic pathway for flavonols originating from p-coumaric acid. The arrows indicate the intermediates that were GC-MS analyzed to confirm the presence of this pathway in the leaf samples of *H. aureonitens.* Only kaempferol and galangin were detected.



Scheme 4. 3. The proposed alternative biosynthetic pathway for galangin production in *Helichrysum aureonitens* originating from cinnamic acid. The arrows indicate the intermediates detected by GC-MS alalysis. This pathway was already tested in *E. coli* by construction of a combinatorial cluster gene (Miyahisa et al., 2006), but it has not been reported from plants

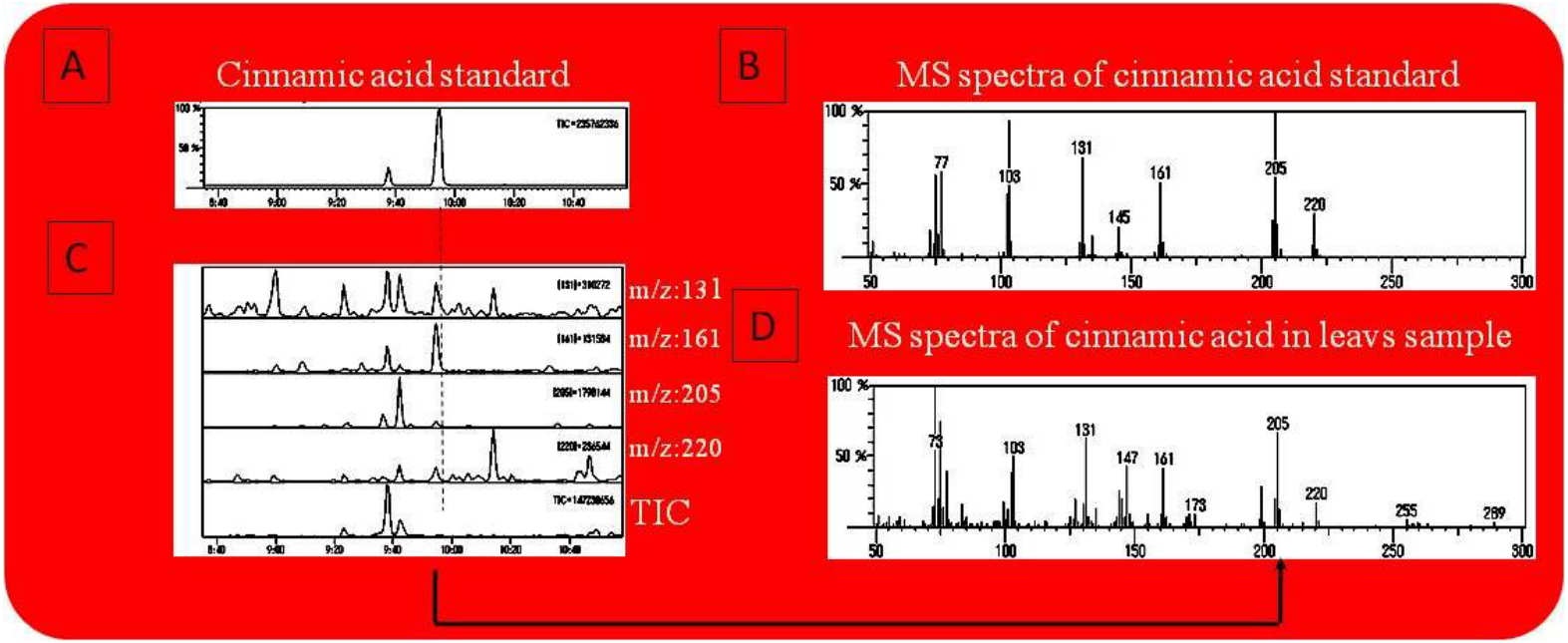


Fig. 4. 3. GC-MS analysis of the presence of cinnamic acid in leaf extracts of *Helichrysum aureonitens*. **A:** Cinnamic acid standard chromatograph; **B:** MS chromatograph of cinnamic acid standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in TIC chromatogram.

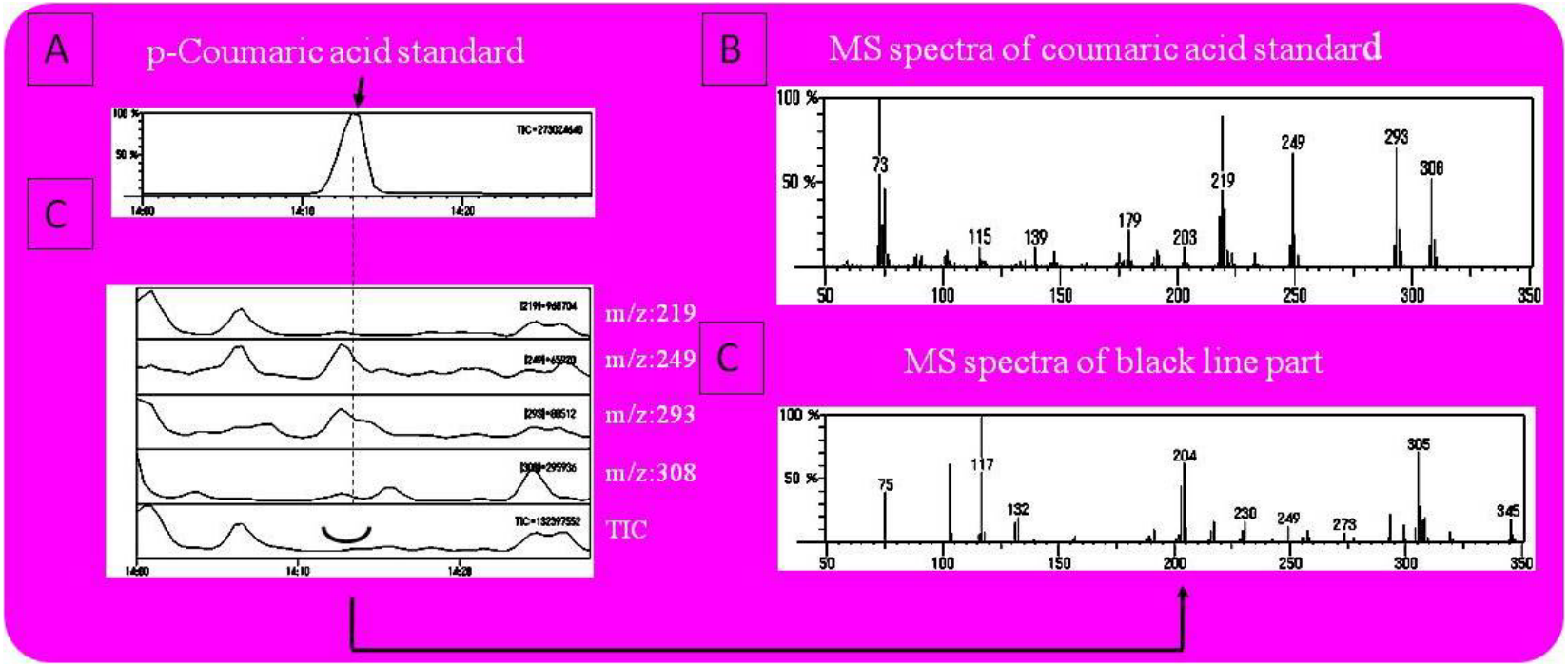


Fig. 4. 4. GC-MS analysis of the absence of *p*-coumaric acid in leaf extracts of *Helichrysum aureonitens*. **A:** *p*-coumaric acid standard chromatograph; **B:** MS chromatograph of *p*-coumaric acid standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in TIC chromatogram.

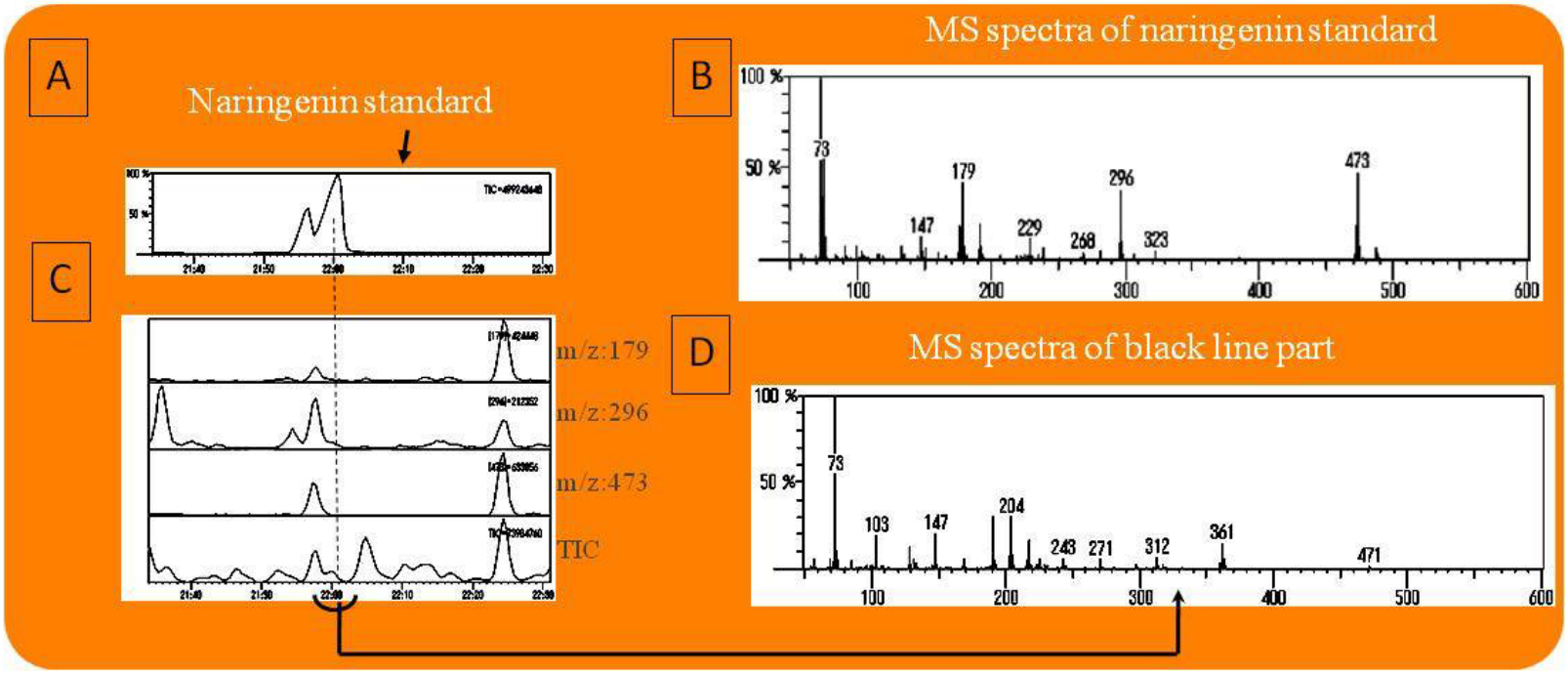


Fig. 4. 5. GC-MS analysis of the absence of naringenin in leaf extracts of *Helichrysum aureonitens.* **A:** Naringenin standard chromatograph; **B:** MS chromatograph of naringenin standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in TIC chromatogram.

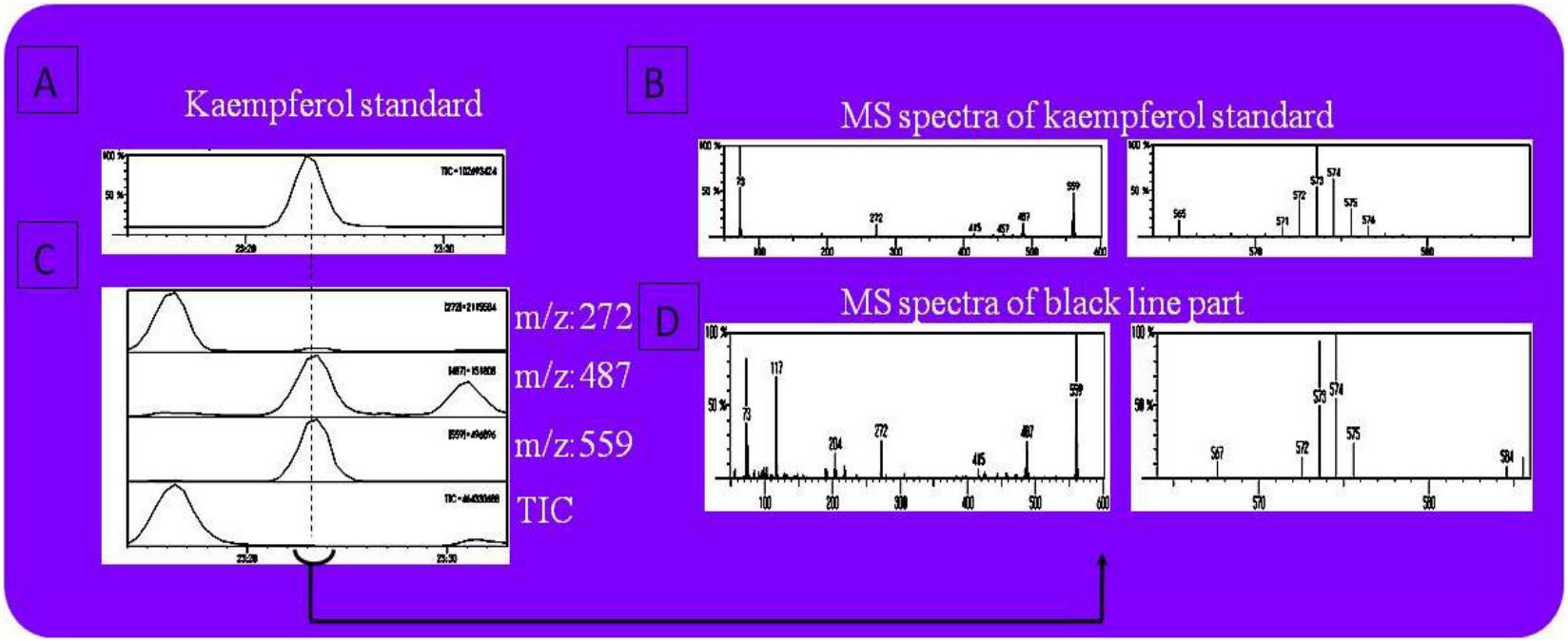


Fig. 4. 6. GC-MS analysis of the presence of kaempferol in leaf extracts of *Helichrysum aureonitens*. **A:** Kaempferol standard chromatograph; **B:** MS chromatograph of kaempferol standard; **C:** Total ion chromatogram. **D:** MS chromatograph of arrowed pick in TIC chromatogram.

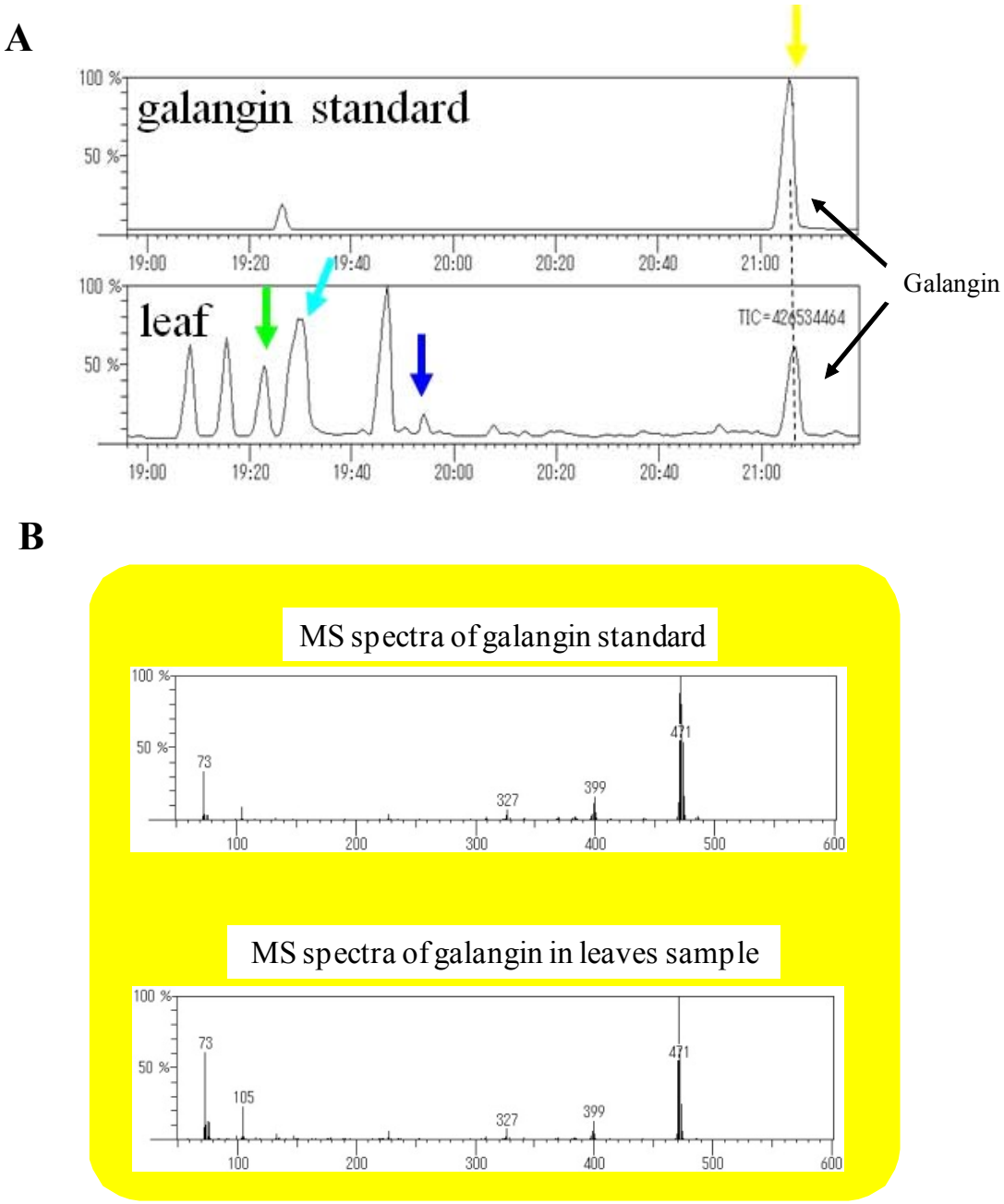


Fig. 4. 7. GC-MS analysis of the presence of galangin in leaf extracts of *Helichrysum aureonitens*. Arrows show the detection of galangin and the precursors (see Fig. 4.8) of the galangin production pathway in leaf extracts by GC-MS. **A:** GC results of standard galangin and detected galangin in the leaf sample; **B:** MS spectra of standard galangin and galangin content in leaf extracts.

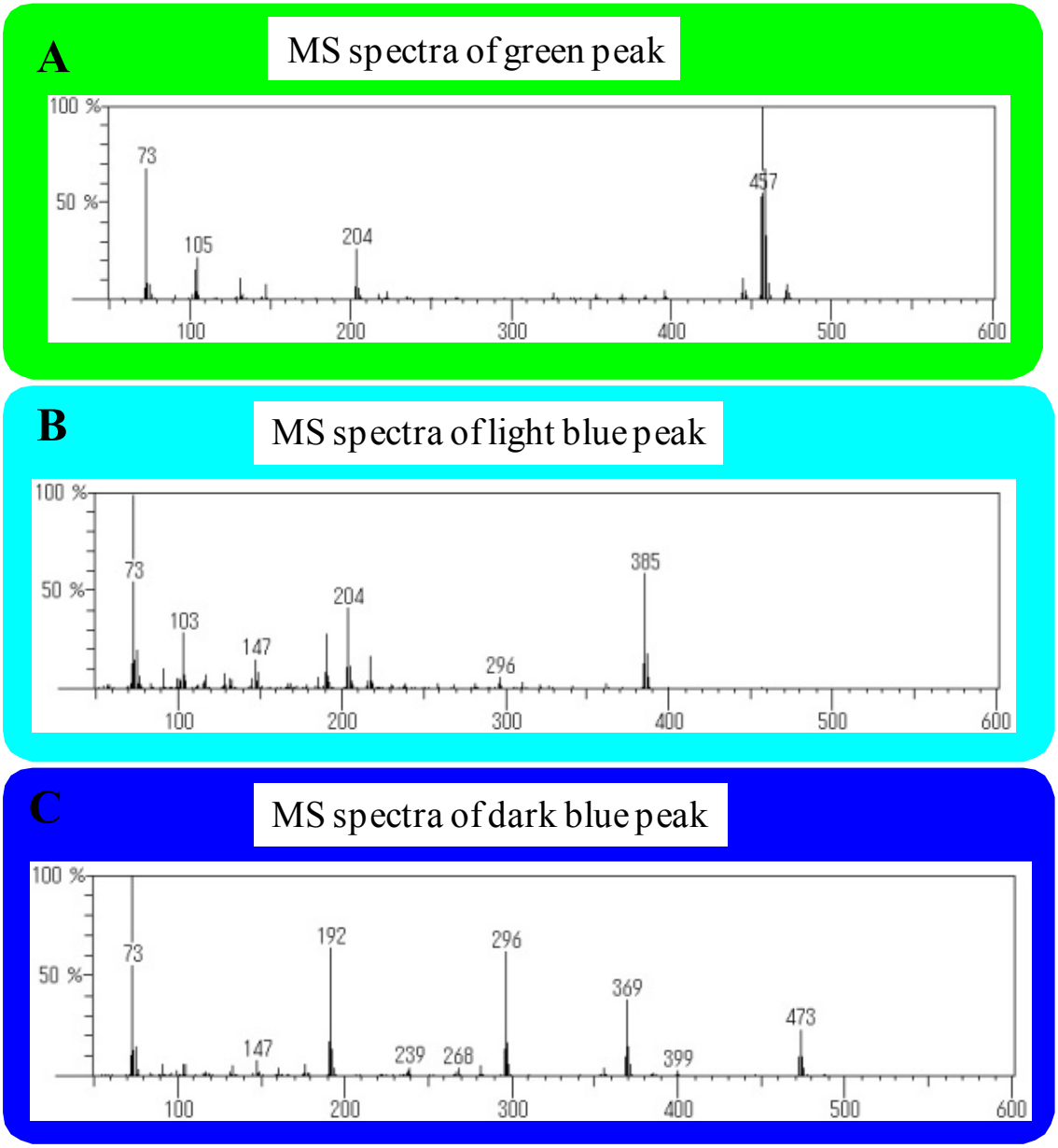


Fig. 4. 8. MS spectra of intermediates (Fig. 4.7) of the non 4'-OH biosynthetic pathway for flavonol production in *Helichrysum aureonitens*. **A:** Pinocembrin chalcone; **B:** Pinocembrin and **C:** Pinobanksin.

4.5 Discussion

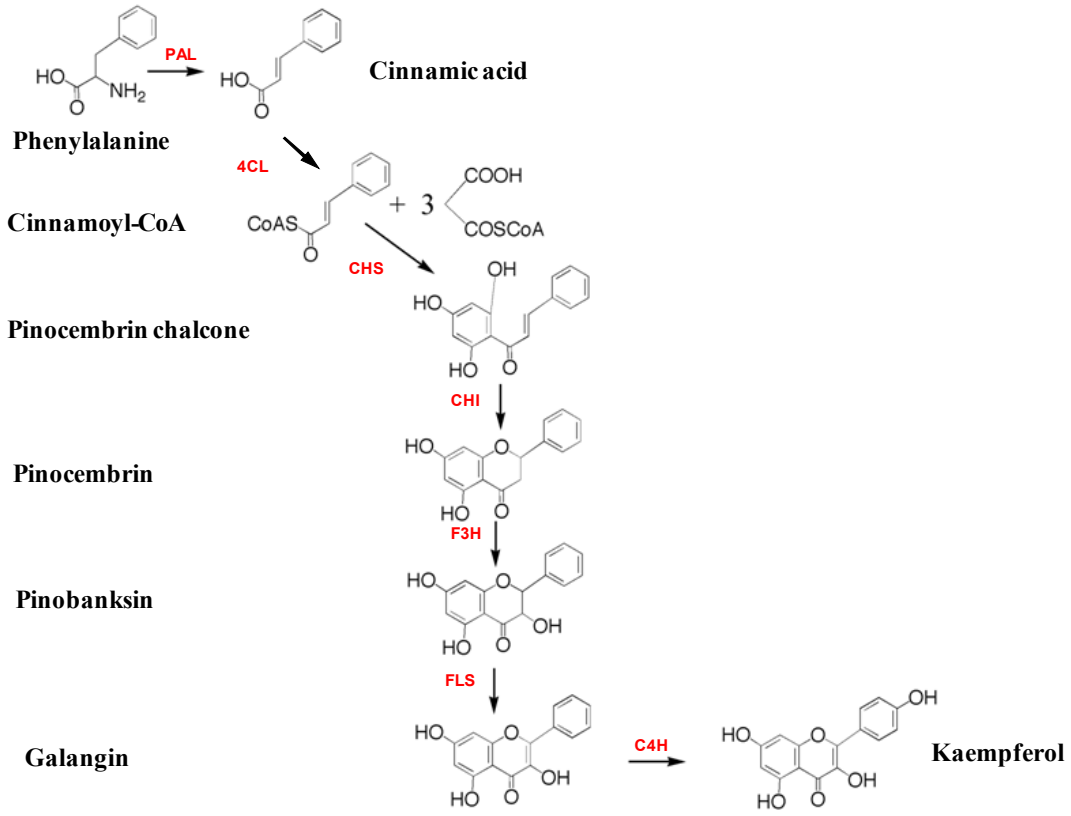
According to scheme 4.1, the production of flavonols are derived from two pathways, one originates from *p*-coumaric acid, with OH at C-4' on the B ring (Fig. 4.2) and the other one originates from cinnamic acid, with a H at the C-4' (Fig. 4.3). Naringenin and pinocembrin from the two separate pathways have been isolated from the bark of *Choerospondias axillaries* (Li et al., 2005), leaves of *Glycyrrhiza glabra* (Biondi et al., 2005), the resinous exudates of *Heliotropium sinuatum* (Torres et at., 1996), the stem extracts of *Viscum liquidambaricolum* (Liu et al., 1993), *Eriodictyon californicum* (Liu et al., 1992), *Artemisia campestris* (Hurabielle et al., 1982) *Prunus cerasus* and *P. domestica* (Nagarajan and Pamar, 1977a; Nagarajan and Pamar, 1977b). In the non 4'-OH pathway cinnamic acid should be converted to cinnamoyl-CoA by the combination with acetyl-CoA. The enzyme that catalyzes this reaction would be *p*-coumarate: CoA-ligase (4CL). One of the questions that can be asked is: Would 4CL accept cinnamic acid instead of *p*-coumaric acid? It has already been reported that 4CL can be activated by cinnamte in some plants (Forkman and Heller, 1999). In cactus elicited cell cultures (*Cephalocereus senilis*), 4CL was activated with cinnamic and *p*-coumaric acids (Liu et al., 1995). Kaneko et al. (2003) also reported 4CL from a Gram-positive filamentous bacterium (*Streptomyces coelicolor* A3) which can convert cinnamic acid to cinnamoyl-CoA, as well as *p*-coumaric acid to *p*-coumaroyl-CoA. It has also been proved that the chalcone synthase (CHS) enzyme is active not only with *p*-coumaroyl-CoA (leading to naringenin), but also with cinnamoyl-CoA (leading to pinocembrin) (Fliegmann et al., 1992; Paré et al., 1992). Chalcone isomerase is the next enzyme involved in the biosynthesis of flavonones and has been reported in the actinomycete, *Streptomyces coelicolor* where it was shown that CHI can also accept pinocembrin chalcone and naringenin chalcone as substrate to produce pinocembrin and naringenin respectively (Liu et al., 1995). In other research on the production of flavonones in *Escherichia coli* an artificial gene cluster

which contained three genes of heteologous origins including PAL, 4CL, CHS and CHI were constructed. *E. coli* cells containing this gene cluster produced two flavonones, pinocembrin from phenylalanine (with 4'-H) and naringenin from tyrosine (with 4'-OH). The same was true for chalcone isomerase (CHI) (Miyahisa et al., 2005). This demonstrates the possibility of the biosynthesis of flavonols from two separate biosynthetic pathways originating from different substrates, cinnamic acid and *p*-coumaric acid.

Based on this information it could be suggested that in some species both pathways are active. According to the results obtained in this study, it is proposed that in the leaves of *H. aureonitens*, the non 4'-OH biosynthetic pathway for the production of galangin and other flavonol compounds is active or that some parts of the 4'-OH pathway is inactive. In the non-4'-OH pathway, cinnamic acid would be the substrate to produce cinnamoyl-CoA directly in a reaction catalyzed by the 4CL enzyme. This means that in this pathway, C4H and *p*-coumaric acid are not involved, but other enzymes, including phenylalanine ammonia lyase (PAL), *p*-coumarate: CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavonone 3-hydroxylase (F3H) and flavonol synthase (FLS) which have similar functions in the 4'-OH pathway, are involved (Scheme 4.3).

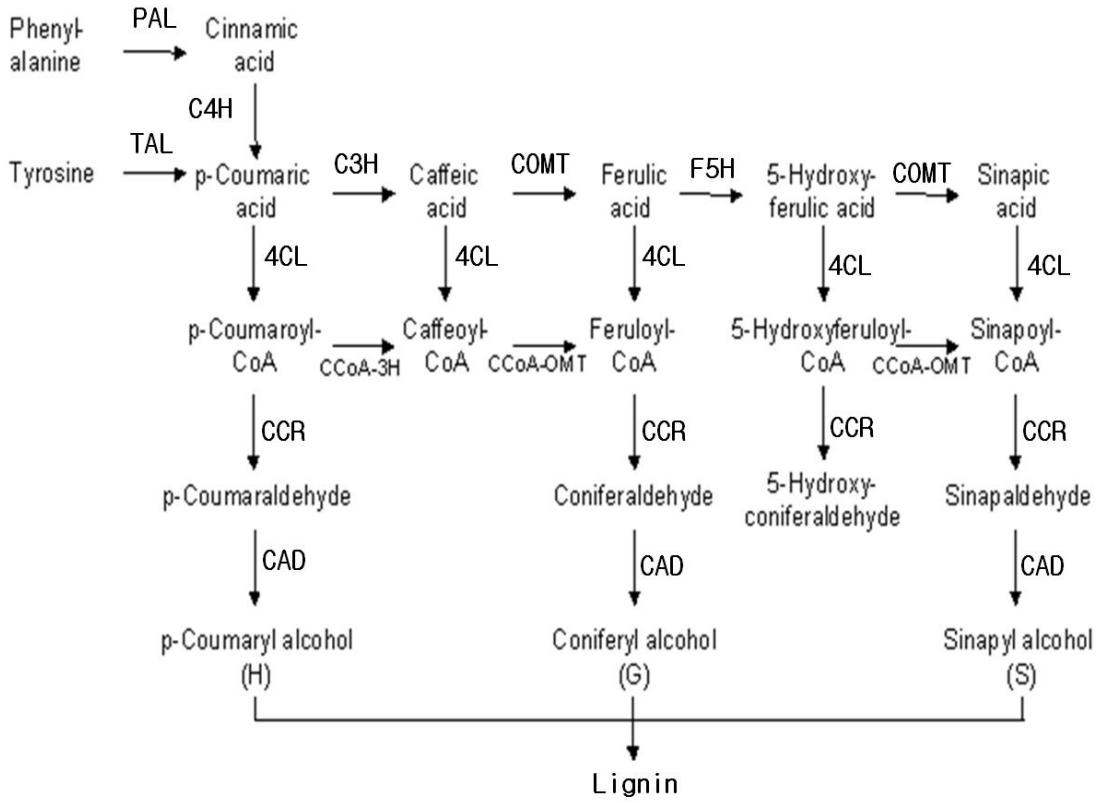
Since we could detect kaempferol in the *H. aureonitens* leaf extracts, the question of whether the proposed pathway for the production of galangin can also produce kaempferol, arose. Although the detection of galangin and kaempferol have been reported in several plants such as *Milletia recemosa* (Ganapaty et al., 1999), *Populus deltoids* and *P. balsamifera* (Kurkin et al., 1990; Kurkin et al., 1990), to date no evidence has been reported to show the conversion of intermediates from the non 4'-OH pathway to the relevant hydroxylated compounds in the 4'-OH pathway. According to the literature, the only enzyme that catalyzes the hydroxylation at the 4' position is cinnamate 4-hydroxylase (C4H) (Boudet, 2003). Based on the results obtained in this part of the study, it is therefore hypothesized that the C4H with the same

function (hydroxylation at 4' position on the B ring) might link the non 4'-OH to the 4'-OH biosynthetic pathways by hydroxylation of galangin to the corresponding intermediate, kaempferol. It is also possible that C4H can make different links between the non 4'-OH and the 4'-OH biosynthetic pathways by hydroxylation of the several intermediates from non 4'-OH pathway to the corresponding intermediates in the 4'-OH biosynthetic pathway (Scheme 4.4).



Scheme 4. 4. Proposed biosynthetic pathway of kaempferol in the leaves of *Helichrysum aureonitens.*

If *p*-coumaric acid is not present in *H. aureonitens* the question arises as to how lignin is synthesized in this species. Briefly, high-resolution microautoradiography of developing hardwood xylem has revealed that three monolignol units are incorporated at different stages of cell wall formation. H (*p*-hydroxyphenyl) units are incorporated in the earliest stages mainly in the cell corner and middle lamella during the period of formation of the S1 layer. The deposition of G lignin continues through the early to late stages (Dixon et al., 2001) (Scheme 4.5). All these branches originate from *p*-coumaric acid. The absence of *p*-coumaric acid in *H. aureonitens* brings up this question as to how lignin is synthesized in this plant?



Scheme 4. 5. The lignin biosynthetic pathway. The lignin biosynthetic enzymes are: PAL phenylalanine ammonia-lyase; TAL tyrosine ammonia-lyase; C4H cinnamate 4-hydroxylase; C3H 4-hydroxycinnamate 3-hydroxylase; COMT caffeic acid 3-O-methyltransferase; F5H ferulate 5-hydroxylase; 4CL 4-coumarate: CoA ligase; CCoA-3H coumaroyl-coenzyme A 3-hydroxylase; CCoA-OMT caffeoyl-coenzyme A O-methyltransferase; CCR cinnamoyl-CoA reductase; and CAD cinnamyl alcohol dehydrogenase (Modified from Boudet et al., 1996; Campbell and Sederoff, 1996).

Although researchers have studied lignin for more than a century, many aspects of its biosynthesis remain unresolved. It is also becoming increasingly clear that lignins are derived from several more monomers than just the three monolignols, as shown in scheme 4.5. Many "normal" plants contain lignins substantially derived from other monomers, and all lignins contain traces of units from apparently incomplete monolignol biosynthesis and other (side-) reactions that occur during that biosynthesis (Ralph et al., 2001). Many of these units have recently been identified in transgenic and mutant plants. For example, researchers have long thought that the hydroxylation and methylation reactions occur at the level of the cinnamic acids and that *p*-coumaric, ferulic, and sinapic acid are subsequently converted to the corresponding monolignols by 4-coumarate:CoA ligase (4CL), CCR, and CAD respectively. There is, however, a number of *in vitro* enzymatic assays with heterologously produced enzymes in which the identification of novel genes implicated in the pathway, and analyses of mutant and transgenic plants modified in monolignol biosynthesis have cast doubt on this route, and the pathway had to be redrawn (Humphreys and Chapple, 2002).

Another level of complexity that may affect the synthesis pathway is about down-regulation of PAL and C4H in tobacco which largely reduces lignin content (Elkind et al. 1990; Bate et al. 1994; Sewalt, et al. 1997). PAL down-regulation reduces mainly G units, whereas C4H down-regulation reduces mainly S units in lignin, an observation that cannot easily be explained by the pathway presented in scheme 4.5.

Dixon et al. (2001) believe that three possible explanations can be proposed for this apparent contradiction: (a) the pathway to G lignin may bypass C4H (Fig. 4.9), (b) C4H still catalyzes reactions other than the 4-hydroxylation of cinnamic acid, or (c) C4H may be part of a metabolic channel committed to S lignin biosynthesis. It is indicated that *p*-coumaric acid might not be the only substrate for the ignin pathway. In fact, the results of this study on *H.*

aureonitens confirms their proposed pathway about lignin production, as *p*-coumaric acid was not detected.

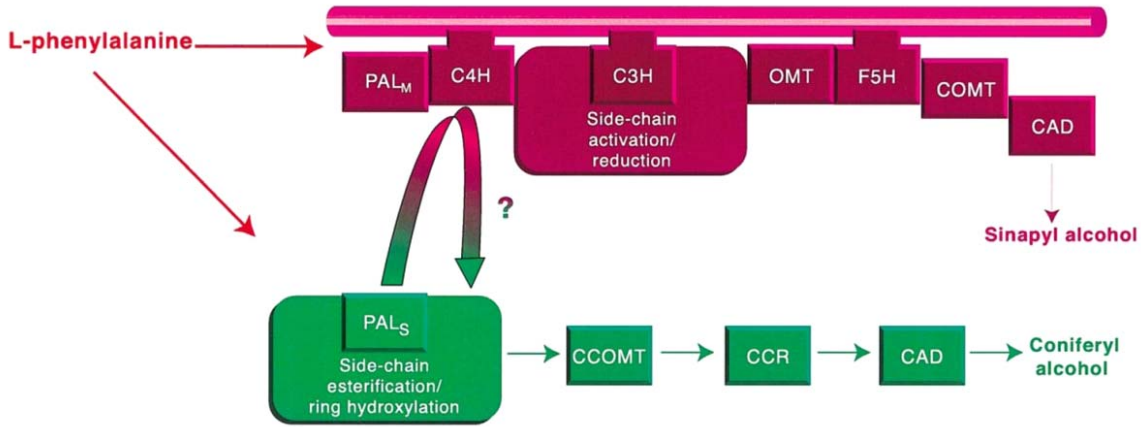


Fig. 4. 9. A metabolic channel model for independent pathways to G and S monolignols (Dixon et al., 2001). The hypothetical metabolic channel leading to S lignin (in red) is proposed to involve a microsomal-associated form of PAL (PAL_M) that directly channels its product, cinnamic acid, to the ER-associated cinnamate 4-hydroxylase (C4H) cytochrome P450. 3- Hydroxylation (possibly via a second P450 enzyme) and CoA esterification and reduction of the side chain then occur, although it is not clear whether hydroxylation occurs before or after side chain modification. These reactions are followed by the first O-methylation reaction (by COMT?) that produces coniferaldehyde, the substrate for the ER-associated F5H. The second methylation reaction is then catalyzed by COMT (perhaps associated with F5H) to yield sinapyl aldehyde, which is reduced by CAD to yield sinapyl alcohol. The hypothetical pathway to G units (in green) is proposed to involve a soluble form of PAL (PAL_S), that either provides cinnamate for C4H in an unchanneled reaction, or by-passes C4H by hydroxylation of the aromatic ring at the level of a side chain esterified intermediate. CCOMT then catalyzes the single O-methylation reaction, and coniferyl alcohol is formed via soluble forms of CCR and CAD. In an alternative model, the pathway to G units might also be membrane associated.

4.6 Conclusions

The results obtained indicated that *p*-coumaric acid and naringenin from the 4'-OH biosynthetic pathway of flavonols are not present, while cinnamic acid, pinocembrin-chalcone, pinocembrin, pinobanksin, galangin and kaempferol were detected in the leaf extracts of *H. aureonitens*. According to these results, we hypothesize that the non 4'-OH biosynthetic pathway for the production of galangin and kaempferol is present in *H. aureonitens*. Since the only enzyme known that can catalyze the hydroxylation at this specific position is C4H, we hypothesize that C4H is present in the leaf extracts of *H. aureonitens* and it is responsible for catalyzing hydroxylation reaction (Scheme 4.4). It is possible that C4H can possibly link the non 4'-OH pathway with the 4'-OH pathway by the hydroxylation of different intermediates. To confirm this hypothesis firstly the presence of C4H in *H. aureonitens* must be proven by the isolation and characterization of the C4H gene, and secondly the functionality of it should be determined.

Chapter 5

Isolation and characterization of cinnamate-4-hydroxylase (C4H) in *Helichrysum. aureonitens*

5.1 Abstract

Cinnamate 4-hydroxylase (C4H) is the second key enzyme of the phenylpropanoid pathway, and synthesizes numerous secondary metabolites that participate in development adaptations. Its function is the hydroxylation at the C-4' position on the B ring. One C4H copy was isolated and cloned from *Helichrysum aureonitens*. It has 1518-base pairs (including the stop codon, TAA) the open reading frame encoding a 506-amino-acid polypeptide. It shows the highest homology to *Echinacea angustifolia* from the Asteraceae's C4H, with 83.6 % identity on the nucleotide level but 93 % identity and 97 % positives on the protein level. The genomic DNA sequence of isolated C4H from *H. aureonitens* indicates the presence of three introns with a longer size compared to the *Arabidopsis thaliana* C4H gene structure. The presence of the first intron has not been reported in the C4H gene from any other species and it is be therefore a new finding from the isolated C4H of *H. aureonitens.*

5.2 Introduction

The second enzyme in the phenylpropanoid pathway, cinnamate 4-hyroxylase (C4H), produces a large number of biologically important secondary metabolites. (Chen et al., 2007). C4H converts *trans*-4-cinnamate to *trans*-*p*-coumarate by catalyzing the hydroxylation at the C-4' position of the B ring. C4H belongs to the P450s family (Russel and Conn, 1967). The cDNA sequence of C4H has been isolated from different plant species and is called CYP73. According to the P450 enzyme database (http://dranderson.utmem.edu/public/73.htm1), 20 different CYP73 members have been identified so far and include alfalfa, *Arabidopsis* and artichoke (Fahrendorf and Dixon, 1993; Mizutami et al., 1997; Teutsch et al., 1993). In cDNA libraries 16 ESTs have been found but pea, *Arabidopsis* and parsley seemingly have only a single gene encoding C4H (Frank et al., 1996; Bell-Lelong et al., 1997; Koopmann et al., 1999).

Galangin is one of the flavonoids produced in *H. aureonitens*. The main difference between the chemical structure of galangin and other members of flavonols is in the existence of the OH group at the C-4' position of the aromatic ring B. According to the results in the previous chapter, one of the possibilities for producing galangin in *H. aureonitens* is the presence of an alternative biosynthetic pathway in which cinnamate activates the 4CL enzyme as a substrate acid to produce cinnamoyl-CoA and not *p*-coumaric via C4H. This means that in the production of galangin in *H. aureonitens*, the C4H enzyme is not involved in this step (Scheme 3.2). Since kaempferol was also detected in the leaf ethanolic extracts of *H. aureonitens* in which the C-4' on ring B is hydroxylated (Fig. 4.2), it is proposed that C4H could be responsible for the hydroxylation at the 4' position to convert galangin to kaempferol. Based on the results obtained in the previous chapters, *p*-coumaric acid, the product of the activity of C4H in plants due to the

4'-OH biosynthetic pathway for flavonols, was absent in the leaf ethanolic extract of *H. aureonitens.*

It was also indicated that some 4'-OH intermediates in this pathway were not detected while some non 4'-OH intermediates were detected which belong to the non 4'-OH biosynthetic pathway (without C4H). The first step in this part of the investigation is the isolation of C4H from *H. aureonitens* leaf samples. The following objectives were set for this part of the study:

- Is C4H present in *H. aureonitens?*
- Characterization of C4H in *H. aureonitens.*
- What is the relationship between C4H in *H. aureonitens* and other plants?

5.3 Materials and methods

5.3.1 Plant materials

Fully expanded mature leaves of intact plants grown in the greenhouse and cells from the cell suspension cultures of *H. aureonitens* were used as plant materials for the gene isolation experiment in this part of the project.

5.3.2 Genomic DNA extraction

Harvested cells from a cell suspension culture of *H. aureonitens* (100 mg) were ground in liquid nitrogen using a mortar and pestle. Genomic DNA was extracted using a modified CTAB-based method (cetyl-trimethyl-ammonium-bromide) according to Gawel and Jarret (1991). To remove any RNA contamination, the extracted DNA was treated with 100 µg/ml DNase free RNase and the DNA was recovered with 96 % ethanol (2-times volume) with sodium-acetate (NaAC) (3M, pH 6.8) (1/10 volume) added. The DNA was finally washed with 70 % ethanol. Geno mic DNA was dissolved in sterile distilled water and stored at -20 °C until used. The quality and concentration of DNA was determined by running the DNA on a 1 % agarose gel to visually test for the purity , and a spectrophotometer (Nanodrop) analysis (A260/280 ratio) was used to determine the quantity and purity.

5.3.3 PCR amplification of an internal fragment of C4H using degenerated primers

To amplify an internal fragment of the C4H gene from *H. aureonitens* two degenerated primers were designed. The upstream primer [5'-AGAAGGGCGAGATCAACGA (A/G) GA (C/T) AA (C/T) GT-3'] and the downstream primer [5'-GGCGTCGTGCAGGTTCAT (A/G) TG (A/C/G/T) GG-3'] were designed using CODEHOP software

(http://blocks.fhcrc.org/codehop.html) based on the conserved area of C4H homologues from some closely related plant species from the Astraceae family (Table 5.1) with amino acid sequences deposited in NCBI database according to Table 5.1. Primer pairs were designed to amplify approximately 500 bp fragment of the C4H gene. A PCR amplification of the internal fragment of C4H was carried out with the genomic DNA. The PCR reaction was performed in a 50 µl reaction mixture containing 100-150 ng genomic DNA, 1.5 µl mM $MgCl_2$ (50 mM), 5 µl 10x *Taq* DNA polymerase buffer, 4 µl dNTP (2.5 mM of each), 1 µl of 20 µ M of each primer and 0.2 µl *Taq* polymerase (5 U/µl, Bio line, Germany). DNA samples were initially denatured for 4 minutes at 94 °C and amplified in two steps with 10 and 25 cycles. DNA denaturation was carried out at 94 °C for 30 seconds, the primer annealing for 30 seconds at 50 °C, and the extension for 1:30 minutes at 72 °C. The second step was done using the same program and increasing the extension period with 10 seconds in each cycle. Cycling was completed with a final extension for 10 minutes at 72 °C in a thermocycler (BioRad icycler-USA). Amplified products were separated on a 1 % (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. The ethidium-bromide stained amplification products were visualized and imaged on a UV transilluminator.

Table 5. 1. List of plants for designing the degenerated primers to amplify the internal fragment in C4H.

Scientific name	Family name	Gene bank accession no.
Helianthus tuberosusm	Asteraceae	Z17369
Zinnia violacea	Asteraceae	U19922
Gossypium arboreum	Malvaceae	AF286648
Nicotiana tobacco	Solanacae	DQ350353
Solanum tuberosum	Solanacae	DQ341174
Petroselinum crispum	Apiaceae	L38898.1
Arabidopsis thaliana	Brassicaceae	NM_128601

5.3.4 Gel recovery and TA cloning

The DNA of the PCR product on the agarose gel was recovered, purified and eluted using the GFXTM PCR DNA and gel band purification kit (GE Healthcare, UK). The quality and concentration of the DNA was determined on a 1 % agarose gel and a UV/VIS spectrophotometer (Nanodrop-ND1000-USA) was used to determine the A260/280 ratio. Gel-eluted DNA was cloned into the TOPO TA vector (Invitrogen, USA) and *E. coli* JM 109 competent cells were transformed with the vector DNA (Invitrogen, USA) by a heat shock treatment based on the manufacturer's instructions. Distinct kanamycin-resistant colonies derived from a Luria-Bertani medium containing plate (LB, 1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl) were inoculated in a liquid LB medium overnight and the plasmids were purified from transformed cells using a commercial plasmid isolation kit (Fermentas, Europe). The plasmids were digested with the restriction enzyme *EcoRI* in a reaction containing 1 µl , 2 µl *EcoRI* and 10x reaction buffer in a total volume of 20 µl. Digestion was performed at 37 °C for 1 hour, and the digested DNA was run on a 1 % (w/v) agarose gel to visualize.

5.3.5 Sequencing and alignment

To confirm the presence of the right insertion and determine the sequence of fragments, the plasmids were sequenced using a M13 universal primer. Sequencing of the insert was carried out by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit, v 4.1 (Perkin Elmer, Applied Biosystems, USA). A 10 µl sequencing PCR mixture reaction containing 2 µl BigDye ready reaction mix, 1 µl 5X dilution buffer, 10 pmol M13 forward or reverse primer (Table 5.2) and 200-300 ng DNA was used for this. The PCR sequencing reactions were performed using denaturation at 96 °C for 10 seconds, primer annealing at 50 °C for 5 seconds and DNA

extension at 60 °C for 4 minutes. The sequencing PCR product was then cleaned by using a Sephadex column (6.65 %) and the DNA was dried before submitting it to the DNA sequencer (3130/xl genetic analyzer, Applied biosystems, Foster City, USA). The NCBI programs, Blastn, blastx and Blastp were used for sequence alignment.

Table 5. 2. List of primers for the identification and amplification of the C4H gene in *Helichrysum aureonitens.*

Primer	Sequence	Comments
Adaptor 1	5'-GGATCCTAATACGACTCACTATAGGGC-3'	Adaptor primer 1
Adaptor 2	5'-AATAGGGCTCGAGCGGC-3'	Adaptor primer 2
CH1-F	5'-AACCTTATGGTCTATCGAAT-3'	Primary first walking primer
CH1-R	5'-AGATTTTATCCGTGTACTAA-3'	
CH1-NF	5'-ATGGCTATTCCTCTTTTAGT-3'	Nested first walking primer-FW
CH1-NR	5'- TTCTCAACAATGTAAAGAAC-3'	
CH2-NR	5'-CATCAAATGATCATGCAAGCCTAC-3'	
CH3-NR	5'-CGCTGCTGCCGGATTCTTCTT-3'	
CH4-NR	5'-GACCGAAATTTTTTGGCGTAATCGGT-3'	
CH5-NR	5'-CCCGAAGATGGGGACGGGGATAA-3'	
CH6-NR	5'-ACGGGGATTGGACCCGGTGGAA-3'	
C4H-F	5'-ATGGATCTACTCCTTTTGGAGAAA-3'	**To amplify full length fragment**
C4H-R	5'-TTAAAGAGATCTTGGTTTGGCAA-3'	

5.3.6 Genome walking

5.3.6.1 Genomic DNA restriction digestion

Isolated genomic DNA (2-2.5 µg) was digested with four blunt-end cutting restriction enzymes (*EcoRV, PvuII, ScaI* and *StuI*) at 37 °C over night in a 100 µl reaction mixture using the modified procedure of Sibert et al. (1995). Inactivation of the enzymes was done by heat treatment. DNA was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) followed by DNA precipitation by adding 1/10 volume of 3 M NaOAc (pH 5.2). Then EtOH 100 % (2.5-times volume) and 1 µl glycogene (20 µg from a 20 mg/ml stock) was added. After incubation at -70 °C for 1 hour, the mixture was centrifuged at 13000 rpm for 15 minutes at 4 ˚C and the resulting pellet was washed with 70 % EtOH. After washing the pellet was air dried and then dissolved in 20 µl sterile water. The dried DNA was stored at -20 ˚C until used.

5.3.6.2 Ligation

Twenty microliter of the restriction enzyme-digested DNA was heated at 50 ˚C for 2 minutes on a heat block and then placed on ice. Adaptor 1 (5'- CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3') and adaptor 2 (5'- ACCTGCCC-3') were used at a concentration of 150 pmol each and were heated at 95 ˚C for 3 min, then chilled slowly. Ligation of the adaptors was accomplished at 20 °C overnight in a 30 µl reaction mixture containing 20 µl heated, digested DNA, 3 µl ligase buffer (10X), 2 µl T4 Ligase (10 U), 1.5 µl of each adaptor (adaptor 1 and 2), and 2 µl sterile water. Ligation was terminated by incubating the mixture at 65˚C for 10 minutes then placed on ice. Finally, the mixture was diluted to 100 µl by adding 70 µl sterile water and then stored at -20 °C until used.

5.3.6.3 PCR amplification

The primary PCR reaction was carried with adaptor primer pair AP1 in a total volume of 25 µl containing 50 mM $MgCl_2$ (1 µl), 0.25 µM each of dATP, dTTP, dCTP, dGTP, 20 pmol of each primer (adaptor-specific and gene-specific primers), 2.5 µl DNA polymerase reaction buffer (10X), 0.5 µl of *Taq* DNA polymerase (5 U/µl, Bio line, Germany). The PCR reaction was run at 94˚C for 4 minutes, followed by 35 amplification cycles (94˚C for 30 seconds, primer annealing temperature based on T_m of the lower primer for 30 seconds, 72 ˚C for 2 minute and 72˚C for 10 minutes for final DNA extension using a thermocycler (BioRad, icycler-USA). The PCR product from the first run was diluted 100- times with sterile the distilled water and 1 µl of diluted PCR product was used as a template for the second (nested) PCR. The reaction product was analyzed by 1 % (w/v) agarose gel electrophoresis.

The nested PCR reaction was performed in a total volume of 50 µl reaction mixture containing $MgCl_2$ (50 mM) 2 µl, 0.2 µM each of dATP, dTTP, dCTP, dGTP, 20 pmol of each primer (adaptor-specific and gene-specific primers), 5 µl DNA polymerase reaction buffer (x10), 1 µl of *Taq* DNA polymerase (5 U/µl, Bio line) and 1 µl of the 100-times diluted PCR product as a template. The nested PCR reaction was performed with the same conditions as outlined above for the primary PCR reaction. The nested PCR product was run on 1 % (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. DNA band/s were visualized and imaged on a UV transluminator. .

5.3.7 Phylogenetic analysis

The phylogenetic relation of the C4H sequence in *H. aureonitens* with the C4H sequences in other plants was created by retrieving the C4H sequences from *Helianthus tuberosusm* (Z17368), *Zinnia elegans* (U19922), *Echinacea angustifolia* (EU676019) from Asteraceae family, *Gossypium arboretum* (AF286648), *Nicotiana tobacco* (DQ350353), *Solanum tuberosum* (DQ341174), *Ammi majus* (AY 219918), *Petroselinum crispum* (L38898),*Arabidopsis thaliana* (cDNA, NM128601), *Arabidopsis thaliana* (gDNA, NC_003071) and *Ammy majous* (AY219918). The alignment was performed using phylogenetic and molecular evolutionary analyses, which were conducted using the Bootstrap consensus for neighbour joining, MEGA version 4.0 software (Kumar et al., 2004).

5. 3. 8 Secondary structure of C4H from *H. aureonitens*

Amino acid sequence and open reading frame (ORF) translation were carried out with MEGA software version 4.0 as well as expasy web site (au.expasy.org/tools/dna/html). Secondary structure of C4H from *H. aureonitens* was predicted on line with Psi-pred (bioinf.cs.ucl.ac.uk/psipred). To find the homology for *H. aureonitens* C4H as a template for modeling of C4H from *H. aureonitens*, the C4H amino acid sequence was submitted to HHPRED web site. The creation of secondary structure model and characterization of C4H from *H. aureonitens* was carried out with SWISS PDB viewer and RASMOL.

Briefly the expasy translated amino acid sequence of C4H was submitted to Psi-pred (to predict secondary structure of C4H) as well as HHPRED web site (to find a closest homology for modeling). Homology modeling was created by HHPRED and the characterization was performed by SWISS PDB viewer. The structure figure was prepared by RASMOL software.

5.4 Results

5.4.1 Amplification of an internal fragment of C4H using degenerated primers

Genomic DNA of *H. aureonitens* was used for the isolation of a full length gene. Based on the database for the C4H sequence of *Arabidopsis thaliana*, degenerated primers were designed for the amplification of an internal fragment, including the second intron (Fig. 5.1). According to the *A. thaliana* C4H structure, the expected size of the internal fragment should be about 500 bp. PCR amplification of the internal fragment for C4H with two degenerated primers resulted in two distinct bands, one about 500 and the other about 750 bp (Fig. 5.2). To find the right fragment, both amplicons were cloned in TA cloning vector and sequenced. NCBI blastn and blastx analysis indicated that the 500 bp band was not identical to the *A. thaliana* C4H, (data not shown). 11 kanamycin-resistant *E-coli* colonies of the upper band (750 bp) were selected and sequenced. Eight clones showed wide homologies to known C4H sequences with highest identities to *A. thaliana* (NC_003071) (Fig.5. 3). Since the gene structure of C4H from *A. thaliana* (NC_003071) is only the reference for the C4H structure map on the database, the major observed difference between *H. aureonitens* and *A. thaliana* in the obtained internal fragment was due to the size of the intron which was found at 472 and 220 bp respectively without regarding the minor differences in nucleotide sequence.

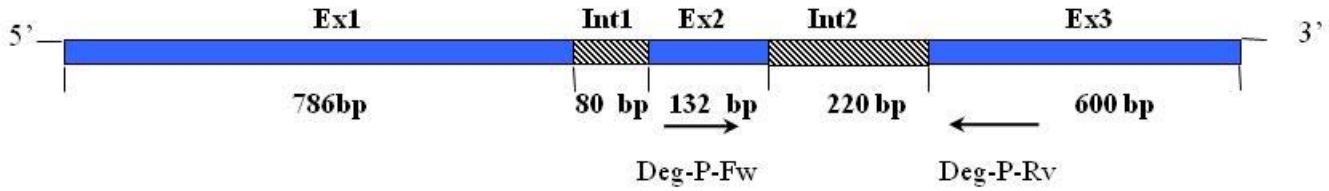


Fig. 5. 1. C4H gene map in *Arabidopsis thaliana* (NC_003071.3). The blue boxes represent the coding region and the grey box an untranslated region. Arrows show the position of the designed degenerated primers used to amplify the internal fragment.

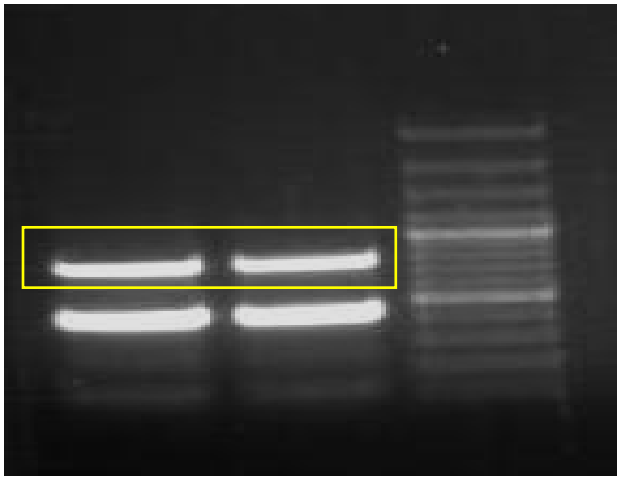


Fig. 5. 2. Amplification of the internal partial fragment of C4H from the genomic DNA of *Helichrysum aureonitens* by degenerated primers. Boxed fragments were cloned and sequences.

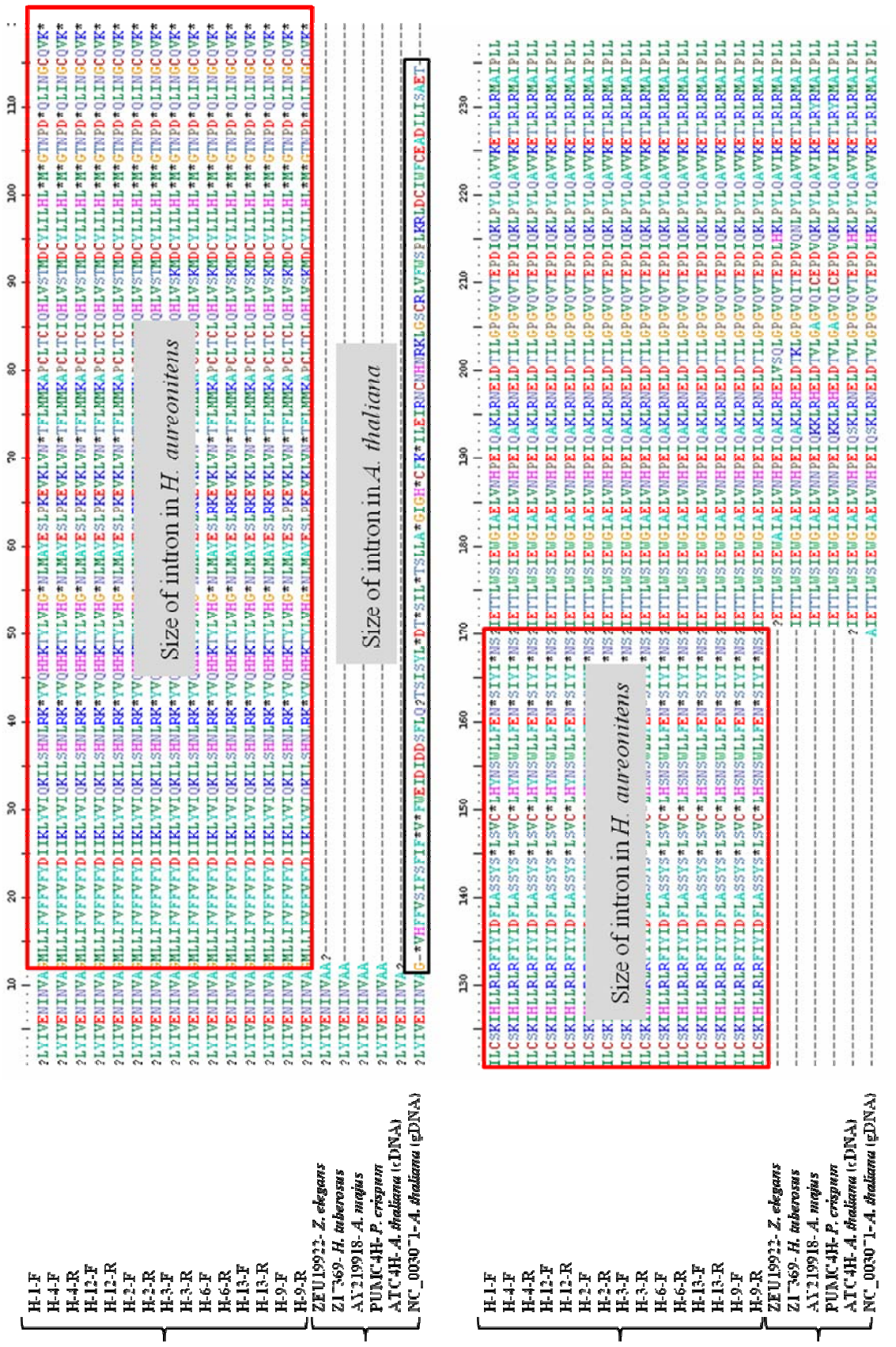


Fig. 5. 3. Multi-alignment of the sequences of the internal fragment of C4H in *Helichrysum aureonitens* from eight colonies with C4H homologies of other plants. The boxed region represents the position of intron.

5.4.2 Genome walking

5.4.2.1 Genomic DNA restriction digestion

The genomic DNA was digested with four blunt-end restriction enzymes, *EcoRV*, *PvuII*, *ScaI* and *StuI*. As shown in figure 5.4, the complete digestion of the genomic DNA with blunt-end restriction enzymes revealed a smear when using gel electrophoresis, which shows a wide range of different fragment sizes of the genomic DNA. The blunt-end digested fragments, which were ligated with adaptors, were used as a DNA template for the primary PCR reaction of the first genome walking to find the upstream and downstream regions of the known area. Two gene specific primers, CH1-F and CH1-R and adaptor primer 1 (AP1) (Table 5.2) were used. Figure 5.5-A shows the PCR product of the primary reaction with AP1 (Adaptor primer 1), CH1-F and CH1-R (Gene specific primers) resulted in a smear in both directions, up and downstream to the known internal fragment. A second PCR (nested PCR) was performed with two other gene specific primers, CH1-NF and CH1-NR, in combination with a adaptor primer 2 (AP2) (Table 5.2). The nested amplification used to identify the downstream region resulted in a bright band of about 750 and 600 bp in *EcoRV* and *ScaI* digested DNA respectively (Fig. 5.5-B) The nested PCR upstream of the known area also resulted in a bright band of about 500 bp only in *ScaI* digested DNA, but no amplification was observed in the *EcoRV* digested DNA. All bands for both directions were recovered from the gel and subcloned into the TOPO-TA vector. The plasmids from kanamycin resistant clones of up and downstream were sequenced from the *ScaI* and *EcoRV* digested DNA experiments. The blastn and blastx analysis for the downstream region indicated that all the clones were highly conserved to known C4H sequences in the database and showed the 3' end sequence of C4H in *H. aureonitens*.

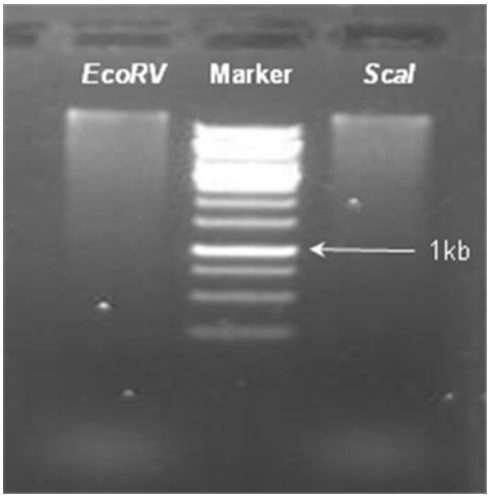


Fig. 5. 4. Complete digestion of the genomic DNA using blunt-end restriction enzymes, *PvuII* and *StuI.* The second lane shows the DNA ladder and the arrow represents the size of 1 kb in the ladder.

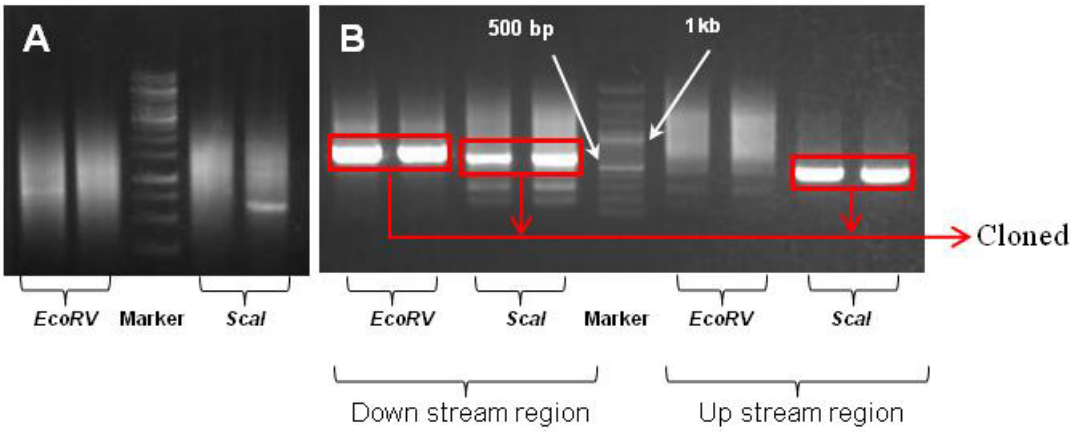


Fig. 5. 5. PCR amplification for the first genome walking with *EcoRV* and *SacI* DNA digestion. **A:** Primary PCR. **B:** Second PCR (nested PCR); the red boxed fragments were cloned and sequenced to identify the up and down stream regions of the known internal fragment.

The results of blastn and blastx for the upstream direction showed that there is a wide similarity among all clones and good homology between clones and known C4H sequences from the other plants on the database. This is true until 119 bp from CH1-NR and after that another intron could start. It means that the sequence of the exon in that partial fragment of *H. aureonitens* is identical to the sequence of genomic DNA in *A. thaliana* (NC_003071.3) (Fig. 5.1).

The second genome walking was performed by using *PvuII* and *StuI* digested DNA as template. Primers CH1-NR and CH2-NR were used for primary and nested PCR respectively. Primer CH2-NR was designed based on the sequences obtained from the first genome walking experiment allowing the identification of the upstream part of the result of first walking. Results of the primary and nested PCR showed a fragment of about 1 kb only in *PvuII* digested DNA and no amplification was observed in *StuI* digested DNA (Fig. 5.6). The blastn and blastx results for the 1 kb fragment showed the presence of a second intron in the genomic DNA of *H. aureonitens.* It further demonstrated that the second intron in the genomic DNA of *H. aureonitens* is longer than the second intron in the genomic DNA of *A. thaliana* (NC_003071.3).

The third walking was carried out based on the result of sequencing in a second genome walking. In this step the CH3-NR primer was used to amplify a 300 bp fragment from *StuI* digested DNA. After sequencing, the blastn and blastx analysis showed that a further intron is present in the genomic DNA of *H. aureonitens.* The size of this intron was determined to be 630 bp. This intron is not present in the genomic DNA of *A. thaliana* (NC_003071.3).

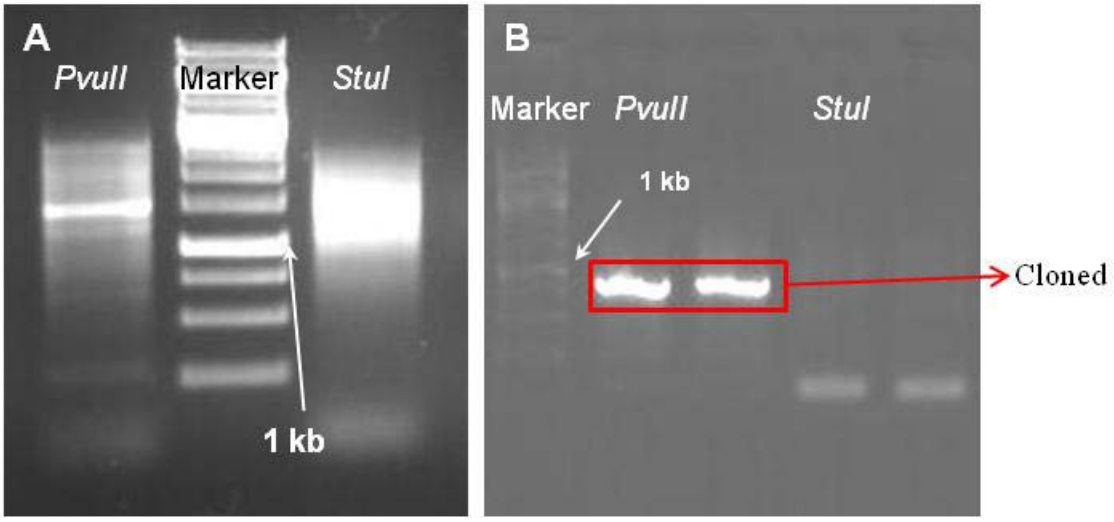


Fig. 5. 6. PCR amplification for the first genome walking with *PvuII* and *StuI* digested DNA for the second genome walking. A: Primary PCR; B: Second PCR (nested PCR); the red boxed fragments were cloned and sequenced to identify the upstream region of known the fragment from the first genome walking.

5.4.3 Amplification of the full length cDNA and genomic C4H from *H. aureonitens*

Amplification of a full length of cDNA and genomic DNA of C4H in *H. aureonitens* by using primers C4H-F and C4H-R yielded a specific band of about 1500 bp in full length C4H amplification of cDNA and another band of about 3400 bp in a full length C4H amplification genomic DNA. These primers were designed based on a obtained sequence of 5' and 3' ends of the genome walking experiments, including a start codon (ATG) and stop codon (TAA). The sequencing results of the full length of cDNA were identical to the genomic DNA sequence except the intron regions.

5.4.4 Molecular characterization of the nucleotide sequence of C4H from *H. aureonitens*

The full length sequence of the genomic DNA of C4H from *H. aureonitens* is 3399 bp. Sequencing results of genomic DNA of C4H from *H. aureonitens* indicated the presence of three introns. Two of them are also present in the genomic DNA of *A. thaliana* (NC_003071.3) and the third one is a new finding in C4H from *H. aureonitens*. The size of the three introns (I, II, III) from the 5' to 3' side is 630 (445-1074), 852 (1420-2196) and 472 bp (2326-2799) respectively (Fig. 5.7 and 5.8).

The synthesized C4H cDNA from total RNA of *H. aureonitens* showed the full length size of 1518 bp (Fig. 5.9). G + C content of the open reading frame (ORF) is 44.4 %, while the introns have a total of 30.27 %.

NCBI Blastn analysis indicated that the open reading frame of isolated C4H from *H. aureonitens* shows high identities to known C4H tags from Asteraceae family with the highest homologies to *Echinacea angustifolia* (EU676019) with a 83 % similarity. The other high similarity was to *Zennia violacea* (U19922), *Helianthus tuberosus* (Z17369) 81 % as inter family and to AtC4H (NM_128601) 74 %. They also show moderate identities to many C4H/CYP73A genes from different families, such as those from *Nicotiana tabacum* (DQ350352), 78 %, *Catharanthus rosous* (Z32563) 77 %, *Gossypium arboreum* (AF286648*),* 77 %. NCBI blastp indicated that the C4H shows very many similarities to the C4H genes from other plants. When pairwise-aligned on the whole molecule scale, the C4H shows identities / positives of 94 % / 97.0 % to intra family *E. angustifolia* (ACF74449); *H. tuberosus* (CAAB78982), 93.0 % / 97.0% to *Z. violacea* (AAB42024), 92.0 % / 96.0 %; to C4H from *N. tabacum* (ABC69412), 88.0 % / 94.0 %, *Solanum tuberosum* 87.0 % / 95.0 %; *G. arboreum* (AAG10197) 88.0 % / 95.0 %; *C. roseus* (CAA83552) 89.0 % / 94.0 % *Arabidopsis thaliana* monocot (AAB58355) 85.0 % / 93.0 % and

to C4H from *Sorghum bicolor* (AAK54447) 75.0 % / 87.0 % and to the gymnosperm C4H of *Ginkgo biloba* (CAA70596) 81.0 % / 90.0 % (Fig. 5. 10).

Fig. 5. 7. C4H genomic DNA sequence from *Helichrysum aureonitens*. The boxd sequences represent the sequence, position and length of introns in the isolated C4H gene from *Helichrysum aureonitens*. The under-lined nucleotides represent the start and stop codons.

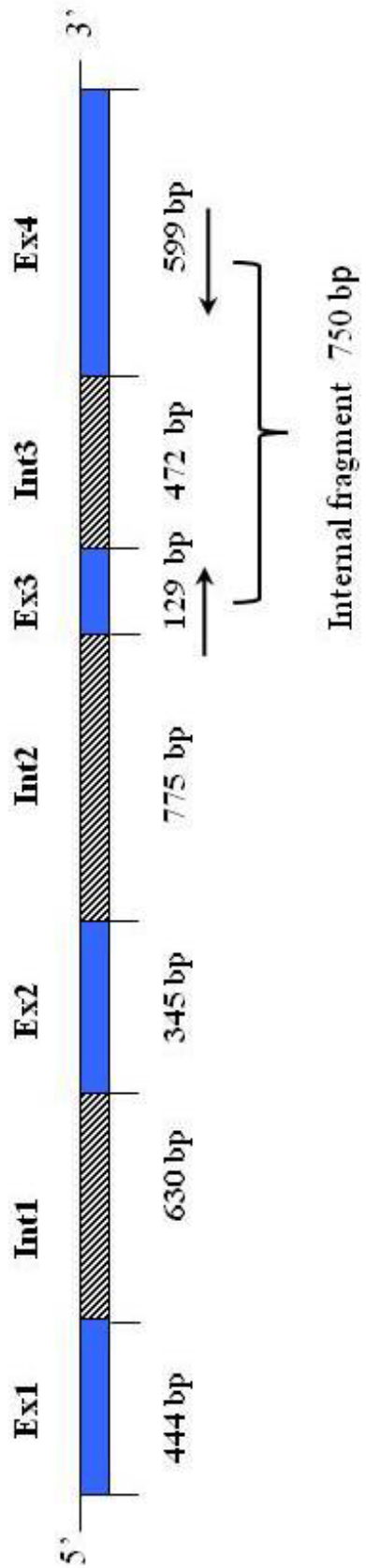


Fig. 5. 8. C4H gene structure in *Helichrysum aureonitens*. Arrows show the position of the designed degenerated primers used to amplify the internal fragment.

Fig. 5. 9. cDNA full length sequence of the C4H in *Helichrysum aureonitens* (1518 bp including stop codon, TAA). Arrows show the position of the attaching place of the specific primers (C4H-F and C4H-R) used to amplify the full length C4H gene. The under-lined nucleotides represent the start and stop codons for the isolated C4H gene from *Helichrysum aureonitens*.

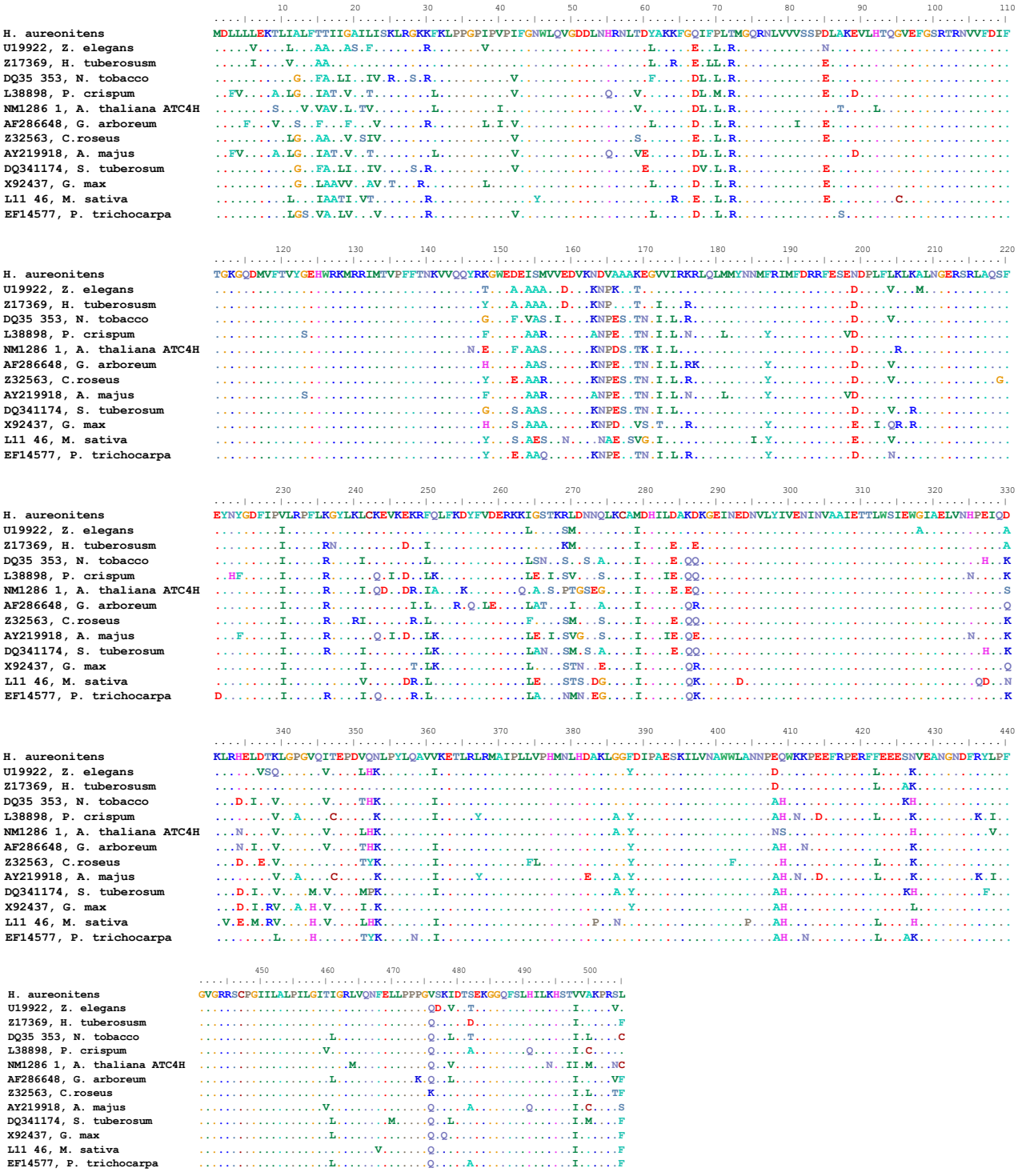


Fig. 5. 10. Multi-alignment of C4H amino acid from *Helichrysum aureonitens* with C4H homologies from other plants. The spots represent the similar amino acid sequences to the C4H from *Helichrysum aureonitens.*

5.4.5 Phylogenetic tree

A preliminary phylogenetic analysis carried out to determine the relationship between C4H from *H. aureonitens* with other C4H genes in other plants revealed that the C4H from *H. aureonitens* can be categorized in the same group of C4H as found in plants belonging to the Asteraceae family including *Z. eleganse* (Ye, et al., 1996), *H. tuberosus* (Teutsch, et al., 1993), and *E. angustifolia* (Feng, et al., 2008), but the C4H in *H. aureonitens* is closest to *Z. violacea* compared to the other inter family tested plants (Fig.5.11). It also indicated that there is a significant difference between C4H from Asteraceae and C4H from others (Fig.5.11).

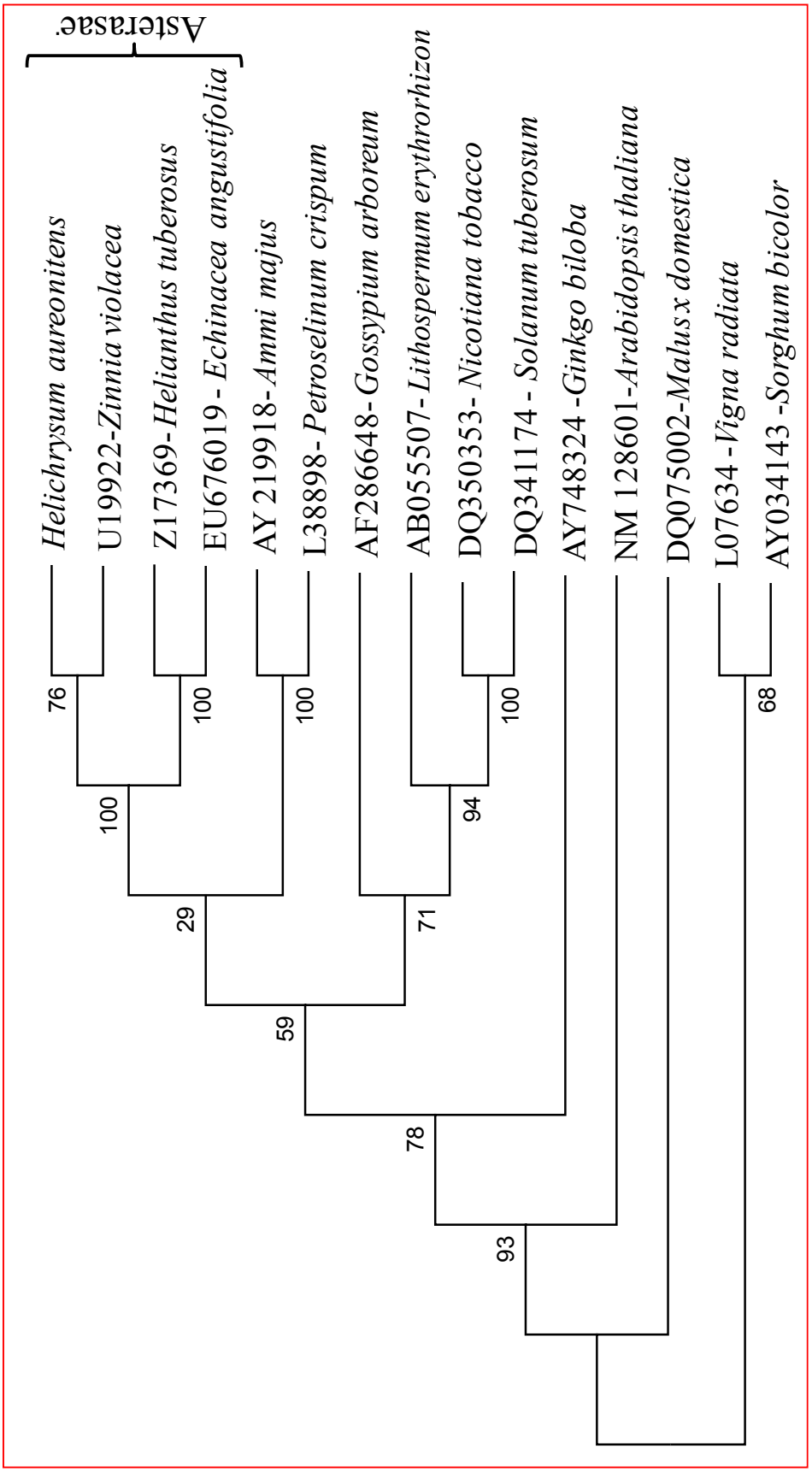


Fig. 5. 11. Phylogenetic tree of the C4H in *Helichrysum aureonitens*.

5.4.6 Secondary and tertiary structures of C4H from *H. aureonitens*

Figure 5. 12 shows the secondary structure of C4H from *H. aureonitens*, which was predicted on line by Psi-pred. In this predicted secondary structure of C4H, it was demonstrated that there are some helixes with different sizes containing 3 to 29 residues. The longest helix located in the middle between two strand regions. The first helix at N-terminus can be attributed to the signal peptide/anchor. To find the homology for the C4H protein from *H. aureonitens*, the translated amino acid sequence of C4H from *H. aureonitens* was applied to HHPRED web site. Figure 5. 13 shows the alignment between amino acid sequences of C4H from *H. aureonitens* and a cytochrome P450, 2C9 monooxygenase, drug metabolizing enzyme, oxidoreductase (IR90-A,) as one of the closest homologies to *H. aureonitens* C4H. As there is no known structure for plant-derived C4H on the database, the closest homologies were selected from human cells. The first 23 amino acids are not included in this alignment because of no homology at the N terminus of C4H from *H. aureonitens* to the IR90-A (Fig. 5. 13). According to this result the secondary structure of *H. aureonitens* C4H modeling was created in the H H PRED online web site (Fig 5. 14). Based on the C4H created model the secondary structure from *H. aureonitens* is composed of 16 alpha helixes (45.35 %), random coils (46.4 %) and strands (8.22 %). Alpha helixes were mainly distributed throughout the protein, containing 2 to 31 amino acids. The longest helix is placed around the middle of C4H protein. Extended strands mainly distributed in two regions: one is the ~100-residue N-terminal region, and another is the ~120-residue region at the C-terminal helix and the central helices with 31 residues. In these 2 regions, extended strands distribute in an interlaced manner with random coils and small alpha helices (Fig. 5.14).

```
Conf:  95699999999999999999985247999999279988844518870999565999999
Pred:  CCHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCCCCCCCCCCEECCHHHCCCCCHHHHHHH
  AA:  MDLLLLEKTLLALFATILGAIFISKLRGKRFKLPPGPIPVPIFGNWLQVGDDLNHRNLTD
               10        20        30        40        50        60

Conf:  99985987799956903899789999999996779645999718999970599608978
Pred:  HHHHHCCEEEEEECCEEEEECCHHHHHHHHHHCCHHHCCCCCCHHHHHHHCCCCCEEEC
  AA:  LAKKFGEIFLLRMGQRNLVVVSSPDLAKDVLHTQGVEFGSRTRNVVFDIFTGKGQDMVFT
               70        80        90       100       110       120

Conf:  88789999999999704799999977799999999999999862679994848999999
Pred:  CCCHHHHHHHHHHHHCCCHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCHHHHHHH
  AA:  VYGEHWRKVRRIMTVPFFTNKVVQQYRYGWEAEAAAVVEDVKKNPAAATEGVVIRRRLQL
              130       140       150       160       170       180

Conf:  99999999809954667848999999999999997234115999999999818588999
Pred:  HHHHHHHHHHCCCCCCCCCHHHHHHHHHHHHHHHHHCCCCCCHHHHHHHHHHCCHHHH
  AA:  MMYNNMFRIMFDRRFESEDDPLFLKLKALNGERSRLAQSFEYNYGDFIPILRPFLRSYLK
              190       200       210       220       230       240

Conf:  99999999999999999999976135678874163899999985478898889999999
Pred:  HHHHHHHHHHHHHHHHHHHHHHHHHCCCCCCCCHHHHHHHHHHHHCCCCCCCHHHHHHH
  AA:  LCKEVKEKRLQLFKDYFVDERRELGSTKSMDNNQLKCAIDHILEAKDKGEINEDNVPYIV
              250       260       270       280       290       300

Conf:  99985220159999999999997598999999999999809999889666157868999 9
Pred:  HHHHHHCCCHHHHHHHHHHHHHHHHCHHHHHHHHHHHHHHHCCCCCCCHHHCCCCHHHHHH
  AA:  ENINVAAIETTLWSIEWGIAELVNHPEIQAKLRNELDTILGPGVQVTEPDIQKLPYLQAV
              310       320       330       340       350       360

Conf:  99998117870255451217871788985289998999607863795547996564832 1
Pred:  HHHHHHHCCCCCCCCCCCCCCCEEECCEEECCCCEEEEEHHHHHCCCCCCCCCCCCCCCC
  AA:  VKETLRLRMAIPLLVPHMNLHDAKLGGFDIPAESKILVNAWWLANNPEQWKKPEEFRPER
              370       380       390       400       410       420

Conf:  25899873234886156357887555856899999999999999764442799987787
Pred:  CCCCCCCEEECCCCEEECCCCCCCCCCCCHHHHHHHHHHHHHHHHHHEECCCCCCCCCCC
  AA:  FFEEESNVEANGNDFRYLPFGVGRRSCPGIILALPILGITIGRLVQNFELLPPPGVSKID
              430       440       450       460       470       480

Conf:  777778623424886379999578 89
Pred:  CCCCCCCCEEECCCCEEEEEECCCC
  AA:  TSEKGGQFSLHILKHSTVVAKPRSLF
              490       500
```

Fig. 5. 12. Secondary structure of C4H from *Helichrysum aureonitens* was predicted in Psi-pred (Psipred V2.6 by David Jones) (bioinf.cs.ucl.ac.uk/psipred). Conf: Confidence (0=low, 9=high); Pred: Predicted secondary structure (H=helix, E=strand, C=coil); AA: Target sequence.

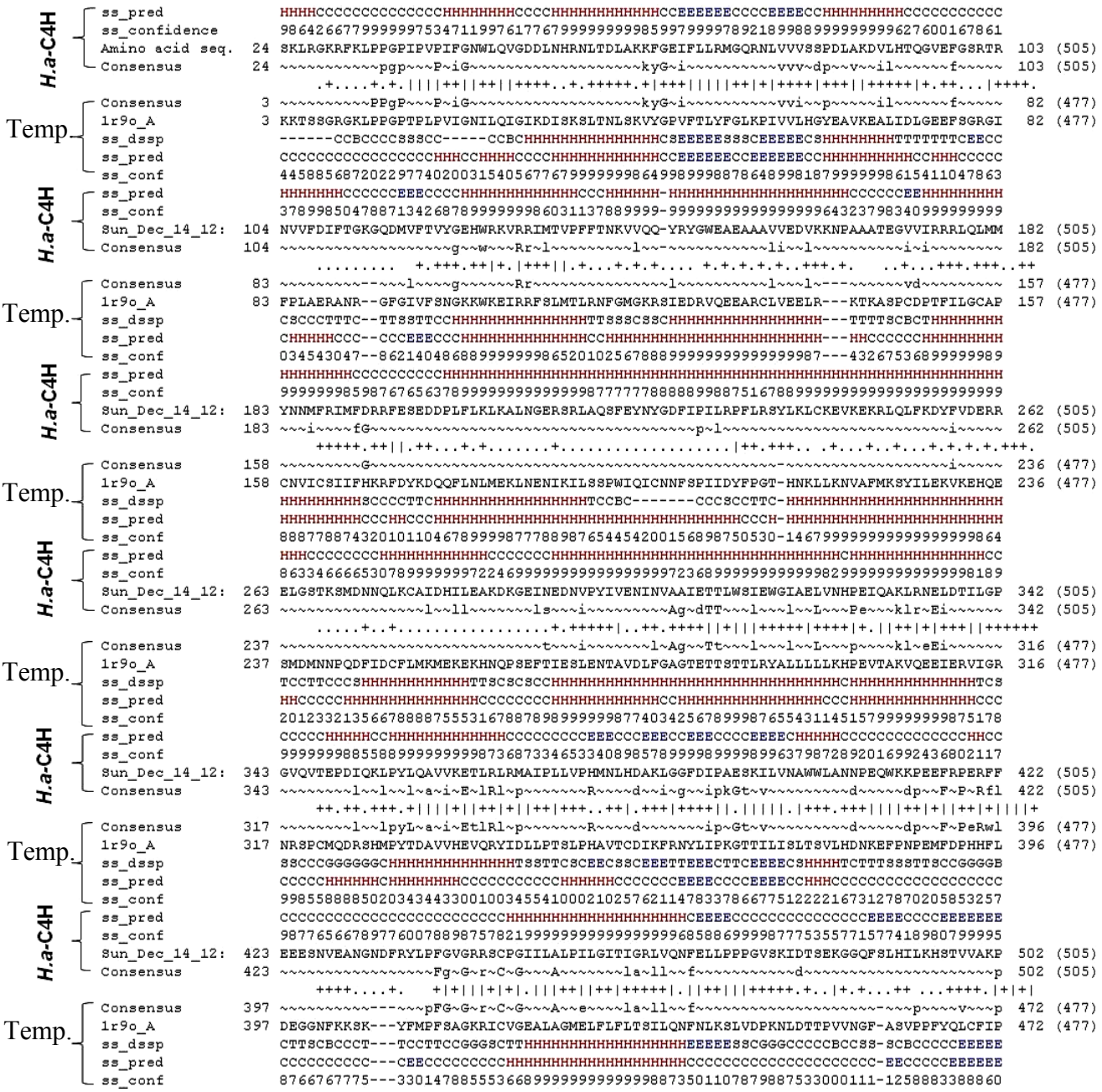


Fig. 5. 13. Protein alignments between C4H amino acid sequences from *Helichrysum aureonitens* and a cytochrome P450. 2C9 monooxygenase, drug metabolizing enzyme, oxidoreductase, heme (IR90-A); HET: HEM FLP; 2.00A {Homo sapiens} SCOP. H: α-Helixes; C: Coils; E: Strands. Confidences show the confidence of predictions. Temp.: The C4H template was used for comparison. *H.a*-C4H: C4H amino acid sequences from *Helichrysum aureonitens*.

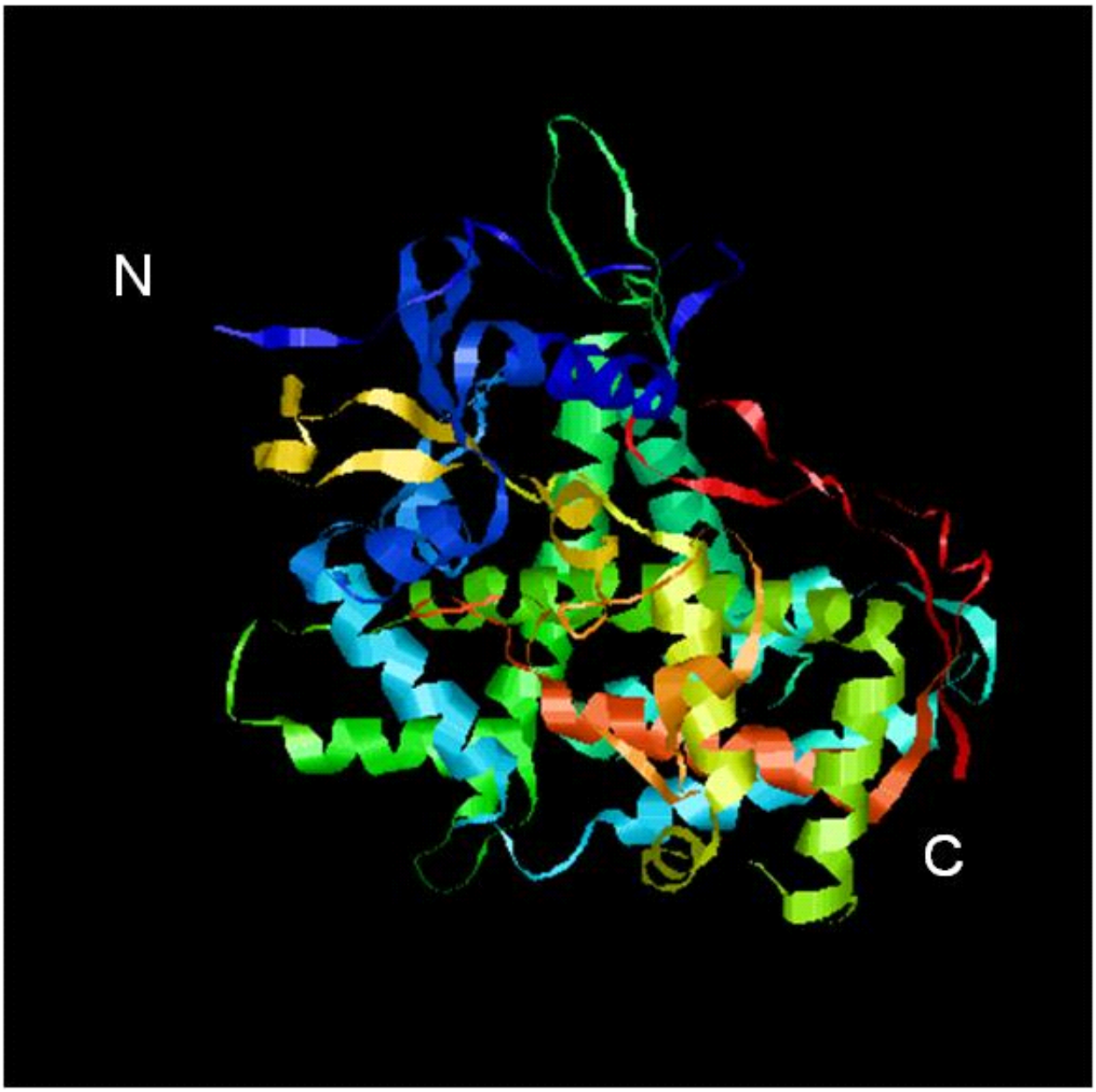


Fig. 5. 14. RASMOL secondary structure model for C4H from *Helichrysum aureonitens*. N: N terminus; C: C terminus.

5.5 Discussion

In this study a copy of C4H from *H. aureonitens* was isolated and sequenced. Although all the transformed colonies from the internal fragment were sequenced and all of them showed the same sequences, there is no clear evidence to conclude that *H. aureonitens* has only one copy of C4H. According to the database there is no report to show that members of the Asteraceae such as, *Z. violacea* (Ye, et al., 1993), *H. tuberosus* (Teutsch, et al., 1993), and *E. Angustifolia* (Feng, et al., 2008) have more than one C4H gene. Pea, *Arabidopsis* and parsley are thought to contain only one copy of C4H (Frank et al., 1996; Bell-Lelong et al., 1997; Mizutani et al., 1997; Koopmann et al., 1999), but in members of the Brassicaceae family, there are some with as much as 6 copies of C4H present in *Brassica napus* (Chen et al., 2007). However to confirm the number of C4H copies in *H. aureonitens* further experiments need to be carried out.

According to the genomic DNA sequencing, the obtained gene structure for *H. aureonitens* C4H is different from all the present data on the database. Based on the C4H gene structure in *A. thaliana* (NC_003071.3) (Fig. 5.1) there are three exons (Ex1, 786 bp; Ex2, 132 bp; Ex3, 600 bp) and two introns (In1, 80 bp and In2, 220 bp), but the C4H gene map obtained for *H. aureonitens* indicates four exons (Ex1, 444 bp; Ex2, 345 bp; Ex3, 129 bp; Ex4, 599) and three introns (In1, 630 bp; In2, 775 bp and In3, 472 bp). The length of the open reading is 1518 bp which is similar to the coding region of C4H in species of the Asteraceae family such *Z. violacea* (U19922) (Ye, et al., 1993), *H. tuberosus* (Z17369) (Teutsch, et al., 1993), and *E. angustifolia* (EU676019) (Feng, et al., 2008) as well as other plants from different families such as *A. thaliana* (AtC4H, NM_128601) (Mizutani et al., 1997). The existence of different sizes of introns of a specific gene in different plants has already been reported. Two copies (BnC4H-1 and BnC4H-2) of C4H were isolated from *Brassica napus*. The genomic DNA sequence of these indicated that there are

two introns in each one, while the size of introns in BnC4H-1 (In1, 70; In2, 378) and BnC4H-2 (In1, 64; In2, 326) are different (Chen et al., 2007). According to the results of this study regarding the gene structure of C4H, it is the first time that the presence of a new intron (Intron 1, Fig. 5.1) for C4H has been reported.

The G+C content of C4H in *H. aureonitens* in the open reading frame and introns showed 44.4 %, and 30.27 % respectively. These results were confirmed by Chen et al. (2007), whose experiment showed higher G+C content in the coding regions than the non-coding regions in the C4H genes isolated from *B. napus.*

The results of the C4H secondary structure model from *H. aureonitens,* in the case of number of helixes, seems different to isolated C4Hs from *Brassica napus* by Chen et al. (2006). They reported 10 to 11 helixes in BnC4H-1 and BnC4H-2 respectively while in this study we reported 16 helixes. The reason for the different number of helixes can be contributed to the different consideration of counted helixes. In this study all helixes with distinct position were considered as separate helixes, but Chen et al. (2006) for example, the longest helix has two separate parts, which joined together with some random coils. The size of longest helix in C4H from *H. aureonitens* in this study was reported 31 residues, while Chen et al. (2006) reported 62 in BnC4H-1 and in BnC4H-2 (two pieces). In C4H from *H. aureonitens,* there are 4 helixes close to each other with a total size of 62 residues (31, 14, 4 and 13) that can consider as a big helix, but it needs more study.

Predictions on the signal peptide/anchor and subcellular localization of C4H from *H. aureonitens* by different software gave inconsistent results. C4H and other microsomal P450s have been extracted from microsomes of various organisms, and it has been presumed that microsomal P450s have a N-terminal hydrophobic helix which serves to anchor the enzyme to the ER

membrane (Winkel-Shirley, 1999; Werck- Reichhart et al., 2002). In C4H the N-terminal hydrophobic helix has been predicted with multiple identities, i.e. signal peptide with cutting site, signal anchor, and transmembrane helix, by different software in this species. A second strong transmembrane helix was also predicted at the C-terminus. Another fact is that the predicted tertiary structures of the C4H protein from *H. aureonitens* do not contain the first 23 residues, and no N-terminal helix can be seen in Fig. 5. 13, although figure 5. 12 shows a helix region at N-terminal with low confidence. Considering P450s in general, there is more than one report indicating effective or possible plastidic localization. These make it difficult to draw a definite conclusion on the properties of the N-terminal helix and subcellular localization of C4H from *H. aureonitens*. But it is obvious that both the N-terminal and the C-terminal sequences of C4H from *H. aureonitens* do not show essential difference from those of AtC4H and most other C4Hs, so the location and topology of C4H from *H. aureonitens* should be similar to those of typical C4H proteins.

5.6 Conclusions

The results in the previous chapter showed that *p*-coumaric acid, the product of C4H activity, was not detected in *H. aureonitens* samples, and based on the known phenylpropanoid biosynthetic pathway C4H may not be active at that position in this species. However, in the leaf samples of *H. aureonitens,* kaempferol, the hydroxylated compound at the 4' position, was detected. Based on these results the initial question that arose was whether C4H is present or expressed in *H. aureonitens.* The results of this part of the study showed that C4H is present in *H. aureonitens* and its characteristics show the highest homologies to *Echinacea angustifolia* from the Asteraceae C4H with 83.6 % identity on nucleotide level but 93 % identity and 97 % positives on the protein level. Although there are some differences in the case of the gene structure, the full length coding region sequence of C4H from *H. aureonitens* is mostly similar to that of other plants (1518 bp including stop codon, TAA). It was observed that the similarity of isolated C4H from *H. aureonitens* was quite similar to that of other Asteraceae species present in the database. The phylogenetic tree result indicated that there is a close relationship among C4H nucleotide sequences from Asteracae and that they can be grouped in one category, but there is a big difference between C4H sequences of this group and C4Hs from other plants. The predicted secondary structure of C4H from *H. aureonitens* is almost similar to C4Hs from other plants, but the characterization of C4H from *H. aureonitens* still needs more experiments.

In proving the hypothesis for the presence of an alternative biosynthetic pathway for kaempferol production in *H. aureonitens* and the possibility of some link/s between an non 4'-OH pathway and the 4'-OH pathway, the expression of isolated C4H from *H. aureonitens* will be discussed in the next chapter.

Chapter 6

C4H expression in *Pichia pastoris*

6.1 Abstract

Cytochrome P450s are one of the largest superfamilies of enzymes found in almost all living organisms. Cinnamic acid 4-hydroxylase (C4H) is a cytochrome P450-dependent mono-oxygenase widely present in higher plants. According to the known phenylpropanoid biosynthetic pathway, this is the second enzyme in the pathway which catalyzes hydroxylation of transcinnamic acid, the product of phenylalanine ammonia-lyase (PAL) action. The full length cDNA of C4H was isolated from *Helichrysum aureonitens* and for the first time integrated in a secreted expression vector, pPICZαC, and then transformed into *Pichia pastoris*. After 48 hrs of methanol-induction protein was collected, precipitated by ammonium sulphate and purified using column chromatography. SDS-PAGE electrophoresis and Western blot showed the expression of a His-tagged protein with a size of 50-60 kDa. The calculated mass of recombinant C4H, including the polyhistidine tag is about 60.5 kDa. The secreted protein was found to be a suitable system for the expression of recombinant C4H protein.

6.2 Introduction

Cytochrome P450s are one of the largest superfamilies of heme-containing enzymes (Chapple, 1998) found in almost all living organisms. Up to now P450s have been found in bacteria, insects, fish, mammals, plants, and fungi (Chapple, 1998). Cinnamic acid 4-hydroxylase (C4H) is a cytochrome P450-dependent mono-oxygenase and the second enzyme in the common branch of the phenylpropanoid pathway (Hotze, et al., 1995). Biochemical and genetic data have demonstrated that C4H belongs to the CYP73A subfamily of cytochrome P450-dependent mono-oxygenase family of enzymes (Schalk et al., 1998), which are widespread represented in higher plants (Yamamura et al., 2001). According to the 4'-OH phenylpropanoid biosynthetic pathway, this enzyme catalyzes the hydroxylation of transcinnamic acid, which is derived from phenylalanine by the action of phenylalanine ammonia-lyase. The product of the C4H reaction, 4-coumaric acid, is then activated to its CoA thioester by 4-coumarate: CoA ligase, and the 4-coumaroyl CoA is then funneled into branched pathways leading to a wide array of phenolic metabolites, including lignin, flavonoids and coumarins (Yamamura et al., 2001).

All known plant cytochrome P450 mono-oxygenase reactions depend on the associated activity of an NADPH: cytochrome P450 oxidoreductase (CPR) (Koopmann and Hahlbrock, 1997). Most of the cytochrome P450 mono-oxygenase enzymes do not use NADPH directly but interact with a P450 oxidoreductase, a flavoprotein, (Chapple, 1998) that transfers electrons from NADPH via FAD and FMN to the prosthetic heme group of the P450 protein (Koopmann and Hahlbrock, 1997). Although detailed characteristics, including structural properties of CPRs have been reported for animal systems (Kim et al., 1996), only a few of these enzymes have been purified (Meijer et al, 1993) and cloned from plant sources (Urban et al., 1997).

Several expression systems have been developed for heterologous gene expression such as yeast

(*Saccharomyces cerevisiae* and *Pichia pastoris*), mammalian cells, amphibian oocytes (*Xenopus laevis*), insect cells and bacteria (*Escherichia coli*). Plant cells are useful as hosts if mutants are available (Holton et al., 1993), but in many cases the distinction from resident activities may present a problem. Expression of the prokaryotic heterologous genes in *E. coli* is the most popular and has been widely used for the last three decades (Daly and Hearn, 2005). However, the expression of the active, soluble P450 hydroxylases in *E. coli* has proven to be challenging in the past due to the difficulty of prokaryotic organisms to harbor these membrane-associated mono-oxygenases (Hotze et al. 1995), protein insolubility (Oeda et al., 1985), lack of P450-reductase function (Porter et al., 1987), or due to limitations in heme biosynthesis (Gallagher et al., 1992; Sinha and Ferguson, 1998). Indeed, the selection of an expression system is affected by different factors including the type of protein being expressed (eg. prokaryotic vs. eukaryotic, soluble vs. membrane bound) and the experimental purpose (eg. kinetics interaction studies or antibody production) (Stutzer `, 2008).

Expression of eukaryotic heterologous genes in prokaryotic cells can be very difficult when a functional protein is required, due to the fact that they lack the molecular machinery required for post-translational modifications (glycosylation, proline cis / trans isomerisation, disulphide isomerisation, lipidation, phosphorylation, etc.). *E. coli* is therefore not suitable for the expression of eukaryotic derived-proteins with a high demand for disulphide bonding and other types of post-translational modifications (Daly and Hearn, 2005).

Protein expression in yeast not only has all the advantages of eukaryotic protein processing such as folding and post-translational modifications of eukaryotes, but also has similar molecular manipulation and growth characteristics of prokaryotes (Cregg et al., 1993). The first yeast species that had been developed as an expression system was the baker's yeast

Saccharomyces cerevisiae, but it is not always a successful expression host (Cregg et al., 1993). The yeast, *Pichia pastoris*, was selected and developed as an alternative yeast expression host, since it displays a lower degree of glycosylation of the recombinant protein than that associated with expression in *S. cerevisiae* (Invitrogen Corporation, 2001). It also contain methanol-regulated promoters which easily regulated during fermenting conditions. As a eukaryote, *P. pastoris* has many of the advantages of the higher eukaryotic expression systems such as protein processing, protein folding, and post-translational modification, as well as being as easy to manipulate as *E. coli* or *S. cerevisiae*. It is faster, easier, and less expensive to use than other cell-culture based eukaryotic expression systems and generally offer higher expression levels (10- to 100-fold higher heterologous protein expression levels than *Saccharomyces*). These features make *Pichia* very attractive as a heterologous protein expression system.

P. pastoris is methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. This is achieved by the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase (AOX). The promoter regulating the production of alcohol oxidase is the one used to drive the MeOH-induced protein expression in *Pichia* (Invitrogen Corporation, 2001). Two genes, AOX1 and AOX2 encode the AOX enzyme. The AOX1 gene is responsible for the production of the majority about 85% the alcohol oxidase activity (Daly and Hearn, 2005). This gene is tightly regulated by the AOX1 promoter that has been isolated and introduced into the plasmid-borne versions to drive the heterologous protein expression. The AOX2 gene, though highly homologous to AOX1 (97%), gives a much slower growth on methanol in comparison to AOX1. Therefore, the loss or mutation of the AOX1 gene results in a strain that must rely on the AOX2 gene for expression. These results in a phenotype where methanol utilisation slow or Mut^S, while the wild-type phenotype is designated as Mut^+ (Invitrogen Corporation, 2001).

Expression of homologous proteins in *P. pastoris* is performed by the integration of DNA by a single crossover at a specific locus (eg. AOX1 gene) of the genome (Fig. 6.1). Multiple gene insertion into the pPICZ plasmid expression at a single locus in a cell is also possible. Due to the low frequency of multiple gene insertion events, the rate of transformation is between 1 and 10%, and is detected in Zeocin resistant transformants (Invitrogen Corporation, 2001). Multi-copy events can occur as gene insertions either at the AOX1 or the aox1: ARG4 loci (Fig. 6.2).

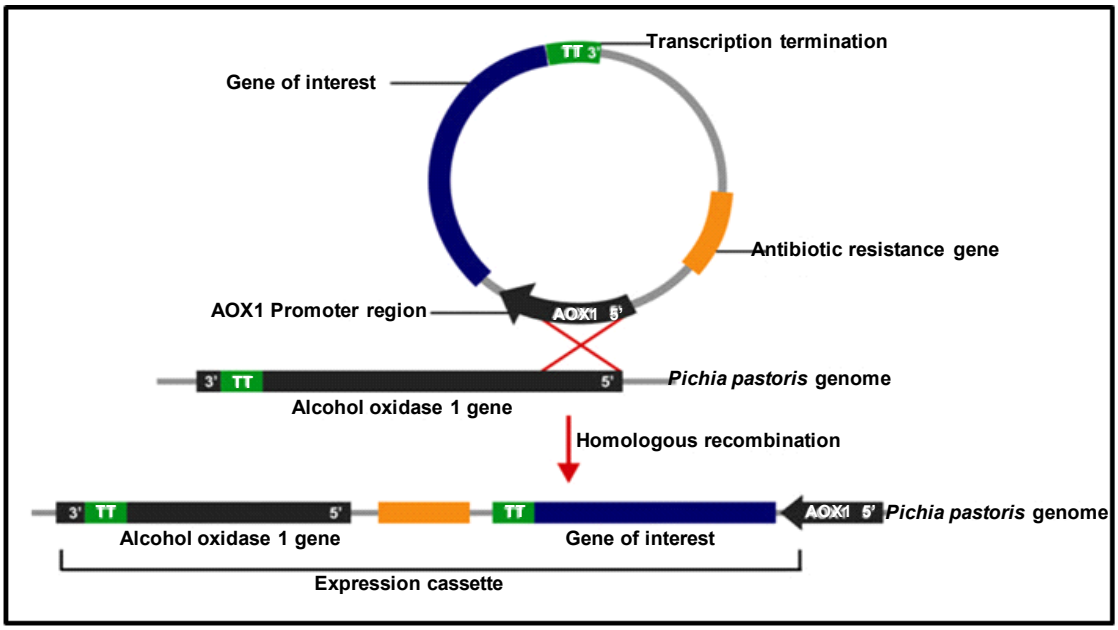


Fig. 6. 1. Insertion of the homologous genes into the AOX1 locus of *Pichia pastoris* (adapted from Invitrogen Corporation, 2001). The red cross indicates a single crossover between the promoter region of an expression vector containing the gene of interest and the promoter region in the yeast genome. This leads to the integration of the homologous gene to the intact genome of *Pichia pastoris*. This integration yields a stable transformant that encodes the target gene and a selected marker (antibiotic resistance), regulated by the AOX1 promoter.

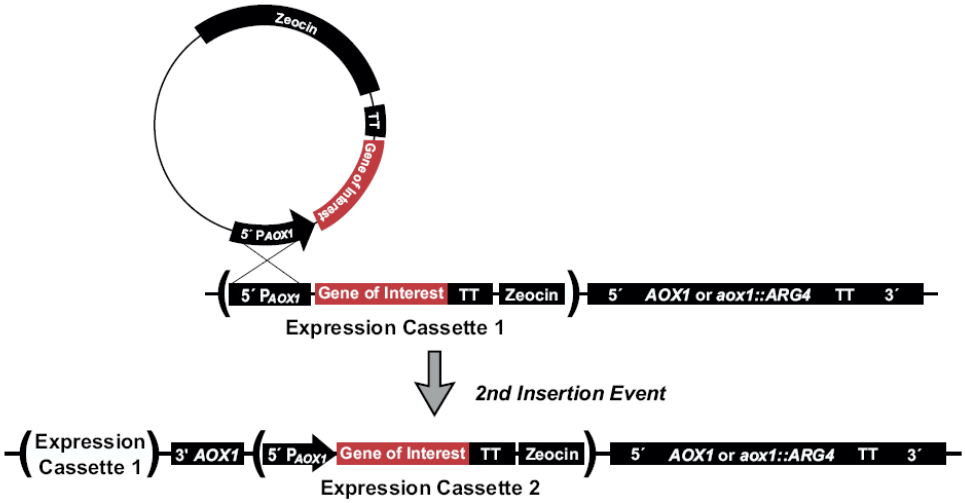
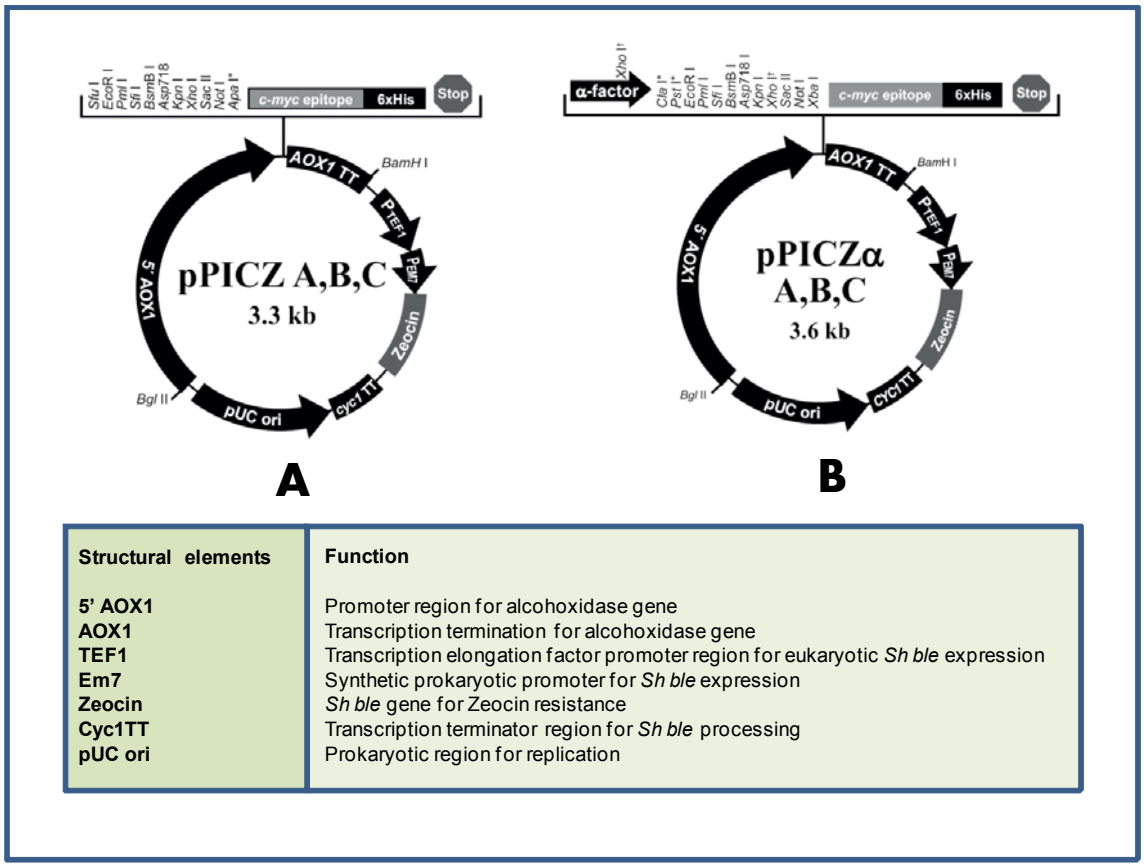


Fig. 6. 2. Insertion of the multiple heterologous genes into the expression pPICZ plasmids. Multi-copy events can occur as gene insertions either at the AOX1 or the aox1::ARG4 loci (Invitrogen Corporation, 2001).

Both intracellular and extracellular secreted expression of proteins can be performed in *P. pastoris*. The secreted expression requires a secretary signal sequence allowing secretion of the expressed protein. The secretion signal sequence from the α-factor peptide of *S. cerevisiae* has been found to give the highest success rate (Cregg et al., 1993). The low levels of exogenous native proteins produced by *P. pastoris* are the main advantage of this secreted expression. This means that the secreted expressed protein comprises the majority of the total protein in the induction medium.

Prior to integration of the gene of interest into a *P. pastoris* expression plasmid, a suitable expression vector should be chosen, based on type and the purpose of expression of the protein of choice. Two plasmids are supplied; intracellular- (pPICZ vectors) and secreted expression (pPICZα vectors) (Fig. 6.2). Both these vectors contain the origins of replication for the amplification of plasmids in a prokaryotic host, like *E. coli* (strains TOP10F', JM109,

DH5αF), before transformation into yeast. Each vector also contains unique restriction sites (*Sac I, Pme I and BstX I*) that can be used for the linearisation of the plasmids for an effective homologous recombination into the *P. pastoris* genome. The pPICZα vectors encode the α-factor mating signal sequencing from *S. cerevisiae* that allows expression via the secretory pathway (Brake et al., 1984). During secretion, the signal sequence is processed in two steps by cleavage with *P. pastoris* specific signal peptidases, KEX2 and STE13.



Structural elements	Function
5' AOX1	Promoter region for alcohoxidase gene
AOX1	Transcription termination for alcohoxidase gene
TEF1	Transcription elongation factor promoter region for eukaryotic *Sh ble* expression
Em7	Synthetic prokaryotic promoter for *Sh ble* expression
Zeocin	*Sh ble* gene for Zeocin resistance
Cyc1TT	Transcription terminator region for *Sh ble* processing
pUC ori	Prokaryotic region for replication

Fig. 3. 3. Structures of pPICZ vectors used for the expression of the heterologous gene in an EasySelect™ *Pichia* expression system (Invitrogen Corporation, 2001). **A:** pPICZ, intracellular expression system. **B:** pPICZα, secreted expression system.

This part of the study was carried out to investigate the second step of the hypothesis which is the expression of C4H. This step is important to determine that the isolated C4H from *H. aureonites* is a putative gene? For that purpose a secreted expression systems in *P. pastoris* was chosen. The objective of this experiment was planned as follows:For expression, yeast (*P. pastoris*) was investigated as a model system for the heterologous expression of *H. aureonites* transcripts, using the open reading frame identified for C4H. This would allow insight into yeast-mediated post transcription modification such as early termination and possible overglycosylation. Finally, confirmation of the amino acid sequence of recombinant C4H will be performed to determine full-length expression.

6.3 Materials and methods

6.3.1 Plant materials

Greenhouse grown plants of *H. aureonitens* were used for the total RNA isolation and subsequent cDNA synthsis.

6.3.2 Total RNA isolation and cDNA synthesis

Fully expanded leaves harvested from the greenhouse grown plants were used for total RNA isolation, using a TriPure total RNA isolation kit. Genomic DNA contamination was eliminated by digestion with a RNase free DNase (Fermentas, Europe). Total RNA was quantified and qualified using NanoDrop® spectrophotometer and by analyzing it on 1 % agarose gel containing ethidium bromide. Approximately 1 µg total RNA was used to synthesize cDNA. The standard reverse transcription protocol was used to synthesize the first strand of cDNA according to the manufacturer's recommendations (Promega, USA).

6.3.3 C4H full gene amplification and digestion

The open reading frame (ORF) of the mature gene of interest should be cloned in a frame and downstream of the α-factor signal sequence and in the frame with the C-terminal tag (Invtrogen Corporation, 2001). For this purpose a set of gene specific oligonucleotides were designed to amplify the ORF of the C4H and incorporation of two restriction site at the ends for directional cloning, including *Xho I* at 5' side and *Sac II* at 3' side. The reverse primer contains a *Sac II* restriction enzyme site and a silenced stop codon to ensure incorporation of the C-terminal myc and His-tags.

Table 6. 1. Primers for the amplification and directional cloning of the C4H into the pPICZαC plasmid. Restriction enzyme sites are underlined. The α-factor signal sequence cleavage sites for KEX2 and STE13 and part of the insert are indicated by arrows and italics respectively. The bold three- nucleotide at the 5' side of the primers are extra nucleotides for increasing the efficiency of digestion.

Primer name	Primer sequence 5'-3	Tm ˚C
CHexp-FW	**CCG**CTCGAGAAGAGA↑GAGGCT↑GAAGCA*GATCTACTCCTTTTG* Xho I kex2 Ste13 Insert	74.47
CHexp-RV	**TCC**CCGCGG*CAAGAGATCTTGGTTTGGC* Insert	71.94

A TOPO-TA plasmid (Invitrogen, USA) containing the full-length C4H was used as a template for the PCR amplification of the C4H ORF. Amplification was performed in a reaction containing 1 µl of a 100x dilution of the plasmid DNA template (240 ng / µl), 1 µl of 20 µM of both CHexp forward and reverse primers (Table 6.2), 2 µl of 10x bio line buffer, 2 µl of 50 mM $MgCl_2$, 2 µl dNTPs (2.5 mM of each) and 0.2 µl Taq polymerase (5 U / µl, Bio line). The master mix was made up to a final volume of 25 µl by adding double distilled deionised water. A hot-start PCR amplification was performed for 30 cycles with denaturation at 94 ˚C for 30 seconds and annealing and extension at 72 ˚C for 2 minutes. It was followed by a final extension for 30 minutes at 72 ˚C, as described in chapter 5.

6.3.4 Preparation of the pPICZαC expression plasmids

A frozen stock of *E. coli* strain JM109 containing pPICZαC, was kindly supplied by Dr Christine Maritz-Oliver (Department of Biochemistry, University of Pretoria). One microliter of the frozen stock was inoculated into 4 µl liquid low salt LB medium (1% tryptone, 0.5%

NaCl, 0.5% yeast extract, pH 7.5) and incubated overnight at 30 ˚C with shaking at 200 rpm. Selection screening was accomplished by adding ZeocinTM at a final concentration of 12 µg / ml. Since Zeocin^{TM} is sensitive to high salt concentrations, all culturing was done using low salt LB. The pPICZαC plasmid was subsequently isolated according to manufacturer's instructions from the over-night culture using a plasmid purification kit from Fermentas (Europe).

6.3.5 Directional cloning of C4H ORF into pPICZαC

Both the pPICZαC plasmids and the open reading frame for C4H were digested with *Sac* II and *Xho I* in two separate reactions. Firstly, *Sac II* digestion was performed in a reaction mixture containing 2 µg of plasmid or insert, 1 µl of *Sac II* restriction enzyme (10 U / µl, Fermentas, Europe), 2 µl of 10x restriction enzyme buffer B (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg / ml BSA) and sterile distilled water was then added to a final volume of 20 µl. Reactions were incubated at 37 ˚C for 4 hours and the enzyme inactivated by incubation at 70 ˚C for 10 minutes. The digested DNA was precipitated with the addition of 1 / 10 volume sodium-acetate (3 M, pH 4.6) and 3 volume ethanol, followed by incubation at -20 ˚C for 30 minutes and then spinning at 16 000 rpm for 30 minutes at 4 ˚C. After precipitation, the DNA was washed with 70 % ethanol and dried using vacuum drier. The pellet was resuspended in 19 µl of sterile distilled water, and the quantification and qualification of the isolated plasmids was carried out using 1 µl for running on 1% agarose gel and 1 µl for Nanodrop.

Digestion with *Xho I* was performed in the second step, by preparing a similar reaction mixture containing the total purified *Sac II* digested DNA (17 µl) for plasmid and insert as template, 1 µl of *Xho I* restriction enzyme (10 U / µl, Fermentas, Europe), 2 µl of 10x restriction enzyme buffer R (10 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg

/ ml BSA) and the volume of mixture was adjusted to a final volume of 20 µl by adding sterile distilled water. This process was followed by incubation at 37 ˚C for 4 hours and then the enzyme again inactivated by incubation at 70 ˚C for 15 minutes. Digested products were purified, resuspended and quantified as described previously. To avoid self-ligation of the plasmid, the digested pPICZαC plasmid was dephosphorylated with the addition of 2 µl alkaline phosphatase (3 U / µl) and incubated at 37 ˚C for 1 hour. The inactivation of the enzyme was performed by incubation at 65 ºC for 15 minutes.

6.3.6 pPICZαC plasmid and C4H insert ligation

The double / directional digested pPICZαC and C4H were ligated at a ratio of 1: 3 in a ligation mixture containing 50 ng of dephosphorylated pPICZαC plasmid, 150 ng C4H insert, 2 µl of a 10x ligation buffer (300 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 10% PEG, pH 7.8) and 2 µl T4 DNA ligase (3 units / µl) in a final volume of 20 µl. The ligation reaction was incubated overnight at 4 ˚C. T4 DNA ligase was subsequently inactivated by incubation at 65 ˚C for 15 minutes.

Ligation products were precipitated by adding 1 / 10 volume of tRNA (10 mg / ml), 1 / 5 volume of sodium acetate (3 M, pH 5) and 3 volumes absolute EtOH, followed by 45 minutes centrifugation 16 000 rpm at 4 ˚C. After discarding the supernatant, it was washed with 70 % EtOH and dried in a vacuum dryer, and dissolved in 20 µl sterile H_2O.

6.3.7 Preparation of competent TOP 10F' *Escherichia coli*

Heat-shock competent TOP 10F' *E. coli* (Invitrogen , USA) was prepared from an overnight culture grown at 30 °C on a shaker (250 rpm) in 1 ml SOB (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 0.0187% KCl, 0.0095% anhydrous $MgCl_2$). This culture was then inoculated into a 500 ml flask containing 50 ml SOB and grown at 30 °C with constant agitation (250

rpm) until it reached an $OD600_{nm}$ of 0.3. Fifty milliliters aliquots of the cultures were placed in the pre-chilled centrifuge tubes and incubated on ice for 10 minutes. The chilled cells were then centrifuged at 1000 g for 15 minutes at 4 °C and the cell pellets were resuspended in 16.7 ml ice cold CCMB media (1.18% $CaCl_2$, 0.4% $MnCl_2.4H_2O$, 10 ml of 1 M KAc, 0.2% $MgCl_2.6H_2O$, 100ml 100% glycerol, in 1 litre with pH at 6.4). Cells were incubated on ice for 20 minutes and collected by centrifugation (1 000 x g, 15 minutes, 4 °C) followed by resuspending the cell pellets in 4.2 ml cold CCMB media. The competent cells suspension was divided into 200 µl aliquots and stored at –70 °C.

6.3.8 Transformation of *E. coli* (competent TOP 10F') with ligated pPICZαC

Ten microliters of the ligated plasmid DNA, prepared by the ligation step (6.3.6), was added to CCMB competent TOP 10F' *E. coli*. The entire mixture was incubated on ice for 30 minutes. Following heat-shock transformation at 42 ˚C for 90 seconds, the cells were incubated on ice for two minutes. Nine hundred microliters of SOB media (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 0.0187% KCl, 0.0095% anhydrous $MgCl_2$, in a 100 ml) containing 50 mM D-glucose was added to the transformed cells and incubated at 30 ˚C for 1 hour on a shaker. One hundred micoliters of the incubated culture were plated on low salt LB-agar medium containing 12.5 µg / ml Zeocin™.

6.3.9 Selection of transformed TOP 10F' *E. coli* clones

Transformed *E. coli* cells were diluted with LB broth supplemented with D-glucose and 12.5 µg / ml ZeocinTM (Invitrogen, USA) at a ratio of 1 / 5 and 1 / 10. The cells (100 µl) were plated on low salt LB-agar medium containing 12.5 µg / ml ZeocinTM and incubated overnight at 37 ˚C. Sixteen colonies were selected for colony PCR screening using a master mix containing 1ul of 5' AOX1 and 3' AOX1 primers (15 µM each) (Table 6.2), 2.0 µl 10x

Bio Line buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1.0 µl $MgCl_2$ (50 mM) and 2 µl dNTPs (2.5 mM of each), 1 µl of liquid culture and 12 µl sterile H_2O to a final total volume of 20 µl. The DNA *Taq* polymerase solution consisted of 0.25 µl *Taq* polymerase (*Takara Taq*^{TM}, Japan), 0.5 µl 10x Bio Line *Taq* DNA buffer and by adding 4.25 µl sterile H_2O was made up a total volume of 5 µl. To confirm the presence of the C4H insert, PCR amplification was performed. An initial denaturation step was performed at 94 ˚C for 7 minutes, the reaction was paused and the *Taq* polymerase solution added to the tubes. The PCR was completed at 80 ˚C for 30 seconds and 30 cycles for denaturation at 94 ˚C for 30 seconds, annealing at 55 ˚C for 30 seconds and extension at 72 ˚C for 2 minutes, followed by a final extension at 72 ˚C for 10 minutes.

Positive clones showing the correct insert size during electrophoresis were grown overnight at 30 ˚C on a shaker in 50 ml tubes containing 5 ml low salt LB-Broth with 12.5 µg / ml ZeocinTM. Glycerol stocks were prepared by adding 400 µl 50% glycerol to 600 µl culture for storage at -70 ˚C. The rest of the cultures were used for plasmid isolation using GeneJET plasmid miniprep kit (Fermentas, Europe). Insert sequences were confirmed by sequencing the transformed plasmids using 5'-AOX1, 3'-AOX1.

Table 6. 2. Primers used in colony PCR to detect positive clones harboring the C4H insert.

Primer Name	Primer Sequence (5' to 3')	Tm (˚C)	Degeneracy
5' AOX1	GAC TGG TTC CAA TTG ACA AGC	57.87	0
3'AOX1	GCA AAT GGC ATT CTG ACA TCC	57.87	0

6.3.10 Linearisation of the expression vector for transformation into *Pichia pastoris*

According to EasySelect™ *Pichia* expression kit manual (Invitrogen Corporation, 2001) if a successful transformation of the plasmid harboring the insert into the yeast is required, the vector should be digested with a restriction enzyme that does not cut within the insert. These enzymes can be either *Pme I, SacI* or *BstX I*. For C4H the only enzyme that does not have the cleavage site is *Pme I*.

pPICZαC linearization with *Pme I* was performed in a reaction mixture containing 10 µg expression plasmid carrying the C4H insert, 20 µl 10x restriction enzyme buffer 4 (20 mM Tris-acetate, 50 mM potassium acetate, 1 mM DTT, pH 7.9), 1 µl of *Pme I* (10U / µl), in a final volume of 200 µl. Incubation was performed at 37 ˚C for 16 hours using gel electrophoresis screening several different times to monitor digestion efficiency (linearization). *Pme I* was heat-inactivated by incubation at 65 ˚C for 20 minutes to terminate the reaction.

Digestion efficiency was determined by DNA gel electrophoresis prior to precipitation of the DNA. Precipitation of DNA was performed by adding 200 µl phenol, chloroform, isoamylalcohol at the ratio of a 25:24:1. After centrifugation for 1 minute at 13 000 rpm, the aqueous phase was transferred to a new 1.5 ml tube, then 1 / 4 volume of sodium-acetate (3M, pH 5.2) and 3 volume 100 % of ethanol was added. After centrifugation at 4 ˚C at 13 000 rpm for 60 minutes, the upper phase was discarded and the pellet washed with 70 % EtOH. Afterwards the pellet was dried using a vacuum dryer at room temperature for 5 minutes and the DNA dissolved in 20 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0).

6.3.11 Transformation of *Pichia pastoris* with linearized plasmids

Electrocompetent yeast cells preparation were carried out according to the EasySelect™ *Pichia* expression kit manuals instruction (Invitrogen Corporation, 2001). An overnight

culture of 0.5 ml was inoculated in 500 ml YPD and grown overnight again to an OD 600 nm 1.3- 1.5. Cells were collected by centrifugation at 1 500 x g (5 minutes, 4 ˚C) and resuspended in 500 ml ice-cold sterile distilled water. Cells were washed again in 250 ml ice-cold water then resuspended in 20 ml ice-cold 1 M sorbitol. Following centrifugation at 1 500 x g (5 minutes, 4 ˚C), the pellet was resuspended in 1 ml 1 M sorbitol. Eighty microliter of electrocompetent GS115 cells and 20 µl linearized plasmids of the C4H clone and the native plasmids were loaded into a pre-chilled MicfroPulser® electroporation cuvette (0.2 cm gap, BIO-RAD) and incubated on ice for 5 minutes. The electrocopetent cells were performed at 1500 volts for 4.8 ms. The electroported cells were immediately transferred into 1 ml 1 M ice-cold sorbitol in a 15 ml tube and incubated at 30 ˚C for 1-2 hours without shaking. Cells were plated out on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol) containing 100 µg / ml zeocin™, and incubated at 30 ˚C for 4 days or until the colonies become visible.

6.3.12 PCR screening of recombinant GS115 clones

Integration of the C4H gene into the genome of *P. pastoris* GS115 cells was determined by PCR amplification after isolation the genomic DNA from overnight cultures of all 23 colonies resistant to Zeocin™ (Zeo^R) selected from the YPDS plates.

6.3.12.1 Genome DNA isolation

Ggenomic DNA was isolated using a modified protocol adapted from Harju et al. (2004) in which 1 ml of culture was centrifuged at high speed at room temperature for 5 minutes. To the transformed supernatant, 200 µl lyses buffer (2 % Triton X-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) was added. Tubes were placed at -80 ˚C until it was completely frozen then immersed in a 95 ˚C water bath for 1 minute to thaw quickly. This process was repeated twice. After thawing the tubes were placed in a vortex for 30

seconds. Two hundred microliters of chloroform were added to the tubes and centrifuged for 5 minutes at high speed. The aqueous layer was transferred to 400 µl ice-cold pure ethanol. Samples were allowed to precipitate at room temperature for 5 minutes and then centrifuged at high speed at room temperature for 5 minutes. The supernatant was discarded and the pellet washed with 0.5 ml of 70 % ethanol followed by drying using vacuum dryer at room temperature for 5 minutes. DNA was dissolved in 50 ml sterile distilled water followed by DNA gel electrophoresis for determining the quality.

6.3.12.2 PCR screening of recombinant GS115 cells from the isolated genomic DNA

PCR amplification was performed with 100 to 150 ng of the genomic DNA isolated from the colonies using a hot-start PCR protocol. Cell lysis was done with an initial denaturation at 95 ˚C for 5 minutes then 80 ˚C for 30 seconds during which *taq* added followed by 30 cycles of denaturation at 94 ˚C (30 seconds), annealing at 54 ˚C (30 seconds) and extension at 72 ˚C (2 minutes) ending in a final extension at 72 ˚C (10 minutes). PCR products were analysed by DNA gel electrophoresis as described previously. The glycerol stock solution was prepared for 5 positive cells by adding 300 µl 50% glycerol to 700 µl cells. Stocks were stored at -70 ˚C. Two of them plus one native plasmid (pPICZαC as negative control) and GS115 Mut^{s} almumin (a secretion control which express secreted albumin) were inoculated into 50 ml of buffered glycerol-complex medium BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, 4 x 10-5 biotin, 1% Glycerol) and incubated overnight with vigorous shaking (300- 400 rpm) at 30 ˚C until the cultures reached an OD_{600} of at least 2. Glycerol stocks of selected positive colonies were prepared by adding 300 µl 50% glycerol to 700 µl and the stocks were stored at -70 ˚C.

6.3.13 Optimization of the conditions for the expression of C4H in the GS115 cells

Expression of C4H was performed by inoculating 50 ml of BMGY culture into a 1000 ml flask containing 100 ml BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% YNB, 4 x 10-5 biotin, 0.5% methanol). Cultures were incubated at 30 ˚C on the shaker at 300-400 rpm for 3 days. Induction was maintained continuously by adding 100 % methanol to the cultures to a final concentration of 0.5 % every 24 h. To find out the optimum time of expression, 1 ml sample was drawn every 24 h followed by centrifugation at 1300 rpm for 5 minutes and the supernatant stored at -70 ˚C for subsequent SDS-PAGE- and Western blot analysis. To avoid any protein degradation a mixture of protease inhibitors, including phenylmethylsulphonyl fluoride (PMSF) and EDTA at a final concentration of 10 µg / ml and 1 mM respectively was added to each sample prior to storage.

6.3.14 Sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) analysis

The secreted expression of C4H in the medium was analysed by running samples on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). SDS-PAGE gel containing a 4% stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS) and a 12% separating gel (375 mM Tris-HCl, pH 8.8, 0.1% SDS) was prepared from an acrylamide stock solution (30% Acrylamide, 0.8 % N'N'- Bis-methylene-acrylamide). The gel solutions were degassed prior to polymerisation by the addition of 50 µl freshly prepared 10% ammonium persulphate and 5 µl TEMED (N,N,N',N'-tetramethyl-ethylenediamine). The collected supernatants were diluted 1:1 in SDS sample buffer (60 mM Tris-HCl, 2% SDS (w / v), 0.1% glycerol (v / v), 0.05% b-mercaptoethanol (v / v) and 0.025% bromophenol blue, pH 6.8) and boiled at 95 °C (5 minutes). Electrophoresis was performed in a running buffer (0.02 M Tris-HCl, 0.1 M glycine, 0.06% SDS, pH 8.3) using a Hoeferâ mini VE vertical gel electrophoresis system (Amersham Pharmacia biotech, USA). All gels were run

with a prestained protein ladder PageRuler^{TM} (#0441) (Fermentas, Europe). The initial voltage was 40 V for 45-60 minutes and then with an increased voltage of 100 V for 2 hours.

6.3.15 Silver staining

Protein profiles from the gels were visualized using a modified silver staining method as described by Blum et al. (1987) and Schevchenko et al. (1996).

The silver staining method utilised for the protein visualisation was adapted from Blum et al. (1987) and Schevchenko et al. (1996). Gels were incubated for 30 minutes at room temperature in a fixing solution (50% methanol, 10% acetic acid) on a rocking platform, followed by incubating in a sensitisation solution (0.02% $Na_2S_2O_3.5H_2O$ (w / v) in a 100 ml) for 30 minutes. The gels were then washed three times (for 10 minutes each) in double distilled deionised water before incubating in a silver nitrate solution ($AgNO_3$, 0.2% w / v) for another 30 minutes. To remove the excess of the silver nitrate, the gels were washed shortly with distilled water and gels placed in a developing solution (6% Na_2CO_3 (w / v) in 100ml water) containing 50 µl 37% formaldehyde and 2 ml sensitisation solution until protein bands appeared. The developing reaction was terminated by adding 0.05 M EDTA. For storage, the gels were placed in distilled water.

6.3.16 Western blot analysis of the expressed C4H in *Pichia pastoris*

Western blotting or immunoblotting allows determining, with a specific primary antibody, the relative amounts of the protein present in various samples. For Western blotting the samples are firstly prepared from tissues or cells that are homogenized in a buffer that protects the protein of interest from degradation. Secondly, the sample is separated using SDS-PAGE and transferred to a membrane for detection. The membrane is then incubated with a generic protein (such as a milk protein) to allow blocking of non-specific places on the membrane. A

primary antibody which is able to bind to a specific protein is then added to the solution. Finally, a secondary antibody-enzyme conjugate, which recognizes the primary antibody, is added localized bound to the primary antibody.

In this experiment, the SDS-PAGE gel was equilibrated for 10 minutes in transfer buffer (25mM Tris/192 mM glycine in 15 % methanol, pH 8.2) followed by electroblotting. A Immun-Blot^{TM}-P polyvinylidene flouride (PVDF) transfer membrane (BIO-RAD Laboratories, Inc., USA) was pre-wetted in 100% methanol for 5 minutes and then equilibrated in transfer buffer for 10 minutes. Transfer of proteins from the gel onto the PVDF membrane was carried out in a BioRad Mini protein II transfer apparatus filled with transfer buffer, at 4 ºC and 200 mA (around 40V) for 2 hrs. After transfer, the membrane was blocked by incubating at 4 ºC overnight with gentle agitation in TBS-blocking buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4-7.6) containing 2.5 % skim milk powder, 0.1 % Tween 20 in combination with a 1: 1000 dilution of primary rabbit polyclonal IgG antibody (His-prob, H-15) (Santa Cruz Biotechnology, USA) (Mans *et al.*, 2004). To remove all unbound antibodies, the membranes were washed three times (each time for 15 minutes.) at room temperature in TBS blocking buffer containing 1 % skim milk powder and 0.1 % Tween 20. Subsequently a 1: 2 500 dilution of secondary conjugated goat anti-rabbit lgG-AP (alkaline phosphatise conjugate) (Santa Cruz Biotechnology, USA) was added and incubated for 50 minutes at room temperature. To remove the unbound antibodies, the membrane was washed again three times (each time for 15 minutes) with TBS buffer containing 0.1 % Tween 20 and then visualized.

Detection was done as outlined by the supplier using the Alkaline phosphatase-cojugate substrate kit (BIO-RAD Laboratories, Inc., USA).

The colour development buffer was prepared by 25x dilution of AP colour development buffer by sterile distilled water. It was also supplemented with AP colour reagent A (nitroblue

tetrazolium in aqueous dimethylformamide [DMF], containing magnesium chloride) and immediately before use with the AP colour reagent B (5-bromo-4-chloro-3-indoyl phosphate in DMF) at a total concentration of 1 % was added. The membrane was immersed in the colour development solution and incubated at room temperature with gentle agitation until the colour development was completed. The reaction was terminated by washing the membrane with double distilled water for 10 minutes with gentle agitation. Afterwards the membrane was air dried.

6.3.17 Ammonium sulphate precipitation

Protein precipitation is a useful method of concentrating proteins from a large volume and is therefore ideal as an initial step in purification. Salting out of proteins, particularly by use of ammonium sulphate, is one of the best known and most used methods for purifying and concentrating enzymes. It is also convenient and effective because of its high solubility, cheapness, lack of toxicity to most enzymes and its stabilizing effect on some enzymes. In this part of the study, to precipitate the secreted expressed C4H from the medium, different fractions containing 10, 20, 30, 40 and 50% (w / v) of ammonium sulphate respectively were prepared and the fractions incubating overnight at 4 °C with gently shaking. The proteins were collected by centrifugation at 13 000 rpm at 4 °C for 15 minutes. Pellets were then resuspended in TBS buffer after decanting the supernatant. As the dissolved precipitates contained a lot of ammonium sulphate an overnight dialyzing with stirring was performed with TBS buffer (5 mM Tris and 20 mM NaCl, pH 7.2) using a dialysing tubing-visking membrane (MW company, USA).

6.3.18 Purification of the polyhistidineine-tagged protein

To purify the expressed C4H-tagged protein from dialysed samples, a Protino® Ni prepacked column kit (Macherey-Nagel, Germany) was used according to the manufacturer's instructions. The freeze-dried or precipitated proteins were resuspended in 1x LEW buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0) and applied to a pre-equilibrated Protino® 1000 Ni prepacked column by using ml 1x LEW and which was then allowed to drain by gravity. Washing the column twice with 1x LEW (2 x 2 ml) was carried out before a three times (1.5 ml) elutions of the polyhistidineine-tagged C4H protein by 1x elution buffer (50 mM NaH_2PO_4 and 300 mM NaCl, 250 mM imidazole pH 8.0). The eluted protein was freeze-dried to concentrate it. Finally it was dissolved in TBS buffer and stored at -20.

6.3.19 Protein determination

The protein concentration was determined using the Bradford method in association with the Quick Start™ Bradford Dye Reagent (Bio-Rad Laboratories Inc., CA, USA). Bovine Serum Albumin (BSA) was used as a standard reference protein. Fifty microliters of BSA with serial dilutions starting from 100 µg / ml of the BSA, or isolated protein sample were pipetted into a microtitre plate well and 50 µl dye reagent was added. The plate was incubated for 15 minutes at room temperature before determining the absorbance at 595 nm with a Spectra max (384 plus) multiplate scanner (Molecular Devices, USA). Reactions were performed in triplicate. The protein concentration was determined based on the prepared standard curve for serial dilutions of BSA.

6.4 Results

6.4.1 Total RNA isolation and cDNA synthesis

The total RNA isolated from leaf of *H. aureonitens* (Fig. 6.4-A) was used for single strand cDNA synthesis followed by double strand cDNA synthesis. As shown in Figure 6.4.-B, PCR amplification of the C4H from cDNA represents the expression of C4H in the leaves of *H. aureonitens* at a size of 1518 bp which is similar to the C4H sizes in other plants.

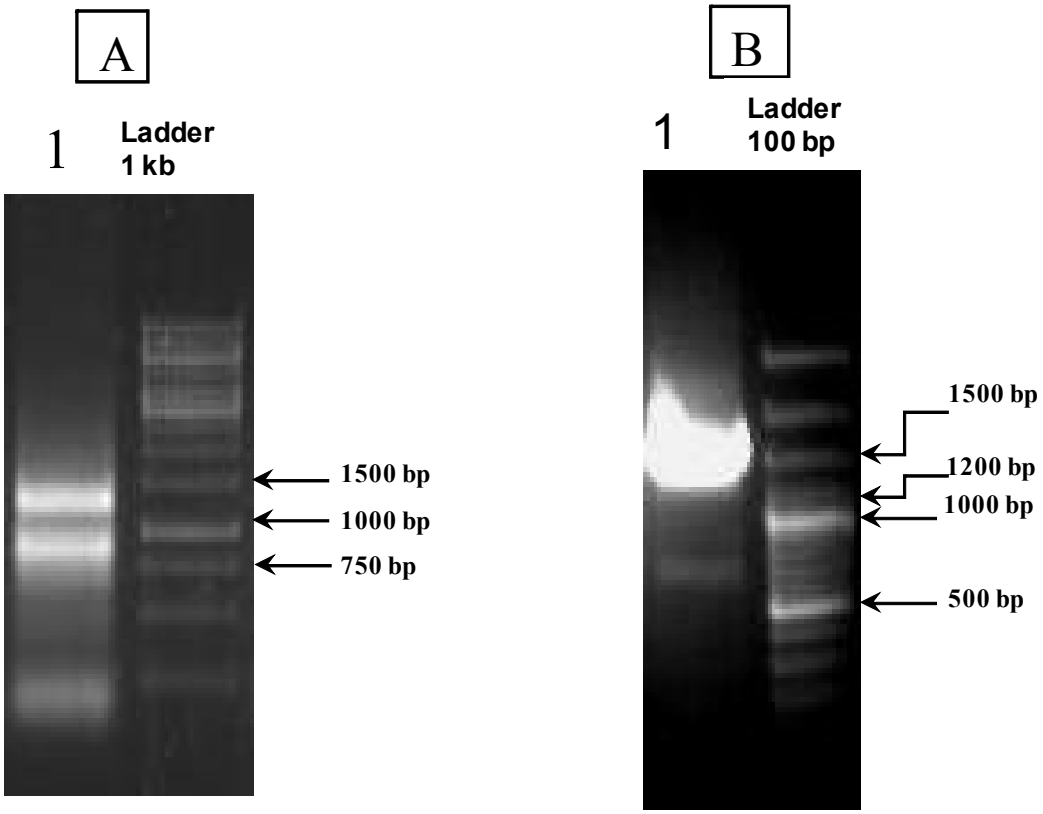


Fig. 6. 4. Total RNA and synthesized cDNA from plants of *Helichrysum aureonitens*. **A:** Total RNA isolation from leaves of greenhouse grown plants of *H. aureonitens.* **B:** PCR product of C4H from cDNA with C4H-FW and C4H-RV primers

6.4.2 Cloning of C4H with restriction sites (*XhoI* and *Sac II*) in a pGEM cloning vector

To clone the PCR amplified fragment, DNA was recovered from the gel and ligated into a pGEM cloning vector (Promega, USR) followed by transformation into the *E. coli* strain JM 109 by heat shock. As shown in Figure 6.5, the cut lane demonstrates that around 1.5 kb DNA insert was successfully ligated into the pGEM plasmid, and removed by digestion with *XhoI* and *SacII* restriction enzymes. The result of the sequencing of the pGEM plasmid carrying the C4H insert also confirmed that the insert was in frame (data not shown). Finally the insert was ligated into the pPICZαC expression vector at a ratio of 3:1 and 5:1 after recovering it from the gel (Fig. 6.5).

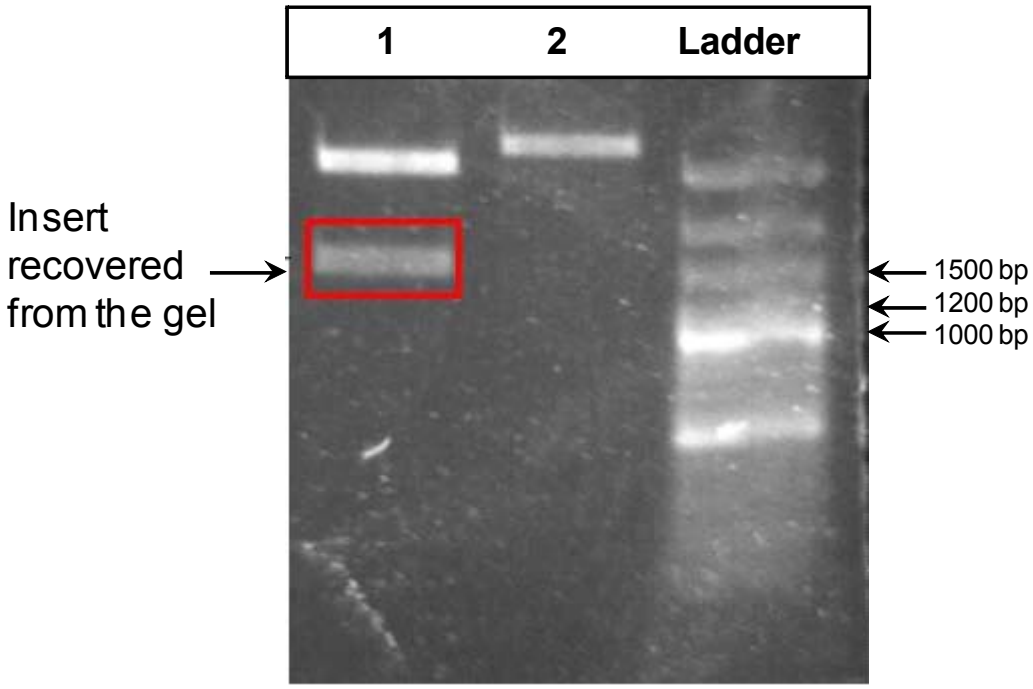


Fig. 6. 5. Double digestion of the pGEM cloning vector with and without the insert (C4H) with *XhoI* and *SacII.* Digested plasmid (lane 1); Native plasmid (lane 2).

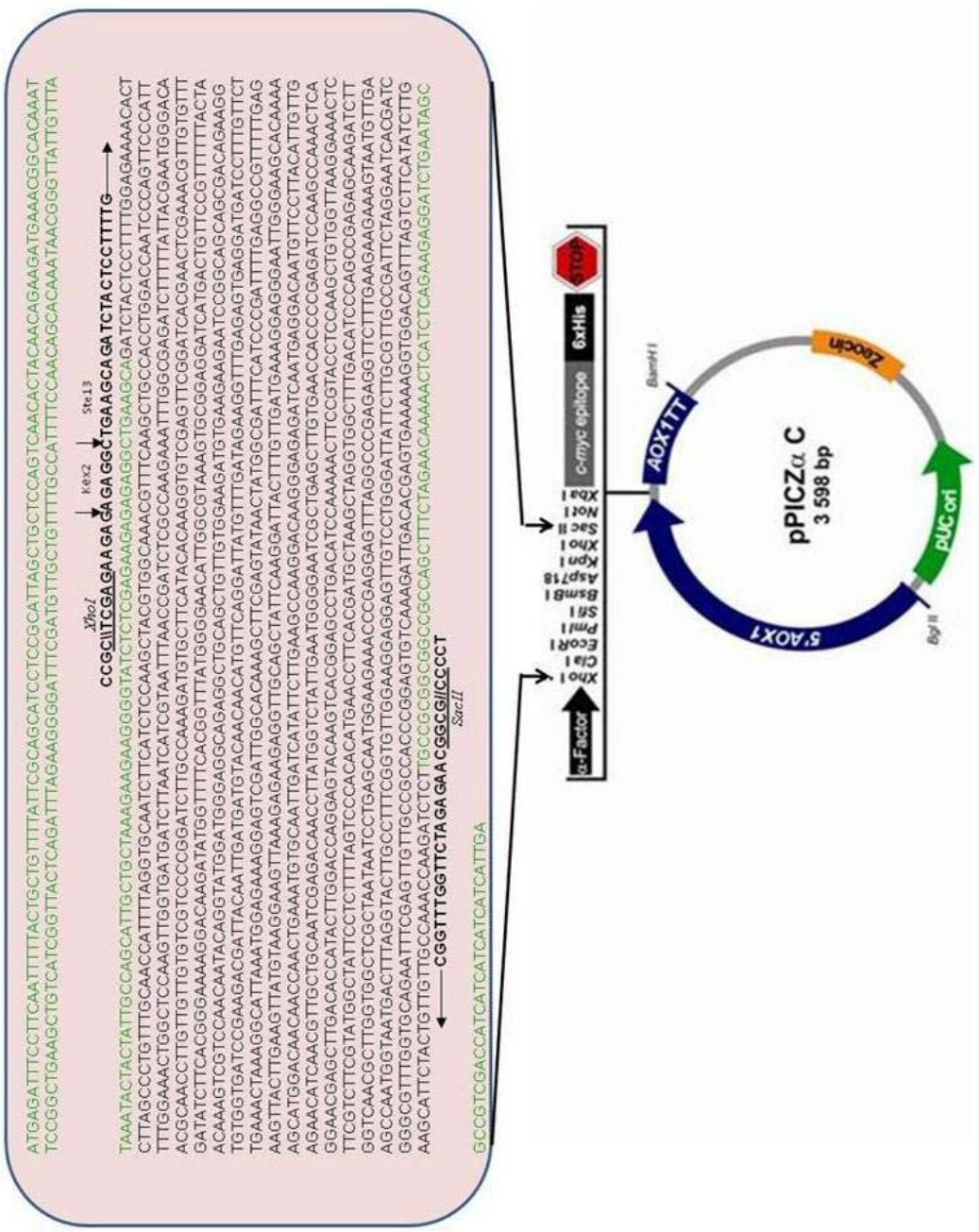


Fig. 6. 6. Drectional cloning strategy of C4H into pPICZαC for expression in *Pichia pastoris*

6.4.3 Transformation of the pPICZαC containing the C4H ORF into *E. coli* TOP 10F'

Following directional ligation, transformation of competent TOP10F' *E. coli* cells by electroporation was performed. PCR screening of clones was done using a hot-start PCR protocol. A number of positive clones (~2 kp band) with low amplification at the lower bands (600bp) were identified for both vector to insert ratios (Fig. 6.7). The lower bands are related to the amplification of the multiple cloning site of the pPICZαC plasmid. As there is a unspecific amplification in positive clones, the isolated plasmid was verified with double digestion (*XhoI* and *SacII* l, Fig. 6.8), automated DNA and sequencing. According to the DNA sequencing of the pPICZαC plasmid, the amino acid sequence of the gene AOX1, includes the α-factor signal, C4H and polyhistidine tag.

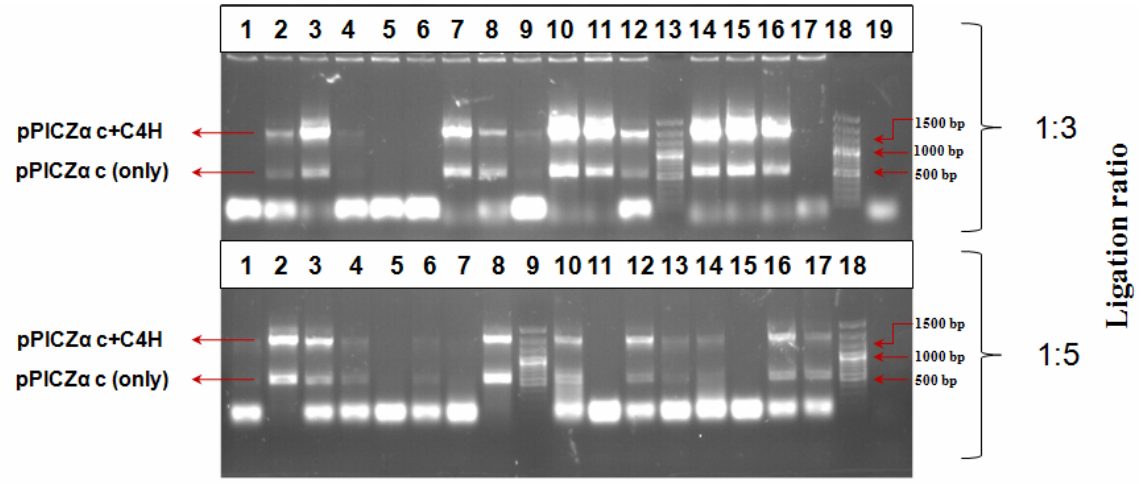


Fig. 6. 7. Colony screening of the *E. coli* strain TOP 10 after transformation by electroporation with AOX1 primers.

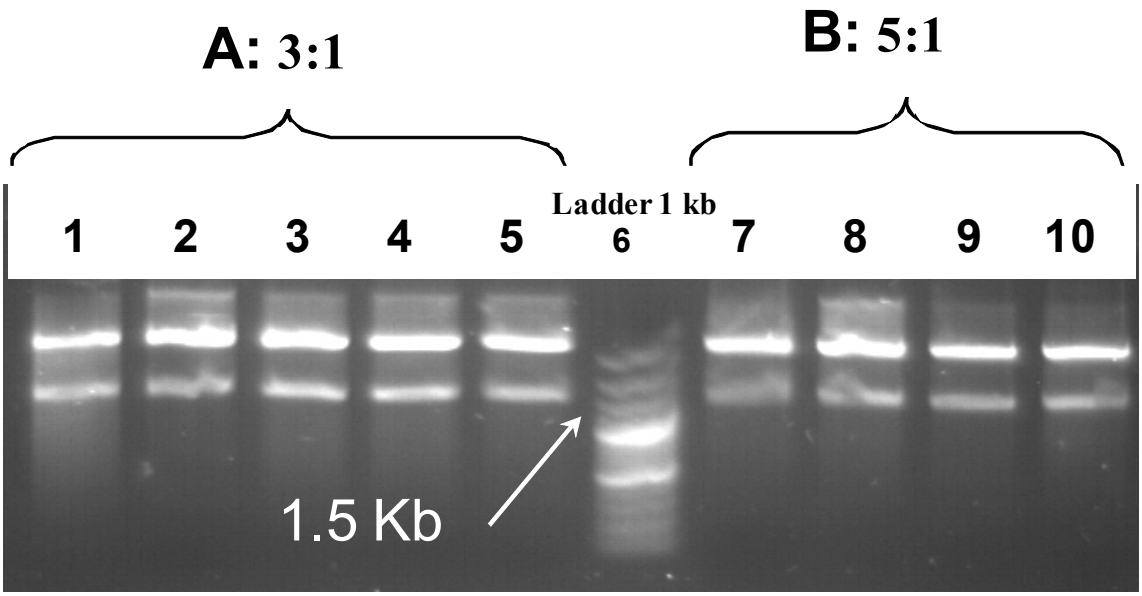


Fig. 6. 8. Double digestion with *XhoI* and *SacII* of the selected plasmids containing C4H. **A:** Ratio of 3:1 (Lane 1-5); **B:** Ratio of 5:1 (Lane 7-10).

Fig. 6. 9. Transformation of pPICZ*α*C into *Pichia pastoris* GS115 cells by electroporation.

Since transformation of the yeast cells with linearized plasmids requires smaller amounts of plasmid, the pPICZαC was digested with *PmeI* restriction enzyme which has no restriction site within the insert. Figure 6.10 indicates that most of the plasmids were digested with the *PmeI* restriction enzyme when compared with the native plasmids. After linearalization, 5-10 µg digested DNA was added to 80 µl of GS115 competent cells and electroporated at 1500 v for 4-5 milliseconds.

After 3 days of growth all the colonies were tested for the presence of C4H in the genome of the yeast cells by the isolation of the genomic DNA. Figure 6.11 shows the isolated genomic DNA from *P. pastoris.* To confirm the existence of the C4H insert in the genome of *P. pastoris*, a PCR amplification was performed with AOX1 primers which amplifies the fragment that is approximately 2.1 kp, and includes the C4H and α-factor fragments with sizes of 1.5 and 0.6 kb respectively. Figure 6.12 confirms the presence of C4H and α-factor in the genome of *P. pastoris,* based on the expected fragment size.

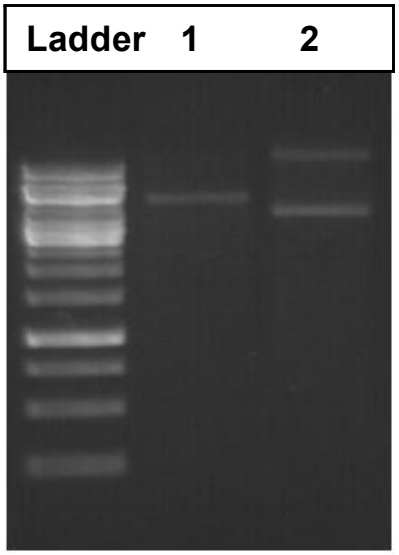


Fig. 6. 10. Linearalization of pPICZα C with *PmeI*. A: Digested plasmid containing C4H (lane 1); B: Undigested plasmid (lane 2).

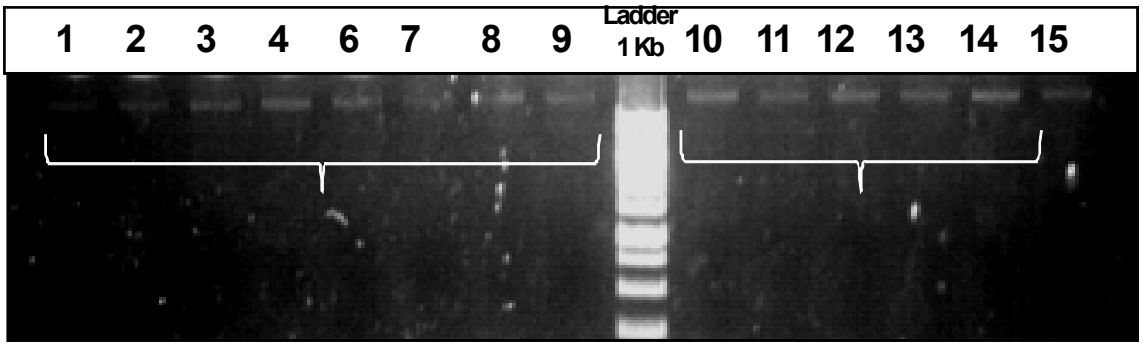


Fig. 6. 11. Genomic DNA isolated from selected colonies of *Pichia pastoris* after transformation with pPICZαC which contains the C4H inserted by electroporation.

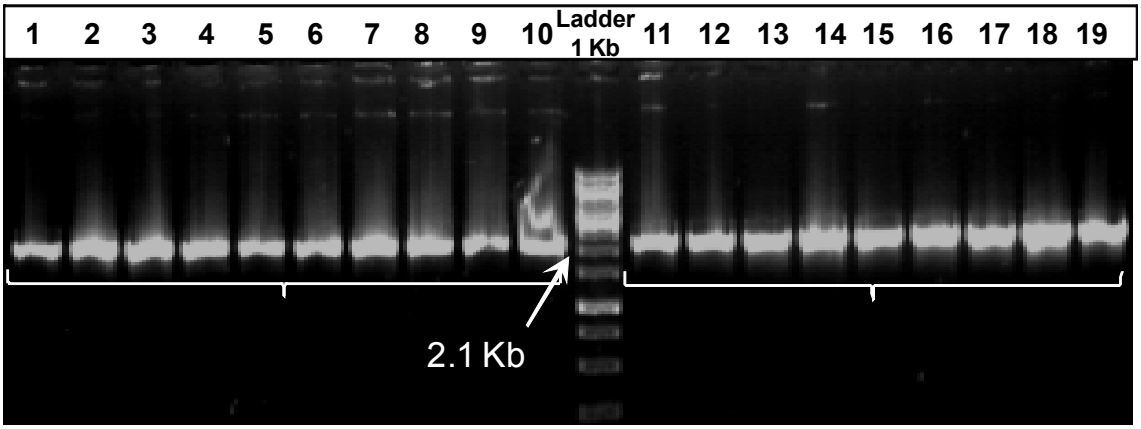


Fig. 6. 12. PCR amplification of the α-factor plus C4H from the genomic DNA of GS115. Arrow shows the size about 2.1 Kb of the PCR amplified fragment of the isolated gDNA of *Pichia pastoris* GS115 cells.

6.4.4 C4H expression in the GS115

To investigate the soluble extracellular expression, a control expression system with the secretion expression of albumin (GS115 / His+ Muts albumin) was included in this study. The albumin expression was achieved with the GS115 secretion control, producing a band at the expected molecular mass of about 67 kDa (Fig. 6.13 -A). Expression of C4H in a secretion pPICZαC plasmids showed a band of a size of approximately 60.5 kDa (Fig. 6.13 -B) which included C4H (58 kDa) and a further 2.5 KDa contributed by the C-terminal histidine and *myc* epitope tags. The result of the Western blot also confirmed the existence of the C4H polyhistidine tagged protein at the expected size on the PVDF membrane (Fig. 6.13 -C).

6.4.5 Precipitation of C4H expressed in the GS115

Figure 6.14-A shows the precipitation of the total protein content of the BMMY medium after 48 hrs of incubation at different concentrations of ammonium sulphate. As shows that C4H protein precipitates over a wide range of ammonium sulphate concentrations (20 – 50 %). Western blot analysis also confirmed that the tagged-protein detected on SDS-PAGE electrophoresis gel was the correct protein (Fig. 6.14-B). Another way to confirm that the expressed protein is the protein of interest is by doing protein sequencing (peptide fingerprinting), but this was not done in this study.

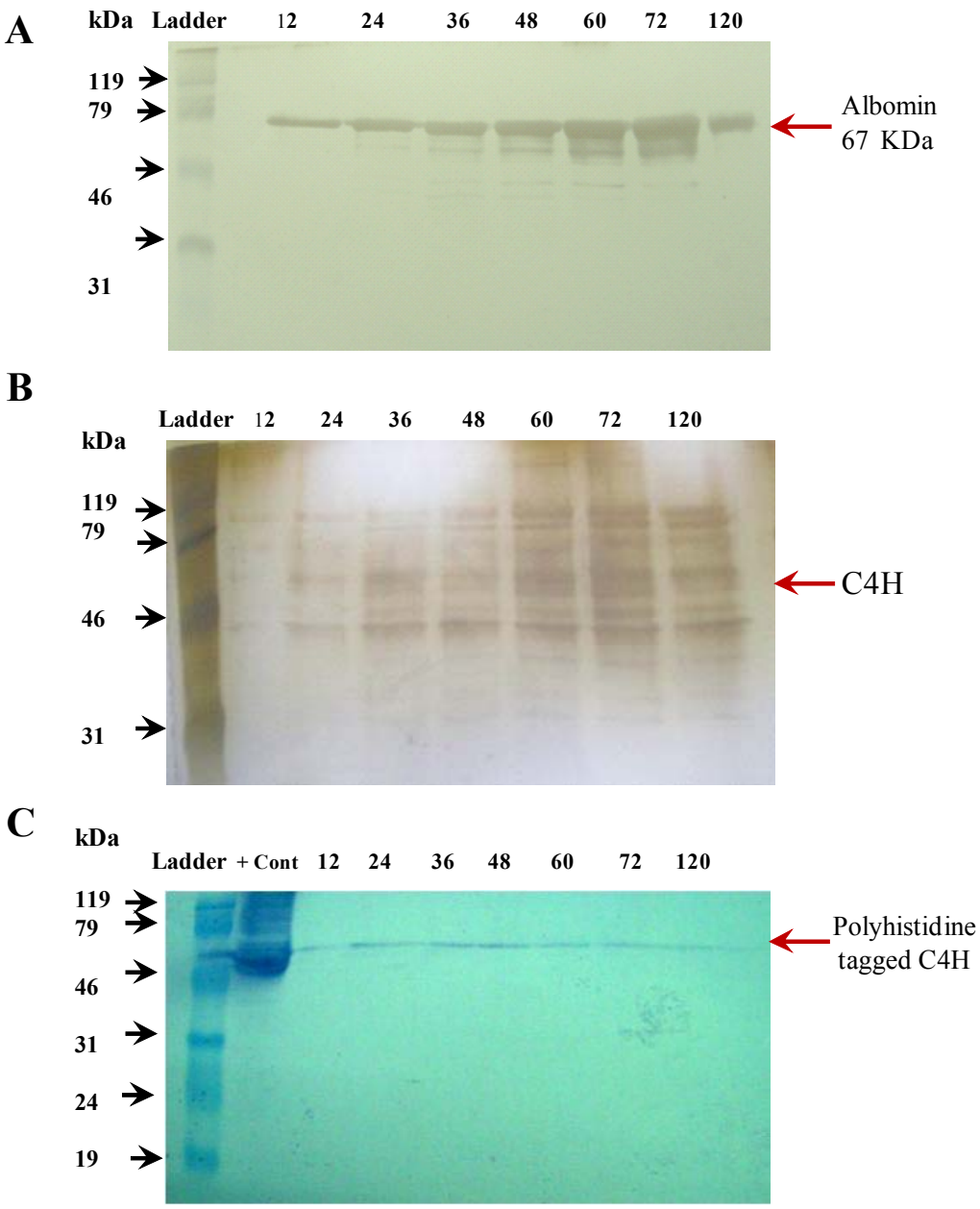


Fig. 6. 13. Expression of albumine and of C4H integrated in a secretion pPICZαC plasmids in *Pichia pastoris* GS115. The cells were harvested in a time course of 12 to 120 hrs. Red arrows show the observed size of the protein of interest. **A**: Silver stained SDS-PAGE electrophoresis of the albumine expression. **B**: Silver stained SDS-PAGE analysis of the C4H expression with a size of about 58 K Da. **C**: Western blot of the polyhistidine tagged C4H expressed in a pPICZαC plasmids in *Pichia pastoris* GS115 cells.

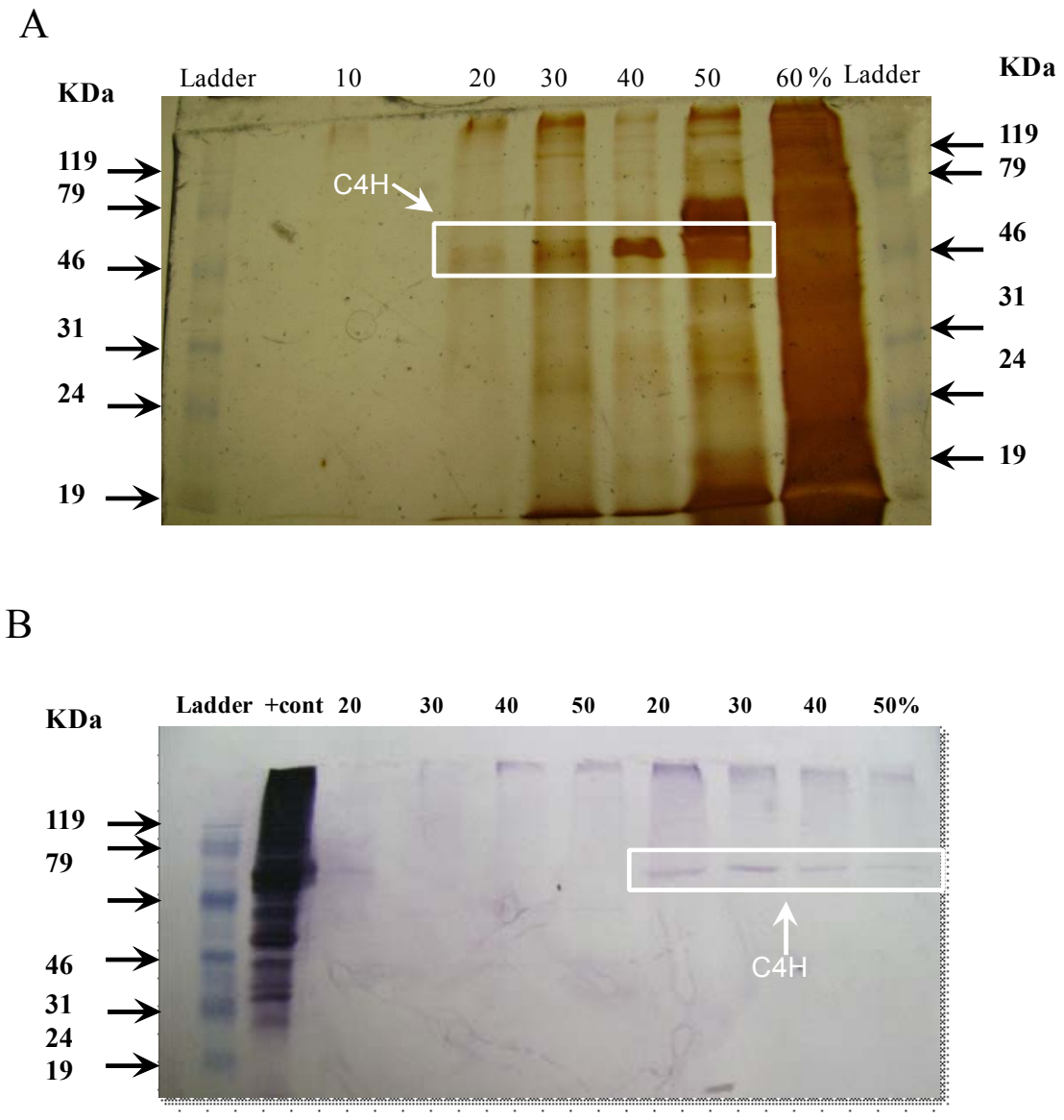


Fig. 6. 14. Ammonium phosphate precipitation of the expressed C4H in *Pichia pastoris* GS115 cells. Boxed bands show the size of the expressed C4H protein. **A**: Silver stained SDS-PAGE electrophoresis analysis of the total precipitated proteins separation from the BMMY medium at different concentrations of ammonium sulphate. **B**: Western blot of the polyhistidine tagged C4H expressed in the pPICZαC plasmids of *Pichia pastoris* GS115 cells which derived derived from two different cell lines.

6.4.6 Histidine-tagged C4H purification and quantification

Histidine tagged C4H was purified from the dialyzed precipitants using the Protino® Ni prepacked columns kit (Figure 6.15). This result also confirmed the previous results of the expression, precipitation of C4H with regards to size. It also showed that C4H can precipitate in different concentrations of ammonium sulphate and that concentrations of 30 to 40 % are the best concentrations to collect the C4H.

To have more protein for quantification, purified tagged-C4H of both fractions from the column purification were mixed together, freeze dried and suspended in 1 ml sodium phosphate buffer.

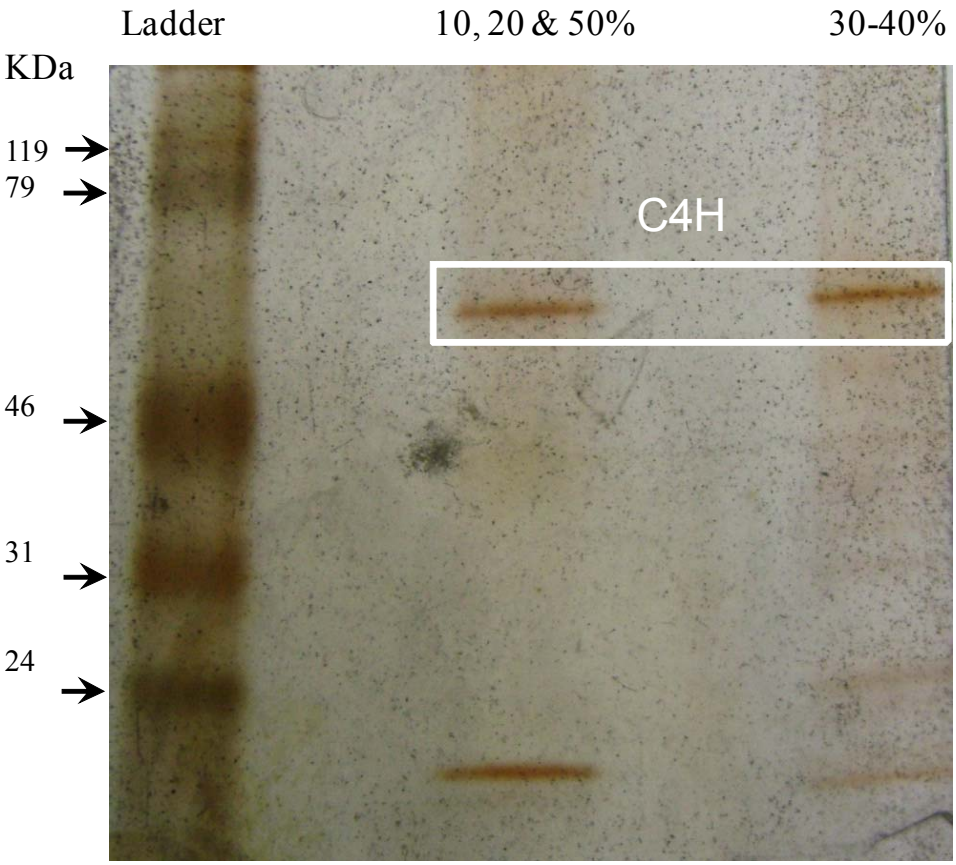


Fig. 6. 15. Silver stained SDS-PAGE analysis of the purified tagged-C4H through the column. The dialyzed precipitants of C4H from the different concentrations of ammonium sulphate were combined together and were purified the column. Lane 3: 10, 20 and 50% together; Lane 5: 30 – 40 % ammonium sulphate.

6.5 Discussion

According to the construct made for the secretory expression (pPICZαC) of C4H in *P. pastoris*, the expressed protein should contain 622 amino acids. The protein should also include the α-factor (signal sequence), C4H and the polyhistidine tag (Fig. 6.3) with a calculated mass of 70.221 kDa. In a secretion expression system the expressed protein would be cleaved in two steps at the signal sequence by the KEX2 and STE13 gene products respectively. This indicates that after cleavage only the C4H and polyhistidine tag, which consists of 533 amino acids with a calculated mass of 60.900 kDa should remain. This protein would be secreted into the growth medium. The calculated mass for the C4H protein excluding the polyhistine tag is approximately 57.590 kDa. As the expected size of most eukaryotic P450 proteins is 50 to 60 kDa (Sakaguchi et al., 1984), this confirms the results of this study. Another method to show that the C4H protein was expressed properly is the Western blot. In the Western blot a primary antibody which is able to bind to its specific protein on the membrane, is added to the solution. This is followed by adding a secondary antibody-enzyme conjugate, which recognizes the primary antibody by finding the locations where the primary antibody is bound and allows for the detection of the fusion protein. In this experiment only the C4H was expressed as polyhistidine tagged protein. The result of Western blot confirms the presence of the expressed C4H tagged-protein.

According to the size of the expressed protein, which was secreted into the medium and developed on a SDS-PAGE gel as well as the detection on the Western blot membrane, it can be concluded that C4H had been successfully expressed in the *P. pastoris* cells.

6.6 Conclusions

In this study *P. pastoris* was used for the first time for the transformation and expression of the C4H enzyme. It was also the first time that a secretion expression system has been used for this enzyme. According to the results of this part of the study, the secretion system was found to be a suitable system for the expression of soluble recombinant His-tagged C4H. Other advantages identified with respect to the system include:

1. Soluble expression of C4H in the media. This is essential since it produces large quantities of enzyme for downstream functional assays.
2. Expression of Histidine-tagged C4H resulted in fast and convenient purification of the recombinant C4H protein.
3. Since C4H protein is secreted into the medium during expression, it was isolated from a less complex media and therefore less contaminated with other proteins.

Chapter 7

General discussion and perspectives

7. 1 Introduction

During the twentieth century, the emphasis gradually shifted from isolating plant natural compounds to synthesized compounds or their chemical analogues. However, the natural products were widely considered as templates for structure optimization programs designed to make perfect new drugs (Raskin et al., 2002). Although the synthetic chemical drugs have a significant market share, the contribution of plants to disease treatment and prevention is still enormous. This is due to the fact that herbal medicine is natural and they are therefore generally considered to be safe for humans. In the twenty-first century attention shifted back to botanical pharmaceuticals as sources of human health products. The relationship between plants and human health is not limited only to medicine. Medicinal plant-derived products now have a considerable share in the market. This includes phytopharmaceuticals, herbal remedies, dietary supplements, homeopathic remedies, medicinal and herbal teas, spirits, aromas and essences, perfumes, cosmetics, colouring agents, varnishes, and detergents. As a consequence, there is an enormous demand for plant products resulting in a huge trade on the local, regional, national and international level for domestic use and commercial trade. This raises the question of where the plant material is obtained from. Unfortunately most of the raw materials are collected from the wild. In China 60 % of the quantities in trade are wild-collected (He and Sheng, 1997). Uncontrolled harvesting from the wild has resulted in near extinction of some medicinal plant species.

There are some methods to meet the increasing demand for natural product without threatening the medicinal plant species. These methods are cultivation in controlled growth systems or application of biotechnology techniques. Biotechnology in different ways can help to increase the production of the desired natural products or in some cases, induction of new compounds. This will also help to preserve the population of wild medicinal plants. The different techniques are micropropagation through tissue and cell suspension culture and

somaclonal variation, or molecular biology through molecular markers, genetic engineering or hairy root induction.

7.2 Cell suspension cultures of *Helichrysum aureonitens*

In this study cell suspension cultures were initially used to attempt to increase the amount of galangin in *Helichrysum aureonitens*. Although galangin was not detected in the cells of *H. aureonitens*, another compound was detected in considerable amounts. This compound was isolated and purified and was identified as a novel chlorophenol compound, 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol, which had not been isolated from any species before. The chemical analysis of *H. aureonitens* cell culture samples also indicated that the selected intermediates based on the 4'-OH flavonoid biosynthetic pathway are not present in the *H. aureonitens* cell samples. These results indicated that the flavonoid biosynthetic pathway is not functional in the cells of the *H. aureonitens* suspension cultures. However the acetylene biosynthetic pathway is active and produces a triyne in the cells of *H. aureonitens* suspension cultures. Hence it can be declared that the results of this part of the study support our first hypothesis of the induction of a new compound by growing the cells of *H. aureonitens* suspension culture in dark conditions as has been hypothesised. The crude extract and pure triyne were tested for bioactivity against *Mycobacterium tuberculosis* (TB) H37Rv as well as evaluation for their cytotoxicity against monkey kidney Vero (Vero cells) and human prostate epithelial carcinoma (DU145) cell lines *in vitro*. *H. aureonitens* cell suspension culture crude extracts showed that the MIC and MBC against *M. tuberculosis* to bet 1.0 mg/ml and 2.0 mg/ml respectively. The triyne was not active at the highest concentration.

7.3 Characterization of flavonoid biosynthesis in *Helichrysum aureonitens*

Based on the results obtained from the chemical analysis of the cells of *H. aureonitens* suspension cultures, the same chemical analysis was carried out on the leaves to analyse the selected intermediates based on the 4'-OH flavonoid biosynthetic pathway, including cinnamic acid, p-coumaric acid, naringenin, galangin and kaempferol. The results showed that *p*-coumaric acid and naringenin are not present in the *H. aureonitens* leaf samples. Some other compounds were detected which were similar to the intermediates in the 4'-OH flavonoid pathway, except that they were not hydroxylated at the C-4' position on the B ring. These intermediates might belong to the non 4'-OH flavonoid pathway which originates from cinnamic acid. All the enzymes in these two pathways are similar and have the same function except cinnamate 4-hydroxilase (C4H), which is the only enzyme that specifically catalysis the hydroxylation at the C-4' position. In the non 4'-OH flavonoid pathway C4H is not involved. Based on these results it was concluded that galangin may be produced through the non 4'-OH pathway for flavonoids in *H. aureonitens.* Although most parts of this alternative pathway has been proven in *E. coli* by construction of a cluster gene, there is still no clear evidence to show that this pathway is also active for the production of galangin in plants. It has been proven that hydroxycinnamoyl:CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) can accept the intermediates without OH- groups at the C-4' position on the B ring. It is, however, not known whether the last two enzymes involved in galangin production, flavonone 3β-hydroxylase (F3'H) and flavonol synthase (FLS), can accept pinocembrin and pinobanksin respectively as a substrate to synthesize galangin.

Kaempferol, the C-4' hydroxylated derivative of galangin was present in the *H. aureonitens* leaf samples. Although these obtained results support our second hypothesis in which the production of kaempferol has been assumed not to be from the 4'-OH pathway, our evidence is not suffecient to state that kaempferol is directly synthesized from galangin by activation of

C4H. To confirm this it is therefore necessary to set up an *in vitro* reaction consisting of galangin as a substrate, purified C4H protein and the necessary biochemical factors to see whether C4H is able to convert galangin to kaempferol or not?

The results of this study also supported previous findings that lignin can be induced even if there is no *p*-coumaric acid present. Although the results clearly showed the presence of an alternative pathway, still more research is needed.

7.4 Characterization of cinnamate 4-hydroxylase in *Helichrysum aureonitens*

Cinnamate 4-hydroxylase is the second enzyme in the phenylpropanoid biosynthetic pathway. Since this pathway is involved in lignin production, it is very important for plants. According to results in this study, C4H was isolated from *H. aureonitens* plant samples and its characterization at the level of nucleic acids and amino acids was performed. The results demonstrated that the isolated C4H gene in *H. aureonitens* plants contains 1518-base pairs (including start and stop codon, ATG and TAA respectively) and an open reading frame encoding a 506-amino-acid polypeptide. It also showed the highest homologies to the species from the Asteraceae.

To make sure that the isolated C4H from *H. aureonitens* plants is a putative gene and can produce a protein, it was expressed in a secretion expression system in *Pichia pastoris* cells. The results of SDS-PAGE electrophoresis and Western blot showed that the expressed protein in *P. pastoris* cells has the same size as mentioned in literature. It was also found that the secretary expression in *P. pastoris* cells is an easy purification system for expression of soluble recombinant C4H.

7.5 Future work

Based on the results of this study, the following experiments can be considered in future work:

1. As the triyne (polyacetylene) was found to be a novel compound, there is a little information about its medicinal properties apart from that reported in this study. More bioactivity assays for this compound are recommended as a wide range of medicinal activity has been reported for polyacetylene compounds including antitumor, antibacterial, antimicrobial, antifouling, antifungal, pesticidal, phototoxic, HIV-inhibitory and immunosuppressive properties..

2. Since most medicinal plants are collected from the wild and there is some genetic variation between the collected plants, it is advisable to investigate the biosynthetic pathway of flavonoids in different species of *Helichrysum* and the results should be compared with that obtained in *H. aureonitens.*

3. It should be determined if *H. aureonitens*'s C4H enzyme can hydroxylate galangin to form kaempferol. It is also recommended to consider the other intermediates from the non 4'-OH pathway as substrates for C4H. Because it is also possible that the non 4'-OH biosynthetic pathway is not active in *H. aureonitens* and instead some non 4'-OH intermediates can be converted to the their hydroxylated forms in the 4'-OH pathway such as pinobanksin or pinocembrin by C4H to produce kaempferol.

4. C4H does not use NADPH directly but instead interact with a flavoprotein known as a P450 reductase (CPR) that transfers electrons to the P450 from the nicotinamide cofactor. To do a functional assay, it is recommended to express CPR in yeast and

then the *H. aureonitens*'s purified C4H from this study in association with substrates (galangin) that can be added to the culture.

5. Investigation on the lignin pathway in *H. aureonitens*, and the presence of an alternative pathway for lignin production.. According to the literature the main substrate for lignin synthesis is *p*-coumaric acid while in *H. aureonitens p*-coumaric acid was not detectable. *H. aureonitens* therefore provides a good opportunity to address the possibility of lignin synthesis without using *p*-coumaric acid.

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