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The occurrence of the toxins, streptol, kirkamide and pavettamine in the gousiekte-causing Rubiaceae species

By

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Submitted in partial fulfilment of requirements for the degree

Magister Scientiae

In

Medicinal Plant Science

Department of Plant and Soil Sciences

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November 2023

Declaration

I, Monique Blignaut, affirm that I independently carried out all the work in this study, following the guidance of my supervisor, Professor J.J.M. Meyer. I assert the accuracy of all presented results, with no instances of falsification or manipulation. I am confident that there is no plagiarism, and proper credit has been given where it is deserved.

Publication resulting partially from this MSc thesis

Received: 24 August 2022 | Accepted: 28 November 2022

DOI: 10.1111/1462-2920.16292

RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY Applied Microbiology International

Cyclitol metabolism is a central feature of *Burkholderia* leaf symbionts

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Funding information
Agence Nationale de la Recherche, Grant/Award Numbers: ANR-10-LABX-41, ANR-11-IDEX-0002-02, ANR-19-TERC-0004-01, MetaboHUB-ANR-11-INBS-0010; Fonds Wetenschappelijk Onderzoek, Grant/Award Number: G017717N; Wellcome Trust, Grant/Award Number: 203141/Z/16/Z

Abstract
The symbioses between plants of the Rubiaceae and Primulaceae families with *Burkholderia* bacteria represent unique and intimate plant–bacterial relationships. Many of these interactions have been identified through PCR-dependent typing methods, but there is little information available about their functional and ecological roles. We assembled 17 new endophyte genomes representing endophytes from 13 plant species, including those of two previously unknown associations. Genomes of leaf endophytes belonging to *Burkholderia s.l.* show extensive signs of genome reduction, albeit to varying degrees. Except for one endophyte, none of the bacterial symbionts could be isolated on standard microbiological media. Despite their taxonomic diversity, all endophyte genomes contained gene clusters linked to the production of specialized metabolites, including genes linked to cyclitol sugar analog metabolism and in one instance non-ribosomal peptide synthesis. These genes and gene clusters are unique within *Burkholderia s.l.* and are likely horizontally acquired. We propose that the acquisition of secondary metabolite gene clusters through horizontal gene transfer is a prerequisite for the evolution of a stable association between these endophytes and their hosts.

INTRODUCTION

Interactions with microbes play an important part in the evolution and ecological success of plants. For example, mycorrhizal associations are present in a vast majority of land plants, and the association with nitrogen-fixing bacteria provided legumes with an important evolutionary advantage (Brundrett, 1991; Smith & Read, 2008; van Rhijn & Vanderleyden, 1995; Vessey et al., 2005). Nevertheless, microbes may also be harmful for plants as microbial pathogen interactions are responsible for major crop losses (Dangl & Jones, 2001; McCann, 2020). Many plant–microbe interactions only occur temporarily: contacts between microbes and the host are often limited to a subpopulation or a specific developmental phase of the host. However, in some associations, microbes are transferred from parents to offspring in a process called vertical transmission, resulting in permanent associations with high potential for co-evolution (Gundel

The article included all chemical analyses and botanical aspects of streptol outlined in the research done during the study.

Acknowledgments

- I would like to acknowledge my supervisor Professor J.J.M. Meyer, for all the guidance and assistance throughout my time at the department. His passion for his work is inspiring. He has provided me with opportunities to learn and allowed me to see parts of the world I could only dream of. The past four years are filled with happy memories from Namibia to St. Lucia. Thank you, Prof., for the incredible adventures.
- Heartfelt thanks go to my family, Tia and Andre Blignaut, whose unwavering support sustained me throughout my university journey. I appreciate your patience in listening to my frustrations and the encouragement that kept me motivated.
- I am grateful to my university colleagues for their collaborative spirit, shared ideas, and assistance during moments of uncertainty. Thank you to especially Sewes Alberts, for listening to me when I needed someone to talk to, for always being there when I needed you and for staying positive no matter the situation.
- Finally, my heartfelt appreciation goes to all the lecturers at the university who steered me toward Plant Science. This guidance has enabled me to establish a solid foundation for a promising career that I am confident awaits me.

Summary

Gousiekte (quick disease) is a cardiotoxicoses commonly found in ruminants and is caused by at least six species of the Rubiaceae family namely, *Fadogia homblei*, *Pavetta harborii*, *Pav. schumanniana*, *Vangueria latifolium*, *V. pygmaea* and *V. thamnus*. Bacterial endophytes are present in all plants that cause *gousiekte* and it might be possible that the bacterial species play a role in the production of the toxin. As a result of varying toxicity of plants that cause *gousiekte*, the parameters of the disease have not been confidently established. Pavettamine was previously identified as the *gousiekte*-causing toxin and shown to be present in all these species, however, the results obtained from experiments regarding pavettamine, have been very inconsistent. The toxins, kirkamide, streptol and streptol glucoside were previously found in *Psychotria kirkii* (Rubiaceae) and reported for their toxicity, the endophyte genome contains the genes responsible for encoding these toxins. The main aim of this study was to determine if kirkamide, streptol and its glucoside occur in some of the *gousiekte*-causing species and to investigate the role of the endophytes in the production of the toxin. Nine non-toxic species of the Rubiaceae family were analysed together with three *gousiekte*-causing species (*F. homblei*, *V. pygmaea* and *V. thamnus*) by means of ^{1}H-NMR, GC-MS, UPLC-QToF and LC-MS analyses. The chemical analysis results were compared with genetic studies done by collaborators who determined if the bacterial endophyte genomes contain the genes for the biosynthesis of these toxins. None of the species carried the kirkamide gene, it was also not detected in the extracts during chemical analyses. The gene encoding streptol was identified in a few species namely, *V. infausta*, *V. madagascariensis*, *V. pygmaea* and *V. randii*. Streptol was detected in two positive controls namely *V. pygmaea* and *V. infausta* however in very low intensities although as expected not in the negative control *F. homblei*. Based on the results, it cannot be confidently said that either kirkamide or streptol plays a role in causing *gousiekte*. However, the results provide compelling evidence that the endophyte contributes to the production of the toxin streptol, as the toxin was only found in the species containing the endophyte gene for streptol.

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Chapter 1: Introduction and literature review

1.1. Introduction

Gousiekte (quick disease) is a disease that affects ruminants and is one of South Africa's six most important plant poisonings of livestock (Fourie et al., 1995). Ruminants are herbivorous cloven-hoofed animals that contain several stomach chambers, that will regorge food and will chew the swallowed food at a later stage, they are characterised as grazing or browsing animals (Myers, 2005; Niwinska, 2012). The species that have been implicated as the cause of gousiekte are (in order of most to least reported for toxicity) *Vangueria pygmaea* (Schltr.) Robyns, *Fadogia homblei* De Wild., *Pavetta harborii* S. Moore, *V. thamnus* Robyns, *Pav. schumanniana* F.Hoffm. ex K.Schum. and *V. latifolia* Sond. (Stanton et al., 2013; Verstraete et al., 2011). Gousiekte mainly affects the heart of the animals (Hurter et al., 1972). There are no definite symptoms that can be seen by visual inspection in the field and as a result gousiekte can only be diagnosed through post-mortem investigations (Fourie et al., 1989). Animals usually dies four to eight weeks after ingestion of toxic material as a result of heart failure (Hurter et al., 1972).

There are several cases where farmers had severe stock losses due to gousiekte. A gousiekte case was reported in the Vaalwater area where a farmer lost more than half of his flock (1 000 sheep out of 1 700). Between 1959 and 1966, 2 051 gousiekte cases were reported within a radius of approximately 48 km of each other. In another case in the Delmas area a farmer reported 37 out of 60 sheep dying from gousiekte (Hurter et al., 1972; Prozesky et al., 1988; Theiler et al., 1923). These are merely a few instances among many, underscoring the urgency of identifying the root cause of the disease and developing a remedy to mitigate substantial livestock losses. Consequently, it is crucial to expand the quest for an alternative explanation for gousiekte, as there is currently no definitive proof that pavettamine is the sole causative factor.

The cause of gousiekte has been determined to be due to a toxic compound known as pavettamine (Bode et al., 2010; Fourie et al., 1995; Van Elst et al., 2013a), but there is some doubt that pavettamine is the main or possibly the only cause of gousiekte. The inability to understand the disease and confidently identify pavettamine as the cause of the disease has been attributed to several aspects, including the variance in the concentration of the toxin in plants. Although, the possibility exists that pavettamine is not

the cause of gousiekte and it could be important to determine if other poisons are present in the gousiekte-causing species.

The toxic gousiekte-causing plants contain endophytes which all belong to the *Burkholderia* genus (Verstraete et al., 2011). Scientists have hypothesised for long that these endophytes play a role in the cause of gousiekte by possibly synthesising the toxin (Van Wyk et al., 1990; Verstraete et al., 2011). If that scenario holds true, it is highly probable that the bacteria harbour the genes responsible for producing the toxin within their genome. This discovery could potentially offer a valuable starting point for devising strategies to prevent animal fatalities caused by gousiekte.

Two toxins, streptol glucoside and kirkamide were isolated from *Psychotria kirkii*, also an endophyte containing species of the Rubiaceae family (Hsiao et al., 2019; Sieber et al., 2015). This species contains visible nodules that house an endosymbiotic bacterial species that are from the same genus as those found in the gousiekte-causing species (*Burkholderia*) (Georgiou et al., 2021; Hsiao et al., 2019). This correlation could be significant since all of the gousiekte-causing species contain endosymbionts and it is hypothesised that these bacterial species play a role in the production of the toxin(s) that cause gousiekte (Van Elst et al., 2013b). It could thus be possible that these toxins assist in causing gousiekte.

1.1.1. Co-occurrence of toxic plants and gousiekte

Gousiekte results in extensive economical losses (Fourie et al., 1995). Cases of gousiekte have been found in South Africa, Botswana, and southern Zimbabwe (Van Elst et al., 2013a). The most cases in South Africa have been recorded in the north eastern part of the country, however, the distribution of the toxic species are not restricted to these areas (Van Elst et al., 2013a). Therefore, it is possible that cases of gousiekte exist in other regions but have not been documented or reported.

Gousiekte is most commonly found in domesticated ruminants such as goats, sheep and cattle (Fourie et al., 1995). Domesticated ruminants are more susceptible to gousiekte when compared to wild animals as is well explained by Fowler (1983); animals other than domesticated ruminants are believed to be adapted to their environment and would often avoid toxication by not feeding on toxic plants, they can also decrease the effect of the toxic species by eating large quantities of other plant material and as a result dilute the toxic compounds and some animals detoxify the compounds in a systemic manner or through rumen degradation (Fowler, 1983; Lawrence et al., 2010). Some animals are, however, more likely to

consume toxic plant species if no other options are available or if there are limited resources. It is thus possible under certain circumstances that wildlife can develop gousiekte. Lastly, an animal that receives nutritious food has a healthy gut with the required gastrointestinal microflora, which might help to degrade the toxic compounds and create resistance against the toxic plant species (Fowler, 1983). Basson (1987) also stated that in some cases when an animal is found in the same habitat as the toxic plant species, the animal can develop a detoxifying mechanism or innate resistance which protects the animal against the toxic compounds. The resistance can often even be found within some individuals of a species.

There are two exceptions where gousiekte have been diagnosed in free living ruminants, springbok and buffalo (Lawrence et al., 2010). In both cases of wildlife contracting gousiekte, the animals were in unfamiliar environments and due to this they could have acted out of the norm and contracted gousiekte by feeding on the toxic plants as explained by Lawrence. The first case identified in wildlife was in springbok which were confined to camps with scarce vegetation due to overgrazing. It is hypothesised that due to low levels of vegetation the animals fed on *V. pygmaea* as soon as the new young leaves emerged after the first seasonal rain (Lawrence et al., 2010). The second case of gousiekte in wildlife was found in buffalo. It was speculated to be due to competition for grazing opportunities between buffalo, rhino, and hippo in an enclosed area where *Pav. schummannia* was abundantly present (Lawrence et al., 2010; Van Elst., 2013a).

In areas where animals contract gousiekte, other species from the Rubiaceae family besides those known to cause gousiekte, are often ignored once gousiekte-causing species are found in the area (Van Wyk et al., 1990). It is therefore possible that other species might cause gousiekte but were never investigated.

1.1.2. Gousiekte symptoms and diagnoses

It is often difficult to diagnose an animal with gousiekte because very few to no symptoms are shown. Gousiekte can only confidently be diagnosed through histopathological investigation, thus during post-mortem investigation of the animal (Fourie et al., 1989). Some of the symptoms sometimes found in animals with gousiekte are for example them struggling to breath, lying with their heads and neck extended, struggling to keep up with the flock or coughing. These symptoms are then followed by sudden death (Pretorius and Terblanche, 1967). The latent period is also very long, often between two to six weeks, which also makes the diagnoses of gousiekte difficult (Theiler et al., 1923). From all the major types

of plant poisonings in South Africa, gousiekte was the last to be investigated by scientists (Bode et al., 2010). There are many factors that influenced gousiekte research, some of which are the variation in clinical signs between infected animals, the inconsistency in the susceptibility of infected animals and fluctuation in the toxicity of the toxic plants that causes gousiekte (Bode et al., 2010; Fourie et al., 1995).

Some characteristic symptoms shown by animals during post-mortem investigations are the development of myocardial lesions which develop into myocardial fibrosis and mild to moderate round cell infiltration (Pretorius and Terblanche, 1967 and Theiler et al., 1923). Round cell infiltration is commonly found in macrophages and lymphocytes and occurs mostly in the endocardium of the apex, left ventricular wall and interventricular septum (Prozesky et al., 1988). It is common for animals with gousiekte to die suddenly when under stress, for example during capture and transport of animals (Lawrence et al., 2010).

Diagnosis of heart disease in animals is a continuous struggle, not just due to the low occurrence heart disease in animals, but also because in the case of gousiekte animals often respond differently to the ingestion of plant material and the toxicity of plant material differs (Buczinski et al., 2010; Fourie et al., 1989). Gousiekte produces similar symptoms as rheumatic fever in humans, which is an immunopathological syndrome and led to the hypothesis that the immune system might influence the pathological effect of gousiekte, however, it was confirmed in an experiment on sheep that there were no humoral or cellular response (Fourie et al., 1995). In humans, heart disease is often diagnosed through electrocardiograms, however, this is not a successful method of diagnosing gousiekte in animals. Studies on sheep where gousiekte was induced and electrocardiograms of the sheep recorded close to the death indicated that there were nothing unusual recorded, which indicates that changes in electrical activity are not necessarily found in animals that suffer from gousiekte (Fourie et al., 1989).

Cardiac enzymology is a method of diagnoses that is used in humans to detect heart disease. The heart releases certain enzymes once it undergoes certain types of injuries such as a heart attack and the elevated levels of enzymes serve as a signal that the heart muscle is injured or that the heart is not receiving enough oxygen (Fletcher, 2018). For this reason, it is very common in humans to measure the serum activity of enzymes and isoenzymes when diagnosing a patient with myocardial infarction (Fourie et al., 1989). These authors investigated the enzyme levels of sheep to determine if this could be used as a method to diagnose an animal with gousiekte. They investigated the levels of aspartate transaminase (AST) as well as elevated serum lactate dehydrogenase (LD) as these are enzymes used in humans as an

indication of myocardial injuries. The overall results indicated that AST is a better method for diagnosis because out of a flock of 15 sheep, 14 showed signs of elevated AST levels, whereas the LD activity of only 10 out of 15 sheep showed elevated levels. They also investigated isoenzyme levels; however, the various related isoenzymes showed no pattern and can thus not be used for diagnoses. However, they concluded that a rise in enzyme concentration can still not serve as a mechanism to confidently confirm gousiekte in animals (Fourie et al., 1989).

Prozesky and his colleagues (2005) attempted to determine the difference in myocardial lesions between animals with short, medium, and long latent periods. The latent periods were defined as the time since the animals were first exposed to the toxic plant up until the death of the animal. Through determining the difference in the myocardial lesions future diagnoses will be much easier and more accurate, so that even in atypical cases of gousiekte, the diagnosis can be made without doubt.

During dosing trials, 10 sheep were given a quantity of *V. pygmaea* relative to their weight every day for differing numbers of days, varying between 23 to 31 days. A wether (castrated ram) that weighed 28 kg was given a dose of 10 g/kg for 31 days (total mass of 8.68 kg plant material), experienced a long latent period and died on day 51. The sheep developed tachycardia, a heart condition causing the heart to pump more than 100 times per minute on day 42 and died 9 days later. During post-mortem investigations they found that the heart of the sheep was dilated and suffered from a collapsed right ventricle (Figure 1.1).

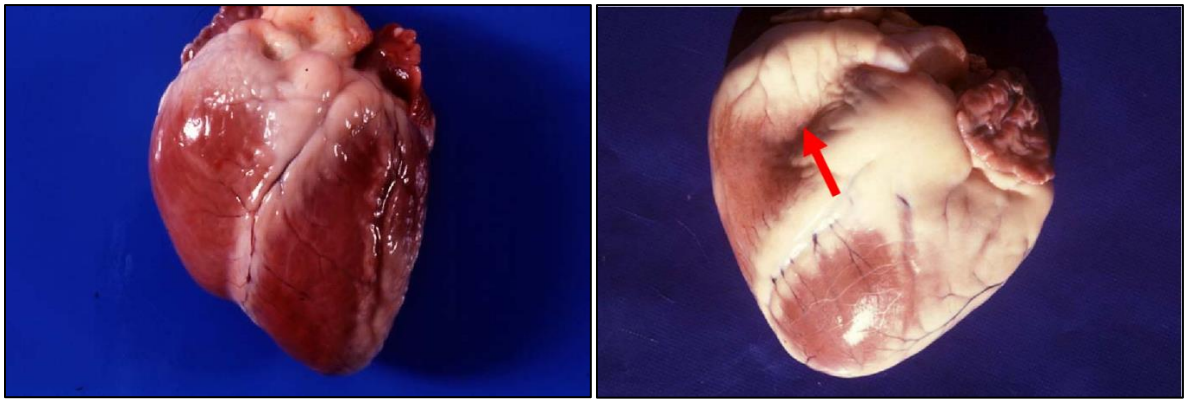

Figure 1.1: The heart of a healthy sheep (left) and the heart of a sheep that received a dose of *Vangueria pygmaea* (right) for 31 days (Prozesky et al., 2005).

1.1.3. The toxins, streptol, streptol glucoside and kirkamide

Kirkamide, streptol and streptol glucoside (Figure 1.2) were first identified in plant material from the extract of the leaves of *Psy. kirkii* (Hsiao et al., 2019; Sieber et al., 2015). The toxin streptol showed plant growth inhibitory affects when it was tested against lettuce seedlings (Hsiao et al., 2019). Interestingly, streptol glucoside is usually present in much higher quantities which might indicate that the glucosylation of streptol protects the plant against the toxic effect of streptol (Hsiao et al., 2019). Kirkamide showed insecticidal activity when tested against pollen beetles (*Meligethes aeneus*). Biological activities such as those possessed by kirkamide and streptol are commonly found under compounds that belong to the C_7N aminocyclitol family (Sieber et al., 2015).

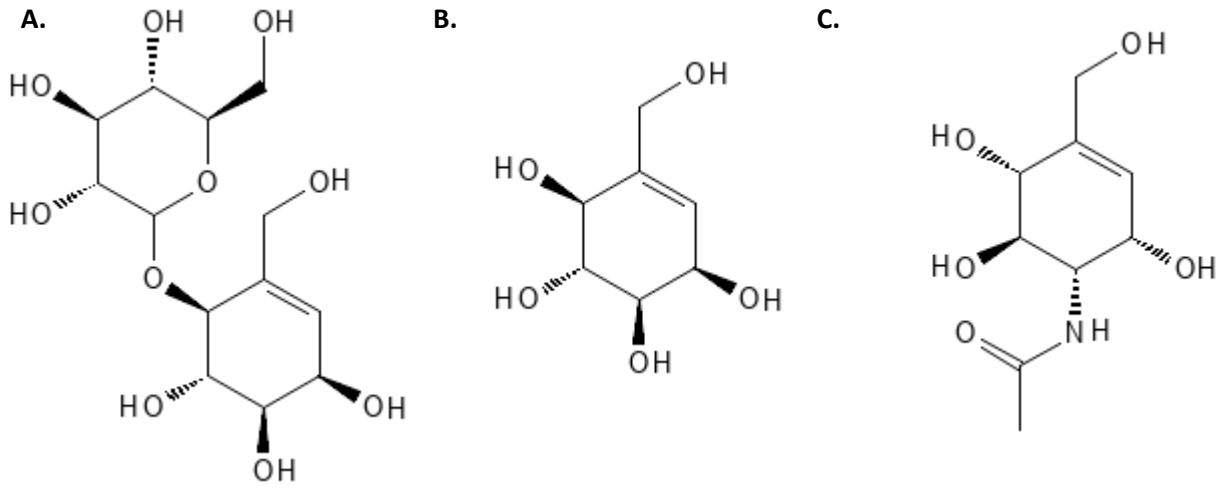

Figure 1.2: Chemical structures of streptol glucoside (A), streptol (B) and kirkamide (C), three toxins identified in *Psychotria kirkii* (Hsiao et al., 2019).

Genes involved in the biosynthesis of the toxin, kirkamide was identified on the genome of uncultured Gram-negative bacterium known as *Candidatus Burkholderia kirkii*. The toxin was at the time of isolation an unknown C_7N aminocyclitol and following the identification of the fully preserved gene in the endophyte's genome, the structure of kirkamide was elucidated through NMR-guided fractionation. After the identification of the structure, synthetic kirkamide was made through a series of reactions (Figure 1.3). The synthesis of kirkamide yielded more than a gram of the compound, indicating the reliability of the procedure (Sieber et al., 2015). The synthesis of kirkamide requires several reactions which included

a Garegg-Samuelsson reaction to produce an enol ether intermediate, kirkamide was then synthesised through Ferrier carboxylation and a Stille cross-coupling.

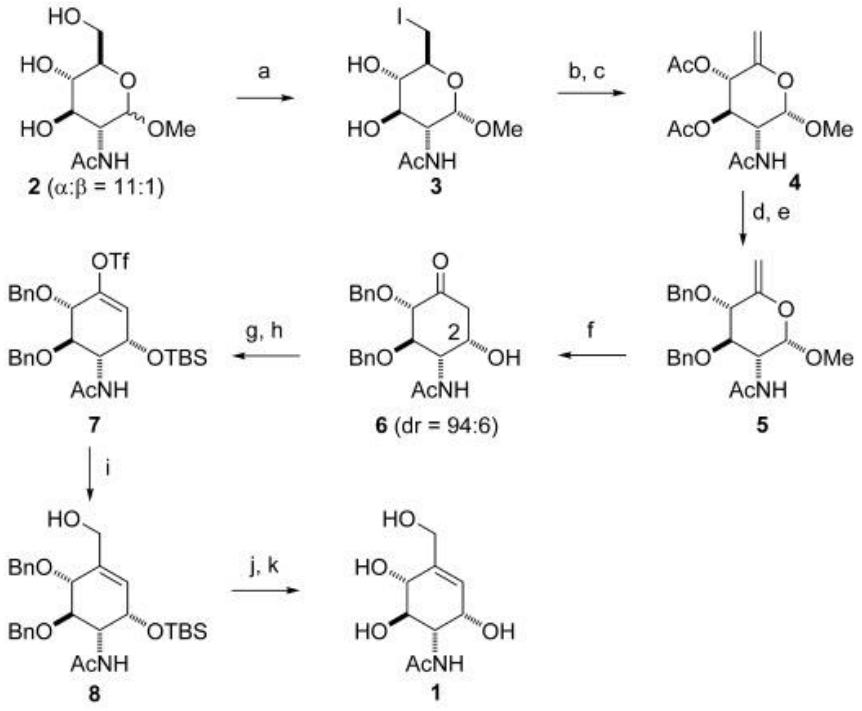

Figure 1.3: Chemical synthesis of kirkamide, a toxin isolated from *Psychotria kirkii*, a species from the Rubiaceae family (Sieber et al., 2015).

Streptol glucoside and its diastereoisomer can be synthesised from a protected form of the streptol molecule through a previously published procedure (Mehta et al., 2005). The protected form of streptol can be prepared from norbornene (Figure 1.4), this procedure yields two important intermediates, 10 and 11 (Figure 1.5). The two intermediates were separated through an enzymatic kinetic reaction and can then be used to synthesise both diastereoisomers. The synthesis of the protected streptol through the intermediates to streptol glucoside, is achieved by steps that involves epoxidation, an adol reaction and an acetate-assisted epoxide ring opening reaction (Hsiao et al., 2019).

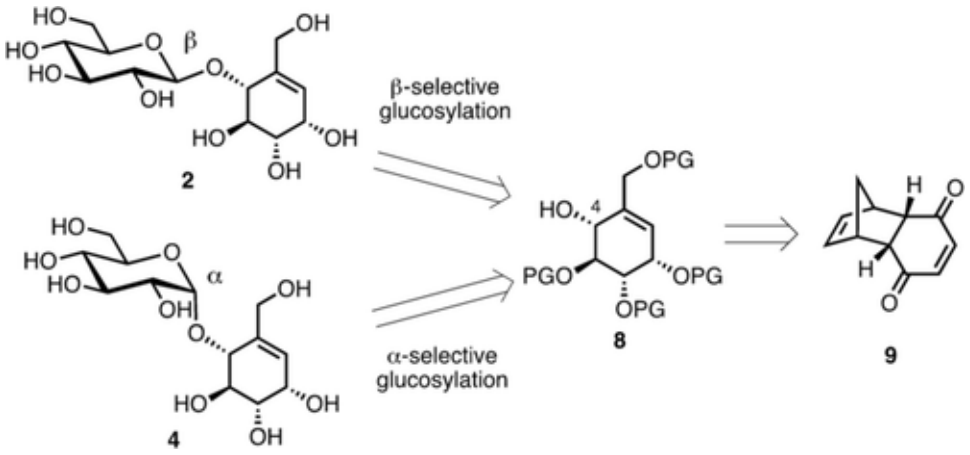

Figure 1.4: Retrosynthetic route of the synthesis of a protected streptol molecule (8) from norbornene (9) to form (+) -streptol glucoside (2) and its diastereoisomer namely A-79197-2 (4), (PG- Protecting group) (Hsiao et al., 2019).

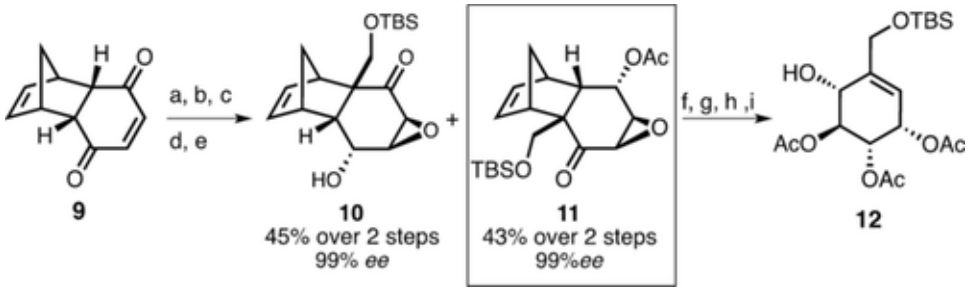

Figure 1.5: Chemical synthesis of the protected streptol molecule (12), from norborene (9) through two intermediates (10 and 11) (Hsiao et al., 2019).

It is possible that streptol and kirkamide could have a role in the development of gousiekte. Given the inconsistent results associated with pavettamine, which makes it imperative to expand the scope of the investigation to explore other potential toxins as well. The occurrence of these toxins in the gousiekte-causing plants and other *Vangueria* species, have not been reported yet. Three of the species known to be pathogenic, namely *F. homblei*, *V. pygmaea* and *V. thamnus* will be investigated in this study along with other species also belonging to the Rubiaceae family.

1.1.4. Endophytes

Bacterial leaf nodules are commonly found in plants belonging to the Rubiaceae family (Verstraete et al., 2011). It has been suggested that the bacterial symbionts have been present in its host for millions of years (Pinto-Carbó et al., 2016).

The bacterial symbiont is vertically transmitted and its presence is critical for the host in order to complete its development, this symbiosis is only known to be found in one other plant family namely Primulaceae (Pinto-Carbó et al., 2016). The mechanism of how the bacteria was transmitted to the next generation was investigated by Miller (1990). He not only found that the bacteria are transmitted to the next generation through the seeds but also indicated that the bacteria are required throughout the life cycle of the plants. Miller (1990) did this by heat treatment of the seeds, through this treatment he killed the bacteria in the seeds and found that the seeds without bacterial symbionts does not reach maturity and die after a few months. Miller (1990) also mentioned that the nodules which houses the bacteria are just a small part of a more complex system.

All the plant species known to cause gousiekte, contain endosymbionts from the *Burkholderia* genus (Figure 1.6) (Verstraete et al., 2011). The two species, *Pav. haborii and Pav. schumanniana* are the only two of the six gousiekte-causing plants that contain visible nodules (Verstraete et al., 2011). A study by Verstraete and associates (2011) investigated specimens from Zambia and the Democratic Republic of Congo and found that the endophytes in the leaves of *Pav. schumanniana* from Zambia are identical to those found in the leaves that were collected in the D.R. Congo. This indicated that the endosymbionts are identical irrespective of where the plant individual occurs. This was confirmed again in the remaining four gousiekte-causing species (*F. homblei, V. latifolia, V. pygmaea and V. thamnus*) which contains non-nodulating endophytic bacteria (Verstraete et al., 2011). Molecular DNA experiments conducted on the plant specimens also confirmed that the endophytes are host specific (Verstraete et al., 2011).

In addition to the six gousiekte-causing species, Verstraete and co-authors (2011) investigated 24 other non-toxic species from the Vanguerieae tribe, the tribe to which four of the gousiekte-causing plants belong to. None of the species contained visible nodules and results indicated that none of the species contains bacteria in their leaves. One of the additional 24 species they investigated was, *Pyg. zeyherii* which was also investigated in this study, making it the only species of the study confirmed to not contain endophytic bacteria in their leaves. It's been hypothesised for a long time that the endosymbiont plays a role in the synthesis of the toxin (Verstraete et al., 2011). The possibility of a connection between the

endosymbiont and toxin production was first suggested by Van Wyk et al. (1990). The endosymbiont of *F. homblei* is the only bacterial endophyte from the gousiekte-causing species that have been cultured successfully outside the host (Van Elst et al., 2013b). Studies regarding *in vitro* cultures of the endosymbiont of *F. homblei* indicated that the bacteria alone cannot produce pavettamine, it is thus believed that both the plant and the bacteria are needed for toxin production (Van Elst et al., 2013b). Studies on the bacterial endophytes in culture has been difficult since the endosymbionts may have lost its ability to survive without its host and thus can only be studied through culture-independent studies (Pinto-Carbó et al., 2016).

In a study by Stanton et al., (2013) non-toxic plant species of the Vangueriea tribe was investigated, the results indicated that three of the species namely *V. infausta*, *V. madagascariensis* and *V. macrocalyx* contains endophytes. TEM micrographs indicated that there is a noticeable difference between the morphology of the endophytes found in the gousiekte-causing plants in comparison to the non-toxic plants (Figure 1.7). The bacteria of *V. infausta* (Figure 1.7; A) are rod shaped and has a width of 1.0-1.5 μm and a length of 4.0 μm. Whereas the bacteria of *V. madagascariensis* has a width of 0.5-1.0 μm and a length of 1.0-1.5 μm (Figure 1.7; C). The rod-shaped bacteria of *V. macrocalyx* has a width of 1.0 μm and a length of 2.5-3.0 μm (Figure 1.7; B). Both the gousiekte-causing endophytes they investigated *V. pygmaea* and *V. thamnus* have a very similar morphology in both size and shape. They were approximately 0.5 μm in width and 2.0 μm in length. The endophytes of *V. pygmaea* (Figure 1.7; D) are mucus like fluid and contains white polyhydroxy butyrate granules.

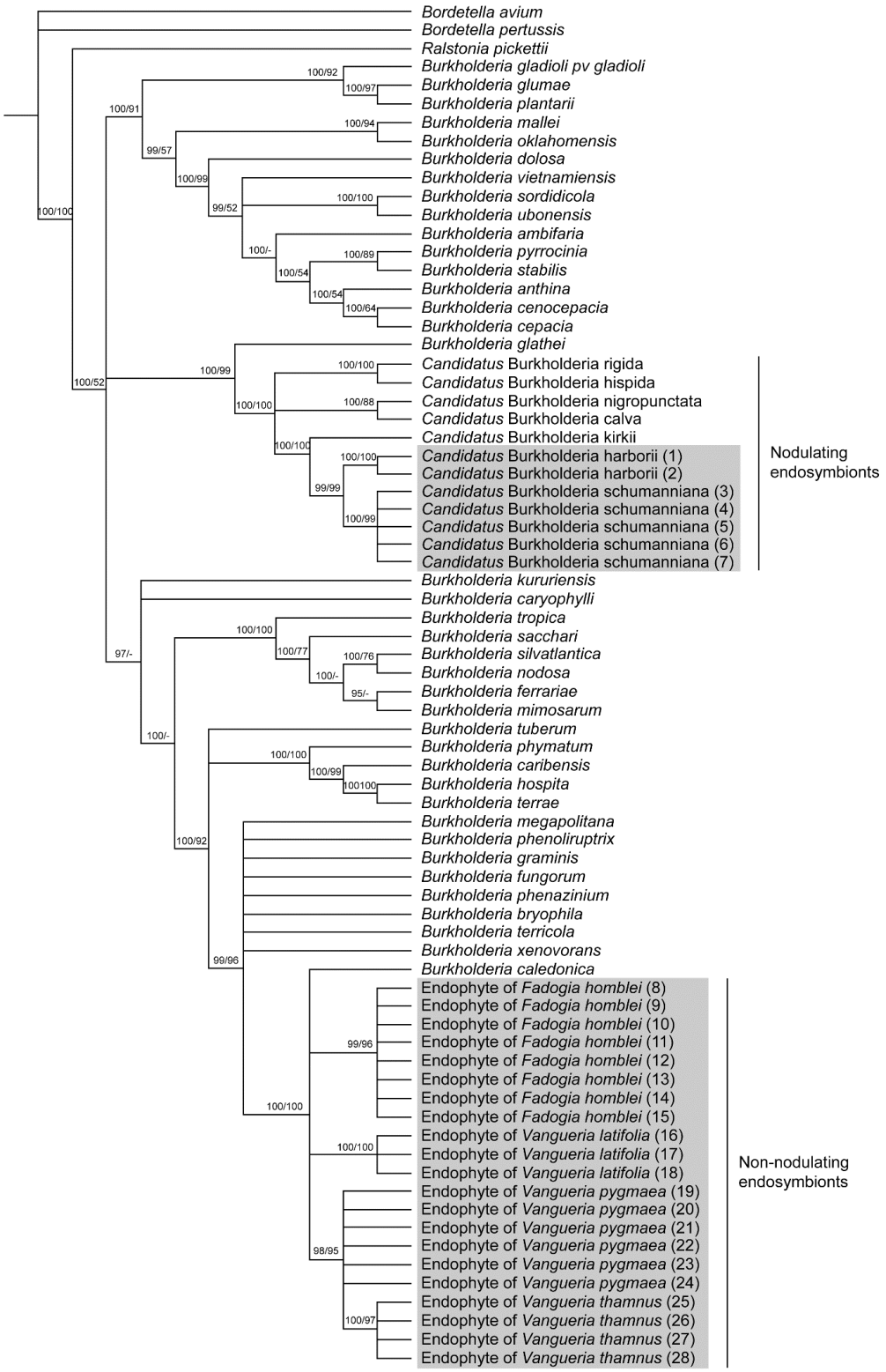

Figure 1.6: Phylogenetic tree of bacterial endophytes found in plants from the Rubiaceae family. The tree is based on results from 16S rDNA, gyrB and recA data. The grey boxes indicate the species that cause gousiekte (Verstraete et al., 2011).

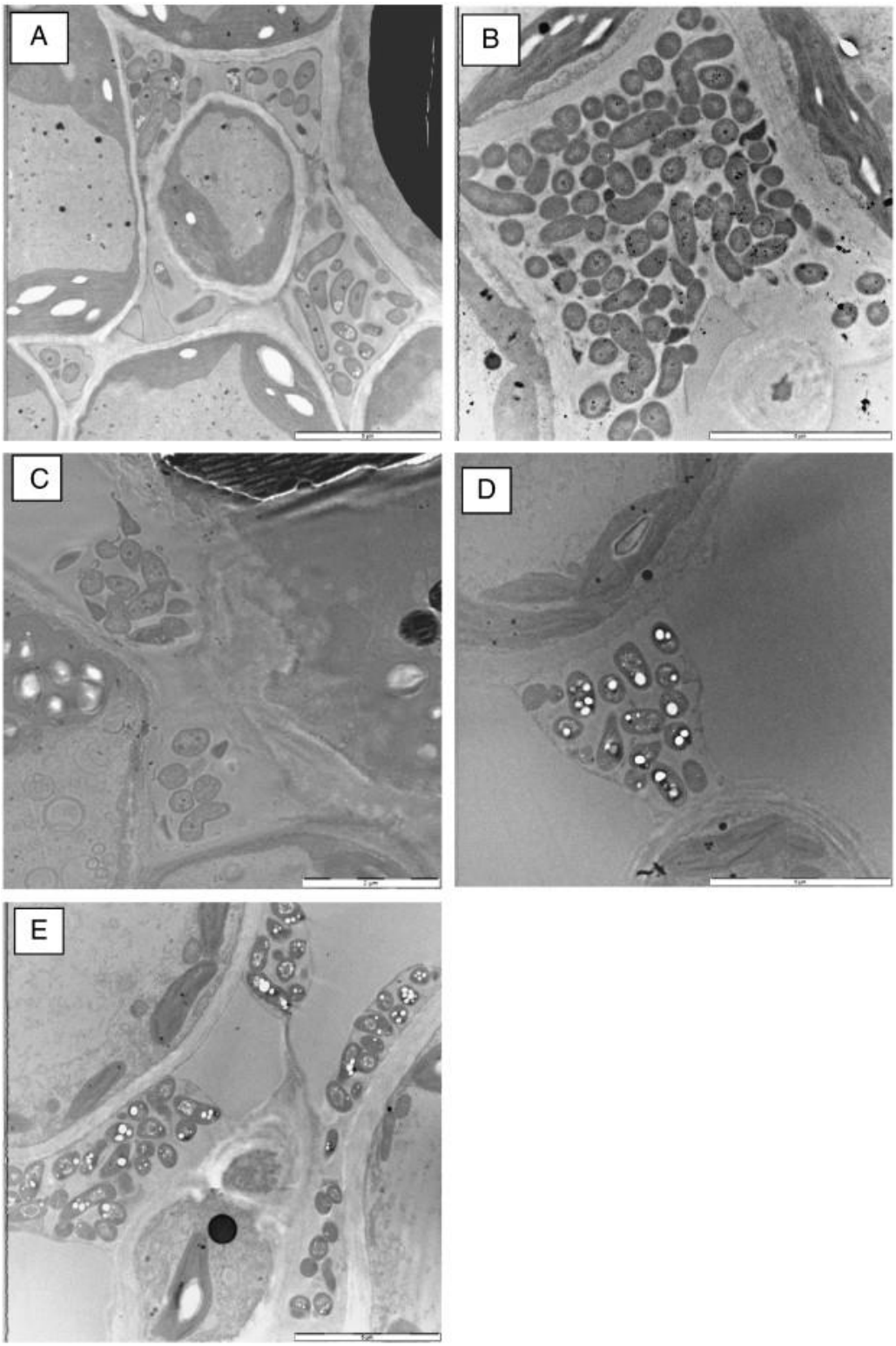

Figure 1.7: TEM micrographs show the endophytes of A) *Vangueria infausta*, B) *V. macrocalyx*, C) *V. madagascariensis*, D) *V. pygmaea* and E) *V. thamnus*, micrographs indicate the variations in the morphology of the endophytes between the different species (Stanton et al., 2013).

1.2. Objectives and hypotheses

This project was conducted through a collaborated study with molecular biologists at the University of Toulouse (France), which provided the opportunity to correlate the genetics of the endophytes present in the plant species under investigation with the corresponding occurrence of toxic compounds in the extracts of the plant species. The overall aim for the study was to provide a connection between the genetics of the plants species by identifying which plants contains the gene for kirkamide and streptol, and comparing this with the chemistry of the plant extracts by identifying which plants expresses the chemistry of the toxins. The study was thus undertaken with the objectives listed directly below:

Objectives

- Determined whether the toxin kirkamide is present only in plants known to be gousiekte-causing or also in the species that have not been recorded as toxic. Analyses were done through ^{1}H-NMR and GC-MS.
- Determined if the selected species *V. pygmaea*, *V. infausta* and *F. homblei* produces streptol and streptol glucoside. This aim was conducted using LC-MS and UPLC-QToF analyses.
- Correlated the chemistry of the plant extracts with the genetics of the bacterial endophytes of all the respective species investigated.
- Samples of *V. pygmaea* was also collected during early and late summer to determine if there is a seasonal difference in the concentration of the toxins.
- Extracts was partially purified through chromatographic methods for the detection of streptol and streptol glucoside by ^{1}H-NMR analyses and UPLC-QToF analysis.

The hypothesis for the study was as indicated below:

Hypotheses

- Kirkamide are present in the toxic species known to cause gousiekte but not in the non-toxic species. However, it is also possible that the non-toxic species contains trace amounts of these toxins, but they are present in such low quantities that they do not affect the animals when they consume the plant material.
- The leaves of *V. pygmaea* collected in March (Autumn) contains significantly lower toxin levels compared to the samples collected in October (Spring).

- The presence of toxins such as kirkamide and streptol, or streptol glucoside, aligns with the genetics of the endophytes. Therefore, if the genes are present in the endophyte's genome, the plant extract will also contain these toxins.

1.3. The Rubiaceae family and species investigated

The Rubiaceae family is one of the most species-rich flowering plant families, consisting of approximately 620 genera which diverges into about 13 000 species (Verstraete et al., 2011). All of the six gousiekte-causing species belong to the Rubiaceae family (Van Elst et al., 2013a). Plant individuals from this family can be found on all continents, a few species of the *Coprosma*, *Galium* and *Sheradia* genera can even be found in Antarctica, although members of the family are more commonly found in tropical and subtropical areas (Verstraete et al., 2011). The species inhabit many different types of biogeographical regions. They are very common in tropical forest and decrease in numbers towards the subtropical areas through to the poles (Davis et al., 2009). The Rubiaceae species can be found with various growth forms that are adapted for different dispersal mechanisms, pollinators and growth forms include woody shrubs, small herbs, lianas and in some cases even large rainforest trees (Davis et al., 2009; Robbrecht, 1988).

Most of the gousiekte-causing species and many of the species under investigation contains woody rhizomes which assist the plants in survival during extreme conditions such as a drought. This characteristic is very advantageous as in the case of *V. pygmaea*, which contains an underground root system, if the area is suffering from drought conditions the species would most likely survive rather than the normal forage (Steyn, 1938).

There is a large diversity of compounds present in plants of the Rubiaceae family such as iridoids, indole alkaloids, anthraquinones, flavonoids, phenolic derivatives and terpenoids (Figure 1.8) (Martins and Nunez, 2015). The species from the Rubiaceae family have a few characteristics that can be used to identify them. They have simple opposite or decussate arranged leaves and contains connate stipules, the inflorescence has a cyme arrangement and are often found to be bisexual and epigynous (Simpson, 2010).

The classification of the tribe Vanguerieae have been difficult but molecular data collected over the past 30 years have assisted in the understanding of the phylogeny of the plants from the Rubiaceae family (Figure 1.9) (Lantz and Bremer, 2005). Robyns (1928) also made important contribution to the classification of the group, he revised all the species form the Vanguerieae tribe and described several

new genera which contributed to new combinations and descriptions of the species from the tribe. This tribe contains more than 180 species and 13 genera (Lantz and Bremer, 2005). Until the study reported by Lantz et al. (2002) and Lantz and Bremer (2004) the tribe Vanguerieae, to which most of the plants in this study belongs to, have been one of the poorest understood tribes regarding phylogeny in the Rubiaceae family. Two of the genera investigated in this study, *Vangueria* and *Fadogia* are part of the 'large-flowered group'. They are classified in this way due to the longer corolla tubes and lobes when compared to other members of Vanguerieae. Individuals from the 'large-flowered group' are difficult to distinguish by morphological characteristics. Earlier studies using genetic information have also failed to confirm the phylogeny of the group, new data sets of a fast evolving region found in the chloroplast was then added in the latest studies which contributed to the successful determination of the phylogeny of the group (Lantz and Bremer, 2005).

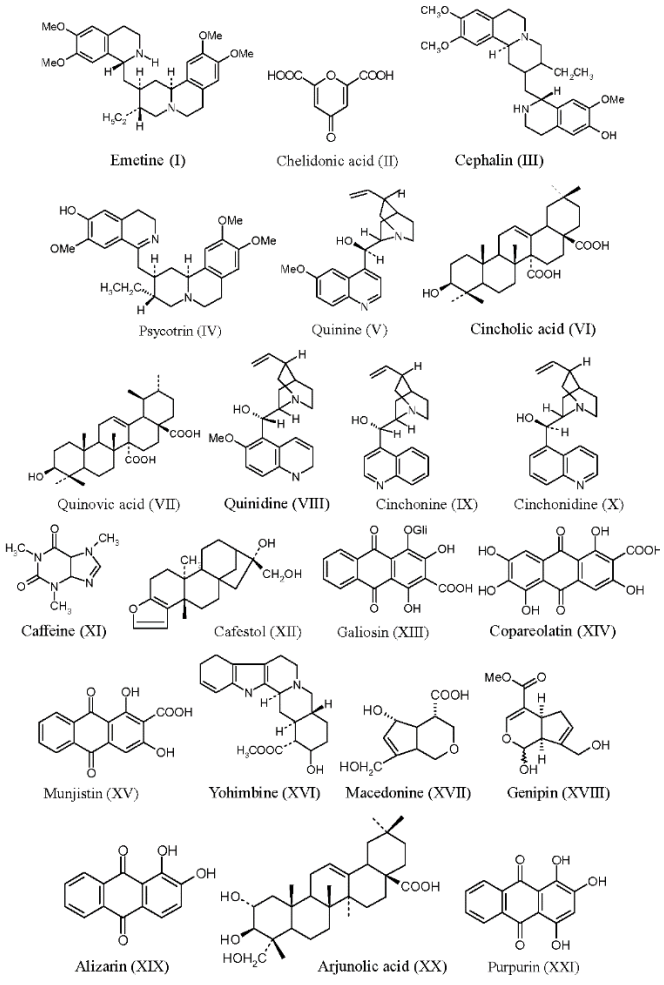

Figure 1.8: Compounds from a diversity of classes that has been isolated from the species of the Rubiaceae family (Martins and Nunez, 2015).

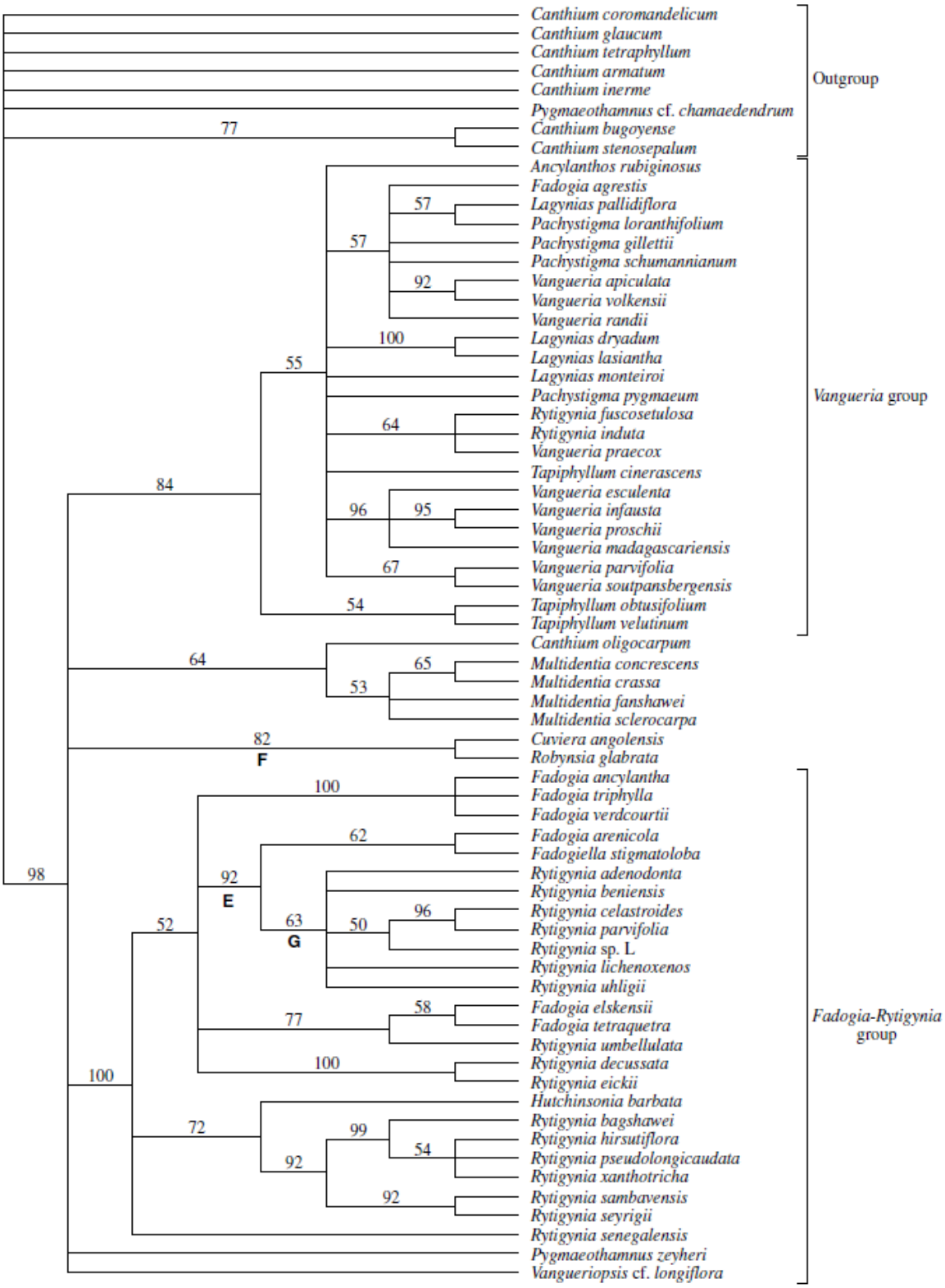

Figure 1.9: Phylogeny of plants from the *Vangueria* genus and other closely related genera (Lantz and Bremer, 2005).

1.3.1. *Fadogia homblei* De Wild.

This species is an erect suffrutex containing many stems that emerges from a rhizome. The stems are often red in colour and contains pubescence that are sparsely spread across the stems. The leaves are elliptic in shape, dark green on top and a light to grey colour on the bottom (Figure 1.10). The stems contain a stipule at the base where the leaves emerge. The flowers are a yellow colour, and the fruit are black and contains a calyx limb. The species is found in South Africa and several other countries throughout Africa for example Tanzania and Angola (Bridson, 1998a).

Figure 1.10: *Fadogia homblei*, a known *gousiekte*-causing species and its distribution in Africa (GBIF Secretariat, 2021a).

1.3.2. *Pavetta revoluta* Hochst.

The small tree or shrub is evergreen and grows up to 6 m tall. The leaves are characteristic to the Rubiaceae family, as oppositely arranged. The leaves have an oval shape and a leathery texture, they are green above and pale on the bottom containing hairy pockets. The flowers are white and found in clusters, flowers can be found during late spring to early summer. The fruit are green and turns black as they ripen (Figure 1.11) (Mbambezeli, 2007). This species contains nodules that houses endophytes that appears as black spots on the leaves (Carstensen, 2012; Herman et al., 1986).

Figure 1.11: *Pavetta revoluta*, a non-toxic plant species containing small green fruit and visible nodules on the leaves and its distribution in Africa (GBIF Secretariat, 2021b).

1.3.3. *Psychotria kirkii* Hiern

This species is a shrub, with branches that are reddish in colour and are scarcely pubescent which are more concentrated towards the end of the branches. The leaves are elliptic in shape and pointed towards the end, containing short hairs and bacterial nodules. The flowers are a cream-yellow and the fruits are red (Figure 1.12). A young tree contains yellow pubescent and loses the pubescence when the tree is fully grown. The species can be found in wooded grassland and riverine habitats (Hyde et al., 2002a; Verdcourt, 1989).


Figure 1.12: *Psychotria kirkii*, a species containing visible nodules that appears as dark spots on the leaves and its distribution in Africa (GBIF Secretariat, 2021c; Leguil, 2008).

1.3.4. *Pygmeothamnus zeyherii* (Sond.) Robyns var. *zeyheri*

This species is characterized as a suffrutex, grows up to 17-30 cm and contains a horizontal rhizome. The slim stems are covered with short hairs that are closely brushed against the stems. The leaves are often found in whorls, oppositely arranged and are elliptic with no hairs. The corolla of the inflorescence is red, green in colour. The fruits are pyriform in shape, green and hairless (Figure 1.13). The species can be found in grassland or in open woodlands and is distributed throughout Gauteng, Limpopo, Mpumalanga and the North West, it can also be found in other countries outside of South Africa such as Angola, Namibia and Zimbabwe (Bridson, 1998b; Foden and Potter, 2005; Hyde et al., 2018).

Figure 1.13: *Pygmaeothamnus zeyherii*, a non-toxic suffrutex of the Rubiaceae family and its distribution in Africa (GBIF Secretariat, 2021d; Wursten, 2013).

1.3.5. *Vangueria dryadum* S.Moore

This species grows between 2.5 to 5m tall and is classified as a small tree or shrub (Figure 1.14). The bark is a pale grey to brown colour, contains long narrow breaks, are heavily covered with hair when juvenile which turns into a brown-red colour and contains no hair when mature. The leaves are thick, discoloured and contains dense pubescence on both sides of the leaf. The flowers are yellow velvety, and the fruits are brown in colour. The species are found in woodlands, riverine thickets or dry riverbeds (Bridson, 1998c).

Figure 1.14: *Vangueria dryadum*, a non-toxic small tree of the Rubiaceae family and its distribution in Africa (GBIF Secretariat, 2021e).

1.3.6. *Vangueria infausta* Burch. subsp. *infausta*

This species is native to several countries in Africa such as Angola, Botswana, Malawi, Mozambique, South Africa, Uganda, Zambia, and Zimbabwe. They are often found in woody grasslands, rocky hillsides, dry forests, and sandy valleys (Maroyi, 2018) It grows between 3 to 8 m tall and is classified as a deciduous shrub or small tree, the trunk is short and multi-stemmed which aids in assisting the hanging branchlets (Palgrave and Palgrave, 2013). Short hairs can be found covering the entire length of the branchlets. The colour of the bark ranges between grey to a yellow-brown colour, it is smooth in texture, and peeling of the bark is also very common to this species. The leaves of this species are oppositely arranged, light green in colour, between 5 and 10 mm long, thick and covered in short velvet textured hairs (Maroyi, 2018). The small flowers are green-white to yellow in colour, they are often found in dense groups on lateral branches (Palgrave and Palgrave, 2013). The fruit can be found as dark, green, shiny and round when young and as they ripen it changes colour to light brown (Figure 1.15) (Maroyi, 2018).

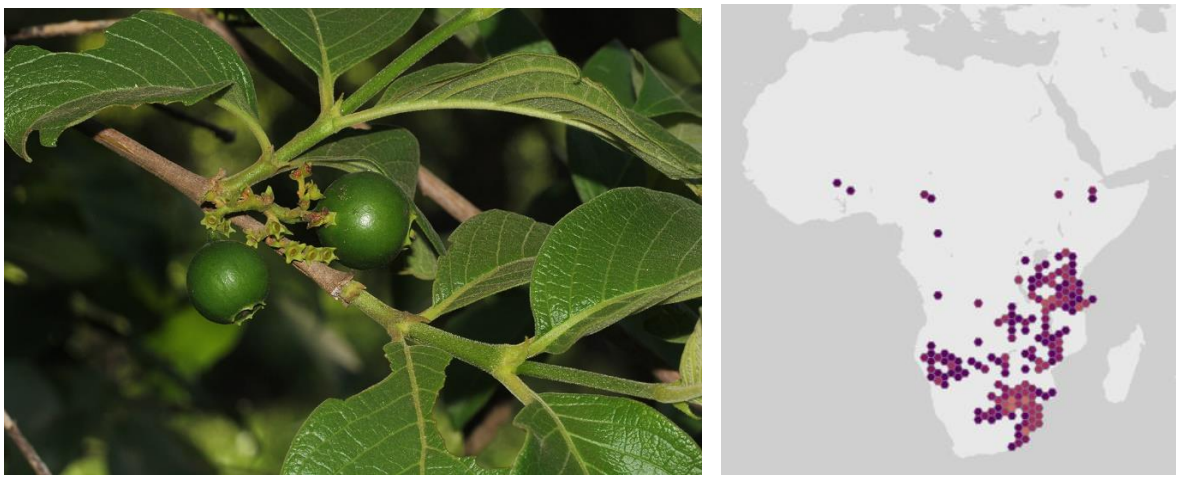

Figure 1.15: *Vangueria infausta*, a non-toxic tree of the Rubiaceae family and its distribution in Africa (GBIF Secretariat, 2021f).

1.3.7. *Vangueria lasiantha* (Sond.) Sond.

This species has a grey smooth bark and grows between 2 to 6 m tall. Younger stems are densely covered with hairs, older stems are smooth with no hairs and grey to red brown in colour. The leaves have an elliptic shape, rounded at the base, and are oppositely arranged. A stipule can also be found in between two oppositely arranged leaves. The leaves are a dark green on the top and light green to grey on the bottom, with pubescence occurring sparsely on the abaxial side. The flowers are green to yellow in colour and can usually be found in dichasially branched chymes which contains five to many flowers each. The fruit are green and turn yellow to brown when ripe (Figure 1.16) (Bridson, 1998d).

Figure 1.16: *Vangueria lasiantha*, a non-toxic species of the Rubiaceae family, containing an immature unripe fruit of green colour and the distribution of the species in Africa (GBIF Secretariat, 2021g; Lifestyle seeds, 2021).

1.3.8. *Vangueria macrocalyx* Sond.

This species is classified as a shrub or small tree and grows between 0.6 to 3 m tall. Spine-like structures can often be found on the trunk of the shrub which are a result of the remains of axillary branches. A young tree will often be found with yellow pubescence on the branches, the hairs are lost as the tree ages and the bark becomes grey. The leaves contain dense yellow pubescence and are elliptic in shape. The inflorescence are cream in colour and can be found in clusters, the colour of the fruits are orange-yellow and round in shape (Figure 1.17) (Bridson, 1998e).

Figure 1.17: *Vangueria macrocalyx*, a non-toxic small tree containing green round immature unripe fruit and ripe orange fruit and its distribution in Africa (GBIF Secretariat, 2021h).

1.3.9. *Vangueria madagascariensis* J.F.Gmel.

The species grows throughout Africa and Asia in riverine-lowland forests, including areas such as Mauritius. This species is a multi-stemmed shrub or small tree, that grows between 1.5 to 15 m tall. The stems contain no hair, pale in colour and except for one variant in Tanzania which can often be found with the bark peeling, the stems are more often found to be unpeeled. The leaves are broad at the base and becomes smaller to the end of the leaves, they are dark green in colour on the top and paler on the abaxial side of the leaf. The flowers are small and yellow in colour and the fruit are smooth and the colour can vary from green to brown, with brown fruit being ripe (Figure 1.18) (Bridson, 1998f; Ramalingum and Mahomoodally, 2014).

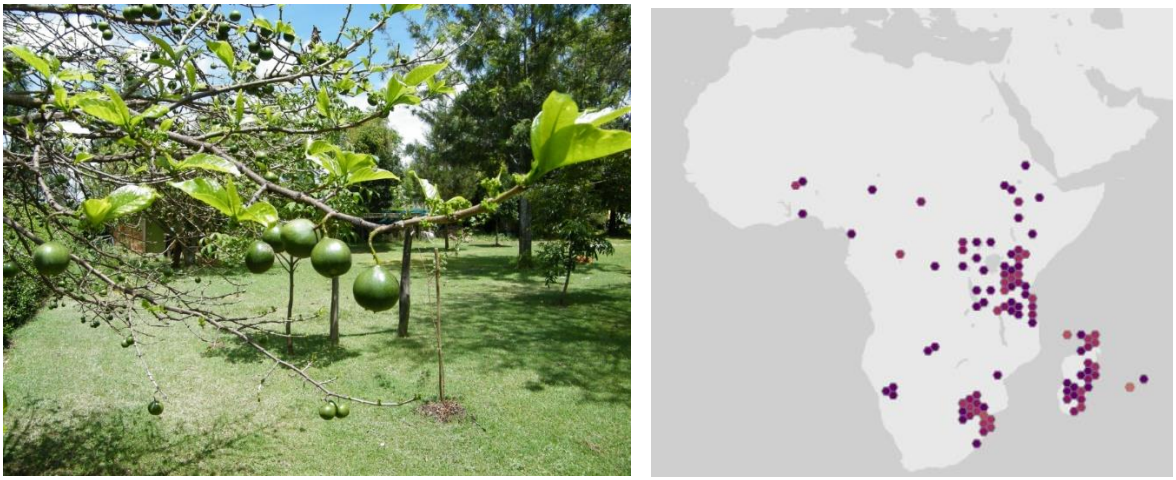

Figure 1.18: *Vangueria madagascariensis*, a non-toxic small tree of the Rubiaceae family, contains green immature fruit and its distribution in Africa (GBIF Secretariat, 2021i; Scamperdale, 2014).

1.3.10. *Vangueria pygmaea* Schltr.

The leaf of the short deciduous shrub emerges from the large underground stem and root system. The shrub grows to approximately 15 cm and can be found in sunny to semi-shaded open grasslands (Figure 1.19). The leaves are attached directly to the base, they do not have an intervening stalk. The margin runs through the entire length. At both sides of the base of two oppositely arranged leaves, triangular stipules can be found on the stem. The leaves are covered with small yellow hair. The fruit of *V. pygmaea* is small, round and green in colour when not ripe and red to orange when ripe. The fruit can be found from October to February. The flowers are found in clusters and are a yellow to green colour and are star shaped (Botha and Venter, 2002).

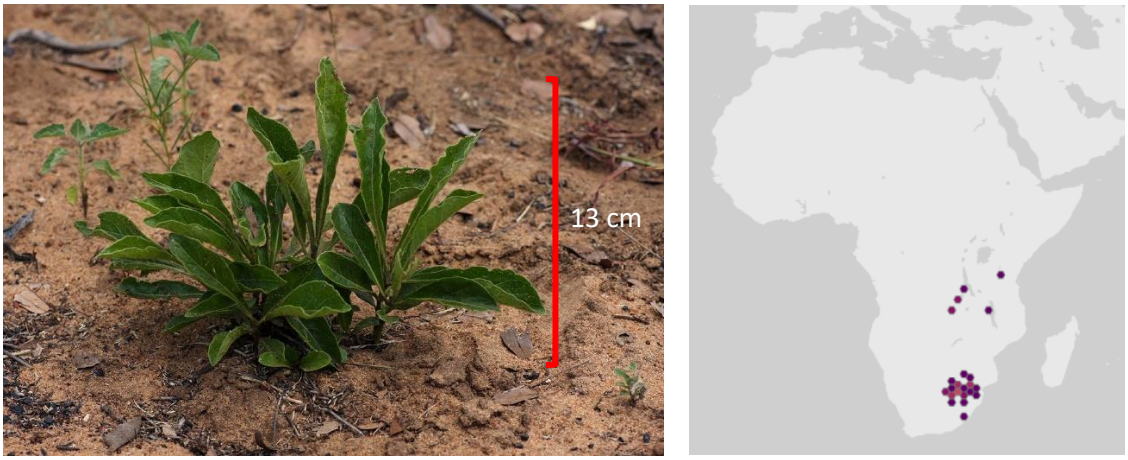

Figure 1.19: *Vangueria pygmaea*, a *gousiekte*-causing suffrutex, this individual was 13 cm tall and its distribution in Africa (GBIF Secretariat, 2021j; Wursten, 2010).

1.3.11. *Vangueria randii* S.Moore subsp. *chartacea* (Robyns) Verdc.

This species is characterised as a small shrub or tree and can be found in woodland areas and alongside rivers. The branches are often a red-brown colour and a smooth texture with no hairs. The leaves are thin and elliptic in shape, with hairs on both the upper and lower surface. The leaves are oppositely arranged with a stipule at the base. The fruits are semi-round in shape, green but turns yellow in colour when ripe (Figure 1.20). The flowers are a white green to golden green colour and can be found between October and March (Hyde et al., 2002b).

Figure 1.20: *Vangueria randii*, a non-toxic small tree of the Rubiaceae family, containing green round unripe fruit and its distribution in Africa (GBIF Secretariat, 2021k; Wursten, 2012).

1.3.12. *Vangueria soutpansbergensis* N. Hahn

This species is known by its common name as Soutpansberg Wild medlar and can be found in the Soutpansberg mountains, it is recorded as rare (Cholo and Kamundi, 2006). It is classified as a deciduous shrub or small tree that can be found at heights of up to 2.5 m. This species is usually found in mixed woodlands in soils containing quartzites, like that of the Soutpansberg. The bark of this small tree is brown-grey and can often be found to be a dark brown colour. The leaves are elliptic but can also be found to be rounded in shape, both the base of the leaf as well as the apex is an oval shape to round. The leaves can often contain small hairs when they are younger. As the small tree grows older it becomes glabrous and the leaves are dark green above and has a pale green-grey colour on the bottom (Hahn, 1997).

The flowers are usually found in cluster of 2-15 flowers. Both the peduncle and pedicel may have a sparse distribution of hairs or be entirely devoid of them. The flowers have a green to lime-green colour (Figure 1.21). The small tree flowers between November and December. The fruit is glabrous and semi-round, the fruits can be found between March and April (Hahn, 1997).

Figure 1.21: *Vangueria soutpansbergensis*, photo taken at Lowveld National Botanical Gardens in Nelspruit during a plant collection excursion (GBIF Secretariat, 2021m).

1.3.13. *Vangueria thamn*us (Robyns) Lantz

This species looks very similar to *V. pygmaea* however, the leaves of *V. thamnus* do not contain any hair (Botha and Venter, 2002; Stanton et al., 2013). This species can be found in grasslands and savannah areas (Verstraete et al., 2014). The flowers are shaped in the form of a star and are yellow green in colour and arranged in dense clusters, the fruit are round but can also often be pear-shaped and are green in colour (Figure 1.22) (Botha and Venter, 2002).

Figure 1.22: *Vangueria thamnus*, a *gousiekte*-causing suffrutex, containing green pear-shaped fruit and its distribution in Africa (GBIF Secretariat, 2021l; Tarboton, 2008).

The plants under investigation can be easily identified by their oppositely arranged leaves and stipules. However, certain species, such as Pygmeothamnus zeyherii, V. thamnus, and V. pygmaea, share similar growth styles and might be prone to misidentification by individuals unfamiliar with these species. The Rubiaceae family comprises a substantial number of members, and the aforementioned species were just a selection with potential links to *gousiekte*. Currently, limited research regarding the potential role of other species to *gousiekte* has been conducted on species outside those already identified as *gousiekte*-causing. Therefore, it is valuable to explore the potential connections of other species from this family to *gousiekte*.

Chapter 2: Isolation and identification of kirkamide, a proposed *gousiekte*-causing toxin produced by endophytes of various species of the Rubiaceae family.

Abstract

Species of five genera from the Rubiaceae family were investigated for the occurrence of the toxin kirkamide in their leaf extracts and for the possible involvement of kirkamide in the cause of *gousiekte*. Three species that have previously been identified as *gousiekte*-causing were included in the study namely, *Fadogia homblei*, *Vangueria pygmaea* and *V. thamnus*. Due to the long-supported hypothesis that the bacteria housed in the plants are largely to blame for the toxicity of the *gousiekte*-causing plants, the endophytes were also investigated for their role in the production of kirkamide. The comparison of the plant extract's chemistry was performed in conjunction with an analysis of the genes identified in the endophyte's genome, which was carried out by colleagues from the University of Toulouse in France. Throughout the duration of the study, limited quantities of a positive control (*Psychotria kirkii*) were used which was previously studied and found to contain kirkamide in the leave extracts. From the overall chemical results, it could be concluded that kirkamide is not present in any of the South African species investigated. The genetic results of our collaborators also showed that none of the *gousiekte*-causing or other *Vangueria* species contained the kirkamide biosynthetic genes on the genome of the endophyte. It has been proposed previously that the toxin that causes *gousiekte* is produced in higher quantities during spring in comparison to summer, this is in correlation to the number of *gousiekte* cases reported between the two seasons. Following this hypothesis one of the toxic species, *V. pygmaea* was analysed during both spring and summer however, kirkamide was not found in *V. pygmaea* extracts during either two of the collections. Although the overall concentration of the compounds in the extract was higher in spring.

2.1. Introduction

The initial discovery of the toxin, kirkamide, in *Psychotria kirkii* served as the catalyst for launching this investigation into the potential involvement of the toxin in the onset of the disease known as *gousiekte* or "quick disease" (Sieber et al., 2015). The identification of the toxin kirkamide meant that the previously identified toxin, pavettamine could potentially not be the cause, or the only cause of *gousiekte* that kills thousands of animals annually.

The toxin, pavettamine was identified over 100 years after *gousiekte* was first discovered (Theiler et al., 1923; Van Elst et al., 2013a). During the search for the toxic compound it was found that the toxin is heat stable, soluble in water and is a cationic compound that forms part of a precipitate in the presence of either ethanol or methanol (Fourie et al., 1995). Since the isolation and identification of pavettamine subsequent related studies have accepted this toxin as the cause of *gousiekte*. However, it is worth noting that progress in understanding *gousiekte* has been slow, since identifying pavettamine. Few studies have been conducted on the chemistry and the effects of pavettamine once it enters animals. The role of pavettamine in the plants themselves also remains unclear, and whether the toxin is produced by the plant, or the endophytes is also still uncertain.

Plants from five genera of the Rubiaceae family namely *Fadogia*, *Pavetta*, *Psychotria*, *Pygmaeothamnus* and *Vangueria* were investigated for the presence of the toxin, kirkamide. Some of the investigated species are known to be toxic, some species are non-toxic, and there are a few species have not yet been investigated. Three of the *gousiekte*-causing species namely *F. homblei*, *V. pygmaea* and *V. thamnus* forms part of this study. The suffrutex *Pyg. zeyheri*, was also included as a negative control because it has previously been shown not to cause *gousiekte* or to contain endophytes (Verstraete et al., 2011). Since kirkamide was previously isolated from the species *Psy. kirkii* it was chosen as a positive control, for method validation and to confirm that the instruments used to analyse the plant extracts can detect the toxin at low concentrations (Sieber et al., 2015). The remaining species have been chosen since they all also belong to the Rubiaceae family and many of these species are found in similar distribution ranges and share many morphological characteristics to the *gousiekte*-causing plant species (Van Elst et al., 2013b; Van Wyk et al., 1990; Verstraete et al., 2011).

Interestingly Van Elst and associates (2013a) found that the species *Psy. kirkii* contains pavettamine in higher amounts in comparison to the traditionally known *gousiekte*-causing species. This species is not found in South Africa but in Zimbabwe where *gousiekte* does occur (Hyde et al., 2002a; Lawrence et al.,

2010). It is important to mention that Van Elst and associates (2013a) grew the plants that were investigated in a greenhouse that do not mimic the natural environment entirely, thus there is a possibility that the concentration of the toxins might differ in their natural environment and in other seasons of the year (Kellerman et al., 2005). The relation between the toxin kirkamide found in *Psy. kirkii* and *gousiekte* is strengthened by the occurrence of visible nodules on the leaves of the plant species, that appears as black dots. It was shown that these nodules house endophytes from the *Burkholderia* genus, the same genus as the endophytes present in *gousiekte*-causing species (Sieber et al., 2015).

It is well-known that plant species from the same family often produce similar compounds (Magnúsdóttir, 2002) and since *Psy. kirkii* belongs to the same family as the *gousiekte*-causing species, other species from this family might also produce kirkamide. The effects of kirkamide on animals such as ruminants is unknown, and it is thus important to investigate its possible role in the cause of the disease.

Psy. kirkii was found in the Mutrikwe Recreational Park where several buffalos died because of *gousiekte*, as mentioned in the introductory chapter. Although, *Psy. kirkii* was ignored once *Pav. schummaniana* was found in the park, since the latter species have previously been recorded as being *gousiekte*-causing (Lawrence et al., 2010; Van Elst et al., 2013a). However, because buffalos are almost exclusively grazing animals it is more likely that they would eat a plant such as *Psy. kirkii* that grows close to the ground in comparison to *Pav. schumanniana* that is a small tree.

Kirkamide was first identified and isolated from *Psy. kirkii* through NMR-guided fractionation from the crude extracts of the leaves (Hsiao et al., 2019). The structure of kirkamide was elucidated in 2015 through high resolution ESI-MS analysis and its exact mass was found to be 240.0844 *m/z* which corresponds to the molecular formula of $C_9H_{15}NO_5Na$, a $[M + Na]^+$ pseudomolecular ion (Sieber et al., 2015). The cytotoxicity of a synthetic form of the compound was tested on brine shrimp and the results indicated that the synthetic kirkamide has a lethal concentration (LC_{50}) of 0.84 μg/ml at 48h (Sieber et al., 2015). The proton of carbon 1 of kirkamide has a characteristic chemical shift between 5.887 and 5.874 ppm (Figure 2.1.) (Pinto-Carbó et al., 2016; Sieber et al., 2015).

One of the key genes that codes for an enzyme involved in biosynthesising C_7N aminocyclitols and several other pseudosugars, 2-epi-5-epi-valione synthase, was identified in the genome of the *Psy. kirkii* endophyte, *Candidatus Burkholderia kirkii* (Hsiao et al., 2019; Mahmud et al., 1999). It has subsequently been shown that this bacterium is responsible for the production of kirkamide and another toxin, streptol (Discussed in chapter 3) in *Psy. kirkii* (Mahmud et al., 1999; Mahmud et al., 2007).

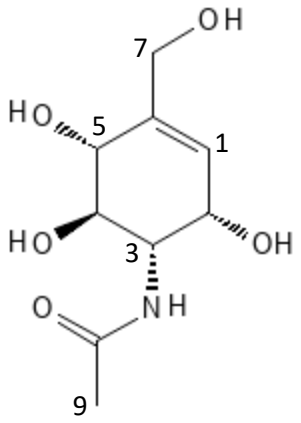

Figure 2.1: Chemical structure of kirkamide, the toxin investigated for its role in *gousiekte* (Sieber et al., 2015).

To establish the connection between kirkamide and *gousiekte*, the presence of kirkamide in both *gousiekte*-causing and non-*gousiekte* causing plant species were investigated. If the toxin is found only in the known *gousiekte*-causing species, it would be a good indication that kirkamide can be associated with *gousiekte*. Apart from identifying kirkamide as a possible cause of *gousiekte* it is also important to note that if the gene for the production of the toxin is found on the endophyte, it is convincing evidence that the endophyte has a big role in the production of the toxin and can provide more evidence of the long-believed hypothesis that it is the endophyte and not the plant that renders the plant toxic. This would assist future research into *gousiekte* in targeting the endophytes to prevent the disease.

2.2. Hypotheses and Objectives

Hypotheses

- The toxin kirkamide is found in the toxic species responsible for causing *gousiekte* but not in the non-toxic species. Although, non-toxic species can contain kirkamide but in such low quantities that it would not affect the animal if the plant material were ingested.
- The leaves of *V. pygmaea* collected in March (late summer) contains significantly lower toxin levels compared to the samples collected in October (early summer).

Objectives

This part of the study is focused on determining if kirkamide is present in the selected species. Our collaborators did the genetic analyses which was focussed on identifying the presence of the gene producing kirkamide in some of the selected species. The objectives were:

- Collect leaf material of all species under investigation during late summer between February and March.
- Determine if the analytical instruments to be used for ^{1}H-NMR and GC-MS analyses, can detect the toxin in the extract of the positive control, *Psy. kirkii*.
- Do a second collection of the toxic species *V. pygmaea* in October to determine if there is a significant difference in the concentration of the toxin between the two seasons.
- Isolate kirkamide using an 80% methanol extraction from the plant leaf material that were collected in both late summer and spring.
- Analyse the extracts with ^{1}H-NMR and GC-MS analyses, to confirm if the plant extracts contain kirkamide.
- Establish a correlation between the chemical findings and the genetic data obtained by our collaborators, who assessed whether the genes responsible for toxin production are present in the endophytes' genomes, potentially rendering the plant poisonous.

2.3. Materials and Methods

2.3.1. Plant collection

We aimed to collect and analyse three individuals of each plant species. For this reason, plants were collected in three different provinces: Mpumalanga, Gauteng and KwaZulu Natal (Table 2.1). Prior to collecting plants, permission was sought and obtained from both the botanical gardens and private landowners. We were successful in collecting three individuals of all the species except for the following: *V. macrocalyx*, *V. madagascariensis* and *V. soutpansbergensis*. The results from these three species were accepted since technical replicates were incorporated throughout the analyses.

Plant samples collected in Mpumalanga and Gauteng:

Fresh leaves of the species *V. dryadum*, *V. infausta*, *V. lasiantha*, *V. macrocalyx*, *V. madagascariensis*, *V. randii* and *V. soutpansbergensis* were collected at the Lowveld National Botanical Gardens in Nelspruit (Mbombela), Mpumalanga during March 2021. Fresh leaf material of *F. homblei* were collected at Roodeplaat, Gauteng during March (2021). Fresh leaf material of *V. pygmaea* were collected at Cullinan, Gauteng, during both March (2021) and October (2021). The species *Pav. revoluta* was collected in the botanical gardens of Hatfield campus of the University of Pretoria, Gauteng in March 2021. *Pyg. zeyheri* was collected at Doornring, Mpumalanga and *V. thamnus* was collected at Lydenburg, Mpumalanga in March 2021. The leaf material was preserved by storing them at 5 °C during transport and throughout the study.

Table 2.1: Plant samples collected in Gauteng and Mpumalanga. Samples of all plants were collected early in March (2021), additional samples of *Vangueria pygmaea* were collected in spring during October of the same year (2021). Samples also analysed for the presence of the kirkamide gene on the genome of the endophyte are indicated with an asterisk.

Plant species	Coordinates	Location of collection	Number of replicates collected	Toxic/ non-toxic
Fadogia homblei*	S 25° 34' 20,5'' E 28° 25' 58,4''	Roodeplaat	3	Toxic
Pavetta revoluta	S 25° 45' 10.84" E 28° 13' 30.16"	Univ. of Pretoria	1	Non-toxic
Pygmaeothamnus zeyheri	S 26° 12' 57.304'' E 27° 47' 30.733''	Doornrig	3	Non-toxic
Vangueria dryadum*	S 25° 26' 35,5'' E 30° 58' 9,3''	LNBG (Lowveld National Botanical Gardens)	3	Non-toxic
Vangueria infausta*	S 25° 26' 35,2'' E 30° 58' 16,4''	LNBG	1	Non-toxic
Vangueria lasiantha*	S 25° 26' 36,5'' E 30° 58' 9,4''	LNBG	2	Non-toxic
Vangueria macrocalyx*	S 25° 26' 34,6'' E 30° 58' 15,6''	LNBG	1	Non-toxic
Vangueria madagascariensis*	S 25° 26' 34,7'' E 30° 58' 16''	LNBG	1	Non-toxic
Vangueria pygmaea*	S 25° 44' 10,2'' E 28° 31' 59,3''	Cullinan	3	Toxic
Vangueria randii *	S 25° 26' 41,4'' E 30° 58' 7,7''	LNBG	1	Non-toxic
Vangueria soutpansbergensis*	S 25° 26' 34,5'' E 30° 58' 15,6''	LNBG	1	Non-toxic

Plant samples collected in KwaZulu-Natal:

Fresh leaves of the species *V. lasiantha*, *V. randii* and *Pav. revoluta* were collected in several areas throughout KwaZulu-Natal during late February (2022) (Table 2.2). The species, *V. lasiantha* and *V. randii* were collected at Pigeon Valley Nature Reserve and the species, *Pav. revoluta* was collected at St. Lucia Nature reserve.

Plant samples provided by French collaborators:

Our collaborators provided a small amount of dried leaf material of *Psy. kirkii,* collected in the Zurich Botanical Garden and it was received in February (2020). The leaves were stored at 5 °C after arrival.

Table 2.2: Plant samples collected in KwaZulu-Natal. Samples were collected in the summer month of February in the second year of the study (2022). These plant samples were collected with the aim to make up three replicates of each species.

Plant species	Coordinates	Location of collection	Number of replicates collected	Toxic/ non-toxic
Pavetta revoluta	N 13° 54' 33.84" E 60° 58' 44.04"	St. Lucia Cape Vidal entrance	2	Non-toxic
Vangueria infausta	S 29° 46' 20.37" E 30° 49' 48.85"	Krantzkloof NR	2	Non-toxic
Vangueria lasiantha	S 29° 51' 52.56" E 30° 59' 12.84"	Pigeon valley NR	1	Non-toxic
Vangueria randii	S 29° 51' 52.56" E 30° 59' 12.84"	Pigeon valley NR	2	Non-toxic

2.3.2. Extraction method

Several extraction methods were tested for the extraction of kirkamide, including methods used by Sieber et al. (2015) during their isolation and synthesis of kirkamide. The following method proved to be the best:

The extraction was carried out in a speed-extractor (Büchi E-914, Switzerland). The cut-up leaf material (5 x 5 mm) was divided into 120 ml metal vials and the extraction done with 80 % distilled methanol (Merck, South Africa). The speed-extractor was set for five cycles with each having a heating phase of 1 min, solvent holding phase of 9 min and a discharge phase of 5 min, making a total of approximately 1 h and 15 min. The extraction was carried out at 50 °C and 10 x 10^3 kPa. The extracts were dried using a Büchi Genevac Plus Centrifugal Evaporator (EZ-2 Plus, England) at the low boiling point setting of 45 °C. After extraction and drying, all extracts were stored at approximately 5 °C throughout the duration of this study.

2.3.3. NMR analysis

Preliminary ^{1}H-NMR analyses were carried out on a 200 MHz NMR (Varian Mercury) at 512 scans. Samples of plant extracts dissolved in 80 % methanol were prepared to a concentration of 50 mg/ml and dried in a Genevac EZ-2 Plus Centrifugal Evaporator. Both deuterated water (D_2O) and methanol (CD_3OD) were tested to see which of the two solvents produced the best NMR results. It was clear that (D_2O) was the best solvent since it produced higher resolution peaks and thus for all analyses the dried 50 mg extracts were redissolved in 970 µl (D_2O), see Calculation 1 below. Maleic acid was used as an internal standard for possible future quantification of the toxin under investigation. Maleic acid was prepared by dissolving 100 mg maleic acid in 600 µl D_2O and left to sonicate until all the maleic acid was dissolved. For the analysis 970 µl of the 50 mg D_2O extract and 30 µl of maleic acid was mixed, thus the final concentration of the plant sample analysed on NMR was 50 mg/ml. All the ^{1}H-NMR results were processed using MestReNova (Mnova) (version 14.2.0 Mestrelab Research, 2020).

Calculation (1) shows an example of the preparation of 50 mg/ml extract solution of *V. macrocalyx*, 0.3127 g).

$$\frac{\text{Weight of extract (mg)}}{\text{Volume required to created 50 mg/ml concentration}}$$

$$\frac{312.7\ mg}{x} = \frac{50\ mg}{1\ ml}$$

$$\frac{312.7\ mg}{50\ mg} = x\ ml$$

$$x = 6.254\ ml$$

Thus 6.254 ml of the extract was transferred to a clean polytop and dried, this was then redissolved in D_2O and maleic acid as described above.

2.3.4. GC-MS analysis

Derivatisation procedure

Where possible three biological replicates were analysed, for the other species two technical replicates were used. The extracts were prepared to a concentration of 1 mg/ml in 2 ml double distilled water. The dissolved extracts were filtered through syringe fitted filters (pore size 0.22 µm, Merck Ltd) and 100 µl transferred to 2.0 ml screw top GC-MS glass vials (Separations Scientific) with 200 µl inserts and dried overnight under a nitrogen stream. The residues were then dissolved in 50 µl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Merck Ltd), vortexed for two minutes and left at 70 °C for one hour. Pyridine was then added as a solvent in 50 µl quantities, making the solution up to a total of 100 µl. Pyridine also assists as a basic catalyst during the derivatisation reaction. During the analysis 5 µl of each replicate was analysed.

GC-MS instrument parameters

After derivatisation of the crude extract, a sample of 1 mg/ml of each species (Calculation 2 below) was analysed in pyridine solvent for targeted metabolomic analysis. Analysis was conducted on a Shimadzu GC-MS-QP2010 (Shimadzu Corporation, Japan) with a 70-eV electric current. During analysis, a 5-MS Rxi column (29.3 m x 250 µm x 0.25 µm i.d.; 0.25 µm df) was used to separate compounds and analyse 1 µl of each replicate with helium as the carrier gas. Before the analyses started pyridine was injected as a blank to ensure that there are no instrumental errors. The analysis was performed under splitless injections of 1 µl and the column flow was set to a linear velocity. The sampling set time was set to 2 min and the solvent cut time 3.5 min. The temperatures of the injector and interface was set to 250 °C. During the GC-MS analysis the oven temperature was held at an initial temperature of 40 °C for 1 min and then increased to a final temperature of 330 °C at a linear rate of 7 °C every minute, the final temperature was kept constant for 10 min, making each run approximately 52 min. The temperatures of the MS ion source were also set to 250 °C. The mass to charge ratio (*m/z*) detection started at 7 min to ensure the solvent does not saturate the detector. Detection ranged from 45 to 650 *m/z* together with a scan speed of 2500 aum per second.

Calculation (2) shows an example of the calculations (for *V. randii*) to prepare 1 mg/ml solutions in 2 ml double distilled water is as follows:

$$\frac{Weight\ of\ extract\ (mg)}{Volume\ required\ to\ created\ 1\ mg/ml\ concentration} = \frac{1\ mg}{1\ ml}$$

$$\frac{489.0\ mg}{x} = \frac{1\ mg}{1\ ml}$$

$$x = 489\ ml$$

$$C_1V_1 = C_2V_2$$

$$(48.9\ mg/ml)(x) = (1\ mg/ml)(2\ ml)$$

$$x = 0.04090\ ml$$

The dissolved extract was then made up to 2 ml with double distilled water.

2.4. Results and discussion

2.4.1. ^{1}H-NMR analyses

The ^{1}H-NMR spectra of plants provide a lot of information regarding the compounds present in a plant since organic molecules all contain protons (Kim et al., 2006). Results were compared to that reported by Sieber et al. (2015) regarding the isolation and detection of kirkamide. During the analysis of the positive control, *Psy. kirkii* signals were found in the area of 5.80 ppm as expected (Figures 2.2). This was an indication that the extraction method was successful, and the instrument can detect the toxin. The results of *Psy. kirkii* indicated that the toxin occurs in low concentrations in comparison to other compounds however, this outcome was anticipated, as Sieber et al. (2015) similarly documented limited amounts of kirkamide. Kirkamide signals were also reported as a doublet of quartets (dq) by Sieber and associates (2015), the signals were not clearly displayed during the ^{1}H-NMR analyses reported in this study due to the lower strength magnet (200 MHz) used in comparison to that of Sieber et al. (2015) (500 MHz). ^{1}H-NMR analyses were used only for preliminary analyses because of its low resolution and complex spectra containing all compounds extracted in 80 % methanol, it is difficult to confidently confirm the results obtained during these analyses. It is therefore a possibility that there are other compounds in the extract that produce similar signals to kirkamide that can lead to wrong conclusions or that it can be shadowed by compounds occurring in higher concentrations and also producing signals in the same range as kirkamide. It is thus important to confirm preliminary ^{1}H-NMR results with additional analyses.

It is also important to note that there is a diminutive chemical shift difference in the signals of kirkamide found in the positive control, *Psy. kirkii* to that reported by Sieber et al., (2015) however, this is possibly due to the acidic nature of maleic acid added. The addition of maleic acid often causes a change in the pH of the sample and thus causes a shift in the data across the spectra (Kukić et al., 2010; Miyataka et al., 2007).

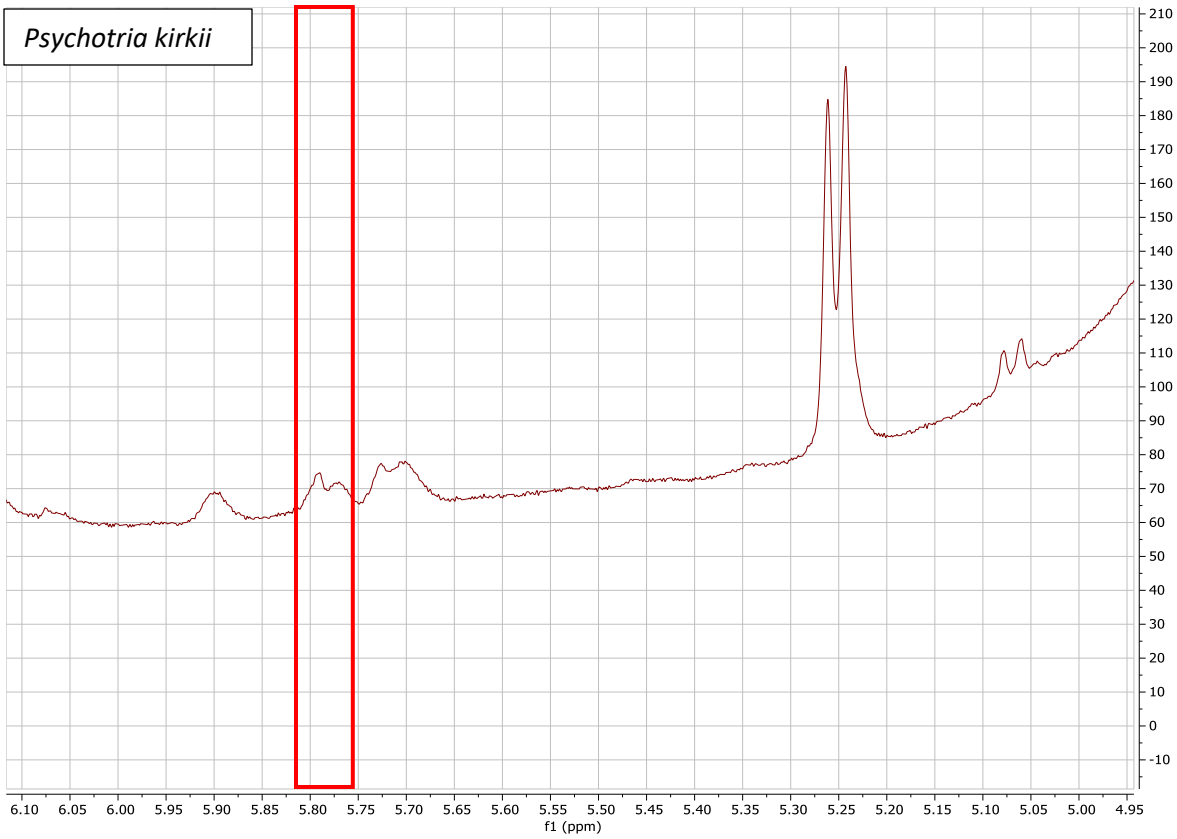

Figure 2.2: ^{1}H-NMR results of *Psychotria kirkii*, the red rectangle indicates the signals at approximately 5.80 ppm, which is proposed to be kirkamide according to Sieber et al. (2015).

During the ^{1}H-NMR analyses no kirkamide was detected in all the other plant species that were investigated. Some species produced small peaks that appeared as unresolved signals around the area of 5.85 ppm however, these signals were not clear enough to conclusively report that kirkamide was present for example the results of *V. infausta* (Figure 2.3). For this reason, all the plant samples were analysed through GC-MS as well because of its much higher detection sensitivity.

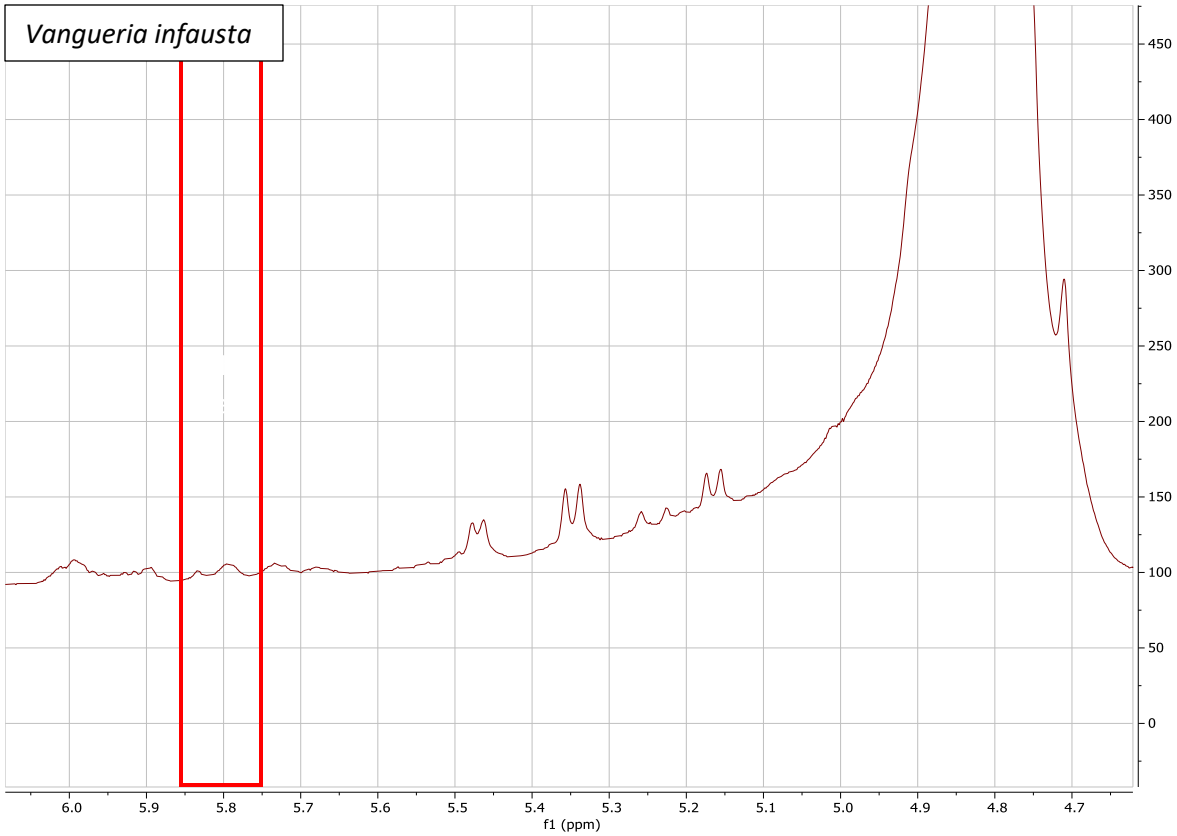

Figure 2.3: ^{1}H-NMR analyses of *Vangueria infausta* with a signal appearing around 5.85 ppm (where kirkamide is expected) however, the resolution was not good enough to confidently correlate the peak to kirkamide.

It has been reported that more cattle die from *gousiekte* during spring, than during other seasons (Hay et al., 2008; Stanton et al., 2014). One potential explanation for this is that animals often graze on the toxic species when they form new leaves in spring, as they sprout before new grass leaves appear after winter (Botha and Penrith, 2008). The concentration of compounds can often fluctuate in the plants, this is often due to seasonal changes, different stages of the life cycle of the plant or in response to abiotic stresses etc. (Liakoura et al., 2001). The effect of seasonal changes on the concentration of kirkamide was investigated in the current study by collecting plant samples in both late summer and spring of *V. pygmaea*. The results of ^{1}H-NMR indicated that there are differences between the concentration of compounds in general produced during spring and late summer months. Both samples analysed were prepared to the same concentration thus the difference in the peak intensity can only be attributed to a change in the concentration of the compounds (Figure 2.4). There are also some compounds produced in

the samples collected in early spring that were not present in the late summer collection. The extracts of the leaves of *V. pygmaea* that were collected in spring had a clear broad singlet signal at 5.813 ppm, which was not present in the extract of the late summer sample (Figure 2.5) however kirkamide should appear as a doublet of quartets. Since the signal was present in detectable quantities, it was expected to split when analysed on the 200 MHz NMR even with its lower resolution. There was thus very little chance that the signal belonged to kirkamide. This was also further investigated by GC-MS.

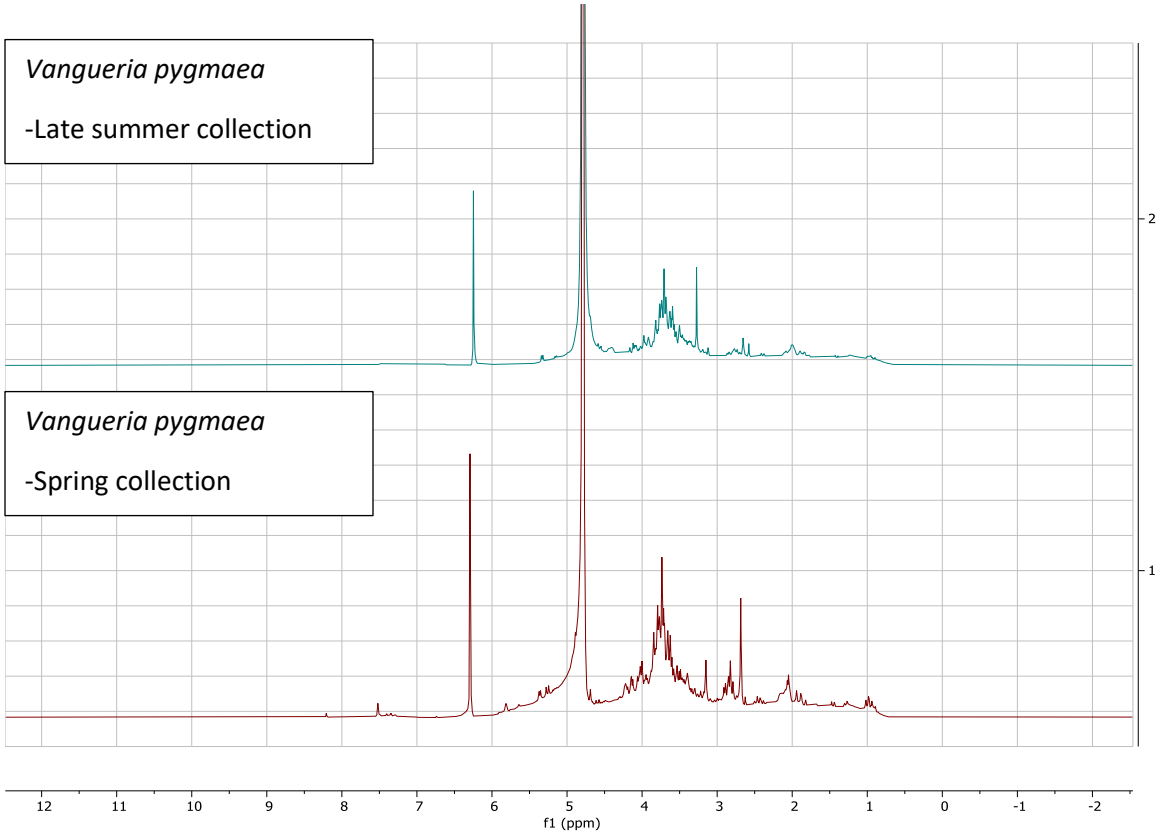

Figure 2.4: ^{1}H-NMR results of *Vangueria pygmaea*; sample collected in late summer during March is at the top (blue) and the spring samples collected in October is at the bottom (red). From the results it is clear that there is a general increase in the concentration of the compounds during spring.

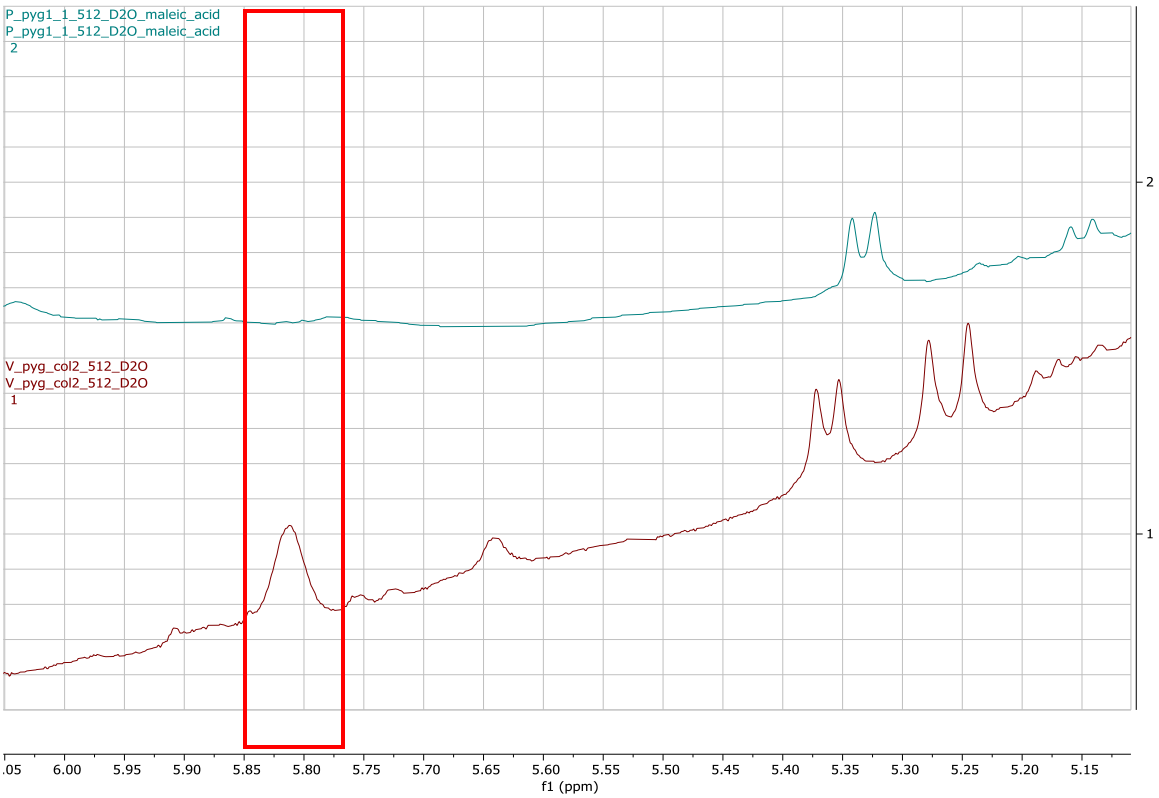

Figure 2.5: A comparison of the expanded ^{1}H-NMR results *of Vangueria pygmaea* collected during the late summer month of March (blue) and during spring in October (red). Indicated in a red rectangle is a signal that could possibly belong to kirkamide however was excluded as a possibility as it did not split into the doublet signal expected.

2.4.2. GC-MS analyses

Due to the separation by column chromatography and the sensitivity of the GC-MS instrument, it has the advantage over NMR that it can detect compounds without being shadowed by other compounds that occur in higher concentrations. *Psy. kirkii* was again used as a positive control in these analyses. The results confirmed those of the ^{1}H-NMR analyses that kirkamide is present in *Psy. kirkii.* Three kirkamide ion fragments at 73, 147, 282 and the molecular mass of 332 *m/z* were expected to be detected according to (Sieber et al., 2015) These fragments are the products formed during the reaction of kirkamide and MSTFA (derivatisation agent). Either one of the following two fragments were also expected, the first being kirkamide bound to four TMS molecules which would then appear as 490 *m/z*, the second was kirkamide bound to five TMS molecules which would then result in a fragment of 577 *m/z*.

From the results of GC-MS analyses, three fragments 73, 147 and 332 *m/z* could be identified in the sample of *Psy. kirkii* together with the 490 *m/z ion* (Figure 2.6). Kirkamide had a retention time (rt) of 27.830 min which corresponds to the rt 27.38 min and 27.73 min of Sieber and Fribourg (2015). Thus, detecting these fragments by GC-MS analysis confirmed the ^{1}H-NMR results that kirkamide is present in *Psy. kirkii*. The results thus concluded that the extraction and chemical detection analyses employed, could detect this poisonous compound in the positive control.

The rest of the samples were analysed under the same conditions however, none of the characteristic fragments were present in any of the plant extracts of the remaining species at the corresponding rt value, indicating that kirkamide is absent in those samples. The chromatograms and MS spectrum of *V. pygmaea* is shown in Figure 2.7. No fragments characteristic to those of kirkamide were also detected during GC-MS analyses of the spring collection of *V. pygmaea*, confirming that kirkamide is not present in this species during either late summer periods or in spring. The peak between 5.80 ppm and 5.85 ppm on the NMR spectrum mention above, thus belongs to another compound that appears in the same area, possibly streptol as will be discussed in Chapter 3.

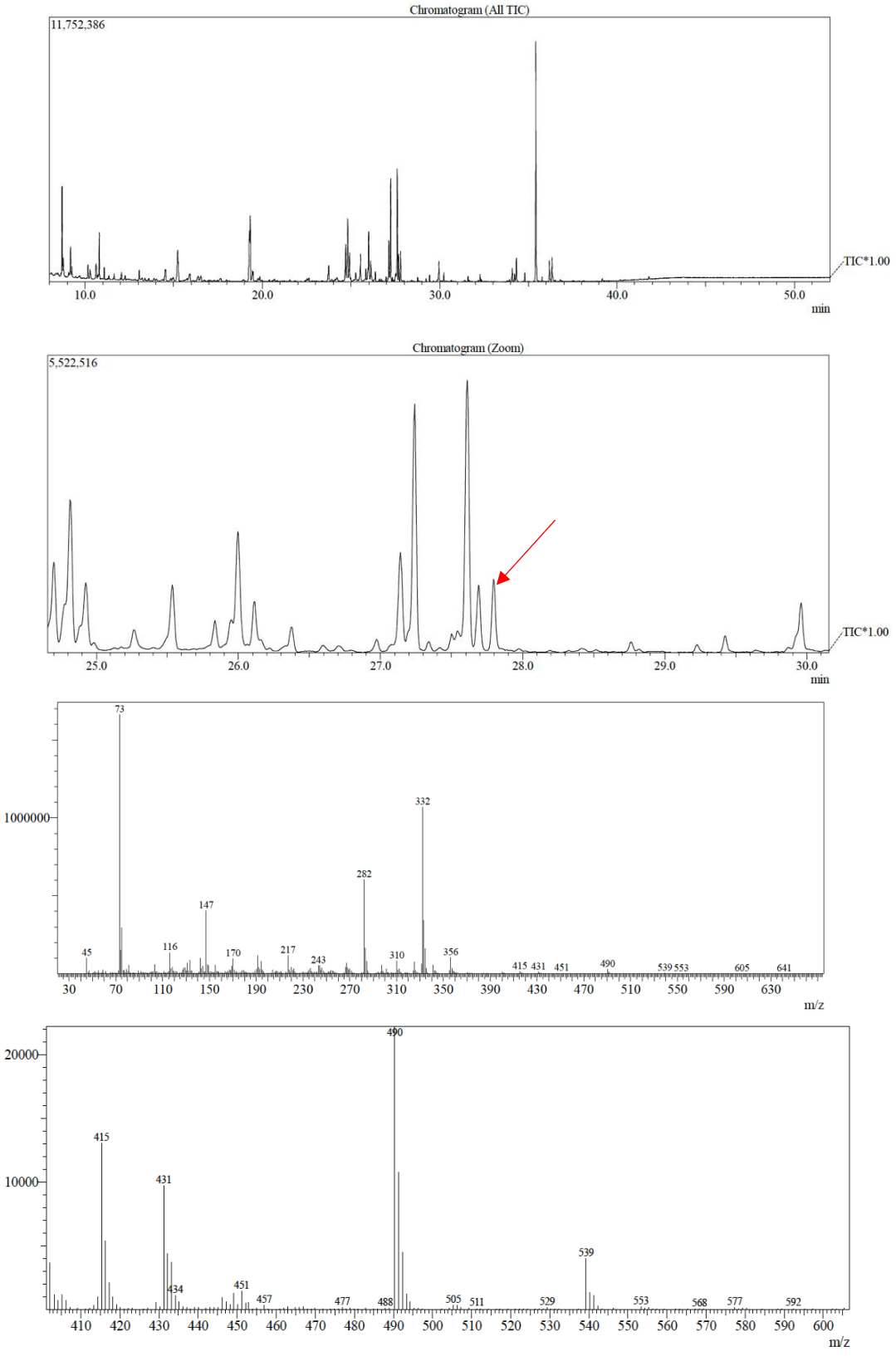

Figure 2.6: GC-MS results of *Psychotria kirkii* showing the presence of kirkamide in the chromatograms and the mass spectra with ion fragments 73, 147, 282, 883, 415, 431, 490 and 505 *m/z* and the enlarged *m/z* region of kirkamide from 410 to 600 *m/z*. The peak denoted by the red arrow signifies the presence of kirkamide.

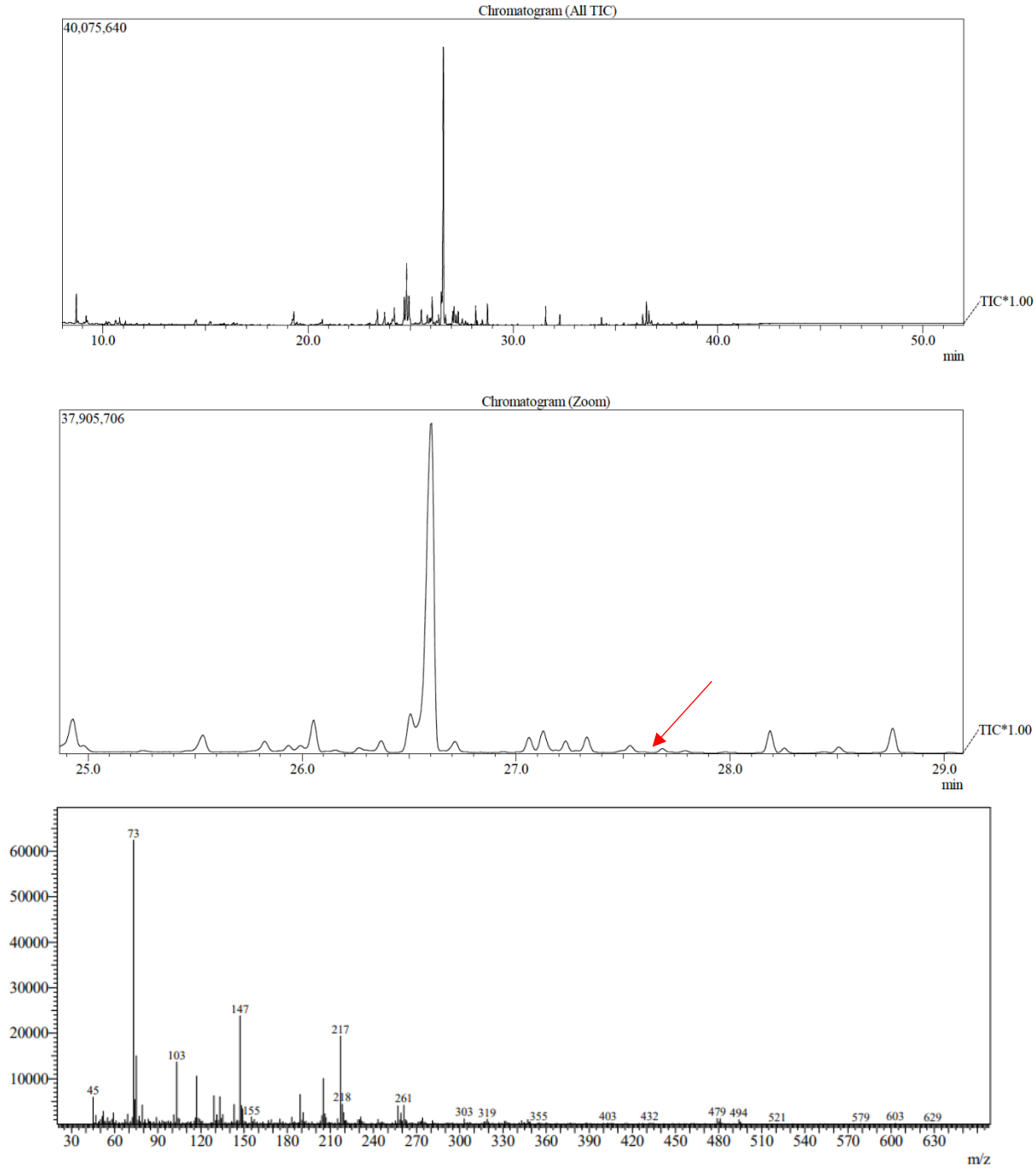

Figure 2.7: GC-MS results from the analysis of *Vangueria pygmaea* samples that were collected during spring. The results indicates that kirkamide was not detected. The peak denoted by the red arrow signifies the presence of kirkamide.

The DNA phylogenetic molecular analyses of the endophytes done by our collaborators showed that all the species (Species indicated with "*" in Table 1) except for *V. lasiantha* contains endophytes from either the *Burkholderia* or *Paraburkholderia* genus (Danneels et al., 2023). As previously stated, the species responsible for causing *gousiekte* harbours endophytes belonging to the same genus, *Burkholderia*. (Lemaire et al., 2011; Van Elst et al., 2013a; Verstraete et al., 2011).

The phytochemical results reported here from the GC-MS analyses corresponds to the genetic studies by our collaborators as they also found that the kirkamide biosynthesis gene is absent on the genome of the endophytes of the species investigated except for *Psy. kirkii*. The toxin was also only detected in the positive control *Psy. kirkii*. None of the other species investigated have the kirkamide gene on the endophyte genome and therefore could not synthesise kirkamide. This is further evidence of the hypothesis that the endophyte is responsible for the toxin production.

The hypothesis stating that kirkamide plays a role in the cause of *gousiekte* can be rejected since it could not be detected in any of the toxic species. The hypothesis suggesting a variance in kirkamide concentration between spring and summer is dismissible, as no kirkamide was identified in either season. Nevertheless, a distinct contrast in the overall concentration of compounds produced in the two seasons was observed, warranting further investigation.

Chapter 3: The partial purification and detection of the toxins, streptol and its glucoside in selected species of the Rubiaceae family.

Abstract

The presence of the toxin, streptol, and its derivative, streptol glucoside, had previously been documented in a plant species of the Rubiaceae family, *Psychotria kirkii*. It was also shown that the genes for its synthesis are present on the genome of the endophyte found in this species. The potential association of these two toxins with *gousiekte* was explored in the current study by determining their presence in the Rubiaceae species responsible for causing *gousiekte*. Considering the findings from previous results and a recent published paper, the leaf extracts of three species – *Fadogia homblei*, *Vangueria infausta*, and *V. pygmaea* were selected for chemical analysis. Previous published results revealed that *F. homblei* lacks the streptol-synthesising genes, while both *V. pygmaea* and *V. infausta* possess these genes. Due to the low concentrations of the toxins being present in the leaves, the extracts were semi-purified through a Sephadex column. Following partial purification, the fractions were subjected to analysis using both ^{1}H-NMR and UPLC-QToF techniques to detect the presence of streptol and streptol glucoside. The toxin was detected in the two investigated *Vangueria* species, while the *Fadogia* species, lacking the streptol genes, showed an absence of the toxin in the leaf extract. This provides supporting evidence that the endophytes are responsible for the toxin production.

3.1. Introduction

The interactions between plants and microbes often impact the development and overall success of plants in their natural environment (Danneels et al., 2023). There are many associations that provide important evolutionary advantages for example the interaction between nitrogen fixing endophytes and legumes (Vessey et al., 2005). Endophytes are defined as microorganisms that colonise a healthy plant and has a close mutualistic relationship with the host plant (Hardoim et al., 2008; Stanton et al., 2014). Microbes often assist plants in growth and development when the plant is affected by biotic and abiotic stresses (Kumar and Verma, 2018). The microbes receive a stable environment and the required nutrition from the host while they provide benefits to the plant through the production of plant growth regulators or by protecting the plant by assisting in defence against pathogens (Rashid et al., 2012; Stanton et al., 2014). However, endophytes can also have detrimental effects and lead to significant economic losses (McCann, 2020). When *gousiekte* was first noticed in 1908 several field trials followed in an attempt to identify the cause of the disease (Fourie et al., 1995). *Vangueria pygmaea* (Schltr.) was the first identified *gousiekte*-causing plant species, and subsequently five additional species were identified namely, *Fadogia homblei* De Wild., *Pavetta harborii* S. Moore, *V. thamnus* Robyns, *Pav. schumanniana* F.Hoffm. ex K.Schum. and *V. latifolia* Sond. (Stanton et al., 2013; Verstraete et al., 2011). In 1995, the suspected toxic compound, pavettamine was identified. The identification and structure elucidation were determined through a combination of a number of studies. (Bode et al., 2010; Fourie et al., 1995; Hurter et al., 1972; Theiler et al., 1923). It was subsequently shown that all six of the *gousiekte*-causing species contains endophytes, which led to the belief that the endophytes play a role in the cause of *gousiekte* (Compant et al., 2005; Van Wyk et al., 1990; Verstraete et al., 2011). This was however not conclusively proven, but the recent publication by Danneels et al. (2023), added much weight to this possibility. Thus, the question remains if it's a coincidence that all the *gousiekte*-causing species contains endophytes that synthesise toxic compound(s) harmful to animals?

Gousiekte poses a serious threat to the agricultural industry, as numerous farmers have previously faced significant losses due to this disease. According to records from 2008, farmers experienced losses amounting to R9 million in cattle and R5 million in sheep (Verstraete et al., 2011).

Having interviewed a few farmers that encountered these species, it is interesting to hear their approach towards the toxic plants and what they believe cause the disease, although their theories are not based on scientific research. There are farmers that allowed their cattle to feed on *V. pygmaea* as they believed

it fattened them very fast due to high protein levels in the leaves. However, during this period they keep the cattle calm as cattle would often die when frightened. They also believe that the species is only toxic when the flowers are present. Many farmers also keep donkeys close to cattle, since the donkeys can feed on the toxic plants without any harm. This is especially interesting since donkeys are not ruminants. They also mentioned that there are usually no *gousiekte* reports occurring during winter times and most cases are reported during the start of a new growing season, spring and throughout summer. This was easily explained by the absence of the plant species during winter.

Pavettamine, the proposed toxic compound has not been definitively established as the causative agent of *gousiekte*. One compelling reason for this uncertainty is that pavettamine has been detected in only four of the six species known to cause *gousiekte*. Another significant factor to consider is that pavettamine has been found in non-*gousiekte* causing plant species as well, and in certain cases, its concentration has been higher in the non-toxic plants, of which *Psychotria kirkii* (Rubiaceae) is an example of (Bode et al., 2010). There is a compelling likelihood that this plant species (*Psy. kirkii*) is indeed toxic (Hisiao et al., 2019). Its geographical distribution often coincides with that of other well-established toxic species and its toxicity has therefore probably been disregarded in certain *gousiekte* cases, because other well-known *gousiekte*-causing species are present in the same location.

The toxin and plant growth inhibitor, streptol glucoside was first isolated from *Psy. kirkii* by Hsiao and associates (2019), its aglycone, streptol, also known as valienol, was previously isolated from a *Streptomyces sp.* (Isogai et al., 1987). The glucosylated compound can often be found in approximately ten-fold higher concentrations in *Psy. kirkii* than that of its aglycone, streptol (Hsiao et al., 2019). It seems as if by the process of glucosylation of streptol, the toxicity of the compound is lowered to the plant to protect it against the toxic effects (Hsiao et al., 2019). The effect of streptol and its glucoside have been tested on lettuce seedlings and results showed that the aglycone possesses growth inhibitory characteristics (Hsiao et al., 2019). Streptol has an IC_{50} of 17 μM and streptol glucoside an IC_{50} value of between 28 μM and more than 100 μM, the IC_{50} was determined by assessing the root growth of lettuce seeds that had been treated with streptol in comparison to the control group. The molecular weight and chemical formula of streptol are 176.17 *m/z* and $C_7H_{12}O_5$ whereas streptol glucosides are 338.31 *m/z* and $C_{13}H_{22}O_{10}$ (Hsiao et al., 2019; Isogai et al., 1987).

3.2. Hypothesis and objectives

This chapter of the study focuses on the isolation and identification of streptol and its glucoside and was directed by previously published genetic investigations (Danneels et al., 2023) in which the endophytes of two *gousiekte*-causing species of the Rubiaceae family was shown to contain the streptol genes, namely *V. pygmaea* and *V. infuasta* whereas these genes were not present on the DNA of *F. homblei.* The emphasis was placed on the partial purification and identification of streptol, as it was hypothesized that its glucoside would likely be present if streptol was detected.

Hypothesis

- The bacterial endophytes of the *gousiekte*-causing plant species play a role in the synthesis of the toxins, streptol and its glucoside and render their host plants poisonous.

Objectives

- Collect the leaves of *V. pygmaea*, *V. infuasta* and *F. homblei* during spring when the plants are reported to be the most poisonous.
- Extract streptol and streptol glucoside from the leaves using 80 % methanol.
- Partially purify plant extracts through a Sephadex LH-20 column.
- Identify promising fractions through TLC analyses.
- Do preliminary analysis by ^{1}H-NMR and confirm the presence of the toxin in the three plant species through UPLC-QToF analyses.
- Compare the chemical results with that of the genetic studies, thus providing evidence that the endophyte plays an important role in the production of the toxin.

3.3. Materials and methods

3.3.1. Plant collection and extraction

Plant material was extracted as described in Chapter 2. Additional samples of the three species, *F. homblei*, *V. infausta* and *V. pygmaea* were collected in spring (Table 3.1).

Table 3.1: The locations where leaf material of *Vangueria pygmaea* was collected during spring (October, 2021) and those of *Fadogia homblei* and *V. infausta* in October, 2022.

Plant species	Coordinates	Location of collection	Number of replicates collected	Toxic/non-toxic
Fadogia homblei	S 25° 34' 20.5'' E 28° 25' 58.4''	Roodeplaat	3	Toxic
Vangueria infausta	N 25° 44' 59.28'' E 28° 14' 53.88''	LC De Villiers (Univ. of Pretoria)	3	Unknown
Vangueria pygmaea	S 25° 44' 10.2'' E 28° 31' 59.3''	Cullinan	3	Toxic

3.3.2. Sephadex LH-20 chromatography

Approximately 100 g of the stationary phase, Sephadex LH-20 (Sigma-Aldrich, Vienna, Austria) was equilibrated by emerging in distilled water. The slurry was poured into a glass column (55 cm x 4 cm, Scott Duran, Germany) and the stationary phase was allowed to settle and then washed repeatedly with distilled water.

Distilled water was used to dissolve 2 g of the crude extract and the sample was then added carefully to the chromatography column. Elution was carried out with 400 ml water, fractions of 10 ml were collected in separate polytops until the visible compounds started to elute, from there on fractions were collected in increments of 5 ml in weighed polytops.

After 400 ml water eluted, the eluent was changed to 200 ml of 30 % methanol and collected again in quantities of 5 ml. The column was then washed with 50 % methanol, followed by 100 % methanol. The samples were dried and then analysed by thin-layer chromatography (TLC) as described below and

fractions that contained polar compounds (e.g., sugars) were subjected to ^{1}H-NMR and UPLC-QToF analyses.

3.3.3. TLC chromatography

The semi-purified fractions were analysed using TLC Silica gel 60 F245 plates (Merck, Darmstadt, Germany). The results of the TLC plates served as a guide for selecting which samples to analyse by ^{1}H-NMR spectrometry. Several solvent systems were tested and analysed. A mixture of ethyl acetate, acetic acid, formic acid, and distilled water (95:5:5:5) gave the best results as the mobile phase. The plates were prepared in duplicates. After development through the mobile phases, the plates were treated with vanillin heated and analysed visually for the presence of streptol or other sugars.

3.3.4. NMR analysis

Preliminary ^{1}H-NMR analyses were carried out on a 200 MHz NMR spectrometer (Varian Mercury, 2002). Varying quantities of samples were analysed depending on the amount obtained during semi-purification (20 mg to 120 mg). All the analyses were carried out using 600 µl D_2O as a solvent, in some cases very little sample were recovered. The instrument was set for 512 scans and both proton and carbon analyses were conducted.

After preliminary analysis of the fractions, the fractions containing signals in the areas expected were analysed on a higher resolution 400 MHz Bruker NMR spectrometer. The parameters were kept the same. All the ^{1}H-NMR results were analysed using MestReNova (Mnova) version 14.2.0 analytical software (Mestrelab Research, 2020).

3.3.5. UPLC-QToF-MS analyses of streptol and streptol glucoside

The presence/absence of underivatised streptol and its glucoside in the plant extracts were determined by using a Waters Synapt G2 high-definition mass spectrometer (HDMS) system (Waters Inc., Milford, Massachusetts, USA). The apparatus consisted of a Waters Acquity UPLC connected to a quadropole-time-of-flight (QToF) instrument. The method of Georgiou et al. (2021) was followed for the detection of streptol in negative mode [M-H]. The samples were analysed using a Luna Omega 1.6 µm C_{18} 100 A, 100 x

2.1 mm (Phenomenex, Separations) column and a solvent system that consisted of MeCN: H2O with 0.1 % NH_4OAc (A, 8:2,) and MeCN: H2O with 0.1 % NH_4OAc (B, 2:8). The gradient was set to start at 95 % of B and to decrease to 50 % of B in 7 min, for the next 2 min the gradient was kept at 50 % of B, the gradient was then gradually decreased from 50 % to 5 % of B for the next 3 min and was followed by a column wash for the next 2 min giving a total run time of 12 min. The column temperature was 40 °C, injection volume 7 µl and the flow rate 0.3 ml min^{-1}.

Mass to charge ratios (*m/z*) were recorded between 50 and 1 200 Da. High energy collision induced dissociation (CID) was used for tandem MS fragmentation. The collision energy for the ramping was set to increase from 10 V to 20 V to get a range of data (MassLynx V4.1 SCN 803; Waters Inc.).

3.4. Results and discussion

3.4.1. *V. pygmaea* TLC and ^{1}H-NMR Results

TLC analyses of fractions from the *V. pygmaea* extract indicated that fractions 15 to 23 could possibly contain streptol and its glucoside. Reason for this being that upon treatment with vanillin, sucrose exhibited a green colouration which was then used, together with the Rf value, to compare to the fractions to determine which samples could possibly contain sugars (Figures 3.1). Those samples were then preliminary analysed through ^{1}H-NMR (200 MHz). The chemical structure of streptol and its glucoside is indicated in Figure 3.2. The preliminary ^{1}H-NMR results indicated that the strongest signal for streptol was in fraction 19. The fractions that seemed promising were also analysed through UPLC-QToF.

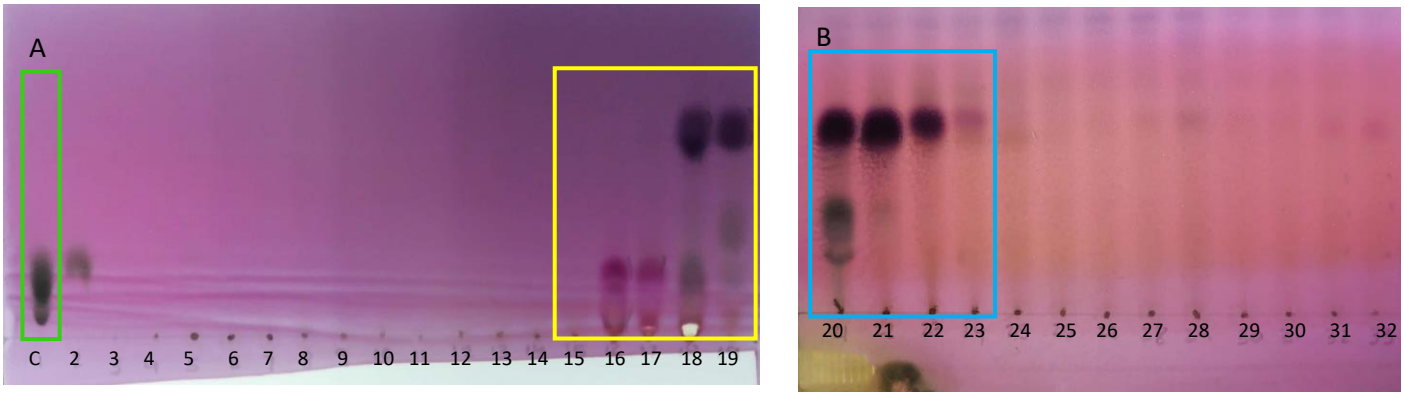

Figure 3.1: **A** - TLC results from the Sephadex purification of *Vangueria pygmaea*. Plates were treated with vanillin, which produced a dark green colour as seen in the control compound, sucrose in the first lane (Control (C), green rectangle) The results served as an indication of which samples to analyse through ^{1}H-NMR (Fractions 15 - 19, yellow rectangle). A mixture of ethyl acetate, acetic acid, formic acid, and distilled water (95:5:5:5) was used as the mobile phase. **B** – TLC results of the remaining fraction captured during Sephadex purification. The results were utilized to identify the fractions that needed further investigation (Fractions 21 - 23, light blue). The same eluent was used and vanillin colouration were used again as in figure A.

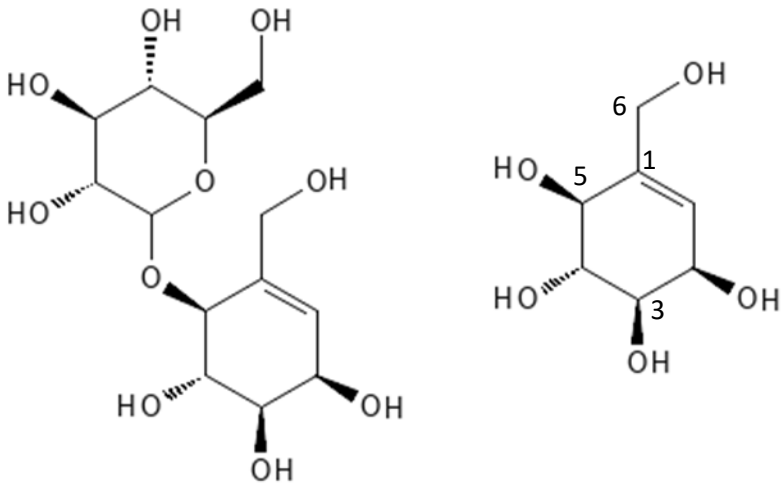

Figure 3.2: Chemical structure of streptol and streptol glucoside (Hsiao et al., 2019).

The quantity of fraction 19 (0.7690 g), was adequate for a second purification employing the identical Sephadex LH-20 column method as the initial purification. After the second purification, fraction 19.14 to 19.25 were preliminary analysed using a 200 MHz NMR apparatus. Fractions 19.17 to 19.19 provided the best results displaying possible signals resembling that of streptol, and were analysed by a 400 MHz NMR instrument. Hsiao et al. (2019) reported a doublet of doublets (dd) at 5.91 with *J*-values of 5.7, 1.7 Hz. for streptol. A possible signal for streptol was present in fractions 19.17 and 19.18 (Full spectrum: Figure 3.3 and 3.4) between 5.70 and 5.68 ppm (Figures 3.5 and 3.6). In fraction 19.17, ^{1}H-NMR results revealed a dd at 5.69 ppm, with a *J*-values of 5.66 and 1.68 Hz (Figure 3.5 and Table 3.2). A small shift from the reported data was expected, this is often due to the use of different instruments and materials as well as pH differences which could be a result of sample preparation as mentioned in Chapter 2. The ^{1}H-NMR results of fraction 19.18 (Figure 3.6) indicated a dd at 5.69 ppm with a *J*-value of 5.63, 1.71 Hz (Table 3.2). The values were almost identical in both fraction (19.17 and 19.18) to that of Hsiao et al. (2019). It appears as if the signals belong to the same compound resulting in a dd signal in both fractions (Figure 3.7).

A dd was also reported at 3.76 ppm by Hsiao and associates (2019) however, there is no clear signal in either two of the fractions, signals for sugars are detected within that range, and it is not possible to definitively identify the specific types of sugars present in this semi-purified extract, especially as the target toxin, streptol is possibly present in small quantities and its signal at 3.76 ppm is shadowed by the more abundant primary sugars.

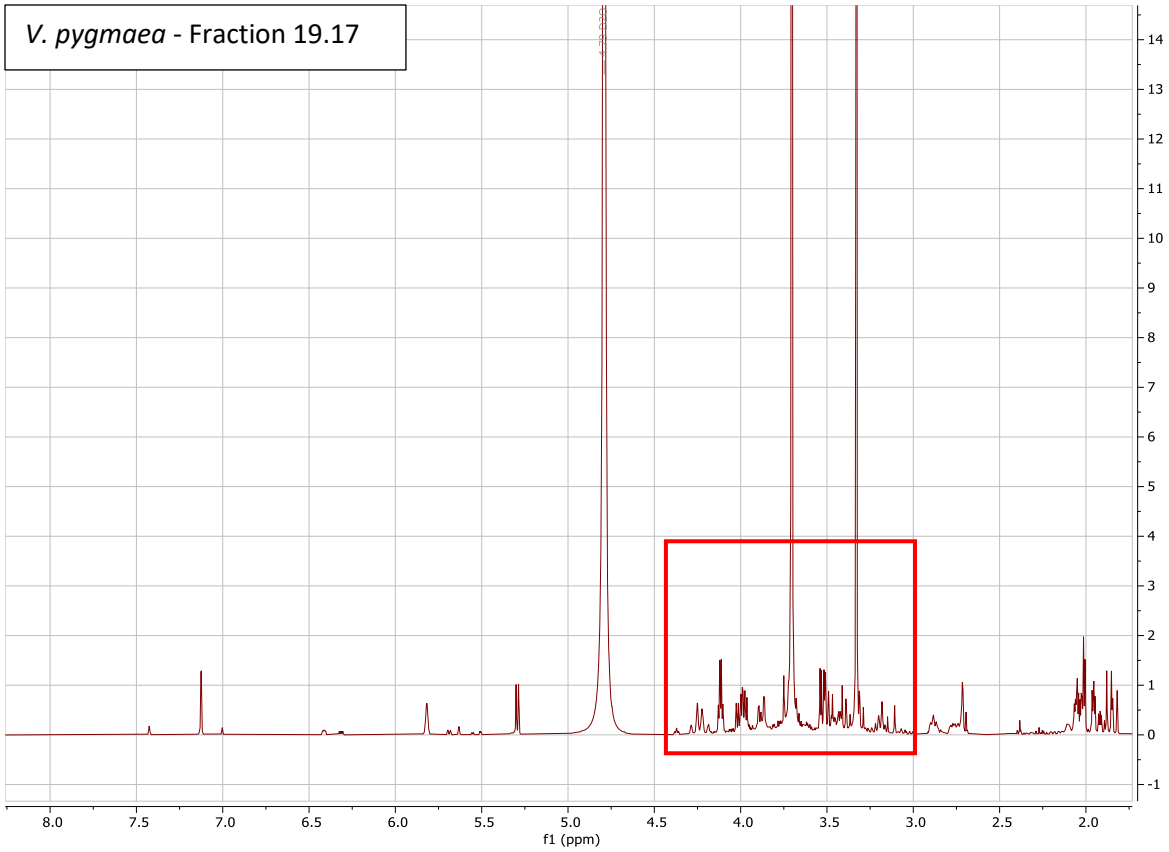

Figure 3.3: Full spectrum of fraction 19.17 analysis by ^{1}H-NMR (D_2O) of *Vangueria pygmaea*. Red rectangle indicating sugars present in the fraction.

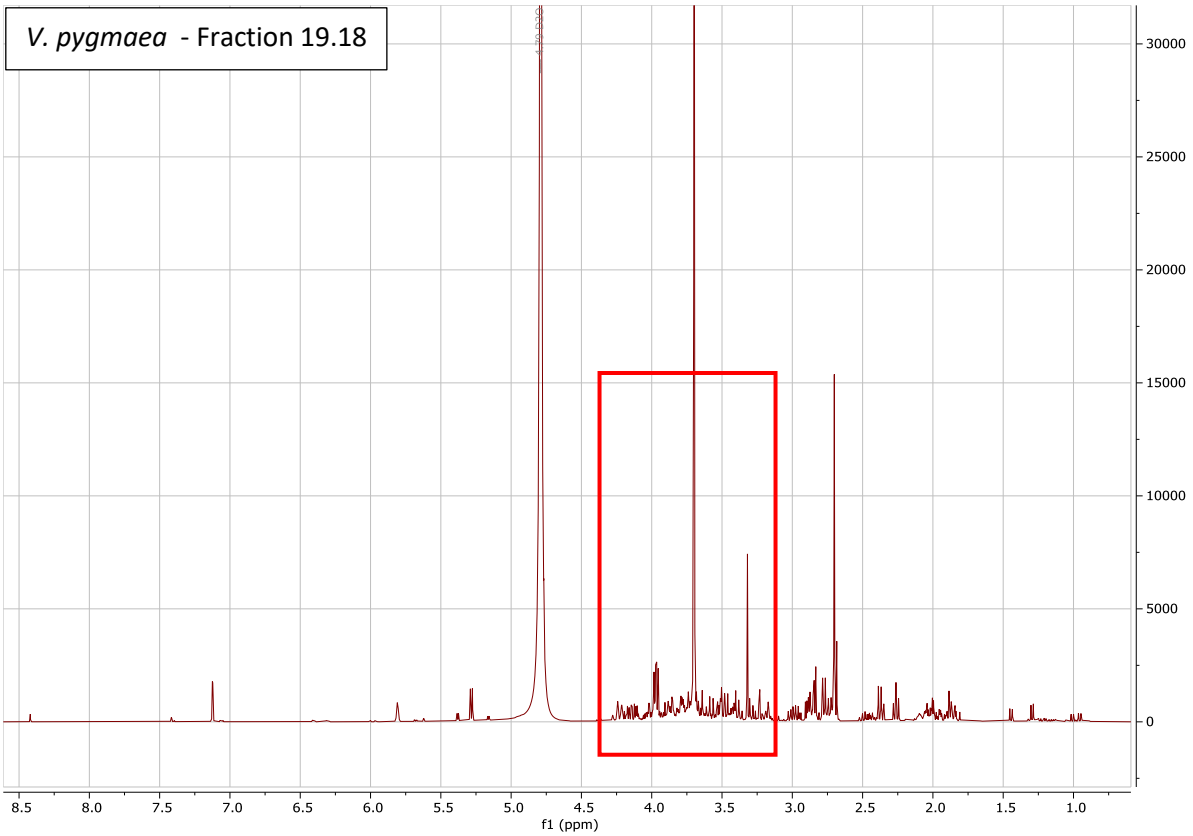

Figure 3.4: Full spectrum of fraction 19.18 analysis by ^{1}H-NMR (D_2O) of *Vangueria pygmaea*. Red rectangle indicating sugars present in the fraction.

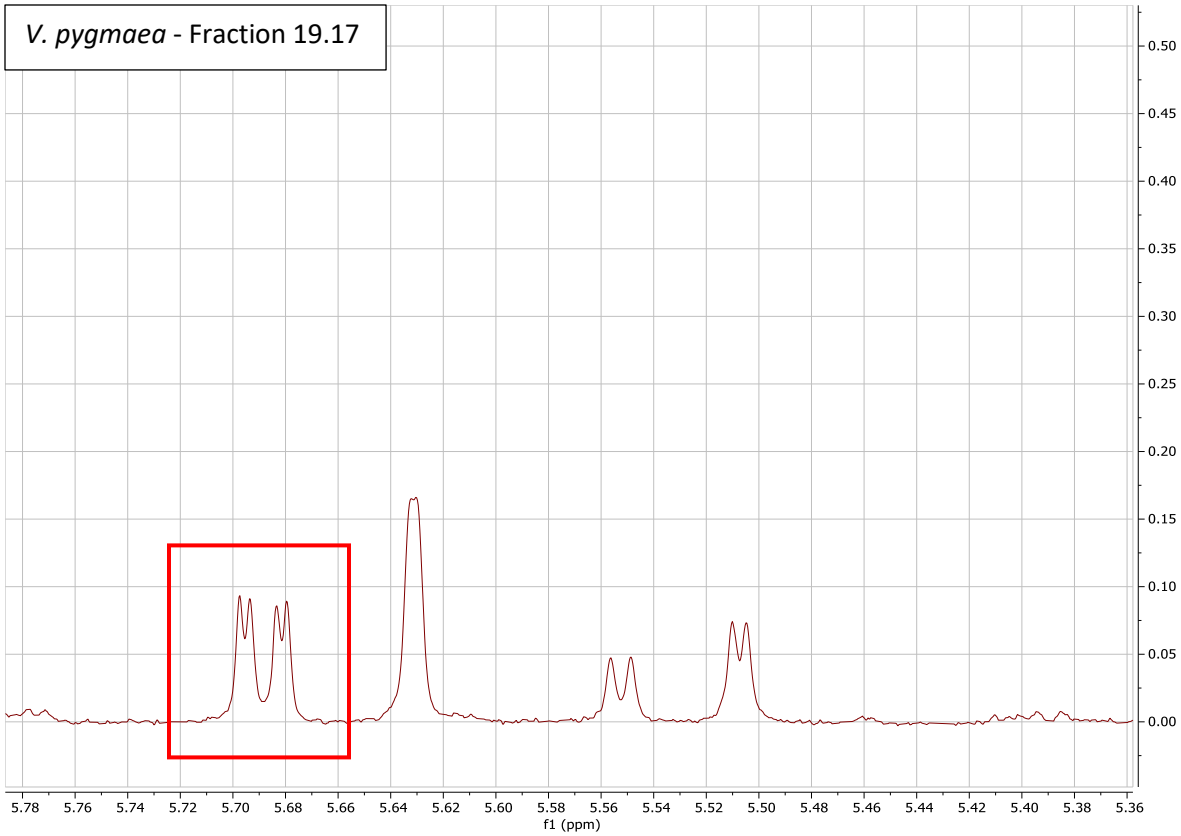

Figure 3.5: Enlarged spectrum from 5.36 to 5.78 ppm (D_2O) of analysis by ^{1}H-NMR of fraction 19.17 of *Vangueria pygmaea*, possibly containing streptol. Doublet of doublets (dd) indicated by red rectangle; characteristic peaks expected for streptol. The signals of the dd have *J*-values of 5.66 and 1.68 Hz.

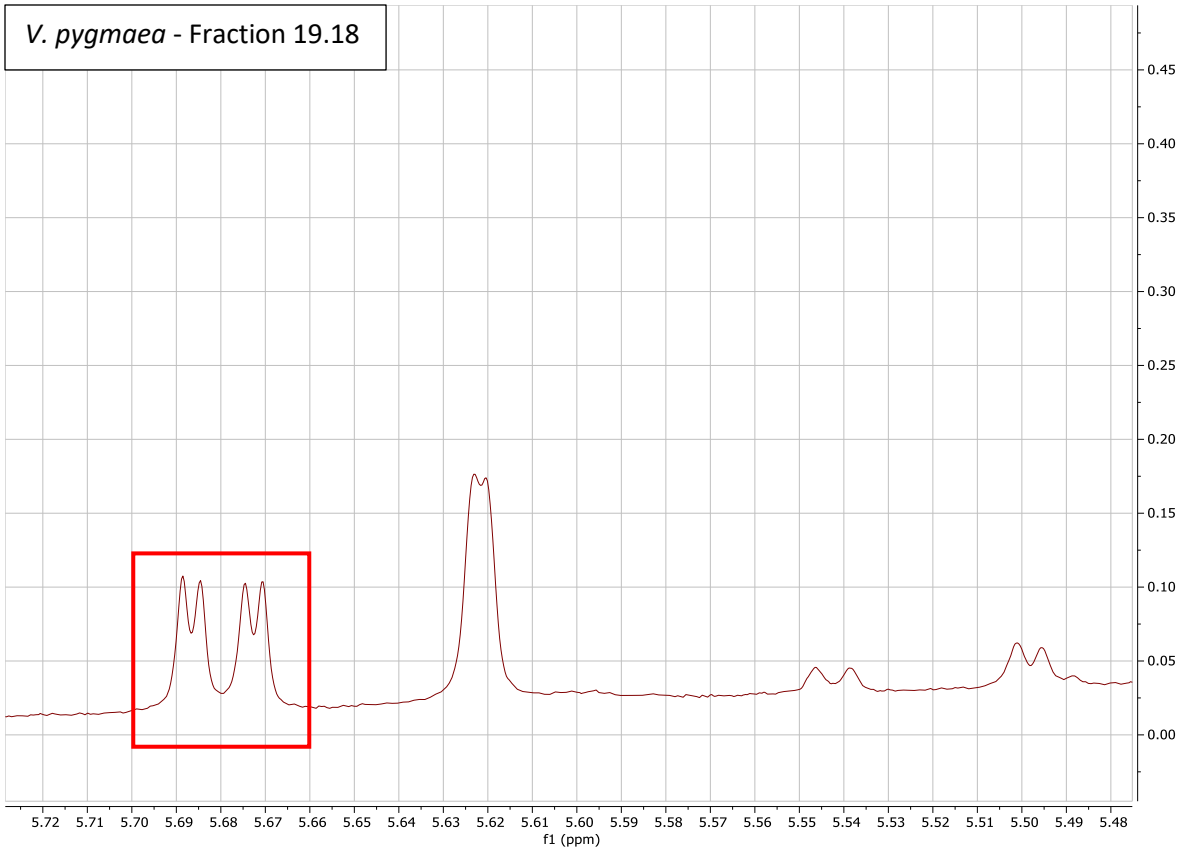

Figure 3.6: Enlarged spectrum from 5.48 to 5.72 ppm of results from ^{1}H-NMR analyses of fraction 19.18 of *Vangueria pygmaea*, possibly containing streptol. Characteristic doublet of doublet peaks expected for streptol, indicated by red rectangle, with *J*-values of 5.63 and 1.71 Hz.

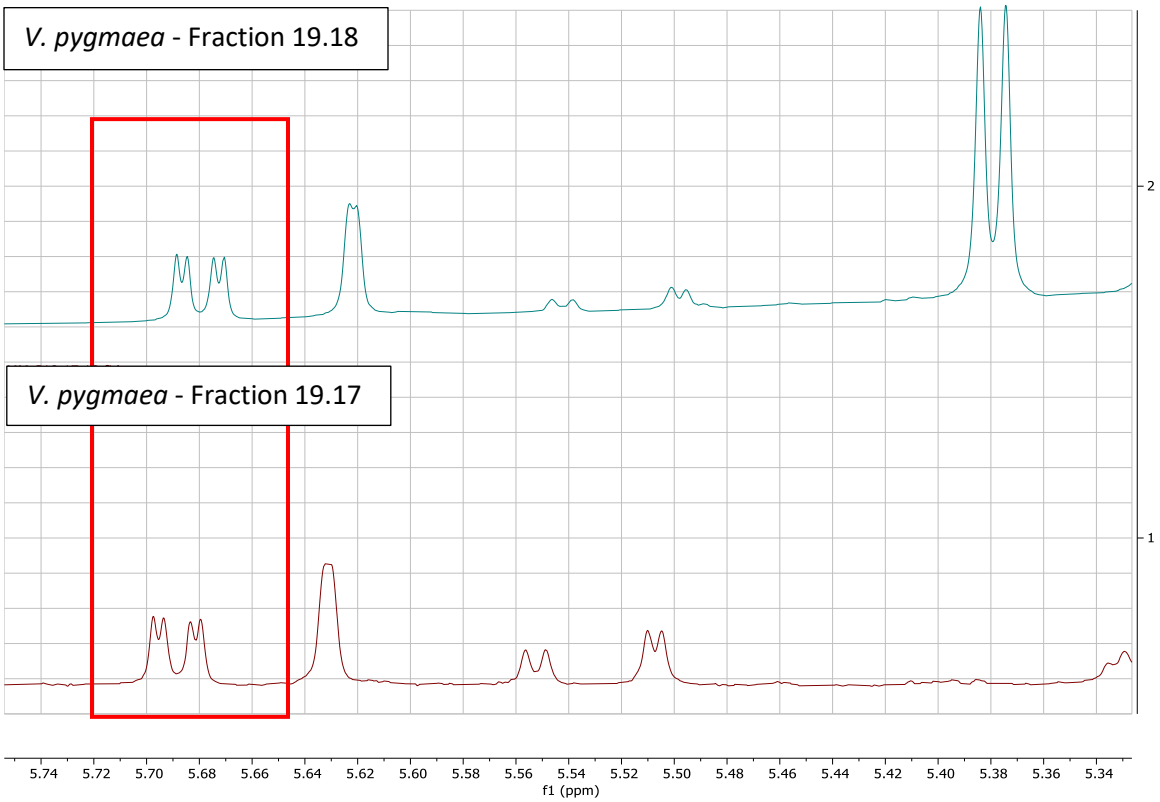

Figure 3.7: Doublet of doublets of fraction 19.17 and 19.18 indicated by red rectangle, *J*-values are almost identical. Both fractions of *Vangueria pygmaea* probably contain streptol.

Table 3.4: *J*-values of the inner and outer peaks of fractions 19.17 and 19.18 of the characteristic doublet of doublets exhibited by streptol during ^{1}H-NMR analyses of the *Vangueria pygmaea* purified fractions.

		Outside peaks		**Inside peaks**	
		ppm	**Hz**	**ppm**	**Hz**
Fraction 19.17	**Peak 1**	5.6813	2273.70	5.6934	2278.56
	Peak 2	5.6954	2279.36	5.6976	2280.24
	J-values	5.66		1.68	
Fraction 19.18	**Peak 1**	5.6866	2275.84	5.6844	2274.95
	Peak 2	5.6725	2270.21	5.6887	2244.95
	J-values	5.63		1.71	

3.4.2. *V. pygmaea* UPLC-QtoF results

The first purified fraction 19 of *V. pygmaea* were analysed through UPLC-QToF. Results indicated that the fraction contained the exact molecular weight of streptol of 175.06 *m/z* when done in the negative mode (Figures 3.8 and 3.9). The compound eluted at 0.81 min. It was expected that the compound would elute at the beginning of the program based on its polarity. The MassLynx (V4.1 SCN 803) software also suggested the best fit to the molecular formula to be $C_7H_{11}O_5$, matching streptol. Both the mDa and PPM confidence values were also below the required value of 5, further strengthening the identification of the toxin, streptol to be present in the semi-purified fraction 19 of *V. pygmaea*.

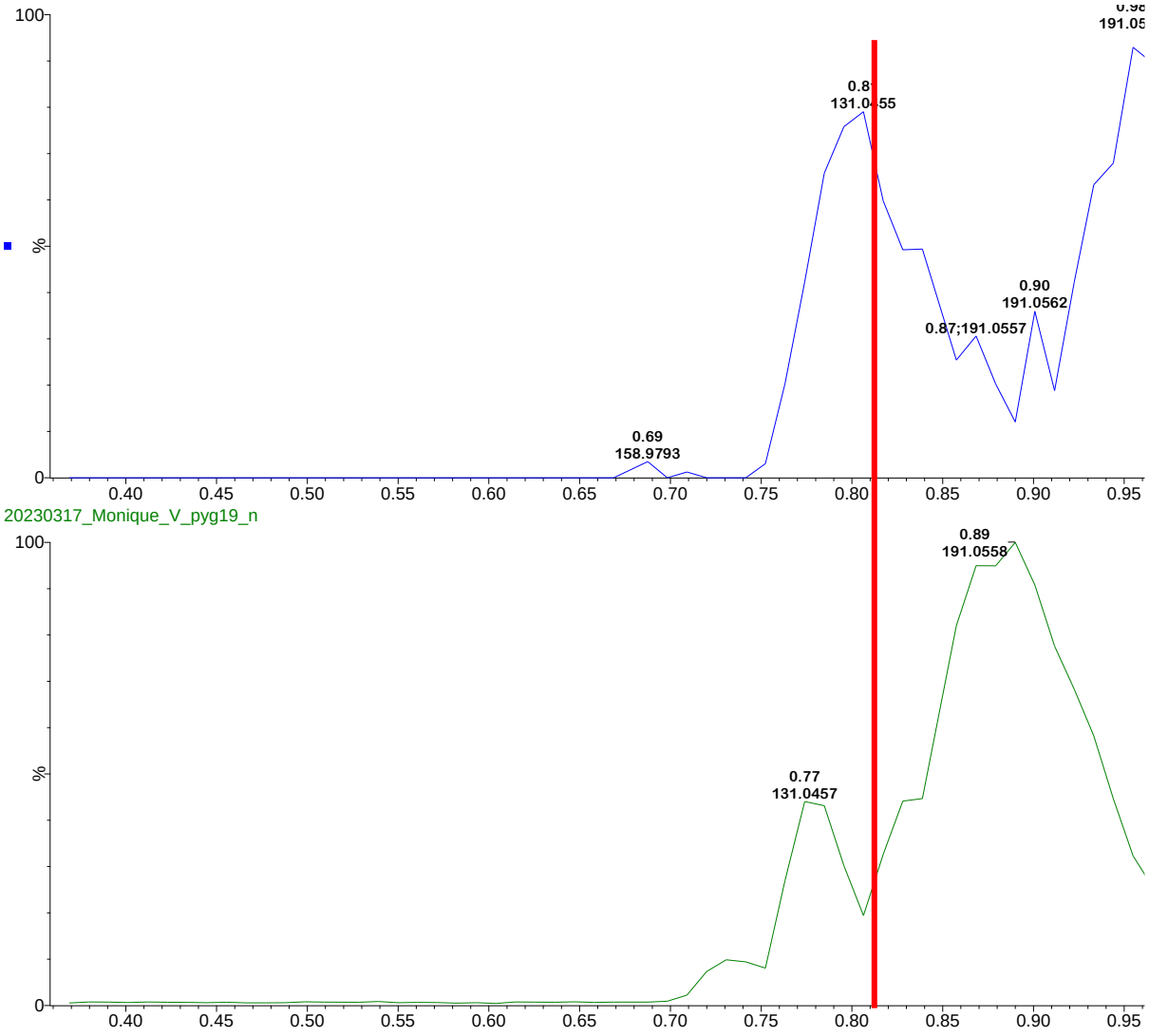

Figure 3.8: UPLC-QtoF results of *Vangueria pygmaea* fraction 19. Red line indicating the retention time at which streptol was found in the fraction (0.81 min). The results displayed above include both the complete chromatogram of fraction 19 (green) and the chromatogram of the exact mass searched for 175.06 *m/z* (blue).

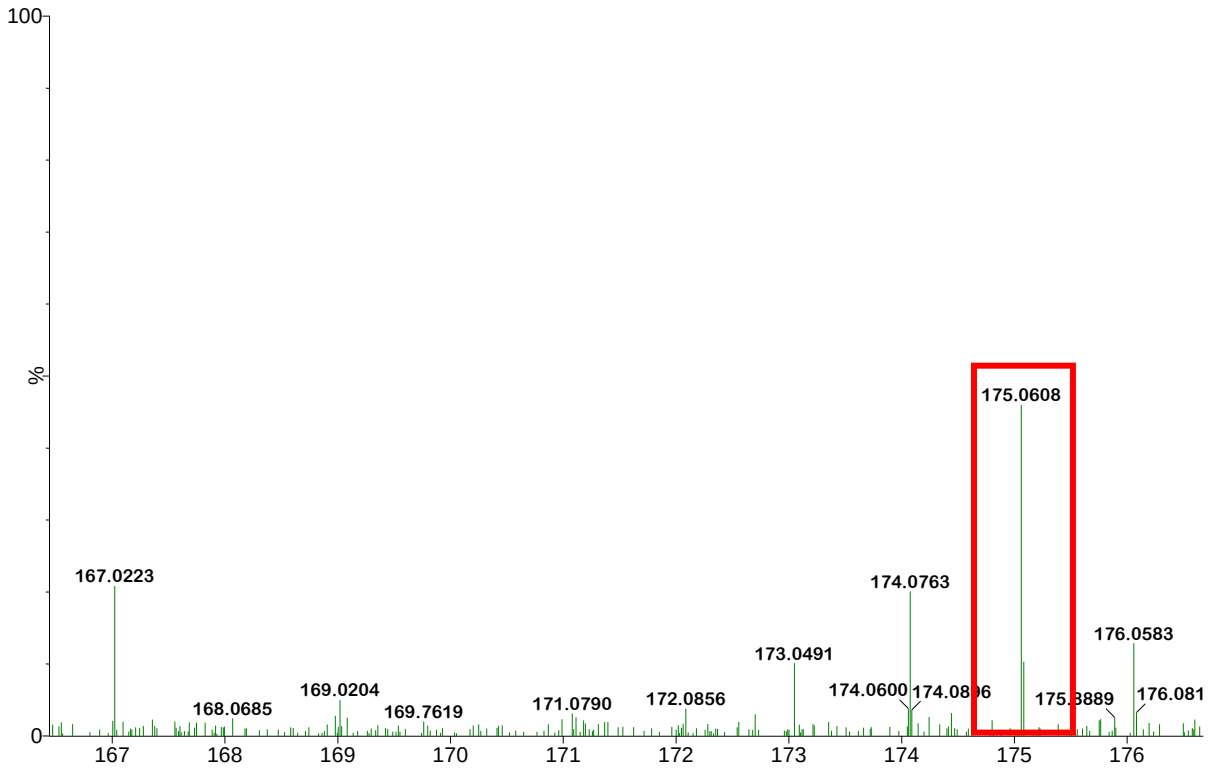

Figure 3.9: UPLC-QToF results of fraction 19 from *Vangueria pygmaea* indicated a strong peak for streptol. The exact mass of streptol was calculated as 175.06 *m/z*, which is shown in the red rectangle.

3.4.3. *F. homblei* ^{1}H-NMR results

Based on the chromatographic, ^{1}H-NMR and UPLC-QtoF results of *V. pygmaea*, fractions 14 to 25 of *F. homblei* were preliminarily analysed through ^{1}H-NMR (200 MHz). Fractions 18 and 19 exhibited signals at 5.90 ppm that could possibly be attributed to streptol however, this required further confirmation.

Although the signal appeared as a doublet (not a doublet of doublets), it eluted in similar fractions as *V. pygmaea* and was found in a fraction also containing other sugars (Figure 3.10). There was a significant shift in the signals when comparing the results of *V. pygmaea* and *F. homblei*. The results of *V. pygmaea* fractions 19 during NMR results, showed the doublet of doublet signals at 5.75 ppm whereas a doublet signal was present at 5.90 ppm in *F. homblei* in fraction 18 and 19 (Figure 3.11 and 3.12). The signals at 5.90 ppm also appears to be two individual peaks, as the height of the peaks differ which is not commonly seen in signals that appear as doublets and thus the conclusion was made that the signals belong to two different compounds and that *F. homblei* does not contain streptol. This outcome was anticipated since

the endophyte of this species lacked the gene required for toxin production. These fractions were then also analysed under the higher resolution and sensitivity, UPLC-QtoF apparatus to confirm the results.

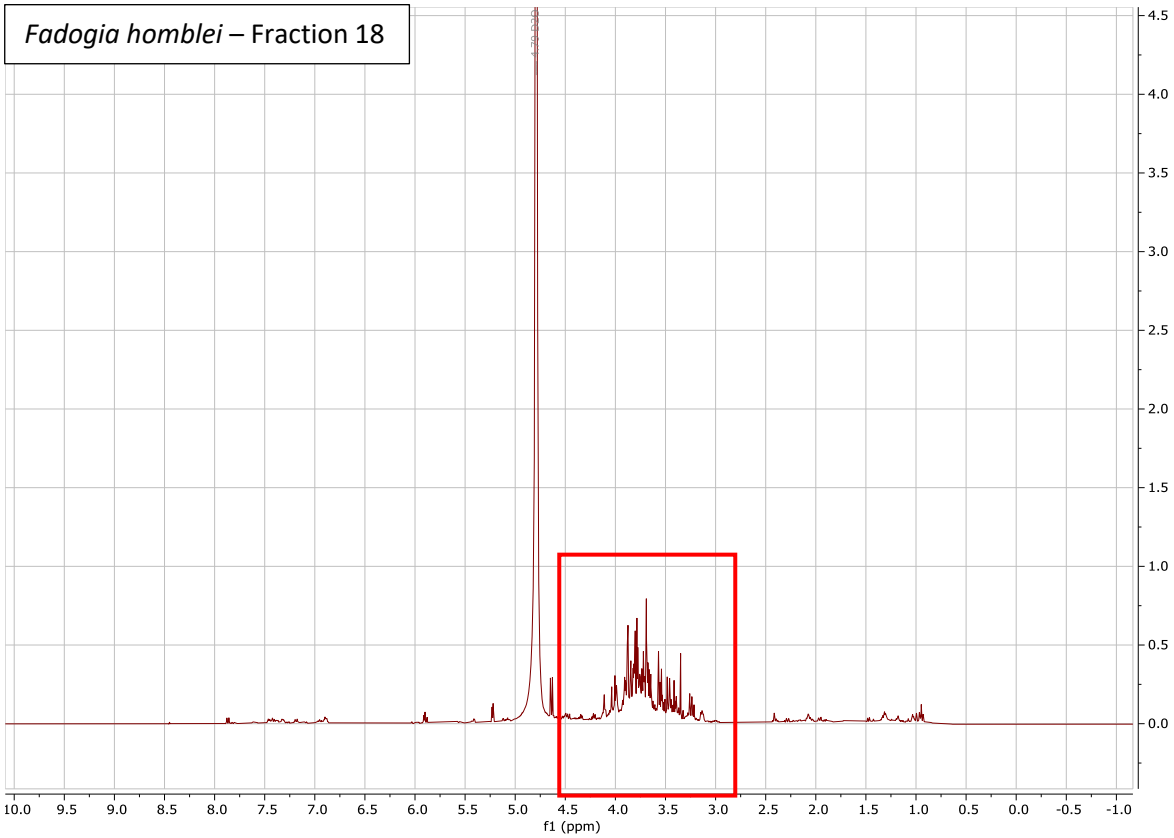

Figure 3.10: Full ^{1}H-NMR spectrum of the purified fraction 18 of *Fadogia homblei*. Red rectangle indicating sugars present in the fraction, which is expected in the fraction containing streptol.

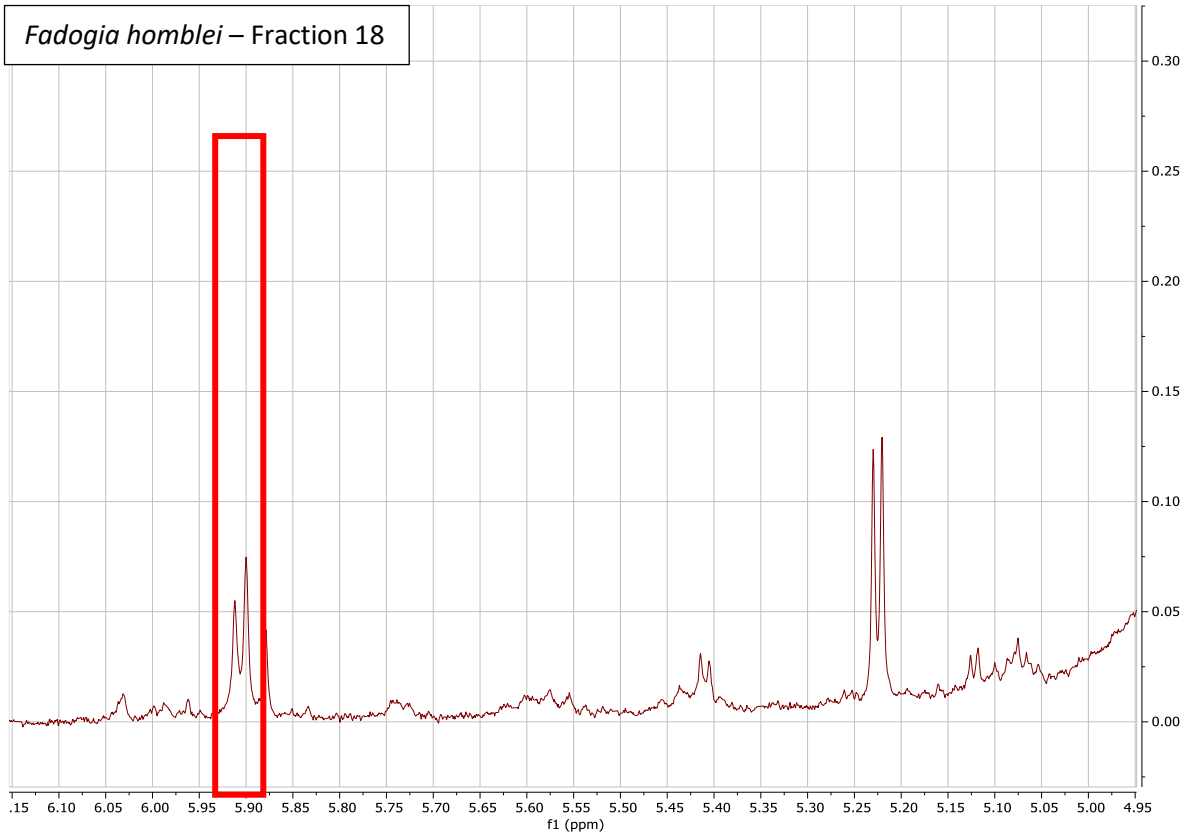

Figure 3.11: The 200 MHz ^{1}H-NMR spectrum of the purified fraction 18 of *Fadogia homblei*. From the results it was concluded that the species did not contain any streptol. A potential signal (red rectangle) was detected, but it was ruled out as a candidate signal for the toxins. The signal is far from where that of streptol is expected, the peaks also appear to belong to two separate compounds due to the height difference of the two peaks.

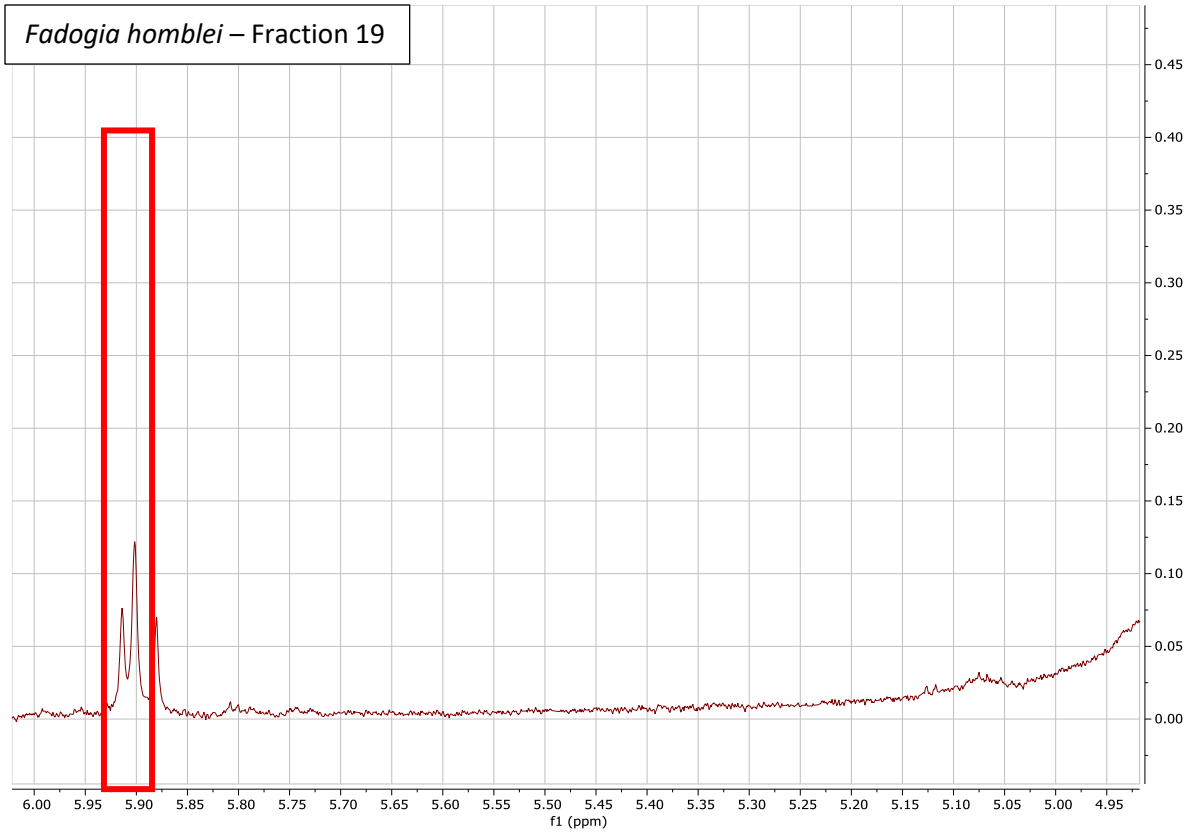

Figure 3.12: ^{1}H-NMR spectrum of the purified fraction 19 of *Fadogia homblei*, showing a doublet at 5.90 ppm.

3.4.4. *F. homblei* UPLC-QToF results

A compound with the same exact mass as streptol eluted at a retention time of 0.785 min, however, the intensity of it was very low. The MassLynx (V4.1 SCN 803) software could not predict the chemical formula of this compound, which makes it likely that it was part of background noise. The UPLC-QToF results supports the ^{1}H-NMR results and confirms that *F. homblei* does not contain streptol. The question can therefore be asked which compound renders this species toxic? Since it belongs to a different genus than all the other *gousiekte*-causing species, it is possible that *F. homblei*'s toxin is a derivative of streptol. Nonetheless, the findings support the hypothesis that the endophyte plays a role in streptol production because the absence of streptol in this species, was expected since it was shown that its endophyte lack the streptol genes (Danneels et al., 2023).

3.4.5. *V. infausta* ^{1}H-NMR results

Fractions 14 to 25 were subjected to NMR analysis, and from the results, it appeared that fraction 18 contained signals matching streptol's. However, the analyses showed a noticeable difference between the results observed between the 200 MHz and 400 MHz instruments. When fraction 18 was analysed using the weaker instrument a doublet signal with a *J*-value of 5.6 Hz was present at 5.80 ppm. When the same sample was analysed with the higher resolution instrument the signal appeared as a multiplet (Figure 3.13). The doublet signal might belong to streptol but due to the low concentration of streptol and the lower strength of the magnet, the peaks do not split into a dd signal on the 200 MHz NMR. It can also be argued that when using an instrument with stronger magnetic strength (400 MHz), peaks that appear as a multiplet, is possibly the peaks of another compound which is shadowing the results of streptol. It is thus unclear from the ^{1}H-NMR analyses if streptol is present in *V. infausta* leaves, and due to the small quantity of sample obtained after the first purification, further purification was not possible.

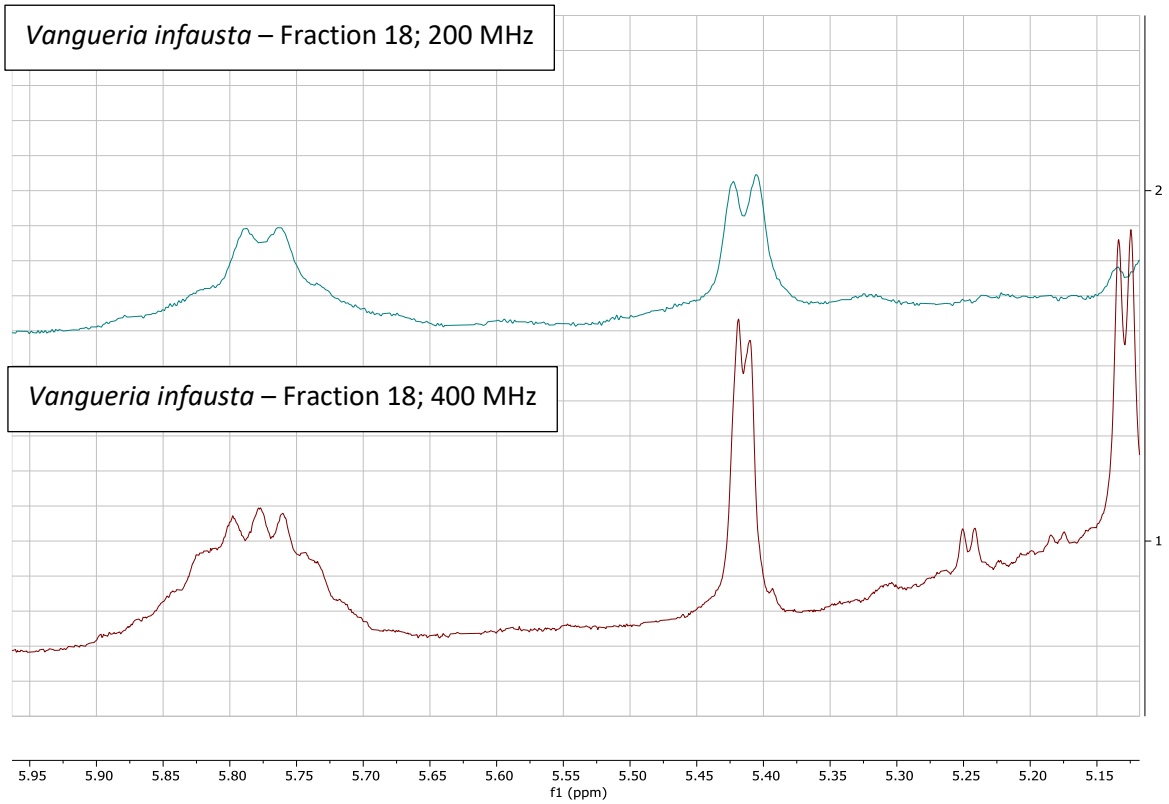

Figure 3.13: ^{1}H-NMR results of *Vangueria infausta*, fraction 18. Results of the 200 MHz (Blue) and 400 MHz (Red) instruments.

Because the results from the ^{1}H-NMR analyses were inconclusive, ^{13}C-NMR and UPLC-QToF analyses were also conducted, and the results compared to those of Hsiao and associates (2019). The ^{13}C-NMR signals (Figure 3.14 and Table 3.3) at 72.57, 72.24 and 71.32 ppm were corresponding that of Hsiao et al. (2019) but two signals reported by these authors were not observed in the analyses; 120.83 ppm and 142.56 ppm. The absence of the peaks could again be attributed to the very small amount (0.1036 g) analysed of the semi-purified sample.

Table 3.5: Results reported by Hsiao and associates (2019) and the results obtained during ^{13}C-NMR analyses of fraction 18 from the purified extract of *Vangueria pygmaea*.

Carbon atom	**Hsiao et al., 2019 signals**	**Observed signals**
C-2	142.56	-
C-1	120.81	-
C-6	72.41	72.57
C-2	72.15	72.24
C-5	71.26	71.32
C-3	65.84	65.82
C-6	60.93	60.88

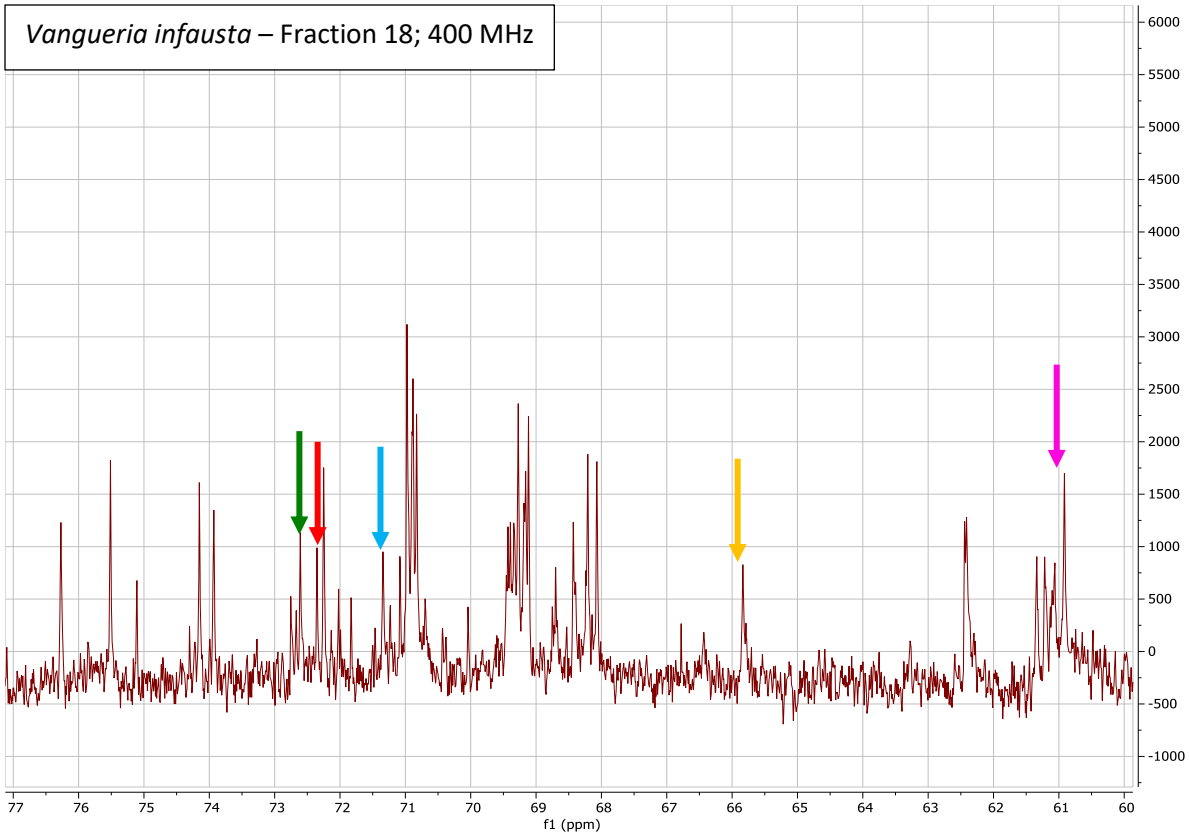

Figure 3.14: ^{13}C-NMR results of *Vangueria infausta*, arrows indicating signals that is believed to be associated with streptol; 72.57 ppm (green), 72.24 ppm (red), 71.32 ppm (blue), 65.82 ppm (orange) and 60.88 ppm (pink). The signals observed are like those reported by Hsiao and associates (2019).

3.4.6. *V. infausta* UPLC-QToF results

UPLC-QToF MassLynx (V4.1 SCN 803) analysis of *V. infausta*'s fraction 18 indicated the presence of a compound with a molecular formula of $C_7H_{11}O_6$, which corresponds to that of streptol, although in very low concentrations. The exact molecular weight was also correct at 175.06 *m/z* (Figures 3.15 and 3.16). The compound eluted at a rt of 0.92 mins. The value of the mDa and the PPM were both 0.00 which also means it is confidently predicted the be a compound of this molecular formula (value being smaller than the required 5).

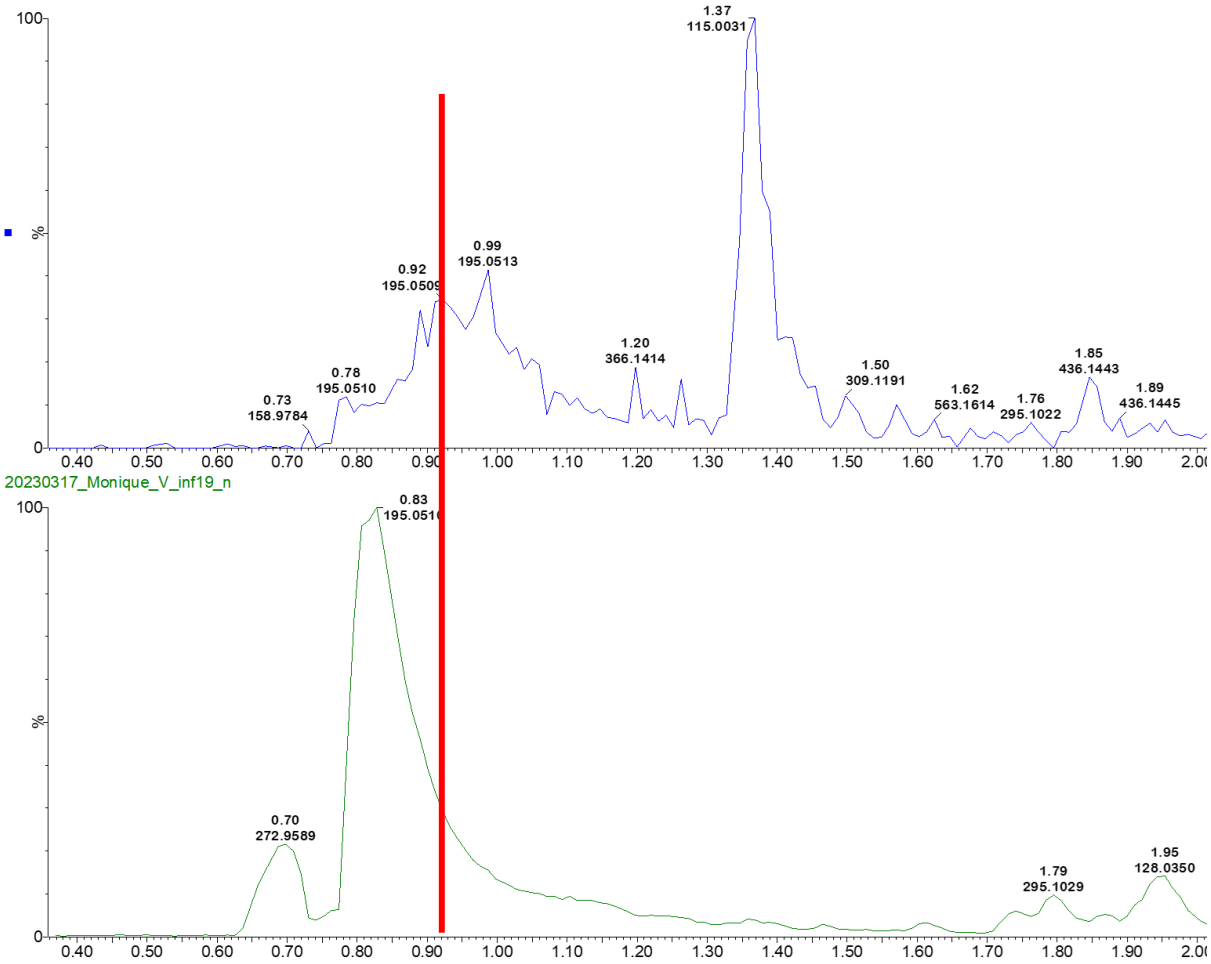

Figure 3.15: The UPLC-QToF chromatogram of the semi-purified fraction 19 of *Vangueria infausta*. The red line indicates the retention time (0.92 mins) where a compound with the exact mass of 175.06 *m/z*, which is probably the toxin, streptol.

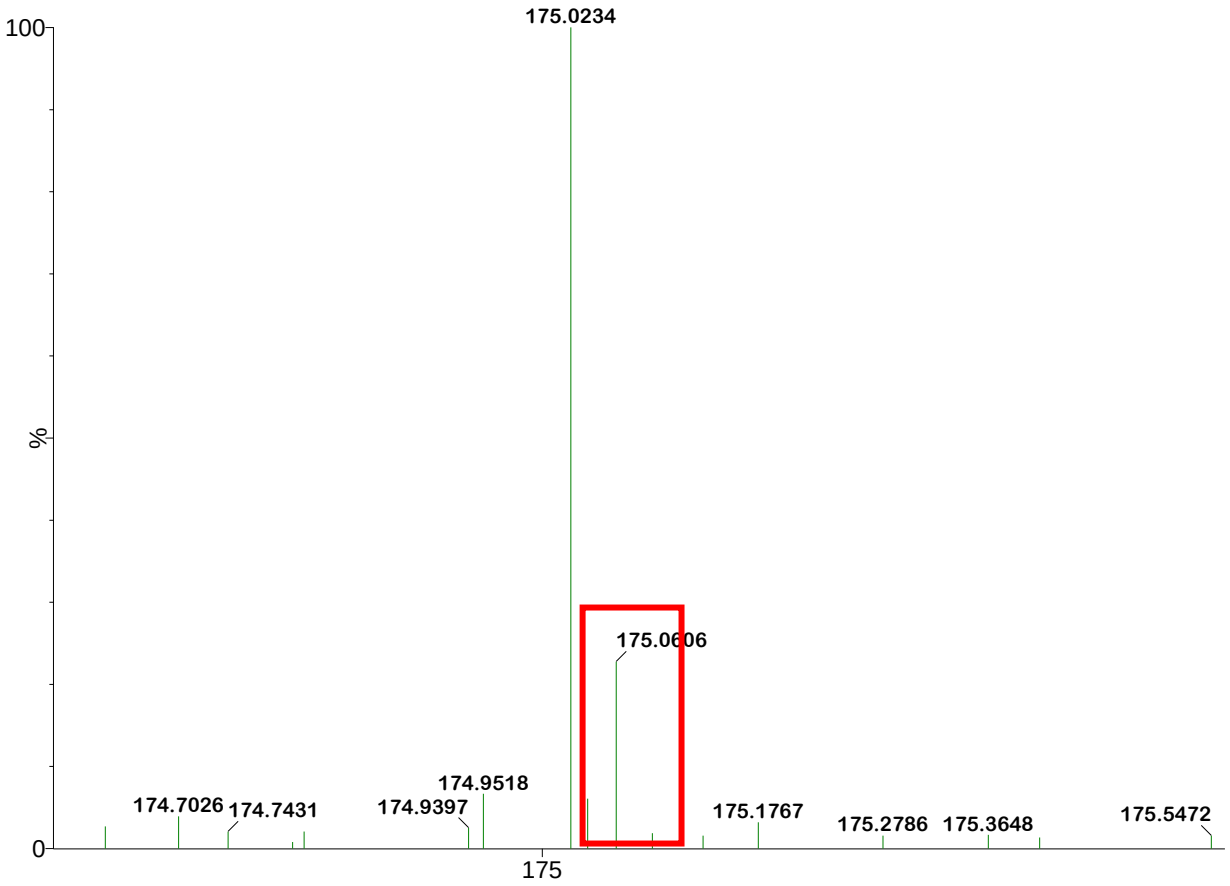

Figure 3.16: UPLC-QToF results of fraction 18 from *Vangueria infausta* indicated a peak, although relatively weak, for streptol. The exact mass of streptol was calculated as 175.06 *m/z*, which is indicated in the red rectangle.

Conclusive proof linking pavettamine to the onset of *gousiekte* is still lacking. Conducting a similar study as the current one on pavettamine, with a focus on the genes responsible for pavettamine synthesis and linking it with a chemical analysis for pavettamine of the *gousiekte*-causing plants, could provide further insight into the role of pavettamine in relation to *gousiekte*.

The intensity of the peak for streptol which correlates to the concentration found in the extract is higher in *V. pygmaea* when compared to *V. infausta*. Interestingly, *V. infausta* is not classified as a species that causes *gousiekte* and thus the low concentration of the toxin was anticipated even though the gene for streptol is carried on the genome of the endophyte. Another noteworthy discovery, as relayed to me in a personal communication with Dr. Johanna Bapela from the University of Pretoria, is that according to

indigenous knowledge, the fruit itself is non-toxic and can be safely consumed. However, there has always been a cautionary advisory against consuming the fruit's seeds.

4. Chapter 4: Conclusion and future prospects

4.1. Conclusion of the study

Gousiekte has been known as a plant-causing toxicoses since 1908 and has cost farmers tremendous losses (Fourie et al., 1995; Hurter et al., 1972; Verstraete et al., 2011). The various obstacles when researching *gousiekte* has made it difficult to fully understand the disease and which plant species cause it (Fourie et al., 1989; Theiler et al., 1923). It has since been shown to be caused by six species of the Rubiaceae family which all harbour an endophyte from the *Burkholderia* genus (Stanton et al., 2013). The initially proposed toxin was identified as pavettamine however, this has not been proven conclusively because there are many unexplained aspects with regards to pavettamine (Bode et al., 2010; Van Elst et al., 2013b). Therefore, other possibilities were considered which led to this study on the toxicity of the toxins, kirkamide, streptol and its glucoside in relation to *gousiekte*.

The first objective of the study was to determine if kirkamide is produced only by the toxic *gousiekte*-causing species. This was determined by means of ^{1}H-NMR and GC-MS analyses. These results were then correlated with those of our colleagues who investigated the presence of the kirkamide biosynthetic genes in the endophyte genomes of the *gousiekte*-causing species. In other words, is the toxin only present in plant species which harbour endophytes with genes to biosynthesise kirkamide. This would then provide compelling evidence for the long-proposed hypothesis that the endophytic bacteria are responsible for *gousiekte*.

The species analysed in the current study for kirkamide were *F. homblei*, *V. dryadum*, *V. infausta*, *V. lasiantha*, *V. macrocalyx*, *V. madagascariensis*, *V. pygmaea*, *V. randii* and *V. soutpansbergensis*. An additional species was analysed which served as a positive control namely *Psy. kirkii*, from which kirkamide was first isolated (Hsiao et al., 2019).

One of the important questions in *gousiekte*-research is the varying toxicity of the plants during seasons. Therefore, another objective was to assess whether there exists a variance in the concentration of kirkamide between autumn and spring. The plant extracts were examined for the presence of kirkamide using ^{1}H-NMR analysis and validated by GC-MS analysis.

The ^{1}H-NMR analyses indicated that none of the species analysed contains kirkamide except the positive control, *Psy. kirkii*. This was confirmed by the GC-MS results. This finding correlated with the genetic

results of our collaborators (Danneels et al., 2023) which showed that only *Psy. kirkii* contains the genes on its endophyte genome responsible for kirkamide biosynthesis. This supports the hypothesis that the endophyte is responsible for the biosynthesis of the toxin kirkamide. However, the hypothesis regarding kirkamide being responsible for the cause of *gousiekte* can be rejected since it was not detected in the toxic species and also not its biosynthetic genes.

No kirkamide was detected in the plant samples collected of *V. pygmaea* in either autumn or spring. It was however noted that the concentration of the compounds in general present in the extracts collected in spring was much higher than that of autumn. Thus, during future studies, it could possibly be advisable to collect plant material during spring when the overall concentration of the compounds is much higher and the concentration of the toxin responsible for *gousiekte* could possibly be higher. These results also correlate to the amount of *gousiekte* cases reported, which is much higher in spring.

The objectives with regards to streptol and its glucoside were similar to that of kirkamide, but since the initial analyses showed that partial purification by column chromatography was required, only three species (*V. infausta, V. pygmaea* and *F. homblei*) were selected for chemical analysis. The focus was set on the partial purification and detection of streptol since it was hypothesised that its glucoside would probably also be present if the aglycone was detected. The objective was to determine if only the species containing the gene of the toxin on the endophyte genome also contains the toxin in the plant extract. The extracts were semi-purified, this was achieved by Sephadex column chromatography and fractions selected for further analyses were based on TLC results. The chemical analyses were done by ^{1}H-NMR, UPLC-QTof and in the case of *V. infausta*, confirmed by ^{13}C-NMR spectrometry.

The ^{1}H-NMR streptol results were compared to Hsiao et al. (2019) but were not conclusive. From the ^{1}H-NMR results of *V. pygmaea* it was relatively clear that the extract contained streptol, since the doublet of doublets (dd) was found in a similar chemical shift area as expected (5.7-5.8 ppm) and had the same *J*-values as that of Hsiao et al. (2019). The UPLC-QTof results of *V. pygmaea* confirmed the significant finding of the presence of streptol. A chromatogram peak with the exact mass of streptol, 175.06 *m/z* was detected, and it was suggested by the software to belong to a compound with the chemical formula of streptol. The peak was also detected at the retention time it was expected to elute, 0.9 mins.

The ^{1}H-NMR results of *V. infausta* was also not conclusive but were confirmed through both ^{13}C-NMR and UPLC-QTof analyses. The ^{1}H-NMR results did have a signal that could belong to streptol and the ^{13}C-NMR results provided further evidence that the semi-purified compound was streptol. The UPLC-QTof results

confirmed that the species contains streptol since it had a chromatogram peak with the exact mass of streptol at a similar retention time as expected.

The ^{1}H-NMR results of *F. homblei* indicated that the extract did not contain streptol. The two signals found in the chemical shift area of streptol seemed to belong to two different compounds since the heights of the peaks differed and were thus not clearly a doublet of doublets signal. The absence of streptol in *F. homblei* was confirmed by UPLC-QTof analyses since the exact mass of streptol could not be detected.

From the results of the chemical analyses, it was concluded that streptol occurs in both the positive controls. The hypothesis is thus supported by the results, that all plant species containing an endophyte that carries the required genes for streptol, synthesises the toxin (Danneels et al., 2023). This provides further evidence to supports the long-standing hypothesis that the endophyte assists in toxin production.

This study provided much supporting evidence that the *Burkholderia* endophyte assists significantly in the synthesis of the toxin, streptol in most *gousiekte*-causing species. Due to the absence of streptol in F. homblei, one cannot assert with confidence that it contributes to the development of *gousiekte* in this species. Since this species is morphologically significantly different from the other *gousiekte*-causing species and therefore classified in another genus than the others, it's possible that it produces a derivative of streptol. Streptol is thus a probable cause of *gousiekte*, although it doesn't rule out the fact that pavettamine might also play a role in causing *gousiekte*.

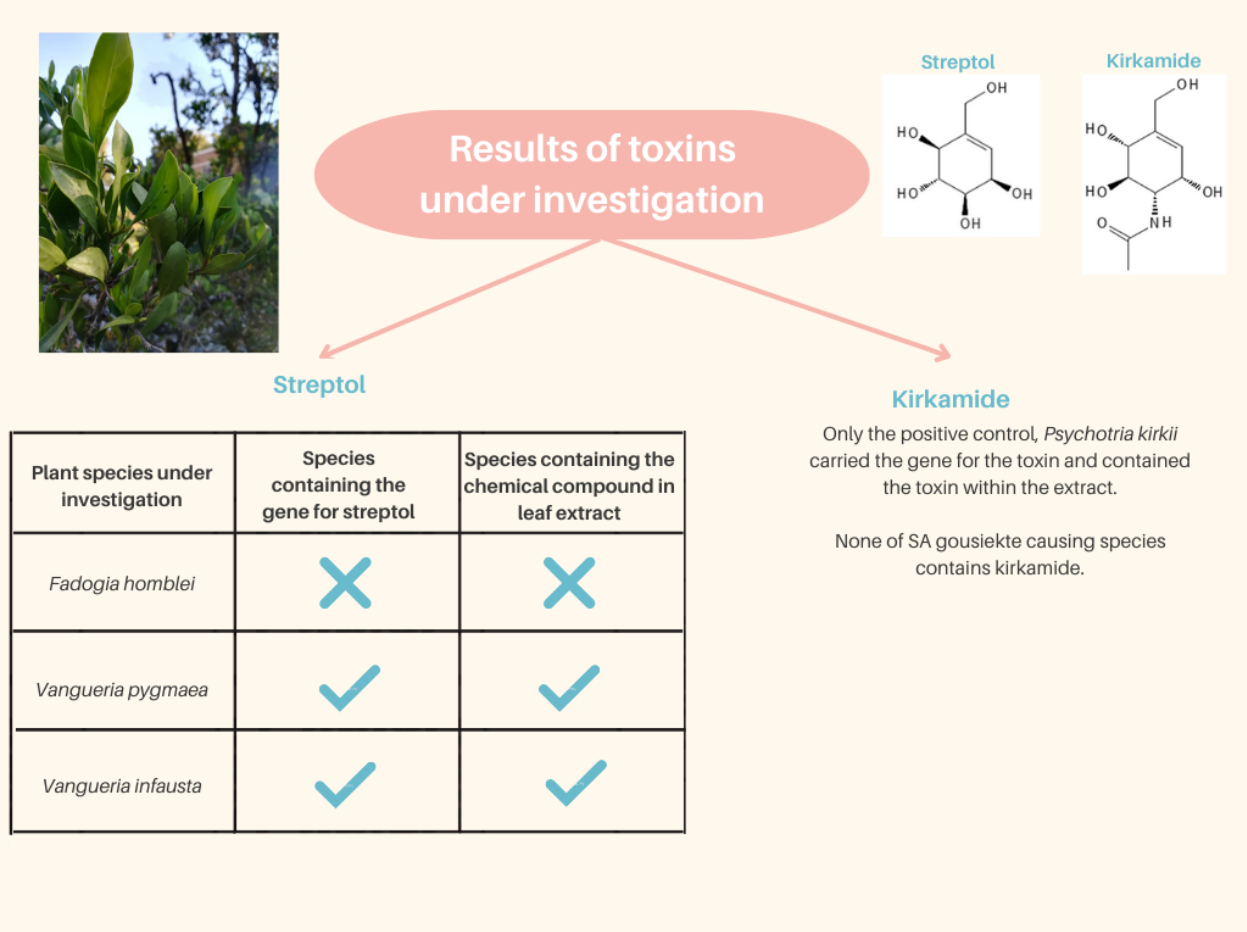

Figure 4.1: Visual representation summarizing the overall findings acquired throughout this research.

4.2. Future prospects into continuing the study

Given that one of the major challenges in *gousiekte* research lies in the fluctuating toxicity of the plants, research in defining the parameters responsible for this variance in toxicity is important. Therefore, determining what the factors are which enhance the production of the toxic compound(s), how external factors for example temperature, rainfall, herbivory, and competition affect the concentration of the toxic compound(s) are all factors that if defined can assist to not only protect the animals by warning farmers to keep them away from the plant species in the specific time frame but could also assist scientists in their studies by knowing when to collect plant material, and when the best time for feeding trials would be.

Since technology has improved significantly over the years it could be beneficial to repeat previous experiments done by scientists on *gousiekte*. For example, identifying the chemical parameters of the

toxic compound(s), if the compound(s) is heat stable, a cationic compound and if it forms a precipitate in the presence of ethanol or methanol (Fourie et al., 1995). Based on these results it would be easier to isolate the compound(s) from the plant extract. It is conceivable that numerous compounds fall under this category; however, in the current era with a wealth of available research, many of these compounds could be excluded based on previous studies. Once left with a few possible compounds it could individually be investigated for being the cause of the disease.

It would also be advisable to reconfirm the occurrence of pavettamine in the *gousiekte*-causing species since the toxin could previously only be isolated from four of the six *gousiekte*-causing species (Bode et al., 2010).

If a well confirmed compound is identified, it would be interesting to see how the toxin affects the animals itself. How various hormone levels changes, how the compound moves through the body of the animals, how it is absorbed and if the chemistry of the compound(s) changes as soon as it enters the animal. If these could be determined it would create an opportunity to identify further methods of preventing the animals from dying or creating a cure.

This would all be very interesting to investigate, but it requires a lot of animals to be sacrificed, many ethical problems, years of research and collecting data of not only the concentration of compounds within the plant but also rainfall patterns, weather conditions, several occasions of collecting plant material in various areas, this would also include analysing all of the data gathered.

Several scientists support the hypothesis that the bacteria are responsible for toxin production as was also indicated in the current study. It would therefore be interesting to test the application of antibiotics to determine how it affects the endophytes and the plant itself. If the plant would be able to absorb the antibiotic, or if it could be administered in other ways such as injecting the antibiotics into the roots or even the flowers or seeds since the bacteria are transferred through the seeds of the plants. Investigations into how the plants are affected when they do not contain the endophytes is also important. All species in the environment plays an important role and trying to remove these plants would also not be advisable. Thus, it would probably be better to find a method of preventing *gousiekte* by targeting the endophytes, and not by attempting to remove the species from the environment.

There are still many uncertainties regarding *gousiekte* and I believe it still requires many years of investigations. However, it causes a concerning number of animal losses and finding a cure is important.

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6. Appendix - GC-MS results of kirkamide analysis

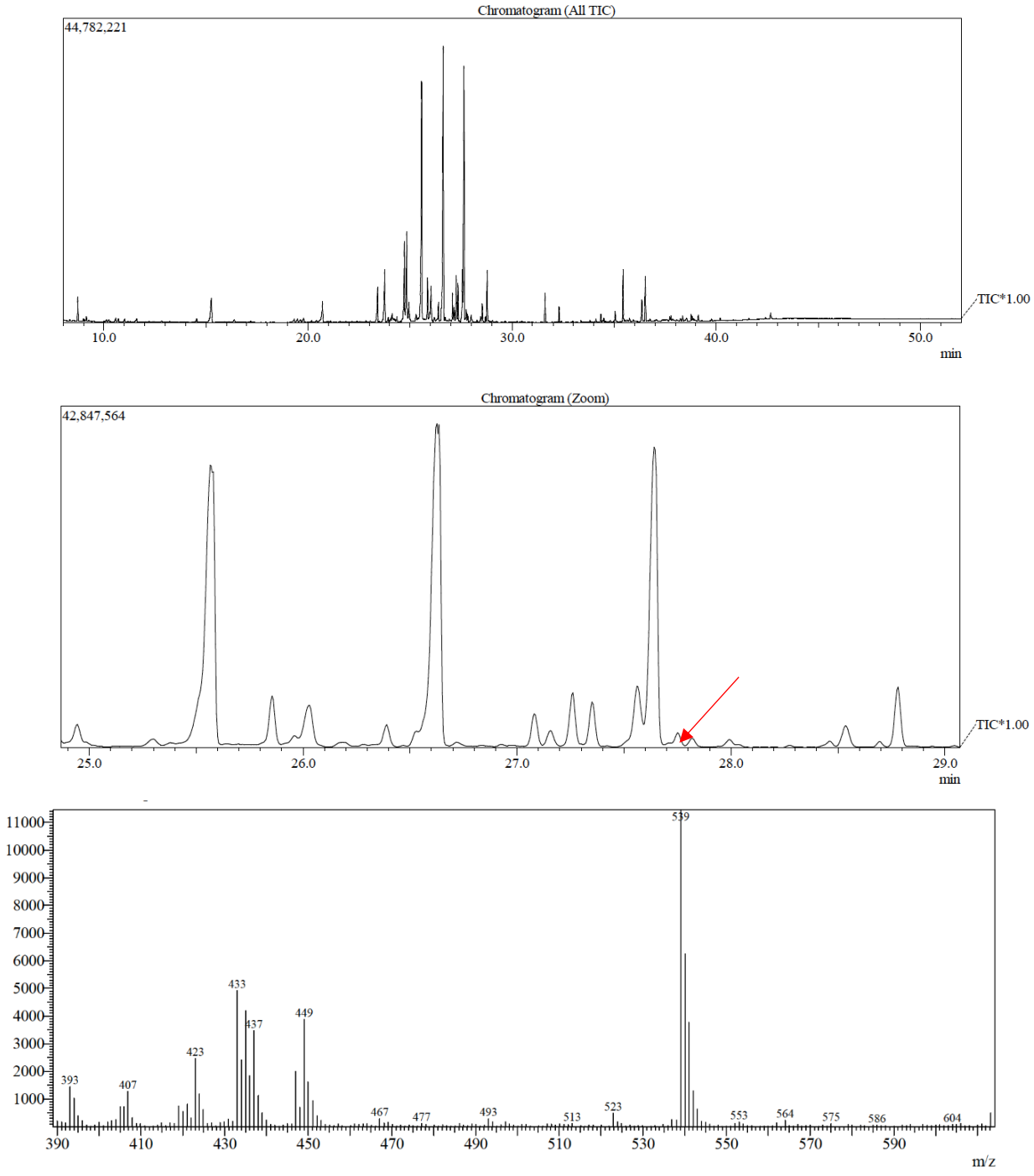

Figure 6.1: GC-MS results of *Fadogia homblei* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

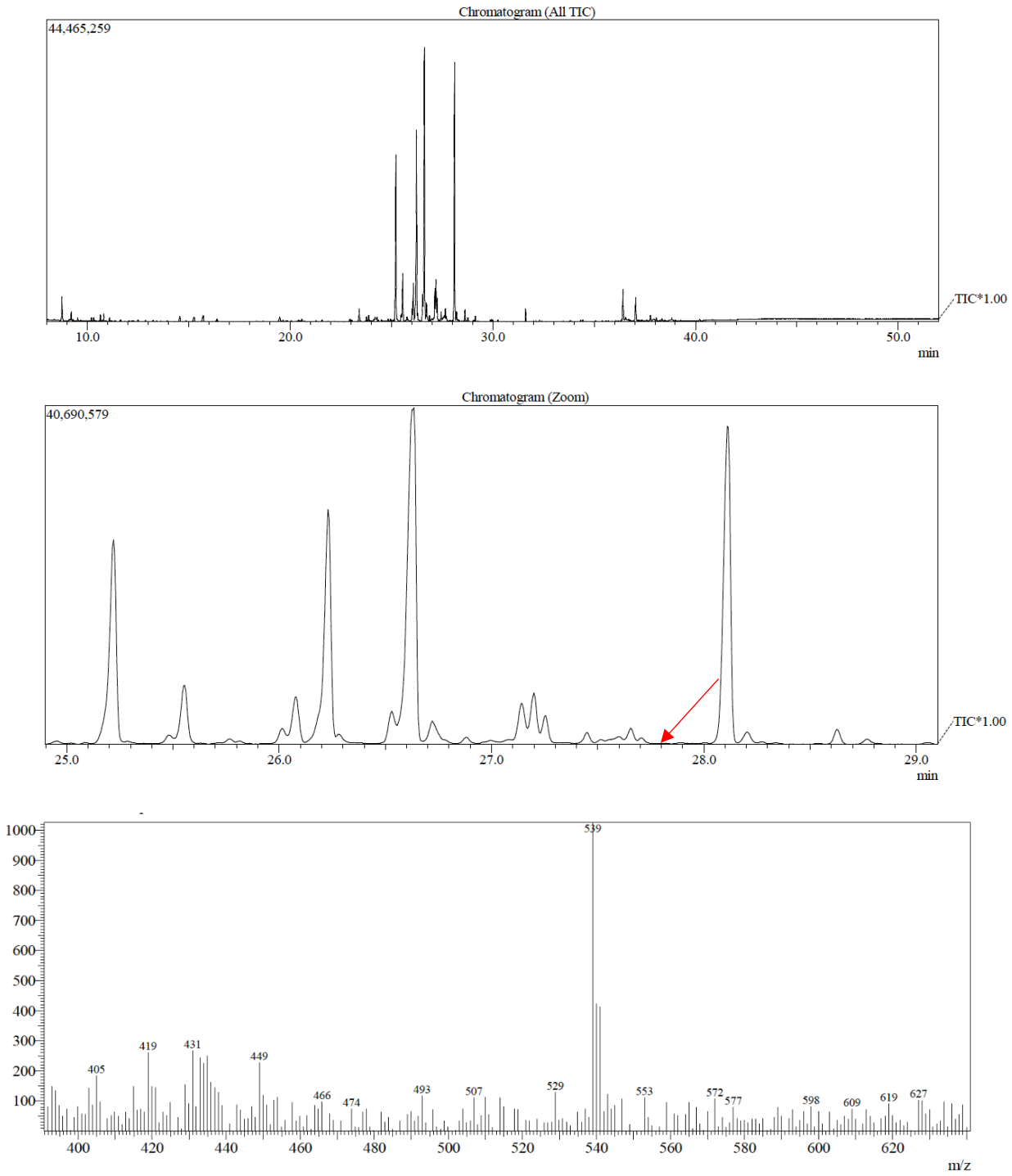

Figure 6.2: GC-MS results of *Vangueria dryadum* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

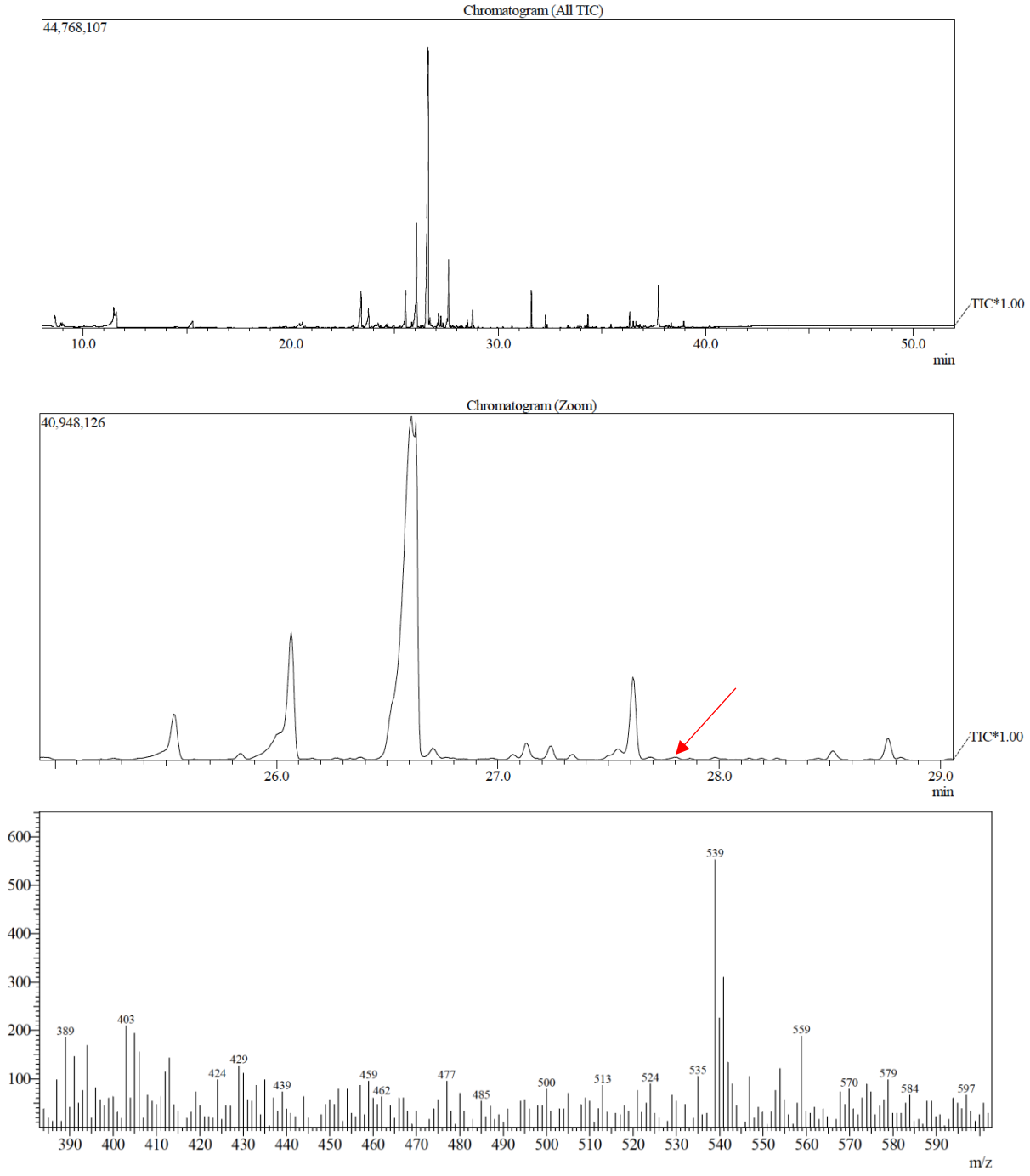

Figure 6.3: GC-MS result of *Vangueria infausta* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

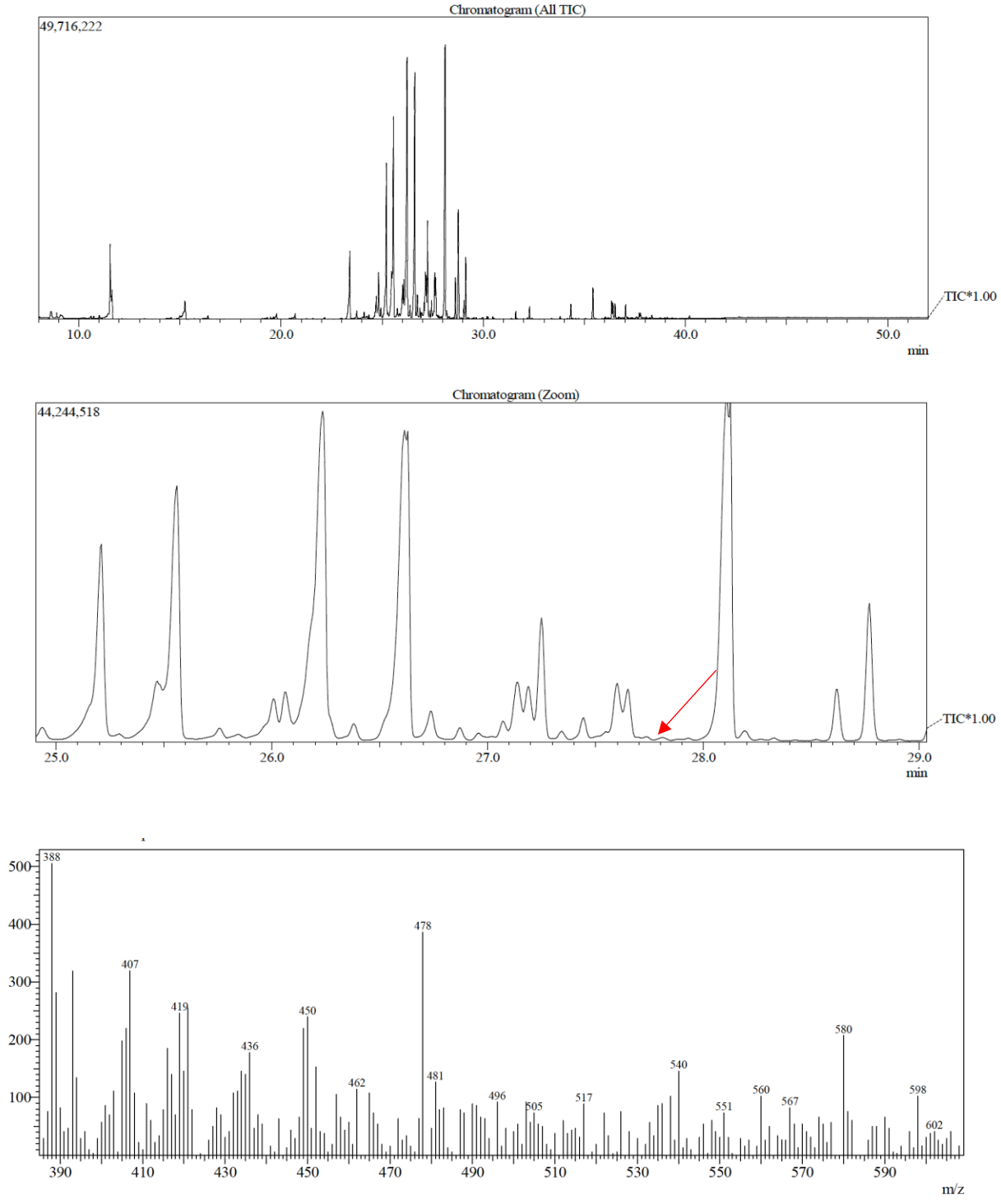

Figure 6.4: GC-MS results of *Vangueria lasiantha* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

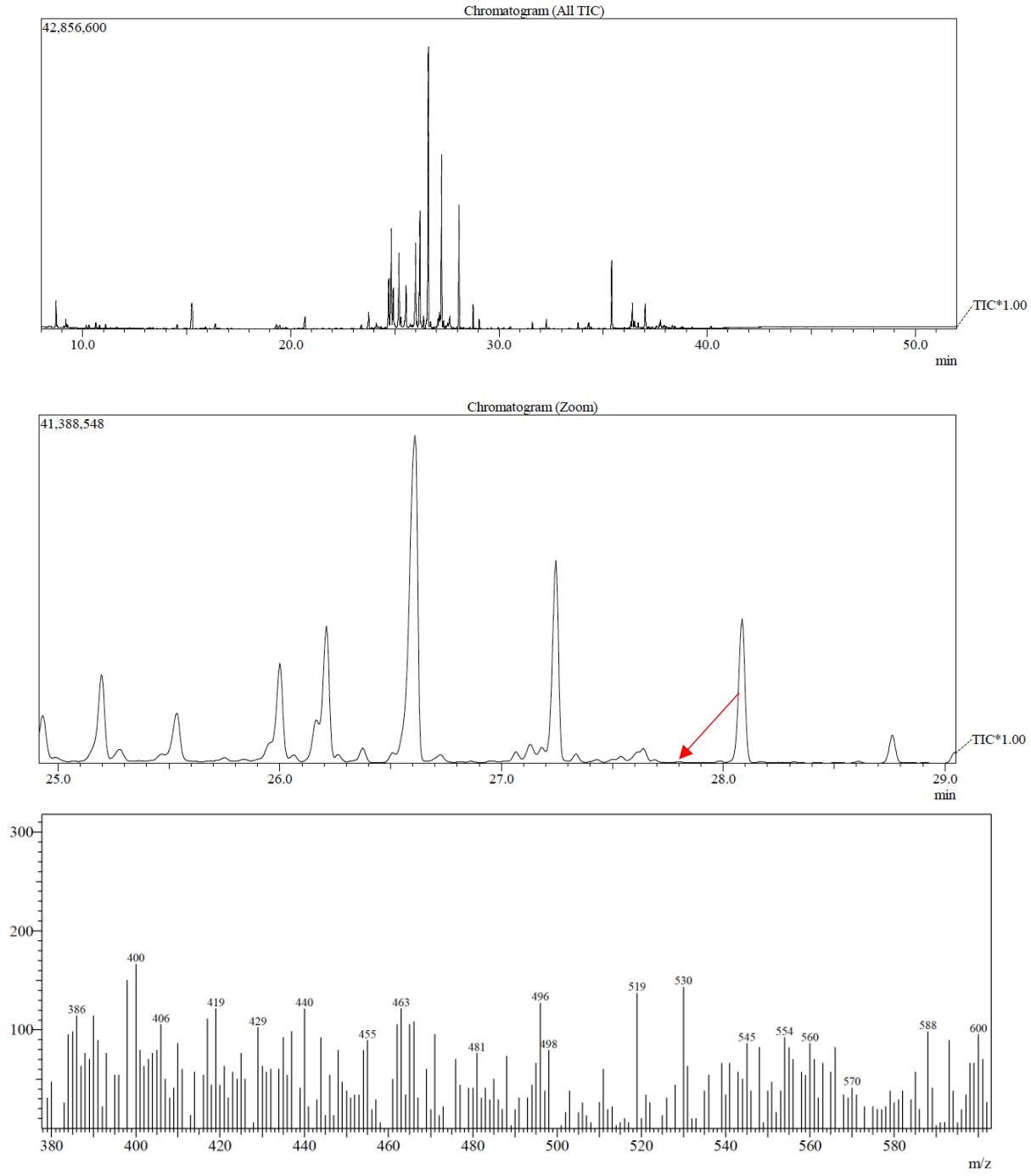

Figure 6.5: GC-MS results of *Vangueria macrocalyx* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

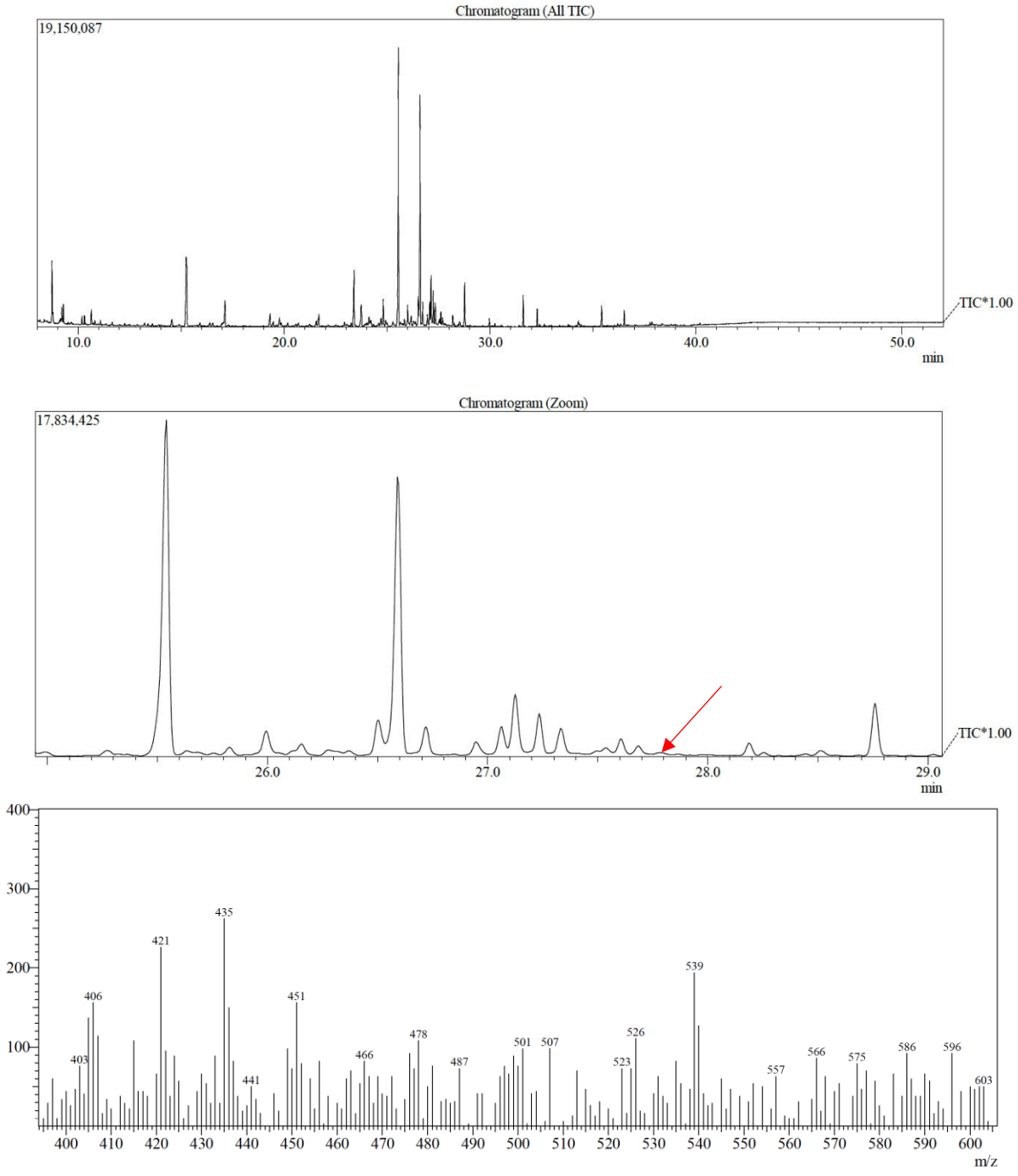

Figure 6.6: GC-MS results of *Vangueria madagascariensis* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

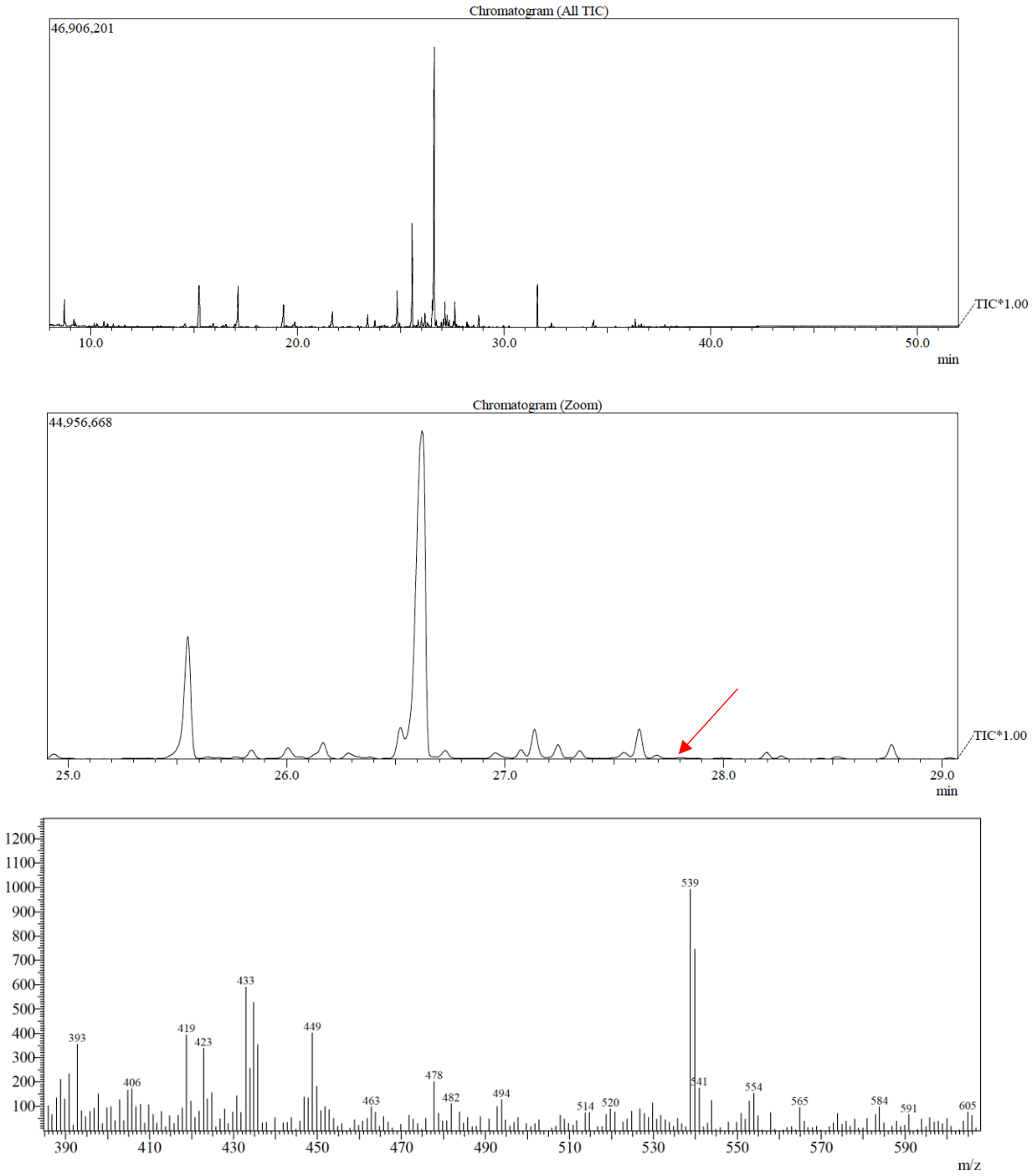

Figure 6.7: GC-MS results of *Vangueria randii* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.

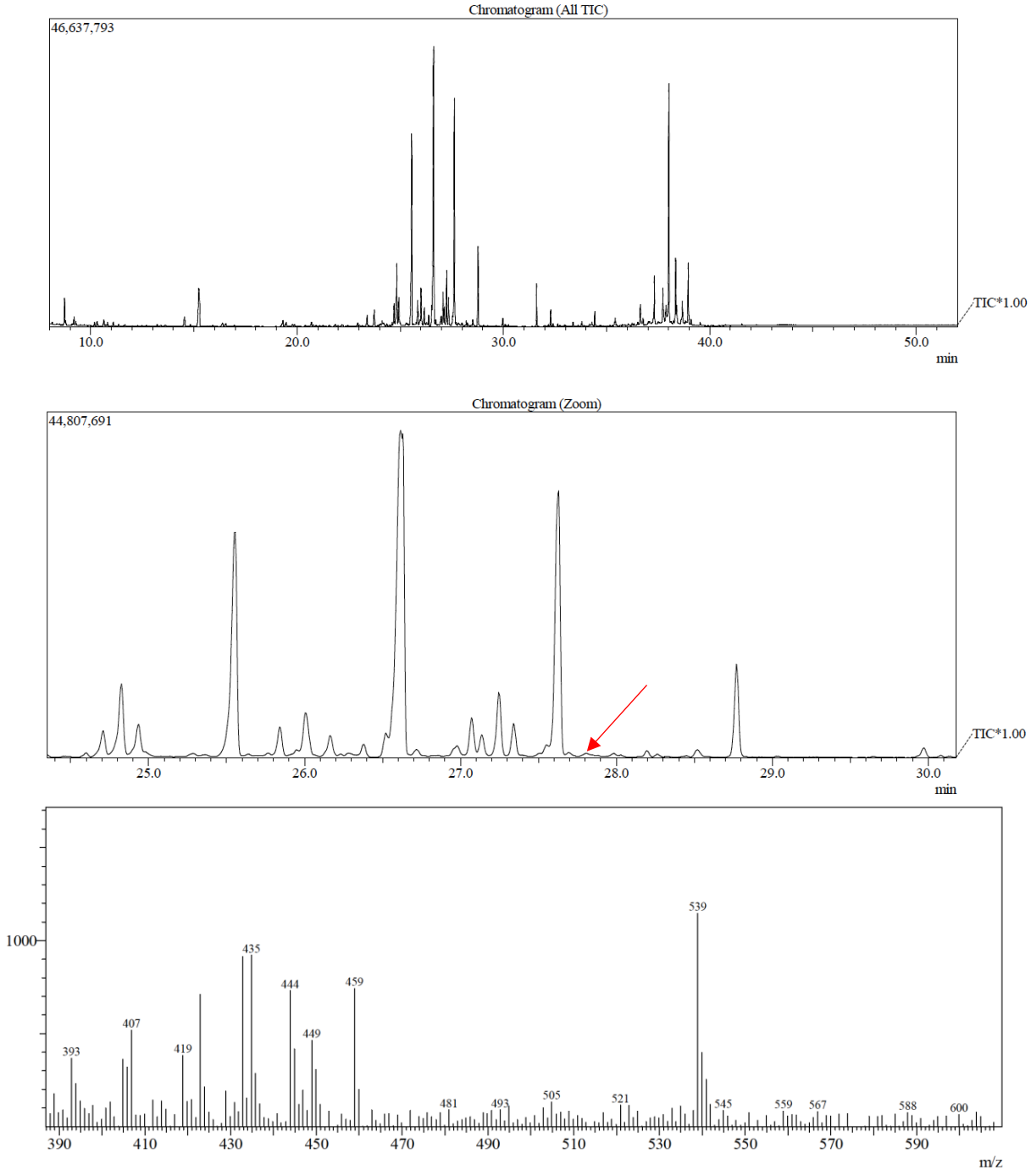

Figure 6.8: GC-MS results of *Vangueria soutpansbergensis* showing the absence of kirkamide in the chromatograms and the enlarged *m/z* region from 400 to 600 *m/z*.