ANTIBACTERIAL ACTIVITY OF PLANTS THAT ARE USED IN THE TREATMENT OF HEARTWATER IN LIVESTOCK AND THE ISOLATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS FROM *PETALIDIUM OBLONGIFOLIUM* AND *IPOMOEA ADENIOIDES*

By

Phatlane William Mokwala

Promoter: Prof. JJM Meyer

Submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy in the Faculty of Natural and Agricultural Sciences

Department of Botany

University of Pretoria

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ABSTRACT

The general antibacterial activity of *Drimia delagoansis, Petalidium oblongifolium* and *Ipomoea* adenioides was determined using selected Gram-positive and Gram-negative bacteria. Only extracts or compounds with high antibacterial activity were then tested against the causative agent of heartwater, *Ehlrichia ruminantium*, since the latter requires specialised culturing conditions. The crude aqueous extract of *D. delagoansis* had low antibacterial activity with its highest MIC against Gram-negative bacteria being 20.0 mg ml^{-1} while the crude methanolic extracts of *P. oblongifolium* and *I.* adenioides had their highest antibacterial activity against Gram-negative bacteria at MIC's of 5.0 and 10.0 mg ml^{-1} respectively. Two compounds were isolated and identified from *I. adenioides* and an unidentified one was isolated from *P. oblongifolium*. The two compounds from *I. adenioides* proved to be caffeic acid with MIC's of 0.8 and 1.0 mg ml^{-1} against Gram-positive and Gram-negative bacteria respectively; and ethyl caffeate with MIC's of 0.4 and 1.0 mg ml^{-1} against Gram-positive and Gram-negative bacteria respectively. Synergism between the two compounds increased the respective MIC's to 0.4 and 0.2 μg ml^{-1} against Gram-positive and Gram-negative bacteria. The unidentified compound isolated from *P. oblongifolium* had a very low MIC of 2.5 μg ml^{-1} against *E. ruminantium*.

Dedicated to:

My wife and the children for their un-ending support

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CHAPTER 1

Plant Uses in Ethnoveterinary Medicine

1.1 **Introduction**

Human beings owe their existence, to a great extent, to plant life. In a normal day, they are fed, clothed, beautified, transported and sheltered by plants. Through the ages plants enabled the human being to survive the wrought of the elements: droughts, floods, famines, plagues etc. The uses of plants in various applications differ among different cultures due to availability of different kinds of plants in different parts of the world (Simpson and Ogorzaly 1995). The study of how different cultures use plants is called ethnobotany. Development occurred through the human being's improvement of the uses and utensils derived from plants.

1.2 **Medicinal uses of plants**

Plants have been used as medicine from time immemorial and in several developing countries they are still the mainstay of health care (Hoareau and Da Silva 1999). According to Weiner and Weiner (1994) herbal medicine was human's first line of defense before the dawn of history. We learnt the use of plants from instinct and from the observation of animals and birds. Through trial humans learnt which plants and preparations served them best. The knowledge was transferred to helping others and eventually rescuing his animals. Initially plants were collected from the wild. Later the plants were planted in herbal gardens and ultimately pharmaceutical companies isolated bioactive compounds from plants and then synthesised them chemically.

According to Hoareu and Da Silva (1999), developed countries are turning to the use of traditional medicinal systems, with about 1 400 herbal preparations being in use. Despite the progress in synthetic chemistry and biotechnology, plants are still an indispensable source of both preventative and curative medicine (Srivastava *et al.* 1996).

1.2.1 Ethnoveterinary plant use

The high incidence of stock diseases is one of the principal constraints to African smallholder livestock systems (Guèye 1997). This holds true with other developing countries. Traditional animal health care practices, referred to as ethnoveterinary medicine, provide low cost alternatives in situations where Western type drugs and veterinary services are not available or are not affordable (Mathias 1996). The practice is widespread among resource-poor small-scale farmers in rural areas of the world. In a survey of the rural areas of the Eastern Cape Province of South Africa, Masika *et al.* (2002) found that 73% of the small-scale farmers interviewed used ethnoveterinary medicine. According to Mathias (1996), ethnoveterinary medicine consists of local people's knowledge pertaining to animal health and production. This involves recognition of sick animals and knowing which plants to use and how to prepare them.

Advantages of the use of ethnoveterinary medicine are: they are often cheaper than comparable Western drugs; locally available and easily accessible; and culturally appropriate and understood (Mathias 1996).

Some of the disadvantages of ethnoveterinary medicine are: inconvenience involved in the use or preparation of certain remedies; cures are variable in their effectiveness according to season; seasonal availability of certain medicinal plants; ineffectiveness against viral diseases; difficulty of standardisation (volumes and concentrations often variable); existence of harmful components or practices (Mathias 1996, Masika *et al.* 2000, Fielding 2001).

In developed or Western countries synthetic drugs are used for the treatment of animal diseases. Even in underdeveloped countries the same types of drugs are used, at times in combination with traditional medicines. Some developed countries make money available for the improvement of the health of livestock in other underdeveloped countries. At times donor aid can be used to maintain inappropriate and unsustainable systems as long as the funds continue to flow (Fielding 2001). When the financial support is terminated, economic reality prevails. The cost of the treatment is compared to the cost of the animal being treated. This leads to farmers resorting to traditional medicine or diluting drugs which then become inefficient. Therefore, ethnoveterinary medicine will remain part of farming systems in the world for some time.

People in developed or Western countries have become concerned about their health, the food they eat and the environment they live in. This has resulted in the rising of organic food production systems. These systems are regulated through guidelines laid down by government agencies like the United States Department of Agriculture (USDA) in the USA and the Advisory Committee on Organic Standards (ACOS) in the UK. The guidelines prohibit the use of pesticides, herbicides, antibiotics and hormones in food (animal and plant) production systems. In some instances only minimal uses are allowed. This necessitates the use of plant derived medicinal products. Homeopathic and other alternative therapies are encouraged. Ethnoveterinary medicine will play an important role in this regard. The cultivation of medicinal plants in rotationally grazed pastures where animals self-medicate, has also been suggested (Minar 2002).

1.3 **Livestock diseases**

Livestock like all other animals, are affected by many diseases. Some of the diseases are caused by: bacteria, viruses, parasites (internal and external) as well as by poisoning. External parasites include blood-sucking organisms like

ticks, flies, maggots etc. Tick-borne diseases are those whose causative agents are transferred by ticks from one animal to another. One of the tick-borne diseases is gallsickness. The name gallsickness, as used by farmers, usually refers to any of the three diseases: heartwater, anaplasmosis and redwater (Mönnig and Veldman 1981). Common symptoms among the three diseases are: anemia, high fever, rapid breathing and in postmortem analysis a distended gallbladder (from which the name derives). These diseases all affect ruminants. Heartwater is caused by the rickettsial bacterium *Ehrlichia ruminantium* (formally *Cowdria ruminantium*) and is transmitted by three-host ticks that belong to the genus *Amblyoma* (Mönnig and Veldman 1981, Bezuidenhout 1994).

Anaplasmosis, which is also called true gallsickness or tick-borne gallsickness, is also caused by rickettsial bacteria belonging to the genus *Anaplasma.* Its vector is the tick *Boophilus decoloratus*. On the other hand, redwater is a disease of cattle. It is caused by the protozoa *Babesia bigemia* and *B. bovis*. It is transmitted by the blue tick (Mönnig and Veldman 1981).

1.3.1 Heartwater

Heartwater (cowdriosis) is an acute infectious and noncontagious tick-borne disease of domesticated and wild ruminants (cattle, sheep, goats, deer, antelope and buffalo).

Symptoms of heartwater include: fever, loss of appetite, respiratory distress, nervousness (inco-ordination), upwards-tilting head, paddling and high-stepping gait movements, rigid posture, hydrothorax, ascites, edema of the lungs and hydropericardium (accumulation of fluid in the sac surrounding the heart), from which the name derives (Bezuidenhout 1994).

1.3.1.1 The causative agent of heartwater

The disease is caused by the rickettsial bacterium *E. ruminuntium*. It belongs to the order Rickettsiales, family Rickettsiaceae, tribe Ehrlichieae and genus *Ehrlichia* (Bezuidenhout *et al*. 1994). The rickettsial bacteria are obligate intracellular Gram-negative bacteria found in ticks, fleas, mites, chiggers and mammals. They are nonsporing, non-acid fast, short rods (0.3 – 10.5 μm by 0.8 – 2.0 μm), can be pleomorphic and they are non-motile. They are aerobes and grow exclusively intercellularly or in close association with eukaryotic cells. The bacteria are found in the endothelial cells of the brain and of capillaries. They usually occur in clumps of five to several thousand organisms. The colonies are usually found close to the nucleus (Bezuidenhout *et al*. 1994).

A B

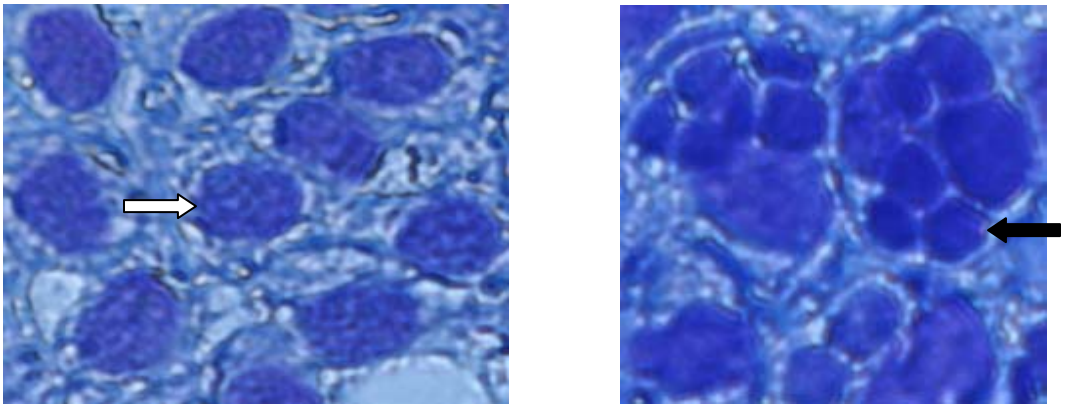

Figure 1.1 Endothelial cells **A** not colonized and **B** colonised by *E. ruminantium*. In **A** the arrow (⇨) shows the nucleus of an uninfected endothelial cell and in **B** the arrow points at colonies of *E. ruminantium* inside an infected cell (➡).

E. ruminantium isolates are normally referred to as stocks and not strains, a terminology used for trypanosoma populations, because they have not been fully characaterised. The bacterial stock is usually named after the farm or country from which it has been isolated. For example, Gardel stock from Guadeloupe, Caribbean Islands; Nigerian D225 stock from Nigeria and Welgevonden stock

from Welgeveonden farm in the Limpopo province of South Africa (Bezuidenhout *et al*. 1994)

The rickettsial bacteria are classified according to their morphology, ecology, epidemiology and their clinical characteristics. It has recently been suggested that these bacteria be re-classified based on information from genetic studies on their 16S rRNA and surface proteins. *Ehrlichia ruminantium* was formerly known as *Cowdria ruminantium* (Dumbler *et al.* 2001, Paddock 2003). The following species fall within the amended genus *Ehrlichia*: *E. canis, E. chaffeensis, E. ewingii, E. muris and E. ruminantium*. They are about 98% similar in their 16S rRNA sequences and all reside and multiply in cytoplasmic vacuoles of host cells (Paddock and Childs 2003).

1.3.1.2 The heartwater vector

Heartwater is transmitted by ticks that belong to the genus *Amblyoma*. These are four-host ticks whose life cycle takes one to four years to complete. The major vectors are *A. variegatum* (with wide distribution in Africa), *A. hebraeum* (which is the main vector in southern Africa) and *A. lepidum* (in East Africa and Sudan) (Bezuidenhout *et al*. 1994). The disease is transmitted by the nymphal and adult stages. The nymph feeds on wild ungulates, ground birds, small mammals, reptiles and amphibians.

1.3.1.3 Distribution of heartwater

The disease occurs only where its vectors occur naturally. It occurs wide-spread in sub-Saharan Africa (Figure 1.2) and the eastern Caribbean Islands. In South Africa heartwater occurs in the northern provinces (Limpopo province and the northern part of North-west province as well as along the eastern coast down to Eastern Cape province (Figure 1.3).

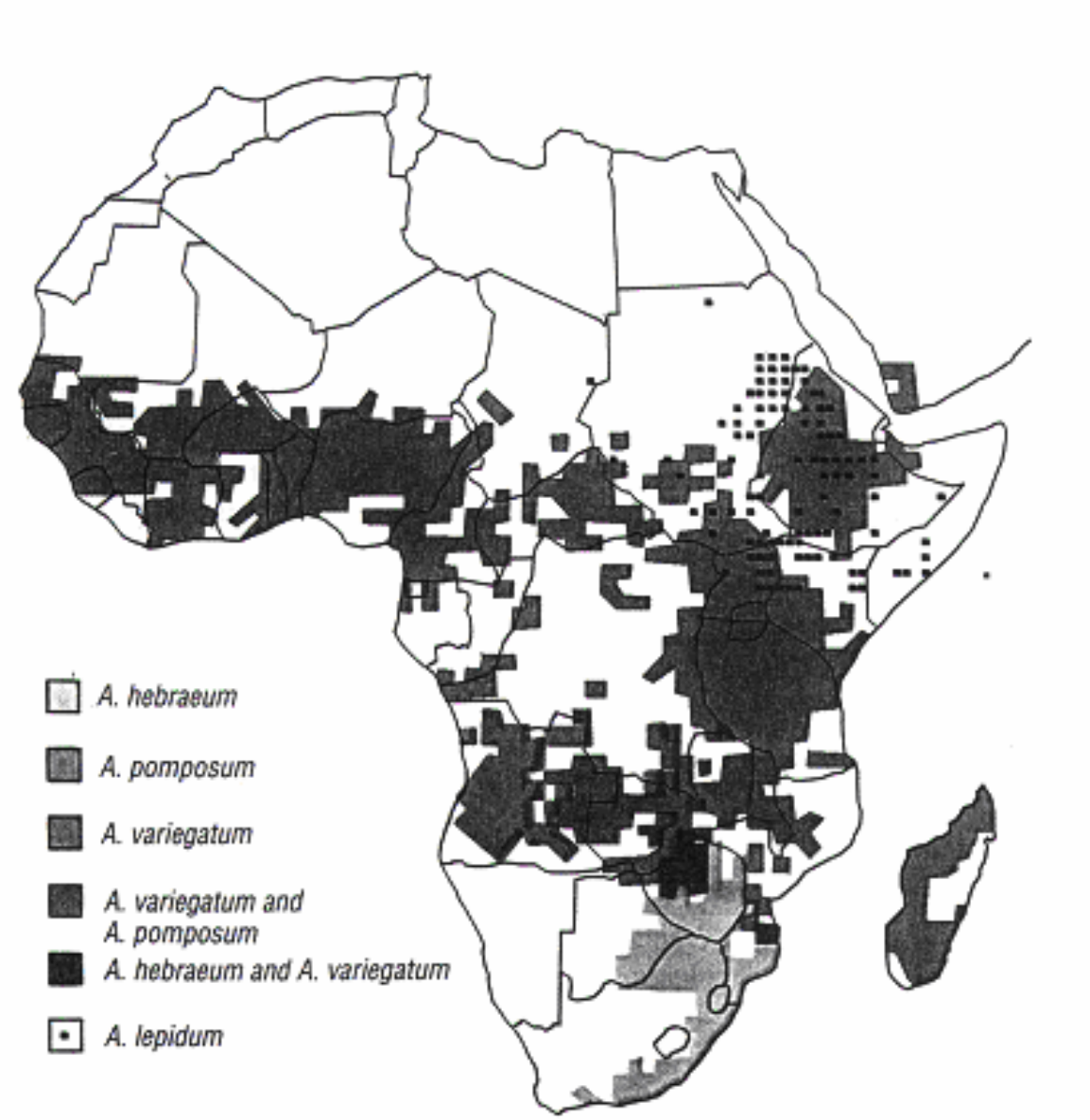

Figure 1.2: Distribution of the host ticks for *E. ruminantium* in Africa (Bezuidenhout *et al.* 1994).

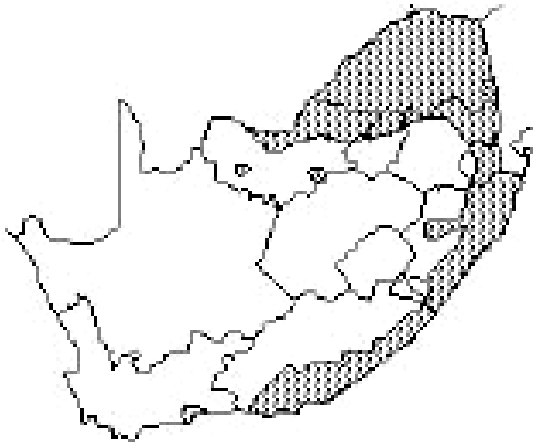

Figure 1.3: Occurrence of heartwater in South Africa [Information Directorate; SA National Department of Agriculture]

1.3.1.4 Diagnosis of heartwater

There are a number of symptoms that can indicate that an animal is infected with heartwater. However, some of the symptoms can be confused with those caused by other diseases like rabies and tetanus. DNA-based probes, especially those based on the polymerase chain reaction (PCR) (Peter *et al.* 2000) and enzyme-linked immuno-sorbent assays that use two major antigenic protein 1 (MAP 1) antigens can reveal the presence of *E. ruminantium* in the blood of heavily infected animals. Only the presence of the causative organisms in brain smears can act as a confirmation of infection with heartwater (Bezuidenhout *et al.* 1994, World Organisation for Animal Health 2004). There is no adequate serodiagnostic test developed yet.

1.3.1.5 Control of heartwater

Heartwater is mainly controlled through: tick control, vaccination and drug administration. Though the causative agent is fragile and does not survive outside the host, the disease is difficult to eradicate through dipping of livestock. Complete vector elimination is problematic because the tick uses different hosts

to complete its life cycle. Further, vaccinated animals need to be challenged (bitten by ticks) in order to boost their immunity. This requires presence of some ticks in the veld.

Tetracycline drugs are quite effective against *C. ruminantium*. When the drugs are administered before signs appear, they suppress the disease and allow development of immunity. One disadvantage of relying on the use of drugs is that most often signs are observed when the disease has already reached advanced stages.

A vaccine, in the form of the inactivated bacterium, has not been developed yet. Immunisation involves the "infection treatment" method. This involves the administration of reacting or infected blood from sheep or cattle. This form of vaccination prompts the animal to develop immunity against the introduced organism. It requires a constant monitoring of the animals following vaccination and to immediately administer a drug when an animal shows signs of infection. Another disadvantage of this form of vaccination is that the vaccine (infected blood) needs to be used within eight hours of packing. This becomes problematic for farmers in rural areas. According to the World Organisation for Animal Health (2004) a first generation vaccine is currently being developed. The vaccine consists of elementary bodies of *E. ruminantium* emulsified in Montanide ISA 50 adjuvant. This has given promising results under controlled experimental conditions and is being evaluated in the field.

1.4 **Medicinal plant use in the control of heartwater and related diseases in South Africa**

It has been observed that some small-scale or subsistence livestock farmers use plants to treat their livestock against heartwater and related sicknesses (gallsickness). Some farmers in the Limpopo province use *Drimia delagoansis* (formerly *Urgenia lydenburgensis*) to treat heartwater in goats and kids. Fresh

bulbs of the plant are macerated in water and soaked overnight. The extract is then administered orally (personal observation).

Small-scale farmers in the Eastern Cape province of South Africa use a number of plants for the treatment of heartwater, redwater and anaplasmosis (Masika *et al.* 2000, Dold and Cocks 2001) examples are: *Arctotis arctotoides, Cussonia spicata, Ledebouria revolute, Heteromorpha trifoliata, Podocarpus latifolius, Verninia mespilifolia.* Few of these plants have proven antibacterial activity. However, according to Masika *et al.* (2000) most of the plants used have purgative properties. The belief is that it washes off excess bile from the animal's body.

The shrub, *Petalidium oblongifolium,* is utilised in the Limpopo province, for its medicinal properties. Livestock (cattle and goats) and game browse on the shrub especially in autumn and winter when grazing becomes scarce. Some farmers claim that the animals cure themselves when they browse on the plant. Livestock, as well as game, thrives in areas where this plant occurs. Other farmers believe that it is only the nutritional properties of the plant that make the animals thrive. *P. oblongifolium* occurs most of the time in association with *Ipomoea adenioides*. However, *I. adenioides* is rarely eaten by animals. It appears as though it might be browsed only in "self-medication".

1.5 **Self-medication in animals**

Animals seem to know sometimes how to take care of themselves. In a study of the African great apes, Huffman (1997) noticed that these animals ingest certain non-nutritional or nutrition poor parts of plants in order to medicate themselves. The self-medicative behaviours of the animals include: bitter pith chewing, leaf swallowing, fur rubbing and geophagy (Huffman 1997). Self-medication in animals also called "zoopharmacognosy" (Huffman 2001) is the use of plants by animals for the treatment as well as prevention of diseases. The efficacy of

plants in animal self-medication can be scientifically proved and the plants then used in the treatment of animals in organic production systems. Huffman (2001) cites an example where chimpanzees in Tanzania chew the bitter-pith of *Vernonia amygdaline* when infected with the nodular worm (*Oesophagostomum stephanostomum*). The chimpanzees were found to recover 20 – 24 hours after ingestion of the bitter-pith and the number or worm eggs per gram of chimpanzee faeces dropped from 130 to 15 during the same period. *V. amygdaline* is used ethnobotanically for the treatment of schistosomiasis, amoebic dysentery and for internal parasites in humans (Huffman 2001). Phytochemical analysis of the plant showed it to have two classes of compounds: sesquiterpene lactones and steroid glucosides. The sesquiterpene lactones have anthelmic, antiamoebic, antitumour and antibiotic properties. An interesting observation in the study mentioned is that the active compounds occur in large quantities in leaves which the chimpanzees avoid. This shows that they selectively use the pith to avoid poisoning. Krief *et al*. (2004) isolated two novel limonoids from the methanolic extract of the leaves of *Trichilia rubescens*. The leaves of this plant were also observed to be taken by chimpanzees in self-medication. The methanolic extract of the plant as well as the isolated compounds had anti-malarial activity *in vitro*. The crude extract had IC_{50} of 12.0 $\mu g\ ml^{-1}$ while the isolated compounds, trichirubine A and trichirubine B had IC_{50} values of 0.3 and 0.2 $\mu g\ ml^{-1}$ respectively.

1.6 **The future of ethnoveterinary medicine**

The use of ethnoveterinary medicine is gaining momentum in both developed and undeveloped countries due to health concerns and availability, as well as affordability of synthetic drugs. This requires that their effectiveness be proved and their application be validated as is the case with Western medicines.

1.7 **Structure of thesis**

The aim of the study was to determine if extracts from ethnobotanically-selected plants and compounds isolated from these have *in* vitro activity against heartwater-causing bacteria. The thesis is composed of six chapters. Chapter 1 is an introduction on plant uses in ethnoveterinary medicines and on heartwater. Chapter 2 deals with antibacterial activity of the aqueous extract of *Drimia delagoansis*, while chapters 3 and 4 deal respectively with the antibacterial activity and isolation of bioactive compounds from *P. oblongifolium* and *Ipomoea adenioides*. Chapter 5 is a general discussion and chapter 6 contains the summary.

1.8 **Hypothesis**

The hypothesis of this study is: *Ethnobotanically-selected plant extracts and compounds isolated from them do inhibit the growth of both Gram-positive and Gram-negative bacteria*, *including the causative agent for heartwater.*

1.9 **Objectives of the study**

Objectives of the study were to:

- Determine antibacterial activity of crude extracts from selected plants against some Gram-positive and Gram-negative bacteria.
- Isolate bioactive compounds from extracts that show high antibacterial activity.
- Determine antibacterial activity of isolated compounds against selected Gram-positive and Gram-negative bacteria.
- Determine antibacterial activity of isolated compounds with high activity against the causative agent of heartwater.

1.10 **References**

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CHAPTER 2

The Antibaterial Activity of *Drimia delagoensis*

2.1 Introduction

Drimia delagoensis Bak. (formerly *Urginea lydenburgensis* R.A. Dyer) is a small bulbous plant belonging to the family Hyacinthaceae (formerly the Liliaceae) and the subfamily Urgineoidea. The genera within the subfamily are poorly defined (Brink and Dold 2003). Jessop (1997) suggested synonymy between the genera Urginea and Drimia. According to Pohl *et al*. (2001), *Urginea* is a heterogeneous genus that is poorly understood and in need of revision. Luyt *et al.* (1999) stated that the genera *Drimia* and *Urginea*, are closely related and are often used as synonyms. In a revision of these genera, Manning and Goldblatt (2003) suggest that *U. lydenburgensis* be renamed *D. delagoensis*. It has an ovoid or pyriform bulb (Figure 2.1), occurs solitarily or in clusters, with the pink bulb being underground and having one or two semi-circular to cylindrical leaves (Jessop 1977, Retief and Herman 1997).

According to Jessop (1977), *D.delagoensis* occurs in the lowveld regions of the Limpopo and Mpumalanga provinces of South Africa (formerly Transvaal), KwaZulu-Natal province and Swaziland.

Figure 2.1: *Drimia delagoensis*

Subsistence farmers in some rural areas (Nebo district) of the Limpopo province use it in the treatment of heartwater in goats and goat kids. Fresh bulbs are macerated in water and soaked overnight. The extract is administered orally to goats. This is usually done when kids start to show symptoms of heartwater.

2.1.1 Bioactive compounds in the subfamily Urgineoidea

According to Pohl *et al.* (2001) plants that belong to the subfamily Urgineoidea are phytochemically characterised by the presence of the cardiac glycosides, bufadienolides. Cardiac glycosides of the bufadienolide type found in *U. maritima* are: scilliroside (industrially used as a rodenticide, Figure 2.2), scillare-3-O-beta-glucoside, proscillaridine A, scilliphaeoside-3-O-beta-D-glucose, scilliglaucoside, and scilliphaeoside. *U. maritima* also contains calcium oxalate crystals. The Cardiac glycosides of *U. numidica* (also of the bufadienolide type) include: proscillaridine A, scilliphaeosidine, 12-epi-scilliphaenosidine and scilliglaucoside (Pascal-Villalobos 2002).

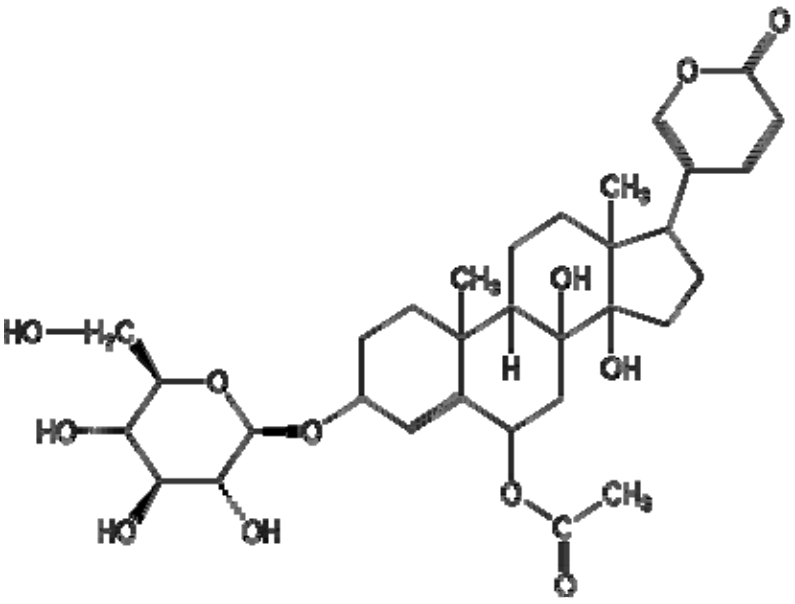


Figure 2.2: Structure of scilliroside, a bufadienolide (Wood 2005)

Cardiac glycosides affect the cardiovascular, neurological and gastrointestinal systems. The worst effect is on the cardiac system. They bind to a site on the cell membrane where they produce a reversible inhibition of the sodium (Na^+)-potassium (K^+) ATPase pump. This results in an increase in intracellular sodium

ions and a decrease in intracellular potassium ions. This in turn results in elevated sodium-calcium ion exchange. The overall effect is increased cardiac contractions (Gao *et al*. 2002, Marx *et al.* 2005). Cardiac glycosides also have vagotonic effects that cause cardiac blocks.

U. maritima (L.) Baker (common name, squill) is also used in veterinary homoeopathy. A diluted tincture (1: 1 000) of a methanolic extract of the bulb is administered orally to food-producing animals. According to the European Medicines Agency, dosage should depend on the patterns of clinical signs and body mass of the animal (EMEA 1999). Standardised squill powder has an oral LD_{50} value of 100 – 500 mg/kg body mass for cattle and 250 – 500 mg/kg body mass for sheep. *U. sanguinea* poisoning in humans, affect the gastro-intestinal tissues, the urinary and the central nervous system (Foukaridis *et al.* 1995, Marx *et al.* 2005). There is a lack of published information on the use of *D. delagoensis.*

2.2 Materials and Methods

2.2.1 Bacteria

The bacteria that were used as test organisms were obtained from the department of Microbiology and Pathology, University of Pretoria. These were Gram-positive (*Bacillus cereus, Bacillus subtilis, Bacillus pumilus and Staphylococcus aureus*) and Gram-negative (*Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Serratia marcescens*). They were maintained on a Nutrient Agar medium (Biolab).

2.2.1.1 Bacterial cultures for antibacterial activity

An inoculum for antibacterial activity was prepared by culturing each type of bacteria in 50 ml nutrient medium (Biolab). The bacterial cultures were shaken

for 24 hours on a horizontal shaker at 150 rounds per minute. For inoculation 50 μl of the bacterial cultures was pipetted into 50 ml fresh nutrient medium. These were then streaked on the nutrient agar that was mixed with plant extract or bioactive compound.

2.2.1.2 Bacterial cultures for bioautography

Bacteria (*B. cereus)* were cultured in 50 ml nutrient medium as above and shaken for 24 hours (2.2.1.1). Each culture was divided into two portions in centrifuge tubes and centrifuged for 20 minutes at 3 000g (Dilika *et al*. 2000). One of the pellets was shaken in 50ml fresh nutrient medium. Fresh cultures were used for spraying onto thin layer chromatograms (TLC).

2.2.2 Plant material

Fresh bulbs of *D. delagoensis* were collected on the farm Proberen 785 in Nebo district of the Limpopo province. A specimen (PRU #93536) was identified by the South African National Botanical Institute (SANBI) in Pretoria and deposited at the herbarium of the University of Pretoria.

2.2.3 Extraction

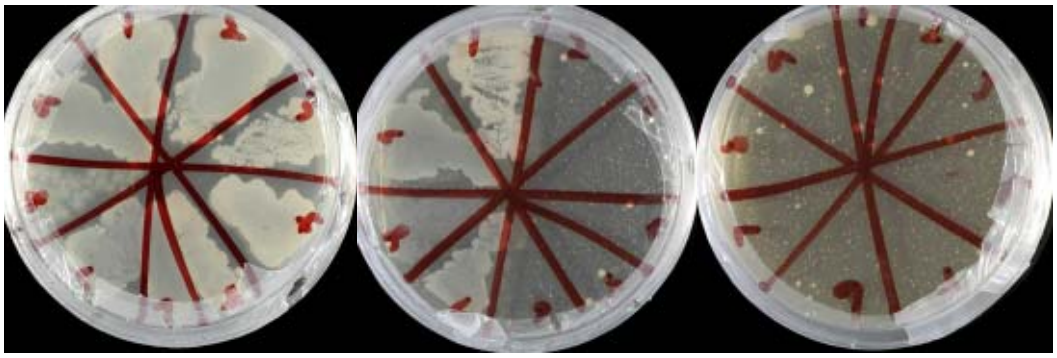
Fresh bulbs (85 g) were cut into thin slices and macerated in water using an Ultra-turrax (Model T45 OPTOLABOR). This was extracted on a horizontal shaker (150 rpm) overnight in 2 l of distilled water. The extract was then filtered through Whatman No. 1 filter paper and washed three times with 200 ml volumes of water. The filtrate was evaporated until dry under reduced pressure on a rotary evaporator at 50°C. This produced 2.93 g dry extract. The extract was dissolved in 25% aqueous dimethyl sulfoxide (DMSO) to produce a stock solution of 100 mg ml^{-1}.

2.2.4 Agar-diffusion bioassay

Aliquots of the stock solution were mixed with melted-autoclaved agar (Dilika *et al.* 2000) to produce a series of concentrations of: 1.0, 5.0, 10.0, 20.0, 30.0, and 40.0 mg ml^{-1} in Petri dishes in triplicates. 10% DMSO was used as control. Each Petri dish was divided into ten sections by drawing lines on the back of the Petri dishes. Different bacteria (Table 2.1) were streaked in the sections (Figure 2.3) and the cultures incubated for 24 hours at 37°C. The cultures were then observed for bacterial growth.

2.3 Results and Discussion

The minimum inhibitory concentrations (MIC's) of the crude extract ranged from 20.0 to 40 mg ml^{-1} (Table 2.1). The highest antibacterial activity of 20 mg ml^{-1} was against most Gram-positive bacteria and the lowest of 40 mg ml^{-1} against Gram-negative bacteria.



A **B** **C**

Figure 2.3: Agar-diffusion antibacterial bioassay. **A**= negative control where all bacteria are not inhibited; **B**= *D. delagoensis* crude extract (20 mg ml^{-1}) where most Gram-positive bacteria are inhibited; and **C**= *D.delagoensis* extract (40 mg ml^{-1}) where all bacteria are inhibited. 1= *B. cerues*, 2= *B. pumilus*, 3= *B. subtilis,* 4= *S. aureus*, 5= *E. cloacae*, 6= *E. coli*, 7= *K. pneumoniae*, 8= *P. aeruginosa*, 9= *S. marcescens*.

Table2.1: Minimum inhibitory concentrations (MIC's) of the crude extract of *D. delagoensis* against selected Gram-positive and Gram-negative bacteria.

Bacterium	Gram status (±)	MIC in mg ml^{-1}
Bacillus cereus	+	40.0
B. pumilus	+	20.0
B. subtilis	+	20.0
Staphylococcus aureus	+	20.0
Enterobacter cloacae	+	20.0
Escherichia coli	-	20.0
Klebsiella pneumoniae	-	30.0
Pseudomonas aeruginosa	-	20.0
Serratia marcescens	-	40.0

The antibacterial activity of the crude extract of *D.delagoensis* is moderate to relatively low though the plant is used in ethnoveterinary medicine. According to Fourie *et al.* (1992), there is a better chance to find bioactivity in plants that are used in folk medicine. In their investigation of 300 ethnobotanically selected plant species for pharmacological activity, 31% showed marked activity, 48% moderate activity and 21% did not have any bioactivity. This indicates that there are some plants used in traditional medicine that do not show any *in vitro* bioactivity.

There could be several reasons why the antibacterial activity of the extract is moderate. Water was used as an extractant as is the ethnoveterinary practice. It could be that the active substances are not effectively extracted in water. During the determination of the antibacterial activity of *Drimia robusta,* Luyt *et al*. (1999) found that the water and methanol extracts did not show any antibacterial activity while the ethyl acetate extract did. They further stated that the *Urginea* species produce cardiac glycosides of the bufadienolide type which upon enzymatic hydrolysis yield medicinally important bufadienolide proscillaridin. This may

suggest that upon absorption into the blood stream and tissues of animals, the cardiac glycosides are acted upon by enzymes to release bioactive molecules.

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CHAPTER 5

General Discussion

In general, plant extracts have low antibacterial activity though many of them are used in traditional medicine. McGaw *et al.* (2002) found that the minimum inhibitory concentration of the leaf extracts of *Schotia brachypetala* was lower than 10.0 mg ml^{-1} for both Gram positive and Gram-negative bacteria. A high antibacterial activity was recorded for *Helichrysum odoratissimum* with a MIC of 0.01 mg ml^{-1} against Gram-positive bacteria (Mathekga and Meyer 1998). *Compretum zeyheri* and *C. erythrophyllum* were reported to contain compounds with antibacterial activity higher than that of the antibiotics chloramphenicol and ampicillin (Kotze and Eloff 2002). The antibacterial activity of the extracts of the three plants (*Drimia delagoansis, Petalidium oblongifolium* and *Ipomoea* anadioides) investigated in this study varied. The minimum inhibitory concentration (MIC) values of their extracts were higher than 10.0 mg ml^{-1} for Gram-negative bacteria. The antibacterial activity of the extract from *U. lydenburgensis* was so low that it was decided not to proceed with isolation of bioactive compounds from it.

The isolated compounds are also of moderate antibacterial activity except for the one isolated from *P. oblongifolium* which could not be identified and has a MIC value of 10.0 µg ml^{-1}aginst *E. ruminantium.* This compound has some cumulative effect in that when it is applied sequentially its MIC decreases to 2.5 µg ml^{-1}. This can be of benefit to browsing animals if the concentration of the compound increases in the circulatory system of the animals provided it does not reach toxic levels. The other isolated compounds were not tested against *E. ruminantium* because they had relatively low activity when compared to tetracycline hydrochloride and because the bacterium is so difficult to culture.

An effective compound against a rickettsial pathogen can possibly be useful for humans also since it is not only livestock that become infected with these organisms. Rickettsial diseases also affect humans (Vincent and Angeloni (1994). The rickettsial organisms are maintained in nature by a cycle involving an animal reservoir, an insect or arthropod vector that equally infests as well as humans.

Major tick-borne Ehrlichiae and Rickettsiae diseases of dogs are: canine monocytic ehrlichiosis, canine granulolytic ehrlichiosis, cyclic canine thrombocytopenia and Rocky Mountain spotted fever (Varela 2003). In dogs the tetracycline and oxytetracycline drugs used for the treatment of the diseases are currently being replaced with doxycycline and minocycline (Varela 203).

Animals benefit from eating plants in addition to the nutritional requirements that they get. Plants contain many compounds in addition to the major nutrients (carbohydrates, lipids and proteins), vitamins and mineral nutrients. These compounds include the terpenoids, phenolics and alkaloids. Phenolics are hydroxylated derivatives of benzoic and cinnamic acids.

Phenolic compounds are found in both edible and non-edible plants. They have multiple biological effects, including antioxidant activity. They improve the nutritional quality of foods by retarding oxidative degradation of lipids. Antioxidant constituents of plant material are important for the maintenance of health and protection from coronary heart diseases and cancer. Flavonoids and other phenolics are important for the plant growth and defense against infection and injury. The phenolics act as reducing agents, hydrogen donators, singlet oxygen quenchers and they have metal chelation properties (Cos *et al*. 2001, Kähkönen *et al*. 1999, Park *et al*. 2000, Shahat *et al*. 2002).

A plant-derived cure for heartwater will benefit resource-poor small-scale livestock keepers, especially when the plant is browsed and liked by the animals.

The cure might involve a combination of plants (*Ipomoea adenioides* and *Petalidium oblongifolium*). This will not only benefit people in underdeveloped countries but also in developed ones. It will play a crucial role in organic farming where the use of synthetic chemicals is prohibited.

Such cures can become additives for animal feed, commercially available without processing. These will without doubt need to be scientifically validated like the D'Ayu-Relief product (Silver 2004). The product is a polyherbal compound that contains ten Eastern Indian herbs. It is used for the management of acute diarrhea in cats and dogs. The formulation is said to enhance benefits and minimize detrimental side effects. It contains herbs with antimicrobial and antiprotozoal activity as well as herbs that help to alleviate diarrhea and to assist in mucosal maintenance and repair. The product was given to thirty-four American and Canadian veterinarians for evaluation. 23% of the veterinarians found the product excellent; 62% found it moderately well to good and 15% found it to be poor. Such validation of ethnoveterinary medicines will bring ethnoveterinary medicines to the same level as orthodox veterinary medicine.

The trend of a preference for organically produced food is increasing. This gives a chance for the revival of ethnoveterinary medicines and anti-tick ethno-practices where tick-repellent plants are used; zero-grazing is practised; and taking animals to grazing between 10:00 and 15:00 when tick activity is at its lowest (Wanzala). When everybody demands organically produced food, civilization will have completed a full cycle. The prediction of Van Wyk and his colleagues (2000) shall have been fulfilled that medicinal plants belong to the future.

5.1 References

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CHAPTER 6

Summary

The antibacterial activity of the crude extracts of the ethnobotanically selected plants, *Drimia delagoensis, Petalidium oblongifolium* and *Ipomoea adenioides* varied. *P. oblongifolium* had the highest antibacterial activity, with respective MIC's of 1.0 mg ml^{-1} against Gram-positive bacteria and 20.0 mg ml^{-1} against Gram-negative bacteria. *D. delagoensis* had the lowest antibacterial activity with respective MIC's of 20.0 mg ml^{-1} against Gram-positive bacteria and 40.0 mg ml^{-1} against Gram-negative bacteria. *I. adenioides* had intermediate antibacterial activity.

Using the antibacterial activity of the crude extracts as a guide, bioactive compounds were isolated from *P. oblongifolium* and *I. adenioides*. The compound isolated from *P. oblongifolium* could not be identified. It is the most bioactive of the isolated compounds with a MIC of 2.5 µg ml^{-1} against *Ehrlichia ruminantium*, the causative agent of heartwater. The compounds isolated from *I. adenioides* were the flavonoids quercetin-3-rhamnoside, quercetin-3-galactoside and quecetin-3-arabinoside and the phenolics caffeic acid and its ethyl ester derivative. Caffeic acid has a lower antibacterial activity than the ethyl ester, with respective MIC's of 0.8 mg ml^{-1} against Gram-positive bacteria and 1.0 mg ml^{-1} against Gram-negative bacteria. The ethyl ester has respective MIC's of 0.4 and 0.6 mg ml^{-1} against Gram-positive and Gram-negative bacteria. There is an antibacterial synergy between these compounds. When combined in equal amounts the antibacterial activity increases with respective MIC's of 0.2 and 0.4 mg ml^{-1} against Gram-positive and Gram-negative bacteria.

The *in* vitro results of this preliminary study show that some plants like *P. oblongifolium*, can probably be used effectively in ethnoveterinary medicine in the treatment of heartwater, while others like *D. delagoensis* might not be as effective. The antibacterial activity of plant extracts might not be attributed to individual or single compounds. A combination of one or more compounds might bring about the effect. A complete analysis of the chemical constituents of the plants is necessary to give an indication of the potential of the plants in ethnoveterinary medicine.

APPENDIX A

UV-VIS Absorption spectra of PC1, PC2 and PC3

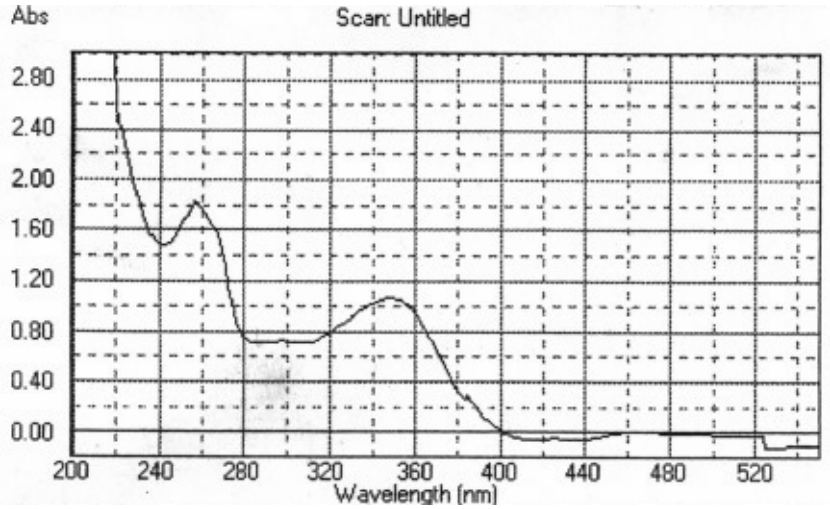

Figure A1: Absorption spectrum of PC1 in methanol

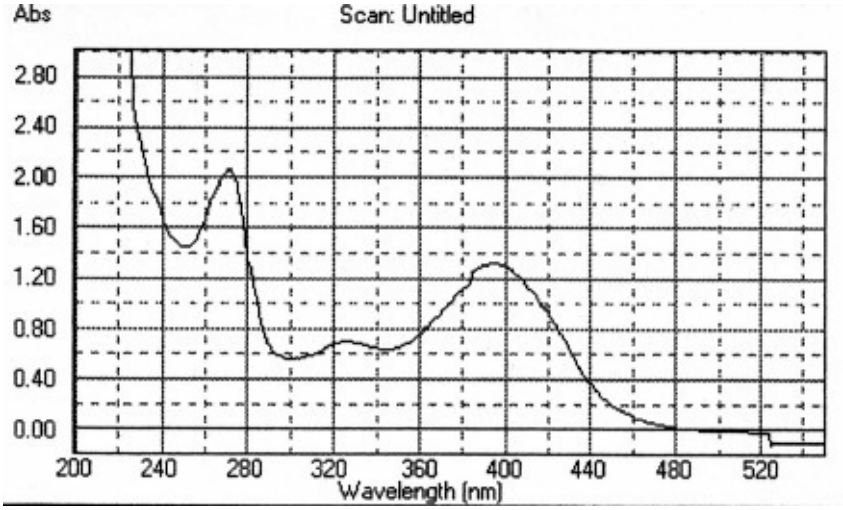

Figure A2: Absorption spectrum of PC1 in methanol and sodium methoxide

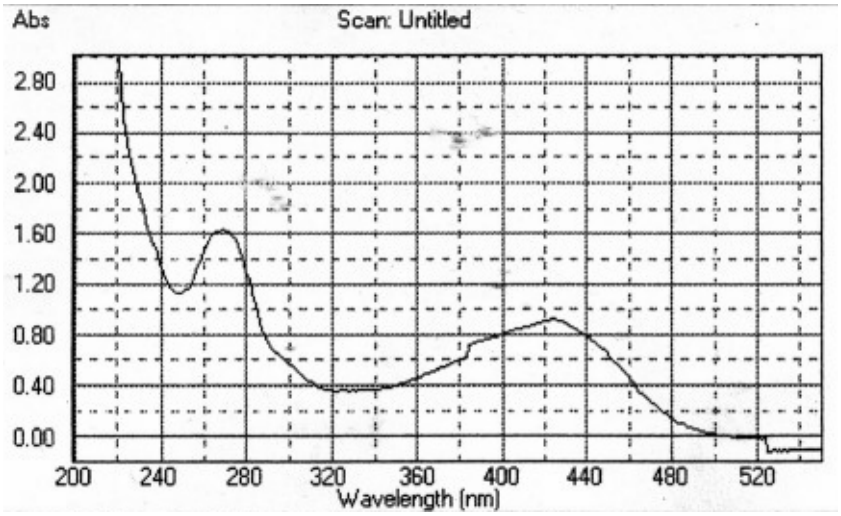

Figure A3: Absorption spectrum of PC1 in methanol and $AlCl_3$

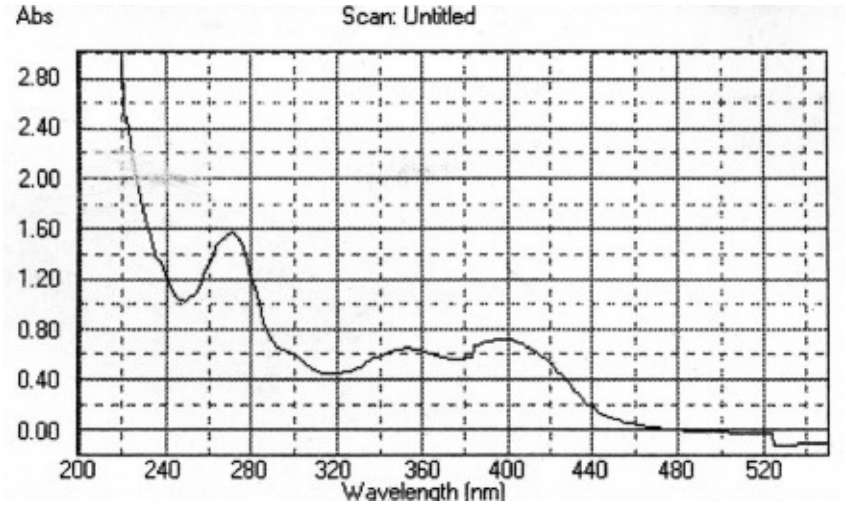

Figure A4: Absorption spectrum of PC! In methanol $AlCl_3$ and HCl

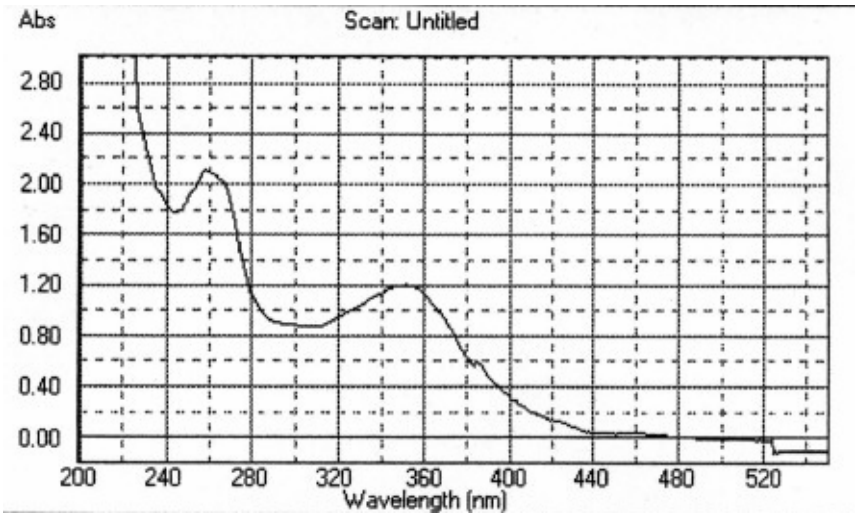

Figure A5: Absorption spectrum of PC1 in methanol and NaOAc

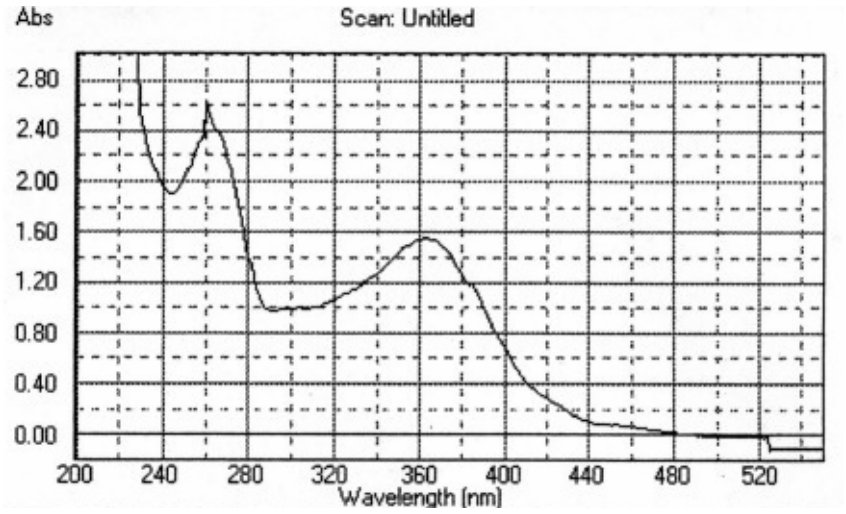

Figure A6: Absorption spectrum of PC1 in methanol, NaOAc and H_3BO_3

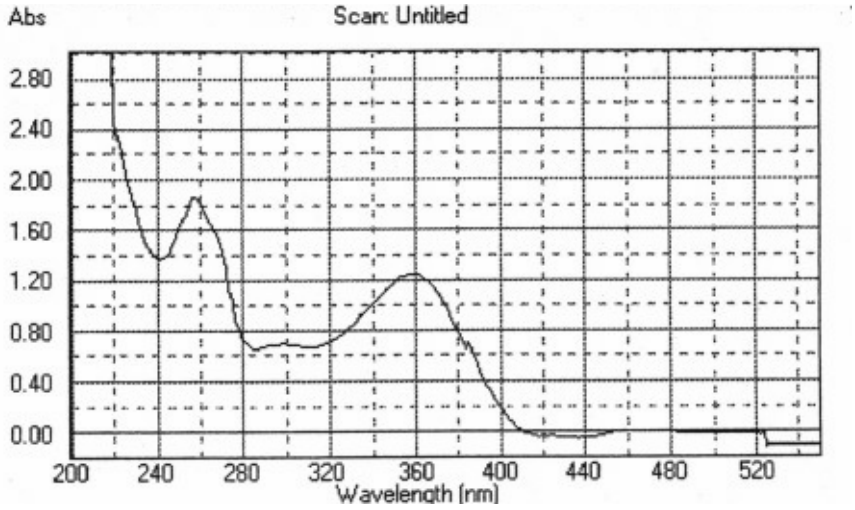

Figure A7: Absorption spectrum of PC2 in methanol

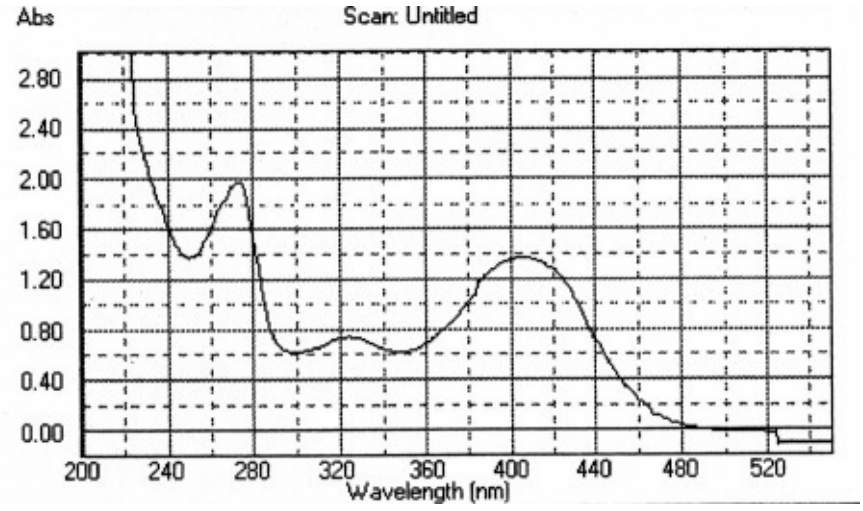

Figure A8: Absorption spectrum of PC2 in methanol and NaOMe

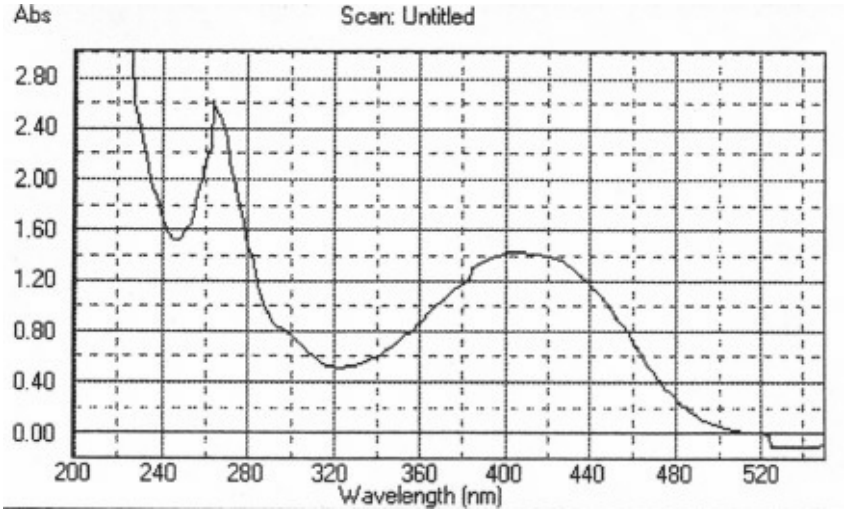

Figure A9: Absorption spectrum of PC2 in methanol with $AlCl_3$

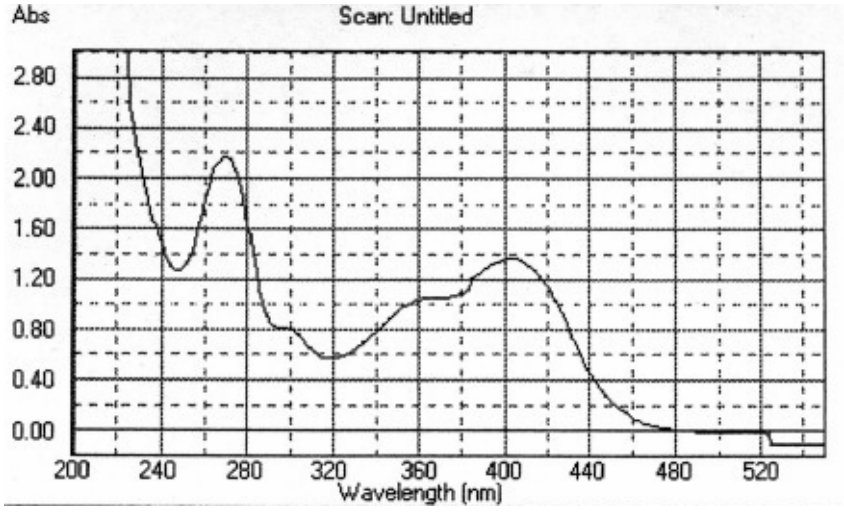

Figure A10: Absorption spectrum of PC2 in methanol with $AlCl_3$ and HCl

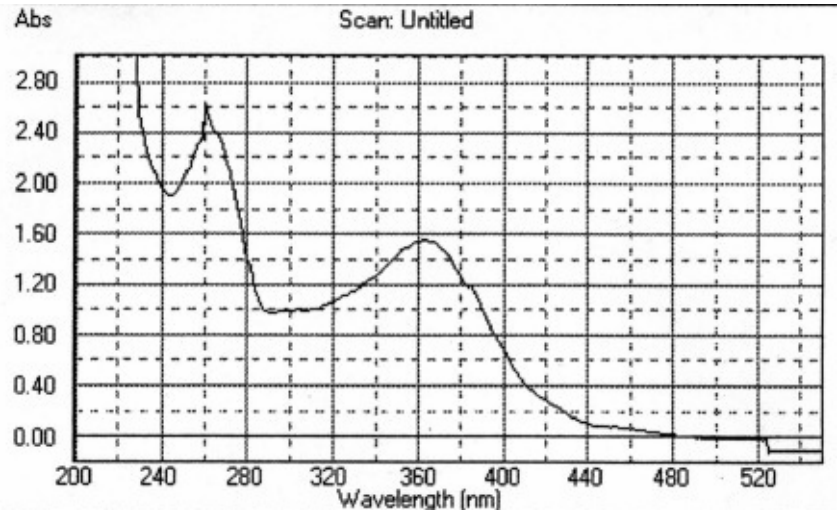

Figure A11: Absorption spectrum of PC2 in methanol with NaOAc

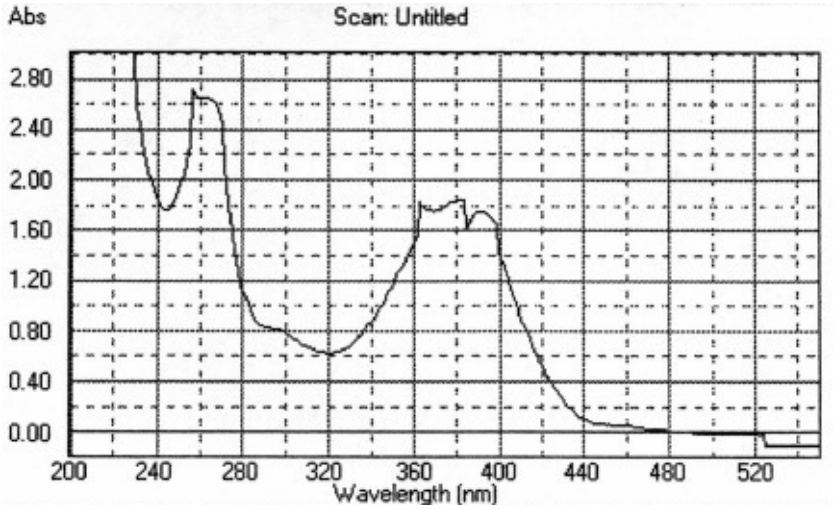

Figure A12: Absorption spectrum of PC2 in methanol with NaOAc and H_3BO_3

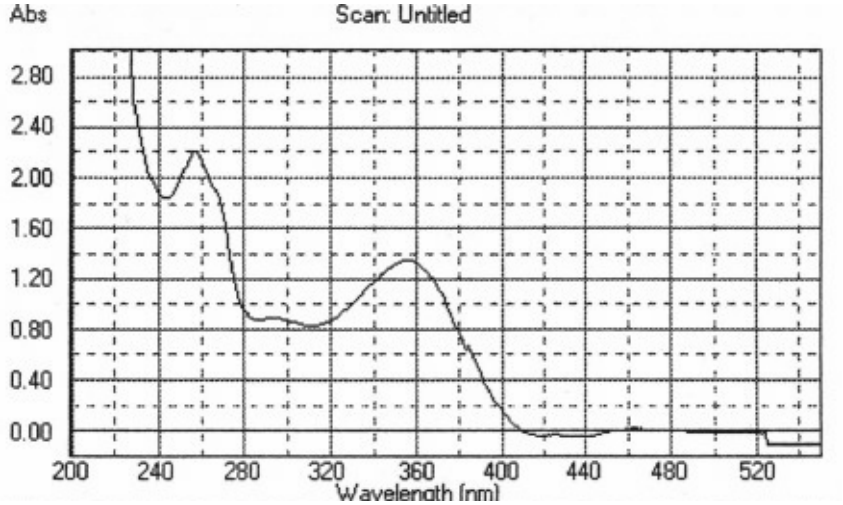

Figure A13: Absorption spectrum of PC3 in methanol

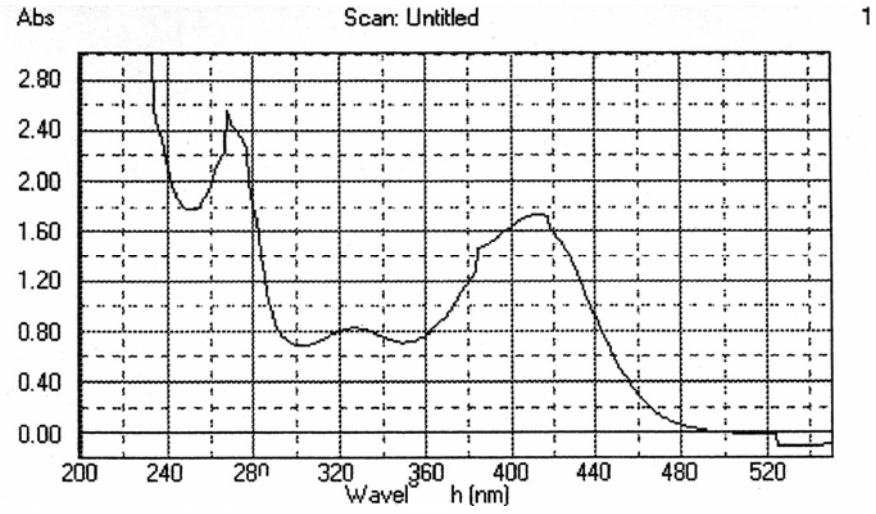

Figure A14: Absorption spectrum of PC3 in methanol with NaOMe

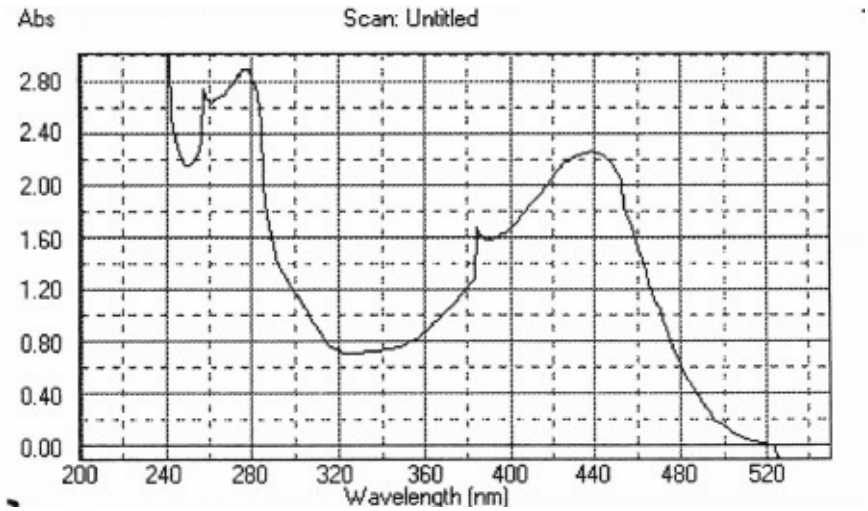

Figure A15: Absorption spectrum of PC3 in methanol with $AlCl_3$

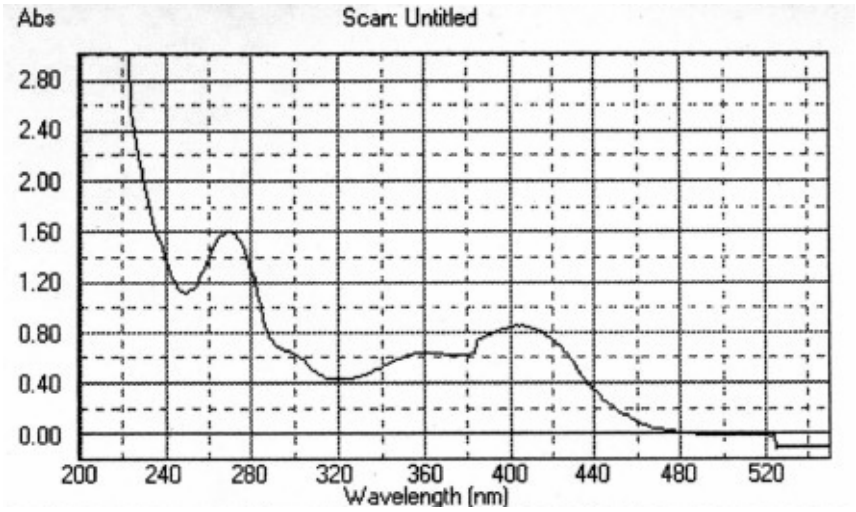

Figure A16: Absorption spectrum of PC3 in methanol with $AlCl_3$ and HCl

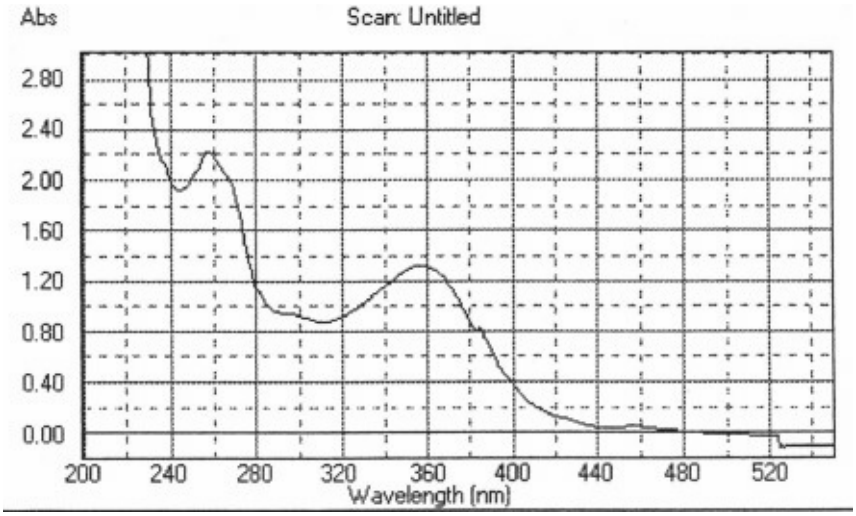

Figure A17: Absorption spectrum of PC3 in methanol with NaOAc

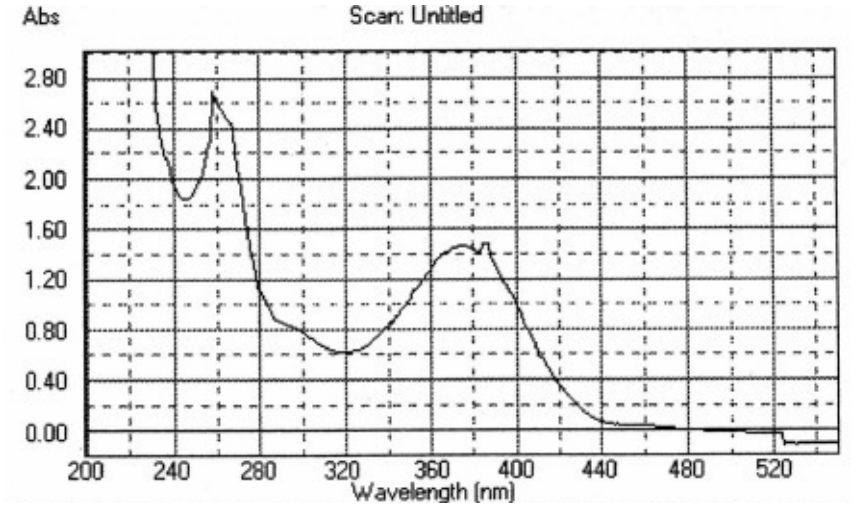

Figure A18: Absorption spectrum of PC3 in methanol with NaOAc and H_3BO_3

APPENDIX B

UV-VIS Absorption Spectra of SC1 and SC2

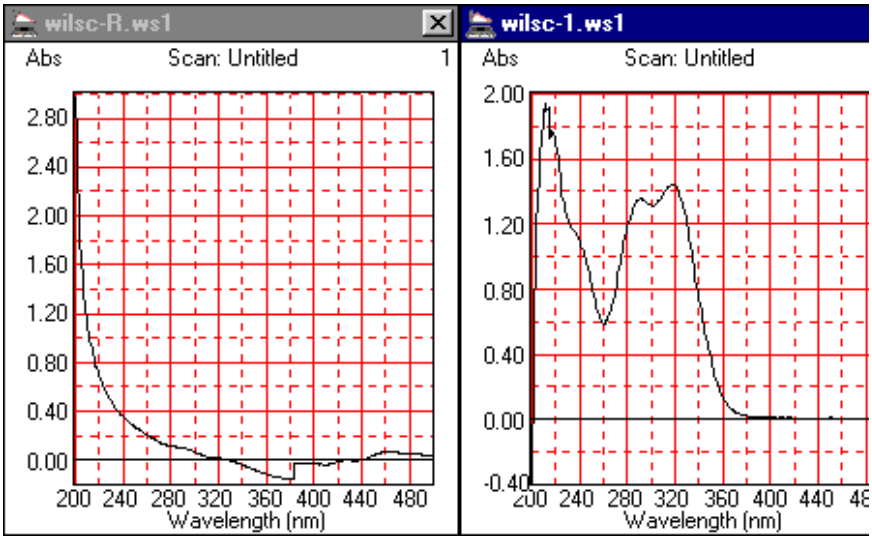

Figure B1: Absorption spectrum of SC1 in MeOH

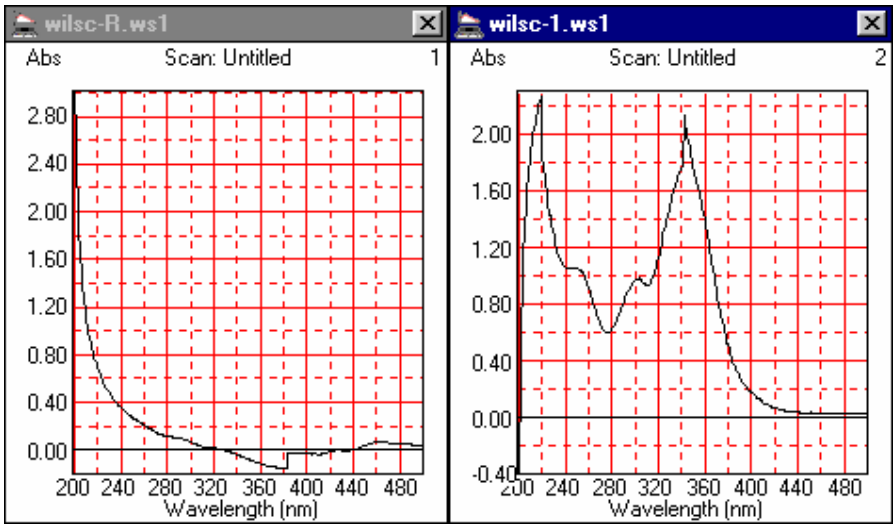

Figure B2: Absorption spectrum of SC1 in MeOH + NaOMe

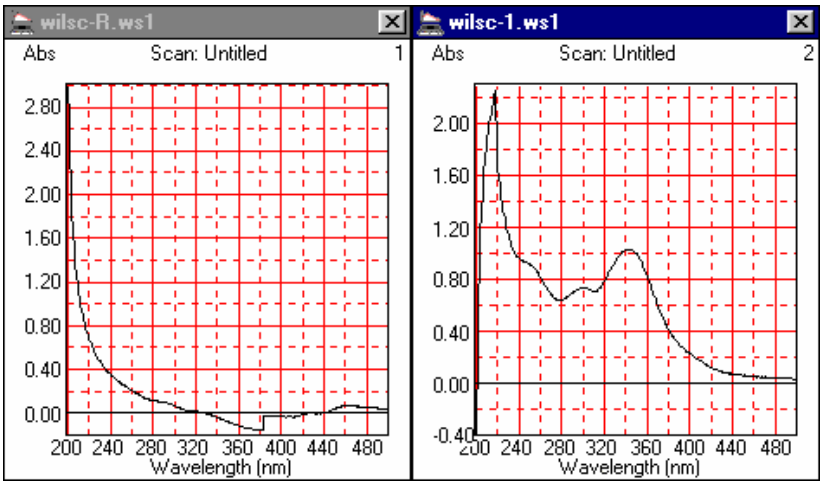

Figure B3: Absorption spectrum of SC1 in MeOH + NaOMe (20 min)

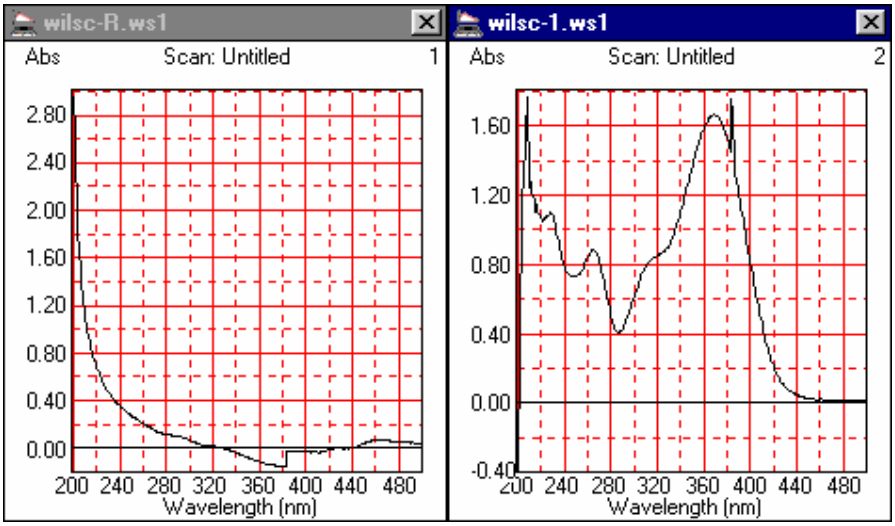

Figure B4: Absorption spectrum of SC1 in MeOH + $AlCl_3$

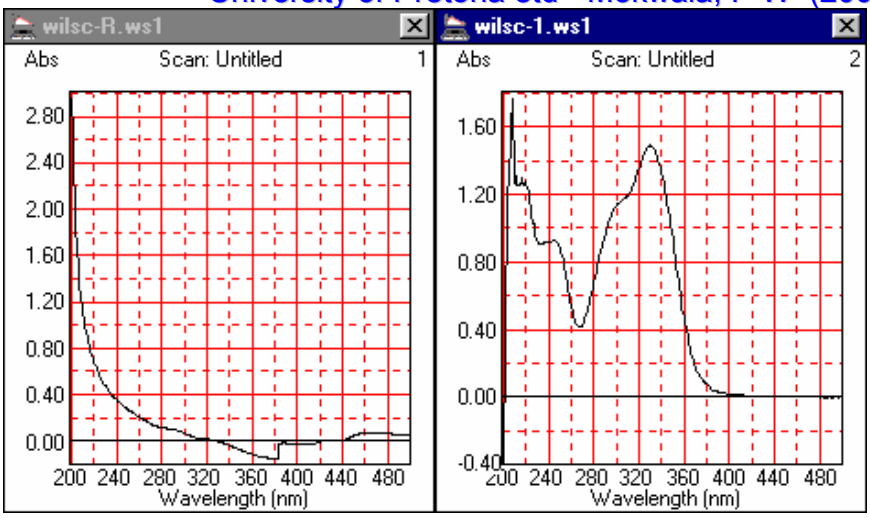

Figure B5: Absorption spectrum of SC1 in AlCl + HCl

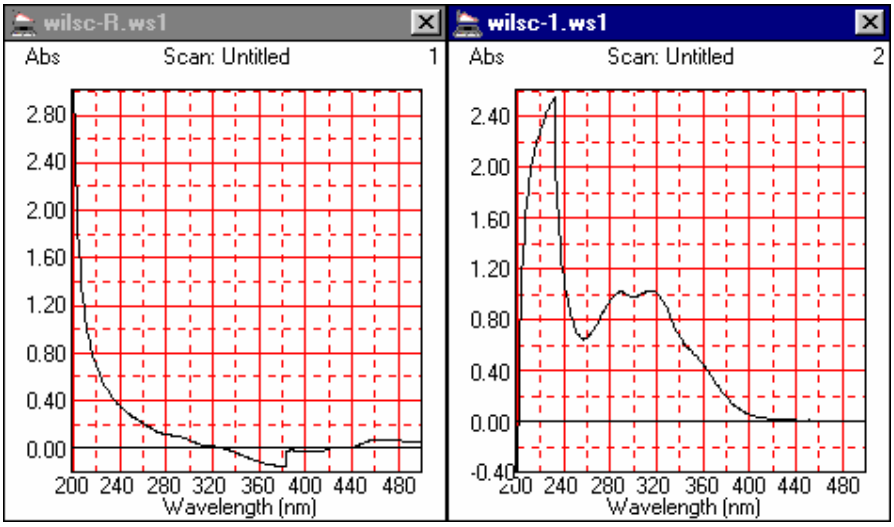

Figure B6: Absorption spectrum of SC1 in MeOH + NaOAc

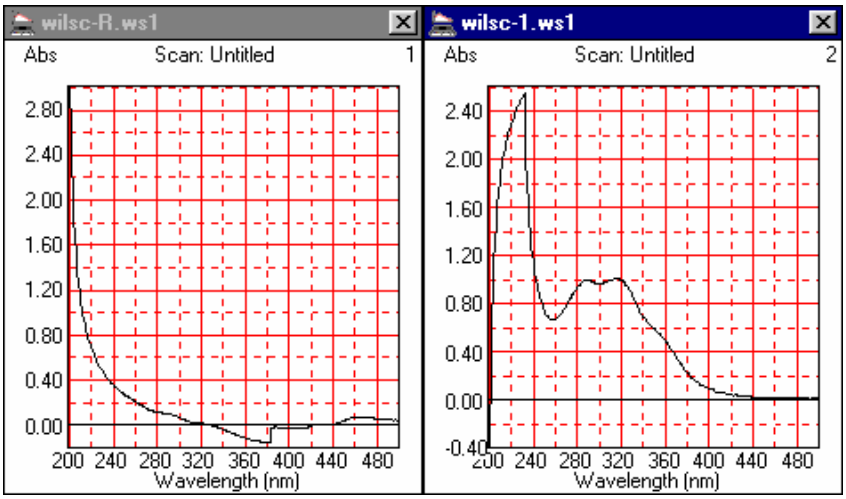

Figure B7: Absorption of SC1 in MeOH + NaOAc (20 min)

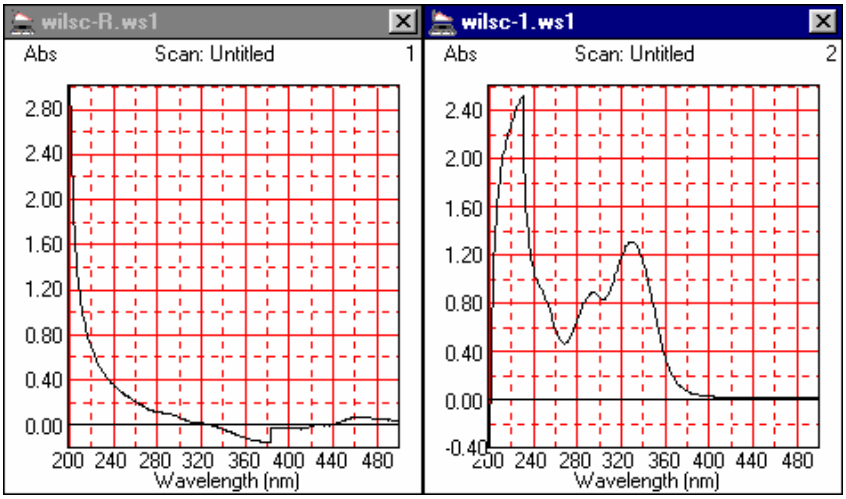

Figure B8: Absorption spectrum of SC1 in NaOAc + H_3BO_3

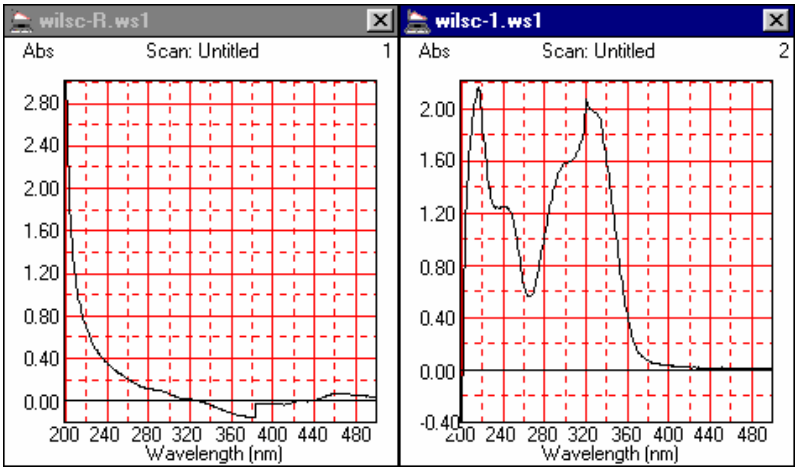

Figure B10: Absorption spectrum of SC2 in MeOH

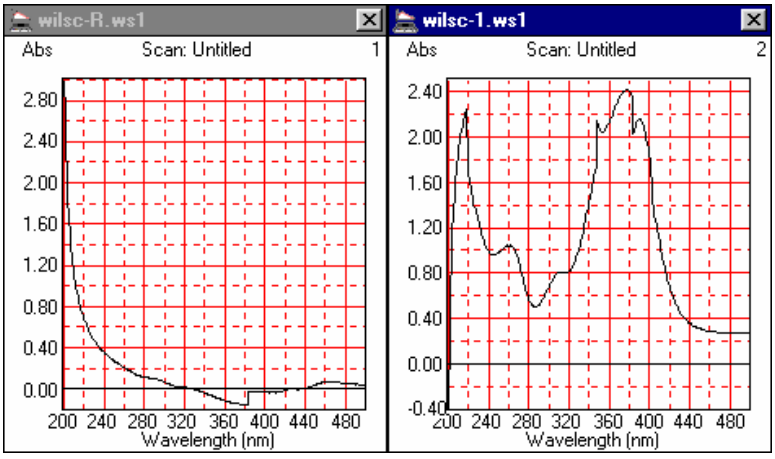

Figure B11: Absorption spectrum of SC2 in MeOH + NaOMe

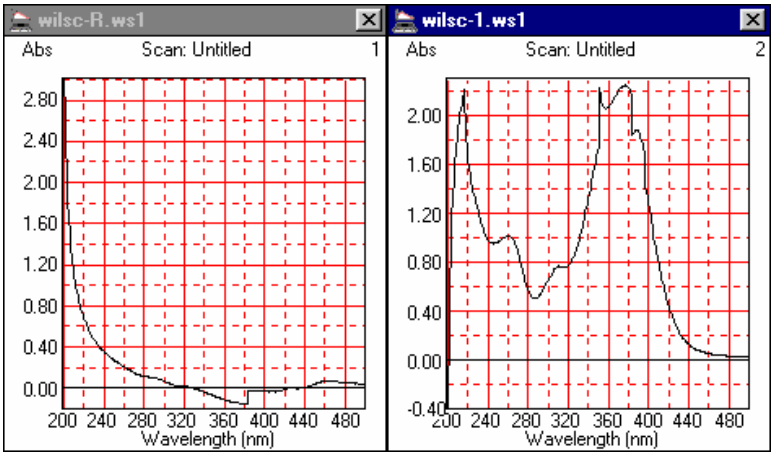

Figure B12: Absorption spectrum of SC2 in MeOH + NaOMe (20 min)

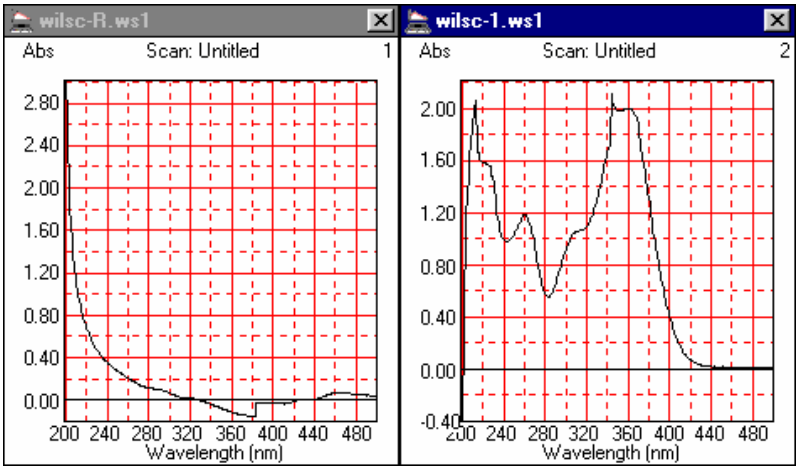

Figure B13: Absorption spectrum of SC2 in MeOH + Al_3Cl

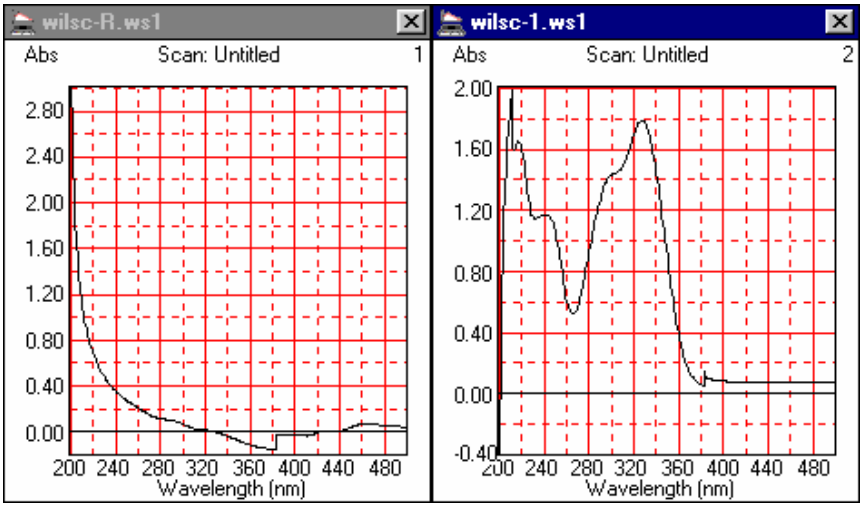

Figure B14: Absorption of SC2 in MeOH + Al_3Cl_3 + HCl

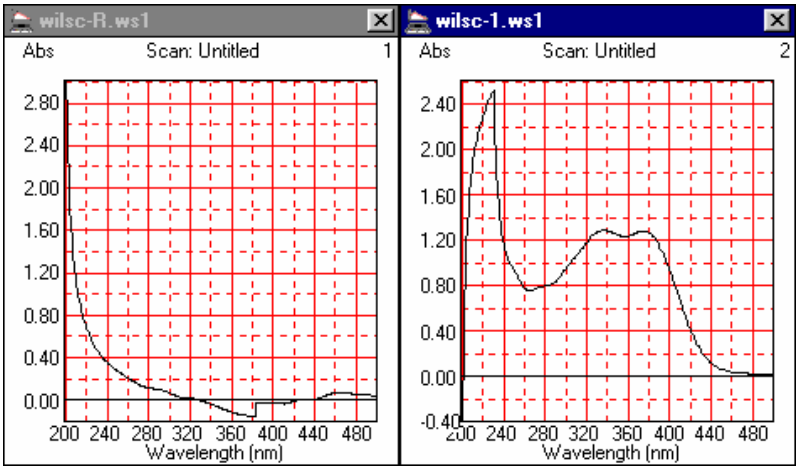

Figure B15: Absorption spectrum of SC2 in MeOH + NaOAc

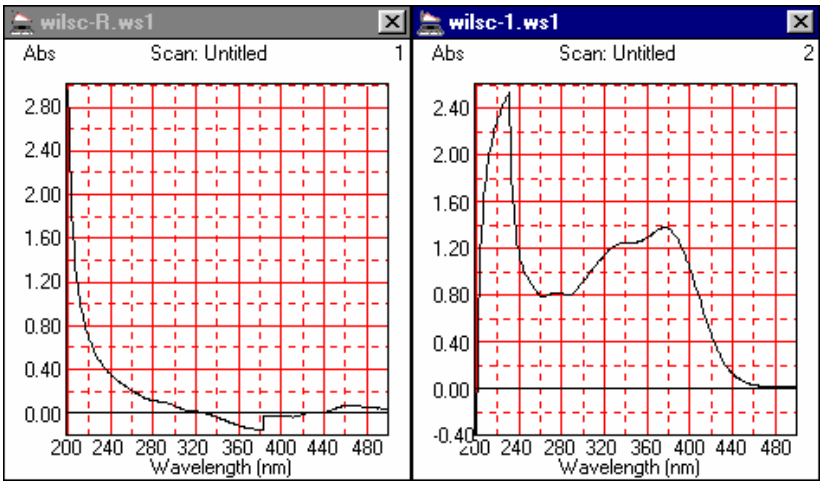

Figure B16: Absorption Spectrum of SC2 in MeOH + NaOAc (20 min)

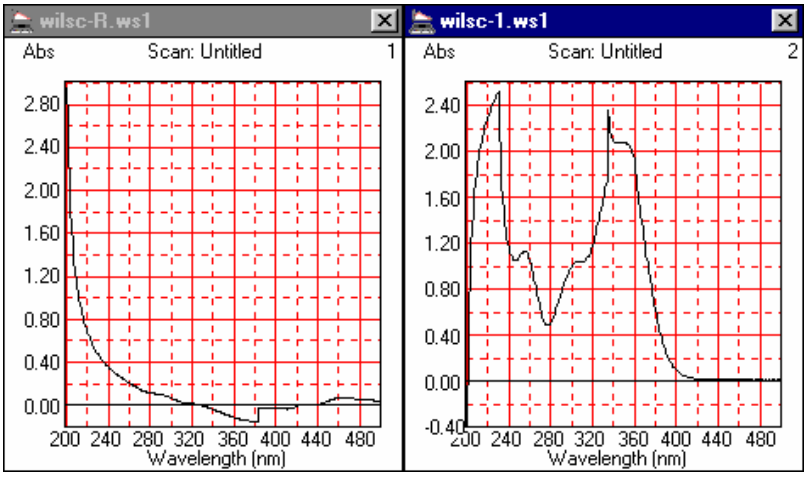

Figure B17: Absorption spectrum of SC2 in MeOH + NaOAc + H_3BO_3

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