The polyamines and endophytes of gousiekte-causing and non-pathogenic *Vangueria* and *Pavetta* species

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Declaration

I Sarah Lillian Stanton declare that all the work conducted in this study was performed by myself under the instruction of my supervisor, Professor J.J.M. Meyer and co-supervisor Professor T.A. Coutinho. I declare that all the results shown are true and no falsification or alteration has occurred. I believe no plagiarism has occurred and credit has been paid where credit is due.

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Summary

Fadogia homblei, *Pavetta harborii*, *P. schumanniana*, *Vangueria latifolia*, *V. pygmaea* and *V. thamnus* belong to the family Rubiaceae and induce the sickness gousiekte in ruminants within southern Africa. Gousiekte is a plant induced cardiomyopathy with a latent period of 3-8 weeks before death of the ruminant occurs (Ellis et al., 2010a; Van der Walt et al., 1990; Fourie et al., 1989; Prozesky et al., 1988). Gousiekte was discovered in 1908, 87 years later the causative compound, pavettamine, was isolated; a further 15 years saw pavettamine elucidated in 2010 (Bode et al., 2010; Fourie et al., 1995). The extraction method to isolate pavettamine is very cumbersome as is the diagnosis of gousiekte due to many factors (Fourie et al., 1995). Bacterial endophytes have been confirmed present in *F. homblei*, *V. latifolia*, *V. pygmaea* and *V. thamnus*, however, to date no bacterial endophyte isolated from a gousiekte-inducing plant produces pavettamine (Van Elst et al., 2011; Van Wyk et al., 1990). There is a theory that the bacterial endophytes present within the gousiekte inducing plants play a key role in synthesis of pavettamine (Van Wyk et al., 1990). Pavettamine was not isolated from any of the plants focused on in this study (*Pavetta gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* or *V. thamnus*). Cytotoxicity screening on H9c2 cells (derived from rat cardiac cells) gave unexpected results with *P. schumanniana* as the most toxic followed by *P. gardeniifolia*, *V. pygmaea*, *V. thamnus*, *V. macrocalyx* and lastly *V. infausta*. Using transmission electron microscopy bacterial endophytes were located in *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* revealing that not only gousiekte-inducing plants contain bacterial endophytes, however, the bacterial endophytes present in the gousiekte-inducing plants (*P. schumanniana*, *V. pygmaea* and *V thamnus*) all appeared morphologically similar. Seasonal colonisation fluctuations of bacterial endophytes was observed within *V. pygmaea* using transmission electron microscopy linking the season (summer) which has the highest quantity of bacterial colonies

to the season when gousiekte cases are reported most frequently. Twelve culturable bacterial endophytes were isolated from *V. pygmaea*, seven from *V. thamnus* and a single bacterium was isolated from both plants. The bacteria were subjected to cytotoxicity screening on H9c2 cells (susceptible to pavettamine) which resulted in two toxic bacteria. One isolated from *V. pygmaea* and the other was isolated from both *V. pygmaea* and *V. thamnus*. The DNA of the toxic bacterium isolated from both *V. pygmaea* and *V. thamnus* was sequenced and found to belong to the family *Bacillus*. The isolation of a toxic bacterium from gousiekte-inducing plants supports the theory that bacterial endophytes play a role in pavettamine synthesis.

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Chapter 1

Literature review

The polyamines and endophytes in gousiekte-causing and non-pathogenic *Vangueria* and *Pavetta* species

1.1. Rubiaceae family

The Rubiaceae is the fourth largest angiosperm family; it has over 600 genera and 10 000 species as members (Van Wyk et al., 1990). Species of the Rubiaceae are located on all continents with the exception of Antarctica, mainly in the subtropical or tropical regions. The species of this family vary significantly in growth forms, habitats and morphology (Verstraete et al., 2011; Van Wyk et al., 1990). Growth forms include woody shrubs, rainforest trees, small herbs, lianas, and geofructices to name a few (Verstraete et al., 2011). The phylogeny of tribe Vanguerieae which belongs to the subfamily Ixoroideae, the focus of this study, was previously poorly understood. Based on research conducted by Lantz and Bremer (2005), a clear morphological characteristic can now distinguish the Vanguerieae species from all the other Rubiaceae tribes. This distinguishing feature is the presence of a pollen presenter at the apex of the style. It is now known that over 180 species belong to the Vanguerieae tribe. Another tribe included in this study is the Pavetteae which is similar in morphology to the Vanguerieae tribe (Verstraete et al., 2011; Lantz and Bremer, 2005).

Many members of the Rubiaceae are non-toxic to humans and animals. However, there are some Rubiaceae species which are poisonous to humans, animals or both. Plant poisonings which occur in either humans or animals can differ quite significantly depending on the toxic plant ingested and its effect on different host organ systems be it the liver, rumen or another organ system. Some species contain toxins which can affect many different hosts whereas other toxins are host specific, such as pavettamine which only affects ruminants (Botha and Penrith, 2008).

The three Rubiaceae genera, *Pavetta*, *Psychotria* and *Sericanthe*, include some species which contain a very distinctive morphological characteristic, i.e. a bacteriocecidia or otherwise referred to as bacterial leaf nodules. This characteristic can be seen with the naked eye and is

a modified substomatal chamber which are inhabited by bacterial endophytes. The mesophyll cells themselves are modified in order to accommodate the bacterial endophytes. The nodule is surrounded by a sheath which is made up of a layer of tightly fitted parenchyma cells, thus the nodule is closed off from the rest of the leaf interior (Verstraete et al., 2011; Van Wyk et al., 1990).

Other members of the Rubiaceae can contain bacterial endophytes but unlike *Pavetta*, *Psychotria* and *Sericanthe* these plants do not form bacterial nodules. Bacterial endophytes are found within the intercellular spaces of the leaves of members of the *Vangueria* and *Fadogia* tribes with no internal modification of the leaves observed (Van Elst et al., 2012; Van Wyk et al., 1990).

A group of Rubiaceae species have been known to induce a disease called gousiekte ("quick disease") in domestic ruminants. The species known to cause gousiekte include *Fadogia homblei*, *Pavetta harborii*, *P. schumanniana*, *Vangueria latifolia*, *V. pygmaea* and *V. thamnus* (Verstraete et al., 2011; Hay et al., 2008; Fourie et al., 1995; Van Wyk et al., 1990). Figure 1.1 shows *P. schumanniana*, *V. pygmaea* and *V. thamnus* during flowering.

Figure 1.1.Photographs of *P. schumanniana*, *V. pygmaea* and *V. thamnus* from left to right.

Of these six species five, *F. homblei*, *P. harborii*, *V. latifolia*, *V. pygmaea* and *V. thamnus*, are geoxylic suffrutices and the remaining plant, *P. schumanniana* is a small tree or shrub. Other closely related Rubiaceae species such as *P. gardeniifolia*, *V. infausta*, *V. madagascariensis* and *V. macrocalyx* resemble *P. schumanniana* but have not been reported to cause gousiekte. Geoxylic suffrutices are otherwise known as underground trees as they have their extensive below ground woody stems leaving only a small protrusion of leaves above ground. The aerial leaves die in winter but are the first to sprout new leaves in spring. Underground trees are easily grazed by domestic ruminants. It is currently accepted that all gousiekte-inducing plants contain bacterial endophytes within their leaves (Van Elst et al., 2012; Verstraete et al., 2011; Van Wyk et al., 1990).

1.2. Bacterial endophytes

Bacterial endophytes can be defined as bacteria which inhabit the internal tissue of a plant host whereby the host does not display any negative signs due to bacterial colonisation. This relationship is usually a mutualistic relationship whereby both individuals involved benefit from the relationship (Verstraete et al., 2011). The bacterial endophyte will benefit from the relationship by inhabiting an environment which is both protective and climatically at an optimum for growth in return the bacterium prevents the plant from being infected by phytopathogenic organisms (Verstraete et al., 2011) or by producing plant growth regulators such as the hormones auxins and cytokinins or the compound indole-3-acetic acid which also supplies the plant with biologically fixed nitrogen (Tiwari et al., 2012; Phetcharat and Duangpaeng., 2011). Within the family Rubiaceae there are many species which have not been shown to contain bacterial endophytes including members of the genera *Afrocanthium*, *Canthium*, *Keetia*, *Psydrax*, *Pygmaeothamnus* and *Pyrostria* (Verstraete et al., 2011).

However Van Wyk et al., confirmed the presence of bacterial endophytes within *F. homblei*, *V. bowkeri, V. latifolia, V. macrocalyx, V. pygmaea* and *V. thamnus* by the means of both light and transmission electron microscopy (Van Wyk et al., 1990). At the time it was not widely known that symbiotic bacteria could inhabit healthy plant tissue without the need of a modified structure such as a leaf nodule (Van Wyk et al., 1990). Since then many studies have been conducted in numerous plant species to detect the presence of bacteria within plant tissues (Tiwari et al., 2012; Phetcharat and Duangpaeng., 2011; Rashid et al., 2011;Hardoim et al., 2008).

The two *Pavetta* species as previously mentioned contain the endophytes within bacterial nodules whereas in the remaining gousiekte-causing plants the bacterial endophytes occur within the intercellular spaces of the leaves (Van Elst et al., 2012). The 16S rDNA region of the genome of bacterial endophytes isolated from both *P. harborii* and *P. schumanniana* was analysed and two species identified, viz. *Candidatus Burkholderia harborii* and *Candidatus Burkholderia schumanniana,* respectively (Verstraete et al., 2011). A bacterial endophyte isolated from all the non-nodulating gousiekte inducing plants was found to belong to the genus *Burkholderia.* Comparing the 16S rDNA region of the endophytic bacteria isolated from the six known gousiekte-inducing plants showed that the bacteria were not identical. The nodulating bacteria and the non-nodulating bacteria belong to the genus *Burkholderia,* however, they occur in different clades of this genus (Van Elst et al., 2012; Verstraete et al., 2011). There is a suggestion that the bacterial endophytes present within the gousiekte-inducing plants are host specific and may possibly produce the toxin pavettamine independently (Verstraete et al., 2011; Van Wyk et al., 1990). To date however no bacterial endophyte isolated from any of the six gousiekte-inducing plants produces pavettamine *in vitro* (Van Elst et al., 2012).

1.3. Gousiekte

Gousiekte is characterised by sudden death of the animal without any pre-warning symptoms, and is a plant-induced cardiomyopathy. It is induced by consumption of any of the six known gousiekte inducing plants. Gousiekte translated from Afrikaans means 'quick disease' although there is a 3-8 week latent period after initial ingestion no symptoms are shown and the animal drops dead from cardiac arrest (Ellis et al., 2010a; Van der Walt et al., 1990; Fourie et al., 1989; Prozesky et al., 1988; Schutte et al., 1984a)

Gousiekte is classified as one of the most important plant toxicosis in southern Africa. Most often livestock poisoning due to plants occurs when the pastures are poor and fodder is absent, and the only greenery available is the toxic plants. Isolation of the active compounds and the mode of action of death have been discovered in many toxic plants. However, much is still unknown (Botha and Penrith, 2008; Fourie et al., 1995). The disease gousiekte most commonly affects domestic ruminants in southern Africa; including Zimbabwe, Zambia, Swaziland, Mozambique and Botswana. However, cases as far as the Democratic Republic of Congo have been recorded (Verstraete et al., 2011; Van Elst et al., 2012; Schutte et al., 1984b).

It has been shown that small amounts of the toxic plants can result in death of the ruminant, however, most often large quantities of fresh plant material of the gousiekte-inducing plant is required for death to occur (Bode et al., 2010). Field studies on gousiekte revealed that animals which have died from the disease show ventricular dilation, degradation of the ventricle walls and extra-cardiac signs of heart failure. At present the only way of diagnosing gousiekte is histo-pathologically and major characteristics of gousiekte poisoning is disintegration of the myofibres, collagen formation, replacement fibrosis, lymphocytic infiltrates, congestion and oedema of the lungs (Prozesky et al., 2005; Van der Walt et al.,

1990; Fourie et al., 1989; Schutte et al., 1984b). The formation of collagen cuts off intercellular connections which impairs normal transmission of impulses within the myocardium thus explaining the abnormalities observed on electrocardiography (ECG) reports (Schutte et al., 1984b).

1.4. Economic importance of gousiekte

There are a vast number of toxic plants amongst the southern African flora. The high economic implications poisonous plants have on society in general are very significant in terms of livestock loss and gross capital loss. Research in both medical and veterinary science has provided more information about these toxic plants but there are still many questions left unanswered (Botha and Penrith, 2008). The disease gousiekte is classified as one of the six most important plant toxicoses known in South Africa and it was the last to be fully evaluated (Bode et al., 2010; Fourie at al., 1995).

Although the disease was first discovered in 1908 no research was taken in understanding gousiekte until 1915. In 1915 a severe outbreak occurred where a sheep farmer lost more than half of his flock (Bode et al., 2010). In another field study in 1988 a farmer near the town Ventersdorp succumbed to a loss of two thirds of his flock to gousiekte poisoning by *V. pygmaea* (Fourie at al., 1989). An outbreak in the Delmas district in the Gauteng highveld saw a farmer lose 37 sheep out of his flock of 60 Ile-de-France sheep. The 37 sheep were poisoned during March, April and May in 1986. On inspection of the fields it was evident that sheep had consumed *V. pygmaea* (Prozesky et al., 1988). One detrimental outbreak saw a farmer lose almost half of his livelihood (Fourie et al., 1995). In South Africa at the end of 2008 the total loss due to mortalities in domestic livestock was R9 million in the case of bovine and just over R5 million in the case of sheep and goats. Thus the total of R14 million was lost in 2008 due to gousiekte alone, this knowledge deems this disease significant in

terms of research and eradication (Verstraete et al., 2011). Many researchers have studied the disease and the gousiekte inducing plants in order to better understand and eradicate the disease. A break-through occurred in 1995 when the toxin was isolated and subsequently identified in 2010 as pavettamine (Bode et al., 2010; Fourie et al., 1995).

1.5. Pavettamine

The disease gousiekte was first discovered in 1908 and it took researchers one hundred and two years to elucidate the toxic compound responsible for gousiekte poisoning. Pavettamine was extracted from *P. harborii* and elucidated as the causative toxin for the gousiekte disease. It is assumed that all gousiekte inducing plants contain pavettamine due to identical symptoms from all six toxic plants (Ellis et al., 2010b). The extraction method needed to extract pavettamine from the gousiekte inducing plants is highly troublesome and numerous factors play a role in the amount extracted. Prior to extraction it was known that the causative toxin was a heat stable, water soluble and cationic compound which could be precipitated with either ethanol or methanol. Initial extraction occurred in 1995 by Fourie et al. It took these scientists 30 sheep and four years to extract pavettamine from *P. harborii*. Many varieties of extraction methods and experiments were employed in order to finally achieve the isolated toxic compound responsible for gousiekte poisoning (Fourie at al., 1995). The same active compound appeared to be present on thin layer chromatography plates of *F. homblei*, *P. harborii*, *P. schumanniana* and *V. pygmaea* and was named pavettamine(Bode et al., 2010; Ellis et al., 2010a; Fourie et al., 1995). Researchers used the Electrospray Ionisation Mass Spectrometry (ESIMS) technique to determine pavettamine's molar mass as 251 and molecular formula as $C_{10}H_{25}N_3O_4$ as seen in Figure 1.2 (Bode et al., 2010).

Figure 1.2: Structure of pavettamine.

The noval compound pavettamine was confirmed as a polyamine similar to spermidine, spermine and putrescine. Polyamines in general play a role in differentiation, normal cell growth and proliferation, roles which include development of flowers, root growth, somatic embryogenesis, development of fruits and plant responses to biotic and abiotic stresses. However, the polyamine pavettamine can be responsible for cell death and neoplastic transformation. To date the only polyamine known to be a poison is pavettamine; with the highest concentration of pavettamine found in the young leaves (Van Elst et al., 2012; Ellis et al., 2010a). Nuclear magnetic resonance (NMR) analysis revealed that the carbon spectrum displayed five distinct signals whereas the proton spectrum expressed multiplet signals for only eight protons. From this knowledge it is evident that pavettamine contains a symmetry element possibly either a symmetry plane or a C_2 axis (Bode et al., 2010). Due to the positive charge of polyamines it is possible for the polyamine to bind to negatively charged molecules such as phospholipids, proteins or nucleic acids. Polyamines are located in every plant cell either in a free non-bonded form or bound to another molecule (Van Elst et al., 2012).

1.6. Factors limiting gousiekte diagnosis

It is well known that many plants, both toxic and medicinal, undergo concentration fluctuations in compounds. Factors including growth stage of the plant, part of the plant consumed, amount of plant ingested, size and sex of consumer and susceptibility of animal or human can determine the lethal concentration of active compound (Botha and Penrith, 2008). Many different factors may influence the toxicity of the gousiekte inducing plants or render

gousiekte diagnosis cumbersome, thus many different factors must be considered before conclusions are drawn. Factors such as lack or varying of symptoms, seasonal toxicity fluctuations, variations between habitats, animal susceptibility differences, climate conditions, locality, soil type, freshness of leaf and virulent bacterial population differences, are just a few (Van Elst et al., 2012; Bode et al., 2010; Hay et al., 2008; Van Wyk et al., 1990; Fourie et al., 1989). No two cases of gousiekte poisoning are the same, the heart tissue is affected differently each time, this fact makes gousiekte diagnosis extremely troublesome and understanding the disease even more problematic (Prozesky et al., 2005).

The seasonal fluctuations in toxicity of the gousiekte inducing plants could be due to bacterial habitation within the leaves (Van Wyk et al., 1990). The bacterial strain responsible for gousiekte induction could become non-virulent during the winter months thus rendering the plants non-toxic which would result in toxic compound concentration differences during the year. Light and transmission electron microscopy conducted by Van Wyk et al. (1990) seemed to indicate variations in numbers of bacterial colonies within the leaves of *F. homblei*, *Pachystigma bowkeri*, *P. venosum V. macrocalyx*, *V. pygmaea* and *V. thamnus* and this was possibly due to the fact that the leaves of the plants were collected at different times during the year in different environmental conditions (Van Wyk et al., 1990).

The research on gousiekte and gousiekte inducing plants has caused an array of theories which provide possible explanations and answers to the many questions that arise about the disease and the bacterial endophytes which occur in them. Bacterial endophytes which are present in all the known gousiekte inducing plants are possibly modified once present in the rumen to produce toxins whereby in any other environment the bacterial endophyte would be non-pathogenic (Van Wyk et al., 1990). There is also the theory whereby the bacterial endophytes present within the plants are bio-activated into a lethal form by the rumen flora (Van Wyk et al., 1990). There is no evidence that the toxic compound responsible for causing

gousiekte is produced by the 'toxic plants alone' for there is a possibility that the bacterial endophytes present in all the gousiekte inducing plants may in fact synthesize the toxic compound (Van Wyk et al., 1990). This theory is supported by the many functions made possible by polyamines in bacteria. These functions include biosynthesis of siderophores, acid resistance, biofilm formation and they form components of the outer membrane in Gram-negative bacteria (Van Elst et al., 2012). The extensive latent period between consumption and death of the animal could be the time needed for the toxic bacterial endophyte to multiply in sufficient numbers for death to occur or for sufficient amount of plant material to be consumed, thus accumulating enough toxin for the animal to die (Van Wyk et al., 1990). Fourie et al. (1995) eliminated the probability of the link between rumen flora and toxicosis by intravenously injecting a sheep with pavettamine and thus inducing gousiekte indicating that the rumen flora is not necessary to induce gousiekte. This discovery, however, does not shed any light on the fact that ruminants are the only group affected by the disease. There is a theory that monogastric animals having a low pH in the stomach are not affected by gousiekte because pavettamine is possibly broken down in low pH conditions (Fourie et al., 1995).

Due to the multitude of factors mentioned above one cannot discredit the high possibility that there may be many more Rubiaceae species which can induce gousiekte. These unknown gousiekte inducing plants may have been overlooked due to the presence of a known gousiekte inducing plants in close proximity, or the far simpler reason that these plants are trees thus grazing ruminants have not been subjected to feeding on the unknown gousiekte inducing plants (Van Wyk et al., 1990).

1.7. Electron microscopy

Using both TEM and light microscopy it is possible to view bacterial endophytes within the Rubiaceae species of *F. homblei*, *P. bowkeri*, *P. venosum*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. The bacteria appeared to be embedded in a mucilage-like substance located in the intercellular spaces of the spongy mesophyll cells. The mucilage was tested by researchers and stained PAS-positive identifying it as a polysaccharide-like substance produced by the bacteria. The morphological appearance of the bacterial endophytes located in *F. homblei*, *P. bowkeri*, *P. venosum*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus* was similar. They were identified as Gram-negative bacteria were mucus producing rods of 2.0µm in length and 0.5µm in width (Van Wyk et al., 1990).

Researchers have used electron microscopy to better understand gousiekte. TEM analysis of sheep hearts treated with gousiekte inducing plants expressed abnormalities of the sarcoplasmic reticula and the mitochondria. Micrographs of sheep myofibrils treated with *V. pygmaea* showed a frayed, disintegrating appearance together with replacement fibrosis and disintegration of myosin as seen in Figure 1.3 (Ellis et al., 2010c). Comparing micrographs of normal and gousiekte induced myocardium revealed that the diseased tissue contained many abnormalities including: disintegration of myofibrils which appeared frayed, disorganised arrangement and loss of myofilaments, degeneration of myosin, mitochondria abnormalities and the nuclei which were enlarged and irregularly shaped (Schutte et al., 1984a). Figure 1.3 reveals the work of Ellis et al. (2010c) whereby rat neonatal cardiomyocytes, RNCM, were subjected to double-labelling. Figure 1.3 A shows the control RNCM, the red stain indicating myosin heavy chain the green titin and the blue indicating the nucleus. Figure 1.3 B shows RNCM treated with 200µM of pavettamine for 48 h. It is clear from Figure 1.3 B that the myosin, red, had been degraded and the titin morphology had been altered (Ellis et al., 2010c).

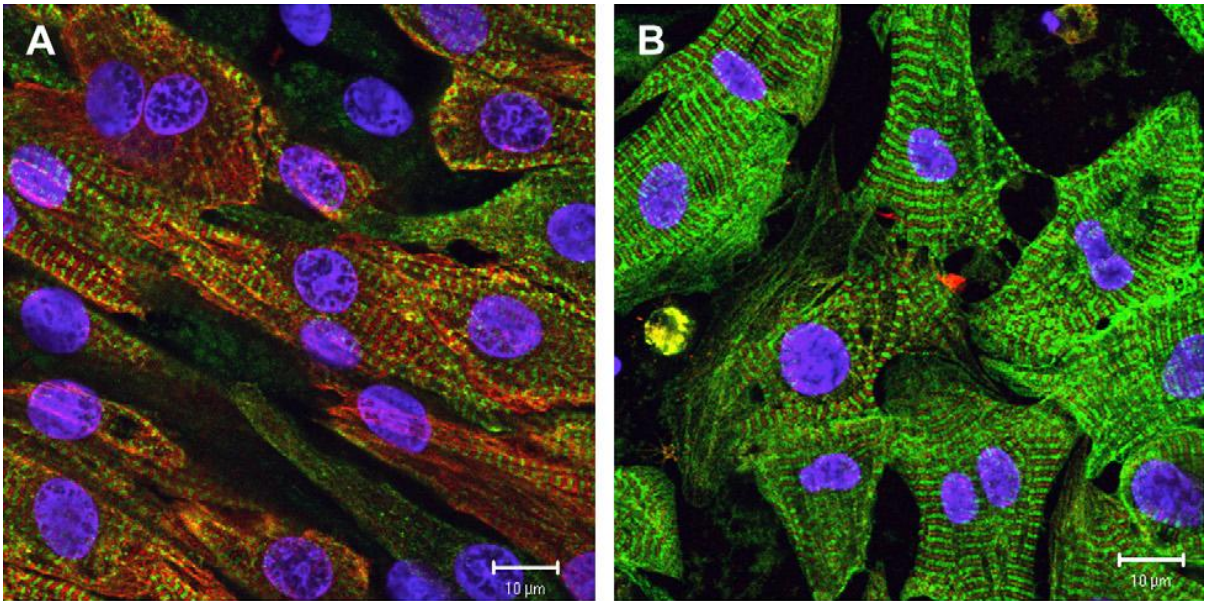

Figure 1.3.Rat neonatal cardiomyocytes (RNCM) revealing myosin (red), titin (green) and the nucleus (blue). (A) indicating control cells. (B) indicating cells treated with 200µM of pavettamine for 48 h (Ellis et al., 2010c).

1.8. Cytotoxicity

Where toxicity is unknown or not clearly understood within an assumed toxic plant, cytotoxicity screening can be carried out. Usually plant poisonings occur due to the plant interacting with bacteria, fungi, insects or helminths which enhance the active compound (Botha and Penrith, 2008). Many plants which do produce toxic compounds are used in moderation for ethnobotanically treating both humans and animals (Botha and Penrith, 2008). Toxic compounds used at a lower dosage can be used pharmaceutically, thus extraction of toxic compounds and evaluation of toxic concentration is of potential economic importance. (Botha and Penrith, 2008).

Pavettamine, the causative toxin responsible for gousiekte poisoning to both cattle and sheep in southern Africa, has been evaluated extensively (Ellis et al., 2010b). Rarely symptoms are shown before death occurs but on the occasion the ruminant may display external symptoms

including: lethargy, straggling, laying down with neck outstretched, coughing, dyspnoea, hyperpnoea, gallop rhythms or tachycardia which are signs of congestive heart failure (Van der Walt et al., 1990; Schutte et al., 1984a). It is common for autopsies of sheep hearts to reveal myocardial fibrosis and mild to moderate round cell infiltration but this is not always the case (Prozesky et al., 1988). Severely affected animals often reveal microscopical lesions in the myocardium including lymphocytic infiltrations and degeneration of myofibres however the severity of the lesions vary per case some animals expressing no noticeable lesions on investigation during autopsy. However, gousiekte poisoning does produce irreversible damage to the myocardium which results in congestive heart failure to ruminants (Prozesky et al., 2005; Van der Walt et al., 1990). Results have revealed that sheep hearts treated with *V. pygmaea* extracts and other dried gousiekte inducing plants usually reveal loss of cardiac myofilaments, lengthening of sarcomeres, cardiac dilatation, replacement fibrosis and disintegration of the myofibres. Research conducted by Ellis et al. (2010) has revealed that pavettamine inhibits protein synthesis in the heart but in no other internal organs in rats (Ellis et al., 2010b; Hay et al., 2008).

An extract from *P. harborii* administered subcutaneously revealed that rats are susceptible to the toxic compound responsible for *P. harborii* poisons and thus gousiekte poisoning (Ellis et al., 2010a). There appears to be Ca^{2+} abnormalities present in rats treated with pavettamine and these abnormalities could lead to myocardial cell degradation (Hay et al., 2008). Reduction in Ca^{2+} uptake by fragmented sarcoplasmic reticula in sheep hearts *in vitro* may be the cause of cardiac failure (Prozesky et al., 1988). This knowledge led researches to explore cell lines such as the H9c2 (2-1) cell line which is derived from embryonic BDIX rat ventricular heart tissue for pavettamine analysis. The H9c2 cell line has retained some of the properties of cardiac muscle thus is susceptible to pavettamine. Experiments conducted on pavettamine exposure to H9c2 cells revealed that the mitochondrion showed abnormalities;

the higher the concentration of pavettamine and the longer the cells were exposed to pavettamine resulted in more extensive abnormalities, the nucleus became fragmented, there was damage to the sarcoplasmic reticula and numerous empty vacuoles were produced. Cell death from pavettamine treatment appeared to be due to necrosis (Ellis et al., 2010a). Pavettamine has strong negative effects on contractile cardiac proteins and cytoskeleton proteins but none as detrimental as on myosin which is degraded first in H9c2 cells (Ellis et al., 2010c). The sarcoplasmic reticula of H9c2 cells treated with pavettamine were less compact, more granular and some were collapsed when comparing the cells to the control cells. The mitochondria of treated cells initially re-located towards one pole and became elongated until 48 hours of treatment revealed the mitochondria disintegrating completely. On closer inspection it was seen that there was an increase in lysosome size and numbers. Furthermore the F-actin was negatively affected by pavettamine treatment; the toxin caused F-actin to lose its mesh-like appearance and it became ruffled around the nuclei (Ellis et al., 2010b). With the use of cytotoxicity screening on the H9c2 cell line it was possible to answer some questions about the elusive disease, gousiekte.

1.9. Aims and Objectives

The major aims and objectives of this study were to:

- Compare the compounds isolated from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*.
- Extract pavettamine from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*.
- Investigate if toxicity was observed *in vitro* from assumed non-toxic plants.
- Evaluate whether *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* are inhabited by bacterial endophytes.

- Study the morphology of the bacterial endophytes isolated from *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* and determine their relatedness visually.
- Investigate whether bacterial endophytes present within *V. pygmaea* undergo seasonal habitation.
- Determine if the culturable bacterial endophytes isolated from *V. pygmaea* and *V. thamnus* were identical.
- Evaluate if any culturable bacteria isolated from either *V. pygmaea* or *V. thamnus* revealed cytotoxicity.

1.10. Hypotheses

The hypotheses of this study links to many of the theories known about gousiekte. The answers are essential in comprehensively understanding the disease and the plants which induce it.

1. *P. gardeniifolia, P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus* produces the polyamine pavettamine which is toxic to H9c2 cells.
2. *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* are inhabited by bacterial endophytes which are morphologically similar and toxic.
3. Bacterial endophytes present within *V. pygmaea* undergo seasonal colonisation fluctuations.
4. An identical bacterial endophyte is present in both *V. pygmaea* and *V. thamnus*.
5. A cytotoxicity study shall reveal if toxic culturable bacteria are present in both *V. pygmaea* and *V. thamnus*.

1.11. Dissertation layout

The dissertation is set out in the following chapters:

- Chapter 2 titled "Compounds isolated from *Pavetta* and *Vangueria* spp. with emphasis on polyamines". This chapter contains information about the chemical compounds extracted and isolated from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. The compounds were concentrated, purified, compared to each other and analysed to determine whether or not there is toxicity present.
- Chapter 3 titled "An evaluation of the endophytic colonies present in pathogenic and non-pathogenic Vanguerieae using electron microscopy". Chapter 3 focuses on the seasonal habitation of bacterial endophytes present in *V. pygmaea* and examines the presence of bacterial endophytes within *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus*. Linking bacterial endophytes as the key factor in causing gousiekte could indicate the presence of bacterial endophytes only in the gousiekte inducing plants.
- Chapter 4 titled "Analysis of bacterial endophyte compounds present in *Vangueria pygmaea* and *Vangueria thamnus*" investigates whether the culturable bacterial endophytes isolated from *V. pygmaea* and *V. thamnus* produce *in vitro* cytotoxicity.
- Chapter 5 titled "Discussion and Conclusion" highlights the key results generated during the study. Chapter 5 indicates where further progress can be made to the study and provides support to theories generated about gousiekte.

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Chapter 2

Secondary compounds isolated from *Pavetta* and *Vangueria* spp. with emphasis on polyamines.

2.1.Abstract

The species investigated in this study, *Pavetta gardeniifolia*, *P. schumanniana*, *Vangueria infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*, all belong to the family Rubiaceae. The plants *P. schumanniana*, *V. pygmaea* and *V. thamnus* are known to cause the sickness "gousiekte". Gousiekte is an important economic disease which mainly affects domestic ruminants within southern Africa. The polyamine, pavettamine, isolated from *P. harborii*, another gousiekte inducing plant, is thought to be present in all gousiekte inducing plants. By using many different chromatography procedures, attempts were made to extract pavettamine from *V. pygmaea*. Using NMR spectrometry analysis two cyclic compounds were extracted from *V. pygmaea* which did not resemble pavettamine. Further compound extraction was conducted on *P. gardeniifolia*, *P. schumanniana*, *V. pygmaea* and *V. thamnus* using a SPE procedure. Due to the possibility that the highly positive polyamine pavettamine may bind to other compounds or molecules, hydrolysis of these bonds were conducted on extracts from *P. schumanniana*. Hydrolysis using heat, basic conditions and acidic conditions were employed in order to determine the most efficient method. A crude compound comparison was done to determine whether the toxic plants contained compounds of a similar nature which were not present in the non-toxic varieties, *P. gardeniifolia*, *V. infausta* and *V. macrocalyx*. The results revealed many compounds present in all of the test plants but no significant similarities between these products were observed in the toxic plants. Lastly, a toxicity assay was conducted on all of the test plants using H9c2 cell lines. Results from the toxicity screening revealed that *P. schumanniana* had the most toxic extract while *V. infausta* was the least toxic.

2.2.Introduction

The genera *Pavetta* and *Vangueria* belong to the Rubiaceae and both genera have known toxic and non-toxic species. *Pavetta* and *Vangueria* also contain some species of which their toxicity status is unknown. The six species to be evaluated in this study are *Pavetta gardeniifolia* A. Rich., *P. schumanniana* F. Hoffm. Ex K. Schum., *Vangueria infausta* Burch.ssp. infausta, *V. macrocalyx* (Sond.) Robyns, *V. pygmaea* (Schltr.) Robyns and *V. thamnus* Robyns (Van Wyk et al., 1990).

It is known that upon consumption, *Fadogia homblei*, *P. harborii*, *P. schumanniana*, *V. latifolia*, *V. pygmaea* and *V. thamnus* induce an asymptomatic cardiac toxicosis referred to as "gousiekte". To date the assumed most toxic species is *V. pygmaea* followed by *F. homblei*, *P. harborii*, *V. thamnus*, *P. schumanniana*, *V. latifolia* (Van Elst et al., 2012). The latent period between consumption and death is 3-8 weeks, thus a positive plant species identification is always inconclusive (Van Wyk et al., 1990; Fourie et al., 1989).Theories that other Rubiaceae species induce gousiekte can thus not be fully supported or denied (Van Wyk et al., 1990). The species *P. gardeniifolia*, *V. infausta* and *V. macrocalyx* share the same distribution ranges and morphological characteristics as some of the known gousiekte inducing species but at present are presumed non-toxic (Van Elst et al., 2012; Verstraete et al., 2011; Van Wyk et al., 1990).

Gousiekte was first discovered over a century ago in South Africa. Fourie et al. (1995) isolated the compound responsible for gousiekte in 1995 from *P. harborii*. The isolation and extraction procedure proved "tedious and the yield of toxin from plants, low," the novel polyamine extracted was named pavettamine (Fourie et al., 1995). Pavettamine was elucidated in 2010 and the chemical structure was found to be $C_{10}H_{25}N_3O_4$ (Bode et al., 2010). Polyamines including spermidine, spermine and putrescine are commonly found in

plants and are needed for proliferation, normal cell growth and differentiation (Ellis et al., 2010a). Although gousiekte and thus pavettamine is only fatal to ruminants, research conducted by Ellis et al. (2010a) revealed that the pure compound pavettamine is susceptible to the cell line H9c2, which is derived from the cardiac cells of rats. H9c2 cell exposure to pavettamine resulted in abnormalities in the mitochondrion and sarcoplasmic reticula which then lead to death via necrosis (Ellis et al., 2010a).

The first aim was to compare the compounds isolated from the six plants. Secondly, to investigate the presence of pavettamine in all six plants. The third aim was to evaluate if the assumed non-toxic plants gave positive cytotoxicity results, and lastly to isolate and elucidate the toxic compound (if identified) from all the test plants which gave positive toxicity results.

The hypotheses of this chapter are significant in the fact that there is a great possibility that many of the non-tested plants may in fact contain pavettamine or a pavettamine-like compound which causes gousiekte in ruminants.

- Pavettamine is present within *Pavetta gardeniifolia*, *P. schumanniana*, *Vangueria infausta*, *V. macrocalyx*, *V. pygmaea* or *V. thamnus*
- *Pavetta gardeniifolia*, *P. schumanniana*, *Vangueria infausta*, *V. macrocalyx*, *V. pygmaea* or *V. thamnus* are toxic to H9c2 cells

2.3.Materials and methods

2.3.1. Plant collection

Collection of leaves of *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus* occurred between January 2010 and November 2012. Voucher specimens were deposited into the H.G.W.J. Schweickerdt Herbarium. Leaves from *P.*

gardeniifolia were collected from the University of Pretoria main campus (GPS 25°45'72"S, 28°13'78"E) in October 2012. The PRU number for the *P. gardeniifolia* specimen is 118676. *P. schumanniana* leaves, PRU number 118677, were collected from Onderstepoort veterinary institute (OVI) facility (GPS 25°38'80"S, 28°11'05"E). The rocky slopes of Blyde River Canyon (GPS 24°34'25"S, 30°47'15"E) was where the leaves of *V. infausta* were collected. The PRU number for the *V. infausta* specimen is 117607.In a black wattle plantation 15km outside the town Piet Retief (GPS 27°09'02"S, 30°59'18"E) was where *V. macrocalyx*, PRU number 117602, was collected. The leaves of *V. pygmaea* were collected from three different sites: the first in Gauteng near Rayton (GPS 25°73'61"S, 28°53'32"E) the PRU number 117989, the second in Mpumalanga near Lydenburg (GPS 25°12'94"S, 30°19'03"E), PRU number 117605 and the third in Mpumalanga within a black wattle plantation near the town Piet Retief (GPS 27°09'06"S, 31°00'08"E) (PRU number 118679). *V. thamnus* was collected from two sites, in both cases it shared a field with that of *V. pygmaea*. The Lydenburg site (GPS 25°12'92"S, 30°19'02"E) has the specimen with a PRU number of 117603 and the Piet Retief (GPS 27°09'05"S, 31°00'08"E) specimen's PRU number is 118678.

2.3.2. Extraction procedures

2.3.2.1.Extraction of secondary compounds from *V. pygmaea*

The assumed most toxic test plant, *V. pygmaea,* was analysed to determine an efficient extraction method that could be applied to each test plant and be reproducible in any laboratory. The extraction method would have to use less plant material, be less time consuming and use fewer laboratory chemicals and resources; but produce a higher quality and quantity of nitrogen containing compounds to that isolated by Fourie et al. (1995).

Leaves from the Rayton site were collected and had a final fresh mass of 3.86kg. The leaves were homogenised with a hand held laboratory blender into small pieces with 3 litres of distilled water and the extract was slowly rotated overnight. The extract was filtered using a vacuum pump and washed with 500ml ethanol to remove all residue soil. The extracted liquid was concentrated to dryness using a BÜCHI rotavapor R-200. The dry weight of the concentrated extract was 60g. 100ml of methanol (100%) was added to the dry extract, the extract was then sonicated for 10 minutes to ensure total solubility. This extraction procedure was conducted at room temperature and is referred to as extraction procedure 1.

2.3.2.2.Extraction of compounds from *V. pygmaea* and *V. thamnus*

The leaves of *V. pygmaea* and *V. thamnus* were collected from the Piet Retief site in March 2012. A total mass of 5g of both *V. pygmaea* and *V. thamnus* fresh leaves were weighed out and ground using a standard grinder for 5 minutes. Once the leaves were ground they were placed in beakers and 20ml of 70% methanol was added to the beakers. They were sonicated for 10 minutes with no heat and thereafter centrifuged for 10 minutes at 10 000rpm. The supernatant was carefully removed and dried using a BÜCHI rotavapor R-200. 6ml of distilled water was added to the dry extracts in order to get the extracts into solution. The same protocol was used for both plants and this method is referred to as extraction procedure 2.

2.3.2.3.Extraction of compounds from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus* using a speed extractor

The materials used for extraction were a combination of old, mature and young leaves. All six species were subjected to the same extraction procedure. Once the leaves of each species were sufficiently dry they were ground and weighed out to exactly 10g. The 10g was then

divided into two glass beakers. Two 40ml metal extraction cylinders of the BUCHI E-916 speed extractor were cleaned in methanol followed by distilled water and dried. A bottom filter was placed in the cylinders followed by the bottom cap. Sand (5g) was added to the two cylinders. To the two glass beakers, each containing 5g of ground leaf material, 5g of sand was added and mixed. The sand/leaf combination was added to each of the cylinders. Finally to each vial 10g of sand was added followed by a top filter. The BUCHI E-916 speed extractor was run on 4 cycles at 50˚C using distilled water only. Once the extraction procedure had completed the two extracts from each plant were combined. Once the process was completed for all the plants, the extracts were placed in labelled polytops and dried using a Genevac EZ-2 vacuum evaporator. This extraction method is referred to as extraction procedure 3.

2.3.2.4.Extraction of compounds from *P. schumanniana* using perchloric acid

Leaves of *P. schumanniana* were separated into young apical leaves and old leaves. The leaves were placed in separate beakers and ground using liquid nitrogen. Once powdered the total weight of the old leaves was 13.83g and the young apical leaves was 18.24g. 2g of powdered leaves were removed from both test beakers and placed in other beakers and labelled as the control. The leaves were then freeze dried for 36h and stored in a -72˚C freezer. A total of 236.6ml of 5% perchloric acid was added to the old leaves and 324.8ml was added to the young apical leaves (2ml of 5% perchloric acid per 100mg of powdered tissue). 40ml of distilled water was added to both control beakers. The extracts were incubated on ice for 30 mins. The extracts were filtered and the powdered plant material that did not go into solution was discarded. The perchloric acid extracts were neutralised using a 2M NaOH solution. This extraction method was labelled as extraction procedure 4.

2.3.3. Separation of secondary metabolites extracted from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*

2.3.3.1. Chromatography and nuclear magnetic resonance analysis of *V. pygmaea*

2.3.3.1.1. Column chromatography

The 100ml of crude extract (60g) from extraction procedure 1 was added to clean silica gel and left overnight in a large porcelain mortar to ensure complete dryness of the silica gel extract. A 1500ml column was washed and rinsed with methanol (100%) and left to dry. One quarter of the column was filled with new silica gel, the plant extract and dry silica was added on top, a 5cm layer of new silica was added on top of the extract silica, and lastly cotton wool was added to the top of the column. Table 2.1 displays the mobile phases which were added to the column.

Table 2.1: Mobile phases of silica gel column.

Hexane	Ethylacetate	
1000ml	0ml	
900ml	100ml	
700ml	300ml	(x2)
500ml	500ml	(x3)
300ml	700ml	(x3)
100ml	900ml	(x3)
0ml	1000ml	(x2)
Ethylacetate	Methanol	
900ml	100ml	(x2)
700ml	300ml	(x2)
500ml	500ml	(x2)
0ml	1000ml	(x2)

Fractions (about 900ml) from the column were collected in 1 litre containers and thereafter concentrated to dryness using BÜCHI rotavapor R-200. A small amount of solvent was added to the dry fractions to produce a concentrated fraction of a final volume of 25ml. Due to the separation by polarity the early fractions were dissolved in hexane, mid fractions with ethylacetate and the later fractions in methanol or distilled water. A total of 33 fractions were concentrated and added to polytops for thin layer chromatography (TLC) analysis.

The nitrogen containing compounds were visualised in fractions 29-33, fraction 29 was subjected to size exclusion chromatography where fractions 5-8 revealed compounds which reacted with ninhydrin, thus were combined and dried. The mixture was subjected to Dowex

ion exchange chromatography which displayed interesting compounds present in wash 2. The dry weight of wash 2 was found to be 0.05g. 3ml of methanol was added to wash 2 and left to dissolve. Silica gel was added to the 3ml of wash 2 and left at room temperature to dry. A 50ml column was washed and rinsed with menthol. A cotton wool bud was added to the column followed by the addition of one third of the column filled with clean silica gel. The dry wash 2 silica gel was added to the column followed by a 2cm layer of clean silica and lastly cotton wool. Table 2.2 shows the solvent system used to change from non-polar to polar. Each solvent system was added to the column to a total volume of 1 litre before commencing onto the next solvent system.

Table 2.2: Solvent system used in 50ml silica column chromatography.

Dichloromethane (ml)	Methanol (ml)	Distilled water (ml)
800	190	10
700	280	20
600	350	50
500	450	50
0	1000	0

The fractions were collected in 100ml vials and labelled per solvent system. The vials were concentrated to a quarter of their volume and prepared for TLC analysis.

From the original column chromatography which contained the crude extract from extraction procedure 1, fractions 29-33 were considered fractions of interest. This is because these fractions contained compounds which reacted with ninhydrin and thus contain a nitrogen atom. This is of significance because polyamines and more importantly pavettamine contains nitrogen atoms. The dry weight of fraction 29 was 15.95g, from this 2g was removed and

placed in a clean polytop tubes. Fractions 30-33 were combined and concentrated using a BÜCHI rotavapor R-200 to a dry weight of 12.2736g. From this 2g was removed and placed in a clean polytop tubes. Between 5-7ml of 70% methanol was added to the dry extracts until they were in solution. Fresh silica gel was added to the extracts and left to dry. Two 500ml columns were washed and rinsed with 70% methanol, and when dry a small cotton wool bud was added to both columns. One third of new silica gel was added to the columns followed by the dry silica extracts. A 2cm layer of fresh silica was then evenly added and lastly cotton wool. Table 2.3 shows the solvent systems used for both columns, each system was added to a total volume of 1 litre.

Table 2.3: Solvent systems for fractions 29 and the combination of 30-33.

Dichloromethane (ml)	Methanol (ml)	Distilled water (ml)
800	190	10
700	280	20
600	350	50
500	450	50
0	1000	0

From both columns the fractions were collected in vials of 50ml. The content of the flasks was concentrated to half the volume and then analysed using TLC.

2.3.3.1.2. Size exclusion chromatography

Fraction 29 was dried using a BÜCHI rotavapor R-200 for further analysis. The Sephadex resin was washed with methanol: distilled water (1:1) and added to a 500ml column until it was about $^{2}/_{3}$ full. The dry weight of fraction 29 was 17.95g, and from this amount 2g was

added to the 500ml column evenly. Table 2.4 reveals the mobile phases which were added to the column.

Table 2.4: Size exclusion column mobile phase.

Methanol	Distilled water
0ml	200ml
10ml	190ml
20ml	180ml

The fractions were collected to a volume of about 50ml; if a colour was noted in the fraction only 20ml was collected. The fractions were then concentrated to half their volume using a BÜCHI rotavapor R-200 and prepared for TLC analysis.

2.3.3.1.3. Ion exchange chromatography

Fractions 5-8 from the size exclusion column containing fraction 29 reacted with ninhydrin and thus were combined and concentrated to dryness. The dry weight of the extract was 0.67g. Dowex 50 was the resin used for further separation. The Dowex 50 resin was prepared by adding hydrochloric acid: distilled water (1:1) to a total volume of 1 litre into a beaker and was left for 2.5 hours at room temperature. The resin was washed with distilled water until the eluted liquid coloured the litmus paper to show neutrality. The Dowex was added to a small (25ml) column and kept damp with distilled water. The combination of fractions 5-8 (total mass of 0.67g) was added to the column evenly. The column was then washed with 200ml of distilled water four times to ensure the sugars were removed. Table 2.5 shows the solvent system used.

Table 2.5: Solvent system of the Ion exchange column.

Washes	Distilled water	25% Ammonia
1	180ml	20ml
2	100ml	100ml
3	0ml	200ml

Washes 1, 2 and 3 were concentrated to dryness using a BÜCHI rotavapor R-200. A total volume of 4ml distilled water: methanol (1:1) was added to each wash. The dissolved fractions were placed into corresponding polytops and labelled for TLC evaluation.

Fractions collected from the column chromatography of the combined fractions 30-33 were analysed using TLC. From the TLC analysis fractions which reacted with ninhydrin were considered compounds of interest and compounds which had the same rf and the same colour were regarded as similar compounds, they were combined and concentrated using a BÜCHI rotavapor R-200. The combined fractions were labelled Com1-Com6. The impurities (non-nitrogen containing compounds) present in Com1 were removed by ion exchange chromatography. The Dowex 50 resin was prepared by adding hydrochloric acid: distilled water (1:1) to a total volume of 1 litre into a beaker and this was left for 2.5 hours at room temperature. The resin was prepared as described previously. The neutral Dowex resin was added to the (25ml) column followed by Com1. 1200ml of distilled water was then added to the column to remove all neutral and negatively charged molecules. A solvent system of distilled water: 25% ammonia solution (9:1) to a total volume of 200ml was added to the column to remove Com1. Com1 was then re-labelled as semi-pure Com1.

2.3.3.1.4. Thin layer chromatography

From the initial column chromatography containing the crude extract from *V. pygmaea*, a total of 33 fractions were collected. The 33 fractions were divided into least polar (1-6), mid polar (7-20) and most polar (21-33). This was necessary because the mobile phase would have to change depending on polarity of the fractions for sufficient separation. Two TLC plates were prepared for each range of fractions in order to highlight the compounds present. One TLC plate for the general indicator vanillin which illuminates many different secondary compounds and the other TLC plate was for ninhydrin which reveals nitrogen containing compounds. The TLC plates were run twice to ensure adequate separation. The fractions 1-6 along with the original extract were spotted on TLC plates and developed with the mobile phase of hexane: ethylacetate (9:1). The original extract with fractions (7-20) was spotted on TLC plates and developed with the mobile phase dichloromethane: methanol (99:1). The more polar fractions (21-33) were spotted with the original extract and the amino acid cysteine and developed with the mobile phase ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). The TLC plates revealed that fractions 29-33 contained compounds which reacted with ninhydrin, thus were deemed compounds of interest.

Fraction 29 was subjected to size exclusion chromatography and the fractions generated from that column were analysed using TLC procedures. The fractions obtained from the Sephadex column were spotted in duplicates on TLC plates for vanillin and ninhydrin detection. The TLC plates were developed twice in a mobile phase of ethylacetate: acetic acid: formic acid; distilled water (6:1:1:1) for better band separation. The fractions were spotted in groups of five. Fractions which reacted with ninhydrin were considered fractions of interest and were combined and concentrated to dryness using a BÜCHI rotavapor R-200.

The size exclusion chromatography column produced fractions 5-8 which reacted with ninhydrin, and these fractions were combined and dried using a Genevac EZ-2 vacuum concentrator. This combination was subjected to ion exchange chromatography which produced washes 1-3. The washes (1, 2, and 3) from the original fraction 29were spotted on TLC plates in duplicates for ninhydrin and vanillin detection. The TLC plates were run twice for sufficient separation with a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). Washes which contained compounds which reacted with ninhydrin were regarded as washes of interest.

Wash 2 eluted compounds which reacted with ninhydrin, thus was sequentially subjected to column chromatography evaluation. The fractions (1-22) collected from the 50ml column of wash 2 were prepared in duplicates for vanillin and ninhydrin detection. The TLC plates were run twice with a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). On inspection the fractions from the methanol solvent system showed compounds of interest. The methanol fractions were later combined as follows:

- 1-3 combined and renamed C1
- 4-7 combined and renamed C2
- 8-13 combined and renamed C3
- 14-22 combined and renamed C4

The dry weight of C1 was 0.0146g, C2 was 0.0062g, C3 was 0.0117g and C4 was 0.0461g. Between 2-5ml of methanol was then added to the dry extracts in order to undergo further TLC analysis. C1, C2, C3, C4, the original wash 2 extract and pavettamine were spotted on a TLC plate and run on the same mobile phase.

From the second column of fraction 29 the fractions generated were closely evaluated and similar fractions were combined as seen below:

- 26-29 combined and named Co1
- 32-35 combined and named Co2
- 36-38 combined and named Co3
- 39-44 combined and named Co4

The combined fractions were then run with the fractions C1, C2, C3 and C4 in order to determine major similarities and differences. The fractions were spotted in duplicates for both vanillin and ninhydrin detection. The TLC plates were run twice for adequate separation on a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1).

The column chromatography column containing the combination of fractions 30-33 underwent the same TLC procedure as the second column chromatography of fraction 29. Fractions were collected to a total volume of 50ml and concentrated down to half of that volume using a BÜCHI rotavapor R-200. Many fractions contained compounds which reacted with ninhydrin, and similar fractions were added together as indicated below:

- 30-32 combined and named Com1
- 33-36 combined and named Com2
- 37-38 combined and named Com3
- 39-44 combined and named Com4
- 45-46 combined and named Com5
- 47-54 combined and named Com6

The combined fractions were then concentrated and re-developed on TLC plates in order to evaluate the compounds and determine single band formation.

2.3.3.1.5. Nuclear Magnetic Resonance (NMR)

Once an extract revealed a single band on TLC it was assumed to be semi-pure or pure. C1 and Com1 was subjected to NMR analysis to determine if peaks were present in the polyamine region (between 2.8-4.5) such as that of pavettamine. The extracts C1 and Com1 were dried using a BÜCHI rotavapor R-200. The dried extracts were then dissolved in 1.5ml of deuterated water. The Varian 200 MHz NMR was set for proton analysis with 12 and 240 scans respectfully. The NMR spectrograph of both C1 and Com1 were compared with that of the pure polyamine, pavettamine. The NMR was re-set for carbon analysis of Com1 with 6000 scans. Com1 was further subjected to both dept and cosy NMR analysis; dept with 5000 repartitions and cosy with 32 scans.

2.3.3.2.Solid Phase Extraction (SPE)

The *V. pygmaea* and *V. thamnus* extracts from extraction procedure 2 were used in the following SPE procedure. Both the *V. pygmaea* extract and the *V. thamnus* extract underwent the same process. The columns used were Strata™X (33µm,85Å) Polymeric RP 500mg / 6ml, tubes, which are polymeric strong cation exchange columns. The SPE procedure was run in a vacuum with a slow flow speed of between 10-30 drops per minute. The column was conditioned with 6ml methanol (100%) and then equilibrated with 6ml of distilled water. Thereafter the samples were loaded. Table 2.6 reveals the solvents used for washes 1-10 from each extract.

Table 2.6: Solvents added to SPE column for both *V. pygmaea* and *V. thamnus*.

Wash	Solvent (6ml)
1	dH_2O
2	dH_2O
3	dH_2O
4	dH_2O
5	dH_2O
6	70% MeOH
7	70% MeOH
8	100% MeOH
9	100% MeOH
10	100% MeOH

The washes from both columns were labelled clearly on polytops and dried to half their volume (3ml) using a Genevac EZ-2 evaporator.

An extract of *P. gardeniifolia* from extraction procedure 3 was also used for SPE analysis. A total of 6ml of distilled water was added to the polytop tube 1 which contained 0.91g of dried extract. The polytop 1 was sonicated for 30 minutes to ensure all the compounds were in solution. The SPE chamber and test tubes were washed with 100% methanol followed by distilled water and left in a low heat oven to dry. The columns used were Strata™X (33µm, 85Å) Polymeric RP 500mg / 6mL, tubes, which are polymeric strong cation exchange columns. The SPE procedure was run under vacuum with a slow flow speed of between 10-30 drops per minute. The cation column was conditioned with 6ml of 100% methanol. The

column thereafter was equilibrated with 6ml of distilled water. The *P. gardeniifolia* extract was loaded into the column. Table 2.7 shows the solvents used per wash collected.

Table 2.7: Solvent system of *P. gardeniifolia* SPE column

Wash	Solvent (6ml)
1	dH_2O
2	dH_2O
3	dH_2O
4	dH_2O
5	dH_2O
6	dH_2O
7	50% MeOH
8	50% MeOH
9	100% MeOH
10	25% NH_4^+
11	25% NH_4^+
12	25% NH_4^+

The 12 washes containing a volume of 6ml were placed into polytops and labelled. The polytops tubes were dried using a Genevac EZ-2 evaporator.

An extract of *P. schumanniana* from extraction procedure 3 was also used for SPE analysis. A total of 0.6g of dried extract was dissolved in 1.5ml of distilled water and 1ml of monopotassium phosphate. The monopotassium phosphate was used to lower the pH of the solution to 1.1. The column used for the SPE procedure was Strata™X (33µm, 85Å) Polymeric RP 500mg / 6mL, tubes, which are polymeric strong cation exchange columns.

The SPE chamber and test tubes to be used were cleaned with 100% methanol followed by distilled water and left in a low heat oven until dry. The extract was centrifuged for 5 minutes at 10 000rpm; the pellet was discarded and the clear solution was used for SPE analysis. The cation exchange column was conditioned with 3ml of 100% methanol and equilibrated with 3ml monopotassium phosphate. A total of 3ml of the extract was then loaded into the column. Table 2.8 displays the solvents used, in both the washes and elutes, in the SPE column of the *P. schumanniana* extract.

Table 2.8: Solvents used in the SPE column of *P. schumanniana*

Stage	Solvent (3 ml)
Wash 1	KH_2PO_4
Wash 2	MeOH
Dry for 5 minutes on full pressure	Dry for 5 minutes on full pressure
Elute 1	NH_4^+ (25%): MeOH (5:95)
Elute 2	NH_4^+ (25%): MeOH (5:95)
Elute 3	NH_4^+ (25%): MeOH (5:95)
Elute 4	NH_4^+ (25%): MeOH (5:95)
Elute 5	NH_4^+ (25%): MeOH (1:1)

Once collected the washes and elutes were placed in polytop tubes and dried using a Genevac EZ-2 vacuum concentrator.

In order to determine the correct SPE method for extracting pavettamine a similar compound, spermidine, was used to test the procedure. Pure spermidine liquid was acquired from SIGMA-ALDRICH. The SPE procedure was run twice using the pure standard spermidine. The initial procedure was conducted on 50mg of spermidine. 2ml of monopotassium

phosphate (KH_2PO_4) was added to the spermidine in a labelled polytop. The column used for the SPE procedure was Strata™X (33µm, 85Å) Polymeric RP 500mg / 6mL, tubes, which are polymeric strong cation exchange columns. The column was conditioned with 3ml of distilled water and equilibrated with 3ml of KH_2PO_4. The spermidine sample was loaded in the column. Table 2.9 reveals the solvents used for the washes and elutes of the initial spermidine SPE column.

Table 2.9: Solvents used in initial SPE column of spermidine

Stage	Solvent (3 ml)
Wash 1	KH_2PO_4
Wash 2	MeOH
Dry for 5 minutes on full pressure	Dry for 5 minutes on full pressure
Elute 1	NaOH pH 10
Elute 2	NaOH pH 10
Elute 3	NaOH pH 12
Elute 4	NaOH pH 12

The washes and elutes collected were placed into polytop tubes and dried using a Genevac EZ-2 evaporator.

For the second SPE procedure of spermidine 25mg of the pure standard spermidine was placed in a polytop and labelled. The same kind of column was used for this procedure as was used for the initial procedure. To the polytop a total of 2ml of hydrochloric acid (HCl) at a pH of 1.1 was added. The column was conditioned with 3ml methanol (100%) and equilibrated with 3ml HCl at a pH of 1.1. The spermidine extract (3ml) was loaded into the column. Table 2.10 displays the solvents used for the washes and elutes of the spermidine

column. The flow rate for this SPE column was reduced to half the standard recommended flow.

Table 2.10: Solvents used in spermidine SPE column

Stage	Solvent (3 ml)
Wash 1	HCl
Wash 2	MeOH
Dry for 5 minutes on full pressure	Dry for 5 minutes on full pressure
Elute 1	NaOH pH 10
Elute 2	NaOH pH 10
Elute 3	NaOH pH 12
Elute 4	NaOH pH 12

The washes and elutes were collected in polytop tubes and dried using a Genevac EZ-2 series for preparation for TLC evaluation.

2.3.3.2.1. Column chromatography of *V. pygmaea* SPE column

Washes 1-10 from the SPE column of *V. pygmaea* revealed many compounds not containing nitrogen; in order to achieve better separation column chromatography was conducted. Washes 1-10 were combined and concentrated to dryness using a BÜCHI rotavapor R-200. . A 100ml column was washed with 100% methanol and left to dry. Between 5-7ml of methanol was added to the dry extract and sonicated until the extract was in solution. Clean dry silica powder was added to the extract and left in a porcelain mortar until dry. A small piece of cotton wool was added to the dry column (50ml) followed by $^{2}/_{3}$ of silica gel. The dry silica gel containing the extract was added evenly and lastly a small piece of cotton wool

was added. Table 2.11 depicts the solvent systems used. Each solvent system was added to a total volume of 400ml.

Table 2.11: Solvent systems used in silica gel column chromatography

Dichloromethane (ml)	Methanol (ml)	dH_2O (ml)
320	76	4
240	144	16
180	180	40
120	240	40
40	320	40

The fractions (1-40) were collected in 50ml vials. The vials were concentrated to about a quarter of their volume using a Genevac EZ-2 evaporator.

2.3.3.2.2. Thin layer chromatography analysis of SPE fractions

Both SPE columns of *V. pygmaea* and *V. thamnus* were prepared and developed using the same procedure. The TLC plates were prepared in duplicates; so for each plant there were two TLC plates, this was for visualisation by vanillin and ninhydrin. The plates were developed on a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). The TLC plates were developed twice within the TLC chamber to achieve better separation the plates were run with pavettamine and the original crude extract from extraction procedure 2.

The fractions generated from the column chromatography of *V. pygmaea* were developed as those mentioned above. The fractions were spotted with the fraction from the load sample of

the SPE column in order to compare the similarities. They were compared with the TLC plates generated from the SPE column of *V. pygmaea*.

A total of 12 fractions were produced from the SPE column of *P. gardeniifolia.* A volume of 1ml of distilled water was added to washes 1-6. A ratio of 1:1 distilled water: methanol was prepared and 1ml of the mixture was added to washes 7 and 8. Wash 9 received 1ml of 100% methanol. A volume of 1ml of 25% ammonia solution was added to washes 10-12. The washes were sonicated for 20 minutes to ensure all compounds were in solution. The washes were then spotted on TLC plates. Six identical TLC plates were prepared containing pavettamine standard, the original crude extract from extraction procedure 3, the load sample and the 12 washes. Two of the six TLC plates were developed with a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). Two of the remaining four TLC plates were developed with a mobile phase of methanol: dichloromethane: ammonia solution (25%) (20:77:3). The last two TLC plates were developed with a mobile phase of distilled water: 2-propanol: acetic acid (60:40:4). All six TLC plates were developed twice to ensure better separation of the bands. Two TLC plates per mobile phase were used; one for detection of vanillin and the other for ninhydrin.

The SPE column of *P. schumanniana* produced 7 fractions. To each of the washes and elutes 10 drops of the corresponding mobile phase was added and sonicated until the dried extract was in solution. The TLC plates were developed in duplicates for both vanillin and ninhydrin detection. The mobile phase used was distilled water: 2-propanol: acetic acid (60:40:4).

The fractions produced from the SPE column of spermidine were developed with spermidine standard and the load sample. The TLC procedure was developed in duplicates for detection by both vanillin and ninhydrin with the mobile phase of distilled water: 2-propanol: acetic acid (60:40:4).

2.3.3.3.Hydrolysis of bound pavettamine present in *P. schumanniana*

An extract of *P. schumanniana* from extraction procedure 3 was subjected to heat treatment. 3ml of room temperature distilled water was added to the 0.88g *P. schumanniana* extract and sonicated for 45min until the extract was in solution. 1ml of the extract was removed and placed in another polytop labelled control. A 1cm magnetic stirrer was added to the extract and heated in a beaker with water to about 90˚C for 4h. The extract was then left at room temperature for 1h to cool.

Another extract of *P. schumanniana* from extraction procedure 3 contained a final mass of 0.80g. This dry extract was dissolved in a 15ml solution of 0.1M NaOH. Once the extract was in solution 2ml was removed and placed in a polytop. The remaining 13ml of extract was placed inside a 50ml separation funnel. 10ml of dichloromethane was added to the funnel and left for 15min in order for the two layers to develop. A slight emulsion was observed; the solution was agitated until the emulsion was removed. The dichloromethane faction was collected in a polytop. A second solution containing dichloromethane was added to the funnel shaken and left for 15min. The funnel was agitated with a pasteur pipette and the faction was collected into a polytop. These two dichloromethane fractions served as the non-polar fractions. 10ml of butan-1-ol was added to the funnel and shaken. The funnel was left for 15min in order for the two layers to develop. The NaOH was collected in a polytop. A second volume of 10ml of butan-1-ol was added to the funnel, shaken and left for 15min. Any NaOH residue was removed and collected in a labelled polytop. The butan-1-ol fractions served as the polar fractions whereas the NaOH fraction served as the alkaline fraction. All fractions were centrifuged at 3000rpm for 10min in order to ensure single solvent fractions were collected. Any fraction containing a layer of unwanted solvent was cleaned by carefully removing the unwanted solvent with a pasteur pipette. All the fractions were then dried using

a Genevac EZ-2 evaporator. Once dried 5 drops of each corresponding solvent was added to the fractions and sonicated until the dry fraction was in solution.

2.3.3.3.1. TLC evaluation of hydrolysis experiments

Once cooled the heated fraction was subjected to TLC analysis. Pure pavettamine standard served as the positive control, and the 1ml of unheated *P. schumanniana* extract was the second control. The two controls were spotted with the heat treated *P. schumanniana* extract on TLC plates. The TLC plates were developed in duplicates for both vanillin and ninhydrin detection. The TLC plates were developed twice for adequate separation with the mobile phase of distilled water: 2-propanol: acetic acid (60:40:4).

The same TLC procedure was conducted on the fractions generated from the basic hydrolysis experiment. Pavettamine and the 2ml control served as the positive controls. The two dichloromethane fractions, one NaOH and the two butan-1-ol fractions were spotted on the TLC plates.

P. schumanniana leaves were subjected to acid extraction (described in point 2.3.2.4.) in order to release pavettamine from its bound state. The extracts from extraction procedure 4; old leaves, young apical leaves, control old leaves and control young apical leaves were spotted on TLC plates with pavettamine standard as the positive control. The plates were prepared in duplicates for both ninhydrin and vanillin detection. The TLC plates were developed twice for adequate separation. The mobile phase was distilled water: 2-propanol: acetic acid (60:40:4).

2.3.3.4.Thin layer chromatography analysis of crude extracts isolated from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*

The plant extracts from extraction procedure 3 were used to compare the compounds isolated from these six plants using TLC procedures. From each of the dry extracts 200mg was removed and placed in a separate polytop and labelled. 1ml of distilled water was added to each polytop. The polytops were sonicated for 30min in order to get the extracts into solution. The standard pavettamine served as the positive control. Two mobile phases were used the first one being ethylacetate: formic acid: acetic acid: distilled water (6:1:1:1) and the second distilled water: 2-propanol: acetic acid (60:40:4). Two TLC plates were prepared for detection by ninhydrin and vanillin.

2.3.4. Cytotoxicity of crude extracts from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*

2.3.4.1. Cell preparation

From extraction procedure 3 a total of 2mg of dried leaf material from each plant was used for cytotoxicity screening. The cell line used for cytotoxicity testing was the H9c2 cell line. These cell lines were obtained from American Type Culture Collection (cat no: CRL-1446™, Manassas, USA). The cells were placed in Dulbecco's Modified Eagle's Medium (DMEM) which had been previously supplemented with 100U/ml penicillin and 100µg/ml streptomycin sulphate and 10% foetal calf serum. This combination will further be referred to as the stock medium (Ellis et al., 2010).

Stock medium (50ml) was placed into a 50ml centrifuge flask and 400µl of ciprobay (excluding fungizone supplement) was added. This combination will be referred to as the complete medium. The H9c2 cells were incubated at 37˚C in 5% CO_2.

Between 48 and 72 hours after initial incubation the cells were 80% confluent and were split to ensure further growth. The complete medium was removed from the cells. The cells were trypsinized twice using a 5x stock trypsin of 0.5% concentration. The cells were placed in a 37˚C incubator for 2 minutes to allow the cells to detach from the flask. 50ml of complete medium was added to the old flask and swirled around to collect all detached cells. 25ml of the complete medium containing cells was added to a new flask placed back into the 5% CO_2, 37˚C incubator.

2.3.4.2.XTT cytotoxicity assay

Once a sufficient number of 80% confluent flasks were grown a XTT cytotoxicity assay could commence. The 80% confluent flasks were trypsinized twice using 0.5% 5x stock trypsin. All the cells were collected into a 50ml centrifuge flask and centrifuged for 5minutes at 980rpm. The cells were re-suspended with 2ml of complete medium. From the 2ml of re-suspended cells 10µl was added to an Eppendorf tube along with 90µl of trypan blue solution (1:10 dilution). From the Eppendorf tube 10µl of the solution was added to the two chambers of the haemocytometer. Using a light microscope the cells were counted using a hand-held tally. The cell concentration was then determined using the following formula:

1. Number of cells counted per square = number of cells counted divided by four
2. Cell suspension (cell concentration) = number of cells counted per square x10 x10000= cells per millilitre
3. Volume added to cell pellet = cell concentration wanted X volume wanted /Concentration of cells in suspension= Total volume
4. Volume wanted = total volume – volume added to cell pellet

Day one: Along with the six test plants pavettamine and actinomycin D both served as a positive control. All together there were 8 samples for testing and four 96 well-plates were used for the experiment (2 samples per 96 well-plate). Into all the outer wells of the plates 200µl of incomplete medium (no foetal bovine serum or 100U/ml penicillin and 100µg/ml streptomycin sulphate) was added. To the inner wells 100µl of cell suspension was added. The 96 well-plates were incubated over night at 37˚C in an atmosphere of 5% CO_2.

Day two: 2mg of each of the six dried crude plant samples were placed in labelled Eppendorf tubes and further dissolved in 100µl of DMSO. The samples were sonicated for between 1-2 hours to ensure all extract were in solution. For the two controls, pavettamine and actinomycin D (both pure compounds); 1mg of each of the controls were placed in an Eppendorf tube followed by 100µl of DMSO. Using 24 well-plates each sample underwent 8 serial dilutions. Each 24 well-plates contained 3 samples' dilutions. For the plant samples 2ml of complete medium was added to the first well and thereafter 1ml in the seven other wells. Pavettamine was treated similar as the test plant samples, however, for actinomycin D, 1ml of complete medium was added to all eight wells. For the six plant samples and pavettamine; from each of the first wells 80µl of complete medium was removed and replaced by 80µl of sample dissolved in DMSO. For the tested positive control, actinomycin D, 1ml of pre-prepared actinomycin D (1µl in 5ml complete medium) was added to the first well. All eight samples were serial diluted by removing 1ml out of the first well and adding it to the second well, mixing the new concentration and then removing 1ml out of the second well and placing it in the third, until a complete serial dilution was achieved. The negative control was prepared by adding 2ml of complete medium to a well, removing 80µl of the complete medium and replacing it with 80µl of DMSO.

All the samples were added into the 96 well-plates in the same fashion. 100µl of each concentration was removed from the 24 well-plate and added to the 96 well-plate. Each of the

8 concentrations per sample was analysed in triplicate. For each sample there was a triplicate DMSO and complete medium control present. The outer wells of the 96 well-plates contained incomplete medium.

Actinomycin D was added to the 96 well-plates in low light intensity due to the compound's photosensitivity. All of the 96 well-plates were incubated for 72 hours.

Day five: The XTT reagent used for this experiment namely, pesto blue, was prepared for all the 96 well-plates by adding 20µl of PMS to every 1ml of pesto blue. A total of 50µl of pesto blue reagent was added to all the test wells; wells containing H9c2 cells. The wells were mixed by pipetting up and down. The 96 well-plates were further incubated for between 4-6 hours. All the bubbles were removed before reading the plates on an ELISA plate reader.

2.4.Results

2.4.1. Chromatography and nuclear magnetic resonance analysis of *V. pygmaea*

2.4.1.1.Column chromatography

All the gousiekte-inducing plants are assumed to contain a toxic, nitrogen containing polyamine which reacts with the indicator ninhydrin. Due to this theory fractions which contained compounds which reacted with ninhydrin were considered fractions of interest. From the TLC analysis of the silica gel column fractions 29-33 showed considerable interest as multiple bands present in these fractions reacted with ninhydrin. Fraction 29 was thus subjected to size exclusion chromatography where fractions 5-8 revealed a reaction with ninhydrin. Fractions 5-8 were combined and run on an ion exchange column which generated washes 1-3. Wash 2 contained compounds which were visualised when sprayed with ninhydrin.

Wash 2 was run through a silica gel column in order to separate the fractions by polarity. This was conducted to achieve semi-pure fractions. Fractions 1-22 reacted with ninhydrin and thus

were considered nitrogen containing compounds of interest. Similar fractions were grouped together, concentrated to dryness and re-spotted on TLC plates with the same mobile phase, Figure 2.1 displays the grouped fractions. [Lane one indicates the pure standard pavettamine. Lane two is the original extract 29 which expresses at least seven different compounds at different rf values and colours also possibly containing pavettamine. Lane 3 shows the semi-pure compound C1. Lane 4 reveals that C2 contains at least four different compounds. Lane 5 shows that C3 was in low concentration and contains at least three different compounds. Lane 6 indicating C4 reveals low concentration and at least six different compounds one being very non-polar seen at the top of the TLC plate.

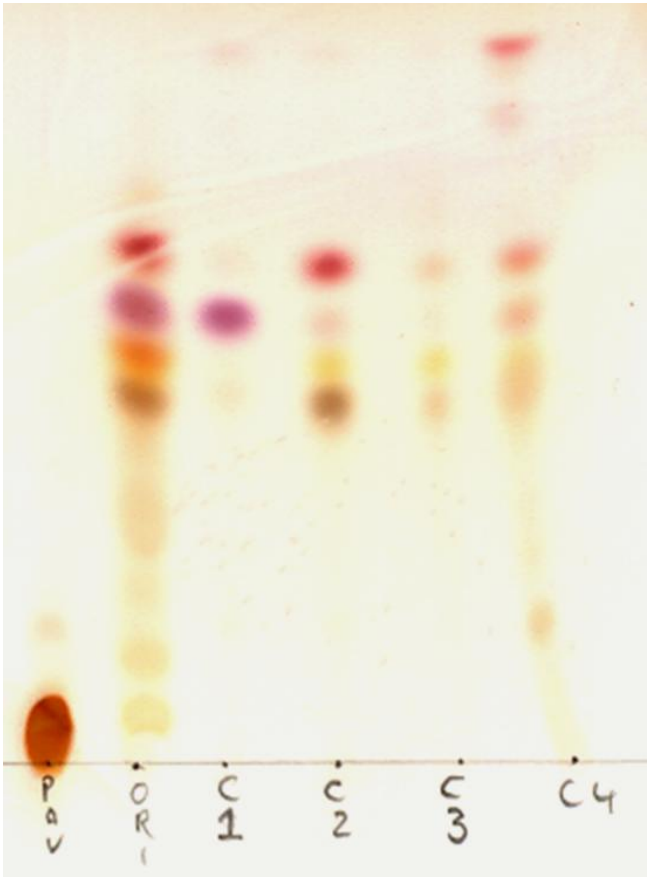


Figure 2.1: Grouped fractions from silica column of wash 2. Lane 1= pure standard pavettamine, lane 2= original extract 29, lane 3= C1, lane 4=C2, lane 5= C3 and lane 6=C4.

From Figure 2.1 it is clear to see that compound C1 appears semi-pure with a single band pattern. Compounds C2-C4 all contain multiple bands thus are still relatively unpure however the band patterns differ between all the fractions.

Column chromatography was repeated for fraction 29 and the combined fractions 30-33 in order to generate semi-pure fractions. Fractions generated from column 29 were analysed on TLC plates and were found to be like the compounds produced in the initial column chromatography experiment. From the combined fractions 30-33's silica gel column, fractions 30-54 reacted with ninhydrin. Figure 2.2 reveals the fractions 30-54.

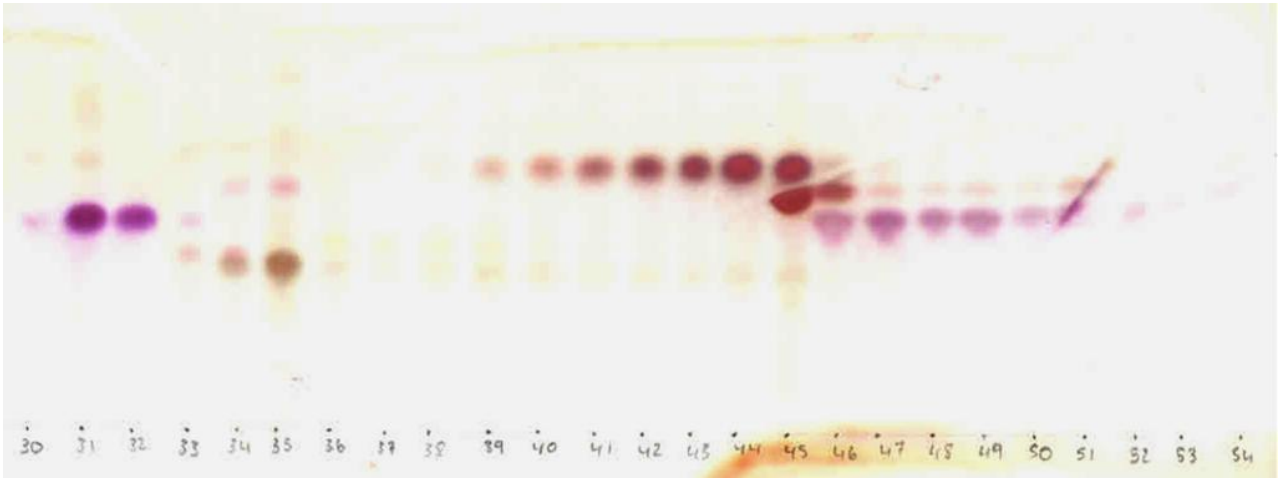


Figure 2.2: TLC of combined fractions 30-33's silica column.

From this initial TLC analysis fractions 30-32 were combined and labelled Com1, 33-36 were combined and labelled Com2, 37 and 38 became Com3, fractions 39-44 which contained a single red band was labelled Com4, fractions 45 and 46 with a double band pattern was labelled Com5 and lastly 47-54 became Com6. These combined fractions were dried and re-spotted; multiple band patterns were noted in Com2-Com6 however all band patterns were different. A single coloured band pattern was present in Com1 however compounds which reacted with long wavelength UV light were present.

2.4.1.2.Size Exclusion Chromatography

The Sephadex column was used to separate the fractions by size. Fraction 29 from the initial column chromatography column was subjected to size exclusion chromatography. On observation of the TLC plates, Figure 2.3, used to analyse the Sephadex column it is clear that there are multiple fractions which contain bands which reacted with ninhydrin and turned a shade of orange. These fractions, 5-8, contain many compounds which contain a nitrogen atom.

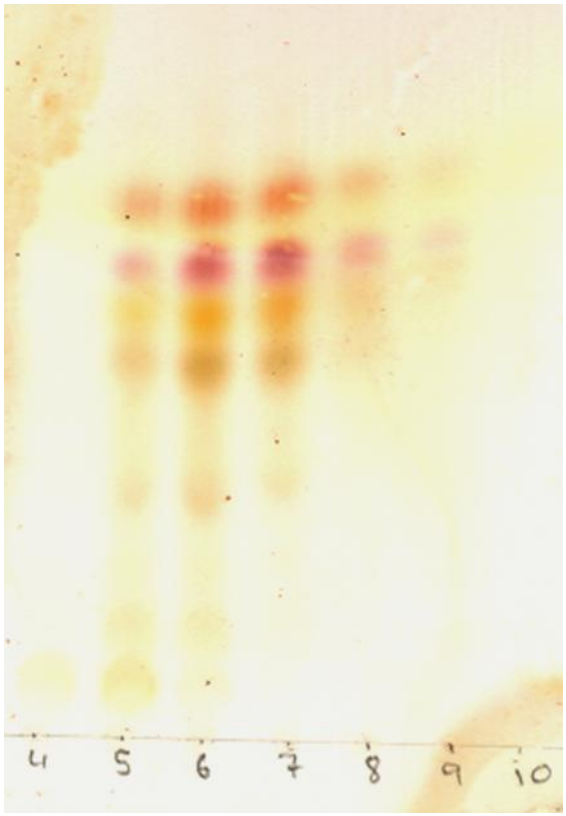


Figure 2.3: TLC plate of size exclusion chromatography column of fraction 29.

2.4.1.3.Ion Exchange Chromatography

The combined fractions 5-8 were run on Dowex 50 resin to separate the compounds by charge. Dowex 50 resin is a cation exchange resin and thus retains compounds with a positive charge allowing sugars and neutral/ acidic amino acids to run through the column. On analysis of the TLC plates generated from the ion exchange column it was clear that

compounds which reacted with ninhydrin eluted from the column in wash 2, 1:1 distilled water: 25% ammonia solution.

As seen in Figure 2.2 fractions 30-32 reveal a single band pattern, these fractions were combined labelled Com1 and subjected to ion exchange chromatography. The cationic exchange resin, Dowex 50 was used to remove the compounds which reacted with long wavelength UV light as seen in Figure 2.4, but which did not react with ninhydrin.

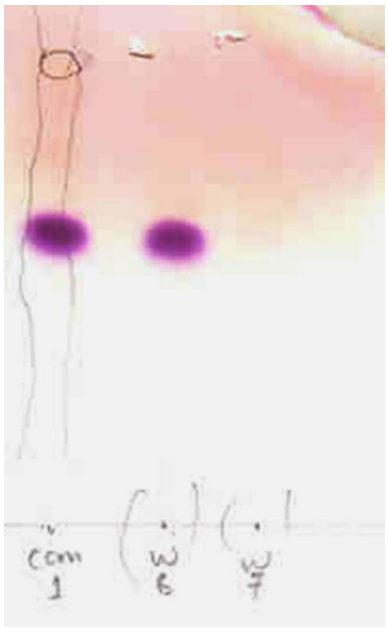


Figure 2.4: TLC of Ion exchange chromatography of Com1, lane 1= original Com1 not subjected to ion exchange chromatography, lane 2= purified Com1, lane 3= no compound Com1 left in the column.

2.4.1.4.Nuclear Magnetic Resonance (NMR)

Compound C1 (Figure 2.1) was subjected to NMR analysis. In order to determine if C1 was pavettamine, the compound responsible for gousiekte poisoning, it was compared with that of a proton scan NMR of pavettamine pure standard. Figure 2.5 shows pavettamine the pure standard.

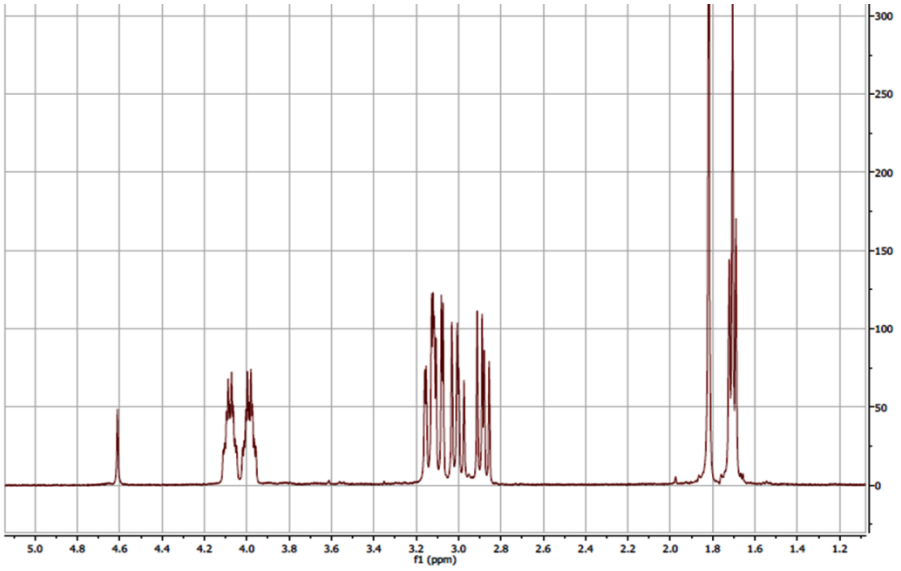


Figure 2.5: NMR spectrum of pure standard pavettamine.

The peak present in the 4.6 region is a reduced water peak. There are doublets in the 4.0, 4.1 region and a multiplet in the 2.9-3.2 region. There are also peaks present in the hydrocarbon region of 1.7 and 1.8. This NMR spectrum, Figure 2.5, was compared to that of C1 which is displayed in Figure 2.6.

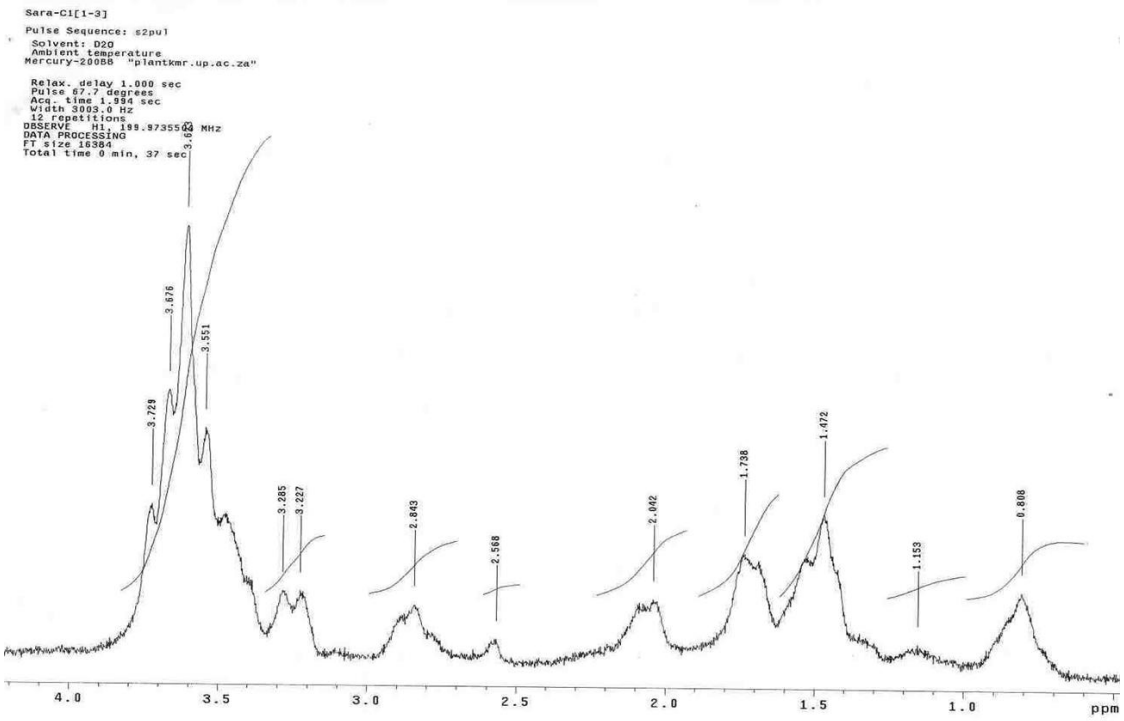


Figure 2.6: NMR spectrum of C1.

The proton NMR spectrum is zoomed in to exclude the water peak, which was present around 4.6. The main peaks in the C1 NMR spectrum include a possible doublet in the 1.4-1.8 region, a doublet in the 3.2 region and a multiplet in the 3.4-3.8 region. No peaks are present in the 4.0, 4.1 region. The compound, C1, therefore is not pavettamine.

The purified extract Com1 seen in Figure 2.4 appeared semi-pure thus was subjected to proton NMR. Figure 2.7 displays the proton NMR which was run with 240 scans.

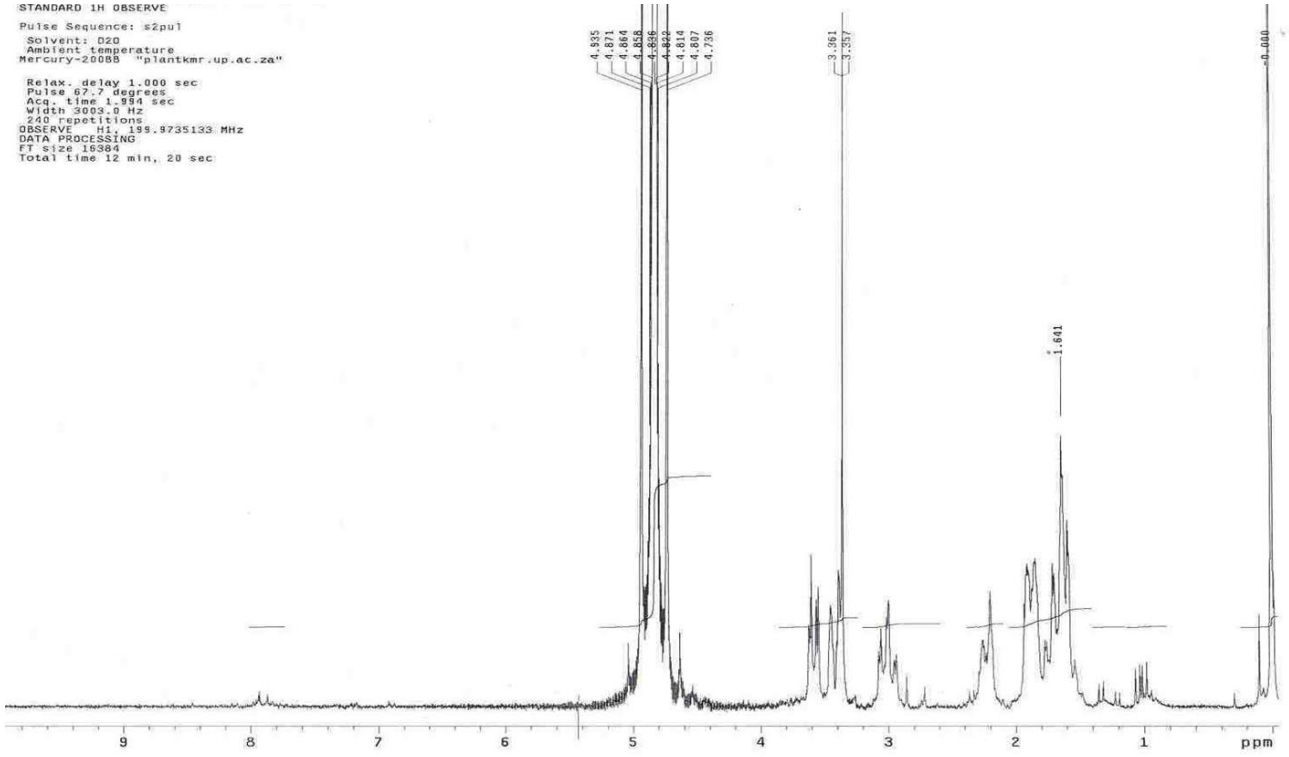


Figure 2.7: Proton NMR spectrum of Com1.

The NMR spectrum of Com1, Figure 2.7, was compared with that of pure pavettamine standard, Figure 2.5. The main peaks of interest include the peaks 1.6-2.0 region, 2.9-3.1 region and the 3.4-3.6 region. Note no peaks are present in the 4.0 region. In order to determine the number of carbon atoms present in this fraction a carbon NMR was conducted and Figure 2.8 reveals the result. From Figure 2.8 it is clear to see that this compound contains a total of 6 carbon atoms. The polyamine pavettamine contains 10 carbon atoms. To further evaluate this compound isolated from *V. pygmaea* a dept NMR was also conducted.

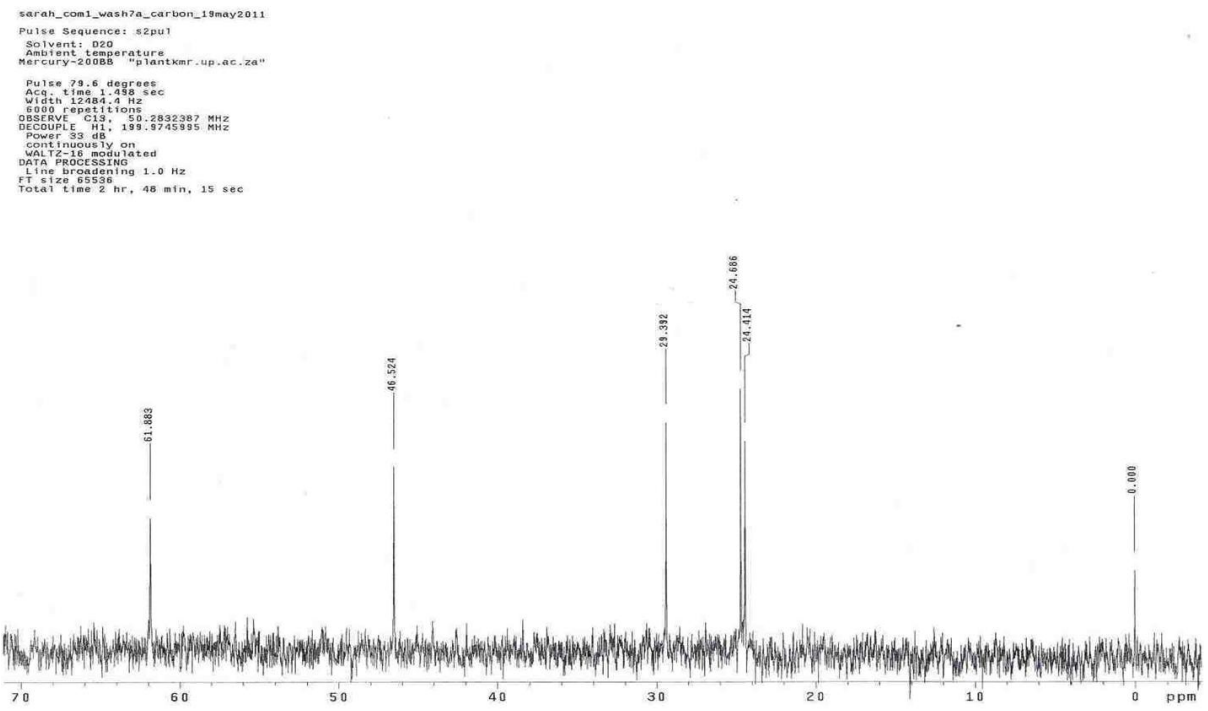


Figure 2.8: Carbon NMR spectrum of Com1.

The main reason for doing a dept NMR is to determine how many protons each carbon atom is bound to. Figure 2.9 reveals the orientation of proton atoms around the 6 carbon atoms of this compound.

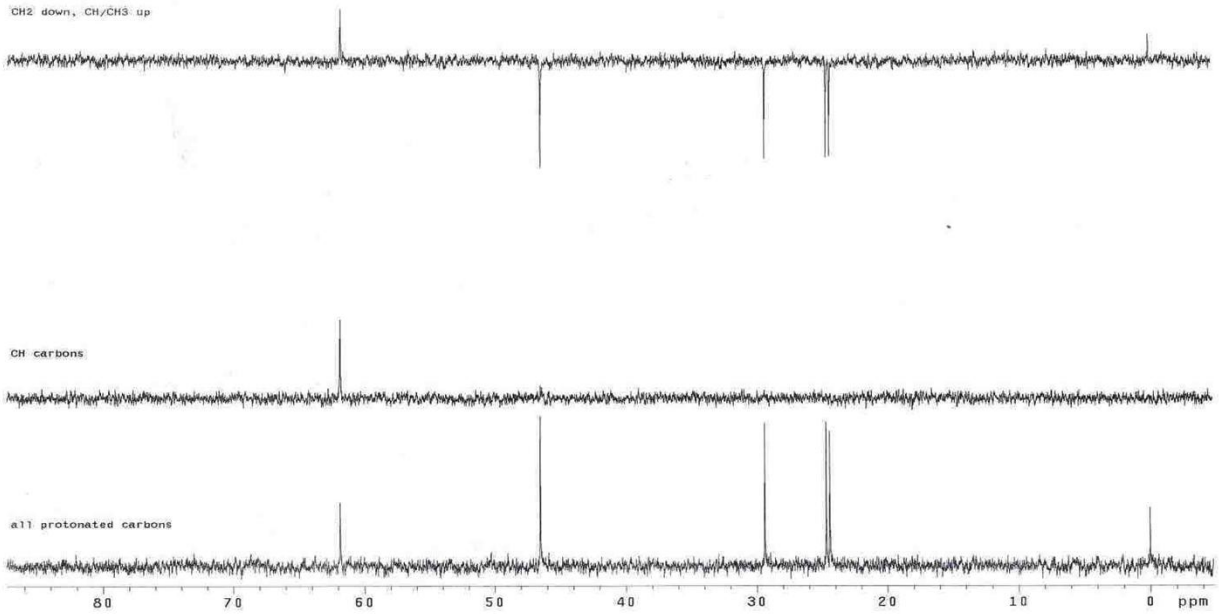


Figure 2.9: Dept NMR of Com1.

The dept NMR indicated that there are 4 CH_2 carbons, 1 CH carbon and 1 CH_3 carbon present in this semi-pure fraction which is Com1. Figure 2.10 (Cosy NMR) uncovers the spacial orientation of the carbon atoms in order to further understand and possibly elucidate the isolated compound Com1.

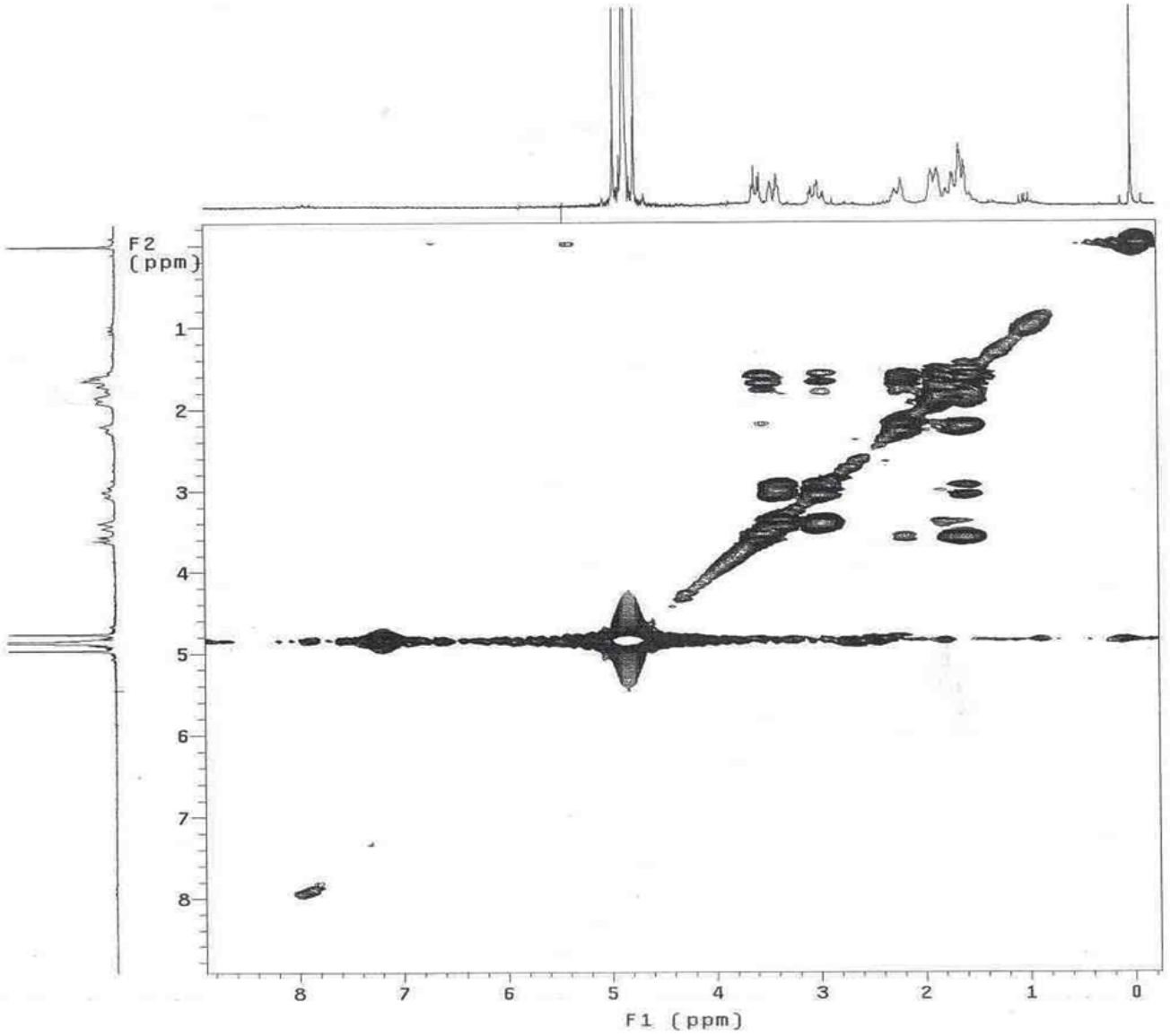


Figure 2.10: Cosy NMR spectrum of Com1.

The Cosy NMR revealed that the 6 carbon compound Com1 appears to be symmetrical, thus possibly a cyclic compound. Due to the fact that this compound did react with ninhydrin there is the assumption that this compound contains at least one nitrogen atom. However information about other possible side chains is not revealed in Cosy NMR.

2.4.2. Solid Phase Extraction (SPE)

The TLC plates generated from the SPE column of *V. pygmaea* contained some compounds which reacted with ninhydrin, thus compounds of interest. However, the majority of ninhydrin reacting compounds eluted with the load sample. The SPE column for *V. thamnus* became blocked due to small residue leaf debris. Thus the TLC analysis for *V. thamnus* was abandoned.

As with the SPE columns of *V. pygmaea*, and *V. thamnus*; the majority of ninhydrin reacting compounds eluted with the load sample in the SPE column of *P. gardeniifolia* as seen in Figure 2.11. However once a relatively strong positive solution of 25% ammonia, was pushed through the column a multitude of compounds of interest eluted as seen with wash 10.

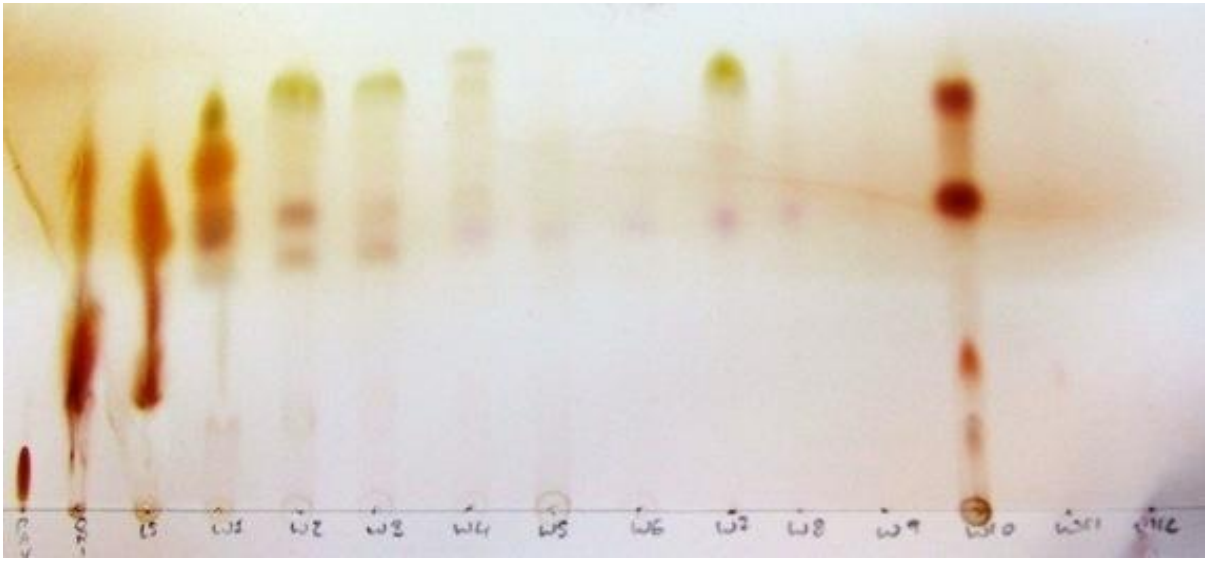


Figure 2.11: SPE column of *P. gardeniifolia* with a mobile phase of distilled water: 2-propanol: acetic acid (60:40:4). Lane 1= pure standard pavettamine, lane 2= original extract, lane 3= load sample (negative and neutral compounds), lane 4= wash 1, lane 5= wash 2, lane 6= wash 3, lane 7= wash 4, lane 8= wash 5, lane 9= wash 6, lane 10= wash 7, lane 11= wash 8, lane 12= wash 9, lane 13= wash 10, lane 14= wash 11 and lane 15= wash 12.

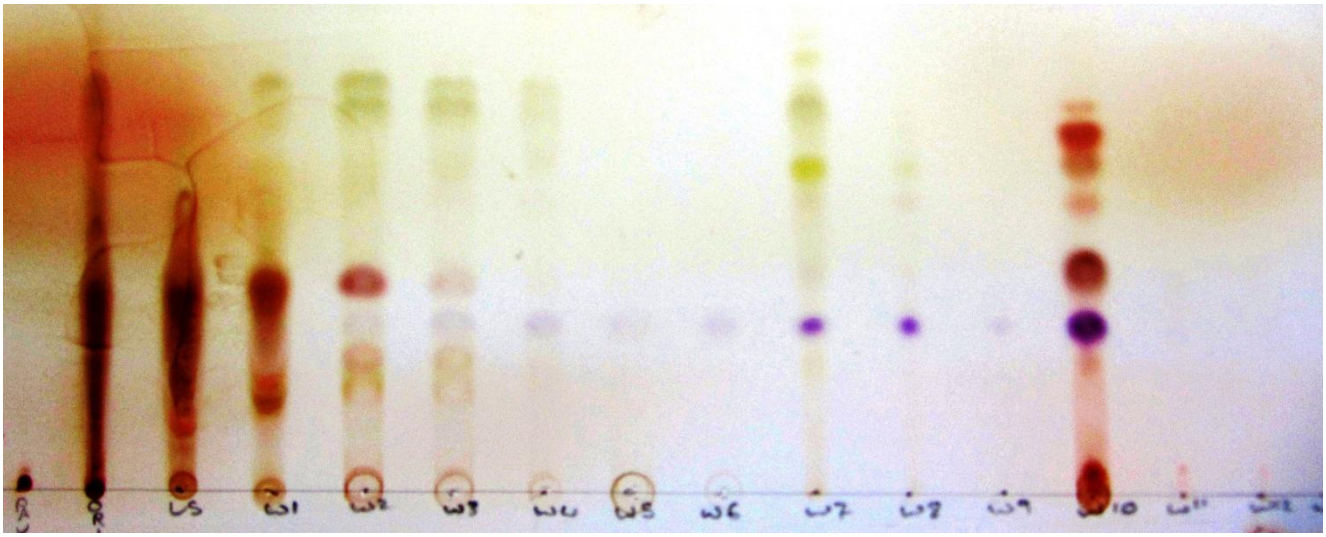


Figure 2.12: SPE column of *P. gardeniifolia* with a mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). Lane 1= pure standard pavettamine, lane 2= original extract, lane 3= load sample (negative and neutral compounds), lane 4= wash 1, lane 5= wash 2, lane 6= wash 3, lane 7= wash 4, lane 8= wash 5, lane 9= wash 6, lane 10= wash 7, lane 11= wash 8, lane 12= wash 9, lane 13= wash 10, lane 14= wash 11 and lane 15= wash 12.

From Figure 2.12 the load sample (third lane), contained a vast majority of compounds which reacted with ninhydrin. The rest of the washes excluding wash 11 and wash 12 (lane 14 and 15 respectfully), all contain compounds which reacted with ninhydrin.

Due to the strong positive charge on free pavettamine, it is expected to remain in the strong cationic column until forced out by a stronger positively charged solution. Figure 2.13 reveals ninhydrin reacting compounds present in *P. schumanniana*.

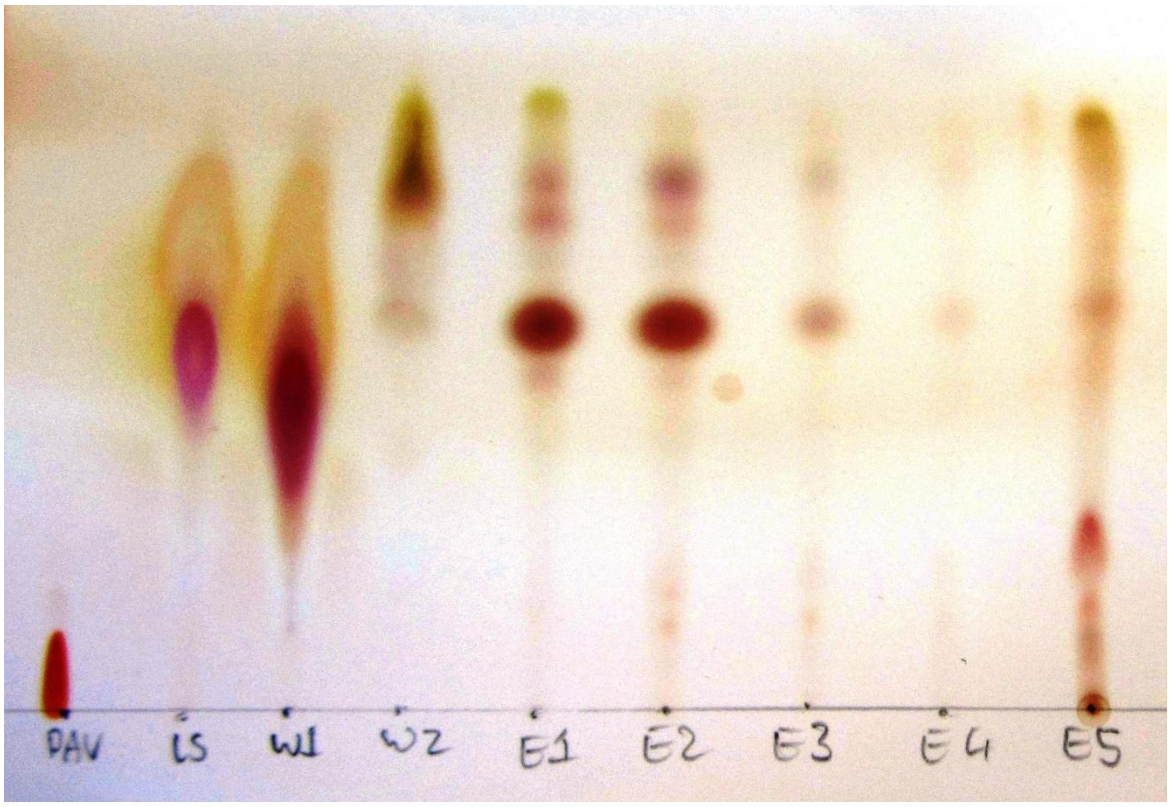


Figure 2.13: SPE column of *P. schumanniana* with the mobile phase of distilled water: 2-propanol: acetic acid (60:40:4). Lane 1= pure standard pavettamine, lane 2= load sample (negative and neutral compounds), lane 3= wash 1, lane 4= wash 2, lane 5= elute 1, lane 6= elute 2, lane 7= elute 3, lane 8= elute 4, lane 9= elute 5.

The mobile phase, distilled water: 2-propanol: acetic acid (60:40:4), did not produce clear band formation, but it is evident from Figure 2.13 that a large amount of ninhydrin reacting compounds eluted in the load sample (lane 2), and wash 1 (lane 3). Elute 5 (lane 9), has promising compounds close to the base line which appear similar in colour and rf value to that of pavettamine (lane 1).

For the initial SPE column of spermidine the TLC plates produced revealed that spermidine eluted in the load sample. The mobile phase for both the initial column and the second column was distilled water: 2-propanol: acetic acid (60:40:4). The TLC plates generated from

the second SPE column of spermidine showed the pure compound eluted in the load sample and wash 1.

2.4.2.1.SPE column chromatography

On analysis of the TLC plates produced from the SPE column of *V. pygmaea* it was clear that majority of compounds were present in the load sample which was run with pavettamine as a control. However, the theory that the original SPE column was overloaded was abandoned due to the few compounds present which eluted after the load sample wash. These compounds were non-polar and reacted to ninhydrin giving a different colour than that of pavettamine. The compounds which eluted after the load sample were of low concentration even after being completely dried before TLC spotting.

2.4.3. Hydrolysis of bound pavettamine in *P. schumanniana*

Heat treatment: The TLC plates produced for detection by both vanillin and ninhydrin were analysed under both short and long wavelengths before being subjected to the colour indicators. There was no noticeable difference in band formation between the non-heated control and the heated test extract. No bands were formed with the same rf value as that of the pure control pavettamine.

Basic hydrolysis: The mobile phase distilled water: 2-propanol: acetic acid (60:40:4) is very polar, thus is able to move pavettamine off the base line of the TLC plate. However, in all three of the fractions, butan-1-ol (polar), NaOH (basic) and dichloromethane (non-polar) there was no band formation with the same rf value as pavettamine.

Acid hydrolysis: Due to the nature of perchloric acid, concentration of the extracts was impossible thus the TLC plates displayed diluted band patterns. The TLC procedure was conducted numerous times in order to remedy this error. Once clear band formation was

achieved it was noted that there were no bands present around the same rf value as that of pavettamine.

2.4.4. Thin layer chromatography analysis of crude extracts isolated from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea*and *V. thamnus*

Extraction procedure 3 was carried out under the same conditions for each of the six plants as was the TLC analysis. The crude compound comparison was conducted in order to determine the chemical relatedness of each of the plants. Figure 2.14 reveals the compounds present in the six test plants.

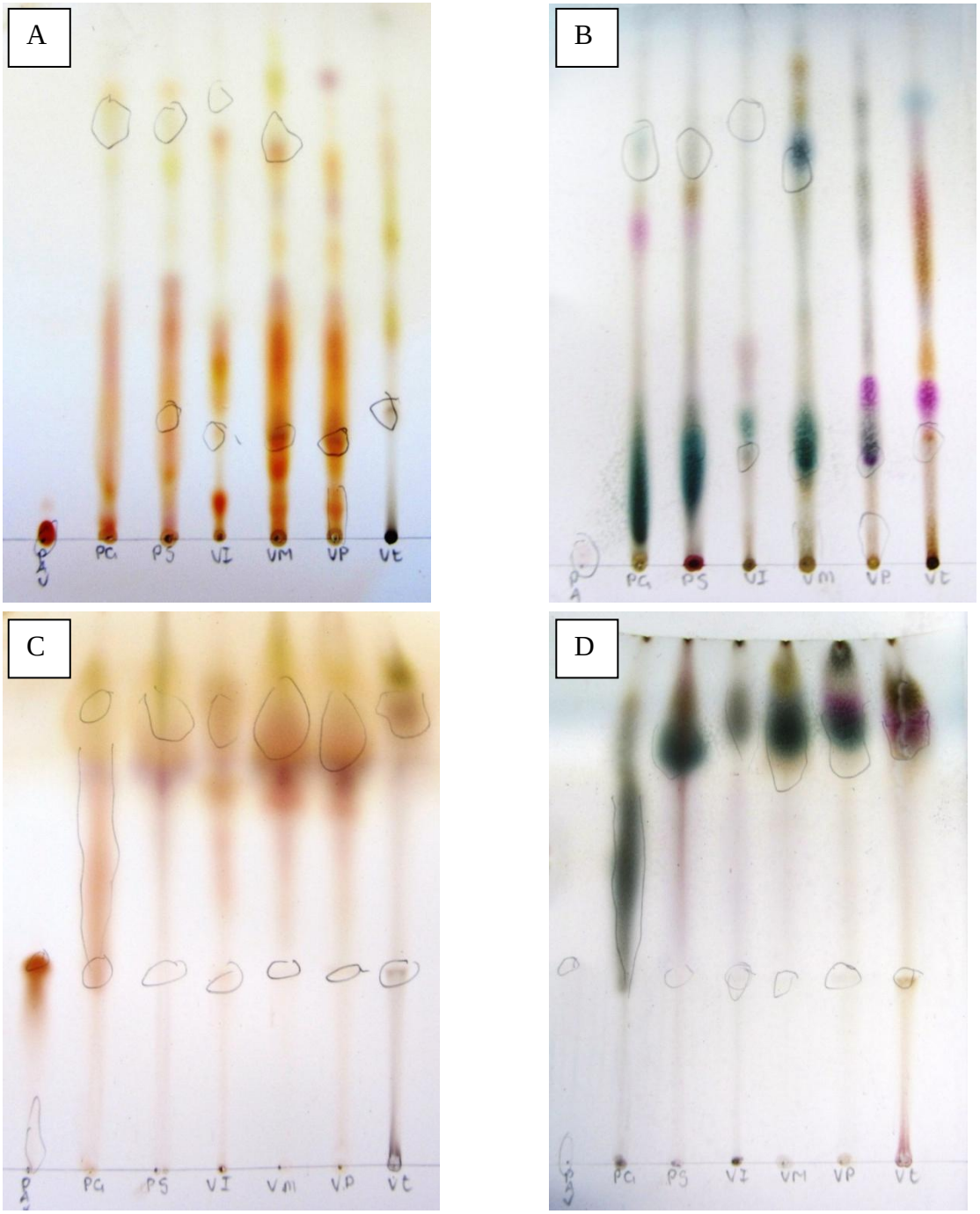
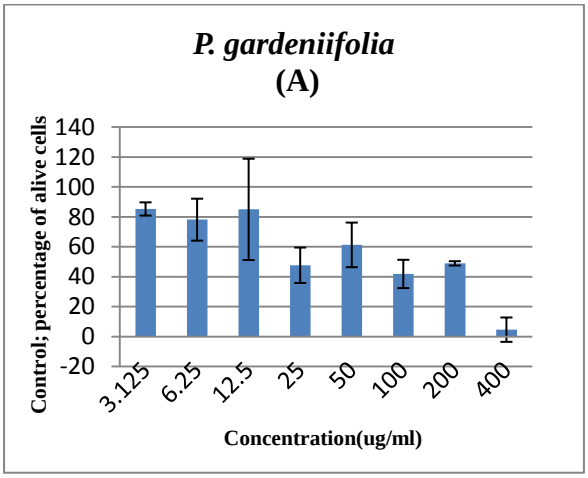


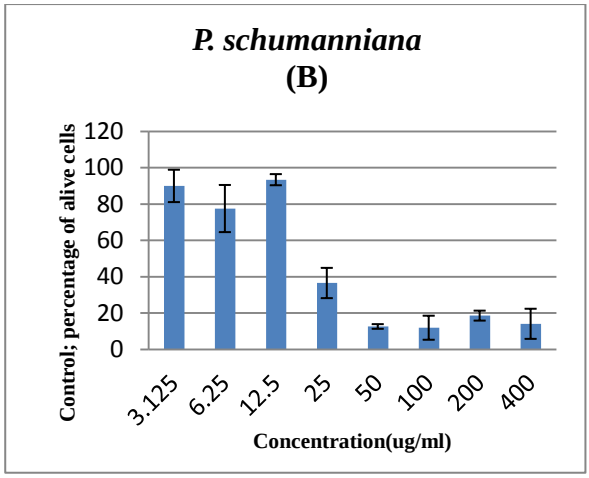
Figure 2.14: TLC analysis of the six plants, from left to right; pure pavettamine as the positive control in the first lane, *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. (A) TLC plate of mobile phase ethylacetate: formic acid: acetic acid: distilled water (6:1:1:1) with ninhydrin indicator. (B) TLC plate of mobile phase ethylacetate: formic acid: acetic acid: distilled water (6:1:1:1) with vanillin indicator. (C) TLC plate of mobile phase distilled water: 2-propanol: acetic acid (60:40:4) with ninhydrin indicator. (D) TLC plate of mobile phase distilled water: 2-propanol: acetic acid (60:40:4) with vanillin indicator.

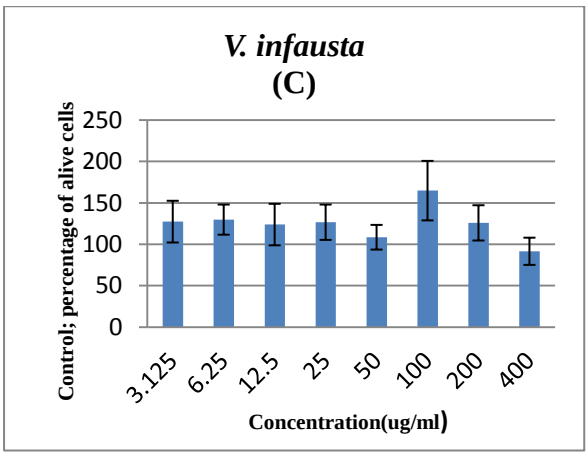
All the TLC plates were evaluated under both short and long wavelength UV light before being dipped in the indicator reagent. The pencil circles seen in Figure 2.14 depict compounds which reacted with either short or long wavelength UV light. The mobile phase ethylacetate: formic acid: acetic acid: distilled water (6:1:1:1) produced TLC plates with better band formation. However, the polar compound, pavettamine, did not move off the line of origin, thus making compound comparison impossible. The mobile phase distilled water: 2-propanol: acetic acid (60:40:4) allowed the polar compounds to move freely off the line of origin. However, single compound, band formation was affected and smearing on the TLC plates occurred. From Figure 2.14 (A and C) it is clear to see that all the plants contain a multitude of compounds which react with ninhydrin thus compounds which contain a nitrogen atom. Figure 2.14 (A and B) revealed that there are significant differences in compound composition between all the test plants; although there were a few compounds which all the plants probably share. This was not surprising due to the fast that all the plants belong to the family Rubiaceae.

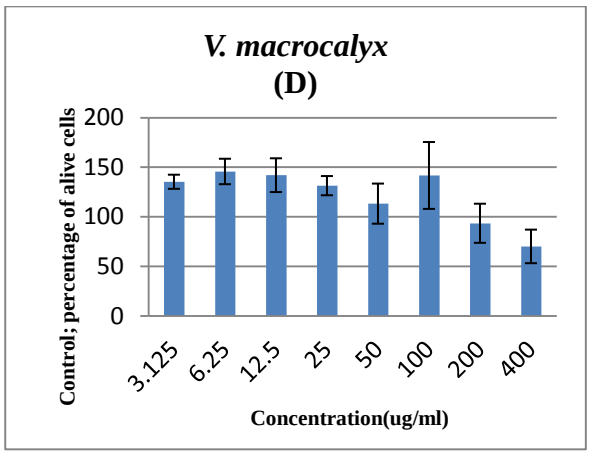
2.4.5. Cytotoxicity

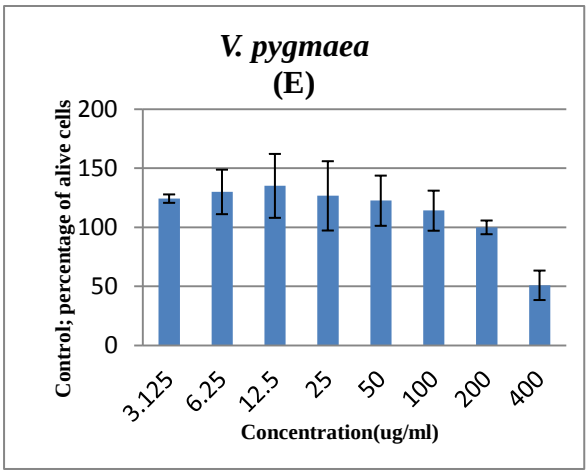
After the 6 hour incubation period the XTT reagent had changed colour in the wells which contained living cells. The plates were read on an ELISA reader and the results were analysed. Figure 2.15 reveals the charts generated from the toxicity screening.

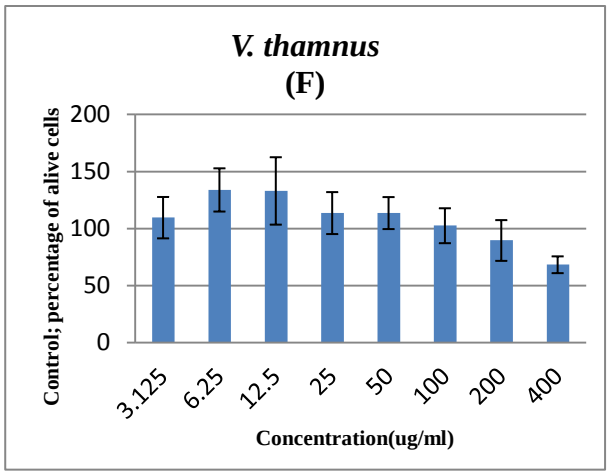












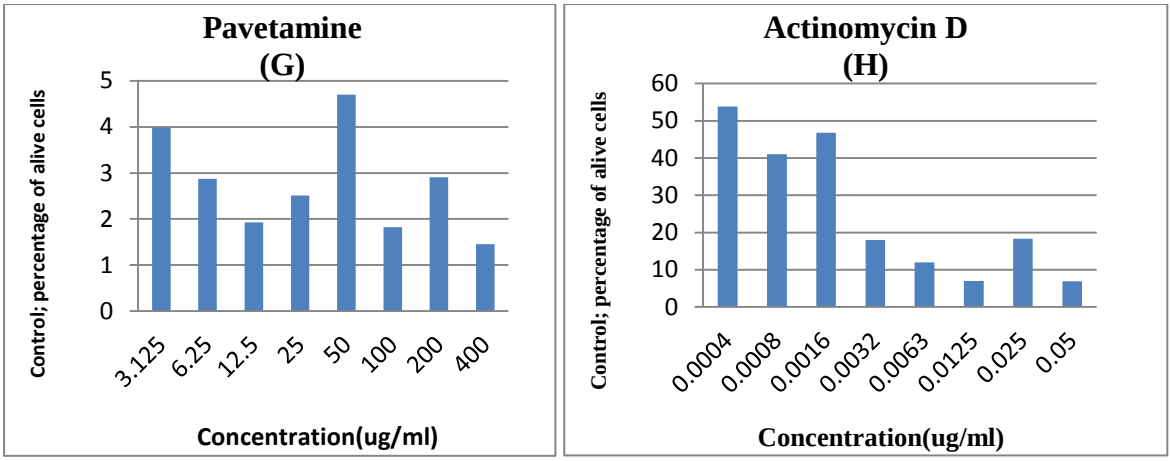


Figure 2.15: Charts displaying the relationship between cell viability and extract concentration. (A) *P. gardeniifolia*. (B) *P. schumanniana*. (C) *V. infausta*. (D) *V. macrocalyx*. (E) *V. pygmaea*. (F) *V. thamnus*. (G) Pavettamine. (H) Actinomycin D.

The IC_{50} value for each chart was determined by locating on the Y axis the 50% cell viability level and then locating on the X axis the concentration where the two lines meet. The lower the IC_{50} value the more toxic the extract. However, extraction procedures and other factors need to be taken into account when determining the toxicity of an extract. Table 2.12 shows the IC_{50} values for the six plants and the two positive controls.

Table 2.12: IC_{50} values of the six plants and the two positive controls

Sample	IC_{50} (µg/ml)
Actinomycin D (H)	0.0008
Pavettamine (G)	< 3.1250
P. gardeniifolia (A)	62.0
P. schumanniana(B)	18.5
V. infausta (C)	> 400
V. macrocalyx (D)	> 400
V. pygmaea (E)	312.0
V. thamnus (F)	> 400

From Table 2.12 it is evident that the most toxic fraction was in that of *P. schumanniana* with an IC_{50} value of 18.5µg/ml. The second and third most toxic fractions were from *P. gardeniifolia* and *V. pygmaea* with IC_{50} values of 62µg/ml and 312µg/ml, respectively. *V. infausta* was the least toxic with an extrapolated IC_{50} value of over 850µg/ml.

2.5.Discussion

Although numerous extraction methods were employed during the study, the polyamine pavettamine was not isolated from any of the following plants: *Pavetta gardeniifolia*, *P. schumanniana*, *Vangueria infausta*, *V. macrocalyx*, *V. pygmaea* or *V. thamnus*. The 'tiresome' method developed by Fourie et al. (1995) did not reveal positive results. TLC evaluation of extracts from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus* did not reveal compounds with the same rf value as the pure standard pavettamine (Fourie et al., 1995; Bode et al., 2010). However, multiple compounds were extracted from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V.*

pygmaea and *V. thamnus* which reacted with ninhydrin and thus contained a nitrogen atom. Pavettamine within plant tissues may thus be present in the bound form. The properties of bound pavettamine would differ from free pavettamine thus all ninhydrin reacting compounds were investigated (Fourie et al., 1995; Van Elst et al., 2012). NMR analysis on nitrogen containing compounds also did not reveal the presence of pavettamine.

According to Verstraete et al. (2011) the gousiekte inducing species with the highest toxicity is *V. pygmaea* followed by *F. homblei*, *P. harborii*, *V. thamnus*, *P. schumanniana*, *V. latifolia* (Verstraete et al., 2011). However, the results from the cytotoxicity screen on H9c2 cells revealed that *P. schumanniana* was the most toxic followed in descending order by *P. gardeniifolia*, *V. pygmaea*, *P. thamnus*, *V. macrocalyx* and lastly *V. infausta*. Using the extraction methods employed *V. pygmaea* and *V. thamnus* did not express high toxicity readings this could be due to the dryness of the leaves before extraction, human error or toxicity fluctuations in season (Van Wyk et al. 1990). The following are the major conclusions from this part of the study:

- The polyamine pavettamine was not isolated from *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*.
- A 6 carbon compound containing $4CH_2$, 1CH and $1CH_3$ was isolated from *V. pygmaea*.
- No clear similarities were visualised in crude compound comparison between *P. gardeniifolia*, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*.
- *P. gardeniifolia* was toxic to H9c2 cells although had not been previously reported to induce gousiekte.

- *P. gardeniifolia* generated higher toxicity readings than known gousiekte inducing plants *Vangueria pygmaea* and *V. thamnus*.

In conclusion it can be said that the first hypothesis can neither be accepted nor rejected as no free pavettamine was observed in any of the test plants. However, this does not mean that the polyamine was not present; just that it was not isolated in this study. The second hypothesis is accepted on the grounds that *P. gardeniifolia*, *P. schumanniana*, *V. pygmaea* and *V. thamnus* revealed varying degrees of toxicity. However, the hypothesis was rejected due to the lack of toxicity displayed in *V. infausta* and *V. macrocalyx*.

2.6.References

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Chapter 3

An evaluation of the endophytic bacteria present in pathogenic and non-pathogenic Vanguerieae using electron microscopy

3.1.Abstract

Fadogia homblei, *Pavetta harborii*, *Pavetta schumanniana*, *Vangueria pygmaea* (=*Pachystigma pygmaeum*), *Vangueria latifolia* (=*Pachystigma latifolium*) and *Vangueria thamnus* (=*Pachystigma thamnus*) all induce one of the most important cardiotoxicoses of domestic ruminants in southern Africa, causing the sickness gousiekte. All the plants which cause gousiekte have previously been shown to contain bacterial endophytes. However, in this study other plants within the Vanguerieae tribe that have not been reported to cause gousiekte; namely *Vangueria infausta*, *Vangueria macrocalyx* and *Vangueria madagascariensis*, have now been shown to also contain endophytes within the inter-cellular spaces of the leaves. The disease gousiekte is difficult to characterise due to fluctuations in plant toxicity. The majority of reported cases of gousiekte poisoning are at the beginning of the growing season; and thus the plants are thought to either be more toxic at this time or the only available green food this early in the season. By using both transmission and scanning electron microscopy the endophytes within these Vanguerieae plants were compared visually. Using the plant reported most often for gousiekte poisoning, *V. pygmaea*, a basic seasonal comparison of the presence of endophytes was done. It was found that the bacterial endophyte colonies were most abundant during the spring season.

3.2.Introduction

Many plant species which have been studied have revealed the presence of bacterial endophytes within their tissues. An endophyte is a microorganism which inhabits a healthy host plant without causing any negative effect to the plant (Hardoim et al., 2008; Phetcharat and Duangpaeng., 2011). The microorganism may benefit the plant by producing growth regulators or defending the plant from pathogens whereas the microorganism is provided with a stable climatic environment and nutrition (Rashid et al., 2011; Tiwari et al., 2012). Within the Rubiaceae the following six plant species were evaluated for the presence of bacterial endophytes, *Pavetta schumanniana* F. Hoffm. ex K. Schum., *Vangueria infausta* Burch. ssp. infausta, *V. macrocalyx* (= *Pachystigma macrocalyx*) (Sond.) Robyns, *V. madagascariensis* J.F.Gmel, *V. pygmaea* **(=** *Pachystigma pygmaeum*) (Schltr.) Robyns and *V. thamnus* (= *Pachystigma thamnus*) Robyns.

The plants of interest in this study have many morphological differences and similarities: *P. schumanniana, V. infausta, V. macrocalyx* and *V. madagascariensis* are all classified as small sized trees. The distribution range of *V. infausta* and *V. madagascariensis* is similar and they are often found in the bushveld and in rocky areas in Kwa-Zulu Natal. However, *V. infausta* has pubescent leaves whereas those of *V. madagascariensis* are globular. Where both *P. schumanniana* and *V. macrocalyx* have pubescent leaves their locality is quite different: *P. schumanniana* is usually located in sandy soils in bushveld regions north of Gauteng whereas *V. macrocalyx* is often found in rocky outcrops in high rainfall areas growing in between rocks (Van Wyk &Van Wyk., 1997). *V. pygmaea* and *V. thamnus* differ significantly from the other plants of interest due to the fact that they are geoxylic suffrutexes otherwise classified as underground trees. The only recognisable difference between these two underground trees is that *V. pygmaea* has leaves which are pubescent whereas those of *V. thamnus* are globular (Van Wyk &Van Wyk., 1997).

The six plants mentioned above have many differences and similarities with regards to their phytochemistry. *V. infausta*, *V. macrocalyx* and *V. madagascariensis* are considered as non-toxic and there has been no reports of poisoning occurring in ruminants after digestion of any of these three species. *P. schumanniana*, *V. pygmaea* and *V. thamnus* along with *P. harborii*, *F. homblei* and *V. latifolium* are responsible for causing the disease "gousiekte" which affects ruminants. *P. harborii*, *F. homblei* and *V. latifolium* were not included in this study.

As mentioned earlier gousiekte is a plant toxicosis which causes cardiomyopathy in ruminants, mainly domestic sheep and cattle. This toxicosis is of economical significance and over R9 million in cattle losses and over R5 million in sheep losses were recorded in 2008 (Verstraete et al., 2011). The difficulty in diagnosing gousiekte is that there are no symptoms of the disease and the animal will die 3-6 weeks after consumption of a gousiekte-inducing plant. The compound pavettamine which is responsible for gousiekte poisoning is a novel polyamine assumed to be present in all the gousiekte-inducing plants (Fourie et al., 1995). The structure of pavettamine; a highly polar, highly positive polyamine was elucidated 15 years after its discovery (Bode et al., 2010). The toxin degrades the animal's myofibres and usually after exercise the animal dies of cardiac failure. The exact cardiac muscle which is affected differs with each case and the latent period is not consistent (Prozesky et al., 2005).

Gousiekte has been known for over a century but due to many factors the disease has been very cumbersome to diagnose and comprehend. The lack of symptoms that the animals display makes understanding the disease in the early stages impossible (Fourie et al., 1995). Livestock varieties bred within southern Africa seem to be hardier to the disease and larger quantities of plant material is required to induce gousiekte (Prozesky et al., 1988). The *Vangueria* gousiekte-inducing plants appear to lose toxicity as the leaves dry. The disease undergoes fluctuations in toxicity based on season. The majority of cases are reported at the

beginning of the growing season, early spring, and all through summer. After this time the number of cases decline with no reports occurring in the winter months (Hay et al., 2008).

The gousiekte-inducing plants and the disease itself have been studied for over a hundred years in order to better comprehend the toxicosis. One discovery by Van Wyk et al. (1990) was the presence of bacterial endophytes in *F. homblei*, *Pachystigma bowkeri*, *P. venosum*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. In the Rubiaceae three genera, namely, *Pavetta*, *Psychotria* and *Sericanthe* have bacterial leaf nodules. These nodules can be seen with the naked eye as black spots on the lamina surface (Van Wyk et al., 1990). In the case of the *Vangueria* species Van Wyk et al. (1990) investigated, bacterial colonies were found to be located in the intercellular spaces of the leaves around the mesophyll cells. These bacteria were seen using transmission electron and light microscopy (Van Wyk et al., 1990).

Both transmission electron (TEM) and scanning electron microscopy (SEM) are methods which allow the researcher to establish the presence or absence of bacterial endophytes in plant tissue. Both methods were used in this study. The following six plant species, *Pavetta schumanniana*, *Vangueria infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus*, were selected due to either their toxic or non-toxic nature. The combination was necessary to evaluate the theory that the presence of bacterial endophytes influences the toxicity of the plant (Verstraete et al., 2011; Van Wyk et al., 1990).

The aims of this study were to determine the presence of bacterial endophytes in *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus*. Evaluate the visual similarities between the bacterial endophytes located in the toxic plants to those located in the non-toxic plants. To determine whether *V. pygmaea* undergoes seasonal habitation of bacterial endophytes.

The hypotheses of this chapter were:

1. All six test plants contain bacterial endophytes within their leaves.
2. The bacterial endophytes present in all six test plants appear morphologically similar.
3. There are seasonal fluctuations in the number of bacterial endophytes present in *V. pygmaea*.

3.3.Materials and methods

3.3.1. Plant collection

Leaves of *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* were collected in the province of Mpumalanga in the same week of October 2011. The leaves of *P. schumanniana* were collected in Gauteng province in October 2012 from the Onderstepoort campus of the University of Pretoria (GPS 25°38'80"S, 28°11'05"E). *V. infausta* (S 24°34'25", E 30°47'15") and *V. madagascariensis* (S 24°33'26", E 30°47'18") were collected in Blyde River Canyon near Hoedspruit. A pine tree plantation near Piet Retief was where *V. macrocalyx* (S 27°09'01", E 30°59'18") was located. *V pygmaea*(S 25°12'94", E 30°19'03") and *V. thamnus* (S 25°12'92", 30°19'02") were found in a field near a railway track about 15km outside Lydenburg. The H.G.W.J. Schweickerdt Herbarium is where the voucher specimens of all the plant species have been deposited. The PRU numbers for the plants are as follows: *P. schumanniana* 118677, *V. infausta* 117607, *V. macrocalyx* 117601, *V. madagascariensis* 117611, *V. pygmaea* 117605 and *V. thamnus* 117603. Where possible leaf samples from different individuals were collected for analysis. From each plant two undamaged mature leaves and two undamaged young apical leaves were collected. The samples were cut on site to a measurement of 4mm by 4mm and placed in a test tube containing 2.5% glutaralderhyde in 0.075M phosphate buffer (Coetzee and Van

der Merwe, 1986). The 24 samples were placed in labelled test tubes and transported back to the laboratory for further preparation.

3.3.1.1.Seasonal collection of *V. pygmaea*

The leaves of *V. pygmaea* were collected from the same site near a small Gauteng town called Rayton (S 25°73'608", E 028°53"321"). The leaves were collected in each season to provide a general seasonal comparison of bacterial endophyte habitation. The leaves were collected during late October 2010 (summer), March 2011 (autumn), June 2011 (winter) and early September 2011 (spring). A voucher specimen of *V. pygmaea*, PRU number 117989, has been placed in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria. Each season six samples were collected for TEM analysis and each sample was collected from a different individual. Three of the samples were of well matured undamaged leaves of length over 40mm and the other three were young undamaged leaves 40mm and less. Four samples were collected for SEM analysis at each sample again coming from a different individual; two samples were mature undamaged leaves of length over 40mm and two were young undamaged leaves 40mm and less.

3.3.2. Sample preparation and fixation

All the samples from the six plants underwent the same initial protocol for sample preparation. All samples were re-analysed and where needed were re-cut into 3.0mm by 3.0mm square TEM samples. The four *V. pygmaea* samples collected for SEM analysis were re-cut into 5.0mm by 5.0mm squares. From the field all the plant samples were immersed in 2.5% glutaralderhyde in 0.075M phosphate buffer they were removed from the buffer and rinsed with 0.075M phosphate buffer for 10 minutes 3 times. The samples were moved to a fume hood were after they were fixed with 0.5% aqueous osmium tetroxide for between 1 and 2 hours. Following the osmium tetroxide treatment the samples were rinsed with distilled

water 3 times at 10 minutes intervals within the fume hood. The samples were dehydrated using a gradient of different concentrations of ethanol (30%, 50%, 70%, 90% 100%, 100%, 100%). The samples remained in each concentration for 10 minutes (Coetzee and Van der Merwe, 1986).

3.3.2.1.Transmission electron microscopy (TEM) preparation

After the dehydration step the separation between TEM sample preparation and SEM sample preparation occurred. The TEM samples were infiltrated with 50% quetol epoxy resin and 50% ethanol for an hour. The mixture was removed and replaced with 100% pure quetol epoxy resin for 4 hours at room temperature, the test tubes were placed on a slow stirrer for the four hours to ensure the samples were completely coated in the quetol epoxy resin. The polymerisation step included placing the samples into a mould with the 100% quetol epoxy resin. The mould was then put into an oven at 60°C for 39 hours. The samples were cut into ultrathin sections using a microtome and placed on copper grids (Coetzee and Van der Merwe, 1986). The TEM used in this study was a JEOL 2100F.

3.3.2.2.Scanning electron microscopy (SEM) preparation

Once all the samples underwent the serial dehydration step the SEM samples were separated from the TEM samples. The SEM samples then underwent critical point drying followed by mounting on a SEM sample stub. In order to make the samples more electrically conductive they were coated with gold particles. The samples were then viewed and after the initial viewing the epidermis from the leaves was removed with cellotape in order to view the sample's interior (Coetzee and Van der Merwe, 1986). The SEM used was a JEOL 840.

3.4. Results

3.4.1. Presence of bacterial endophytes

TEM leaf samples from *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* were viewed. Figure 3.1 shows the TEM micrographs produced from these six samples. All six species contained bacterial endophytes within their leaves.

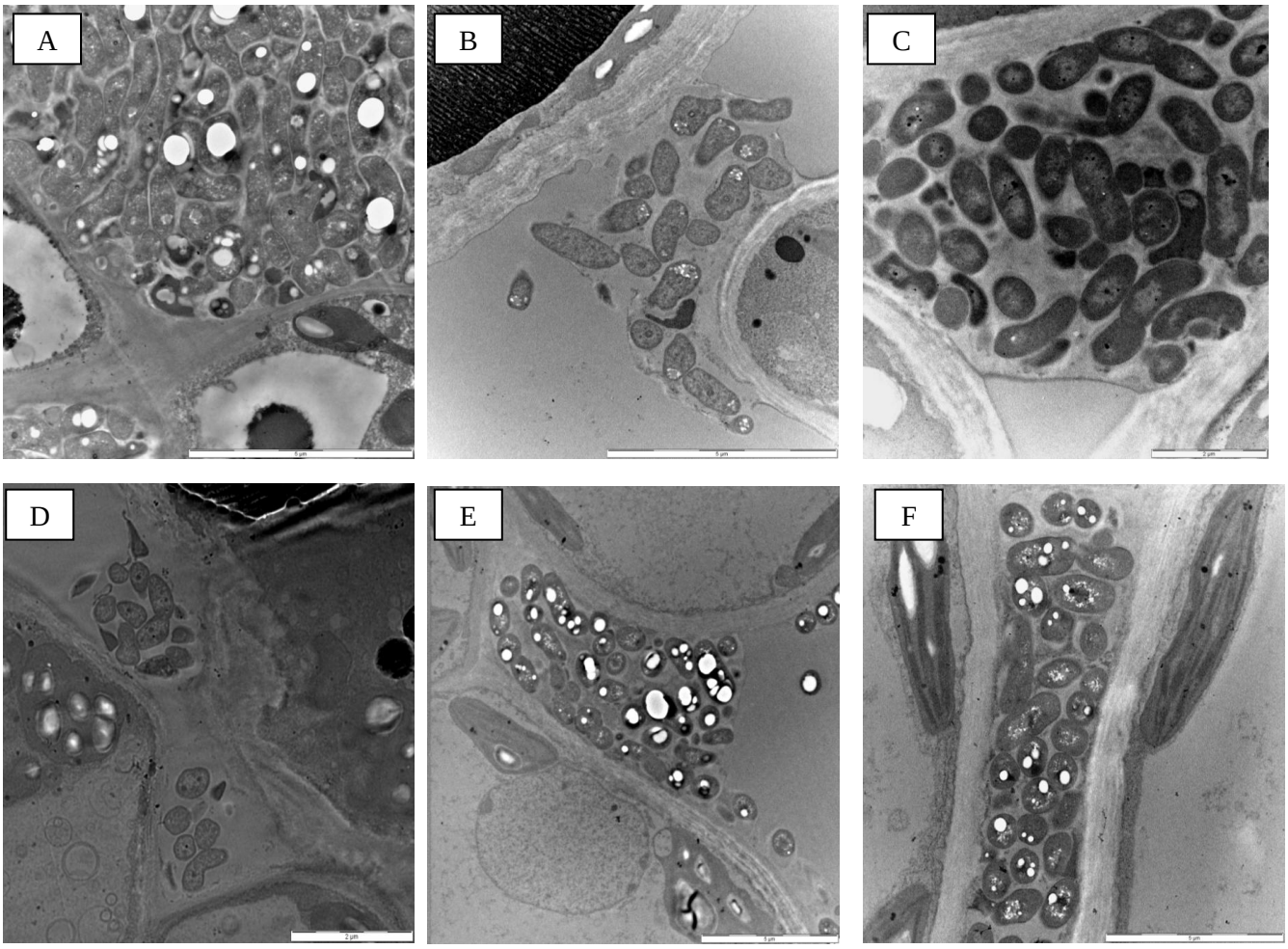

Figure 3.1: TEM micrographs of: (A) *P. schumanniana* 5µm. (B) *V. Infausta* 5µm. (C) *V. macrocalyx* 2µm. (D) *V. Madagascariensis* 2µm. (E) *V. pygmaea* 5µm. (F) *V. thamnus* 5µm.

From the TEM micrographs of the leaves seen in Figure 3.1 it is clear that bacterial endophytes were located in all six species. Bacterial endophytes were only found in the leaf nodules of *P. Schumanniana* (Figure 3.1.A). The leaf nodules contained large numbers of

bacteria. No endophytes were seen in any other part of the leaf in Figure 3.1.A. In the *Vangueria* species, Figure 3.1.B-F, which do not have bacterial leaf nodules, bacterial endophytes were only seen in the intercellular spaces of the leaves. The majority of the endophytes were located around the spongy mesophyll cells.

3.4.2. Bacterial morphological comparison

By using TEM analysis it is possible to visually compare the morphological differences and similarities between the bacterial endophytes located in the six different plant species. The endophytes observed within *P. schumanniana* were rod shaped and on average were 2.0µm long and 0.5µm wide. Located within the endophytes was a white "structure" which was present in both *V. pygmaea* and *V. thamnus* (Figure 3.1A, E and F). This substance appeared to be a polysaccharide-like substance which was probably polyhydroxybutyrate-like granules (Collins et al., 2012). The endophytes within *P. schumanniana* resembled those viewed in both *V. pygmaea* and *V. thamnus* in that their length, width and shape were very similar. The endophytes within the non-toxic *Vangueria* species were morphologically distinct to those occurring in the different gousiekte-inducing plants. The bacterial endophytes within *V. infausta* were rod shaped and had a width of between 1.0-1.5µm and a length of 4.0µm. As seen in Figure 3.1C the bacterial endophytes within *V. macrocalyx* had a well defined rod structure with a length of between 2.5-3.0µm and a width of 1.0µm. The bacterial endophytes observed in *V. madagascariensis* had a length of 1.0-1.5µm and a width of between 0.5-1.0µm and were rod shaped. The bacterial endophytes present in all six of the plant species produced a slime-like polysaccharide, probably EPS, which encased the bacteria within the intercellular spaces.

3.4.3. Seasonal endophyte colonisation of *V. pygmaea*

SEM analysis was used to determine whether healthy bacterial colonies were observed during each collection date. Figure 3.2 reveals the SEM micrographs of *V. Pygmaea* collected during March 2011(autumn), June 2011 (winter) and early September 2011 (spring). Bacterial endophytes were visualised in *V. pygmaea* collected in all three seasons. In both March (autumn) and September (spring) bacteria were observed within the tissue. Extensive colonisation was not observed in tissue collected in June.

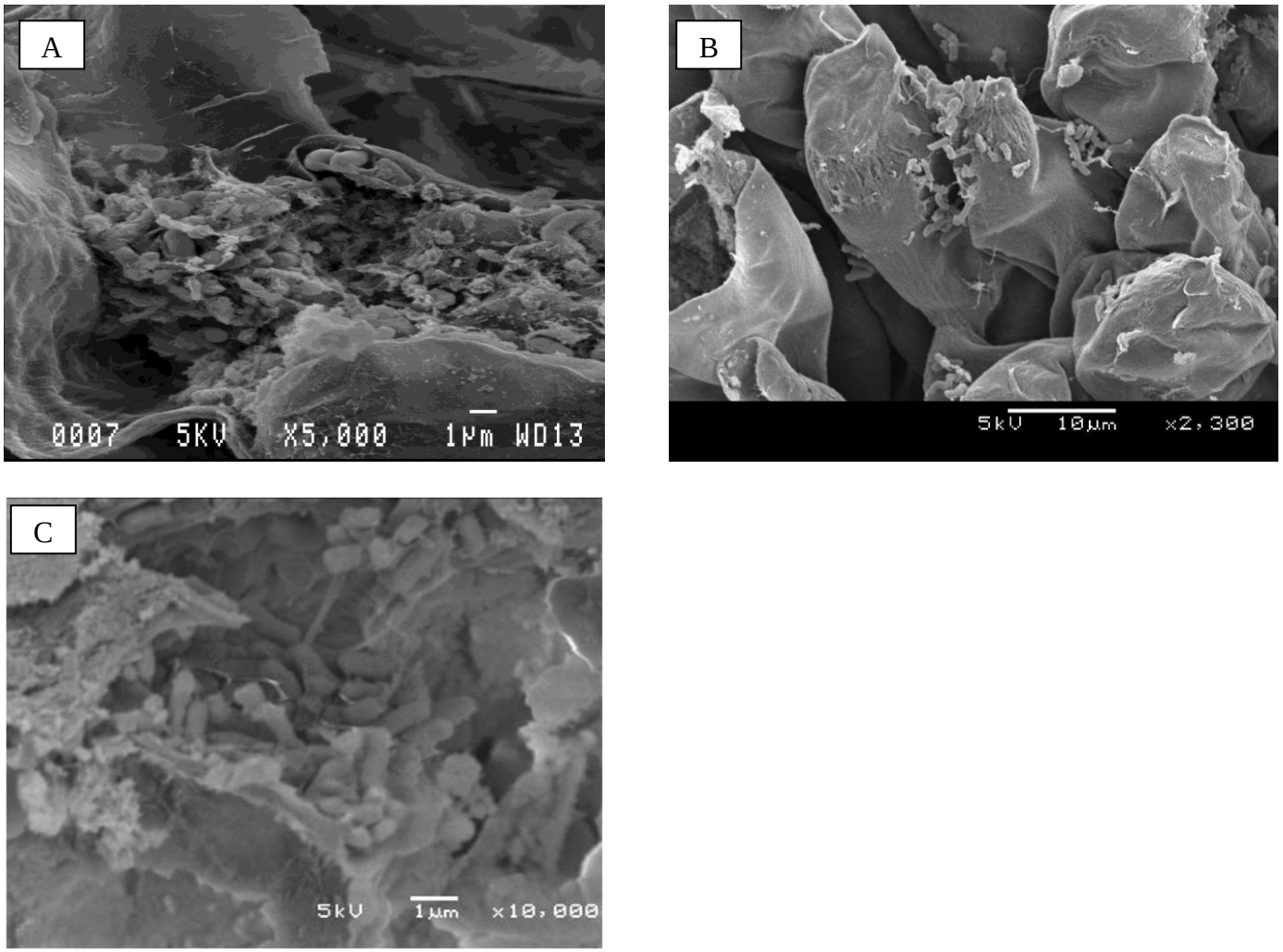

Figure 3.2: SEM micrographs of *V. pygmaea*. (A) March 2011. (B) June 2011. (C) early September 2011.

By using TEM analysis it was possible to determine visually if bacterial colonisation patterns altered with season. It was also possible to visualise the condition of the bacterial endophytes between tissues collected over four seasons. Figure 3.3 shows the similarities and differences between the plant tissues: March 2011 (autumn), June 2011 (winter), early September 2011 (spring) and late October 2010 (summer).

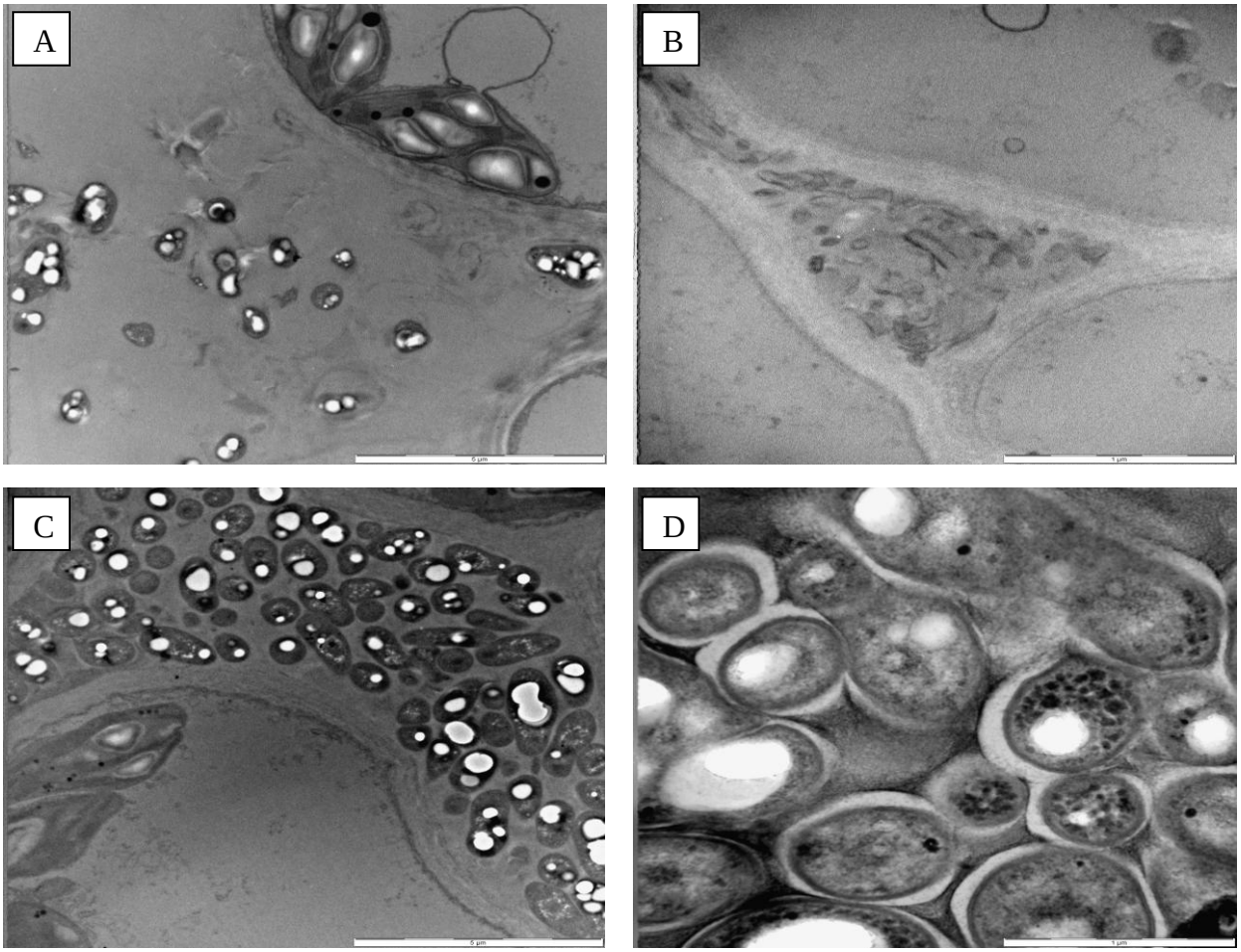

Figure 3.3: Inhabitation of bacterial endophytes using TEM analysis. A) March 2011 (autumn). B) June 2011 (winter). C) early September 2011 (spring). D) late October 2010 (summer).

Bacterial endophytes were seen within the intercellular spaces of the leaves collected in all seasons but the number and health status of the endophytes appeared to differ throughout the year. In March 2011 (the autumn evaluation), examination of the leaf tissue revealed that the

bacteria visually appeared healthy. However, only a few bacteria were observed, (Figure 3.3A). In plant tissue collected in winter (June 2011) even fewer bacterial endophytes were observed and they appeared to be disintegrating (Figure 3.3B).In tissue collected in early September 2011, the beginning of spring and the growing season, the tissue appeared to be extensively colonised by bacteria (Figure 3.3C, D). These slime forming bacteria extensively colonised the intercellular spaces of the leaves during the summer, late October months. From these micrographs the polyhydroxybutyrate-like granules can be clearly seen.

3.5. Discussion

Bacterial endophytes were observed in all six plant species examined in this study, namely, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* as well as in the assumed non-toxic plants, *V. infausta*, *V. macrocalyx* and *V. madagascariensis*. Verstraete et al. (2011) indicated that only toxic plants contained bacterial endophytes within the lamina. The plants they examined included all known gousiekte-inducing plants, *F. homblei*, *P. harborii*, *P. schumanniana*, *V. latifolia*, *V. pygmaea* and *V. thamnus*. Species from other genera, namely, *Afrocanthium*, *Canthium*, *Keetia*, *Psydrax*, *Pygmaeothamnus*, and *Pyrostria*, were also analysed for the presence of bacterial endophytes but none were detected (Verstraete et al., 2011).

The bacterial endophytes in *P. schumanniana* were only located within the leaf nodules. The five *Vangueria* species all contained bacterial endophytes within the intercellular spaces of the leaves. The bacterial endophytes in the three non-toxic species revealed many morphological differences when compared to the gousiekte-inducing plants. The shape, clustering pattern, morphological composition and position in the leaf lamina of the bacterial endophytes located in *V. infausta* were similar to *V. madagascariensis*. The bacterial endophytes present within *V. macrocalyx* were observed less frequently then the other five

species. The morphological differences noted between the endophytes located in *V. macrocalyx* and the other bacterial endophytes included a less defined extracellular polymeric substance (EPS), a more elongated shape and a visually more dense composition. The bacteria present within *P. schumanniana*, *V. pygmaea* and *V. thamnus* resembled each other. The bacteria were similar with respect to size, produced EPS and contained polyhydroxybutyrate-like granules. These similarities support the observation that bacterial endophytes may play a significant role in gousiekte poisonings.

By using SEM analysis it was possible to visualise the presence of bacterial endophytes on the lamina surface. Although the bacteria are suspected of entering the plant leaves via seeds, roots or stomata (Verstraete et al., 2011; Hardoim et al., 2008) this was not observed in this study. Once the epidermis was removed from the SEM samples the interior of the leaves could be scanned. The three evaluation periods March 2011 (autumn), June 2011 (winter) and early September 2011 (spring) all revealed the presence of bacterial endophytes. It was evident that the bacteria observed within the winter month was present as individuals and did not form clusters. A larger number of bacteria were observed in the spring sample compared to autumn sample.

By using TEM analysis it was possible to visually evaluate bacterial colonisation fluctuations over four different seasons, March 2011(Autumn), June 2011 (Winter), early September 2011 (Spring) and late October 2010 (Summer). The micrograph from March 2011, revealed sporadically spaced bacterial endophytes which appeared alive and present within the intercellular spaces of the leaves. The June 2011 evaluation displayed intercellular spaces devoid of bacteria throughout most of the leaves, where bacterial endophytes were seen they appeared to be degraded. In early September 2011 bacterial endophytes colonised the intercellular spaces around the spongy mesophyll parenchyma cells. However, the colonisation of bacterial endophytes was more extensive in late October 2010 where they

were noted in almost all the intercellular spaces of the leaves. These colonisation fluctuations correspond to the time of year when the majority of gousiekte cases are reported, early summer. The bacterial endophytes within *V. pygmaea* are present in their highest number during the season when gousiekte poisoning is at its highest. Whereas in winter when no gousiekte cases are reported the bacterial endophytes within *V. pygmaea* are either absent or degraded (Hay et al., 2008).

In conclusion bacterial endophytes were clearly seen in all six of the species including the three non-toxic plants, *V. infausta*, *V. macrocalyx* and *V. madagascariensis*. This observation suggests that the presence of bacterial endophytes does not indicate toxicity of the plant. As discussed the bacterial endophytes present in the non-toxic plants displayed features which were different from the bacteria viewed in the toxic species. The bacteria present in the three gousiekte-inducing plants resembled each other significantly with respect to their shape and size. Isolation and sequencing of the bacteria would determine if the bacteria belong to the same genus. The other three gousiekte -inducing plant species, *F. homblei*, *P. harborii* and *V. latifolia*, would need to undergo the same TEM experiment in order to conclude if there is indeed a link between the bacterial endophytes morphology and the gousiekte disease. Similar bacteria would then need to be isolated from all six gousiekte-inducing plants in order to confirm this connection. There was a clear population difference between the four collection times with the highest number of bacteria present in *V. pygmaea* in late October (summer) followed in descending order by early September (spring), March (autumn) and lastly June (winter). These results support the theory that bacterial endophytes may indeed be linked in gousiekte poisoning.

3.6. References

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Chapter 4

Evaluation of bacterial endophytes isolated from *Vangueria pygmaea* and *Vangueria thamnus*

4.1.Abstract

Vangueria pygmaea and *V. thamnus* are classified as geoxylic suffixes which appear very much alike and induce the disease "gousiekte" together with *Fadogia homblei*, *Pavetta harborii*, *P. schumanniana* and *V. latifolia*. All gousiekte-inducing plants contain bacterial endophytes within their leaves. The two *Pavetta* species contain the bacterial endophytes within leave nodules which can be seen with the naked eye. However, the remaining four gousiekte-inducing plants contain bacterial endophytes within the inter-cellular spaces of their leaves. Culturable bacterial endophytes were isolated from *V. pygmaea* and *V. thamnus* and grown in bulk in nutrient broth. There were a total of twelve bacterial endophytes isolated from *V. pygmaea* and seven from *V. thamnus*. A single visually identical bacterium based on colour, colony forming pattern, texture, smell and growth rate was isolated from both *V. pygmaea* and *V. thamnus*. The bacterial endophytes were grown to an optical density of 1.3 (CFU/ml) and the compounds produced by the endophytes were subjected to cytotoxicity screening on H9c2 cells. Samples which produced IC_{50} values of less than 50µg/ml were assumed toxic and subjected to the XTT assay for an additional two replications. Endophyte 14, isolated from *V. pygmaea*, and endophyte 20 which was visually compared and assumed to be the same strain isolated from both *V. pygmaea* and *V. thamnus*, expressed IC_{50} values of less than 50µg/ml in all three assays. Ion exchange chromatography analysis was conducted on endophyte 20 in order to identify and purify the toxic compound. Fractions generated from the ion exchange column were subjected to NMR analysis and compared with the pure standard pavettamine. NMR analysis revealed that the fraction was not pavettamine. Endophyte 20 was tentatively identified as a *Bacillus* sp. based on its 16S rDNA sequence.

4.2.Introduction

Bacterial endophytes can be defined as microorganisms which live either their whole or a section of their lives within healthy plant tissue causing no apparent harm to the host plant (Hardoim et al., 2008; Phetcharat and Duangpaeng., 2011; Rashidet al., 2011; Tiwari et al., 2012). Bacteria isolated from surface sterilized tissue or from internal tissue are endophytes and are commonly from soil dwelling genera such as *Bacillus*, *Pseudomonas* and *Azospirillum* (Phetcharat and Duangpaeng., 2011). The relationship between the endophytic bacterium and the host plant can be either 'obligate' or 'facultative'. An endophyte is 'obligate' when it is entirely dependent on the host plant for survival and transmission is conducted through vectors. A 'facultative' endophyte lives a section of its life outside of the host plant and is independent of the host plant (Hardoim et al., 2008). The plant/bacteria relationship is usually beneficial to both parties. The bacteria benefit from plants by being encased in an optimum environment where no external stresses are present and by the increased quantity of nutrients (Hardoim et al., 2008). The plant host benefits from the bacterial inhabitation in many different ways which include promoting growth by metabolizing nutrients such as phosphorous and nitrogen, siderophore biosynthesis, inducing plant growth regulators (indole-acetic acid) and producing phytohormones. Bacterial endophytes defend the host plant from pathogens by competing for space and nutrients and activating plant defence mechanisms (Phetcharat and Duangpaeng., 2011; Rashidet al., 2011; Tiwari et al., 2012). Bacterial endophytes have been found to suppress stress related plant ethylene biosynthesis through 1-aminocyclopropane-1-carboxylate deaminase activity and to play a role in phytoremediation strategies (Phetcharat and Duangpaeng., 2011; Rashidet al., 2011; Tiwari et al., 2012). Bacterial endophytes also have the capability to increase the production of secondary metabolites (Tiwari et al., 2012).

Bacterial endophytes have been identified using cultivation-independent approaches in all six of the gousiekte-inducing plants, *Fadogia homblei*, *Pavetta harborii*, *P. schumanniana*, *Vangueria latifolia*, *V. pygmaea* and *V. thamnus*. The bacterial endophyte observed in *P. schumanniana* has been described as *Candidatus* Burkholderia schumanniana (this is an uncultural bacterium). The difference in the 16S rDNA between the bacteria isolated from *P. harborii* and *P. schumanniana* was only 1% thus the bacteria isolated from *P. harborii* belongs to the *Burkholderia* genus (Verstraete et al., 2011). The 16S rDNA regions of bacteria isolated from *F. homblei*, *V. latifolia*, *V. pygmaea* and *V. thamnus* were found to be 99.9% identical to *Burkholderia caledonica* (Verstraete et al., 2011).

The two plants focused on in this chapter were *Vangueria pygmaea* (= *Pachystigma pygmaea*) Schltr. Robyns and *Vangueria thamnus* (= *Pachystigma thamnus*) Robyns. These two plants belong to the family Rubiaceae and are subdivided into the tribe *Vangueria*. *V. pygmaea* and *V. thamnus* are geoxylic suffrutices otherwise characterised as underground trees. The underground trees only expose between 5-20 cm of growth above the ground, however, extensive growth of stems and roots occurs below ground sometimes reaching 30m of subterranean growth (Van Wyk et al., 1990). Bacterial endophytes have been observed within the intercellular spaces of the leaves of both *V. pygmaea* and *V. thamnus* (Van Wyk et al., 1990). Using transmission electron microscopy, similarities in the bacterial endophytes located in each plant were determined. The bacterial endophytes located in both plants produce slime-like mucus (EPS) and contain polyhydroxybutyrate-like granules (refer to the previous chapter). The bacterial endophytes present within *V. pygmaea* undergo seasonal habitation (chapter 3), the greatest colonisation occurring in late spring (September) through to summer (October-January). In order to determine whether the bacterial endophytes play a significant role in the gousiekte disease they will need to be cultured outside of the plant and tested.

When a ruminant consumes lethal doses of a gousiekte-inducing plant the animal dies of heart failure within 3-8 weeks (Prozesky et al., 1988). Gousiekte is one of the six most important economical plant toxicosis present in southern Africa and significant losses in livestock occur every year. Although the illness was discovered in 1908 the causative toxin, pavettamine, was only extracted in 1995 through a lengthy extraction procedure (Fourie et al., 1995; Bode et al., 2010). The elucidation of pavettamine occurred in 2010 revealing that it was a novel polyamine with the molecular structure of $C_{10}H_{25}N_3O_4$. Factors such as loss of leaf toxicity when dried plant material is consumed, seasonal toxicity fluctuations, variations in symptoms of the disease, breed susceptibility and varying quantities of plant material needed for death to occur, make initial diagnosis and identification of the illness cumbersome (Hay et al., 2008; Bode et al., 2010).

A few theories have arisen to determine why the gousiekte disease only affects ruminants, has a long latent period and expresses fluctuations in toxicity. One theory is that the endophytic bacteria themselves are responsible for gousiekte and not the plant species (Van Wyk et al., 1990). This theory would explain the seasonal toxicity fluctuations in the plant species due to variations in bacterial endophyte habitation; it would also verify the loss of toxicity as the plant material is dried as the endophytes would become non-viable (Van Wyk et al., 1990). Another assumption is that due to the fact that ruminants are only affected, a possible interaction between the plants, bacterial endophytes, rumen conditions and rumen flora may cause gousiekte (Van Wyk et al., 1990). There is the possibility that the bacterial endophytes present in all six gousiekte-inducing plants produce a precursor which is transformed into the active form on contact with the rumen flora or rumen conditions. There is a strong possibility that there are more Rubiaceae species which induce gousiekte that have not yet been evaluated (Van Wyk et al., 1990; Verstraete et al., 2011).

The novel polyamine, pavettamine is the causative toxin responsible for gousiekte expresses cell death to H9c2 cells after 72h of exposure. H9c2 cells are derived from rat cardiac cells and are susceptible to pavettamine screening. The cells display abnormalities in the mitochondria and sarcoplasmic reticula and after 72h of exposure the nucleus becomes fragmented and membrane bedding occurs (Ellis et al., 2010).

The aims of this chapter were to evaluate the similarity of bacteria based on colour, colony forming pattern, texture, smell and growth rate isolated from *V. pygmaea* and *V. thamnus*. To determine if any culturable bacteria isolated from either *V. pygmaea* or *V. thamnus* revealed toxicity by XTT assay.

The hypotheses of this chapter, which were based solely on culturable bacteria, were as follows:

1. *V. pygmaea* and *V. thamnus* contain the same species of bacterial endophyte.
2. The XTT assay will reveal positive toxicity results from the culturable bacteria isolated from both *V. pygmaea* and *V. thamnus*.

4.3.Materials and Methods

4.3.1. Isolation of bacterial endophytes from *Vangueria pygmaea* and *V. thamnus*

4.3.1.1. Plant collection

Fresh leaves from *V. pygmaea* and *V. thamnus* were collected from the same field in Mpumalanga near the town Piet Retief. *V. pygmaea* leaves (GPS 27°09’06”S, 31°00’08”E) and *V. thamnus* leaves (GPS 27°09’05”S, 31°00’08”E) were collected from different individuals within the colony. Voucher specimens from both plants were deposited into the

H.G.W.J. Schweickerdt Herbarium within the University of Pretoria. The PRU number for *V. pygmaea* is 118679 and the PRU number for *V. thamnus* is 118678.

4.3.1.2.Sample preparation

The leaves were carefully separated into labelled beakers to ensure no cross contamination occurred between the plants. The fresh leaves from both plants were rinsed in order to remove all soil debris. The leaves from each beaker were further divided into undamaged young and mature leaves; any damaged leaves were discarded. The undamaged young and mature leaves, test leaves, from each plant were treated in the same way. The leaves were initially dipped in a solution of 0.5% sodium hypoclorite and placed on paper towel to dry. The test leaves once dry were placed in a magnetic stirring beaker containing 70% ethanol for 5 minutes in order to destroy any bacteria on the phylloplane. The leaves were removed from the beaker and rinsed in double distilled water for a minute. Thereafter they were examined and damaged leaves discarded. The undamaged leaves were placed on a sterile surface within a lamina flow bench.

4.3.1.3.Isolation of bacterial endophytes from leaf samples

The materials used for isolation of endophytes were autoclaved at 121˚C for 15 mins in order to sterilise all equipment. This material together with 18 sterile Petri dishes containing nutrient agar were placed within a laminar flow hood with the ultra-violet light on. The leaves were evaluated for damage to the leaf lamina, damaged leaves were discarded. The surface of the leaves were sterilized in a 10% sodium hypochlorite solution for 1 min followed by a 70% ethanol solution for another minute and finally rinsed in distilled water to remove remaining ethanol. One leaf from each test plant, *V. pygmaea* and *V. thamnus*, was used as a positive control by producing a leaf print in two of the Petri dishes. Two Petri dishes were sealed after sterilisation and served as a closed control. Each leaf was evaluated under the

same sterile conditions. The leaf was sliced in a cross-sectional manner with a sterile blade. The cut side of the leaf was pressed into the nutrient agar twice. The leaf was then cut again and the cut side was pressed into the nutrient agar (sigma) dish an additional time. The Petri dish was sealed and labelled. This similar procedure was conducted using all leaf samples. The Petri dishes were all placed into an incubator set at 37˚C with the control Petri dishes. The Petri dishes were evaluated every 24h for bacterial growth.

4.3.1.4.Pure colony formation

The two closed control Petri dishes revealed no growth after 24, 48 or 72 h incubation. The leaf print controls of both the young leaf and the mature leaf from *V. thamnus* displayed no bacterial growth at 24, 48 or 72 h incubation. The leaf prints from both the young leaf and the mature leaf of *V pygmaea* revealed the growth of at least two different bacteria. The test leaves from the four different categories, *V. pygmaea* young leaves, *V. pygmaea* mature leaves, *V. thamnus* young leaves and *V. thamnus* mature leaves, all had different bacteria after 72 h incubation. A single colony from the young leaf print of *V. thamnus* was streaked on to fresh nutrient agar. This procedure was repeated for the mature leaf of *V. thamnus*. The two control leaf prints from *V. pygmaea* were visually analysed and it was concluded that the mature leaf print contained a single bacterial colony while the young leaf print contained two different bacteria. Three new sterile petri dishes containing nutrient agar were used to re-plate the bacterial growth forms isolated from the leaf prints of *V. pygmaea*. The bacteria were re-plated and streaked onto fresh nutrient agar. The petri dishes were placed inside the incubator which was set on 37˚C. After 72 hours of growth the petri dishes were re-analysed and re-plated. If a petri dish contained more than one bacterium they were re-plated onto media in different Petri dishes. The bacterial endophytes were visually assessed and those with similar bacteria colony morphology grouped together. Bacteria were Gram stained and viewed using a light microscope.

4.3.1.5.Bulk bacterial endophyte growth

Once pure colony formation occurred on nutrient agar, the endophytes were counted based on colony formation from each plant. There appeared to be a total of twelve different endophytes isolated from *V. pygmaea* and seven isolated from *V. thamnus*. One endophyte was visually identical which appeared present in both *V. pygmaea* and *V. thamnus*. Twenty two 500ml conical flasks were washed, dried and 250ml of nutrient broth added to each flask. The flasks were then sealed with tin foil. The flasks along with 2 large glass petri dishes and tweezers were autoclaved. Once autoclaved the equipment was placed in a sterilized laminar flow hood. Two control flasks were placed into the incubator without breaking the sealed lid. A pure colony was selected from each Petri dish and added to a corresponding flask containing nutrient broth. The flasks were placed into a shaking incubator set at 37˚C.

After 72 h growth was observed in all the flasks except the two control flasks, thus inoculation into fresh media was conducted as described above.

4.3.2. Photospectrometry to determine optimum growth

The 20 conical flasks containing bacterial endophytes were removed from the shaking incubator after 48 hours. Using sterilized Pasteur pipettes 2ml of nutrient broth was removed from each flask and placed in corresponding curvettes. The optical densities (OD) of the 20 endophytes were read using a photospectrometer set at 600nm. If the OD of an endophyte was lower than 1.3 the flask was placed back into the shaking incubator to allow growth to continue. Once an OD of 1.3 was reached the bacteria were considered to have reached their optimal growth. All the flasks were evaluated under the same conditions. The control flask containing autoclaved nutrient broth was analysed as a negative control in order to ensure that no bacterial growth had occurred. The contents of all the flasks (nutrient broth containing the bacterial endophytes) were placed into sterilized 500ml round bottom flasks. The nutrient

broth was concentrated to a total volume of 25ml using a BÜCHI rotavapor R-200. The concentrated 25ml of the flasks were placed into sterilised vials. Once all 20 test flasks and the single control flask had been concentrated to 25ml they were centrifuged at 10000rpm for 5 minutes. The supernatant of each flask was removed and placed into new sterilised labelled vials. The remaining pellet within each flask was discarded. A total volume of 5ml was removed from each vial and placed in sealed sterilized polytops. The 21 vials were stored in a cold room at a temperature of 4˚C. The 21 polytops were placed in a sterilized laminar flow hood. A total volume of 4ml of endophytic nutrient broth from each polytop was passed through sterilized 2µm diameter tips using unopened sterile syringes. The filtered nutrient broth containing endophytic compounds was collected into new sterilized labelled polytops. The new polytops containing the nutrient broth and the endophytic compounds were placed into a Genevac EZ-2 series and dried. The polytops containing 1ml of nutrient broth and endophytes was used for thin layer chromatography (TLC) analysis.

4.3.3. Thin layer chromatography analysis

The toxic compound responsible for inducing gousiekte, pavettamine, was used as the positive control. The polytop containing the nutrient broth void of endophytic growth served as the negative control. With the exception of pavettamine which was only spotted with one drop due to the relatively high concentration of the pure compound, five drops of all 20 nutrient media that had contained endophytes along with the negative control were spotted. It was essential that each drop's spot was dry before a new drop was added. The spotting was conducted in a band formation. Four identical TLC plates were prepared, two for detection by vanillin and two for detection by ninhydrin. The indicator ninhydrin colours compounds which contain a nitrogen atom whereas vanillin is a more general indicator. Two plates, one for vanillin and one forninhydrin, were placed in the mobile phase of ethylacetate: formic acid: acetic acid: distilled water (6:1:1:1). The remaining plates were added to the mobile

phase of distilled water: 2-propanol: acetic acid (60:40:4). The four TLC plates were run twice for sufficient separation of the compounds. The plates were dried before being dipped into the indicator solutions.

4.3.4. Cytotoxicity

4.3.4.1. Cytotoxicity preparation

From each of the dried sterilised polytops, 1-20, a total of 2mg of dried nutrient broth containing the endophyte compounds was used for cytotoxicity screening. The cell line H9c2 was used to test the endophyte compounds present in the nutrient broth. This cell line was obtained from American Type Culture Collection (cat no: CRL-1446™, Manassas, USA). As described in chapter 2, the cells were placed in Dulbecco's Modified Eagle's Medium (DMEM) which had been previously supplemented with 100U/ml penicillin and 100µg/ml streptomycin sulphate and 10% foetal calf serum. This combination of antibiotics and medium will be referred to as the stock medium (Ellis et al., 2010).

From the stock medium a total of 50ml was removed and placed into a 50ml centrifuge flask and 400µl of ciprobay (excluding fungizone supplement) was added. This combination will be referred to as the complete medium. The complete medium was shared between two large 50ml incubation flasks containing suspended cells. The H9c2 cells were incubated at 37˚C in 5% CO_2.

Halving samples: Once the cells had been incubated between 48 and 72 hours the cells were 80% confluent and were halved. The H9c2 cells adhered to the flask thus the complete medium was discarded. The cells were detached from the flask by the use of trypsin, this was conducted twice, for approximately 15 seconds of swirling, using a 5x stock trypsin at a 0.5% concentration. The cells were placed in a 37˚C incubator for 2 minutes to allow all of the cells to detach from the flask. A total volume of 50ml of complete medium was added to the

initially incubated flask and swirled to collect all detached cells. 25ml of that complete medium containing cells was placed into a new sterile flask. The two flasks were labelled and returned to the 5% CO_2 37˚C incubator.

4.3.4.2.XTT assay

Hemocytometer cell count: The 80% confluent flasks were trypsinized twice using 0.5% 5x stock trypsin. All the detached cells were collected into a 50ml centrifuge flask and centrifuged for 5mins at 980rpm in order to form a pellet. The used complete medium was discarded and the cells were re-suspended using 2ml of freshly prepared complete medium. From the 2ml of re-suspended cells 10µl was added to an Eppendorf tube along with 90µl of trypan blue solution (1:10 dilution). From the Eppendorf tube 10µl of the solution was added to the two chambers of the hemocytometer. Using a light microscope the cells were carefully counted using a hand-held tally. The cell concentration was then determined using the following formula.

1. Number of cells counted per square = number of cells counted divided by four
2. Cell suspension (cell concentration) = number of cells counted per square X10 X 10000 = cells per millilitre
3. Volume added to cell pellet = cell concentration wanted X volume wanted /concentration of cells in suspension= Total volume
4. Volume wanted = total volume – volume added to cell pellet

Toxicity screening of endophyte compounds from *V. pygmaea* and *V. thamnus*. The toxicity screening of the endophyte compounds 1-20 was conducted under the same conditions as described in Chapter 2.The pure compounds pavettamine and actinomycin D both served as the positive control. There were 22 samples for testing, 20 endophyte compounds a negative control which was pure sterile nutrient broth and the positive control pavettamine. A total of

eleven 96 well-plates were needed for the experiment (2 samples per 96 well-plate). Into all the outer wells of the 96 well plates, 200µl of incomplete medium (no foetal bovine serum or 100U/ml penicillin and 100µg/ml streptomycin sulphate) was added. To the inner wells 100µl of cell suspension was added. The 96 well-plates were incubated overnight at 37˚C in an atmosphere of 5% CO_2 in order for the cells to attach to the bottom of the wells.

After 24h incubation from each of the 20 polytops containing dried nutrient broth and endophyte compound a total of 2mg of sample was removed and placed in labelled Eppendorf tubes which were further supplemented with 100µl of DMSO. The 20 samples were sonicated for between 1-2 hours to ensure all extract dissolved in the DMSO. For the two positive controls, pavettamine and actinomycin D, which are both pure compounds; 1mg of each of the controls were placed in a labelled Eppendorf tube followed by 100µl of DMSO.

Using 24 well-plates each sample underwent 8 serial dilutions. Each 24 well-plate contained 3 sample's dilutions. For the 20 endophyte compound samples 2ml of complete medium was added to the first well and thereafter 1ml in the seven other wells. Pavettamine was treated under the same conditions as the endophyte compound samples. However, for actinomycin D 1ml of complete medium was added to all eight wells. All 20 endophyte compound samples and pavettamine underwent a serial dilution. The negative control was prepared by adding 2ml of complete medium to a well, removing 80µl of the complete medium and replacing it with 80µl of DMSO.

All 22 samples, 20 endophyte compounds, pavettamine and negative control, excluding actinomycin D were added into the 96 well-plates using the same method. 100µl of each concentration was removed from the 24 well-plate and added to the 96 well-plate. Each of the 8 concentrations per sample was carried out in triplicates. For each sample there was a

triplicate DMSO and complete medium control used. The outer wells of the 96 well-plates contained incomplete medium to ensure the plate remained hydrated.

Actinomycin D was added to the 96 well-plates in the same way as the test samples the only difference being that the positive control was subjected to as little light as possible due to its photosensitivity. All of the 96 well-plates were incubated for 72 hours in an incubator set at 37˚C and 5% CO_2.

The XTT reagent, pesto blue, was prepared for all the 96 well-plates by adding 20µl of PMS to every 1ml of pesto blue and added to the wells on day five. A total of 50µl of pesto blue reagent was added to all the test wells i.e. those containing H9c2 cells. The 96 well-plates were further incubated for between 4-6 hours. The plates were read on an ELISA plate reader.

4.3.5. Ion exchange chromatography

The polyamine spermidine was used to develop a standard protocol for extraction of nitrogen containing compounds from the endophyte containing nutrient broth. Dowex 50 cation exchange resin was used for the ion exchange chromatography column. The resin was prepared as previously described. A 25ml column was washed with 100% methanol and rinsed with distilled water and the column was then left to dry. A small cotton wool ball was placed in the column at the base. Dowex 50 cation exchange resin was added to the column until about $^2/_3$ of the column was filled and the resin was kept moist with distilled water. A total of 25mg of pure standard spermidine was added evenly to the column. A small cotton wool ball was then placed over the resin. Table 4.1 displays the solvent system used for the ion exchange column of spermidine.

Table 4.1: Solvent system of Ion exchange chromatography column for spermidine

Fraction	Solvent	Volume (ml)
Wash 1-4	Distilled water	40
Elute 1-2	Distilled water: ammonia solution (23%) 9:1	20
Elute 3-4	Distilled water: ammonia solution (23%) 1:1	20
Elute 5-9	Ammonia solution (23%)	50
Elute 10-19	Sodium hydroxide (NaOH) 1M solution	100

All elutes and washes were collected in labelled polytops and dried using a Genevac EZ-2 apparatus. Five drops from the corresponding solvent system was added to the correct polytop.

The ion exchange column of endophyte 20 was prepared in a similar way to that of the spermidine column. The polytop containing the dried sterilized compounds of endophyte 20 was scraped with a sterilized spatula and tweezers in order to lift the compounds from the bottom of the polytop. The nutrient broth containing compounds of endophyte 20 were weighted to a mass of 100mg and placed into an Eppendorf tube thereafter 1.5ml of distilled water was added. The Eppendorf tube was sonicated for 30 minutes in order for the compounds of endophyte 20 to go into solution. The solution was then added to the 25ml column evenly. Table 4.2 shows the solvent system used for the endophyte compounds 20 column.

Table 4.2: Solvent system used for the ion exchange column containing the compounds in the broth of endophyte 20

Fraction	Solvent	Volume (ml)
Washes 1-6	Distilled water	60
Elutes 1-4	Distilled water: ammonia solution (23%) 9:1	40
Elutes 5-8	Distilled water: ammonia solution (23%) 1:1	50
Elutes 9-10	Ammonia solution (23%)	20
Elutes 11-14	Sodium hydroxide (NaOH) 1M solution	40

The fractions were collected in labelled polytops. The polytops were dried using a Genevac EZ-2 apparatus; five drops of the corresponding solvent system was added to each polytop.

4.3.6. Thin layer chromatography analysis

Two identical TLC plates were prepared for two different mobile phases; ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1) and distilled water: 2-propanol: acetic acid (60:40:4). The pure standard spermidine was spotted as the positive control followed by washes 1-4 and elutes 1-19. The TLC plates were run twice for adequate separation and the indicator used once the plates were dry was ninhydrin. The fraction where the pure spermidine was eluted from the column was noted for comparison of the endophyte 20 compound column.

Analysis of the fractions generated from the column was identical to that of the TLC evaluation of the spermidine column. Two TLC plates were prepared in the same fashion for two different mobile phases; ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1) and distilled water: 2-propanol: acetic acid (60:40:4). The TLC plates were spotted with

pavettamine as the positive control, pure nutrient broth and the original endophyte 20 compounds as the negative control. Washes 1-6 and elutes 1-11 were spotted twice to ensure high concentration. Fractions 12-14 did not reveal compounds present thus were not spotted in final TLC plate evaluation. Both TLC plates were run twice for adequate separation, once dried the TLC plates were viewed under both long and short wavelength ultra violet light before being dipped in ninhydrin.

4.3.7. Nuclear magnetic resonance (NMR) analysis

From the endophyte 20 compounds column all fractions were dried using a Genevac EZ-2 evaporator once TLC analysis had been conducted. A total of 100mg of the dried nutrient broth containing endophyte 20 compounds was weighed and placed into an Eppendorf tube. To the Eppendorf tube 0.8ml of deuterated water was added. To the dried washes 1-6 and elutes 1-11 a volume of 0.8 ml of deuterated water was added to the polytops. The polytops and the Eppendorf tube containing the original compounds of endophyte 20 were sonicated for 20 minutes to ensure all solid was in solution. The NMR used was a Varian 200 MHz NMR and all the samples were loaded into freshly cleaned NMR tubes. The NMR was set for proton analysis and all the samples underwent 256 scans. All NMR graphs were compared to that of the pure compound pavettamine.

4.3.8. Endophyte 20 Identification

4.3.8.1. DNA extraction

Pure colonies of endophyte 20 were developed on nutrient agar plates. Endophyte 20 was subjected to standard procedures of DNA extraction and sequencing. From the nutrient agar plates 2 colonies were selected and suspended in 1ml of PBS buffer in an Eppendorf tube. Standard procedures were used to achieve un-fragmented DNA from the bacterial colonies. The DNA sample was now present in the Eppendorf tube and a total of 5µl of the DNA

sample belonging to endophyte 20 was removed from the Eppendorf tube and added to 1µl of gel red this solution was mixed and added to 2µl of ladder 100bp. The mixture was run on a 1% agarose gel for 30 minutes at an 80V setting in order to analyse the presence of DNA. The remaining endophyte 20 DNA sample within the Eppendorf tube was stored at -20°C.

4.3.8.2.16S PCR set up

One forward primer and one reverse primer were used for sequencing and amplification of the 16S rRNA gene. Table 4.3 displays the components of the master solution which was prepared within the sterilised fume hood.

Table 4.3: Solvents quantities present in PCR master solution

Solvent	Volume (µl)
Buffer	5.0
$MgCl_2$	4.0
dNTPs	4.0
Primer pH (1:10)	1.0
Primer pA (1:10)	1.0
Taq (Southern)	0.3
DNA template	1.0
NF water	33.7
Total	50.0

4.3.8.3.Sequencing PCR

A master mix solution was compiled within a sterilised fume hood using a standard kit and sequencing protocol. A total volume of 6.0µl of master mix and 4.0µl of cleaned DNA

sample were placed into a PCR Eppendorf tube which was stored at -20°C. The endophyte 20 sample was placed into the PCR sequencing machine.

4.3.8.4.DNA sequencing

The sequencing tube containing endophyte 20 DNA was stored at -20°C. The sample was now prepared for submission to the sequencing facility to be sequenced. Once sequences were returned they were analysed with the forward and reverse primer. The DNA sequences were submitted to DNA databases in order to identify which bacterial family endophyte 20 belonged.

4.4.Results

4.4.1. Isolation of bacterial endophytes from *V. pygmaea* and *V. thamnus*

The two closed control Petri dishes revealed no growth at 24, 48 or 72 h evaluations. The leaf print controls of both the young leaf and the mature leaf from *V. thamnus* displayed no bacterial growth at 24, 48 or 72 h. The leaf prints from both the young leaf and the mature leaf of *V. pygmaea* revealed at least two different bacterial colonies present on the media. The leaves from the four different categories: *V. pygmaea* young leaves, *V. pygmaea* mature leaves, *V. thamnus* young leaves and *V. thamnus* mature leaves all revealed many different bacterial colonies after 72 h. There appeared to be a larger variety of bacteria present particularly in the young leaves from both plants.

A total of 50 bacterial endophytes were re-plated after initial growth. The similarity based on colour, texture, size, growth form, and smell reduced the total from 50 bacterial endophytes to 28 bacterial endophytes. On further evaluation it was assessed that some bacterial endophytes were present in duplicates thus the total endophytes was reduced from 28 to 20. The endophytes labelled 1-7 were isolated from *V. thamnus* and the endophytes labelled 8-19

were isolated from *V. pygmaea*. The colour, texture, size, growth form, and smell of endophyte 20 was identical in both strains isolated from *V. pygmaea* and *V. thamnus* thus the endophyte was assumed to be the same species.

The pure bacterial colonies from the nutrient agar plates were grown up in bulk in nutrient broth in order to ensure that any compounds produced by the bacterial endophytes would be in solution. Once the flasks containing the endophytic growth had been re-cultured into new flasks analysis could commence. The growth rate of the endophytes were analysed and on visual basis; the endophytes were placed in categories of fast, medium and slow growth as described below.

4.4.2. Optical density

After 48 hours of growth the initial OD was read and the 20 endophytes were placed into the categories of fast (F, OD of above 0.9), medium (M, OD of above 0.6 and below 0.9) and slow (S, OD of below 0.6) growth rate. Table 4.4 displays the initial OD readings.

Table 4.4: Optical density readings of all isolated endophytes after 48 hours using a spectrophotometer

Endophyte	Optical density (OD) reading	Growth rate categories
1	0.965	F
2	0.874	M
3	0.774	M
4	0.979	F
5	1.207	F
6	1.017	F
7	0.385	S
8	0.701	M
9	0.954	F
10	0.808	M
11	0.761	M
12	1.285	F
13	0.985	F
14	0.472	S
15	1.292	F
16	0.970	F
17	0.581	S
18	1.215	F
19	0.643	M
20	1.215	F

The endophytes belonging to the F category were monitored closely in order to ensure the OD of 1.3, the optimum growth stage, was achieved. The endophytes belonging to the M and S category were replaced into the shaking incubator and analysed every 12 hours. Once an OD of 1.3 was achieved the endophyte was removed from the incubator for further analysis.

The compounds which the endophytes produced were concentrated using a BÜCHI rotavapor R-200 from a volume of 200ml to 25ml. The concentrated nutrient broth was centrifuged to remove endophyte debris. The remaining supernatant would contain the compounds which the endophytes produced and only a few bacteria. In order to ensure no bacterial presence within the polytops containing 4ml of the compounds; the nutrient broth was passed through 0.22µm diameter filters. The 0.22µm diameter filters were significant for the majority of bacteria are too large to pass through the filters.

4.4.3. Thin layer chromatography analysis

From the four TLC plates, conducted with the 20 isolated bacteria, it can be concluded that it appears that compounds are being produced by the endophytic bacteria that are not present in the negative control and that the bacteria appear to be producing different compounds when compared to each other. However, due to the makeup of the nutrient broth containing sugars and amino acids clear conclusive results cannot be stated due to multitude of band patterns the nutrient broth produces on the TLC plates. Compound bands produced by the bacteria could be concealed or overlooked due to the extensive band formation. No band pattern present in endophytes 1-20 was produced with the same rf value as that of the positive control, pavettamine. No one endophyte clearly differed in band pattern formation from the other endophytes.

4.4.4. Cytotoxicity

4.4.4.1.Cytotoxicity of endophyte compounds from *V. pygmaea* and *V. thamnus*

After 4-6 hours of incubation the indicator pesto blue would have changed to a pink colour if cells lost viability. Using the ELISA plate reader qualitative results were generated. The initial cytotoxicity screening revealed a diversity of IC_{50} values between the endophytes. Table 4.5 displays the IC_{50} values of the two positive controls, pavettamine and actinomycin D, the negative control and the 20 test endophytes.

Table 4.5: IC_{50} values of the 20 endophytes

Test sample	IC_{50} values (µg/ml)
DMSO negative control	>200
Actinomycin D positive control	0.003096
Pavettamine positive control	<3.125
Endophyte 1	280.4
Endophyte 2	228.7
Endophyte 3	397.8
Endophyte 4	>200
Endophyte 5	<3.125
Endophyte 6	>400
Endophyte 7	>400
Endophyte 8	>300
Endophyte 9	249.5
Endophyte 10	250.6
Endophyte 11	>300

Endophyte 12	>300
Endophyte 13	>300
Endophyte 14	<37.5
Endophyte 15	>200
Endophyte 16	>200
Endophyte 17	>150
Endophyte 18	>200
Endophyte 19	>400
Endophyte 20	<25

Endophytes 1-7 were isolated from *V. thamnus*, endophytes 8-19 were isolated from *V. pygmaea* and endophyte 20 was isolated from both *V. pygmaea* and *V. thamnus*. From the initial cytotoxicity screening any endophytic compound revealing an IC_{50} value of less than 50µg/ml was deemed toxic. From Table 4.6 it is clear that 3 endophytes, namely endophyte 5, 14 and 20, produced IC_{50} values of less than 50µg/ml. The analysis of endophytes 5, 14 and 20, was repeated in another XTT assay in duplicate in order to determine the accuracy of the cytotoxicity screening. Figures 4.1 and 4.2 reveal the results generated for endophyte 5.

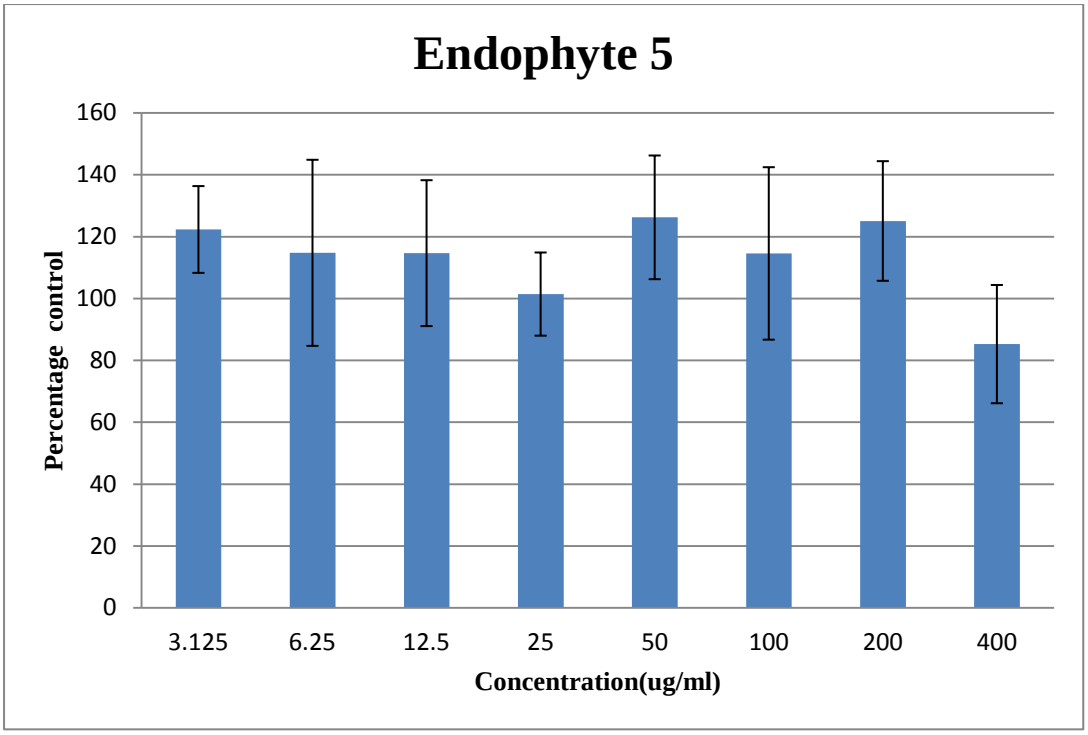

Figure 4.1: Chart displaying the percentage of cell death with concentration of broth and compounds from endophyte 5, first repetition.

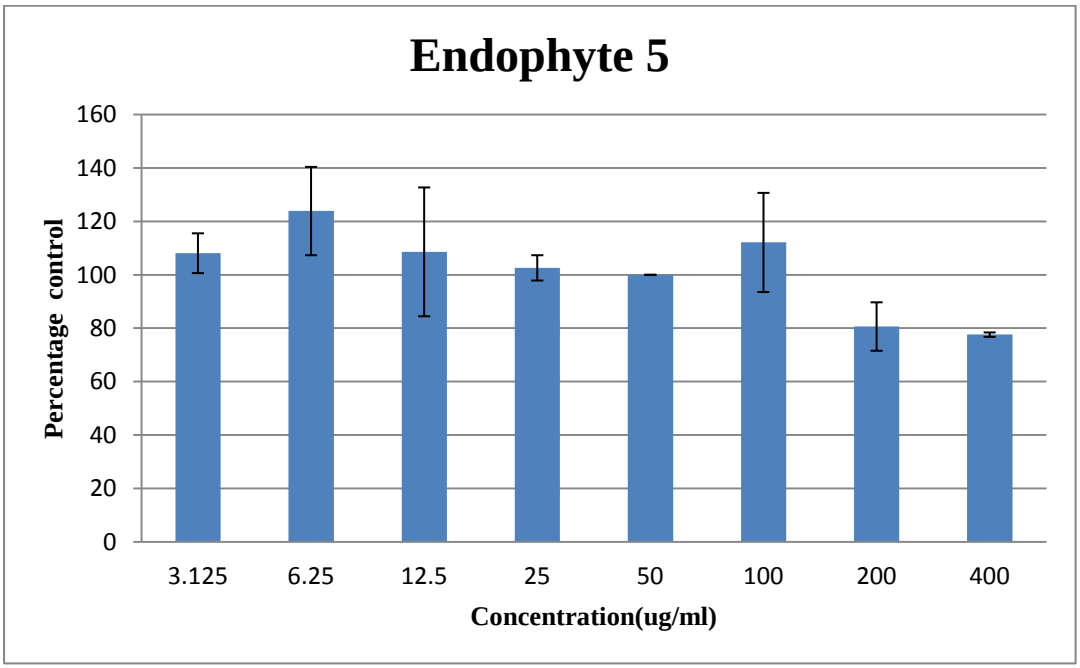

Figure 4.2: Chart displaying the percentage of cell death with concentration of broth and compounds from endophyte 5, second repetition.

The results reveal slight differences in the peak positions but the IC_{50} values for both figures is > 400µg/ml. This analysis reveals that the original XTT assay generated incorrect results. With an IC_{50} value of over 50µg/ml endophyte 5 was deemed no longer toxic. Figures 4.3 and 4.4 display the results produced for endophyte 14.

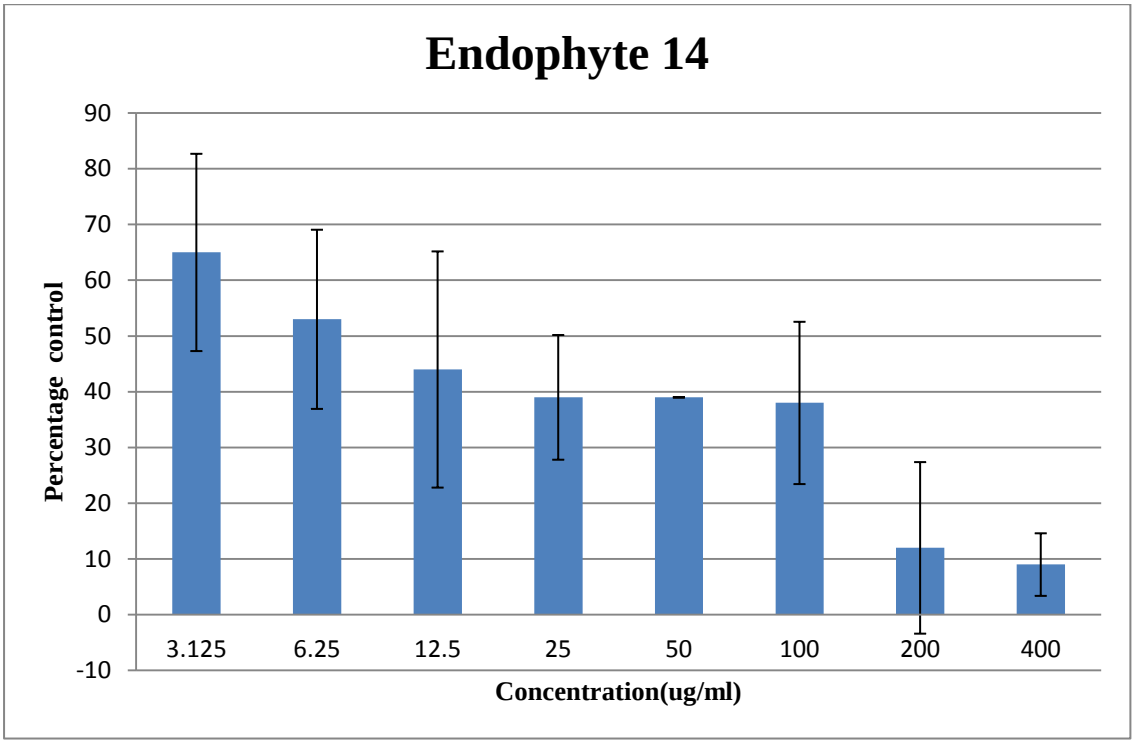

Figure 4.3: Bar chart showing H9c2 cell death vs broth and compound concentration on endophyte 14, first repetition.

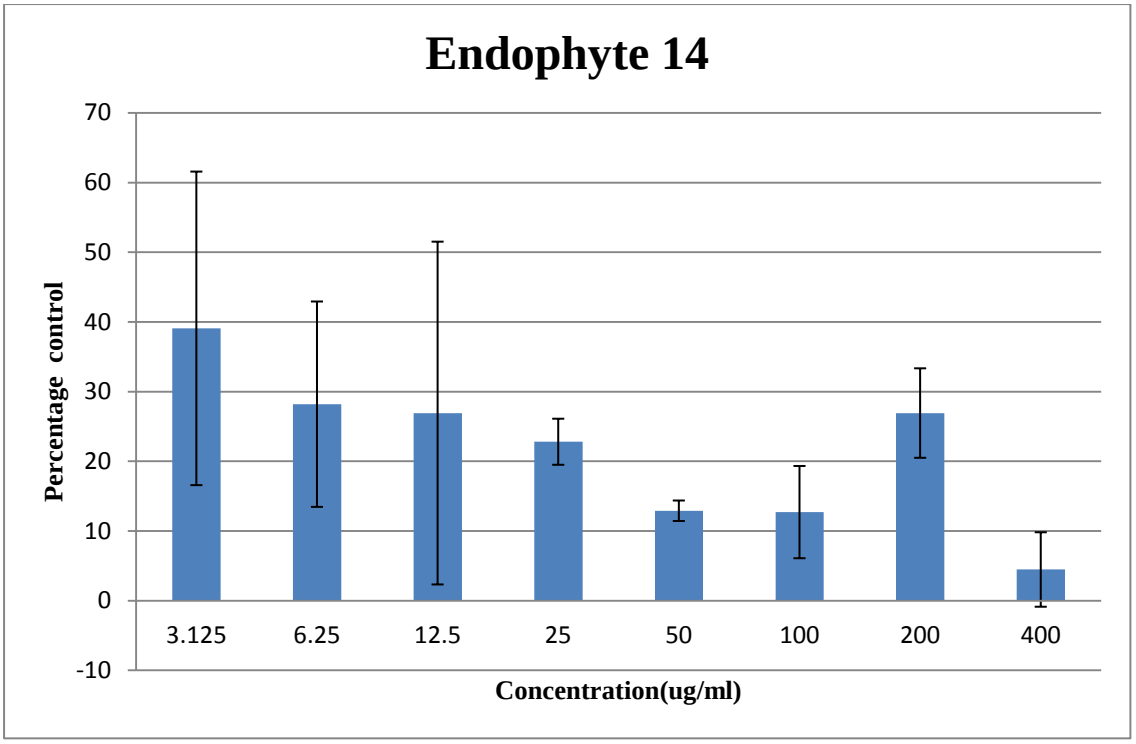

Figure 4.4: Bar chart showing H9c2 cell death vs broth and compound concentration on endophyte 14, second repetition.

From Figures 4.3 and 4.4 it is clear that Figure 4.4 expresses lower values with an IC_{50} of <3.125µg/ml whereas Figure 4.3 has an IC_{50} value of <9µg/ml. Initially the IC_{50} value for endophyte 14 was much higher at a value of <37.5µg/ml. Although the differences in the results for endophyte 14 are significant, the main conclusion is that all three of the results revealed an IC_{50} value of less than 50µg/ml thus endophyte 14 is toxic. Figures 4.5 and 4.6 shows the results generated for endophyte 20 which was isolated from both *V. pygmaea* and *V. thamnus*.

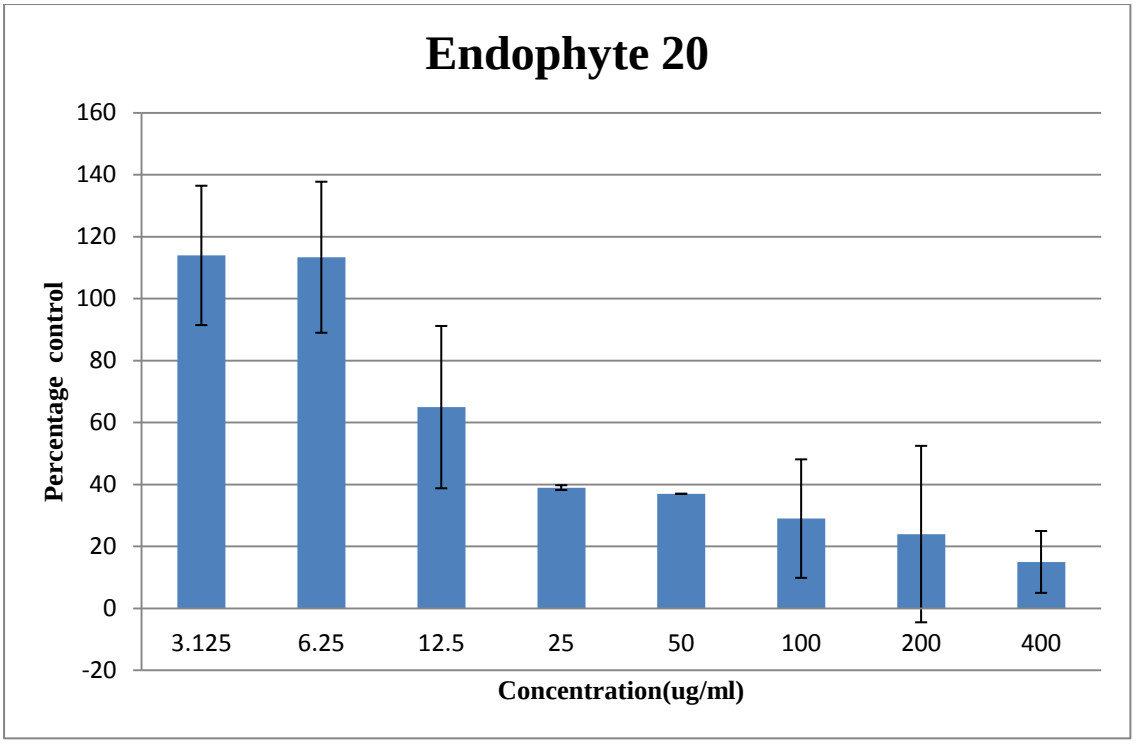

Figure 4.5: Chart expressing the IC_{50} values for the first repetition from endophyte 20.

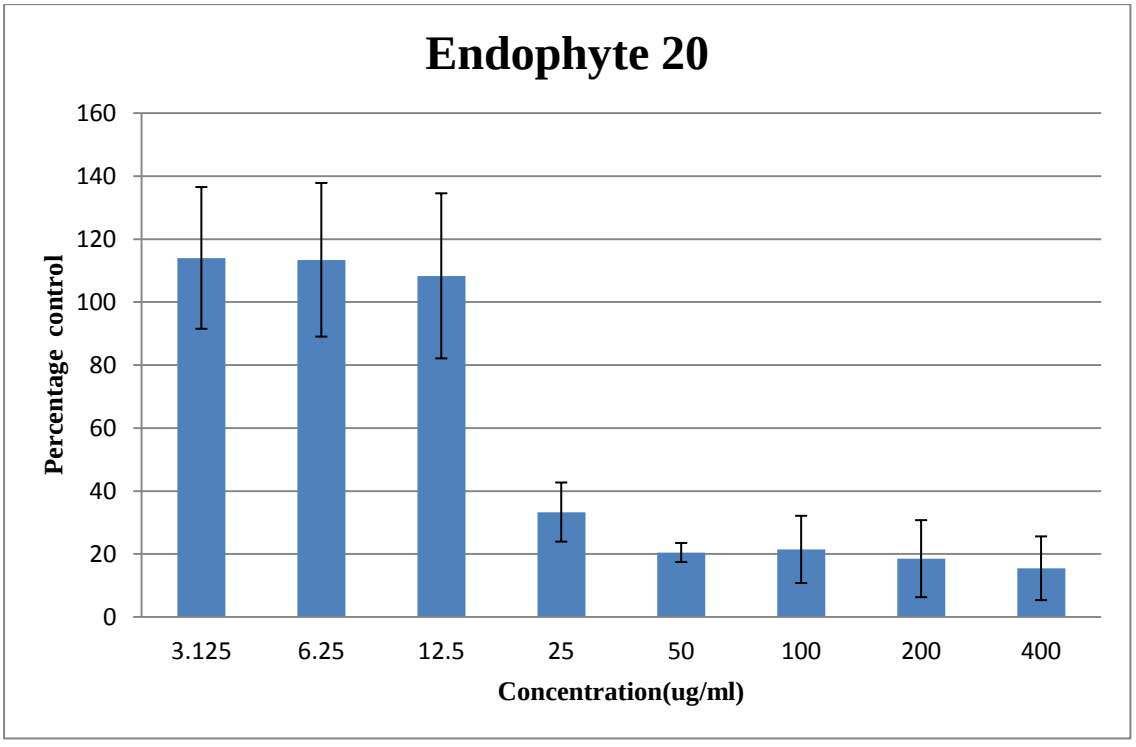

Figure 4.6: Chart expressing the IC_{50} values for the second repetition from endophyte 20.

Comparing Figure 4.5 and 4.6 there were little differences in the values at which concentration of endophyte compound and broth solution the H9c2 cells became less viable. It is clear from both figures and the initial results from endophyte 20 that a concentration of <25µg/ml is needed to induce death in H9c2 cells. Due to the fact that the IC_{50} value of endophyte 20 is less than 50µg/ml, endophyte 20 can be said to produce a toxic compound(s).

4.4.5. TLC analysis of ion exchange chromatography columns

Method determination by using the pure compound spermidine revealed that once the TLC plates were dried after being run twice to ensure adequate separation the pure compound eluted the column in fractions 16 and 17. This was seen in both the TLC plates although two different mobile phases were used. This information was significant in comparing the TLC plates from the spermindin column to those generated from the endophyte 20 column.

Ion exchange chromatography is used to separate compounds based on charge. The sugars and negatively charged amino acids needed by the bacteria will in theory be washed out of the column first. The more positive compounds will be retained as is the case with spermidine. The mobile phase distilled water: 2-propanol: acetic acid (60:40:4) revealed the presence of more compound bands, pavettamine moved from the origin and with less smearing then the less polar mobile phase of ethylacetate: acetic acid: formic acid: distilled water (6:1:1:1). Figure 4.7 displays the nitrogen containing compounds present in endophyte 20.

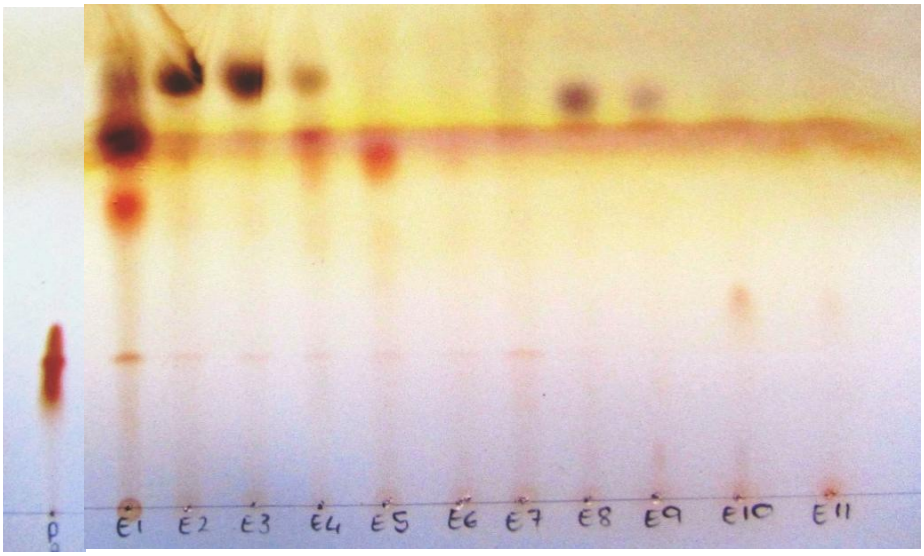

Figure 4.7: TLC evaluation of endophyte 20 ion exchange column. Lane 1= pavettamine standard, lane 2= elute 1, lane 3= elute 2, lane 4= elute 3, lane 5= elute 4, lane 6= elute 5, lane 7= elute 6, lane 8= elute 7, lane 9= elute 8, lane 10= elute 9, lane 11= elute 10 and lane 12= elute 11.

Elutes 12-14 did not reveal compounds which reacted with either short or long wavelength light, nor was any compound visualised once the plate was dipped in ninhydrin. It was concluded that all compounds produced by endophyte 20 had eluted the column by elute 11 (lane 12). Pavettamine (lane 1) formed a streak which appears to have the same rf value as compounds in, elute 1 (lane 2), elute 10 (lane 11) and elute 11 (lane 12), however it is possible that the compound(s) may come from the nutrient broth. The large quantity and highly concentrated compounds present in the nutrient broth can be credited to substances needed for bacterial growth and health. All the washes and elutes present in Figure 4.7 display compounds which contain a nitrogen atom. Although many of the compounds have different rf values to that of pure pavettamine none of the compounds can be excluded as non-toxic. The compounds present in elute 9 (lane 10), 10 (lane 11) and 11 (lane 12) appear to be at a lower concentration then the compounds present in the other elutes and washes.

4.4.6. Nuclear magnetic resonance (NMR) analysis

The original extract from endophyte 20 revealed a high concentration of sugars in the NMR graph, these sugar peaks were in the region of 3.5-4.5 which overlaps with the polyamine region of 3.6-4.3. The ion exchange column was used to remove the sugar molecules in order to expose the compounds beneath. This was achieved with wash 1; the NMR spectrum displayed a large quantity of the sugar molecules and no other compounds. The NMR spectrum was compared to that of the original and peaks in the same sugar region were present on both spectra's. The peaks present on the NMR spectra's of wash 2, 4, 5 and 6 were of low concentration and no clear findings could be made. The NMR spectra's from elute 1, 2 and 3 showed no clear similarities with the pure standard pavettamine.

4.4.7. Endophyte 20 identification

Using the program database Genbank it was established that the 16S rDNA sequence generated identified bacterial endophyte 20 as a member of the genus *Bacillus*. The DNA sequence did not match any known sequence in the database thus endophyte 20 is assumed to be a novel bacterial species present in both *V. pygmaea* and *V. thamnus*.

4.5.Discussion

In this study it was possible to isolate numerous bacterial colonies from either the leaf's phylloplane or within *V. pygmaea* and *V. thamnus*. Both *V. pygmaea* and *V. thamnus* contain bacterial endophytes within their leaves. In this study a total of twelve bacterial endophytes were isolated from *V. pygmaea* and seven from *V. thamnus*. Endophyte 20 which was isolated from both *V. pygmaea* and *V. thamnus* was visually analysed with respect to colour, texture, growth pattern, colony formation and smell and was assumed to be the same strain isolated from both plants. It was expected that culturable bacterial endophytes would be isolated from

both *V. pygmaea* and *V. thamnus* due to previous studies by Verstraete et al. (2011**)** whereby bacterial endophytes belonging to the genus *Burkholderia* were isolated from all the gousiekte-inducing plants, although the bacteria isolated were unculturable (Verstraete et al., 2011). Extensive work conducted on *P. harborii* and *P. schumanniana* revealed that these two gousiekte-inducing plants contain *Candidatus* Burkholderia schumanniana (Verstraete et al., 2011).

The only clear way to be sure that both *V. pygmaea* and *V. thamnus* contain the same bacterial endophyte would be to sequence all isolated endophytes from the two plants and compare the DNA sequences. There is a possibility that more bacterial endophytes are shared between the two plants and additional analysis would either confirm or deny this observation. Due to the extensive overlap in the plants distribution ranges it is not unexpected for the two plants to contain the same bacterial endophytes. The non-culturable bacteria present within the plants needs to be isolated and sequenced as do all the culturable bacteria. Evaluating the culturable bacteria only gives the experiment a one-sided approach. The use of nutrient broth as a growth medium could be expanded to include many other media in order to isolate the maximum number of culturable bacteria. All bacterial organisms isolated from the plant's phyllosphere or from within the plant needs to be sequenced in order to avoid any replications.

Samples from all 20 endophytes isolated were used for thin layer chromatography analysis which generated unclear results due to the presence of the growth medium, (nutrient broth). Nutrient broth contains sugars and amino acids which generate band patterns in the same region as polyamines, thus the nutrient broth will need to be removed from the bacteria endophytes before future evaluation. There was, however, no band patterns produced with the same rf value as that of pavettamine. This possibly indicates the absence of pavettamine produced from all of the bacteria isolated.

All culturable bacteria were submitted to an XTT assay to determine the toxicity of the compounds the endophytes produce. The XTT assay was conducted as that of Ellis et al. (2010) with a few minor changes (Ellis et al., 2010). In the initial screening 3 endophytes revealed toxicity readings. For more accurate results all 20 endophytes should be submitted to at least triplicate XTT evaluation. The initial 3 were re-evaluated in duplicates and only 2 endophytes were considered toxic, an endophyte isolated only from *V. pygmaea* and an endophyte isolated from both *V. pygmaea* and *V. thamnus*. The second hypotheses was accepted, an endophyte isolated from both *V. pygmaea* and *V. thamnus* expressed toxicity. The toxic bacterium isolated from both *V. pygmaea* and *V. thamnus* did not belong to the genera *Burkholderia* as expected but belongs to a common soil dwelling genus *Bacillus* (Verstraete et al., 2011; Phetcharat and Duangpaeng., 2011). However in order to determine whether or not this bacterium is producing the toxin responsible for inducing gousiekte further field trials will need to be performed. Gousiekte is identified by a 3-8 week latent period, thus toxicity itself does not conclude gousiekte toxin (Prozesky et al., 1988).

A more conclusive study would be to isolate culturable endophytes and using appropriate techniques such as DGGE to detect non-culturable endophytes from all six known gousiekte inducing plants, *F. homblei*, *P. harborii*, *P. schumanniana*, *V. latifolia*, *V. pygmaea* and *V. thamnus*. Identify and sequence all bacteria isolated from all of the gousiekte inducing plants. Conduct a cytotoxicity XTT assay on all bacteria isolated and compare similar bacteria between the plants. Toxic bacteria would be fractionated and fractions run on XTT assay in order to isolate toxic compounds. Toxic compounds would be analysed and elucidated. Bacterial fractions which express toxicity would be subjected to transmission electron microscopy in order to determine the mode of death the H9c2 cells are experiencing. Bacteria which displayed similarities in mode of action would be used for field trials. The findings

would reveal if there is a link between the bacterial endophytes and the induction of gousiekte.

In conclusion, culturable bacterial endophytes were isolated from both *V. pygmaea* and *V. thamnus*. A single culturable bacterial endophyte appeared to be isolated from both *V. pygmaea* and *V. thamnus.* Two of the bacterial endophytes were deemed toxic due to IC_{50} values lower than 50µg/ml one of these bacterial endophytes was assumed to be isolated from both test plants, *V. pygmaea* and *V. thamnus* and was found to belong to *Bacillus.*. This result concludes that a toxic bacterium inhabits both *V. pygmaea* and *V. thamnus* and there may be a possibility that this bacterium produces the toxin which induces gousiekte.

4.6.References

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Chapter 5

Discussion and Conclusion

5.1. Discussion and Conclusion

Although a great many extraction and separation techniques were employed during this study pavettamine was not isolated from *V. pygmaea*. However, a number of other nitrogen containing compounds were isolated and purified. These results proved that the pavettamine extracting procedure is indeed extremely cumbersome and that the compound is present in very low concentrations (Fourie et al., 1995). There is the possibility that pavettamine within the plant extract was bound to another compound due to its high positive charge, or the simple explanation is that pavettamine was not present in *V. pygmaea* or is present in such low quantities that it could not be detected.

From all the gousiekte-inducing plants *V. pygmaea* and *V. thamnus* appear the most similar and to the untrained eye the two species look identical. The only defining feature is the leaf pubescence of *V. pygmaea* whereas *V. thamnus* is globular. Due to this close resemblance many taxonomists expected the chemistry of these two plants to be very similar. However, this was not the case. Thin layer chromatography results revealed that these two plants contain very different compounds when using detection by vanillin, a general indicator, and ninhydrin, a nitrogen indicator. Using the same extraction procedures and two mobile phases, *V. pygmaea* and *V. thamnus* share only a few compounds which were similar.

The six plants subjected to cytotoxicity screening included *P. schumanniana*, *P. gardeniifolia*, *V. infausta*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. Before evaluation commenced it was thought that *P. gardeniifolia*, *V. infausta* and *V. macrocalyx* were all non-toxic as no literature had stated otherwise. The results generated were quite unexpected. The XTT assays revealed that *P. schumanniana* was the most toxic of all the plants; this in itself was unexpected for *V. pygmaea* was assumed the most toxic of all the gousiekte -inducing plants. *P. gardeniifolia* was the second most toxic plant followed by *V. pygmaea*, *V. thamnus*,

V. macrocalyx and lastly the least toxic was *V. infausta*. The low IC_{50} value for *P. gardeniifolia* deems the plant toxic which is the first report of such a result. The XTT assay will need to be conducted several more times with different indicators to ensure accurate results. Once toxicity is ensured in the H9c2 cell line *P. gardeniifolia* would have to be tested on live ruminants to determine whether gousiekte is induced in field studies.

Six plants, namely, *P. schumanniana*, *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus*, were evaluated for the presence of bacterial endophytes. Culturable bacterial endophytes were observed in all six plant species. With the exception of *P. schumanniana*, the bacteria were located within the intercellular spaces of the leaves mainly around the spongy mesophyll parenchyma cells. In the case of *P. schumanniana* the endophytes were located within the bacterial leaf nodules. It is important to note that bacterial endophytes were viewed in both the toxic *P. schumanniana*, *V. pygmaea* and *V. thamnus*, and the non-toxic, *V. infausta*, *V. macrocalyx* and *V. madagascariensis*, species. This result disproves the theory that only toxic gousiekte causing plants contain bacterial endophytes.

On close inspection of the morphology of the bacterial endophytes there appears to be significant similarities between them in *V. pygmaea* and *V. thamnus*, *i.e.* they display similar size, distribution, production of a slime-like mucus and contain polyhydroxybutyrate-like granules. The polyhydroxybutyrate-like granules appear to be present in bacteria observed in *P. schumanniana* but in no other test plant. The bacteria present within the non-toxic plants do not display significant similarities to the toxic plants. Isolation of all the endophytes present within the test plants and other gousiekte-inducing plants is vital in determining whether there is a link between the bacterial endophytes and the disease gousiekte. However, the identity of these endophytes needs to be confirmed.

Bacterial endophytes present within *V. pygmaea* undergo seasonal colonisation fluctuations. Late October, early summer, is when bacterial colonisation is greatest and bacteria can be seen throughout the leaf's intercellular spaces. Bacteria seen in June appear to be disintegrating or dying. One can thus conclude that bacterial colonisation is lowest during the winter months. The seasonal bacterial habitation fluctuations does co-inside with gousiekte cases reported. The vast majority of cases are reported during early summer where bacterial habitation is highest whereas in winter, cases are rare. In order to determine if in fact the bacterial habitation fluctuations corresponds to seasonal toxicity fluctuations the other gousiekte inducing plants would have to be studied along with *V. pygmaea*. Cytotoxicity screening of the plant extracts would have to be undertaken monthly together with the bacterial fluctuation study.

In terms of culturable bacteria, twelve bacterial endophytes were isolated from *V. pygmaea* and a further seven were isolated from *V. thamnus*. Preliminary results indicate that only one bacterial endophyte was common and isolated from both *V. pygmaea* and *V. thamnus* and labelled endophyte 20. These results need further confirmation. Sequencing non-culturable bacteria would determine the total number of bacterial endophytes present in both plants and evaluate if any more bacterial endophytes are shared between the two plants.

The isolated bacteria were subjected to cytotoxicity screening which resulted in two bacterial endophytes being toxic to H9c2 cells after a 72h time re-laps. One was isolated from *V. pygmaea* and the second was the bacterial endophyte isolated from both plants, endophyte 20. This result is the first report of a bacterial endophyte isolated from gousiekte-inducing plants expressing toxicity. Endophyte 20 generated IC_{50} values of less than 25µg/ml in all three of the XTT assays performed on the bacterium, concluding that the bacterium produces a toxic compound. Endophyte 20 would need to be grown in bulk and tested on domestic ruminants to ensure that the toxin the bacteria produces is responsible for inducing gousiekte.

Although *Burkholderia* have been identified as bacterial endophytes in *F. homblei*, *P. harborii* and *P. schumanniana*, there may be other endophytes present within the gousiekte-inducing plants that has not yet been isolated (Verstraete et al., 2011). The 16S rDNA of endophyte 20 was evaluated and it was concluded that the novel bacterium belongs to the genus *Bacillus*.

This study has provided some support to the theory that bacterial endophytes play a role in inducing gousiekte. The habitation of bacterial endophytes co-insides with the cases reported and the fact that a toxic bacterium was isolated from both *V. pygmaea* and *V. thamnus* links endophytic bacteria to the gousiekte disease. All in all many of the questions surrounding the gousiekte inducing plants and the disease itself has been answered in this study. However, there are still a great number of questions which have not been answered.

5.2. References

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Chapter 6

Appendix

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An evaluation of the endophytic colonies present in pathogenic and non-pathogenic Vanguerieae using electron microscopy

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ABSTRACT

Fadogia homblei, *Pavetta harborii*, *Pavetta schumanniana*, *Vangueria pygmaea* (=*Pachystigma pygmaeum*), *Vangueria latifolia* (=*Pachystigma latifolium*) and *Vangueria thamnus* (=*Pachystigma thamnus*) all induce one of the most important cardiotoxicoses of domestic ruminants in southern Africa, causing the sickness gousiekte. All the plants which cause gousiekte have previously been shown to contain bacterial endophytes. However, in this study other plants within the Vanguerieae tribe that have not been reported to cause gousiekte; namely *Vangueria infausta*, *Vangueria macrocalyx* and *Vangueria madagascariensis*, have now been shown to also contain endophytes within the inter-cellular spaces of the leaves. The disease gousiekte is difficult to characterise due to fluctuations in plant toxicity. The majority of reported cases of gousiekte poisoning are at the beginning of the growing season; and thus the plants are thought to be more toxic at this time. By using both transmission and scanning electron microscopy the endophytes within these Vanguerieae plants were compared visually. Using the plant reported most often for gousiekte poisoning, *V. pygmaea*, a basic seasonal comparison of the presence of endophytes was done. It was found that the bacterial endophyte colonies were most abundant during the spring season.

1. Introduction

The family Rubiaceae is the fourth largest flowering family with over six hundred genera, of which *Vangueria* is one such genus comprising of over fifty different species (Verstraete et al., 2011). Many of the members of the Rubiaceae family contain endophytes; some endophytes form nodules and others are present within the inter-cellular spaces of the leaves (Van Wyk et al., 1990; Verstraete et al., 2011). Some plant species in the *Vangueria* genus, namely *Vangueria latifolia* Sond. (=*Pachystigma* cf. *latifolium*), *Vangueria pygmaea* (Schltr.) Robyns (=*Pachystigma pygmaeum*) and *Vangueria thamnus* Robyns (=*Pachystigma thamnus*) are known to be pathogenic whereas *Vangueria infausta* Burch. ssp. *infausta*, *Vangueria macrocalyx* (Sond.) Robyns and *Vangueria madagascariensis* J.F. Gmelin (Lantz and Bremer, 2005) are assumed to be non-pathogenic.

Bode et al. (2010) isolated pavettamine from *Pavetta harborii* which is assumed to be the causative toxin responsible for all gousiekte poisoning. Other plants responsible for gousiekte poisoning include *Fadogia homblei*, *P. harborii* and *Pavetta schumanniana* (Fourie et al., 1989; Verstraete et al., 2011). Gousiekte occurs mainly in southern Africa but cases as far as the Democratic Republic of Congo have been reported (Verstraete et al., 2011). The gousiekte disease affects domestic ruminants, mainly cattle and sheep and is a plant induced cardiomyopathy (Botha and Penrith, 2008; Ellis et al., 2010a). After 3–6 weeks of ingestion of one of the plants the ruminant will suddenly die, usually after physical activity. The word 'gousiekte' was translated from Afrikaans literally means 'quick disease' for there is no pre-warning before the ruminant dies from cardiac failure (Fourie et al., 1989; Ellis et al., 2010b). Van Wyk et al. (1990) reported that endophytes were present in *V. latifolia*, *V. macrocalyx*, *V. pygmaea* and *V. thamnus*. One of the important remaining questions is whether the bacterial endophytes are pathogenic and thus inducing gousiekte or if it's just coincidental that endophytes are located in all the gousiekte inducing plants.

Gousiekte was first identified in 1908 but due to the following factors this disease has proved hard to diagnose; varying or lack of symptoms, animal susceptibility differences, loss of toxicity as the plant dries and apparent seasonal toxicity of the plants (Van Wyk et al., 1990; Hay et al., 2008; Bode et al., 2010). Considering that gousiekte inducing plants apparently undergo seasonal toxicity it would be expected that there is a change during the year in either the concentration of toxic compounds present or the number of endophytes which inhabits the inter-cellular spaces of the leaves during the year. We report here on the occurrence of endophytes within the leaves of the toxic and non-toxic *Vangueria* species and the seasonal endophyte variation in the most often reported gousiekte inducing species, *V. pygmaea*.

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2. Materials and methods

2.1. Plant collection

Freshly collected leaf material of *V. infausta*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea* and *V. thamnus* was prepared for transmission electron microscopy. All the leaves were collected in the same week of October 2011 in Mpumalanga province. *V. macrocalyx* leaves were collected near Piet Retief (S 27°09′01″, E 30°59′18″). A field outside Lydenburg was the site for both *V. pygmaea* (S 25°12′94″, E 30°19′03″) and *V. thamnus* (S 25°12′92″, 30°19′02″). The rocky hills near Blyde River Canyon were the site

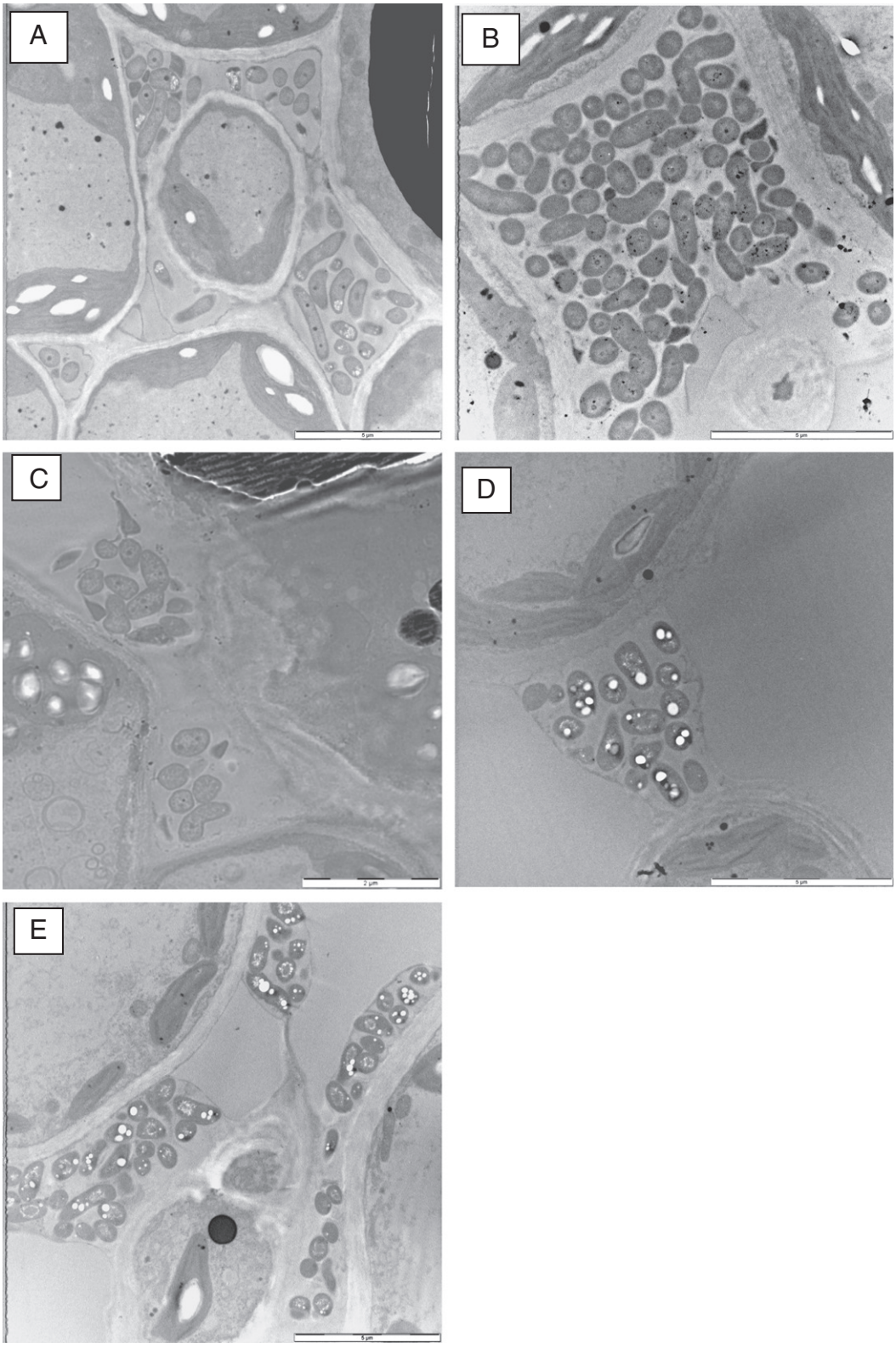

Fig. 1. Endophyte comparison; TEM micrographs of A) *Vangueria infausta*, B) *Vangueria macrocalyx*, C) *Vangueria madagascariensis*, D) *Vangueria pygmaea* and E) *Vangueria thamnus*.

where *V. infausta* (S 24°34′25″, E 30°47′15″) and *V. madagascariensis* (S 24°33′26″, E 30°47′18″) were found. Voucher specimens have been deposited in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria. The PRU numbers for the plants are: *V. infausta* ssp. *Infausta*, 117607; *V. macrocalyx*, 117601; *V. madagascariensis*, 117611; *V. pygmaea*, 117605; and *V. thamnus*, 117603. Four samples were collected of each plant species, 2× mature fresh leaf and 2× young fresh leaf, and immediately immersed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (Coetzee and Van der Merwe, 1986). These 20 samples were prepared for transmission electron microscopic analysis by following a standard procedure for TEM samples as described below.

2.2. Seasonal plant collection of *V. pygmaea*

V. pygmaea leaves were collected from a site near the town Rayton in Gauteng (S 25°73′608″, E 028°53′321″), during late October 2010, March 2011, June 2011 and early September 2011. A voucher specimen was placed in the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria and the PRU number is 117989. Six samples were prepared per seasonal comparison for TEM viewing; 3× mature leaves and 3× young leaves. Four samples were also prepared for SEM viewing during each season; 2× mature leaves and 2× young leaves.

2.3. Sample preparation

All five of the *Vangueria* species were prepared as follows. The leaves were cut into 0.5 mm^2 squares. The plant parts were placed into test tubes and fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer at a pH of 7.4 for between 1 and 2 h at room temperature. Each of the tubes was then rinsed 3 times, 10 min each in 0.075 M phosphate buffer. The samples were fixed in 0.5% aqueous osmium tetroxide in a fume hood and left at room temperature for between 1 and 2 h. The test tubes were rinsed 3 times, 10 min each in distilled water in a fume hood. The samples were dehydrated in ethanol at concentrations of 30%, 50%, 70%, 90% and 3× 100% for 10 min each (Coetzee and Van der Merwe, 1986).

2.4. TEM preparation

The TEM samples were infiltrated with 50% quetol epoxy resin in ethanol for between 30 min and an hour and then infiltrated with 100% pure quetol epoxy resin for 4 h at room temperature. The samples were then polymerised at 60 °C for 39 h and cut into ultrathin sections using a microtome and placed on small copper grids (Coetzee and Van der Merwe, 1986). The TEM used was a JEOL 2100 F.

2.5. SEM preparation

After dehydration the samples underwent critical point drying and were mounted onto the SEM sample stubs. The samples were then coated with gold which makes the sample electrically conductive. After initial viewing of the SEM samples they were split using cello tape to view the interior of the leaves (Coetzee and Van der Merwe, 1986). The SEM used was a JEOL 840.

3. Results

3.1. Endophyte comparison

The five different *Vangueria* species were viewed in order to determine if there were any morphological differences in the endophyte colonies when observed under the transmission electron microscope (Fig. 1). The micrographs of all the *Vangueria* species reveal bacterial endophyte colonisation in the inter-cellular spaces of the leaves. However, the morphology of the colonies appears to be significantly different between the species. As seen in Fig. 1A the bacteria of *V. infausta* are rod shaped, about 1.0–1.5 μm in width and 4.0 μm in length. The morphology of the bacteria present within *V. madagascariensis* (Fig. 1C) appears to be different to that of *V. infausta*; the width is between 0.5 and 1.0 μm and the length 1.0–1.5 μm. This is the first report of endophytes occurring in *V. madagascariensis*. The bacterial endophytes within *V. macrocalyx* are rod shaped, width of 1.0 μm and length of between 2.5 and 3.0 μm as seen in Fig. 1B. The bacterial endophytes within the two gousiekte-causing species, *V. pygmaea* and *V. thamnus* are morphologically quite different from the other three species. The endophytes from the gousiekte-causing species are similar in size and shape, about 0.5 μm wide and 2.0 μm long. Both species' bacterial endophytes are embedded in slime-like mucus and contained white polyhydroxybutyrate-like granules (Collins et al., 2012) as shown in Fig. 1D and E.

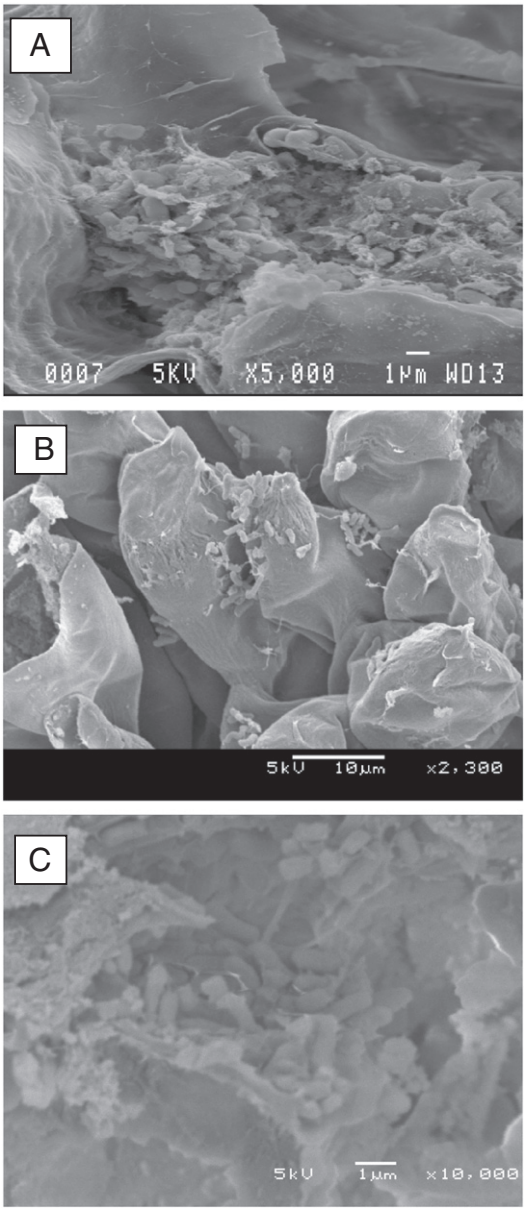

Fig. 2. Seasonal inhabitation of *Vangueria pygmaea* bacterial endophytes as shown by SEM. Micrographs of colonies observed in A) March B) June and C) early September 2011.

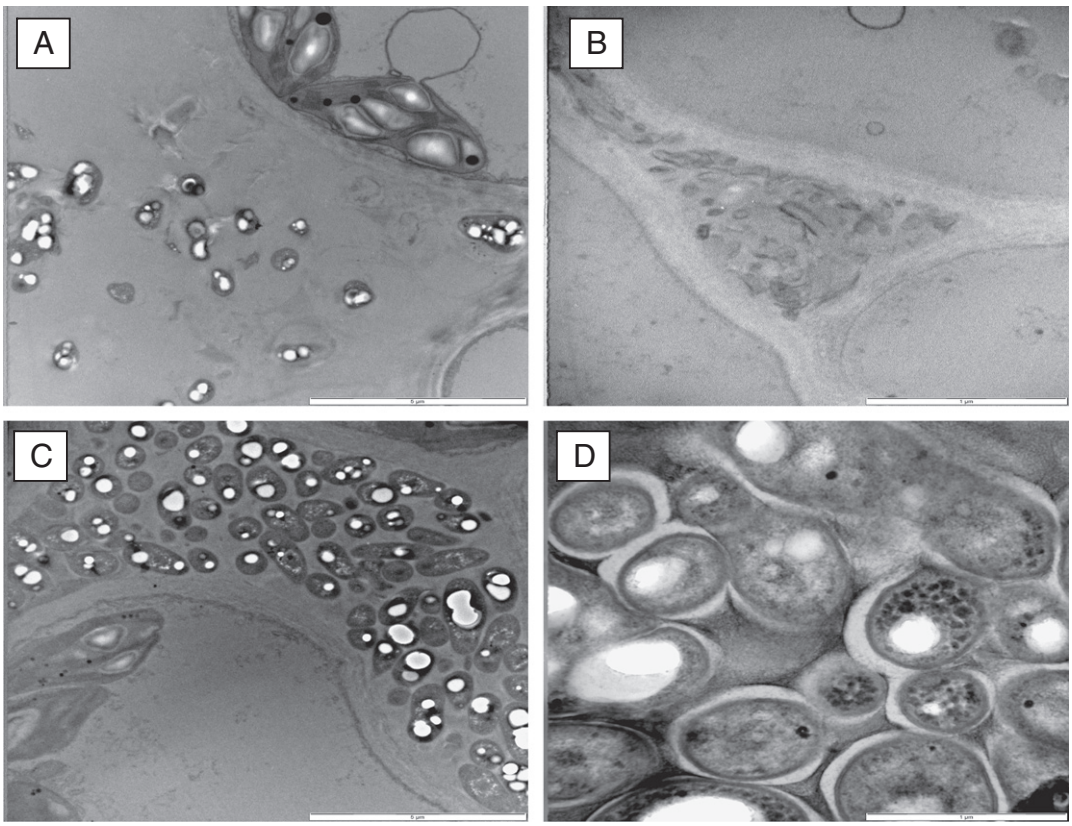

Fig. 3. Seasonal inhabitation of *Vangueria pygmaea* endophytes using TEM. A) March B) June C) early September and D) late October.

3.2. Seasonal endophyte colonisation of V. pygmaea

The SEM micrographs of plants collected during March 2011, June 2011 and early September 2011 are shown in Fig. 2. A change in the number of healthy endophyte colonies could clearly be seen within *V. pygmaea* during different times of the year. Fig. 2A (March) shows a moderate number of bacteria present. Comparing the number of colonies in Fig. 2A with those seen in Fig. 2B (June) it can be noted that there is a significant decrease in the number of bacteria present in the winter. Fig. 2C (September) shows high numbers of bacteria present within the leaves; clearly showing that early September has more endophytic bacteria present than the other months. This was also observed in plants collected in late October.

Fig. 3 shows the TEM micrographs from March 2011, June 2011, early September 2011 and late October 2010. It can be seen that bacterial endophytes are present within the inter-cellular spaces of the leaves of *V. pygmaea* throughout the year. Fig. 3A (March) shows bacterial colonies sporadically spaced during the autumn season whereas very few if any living bacteria can be seen during June, one of the winter months. From these observations it can be said that early September (Fig. 3C) and late October (Fig. 3D) contain the highest numbers of endophytes whereas June (Fig. 3B) contains the least and the endophytes that were present appear to be dead.

4. Discussion

4.1. Endophyte comparison

Bacterial endophyte colonies were observed for the first time in *V. madagascariensis* and are present in the four other *Vangueria* species. Due to the fact that none of these species produce nodules for the bacterial endophytes to reside in; it is not a surprise that the bacterial colonies were observed within the inter-cellular spaces of the leaves. The bacteria are rod shaped and were located throughout the leaves but were more commonly found around the spongy mesophyll parenchyma cells. The bacterial colonies were visually compared with each other and bacteria observed within *V. infausta* were the largest and appeared most similar to those seen within *V. madagascariensis*. It is possible that the bacteria within *V. infausta* and *V. madagascariensis* belong to the same genus. These bacterial colonies did not resemble any of the other endophytes observed within the other three *Vangueria* plants. The bacterial endophytes within the two gousiekte-causing species, *V. pygmaea* and *V. thamnus* resembled each other quite significantly. They contained white possibly polyhydroxybutyrate-like granules, produced a mucus-like substance and they were roughly the same size. The bacteria within *V. macrocalyx* were also observed within the inter-cellular spaces of the leaves. The bacteria appeared different morphologically when compared to the other *Vangueria* species, they were larger and were not embedded in a mucus-like substance as seen in Fig. 1B.

The number of bacterial endophytes generally seen in *V. infausta*, *V. macrocalyx* and *V. madagascariensis* was significantly less than that observed in *V. pygmaea* and *V. thamnus*. All these observations might be significant due to the fact that *V. pygmaea* and *V. thamnus* are the species reported to cause the sickness gousiekte, whereas *V. infausta*, *V. macrocalyx* and *V. madagascariensis* are assumed to be non-toxic. The two species reported to cause gousiekte, *V. pygmaea* and *V. thamnus*, contain endophytes which are very similar; whereas the species which are assumed to be non-toxic, *V. infausta*, *V. macrocalyx* and *V. madagascariensis*, contain endophytes which appear very different to the toxic species.

4.2. Seasonal colonisation

V. pygmaea has been reported to undergo seasonal toxicity fluctuations; being most toxic at the beginning of the growing season

(September) and least toxic during the winter months of June and July (Hay et al., 2008). In order to evaluate if the bacterial endophytes have any correlation in the toxicity of *V. pygmaea* and *V. thamnus* a seasonal comparison of the numbers of bacterial colonies present within the leaves was conducted. In Fig. 3A (March) it can be seen that the condition of the bacteria seems to be 'healthy' and they are present in a moderate number but not tightly packed together. When comparing this observation with that of Fig. 3B, it is clear to see that when bacteria were observed in the winter (June) they appeared to be dying and non-functional. Fig. 3C from early September shows extensive bacterial colonisation within the inter-cellular spaces of the leaves and reveals that the bacteria are encapsulated within a mucilage that appears to be produced by the bacteria. Fig. 3D shows the abundance of bacterial endophytes packed tightly into an inter-cellular space, the bacterial colonies appear to be 'healthy' morphologically during late October (summer).

The results revealed that endophytes are most abundant at the beginning of the growing season, early September, and the least during the winter months in *V. pygmaea*. This correlates with the fact that the plant is reported to be most toxic at the beginning of the growing season, as most poisoning cases are reported during this time and in comparison very little number of cases is reported during the winter. It can be said that these experiments may possibly support the theory that the bacteria and not the plant produces the toxic compound(s) or precursor(s) which make these plants toxic. Further studies need to be conducted to determine if toxic compounds are actually produced by the endophytes.

Another theory which needs to be further evaluated is that gousiekte only affects ruminants and it is possible that the bacterial endophytes produce a precursor compound which only becomes toxic after being metabolised in the rumen. The long period it takes for the ruminant to die could be due to either the toxin having to accumulate in large quantities by addition of more plant material or the bacteria having to multiply in sufficient numbers to produce enough toxin to kill the animal.

In conclusion it can be said that there are still many questions surrounding the plants which induce the sickness gousiekte. It is clear however that other plant species belonging to the Vanguerieae which are reported to be non-toxic, also contain bacterial endophytes within the inter-cellular spaces of the leaves. These might however be of a different species. In order to support the theory that the bacterial endophytes produce the toxin, the same bacterial species or similar compound-producing bacteria need to be isolated from *F. homblei*, *P. harborii*, *P. schumanniana*, *V. latifolia*, *V. pygmaea* and *V. thamnus*.

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