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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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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

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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