In vitro production of phytoalexins by *Helichrysum kraussii*

by

Gerhard Prinsloo

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Supervisor: Prof. J.J.M. Meyer

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Introduction and background

Introduction

1.1 General introduction and background

It is estimated that there are about 250 000 species of higher plants on earth. The interaction between plants and their pathogens is complex and may be very specific to a given plant. The defence strategies of plants against their pathogens are manifold and include the use of antimicrobial compounds (Graver & Kokubun, 2001).

The genus *Helichrysum* Mill. (Asteraceae), has been found to possess many active compounds with activity against several pathogens. Plants of this genus have been used traditionally as a cure for many different diseases and illnesses. The manner in which plants protect themselves, and the mechanisms that they have developed is complex, and more is learned of their adaptations every day.

Plants, being immobile, posses many different defence mechanisms to protect themselves against pathogens, insects, herbivore attacks and certain stress situations. Plants use the hypersensitive response (HR) or the systemic acquired resistance (SAR) response to protect themselves. These responses are responsible for the production or synthesis of additional compounds not part of the normal metabolism that act as an antimicrobial defence mechanism (Van Loon, 1997).

The HR involves programmed cell death, by producing active oxygen species (AOS) that will destroy the cells around the site of infection. The plant destroys a few of its own cells, thereby preventing the pathogen from invading the rest of the plant. This will lead to the recognition of certain elicitors and eventually a cascade of reactions follow to produce the resistance products. The plant does not only protect itself around the site of infection, but also systemically (SAR). This involves the production or use of a signal transduction mechanism that leads to the production of resistance products throughout the whole plant. The plant will therefore be resistant, not only around the site of infection, but also throughout the whole plant. This helps to prevent secondary infections (Graver & Kokubun, 2001).

Micro-organisms, wounding, stress, ultraviolet light and freezing, are just a few examples of reactions that could lead to the production of elicitors. These elicitors are recognised by the plant, by means of an avirulence resistance ligand, that forms after recognition. The plant produces a resistance gene product that binds to the avirulence gene product of the invader, and this leads to the activation of the resistance system of the plant (Taiz & Zeiger, 1998). A cascade of reactions follows the recognition of the elicitors produced by the invader. This involves a lot of reactions to synthesise the resistance products. Compounds like salicylic acid, ethylene and jasmonic acid, are well-known compounds responsible for signal transduction as part of the cascade. These important messengers are also produced upon recognition, but are not necessarily the end product; and are most of the time, just an important link in the production of resistance compounds by the plant (Daniel & Purkayasta, 1995).

The aim of the recognition system is to produce resistance products that will be effective in protecting the plant against attack or stress situations, to ensure survival. Compounds like proteinase inhibitors, glucanases and phytoalexins are produced to protect the plant, and are part of the HR and SAR responses (Dixon & Harrison, 1994).

Phytoalexins is a well-known group of antimicrobial compounds in plants, and form a very important part of the resistance mechanism. These small molecular mass compounds are produced whenever they encounter certain stress situations, for instance pathogen attacks. It seems as if these compounds are not part of normal plant metabolism, and are quickly produced only when the plant needs resistance to ensure survival (Taiz & Zeiger, 1998).

The aim of the study was to determine whether, certain elicitors are responsible for the *in vitro* production of specific phytoalexins in *H. kraussii*; and also to determine the antimicrobial effect of the synthesised compounds.

The study could not be performed on plants harvested from the field, because of the continuous risk of pathogen and insect attack on the plant. Tissue cultures were therefore produced to create a stable and controlled environment. Cell suspension cultures were produced from the tissue cultures, to enhance the uptake of jasmonic acid. Jasmonic acid, being a potent activator and part of the signal transduction system in many plants, was used to elicit the resistance system, in order to produce phytoalexins (Figure 1.1).

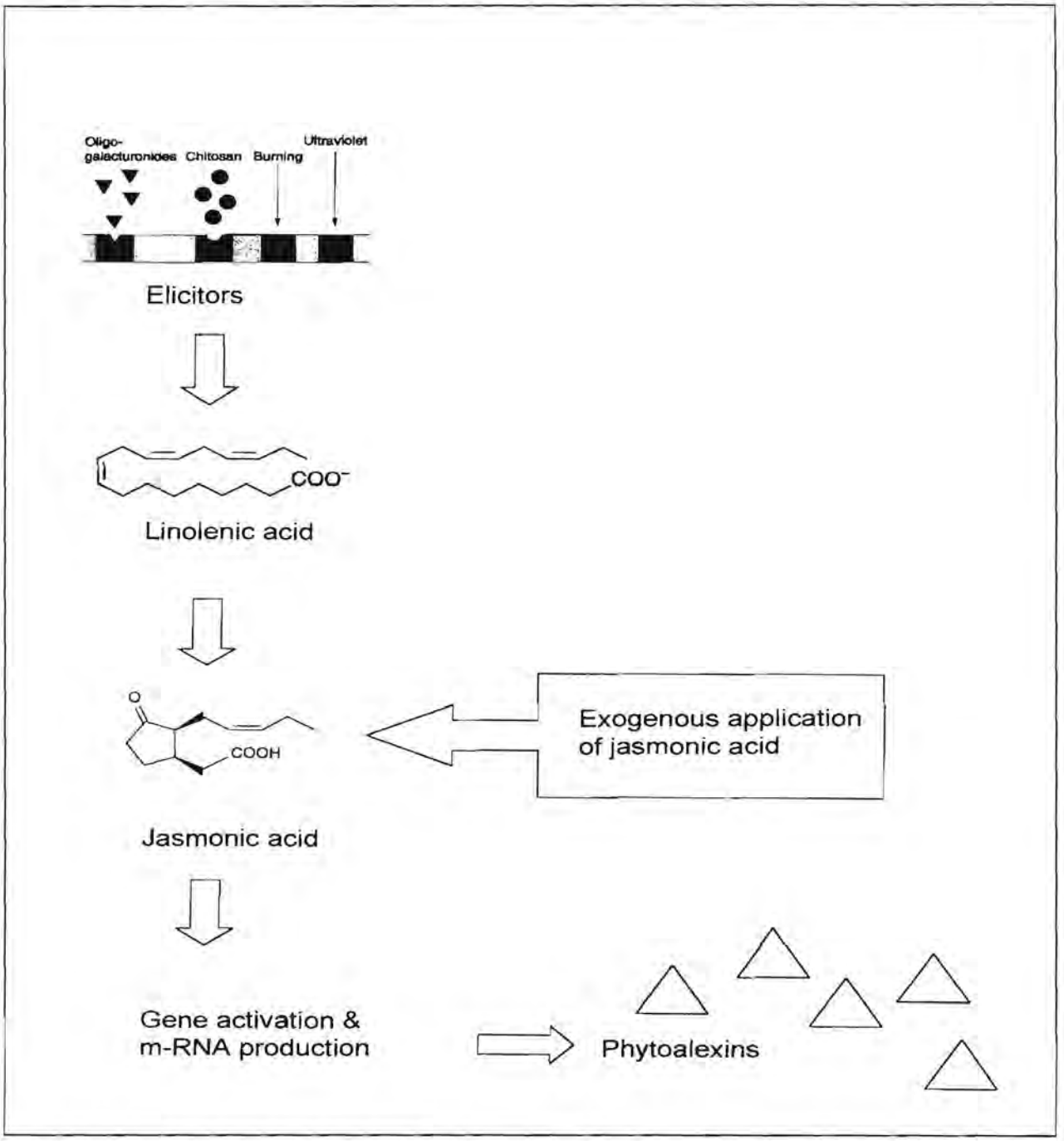

Figure 1.1: Activation of the resistance system by exogenous application of jasmonic acid to the plant material.

It was important to determine if the phytoalexins produced in cell culture, were present in the plant extract from plants collected from the field. It would have been easier to obtain these compounds form the plants, which grows abundantly around Pretoria, than to

extract them from plant material, produced by cell suspension cultures. The phytoalexins were however not found in the plants, but several other compounds were isolated and identified, of which some exhibit potent antibacterial activity.

The dissertation comprises of nine chapters. The genus *Helichrysum* and specifically the species *H. kraussii* that this study has focussed on is described in chapter one. This section focuses on the medicinal value and potential of these plants. The second chapter discusses the general uses of tissue- and cell suspension cultures. The chapter also mentions some of the advantages and disadvantages, as well as the application of these techniques in this study. The third chapter gives a broad discussion on phytoalexins. The chapter gives background information on phytoalexins, and a few examples of phytoalexins isolated. The rest of the chapter discusses the process in activating the resistance system in *H. kraussii*, and the steps followed to isolate the phytoalexins synthesised. A description and discussion follows on the isolation and identification of these compounds. Chapter four deals with some of the potent antibacterial compounds present in the plants obtained from the field and are followed by a discussion on the isolation and identification of these compounds. The general discussion and conclusions follow in chapter five. Problems, and future prospects are discussed in this chapter. The summaries are given in chapters six (English) and seven (Afrikaans), and the acknowledgements and references are given in chapters eight and nine.

1.2 The genus *Helichrysum* Mill.

The genus *Helichrysum* is part of the Asteraceae family, and was already described in 1691–1771 by Philip Miller. There are about 500 *Helichrysum* species all over the world, of which 245 occur in South Africa. The description of this genus is extremely broad because of its numerous members and the wide variation between the members. These plants may be annual, biennial or perennial herbs, suffrutices or shrubs. They are generally erect, sometimes prostate and often glandular. The leaves are variously shaped, and the flowers may vary from 2–2500 (Hilliard, 1983).

Many different uses for *Helichrysum* species have been reported. The South African *Helichrysum* species are used medicinally for a range of conditions that include wound dressing to guard against infections (Van Wyk *et al.*, 1997). *Helichrysum* species have also been used for common diseases like flu, infections and as a treatment for a lot of conditions such as pain. The medicinal uses of some of the interesting species investigated by other authors are given below:

H. callicomum Harv., has been used as a leaf infusion for colds, body pain and as an ingredient in an enema for colic by the Basothos in the QwaQwa district (Watt & Breyer-Brandwijk, 1962).

H. odoratissimum (L.) Sweet., has been used for the relief of abdominal pains, heartburn, coughs, colds, and to treat female sterility, eczema and wounds (Van Puyevelde *et al.*, 1989).

H. glomeratum Klatt, has been used as a leaf infusion that was administered to children with stomach aches, to treat burns, and as an ingredient in a colic remedy by the Basothos (Watt & Breyer-Brandwijk, 1962).

H. rugulosum Less., has been used as a root decoction, and were used to treat children suffering from diarrhoea, and to fumigate huts. It is similarly used as an ingredient in an enema for colic (Watt & Breyer-Brandwijk, 1962).

H. hypoleucum Harv., has been used as a tea as an emetic, and for chest problems by the Basothos. It has also been used as a poultice for swelling and as an antibiotic (Hutchings & Van Staden, 1994).

H. pilosellum (L.f.) Less., has been used as a leaf infusion for stomach ache, coughs, and colds and is also used as an ingredient in colic remedies by the Basothos (Hutchings & Van Staden, 1994).

1.3 *Helichrysum kraussii* Sch. Bip.:

H. kraussii is a twiggy perennial shrublet with small yellow flowers. It is up to 1 m high, much branched, densely covered with leaves and very aromatic with a strong curry-like smell. It is found in grassland and bushveld, usually in dense stands, particularly on the summits of rocky ridges (Van Wyk & Malan, 1998). It has a South African distribution (Figure 1.2) predominantly in Mpumalanga, Northern Province, Gauteng and also coastal KwaZulu-Natal (Hilliard, 1983). Medicinally, *H. kraussii*, has been smoked in a pipe for cough relief and as a remedy for pulmonary tuberculosis (Watt & Breyer-Brandwijk, 1962).

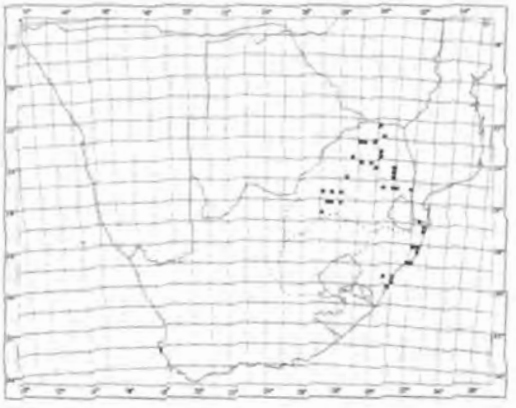

Figure 1.2: Distribution map of *H. kraussii* in Southern Africa.

Chapter 2

Initiation of tissue- and cell suspension cultures of *H. kraussii*

Chapter 2

Initiation of tissue- and cell suspension cultures of *H. kraussii*

2.1 Introduction

The technique of tissue culturing involves the growth of plants or plant material on a growth medium. The environment is aseptic and regulated to support maximum growth of the culture. The plant material is "synthetically" grown on the medium to produce plants, plant material or plant products by manipulating one or more of the natural plant processes.

Tissue- and cell suspension cultures are a convenient way of designing and executing controlled experiments and can support research in various ways. Tissue cultures are especially helpful in the vegetative production of certain plants. All the material obtained from the cultures will be a clone of the original material. Thousands of plants could be produced by using only a small piece of plant material such as a leaf or a shoot. This method is also very helpful in propagating pure lines, eliminating the fertilisation process and mixing of genetic material (Stafford & Warren, 1991).

2.1.1 Tissue cultures

Tissue cultures can be used very effectively in research programs, to maintain a stable line of a certain species or plant. In this study the tissue cultures were used to propagate a lot of the plant material in the laboratory. Plant material was therefore available throughout the year, and the availability of fresh material from the field in the winter season was not a problem. Tissue cultures are also very useful when they are used to produce cell suspension cultures, which have some added benefits of their own.

Initiation of the cultures is one of the most important steps in the successful establishment of tissue cultures. Contamination, the medium itself and the external conditions all play a role in the success of this very important first step. Tissue cultures will not grow and divide if all the different factors are not optimal for maintenance and growth of the plant. Once the initiation phase is finished, the tissue culture will maintain itself, and should be strong enough to be subcultured.

Subculturing of the tissue cultures is important for the maintenance of the calli. The growth medium contains all the sugars, water, micro- and macro-elements, vitamins and growth hormones that the calli need for growth and development. The medium serves as a synthetic soil and also supports the plant when growing. If the cultures are left on a medium for too long, the medium will become depleted of nutrients, water and elements. This will inhibit the growth and health of the calli. Subculturing prevents the calli from becoming stressed, which is a major factor in the inhibition of growth of the calli. It is important to work aseptically when the tissue cultures are subcultured. The laminar flow

cabinet is used for subculturing, and the same rules apply as with the initiation of tissue cultures.

Tissue culture is a widely applicable method for maintaining plant material, production and also multiplication of specific individuals. Tissue cultures are widely used because it forms the basis of many other techniques and methods like somatic embryogenesis and gene transfer. The creation of cell suspension cultures from tissue cultures is discussed below.

2.1.2 Cell suspension cultures

Cell suspension cultures involve the cutting of the tissue culture into little pieces, and adding it to a liquid medium. This enhances the growth rate, because the single cells divide much quicker with the medium surrounding every cell. This is however a very stressful situation for the plant material, as the cells in the medium need to be provided with sufficient oxygen. This can be achieved by keeping the cultures on a shaker. This creates stress conditions, and could lead to the inhibition of cell division and growth. Contamination is a much bigger problem in cell suspension cultures than in tissue cultures, because one microbe spore can contaminate the whole cell suspension culture. In the case of tissue cultures, the calli are separated, and contamination can easily be eliminated, if spotted early enough.

Cell suspension cultures are however very useful when certain compounds need to be added to the plant material. Growth regulators and vitamins can be added throughout the

growth process, without changing the whole medium. In this study, it was very effective to use cell suspension cultures, as jasmonic acid could be applied directly to the cell suspension culture medium. Every cell was exposed to this compound, which theoretically should lead to the production of a higher concentration of resistance compounds. In the case of an intact plant, only a certain amount of the compound will be formed, and in a lot of cases the compounds are only formed in a certain part of the plant (Hyvarinen *et al.*, 2000).

One needs to be aware of the fact that the plant material could mutate during one of the stages. This could lead to the propagation of different plant material, and the differences could lead to wrong conclusions about the plant that has been studied. The aseptic conditions of the cultures make it a sophisticated method that can not be used extensively in all applications.

2.2 Materials and methods

Plant material was collected in April 1999 south of Strubenkop, Pretoria, on the experimental farm of the University of Pretoria. Herbarium specimens (G. Prinsloo 2 & 3) have been deposited in the H.G.W.J. Schweickerdt Herbarium (PRU) at the University of Pretoria. Young green plants were collected, and plants with young auxiliary buds were chosen to initiate the tissue cultures.

One litre agar medium was prepared containing half strength Murashige & Skoog, 3% sucrose, 1mg/ml 1-naphthylacetic acid (NAA), 0.05 mg/l kinetin and 0.3 % agar. Kinetin did not dissolve easily in water, and it was necessary to use a concentrated sodiumhydroxide (NaOH) solution to dissolve it. The pH was adjusted to 5.8 before the agar powder was added. The medium with the vitamins and hormones was then autoclaved for 20 minutes, to sterilise it (Dilika & Meyer, 1998).

Growth points and auxiliary buds were dissected. The surface was then decontaminated in 1% sodium hypochlorite (NaClO) for 20–40 seconds, and transferred to sterilised water. The plant material was then washed twice in sterilised water for five minutes each. The plant material was then removed from the water, and cut into smaller pieces to expose the living cells on the inside of the plant. These plant parts were then transferred to sterilised agar medium, and stored in a growth cabinet at 27.5°C, and 16 hours light per 24 hours. Contaminated plant material was frequently removed to ensure aseptic conditions inside the containers.

The same medium that was used for the tissue cultures was also used for the cell suspension cultures but the agar component was omitted. Two or three of the calli pieces were used to produce a cell suspension culture in 75 ml medium in Erlenmeyer flasks. The calli were cut into small pieces, and then transferred to 75 ml of the cell suspension medium. The cell suspension cultures were then stored again in the same growth chamber, but this time on a shaker set to a speed of 160 rpm.

2.3 Results

The first initiation of tissue culture was unsuccessful. The pH was believed to be the problem, as it was not adjusted before the medium was autoclaved. The second time the pH was adjusted to 5.8 and autoclaved afterwards to sterilise the medium. The pH change played an enormous role, because after three weeks, the first callus growth could be observed, and after two months, the first calli were subcultured and divided. The cultures were subcultured every three weeks to prevent nutrient depletion. The calli were initially green in colour, but as it got older, it changed to a dark brown colour (Figure 2.1). After subculturing, some of the calli would change to the original green colour as new tissue was formed. George (1996) noted that even when it is established, calli turned brown and died if left on the medium for too long. The most widely used method for preventing tissue blackening is to subculture explants frequently.

Cell suspension cultures were easily produced from the tissue cultures (Figure 2.2). These cultures also needed regular subculturing, but it was not as predetermined as was the case with the tissue cultures. The cell suspension culture's growth was much more variable than the tissue cultures, and it was only necessary to subculture if the medium was changing colour.

Subculturing was performed by taking the cell suspension cultures from the shaker, and leaving it for a few minutes to settle. The old medium was then decanted from the flask, and 75 ml fresh medium was added to the cells. It was necessary to subdivide the cells from one culture into other cultures, as the cells became too much for a single flask.

Figure 2.1: Tissue cultures in a growth chamber at optimum conditions.

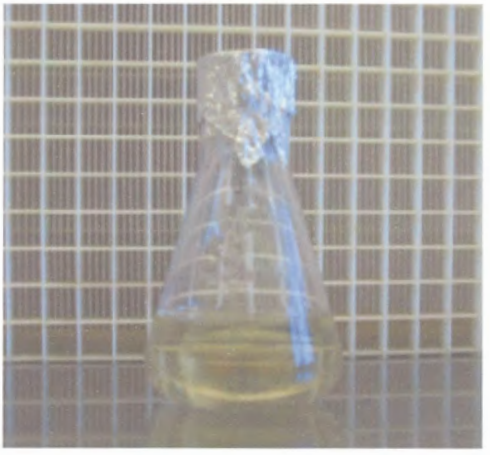

Figure 2.2: A cell suspension culture.

2.4 Discussion

The initiation of tissue- and cell suspension cultures was obtained with relative ease. The pH adjustment however, proved to be a very important step in the success of tissue- and cell suspension cultures. Tissue- and cell suspension cultures need a lot of care and attention, because of the continuous risk of contamination, the risk of power failures and depletion of the medium before subculturing. The growth and success of the cultures depend primarily on the input of the researcher and the continuous care and precautions taken to ensure optimum growth.

It is a very time consuming technique, especially at the initiation phase. Although very effective in the study in terms of availability of plant material, and the ease of adding jasmonic acid to the plant material, it took a long time to produce a sufficient amount of plant material to study. Cell suspension cultures were subcultured frequently and it took more than six months to produce enough plant material for the study. Contamination is also a very big problem, and makes it almost impossible for upscaling of the whole production process of cell suspension cultures without the proper bioreactor apparatus.

The cell suspension cultures proved to be very useful in the rest of the study in producing phytoalexins. It was also very useful in the addition of jasmonic acid as the elicitor molecule for the production of phytoalexins.

Chapter 3

Isolation of phytoalexins from *H. kraussii* cell suspension cultures

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3.1 Introduction

Phytoalexins are small molecular mass compounds produced in the plant after certain stress situations occurred in its environment. These stress situations could be a pathogen attack, insects feeding on the plant, UV light, etc. All these factors could be responsible for the activation of the resistance system, and could eventually lead to the production of phytoalexins as the resistance compounds of the plant (Dixon & Harrison, 1994).

Jasmonic acid (Figure 3.1) and its methyl ester are widely distributed in the plant kingdom. It has been suggested that jasmonic acid acts as a signal molecule in response to various environmental stimuli including wounding and infection by pathogens, which leads to the activation of stress related genes and enzymes. Treatment with jasmonic acid or methyl jasmonic acid affect secondary metabolism in plants. Jasmonic acid strongly elicits the production of rice phytoalexins, oryzalexins, momilactones, and sakuranetin, in leaves and cell suspension cultures of rice plants (Daniel & Purkayasta, 1995).

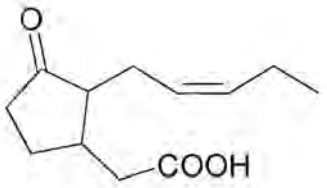


Figure 3.1: Structure of jasmonic acid.

3.1.1 Micro-organism resistance

Micro-organisms tend to develop resistance against many antimicrobial compounds used as medication to cure illnesses and diseases. A big percentage of antibiotics are no longer useful, because the micro-organisms have developed resistance against them, and they are only useful when given as part of a combined medication. It is therefore necessary to search continuously for novel antimicrobial compounds to replace current failing antibiotics (Rang *et al*, 1995).

The head of the World Health Organisation (WHO)—Dr. Gro Harlem Brundtland—warned that if there is not a serious attempt to eliminate and decrease the occurrence of contagious diseases, antimicrobial resistance will continue and increase and it will take us back to a time when there were no antibiotics to cure diseases. We accept too easily that there will always be medicine available to cure diseases like tuberculosis (TB), malaria, syphilis, and other infectious diseases. All these medicines could very soon be worthless (Beeld, 10 Augustus, 2001; Schentag, 2000).

The first micro-organisms started to develop resistance against antibiotics four years after the production of penicillin by pharmaceutical companies in 1943. *Stapphylococcus aureus* was one of the first bacteria to develop resistance, and by 1967 *Streptococcus pneumoniae* also developed resistance. These bacteria are responsible for dangerous diseases like pneumonia. In Thailand, three of the general medicines used for curing malaria are no longer effective anymore and the increase in resistance of TB is one of the major problems in South Africa (South African Tuberculosis Association, 1998).

The misuse of antibiotics is responsible for an impending disaster. There are many resistant organisms, and only a few medicines available to cure infectious diseases. In a lot of cases, doctors cannot do anything about diseases like HIV, because there is nothing available to cure the disease. There is a world-wide report of diseases in hospitals that cannot be cured, and all these people eventually die from these illnesses (Zinner, 2000).

There is a misconception under the public that pharmaceutical companies are constantly developing new medicines to replace those that are not effective anymore. This is however not the case, and many people would ask why not. There is one major reason for this. The WHO said that it costs up to R4 milliard and about twenty years of research before new cures are developed. The world is not paying attention to the fact that resistance against antibiotics has been increasing rapidly (Tan Yu, *et al.*, 2000).

People are not only affected directly by pathogens, but also indirectly. Millions of dollars are wasted each year due to a loss in production of crops and millions are spent each year by farmers to control pests and pathogens on commercially important crops. An epidemic of southern corn leaf blight in the US in 1970, led to a loss of $1 billion. These compounds could therefore not only be a solution to the micro-organism resistance development against human diseases, but also the problems with pathogens and pests on farms that produce economically important crops (Agrios, 1997).

Phytoalexins, being antimicrobial compounds and only produced when they are really needed, are therefore an attractive target in the search for antimicrobial compounds. These compounds are only produced to protect the plant in situations where the plant may not survive. These compounds are therefore very effective in the fight against pathogens and stress situations (Taiz & Zeiger, 1998).

3.2 Brief history on the discovery of phytoalexins

In 1911 the French botanist Noel Bernard discovered that plants can produce antifungal substances which are specifically formed when the plant is attacked by fungi. He found that the tubers of two orchid species became resistant to further fungal attack after they had been infected by the fungus *Rhizoctonia repens*. By placing the infected tuber tissues on agar and introducing fungi onto the medium, Bernard found that the fungus-infected tissue produced a diffusible inhibitor of fungal growth (Graver & Kokubun, 2001).

The same phenomenon was observed with potato tubers infected by *Phytophtora infestans*. These compounds were called phytoalexins. Phytoalexins were defined by Müller and Börger (1940) as "chemical compounds produced as a result of invasion of living cells by a parasite". This definition has been modified frequently whenever new evidence revised earlier concepts. It soon became clear that phytoalexins were not only formed in plants after exposure to fungi, but also by various non-biological stress factors such as irradiation with short wavelength UV light, or treatment with heavy metal ions such as copper or mercury salts. For this reason Ingham (1973) redefined phytoalexins as "antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors". The term phytoalexin is generally limited to secondary metabolites of low molecular weight, usually below 1000, and does not apply to antifungal peptides and proteins produced by plants (Graver & Kokubun, 2001).

Since 1940, phytoalexin research progressed to reach different stages. Daniel & Purkayasta (1995) defined the different stages, and the progress in phytoalexin research. The different stages are given below.

- 1940–1959: Detection, isolation, characterization, and identification of new antifungal compounds and bioassays.

- 1960–1969: Mechanisms of phytoalexin induction, host specificity of phytoalexins, and biosynthesis of phytoalexins.

- 1970–1979: Phytoalexin production by bacteria and viruses, metabolism of phytoalexins, disease control and elicitation of phytoalexins.

- 1980–1989: Race-specific phytoalexin elicitors, mechanisms for phytotoxicity and factors affecting elicitation of phytoalexins.

- 1990: Sensitive and rapid assays for antibacterial activity of phytoalexins. Production and gene-encoding enzymes of phytoalexin biosynthesis.

In the past half-century phytoalexins have been fully established as one of the major factors of disease resistance in plants. Its application in plant protection, plant breeding, germplasm screening, and the study of biogenetic relationships among plants is fairly well understood; but whether higher rate of production or accumulation of phytoalexins or inability of a parasite to degrade or detoxify phytoalexins is more important in plant disease resistance is not clear. Since phytoalexin detoxifying mechanisms of parasites differ in many respects, it is not unusual to find differential tolerance of pathogens to a phytoalexin (Daniel & Purkayasta, 1995).

Phytoalexins are today regarded as a group of structurally diverse molecules that are generally lipophilic, nonspecific in their antimicrobial activity, and particularly potent. The mechanisms of their activity are not well understood and the basis of their toxicity is likely to involve disruption of membranes, although a variety of physiological and

biochemical processes other than changes in membrane permeability may also be affected. In contrast to preformed antifungal compounds, phytoalexins are not present in healthy plant tissue and are synthesised from remote precursors in response to pathogen attack or stress— probably as a result of *de novo* synthesis of enzymes. Although both disease resistant and susceptible plants may respond to pathogen attack by producing phytoalexins, these compounds generally accumulate more rapidly and in higher concentrations in resistant plants (Morrissey & Osbourn, 1999).

It is often difficult to determine whether a compound is constitutive or induced, for the compound may normally be present in very small quantities, but may increase dramatically in concentration after infection. Strictly speaking this is not a phytoalexin because it is not synthesised *de novo*. To compensate for this problem, Stoessl (1980) proposed a new definition for phytoalexins: "Products of higher plant metabolism, absent from healthy tissues or present only in negligible traces, which accumulate in significant amounts in response to fungal or bacterial challenge".

Another point of confusion between constitutive and induced antifungals is that the same compound may be a preformed antifungal compound in one species and a phytoalexin in another. Some compounds may be a phytoalexin in one organ and constitutive in another part of the same plants such as momilactone A, which occurs in rice husks and in rice stems, but is a phytoalexin in rice leaves (Graver & Kokubun, 2001).

The term phytoalexins is usually restricted to the antibiotic compounds that require *de novo* expression of the enzymes involved in the metabolic pathway. This is a very economical way to counteract pathogens, because the carbon and energy resources are diverted to phytoalexin synthesis only at the early period of infection, and only at this site. Unchallenged plants use this energy for the normal processes in the plant such as production of seeds, flowers and the growth of new leaves (Graver & Kokubun, 2001).

Some plants do not synthesise phytoalexins, but they release toxins that are normally stored as less toxic glycosides in the vacuole. When this glycoside comes in contact with hydrolising enzymes, it produces a toxic aglycone. Although this aglycone is released after an attack and therefore not normally present in the plant, is it strictly speaking not a phytoalexin, because the enzymes involved were already present in the plant before the attack (Graver & Kokubun, 2001).

3.2.1 Structural classes of phytoalexins

All the major structural classes such as phenolics, terpenoids and alkaloids are represented, and the structures are often unique at the family level. The majority of phytoalexins produced by members of the family Leguminosae (Fabaceae *sensu lato*) are isoflavonoids, and in the Cruciferae (Brassicaceae) uniquely sulfur-containing indoles are found (Graver & Kokubun, 2001).

Kievitone and phaseollidin (Figure 3.2) are isoflavonoid phytoalexins that are produced by the legume *Phaseolis vulgaris* (Morrissey & Osbourn, 1999). The pea plant (*Pisum*

sativum) produces the isoflavonoid phytoalexin, pisatin (Figure 3.3), and chickpea (*Cicer arietinum*) produces maackiain and medicarpin (Figure 3.4) (Morrissey & Osbourn, 1999). In red clover (*Trifolium pratense*), a number of secondary metabolites have been identified. Among them, maackiain has been characterised as a pterocarpanoid phytoalexin, and its glucoside and malonyl glucoside occurs as constitutive components. It has also been shown that clovamide congeners are increased by jasmonic acid treatment in roots of red clover (Tebayashi *et al.*, 2000).

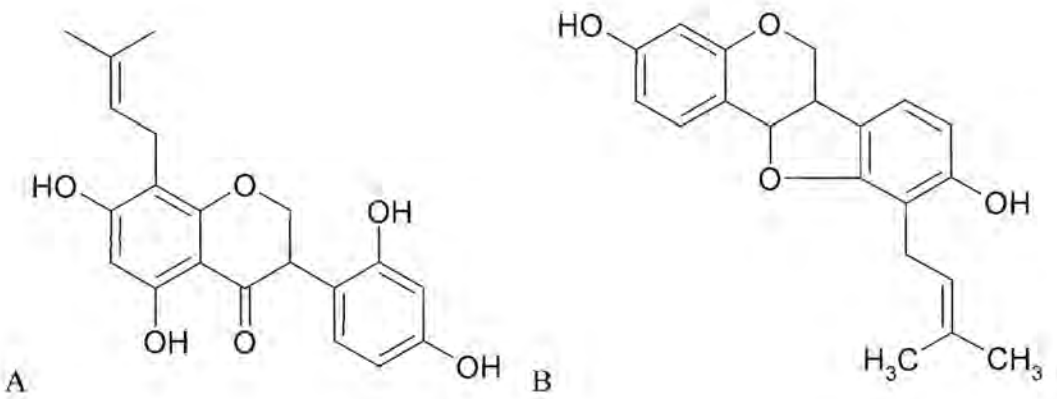


Figure 3.2: Structures of kievitone (A) and phaseollidin (B) (Morrissey & Osbourn, 1999).

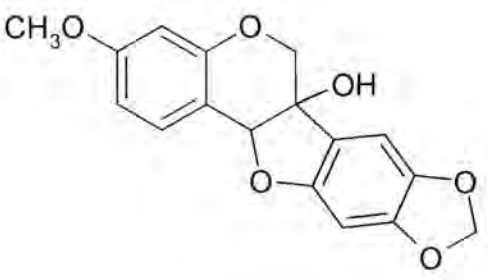
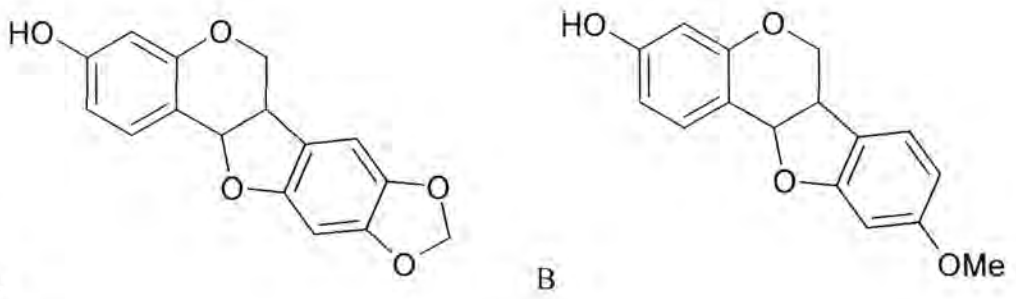


Figure 3.3: Structure of pisatin (Daniel & Purkayasta, 1995).



A B

Figure 3.4: Structures of maackiain (A) and medicarpin (B) (Daniel & Purkayasta, 1995).

Although phytoalexins from 30 different plant families have been isolated since 1940, cruciferous phytoalexins were the first reported sulfur-containing phytoalexins. Most interestingly, Brassiceaea species appear to be the only plant family producing sulfur-containing phytoalexins (Pedras *et al.*, 2000). Cultivated Brassicaceae species produce a different group of sulfur-containing phytoalexins belonging to the brassinin family (Figure 3.5) following challenges with pathogens (Morrissey & Osbourn, 1999).

Figure 3.5: Structure of brassinin (Morrissey & Osbourn, 1999).

The resistance of the wild crucifers to different pathogens depends on the production of the phytoalexin camalexin (Figure 3.6). Camalexin and related compounds are structurally similar to the synthetic systemic fungicide thiabendazole, which is used extensively for control of plant diseases (Morrissey & Osbourn, 1999).

Arabidopsis thaliana (a crucifer) also synthesises camalexin (Figure 3.6) on challenge with bacterial and fungal pathogens, and this appears to be the only phytoalexin to be produced in the plant (Morrissey & Osbourn, 1999).

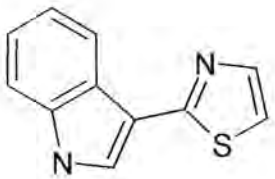


Figure 3.6: Structure of camalexin.

Ayapin and scopoletin accumulated in *Helianthus tuberosus* after elicitation with $CuCl_2$ or sucrose. Ayapin and scopoletin are phenolic compounds belonging to the family of 7-hydroxylated coumarins (Figure 3.7). They have been described as phytoalexins; their accumulation being correlated with resistance to microbial pathogens in tobacco and sunflower, as insect feeding detergents and as inhibitors or inducers of parasitic weed germination. In addition, physiological effects like promotion of stomatal closure in sunflower, or inhibition of bud growth in pea, have also been associated with scopoletin at very low concentrations (Cabello-Hurtado *et al.*, 1998).

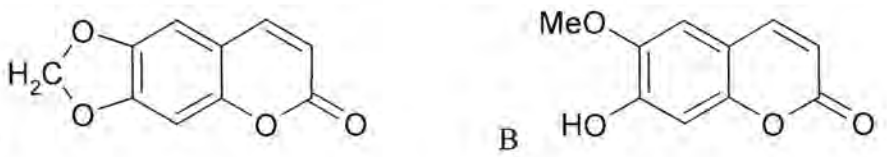


Figure 3.7: Structures of ayapin (A) and scopoletin (B) (Cabello-Hurtado *et al.*, 1998).

During infection of potato tubers, preformed steroidal glycoalkaloids, and phenolics such as chlorogenic acid, caffeic acid, and scopoletin, as well as a number of sesquiterpenes were found. The sesquiterpenes rishitin, lubimin and solavetivone are the major phytoalexins that accumulate in potato tubers upon fungal infection (Figure 3.8).

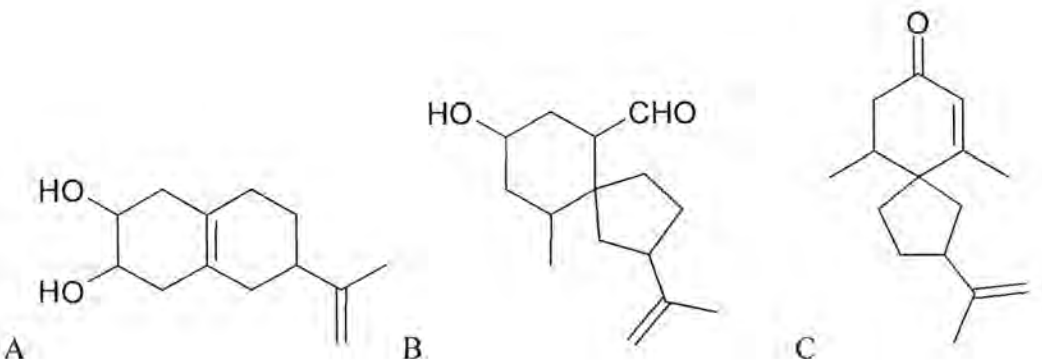


Figure 3.8: Structures of rishitin (A), lubimin (B) and solavetivone (C) (Morrissey & Osbourn, 1999).

Gossypium spp. (cotton) produce an array of sesquiterpenoid defense compounds, some of which accumulate in the pigment glands and in the root epidermis of healthy plants, and others that function as phytoalexins (Abraham *et al.*, 1999).

After elicitation of *Platanus X acerifolia* cell suspension cultures, induction of the synthesis of xanthoarnol (Figure 3.9), a dihydrofuranocoumarin phytoalexin was observed. This compound accumulated in the growth medium of the cell suspension cultures (Alami *et al.*, 1999).

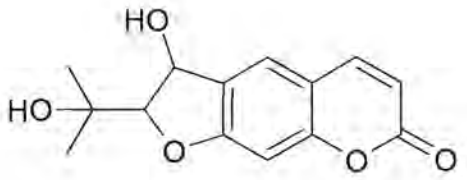


Figure 3.9: Structure of xanthoarnol

Investigations on the resistance mechanisms of unripe nectarine (*Prunus persica*) fruit, found that the fruit produced phytoalexins upon wounding and infection. Seven phytoalexins from this fruit were identified (Lahlou *et al.*, 1999).

In most plants, induction of phenylpropanoid phytoalexins is the result of the activation of biosynthetic enzymes such as phenylalanine ammonia-lyase and chalcone synthase. However in several leguminous plants, release of phytoalexins or their precursors from glycosides has been reported. Avenanthramides, the phytoalexins from oats, are biosynthesised from coumaric acid (Ishihara *et al.*, 1999).

Combinations of different compounds may have synergistic effects. Some steroidal glycoalkaloids have little antifungal activity when tested separately, but exhibit pronounced synergistic activity when mixed in pairs. Structurally unrelated compounds are also likely to have in combination a synergistic antifungal activity (Osbourn, 1999).

It is clear that phytoalexins are not restricted to some plant families, or to some structural groups. Phytoalexins from a large number of families have been identified over the past years, and more phytoalexins are isolated daily.

3.2.2 *Trans*-resveratrol

Trans-resveratrol (Figure 3.10), a phytoalexin found at high levels in grapes and in grape products such as red wine, has been shown to have anti-inflammatory and antioncogenic properties. This data may also explain aspects of the "French-Paradox" that is associated with reduced mortality from coronary heart disease and certain cancers among the French—avid red wine drinkers—and provide a rationale for the role of a potent chemopreventive compound in blocking the initiation of inflammation and oncogenesis (Holmes-McNary & Baldwin, 2000).

In 1997, resveratrol was reported to be one of the most potent chemopreventive agents able to block all three phases of tumor development that includes initiation, promotion and progression. Recently this drug was reported to be a phytoestrogen that behaves as a superagonist of estrogen receptors and thereby an inducer of tumor cell proliferation. Its structural similarity with estrogen may also account for its cardioprotective effects (Manna *et al.*, 2000). Resveratrol was found to also inhibit herpes simplex virus types 1 and 2 replication in a dose-dependent, reversible manner. Its antioxidant activity is thought to protect against coronary artery disease through the modulation of lipid synthesis, and inhibition of platelet aggregation. It also down-regulates prostaglandin and prostacyclin synthesis probably due to the inhibition of cyclo-oxygenase and

hydroperoxidase (Docherty *et al.*, 1999). Resveratrol also inhibits endothelial adhesion. Resveratrol modulates endothelium activity; its function could be that of preserving endothelial function by inhibiting the impairment of release of nitric oxide (Ferrero *et al.*, 1998).

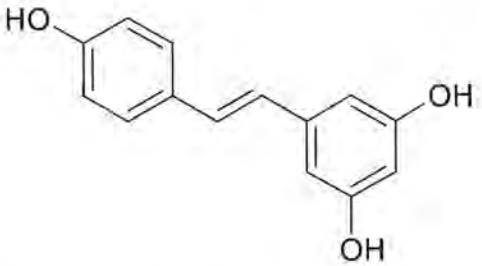


Figure 3.10: Structure of *trans*-resveratrol

3.3 Materials and methods

To study the effect of phytoalexin production, all natural causes of activation of the plant's resistance system must be eliminated. Plants are under constant risk of attack or stress when they are in the open field and it was therefore necessary to provide a stable and controlled environment by utilising cell suspension cultures, to be able to study the effect of phytoalexin production in *H. kraussii*. The cultures were stored in a growth cabinet at 27.5°C, and 16 hours light per 24 hours. The cultures were kept on a shaker to ensure sufficient aeration of the plant material in the liquid medium.

3.3.1 Time intervals and concentrations

The investigation commenced with testing the effect of exposure to jasmonic acid over different time intervals, because there was no time-framework to work from. Time intervals of one week, two weeks and three weeks were used to get an estimation of when

the most of the resistance compounds will be formed. After determining at what stage the highest concentration of compounds were produced, the effect of different concentrations of jasmonic acid on the plant material were tested. Three concentrations, 1.0, 5.0 and 10.0 µM, of jasmonic acid in the cell suspension cultures were used. After determining the best results, the study focussed on the compounds formed after application of jasmonic acid.

3.3.2 Isolation of phytoalexins

Ellard-Ivey & Douglas showed in 1996 the relative RNA amount produced after jasmonic acid was added; with 1.0 µM of jasmonic acid giving the best results. Jasmonic acid (100.0 mg) was dissolved in 100% Triton X-100, and after filter sterilisation, 100.0 µl of the solution was added to the cell suspension cultures.

After exposure to jasmonic acid the cell suspension cultures were filtered and washed to remove the medium from the cells. The medium was discarded, and the cells were homogenised in a small amount of acetone. The acetone mixture was then filtered to remove the unwanted cell rests from the extract. This filtration process was repeated three times, to ensure that all the compounds were extracted. The acetone extract was then concentrated under reduced pressure on a rotary evaporator to dryness. The extract was resuspended in chloroform, and the undissolved material was dissolved in water, because some of the polar compounds did not dissolve in the relatively apolar solvents. These extracts were chromatographed on an aluminium silica gel 60 thin layer chromatography (TLC) plate with three controls. The one control was an extract from

the cell suspension cultures that did not receive any jasmonic acid treatment. The other controls were an extract from the tissue culture calli, and the third control was jasmonic acid. The calli were also filtered and extracted in acetone, and afterwards dissolved in water and chloroform. This was done to ensure that the stress situations (shaking) in the cell suspension culture controls did not give misleading results. These controls were kept under the same conditions, and were also extracted at the same time as the rest of the cell suspension cultures. After development on the TLC plate with equal parts of chloroform and ethyl acetate as the solvent mixture, the plates were tested with bacteria to determine if any antibacterial compounds were formed after addition of jasmonic acid.

The plates were sprayed with *Bacillus cereus* as an indicator for the presence of antibacterial compounds. A 24 hour old *B. cereus* suspension, cultured in nutrient agar was sprayed onto the TLC plates (Dilika and Meyer, 1996). The plates were then stored in a growth chamber at 30°C and high humidity (Hamburger & Cordell, 1987). After 24 hours of growth, the TLC plates were sprayed with 2 mg/ml 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tertrazolium chloride (INT). In response to spraying with INT, the plates changed colour to a reddish-pink, where bacterial growth occurred (Dunigan *et al.*, 1995).

After comparison of the TLC plates the extract was separated on a dry silica column. The extract was mixed with a small amount of the silica gel, and applied on top of the silica gel in the column. A small amount of dry silica gel was used to cover the extract, to ensure that the concentrated extract did not dissolve in the solvent. A 70 % hexane:

ethyl acetate solvent was used for separation. To separate the closely related phytoalexins from each other, it was necessary to use a smaller, slow silica column. A more apolar solvent of 90% hexane: ethyl acetate was used for separation. The fractions were evaluated each time on TLC plates.

After separation of the two phytoalexins, it was necessary to elucidate the structures with data from ^{1}H-NMR, ^{13}C-NMR and GC-MS. Previously published spectral data were compared with our data to confirm the structures of the phytoalexins.

3.4 Results

The highest amount of phytoalexins was synthesised after two weeks of exposure to jasmonic acid. The rest of the study was therefore based on an activation period of two weeks after jasmonic acid was added. The controls were kept under the same conditions but the jasmonic acid treatment was omitted.

A concentration of 5.0 μM of jasmonic acid added to the cultures was the most effective concentration to activate the resistance system of the plant. The other two concentrations were also effective in activating the resistance system, but to a lesser extent.

3.4.1 Antibacterial activity

A part of the definition of phytoalexins states that it should have antimicrobial activity. It was therefore crucial that these compounds had to be antimicrobial for them to be classified as phytoalexins.

Bacterial bioassays on TLC plates were performed to determine the activity of these compounds. After spraying the TLC plates with *B. cereus*, inhibitory activity on the growth of *B. cereus* was observed (Figure 3.11). Both the water and chloroform extracts showed biological activity against the bacteria. There was however a small amount of the compounds formed in the control of the cell suspension cultures (CC) as well. From the bioassay with *B. cereus*, it is evident that there is a lot of antibacterial activity present. We were however only looking for compounds produced by the activated cell suspension cultures, which were not present in the controls. The inhibition from the water extracts is present in the controls (WT & WC), and those were therefore not compounds formed as a result of the jasmonic acid added to the cultures. In the chloroform extract there was however inhibition in the activated cell suspension culture extract (CJ) that was not present in the controls (CC & CT). These compounds (A & B) were possibly phytoalexins produced as a result of activation.

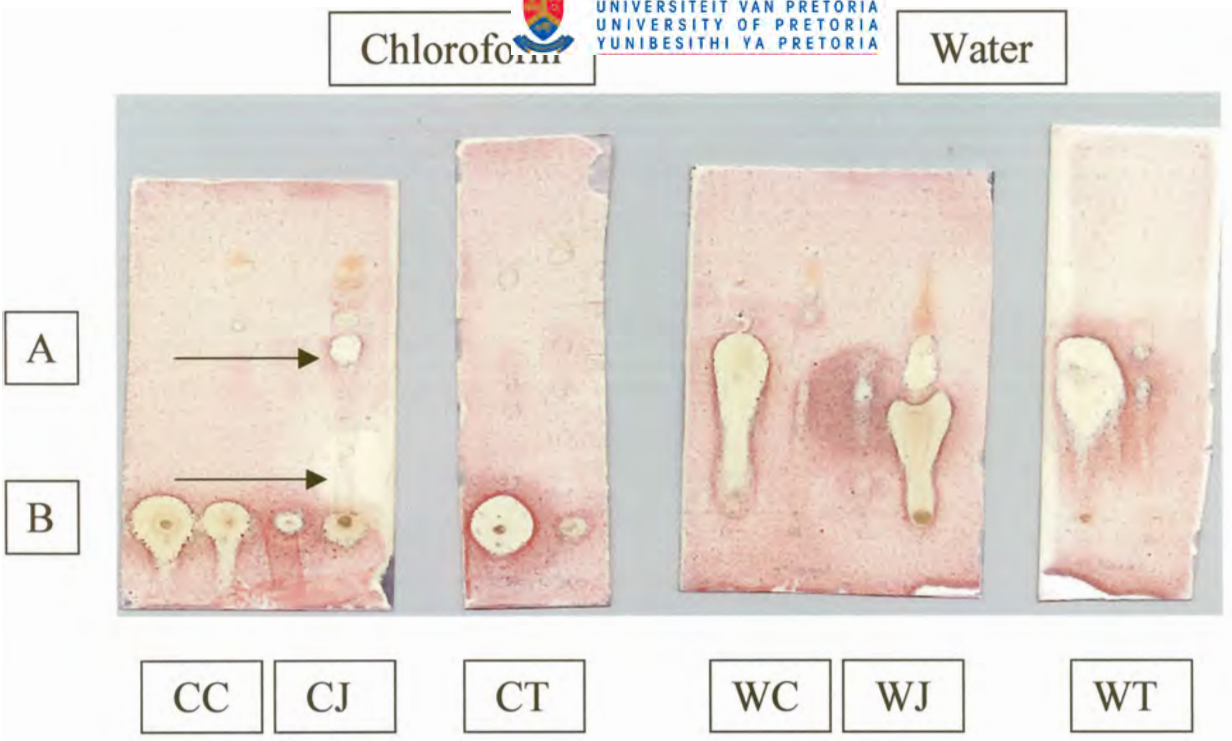


Figure 3.11: Bioassay of antibacterial activity in the water (W) and chloroform (C) extracts. Two concentrations were used for each sample. Two different solvents were used for the two extracts. For the chloroform (C) extract a solvent of 1:1 chloroform : ethyl acetate was used, and for the water (W) extract a solvent of pure ethanol was used. CC = cell suspension culture control, CJ = cell suspension culture activated with the addition of jasmonic acid and CT = tissue culture control. WC = cell suspension culture control, WJ = cell suspension culture activated with the addition of jasmonic acid and WT = tissue culture control. A & B are compounds produced by the activated cell suspension cultures, not present in the controls.

3.4.2 Isolation of compounds

After determining the optimum range for the production of these compounds and the determination of its antibacterial activity, the study focused on the isolation of compounds A & B. It was only possible to obtain 93 g of plant material after filtration of the cell suspension cultures. The material was extracted in acetone, and after filtration and concentration, the mass of the concentrated extract was 4.5 g.

The activated cell suspension extract was compared with the control (which did not receive jasmonic acid treatment) on TLC with different solvent systems. At least two different compounds were again produced in cell suspension cultures that were not present in the control (Figure 3.12). The compound that was targeted as the first compound to be isolated and identified (A) are also indicated (red arrow) in the figure. The possibility existed that the compounds produced were compounds that are known to be part of the defence system for instance salicylic acid or jasmonic acid which were added to the cell suspension cultures. The extract was compared with jasmonic acid, salicylic acid (SA), galangin (compound previously isolated from *Helichrysum* by Mathekga & Meyer, 1998) and umbelliferone (a phytoalexin) on a TLC plate to see if it was not one of these compounds in the cell suspension cultures. The compound produced in the cell suspension cultures did not match one of these compounds, and it was therefore worth separating the compound from the extract.

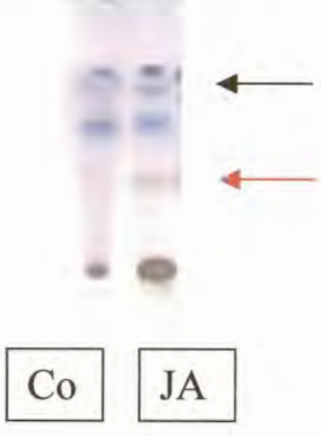


Figure 3.12: TLC plates showing compounds produced in cell suspension culture (Co = control, JA = activated cell suspension cultures).

Separation was obtained with a silica column with a solvent system of hexane and ethyl acetate (7:1), and the fractions containing the compound were combined. When these fractions were evaluated on TLC, it became clear that there was more than one

compound present in the spot that was previously identified as one compound (Figure 3.13). The fractions were concentrated and only 64 mg of these fractions were obtained. It was necessary to use another smaller silica column to separate the two main compounds in the fraction. A very slow small column was used, with a solvent system of hexane : ethyl acetate (9:1). From the fractions on the TLC, it looked as if the two compounds were separated from each other and it looked relatively pure (Figure 3.14). The fractions containing the same compounds were combined and concentrated to determine how much of the compounds were available for identification of the compounds. The mass of the two compounds were 21 mg and 8 mg.

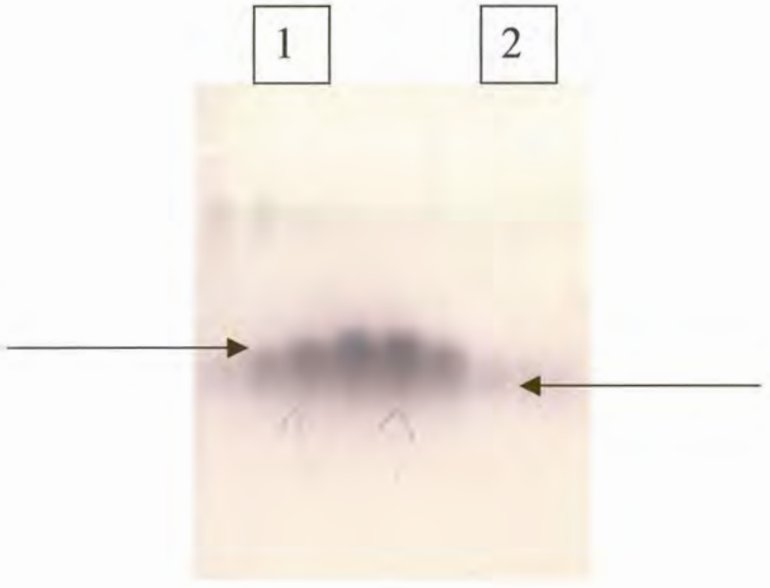


Figure 3.13: TLC plate showing separation between compounds 1 & 2. A solvent system of hexane : ethyl acetate was used (1:1).

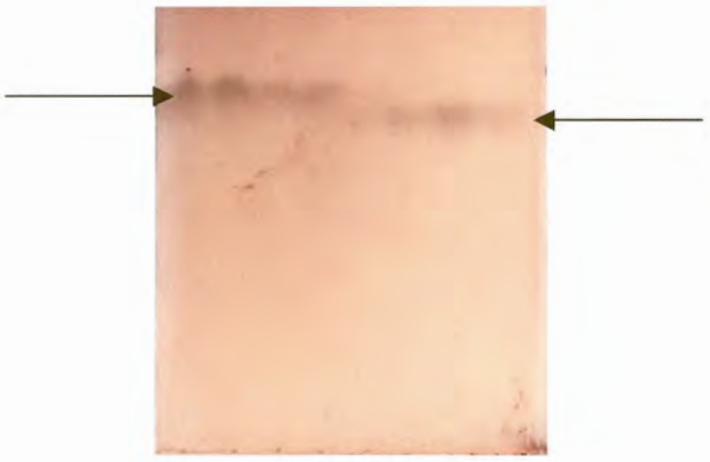


Figure 3.14: TLC plates showing compounds (1 & 2) obtained from separation on a silica column. A solvent system of hexane : ethyl acetate (3:7) was used.

After separation of the compounds, they were tested again for antibacterial activity. Figure 3.15 shows the antibacterial activity of the compounds isolated from the activated cell suspension cultures. Both the compounds had inhibitory activity against the growth of *B. cereus*.

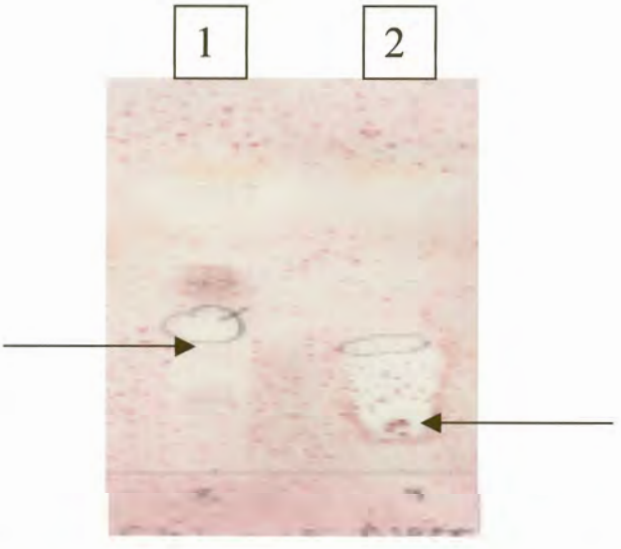


Figure 3.15: Antibacterial activity of isolated compounds 1 & 2 separated with a solvent system of hexane : ethyl acetate (7:3).

3.4.3 Identification of compounds

To identify these compounds, nuclear magnetic resonance (NMR) and mass spectroscopy (MS) analysis were used, and the spectra are shown in Figures 3.16–3.22. Unfortunately there was not enough material isolated compound 2, and a proper ^{13}C-NMR was not obtained. It was therefore not possible to confirm the structure of it. The ^{1}H-NMR and MS are given in Figures 3.21 & 3.22.

Compound 1 was identified as β-amyrin, and compound 2 was preliminary identified as α-amyrin. β-amyrin was identified by comparison with previously published ^{13}C-NMR data (Knight, 1974 & Seo *et al.*, 1975), ^{1}H-NMR (Linde, 1979) and MS data (Aexel *et al.*, 1972). The other compound α-amyrin was compared with previously published ^{1}H-NMR and MS data (Seo, *et al.*, 1975).

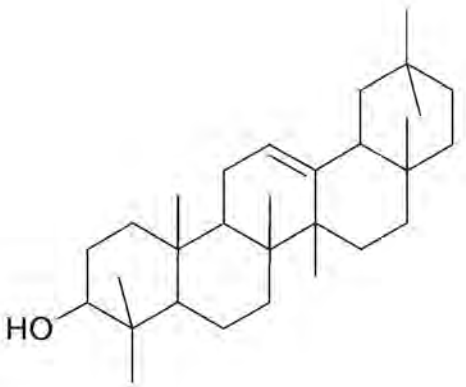


Figure 3.16: Structure of β-amyrin (compound 1).

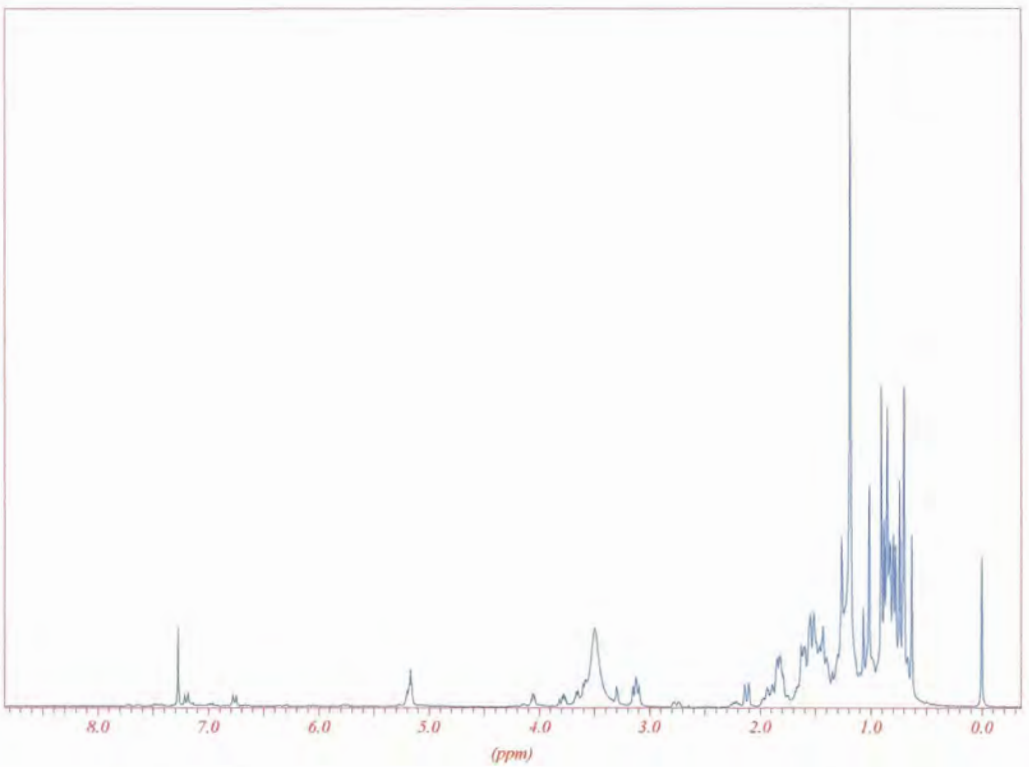


Figure 3.17: ^{1}H-NMR spectrum of β-amyrin. ^{1}H-NMR (300 MHz): δ = 5.17 (m,1H), 3.1 (t, J=9, 1H), 2.1(d, J=11, 1H), 1.18(s, $1CH_3$), 1.06(s, $1CH_3$) 1.01(s, $1CH_3$), 0.90(s, $1CH_3$), 0.88(s, $1CH_3$), 0.85(s, $1CH_3$), 0.70(s, $1CH_3$).

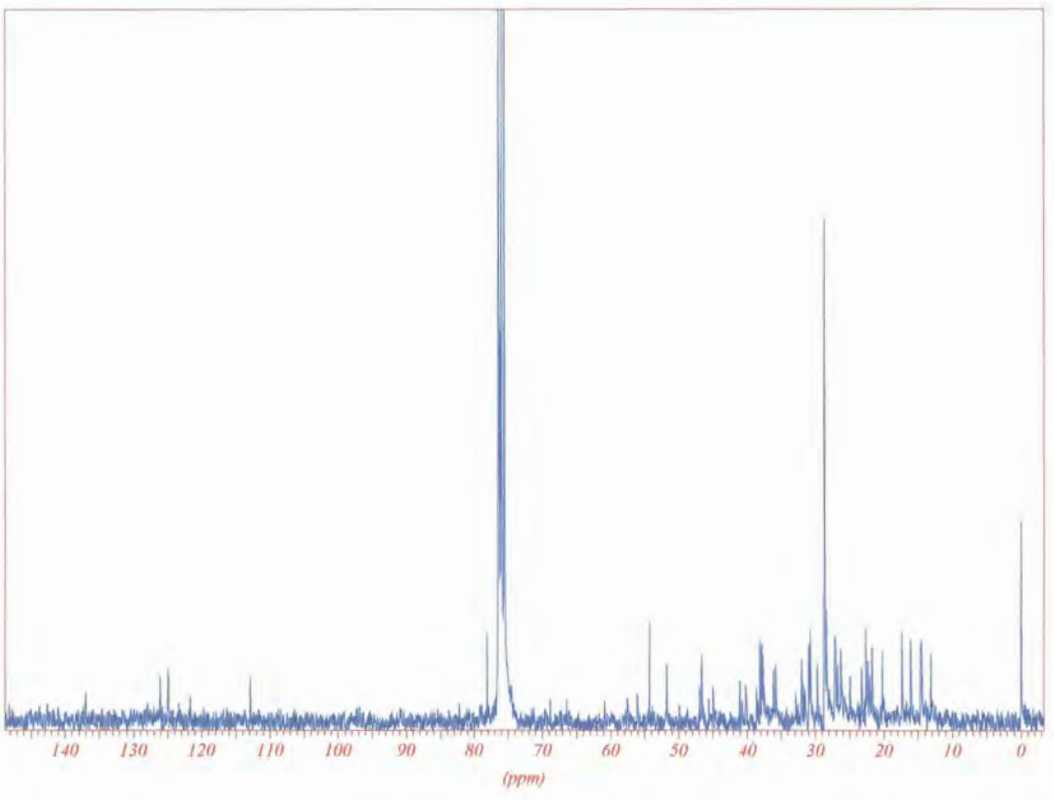


Figure 3.18: ^{13}C-NMR spectrum of β-amyrin. ^{13}C-NMR (75 MHz): 14.07 (s), 15.46 (t), 17.05 (d), 18.30 (s), 21.14 (s), 22.66 (s), 23.00 (s), 23.30 (s), 23.58 (s), 24.17 (s), 25.90 (s), 27.24 (s), 27.70 (d), 28.13 (s), 29.33 (s), 29.68 (s), 30.75 (t), 32.97 (s), 36.71 (d), 37.01 (d), 38.76 (t), 39.06 (s), 46.59 (s), 52.70 (s), 55.25 (s), 79.07 (s), 125.87 (s), 127.09 (s).

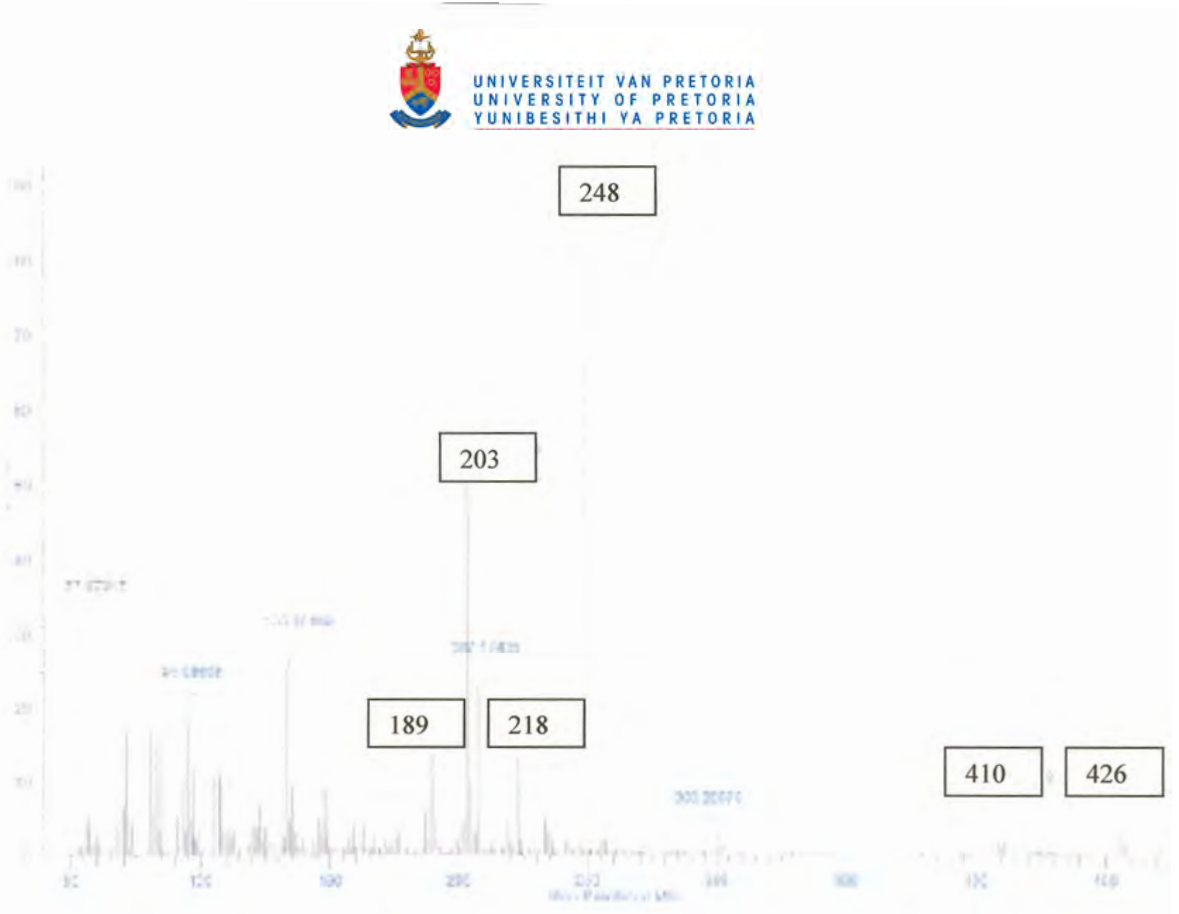


Figure 3.19: MS analysis of β-amyrin. EIMS *m/z*: 426, 410, 248, 218, 203, 189.

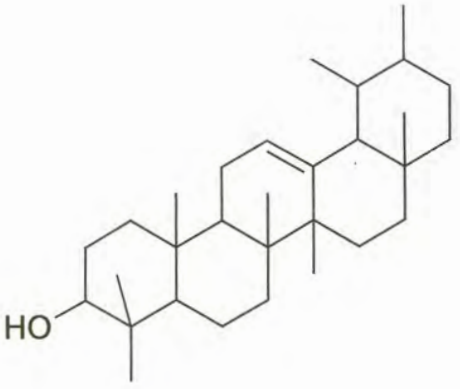


Figure 3.20: Structure of α-amyrin (compound 2)

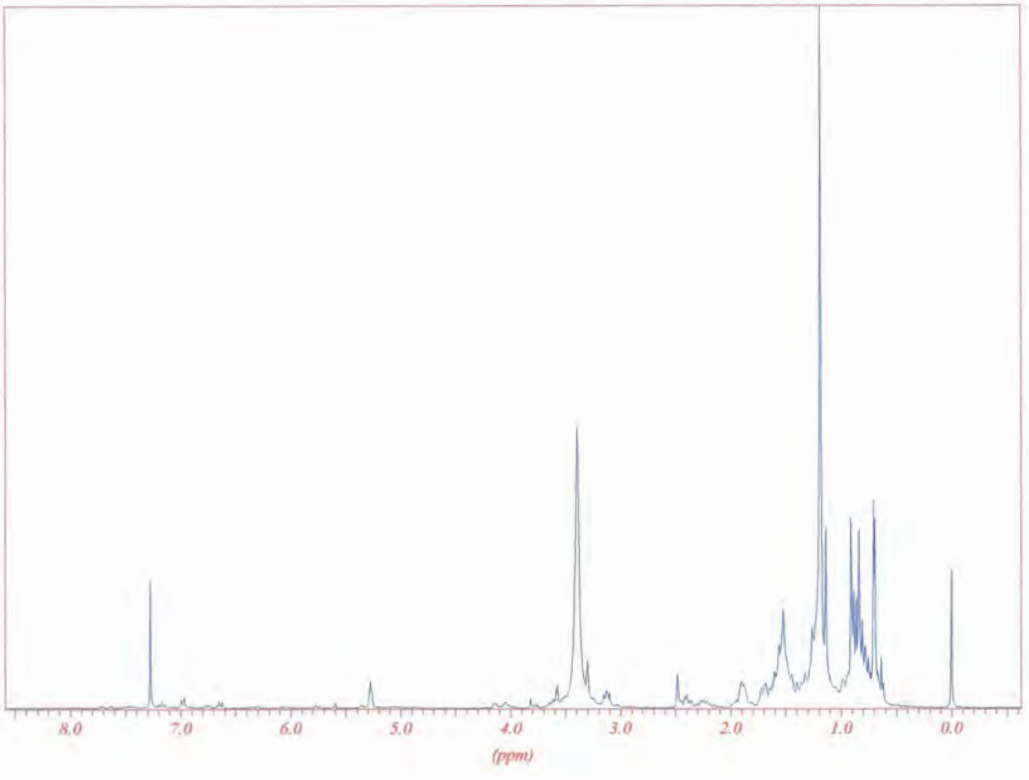


Figure 3.21: ^{1}H-NMR spectrum of α-amyrin. ^{1}H-NMR (300 MHz): δ = 5.26 (m,1H) 3.29 (m, 1H) 2.39 (d, J=11, 1H), 1.18 (s, 1CH_3), 1.12 (s, 1CH_3), 0.91 (s, 2CH_3), 0.88 (s, 1CH_3), 0.86(s, 1CH_3), 0.84(s, 1CH_3).

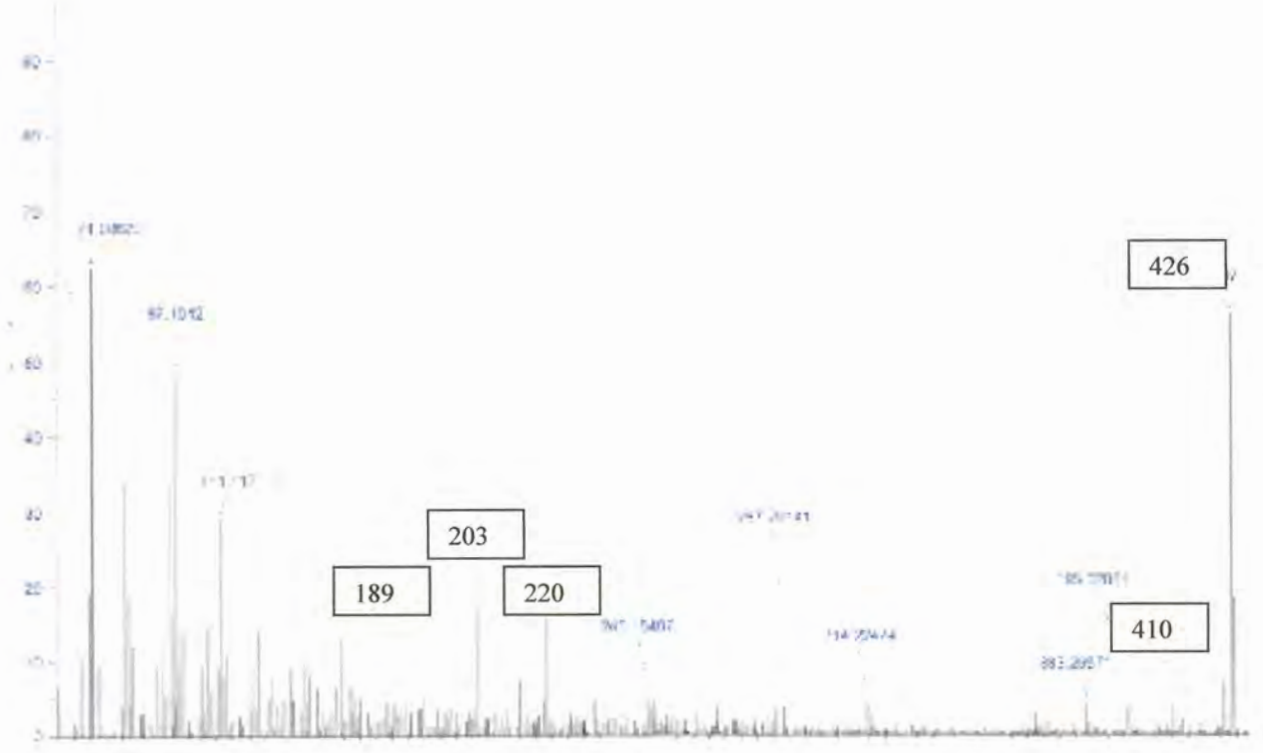


Figure 3.22: MS data for α-amyrin. EIMS *m/z*: 426, 410, 218, 203, 189.

3.5 Discussion

The definition of phytoalexins states that they are small molecular mass compounds that are not normally present in plants. These compounds are produced after elicitation of the defence system of the plant, which are then active as antimicrobial compounds. When all of these criteria are met, one can say that the compound is a phytoalexin.

Not much attention was paid to the optimum ranges for the production of phytoalexins, because it was not the major focus of the study. What was important however was to make sure that there are indeed compounds formed after jasmonic acid was added to the cultures, which were not present in the controls.

There were compounds produced in the activated cell suspension culture that was not produced in the cell suspension culture control, and these were targeted for isolation. By comparing the cell suspension cultures and the tissue cultures, many differences could be observed on the TLC plates.

In this study we used jasmonic acid as an elicitor to activate the defence system, in order for the plant to produce phytoalexins. Not much work has been done on jasmonic acid as an elicitor, and most of this work has been based on the findings obtained with salicylic acid. In this study, jasmonic acid has proved to be a potent activator of the defence system in *H. kraussii*.

Two compounds were isolated with the use of silica columns. The final separation of the two compounds was done on a very small slow silica column, because the two compounds are very similar. The two compounds were successfully separated on the column, and the next step was to elucidate their structures.

Very small amounts of the compounds were isolated and made it very difficult to elucidate the structures. A ^{1}H- NMR, ^{13}C-NMR and a GC-MS analysis were performed on both these compounds. Both the compounds' ^{1}H-NMR was very useful, and proved to be a very important tool in structure elucidation. It is however impossible to determine the structures of terpenoids with only a ^{1}H-NMR. The ^{13}C-NMR of α-amyrin was not obtained, because the amount of this compound isolated was not enough for this analysis. From the ^{1}H-NMR, ^{13}C-NMR and GC-MS was it possible to elucidate the structure of β-amyrin. Because of the similarity between the two compounds, it was possible to identify α-amyrin with ^{1}H-NMR and GC-MS data

only. The data corresponded very well to the published data, and simplified identification of this compound.

The two amyrins isolated were previously described by Fulton *et al.*, (1994), Fulton & Threlfall (1993) and several others, as key compounds in triterpene phytoalexin production. These compounds are the first compounds to be formed as part of the redirected biosynthetic pathway, leading to the production of phytoalexins rather than the normal production of phytosterols (Figure 3.23). These compounds are also antimicrobial compounds produced upon infection, and are therefore not only important in resistance as phytoalexins, but also as an important compounds involved in the production of other compounds as part of the resistance system.

The amyrins are well-known and abundant compounds throughout the plant kingdom. β-amyrin together with α-amyrin are probably the two most important triterpenoids which occur in nature (Knight, 1974). The two amyrins are widely distributed in the vegetable kingdom, occuring in the free state, but more frequently as esters, particularly as acetates in several families such as the Burseraceae, Rutaceae, Moraceae and several others. The two compounds occur together in many plant exudates, resins, waxes and latex (Simonsen & Ross, 1957).

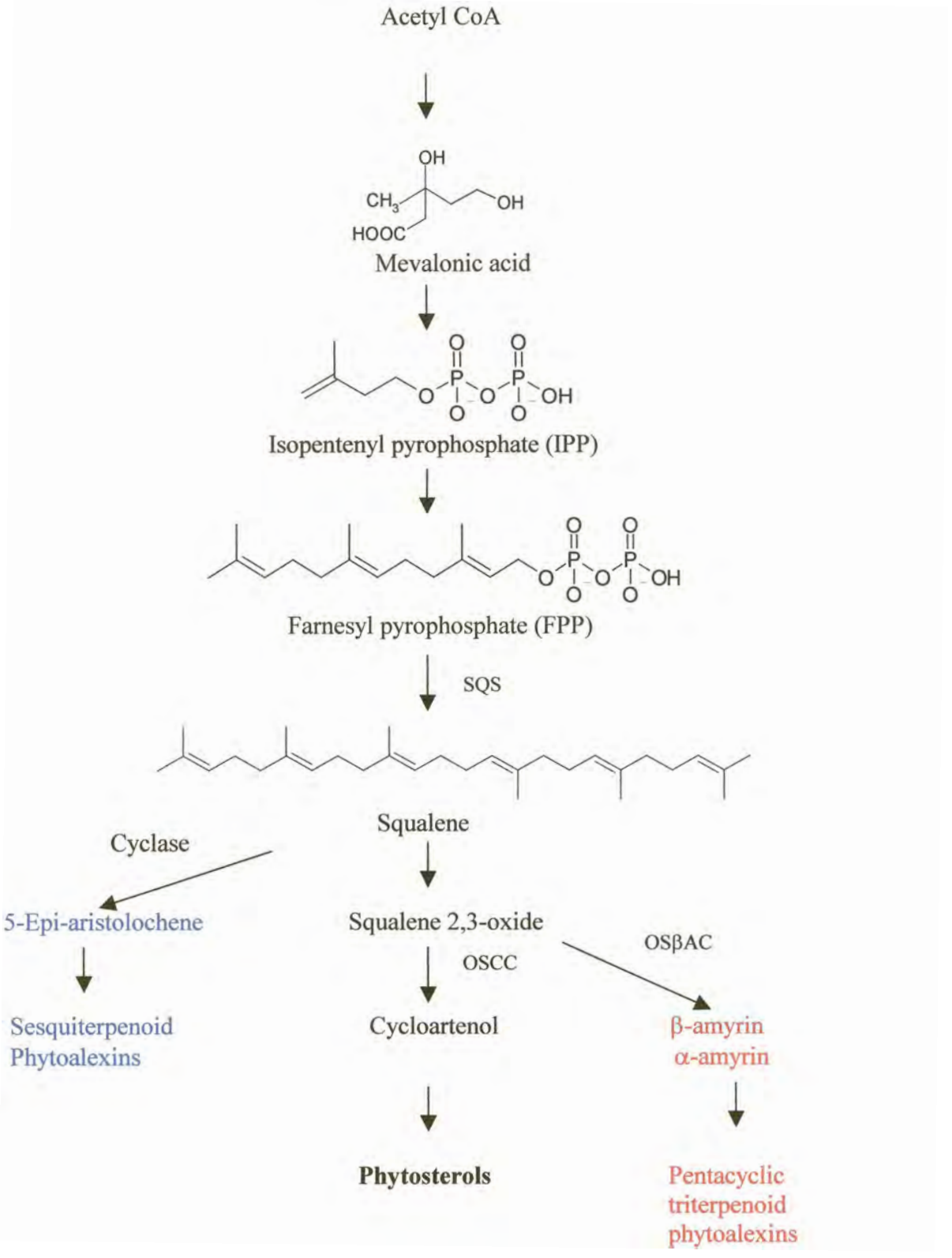


Figure 3.23: A simplified diagram of some of the important biosynthetic steps in the production of phytosterols is shown. The effect of elicitors on the pathway is indicated by the red and blue pathways. The blue pathway indicates the effect of

elicitors on plants from the Solanaceae family, and the red pathway indicates the effect on *Tabernaemontana divaricata*. SQS = squalene synthetase, OSCC = squalene 2,3-oxide:cycloartenol cyclase and OSβAC = squalene 2,3-oxide:β-amyrin cyclase (Trojanowska *et al.*, 2001 & Fulton *et al.*, 1994).

β-Amyrin is the basic structure of the β-amyrins and include compounds like germanicol and oleanolic acid. This basic structure is an important link in the production of several terpenoids necessary for survival of the plant (De Mayo, 1959).

α-Amyrin is not as abundant as β-amyrin, and is always found in a lower concentration than β-amyrin. α-Amyrin does not appear to have such a wide variety of naturally occurring derivatives as does β-amyrin. Naturally occurring derivatives include well-known compounds like asiatic acid and ursolic acid (De Mayo, 1959).

Several authors described the role of amyrins in the production of phytoalexins. Different elicitors and plant material have been used to illustrate the basic mechanism of induction and inhibition of certain enzymes after elicitation. The mevalonic acid pathway is used for the normal production of terpenes in the plant. In the case of activation, there is a slight change in the concentrations of key enzymes, to direct the metabolic pathway toward phytoalexin production (Figure 3.23).

Trojanowska, *et al.*, (2001), investigated the production of avenacin (synthesised from β-amyrin) in an avenacin-deficient mutant of *Avena strignosa* (Poaceae). Several avenacin antifungal saponins have been isolated before, with a common pentacyclic triterpenoid skeleton that is, as in the case of phytosterols, derived from mevalonic

acid. A series of reactions lead up to and including squalene 2,3-oxide, which on cyclisation give rise to either β-amyrin, or cycloartenol, the precursor of phytosterols and their derivatives (Figure 3.23). Fulton and Threlfall (1993) used elicitor treated tobacco cultures to determine the major regulatory events in the rapid and efficient accumulation of sesquiterpenoid phytoalexins. Almost total inhibition of squalene synthetase was found in cultures activated by the addition of cellulase as an elicitor. This ensures that the newly synthesized cytosolic farnesyl diphosphate (FPP) that is used for phytosterol biosynthesis in unelicited cells is channeled into the biosynthesis of sesquiterpenoid phytoalexins. The phytoalexin capsidiol was therefore rapidly accumulated in the culture after a decline in the squalene synthetase activity, but the squalene 2,3 oxide-cycloartenol cyclase activity showed little change. The mechanism by which this rapid loss of squalene synthetase activity is brought about is however not understood. Several other increases in concentration of important enzymes have been found by Fulton *et al.*, (1994), with the use of elicitor-treated cultures of *Tabernaemontana divaricata* (Apocynaceae). The induced and inhibited enzymes are different when compared to the findings of Fulton and Threlfall (1993) with their work on Solanaceae. These findings are however still entirely consistent with an elicitor-induced redirection of terpenoid biosynthesis away from phytosterol biosynthesis and towards pentacyclic triterpenoid phytoalexin biosynthesis. The switch being mediated as a direct result of the rapid inhibition of squalene 2,3-oxide:cycloartenol cyclase activity prior to the *de novo* synthesis of squalene 2,3-oxide:amyrin cyclase (Figure 3.23).

The cell cultures activated by the addition of a *Candida albicans* elicitor preparation were also accompanied by an inhibition of cell growth, the cessation of both

phytosterol biosynthesis and indole alkaloid production, and a five fold increase in the cellular level of squalene synthetase activity. A rapid and transient increase was found in several enzymes involved in the mevalonic acid pathway, for instance HMG-CoA reductase, IPP isomerase, prenyl transferase and squalene transferase activity. An inhibition was found in the activity of squalene 2,3-oxide:cycloartenol cyclase, which is important for the lower production of phytosterols and a increased production of phytoalexin production. Similar behaviours have been reported for HMG-CoA reductase, IPP isomerase and prenyl transferase activity in elicitor-treated cultures of tobacco (Fulton *et al.,* 1994).

Both isolated compounds, β-amyrin and α-amyrin can therefore be classified as phytoalexins produced by *H. kraussii,* but even more important, can act as precursors for other phytoalexins to be produced. This once more demonstrates the elicitation function of jasmonic acid as part of the defence system in plants, and especially in *H. kraussii.*

Chapter 4

Isolation of compounds from wild *H. kraussii* plants

Chapter 4

Isolation of compounds from wild *H. kraussii* plants

4.1 Introduction

The aim of this part of the study; was to relate the phytoalexin production observed in cell suspension cultures, to the plants in the field. Phytoalexins present in plants collected from the field would have simplified further extraction and isolation of the compounds.

The medicinal uses reported for *H. kraussii* include the use in a pipe for cough relief and as a remedy for pulmonary tuberculosis (Watt & Breyer-Brandwijk, 1962). Relatively few published data is available on the compounds and activity of this plant. Bougastos *et al.* (2002), identified caryophyllene and K-pinene as the main compounds from the oil extracted from *H. kraussii*. These compounds inhibited the growth of several bacteria and pathogenic fungi. Prenyl-butyrylphloroglucinol and kaurenoic acid were also isolated from *H. kraussii*, and showed antibacterial activity against five Gram positive and two Gram negative bacteria (Bremner & Meyer, 2000).

4.2 Materials and methods

Two kilograms of plant material were collected in May 2001 from the field on the experimental farm of the University of Pretoria, south of Strubenkop. A herbarium specimen (G. Prinsloo 1) was deposited in the H.G.W.J. Schweickerdt herbarium (PRU) at the University of Pretoria.

4.2.1 Plant extract preparation

The plant material was cut into small pieces and homogenised in methanol and left for 24 hours. The extract was then filtered and the remaining plant material extracted with acetone for 48 hours. The methanol and acetone extracts were then combined, and the combined extract was once again filtered and concentrated to obtain 203 g of concentrated extract.

This extract was tested for biological activity using *Bacillus cereus*. The silica gel 60 TLC plates were developed with a solvent of hexane : ethyl acetate (1:1), sprayed with bacteria, and coloured with the INT colour reagent after 24 hours as described in chapter 3. The coloured plates were then evaluated to determine the antibacterial activity of the *H. kraussii* extract.

4.2.2 Separation of extract into different fractions

Significant antibacterial inhibition was observed and the extract of *H. kraussii* was separated by means of a dry silica column. Forty grams of this extract was mixed with a small amount of silica gel, and applied to the top of the silica column. The top of the

column was then covered with dry silica gel to ensure that the concentrated extract did not dissolve and dilute in the solvent applied to the column. The solvent system used on the column was: 2000 ml of 100% hexane, 2000 ml of 25% hexane: ethyl acetate, 2000 ml of 50% hexane: ethyl acetate, 2000 ml of 70% hexane: ethyl acetate, 1000 ml of 100% ethyl acetate. The rest of the compounds still remaining on the column were washed out with 2000 ml of 10% ethyl acetate: methanol.

The separated compounds were tested on a TLC plate with different solvent systems to confirm the purity of the compounds. After the purity of the compounds was confirmed, the compound structures were elucidated. ^{1}H-NMR, ^{13}C-NMR, UV and MS analysis were performed to identify the isolated compounds.

4.3 Results

The phytoalexins isolated from the cell cultures were not found in the extract of the intact wild plants. Several crystals of pure compounds were obtained from the fractions separated on the column. As this plant has good antibacterial activity, these crystals were further purified and identified by bioassay-guided fractionation.

The extract, fractions and compounds isolated, were tested for biological activity against *B. cereus*. From Figure 4.1 it could be observed that the plant extract has many antibacterial compounds and that there are only small areas of growth of bacteria on the TLC plate.

Figure 4.1: Antibacterial activity of *H. kraussii*. A solvent system of hexane and ethyl acetate (1:1) were used.

4.3.1 Purification of the extract

Separation of the extract was obtained with the silica column, and the different fractions were applied to a TLC plate. The fractions were concentrated as quickly as possible to prevent the compounds in the fractions from breaking down. After concentration, a few of the fractions showed crystal formation. These crystals were separated from the rest of the fractions and the separated crystals, and the fractions were developed on a TLC plate to evaluate their purity. From these TLC plates, similar compounds in the different fractions could be identified, and some of the fractions were combined. The combined fractions and crystals were then compared with the cell suspension culture fractions, to identify compounds that could be present in the plant, but absent from the cell suspension cultures (Figure 4.2). It looked as if there were indeed compounds present in both the plants and activated cell suspension culture that were not present in the cell suspension culture controls (indicated by the arrows). It was however, not very clear, and the similarities could also be accounted for by the concentration differences on the TLC plate.

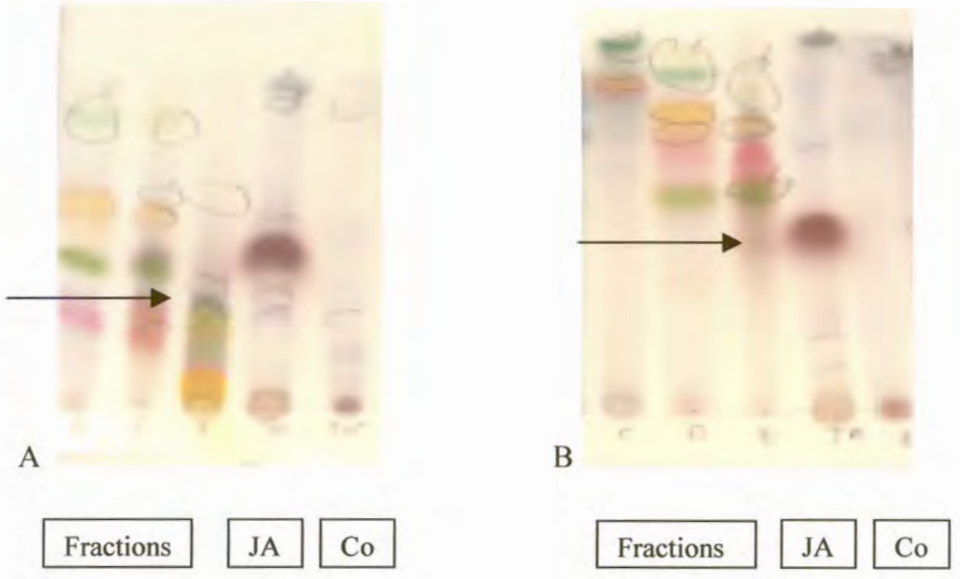


Figure 4.2: Comparison between fractions of the plant extract, activated cell suspension culture (JA) and cell suspension culture control extracts (Co). TLC plate A was developed with a solvent system of chloroform and methanol (95:5), and TLC plate B was developed with a solvent system of chloroform and ethyl acetate (1:1).

4.3.2 Identification of isolated compounds

Four different solvent systems were used to make sure that there was no possibility of compounds lying on top of each other with the use of a specific solvent system. Different fractions were combined when it contained the same pure compounds. Only two of the compounds seemed pure (indicated by the arrows in Figure 4.3), and the rest of the compounds contained varied amounts of impurities. Some of the compounds were purified after recrystallisation, and with some of the compounds it was necessary to do further separation on columns. Five pure compounds were further analysed by NMR, UV and MS (Figures 4.5–4.21) analysis. Four flavonoids were isolated and identified as

3,7-dihydroxy-5-methoxyflavone (1), 5,7-dihydroxy-3-methoxyflavone (2), 5-hydroxy-6,7,8-trimethoxy-flavone (alnetin) (3) and 5-hydroxy-3,7,8-trimethoxyflavone (methylgnaphaliin) (4). One terpenoid was also identified as pleuchiol (5).

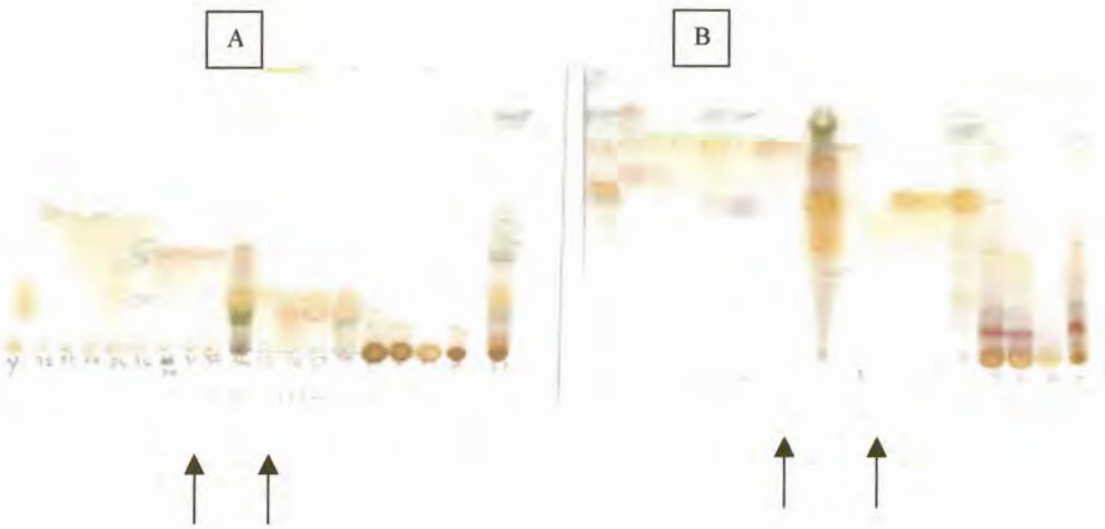


Figure 4.3: TLC plates developed with different solvents (pure compounds indicated by arrows). TLC plate A was developed with a solvent system of hexane and ethyl acetate (8:2), and TLC plate B was developed with a solvent system of chloroform and methanol (95:5).

4.3.2.1 3,7-Dihydroxy-5-methoxyflavone

Although the compound has been isolated previously, no published data on its biological activity could be found. The compound was isolated from *Pityrogramma triangularis* and *Populus gemma* (Wollenweber & Volker, 1981). The structure was confirmed by using ^{13}C-NMR and comparing the data for this compound to data obtained from the other compounds isolated.

Figure 4.4: Structure of 3,7-dihydroxy-5-methoxyflavone.

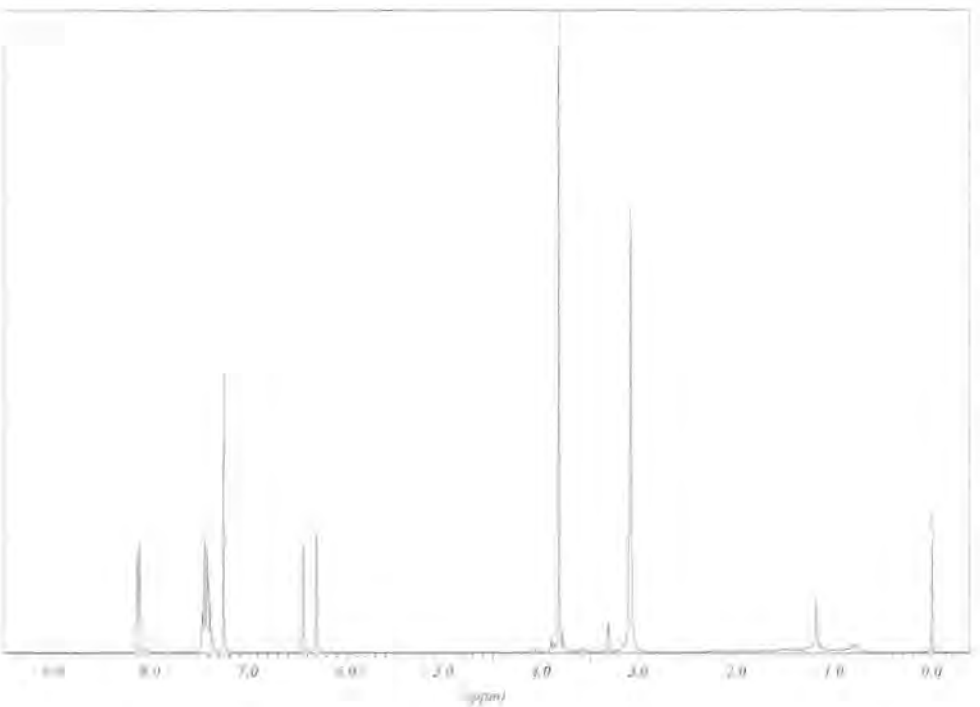


Figure 4.5: ^{1}H-NMR spectrum of 3,7-dihydroxy-5-methoxyflavone. ^{1}H-NMR (300 MHz): δ 8.12 (2H, m, H-2', H-6'), 7.45 (3H, m, H-3', H-4', H-5'), 6.44 (1H, d, J=2, H-6), 6.30 (1H, d, J=2, H-8), 3.8 (3H, s, 5-OMe).

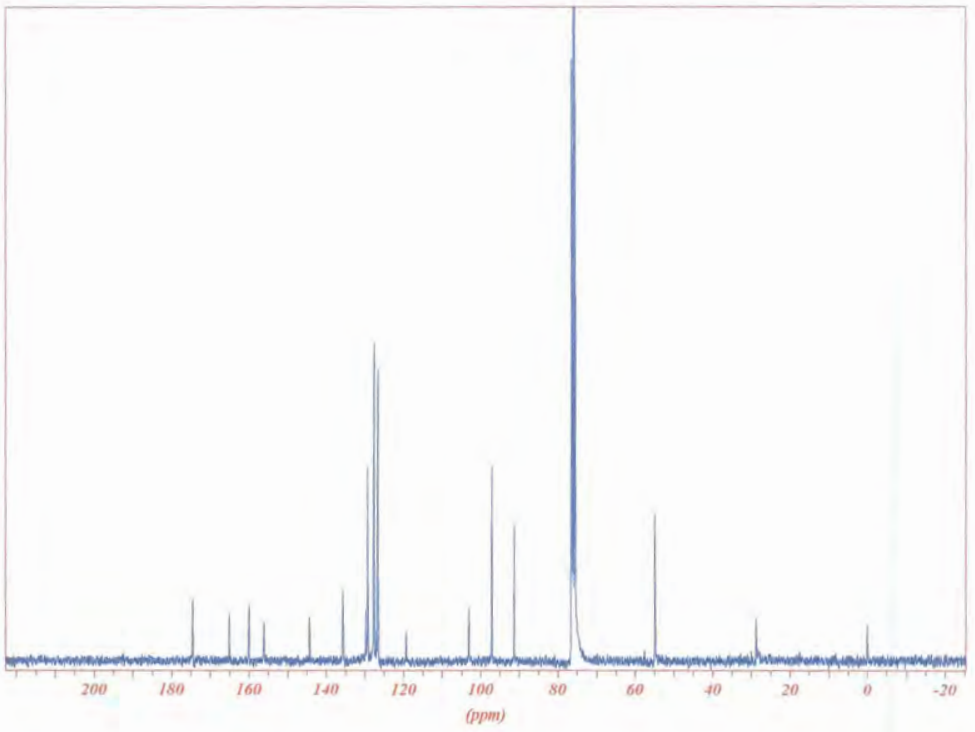


Figure 4.6: ^{13}C-NMR spectrum of 3,7-dihydroxy-5-methoxyflavone. ^{13}C-NMR (75 MHz): 174.5 (s), 165.0 (s), 160.0 (s), 156.0 (s), 144.3 (s), 135.6 (s), 129.8 (d), 127.6 (d), 119.5 (s), 103.0 (s), 97.0 (s), 91.3 (s), 76.2 (s), 76.0 (t), 54.8 (s), 28.7(s).

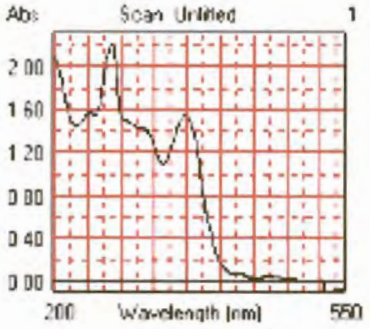


Figure 4.7: UV analysis of 3,7-dihydroxy-5-methoxyflavone. UV λ_{max} nm: 270 and 348

4.3.2.2 5,7-Dihydroxy-3-methoxyflavone

Limited published data is available on this compound that has been isolated from *Populus nigra* (Wollenweber & Volker, 1981). The data was also compared with the other isolated compound's data, and confirmed by ^{13}C-NMR.

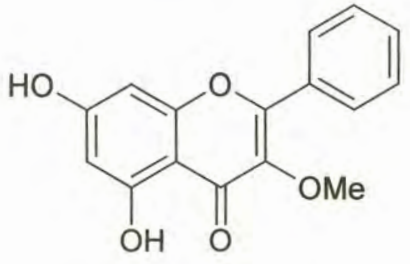


Figure 4.8: Structure of 5,7-dihydroxy-3-methoxyflavone.

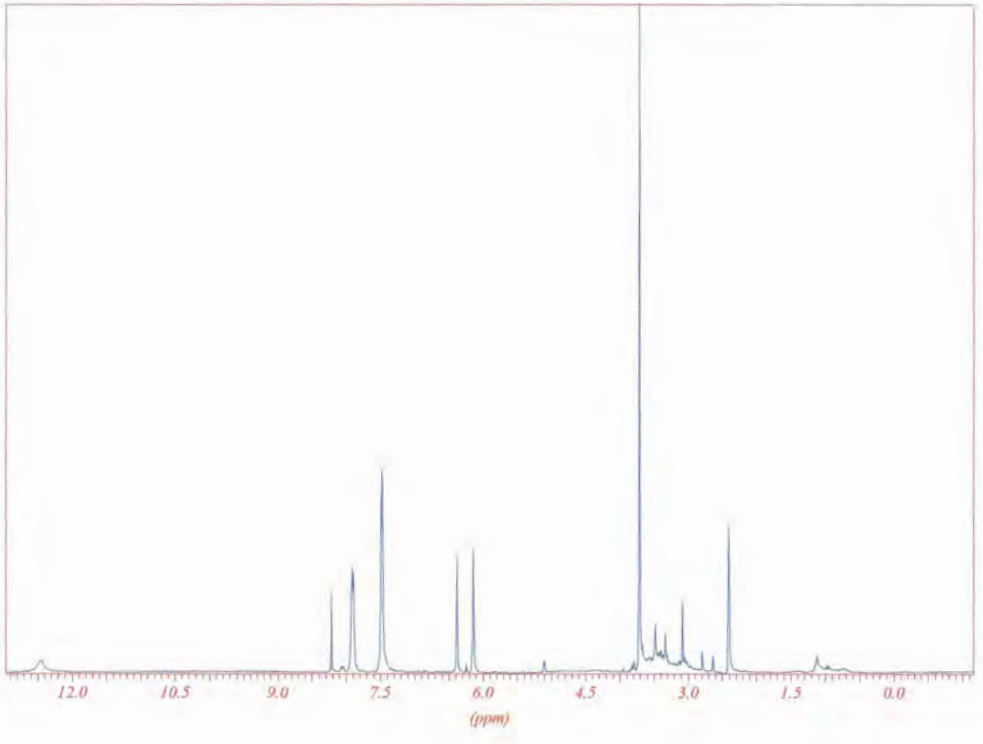


Figure 4.9: ^{1}H-NMR spectrum of 5,7-dihydroxy-3-methoxyflavone. ^{1}H-NMR (300 MHz): δ 12.5 (1H, s, OH-5), 7.90 (2H, m, H-2', H-6'), 7.47 (3H, m, H-3', H-4', H-5), 6.38 (1H, d, J=2, H-6), 6.14 (1H, d, J=2, H-8), 3.70 (3H, s, 3-OMe).

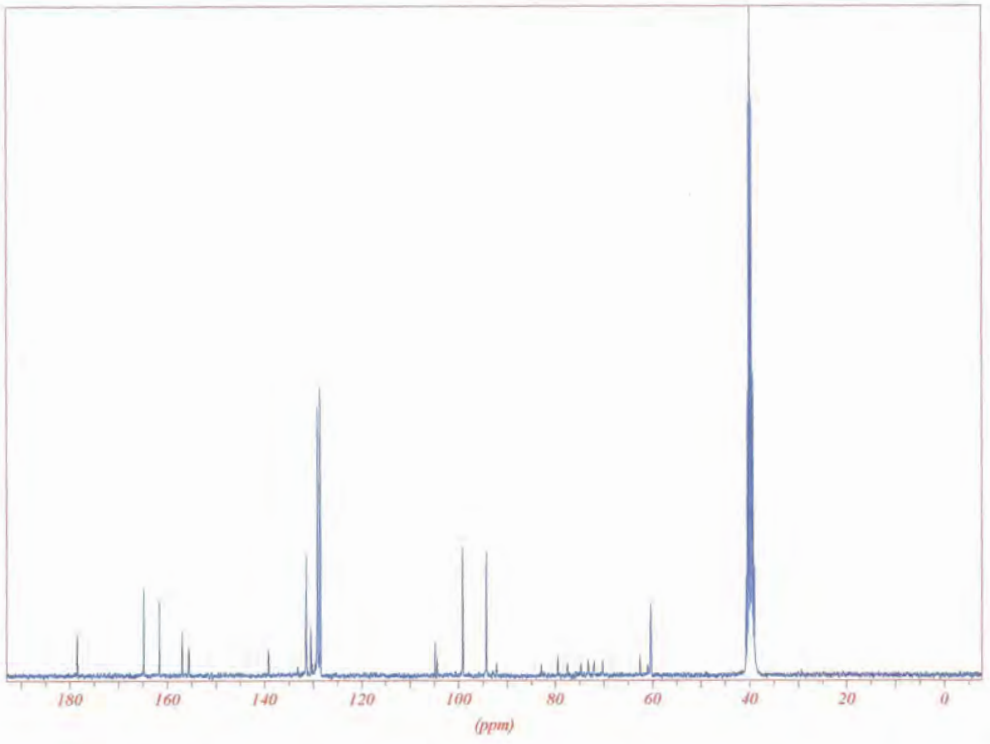


Figure 4.10: ^{13}C-NMR spectrum of 5,7-dihydroxy-3-methoxyflavone. ^{13}C-NMR (75 MHz): 60.36(s), 94.22 (s), 99.11 (s), 104.8 (d), 128.49 (s), 129.08 (s), 130.40 (d), 131.56 (s), 133.13 (s), 139.12 (s), 155.54 (s), 156.94 (s), 161.63 (s), 164.84 (s), 178.47 (s).

4.3.2.3 5-Hydroxy-6,7,8-trimethoxyflavone

The data obtained from NMR and UV analysis were compared with data previously published by Leong *et al.* (1998). The data was also compared to the data of the very similar structures isolated.

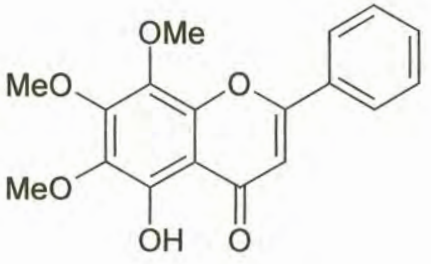


Figure 4.11: Structure of alnetin (5-hydroxy-6,7,8-trimethoxyflavone) (Leong, *et al.*, 1998).

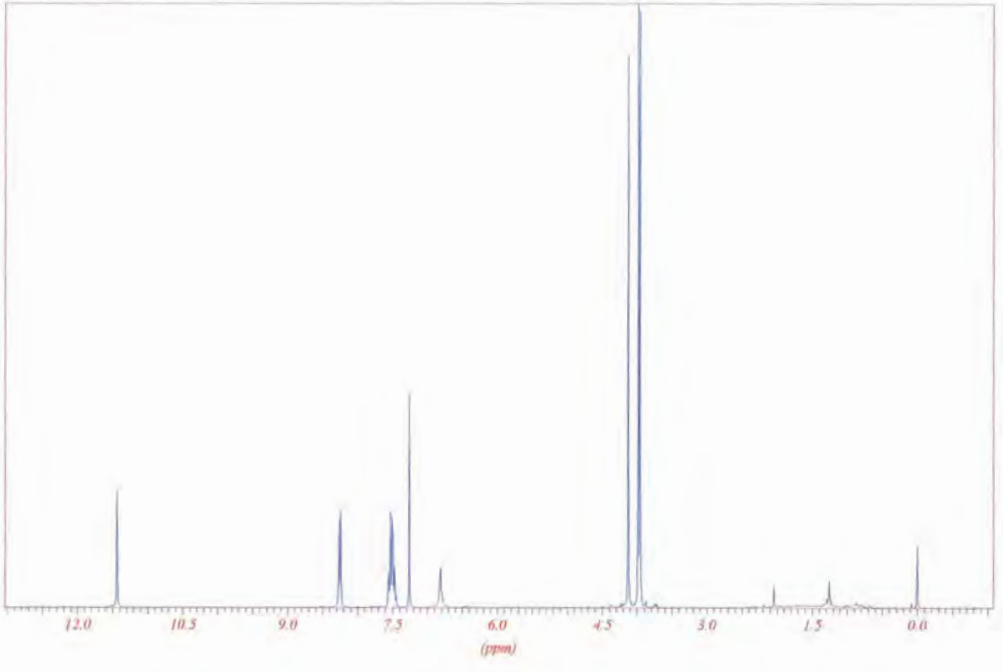


Figure 4.12: ^{1}H-NMR spectrum of alnetin. ^{1}H-NMR (300 MHz): δ 11.45 (1H, s, OH-5), 8.25 (2H, m, H-2', H-6'), 7.51 (3H, m, H-3', H-4', H-5'), 6.81 (1H, s, H-3), 4.12 (3H, s, 6-OMe), 3.98 (3H, s, 8-OMe), 3.96 (3H, s, 7-OMe).

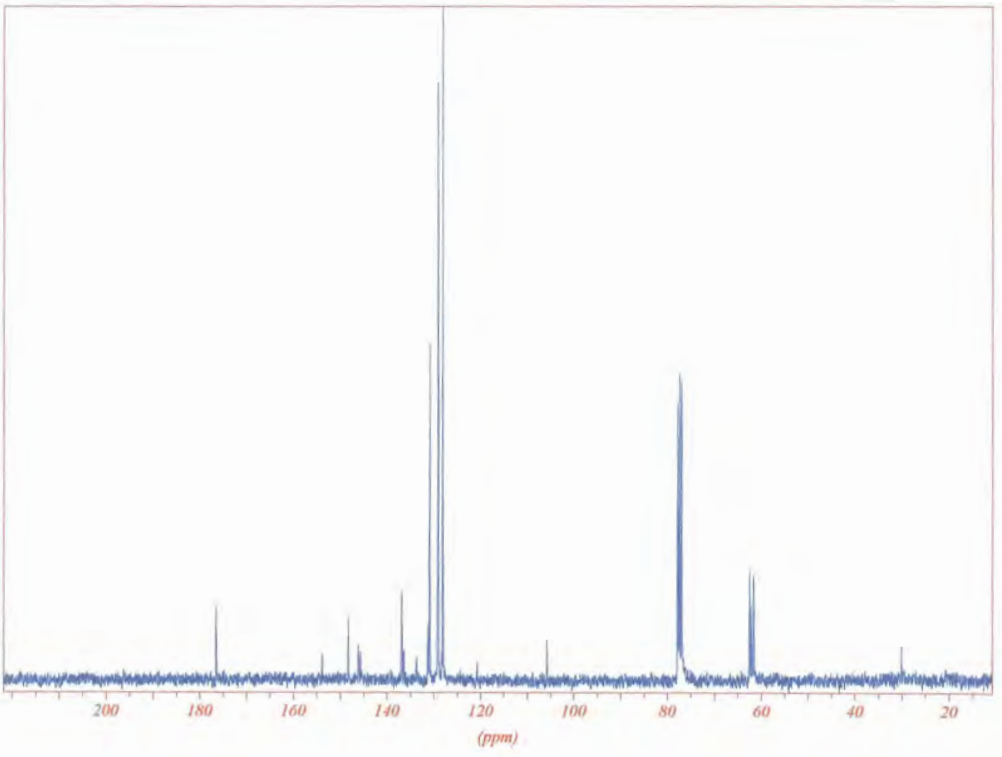


Figure 4.13: ^{13}C-NMR spectrum of alnetin. ^{13}C-NMR (75 MHz): 176.5 (s), 153.8 (s), 148.2 (s), 146.1 (s), 145.6 (s), 136.8 (s), 133.7 (s), 131.2 (s), 130.8 (s), 129.1 (d), 120.7 (s), 105.8 (s), 77.8 (d), 77.0 (d), 62.1 (s), 61.6 (d).

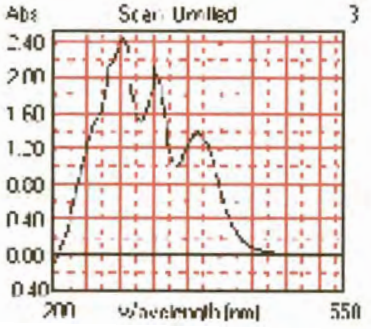


Figure 4.14: UV analysis of alnetin. UV λ_{max} nm: 282, 318 and 368.

4.3.2.4 5-Hydroxy-3,7,8-trimethoxyflavone

Only one published paper was found on the isolation of this compound from *Gnaphalium obtusifolium* (Hänsel & Ohlendorf, 1969). The ^{1}H-NMR and UV data were compared with the published data.

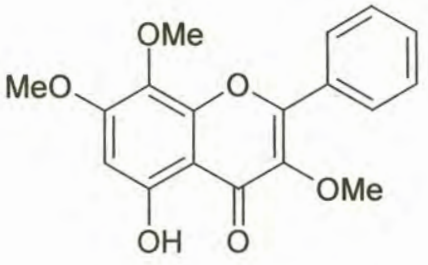


Figure 4.15: Structure of methylgnaphaliin (5-Hydroxy-3,7,8-trimethoxyflavone) (Hänsel & Ohlendorf, 1969).

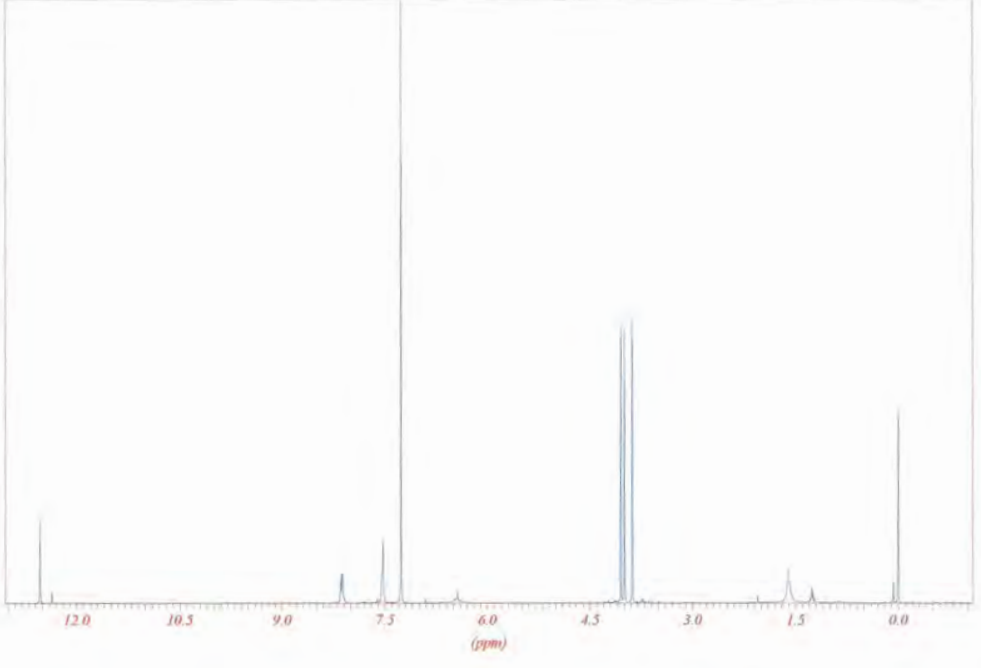


Figure 4.16: ^{1}H-NMR spectrum of methylgnaphaliin. ^{1}H-NMR (300 MHz): δ 12.53 (1H, s, OH-5), 8.13 (2H, m, H-2', H-6'), 7.54 (3H, m, H-3', H-4', H-5'), 6.43 (1H, s, H-6), 4.05 (3H, s, 3-OMe), 3.99 (3H, s, 7-OMe), 3.88 (3H, s, 8-OMe).

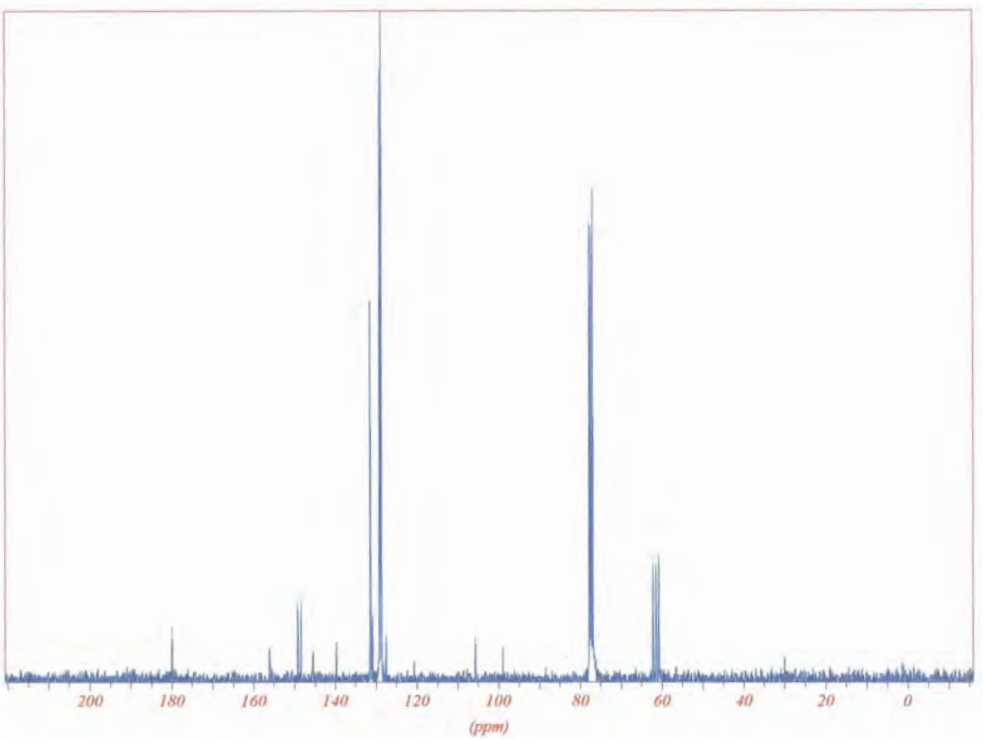


Figure 4.17: ^{13}C-NMR spectrum of methylgnaphaliin. ^{13}C-NMR (75 MHz): 179.9 (s), 156.1 (s), 149.2 (s), 148.4 (s), 145.4 (s) 139.7 (s), 131.4 (s), 130.9 (d), 128.9 (d), 127.6 (s), 120.7 (s), 105.6 (s), 98.9 (s), 77.4 (s), 77.0 (d), 61.8 (d), 60.7 (s), 30.0 (s).

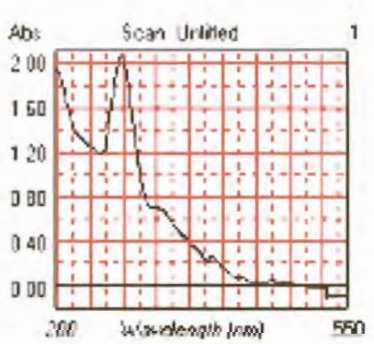


Figure 4.18: UV analysis of methylgnaphaliin. UV λ_{max} nm: 273 and 358

4.2.3.5 Pleuchiol

The data for pleuchiol had been compared with previously published data by Alam *et al.*, (1994) to confirm the structure. No published data is available on the biological activity of this compound.

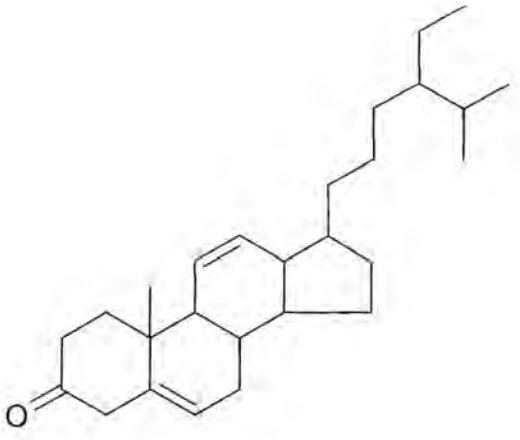


Figure 4.19: Structure of pleuchiol (Alam *et al*, 1994).

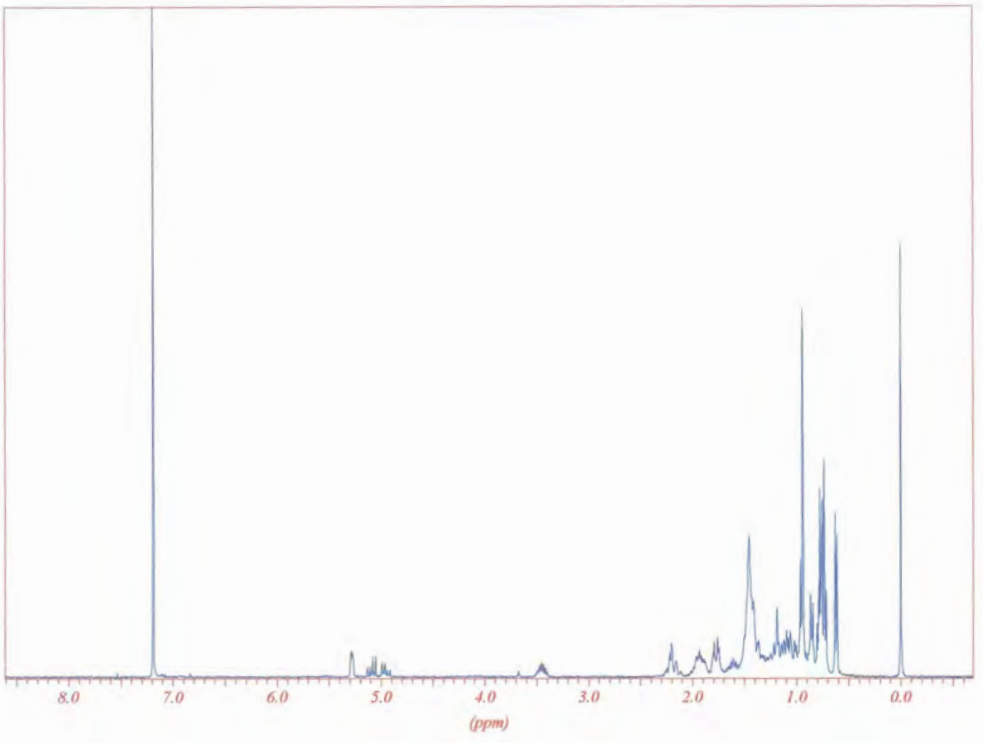


Figure 4.20: ^{1}H-NMR spectrum of pleuchiol.

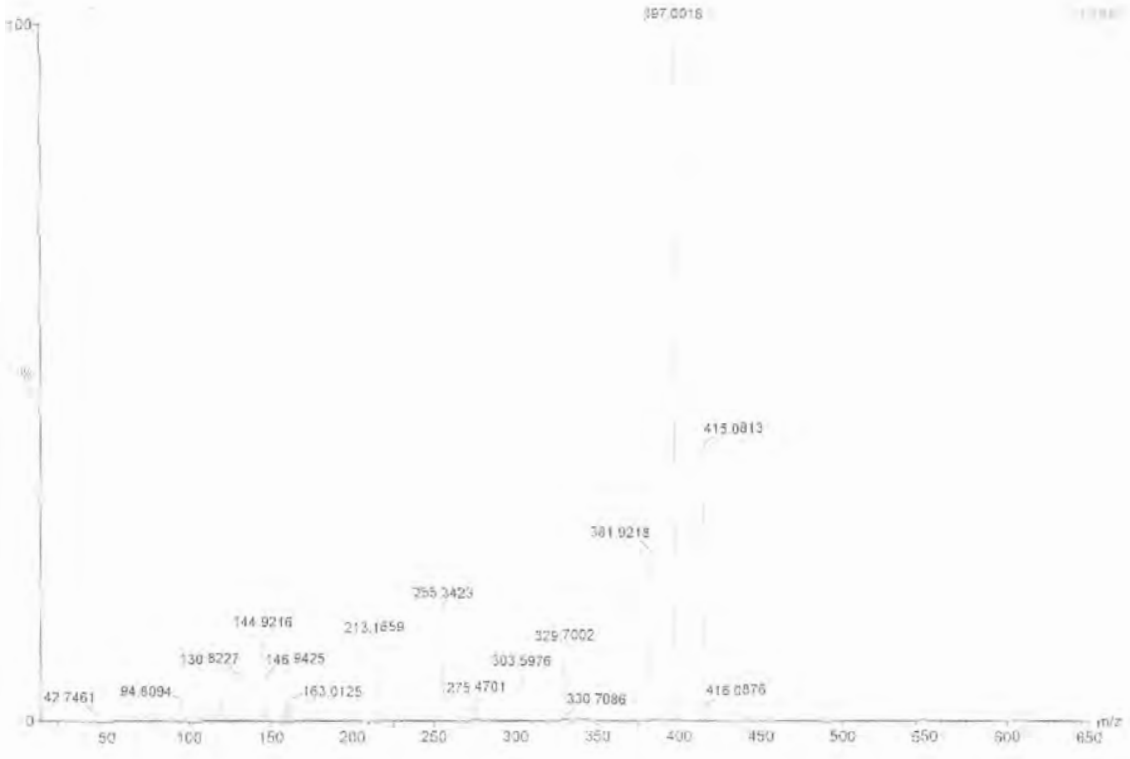


Figure 4.21: MS analysis of pleuchiol. MS *m/z*: 397, 394, 381, 351, 327, 300, 273, 271, 253, 229, 213, 164.

4.3.3 Antibacterial activity

The pure compounds were tested for biological activity against *B. cereus*. Not all of the compounds isolated from the extract possessed antibacterial activity against *B. cereus*, and most of the compounds only gave a slight indication of inhibition. Methylnaphaliin was however very active and inhibited the growth of bacteria very effectively and is showed in Figure 4.22.

Figure 4.22: Antibacterial activity of methylgnaphaliin.

4.4 Discussion

The extract obtained from plants from the field contained similar compounds when compared to the cell culture extracts. Fewer compounds were produced in cell cultures. In the extract obtained from the wild plants, many more compounds are present, and often in much higher concentrations than the cell culture material. This is in accordance with the findings of Stafford & Warren (1991), where many differences between cell cultures and wild plants were found.

The phytoalexins found in the cell cultures were not present in the wild plants. It is possible that the plants were not stressed, and probably only produce them when needed. This is in accordance with Stoessl (1980) and many others who stated that the production of phytoalexins is an economical mechanism for defence. This however, meant that we could not obtain enough of the compounds for a full bioactivity analysis. As mentioned

before, it is extremely time consuming to produce plant material in cell culture–especially if compounds in low concentrations need to be isolated from the material.

Although the two compounds were not found in the wild plants, a number of other interesting compounds were isolated from the extract, and valuable experience was obtained with the isolation, separation and identification of these compounds.

A lot of intact plant material was available for this part of the study, and with the use of a big column, enough plant material could be separated to obtain sufficient amounts of the compounds isolated. Four flavonoids and one terpenoid were isolated successfully and identified from *H. kraussii*.

The two flavonoids 3,7-dihydroxy-5-methoxyflavone and 5,7-dihydroxy-3-methoxy-flavone are not well-known compounds, and very little information is available on these compounds. It is known that hydroxyl groups at positions 3 and 5 are essential for high-affinity binding to a plasma membrane glycoprotein (P-gp). P-gp acts as a pump by extruding anticancer compounds and preventing their antitumour effect. Multidrug resistance phenotypes have been correlated with increased expression of this protein. It has also been shown that a hydrophobic substituent on the B-ring considerably enhanced the binding affinity, and eventually inhibiting protein function (Boumendjel, *et al.*, 2001).

Alnetin is a flavonoid isolated previously from *Alnus sieboldiana* (Betulacaea) (Asakawa, 1971). It has also been isolated from *Lindera lucida* (Leong, *et al.*, 1998). Related

compounds were isolated from *Helichrysum nitens* (Tomas-Barberan, *et al.*, 1988), and these compounds had an inhibitory affect against fungal growth. All these compounds isolated were of external occurrence (glandular trichomes) that could possibly support the defence system of these plants.

Methylgnaphaliin was the only isolated compound to show antibacterial activity, and from the bioassays it seems as if it is effective in very low concentrations. Only one publication was found reporting on the isolation of this compound from *Gnaphalium obtusifolium* (Hänsel & Ohlendorf, 1969). No biological activity for it has been described before, and more antimicrobial work could lead to interesting results.

Chapter 5

Conclusion

Chapter 5

Conclusion

Tissue- and cell suspension cultures were used in this study to propagate *Helichrysum kraussii* and to manipulate its metabolic processes. Tissue cultures were initiated fairly easy with the use of a medium described by Dilika & Meyer in 1998. The tissue cultures were subcultured frequently to ensure optimum growth, and were used to produce cell suspension cultures. The same medium was used, only the agar component was omitted. The cell suspension cultures were subcultured when the medium changed to a darker brown colour, or if the cell production was so much that the movement of the shaker did not aerate the cells sufficiently. The maintenance of cell cultures is a very labour-intensive technique, and a lot of factors play a role in the successful growth of the cultures. It was however a very useful technique in this study as it provided a stable and controlled environment that would not have been the case with a study of plants in the field.

It was not necessary to infect the plant material with pathogens, or spray the elicitors on the leaf area of the plant. With the addition of the jasmonic acid to the cultures, a pathogen attack was mimicked, and the same results were probably obtained that would have been the case with an infection in the field.

After an introduction to the *Helichrysum* genus, and phytoalexins in the first two chapters, the third chapter discussed the activation of the cell cultures and the production of phytoalexin precursors and other phytoalexins. It also discussed the isolation of two key compounds in the production of phytoalexins, and the redirection

of the mevalonic biosynthetic pathway towards phytoalexin production. α- and β-amyrin are found throughout the plant kingdom. The compounds are also used to synthesise several other important triterpenes. Both compounds were shown to have antibacterial activity against *B. cereus* and functions as phytoalexins in *H. kraussii.* The most important function of the amyrins is the synthesis and production of other phytoalexins in the plant. Fulton *et al.* (1994), Trojanowska (2001) and several other authors illustrated that the amyrins are the first compounds to be produced in the redirected mevalonic biosynthetic pathway, producing phytoalexins in stead of the normal production of phytosterols.

Several analyses were used to elucidate the structures of α- and β-amyrin and involved the extensive use of ^{1}H-NMR, ^{13}C-NMR and GC-MS. Several problems were encountered, for instance the amount of compound to be used for each analysis. ^{13}C-NMR needs more (about 12 mg) of the compounds for an analysis than is the case with ^{1}H-NMR, where only a very small amount is needed. It was therefore not possible to analyse α-amyrin with ^{13}C-NMR. The results of the different analysis were carefully interpreted and compared to published data to obtain the structures of the two compounds.

Jasmonic acid was used to activate the resistance system of the plant. Although it is not sure which type of activation would lead to the production of jasmonic acid in the plant, it is clear that jasmonic acid plays a very important role in resistance of the plant. Jasmonic acid was effectively used in the activation of the resistance system, and also the production of the resistance products in the plant. The results suggest that jasmonic acid had directly or indirectly an effect on certain enzymes in the

mevalonic acid pathway. If compared to the results from Fulton *et al.* (1994) in activated cell cultures of *T. divaricata* (Apocynaceae), it is probable that the same inducing activity is present in *H. kraussii* plants to produce the two amyrins. The high concentration of the compounds in comparison to the other compouns produced in the activated cultures is supporting the fact that these compounds are important compounds for the synthesis of other phytoalexins to be produced upon activation. The resistance products involves the production of at least two terpenoids, and although only the two amyrins were isolated in this study, there were more compounds produced after activation that could have been studied and identified.

The whole idea of plants—representing lower life forms on earth—having their own "immune system", switched on and off as it is necessary, is a very interesting and intelligent mechanism. This mechanism ensures survival at an economical level to maintain plant processes. These compounds are not always present in the plant, and are therefore a target for the production of novel compounds, and also the production of novel antimicrobial compounds. Although only two compounds have been identified, more compounds have been produced upon elicitation of the cultures. The applications and uses of phytoalexins could be an interesting study in future. The applications of these compounds in finding solutions for both human and plant diseases could be one of the main focus areas of phytoalexin research in future.

The aim of the study described in chapter four was to determine if the compounds produced in cell culture were present in the extract obtained from the plants from the field. It would have been much cheaper, time- and labour efficient to isolate these compounds from the plants from the field, because of the low concentration present in

the cell cultures, and the mass of cultures available. The phytoalexins were however not found in the plants from the field. It was therefore not possible to isolate these compounds from the plant extracts, and more of these compounds could only be obtained by the activation of the cell cultures. It is however a confirmation that these compounds are not normally present in the plant. It is also possible that these compounds might have been present in the plant extract—but as expected—it was in such low concentrations that it was not detectable.

The compounds isolated from the intact plants—four flavonoids and one terpenoid—were isolated with a silica column and identified with the use of ^{1}H-NMR, ^{13}C-NMR, MS and UV analyses. All the data obtained from the different techniques were compared with previously published data, to confirm the structures of these compounds. All five compounds have been isolated before, although most of these compounds have not been tested for biological activity.

Methylgnaphaliin was the only compound effective against *B. cereus*. Alnetin and other related compounds have been tested previously and these compounds were very effective against fungal growth. It is therefore still a possibility that the compounds isolated might have biological activity, but not against *B. cereus*.

Many of the mechanisms involving phytoalexins are not well understood, and the toxicity, physiological and biochemical uses still need a lot of attention. The isolation and identification of these compounds in this plant gives an idea of the widespread production of phytoalexins by plants growing in the field.

As was stated by Morrissey & Osbourn in 1999, the production of phytoalexins was much higher, and more rapid in resistant plants, than in susceptible plants. They also observed different phytoalexins being produced in cultivated *Brassica* species in comparison to wild brassinin members. This supports the fact that these compounds might be an important link in the solution to pathogen infection of crops—and could also find their way into curing human diseases and combating pathogens.

One can conclude that many interesting compounds have been isolated and classified as phytoalexins. The uses and effects of these compounds on cultivated and wild plants are getting more attention today, and application of these mechanisms could maybe lead to a solution to fight parasites and pests on commercially important plants.

Chapter 6

Summary

Summary

In vitro production of phytoalexins by *Helichrysum kraussii*

by

Gerhard Prinsloo

Supervisor: Prof. J. J. M. Meyer

Degree: Magister Scientiae

Many *Helichrysum* species have been screened and tested for biological activity because of their uses to cure many diseases. *H. kraussii* Sch. Bip. has been used to cure several illnesses, for instance coughs and tuberculosis (Van Wyk *et al.*, 1997).

This study focused on the production of phytoalexins in *H. kraussii*. To activate the resistance system of the plant and to initiate the synthesis of resistance compounds, jasmonic acid was used as an elicitor. Jasmonic acid is a well-known signaling compound, and its involvement in the resistance system of plants is not a new concept.

To eliminate the risk of attack and activation of plants in the field, tissue cultures were initiated with the aim of producing cell suspension cultures, which in turn made the uptake of jasmonic acid by the plant material more effective. Different time intervals and concentrations of jasmonic acid were tested to determine the strongest activation.

Two phytoalexins: α-amyrin and β-amyrin, were subsequently isolated from the activated cell suspension cultures. These compounds are important for the production

of other triterpenoid phytoalexins, and are the first compounds of the redirected biosynthetic pathway leading to phytoalexin production. This supports the findings that the normal mevalonic acid pathway that produce phytosterols are inhibited, which leads to increased production of the amyrins to synthesise other phytoalexins. These compounds were also tested against bacteria to determine if they are antibacterial. They were indeed active against *B. cereus*, which supports the definition of phytoalexins being antimicrobial compounds in the resistance system.

The biological activity of *H. kraussii* plants obtained from the field was also studied. It was necessary to relate the production of phytoalexins back to the plants in the field, and isolate the compounds from the plant material in future. The isolation from intact plant material, would have been more time- and labour efficient than isolation form cell culture material. The phytoalexins were however not present, but several compounds were isolated from the plants. The compounds isolated—one terpenoid and four flavonoids—were tested for the possibility of biological activity against bacteria. Variable results were obtained, with some compounds totally inhibiting the growth of the bacterium *B. cereus*.

Chapter 7

Summary in Afrikaans

Opsomming

In vitro produksie van fitoaleksiene in *Helichrysum kraussii*

deur

Gerhard Prinsloo

Promotor: Prof. J.J.M. Meyer

Graad: Magister Scientiae

'n Groot aantal *Helichrysum* Mill. spesies is al getoets vir biologiese aktiwiteit omdat die plant tradisioneel gebruik word vir die behandeling van verskeie siektes. *H. kraussii* Sch. Bip. is hoofsaaklik gebruik in die behandeling van hoes en tuberkulose.

In hierdie studie is die produksie van fitoaleksiene in *H. kraussii* ondersoek. Om die weerstandsisteem van die plant en sintese van weerstandsverbindings te inisieer is jasmoonsuur gebruik as 'n elisitor. Jasmoonsuur is 'n bekende verbinding in die vervoer van seine in die plant, en die verbinding se betrokkenheid in die weerstandsisteem van die plant is nie meer 'n nuwe konsep nie.

Om die kompleksiteit van die omgewing van die plante in die veld te elimineer, is weefselkulture geïnisieer met die oog op die produksie van selsuspensie kulture. Verskillende tydsintervalle en konsentrasies van jasmoonsuur is getoets om die meeste aktivering te bepaal.

Twee fitoaleksiene naamlik α-amirien en β-amirien is geïsoleer van die geaktiveerde selkultuur plantmateriaal. Die terpenoïede is belangrike verbindings betrokke by die sintese van ander triterpenoïed fitoaleksiene, en is ook die eerste verbindings wat gevorm word as deel van die gewysigde biosintetiese pad wat verantwoordelik is vir fitoaleksien produksie. Dit ondersteun ook die bevindinge dat die mevaloniensuur pad wat normaalweg fitosterole vorm, geïnhibeer word, en dan lei tot 'n verhoogde produksie van amiriene, en later ook ander fitoaleksiene. Die geïsoleerde verbindings is ook getoets teen bakterieë, om die biologiese aktiwiteit van die verbindings te bepaal. Die verbindings het aktiwiteit getoon teen die groei van *B. cereus*, en ondersteun die definisie van fitoaleksiene dat hulle antimikrobiese verbindings is, en deel vorm van die weerstandsisteem van plante.

Om die produksie van fitoaleksiene in verband te bring met die produksie in die plant, is plante in die veld versamel om te bepaal of die fitoaleksiene in die plante voorkom. Dit sou baie makliker, goedkoper en meer tyd effektief gewees het, as die verbindings uit die plante in die veld versamel en geïsoleer kon word. Die verbindings is nie gevind nie, en tydens die proses is verskeie ander verbindings geïsoleer. Die geïsoleerde verbindings—een terpenoïed en vier flavonoïede—is getoets vir die moontlikheid van biologiese aktiwiteit teen bakterieë. Van die verbindings wat geïsoleer is, was hoogs effektief in die inhibering van die groei van die bakterieë *B. cereus*.

Chapter 8

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Chapter 9

References

References

ABRAHAM, K. J., PIERCE, M. L. & ESSENBERG M. 1999. The phytoalexins deoxyhemigossypol and hemigossypol are elicited by *Xanthomonas* in *Gossypium* cotyledons. Phytochemistry, Vol. 52, pp. 829–836.

AEXEL, R. T., RAMSEY, R. B. & NICHOLAS, H. J. 1972. Sterols and diterpenes of *Pelargonium hortorum*. Phytochemistry, Vol. 11, pp. 2353–2354.

AGRIOS, G. N. 1997. Plant pathology. Fourth edition. Academic press. New York.

ALAM, S., CHOPRA, N., ALI, M., NIWA, M. & SAKAE, T. 1994. Ursane and sterol derivatives from *Pleuchea lanceolata*. Phytochemistry, Vol. 37, No. 2, pp. 521–524.

ALAMI, I., CLERIVET, A., NAJI, M., VAN MUSTER, M. & MACHEIX, J. J. 1999. Elicitation of *Platanus X acerifolia* cell-suspension cultures induces the synthesis of xanthoarnol, a dihydrofuranocoumarin phytoalexin. Phytochemistry, Vol.51, pp. 733–736.

ASAKAWA, Y. 1971. Chemical constituents of *Alnus sieboldiana* (Betuleaceae). The isolation and structure of flavonoids and stilbenes. Bulletin of the chemical society of Japan, Vol. 44, pp. 2761–1766.

BEELD. Draai rem aan met antibiotika of keer terug na donker verlede. 10 August 2001, p. 11. Berig deur Liezel de Lange.

BOUGASTOS, C., MEYER, J. J. M., MAGIATISM P., VAGIAS, C. & CHINOU, I. B. 2002. Composition and antimicrobial activity of the essential oils of *Helichrysum kraussii* and *H. rugulosum* from South Africa. Flavour and Fragrance Journal. In press.

BOUMENDJEL, A., BOIS, F., BENEY, C., MARIOTTE , A-M., CONSEIL, G. & DI PIETRO, A. 2001. B-ring substituted 5,7-dihydroxyflavonols with high-affinity binding to P-glycoprotein responsible for cell multidrug resistance. Bioorganic & medicinal chemistry letters 11, pp. 75–77.

BREMNER, P. D. & MEYER, J. J. M. 2000. Prenyl-butyrylphloroglucinol and kaurenoic acid: Two antibacterial compounds from *H. kraussii*. South African Journal of Botany, Vol. 66(2), pp. 115–117.

CABELLO-HURTADO, F., DURST, F., JORRIN, J. V. & WERCK-REICHHART, D. 1998. Coumarins in Helianthus tuberosus: characterization, induced accumulation and biosynthesis. Phytochemistry, Vol.49, pp. 1029–1036.

DANIEL, M. & PURKAYASTA, R. P. 1995. Handbook of phytoalexin metabolism and action. Marcel Dekker Inc., New York.

DE MAYO, P. 1959. The higher terpenoids. The chemistry of natural products, Volume III. Interscience Publishers, Inc. New York.

DILIKA, F. & MEYER, J. J. M. 1996. Antibacterial activity of *Helichrysum pedunculatum* used in circumcision rites. Journal of Ethnopharmacology, Vol. 53, pp. 51–54.

DILIKA, F. & MEYER, J. J. M. 1998. Antibacterial activity of *Helichrysum pedunculatum* callus cultures. South African Journal of Botany, Vol. 64 (5), pp. 312–313.

DIXON, R. A. & HARRISON, M. G. 1994. Early events in the activation of plant defense responses. Annual review of phytopathology, Vol. 32, pp. 479–501.

DOCHERTY, J. J., FU, M. M. H., STIFFLER, B. S., LIMPEROS, R. J., POKABLA, C. M. & DE LUCIA, A. L. 1999. Resveratrol inhibition of herpes simplex virus replication. Antiviral Research, Vol. 43, pp. 145–155.

DUNIGAN, D. D., WATERS, S. B. & OWEN, T.C. 1995. Aqueous soluble tetrazolium/Formazan MTS as an indicator of NADH-and NADPH-dependent dehydrogenase activity. BioTechniques 19:640–649.

ELLARD-IVEY, M. & DOUGLAS, C. J. 1996. The role of jasmonates in the elicitor- and wound-inducible expression of defense genes in Parsley and transgenic Tobacco. Plant Physiology Vol. 112, p 183–192.

FERRERO, M. E., BERTELLI, A. A. E., FULGENZI, A., PELLAGATTA, F., CORSI, M.M., BONFRATE, M., FERRARA, F., DE CATERINA, R., GIOVANNINI, L. & BERTELLI, A. 1998. Activity in vitro of resveratrol on granulocyte and monocyte adhesion to endothelium. American Journal of Clinical Nutrition, Vol. 68, pp. 1208-1214.

FULTON, D. C., KROON, A. P. & THRELFALL, D. R. 1994. Enzymological aspects of the redirection of terpenoid biosynthesis in elicitor-treated cultures of *Tabernaemontana divaricata*. Phytochemistry, Vol. 35, No. 5, pp. 1183–1186.

FULTON, D. C. & THRELFALL, D. R. 1993. 2,3-Oxidosqualene-cyloartenol cyclase activity is not inhibited in elicitor-treated tobacco cultures. Phytochemistry, Vol. 32, No. 3 pp. 705–707.

GEORGE, E. F. 1996. Plant propagation by tissue culture. Part 2. In practice. Second edition. Exegetics Ltd. England.

GRAVER, R. J. & KOKUBUN, T. 2001. Review. Plant-fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochemistry, Vol 56 (3), pp 253–263.

HAMBURGER, M. O., & CORDELL, G. A. 1987. A direct bioautographic TLC assay for compounds posessing antibacterial activity. Journal of Natural Products, Vol., No. 1, pp.19–22.

HÄNSEL, R. & OHLENDORF, D. 1969. Im B-ring unsubstituierte flavone aus *Gnaphalium obtusifolium*. Tetrahedron letters No.6, pp. 431–432.

HILLIARD, O. M. 1983. In: Flora of Southern Africa. Ed. O.A. Leistner, Vol. 33, part 7 (Asteraceae), pp. 93–95. Botanical Research Institute of Southern Africa.

HOLMES-McNARY, M. & BALDWIN, A. S. 2000. Chemopreventive properties of *trans*-resveratrol are associated with inhibition of activation of the IkB kinase. Cancer Research, Vol. 60, pp. 3477–3483.

HUTCHINGS, A. & VAN STADEN, J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine1. Plants used for headaches. Journal of Ethnopharmacology, 43, pp. 89–124.

HYVARINEN, M., KOOPMANN, R., HORMI, O. & TUOMI, J. 2000. Phenols in reproductive and somatic structures of lichnens: A case of optimal defence? Oikos, Vol. 91 (2), pp. 371–375.

INGHAM. 1973. Disease resistance in higher plants. The concept of pre-infectional and post-infectional resistance. Phytopathologische Zeitschrift 78, pp. 314–335.

ISHIHARA, A., OHTSU, Y, & IWAMURA, H. 1999. Biosynthesis of oat aventhramide phytoalexins. Phytochemistry, Vol.50, pp. 237–242

KNIGHT, S. A. 1974. Carbon-13 NMR spectra of some tetra- and pentacyclic triterpenoids. Organic Magnetic Resonance, Vol. 6, pp. 603–611.

LAHLOU, H. E., HIRAI, N., TSUDA, M. & OHIGASHI, H. 1999. Triterpene phytoalexins from nectarine fruits. Phytochemistry, Vol.52, pp. 623–629.

LEONG, Y-W., HARRISON, L. J., BENNETT, G. J., KADIR, A. A. & CONNOLLY, J. D. 1998. A dihydrochalcone from *Lindera lucida*. Phytochemistry, Vol 47, No. 5, pp. 891–894.

LINDE, H. 1979. Über inhaltsstoffe der perikarpen von *Sapindus mukorossi* Gaertn. Archiv der Pharmazie (Weinheim), Vol. 312, pp. 416–425.

MANNA, S. K., MUKHOPADHYAY, A. & AGGARWAL, B. B. 2000. Resveratrol suppress TNF-induced activation of nuclear transcription factors NF-kB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. The Journal of Immunology, Vol. 164, pp. 6509–6519.

MATHEKGA, A. D. M. & MEYER J. J. M. 1998. Antibacterial activity of South African *Helichrysum* species. South African Journal of Botany 1998, 64 (5) 293–295.

MORRISSEY, J. P. & OSBOURN, A. E. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiology and molecular biology reviews. Vol. 63, No.3, pp. 708–724.

MÜLLER, K. O. & BÖRGER, H. 1940. Experimentelle untersuchungen – ber die Phytophtore-Resistenz der Kartoffel. Arbeiten aus der Biologischen Reichsanstalt für Land-und Forstwirtschaft, Berlin-Dahlem 23, pp. 189–231.

OSBOURN, A. E. 1999. Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal genetics and biology, Vol.26, pp. 163–168.

PEDRAS, M. S. C., OKANGA, F. I., ZAHARIA, I. L. & KHAN, A. Q. 2000. Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation. Phytochemistry, Vol. 53, pp. 161–176.

RANG, H. P., DALE, M. M. & RITTER, J. M. 1995. Pharmacology. International student edition. Third edition. Churchill Livingstone.

SCHENTAG, J. J. 2000. Sparfloxacin: A review. Clinical therapeutics, Vol. 22 (4), pp. 372–387.

SEO, S., TOMITA, Y. & TORI, K. 1975. Carbon-13 NMR spectra of urs-12-enes and application to structural assignments of components of *Isodon japonicus* Hara tissue cultures. Tetrahedron Letters, No. 1, pp. 7–10.

SIMONSEN, J. & ROSS, W. C. J. 1957. The Terpenes. Volume IV. Cambridge at the university Press. London.

SOUTH AFRICAN TUBERCULOSIS ASSOCIATION. 1998. Tuberculosis. Sunsite, Southern Africa.

STAFFORD, A. & WARREN, G. 1991. Plant cell- and tissue culture. Open university press, Biotecnology series, Manchester.

STOESSL, A. 1980. Phytoalexins – a biogenetic perspective. Phytopathologische zeitschrift 99, pp. 251–272.

TAIZ , L. & ZEIGER, E. 1998. Plant physiology, second edition. Sinques Associates, Inc. Sunderland, Massachusetts.

TAN-YU, T., TILLETT, D. J. & McKAY, I. A. 2000. Molecular strategies for overcoming antibiotic resistance in bacteria. Molecular medicine today, Vol. 6 (8), pp. 309–314.

TEBAYASHI, S., ISHIHARA, A., TSUDA, M. & IWAMURA, H. 2000. Induction of clovamide by jasmonic acid in red clover. Phytochemistry, Vol. 54, pp. 387–392.

TOMAS-BARBERAN, F. A., MSONTHI, J. D. & HOSTETTMANN, K. 1988. Antifungal epicuticular methylated flavonoids from *Helichrysum nitens*. Phytochemistry, Vol. 27, No. 3, pp. 753–755.

TROJANOWSKA, M. R., OSBOURN, A. E., DANIELS, M. J. & THRELFALL, D. R. 2001. Investigation of the avenacin-deficient mutants of *Avena strignosa*. Phytochemistry, Vol. 56, pp. 121–129.

VAN LOON, L. C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. European Journal of Plant Pathology, Vol. 103, pp. 753–765.

VAN PUYEVELDE, L., DE KIMPE, N., COSTA, J., MUNYJABO, V., NYIRANKULIZA, S., HAKIZAMUNGU, E. & SCHAMP, N. 1989. Isolation of flavonoids and a chalcone from *Helichrysum odoratissimum* and synthesis of helichrysetin. Journal of Natural Products, 52(3), pp. 629–633.

VAN WYK, A. E. & MALAN, S. 1998. Field guide to the wild flowers of the highveld. Struik, Pretoria.

VAN WYK, B-E., VAN OUDTSHOORN, B. & GERICKE, N. 1997. Medicinal plants of South Africa. Briza, Pretoria, pp. 148-149.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. The medicinal and poisonous plants of Southern and Eastern Africa. E. & S. Livingstone Ltd., Edinburgh and London.

WOLLENWEBER, E. & VOLKER, H.D. 1981. Review. Occurrence and distribution of free flavonoid aglycones in plants. Phytochemistry, Vol. 20, pp. 869–932.

ZINNER, S. 2000. New pathogens in neutropenic patients with cancer: An update for the new millennium. 2000. International journal of antimicrobial agents, Vol. 16 (2), pp. 97–101.